Supplemental Figures and Legends

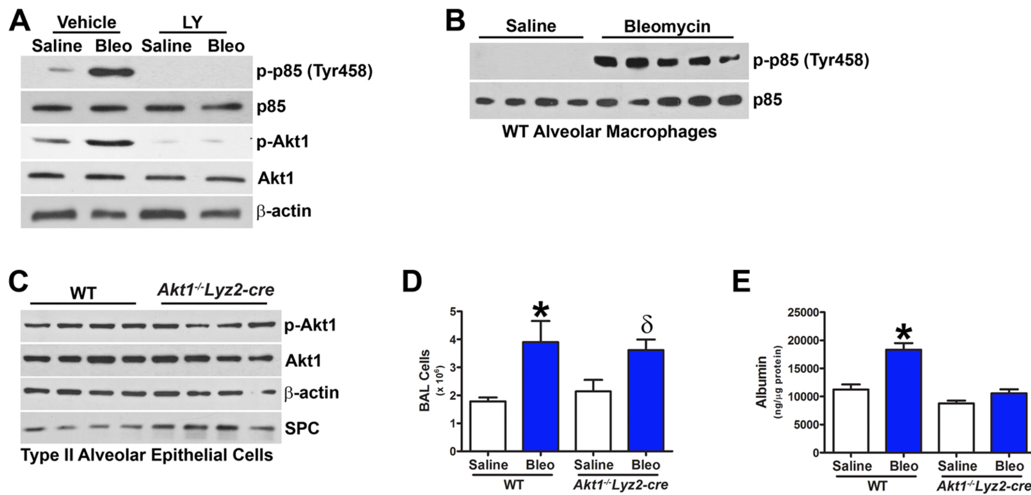

Figure S1, related to Figure 1. (**A**) Immunoblot analysis for p-p85 and p-Akt1 in macrophages exposed to saline or bleomycin (bleo) (12.5 mU/ml) in the presence of vehicle or 50 μM LY294002 (LY). (**B**) Immunoblot analysis for p-p85 in alveolar macrophages isolated from WT mice exposed to saline or bleomycin. (**C**) Immunoblot analysis for p-Akt1 in type II alveolar epithelial cells isolated from WT and *Akt1$^{-/-}$Lyz2-cre* mice. (**D**) Total number of BAL cells in WT (n = 4 saline; n = 5 bleo) and *Akt1$^{-/-}$Lyz2-cre* mice (n = 4 saline; n = 7 bleo). (**E**) Albumin levels measured in BAL fluid from WT (n = 4 saline; n = 5 bleo) and *Akt1$^{-/-}$Lyz2-cre* mice (n = 4 saline; n = 6 bleo). *, $p < 0.05$ vs WT+saline; δ, $p < 0.05$ vs *Akt1$^{-/-}$Lyz2-cre*+saline. One-way ANOVA with Tukey's comparison. See Figure 1 of main text.

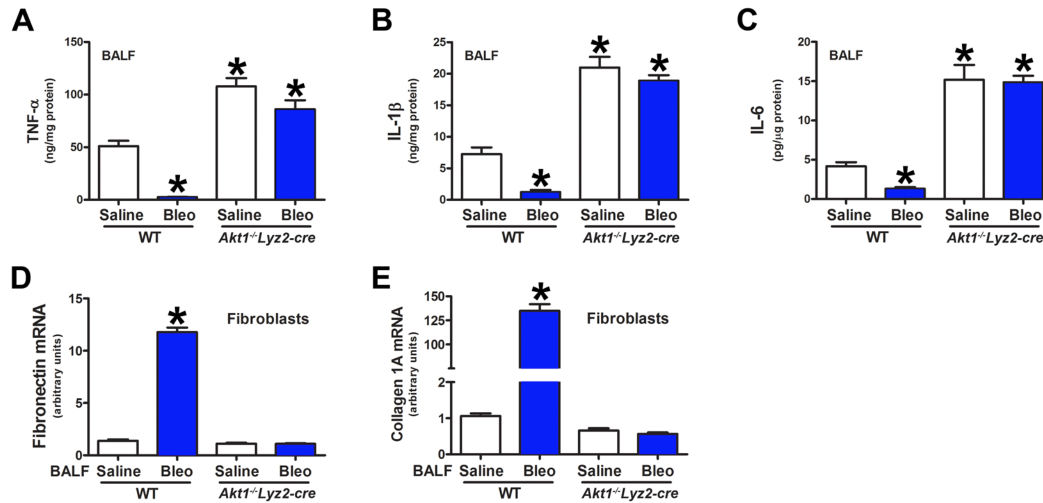

Figure S2

Figure S2, related to Figure 2. (**A**) TNF-α, (**B**) IL-1β, and (**C**) IL-6 levels were measured in BAL fluid from WT and *Akt1$^{-/-}$Lyz2-cre* mice after saline or bleomycin exposure. WT (n = 4 saline, n = 5 bleo) and *Akt1$^{-/-}$Lyz2-cre* (n = 4 saline, n = 5 bleo). Total RNA was isolated from normal lung fibroblasts conditioned with BAL fluid from exposed WT and *Akt1$^{-/-}$Lyz2-cre* mice. (**D**) Fibronectin and **(E)** collagen 1A mRNA were measured and expressed in arbitrary units. n = 4 per saline group and n = 5 per bleomycin group. *, $p < 0.05$ vs WT+saline. One-way ANOVA with Tukey's comparison. See Figure 2 of main text.

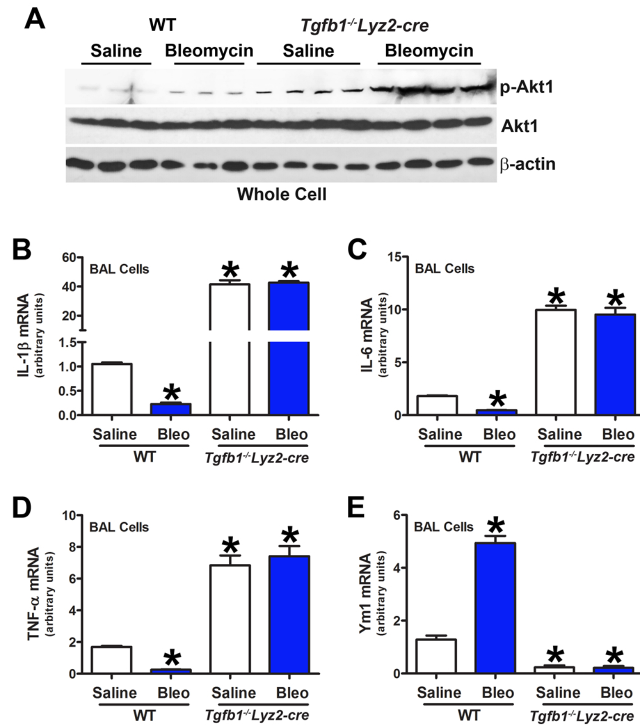

Figure S3, related to Figure 3. (**A**) Representative immunoblot analysis for p-Akt1, Akt1, and β-actin exposure (n = 6) in BAL cells from WT and *Tgfb1$^{-/-}$Lyz2-cre* mice were exposed to saline or bleomycin. (**B**) Il-1β, (**C**) IL-6, (**D**) TNF-α and (**E**) Ym-1 mRNA levels were measured in BAL cells from WT and *Tgfb1$^{-/-}$Lyz2-cre* mice after saline or bleomycin exposure (n = 6). *, $p < 0.05$ vs WT+saline. One-way ANOVA with Tukey's comparison. See Figure 3 of main text.

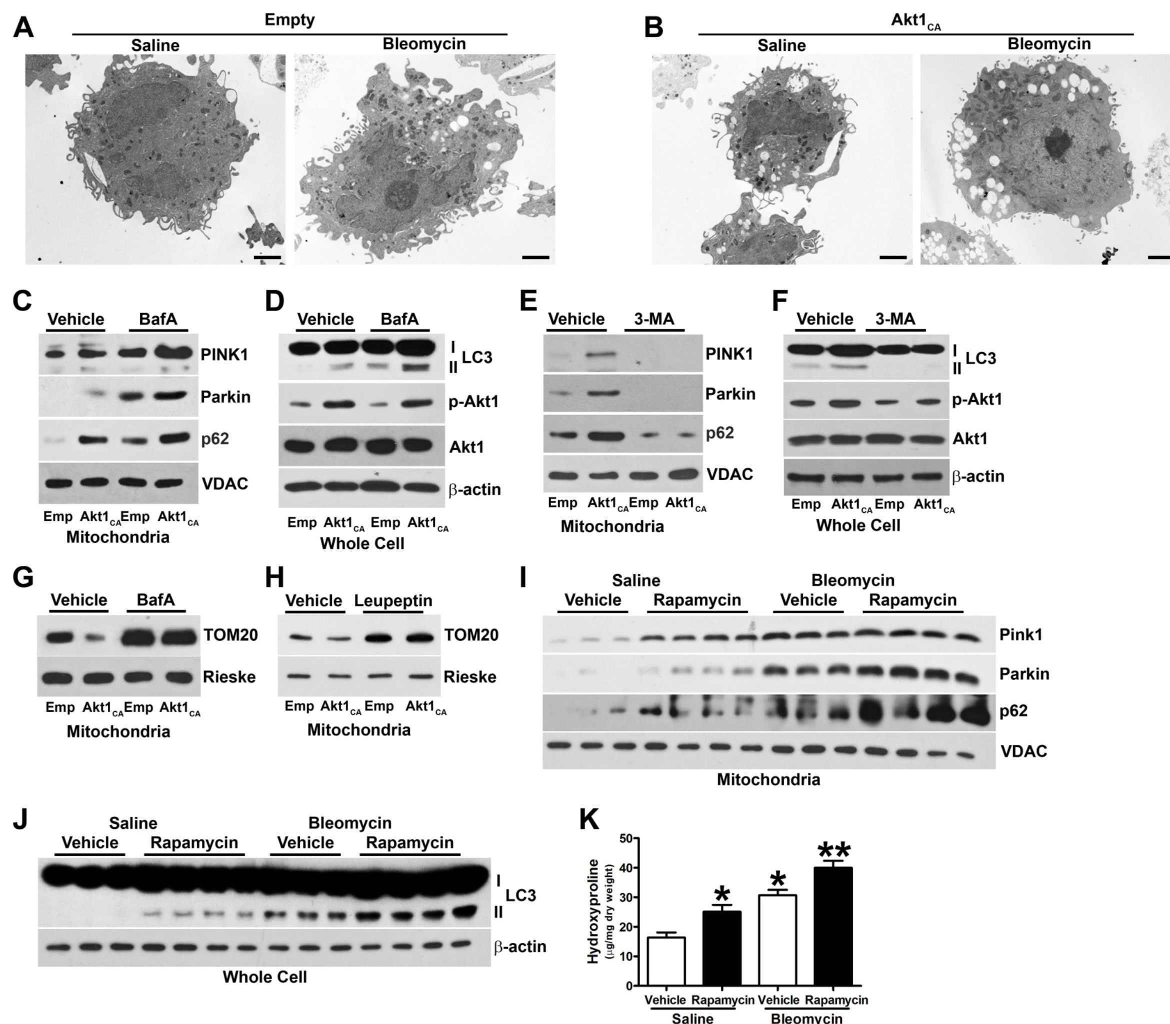

Figure S4, related to Figure 4. THP-1 cells expressing an empty vector or $Akt1_{CA}$ were treated with bleomycin and analyzed by transmission electron microscopy. (**A** and **B**) Bar = 2 μm. (**C**) Mitochondrial PINK1, Parkin, and p62, and (**D**) LC3-I and –II expression were determined in MH-S cells expressing an empty vector or $Akt1_{CA}$ and treated with vehicle or Bafilomycin A (Baf A) (100 nM) for 5 h. (**E**) Mitochondrial PINK1, Parkin, and p62, and (**F**) LC3-I and –II expression were determined in MH-S cells expressing an empty vector or $Akt1_{CA}$ and treated with vehicle or 3-methyladenine (3-MA) (10 mM). Mitochondrial TOM20 expression was determined by immunoblot analysis in MH-S cells expressing an empty vector or $Akt1_{CA}$ and treated with vehicle or (**G**) Bafilomycin A (Baf A) or (**H**) leupeptin (50 μg/ml). (**I**) Mitochondrial PINK1, Parkin, and p62, and (**J**) LC3-I and -II expression were measured in alveolar macrophages isolated by BAL in saline or bleomycin-exposed WT mice treated with daily i.p. injections of vehicle or rapamycin (3 mg/kg). (**K**) Hydroxyproline assay in excised lungs, $n = 5$ for Saline+vehicle, rapamycin, and Bleomycin+Vehicle, $n = 4$ for Bleomycin+rapamycin. *, $p < 0.05$ vs Saline+Vehicle, ** $p < 0.05$ vs all conditions. One-way ANOVA with Tukey's comparison. See Figure 4 of main text.

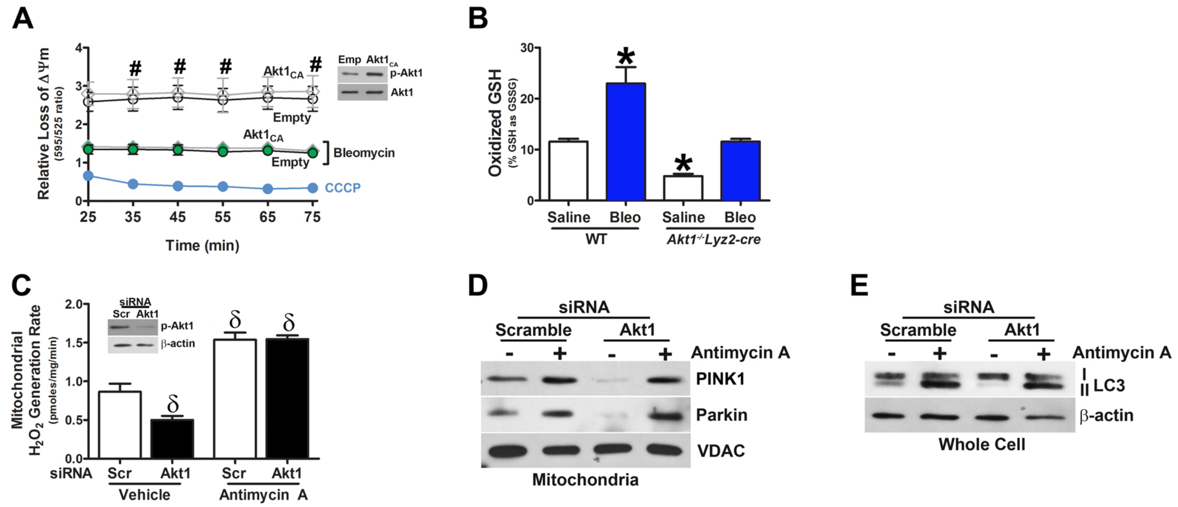

Figure S5, related to Figure 5. (**A**) THP-1 cells expressing empty or Akt1$_{CA}$ vectors were treated with bleomycin (12.5 mU/ml). Mitochondrial membrane potential was measured using JC-1 dye (10 µg/ml) at indicated times. Inset, Akt1 overexpression was verified by immunoblot analysis. $n = 6$ per group. Carbonyl cyanide m-chlorophenyl hydrazone (CCCP). BAL was performed on exposed WT and *Akt1$^{-/-}$Lyz2-cre* mice. (**B**) GSH and GSSG were measured by HPLC and mass spectrophotometry and expressed as oxidized glutathione (GSSG) relative to its reduced form (GSH). WT ($n = 4$ saline; $n = 5$ bleo) and *Akt1$^{-/-}$Lyz2-cre* mice ($n = 4$ saline; $n = 6$ bleo). (**C**) Mitochondrial H_2O_2, (**D**) mitochondrial PINK1 and Parkin, and (**E**) LC3-I and -II expression were measured in macrophages transfected with scrambled or Akt1 siRNA and treated with Antimycin A. Inset, Akt1 silencing verified by immunoblot analysis. $n = 6$. *, $p < 0.05$ vs WT+saline; #, $p < 0.05$ vs Empty+bleo and Akt1+bleo; δ, $p < 0.05$ vs Vehicle+scr. One-way ANOVA with Tukey's comparison. See Figure 5 of main text.

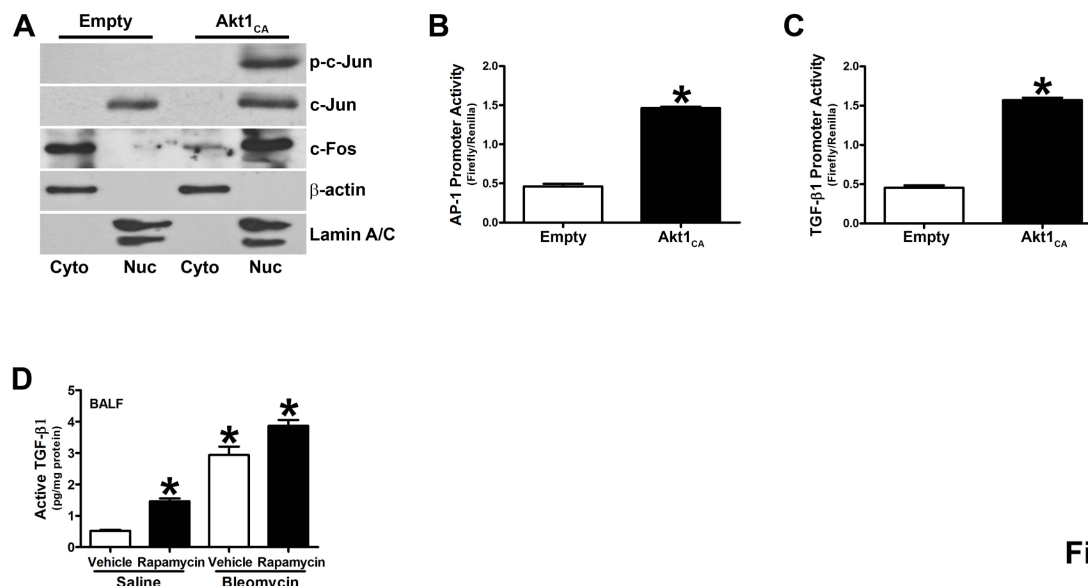

Figure S6, related to Figure 6. (**A**) MH-S cells expressing empty or Akt1$_{CA}$ vectors were isolated into cytoplasmic (cyto) or nuclear (nuc) fractions. P-c-Jun, c-Jun, and c-Fos were analyzed by immunoblot analysis. (**B**) AP-1 and (**C**) TGF-β1 promoter activity were measured in MH-S cells expressing empty or Akt1$_{CA}$. $n = 6$. (**D**) Active TGF-β1 measured in BAL fluid from WT mice exposed to saline or bleomycin and treated with vehicle or rapamycin (3 mg/kg, i.p. daily). $n = 5$ per group. *, $p < 0.05$ vs all conditions. One-way ANOVA with Tukey's comparison. See Figure 6 of main text.

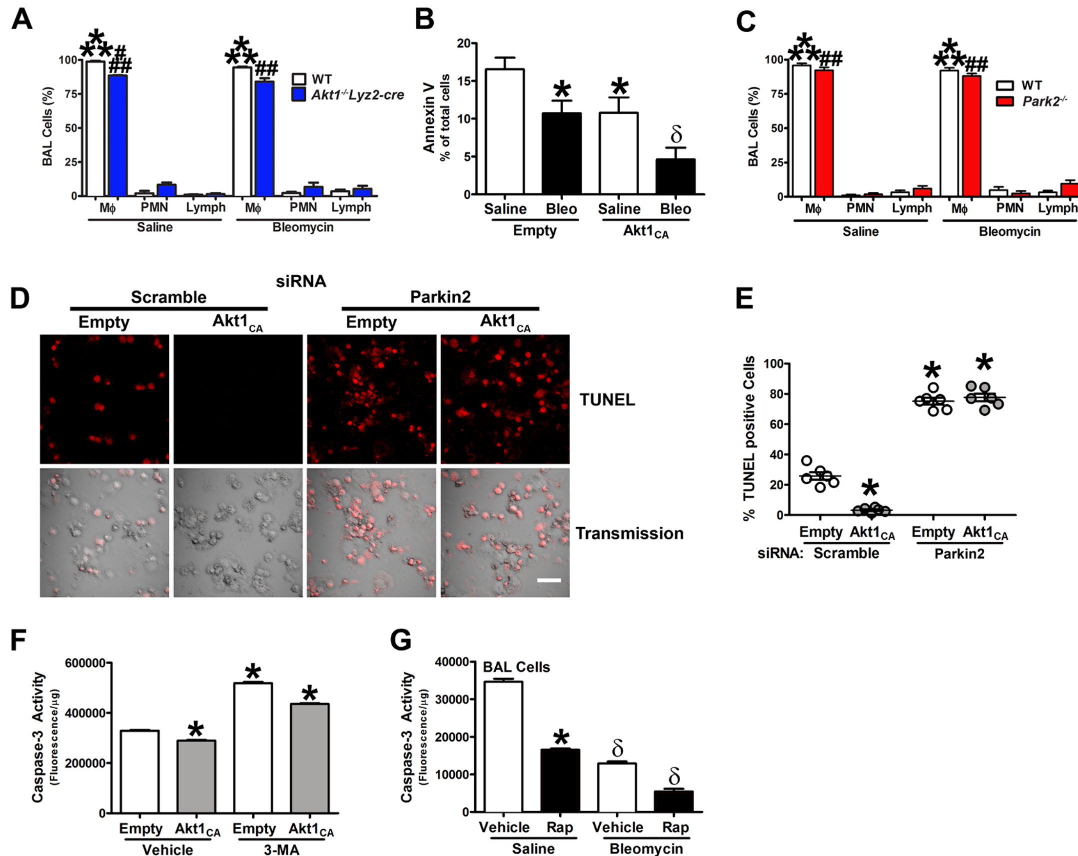

Figure S7

Figure S7, related to Figure 7. (**A**) Cell differential determined using Wright-Giemsa stain from BAL performed in WT (n = 4 saline; n = 5 bleo) and *Akt1*$^{-/-}$*Lyz2-cre* mice (n = 4 saline; n = 7 bleo). Polymorphonuclear (PMN). (**B**) Flow cytometry of Annexin V in MH-S cells expressing empty or $Akt1_{CA}$ and treated with saline or bleomycin, n = 5. (**C**) Cell differential determined using Wright-Giemsa stain from BAL performed in WT and *Park2*$^{-/-}$ mice (n = 5). (**D**) Representative images of TUNEL staining and (**E**) quantification of TUNEL positive cells in THP-1 cells transfected with scramble or Parkin2 siRNA in combination with empty or $Akt1_{CA}$ vectors. n = 6, each dot represents an average of 6 images. Bar = 40 µm. (**F**) Caspase-3 activity measured in MH-S cells expressing empty or $Akt1_{CA}$ and treated with 3-MA, n = 5. (**G**) Caspase-3 activity measured in alveolar macrophages isolated by BAL from exposed WT mice given vehicle or rapamycin, n = 5 per group. *, $p < 0.05$ vs Empty+saline, Empty+scramble, Vehicle+empty or Saline+vehicle; δ, $p < 0.05$ vs all conditions; ***, $p < 0.0001$ vs PMN and Lymph; ##, $p < 0.01$ vs WT+saline or WT+bleomycin; ###, $p < 0.0001$ vs WT+saline. One-way ANOVA with Tukey's comparison. See Figure 7 of main text.

Supplemental Experimental Procedures

Materials
Bleomycin was obtained from the University of Iowa Hospitals and Clinics hospital stores. α-ketoglutarate, p-Hydroxylphenyl acetic acid (pHPA), antimycin A, bafilomycin A (Baf A), carbonyl cyanide m-chlorophenylhydrazone (CCCP), horseradish peroxidase (HRP) and LY294002 (LY) were purchased from Sigma Chemical Company (St. Louis, MO). Leupeptin and 3-Methyladenine (3-MA) were purchased from Millipore.

Mice
Akt1$^{-/-}$*Lyz2-cre* mice were generated by selective disruption of Akt1 gene in the cells of the granulocyte/monocyte lineage by crossing *Akt1*$^{fl/fl}$ mice (a generous gift from Dr. Morris Birnbaum, University of Pennsylvania, Philadelphia, PA) with mice containing a Cre recombinase under the control of the Lysozyme M promoter. *Tgfb1*$^{-/-}$*Lyz2-cre* mice were generated in a similar manner. Mice were intratracheally administered 1.75-2 U/kg of bleomycin or saline, as a negative control, after being anesthetized with 3% isoflurane using a precision Fortec vaporizer (Cyprane, Keighley, UK). Rapamycin (LC Laboratories) was diluted in DMSO and administered by intraperitoneal (i.p.) injections daily of 3 mg/kg. Rapamycin or DMSO control i.p. injections were given 6 h post administration of bleomycin or saline and continued for 20 days.

Cell Culture
Macrophages were maintained in RPMI 1640 media with 10% fetal bovine serum and penicillin/streptomycin supplements. IMR-90 normal human lung fibroblast and IPF fibroblasts were cultured in DMEM supplemented with 10% fetal bovine serum, streptomycin, and amphotericin B. All experiments were conducted in RPMI containing 0.5% serum.

Isolation of Mitochondria and Cytoplasm
Cell fractions were prepared as described previously (He et al., 2011).

Quantitative Real Time PCR
Human TGF-β1, human hypoxanthine-guanine phosphoribosyltransferase (HPRT), human IL-10, human mannose receptor, mouse β-actin, mouse IL-1β, mouse TGF-β1, mouse TNF-α, and mouse Ym1 primers are previously described (Larson-Casey et al., 2014; Murthy et al., 2015). The following primer sets were also used: human collagen 1α, 5'-GCT CGT GGA AAT GAT GGT GC-3' and 5'-ACC AGG TTC ACC GCT GTT AC-3'; human fibronectin, 5'-TGT TAT GGA GGA AGC CGA GGT T-3' and 5'-CGA TGC AGG TAC AGT CCC AGA-3'; mouse IL-6, 5'-CAG AAT TGC CAT CGT ACA ACT CTT TTC-3' and 5'-AAG TGC ATC ATC GTT GTT CAT ACA-3'. Data was calculated by the cycle threshold (ΔΔCT) method. The mRNA measurements were normalized to β-actin or HPRT and expressed in arbitrary units.

Immunoblot Analysis
Primary antibodies used: rabbit monoclonal anti-phospho-SMAD2 (Ser465/467) (138D4); rabbit monoclonal anti -SMAD2 (D43B4), rabbit polyclonal anti-phospho-Akt (Ser473), rabbit polyclonal anti-Akt, rabbit monoclonal anti-Caspase-3 (8G10), rabbit polyclonal anti-LC3A/B, rabbit polyclonal anti-Lamin A/C, rabbit polyclonal anti-phospho-PI3 Kinase p85 (Tyr458), rabbit polyclonal anti- PI3 Kinase p85, and rabbit polyclonal anti-VDAC (Cell Signaling); rabbit polyclonal anti-Parkin, rabbit polyclonal anti-PINK1, mouse monoclonal anti-Mitofusion-2, mouse monoclonal anti-UQCRFS1 (5A5) (anti-Rieske), and mouse monoclonal anti-SQSTM1/p62 (Abcam); rabbit polyclonal anti-Prosurfactant Protein C (Millipore); rabbit polyclonal anti-c-Fos (4), rabbit polyclonal anti-c-Jun (H-79), mouse monoclonal anti-p-c-Jun (KM-1), rabbit polyclonal anti-TOM20 (FL-145) (Santa Cruz); mouse monoclonal anti-alpha-smooth muscle actin (α-SMA) (American Research Products); and mouse monoclonal anti-β-actin (AC-15) (Sigma).

Glutathione Assay
Reduced (GSH) and oxidized glutathione (GSSG) in lung were measured by high-performance liquid chromatography (HPLC) and mass spectrometry analysis as previously described (Murthy et al., 2015).

TUNEL Assay
Detection of apoptosis was determined in macrophages and in BAL cells from WT, *Akt1*$^{-/-}$*Lyz2-cre*, and *Park2*$^{-/-}$ mice exposed to saline or bleomycin by TUNEL analysis as previously described (Larson-Casey et al., 2014).

Mitochondrial Membrane Potential
Cells were loaded with JC-1 dye (Molecular Probes) at a final concentration of 10 μg/ml for 15 min at 37 °C. After incubation, cells were washed twice. Fluorescence was measured using a SpectraMax M2 plate reader. CCCP, at a final concentration of 30 μM, was used as a positive control in all experiments.

Hydroxyproline Determination

Lung tissue was dried to a stable weight and acid hydrolyzed with 6N HCl for 24 h at 112 ˚C. Hydroxyproline concentration was normalized to the dry weight of the lung, as described previously (Murthy et al., 2009).

References and Notes

Larson-Casey, J.L., Murthy, S., Ryan, A.J., and Carter, A.B. (2014). Modulation of the mevalonate pathway by Akt regulates macrophage survival and development of pulmonary fibrosis. J Biol Chem *289*, 36204-36219.

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Murthy, S., Larson-Casey, J.L., Ryan, A.J., He, C., Kobzik, L., and Carter, A.B. (2015). Alternative activation of macrophages and pulmonary fibrosis are modulated by scavenger receptor, macrophage receptor with collagenous structure. FASEB J.

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