Sestrin 2 Protein Regulates Platelet-derived Growth Factor Receptor β (Pdgfrβ) Expression by Modulating Proteasomal and Nrf2 Transcription Factor Functions*

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Background: Sestrin 2 is a redox-dependent repressor of Pdgfrβ signaling and thereby interferes with lung injury repair. Results: Sestrin 2 is a positive regulator of proteasomal function and activates transcription of Nrf2-regulated antioxidant genes. Conclusion: Sestrin 2 is a component of a novel Sestrin2/Pdgfrβ repressor pathway. Significance: The Sestrin2/Pdgfrβ repressor pathway is likely to play role in the pathogenesis of chronic obstructive pulmonary disease (COPD).

We recently identified the antioxidant protein Sestrin 2 (Sesn2) as a suppressor of platelet-derived growth factor receptor β (Pdgfrβ) signaling and Pdgfrβ signaling as an inducer of lung regeneration and injury repair. Here, we identified Sesn2 and the antioxidant gene inducer nuclear factor erythroid 2-related factor 2 (Nrf2) as positive regulators of proteasomal function. Inactivation of Sesn2 or Nrf2 induced reactive oxygen species-mediated proteasomal inhibition and Pdgfrβ accumulation. Using bacterial artificial chromosome (BAC) transgenic HeLa and mouse embryonic stem cells stably expressing enhanced green fluorescent protein-tagged Sesn2 at nearly endogenous levels, we also showed that Sesn2 physically interacts with 2-Cys peroxiredoxins and Nrf2 albeit under different reductive conditions. Overall, we characterized a novel, redox-sensitive Sesn2/Pdgfrβ suppressor pathway that negatively interferes with lung regeneration and is up-regulated in the emphysematous lungs of patients with chronic obstructive pulmonary disease (COPD).

Sestrin 2 belongs to a family of highly conserved, stress-inducible proteins implicated in the regulation of metabolic homeostasis. Mammalian cells express three sestrin isoforms: sestrin 1 (Sesn1); also known as PA26, sestrin 2 (Sesn2); also known as Hi95), and sestrin 3 (Sesn3) (1, 2); Sesn1 and Sesn2 are involved in the defense against reactive oxygen species (ROS). The antioxidant function of the sestrins was originally attributed to their sulfenic acid reductase activity required for regenerating over-oxidized 2-Cys-peroxiredoxins (Prxs) (3). Prxs are highly conserved and ubiquitously expressed antioxidant proteins that are inactivated by hyperoxidation of their peroxidatic cysteine, thus yielding sulfenic acid in the presence of high peroxide concentrations (4, 5). However, it was later shown that the primary Prx reactivating enzyme is sulfiredoxin, which unlike the typical cellular reductants such as glutathione or thioredoxin can reduce sulfenic acid to thiol (6, 7). Therefore, the ability of Sesn1 and -2 to catalyze sulfenic to sulfenic acid is still a matter of debate.

Antioxidant activities of Sesn1 and -2 were recently attributed to their activation of the Nrf2 (nuclear factor erythroid 2-related factor 2)-Keap1 (Kelch-like ECH-associated protein 1) pathway (7). Nrf2 is a global antioxidant gene inducer whose activity is tightly controlled by cytoplasmic association with its inhibitor, Keap1. Keap1 targets Nrf2 for polyubiquitination and degradation by the 26 S proteasome, thereby keeping its basal levels low. Oxidative stress disrupts the Nrf2-Keap1 protein complex, and Nrf2 translocates into the nucleus where it heterodimerizes with its small Maf binding partner to transactivate antioxidant gene expression by binding the antioxidant response elements typically present in promoter regions of catalytic subunit; nLAP, (N-terminal) localization and affinity purification tag; NAC, N-acetylcysteine; Nf-Y, nuclear transcription factor complex Y; Pdgfr, platelet-derived growth factor; Pdgfrβ, platelet-derived growth factor receptor β; ROS, reactive oxygen species; SV40, simian virus 40; Prxs, peroxiredoxins; IF, immunofluorescence; IP, immunoprecipitate; MLF, mouse lung fibroblast; CMH, 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrroliodine; Sod, superoxide dismutase; Ho1, hemoxygenase 1; Nrf2, nuclear factor erythroid 2-related factor 2; Keap1, Kelch-like ECH-associated protein 1.
antioxidant genes (8). Sesn2 interfered with this pathway by stimulating the autophagic degradation of Keap1 (7).

Recently, we and others identified Sesn2 as a repressor of Pdgfrβ signaling (9, 10) and Pdgfrβ signaling as an inducer of lung tissue regeneration and injury repair (9). In mice, mutational inactivation of Sesn2 prevents development of cigarette smoke-induced pulmonary emphysema by up-regulating Pdgfrβ-controlled alveolar maintenance programs (9). Although we showed that this up-regulation is mediated by ROS (9), the molecular mechanism(s) of this regulation was not determined.

The proteasome is the major site for removal of short-lived, unfolded, and damaged proteins. With a molecular weight of 150 MDa, the 26 S proteasome is one of the largest known catalytic complexes inside all eukaryotic cells. Composed of the catalytically active 20 S core and 19 S regulatory particles, the 26 S proteasome governs ubiquitin- and ATP-dependent protein degradation (11, 12). Three subunits of the 19 S proteasome (Rpt5, Rpn10, and Rpn13) bind polyubiquitinated substrates that earmark them for degradation (13–16). The catalytic centers are located inside the 20 S proteasome where three of its seven β-subunits show proteolytic activity, namely β1, β2, and β5, exhibiting caspase-, trypsin-, and chymotrypsin-like activities, respectively (17, 18). Oxidative stress leads to proteasome disassembly into its 19 S and 20 S subunits and to the accumulation of polyubiquitinated proteins (19).

Here we identify Sesn2 as a positive regulator of proteasomal function and the proteasome as a major component of a redox-sensitive signal transduction pathway controlling Pdgfrβ signaling. Within this pathway, we show that Nrf2 provides a crucial redox switch that, when activated by Sesn2, down-regulates Pdgfrβ by enhancing its proteasomal degradation. Finally, we show that this Sesn2/Pdgfrβ suppressor pathway is up-regulated in the emphysematous lungs of individuals with advanced chronic obstructive pulmonary disease (COPD), where it likely interferes with lung regeneration.

EXPERIMENTAL PROCEDURES

Reagents, Antibodies—Rabbit polyclonal antibodies against Sesn2 and Gclc were purchased from Proteintech Group (Manchester, UK). Rabbit polyclonal antibodies against Pdgfrβ (958; used for immunofluorescence (IF) and immunoprecipitation (IP)), PSMD1 (H-300), 20 S proteasome (9739; used for immunoprecipitation (IP)), PSMβ6; NM_002798) was cloned into the BamHI site of pEGFP-N1. Ubiquitin-like (UBL) domain of hHR23B was cloned into the BamHI site of pGEX-4T1. The large T-antigen expression plasmid pSG5 was obtained from Addgene (#9053) (Cambridge, UK) (20). The doxycycline-inducible pRRL-pPT-SV40 large T antigen lentivirus used for the conditional immortalization of ES cell derived fibroblasts was a gift from Oezdem Demirel (Goethe University Frankfurt).

Cell Cultures and Cell Transductions—MLFs and HeLa, MRC5, and HEK293T cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies) supplemented with 10% (v/v) fetal calf serum (Life Technologies), 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies). HTB87-tagged hRpn11 expressing HEK293T cells (provided by L. Huang, University of California, Irvine) were used for affinity purification of human proteasomes. nLAP-Sesn2, Prx1, and Prx2 HeLa cell lines obtained from the NCI’s Cell Line Sharing Program were used for transduction experiments. Mouse monoclonal antibodies against DJ1, GAPDH, α-tubulin, hemoxygenase 1 (Hoe1; P109), Keap1 (D6B12), MAP1LC3B (D11), and Pdgfrβ (28E1; used for Western blot) and mouse monoclonal antibody against p53 (1C12) were from Cell Signaling (Danvers, MA). Mouse monoclonal antibody against the His tag (C terminus) and β-actin were from Life Technologies and Sigma, respectively. Rabbit polyclonal antibodies against peroxiredoxin 1 (Prx1) and Prx-SO3 used for Western blots and IF were purchased from Abfrontier (Hamburg, Germany) and Abcam (Cambridge, UK), respectively. Mouse monoclonal antibodies against Prx2 were from Abfrontier (Hamburg, Germany). Cy3-conjugated goat anti-rabbit and Alexa Fluor 488 antibodies were from Dianova (Hamburg, Germany) and Molecular Probes (Darmstadt, Germany), respectively. Phalloidin–FITC was purchased from Molecular Probes and Cy3-streptavidin was from BioLegends (San Diego, CA). Secondary goat anti-mouse and goat anti-rabbit antibodies coupled to horseradish peroxidase (HRP) were from Santa Cruz Biotechnology and Sigma, respectively. Cytokines: hPDGF-BB (Sigma). Reagents Tempol (Tocris, Bristol, UK), H2O2, N-acetylcysteine (NAC), MG132, and chloroquine were all from Sigma.
Sesn2 and Pdgfrβ Expression

pBabe-Puro retrovirus and selecting in 2 μg/ml puromycin (Life Technologies), shRNA knockdown of Sesn2 in MRC5 cells or Nrf2 in HeLa cells was performed by using the Mission Lentiviral shRNA system (Sigma).

Cell Exposure to Growth Factors, Translational Inhibitors, Oxidants, and Antioxidants—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 (9). For Pdgfrβ half-life measurements, cells were exposed to 10 μg/ml cycloheximide (CHX with and without 5 mM Tempol or 10 μM MG132 or 100 μM chloroquine). Finally, for ROS level manipulations, cells were incubated with 125 μM H2O2, 6 h unless otherwise stated or with 5 mM Tempol or NAC for 7 and 8 h, respectively.

Glutathione S-Transferase (GST) Protein Expression and Pulldown Assays—GST fusion proteins expressed in BL21 Escherichia coli strain were purified and coupled to glutathione-Sepharose beads (GE Healthcare) as previously described (25). For GST pulldown assays, MLFs and HEK293T cells were lysed on ice for 30 min in 50 mM Tris, pH 7.4, 0.15M NaCl, 2 mM (25). For GST pulldown, MLFs and HEK293T cells were lysed on ice for 30 min in 50 mM Tris, pH 7.4, 0.15M NaCl, 2 mM EDTA, 1% (v/v) Nonidet P-40 lysis buffer supplemented with Protease Inhibitor Mixture (Roche Applied Science). Cell lysates were incubated with either GST or GST-tagged proteins immobilized on glutathione-Sepharose beads overnight at 4 °C. The beads were washed 3 times with 1 ml of lysis buffer. The samples were resuspended in loading buffer containing 10% (v/v) β-mercaptoethanol, boiled for 5 min at 95 °C and separated by SDS-PAGE.

Immunofluorescence—Cells cultured on coverslips were fixed with 4% paraformaldehyde (Carl Roth), blocked, and permeabilized with 1% (v/v) BSA in PBS containing 0.5% (v/v) Triton X-100 (Carl Roth) or with 50 μg/ml digitonin (in DMSO; Carl Roth, Karlsruhe, Germany) for 15 min at room temperature. 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) supplemented with 1,4-diazadiyclo(2,2,2)-octane (50 mg/ml; Fluka, Taufkirchen, Germany). 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.

Co-immunoprecipitation—Cells were lysed for 30 min on ice in IP lysis buffer (10 mM Tris-HCl, pH 8, 150 mM NaCl, 5 mM EDTA, pH 8, 0.5% (v/v) Triton X-100, 60 mM N-octylglycyr pantoside) supplemented with Roche Applied Science protease inhibitor mixture. For HTBH-hRpn11 IPs, lysates from HTBH-hRpn11 HEK293 cells were incubated with streptavidin agarose beads (Chromotek, Martinsried, Germany) and subsequently washed in IP lysis buffer. IPs were analyzed by SDS-PAGE and Western blotting.

Proteasome and Nrf2 Activity Assays—For measuring proteasome activity, cells were lysed on ice in proteasome activity lysis buffer (100 mM NaCl, 50 mM Na2PO4, 10% (v/v) glycerol, 5 mM MgCl2, 0.5% (v/v) Nonidet P-40) supplemented with Protease Inhibitor Mixture (Roche Applied Science), 1 mM sodium orthovanadate, and 5 mM ATP (Sigma). The lysates were homogenized on ice by using a 23-gauge needle and cleared by centrifugation at 13,000 rpm for 15 min. Chymotrypsin-like activity was measured in duplicate using 50 μg of proteins and 100 μM succinyl-LLVY-aminomethylcoumarin (Enzo Life Sciences) fluorogenic substrate per reaction as previously described (14). Assays were carried out at 37 °C for 30 min, and activities were measured using a Viktor2 (PerkinElmer Life Sciences) spectrophotometer with 380-nm excitation and 460-nm emission filters. For measuring proteasome activity in human lung samples, proteasome was purified from the tissue lysate by using the GST-tagged ubiquitin-like (UBL) domain of hHR23B. GST pulldown assays were performed overnight at 4 °C before proteasome activity measurements.

Nrf2 transactivation activity was estimated by using the Cignal Lenti antioxidant response element reporter (luc) assay (CLS-2020L) (Qiagen, Leipzig, Germany) 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, Bad Wildbad, Germany).

ROS Measurements—Superoxide release from MLFs was measured by EPR as described (26). Briefly, EPR measurements were performed at −170 °C using an EMXelectron Electron Spin Resonance (ESR) spectrometer (Bruker, Karlsruhe, Germany) and 1-hydroxy-3-methoxy carbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) (Noxygen, Elzach, Germany) as the spin probe for detecting intra- and extracellular superoxide production. Because CMH reacts with superoxide and peroxynitrite, parallel samples containing either CMH alone or CMH and superoxide dismutase (SOD) conjugated to polyethylene glycol (PEG-SOD) were measured. This enabled the assessment of the superoxide signal as part of the total CMH signal. Thus, duplicate samples of 2 × 105 cells were incubated with 15 units/ml PEG-SOD (Sigma) for 2 h at 37 °C followed by the addition of CMH (500 μM) ± PEG-SOD. After incubating for another 20 min, the samples were shock-frozen and stored in liquid nitrogen. Spectrometry was performed on frozen samples using a g-factor of 2.0063, a center field of 3349.95G, a microwave power of 200 milliwatt, a sweep time of 20 s, and a sweep number of 5.

Nucleic Acids and Protein Analyses—Total RNA was isolated by using the TriReagent kit (Sigma) 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 of reverse transcription buffer containing 2 μM random primers (New England Biolabs, Frankfurt am Main, Germany) and 200 units of RevertAid H Minus Reverse Transcriptase (Thermo Fisher Scientific). Real time PCRs (Opticon 2 quantitative PCR machine, MJ Research, Ramsey, MN) were performed in duplicate in 25 μl of quantitative PCR SYBR Green Mix (Thermo Fisher Scientific) containing 5 μl of 5-fold diluted reverse transcription product using an annealing temperature of 60 °C. RNA polymerase II was used for normalization. Primer sequences are available on request. Cell lysates and Western blotting for protein quantification were performed as previously described (9, 27). For protein
analysis in PDGF-BB stimulation experiments, lysis buffers were supplemented with 1 mM each of sodium fluoride and sodium orthovanadate.

**Patient Characteristics**—Human lung tissues were obtained from COPD transplant patients (GOLD stage IV) and healthy donor controls (Table 1). The studies were approved by the Ethics Committee of the Justus-Liebig-University School of Medicine (AZ 31/93), Giessen, Germany.

**Statistics**—Comparisons between more than two groups were performed using analysis of variance (ANOVA) with the Bonferroni post-hoc test. For comparisons of two groups, Student’s two-tailed t test was used. p values below 0.05 were considered significant.

**RESULTS**

*Sesn2-depleted Mouse Lung Fibroblasts Accumulate Proteasomal Target Proteins*—We and others showed that ROS and Pdgfrβ accumulate in MLFs from Sesn2 knock out mice (Sesn2 KO MLFs) (3, 9, 24). To test whether Pdgfrβ accumulation results from its decreased turnover, we estimated Pdgfrβ levels in MLFs at various time intervals after exposing the MLFs to the protein synthesis inhibitor CHX. Fig. 1A shows that Pdgfrβ levels in WT and Sesn2 KO MLF exposed to CHX (10 μg/ml) for the indicated time intervals. Left panel, representative Western blot. Right panel, nonlinear regression analysis (one-phase exponential decay) of relative Pdgfrβ levels quantified by densitometry. B, Pdgfrβ levels in WT and Sesn2 KO MLF exposed to CHX and Tempol. Left panel, representative Western blot. Right panel, nonlinear regression analysis of relative Pdgfrβ levels quantified by densitometry. C, Pdgfrβ levels in WT and Sesn2 KO MLF exposed CHX ± MG132. Left panel, Western blot where p53 served as a positive control for proteasomal inhibition. Right panel, nonlinear regression analysis of relative Pdgfrβ levels quantified by densitometry. D, Pdgfrβ levels in WT and Sesn2 KO MLF exposed to CHX ± chloroquine. Left panel, Western blot where LCB served as a positive control for lysosomal inhibition. Right panel, nonlinear regression analysis of relative Pdgfrβ levels quantified by densitometry.

![Graph A: CHX / h](image)

**FIGURE 1.** Pdgfrβ half-life in Sesn2 KO MLF. A, Pdgfrβ levels in WT and Sesn2 KO MLF exposed to CHX (10 μg/ml) for the indicated time intervals. Left panel, representative Western blot. Right panel, nonlinear regression analysis (one-phase exponential decay) of relative Pdgfrβ levels quantified by densitometry. B, Pdgfrβ levels in WT and Sesn2 KO MLF exposed to CHX and Tempol. Left panel, representative Western blot. Right panel, nonlinear regression analysis of relative Pdgfrβ levels quantified by densitometry. C, Pdgfrβ levels in WT and Sesn2 KO MLF exposed CHX ± MG132. Left panel, Western blot where p53 served as a positive control for proteasomal inhibition. Right panel, nonlinear regression analysis of relative Pdgfrβ levels quantified by densitometry. D, Pdgfrβ levels in WT and Sesn2 KO MLF exposed to CHX ± chloroquine. Left panel, Western blot where LCB served as a positive control for lysosomal inhibition. Right panel, nonlinear regression analysis of relative Pdgfrβ levels quantified by densitometry.
levels declined about five times slower in Sesn2 KO MLFs than in WT MLFs. However, the extended Pdgfrβ half-life in Sesn2 KO MLFs could be readily reversed to normal by the ROS scavenger Tempol (Fig. 1B), indicating that the Pdgfrβ half-life prolongation is ROS-dependent.

Earlier studies showed that Pdgfrβ can undergo both lysosomal and proteasomal degradation (28, 29). To distinguish between these possibilities in our system, we treated MLFs exposed to CHX with chloroquine or MG132 to selectively inhibit lysosomal and proteasomal degradation, respectively. Fig. 1, C and D, show that MG132, but not chloroquine, prolonged the Pdgfrβ half-life in WT MLFs, indicating that Pdgfrβ degradation is primarily proteasomal.

Because excessive ROS negatively interfere with proteasomal function (19), we assumed that Sesn2 KO MLFs exhibit defects in the proteasomal protein degradation system. To test this, we first estimated c-myc and p53 protein levels in WT and Sesn2 KO MLFs by Western blotting. Because c-myc and p53 are exclusively degraded by the proteasome (30, 31), their steady state levels indirectly reflect proteasome activity. As shown in Fig. 2A, both c-myc and p53 accumulated in Sesn2 KO MLFs, suggesting reduced proteasomal activity. This effect was independent of Pdgfrβ signaling, as c-myc and p53 levels remained unaffected by PDGF-BB ligand stimulation (Fig. 2A). Consistent with the reduced proteasomal activity, Sesn2 KO MLFs accumulated polyubiquitinated proteins including Pdgfrβ (Fig. 2, B and C).

Next, we tested whether decreased receptor endocytosis might contribute to the elevated Pdgfrβ levels in Sesn2 KO MLFs by monitoring subcellular Pdgfrβ localization at various time intervals after PDGF-BB stimulation. As shown in Fig. 2D, the kinetics of Pdgfrβ internalization after ligand stimulation were similar in WT and Sesn2 KO MLFs, suggesting that its endocytosis is not affected by the Sesn2 mutation. Taken together, the results suggest that Pdgfrβ accumulates in Sesn2 KO MLFs as a result of a ROS-induced proteasome dysfunction.

Sesn2 Binds the Proteasome and Modulates Its Function—To confirm that proteasomal activity is reduced in the Sesn2-de-
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To test whether Sesn2 binds to the proteasome, we used HEK293T cells stably expressing a His-tagged, biotinylatable Rpn11 (HTBH-hRpn11) subunit of the 19 S proteasome (32) and pulled down Rpn11 by using streptavidin-coupled agarose beads. Western blot analysis identified Sesn2 in the pulldown in addition to the 19 S PSMD1 and 20 S α and β1 subunits, indicating that Sesn2 physically associates with the native proteasome (Fig. 4A). This association was supported further by IF stainings, which are suggestive of partial cytoplasmic and nuclear Sesn2/proteasome co-localization (Fig. 4B).

To investigate which proteasomal subunits bind to Sesn2, we performed GST pulldown assays from WT MLFs using a purified Sesn2-GST fusion protein bound to glutathione-Sepharose beads. As shown in Fig. 4C, Sesn2 bound to the α and catalytically active β1 and β5 subunits but not to the β2 and PSMD1 subunits of the 20- and 19 S proteasome, respectively. However, when testing β1 subunit binding, we observed a β1 band shift in the Sesn2-GST pulldown when compared with input (Fig. 4C). To verify that β1 indeed interacted with Sesn2, we performed additional GST pulldown assays from HEK293T cells transiently transfected with a GFP-tagged human β1 subunit expression plasmid. Fig. 4D shows that Sesn2-GST readily pulled down β1-GFP, but not GFP, confirming that Sesn2 physically interacts with the β1 subunit of the 20 S proteasome. Overall, these results suggest that Sesn2 may directly interfere with the proteolytic functions of the proteasome.

Sesn2 Interacts with 2-Cys Peroxiredoxins in a Redox-dependent Manner—It was suggested that Sesn2 reduces oxidative stress by binding to and reducing over-oxidized 2-Cys peroxiredoxins, in particular Prx1 and Prx2 (3). Because the Sesn2/Prx interactions were thus far detected only in exogenous protein overexpression experiments (3), we asked whether the interactions could also be detected under physiological conditions. For this, we used a HeLa cell line from the Bacterial Artificial Chromosome (BAC) Interactomics Resource stably expressing a BAC transgene in which the N terminus of SESN2 containing a localization and affinity purification (LAP) tag (nLAP-SESN2) (33). As nLAP-SESN2 is expressed and regulated like endogenous SESN2, these cells were amenable to generic tag-based proteomics under nearly physiological conditions.

We immunoprecipitated nLAP-SESN2 from the nLAP-Sesn2 HeLa cells using GFP-TrapA beads and probed the IPs with anti-Prx1, Prx2, and over-oxidized Prx (Prx-SO3) antibodies. Fig. 5A shows that PRX1 and PRX-SO3, but not PRX2, co-immunoprecipitated with nLAP-SESN2. Consistent with this finding, IF stainings of nLAP-Prx1 and nLAP-Prx2 HeLa cells obtained from the same BAC Interactomics resource showed cytosolic SESN2-PRX1 but not SESN2-PRX2 co-localization (Fig. 5B). Similarly, in nLAP-Sesn2 HeLa cells, SESN2 co-localized with endogenous PRX1 and PRX1-SO3 (Fig. 5C). However, this Sesn2/Prx interaction was inhibited by hydrogen peroxide (H$_2$O$_2$) (Fig. 5, A and C), which also induced translocation of SESN2 from the cytoplasm into the nucleus (Fig. 5C), implying that Sesn2 is not a Prx reductase.

We repeated these experiments using conditionally immortalized murine fibroblasts derived from an embryonic stem cell line expressing nLAP-Sesn2 from its endogenous locus (nLAP-
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Sesn2-ESC) (21). Unlike the HeLa cells from the BAC Interactomics resource, the nLAP-Sesn2-ESCs were obtained by inserting the nLAP tag into a multipurpose Sesn2 gene trap allele by recombinase-mediated cassette exchange (21). Similar to the HeLa cells, the nLAP-Sesn2 of the conditionally immortalized fibroblasts (nLAP-Sesn2-MF) interacted with Prx1 and Prx-S03 in a redox-dependent manner (Fig. 5D). However, although nLAP-Sesn2-MFs did not express higher levels of Prx2 than the corresponding HeLa cells (Fig. 5E), Sesn2 interacted with Prx2 (Fig. 5D), suggesting that the basal Sesn2/Prx2 interaction is likely cell type-specific.

Nrf2 Activity Is Reduced by Sesn2 Inactivation—A recent study showed that Sesn2 activates the antioxidant transcription factor Nrf2 by binding and inactivating its inhibitor Keap1 (7). To test whether Nrf2 activity is compromised in Sesn2-depleted cells, we first estimated Nrf2 and Keap1 expression in WT and Sesn2 KO MLFs by quantitative real-time-PCR and Western blotting. As shown in Fig. 6A, neither Nrf2 nor Keap1 expression was affected by the loss of Sesn2. However, when the MLFs were subjected to antioxidant response elements (ARE)-luciferase reporter assay, Sesn2 KO MLFs developed significantly less luciferase activity than the WT MLFs under both basal and oxidative stress conditions (Fig. 6B) and showed reduced expression of hemoxigenase 1 (Ho1), an endogenous Nrf2 target gene (Fig. 6C). These results suggest that ROS accumulate in Sesn2-deficient MLFs as a result of reduced Nrf2 activity and, consequently, inhibit proteasomal function.

Sesn2 Directly Interacts with Nrf2 in a Redox-dependent Manner—As noted above, Sesn2 activates Nrf2 by directly interacting with its inhibitor Keap1. However, this interaction was described in HEK293T cells overexpressing Sesn2 and Keap1 cDNAs (7). To test whether the Sesn2/Keap1 interaction can be replicated in cells expressing nearly normal levels of Sesn2, we immunoprecipitated nLAP-SESN2 from HeLa cells.

FIGURE 4. Sesn2 associates with the proteasome. A, native proteasome streptavidin pulldown assays from HTBH-hRpn11 HEK293 cells. Western blots were probed with antibodies against His, Sesn2, PSMD1, and 20S β1 and α subunits. B, Rpn11 colocalization with endogenous Sesn2 in HTBH-hRpn11 HEK293 cells visualized by indirect immunofluorescence. Scale bar: 20 μm. C, Sesn2-GST pulldown assays from MLF lysates probed with antibodies against proteasomal subunits (top panel). The purified, GST, and full-length Sesn2-GST fusion proteins used for the pulldown assays are marked by asterisks on the bottom panel. D, Sesn2-GST pulldown assays from HEK293T cells expressing exogenous β1-GFP probed with GFP antibodies.
either treated or untreated with H2O2 and probed the IPs with anti-Keap1 and Nrf2 antibodies. Fig. 6D shows that NRF2, but not KEAP1, co-immunoprecipitated with nLAP-SESN2, especially under oxidative stress conditions. Likewise, as revealed by IF staining, H2O2-induced SESN2-NRF2 co-localization and cytoplasmic nuclear translocation (Fig. 6E), suggesting that SESN2 may act as a transcriptional co-activator of Nrf2 target genes independently of Keap1.

As there is some concern in the literature whether the size of the mRNA predicted Nrf2 translation product (11015 kDa) represents bona fide Nrf2 protein (34), we subsequently validated the anti-Nrf2 antibody employed in these experiments using immortalized MLFs from Nrf2 knockout mice (23) and HeLa cells transduced with anti-Nrf2 shRNA. As shown Fig. 6F, the 65-kDa band detected by the R&D monoclonal antibody was missing in Nrf2 KO MLFs, and its intensity was reduced in NRF2 KD HeLa cells, confirming that this band represents monomeric Nrf2.

Nrf2 Inactivation Replicates the Pdgfrβ Induction Phenotype of Sesn2 KO MLFs—To test whether Nrf2 inactivation replicates the Pdgfrβ induction phenotype of Sesn2 KO MLFs, we estimated ROS levels and Pdgfrβ expression in MLFs derived from Nrf2 KO mice. As shown in Fig. 6G, Nrf2 KO MLFs accumulated ROS and expressed significantly higher Pdgfrβ levels than the corresponding WT MLFs (Fig. 6H and I), resulting in Pdgfrβ signal amplification in response to ligand stimulation (Fig. 6J and K). This suggests that Sesn2, Nrf2, and the proteasome are all part of a redox-sensitive Pdgfrβ-suppressing pathway whose inactivation up-regulates Pdgfrβ. However, unlike previously reported Pdgfrβ activation by ROS-mediated inhibition of dedicated protein-tyrosine phosphatases (35, 36), the elevated phosphorylated Pdgfrβ (p-Pdgfrβ) levels in Nrf2 KO MLFs were largely attributable to Pdgfrβ accumulation rather than enhanced phosphorylation. Thus, the Pdgfrβ/Gapdh ratio but not the p-Pdgfrβ/Pdgfrβ ratio was elevated in Nrf2 KO MLFs (Fig. 6K).

Sesn2 Inactivation Up-regulates Nf-γ—We showed previously that the up-regulation of Pdgfrβ in the lungs of Sesn2 KO mice occurred at both the mRNA and protein level (9). To test the possibility that mRNA induction is mediated by a Pdgfrβ transcription factor whose proteasomal degradation is delayed in Sesn2-depleted cells, we focused on the Nf-γ subunit of the Nf-γ transcription factor complex, which controls basal Pdgfrβ gene transcription (37) and is degraded exclusively by the proteasome (38). Fig. 7A and B, shows that Nf-γ and Pdgfrβ levels...
were significantly increased in Sesn2 KO MLFs. Most importantly, Nf-ya was also up-regulated in Nrf2 KO MLFs (Fig. 7, C and D), suggesting that Nf-ya and Nrf2 operate within the same Sesn2/Pdgfrβ suppressor pathway (Fig. 7E).

Nrf2 Is Up-regulated in the Lungs of Individuals with COPD—Recently, we showed that SESN2 is up-regulated and PDGFRβ expression repressed in the lungs of individuals with advanced COPD (9), suggesting that the entire Sesn2/Pdgfrβ suppressor
pathway (Fig. 7E) might be up-regulated in COPD lungs. Indeed, in contrast to previous reporting (39), we found NRF2 significantly up-regulated and KEAP1 repressed in COPD lungs when compared with healthy donor lungs (Fig. 8, A and B; see Table 1 for patient characteristics). Moreover, consistent with enhanced antioxidant defense, Nrf2 target genes such as GCLC, HO1, and PRX2 were also up-regulated in the COPD lungs, whereas the expression of the positive Nrf2 regulator DJ1 was not significantly different from normal lungs (Fig. 8, A and B). Finally, consistent with recent reporting (40) and in contrast to a previous study (53), proteasomal activity and subunit expression were not decreased in COPD lungs (Fig. 8, C–F).

**DISCUSSION**

We recently reported that Sesn2 interferes with the development of pulmonary emphysema by suppressing Pdgfrβ-regulated alveolar maintenance programs (9). Here, we identified the transcription factor Nrf2 and the 26 S proteasome as key components of a Sesn2/Pdgfrβ suppressor pathway and found that this pathway is highly up-regulated in lungs of individuals with advanced COPD.

In lung fibroblasts of mouse and human origin, inactivation of Sesn2 decreased proteasomal activity by inducing ROS formation via inhibition of Nrf2-controlled antioxidant gene expression. Decreased proteasomal degradation of Pdgfrβ and of its transcriptional activator Nrf-ya resulted in Pdgfrβ accumulation and signal amplification in response to ligand stimulation (9).

**In vitro and in vivo** protein binding studies revealed that Sesn2 physically interacts with several 20 S proteasomal subunits, including the catalytically active β1 and β5 subunits. Other oxidoreductases were also reported to associate with the 26 S proteasome. For example, thioredoxin-like 1 (Txn1) or thioredoxin-related protein of 32 kDa (TRP32) interacts with the 20 S regulatory subunit near the substrate binding site and was suggested to play a role in proteasome assembly (41–43). Likewise, the human flavin-dependent NADPH:quinone reductase (NQO1) interacts with the 20 S proteasome and protects transcription factors p53 and p73 from proteasomal degradation (31). Accordingly, Sesn2 could directly stimulate proteasomal activity by serving as a maturation factor that recruits proteasomal subunits for assembly. To our knowledge, the only other known interaction partner of the catalytically active proteasomal β subunits is proteasemembrin (Ump1), which is involved in 20 S proteasome assembly (44, 45). In yeast, inactivation of the Ump1 homolog causes disordered proteasome assembly with abnormal subunit stoichiometry, incomplete propeptide processing, and reduced proteolytic activity (44). Similarly, the inactivation of Sesn2 might cause proteasome dysfunction by preventing its orderly assembly. Alternatively, Sesn2 interaction with 20 S proteasome might affect the structure of 20 S active sites and thus have an impact on proteasomal activity. Although this cannot be excluded by the present experiments, the recovery of proteasomal function in Sesn2-depleted cells by treatment with antioxidants such as NAC and Tempol suggests that Sesn2 also interferes indirectly with proteasomal function via ROS.

To scrutinize possible mechanisms of ROS accumulation in Sesn2-depleted cells, we used HeLa cells and mouse ESC-derived fibroblasts expressing EGFP-tagged Sesn2 from its endogenous chromosomal location, which enables generic tag-based proteomics under nearly physiological conditions. Although we found that Sesn2 physically interacts with peroxiredoxins, including over-oxidized peroxiredoxins, these interactions were effectively blocked by ROS. Thus, although the biological significance of the Sesn2/Prx interactions remains to be established, their inhibition by oxidative stress does not support the sulfenic acid reductase activity previously attributed to sestrins (3).

Figure 6. **Nrf2 is a component of the Sesn2/Pdgfrβ suppressor pathway.** A, Nrf2 and Keap1 expression in WT and Sesn2 KO MLFs. Upper panel, Nrf2 protein expression in response to PDGF-BB. Lower left panel, relative Nrf2 mRNA levels in WT and Sesn2 KO MLFs quantified by quantitative real-time PCR in three independent experiments. ns, not significant. Lower right panel, Keap1 protein expression in WT and Sesn2 KO MLFs. B, suppression of Nrf2 activity in Sesn2 KO MLFs. MLFs were transiently transfected with the pCignal Lenti-TRE-Reporter. After 48 h aliquots were treated with 125 μM H2O2 for 6 h, and luminescence was measured using a Mithras LB 940 plate reader. ALU, relative light units. C, Ho1 expression in WT and Sesn2 KO MLFs. Left panel, Western blot. Right panel, densitometric quantification. D, nLAP-Sesn2 IPs from HeLa cells probed with antibodies against Sesn2, Nrf2, and Keap1. Where indicated, cells were exposed for 6 h to H2O2 before the IPs. E, subcellular localization of Sesn2 and Nrf2 in nLAP-Sesn2 HeLa cells visualized by EGFP autofluorescence and indirect immunofluorescence staining before and after treatment with 125 μM H2O2. Scale bar: 20 μm. F, Western blot probed with monoclonal anti-Nrf2 antibody (R&D) showing absent and reduced Nrf2 expression in Nrf2-KO MLFs and Nrf2 knockdown HeLa cells, respectively. G, increased superoxide anion levels in Nrf2 KO MLFs measured by EPR spectroscopy. Results are represented as the means ± S.E. of six independent measurements. H, representative Western blot showing expression levels of Pdgfrβ, Gclc, and Sesn2 in WT and Nrf2 KO MLFs. I, densitometric quantification of Pdgfrβ (left panel) and Gclc (middle panel) and Sesn2 (right panel) expression in WT and Nrf2 KO MLFs. J, representative Western blot showing phosphorylated Pdgfrβ (pPdgfrβ) in WT and Nrf2 KO MLFs after treatment with 25 ng/ml recombinant human PDGF-BB. K, densitometric quantification of Pdgfrβ relative to GAPDH (left panel) and total Pdgfrβ (right panel). All results are represented as the means ± S.D. of three independent experiments. **, p < 0.01; ***, p < 0.001.
FIGURE 7. Nf-ya is a component of the Sesn2/Pdgfrβ suppressor pathway. A, expression of Nf-ya and Pdgfrβ in WT and Sesn2 KO MLFs. Left panel, representative Western blot. Right panel, densitometric quantification. B, Nf-ya subcellular localization in WT and Sesn2 KO MLFs visualized by immunofluorescence staining. Actin filaments were stained with fluorochrome-conjugated phalloidin. Note the nuclear accumulation of Nf-ya in Sesn2 KO MLFs. Scale bar: 20 μm. C and D, expression of Nf-ya in WT and Nrf2 KO MLFs. Shown is a representative Western blot (C) and densitometric quantification (D). All results are represented as the means ± S.D. of three independent experiments. *p < 0.05, ***p < 0.001. E, schematic illustration of the Sesn2/Pdgfrβ suppressor pathway. Left panel, up-regulation of Sesn2 activates Nrf2 and the proteasome by direct binding and by suppressing ROS. The increased proteosomal degradation of Pdgfrβ and its transcriptional inducer Nf-ya reduces Pdgfrβ levels and Pdgfrβ signaling in response to ligand stimulation. Right panel, Sesn2 inactivation inhibits Nrf2 and proteasome functions resulting in Pdgfrβ accumulation and signal activation in response to ligand stimulation.
FIGURE 8. Induction of the Sesn2/Pdgfrβ suppressor pathway in lungs of individuals with late stage COPD. A, representative Western blots showing the expression of NRF2 (65 kDa), KEAP1, DJ1, PRX2, GCLC, and HO1 in lungs from healthy human donor and of individuals with late-stage COPD. B, densitometric quantification of protein levels: healthy donor (n = 16); COPD (n = 20). ns, not significant. C, CLA in lungs of healthy donors and individuals with advanced COPD: healthy donor (n = 8); COPD (n = 10). D, Western blot showing proteasomal subunit expression in lungs of healthy donors and individuals with advanced COPD. E, densitometric quantification of proteasomal subunit expression in lungs of donors and individuals with COPD: healthy donor (n = 8); COPD (n = 10). Each entry corresponds to one individual (for patient characteristics). Mean values ± S.E. are indicated by straight lines. **, p < 0.01; ***, p < 0.001.
in COPD lungs is still a matter of debate. Although induction of antioxidant genes, including Nrf2, by chronic exposure to cigarette smoke was clearly documented in both airway epithelial cells and lung tissue (46 – 49), the picture is less clear in lungs of patients with COPD. Several genome-wide expression studies showed either up-regulation or no change in the expression of some (but not all) Nrf2-related antioxidant genes in COPD lungs (46, 50, 51), whereas other studies reported a decline in Nrf2 activity correlating with the severity of the disease (39). Our data showing that NRF2 is up-regulated in the lungs of individuals undergoing transplantation for late-stage COPD suggest that pharmacological NRF2 activators recently proposed for COPD treatment (52) should be used with caution.

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REFERENCES
1. Budanov, A. V., Shoshani, T., Faerman, A., Zelin, E., Kamer, I., Kalinski, H., Gorodin, S., Fishman, A., Chajut, A., Einat, P., Skaliter, R., Gudkov, A. V., Chumakov, P. M., and Feinstein, E. (2002) Identification of a novel stress-responsive gene H95 involved in regulation of cell viability. *Oncogene* 21, 6017–6031
2. Velasco-Miguel, S., Buckbinder, L., Jean, P., Gelbert, L., Talbott, R., Laidlaw, J., Sezinger, B., and Kley, N. (1999) PA26, a novel target of the p53 tumor suppressor and member of the GADD family of DNA damage and growth arrest inducible genes. *Oncogene* 18, 127–137
3. Budanov, A. V., Sablina, A. A., Feinstein, E., Koonin, E. V., and Chumakov, P. M. (2004) Regeneration of peroxiredoxins by p53-regulated sestrins, homologs of bacterial AhpD. *Science* 304, 596–600
4. Rhee, S. G., Chae, H. Z., and Kim, K. (2005) Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling. *Free Radic. Biol. Med.* 38, 1543–1552
5. Nystrom, T., Yang, J., and Molin, M. (2012) Peroxiredoxins, gerontogenes linking aging to genome instability and cancer. *Genes Dev.* 26, 2001–2008
6. Woo, H. A., Bae, S. H., Park, S., and Rhee, S. G. (2009) Sestrin 2 is not a reductase for cysteine sulfinic acid of peroxiredoxins. *Antioxid. Redox Signal.* 11, 739–745
7. Bae, S. H., Sung, S. H., Oh, S. Y., Lim, J. M., Lee, S. K., Park, Y. N., Lee, H. E., Kang, D., and Rhee, S. G. (2013) Sestrins activate Nrf2 by promoting p62-dependent autophagic degradation of Keap1 and prevent oxidative liver damage. *Cell Metab.* 17, 73–84
8. Kessler, T. W., Wakabayashi, N., and Biswal, S. (2007) Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu. Rev. Pharmacol. Toxicol.* 47, 89–116
9. Heidler, J., Fysikopoulos, A., Wempe, F., Semitz, M., Bangsow, T., Tomasovic, A., Veit, F., Scheibe, S., Pitchl, A., Weisel, F., Lloyd, K. C., Jaksh, P., Klepetko, W., Weissmann, N., and von Melchner, H. (2013) Sestrin-2, a repressor of PDGFRβ signalling, promotes cigarette-smoke-induced pulmonary emphysema in mice and is up-regulated in individuals with COPD IV, global initiative for chronic obstructive lung disease (GOLD) stage IV; FEV1, forced expiratory volume per second; FVC, forced vital capacity.

### Table 1

Clinical characteristics of normal donors and COPD patients

| No. | Patient | Age | Sex | Pack/year | Diagnosis | FEV1/FVC | FEV1(l) | FEV1/predicted |
|-----|---------|-----|-----|-----------|-----------|----------|---------|---------------|
| 1   | Donor   | 24  | M   |           | COPD      | 49       | 1.56    | 14            |
| 2   | Donor   | 52  | F   |           | COPD      | 45       | 0.66    | 19            |
| 3   | Donor   | 61  | F   |           | COPD      | 31       | 0.86    | 20            |
| 4   | Donor   | 26  | M   |           | COPD      | 63       | 0.76    | 22            |
| 5   | Donor   | 29  | F   |           | COPD      | 29       | 0.56    | 16            |
| 6   | Donor   | 55  | F   |           | COPD      | 38       | 0.55    | 13            |
| 7   | Donor   | 40  | F   |           | COPD      | 61       | 0.67    | 23            |
| 8   | Donor   | 42  | M   |           | COPD      | 40       | 0.93    | 20            |
| 9   | Donor   | 23  | M   |           | COPD      | 42       | 0.59    | 16            |
| 10  | Donor   | 63  | F   |           | COPD      | 47       | 0.57    | 23            |
| 11  | Donor   | 87  | M   |           | COPD      | 64       | 1.11    | 35            |
| 12  | Donor   | 31  | M   |           | COPD      | 60       | 1.00    | 20            |
| 13  | Donor   | 37  | F   |           | COPD      | 56       | 0.59    | 16            |
| 14  | Donor   | 34  | F   |           | COPD      | 50       | 0.57    | 23            |
| 15  | Donor   | 48  | M   |           | COPD      | 35       | 0.42    | 12            |
| 16  | Donor   | 50  | F   |           | COPD      | 57       | 0.63    | 14            |
| 17  | Donor   | 54  | M   |           | COPD      | 57       | 0.56    | 13            |
| 18  | Donor   | 52  | M   |           | COPD      | 58       | 0.55    | 17            |
| 19  | Donor   | 53  | M   |           | COPD      | 54       | 0.56    | 13            |
| 20  | Donor   | 55  | M   |           | COPD      | 60       | 0.82    | 24            |
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J. G., Hackett, N. R., and Crystal, R. G. (2009) Coordinate control of expression of Nrf2-modulated genes in the human small airway epithelium is highly responsive to cigarette smoking. *Mol. Med.* **15**, 203–219

Müller, T., and Hengstermann, A. (2012) Nrf2: friend and foe in preventing cigarette smoking-dependent lung disease. *Chem. Res. Toxicol.* **25**, 1805–1824

Zhu, L., Barrett, E. C., Barret, E. C., Xu, Y., Liu, Z., Manoharan, A., and Chen, Y. (2013) Regulation of Cigarette Smoke (CS)-Induced Autophagy by Nrf2. *PLoS ONE* **8**, e55695

Comandini, A., Marzano, V., Curradi, G., Federici, G., Urbani, A., and Saltini, C. (2010) Markers of anti-oxidant response in tobacco smoke exposed subjects: a data-mining review. *Pulm. Pharmacol. Ther.* **23**, 482–492

Brandsma, C. A., van den Berge, M., Postma, D. S., Jonker, M. R., Brouwer, S., Paré, P. D., Sin, D. D., Bossé, Y., Laviolette, M., Karjalainen, J., Fahrnmann, R. S., Nickle, D. C., Hao, K., Spanjer, A. L., Timens, W., and Franke, L. (2015) A large lung gene expression study identifying fibulin-5 as a novel player in tissue repair in COPD. *Thorax* **70**, 21–32

Biswal, S., Thimmulappa, R. K., and Harvey, C. J. (2012) Experimental therapeutics of Nrf2 as a target for prevention of bacterial exacerbations in COPD. *Proc. Am. Thorac. Soc.* **9**, 47–51

Malhotra, D., Thimmulappa, R., Vij, N., Navas-Acien, A., Sussan, T., Merali, S., Zhang, L., Kelsen, S. G., Myers, A., Wise, R., Tuder, R., and Biswal, S. (2009) Expression of concern: Heightened endoplasmic reticulum stress in the lungs of patients with chronic obstructive pulmonary disease: the role of Nrf2-regulated proteasomal activity. *Am. J. Respir. Crit. Care Med.* **180**, 1196–1207