Supporting Information

Oruqaj et al. 10.1073/pnas.1415111112

SI Materials and Methods

Cell Culture and Tissue Sections. In this study, we have used human lung tissue (cryosamples and paraffin-embedded tissue) and fibroblasts from patients with IPF or control organ donors, obtained from the Giessen DZL-biobank at University of Giessen and Marburg Lung Center. Directly after lung transplantation tissue samples were snap frozen in liquid nitrogen or placed in 4% (wt/vol) paraformaldehyde pH 7.4. Control and IPF fibroblasts were cultured in Dulbecco's Modified Eagle's Medium (DMEM) low-glucose media supplemented with 2 mM L-glutamine, 10 U of penicillin per mL, 100 μg of streptomycin per mL, 10% FBS and maintained at 37 °C with 5% (vol/vol) CO_2. Treatment of cells with rhTGF-β1 (R&D catalog no. 240-B), LY364947 (Tocris catalog no. 2718), rHuTNF-α (Biomol catalog no. 50435), interleukin 6, human recombinant (rHuIL-6) (Biomol, catalog no. 50436), SR11302 (Tocris, catalog no. 2476), inhibitor of activator protein-1 (AP-1) transcription factor activity, Luteolin (Sigma, L9283), an Nrf2 inhibitor, ciprofibrate (Sigma-Aldrich Chemie), WY14643, a selective PPAR-α agonist (Tocris, catalog no. 1312), GW6471 (Tocris catalog no. 4618), a PPAR-α antagonist, human *PEX13* siRNA (si-1) (Ambion, catalog no. AM16708), human *PEX13* siRNA (si-2) (Ambion, catalog no. AM16773), silencer select negative control siRNA (Ambion, catalog no. 4390843), Interferin siRNA transfection Reagent (Peqlab, catalog no. 130409-10), TransIT-LT1 (Mirus Bio), Attractene transfection reagent (Qiagen, catalog no. 1051561).

Isolation of Control/IPF Fibroblasts. Primary human control ($n = 10$) and IPF fibroblasts ($n = 10$) were established from human lung tissue biopsies obtained from patients undergoing lobectomy or pneumectomy. Tissues were cut in small slices, placed in cell culture flasks, regularly grown out from the tissue slices after 1 wk and passaged by standard trypsinization. Isolated fibroblasts were frozen in passage 3 or 4 until use. Fibroblasts were plated onto 10-cm^2 cell culture dishes until confluent, and then passaged or plated for the respective experiments. For all experiments cells were used before their eighth passage. After passaging, cells were grown for 24 h before undergoing any treatment with above mentioned reagents or siRNA transfection.

Mice. C57BL/6J wild-type mice, 8 wk of age, were kept under specific pathogen-free conditions in the University of Southern California (USC) animal facility until use. Floxed TGF-β receptor II (TβRII) mice were provided by Harold Moses (Vanderbilt University, Nashville, TN; ref. 1). The genomic clone 129/Sv containing Exon 2 and Exon 3 of the TβRII gene was used to generate the targeting vector (1, 2). Heterozygous male mice were bred to wild-type C57BL6 females and embryos were collected at the one-cell stage and injected with a Cre-expressing plasmid (pBS185) (2). Mice were monitored regularly and received food and water ad libitum. Lungs were removed and immersion fixed in 4% paraformaldehyde fixative in PBS (pH 7.4) and embedded in paraffin (Paraplast Plus) for histological analysis. Animal protocols used in the experiments were approved by the USC Institutional Animal Care and Use Committee and were in accordance with the National Institutes of Health (NIH) guidelines for animal care.

Bleomycin-Induced Pulmonary Fibrosis. Female C57BL/6J control and TGF-β receptor II (TβRII) mice 8-wk-old, anesthetized with pentobarbital sodium (30–40 μg/g ip) were administered with 4 U/kg bleomycin (BLM) (Sigma) diluted in 120 μL of saline, or saline alone by intratracheal instillation using an intratracheal aerosolizer (MicroSprayer Aerosolizer, Model IA, Penn-Century; ref. 3) on day 0 (4). The mouse lungs were then harvested 7, 14, and 28 d after BLM treatment. The bleomycin dose used in the experiment was shown to produce pulmonary fibrosis consistently with a low mortality rate (10%) (4). Lung tissues were fixed in 4% PFA in PBS (pH 7.4) at 4 °C overnight, then dehydrated and embedded in paraffin. Tissue sections of 5 μm thickness were cut on a microtome and used for immunofluorescence stainings.

***PEX13* siRNA Transfection of Control and IPF Fibroblasts for Western Blot and qRT-PCR Analyses and Cytokine Measurements.** Human control and IPF lung fibroblasts were cultured in 12-well or 24-well plates (BD Falcon catalog no. 353043), at 8×10^4 cells per well or 4×10^4 for 24 h in normal media (DMEM 1×, Gibco) low glucose medium supplemented with 2 mM L-glutamine, 10 U of penicillin/mL, 100 μg of streptomycin/mL, 10% FBS) and maintained at 37 °C with 5% (vol/vol) CO_2. Briefly, siRNA pools for *PEX13 knockdown* (same amount of *PEX13* siRNA 1 and 2) were incubated with Interferin siRNA Transfection Reagent (Peqlab, catalog no. 13-409-10) in basal media with no serum or antibiotics and allowed to form the complex for 15 min at room temperature. The complex was then added to the cell suspension for each well (final siRNA concentration of 15 nM). After 24 h, cells were transfected for the second time with *PEX13* siRNA. After 72 h from the first transfection, cells were collected by centrifugation (200 × *g* for 5 min at room temperature) and the pellet processed further for RNA or protein isolation. The supernatants were used for cytokine and collagen assays. For immunofluorescence and DHE staining, cells were grown on poly-L-lysine coated coverslips. After siRNA treatment, they were fixed with the above mentioned 4% PFA-fixative and processed as described in IF (5).

rhTGF-β1 Treatment. The human control and IPF pulmonary fibroblasts were cultured in the same manner as described above for the rhTGF-β1 treatment studies. After 24 h, cells were challenged with 5 ng/mL rhTGF-β1 for an additional 24 h. 1 h before TGF-β1 treatment cells were treated with specific inhibitors such as LY364947 5 μM (Tocris catalog no. 2718) TGF-β1 inhibitor, SR11302 10 μM (Tocris, catalog no. 2476) inhibitor of activator protein-1 (AP-1) transcription factor activity, or Luteolin 25 μM (Sigma, L9283) an Nrf2 inhibitor. Cells were further processed for immunofluorescence, RNA and protein isolation and supernatants were collected for cytokine assays by ELISA according to manufacturer's instructions. For TGF-β1–induced ROS production studies, cells were treated for 30–60 min, then undergoing staining with dihydroethidine (DHE) in a final concentration of 5 μM (see below).

TNF-α Treatment. Cells were seeded as described previously at a density of 8×10^4 cells per well in 12-well plates. After 24 h, they were challenged with 10 ng/mL human rHuTNF-α (Biomol catalog no. 50435), for different time points: 0 h, 1 h, 4 h, and 6 h duration (6). At the end of incubation period, the cells were processed for protein, RNA isolation, and luciferase reporter assays.

IL-6 Treatment. IPF fibroblasts were seeded as described at a density of 8×10^4 cells per well in 12-well plates. After 24 h, they were challenged with 20 ng/mL human IL-6 (Biomol, catalog no. 50435) for 6 h duration. At the end of incubation period, the cells were processed for protein analysis with Western blotting.

PPAR-α Agonist (Ciprofibrate, WY14643) and PPAR-α Antagonist (GW6471) Treatment. The experiments were performed on IPF fibroblasts from the second-eighth passages. Cells were seeded for 24 h in 12-well and 24-well plates, as described above. Cells were then treated with ciprofibrate (Sigma-Aldrich Chemie), for 48 h, with the indicated concentrations: 0 μM, 150 μM, 300 μM, 600 μM, WY14643 (Tocris, catalog no. 1312) for 48 h with the indicated concentrations: 0 μM, 50 μM, 100 μM, 200 μM, or with GW6471 (Tocris catalog no. 4618), for 24 h with the indicated concentration, 10 μM. After respective treatments, cells were processed for immunofluorescence, and protein isolation. For the experiment of TGF-β1 treatment with combined PPAR-α agonists or antagonist, IPF cells were either pretreated with ciprofibrate (200 μM) or WY14643 (100 μM) for 48 h after which the medium was replaced with serum free medium alone or serum free medium containing GW6471 (10 μM) wherever indicated for an additional 1 h. Treatment with ciprofibrate (200 μM), WY14643 (100 μM) for 2 h was done in serum free medium. This treatment was followed by the addition of TGF-β1 (5 ng/mL) to the respective wells indicated.

Poly-L-Lysine Coating. Coverslips were placed in 12 well or 24 well Petri dishes. 10 mg of poly-L-lysine hydrobromide was dissolved in 100 mL of 0.1 M borate buffer and filtered with Millipore syringe filters of 0.22 μm, thereafter 2 mL of the solution was added to the sterile coverslips. The incubation of Petri dishes with coverslips was done overnight under a laminar flow. After 24 h the dishes were washed twice with dd H_2O, left in ddH_2O for 5 h and thereafter washed again 2 × 5 min with dd H_2O, followed by aspiration of water drops and air dried before storage.

Immunofluorescence. Control and IPF fibroblasts were plated on poly-L-lysine (Sigma Aldrich) coated coverslips in 24 well plates for 24 h and thereafter treated with rhTGF-b1 (R&D catalog no. 240-B), LY364947 (Tocris catalog no. 2718), rHuTNF-a (Biomol catalog no. 50435), ciprofibrate (Sigma), WY14643 (Tocris, catalog no. 1312), GW6471 (Tocris, catalog no. 4618) for the indicated time points. Thereafter, they were subjected to an indirect immunofluorescence staining protocol as described (5, 7). Briefly, cells were washed with PBS, fixed in 4% paraformaldehyde and 2% saccharose in PBS buffer pH 7.4, after which they were permeabilized using 1% glycine containing 0.02% Triton X-100. Nonspecific binding sites were blocked with 1% BSA in PBS containing 0.05% Tween 20 for 1 h at room temperature. Then, coverslips were incubated overnight at 4 °C with the primary antibodies against PEX13p (D. I. Crane, Griffith University, Nathan, Australia, rabbit anti-mouse, 1:2,000); PEX14p (D. I. Crane, rabbit anti-mouse, 1:4,000); ACOX1 (P.P. Van Veldhoven, dilution 1:1,000); catalase (D. I. Crane, rabbit anti-mouse, 1:4,000, Polyscience rabbit anti-mouse catalog no. 23728, 1:250), SOD-2 (polyclonal, Abcam, rabbit anti-mouse, 1:2,000); Nrf2 (H-300; Santa Cruz, rabbit anti-mouse, 1:200); HO-1 (Stressgen, rabbit anti-mouse, 1:1,000); Glutathione reductase (Abcam/Biozol, ab16801, rabbit anti-mouse, 1:500); SMA (Sigma, mouse anti-rabbit, 1:2,000); collagen I (Novus Biologicus, rabbit anti-mouse, 1:500); collagen III (Novus Biologicus, rabbit anti-mouse, 1:500); prolyl 4-hydroxylase (PDI) (Acris, AP08767-PU-N, rabbit anti-mouse, 1:100), total Smad3 (Cell signaling, Cat;9523, rabbit anti-mouse, 1:50); K_i-67 mouse Clone MIB-1 (DakoCytomation, F7268, catalog no. 55960, human anti-mouse, 1:30). Coverslips were then washed and incubated with Alexa Fluor 488-conjugated secondary Ab at 1:1,000 dilution (Invitrogen). Nuclei were visualized with 1 μM Hoechst 333424 and TOTO-3-iodide 1:1,000 at room temperature embedded in Mowiol 4–88 with *N*-propyl gallate as an antifading agent. Images were captured using a confocal microscope (Leica TCS SP2, Leica). Pictures were processed with Adobe Photoshop version 9.

QRT-PCR. Control and IPF cells were grown in basal conditions as well as treated with *PEX13* siRNA, control siRNA, rHuTGF-b1, LY364947, rHuTNF-α as mentioned above and cells were harvested after the respective time points. RNA was isolated using the RNeasy kit (Qiagen), and cDNA was synthesized by reverse transcription using the high-capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative RT-PCR analysis was carried out using SYBR premix on a qPCR machine (Bio-Rad iCycler) according to the manufacturer's instructions. Normalization for cDNA quantity was done using *HPRT* and *28S rRNA* control primers for each template. The relative expression, fold change of a defined gene was calculated using the ddCT method. PCR amplification of the cDNA was done with the following primers:

PEX13: For: CCATGTAGTTGCCAGAGCAG, Rev: CATCAAGGCTAGCCAGAAGC;

TGF-β1: For:GGATAACACACTGCAAGTGGAC, Rev: GGGTTATGCTGGTTGTACAGG

COL1A2: For:TCAGAACATCACCTACCACTGC, Rev:GTCCAGAGGTGCAATGTCAAG

TNF-α; For: TCTACTCCCAGGTCCTCTTCAA, Rev: AGACTCGGCAAAGTCGAGATAG

IL-6: For: AGGAGACTTGCCTGGTGAAA, Rev: CAGGTTTCTGACCAGAAGAAGG

28S rRNA: For: AAACTCTGGTGGAGGTCCGT, Rev: CTTACCAAAAGTGGCCCACTA

Semiquantitative RT-PCR Analysis. Total RNA from control and IPF fibroblasts was prepared by using the RNeasy Mini kit (Qiagen) after indicated treatments described above. One μg of cDNA was synthesized from DNase-I-treated total RNA using the SuperScript II First-Strand Synthesis System plus RNaseOut (2 h at 42 °C). For semiquantitative analysis, specific primers for respective genes were designed using the PRIMER3 program (www.ncbi.nlm.nih.gov/tools/primer-blast) and synthesized by Eurofins, MWG or Operon. PCR was performed using 50 ng pf cDNA, 100 nmol forward and reverse primers and, 5′ TaqDNA polymerase in a final volume of 25 μL. Primers used in this study for the semiquantitative RT-PCR are described below:

PEX13: For: TCAGCAAGCTGAAGAAAGCA, Rev: CTGCAGGCAAACATGAAAGA

TGF-β1: For:GGATAACACACTGCAAGTGGAC, Rev: GGGTTATGCTGGTTGTACAGG

COL1A2: For:TCAGAACATCACCTACCACTGC, Rev:GTCCAGAGGTGCAATGTCAAG

IL-6: For: AGGAGACTTGCCTGGTGAAA, Rev: CAGGTTTCTGACCAGAAGAAGG

MMP2: For: TACTGGATCTACTCAGCCAGCA, Rev: CTTCAGGTAATAGGCACCCTTG

28S rRNA: AAACTCTGGTGGAGGTCCGT, Rev: CTTACCAAAAGTGGCCCACTA

HPRT: For: AAGCTTGCTGGTGAAAAGGA, Rev: AAGCAGATGGCCACAGAACT

Western Blotting. Control and IPF fibroblasts were washed with 1× PBS, lysed and the protein quantification done with the Bradford protein assay (Bio-Rad) as described (5). Protein samples (30 μg per lane) were separated on 12% SDS/PAGE and further processed to blotting onto polyvinylidene difluoride membranes (PVDF, Millipore). Membranes were blocked with 5% skim milk or 5% BSA, 50 mM Tris·HCl (pH 7.6), 150 mM NaCl, and 0.1%

Tween 20 for 1 h at room temperature or at 4 °C overnight. The primary antibodies were used against PEX13p, (polyclonal rabbit, D. I. Crane, 1:2,000), Collagen I (polyclonal rabbit, Novus biologicus, 1:500), α-SMA (monoclonal mouse, Sigma, 1:2,000). Anti-GAPDH was used to obtain a loading control (monoclonal mouse, Hytest, 1:10,000; catalog no. 5G4. For secondary antibody detection, alkaline phosphatase labeled goat anti-rabbit IgG or anti-mouse IgG alkaline phosphatase (DPC Bierman) were used at 1:20,000 or 1:30,000 dilutions, respectively. Antigen–antibody complexes were visualized with chemiluminescence detection using the Immun-Star alkaline phosphatase substrate from Bio-Rad according to the manufacturer's instructions. The bands were visualized by exposing the blots to Kodak Biomax Films and quantified with a Bio-Rad Gel Doc 2000 system (Bio-Rad).

Plasmid Constructs. The luciferase reporter gene construct for the SBE (Smad binding element) was a gift from Bert Vogelstein (The Ludwig Center and the Howard Hughes Medical Institute at Johns Hopkins Kimmel Cancer Center, Baltimore, MD); COL1A2 luciferase construct was from Eunsun Jung (BioSpectrum Life-Science Institute, Seoungnam, Korea), p-ARE, luc was from William E. Fahl (University of Wisconsin, Madison). The plasmid pAP-1-luc with three AP-1 repeats in front of a minimal *fos*-promoter was a gift from Dr. C. A. Hauser (The Burnham Institute, La Jolla, CA) (8). The empty control vectors pGL2-basic, pGL3-basic was purchased from Promega. PPAR reporter plasmid was from Qiagen Cignal PPAR Reporter (luc) kit, catalog no. CCS-3026L. PPAR-alpha expression plasmid pSG5 PPAR alpha was a gift from Bruce Spiegelman (Addgene plasmid no. 22751).

Transfection and Dual Luciferase Assay. Control and IPF fibroblasts were cultured overnight. Transfection of plasmid DNA into the cells was performed with TransIT-LT1 (Mirus Bio) as described (7) or Attractene transfection reagent catalog no. 1051561 as described in the manufacturer's instructions (Qiagen). Cells were transfected with 1 μg of the reporter plasmid and in cotransfection experiments with 1 μg of the indicated expression vectors.

Cells were transfected with plasmids and after 24 h treated with respective reagents for certain time-points. For the PPAR reporter assay, cells were cotransfected with PPAR-α expression plasmid and PPAR reporter plasmid and after 24 h treated with the respective reagents for the indicated times. Cells were lysed with luciferase lysis reagent (Promega), and firefly or Renilla luciferase activity was determined with the dual luciferase reporter assay system (Promega) as described in the manufacturer's instructions.

Measurement of Reactive Oxygen Species. Generation of reactive oxygen species (ROS) was detected with dihydroethidine (DHE) at a final concentration of 5 μM. DHE is oxidized by superoxide to its fluorescent product, ethidine. Ethidine remains intracellularly after it is oxidized, thus allowing quantitative estimations of the intracellular ROS level (Ahlemeyer et al., 2007). Control and IPF fibroblasts were grown on coverslips and transfected with *PEX13* siRNA, control siRNA, or treated respectively with the above mentioned compounds. DHE was added to the cells and incubated for 20 min. Thereafter cells were washed with 1× PBS and fixed with 4% paraformaldehyde in PBS, pH 7.4 for 20 min at room temperature. The coverslips were mounted for measuring cellular ethidine fluorescence under a confocal laser-scanning microscope (Leica TCS SP2, Leica) (40× objective). Ethidine fluorescence intensity was quantified individually in all cells using the Leica Confocal Software program (Leica). The measured values represent the mean fluorescence intensity (MFI) of ethidine per cell.

Sircol Collagen Assay. To examine the release of collagens into the cell culture medium, control and IPF fibroblasts were grown in 12 well plates for 24 h, followed by *PEX13* siRNA or control siRNA transfection using Interferin (4 μl or 2 μl). After 72 h, the supernatant was collected and collagen production was analyzed by using the Sircol Assay according to the manufacturer's instructions (Biocolor; Cat. No. S1000).

Cytokine ELISAs. Control and IPF fibroblasts were transfected with *PEX13* or control siRNA and 72 h after transfection the supernatants were collected and levels of secreted cytokines were analyzed using the human TGF-β1 immunoassay (R&D, Cat. No. DB100B), or the human IL-6 Quantikine ELISA Kit (R&D, Cat. No. D6050). In addition, cells were also treated with rHuTGF-β1, LY364947, ciprofibrate, GW6471 as mentioned above for 24 h and the secreted levels of IL-6 were analyzed in the collected supernatant at the respective time point with the Quantikine ELISA kit according to the manufacturer's instructions (R&D, Cat, No. D6050).

1. Chytil A, Magnuson MA, Wright CV, Moses HL (2002) Conditional inactivation of the TGF-beta type II receptor using Cre:Lox. *Genesis* 32(2):73–75.
2. Sauer B (1993) Manipulation of transgenes by site-specific recombination: use of Cre recombinase. *Methods Enzymol* 225:890–900.
3. Bivas-Benita M, Zwier R, Junginger HE, Borchard G (2005) Non-invasive pulmonary aerosol delivery in mice by the endotracheal route. *Eur J Pharm Biopharm* 61(3):214–218.
4. Zhao J, et al. (2002) Smad3 deficiency attenuates bleomycin-induced pulmonary fibrosis in mice. *Am J Physiol Lung Cell Mol Physiol* 282(3):L585–L593.
5. Nenicu A, et al. (2007) Peroxisomes in human and mouse testis: differential expression of peroxisomal proteins in germ cells and distinct somatic cell types of the testis. *Biol Reprod* 77(6):1060–1072.
6. Sullivan DE, Ferris M, Nguyen H, Abboud E, Brody AR (2009) TNF-alpha induces TGF-beta1 expression in lung fibroblasts at the transcriptional level via AP-1 activation. *J Cell Mol Med* 13(8B):1866–1876.
7. Vijayan V, Baumgart-Vogt E, Naidu S, Qian G, Immenschuh S (2011) Bruton's tyrosine kinase is required for TLR-dependent heme oxygenase-1 gene activation via Nrf2 in macrophages. *J Immunol* 187(2):817–827.
8. Genovese T, et al. (2005) Role of endogenous and exogenous ligands for the peroxisome proliferator-activated receptor alpha in the development of bleomycin-induced lung injury. *Shock* 24(6):547–555.

Fig. S1. Compromised antioxidative response in IPF fibroblasts. (*A–D*) Double immunofluorescence of the antioxidant proteins SOD1, HO-1, GR, and Nrf2 with α-SMA fibrotic marker in donor and IPF fibroblasts. (*E*) Quantification of peroxisomal biogenesis protein PEX14 per fibroblast from Fig. 1. (*F*) Luciferase reporter activity of ARE and AP1 transcription factors in control and IPF fibroblasts. The activity of firefly luciferase was measured in cell lysates and normalized to the activity of renilla. E.V.: empty vector; CO: Control; IPF: idiopathic pulmonary fibrosis. Data represent ± SD of three independent experiments. *P* value, two tailed Student *t* test. (Scale bar: 10 μm in *A–D*.)

Fig. S2. Higher abundance of fibrotic marker proteins COL1, COL3A1, and PDI induced by PEX13 knockdown. (*A* and *B*) Depiction of the fibrotic marker proteins collagen I (COL1), collagen III (COL3A1) and prolyl 4-hydroxylase (PDI) with immunofluorescence staining in *PEX13* knockdown in control and IPF fibroblast cultures. Nuclei were stained with 1 μM Hoechst 33342. (Scale bar: 10 μm.)

Fig. S3. Increased fibrotic response and MMP2 in *PEX13* knockdown of IPF fibroblasts. (*A*) Expression of *PEX13*, *TGF-β1*, *COL1A2*, and *MMP2* at mRNA level shown by RT-PCR in IPF fibroblasts. Expression of *28S rRNA* gene was used as control. (*B–E*) Quantification of mRNA expression from depicted genes in *A*. Data represent ± SD of three independent experiments. *P* value, two tailed Student *t* test. BC: basal control, IC: interferin control, Sc: scrambled control, si-1: siRNA-1, si-2: siRNA-2.

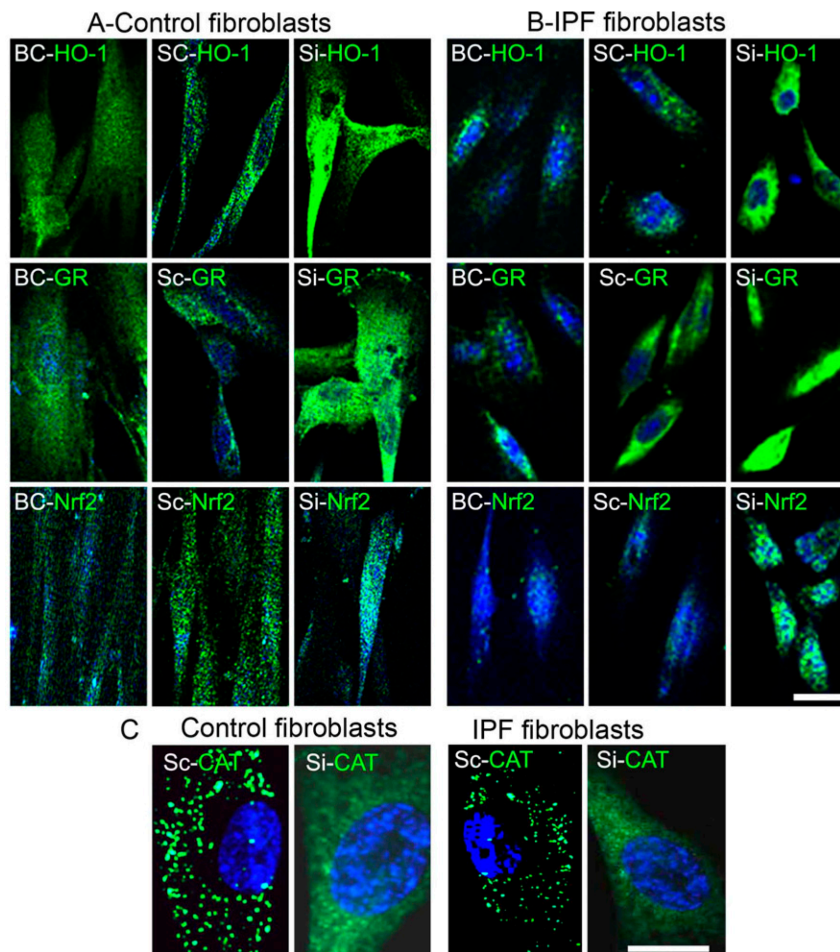

Fig. S4. Antioxidative response in *PEX13* siRNA treated fibroblasts. (*A* and *B*) Antioxidative response in *PEX13* knockdown control and IPF fibroblasts, depicted by immunofluorescence stainings for HO-1, glutathione reductase (GR) and Nrf2. (*C*) Mistargeting of peroxisomal matrix enzyme catalase into the cytoplasm in control and IPF fibroblast. (Scale bar: 10 μm in *A*–*C*.) BC: basal control, Sc: scrambled control, si: siRNA.

Fig. S5. TGF-β induction affects PEX13 in control and IPF fibroblasts. (*A*) IL-6 secretory levels measured by ELISA after treatments with concentrations of TGF-β1 (5 ng/mL) and LY364947 (5 μm) in control and IPF fibroblasts. C: control, T: TGF-β1, LY: LY364947, T+LY: TGF-β1+LY364947 (*B*) SBE dual-luciferase reporter gene assay along with Renilla control reporter. The activity of firefly luciferase was measured in cell lysates and normalized to the one of renilla luciferase. Data represent the results of at least three experiments performed in triplicates. (*C*) Total Smad3 staining of TGF-β1 treated control and IPF fibroblasts. (*D* and *E*) Depiction of *PEX13*, *TGF-β1*, and *IL-6* mRNA levels with RT-PCR, followed by quantification. Data represent ± SD of three independent experiments. *P* value, two-tailed Student *t* test. (Scale bar: 10 μm.)