Supplementary data for

HIF-independent actions of HIF-prolyl hydroxylase inhibitors reveal off-target pathways with therapeutic implications

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Contents

Figure S1. High concentrations of roxadustat and molidustat inhibit cell viability.

Figure S2. Roxadustat and molidustat induce HIF-target gene expression as well as HRE-Luc activity.

Figure S3. High concentrations of roxadustat and molidustat inhibit cell proliferation.

Figure S4. Roxadustat and molidustat do not affect cell adhesion.

Figure S5. Roxadustat and molidustat do not increase reactive oxygen species.

Figure S6. Roxadustat affects angiogenesis in HUVEC cells.

Figure S7. Correlation of the RNA-seq samples.

Figure S8. Venn diagrams of all different treatment groups.

Figure S9. Top 20 up- and downregulated genes in ΔHIF1ΔEPAS1 cells treated with roxadustat.

Figure S10. Top 20 up- and downregulated genes in ΔHIF1ΔEPAS1 cells treated with molidustat.

Table S1: Nucleotide sequences used for CRISPR-Cas9 primers.

Table S2: List of antibodies used in the study.

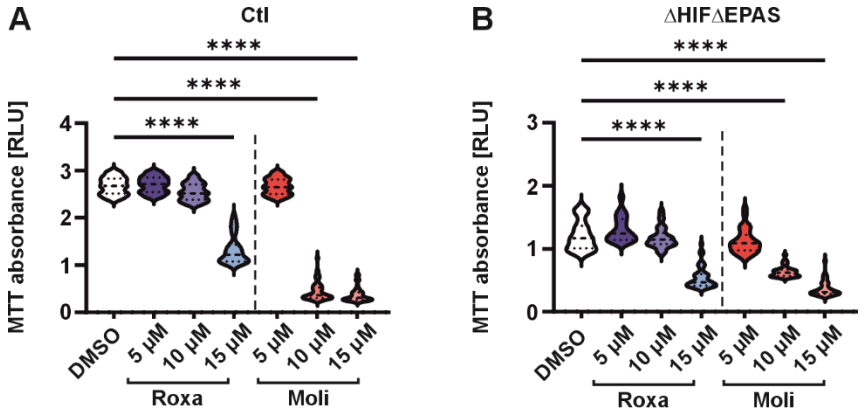


Figure S1. High concentrations of roxadustat and molidustat inhibit cell viability.

(A,B) PC-3 control (Ctl) and ΔHIF1ΔEPAS1 cells were cultured for 24 h, then treated with roxadustat (Roxa) or molidustat (Moli) at different concentrations, and further cultured for 24 h. Thereafter, an MTT assay was performed.

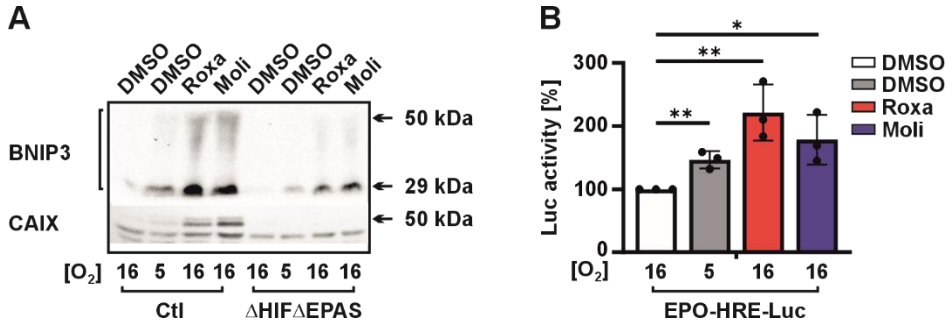


Figure S2. Roxadustat and molidustat induce HIF-target gene expression as well as HRE-Luc activity.

(A) PC-3 control (Ctl and ΔHIF1ΔEPAS1 cells were cultured for 24 h, then treated with either roxadustat (Roxa) or molidustat (Moli) and further cultured under normoxia or hypoxia (5%O_2) for 16 h. Total protein levels were measured by Western blot with antibodies against BNIP3, CAIX and α-tubulin. **(B)** HEK 293 cells were transfected with an EPO-HRE-Luc expression plasmid for 5 h. After a medium change, cells were treated with roxadustat (Roxa) or molidustat (Moli) and further cultured under normoxic or hypoxic (5%O_2) conditions for 24 h.

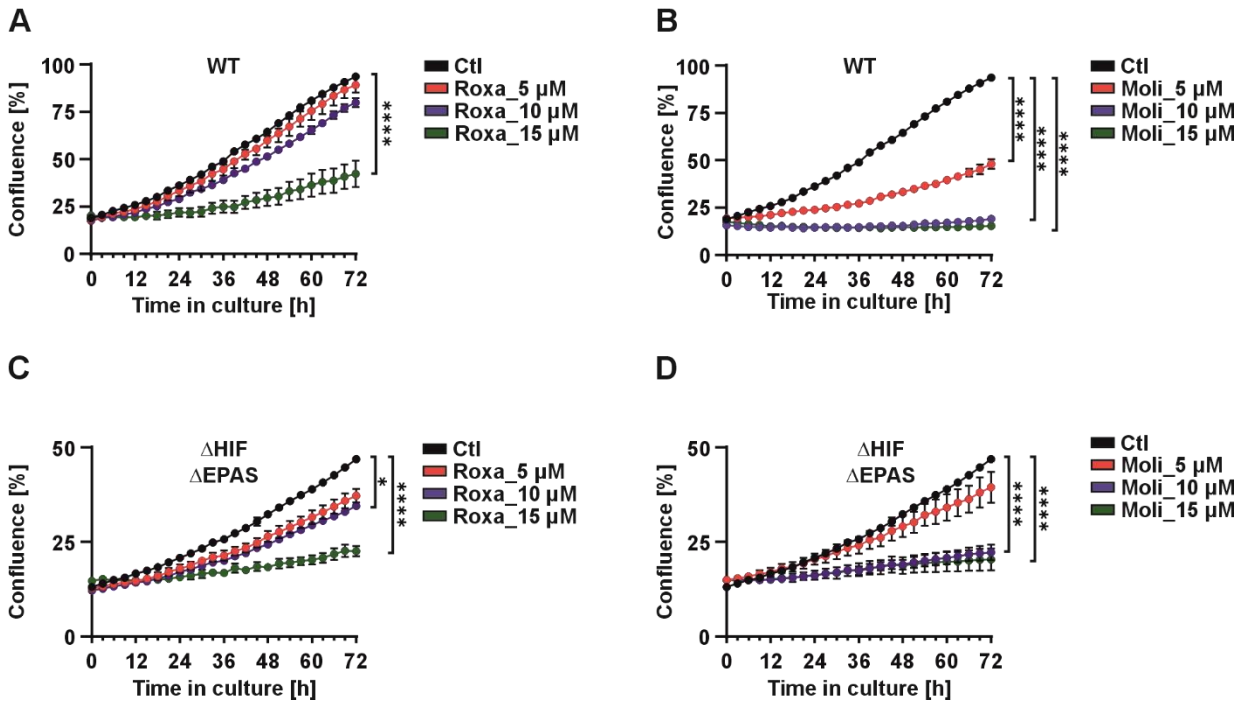


Figure S3. High concentrations of roxadustat and molidustat inhibit cell proliferation.

(A - D) PC-3 control (Ctl) and ΔHIF1ΔEPAS1 cells were cultured for 16 h, after which they were treated with roxadustat (Roxa) or molidustat (Moli) at different concentrations. The live cell proliferation rate was measured with the InCucyte®ZOOM every 3 h for 72 h.

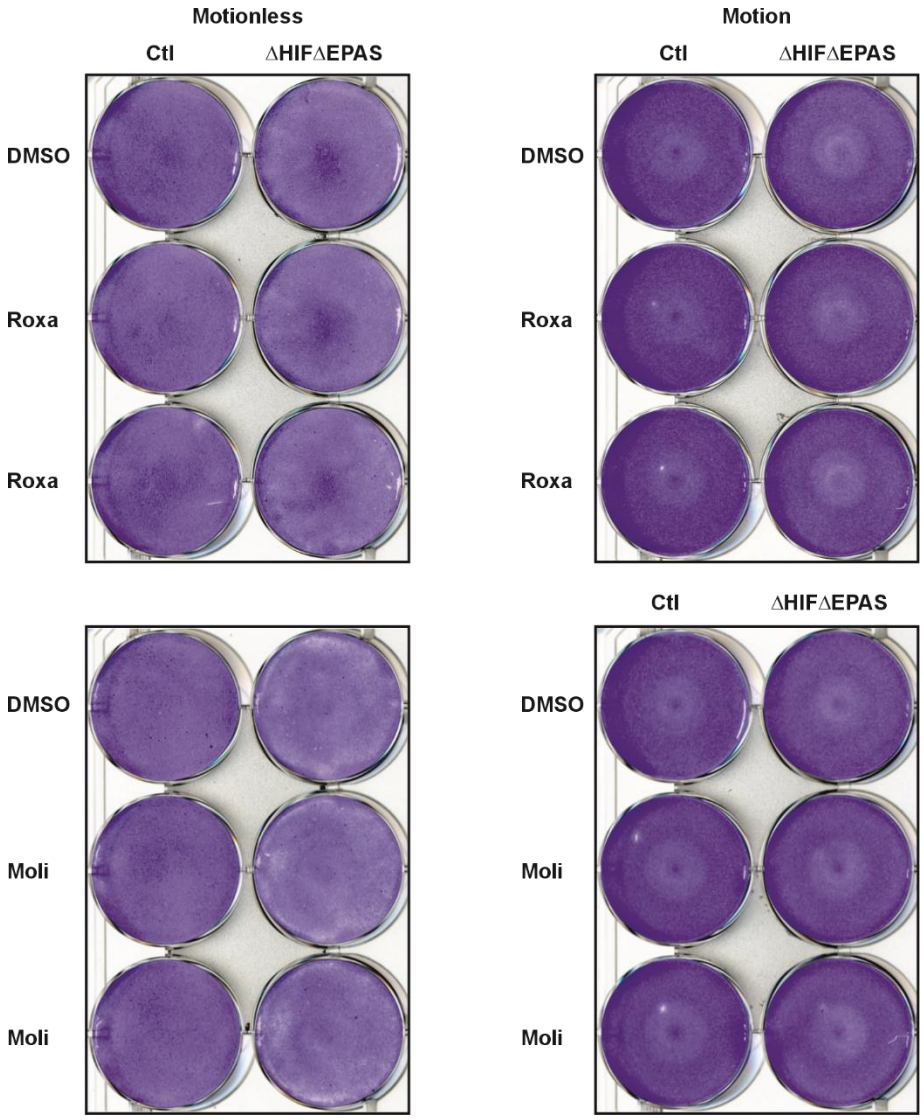


Figure S4. Roxadustat and molidustat do not affect cell adhesion.

PC-3 control (Ctl) and ΔHIF1ΔEPAS1 cells were plated on 6-well plates and grown until confluence was reached. The cells were then treated with roxadustat (Roxa; 10 µM) or molidustat (Moli; 5 µM) and further cultured for 24 h. Thereafter, the plates were transferred to an orbital shaker for an additional 8 h. Control plates remained in the cell incubator.

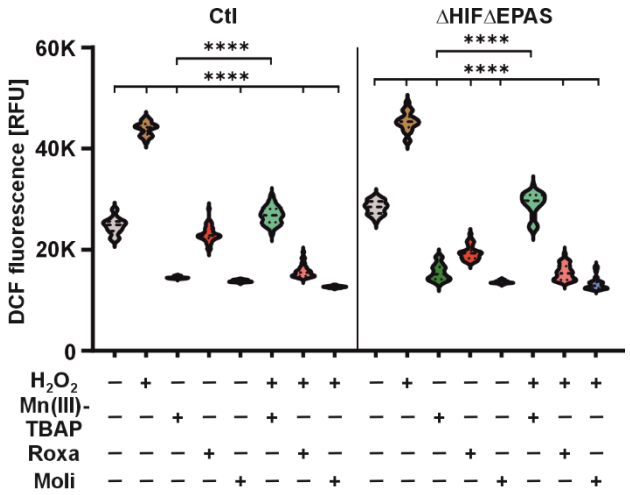


Figure S5. Roxadustat and molidustat do not increase reactive oxygen species.

PC-3 control cells (Ctl) and ΔHIF1ΔEPAS1 cells were treated with roxadustat (10 µM), molidustat (5 µM) or Mn(III)TBAP (20 µM) for 24 h and then stimulated with H_2O_2 (500 µM) for additional 30 min.

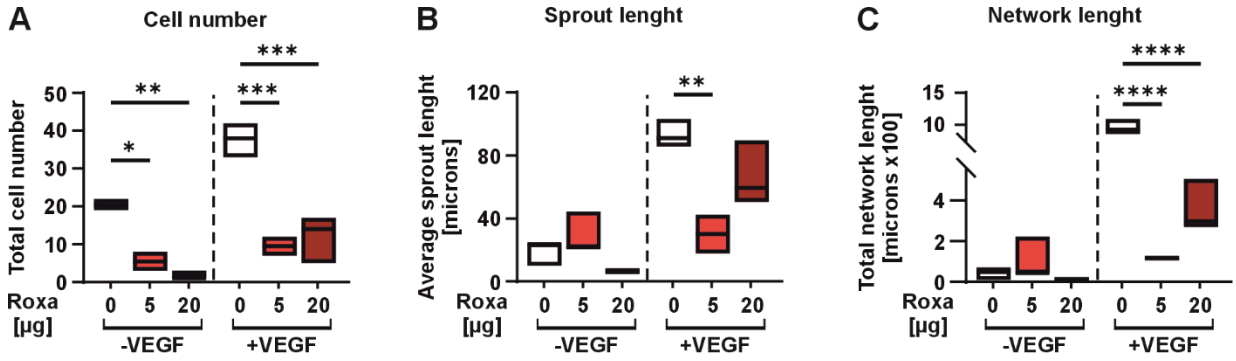
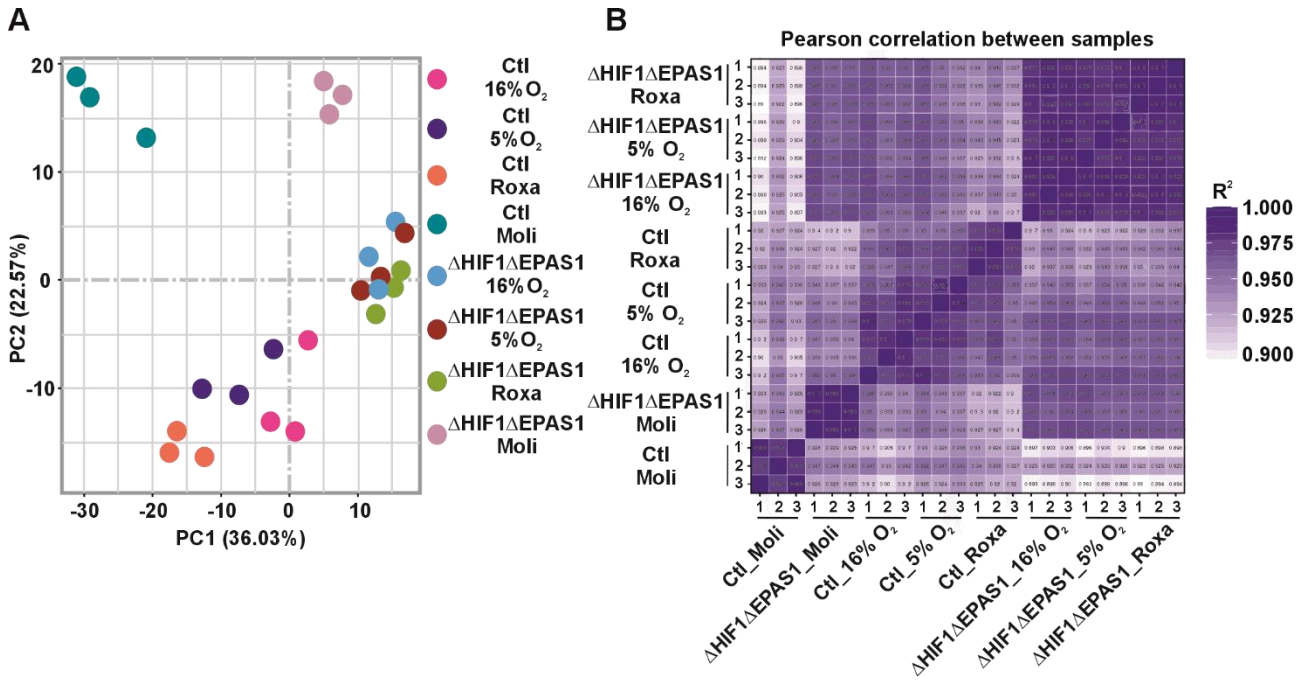


Figure S6. Roxadustat affects angiogenesis in HUVEC cells.

Quantification of the angiogenesis assay in HUVEC cells after 3 days of roxadustat (Roxa) treatment for 3 days: **(A)** cell number, **(B)** sprout length, and **(C)** network length.



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58 **Figure S7. Correlation of the RNA-seq samples.**

59 **(A)** Principal component analysis (PCA) was performed on normalized gene expression data from all
60 samples. Each point represents an individual sample and is colored by experimental group. **(B)** Pearson
61 correlation between the samples.

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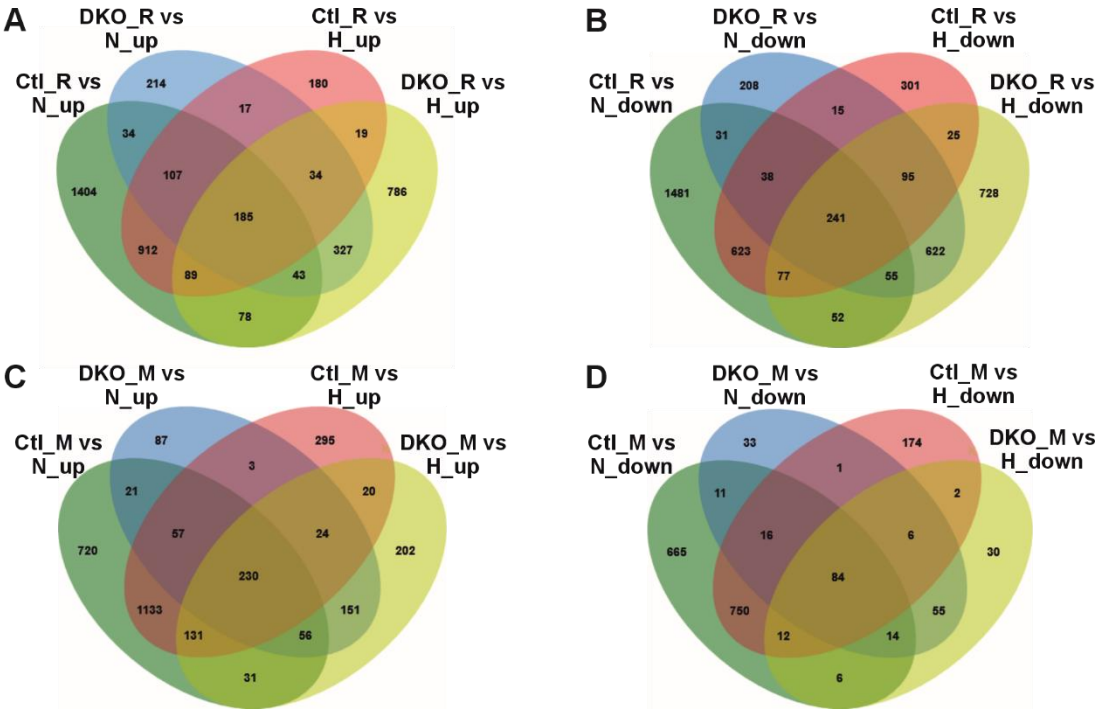

Figure S8. Venn diagrams of all different treatment groups.

Venn diagrams of differentially expressed unique and shared genes. **(A)** Upregulated genes in control (Ctl) or ΔHIF1ΔEPAS1 (DKO) cells upon roxadustat (R) treatment. **(B)** Down-regulated genes in control (Ctl) or ΔHIF1ΔEPAS1 (DKO) cells upon roxadustat (R) treatment. **(C)** Upregulated genes in control (Ctl) or ΔHIF1ΔEPAS1 (DKO) cells upon molidustat (M) treatment. **(D)** Down-regulated genes in control (Ctl) or ΔHIF1ΔEPAS1 (DKO) cells upon molidustat (M) treatment.

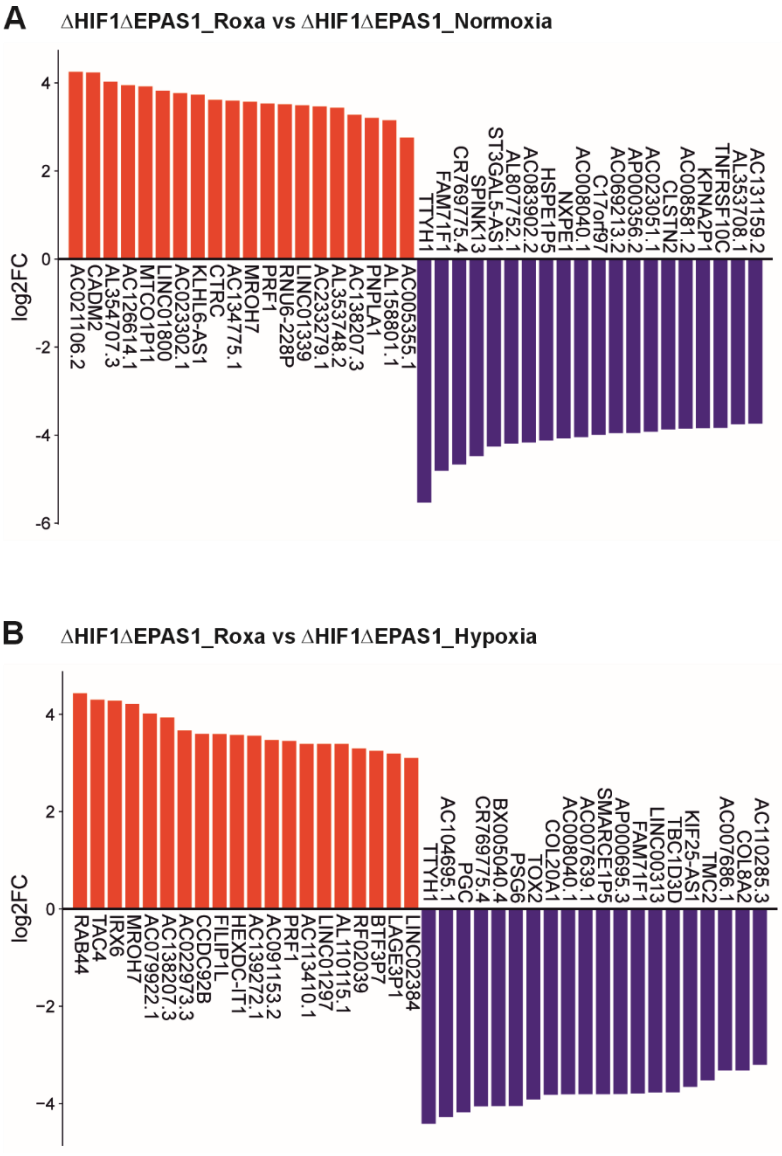

Figure S9. Top 20 up- and downregulated genes in ΔHIF1ΔEPAS1 cells treated with roxadustat.

(A) Most up- and downregulated genes in ΔHIF1ΔEPAS1 cells upon roxadustat (Roxa) treatment compared to normoxia. **(B)** Most up- and downregulated genes in ΔHIF1ΔEPAS1 cells upon roxadustat (Roxa) treatment compared to hypoxic conditions.

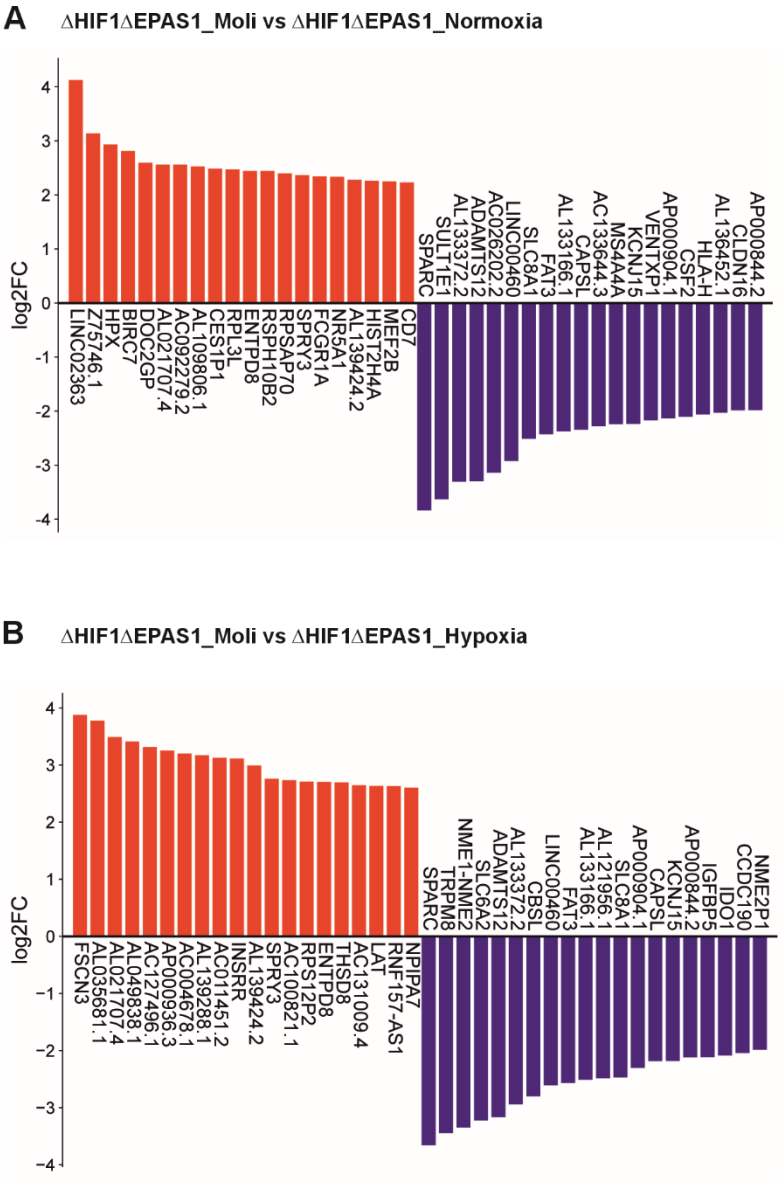

Figure S10. Top 20 up- and downregulated genes in ΔHIF1ΔEPAS1 cells treated with molidustat.

(A) Most up- and downregulated genes in ΔHIF1ΔEPAS1 cells upon molidustat (Moli) treatment compared to normoxia. **(B)** Most up- and downregulated genes in ΔHIF1ΔEPAS1 cells upon molidustat (Moli) treatment compared to hypoxic conditions.

Table S1: Nucleotide sequences used for CRISPR-Cas9 primers.

Gene	Nucleotide sequence (5' to 3')
HIF1A (guide)	CCATCAGCTATTTGCGTGTG
EPAS1 (guide)	CAAGGCCTCCATCATGCGAC
Genotyping/ Sequencing	**Nucleotide sequence (5' to 3')**
HIF1	Forward: GAAAAGTCTCGAGATGCAGCCA
	Reverse: GGGAAAAGCCAGTATCTTATTCCTG
EPAS1	Forward: CCCATGTGAAGCCCTGTTCT
	Reverse: CCCATGTTCTTCCCTGGTCC
CBFA2T3 (off-target HIF1A)	Forward: GAGGAGCTCAACCACTGGG
	Reverse: CTCGGACAAGGTCTGGCTC
ALDH1L1 (off-target EPAS1)	Forward: TGATCTCTCAGTGTCACAGCCA
	Reverse: GCCCCTTCACACCCTTATCC

Table S2: List of antibodies used in the study.

Primary Antibodies	Origin	Clonality	Dilution factor	Company	Catalog nr.
HIF-1α	Mouse	monoclonal	1:1.000	BD Bioscience	#610959
HIF-2α	Rabbit	monoclonal	1:1.000	Cell signaling	#7096
CAIX	Mouse	monoclonal	1:1.000	Santa Cruz	#sc-365900
BNIP3 (D7U1T)	Rabbit	monoclonal	1:1.000	Cell signaling	#44060
α-Tubulin (clone B-5-1-2)	Mouse	monoclonal	1:10.000	Sigma-Aldich	#T5168
Secondary Antibodies	**Origin**		**Dilution factor**	**Company**	**Catalog nr.**
Mouse-HRP	Goat		1:5.000	Bio-Rad	#1706516
Rabbit-HRP	Goat		1:5.000	Bio-Rad	#1706515