

Additional file 2 for

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

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Supplementary Figures

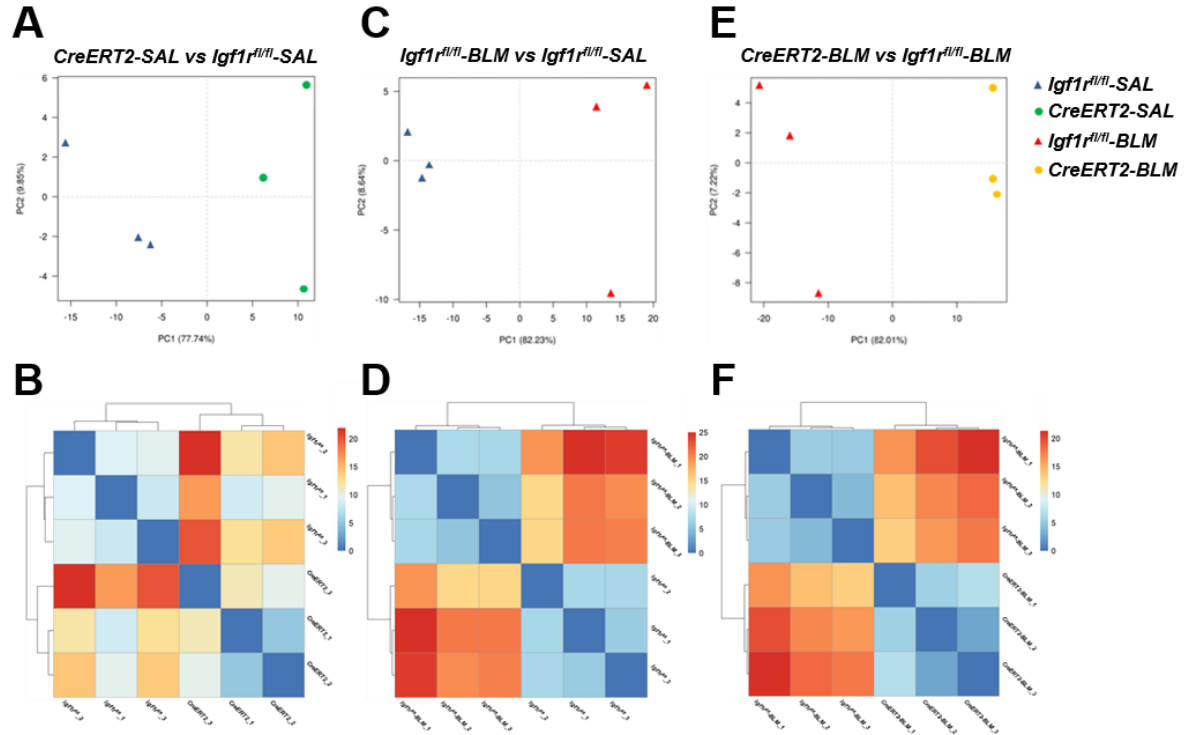


Fig. S1. Clustering of RNA-seq experimental groups. Principal component analysis (top panel) and hierarchical clustering (bottom panel) of lung RNA-Seq differential expression data. (**A, B**) *CreERT2-SAL vs Igf1r^{fl/fl}-SAL* mice. *Igf1r^{fl/fl}-SAL* and *CreERT2-SAL* samples clustered by genotype, although the inter-sample variances and Euclidean distances were high. (**C, D**) *Igf1r^{fl/fl}-BLM, vs. Igf1r^{fl/fl}-SAL* mice. *Igf1r^{fl/fl}-SAL* and *Igf1r^{fl/fl}-BLM* samples were strongly clustered by treatment in both PCA and hierarchical clustering. (**E, F**) *CreERT2-BLM vs. Igf1r^{fl/fl}-BLM* mice. *Igf1r^{fl/fl}-BLM* and *CreERT2-BLM* samples displayed very significant inter-group variances and distances.

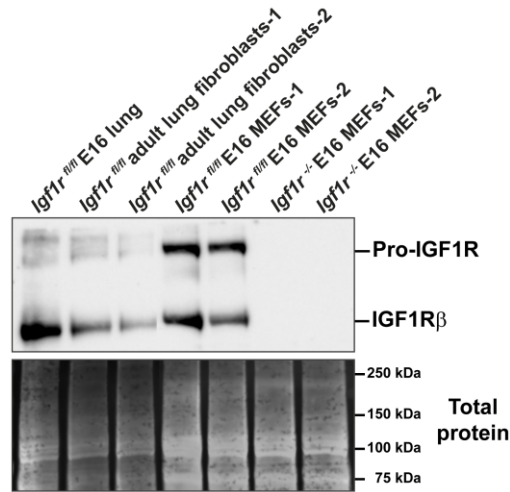
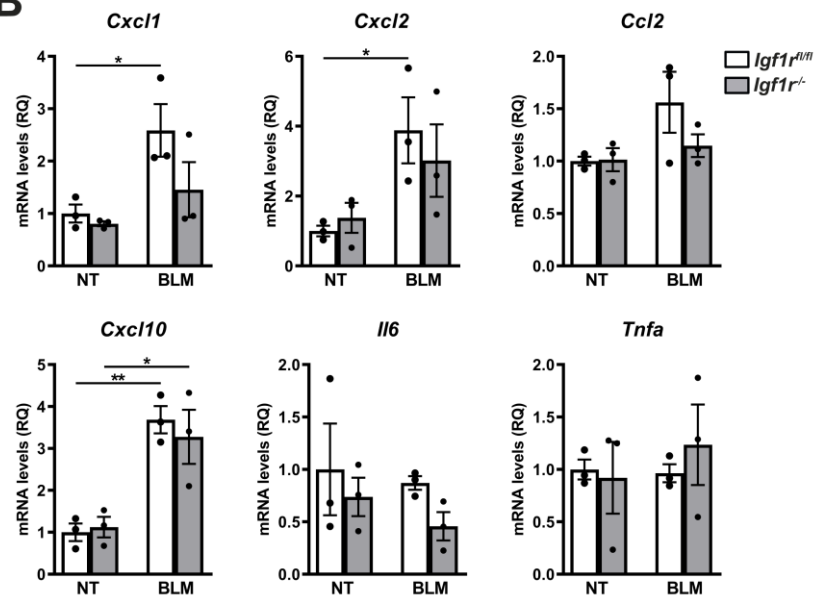
A**B**

Fig. S2. IGF1R protein expression in mouse primary fibroblasts in culture and mRNA expression levels of inflammatory cytokines in MEFs exposed to BLM. (A) IGF1R β expression analyzed by Western blot from protein extracts of *Igf1r^{fl/fl}* embryonic lung at embryonic day 16 (E16) (28 μ g), *Igf1r^{fl/fl}* primary adult lung fibroblasts (19 μ g) and *Igf1r^{fl/fl}/Igf1^{-/-}* primary MEFs (28 μ g). (B) Real-time qPCR analyses of mRNA extracted from primary *Igf1r^{fl/fl}* and *Igf1r^{-/-}* MEFs, either not treated (NT) or treated with BLM (10 μ g/mL) for 24 h. n = 3 experiments per group. Data are expressed as mean $\pm$ SEM. *p < 0.05; **p < 0.01 (Student's *t*-test or Mann-Whitney *U* test).

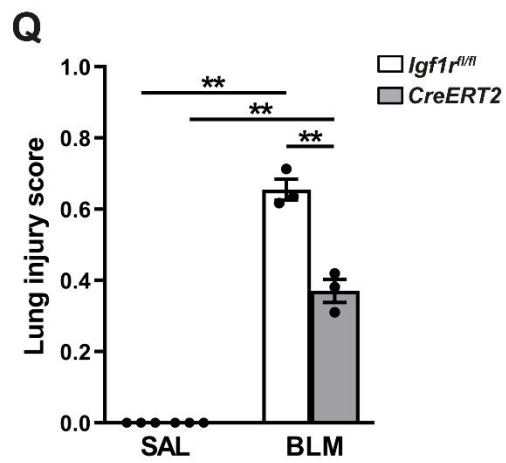
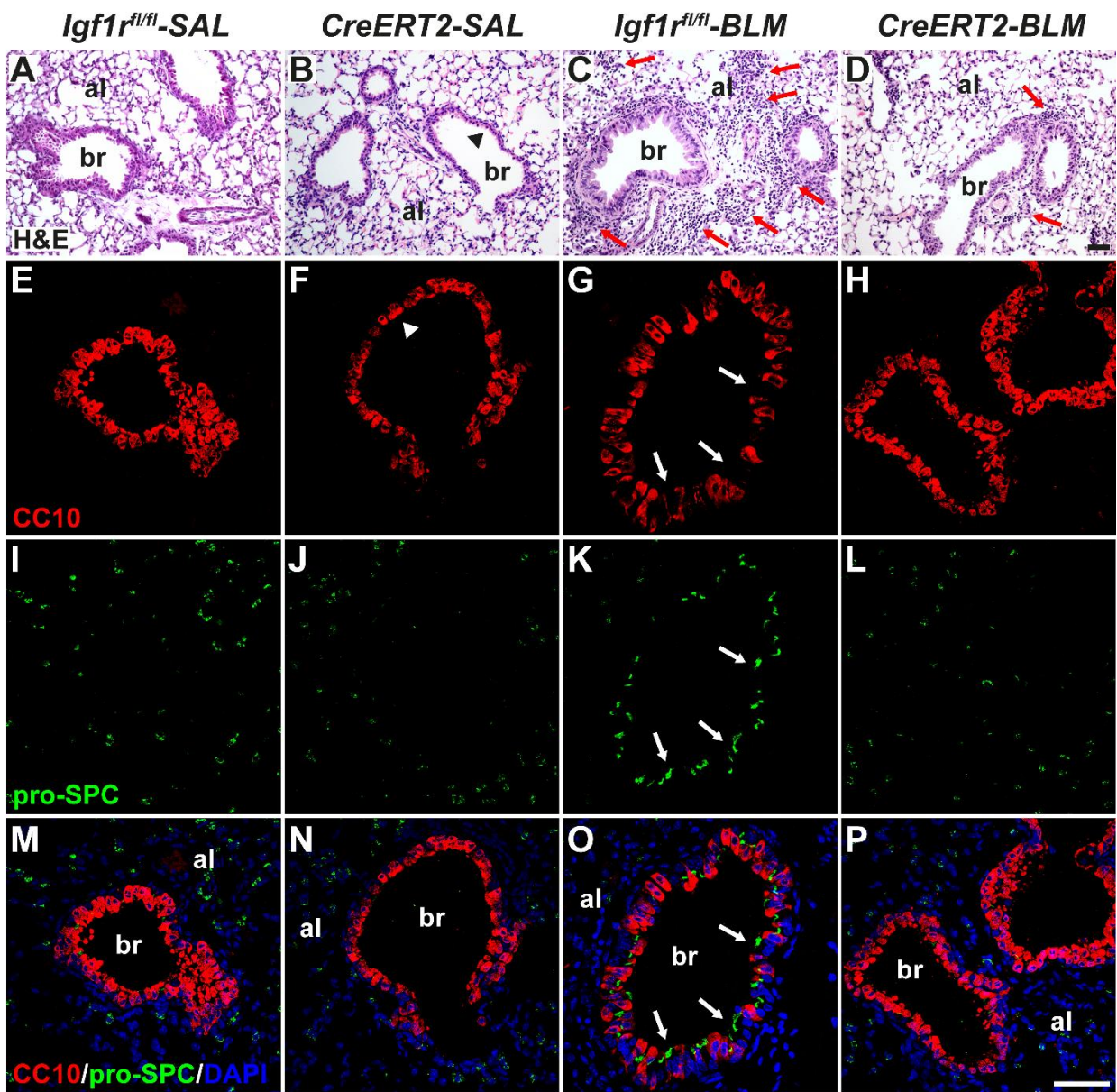


Fig. S3. Reversion of the inflammatory phenotype and protective effect on bronchiolar and type 2 alveolar epithelial cells caused by the BLM-mediated acute lung injury. (A-D) Representative images of H&E stained lung sections of *Igf1^{fl/fl}*-SAL (A), *CreERT2*-SAL (B), *Igf1^{fl/fl}*-BLM (C) and *CreERT2*-BLM (D) mice showing a reduction in inflammatory cells infiltration in peribronchiolar and perivascular areas of *CreERT2*-BLM, more evident in *Igf1^{fl/fl}*-BLM mice than in *CreERT2*-BLM lungs (red arrows). (E-P) Representative images of CC10 (E-H), pro-SPC (I-L) and merged staining on lung sections of the four groups (M-P). *Igf1^{fl/fl}*-BLM lungs (G) presented a decrease in the intensity and density of staining in CC10⁺ club cells, resulting in a discontinuous pattern with the presence of abundant unlabeled cells intermixed in the bronchiolar epithelium (white arrows). CC10 staining of *CreERT2*-BLM lungs (H) was intense and showed a continuous pattern, resembling that of *Igf1^{fl/fl}*-SAL controls (E). CC10 staining also revealed the thinning of the bronchiolar epithelium in *CreERT2*-SAL lungs (B and F) (arrowheads). Density of pro-SPC⁺ stained alveolar cells was reduced in *CreERT2*-SAL alveolar areas (J). BLM treatment further reduced the presence of pro-SPC⁺ cells in *Igf1^{fl/fl}*-BLM alveoli (K), presence that was recovered in *CreERT2*-BLM alveoli (L). pro-SPC staining was barely detected in the bronchioles of *Igf1^{fl/fl}*-SAL (I, M) and *CreERT2*-SAL (J, N) lungs. However, the frequency of pro-SPC⁺ cells was increased in *Igf1^{fl/fl}*-BLM bronchioles (K, O), where pro-SPC did not co-localize with CC10 staining (white arrows). *CreERT2*-BLM lungs did not show this pattern (L, P) being similar to that of *CreERT2*-SAL (J, N). Scale bars: 50 μ m. *al*: alveolar zones. *br*: bronchiolar zones. (Q) Determination of lung injury score as described by Matute-Bello et al. (*Am J Respir Cell Mol Biol* **2011**, 44, 725-738). Increased lung injury score in *Igf1^{fl/fl}*-BLM mice was significantly reduced in *CreERT2*-BLM mice. Data are expressed as mean $\pm$ SEM (n = 3 per group). **p < 0.01 (Student's *t*-test).

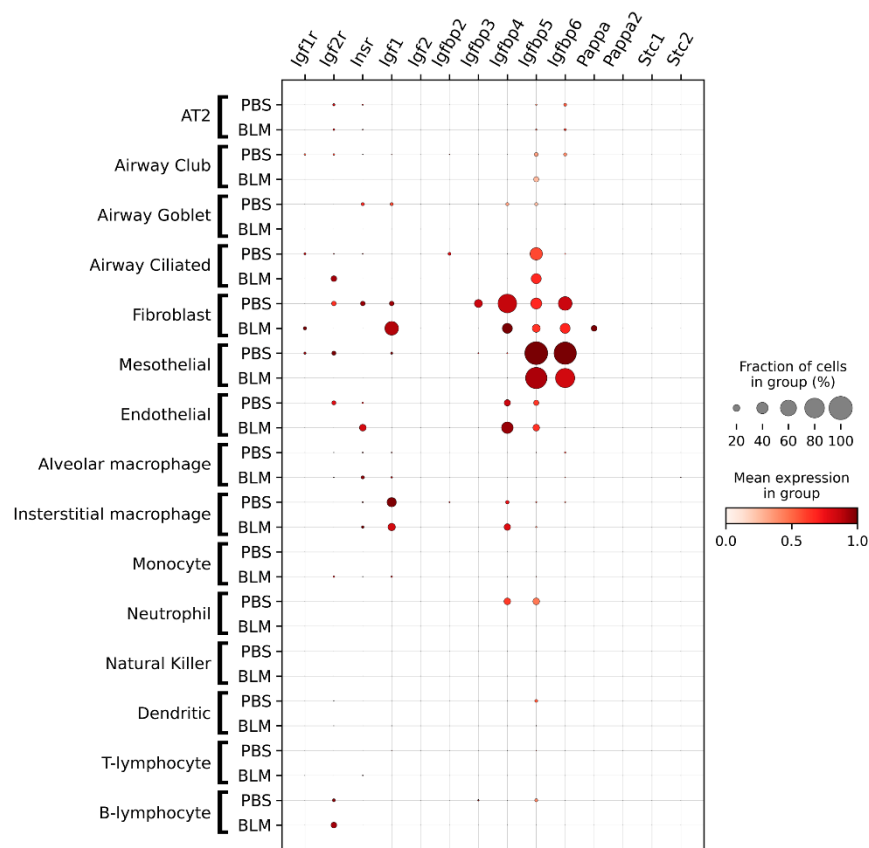


Fig. S4. Cell-type specific mRNA expression of IGF system genes in control and BLM-injured lungs. The dot plot shows expression patterns of IGF system genes across annotated lung cell types and conditions in PBS-treated controls and BLM-challenged lungs three days after BLM treatment (GSE141259 dataset, *Nat Commun* **2020**, *11*, 3559). Dot size indicates the percentage of cells with detected gene expression per cell type on each group; dot color represents the gene-wise scaled mean expression among expressing cells. Only subgroups with detected gene expression in $\geq 1\%$ of cells are included. See Supplementary Methods in Additional File 1 for more details on data filtering and visualization.

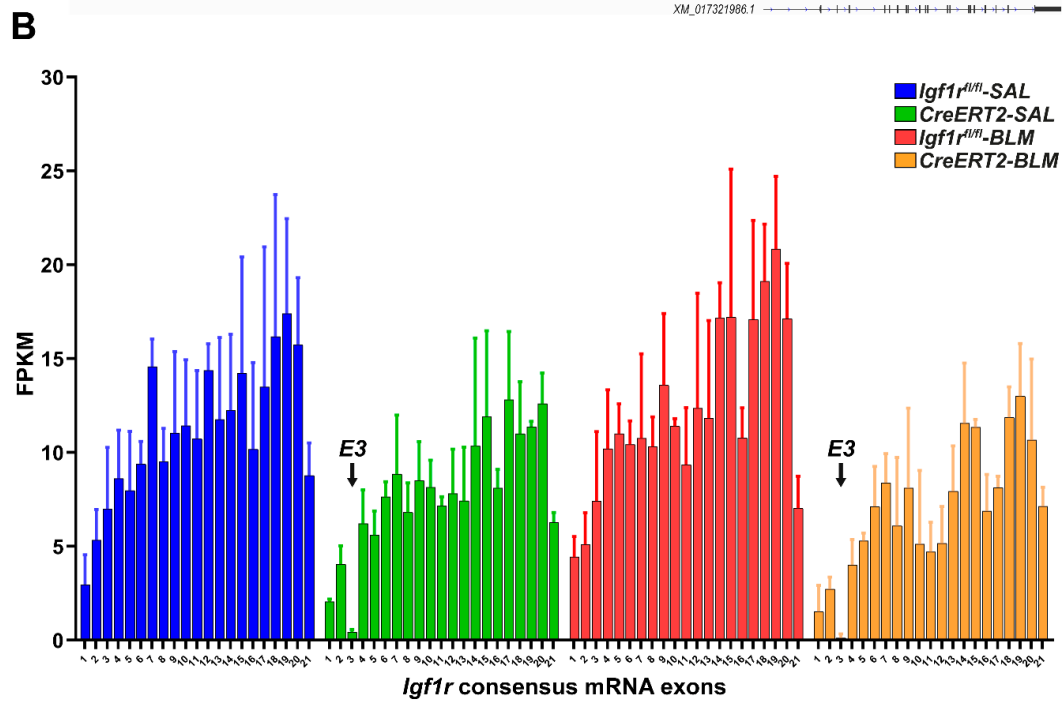
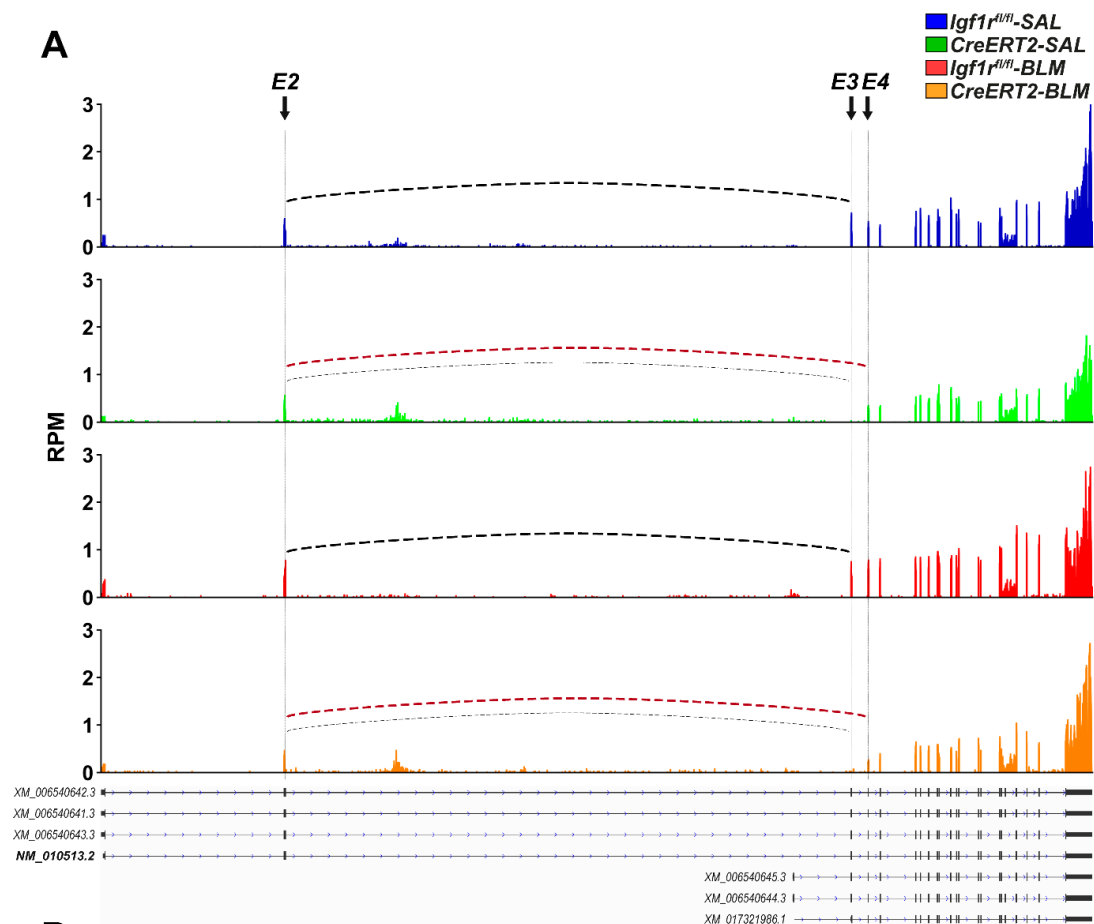


Fig. S5. *Igf1r* full-gene and exon-specific coverage. (A) Read coverage along the full transcribed sequence of the *Igf1r* gene. Reads overlapping each position were counted, normalized to reads per million mapped reads (RPM) in all mouse lung RNA samples and averaged by group. RefSeq *Igf1r* mRNA transcript variants annotated to the *Mus musculus* genome (*mm38.p6* release) are depicted below the x-axis and mean RPM for each nucleotide position in each group represented with vertical colored bars. “E2”, “E3” and “E4” labels point to respective locations of these exons in the *Igf1r* RefSeq curated mRNA sequence (NM_010513.2; highlighted in bold). Black dashed lines indicate the normal splicing event from exon 2 to exon 3 that occurs in *Igf1r^{fl/fl}-SAL* and *Igf1r^{fl/fl}-BLM* lungs, as well as in the few *CreERT2-SAL* and *CreERT2-BLM* lung cells which did not undergo TMX-induced deletion. However, in *CreERT2-SAL* and *CreERT2-BLM* mice, where exon 3 is mostly deleted, splicing can jump to exon 4 (indicated by red dashed lines), rendering transcripts with a successful junction of exons 2 and 4. Note that there is also an over-abundance of reads along an intronic region between exons 2 and 3 in these samples, which may be rendering additional aberrant transcripts. (B) Vertical colored bars indicate exon-specific fragments per kilobase per million mapped reads (FPKM) in the four groups of mice, from exon 1 to 21 of the *Igf1r* RefSeq NM_010513.2 mRNA. Bars indicate standard deviation. “E3” labels point to exon 3 depletion of FPKM in *CreERT2-SAL* and *CreERT2-BLM* mice. Note also the general trend toward reduced FPKM in the lungs of both groups of IGF1R-deficient mice.

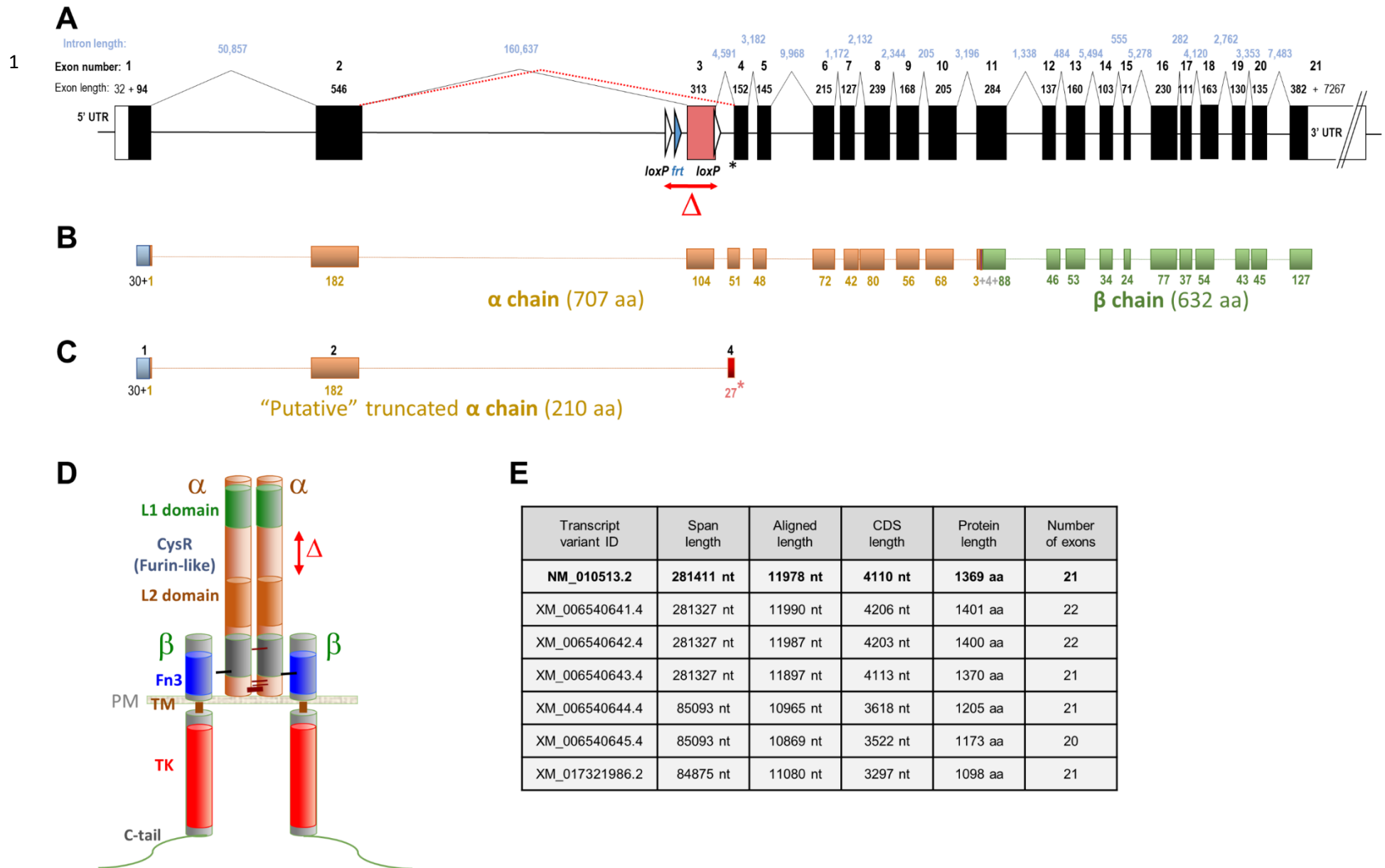


Fig. S6. Schematic representation of the *Igf1r* gene showing exon and intron distribution, and its putative encoded protein before and after Cre-mediated exon 3 deletion in *Igf1r^{fl/fl}* mice. (A)

Representation of the mouse *Igf1r^{fl/fl}* gene showing exon and intron as well as peptide sequence distribution in extension, in base pairs and amino acid respectively, without considering alterations caused by the insertion of the *loxP* and *frt* sites. White boxes indicate noncoding regions (5' and 3'UTR), whereas black boxes represent the ORF and coding exons, which are consecutively numbered and their nucleotide extension specified above. Exon and intron extension are indicative, not in scale. The red box represents exon 3, the blue arrowhead indicates a *frt* site location, and white arrowheads two *loxP* sites flanking exon 3 in the *Igf1r^{fl/fl}* mutation described by Klötting *et al.* (*Diabetes* 57:2074, 2008). Solid lines represent constitutive normal splicing, and red dashed lines the possible alternative RNA processing in case of exon 3 deletion after *Cre* recombination in *loxP* sites (Δ and two headed red arrow). The asterisk under exon 4 refers to the situation of a STOP codon in case of a splicing event occurring between exon 2 and 4 after Cre-mediated exon 3 deletion. (Sequences referred to mRNA NM_010513.2 and protein NP_034643.2 RefSeq entries in Gene Bank data, 2014). (B) Encoded normal IGF1R (upper scheme) showing α (brown) and β (green) chains length (707 and 632 aa, respectively) (Abbot *et al* 1996, *J Biol. Chem.* 267: 10759; See Girnita *et al.* 2014, *Cell. Mol. Life Sci.* 71, 2403–2427 for the human IGF1R protein structure). The first 30 aa (grey box) correspond to the signal peptide. The 4 aa between α and β chains are cleaved by furin and carboxypeptidase D proteases during post-translational processing of IGF1R protein precursor (Han *et al.* 2020, *Nature* 580 (7801), 136-141). (C) Putative truncated α chain (210 aa) that can be generated after exon 3 deletion. Aberrant splicing from exon 2 to 4 causes a change in nucleotide reading frame that encodes for a STOP codon at amino acid position 211, rendering 27 aberrant amino acids at the 3' end of the peptide. (D) IGF1R structure–function relationship representing the $\alpha_2\beta_2$ molecular composition of the receptor anchored to the cellular plasma membrane (PM). Main peptide domains are indicated in different colours: L1, CysR (Furin-like) and L2 for the α subunit; and fibronectin3 (Fn3), transmembrane (TM), tyrosin kinase (TK) and C tail domains in the β subunit (Kavran *et al.* 2014 *eLife* 3, e03772 and Blyth & Forbes 2020, *Cells* 9, 2276 for more detailed information on the human IGF1R protein structure). The sequence corresponding to the deletion of exon 3 (Δ and two headed red arrow) encodes that of the receptor that binds the IGF ligands (Blyth & Forbes 2020, *Cells* 9, 2276). (E) RefSeq *Igf1r* mRNA transcript variants annotated to the *mus musculus* genome (*mm38.p6* release).

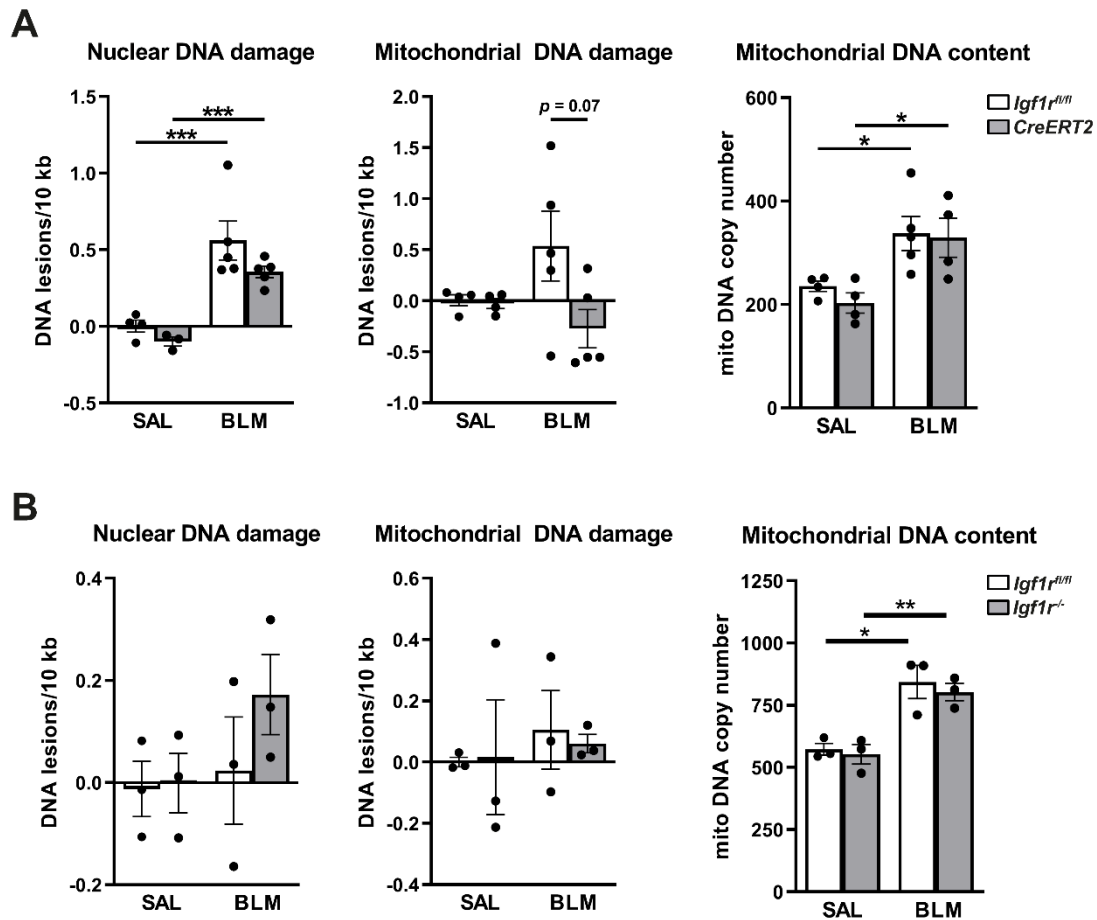


Fig. S7. Nuclear and mitochondrial DNA damage and mitochondrial DNA copy number in mice lungs and MEFs after BLM challenge. (A) Quantification of DNA lesion frequency in nuclear (left) and mitochondrial (middle) DNA, and mitochondrial DNA copy number (right) in lungs of the four experimental groups of mice. (B) Equivalent measurements in *Igf1^{fl/fl}* and *Igf1^{-/-}* MEFs, after either no treatment (NT) or treatment with BLM (10 μ g/mL) for 24 h. Lesion frequency was assessed by long-amplicon quantitative PCR (LA-qPCR). Mitochondrial copy number was determined by RT-qPCR and used to normalize mitochondrial DNA lesion frequency data. All data are represented as mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Student's *t*-test or Mann-Whitney *U* test).

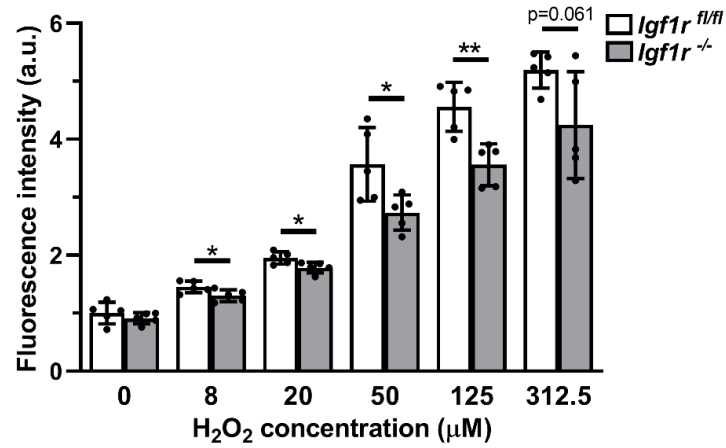


Fig. S8. Primary MEFs lacking IGF1R show reduced ROS levels upon H₂O₂ treatment. ROS levels were measured on *Igf1r*^{-/-} and *Igf1r*^{+/+} MEFs treated with increasing concentrations of H₂O₂, using the ROS-sensitive dye, 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) as an indicator. Data presented correspond to 30 min of incubation and are represented as H2DCFDA fluorescence intensity ratio over control (*Igf1r*^{+/+} MEFs without H₂O₂ addition). Representative results from three experiments with independent cell cultures are shown (mean ± SD; n = 5 per group and condition) **p* < 0.05; ***p* < 0.01 (Student's *t*-test or Mann-Whitney *U* test).

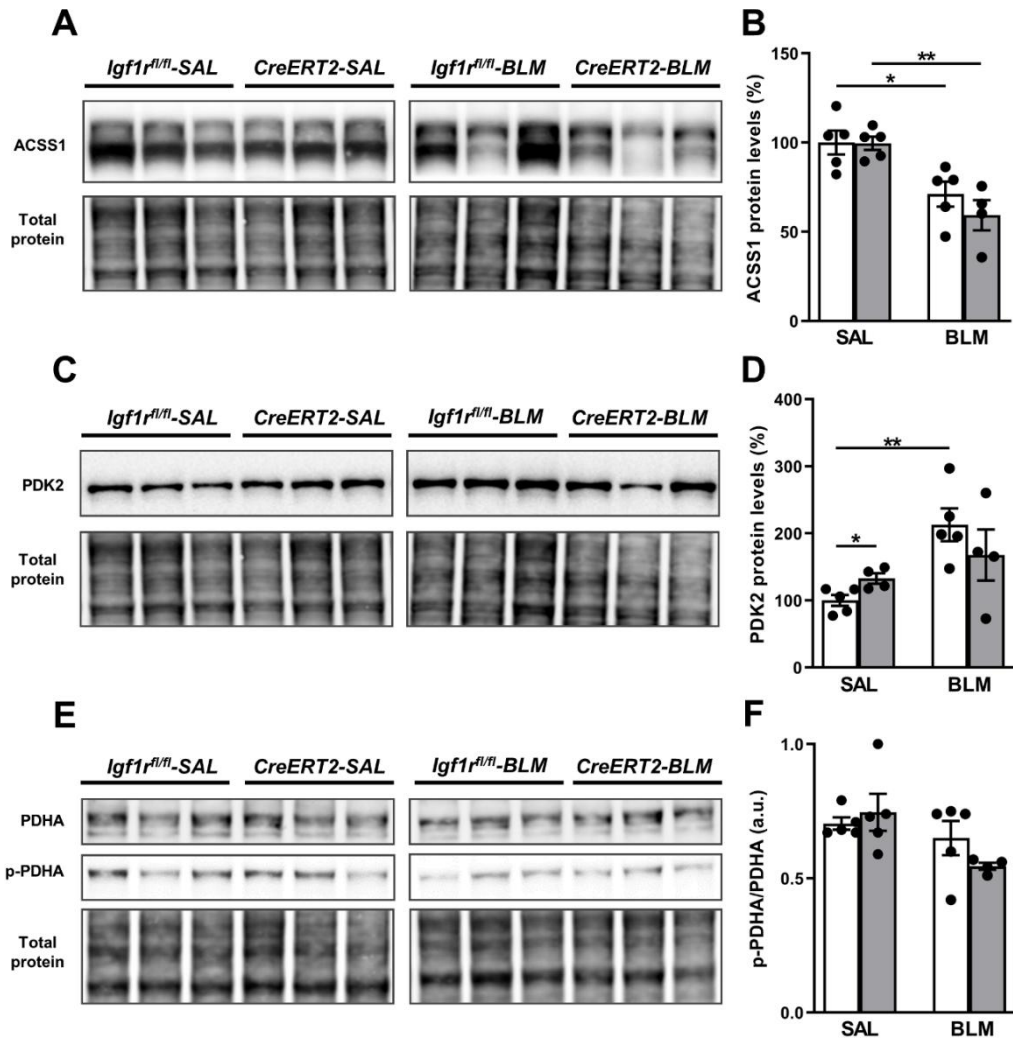


Fig. S9. Protein expression analysis of key metabolic enzymes assessed by western blot.

Western blotting was used to determine levels of ACSS1 (A-B), PDK2 (C-D) and PDHA phosphorylation ratio (E-F) in lung homogenates of the four experimental groups of mice (n = 4-5 per group). (B, D) Quantification of chemiluminescence signals in (A, C) respectively, normalized to total protein and presented as percentage compared to *Igf1^{fl/fl}* controls. (F) Ratio of phospho-PDHA to total PDHA chemiluminescence signals in (E). Data are represented as mean ± SEM. * $p < 0.05$; ** $p < 0.01$; (Student's *t*-test). a.u.: arbitrary units.

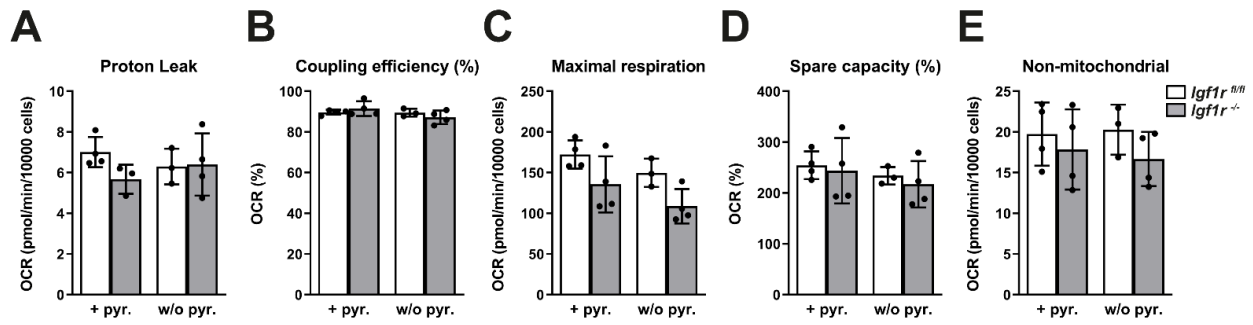
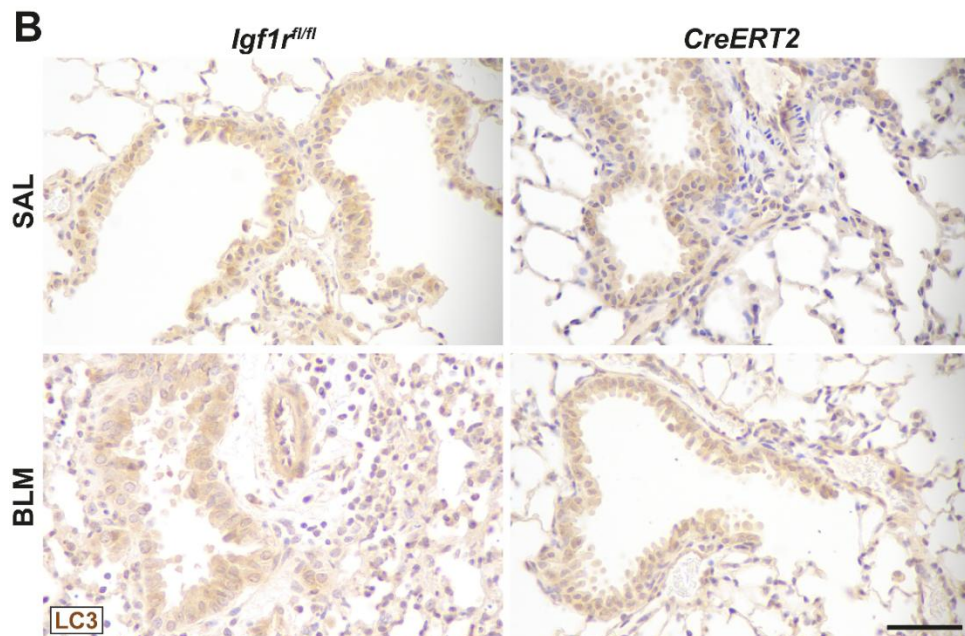
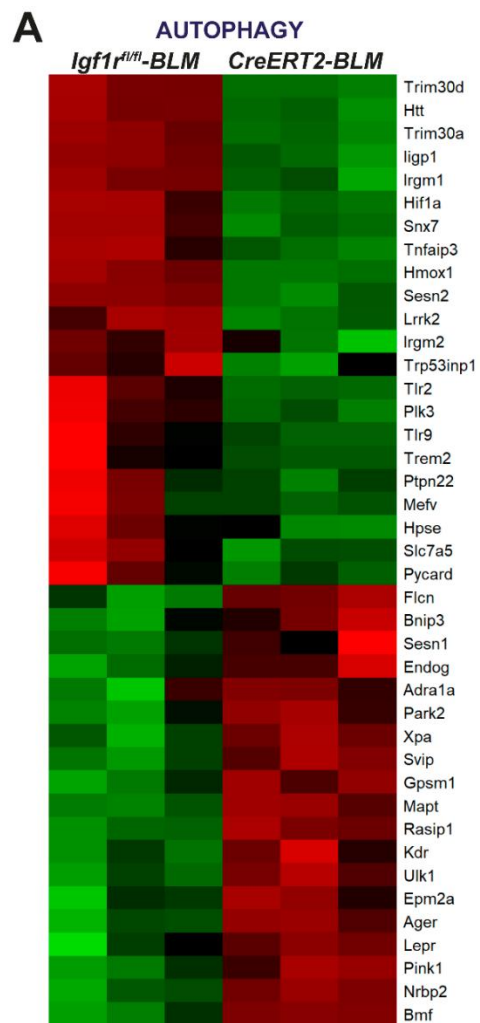


Fig. S10. Additional Seahorse XF metabolic parameters in *Igf1r^{fl/fl}* and *Igf1r^{-/-}* MEFs. Oxygen consumption rate (OCR) was monitored in *Igf1r^{fl/fl}* and *Igf1r^{-/-}* MEFs under basal conditions and after the injection of electron transport chain modulators, with (pyr.) or without (w/o pyr.) pyruvate in the medium. (A) Proton leak. (B) Coupling efficiency. (C) Maximal respiration. (D) Spare respiratory capacity (expressed as % of maximal over basal respiration). (E) Non-mitochondrial respiration. In agreement with the similar pattern of basal and ATP-linked respiration in all conditions (Fig. 6D-E), there was no difference in respiration due to proton leak and, by extension, in the efficiency of electron chain coupling to ATP energy production. Although statistical significance was not reached, the differential pattern of maximal respiration (after addition of FCCP) showed a similar trend to basal respiration across all conditions. There were no differences on the % reserve respiratory capacity between any of the conditions, potentially ruling out any effect of IGF1R absence on the oxidative capacity of the electron transport chain in these conditions. Data are presented as mean $\pm$ S.D. (n = 3-4 replicates per group).



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72 **Fig. S11. Altered mRNA expression of autophagy-related genes in BLM-challenged *CreERT2***
73 **lungs and LC3 immunostaining. (A)** Heatmap showing normalized RNA expression levels (gene
74 centered scale) of genes with central roles in autophagy between *Igf1^{fl/fl}-BLM* and *CreERT2-BLM*
75 samples. **(B)** LC3 IHQ in lungs from the same four groups of mice did not show differences. Scale bar:
76 50 μ m.

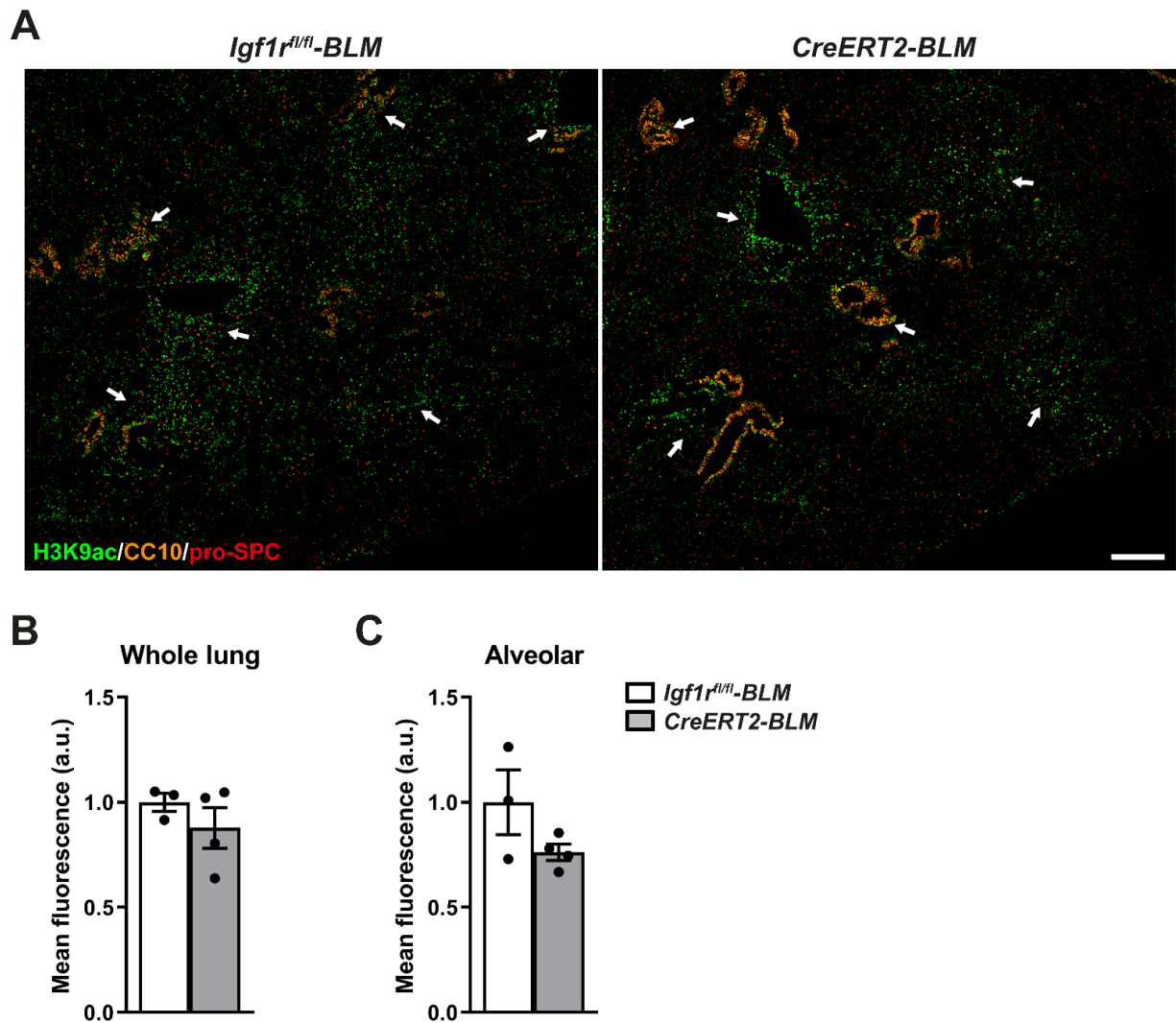


Fig. S12. Histone 3 acetylation in lungs of BLM-challenged mice. (A) H3K9ac (green), CC10 (orange) and pro-SPC (red) co-immunostaining on lung sections from *Igf1^{fl/fl}*-BLM and *CreERT2*-BLM mice. Lungs exhibit heterogeneous H3K9ac staining pattern, with cells generally presenting higher nuclear fluorescence intensity on certain lung areas, particularly surrounding blood vessels and, to a lesser extent, on bronchioles (arrows). **(B-C)** Quantification of H3K9ac mean fluorescence intensity in the whole lung section **(B)** or in alveolar-specific regions **(C)**. H3K9ac nuclear fluorescence intensity was measured using DAPI staining to delimit the measuring surface and to calculate mean fluorescence values. Data are presented as mean ± SEM (n = 3-4 per group). Scale bar: 200 μm.