SUPPLEMENTAL MATERIAL

MicroRNA-126-3p inhibits angiogenic function of human lung microvascular endothelial cell via LAT1-mediated mTOR signaling

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Supplemental Methods

Western Blot

Western blot was performed as previously described [23]. Briefly, following treatments, cultured cells were washed on ice with cold PBS and gently detached with a cell scraper. Cell suspension was spun at 16,000 g for 10 minutes at 4 °C, the supernatant was discarded, and the pellets were lysed on ice in RIPA buffer (R0278, MilliporeSigma) supplemented with phosphatase inhibitor (Roche Diagnostics) and protease inhibitor (Roche Diagnostics). After 30 minutes, the cell lysate was sonicated and centrifuged at 16,000 g for 10 minutes at 4 °C, and the supernatant was used for assays. Protein concentration was determined by a bicinchoninic assay (BCA) (Pierce Biotechnology, Inc.,Rockford, IL) using bovine serum albumin (BSA) as a standard. Western blots were performed by loading equal amount of protein (10-50 µg) resolved by SDS-PAGE and transferred onto a PVDF membrane (EMD Millipore). Membranes were blocked with either 5% BSA in Tris-buffered saline, 0.1% Tween 20 (TBS-T) or Protein-Free T20 blocking buffer (Thermo Fisher Scientific Inc.) and incubated with diluted primary antibodies at 4°C overnight per manufacturers' specifications. All antibodies used are listed in the Major Resource Table.

RNA sequencing

HLMVEC from three individual donors (ID: 0000612039, 0000489936, 0000525116) purchased from LONZA were transfected with miR-126-3p mimics or inhibitors (5nM each, 16hrs). For controls, cells were co-transfected with non-targeting mimics and antisense inhibitors (5nM each, 16hrs). Total RNA was isolated using the miRNeasy mini kit (Qiagen, Germantown, MD), following the manufacturer's instructions. The

isolated total RNA was processed for next-generation sequencing (NGS) library construction as developed in the NJH Genomics Facility for analysis with an Illumina 2500 (San Diego, CA, USA). A Kapa Biosystems (Wilmington, MA, USA) KAPA mRNA HyperPrep kit for whole transcriptome libraries was used to primarily target all polyA RNA. Briefly, library construction started from isolation of total RNA species, followed by mRNA (poly-A) isolation, 1st and 2nd strand cDNA synthesis, adaptor ligation, amplification, and cluster generation. Once validated, the libraries were sequenced as barcoded-pooled samples and ran on the HiSeq 2500. Illumina TruSeq adapters were removed from RNA-seq reads with skewer (version 0.2.2) [24]. After adapter removal, reads with length less than 18 were removed (default setting for skewer). The quality of the reads was assessed using FastQC[25] (version 0.11.5) before and after read trimming. Reads were mapped with the STAR aligner (version 2.5.1b) [28] to the hg19 assembly of the human genome using gene annotations from Ensembl version 75[32]. Reads mapping to each gene of the Ensembl 75 annotation were counted with the featureCounts program from the Subread software package (version 1.6.2) [30]. The counts were transformed using the variance-stabilizing transform (VST) in DESeq2 [31] version 1.20.0. Before comparing expression between groups, genes with expression <1 count per million (CPM) in at least three samples were removed. Statistical modeling was performed using a linear mixed effect model with the lme4 package (version 1.1)[26] for the R statistical software (version 3.6). Hypothesis testing of the regression coefficients was performed using the contest function in the lmerTest package (version 3.1)[29]. The p-values reported were adjusted for multiple testing using the method by Benjamini & Hochberg[27].

All raw data files can be downloaded from the gene expression ombinus, GEO: GSE141129.

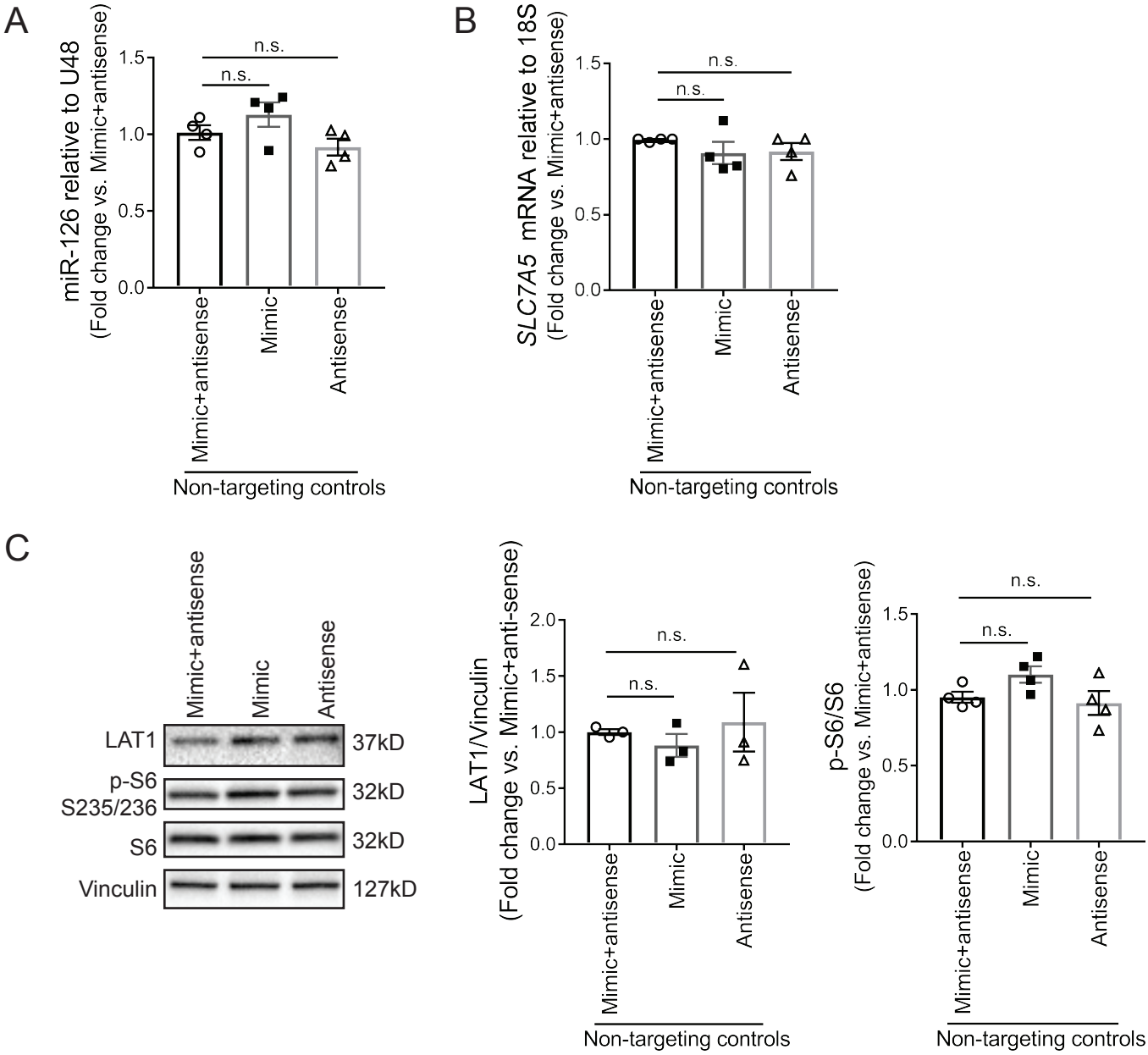
Linear mixed-effect model for regression

Although the RNA-seq data did not accurately measure the amount of miR-126 in each sample, qPCR estimates of the miRNA counts were calculated through RT-qPCR as described earlier (copy numbers listed in Sup table I). This quantitative information was used for a regression analysis. A linear mixed effects model was used to explain the expression of each gene. The dependent variable, the VST of the counts, was modeled as a function of miR-126 amount (specifically, the log2-transformed miR-126 copy numbers) and the sample donor. The miR-126 copy number variable was treated as a fixed effect in the regression, along with a fixed y-intercept, while the donor was treated as a random effect. Intuitively, this fits a line to the gene expression as a function of the miR-126 amount, with an offset (shifting the line up or down) specific to each individual donor. The model terms were fit using restricted maximum likelihood (REML) estimation. The model's fit converged for 13, 360 of the 14, 517 tested genes. The remaining genes went unmodeled and were not included in the hypothesis testing. The regression slope and y-intercept for each gene's regression were extracted from the model. Intuitively, the slope can be interpreted as the predicted change in VST of the counts for a particular gene when the quantity of miR-126 is doubled (it should be noted that the VST itself acts as a log-transformation of the counts in most circumstances). Genes with positive coefficients were positively correlated with miR-126, and genes with negative coefficients were negatively correlated with miR-126. The significance of the slope term in the model was obtained using a t-test, under the null hypothesis that the slope was

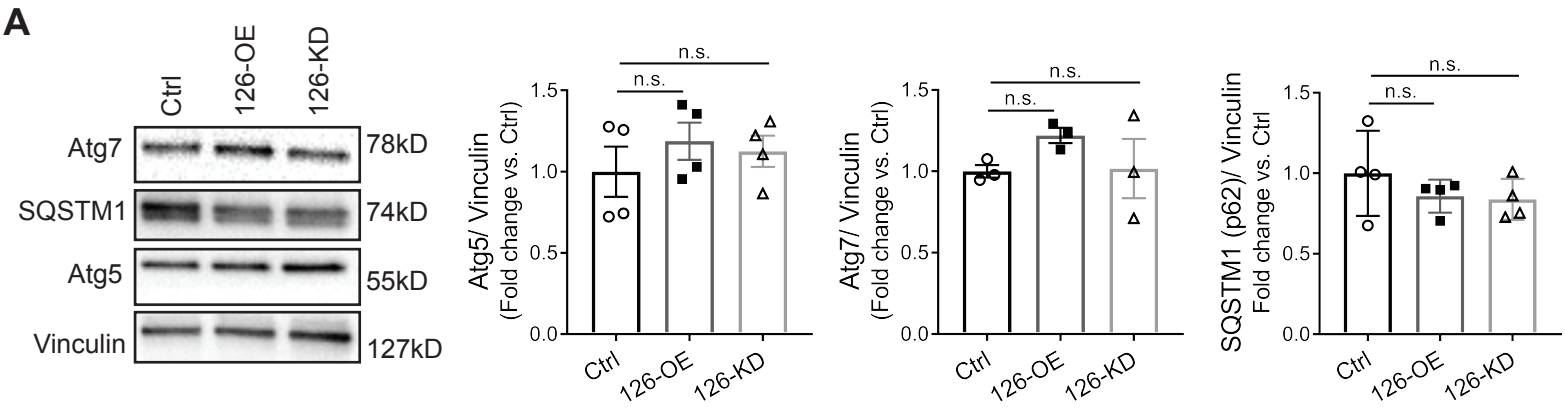
zero. The p-values were adjusted for multiple testing.

Ingenuity Pathway Analysis (IPA)

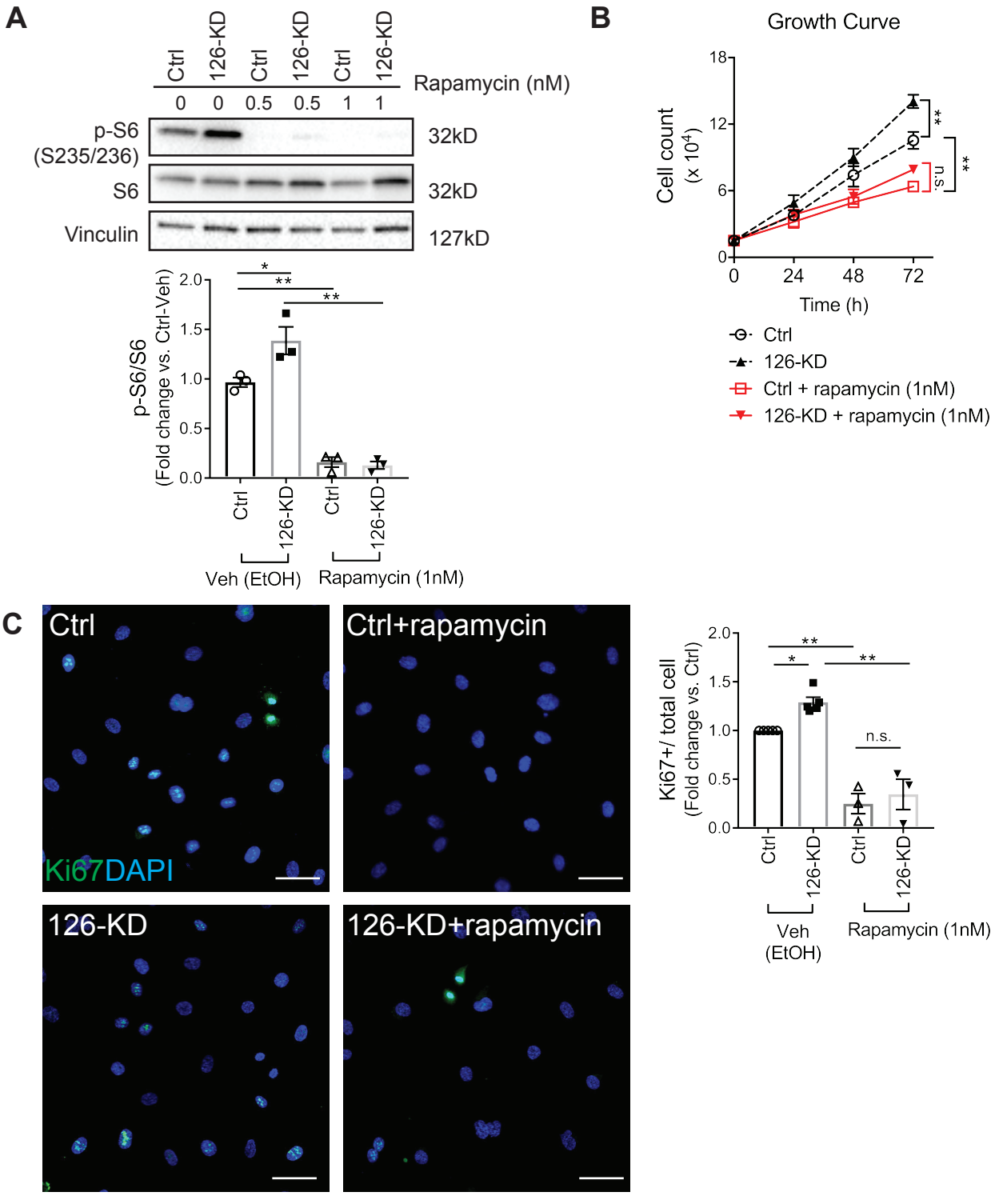
Following RNA sequencing, genes significantly correlated with miR-126 levels (co-efficient $\neq 0$, adjusted-p value <0.05) were uploaded and analyzed using the Ingenuity Pathway Analysis software (IPA, QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis) to investigate signaling pathways regulated by miR-126. A core canonical signaling pathway analysis was performed and the Z-scores and p-values were calculated by the IPA using Fisher's Exact Test. Pathways with p-value less than 0.05 were visualized and ranked by z-scores.



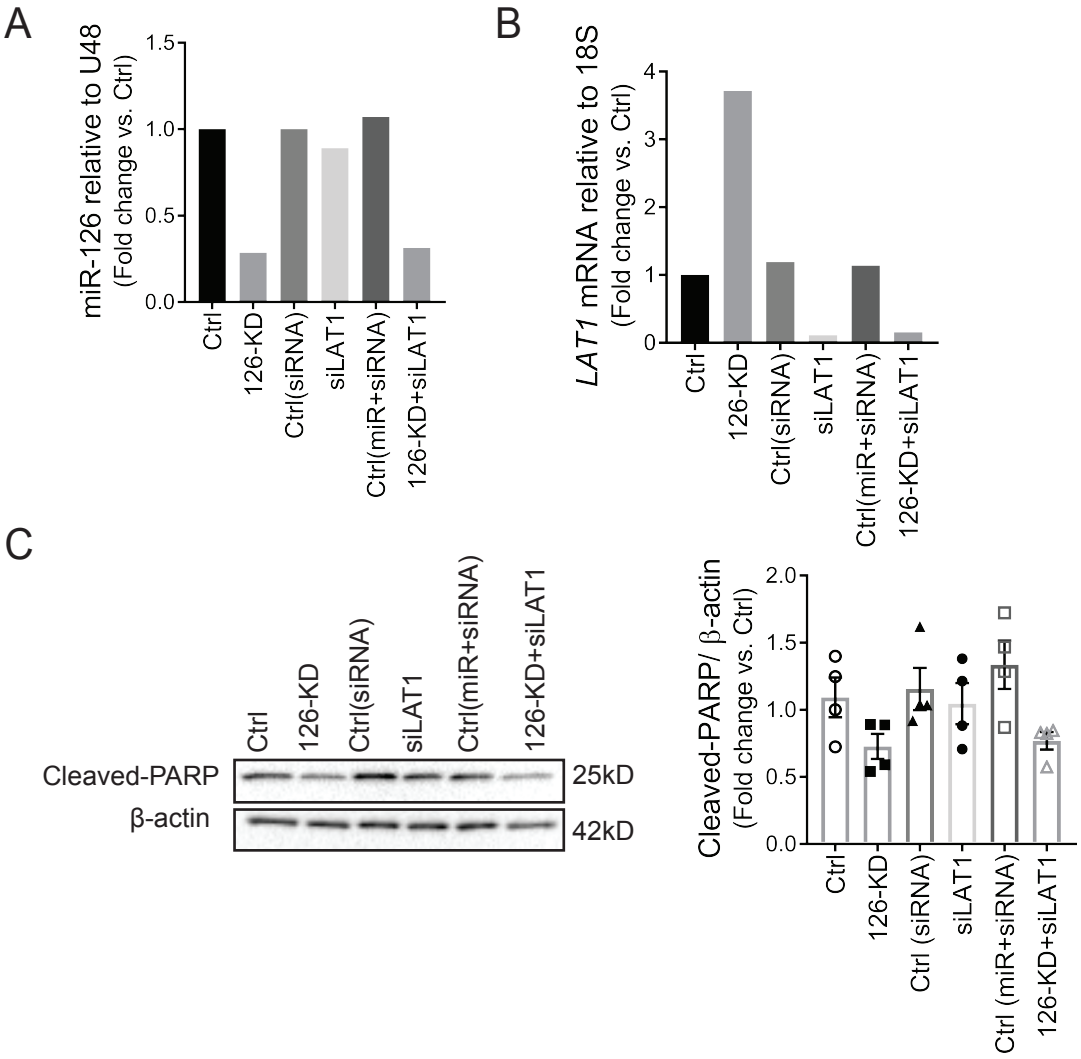
Supplemental figure I. Off-target effects comparison between non-targeting miRNA mimic, antisense inhibitor, and their co-transfection. A-B, Expression levels of miR-126 and its target gene, *SLC7A5*, determined by RT-qPCR. **C,** LAT1 protein expression and S6 activity determined by Western blot. Left: representative Western blot image. Middle, right: densitometry quantification by normalizing LAT1 to vinculin and p-S6 to S6, respectively. Results presented as mean +/- SEM; No statistical significance was detected using One-way ANOVA followed by Dunnett's multiple comparisons test.



Supplemental figure II. Effect of miR-126 on autophagy in HLMVEC. A, Cell autophagy determined by Atg7, Atg5, and SQSTM1 (p62) levels. Left: representative Western blot image. Right: densitometry quantification normalized to vinculin. All results presented as mean +/- SEM; No statistical significance was detected using One-way ANOVA followed by Dunnett's multiple comparisons test.



Supplemental figure III. Role of mTOR signaling in miR-126-regulated cell proliferation. A, Representative Western blot image and quantification of S6 activity after treatment with the mTOR inhibitor rapamycin (0.5nM or 1nM, 6hrs). **B,** Growth curve demonstrated by live cell count of HLMVEC treated with rapamycin (1nM) for 24, 48, or 72hrs. **C,** Left: representative IF images of HLMVEC stained for Ki67 (green) and DAPI (blue). Scale bar: 50μm. Right: quantification of Ki67 expression by first normalizing Ki67 positive cells to the total number of cells (DAPI) and then to the Ctrl condition. 10 images were analyzed per transfection condition per individual experiment. Results presented as mean +/- SEM; One-way ANOVA followed by Tukey's post-hoc multiple comparisons was performed for each time point in **B**. Two-way ANOVA was performed for analyses in **A** and **C**. *p<0.05; **p<0.01; ****p<0.0001;



Supplemental figure IV. Role of LAT1 in miR-126-mediated cell apoptosis. A-B, Transfection efficiencies of miR-126 inhibitor, LAT1 siRNA or co-transfection of both, verified by RT-qPCR. **C,** Cell apoptosis indicated by Western blot measurement of cleaved-PARP. Results presented as mean +/- SEM; No statistical significance was detected using One-way ANOVA followed by Tukey's post hoc test.

Supplemental Table I. Copy numbers of miR-126 in HLMVEC used for RNA-seq.

Donor ID	Ctrl	126-OE	126-KD
0000612039	225423673	3243703876	89204553
0000489936	374103155	6712173364	241181094
0000525116	225423673	4306650709	71200703

Definition of abbreviations: OE = Overexpression; KD = Knockdown

Major Resources Table

Table I. Reagents

Name	Vendor	Catalog #
JPH203	Selleckchem	S8667
Rapamycin	Sigma	R0395-1MG
Caspase-Glo 3/7 Assay System	Promega	PRG8090
TaqMan Universal PCR Master Mix	ThermoFisher	4364338
ProLong Gold Antifade Mountant	ThermoFisher	P36931
Calcein AM Fluorescent Dye	Fisher Scientific	08-774-500

Table II. MicroRNAs and siRNAs

Name	Vendor	Catalog #
MicroRNA Hairpin Inhibitor Transfection Control with Dy547	Dharmacon	IP-004500-01-05
MiRIDIAN microRNA Mimic Transfection Control with Dy547	Dharmacon	CP-004500-01-05
MiRIDIAN microRNA Mimics for hsa-mir-126-3p	Dharmacon	C-300626-07-0002
MiRIDIAN microRNA Hairpin Inhibitors for hsa-mir-126-3p	Dharmacon	IH-300626-08-0002
ON-TARGETplus Non-targeting Pool	Dharmacon	D-001810-10-05
Accell *SLC7A5* siRNA	Dharmacon	E-004953-00-0005

Table III. Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration
Human LAT1	Cell Signaling Technology	5347S	0.026μg/ml
Phospho-ribosomal protein S6 (Ser235/236)	Cell Signaling Technology	2211S	0.0189μg/ml
Ribosomal protein S6	Cell Signaling Technology	2217S	0.037μg/ml

Phospho-p70 S6 kinase (Thr389)	Cell Signaling Technology	9205S	0.03μg/ml
P70 S6 kinase	Santa Cruz	sc-8418	0.4μg/ml
Atg 7	Cell Signaling Technology	8558S	0.04μg/ml
Atg 5	Cell Signaling Technology	12994S	0.3μg/ml
Cleaved- PARP	Abcam	ab32064	0.5μg/ml
SQSTM1 (p62)	Abnova	H00008878-M01	0.1μg/ml
Ki67	Santa Cruz	sc-23900 AF488	1μg/ml
β-actin	Sigma	A5441	1:6000
Vinculin	Abcam	ab18058	0.33μg/ml
GAPDH	Abcam	ab9485	0.33μg/ml
Anti-rabbit IgG HRP	Fisher Scientific	45-000-683	1:10000
Anti-mouse IgG HRP	Fisher Scientific	45-000-679	1:10000

Table IV. Quantitative Real-time PCR

Name	**Vendor or Source**	**Catalog #**
Taqman gene expression assay: *SLC7A5*	ThermoFisher	Hs01001189_m1
Taqman gene expression assay: *ITGA6*	ThermoFisher	Hs01041011_m1
Taqman gene expression assay: *PTPN9*	ThermoFisher	Hs00361739_m1
Taqman gene expression assay: *AKT2*	ThermoFisher	Hs01086099_m1
Taqman gene expression assay: *ADAM9*	ThermoFisher	Hs00177638_m1
Taqman gene expression assay: *CAMSAP1*	ThermoFisher	Hs00251465_m1
Taqman gene expression assay: *BAK1*	ThermoFisher	Hs00832876_g1
Taqman gene expression assay: *LARP6*	ThermoFisher	Hs00217969_m1
Taqman gene expression assay: *CRK*	ThermoFisher	Hs00180418_m1
Taqman gene expression assay: *DLK1*	ThermoFisher	Hs00171584_m1
Taqman gene expression assay: *SERPINE1*	ThermoFisher	Hs00167155_m1

Taqman gene expression assay: 18s	ThermoFisher	Hs99999901_s1
Taqman microRNA expression assay: hsa-miR-126-3p	ThermoFisher	4427975 (assay ID: 002228)
Taqman microRNA expression assay: hsa-miR-126-5p	ThermoFisher	4427975 (assay ID: 000451)
Taqman microRNA control assay: RNU48	ThermoFisher	4427975 (assay ID: 001006)

Table V. Cell culture reagents

Name	Vendor	Catalog #
EBM-2 Endothelial Cell Growth Basal Medium-2	LONZA	CC-3156
EGM-2 MV Microvascular Endothelial SingleQuots Kit	LONZA	CC-4147
Lipofectamine RNAiMax Transfection Reagent	ThermoFisher	13778150
Matrigel GFR Membrane Matrix	Fisher Scientific	CB-40230A

Table VI. Cells

LONZA primary human lung microvascular endothelial cells (HLMVEC) –Cat # CC-2527			
Donor ID (Lot #)	**Sex**	**Age**	**Usage**
0000612039	Female	63	RNA-seq, Western blot analysis of apoptosis
0000525116	Female	52	RNA-seq, Western blot analysis of apoptosis and mTOR activity, proliferation, tube formation, caspase activity, growth curve
0000489936	Female	2	RNA-seq, Western blot analysis of apoptosis, proliferation, tube formation, caspase activity
0000549135	Female	55	Western blot analysis of mTOR activity, tube formation, caspase activity
0000421710	Female	69	Proliferation, Western blot analysis of apoptosis
0000441097	Female	46	Tube formation, caspase activity
0000547317	Male	57	Tube formation, proliferation
18TL204558	Male	40	Tube formation, Western blot analysis of mTOR activity, apoptosis, and growth curve
18TL012117	Male	44	Proliferation, Western blot analysis of mTOR activity