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

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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 (wbOtub1del/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.

Abbreviations: BrdU, bromodeoxyuridine; BW, body weight; clCasP3, cleaved caspase 3; CNS, central nervous system; DEPTOR, DEP domain-containing mTOR-interacting protein; DUB, deubiquitinase; ELISA, enzyme-linked immunosorbent assay; FSC-A, forward scatter-area; FSC-H, forward scatter-height; IF, idiopathic interstitial pneumonia; IFP, idiopathic pulmonary fibrosis; KO, knockout; NTC, non-template control; OD, optical density; OTUB1, ovarian tumor (OTU) domain-containing ubiquitin aldehyde-binding protein 1; Otub1+/−, heterozygous Otub1 deletion; Otub1fl/fl, mice with floxed Otub1 exons 2 and 3; pcOtub1Wt/del, potential of conditional Otub1 deletion; PEI, polyethylenimine; scRNA, single-cell RNA (sequencing); SSC-A, side scatter-area; SSC-H, side scatter-height; TEM, transmission electron microscopy; Tx, tamoxifen; Ub, ubiquitin proteins; UBC-CreERT2, CreERT2 transgene under the control of the ubiquitin C promoter; wbOtub1del/del, induced whole-body Otub1 deletion; Wt, wild-type.
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 cells18 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 (IPPs) 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-Otub1tm1b(EUCOMM)Hmgu/H was purchased from the EUCOMM consortium (http://www.mouse phenotype.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 (wbOtub1del/del) deletion were generated by crossing heterozygous mice with the potential of conditional Otub1 deletion (pcOtub1)Wt/del; strain Otub1tm1a(EUCOMM)Hmgu/IsOri, purchased from the EUCOMM consortium via the EMMA repository and provided by the Institut Clinique
Female 87174624
Male 108166329

This 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.

(a) Total numbers of observed mice and gender distribution within each genotype. En2SA IRES lacZ pA

(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.

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) cells27 were cultured in 50% DMEM and 50% RPMI (Sigma-Aldrich) supplemented with 10% FCS, 100 µg/ml S, and 100 U/ml P. A8B/13 (kidney) cells28 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). A88/13 cells were propagated at 33°C and differentiated for 10–14 days at 37°C. Kelly (neuroblastoma) cells 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% O2 and 5% CO2). For transient transfection of cells, lipofectamine 2000 reagent (Invitrogen) or polyethylenimine (PEI; Polysciences, Warrington) was used as described previously. 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 Na3VO4, 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. 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. 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).

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. 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 (Thr380; 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. 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 auto-stainer (Dako, Glostrup, Denmark) was employed for SP-C and CD31. All antibody blocking steps were performed with H2O2 (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 Reynolds’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 dm³ chamber and allowed to adapt for 1 h. Respiratory parameters were analyzed over a 5 min interval at 21% O₂ and during the first 5 min after reaching 10% O₂ (referred to as “early”). In addition, respiration was assessed after 25–30 min at 10% O₂ (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**

**Otub1**⁺⁻ and **Otub1**⁻⁻:

**Otub1**-5arm-WTF: 5’-TAGATGCTACACAGTGCTTTAGA-3’

**Otub1**-Ctrl-WTR: 5’-TTAGAGATCAGCTCTGGAAATA-3’

5mut-R1: 5’-GAACCTTGGAAATAGAATCTTCG-3’

pc**Otub1** KO

Kr_3209: 5’-CCAAACAGCTTCCCCACACCGG-3’

Ef_5272: 5’-TCCACCCCTTATCCGCTTTCTTCT-3’

**Otub1**⁻⁻KO:

Ef_5272: 5’-TCCACCCCTTATCCGCTTTCTTCT-3’

Er_5277: 5’-CAGACCAGAGCAGATTAAGAAGGCTA-3’

**UBCre**-ERT2:

UBCre 25285: 5’-GACGTCACCCTGTTGCTTT-3’

UBCre-oMR7338: 5’-CTAGGCCACAGTATTGAAAGA-3’

**UbCre-oMR9074**:

5’-AGGCAAAATTTTGTTACGCG-3’

**wbOtub1** KO:

Ef_5273: 5’-GAAGGACACAGGGCCGTCTCAGT-3’

L3r_5274: 5’-TCTACCCATCCCAAACACAGAA-3’

loxP:

Primer 1307 forw: 5’-GCCAGAAGCAGCTTTAATC-3’

Primer 1307 rev: 5’-GACAAGCGTTAGTGGCACAAT-3’

Sex determination:

**Smcx**-1: 5’-CCGCTGCAAATTTCTTTG-3’

**Smc4**-1: 5’-TGAAGCTTTTGGCTTTTGAG-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.
To further assess the underlying cause of the decreased saccular air space observed in \textit{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,
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, 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. 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. 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 µm² 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.
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. 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

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

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.
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 sacculair 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% O2), 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% O2) in the same mice increased tidal and minute volumes in all groups (Figure 6C, 10% O2, early), but *Otub1* KO mice maintained an increased tidal and minute volume relative to both controls (Figure 6C, 10% O2, early). After 25–30 min in hypoxia (Figure 6C, 10% O2, 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, and OTUB1 has been suggested to directly regulate DEPTOR protein stability, a negative regulator of mTORC1 and mTORC2. mTOR signaling via the ribosomal protein S6 kinase (S6K) plays a pivotal role in embryonic body size and weight development, cell proliferation, and lung development, and respiration. AKT activation often occurs upstream of mTOR activation and OTUB1 has previously been suggested to regulate AKT activity (in CD8+ T and NK cells). 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...
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. 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.
OTUB1 has previously been shown to affect RAS, p53, and AMPK activity.\textsuperscript{13,22,53} All of these proteins can in turn lead to a differential regulation of mTOR activity,\textsuperscript{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 factor inhibitorbinding HIF (FIH) interacts with OTUB113,56,57 and regulates OTUB1 enzymatic activity15 as well as its interactor.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 OTUB113,15.

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 treatment16—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.

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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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REFERENCES
1. Clague MJ, Urbe S, Komander D. Breaking the chains: deubiquitylating enzyme specificity begets function. Nat Rev Mol Cell Biol. 2019;20:338-352.
2. Günter J, Ruiz-Serrano A, Pickel C, Wenger RH, Scholz CC. The functional interplay between the HIF pathway and the ubiquitin system - more than a one-way road. Exp Cell Res. 2017;356:152-159.
3. Clague MJ, Heride C, Urbe S. The demographics of the ubiquitin system. Trends Cell Biol. 2015;25:417-426.
4. Edelmann MJ, Iphofer A, Akutsu M, et al. Structural basis and specificity of human otubain 1-mediated deubiquitination. Biochem J. 2009;418:379-390.
5. Wang T, Yin L, Cooper EM, et al. Evidence for bidentate substrate binding as the basis for the K48 linkage specificity of otubain 1. J Mol Biol. 2009;386:1011-1023.
6. Nakada S, Tai I, Panier S, et al. Non-canonical inhibition of DNA damage-dependent ubiquitination by OTUB1. Nature. 2010;466:941-946.
7. Wiener R, Landry MC, Sanches M, et al. OTUB1 co-opts Lys48-linked ubiquitin recognition to suppress E2 enzyme function. *Mol Cell*. 2012;45:384-397.

8. Wiener R, Zhang X, Wang T, Wolberger C. The mechanism of OTUB1-mediated inhibition of ubiquitination. *Nature*. 2012;483:618-622.

9. Sato Y, Yamagata A, Goto-Ito S, et al. Molecular basis of Lys-63-linked polyubiquitination inhibition by the interaction between human deubiquitinating enzyme OTUB1 and ubiquitin-conjugating enzyme UBC13. *J Biol Chem*. 2012;287:25860-25868.

10. Zhu Q, Fu Y, Li L, Liu CH, Zhang L. The functions and regulation of Otubains in protein homeostasis and diseases. *Ageing Res Rev*. 2021;71:101303.

11. Herhaus L, Perez-Oliva AB, Cozza G, et al. Casein kinase 2 (CK2) phosphorylates the deubiquitylase OTUB1 at Ser16 to trigger its nuclear localization. *Sci Signal*. 2015;8:ra35.

12. Li Y, Sun XX, Elferich J, Shinde U, David LL, Dai MS. Monoubiquitination is critical for ovarian tumor domain-containing ubiquitin aldehyde binding protein 1 (Otub1) to suppress UbcH5 enzyme and stabilize p53 protein. *J Biol Chem*. 2014;289:5097-5108.

13. Scholz CC, Rodriguez J, Pickel C, et al. FIH regulates cellular metabolism through hydroxylation of the deubiquitinase OTUB1. *PloS Biol*. 2016;14:e1002347.

14. Wiener R, DiBello AT, Lombardi PM, et al. E2 ubiquitin-conjugating enzymes regulate the deubiquitinating activity of OTUB1. *Nat Struct Mol Biol*. 2013;20:1033-1039.

15. Pickel C, Günter J, Ruiz-Serrano A, et al. Oxygen-dependent bond formation with FIH regulates the activity of the client protein OTUB1. *Redox Biol*. 2019;26:101265.

16. Saldana M, VanderVorst K, Berg AL, Lee H, Carraway KL. Otubain 1: a non-canonical deubiquitinase with an emerging role in cancer. *Endocar Relat Cancer*. 2019;26:R1-R14.

17. Li Y, Yang JY, Xie E, et al. Preventing abnormal NF-kappaB activation and autoimmunity by Otub1-mediated p100 stabilization. *Cell Res*. 2019;29:474-485.

18. Mulas F, Wang X, Song S, et al. The deubiquitinase OTUB1 augments NF-kappaB-dependent immune responses in dendritic cells in infection and inflammation by stabilizing UBC13. *Cell Mol Immunol*. 2021;18:1512-1527.

19. Wang X, Mulas F, Yi W, et al. OTUB1 inhibits CNS autoimmunity by preventing IFN-gamma-induced hyperactivation of astrocytes. *EMBO J*. 2019;38:e100947.

20. Zhou X, Yu J, Cheng X, et al. The deubiquitinase Otub1 controls the activation of CD8(+) T cells and NK cells by regulating IL-15-mediated priming. *Nat Immunol*. 2019;20:879-889.

21. Koschel J, Nishanth G, Just S, et al. OTUB1 prevents lethal hepatocyte necroptosis through stabilization of c-IAP1 during murine liver inflammation. *Cell Death Differ*. 2021;28:2257-2275.

22. Baisetti MF, Simicek M, Abbasi Asbagh L, et al. OTUB1 triggers lung cancer development by inhibiting RAS monoubiquitination. *EMBO Mol Med*. 2016;8:288-303.

23. Xie JJ, Guo QY, Jin JY, Jin D. SP1-mediated overexpression of IncRNA LINC01234 as a ceRNA facilitates non-small-cell lung cancer progression via regulating OTUB1. *J Cell Physiol*. 2019;234:22845-22856.

24. Bisserier M, Milara J, Abdeldeebbar Y, et al. AAV1.SERCA2a gene therapy reverses pulmonary fibrosis by blocking the STAT3/FOXM1 pathway and promoting the SNON/SKI axis. *Mol Ther*. 2020;28:394-410.

25. Horimasa Y, Ishikawa N, Taniwaki M, et al. Gene expression profiling of idiopathic interstitial pneumonias (IIPs): identification of potential diagnostic markers and therapeutic targets. *BMC Med Genet*. 2017;18:88.

26. Dickinson ME, Flenniken AM, Ji X, et al. High-throughput discovery of novel developmental phenotypes. *Nature*. 2016;537:508-514.

27. Gutsche K, Randi EB, Blank V, et al. Intermittent hypoxia confers pro-metastatic gene expression selectively through NF-kappaB in inflammatory breast cancer cells. *Free Radic Biol Med*. 2016;101:129-142.

28. Randi EB, Vervaet B, Tsachaki M, et al. The antioxidative role of cytoglobin in podocytes: implications for a role in chronic kidney disease. *Antioxid Redox Signal*. 2020;32:1155-1171.

29. Orlando IMC, Lafleur VN, Storti F, et al. Distal and proximal hypoxia response elements cooperate to regulate organ-specific erythropoietin gene expression. *Haematologica*. 2020;105:2774-2784.

30. Imeri F, Nolan KA, Bapt AM, et al. Generation of renal Epo-producing cell lines by conditional gene tagging reveals rapid HIF-2 driven Epo kinetics, cell autonomous feedback regulation, and a telocyte phenotype. *Kidney Int*. 2019;95:375-387.

31. Uhlen M, Fagerberg L, Hallstrom BM, et al. Tissue-based map of the human proteome. *Science*. 2015;347:1260419.

32. Du Y, Guo M, Whitsett JA, Xu Y. ‘LungGENS’: a web-based tool for mapping single-cell gene expression in the developing lung. *Thorax*. 2015;70:1092-1094.

33. Du Y, Kitzmiller JA, Sridharan A, et al. Lung gene expression analysis (LGEA): an integrative web portal for comprehensive gene expression data analysis in lung development. *Thorax*. 2017;72:481-484.

34. Du Y, Ouyang W, Kitzmiller JA, et al. Lung at a glance: an integrative web tool of lung ontology, imaging and single cell omics. *bioRxiv*. 2020;2020.06.19.161851.

35. Cowley GS, Weir BA, Vazquez F, et al. Parallel genome-scale loss of function screens in 216 cancer cell lines for the identification of context-specific genetic dependencies. *Sci Data*. 2014;1:140035.

36. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods*. 2012;9:676-682.

37. Schneider CA, Rasband WS, Eliceiri KW. NIH image to ImageJ. 25 years of image analysis. *Nat Methods*. 2012;9:671-675.

38. Gschwend J, Sherman SPM, RidderF, et al. Alveolar macrophages rely on GM-CSF from alveolar epithelial type 2 cells before and after birth. *J Exp Med*. 2021;218:e20210745.

39. Clapcote SJ, Roder JC. Simplex PCR assay for sex determination in mice. *Biotechniques*. 2005;38(5):702-706.

40. Cole-Jeffrey CT, Terada R, Neth MR, Wessels A, Kashara H. Progressive anatomical closure of foramen ovale in normal neonatal mouse hearts. *Anat Rec*. 2012;295:764-768.

41. Ikeda H, Shiojima I, Oka T, et al. Increased Akt-mTOR signalling in lung epithelium is associated with respiratory distress syndrome in mice. *Mol Cell Biol*. 2011;31:1054-1065.

42. Schittny JC. Development of the lung. *Cell Res*. 2017;367:427-444.

43. Goncharov T, Niessen K, de Almagro MC, et al. OTUB1 modulates c-IAP1 stability to regulate signalling pathways. *EMBO J*. 2013;32:1103-1114.
44. Lin JT, Lineberry NB, Kattah MG, et al. Naive CD4 T cell proliferation is controlled by mammalian target of rapamycin regulation of GRAIL expression. *J Immunol*. 2009;182:5919-5928.

45. Zhou K, Mai H, Zheng S, et al. OTUB1-mediated deubiquitination of FOXM1 up-regulates ECT-2 to promote tumor progression in renal cell carcinoma. *Cell Biosci*. 2020;10:50.

46. Zhao L, Wang X, Yu Y, et al. OTUB1 protein suppresses mTOR complex 1 (mTORC1) activity by deubiquitinating the mTORC1 inhibitor DEPTOR. *J Biol Chem*. 2018;293:4883-4892.

47. Caron A, Briscoe DM, Richard D, Laplante M. DEPTOR at the nexus of cancer, metabolism, and immunity. *Physiol Rev*. 2018;98:1765-1803.

48. Magnuson B, Ekim B, Fingar DC. Regulation and function of ribosomal protein S6 kinase (S6K) within mTOR signalling networks. *Biochem J*. 2012;441:1-21.

49. Huang K, Fingar DC. Growing knowledge of the mTOR signalling network. *Semin Cell Dev Biol*. 2014;36:79-90.

50. Land SC, Scott CL, Walker D. mTOR signalling, embryogenesis and the control of lung development. *Semin Cell Dev Biol*. 2014;36:68-78.

51. Manning BD, Toker A. AKT/PKB signaling: navigating the network. *Cell*. 2017;169:381-405.

52. Lopez-Barneo J, Macias D, Platero-Luengo A, Ortega-Saenz P, Pardal R. Carotid body oxygen sensing and adaptation to hypoxia. *Pflugers Arch*. 2016;468:59-70.

53. Sun XX, Challagundla KB, Dai MS. Positive regulation of p53 stability and activity by the deubiquitinating enzyme Otubain 1. *EMBO J*. 2012;31:576-592.

54. Saxton RA, Sabatini DM. mTOR signaling in growth, metabolism, and disease. *Cell*. 2017;168:960-976.

55. Prabhakar NR, Semenza GL. Regulation of carotid body oxygen sensing by hypoxia-inducible factors. *Pflugers Arch*. 2016;468:71-75.

56. Scholz CC, Cavadas MA, Tambuwala MM, et al. Regulation of IL-1beta-induced NF-kappaB by hydroxylases links key hypoxic and inflammatory signaling pathways. *PNAS*. 2013;110:18490-18495.

57. Sulser P, Pickel C, Günter J, et al. HIF hydroxylase inhibitors decrease cellular oxygen consumption depending on their selectivity. *FASEB J*. 2020;34:2344-2358.

58. Zhang N, Fu Z, Linke S, et al. The asparaginyl hydroxylase factor inhibiting HIF-1alpha is an essential regulator of metabolism. *Cell Metab*. 2010;11:364-378.

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