Year: 2021

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RESEARCH ARTICLE

THE **FASEB** JOURNAL

OTUB1 regulates lung development, adult lung tissue homeostasis, and respiratory control

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Abstract

OTUB1 is one of the most highly expressed deubiquitinases, counter-regulating the two most abundant ubiquitin chain types. OTUB1 expression is linked to the development and progression of lung cancer and idiopathic pulmonary fibrosis in humans. However, the physiological function of OTUB1 is unknown. Here, we show that constitutive whole-body *Otub1* deletion in mice leads to perinatal lethality by asphyxiation. Analysis of (single-cell) RNA sequencing and proteome data demonstrated that OTUB1 is expressed in all lung cell types with a particularly high expression during late-stage lung development (E16.5, E18.5). At E18.5, the lungs of animals with *Otub1* deletion presented with increased cell proliferation that decreased saccular air space and prevented inhalation. Flow cytometry-based analysis of E18.5 lung tissue revealed that *Otub1* deletion increased proliferation of major lung parenchymal and mesenchymal/other non-hematopoietic cell types. Adult mice with conditional whole-body *Otub1* deletion (wb*Otub1*^del/del) also displayed increased lung cell proliferation in addition to hyperventilation and failure to adapt the respiratory pattern to hypoxia. On the molecular level, *Otub1* deletion enhanced mTOR signaling in embryonic and adult lung tissues. Based on these results, we propose that OTUB1 is a negative regulator of mTOR signaling with essential functions for lung cell proliferation, lung development, adult lung tissue homeostasis, and respiratory regulation.

1 | INTRODUCTION

The ubiquitin system plays an essential role in most cellular signaling pathways and, hence, in cell and tissue homeostasis.[1] The ubiquitin system regulates cellular proteins by conjugation of ubiquitin proteins (Ub) via Ub-conjugating enzymes (E1s, E2s, E3s).[1] Deubiquitinases (DUBs) are negative regulators of the Ub system, removing or trimming Ub chains and regulating the available pool of free Ub.[1,2] DUBs recently gained interest as therapeutic targets for various diseases.[1] However, the function and regulation of many DUBs is insufficiently understood. The DUB ovarian tumor (OTU) domain-containing ubiquitin aldehyde binding protein 1 (OTUB1) shows one of the highest cellular protein levels of all known approximately 100 DUBs.[1,3] OTUB1 exhibits a unique combination of canonical and non-canonical functions. Canonically, OTUB1 specifically cleaves K48-linked ubiquitin chains, preventing proteasomal degradation of substrate proteins.[4,5] In addition, OTUB1 inhibits E2 enzymes independent of its enzymatic activity, impeding K48- and K63-linked ubiquitin chain formation.[6-9] The function of OTUB1 is regulated in a complex manner by post-translational modifications,[10] including phosphorylation,[11] mono-ubiquitination,[12] and hydroxylation,[13] as well as by the interaction with E2 enzymes.[14,15]

In vitro analyses of OTUB1 have previously linked this DUB to the regulation of a range of different signaling pathways and proteins, including the DNA damage response, inflammatory responses, the anti-viral response, pro-fibrotic signaling, proliferation, and cell death.[10,16] Proteins that are known to be regulated by OTUB1 include p53, FOXM1, phosphorylated SMAD2/3, RAS, AKT, DEPTOR, RhoA, and TRAF3/6.[10,16] However, the function of OTUB1 *in vivo* is only beginning to be understood. Some central nervous system, hepatocyte survival, and immune cell-specific functions of OTUB1 have recently been analyzed in mice.[17-21] Cell-type-specific deletion of *Otub1* demonstrated that OTUB1 can regulate pro-inflammatory signaling in dendritic cells[18] and astrocytes,[19] in B cells,[17] T cells, and natural killer (NK) cells.[20] In hepatocytes, deletion of *Otub1* increased inflammation-induced cell death.[21] Thus, OTUB1 is an important regulator of immune cell activity and inflammation.

Several links exist between OTUB1 and lung pathologies. OTUB1 expression is increased in human lung adenocarcinoma and squamous cell carcinoma,[22,23] and OTUB1 was linked to triggering lung cancer development as well as to facilitating lung cancer progression.[22,23] Mechanistically, OTUB1 regulates RAS mono-ubiquitination in lung cancer, increasing RAS membrane localization and RAS activity.[22] Furthermore, OTUB1 expression is increased in human patients with idiopathic pulmonary fibrosis (IPF), which was functionally linked to increased lung fibrosis.[24] It was suggested that OTUB1 enhanced pulmonary fibrosis by upregulation of pro-fibrotic signaling through an OTUB1-mediated increase in the protein stability of FOXM1 and phosphorylated SMAD2/3.[24] In addition, OTUB1 showed the third-highest increase in its expression level in human idiopathic interstitial pneumonias (IIPs) compared to a total of 98 upregulated genes,[25] indicating relevance of OTUB1 in IPPs. Despite the existing links between OTUB1 and several lung pathologies, it remains unclear if OTUB1 plays a role during lung tissue development, homeostasis, and/or lung function. The relevance of OTUB1 for physiological tissue or whole-body function is generally unknown.

In summary, various links exist between OTUB1 and (lung) pathologies, leading to an increasing interest in OTUB1 as a possible novel therapeutic target. Therefore, we analyzed constitutive and conditional whole-body OTUB1 knockout mouse models and found that OTUB1 is essential for lung development and function.

2 | MATERIALS AND METHODS

2.1 | Mice

Mice with heterozygous *Otub1* deletion (*Otub1*^{+/−}, strain C57BL/6N-Otub1[tm1b(EUCOMM)Hmgu/H]) were purchased from the EUCOMM consortium (http://www.mousephenotype.org; via the EMMA repository, https://www.infrafrontier.eu/)[26] and bred to generate *Otub1*^{−/−} mice (Figure 1A). Mouse Contract Services were provided by the Mary Lyon Centre at MRC Harwell (http://www.har.mrc.ac.uk; UK). Wild-type mice (*Wt*; C57BL/6N, Charles River, MA, USA) were used for breeding with *Otub1*^{+/−} mice. Mice with induced whole-body *Otub1* (wb*Otub1*[del/del]) deletion were generated by crossing heterozygous mice with the potential of conditional *Otub1* deletion (pc*Otub1*[Wt/del]; strain *Otub1*[tm1a(EUCOMM)Hmgu/lcsORl]; purchased from the EUCOMM consortium via the EMMA repository and provided by the Institut Clinique

FIGURE 1 Gross developmental defects in $Otub1^{-/-}$ mice. (A) Genotype of mice with heterozygous or homozygous $Otub1$ deletion ($Otub1^{+/-}$ or $Otub1^{-/-}$). Image shows PCR results determining the genotype. En2SA, engrailed 2 gene splice acceptor; FRT, flippase recognition target; IRES, internal ribosome entry site; lacZ, β-galactosidase gene; NTC, non-template control; pA, polyadenylation; loxP, locus of X (cross)-over in P1; Wt, wild-type. (B) Total numbers of observed mice and gender distribution within each genotype following $Otub1^{+/-}$ mating (E18.5 and P0). (C) Relative distribution of genotypes and genders following $Otub1^{+/-}$ mating. The numbers in the graph indicate the total percentage of each genotype. (D) Representative images of *wild-type* (Wt), $Otub1^{+/-}$ and $Otub1^{-/-}$ mice (P0). (E) Body weight (BW) and body length relative to Wt littermates (E18.5; $Otub1^{+/-}$, $n = 94$; $Otub1^{-/-}$, $n = 27$). Data are presented as mean ± SEM. ***$p < .001$ by two-tailed Student's t-test

de la Souris, ICS, Illkirch, France) with *Flp* deleter mice (129S4/svJaeSor-Gt(Rosa)26Sor$^{tm1(FLP1)Dym}$), leading to the removal of the inserted *lacZ* gene and neomycin resistance cassette and resulting in mice with floxed *Otub1* exons 2 and 3 ($Otub1^{fl/fl}$; Figure S2A). $Otub1^{fl/fl}$ mice were crossed with mice carrying one copy of a Cre^{ERT2} transgene under the control of the ubiquitin C promoter (UBC-Cre^{ERT2}; strain B6.Cg-Ndor1$^{Tg(UBC-cre/ERT2)1Ejb/2J}$; purchased from The Jackson Laboratory; https://www.jax.org/strain/008085). For the induction of *Otub1* deletion, $Otub1^{fl/fl}$ x UBC-Cre^{ERT2} ($Otub1^{fl/fl}$ + Cre) mice were treated with 200 µg of tamoxifen/g BW (Sigma-Aldrich, St. Louis, MO, USA) by oral gavage on 5 consecutive days. Mice were maintained at a constant ambient temperature (21 ± 1°C) with 12-h light/dark cycles, standard chow diet (Provimi Kliba, Gossau, Switzerland) and water available *ad libitum* under optimal hygienic conditions, unless indicated otherwise. Both genders were used for the investigations of developmental phenotypes. For adult mice, female mice were analyzed. Morphological analyses were performed by a mouse pathologist. All investigators were blinded during the analysis of *in vivo*

data. Animal experiments were approved by the veterinary office of the canton Zurich, Switzerland (license number ZH035/16).

2.2 | Cell culture

MDA-MB468 (breast cancer; American Type Culture Collection, ATCC), HepG2 and Hep3B (hepatocarcinoma; ATCC), Caco-2 (colon carcinoma; ATCC), HEK293 (immortalized kidney; ATCC) as well as A549 (epithelial lung carcinoma; ATCC) cells were cultured in DMEM (Sigma-Aldrich) containing each 4.5 g/l sodium pyruvate, glucose and L-glutamine (Sigma-Aldrich), supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco by Life Technologies, Carlsbad, CA, USA), 100 µg/ml streptomycin (S), and 100 U/ml penicillin (P; Sigma-Aldrich). SUM149PT (breast cancer) cells[27] were cultured in 50% DMEM and 50% RPMI (Sigma-Aldrich) supplemented with 10% FCS, 100 µg/ml S, and 100 U/ml P. AB8/13 (kidney) cells[28] were cultured in RPMI supplemented with 10% FCS, 50 IU/ml P, 50 µg/ml S, supplemented with 5 µg/ml

insulin, 5 µg/ml transferrin, and 5 ng/ml sodium selenite (Roche, Mannheim, Germany). AB8/13 cells were propagated at 33°C and differentiated for 10–14 days at 37°C. Kelly (neuroblastoma) cells[29] were cultured in RPMI, 10% FCS, 100 µg/ml S and 100 U/ml P. Cells were maintained under standard conditions (humidified atmosphere at 37°C with 18.5% O_2 and 5% CO_2). For transient transfection of cells, lipofectamine 2000 reagent (Invitrogen) or polyethylenimine (PEI; Polysciences, Warrington) was used as described previously.[27] All cells were regularly tested for mycoplasma contamination.

2.3 | Protein extraction and analyses

Protein extraction was performed with lysis buffer containing 150 mM NaCl, 1 mM EDTA, 25 mM Tris-HCl pH 8.0, 1% NP-40, 1 mM Na_3VO_4, 1 mM PMSF, and 1 mM NaF. For cell lysis, a protease inhibitor cocktail (Sigma-Aldrich) was added to the lysis buffer. For tissue lysis, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin A were included in the lysis buffer, and tissue samples were homogenized using a polytron homogenizer (VWR International, Amsterdam, Netherlands). Protein concentrations were determined by the BCA assay (Thermo Fisher Scientific, Waltham, MS, USA). OTUB1 protein data from murine lung tissue were obtained from the LungMAP consortium (https://lungmap.net/; 1U01HL122638; downloaded on 04/12/2020; Figure 3A).

2.4 | RNA analyses

RNA was extracted from tissues using the guanidine thiocyanate-acid phenol-chloroform method as previously described.[30] The RNA was converted to cDNA by reverse transcription of 2 µg of total RNA using AffinityScript transcriptase (Agilent, Santa Clara, CA, USA). The cDNA was analyzed using the SYBR Green qPCR reagent kit (Kapa Biosystems, London, UK) in a MX3000P light cycler (Agilent). Transcript levels were normalized to mouse ribosomal protein S12 mRNA levels. OTUB1 RNA-sequencing data from various human organs (Figure S1A) were extracted from a previous analysis.[31] OTUB1 RNA-sequencing data from murine and human lung cells/tissue were obtained from the LungMAP consortium (https://lungmap.net/; 1U01HL122638; downloaded on 04/12/2020; Figure 3A) and Lung Gene Expression in Single-cell (LungGENS; https://research.cchmc.org/pbge/lunggens/default.html; Figure 3B–M).[32–34]

2.5 | Cell proliferation analysis

Stable transfection of human A549 cells (shControl or shRNA-targeting OTUB1) was established and the effect of OTUB1 knockdown on proliferation was assessed by bromodeoxyuridine (BrdU) incorporation, using the BrdU Cell Proliferation enzyme-linked immunosorbent assay (ELISA) kit (Abcam, Cambridge, UK; ab126556) according to the manufacturer's description. A doubling time of 22 h was assumed for A549 cells as previously described.[35] Following the addition of BrdU to the cells for the indicated time points, the optical density (OD) was measured at 450 nm (and as a reference at 550 nm) with the Infinite 200 PRO multimode plate reader (Tecan Group Ltd., Switzerland). As a second method for the analysis of cell proliferation, the stably transfected A549 cells were seeded (50 000 cells/sample) and counted at the indicated time points with the Vi-Cell XR cell counter (Beckham, Indianapolis, IN, USA).

2.6 | Immunoblot analysis

Equal protein amounts were separated by SDS-PAGE, transferred to nitrocellulose or polyvinylidene difluoride membranes and detected using anti-OTUB1 (Cell Signaling, Danvers, MA, USA; 3783; 1:1000), anti-DEPTOR (Novus Biologicals; nbp1-49674; 1:1000), anti-p70 S6 kinase (Cell Signaling; CST9202; 1:1000), anti-phospho-p70 S6 kinase (Cell Signaling; 9234; 1:1000), anti-Akt (pan; Cell Signaling; 2920; 1:1000), anti-phospho-Akt (Ser473; Cell Signaling; 4060; 1:1000), anti-phospho-Akt (Thr308; Cell Signaling; 2965; 1:1000), anti-α-tubulin (Cell Signaling; 2144; 1:1000), anti-SMC1 antibodies (Abcam; 9262; 1:2000), and horseradish peroxidase (HRP)-coupled secondary antibodies (Thermo Fisher Scientific; 31430, 31460; 1:5000). Chemiluminescence detection was performed with Supersignal West Dura (Thermo Fisher Scientific) and recorded with a CCD camera (LAS-4000; GE Healthcare, Chalfont, St. Giles, UK) as previously described.[27] Quantification was performed with ImageQuant TL gel analysis software (GE Healthcare, Version 8.1). In addition to the verification by the suppliers, the antibody against OTUB1 was tested against (tag-containing) overexpressed proteins in cell lines and the results were compared with results obtained with anti-tag antibodies. Furthermore, the anti-OTUB1 antibody was confirmed using knockout mouse embryonic fibroblasts and knockout mouse tissues. Antibodies against induced protein phosphorylation were assessed in the presence and absence of a known stimulation of the protein phosphorylation.

2.7 | Lung inflation test

The trachea of P0 mice was tied shut, the lung was excised and its floatability was tested in PBS. Inflated lungs float, non-inflated lungs sink.

2.8 | Tissue staining

Organs were fixed in 4% formaldehyde (Sigma-Aldrich) for 24 h. The sections were deparaffinized with xylene and rehydrated with decreasing concentrations of ethanol in water. Tissue sections (3–5 μm thick) were stained with hematoxylin and eosin (H&E), periodic acid-schiff (PAS) or subjected to immunohistochemistry (IHC) for cleaved caspase 3 (Asp175; Cell Signaling; 9664; 1:400), SP-C (Santa Cruz Biotechnology, Dallas, TX, USA; sc-13979; 1:50), Ki67 (Ventana Medical Systems, Tucson, AZ, USA; 790-4286; undiluted), or CD31 (Santa Cruz Biotechnology; sc-1506R; 1:1000). Discovery XT IHC research instrument (Ventana Medical Systems) was used for the detection of cleaved caspase 3 and Ki67. An autostainer (Dako, Glostrup, Denmark) was employed for SP-C and CD31. All antibody blocking steps were performed with H_2O_2 (Dako) and REAL Antibody Diluent (Dako). Conditioning 1 pre-treatment (cell conditioning 1 (CC1), Ventana Medical Systems) was used for the antigen retrieval for subsequent analysis with the Discovery XT systems, and antigen retrieval with EDTA buffer (pH 9) was used for the Dako autostainer. For IHC signal detection, HRP-coupled secondary antibodies were used (Ventana Medical Systems; 760-4311; Jackson ImmunoResearch; 711-065-152) together with DAB substrate buffer (Dako; K3468). Quantification was performed with ImageJ 1.53a (NIH, MD, USA).[36,37]

2.9 | Transmission electron microscopy

Tissue samples of the cranial right lung lobe were fixed for 24 h in 2.5% glutaraldehyde in 0.2 M sodium phosphate buffer (pH 7.3) for transmission electron microscopy (TEM) examination in the TEM Unit (Institute of Veterinary Pathology, Laboratory for Animal Model Pathology, University of Zurich). Briefly, specimens were washed in sodium phosphate buffer and fixed in 1% osmium tetroxide in phosphate buffer/distilled water for 60 min. Tissues were dehydrated in ascending concentrations of ethanol, followed by propylene oxide. Specimens were subsequently infiltrated with 50% resin (Epoxy embedding medium, Sigma Aldrich) and incubated overnight. The tissues were then incubated in polyethylene embedding capsules filled with 100% resin for 3 days.

Semi-thin sections (1 μm) were prepared using an ultramicrotome (Reichert-Jung Ultracut, Munich, Germany) with a diamond knife (Diatome, Biel, Switzerland), stained with toluidine blue, and areas of interest for the preparation of ultrathin sections were chosen under the light microscope. Ultrathin sections (90 nm) were prepared with a diamond knife, mounted on copper grids, stained with uranyl acetate and Reynold's lead citrate, and examined with a transmission electron microscope (Philips CM10, FEI, Thermo Fisher Scientific) operating with a Gatan Orius Sc1000 digital camera (Digital Micrograph, Gatan, Pleasanton, CA, USA).

2.10 | Tissue processing for flow cytometry

Embryonic lungs were prepared as previously described.[38] In brief, E18.5 embryonic lungs were harvested following timed pregnancies. Dissected lungs were incubated in ice-cold FACS buffer (2% FBS, 0.05% sodium azide in PBS), manually dissociated with scissors, and digested during gentle rocking with 50 μg/ml Liberase TM (Roche) and 25 μg/ml DNase I (Roche) in pre-warmed RPMI-1640 for 15–20 min at 37°C. Following trituration with a P1000 pipette, the tissue was digested for another 15–20 min at 37°C. After the second trituration, the tissue was filtered through 50 μm cell strainers. Red blood cells were lysed (BD Pharm Lyse; BD Biosciences, San Jose, CA, USA), and the remaining cells were washed, filtered, and stained for flow cytometry.

2.11 | Flow cytometry

Flow cytometry was performed as previously described,[38] using Fc block (anti-mouse CD16/32, BioXCell, Lebanon, NH, USA; 2.4G2), anti-mouse CD45 (BD Biosciences; 30-F11), anti-mouse I-A/I-E (BioLegend, San Diego, CA, USA; M5/114.15.2), anti-mouse CD31 (BioLegend; 390), anti-mouse CD140a (BioLegend; APA5), anti-mouse CD326 (BioLegend; G8.8), anti-mouse CD49f (BioLegend; GoH3), anti-mouse/human CD324 (BioLegend; DECMA-1), anti-mouse CD11c (BioLegend; N418), anti-mouse CD11b (BioLegend; M1/70), anti-mouse Ly-6G (BioLegend; 1A8), anti-mouse CD64 (BioLegend; X54-5/7.1), anti-mouse F4/80 (BioLegend; BM8), anti-mouse GR-1 (BioLegend; RB6-8C5), anti-mouse TER-119/ erythroid cells (BioLegend; TER-119), anti-mouse CD19 (BioLegend; 6D5), anti-mouse CD90.2 (BioLegend; 30-H12), anti-mouse FcER1a (BioLegend; MAR-1), anti-mouse CD3 (BioLegend; 17A2), and anti-mouse Ki-67 (Thermo Fisher Scientific; SolA15). Dead cells were

excluded using Zombie Red (BioLegend). Following staining of surface markers, cells were fixed using the FoxP3 fixation kit (Invitrogen), and washed and stained for Ki-67 in permeabilization buffer (Thermo Fisher Scientific; 00-8333-56). Analysis was performed on a FACSymphony (BD Biosciences) with five lasers (355, 405, 488, 561, and 639 nm). To exclude doublets, samples were gated by the forward scatter-height and the forward scatter-area (FSC-A), followed by side scatter-height and side scatter-area (SSC-A); FSC-A and SSC-A gating was also utilized to exclude debris, with subsequent exclusion of dead cells. Lymphocytes were defined as lacking CD11b, CD11c, F4/80, Gr-1, FcεRIα, and Ter119. Data was analyzed using FlowJo (Tree Star, Ashland, OR, USA).

2.12 | Whole-body plethysmography

Basal respiration and the adaptation of respiration to hypoxia were assessed in unrestrained conscious mice using a whole-body plethysmograph and BioSystem XA software (Buxco Research Systems, Wilmington, NC, USA). Each mouse was placed in a 1 dm^3 chamber and allowed to adapt for 1 h. Respiratory parameters were analyzed over a 5 min interval at 21% O_2 and during the first 5 min after reaching 10% O_2 (referred to as "early"). In addition, respiration was assessed after 25–30 min at 10% O_2 (referred to as "late"). To exclude measurements of non-basal respiration, data were not taken into account if ≥3 consecutive respiratory frequency measurements showed ≥400 breaths/min or a variability among the single measurements of >70 breaths/min.

2.13 | Genotyping primers

$Otub1^{+/-}$ and $Otub1^{-/-}$:
Otub1-5arm-WTF: 5′-TAGATGCTACACAGTGCTTTT AGA-3′
Otub1-Ctrl-WTR: 5′-TTAGAGATCAGCTCTGGAAATA TAG-3′
5mut-R1: 5′-GAACTTCGGAATAGGAACTTCG-3′
pc*Otub1* KO$^{Wt/del}$:
Kr_3209: 5′-CCAACAGCTTCCCCACAACGG-3′
Ef_5272: 5′-TCCACCCCTTCATCCTGCTTTCT-3′
$Otub1^{fl/fl}$:
Ef_5272: 5′-TCCACCCCTTCATCCTGCTTTCT-3′
Er_5277: 5′-CAGACCAGAGCAGGATTAAGAAGCCTA-3′
UBC-Cre^{ERT2}:
UBCre 25285: 5′-GACGTCACCCGTTCTGTTG-3′
UBCre-oIMR7338: 5′-CTAGGCCACAGAATTGAAAGA TCT-3′
UBCre-oIMR7339: 5′-GTAGGTGGAAATTCTAGCATC-3′

UBCre-oIMR9074: 5′-AGGCAAATTTTGGTGTACGG-3′
wb*Otub1* KO:
Ef_5273: 5′-GAAGGACAAAGGGCGTGTCTCAGT-3′
L3r_ 5274: 5′-TCTACCCATCCCAACACCAGCAAG-3′
loxP:
Primer 1307 forw: 5′-GGCAGAAGCACGCTTATCG-3′
Primer 1307 rev: 5′-GACAAGCGTTAGTAGGCACAT-3′
Sex determination[39]:
Smcx-1: 5′-CCGCTGCCAAATTCTTTGG-3′
Smc4-1: 5′-TGAAGCTTTTGGCTTTGAG-3′

2.14 | Statistical analysis

For statistical analysis between two different data points, a two-tailed Student's *t*-test was applied as indicated. For the analysis of proliferation time courses, two-way ANOVA followed by Bonferroni's post hoc test was used.

3 | RESULTS

3.1 | Homozygous *Otub1* deletion induces lethal developmental defects

OTUB1 is ubiquitously expressed in humans and mice (Figure S1A–C). To elucidate the OTUB1 function *in vivo*, we characterized mice with constitutive whole-body heterozygous and homozygous *Otub1* deletion (Figure 1A). Homozygous *Otub1* knockout (KO) was lethal, while *Otub1* haploinsufficient mice were viable (Figure 1B,C). The absence of OTUB1 did not affect the sex ratio (Figure 1B,C). Most $Otub1^{-/-}$ mice died perinatally (the death of all of these mice occurred during or immediately after birth), but there were less $Otub1^{-/-}$ mice observed at E18.5 (births occurred on average at E19.5) than expected when assuming a Mendelian distribution (Figure 1B,C). On a few occasions, incomplete placental absorptions were observed at E18.5, two of which could be identified as $Otub1^{-/-}$, suggesting that a small number of $Otub1^{-/-}$ mice died of unknown cause during earlier developmental processes. While $Otub1^{+/-}$ embryos were comparable in size and weight to *Wt* mice, $Otub1^{-/-}$ embryos were significantly lighter and smaller than $Otub1^{+/-}$ mice (Figure 1D,E).

3.2 | Constitutive *Otub1* deletion prevents respiration

We next analyzed the organs of $Otub1^{-/-}$ mice in detail. The ventricular wall diameter and the heart weight of

$Otub1^{-/-}$ animals were increased (Figure 2A,B). Other organs showed no weight differences (Figure 2B) or gross histological abnormalities (Figure S1D). Although the lung of $Otub1^{-/-}$ mice showed no difference in weight and gross morphology, we observed gasping in $Otub1^{-/-}$ newborn pups (P0) and E18.5 embryos. In contrast to Wt and $Otub1^{+/-}$ lungs, $Otub1^{-/-}$ lungs sank during a PBS floating test, demonstrating that $Otub1^{-/-}$ animals were unable to inflate their lungs (Figure 2C). Histological analyses of the lungs showed that the saccular air space was significantly decreased in $Otub1^{-/-}$ mice (Figure 2D). Of note, both the left and the right ventricle were symmetrically altered in $Otub1^{-/-}$ embryos (Figure 2A). Thus, there is no evidence for a specific effect on one particular ventricle by $Otub1$ deletion, which could have occurred by, for example, an increased blood pressure. Because prior to birth the open foramen ovale ensures the right-to-left arterial shunt,[40] it appears unlikely that the morphological alterations in the lung affected the cardiac tissue or, *vice versa*, that the cardiac phenotype influenced lung development.

In summary, we suggest that the decreased saccular air space in mice with $Otub1$ ablation led to the inability to inflate their lungs and caused perinatal lethality by asphyxiation.

3.3 | OTUB1 is particularly highly expressed in the lung during late-stage embryonic development

In order to assess the expression of OTUB1 in lung tissue in more detail, we next analyzed existing data on OTUB1 mRNA and protein levels at different developmental stages in wild-type mice. RNA sequencing and analysis of the proteome showed the highest OTUB1 mRNA and protein levels at E16.5, gradually decreasing at E18.5, P7, and P28 (Figure 3A). Single-cell RNA (scRNA) sequencing from murine lung tissue at the age of E16.5, E18.5, and P1 showed a comparable OTUB1 mRNA expression in all analyzed lung cell types (Figure 3B–J). scRNA sequencing of human lung tissue at the age of P1 also detected OTUB1 mRNA in all cell types (Figure 3K–M). These results indicate that there is no specific expression of OTUB1 in a particular lung cell type, complicating the identification of the mechanisms leading to lung failure.

FIGURE 2 Lung tissue alterations lead to asphyxiation of $Otub1^{-/-}$ mice. (A) H&E staining of frontal heart sections (P0). Rv/Lv, right/left ventricle. (B) $Otub1^{+/-}$ ($n = 27$) and $Otub1^{-/-}$ ($n = 14$) heart, liver, and lung weight corrected for BW and normalized to the corresponding Wt ($n = 15$) littermates (E18.5). The dashed line indicates Wt littermates. (C) Floating test for P0 Wt, $Otub1^{+/-}$ and $Otub1^{-/-}$ lungs. Filled arrowheads indicate the surface, empty arrowheads indicate the lung. (D) Histological analyses and quantification of E18.5 transversal lung sections by H&E. Quantification of H&E staining in E18.5 Wt ($n = 9–14$), $Otub1^{+/-}$ ($n = 14–25$) and $Otub1^{-/-}$ ($n = 11–14$) lung tissues was normalized to total tissue area. pos., positive. Data are presented as mean ± SEM. *$p < .05$, **$p < .01$, ***$p < .001$ by two-tailed Student's t-test

FIGURE 3 OTUB1 expression in lung tissue. (A) Normalized OTUB1 mRNA (detected by RNA sequencing) and OTUB1 protein levels (proteome analysis) from murine whole lung tissue. (B–M) Single-cell RNA (scRNA) sequencing of murine (B–J) and human (K–M) lung tissue at the age of E16.5 (B–D), E18.5 (E–G), P1 (murine; H–J) and P1 (human; K–M). The images depict the identified cell types (B, E, H, K), the detected OTUB1 mRNA expression (red dots; C, F, I, L), and mean mRNA levels within the detected cell types (D, G, J, M). Each filled circle denotes a single cell (B, C, E, F, H, I, K, L). tSNE, t-distributed stochastic neighbor embedding. mE16.5, mE18.5, mP1, murine lung tissue analyzed at the age of E16.5, E18.5, P1. hP1, human lung tissue analyzed at the age of P1. The OTUB1 RNA-sequencing and proteome datasets were obtained from the LungMAP consortium (https://lungmap.net/). The scRNA sequencing data were provided by Lung Gene Expression in Single-cell (LungGENS; https://research.cchmc.org/pbge/lunggens/default.html)

3.4 | *Otub1* deletion increases cell proliferation in the developing lung

To further assess the underlying cause of the decreased saccular air space observed in *Otub1*$^{-/-}$ mice,

the lung tissue was analyzed in detail. First, a possible difference in surfactant production was assessed. Glycogen is necessary for the production of surfactant phospholipids,[41] but we found no difference in glycogen content by staining with PAS (Figure 4A). Also,

FIGURE 4 Lung tissue of adult mice with induced whole-body *Otub1* deletion demonstrates an increased cell proliferation. (A and B) Histological analyses, and quantification of E18.5 transversal lung sections by PAS (*A*) and anti-surfactant C (SPC) antibody (*B*). (C) Transmission electron microscopy of lung tissue from *Wt* and *Otub1*$^{-/-}$ mice (E18.5). lb, lamellar body; n, nucleus. (D–F) Histological analyses and quantification of E18.5 transversal lung sections by anti-cluster of differentiation 31 (CD31) antibody (*D*), anti-cleaved caspase 3 (clCasp3) antibody (*E*) and anti-Ki67 antibody (*F*). Quantifications of the stainings in E18.5 *Wt* (*n* = 9–14), *Otub1*$^{+/-}$ (*n* = 14–25) and *Otub1*$^{-/-}$ (*n* = 11–14) lung tissues were normalized to parenchymal (parench.) tissue area. pos., positive. Arrowheads indicate examples of positive signal. Data are presented as mean ± SEM. *$p < .05$, **$p < .01$, ***$p < .001$ by two-tailed Student's *t*-test

surfactant protein C (SPC) detection by IHC showed comparable levels between the genotypes (Figure 4B), and TEM depicted a normal visual appearance of lamellar bodies in *Otub1*$^{-/-}$ embryos (Figure 4C). These results indicated a normal surfactant production in *Otub1*$^{-/-}$ embryos. IHC detection of CD31, a marker

of vascular endothelial cells, showed a significant decrease in *Otub1*$^{-/-}$ lungs compared to *Wt* but not to *Otub1*$^{+/-}$ lungs (Figure 4D). *Otub1*$^{+/-}$ embryos showed a (non-significant) decrease in CD31 lung staining compared to *Wt* (Figure 4D) without developing a lung phenotype and the difference between *Otub1*$^{+/-}$ and *Otub1*$^{-/-}$ embryos was rather small and also not significant (Figure 4D). Therefore, it appears implausible that the pulmonary vessel density is a major contributor to the *Otub1*$^{-/-}$ lung phenotype.

Morphological analyses revealed that in the three analyzed genotypes the thickness of the alveolar septa ranged from 3 to 6 cells (depending on the assessed region). No remarkable differences were identified in the cellular morphology between the groups and no morphological alterations were observed that would have indicated cellular damage (i.e., degeneration, necrosis, apoptosis) or inflammation. In KO animals, the saccular air spaces appeared narrowed compared to WT and heterozygous animals, and in some cases, the saccular air spaces were completely closed in *Otub1*$^{-/-}$ mice. In all three genotypes, assessment of the anti-CD31 immunostaining (Figure 4D) revealed two layers of capillaries within the septa in agreement with a double-layered capillary network of the immature septa as expected at this developmental age,[42] and no alterations were identified in *Otub1*$^{-/-}$ mice relative to control. *Otub1*$^{-/-}$ mice did also not show any visible morphological alteration in the capillary pulmonary bed (i.e., vascular damage). Thus, it is unlikely that *Otub1* deletion decreased the saccular air space through morphological changes within the endothelial cell layer or the capillary network.

OTUB1 has previously been shown to regulate apoptosis via, for example, increasing cleaved caspase 3 (clCasp3) levels.[43] In E18.5 lungs, clCasp3 levels displayed a large variability (Figure 4E). The clCasp3 levels were comparable between *Wt* and *Otub1*$^{-/-}$ lungs and significantly decreased in *Otub1*$^{+/-}$ lung tissue compared to *Otub1*$^{-/-}$ lungs (Figure 4E). Importantly, the clCasp3 levels were overall very low (Figure 4E). Hence, apoptosis is unlikely to contribute to the *Otub1*$^{-/-}$ lung phenotype.

OTUB1 has also been reported to regulate cell proliferation.[44,45] Analysis of proliferation by Ki67 IHC demonstrated that *Otub1* deletion significantly enhanced proliferation in the developing lung compared to both *Otub1*$^{+/-}$ and *Wt* mice (Figure 4F). Significantly more Ki67-positive cells were observed per μm^2 of the parenchymal area in *Otub1*$^{-/-}$ lungs compared to both controls (Figure 4F). In summary, we suggest that the observed decrease in the saccular air space in *Otub1*$^{-/-}$ lungs is caused by increased cell proliferation.

FIGURE 5 *Otub1* deletion-mediated increases in cell proliferation affects all major parenchymal and mesenchymal cell compartments. (A and B) Flow cytometry analysis of E18.5 lungs isolated from the indicated genotypes, assessing Ki67 staining in the CD45$^-$ compartment. (A) Identified populations include epithelial cells (G1: CD45$^-$EpCAM$^+$CD31$^-$), endothelial cells (G2: CD45$^-$CD31$^+$EpCAM$^-$), and mesenchymal/other non-hematopoietic cells (G3: CD45$^-$EpCAM$^-$CD31$^-$). (B) Quantified Ki67 levels per parental cell population for epithelial cells (G1), endothelial cells (G2), and mesenchymal/other non-hematopoietic cells (G3) in *Wt* ($n = 7$), *Otub1*$^{+/-}$ ($n = 10$), and *Otub1*$^{-/-}$ ($n = 5$) lungs. Data were derived from two independent experiments and are presented as mean ± SEM. ***$p < .001$ by two-tailed Student's *t*-test

3.5 | Constitutive deletion of *Otub1* increases the proliferation of all major lung parenchymal and mesenchymal cell types

We next investigated which lung cell types increased their proliferation in response to the deletion of *Otub1*. E18.5 lungs were digested and the different cell types were profiled and analyzed for Ki67 staining using flow cytometry and three comprehensive marker panels for all major lung parenchymal and mesenchymal cell compartments as well as for cells of myeloid and lymphoid lineages.[38] In the CD45$^-$ compartment, Ki67 positive cells were significantly increased in *Otub1*$^{-/-}$ parenchymal epithelial cells (EpCAM$^+$CD31$^-$) and endothelial cells

(CD31$^+$EpCAM$^-$) as well as in *Otub1*$^{-/-}$ mesenchymal/other non-hematopoietic cells (EpCAM$^-$CD31$^-$) compared to corresponding *Otub1*$^{+/-}$ and *Wt* cell populations (Figure 5). In the endothelial cell compartment, the percentage of Ki67-positive cells was already relatively high in *Otub1*$^{+/-}$ and *Wt* lungs (average of 61.2% and 66.7% per parental population), but *Otub1* KO still significantly increased the percentage of Ki67-positive endothelial cells (85.3% per parental population) by around 1.3-fold (Figure 5). In epithelial and mesenchymal/other non-hematopoietic cells, *Otub1* KO mice showed an increase in the percentage of Ki67 positive cells by approximately threefold relative to both *Wt* and *Otub1*$^{+/-}$ mice (epithelial cells: average of 22.9% in *Wt*, 30.5% in *Otub1*$^{+/-}$ and 75.6% in *Otub1*$^{-/-}$; mesenchymal/other non-hematopoietic

cells: average of 21.0% in *Wt*, 25.1% in *Otub1*$^{+/-}$ and 67.7% in *Otub1*$^{-/-}$; Figure 5).

In order to assess a possible contribution of immune cells to the observed increase in cell proliferation in *Otub1*$^{-/-}$ E18.5 lungs, the CD45$^+$ cell compartment was analyzed. In the myeloid cell populations, *Otub1* deletion had no effect on the proliferation of granulocytes (Ly-6G$^+$CD64$^-$), nor on the combined population of macrophages, monocytes, and dendritic cells (CD64$^+$Ly-6G$^-$; Figure S2). In the lymphoid cell compartment, *Otub1* KO significantly increased the percentage of Ki67 positive B cells (CD19$^+$CD3$^-$) in E18.5 lungs in comparison to both controls (Figure S3). T cells (CD3$^+$CD19$^-$) showed no difference in proliferation across the genotypes. Non-B/non-T lymphocytes (CD3$^-$CD19$^-$) demonstrated an increase in Ki67-positive cells in *Otub1* KO mice relative to *Wt* but not to *Otub1*$^{+/-}$ mice (Figure S3). Of note, the total lung lymphoid cell population at E18.5 is much smaller than the parenchymal and mesenchymal cell populations (data not shown). Thus, the observed significant increase in B cell proliferation is not likely to contribute to the observed tissue phenotype (Figure 4).

In summary, *Otub1* KO increases proliferation in multiple pulmonary cell types, in particular in epithelial and mesenchymal cells, which we propose to lead to the observed decrease in the saccular air space in *Otub1*$^{-/-}$ lungs.

3.6 | Conditional whole-body *Otub1* deletion causes hyperventilation in adult mice

To analyze the physiological relevance of OTUB1 in adult mice, we established a mouse strain with tamoxifen (Tx)-inducible whole-body *Otub1* ablation (Figure S4A). The deletion of *Otub1* was highly efficient with some intra- and inter-organ variation (Figure S4B,C). Five months after *Otub1* ablation, the brain showed a minor weight difference, but quadriceps, spleen, and kidney did not show any weight difference (Figure S5).

Because *Otub1* deletion affected lung development, we analyzed whether deletion of OTUB1 during adulthood impacted upon the lung tissue and functional respiration. Interestingly, the weight of lungs lacking *Otub1* was significantly increased compared to the wild-type lungs (Figure 6A). An increased number of proliferating and total cells were detected in adult *Otub1* KO lungs (Figure 6B), which was comparable to our findings in *Otub1*$^{-/-}$ E18.5 lungs (Figures 4F and 5). Hence, *Otub1* deletion induces proliferation in lung tissue independent of the developmental stage.

Next, we analyzed if *Otub1* deletion affects respiration. Under normal inspiratory oxygen conditions (normoxia; 21% O_2), the respiratory frequency was not significantly altered in *Otub1* KO mice, but the tidal volume was increased relative to Tx-treated control mice and the minute volume was significantly higher in comparison to both controls (Figure 6C), suggesting alveolar hyperventilation in mice lacking *Otub1*. Acute inspiratory hypoxia (10% O_2) in the same mice increased tidal and minute volumes in all groups (Figure 6C, 10% O_2, early), but *Otub1* KO mice maintained an increased tidal and minute volume relative to both controls (Figure 6C, 10% O_2, early). After 25–30 min in hypoxia (Figure 6C, 10% O_2, late), the control mice adapted and showed comparable respiration to normoxia (Figure 6C). *Otub1* KO mice, on the contrary, did not adjust and maintained an increased respiratory frequency, tidal volume, and minute volume (Figure 6C). In summary, our mouse KO data revealed that OTUB1 is essential for lung tissue homeostasis, regulation of respiration, and the adaptation of respiration to hypoxia.

3.7 | *Otub1* deletion increases mTOR signaling

To investigate possible molecular mechanisms underlying the observed phenotypes, we analyzed the effect of OTUB1 on cell proliferation further. In cultured human A549 cells, OTUB1 knockdown significantly increased proliferation in comparison to control cells (Figures 7A,B and S6A), confirming the regulatory function of OTUB1 for lung cell proliferation and indicating that OTUB1 is also relevant for the regulation of proliferation in human lung cells. OTUB1 has previously been linked to the regulation of cell proliferation *in vitro* by affecting mammalian target of rapamycin (mTOR) activity,[46] and OTUB1 has been suggested to directly regulate DEPTOR protein stability,[46] a negative regulator of mTORC1 and mTORC2.[47] mTOR signaling via the ribosomal protein S6 kinase (S6K) plays a pivotal role in embryonic body size and weight development,[48] cell proliferation,[48,49] lung development,[41,50] and respiration.[41] AKT activation often occurs upstream of mTOR activation[51] and OTUB1 has previously been suggested to regulate AKT activity (in CD8(+) T and NK cells).[20] We, therefore, analyzed whether *Otub1* deletion affected AKT or mTOR-dependent S6K activation in lung tissue by assessing the phosphorylation status of both AKT and S6K.

In E18.5 *Otub1*$^{-/-}$ lungs, phosphorylation of AKT Thr308 and Ser473 was not altered, but phosphorylation of S6K and total S6K levels were increased compared to *Wt* mice (Figures 7C and S6B). Interestingly, *Otub1* haploinsufficiency also led to an increase in phosphorylated S6K, but only in some of the tested animals (Figure 7C), indicating a gene dosage-dependent effect. DEPTOR

FIGURE 6 Lung tissue and respiratory alterations in adult mice with induced whole-body *Otub1* deletion. (A) Lung weight normalized to BW of the mice analyzed in (C) (3.5 months after *Otub1* deletion; 5.5 months old mice; *Otub1*$^{fl/fl}$ + Cre + Veh, $n = 6$; *Otub1*$^{fl/fl}$ − Cre + Tx, $n = 8$; *Otub1*$^{fl/fl}$ + Cre + Tx, $n = 5$). (B) Immunohistology and quantification of Ki67 and total cell number in lung tissue (3.5 months after *Otub1* deletion; 5.5 months old mice; $n = 3$). (C) Consecutive analysis of the respiration of unrestrained mice (2 months after *Otub1* deletion; 4 months old mice; *Otub1*$^{fl/fl}$ + Cre + Veh, $n = 6$; *Otub1*$^{fl/fl}$ − Cre + Tx, $n = 7$; *Otub1*$^{fl/fl}$ + Cre + Tx, $n = 6$) determined by barometric pressure plethysmography in normoxia (21% O_2) and hypoxia (10% O_2). V_t, tidal volume; V_I, minute volume; early, first 5 min in hypoxia; late, 25–30 min in hypoxia. Arrowheads indicate examples of positive signal. All data are presented as mean ± SEM. $^{#}$/*$p < .05$; $^{##}$/**$p < .01$; ***$p < .001$ by two-tailed Student's *t*-test. $^{#/##}$, compared to the corresponding measurements of the same mice in normoxia

levels were decreased (Figure 7C), which could explain the increase in phosphorylation of S6K and would be in line with a direct regulation of DEPTOR protein half-life by OTUB1. In summary, these results demonstrated that mTOR-dependent signaling was increased in OTUB1 deficient E18.5 lungs.

In adult lungs with conditional *Otub1* KO, no difference in AKT phosphorylation was observed, but an increase in phosphorylated and total S6K levels (Figures 7D and S6C) comparable to the results in E18.5 lungs. DEPTOR levels showed a tendency to be increased (Figure 7D). Because DEPTOR levels were differentially regulated between E18.5 and adult lungs with *Otub1* KO, whereas phosphorylated and total S6K levels were affected in the same manner, DEPTOR likely plays no role in the observed effects in the lung. Overall, these results indicate that deletion of *Otub1* augments mTOR-dependent S6K activity, increasing cell proliferation in embryonic and adult lungs, which in turn leads to the observed phenotypes.

4 | DISCUSSION

We provide *in vivo* evidence that the DUB OTUB1 plays a pivotal role in embryonic lung development, functional respiration, and adult lung homeostasis. We propose that the failure of *Otub1*$^{−/−}$ mice to inflate their lungs is caused by increased proliferation of parenchymal and mesenchymal cells, which in turn decreases saccular air space and likely augments lung tissue resistance against mechanical stretch. In adult mice, OTUB1 is essential for the control of the oxygen-dependent regulation of respiration, but this effect is unlikely to occur in a lung-autonomous manner. Rather, OTUB1 may play a key role in oxygen sensing by the carotid body, the main sensor of arterial oxygen partial pressure, and regulator of hypoxia-induced alveolar ventilation.[52] Future investigations should assess if *Otub1* deletion specifically in the oxygen-sensing cells of the carotid body contributes to the observed respiratory phenotype.

Lack of *Otub1* enhanced mTOR signaling in embryonic and adult lung tissue independent of AKT or DEPTOR.

FIGURE 7 *Otub1* deletion increases cell proliferation and mTOR signaling. (A) BrdU assay assessing cell proliferation at the indicated hours after seeding in A549 cells stably transfected with shRNA targeting OTUB1 (shOTUB1) or with a shRNA without target (shCtrl). (B) Cell proliferation analysis in the same A549 cells as described above, counting cells at the indicated days after seeding ($n = 4$). (C) Immunoblotting and quantification (normalized to loading control) of the indicated (phospho)proteins in E18.5 mouse lung tissue lysates ($n = 3$). For pS6K and S6K, the most relevant band was quantified (the band with the highest intensity, running at the lower molecular weight); ns, non-specific. (D) Immunoblotting and quantification (normalized to loading control) of the indicated (phospho)proteins in lung tissue lysates following 4 h of starvation during the light cycle (5 months after the conditional *Otub1* KO; 6.5 months old mice; $n = 3$). For pS6K and S6K, the most relevant band was quantified (the band with the highest intensity, running at the lower molecular weight). Representative images are shown and data are presented as mean $\pm$ SEM. $*p < .05$ by Student's t-test

OTUB1 has previously been shown to affect RAS, p53, and AMPK activity.[13,22,53] All of these proteins can in turn lead to a differential regulation of mTOR activity,[54] providing a potential molecular mechanism for the observed increase in mTOR activity. mTOR signaling has been reported to control body weight and size during development through

regulation of S6K,[48] and to play a key role in lung development.[41,50] Increased mTOR signaling causes death by respiratory failure following extraction at E18.5.[41] In the carotid body, increased mTOR activity can lead to an imbalance between the 1α and 2α subunits of the transcription factor hypoxia-inducible factor (HIF), affecting the hypoxia sensitivity of the carotid body.[55] Overall, these findings are in line with the role of OTUB1 as a negative regulator of mTOR signaling, explaining (at least in part) the observed phenotypes in mice with *Otub1* deletion.

We previously reported that the oxygen sensor factor inhibiting HIF (FIH) interacts with OTUB1[13,56,57] and regulates OTUB1 enzymatic activity[15] as well as its interactome.[13] Mice lacking the gene encoding FIH (*Hif1an*) present with a metabolic phenotype and hyperventilation.[58] This is analogous to the observed hyperventilation of adult mice with *Otub1* KO, supporting our hypothesis that FIH is an important regulator of OTUB1.[13,15]

Given the observed phenotype of asphyxiation following birth, mice with constitutive *Otub1* deletion may serve as a novel disease model for lethal respiratory failure in newborns. Based on our flow cytometry analysis, all major lung parenchymal and mesenchymal cell types showed an increase in cell proliferation, with epithelial and mesenchymal cells likely contributing the most to the reported lung malformation that leads to asphyxiation. It would be of interest to test this hypothesis in a mouse model of lung cell type-specific *Otub1* deletion. In addition, adult mice with induced *Otub1* deletion could be exposed to chronic hypoxia to further analyze their (lack of) respiratory adaptation to hypoxia. Moreover, this mouse model may be used to assess the function of OTUB1 in lung fibrosis and/or lung infection in detail. In this regard, it is interesting that mesenchymal/other non-hematopoietic cells (which consist to a significant degree of fibroblasts) showed a strong increase in proliferation, as OTUB1 may thus contribute to the development of lung fibrosis through the regulation of fibroblast proliferation.

In summary, OTUB1 plays a key role in lung tissue physiology during embryonic development and adulthood. Moreover, OTUB1 is important for the control of respiration and its adaptation to hypoxia. These results indicate that pharmacologic targeting of OTUB1—as for example suggested for cancer treatment[16]—may not be without severe side effects. Furthermore, our findings may contribute to the identification of causes of developmental and respiratory diseases, including lung cancer, IPF, and IIPs.

DISCLOSURES
The authors declare that no competing interests exist.

AUTHOR CONTRIBUTIONS
Amalia Ruiz-Serrano, Josep M. Monné Rodríguez, Julia Günter, Samantha P. M. Sherman, Agnieszka E. Jucht, Pascal Fluechter, Yulia L. Volkova, Svende Pfundstein, and Giovanni Pellegrini performed experiments; Amalia Ruiz-Serrano, Josep M. Monné Rodríguez, Julia Günter, Samantha P. M. Sherman, Pascal Fluechter, Giovanni Pellegrini, Christoph Schneider, Roland H. Wenger, and Carsten C. Scholz designed experiments; Josep M. Monné Rodríguez, Giovanni Pellegrini, Carsten A. Wagner, Christoph Schneider, Roland H. Wenger, and Carsten C. Scholz contributed new reagents/analytical tools; Amalia Ruiz-Serrano, Josep M. Monné Rodríguez, Julia Günter, Samantha P. M. Sherman, Agnieszka E. Jucht, Pascal Fluechter, Yulia L. Volkova, Giovanni Pellegrini, Christoph Schneider, Roland H. Wenger, and Carsten C. Scholz analysed data; Carsten C. Scholz designed the overall study and wrote the paper.

DATA AVAILABILITY STATEMENT
Underlying raw data is available under https://dataverse.harvard.edu/dataverse/RuizSerrano_OTUB1-Lung.

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