

Additional file 1 for

IGF1R deficiency mitigates acute lung injury by promoting anti-inflammatory transcriptional profiles

**Alfredo Urtubia¹, Sergio Piñeiro-Hermida², Elvira Alfaro-Arnedo¹, Judith Beni-Ledesma¹,
Marta Canalejo¹, María de Toro³, José G. Pichel^{1,4,*} and Icíar P. López¹**

¹Lung Cancer and Respiratory Diseases Unit, Center for Biomedical Research of La Rioja (CIBIR),
Fundación Rioja Salud, Logroño, Spain

²Telomeres and Tumor Microenvironment Unit, Fundación Rioja Salud (CIBIR), Logroño, Spain

³Genomics and Bioinformatics Core Facility, Center for Biomedical Research of La Rioja (CIBIR),
Fundación Rioja Salud, Logroño, Spain

⁴Spanish Biomedical Research Networking Centre – Respiratory Diseases (CIBERES), ISCIII; Madrid,
Spain

* Correspondence should be addressed to J.G.P. (jgpichel@riojasalud.es)

Supplementary Materials and Methods

Generation of IGF1R-deficient mice, establishment of the BLM-induced acute lung injury murine model and tissue collection

UBC-CreERT2; Igf1r^{fl/fl} mice were crossed with *Igf1r^{fl/fl}* mice to directly generate descendants in equal proportions in the same litter. Tamoxifen (TMX) (Sigma, Burlington, MA) was daily administered for five consecutive days to four-week-old mice of both genotypes to induce a postnatal *Igf1r* gene conditional deletion in *UBC-CreERT2; Igf1r^{fl/fl}* mice [1]. After TMX treatment, *UBC-CreERT2; Igf1r^{Δ/Δ}* (*CreERT2*; IGF1R-deficient) and *Igf1r^{fl/fl}* (control) mice were used for experimental purposes. Six-week-old prepubertal, tamoxifen-treated *UBC-CreERT2; Igf1r^{fl/fl}* (*CreERT2*) and *Igf1r^{fl/fl}* female mice were used for BLM or SAL treatment, as previously reported [2], to respectively generate the four experimental groups: *Igf1r^{fl/fl}*-SAL, *CreERT2*-SAL, *Igf1r^{fl/fl}*-BLM and *CreERT2*-BLM. Briefly, mice of both genotypes were intra-tracheally instilled with either a single dose of 2.5 μL/g body weight of BLM sulfate (5 U/kg) (EMD Millipore, Billerica, MA) in saline (SAL) (2 U/mL) or SAL alone, under a ketamine-xylazine anesthetic combination in saline (100:10 mg/kg respectively; 10 μL/g). Three days later, animals were euthanized with 10 μL/g ketamine-xylazine at 300:10 mg/kg respectively. Left lungs were inflated with formalin fixative, post-fixed by immersion in formalin for 8-10 h, embedded in paraffin and cut into 3 μm sections for histopathological evaluation or immunohistochemistry; and right lobes were separated and snap frozen in liquid nitrogen for RNA, DNA and protein isolation (Fig. 1A).

Lung RNA isolation, cDNA library preparation and high-throughput sequencing

Inferior right lung lobes were homogenized in 1 mL of Trizol® (Invitrogen, Carlsbad, CA) and total RNA was isolated with a RNeasy Mini Kit (Qiagen, Hilden, Germany), following the manufacturers' specifications. Total RNA was treated with 2.72 KU/μl RNase-free DNase (Qiagen). The quantity and quality of RNA were assessed on a Qubit 3.0 (Invitrogen) and RNA integrity was analyzed by capillary electrophoresis in a Fragment Analyzer system with the

Standard Sensitivity RNA Analysis Kit (15 nt) (Agilent Technologies, Santa Clara, CA). Sequencing libraries were prepared by following the TruSeq Stranded mRNA Library Preparation Kit (Illumina Inc.) from 1 µg of total RNA. Before high throughput sequencing, library concentration was measured via Qubit 3.0 (Invitrogen) and library size was assessed in a Fragment Analyzer system with the NGS Fragment Kit (1-6000 bp) (Agilent Technologies). The libraries were then run in a HiSeq 1500 PE100 lane in Rapid mode.

Bioinformatic analyses

Firstly, the quality of RNA-seq reads was assessed using FastQC [3]. The raw reads were then trimmed, filtered with a Phred quality score of at least 25 and all adapters removed with Trimmomatic software [4]. Clean reads were aligned versus the *Mus musculus* GRCm38.p6 reference genome by using HiSat2 [5] with default parameters. Resulting alignment files were quality-assessed with Qualimap2 [6] and sorted and indexed with Samtools [7]. Reads on gene features were counted with FeatureCounts [8]. In order to evaluate at the transcriptomic level the effect of generalized IGF1R deficiency in the context of the BLM challenge, three differential gene expression comparisons were established between experimental groups: i) *CreERT2-SAL* compared to *Igf1r^{fl/fl}-SAL* control lungs (*CreERT2-SAL* vs *Igf1r^{fl/fl}-SAL*, "mutation"); ii) BLM-challenged *Igf1r^{fl/fl}* compared to *Igf1r^{fl/fl}-SAL* mice (*Igf1r^{fl/fl}-BLM* vs *Igf1r^{fl/fl}-SAL*, "injury"); and iii) BLM-challenged *CreERT2-BLM* compared to BLM-challenged *Igf1r^{fl/fl}-BLM* mice (*CreERT2-BLM* vs *Igf1r^{fl/fl}-BLM*, "attenuation") (Fig. 1B). Then, read count normalization and quantitative differential expression analysis was performed with DESeq2 [9]. In order to automate this process and facilitate all group combination analyses, the SARTools pipeline was used [10]. Resulting data was obtained as .csv tables, including raw counts, normalized counts, Fold-Change estimation and dispersion data for the analysis and were annotated with additional data from the Biomart database [11]. Principal Component Analysis (PCA) plots were generated using the *PCAPlot* function from the SARTools package. Hierarchical clustering heatmaps of expression data samples were performed with the Pheatmap package [12], representing Euclidean distances and using the *complete* method for

clustering. To control the False Discovery Rate (FDR), p -values were amended by Benjamini-Hochberg (BH) multiple testing correction. Those features with FDR adjusted p -values below a 0.05 threshold and Fold Change ≥ 1.5 / ≤ 0.67 ($|\log_2 \text{FC}| \geq 0.58$) were considered as significantly up- or down-regulated genes. Volcano plots of differential expression data were plotted using the EnhancedVolcano package [13]. Gene set enrichment analysis (GSEA) was performed with differentially expressed genes using the clusterProfiler package [14]. Gene Ontology enrichment was obtained through its enrichGO tool. Similarly, KEGG and Reactome pathway analysis was conducted with the enrichKEGG and ReactomePA [15] tools. Heatmaps of DEGs were generated with Pheatmap from *txt*. tables containing normalized expression values for the corresponding genes and samples, setting a gene-centered scale and the *complete* method for gene clustering. Coverage along the *Igf1r* gene full transcribed region was obtained using the *pileup* function from the RSamtools package [16], counting properly paired reads overlapping each nucleotide position. Then counts were normalized to reads per million mapped reads (RPM) and averaged by sample group. *Igf1r* exon-specific fragment counts were obtained with FeatureCounts, then normalized to fragments per kilobase per million mapped reads (FPKM) and averaged by sample group. In addition, junction counts were also obtained with FeatureCounts to identify splicing events in all samples.

Single-cell transcriptomics data mining and visualization

Single-cell RNA-sequencing (scRNA-seq) datasets from human and mouse lung samples were analyzed using the Scanpy package [17] in Python. The human COVID-19 dataset (GSE171524; Melms et al., 2021 [18]) and the Tabula Muris mouse lung dataset (GSE109774; Tabula Muris Consortium, 2018 [19]) were obtained directly as *.h5ad* files from the CELLxGENE website (<https://cellxgene.cziscience.com/datasets>). The whole-lung BLM-induced lung injury dataset (GSE141259; Strunz et al., 2020 [20]) was downloaded as a Seurat *.RData* file from the authors' GitHub repository and converted to *.h5ad* using the SeuratDisk [21] package in R, ensuring compatibility with Scanpy-based workflows. All datasets were confirmed to be preprocessed, quality-checked, and curated as reported [18, 19, 20]. High-

resolution cell type annotations were used in each case, with some fine-grained cell types grouped to enable appropriate clustering in the context of this study. For each dataset, subsets of interest were selected by filtering the *AnnData* object by the corresponding categories within the *obs* attribute. Only cells with ≥ 500 unique molecular identifiers (UMIs) or ≥ 200 detected genes were retained for downstream analysis. Combined grouping variables were constructed within the *obs* attribute to enable subgroup-level analyses. To reduce noise, gene expression values were masked in cell type-condition subgroups where expression was detected in fewer than 1% of cells. Custom dot plot visualizations were then generated with the *scanpy.pl.DotPlot* function from normalized, log-transformed expression matrices. Mean expression values were computed from cells with detected expression for each gene (*mean_only_expressed=True*) and scaled by gene (*standard_scale='var'*). For the *Tabula Muris* dataset, only data from mice aged 1 to 3 months were included in the analysis. In the BLM-induced lung injury dataset, analysis was restricted to samples from day 3 after BLM treatment, to match the conditions of this study; and to PBS-treated controls from days 3 and 10 altogether, due to otherwise insufficient data in the control group.

Histology, immunofluorescence and immunohistochemistry

For histological analysis, following formalin fixation, tissues were dehydrated and embedded in paraffin, cut into 3 μm sections, rehydrated and stained with hematoxylin-eosin (H&E). Slides were observed and photo documented using a light microscope (Nikon Instruments Inc., Melville, NY). For immunostaining, paraffin sections of left lung lobes were dewaxed and rehydrated by standard methods. Antigen retrieval was performed by immersing the slides in a boiling solution of 10 mM Citrate (pH 6.0) for 17 minutes, and followed by a 30 min cool down in the same buffer. For fluorescence immunostaining, all slides were blocked with 10% normal donkey serum in PBS-Triton (0.1%) and Glycine (10 mM) for 1 h at room temperature, and incubated at 4°C overnight with the following primary antibodies: CC10 (1:100; clone E-11; cat. no. sc-365992; Santa Cruz Biotechnology, Dallas, TX), Pro-SPC (1:100; clone M-20; cat. no. sc-7706; Santa Cruz Biotechnology), and H3K9ac (1:200; clone

C5B11; Cat. No. 9649; Cell Signalling Technology, Danvers, MA). After washing, sections were incubated for 1 h with secondary antibodies: Alexa Fluor™ donkey anti-rabbit 488, anti-mouse 555 and anti-goat 633, all from Santa Cruz Biotechnology. Then slides were washed and mounted in VECTASHIELD® mounting medium with DAPI (Vector Laboratories, Newark, CA). Images of CC10/pro-SPC immunostaining in bronchiolar and alveolar areas were obtained with a confocal microscope, using a x63 oil immersion objective (Stellaris 8, Leica Microsystems, Concord, ON, Canada). To document and analyze H3K9ac/CC10/pro-SPC immunofluorescence slides, whole-lung sections were imaged by merging tiles of 20x-magnification images taken with a fluorescence microscope (Axio Imager Z2, ZEISS, Oberkochen, Germany). For DAB immunohistochemical analyses, the DAB Novolink Polymer Detection System (Novocastra Laboratories Ltd, Newcastle Upon Tyne, UK) was used according to manufacturer's instructions. p21 (1:100; clone M19; cat. no. sc-471; Santa Cruz Biotechnology) and LC3 A/B (1:150; cat. no. 4108; Cell Signaling Technology) primary antibody incubations were performed at 4°C overnight in TBS and 10% normal goat serum. After DAB staining, the slides were dehydrated by standard methods and mounted in EUKITT mounting medium to be examined and documented under a light microscope (Nikon Instruments).

Lung injury score

The acute lung injury (ALI) score was calculated as described Matute-Bello *et al.* [22]. This score system includes five histological features: A) neutrophils in the alveolar space; B) neutrophils in the interstitial space; C) hyaline membranes formation; D) proteinaceous debris filling the airspace; and E) alveolar septal thickening. Each item was assigned a score of 0, 1, or 2 based on the severity of lung injury. The final ALI score was determined using the following formula: $[(20 \times A) + (14 \times B) + (7 \times C) + (7 \times D) + (2 \times E)]$ divided by the number of fields multiplied by 100, leading to a score between zero (no lung injury) and one (severe injury) (n=4 animals per group). Histological samples were reviewed in a blinded manner to ensure unbiased evaluation.

Generation of *Igf1r* KO mice and isolation and culture of mouse embryonic fibroblasts

To generate embryos lacking *Igf1r* (*Igf1r*-KO or *Igf1r*^{-/-}), *CreERT2* (*UBC-CreERT2*; *Igf1r*^{fl/fl} treated with TMX) mice were crossed with each other to generate heterozygous *Igf1r*^{fl/-} mice with one of the alleles of the *Igf1r* gene deletion (*Igf1r*-KO or *Igf1r*) fixed in the germline. These heterozygous males and females (*Igf1r*^{fl/-}) were fertile and were crossed to generate offspring in the expected Mendelian proportions. These mice were used to maintain a colony of mice in heterozygosis, since total KO mice (*Igf1r*^{-/-}) do not survive postnatally [23]. Deletion in the *Igf1r* gene was verified by PCR of genomic DNA and by Western blot analysis in *Igf1r*^{-/-} embryos obtained from these heterozygous crosses. Heterozygous *Igf1r*^{fl/-} mice were inter-crossed to generate embryos for isolation of primary mouse embryonic fibroblasts (MEFs). At day 16 of gestation, mouse embryos were dissected and genotyped by PCR using DNA extracted from their tails. Homozygous control *Igf1r*^{fl/fl} and *Igf1r*^{-/-} mutant embryo littermates were used to establish primary cultures of MEFs. Briefly, embryos were cleaned of viscera and minced into thin pieces in 35-mm culture dishes, followed by incubation with 0,25% trypsin-EDTA for 20 min. Then, the resulting cell suspensions were plated onto 100-mm culture dishes and cultured in DMEM high glucose GlutaMAX™ supplemented with 10% FBS and 1% Pen Strep (100 U/mL penicillin and 100 µg/mL streptomycin) (all from Gibco/Thermo Fisher Scientific Inc.). 24 h later, cells were washed twice with PBS and the medium was replaced. Once plates were filled with fibroblasts, a 1:3 split was performed, and when these plates reached 80-90% confluence, cells were frozen in 3 vials per plate with DMEM supplemented with 20% FBS and 10% DMSO for later use. For all experiments, MEFs were cultured on the same culture medium described above and used within three to four passages.

MEFs treatments, sample collection and immunofluorescence staining

For experiments assessing BLM-induced cytokine mRNA expression, DNA lesion frequency, and histone H3 acetylation, *Igf1r*^{fl/fl} and *Igf1r*^{-/-} MEFs were seeded in 60 mm cell culture Petri dishes and treated with either 10 µg/mL BLM in DMEM or DMEM for 24 hours. Following the 24-hour period, cells for RNA extraction were washed twice with ice-cold PBS,

scraped into TRIzol[®] reagent (Invitrogen) and stored at -80°C. Cells for genomic DNA extraction were washed twice with PBS, trypsinized, pelleted by centrifugation at 300 × *g* for 5 min at RT, and resuspended in NZY Tissue gDNA Isolation Kit lysis buffer (NZYtech, Lisbon, Portugal) for subsequent DNA isolation according to the manufacturer's instructions. For histone extraction, cells were washed twice with PBS, trypsinized, pelleted by centrifugation at 150 × *g* for 5 min at RT, and processed immediately using the Histone H3 Acetylation Assay Kit (Cat. No. ab115102; Abcam, Cambridge, UK), following the manufacturer's specifications. Resulting histone extracts were stored at -80°C.

For immunofluorescence analyses of BLM-induced DNA damage and mitochondrial homeostasis disruption, *Igf1r^{fl/fl}* and *Igf1r^{-/-}* MEFs were seeded on 35 mm dishes with 14 mm micro-well and #1.5 glass-like polymer coverslip (Cat. No. D35-14-1.5P; Cellvis, Mountain View, CA) in DMEM. The next day after plating, cells were treated with either 20 µg/mL BLM in DMEM or DMEM for 2h. Then, cells were washed once and left to grow in DMEM. 48h after treatment, plates were washed twice and fixed with 4% PFA in PBS for 10 min at RT. For immunostaining, plates were first washed and permeabilized with PBS-Triton (0.1%) for 10 min at RT. Cells were then washed and blocked with 5% BSA and 2% normal goat serum (NGS) in PBS-Triton (0.1%) for 1 h at room temperature and incubated at 4°C overnight with TOM20 (1:1000; Cat. No. 11802-1-AP; Proteintech, Rosemont, IL) or γH2AX (1:100; Cat. No. 05-636; Sigma) antibodies with 2% BSA and 2% NGS in PBS-Triton (0.1%). After washing, cells were incubated with Alexa Fluor[™] Goat anti-rabbit 546 or 647 secondary antibodies (1:500; Santa Cruz Biotechnology) and Phalloidin DyLight[™] 488 (1:500; Thermo Fisher Scientific Inc.). Finally, plates were washed and mounted in VECTASHIELD[®] mounting medium with DAPI (Vector Laboratories).

For longer-term experiments of induced senescence, *Igf1r^{fl/fl}* and *Igf1r^{-/-}* MEFs were seeded in 60 mm cell culture Petri dishes and treated with either 20 µg/mL BLM in DMEM or DMEM for 2h, washed once and maintained in culture. Seven days after treatment, plates were washed twice with ice-cold PBS and scraped with RIPA buffer (Thermo Fisher Scientific, Inc.)

supplemented with protease and phosphatase inhibitor cocktails (Roche, Basilea, Switzerland). Protein samples were then centrifuged at $14000 \times g$ for 20 min at 4°C and the supernatants were collected and stored at -80°C.

MEFs RNA isolation, reverse transcription and real-time qPCR

Total RNA was isolated from TRIzol® homogenates of cultured MEFs using the RNeasy Mini Kit (Qiagen) and reverse-transcribed to cDNA with the SuperScript II First-Strand Synthesis System (Invitrogen), following the manufacturers' instructions. cDNA samples were then amplified by real-time qPCR in triplicate reactions for each primer pair assayed on a QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific Inc.), using NZYSupreme qPCR Green Master Mix (2x), ROX (NZYTech). Data were obtained and analyzed with QuantStudio 3/5 Real-Time PCR Software (Thermo Fisher Scientific Inc.). All primers used are shown below:

Gene	Accession No.	Forward primer (5'-3')	Reverse primer (5'-3')
<i>Cxcl1</i>	NM_008176.3	ATCCAGAGCTTGAAGGTGTTG	GTCTGTCTTCTTTCTCCGTTACTT
<i>Cxcl2</i>	NM_009140.2	ATCCAGAGCTTGAGTGTGACG	GTTAGCCTTGCCTTTGTTTCAG
<i>Ccl2</i>	NM_011333.3	CACCAGCCAACTCTCACTGA	CGTAACTGCATCTGGCTGA
<i>Cxcl10</i>	NM_021274.2	CCAAGTGCTGCCGTCATTTTC	TCCCTATGGCCCTCATTCTCA
<i>Il6</i>	NM_031168.2	ACGGCCTTCCCTACTTCACA	CATTTCCACGATTTCCCAGA
<i>Tnf</i>	NM_013693.3	GCCTCTTCTCATTCCTGCTT	CTGATGAGAGGGAGGCCATT
<i>Hprt1</i>	NM_013556.2	CAGTCCCAGCGTCGTGATTA	GGCCTCCCATCTCCTTCATG

DNA lesion frequency and mitochondrial copy number assessment

Genomic DNA (gDNA) was isolated from the accessory lobes of mouse lungs using the DNeasy® Blood & Tissue Kit (Qiagen), and from MEF cell pellets using the NZY Tissue gDNA Isolation Kit (NZYtech). DNA lesion frequency was assessed by Long-Amplicon Quantitative PCR (LA-qPCR), following the protocols described by Furda *et al.* [24] and González-Hunt *et al.* [25] with minor modifications. The Qubit Fluorometric Quantification System (Thermo Fisher Scientific) was used for accurate quantification of both gDNA samples and LA-qPCR products. LA-qPCR reactions were carried out using 7.5 ng of gDNA in a final volume of 25 µL, containing

1× LongAmp Hot Start Taq 2× Master Mix (New England Biolabs Inc., Ipswich, MA), 0.4 μM of each primer, and nuclease-free water. The thermal cycling profile consisted of an initial denaturation at 94 °C for 2 minutes, followed by repeated cycles of denaturation at 94 °C for 15 seconds and annealing at the fragment-specific temperature for 12 minutes, and a final extension at 72 °C for 10 minutes. Annealing temperatures and cycle numbers were optimized for each target as follows: for the 6.6-kb DNA polymerase β fragment (accession AA79582), 65 °C and 26 cycles; and for the 10-kb mitochondrial fragment, 64 °C with 18 cycles for MEF samples and 21 cycles for lung tissue samples. To enable efficient amplification in mitochondrial DNA assays, DNA samples were linearized by digestion with HaeII (New England Biolabs) for 1 hour at 37 °C, following the manufacturer's instructions. Relative mitochondrial to nuclear DNA content was assessed by real-time qPCR using a short mitochondrial fragment (117 bp) and a short nuclear fragment (113 bp), following the comparative Ct method. Mitochondrial DNA copy number, normalization 10-kb mitochondrial Qubit data and lesion frequency calculations were performed as described in Gonzalez-Hunt *et al.* [25].

Mitochondrial morphology and network connectivity analysis.

Igf1r^{fl/fl} and *Igf1r^{-/-}* MEFs stained for TOM20, actin and DAPI were imaged on a confocal microscope (Stellaris 8, Leica Microsystems). Briefly, z-stacks were obtained using a x63 oil immersion objective (1.4 NA). Pixel size and z-spacing were adjusted as per the calculated optimal Nyquist sampling parameters, and pinhole size was set to 0.75 Airy units. Laser power, detector filtering/gating, and gain were adjusted to maximize signal while also minimizing background signal, cross-fluorescence, and photobleaching, using three frame averages for the TOM20 channel. These z-stacks were then deconvolved using the “Lightning Process” functionality of LAS X Leica software with system-optimized settings and a regularization parameter of 0.05. The *Mitochondria Analyzer* plugin developed by Chaudhry *et al.* was used for mitochondrial object segmentation and morphological and network connectivity analysis [26]. Thus, the settings for thresholding were adjusted for optimal segmentation of

mitochondrial objects on our set of confocal images, with “enhance local contrast” max slope set to 1.25 and “Sigma filter plus” radius to 1.7 pixels to pre-process the images; block size of 1.25 μm and C-value of 5 for adaptive thresholding and the post-processing commands “Despeckle”, “Remove outliers” (1.7 pixel radius) and “Fill 3D holes” activated. The thresholded z-stacks were then subjected to morphological and network connectivity analysis with default parameters.

Measurement of mitochondrial respiration and glycolytic activity

Igf1r^{fl/fl} and *Igf1r^{-/-}* MEFs were seeded on XF24 plates (Agilent Technologies) coated with poly-D-lysine at a density of 25000 cells/well and grown overnight at 37 °C under 5% CO₂. 24 h after seeding, culture medium was washed and replaced by pre-warmed Seahorse XF-DMEM medium (pH 7.4) containing 10 mM glucose, 2 mM glutamine, and 2% FBS, with or without 1 mM sodium pyruvate as appropriate. Cells were incubated at 37 °C for 45 min in a non-CO₂ incubator and then the Seahorse XF24 Analyzer (Agilent Technologies) was used to take three measurements of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) under basal conditions and after the injection of inhibitors. Each assay cycle consisted of 3 min of mixing, 2 min of waiting, and 3 min of measuring. After the last measurement, 1 $\mu\text{g/mL}$ Hoechst was injected to stain cell nuclei. Once the plate was recovered from the Seahorse analyzer, a photo of each well was taken using an inverted fluorescence microscope (Leica Microsystems) and nuclei were automatically counted using the ImageJ2 software. For mitochondrial respiration assessment, sequential injections consisted of 1.5 μM oligomycin (#75351, Sigma), 2 μM FCCP (#C2920, Sigma), and a mixture of 0.5 μM rotenone (#45656, Sigma) and 0.5 μM antimycin A (#A8674, Sigma). Using the Seahorse Analytics software (Agilent Technologies), OCRs were normalized to cell number and used to calculate the following parameters: non-mitochondrial OCR (minimum OCR after rotenone/antimycin A), basal respiration (last OCR before oligomycin – non-mitochondrial OCR), proton leak (minimum OCR after oligomycin – non-mitochondrial OCR), ATP production-coupled respiration (last OCR before oligomycin – minimum OCR after oligomycin), maximal respiration

(maximum OCR after FCCP – non-mitochondrial OCR), % coupling efficiency (ATP production-coupled respiration / basal respiration × 100), and % spare respiratory capacity (maximal respiration / basal respiration × 100). For glycolytic function assessment, the Seahorse XF Glycolytic Rate Assay Kit (Agilent Technologies, cat. no. 103344-100) was used. After baseline measurements, 0.5 μM rotenone/antimycin A and 50 mM 2-deoxyglucose (2-DG) were sequentially injected. Glycolysis-associated proton efflux rate (GlycoPER) was determined from ECAR and OCR; ECAR was converted to total proton efflux rate (PER), and the estimated mitochondrial acidification component was subtracted to yield GlycoPER. Similarly to OCR parameters, cell number-normalized glycoPERs were used to automatically calculate basal glycolysis (last glycoPER before Rotenone/Antimycin), and compensatory glycolysis (maximum glycoPER after Rotenone/Antimycin A) with Seahorse Analytics.

γH2AX foci and nuclear morphology analysis

Igf1r^{fl/m} and *Igf1r^{-/-}* MEFs stained for γH2AX and DAPI were imaged on a confocal microscope (Stellaris 8, Leica Microsystems). Briefly, z-stacks were obtained using a 40x oil immersion objective (1.3 NA). Pixel size and z-spacing were adjusted as per the calculated optimal Nyquist sampling parameters, and pinhole size was set to 1 Airy unit. Laser power, detector filtering/gating, and gain were adjusted to maximize signal while also minimizing background signal, cross-fluorescence, and photobleaching. Using the ImageJ software, confocal stacks were then merged on maximal projection and subsequently processed to obtain accurate segmentation of the γH2AX foci with the *Adaptive Thresholding* plugin. Nuclear regions of interest (ROIs) were then defined and γH2AX foci for each nucleus counted with *Analyze Particles*. To obtain nuclear morphology parameters, ImageJ shape descriptors were applied on those same ROIs. Nuclear atypia classification into “normal”, “mis-shapen”, “micronuclei” and “multinucleated” categories was performed as previously described [27].

Measurement of Reactive Oxygen Species (ROS)

ROS measurement on cultured MEFs was performed using a ROS-sensitive dye, 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA; Invitrogen), as an indicator. Briefly, *Igf1r*^{-/-} and *Igf1r*^{fl/fl} MEFs were seeded at 25000 cells/well in a 96-well plate with 100 μ L of culture medium (DMEM supplemented with 10% FBS and 1% Pen/Strep). 24 h later, medium was removed, cells were washed once with HBSS and incubated in 100 μ L of HBSS-H2DCFDA (10 μ M) with increasing concentrations of H₂O₂ (0, 8, 20, 50, 125 and 312.5 μ M). Finally, fluorescence intensity was measured after 15, 30, 45 and 60 min of incubation at 485/535 nm (excitation/emission), using a microplate reader (Synergy 4; BioTek, Winooski, VT). Data presented correspond to 30 min of incubation and were represented as fluorescence intensity ratio over control (*Igf1r*^{fl/fl} MEFs without H₂O₂ addition). The experiment was repeated three times with five replicates per condition.

Western blotting

Superior mouse lung lobes were solubilized in RIPA lysis buffer (Thermo Fisher Scientific, Inc.) supplemented with protease and phosphatase inhibitor cocktails (Roche). To perform Western blot assays, 20 μ g of lung protein samples or 12 μ g of cell extracts were separated in SurePage 4-20% Bis-Tris Gels (GenScript Biotech, Piscataway, NJ) and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Total protein signals were obtained using the Revert Total Protein Stain kit (LI-COR Biosciences, Lincoln, NE) according to manufacturer's instructions, and imaged with a ChemiDoc imager (Bio-Rad). Membranes were then washed and blocked with TBS with 0.1% tween and 5% non-fat milk or BSA for 1 hour at RT, and incubated overnight with the following primary antibodies in blocking solution: p21 (1:500; clone M19; Cat. No. sc-471; Santa Cruz Biotechnology), ACSS1 (1:1000; Cat. No. 17138-1-AP; Proteintech), PDK2 (1:500; Cat. No. ab68164; Abcam), PDHA (1:1000; Cat. No. ab1103330; Abcam), p-PDHA (Phospho S293; 1:1000; Cat. No. ab177461), TOM20 (1:2000; Cat. No. 11802-1-AP; Proteintech). For p21, ACSS1, PDK2 and TOM20 western blots, following washing, the membranes were incubated with horseradish peroxidase-conjugated

goat anti-rabbit antibody (Cat. No. 7074; Cell Signaling Technology) at a 1:10000 dilution. Chemiluminescence signals were obtained using Advanced ECL Western Blot Substrate (NZYTech). For p-PDHA/PDHA western blot, primary antibodies were applied to the same membrane, which, after washing was incubated with anti-rabbit IRDye 680 (Cat. No. 926-32221) and anti-mouse IRDye 800 (Cat. No. 926-32221) fluorescent antibodies (LI-COR Biosciences), both at 1:5000 dilution. Chemiluminescence or fluorescence signals were detected with a ChemiDoc imager (Bio-Rad), and finally quantified and normalized to total protein signals using the Image Lab software (Bio-Rad).

Lung genomic DNA extraction and global DNA methylation assay

Lung genomic DNA was obtained from the accessory lung lobes using the DNeasy® Blood & Tissue Kit (Qiagen), following manufacturer's guidelines. DNA quantification and quality were determined with the ND-100-Nanodrop Spectrophotometer. The global DNA methylation levels were obtained with an ELISA commercial kit (5-mC DNA ELISA Kit; cat no. D5325; Zymo Research, Irvine, CA) as previously described [28]. Briefly, 250 ng of lung genomic DNA were diluted in a final volume of 250 µL of 5-mC coating buffer and incubated at 98°C for 5 min. After denaturation, 100 µL of solution containing 100 ng of DNA were transferred to the plate in duplicated wells and incubated at 37°C for one hour. Samples were washed three times with 200 µL of 5-MC ELISA buffer and further incubated at 37°C for 30 min in 200 µL of the same buffer. After discarding this buffer, samples were incubated with 100 µL of the antibody mix (Anti-5-methylcytosine and secondary) in ELISA Buffer at 37°C for one hour, wells were washed and incubated with the developer for 30 min, and absorbance read at 450 nm in a PolarStar Omega Spectrophotometer (BMG Lab Tech, Ortenberg, Germany). Quantification of global DNA methylation was obtained from calculating the amount of methylated cytosines in the samples (5 mC) relative to a standard curve including negative and positive controls following manufacturer's protocol.

References

1. Lopez IP, Rodriguez-de la Rosa L, Pais RS, Piñeiro-Hermida S, Torrens R, Contreras J, et al. Differential organ phenotypes after postnatal Igf1r gene conditional deletion induced by tamoxifen in UBC-CreERT2; Igf1r fl/fl double transgenic mice. *Transgenic Res.* 2015; 24:279-94.
2. Piñeiro-Hermida S, Lopez IP, Alfaro-Arnado E, Torrens R, Iniguez M, Alvarez-Erviti L, et al. IGF1R deficiency attenuates acute inflammatory response in a bleomycin-induced lung injury mouse model. *Sci Rep.* 2017; 7:4290.
3. Andrews S. FastQC: a quality control tool for high throughput sequence data. 2010. <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>.
4. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics.* 2014; 30:2114-20.
5. Kim D, Paggi JM, Park C, Bennett C, Salzberg SL. Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nat Biotechnol.* 2019; 37:907-15.
6. Okonechnikov K, Conesa A, Garcia-Alcalde F. Qualimap 2: advanced multi-sample quality control for high-throughput sequencing data. *Bioinformatics.* 2016; 32:292-4.
7. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. *Bioinformatics.* 2009; 25: 2078-9.
8. Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics.* 2014; 30:923-30.
9. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 2014; 15:550.
10. Varet H, Brillet-Gueguen L, Coppee JY, Dillies MA. SARTools: A DESeq2- and EdgeR-Based R Pipeline for Comprehensive Differential Analysis of RNA-Seq Data. *PLoS One.* 2016; 11:e0157022.
11. Durinck S, Spellman PT, Birney E, Huber W. Mapping identifiers for the integration of genomic datasets with the R/Bioconductor package biomaRt. *Nat Protoc.* 2009; 4:1184-91.
12. Kolde R. Pheatmap: pretty heatmaps. R Package Version 1.0.12. 2019. <https://cran.r-project.org/web/packages/pheatmap>
13. Blighe K, Rana S, Turkes E, Ostendorf B, Grioni A & Lewis M. EnhancedVolcano: Publication-ready volcano plots with enhanced colouring and labeling. R package version 1.20.0. 2023. <https://github.com/kevinblighe/EnhancedVolcano>
14. Yu G, Wang LG, Han Y, He QY. clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS.* 2012; 16:284-7.
15. Yu G, He QY. ReactomePA: an R/Bioconductor package for reactome pathway analysis and visualization. *Mol Biosyst.* 2016; 12:477-9.

16. Morgan M, Pagès H, Obenchain V & Hayden N. Rsamtools: Binary alignment (BAM), FASTA, variant call (BCF), and tabix file import. R package version 2.18.0. 2023. <https://bioconductor.org/packages/Rsamtools>.
17. Wolf FA, Angerer P, Theis FJ. SCANPY: large-scale single-cell gene expression data analysis. *Genome Biol.* 2018; 19(1):15.
18. Melms JC, Biermann J, Huang H, Wang Y, Nair A, Tagore S, et al. A molecular single-cell lung atlas of lethal COVID-19. *Nature.* 2021; 595:114–119.
19. The Tabula Muris Consortium. Single-cell transcriptomics of 20 mouse organs creates a Tabula Muris. *Nature.* 2018; 562:367–372.
20. Strunz M, Simon LM, Ansari M, Mattner LF, Angelidis I, Mayr CH, et al. Alveolar regeneration through a Krt8+ transitional stem cell state that persists in human lung fibrosis. *Nat Commun.* 2020; 11:3559.
21. Hoffman P, Satija R. SeuratDisk: interfaces for HDF5-based single cell file formats. R Package Version 0.9021. 2023. <https://github.com/mojaveazure/seurat-disk>.
22. Matute-Bello G, Downey G, Moore BB, Groshong SD, Matthay MA, Slutsky AS, et al. An official American Thoracic Society workshop report: features and measurements of experimental acute lung injury in animals. *Am J Respir Cell Mol Biol.* 2011; 44:725–738.
23. Liu JP, Baker J, Perkins AS, Robertson EJ, Efstratiadis A. Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). *Cell.* 1993; 75:59–72.
24. Furda AM, Bess AS, Meyer JN, Van Houten B. Analysis of DNA damage and repair in nuclear and mitochondrial DNA of animal cells using quantitative PCR. *Methods Mol Biol.* 2012; 920:111–132.
25. Gonzalez-Hunt CP, Rooney JP, Ryde IT, Anbalagan C, Joglekar R, Meyer JN. PCR-based analysis of mitochondrial DNA copy number, mitochondrial DNA damage, and nuclear DNA damage. *Curr Protoc Toxicol.* 2016; 67:20.11.1–20.11.25.
26. Chaudhry A, Shi R, Luciani DS. A pipeline for multidimensional confocal analysis of mitochondrial morphology, function, and dynamics in pancreatic β -cells. *Am J Physiol Endocrinol Metab.* 2020; 318:E87–E101.
27. Hart M, Adams SD, Draviam VM. Multinucleation associated DNA damage blocks proliferation in p53-compromised cells. *Commun Biol.* 2021; 4:451.
28. Ivorra C, Fraga MF, Bayón GF, Fernández AF, Garcia-Vicent C, Chaves FJ, et al. DNA methylation patterns in newborns exposed to tobacco in utero. *J Transl Med.* 2015; 13.