

Supplementary Data

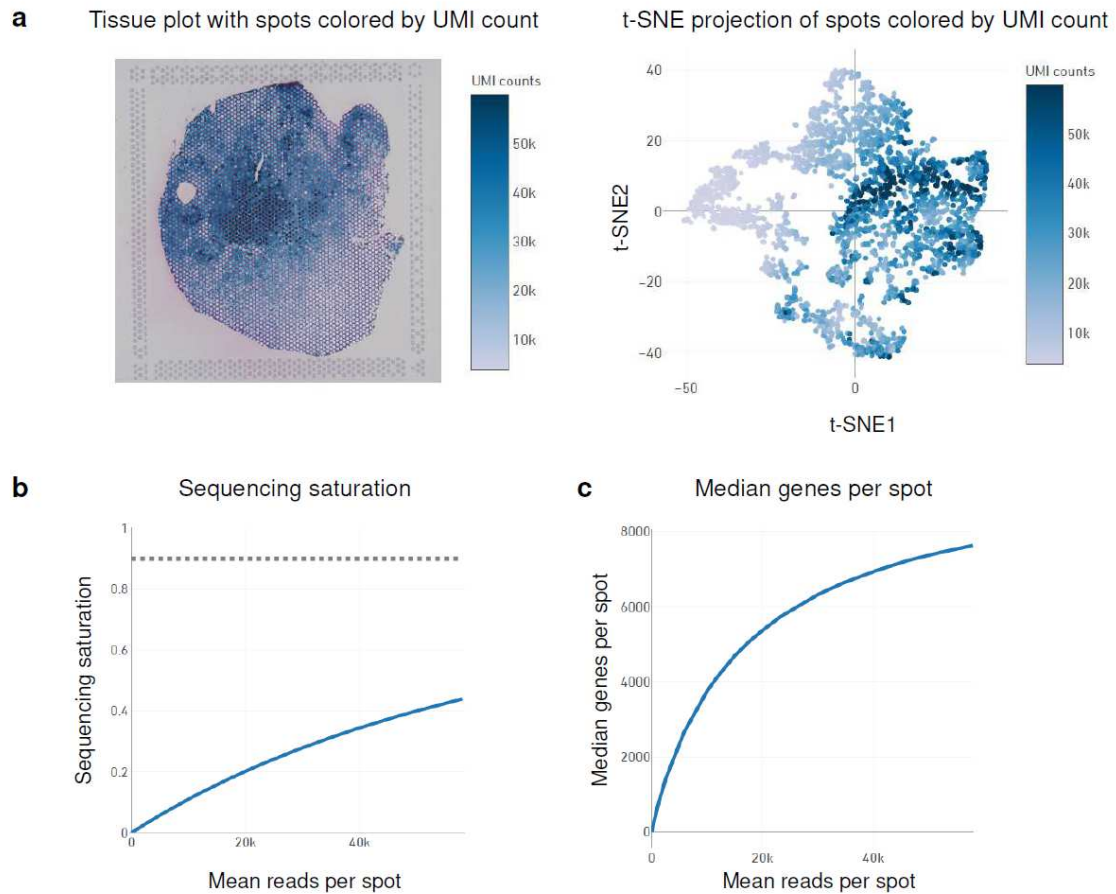
Hypoxia drives the formation of lung micropapillary adenocarcinoma through hypoxia-inducible factor-1 α

Umeda D *et al.*

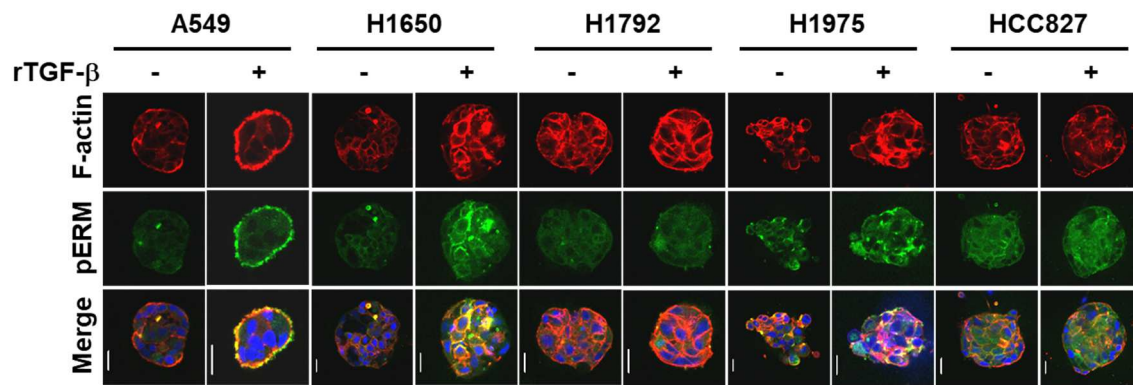
Supplementary materials and methods

Immunocytochemistry

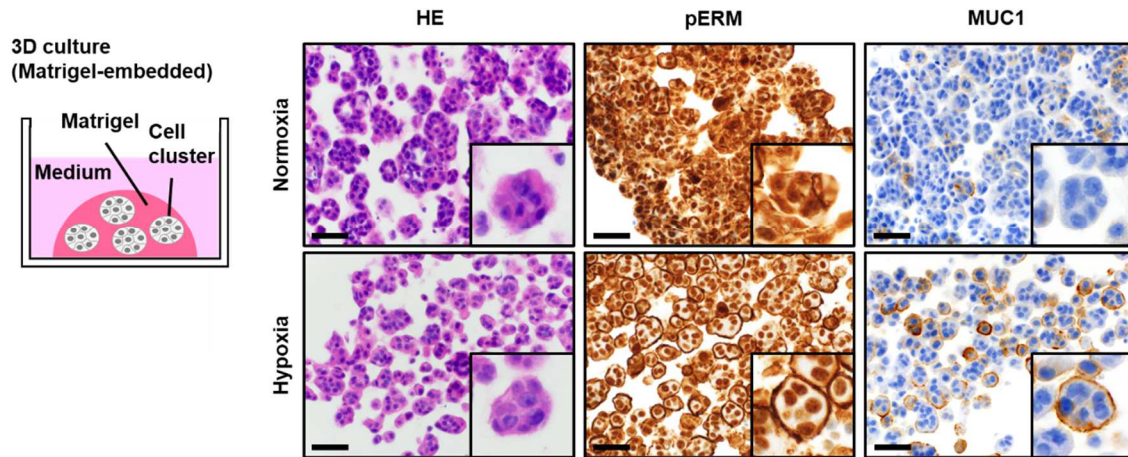
A549 cells cultured in Matrigel under normoxia or hypoxia (1% oxygen) were washed in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 15 min. After centrifugation, the fixed cell pellets were pre-embedded in agarose gel and then embedded in paraffin using the same method used for tissue sample processing. The paraffin-embedded cells were sectioned, processed, and subjected to immunohistochemistry with an anti-phospho-ERM antibody (1:400 dilution) and an anti-MUC1 antibody (1:800 dilution; Abcam, Cambridge, UK) in accordance with the manufacturer's instructions. 3,3'-Diaminobenzidine (DAB) (Dako/Agilent, Santa Clara, CA, USA) was used as the chromogen.



Supplementary Figure S1. Visualization of metrics of Visium spatial gene expression analysis. **a**, Unique molecular identifier count in the spatial transcriptome result and t-Distributed Stochastic Neighbor Embedding (t-SNE) projection of respective spots colored by UMI count. **b**, Sequencing saturation in the spatial transcriptome analysis. **c**, Median number of genes detected per spot under the tissue-associated barcode in the spatial transcriptome analysis.



Supplementary Figure S2. rTGF- β induced formation of MLSs in 3D culture system using A549 cells. Each LUAD cell line was cultured in the 3D culture system for 5 days with 10 ng/mL rTGF- β . Cells were fixed and immunostained for pERM. F-actin and the nucleus were stained with Alexa Flour 594 phalloidin and DAPI, respectively. Scale bar: 20 μ m.



Supplementary Figure S3. Hypoxia induced MLSs even when the entire periphery of the cell cluster was surrounded by Matrigel. Scheme representing the Matrigel-embedded 3D cell culture (left panel). Hematoxylin and eosin cytology and pERM/MUC1 immunocytochemistry of A549 cells embedded in polymerized Matrigel and cultured for 5 days under normoxia (20% oxygen) or hypoxia (1% oxygen) (right panel). Scale bar: 50 μ m. Inset, high magnification.

Supplementary Table S1. Metrics of Visium spatial gene expression analysis in this study

General	
Number of spots under tissue	3,569
Mean reads per spot	57,991
Median genes per spot	7,632
Sequencing	
Number of reads	206,971,505
Valid barcodes	98.5%
Valid UMIs	100.0%
Sequencing saturation	43.9%
Q30 bases in barcode	96.1%
Q30 bases in probe read	87.6%
Q30 bases in UMI	93.7%
Mapping	
Reads mapped to probe set	99.0%
Reads mapped confidently to probe set	98.4%
Reads mapped confidently to the filtered probe set	84.1%
Spots	
Fraction reads in spots under tissue	95.5%
Mean reads under tissue per spot	54,505
Median UMI counts per spot	26,731
Genes detected	17,017

UMIs, unique molecular identifiers.

Supplementary Table S2. Classification of the clusters determined in Visium spatial gene expression analysis based on histology

Cluster No.	Count	Histological classification
Cluster 1	700	PC
Cluster 2	433	Fibrovascular stroma > PC
Cluster 3	411	Fibrovascular stroma > MPC
Cluster 4	410	MPC > Fibrovascular stroma
Cluster 5	272	Blood vessel
Cluster 6	267	Fibrovascular stroma > MPC
Cluster 7	258	MPC > Fibrovascular stroma
Cluster 8	254	Vascular endothelium
Cluster 9	202	MPC
Cluster 10	184	PC > lymphocytes
Cluster 11	178	MPC > Fibrovascular stroma

PC, Papillary adenocarcinoma; MPC, Micropapillary adenocarcinoma

Supplementary Table S3. Top upstream regulators: Cluster 9 (MPC) versus 1 (PC)

Name	<i>p</i> -value
TMPO	1.64e-09
LMNA	4.14e-06
ESR2	7.27e-06
TGFB3	1.26e-05
TP73	3.24e-05

Supplementary Table S4. The top 100 genes whose expression levels were higher in MPC than in PC from visium ST data (the MPC signature genes)

	SYMBOL	Log ₂ FC	P.Value		SYMBOL	Log ₂ FC	P.Value
1	ASPN	1.597784715	1.23E-06	51	MUC1	0.672078532	0.260862921
2	LRRC15	1.465108226	2.84E-05	52	GPM6B	0.662578815	0.503725967
3	CST1	1.461601369	2.84E-05	53	TC2N	0.662344647	0.302500503
4	COL11A1	1.340251079	6.08E-05	54	OLFML2B	0.659705314	0.413888857
5	IPCEF1	1.247093455	0.000314372	55	CHUK	0.657150165	0.443679545
6	COL12A1	1.212922023	0.000682794	56	EEF1D	0.647541601	0.375596035
7	GJA1	1.081786163	0.00549092	57	GJB2	0.646228608	0.613725194
8	HSPA1A	1.063324786	0.004459495	58	LRP2	0.642053004	0.502069645
9	MMP28	1.052564782	0.004459495	59	CCDC78	0.640485045	0.56518238
10	NDRG1	1.039523917	0.007863725	60	HIST1H2BD	0.640352659	0.376220012
11	WFDC3	0.992045272	0.048714236	61	SLC16A9	0.6286917	0.528034682
12	MMP11	0.977089539	0.016693798	62	AGR2	0.627051367	0.42060392
13	SPP1	0.970139478	0.040447352	63	TIMP2	0.623481052	0.405744363
14	PRSS23	0.946056212	0.021172845	64	ZNF431	0.621488336	0.640026264
15	COL10A1	0.934575908	0.02695351	65	DSEL	0.620284324	0.502165627
16	EBP	0.932727323	0.044179611	66	DKK3	0.617681339	0.552128026
17	ANTXR1	0.923588154	0.031826195	67	VKORC1L1	0.614660852	0.419098255
18	ERFL	0.889238629	0.131725358	68	SLC16A3	0.614572367	0.569554298
19	MMP13	0.875694567	0.074293555	69	STEAP4	0.613736511	0.485872858
20	TMEM108	0.841051297	0.178984666	70	PPP4R4	0.60777093	0.614337177
21	COL6A1	0.827373457	0.069649411	71	MAP1LC3A	0.605758379	0.682638369
22	CCND1	0.800126867	0.231177622	72	VCAM1	0.602118605	0.684534865
23	CRIPT	0.797760973	0.216264709	73	PTEN	0.596225778	0.502165627
24	FGFR1	0.793730973	0.144385552	74	ICMT	0.592357724	0.502165627
25	SLC41A2	0.774932013	0.238083172	75	CADM4	0.592018015	0.671368959
26	TIMP3	0.771907136	0.135860705	76	PCOLCE	0.590585445	0.553089898
27	HSPA1B	0.763953649	0.150113933	77	PCBP2	0.589167245	0.526840367
28	STBD1	0.762153482	0.260862921	78	SRI	0.585626472	0.660909095
29	ITGA11	0.756937581	0.216264709	79	COL5A2	0.585045091	0.563969838
30	TGFB1	0.75582752	0.164302354	80	SHOC2	0.585045091	0.653589106
31	ABO	0.749174575	0.250967801	81	PRRX1	0.583148412	0.619782054
32	FTH1	0.738521884	0.157691018	82	C1orf121	0.582017399	0.619782054
33	RAD21	0.732780755	0.207061328	83	NDNF	0.569189082	0.607644984
34	DAP3	0.732640015	0.363841297	84	TGFA	0.566922572	0.685942718
35	FBXO2	0.728277975	0.35074039	85	PLP2	0.561609032	0.619782054
36	ANXA2	0.723732757	0.20277103	86	TM7SF2	0.560825349	0.62966817
37	UBE2K	0.717480816	0.218979696	87	PTPN9	0.559664026	0.780153589
38	MALL	0.714593334	0.216264709	88	FBLN2	0.558560731	0.666928662
39	SLC30A9	0.714402466	0.230838253	89	CCSER2	0.556803322	0.659612157
40	KIFC2	0.701142156	0.226623975	90	PLOD2	0.556065261	0.802385279
41	RCN1	0.699703706	0.246974172	91	DSP	0.555050351	0.617973222
42	TP53I13	0.697053051	0.28026286	92	UNC5B	0.554720555	0.792636704
43	TNFRSF12A	0.696288035	0.236677738	93	ERLIN1	0.553808255	0.7629162
44	ST6GALNAC2	0.687614825	0.375596035	94	ECM1	0.553371041	0.805049244
45	COMTD1	0.68364849	0.411287751	95	ADAM12	0.553280173	0.772909782
46	NT5C3B	0.678508812	0.28026286	96	HIST1H2AC	0.552575938	0.666928662
47	S100A6	0.678296961	0.259814371	97	DPH3	0.5508381	0.805049244
48	SNRPE	0.675959675	0.474493827	98	TPMT	0.549716907	0.742137566
49	ATP5MPL	0.672935334	0.442386196	99	MUC21	0.543580303	0.737664296
50	SMIM13	0.672507933	0.482453194	100	ZDHHC7	0.540968059	0.759892457

Supplementary Table S5. The transcripts per million (TPM) and the log2-transformed fold changes (hypoxia/normoxia) of the MPC signature genes in RNA seq of A549 cells

Among the MPC signature genes listed in Table S4, those with a TPM of less than 1 in RNA-seq were deleted from this list.

SYMBOL	TPM		Log ₂ FC (hypoxia/normoxia)
	Normoxia	Hypoxia	
CST1	52.07366	65.02698	0.320484763
COL12A1	2.26888	12.36605	2.446332998
GJA1	26.72883	66.82206	1.321927857
HSPA1A	126.0405	163.8967	0.378899874
NDRG1	100.0592	1437.467	3.844603208
SPP1	414.9204	531.1583	0.356307306
PRSS23	91.61495	184.6814	1.011383433
EBP	106.1059	127.5394	0.265437051
ANTXR1	24.8687	27.40068	0.139880878
COL6A1	3.789583	10.23509	1.433412214
CCND1	50.85318	80.41875	0.661193993
CRIPT	5.219879	6.706453	0.361533571
FGFR1	45.63689	103.5309	1.181788624
SLC41A2	21.22761	52.19067	1.297849632
HSPA1B	73.34438	89.84545	0.29275892
STBD1	6.596311	13.76234	1.060994885
TGFB1	171.4724	717.2062	2.064411299
FTH1	10457.67	13050.31	0.319523087
RAD21	53.28623	62.29436	0.225338866
DAP3	60.03738	61.77237	0.041100643
FBXO2	16.96757	17.68849	0.060030934
ANXA2	2606.121	5166.749	0.987352812
UBE2K	51.52001	46.32154	-0.153449718
MALL	228.3506	300.2651	0.394986022
SLC30A9	19.74949	19.82454	0.005471993
KIFC2	15.59381	21.24268	0.445992056
RCN1	116.4622	173.371	0.574001012
TP53I13	29.85737	36.57144	0.292630598
TNFRSF12A	65.22985	242.1322	1.892190859
COMTD1	19.12104	28.84996	0.593408826
NT5C3B	51.80475	55.95892	0.111283764
S100A6	3626.793	5060.037	0.480453593
SNRPE	206.7082	204.5621	-0.015057004
ATP5MPL	278.2972	288.8372	0.053629893
SMIM13	9.434674	9.787048	0.052901107
MUC1	3.63628	26.34536	2.85701353
TC2N	17.99783	22.51168	0.322850627
CHUK	12.37265	11.11881	-0.154152635
EEF1D	695.8391	1043.816	0.585042149
CCDC78	4.173984	4.712785	0.175154796
AGR2	1014.213	671.0326	-0.595906468
TIMP2	21.46542	39.52224	0.88065026
ZNF431	1.453693	2.828297	0.960211002
DKK3	20.42132	19.70064	-0.0518337
VKORC1L1	17.63434	25.98787	0.559451195
SLC16A3	7.616937	75.45291	3.308293693
MAP1LC3A	6.938444	9.5656	0.463243301
PTEN	21.70963	20.06784	-0.113449819
ICMT	32.86874	32.33488	-0.023624988
CADM4	10.33891	11.07733	0.099525664
PCOLCE	2.250335	2.506953	0.155795162
PCBP2	393.4977	625.681	0.669072198
SRI	49.26649	134.2385	1.446120008
COL5A2	16.86182	23.92443	0.504724486
SHOC2	12.57472	18.50863	0.55767195
TGFA	8.813962	13.7278	0.639237542
PLP2	264.1127	562.1047	1.089685278
TM7SF2	28.77014	22.9092	-0.328644998
PTPN9	16.60366	27.44005	0.724782026
CCSER2	8.300122	7.958128	-0.060703438
PLOD2	36.47992	292.6487	3.003995779
DSP	18.91706	71.59193	1.920109364
ERLIN1	22.43599	26.74468	0.253437151
ECM1	2.223779	2.297081	0.046788311
DPH3	10.21075	14.42715	0.498697476
TPMT	7.105747	6.216548	-0.192872637
ZDHHC7	35.2832	50.03933	0.504081049

Appendix. The original gel and blot images

Fig. 2g

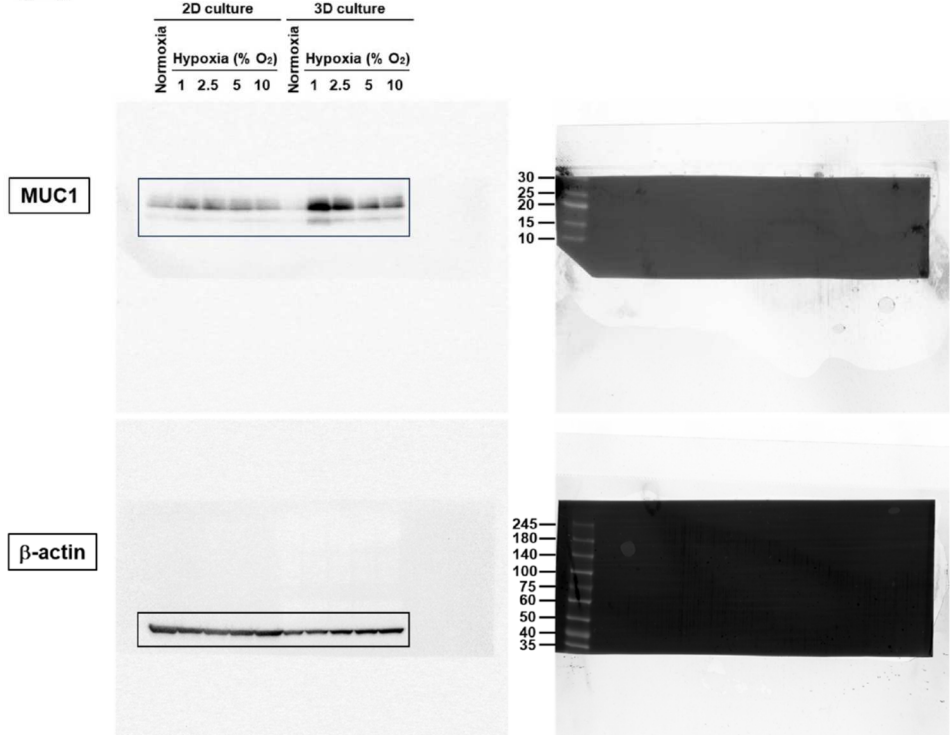


Fig. 2h

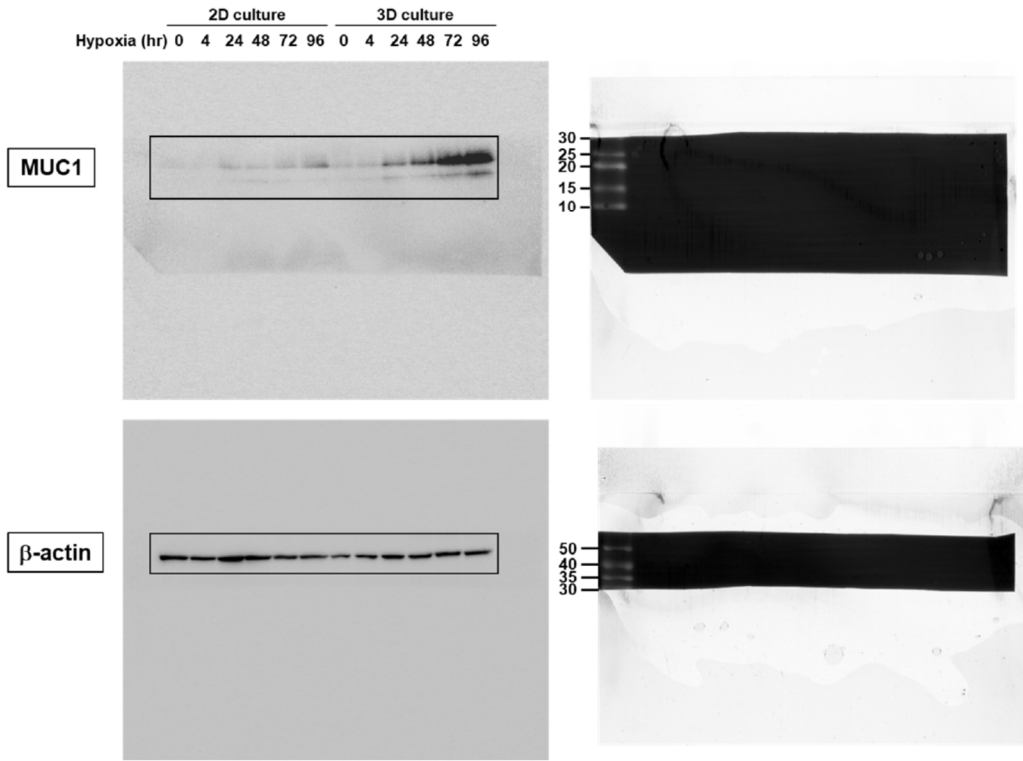


Fig. 2j

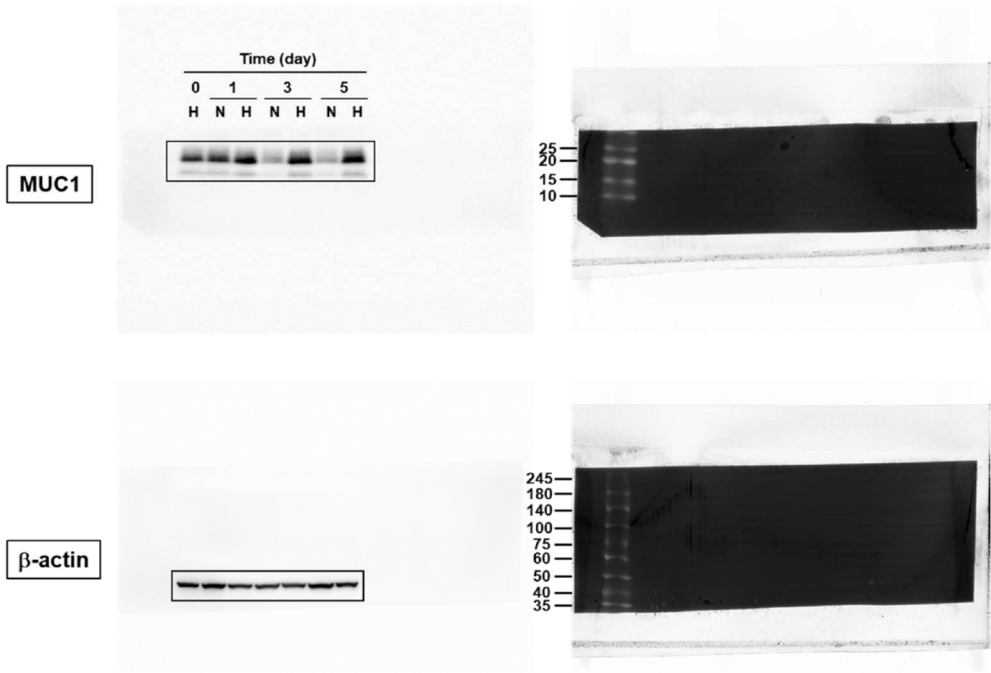


Fig. 4a

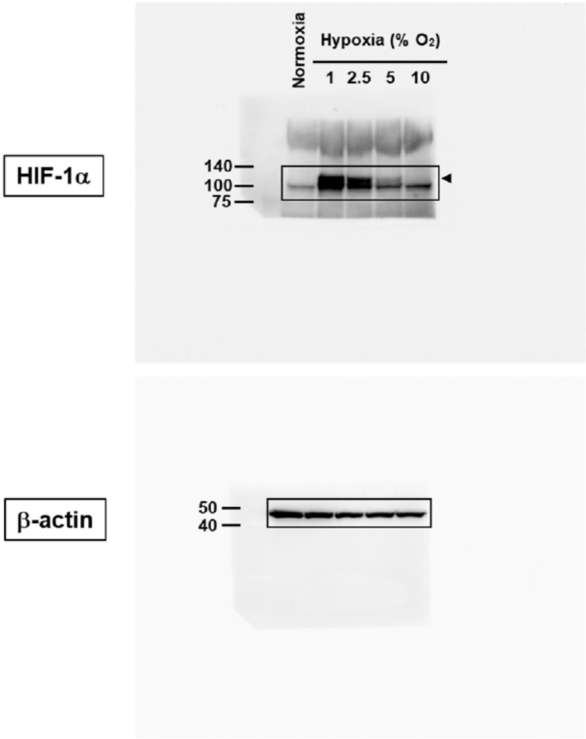


Fig. 4b

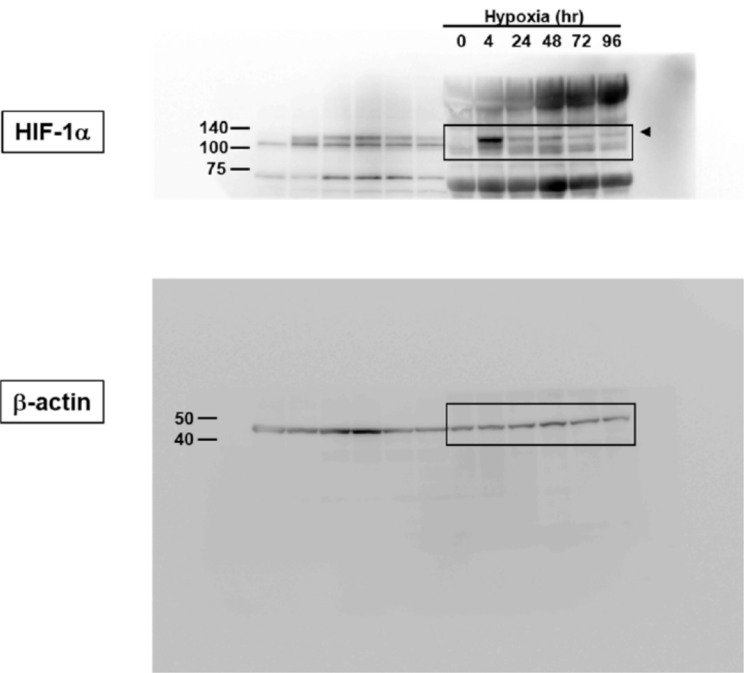


Fig. 4e

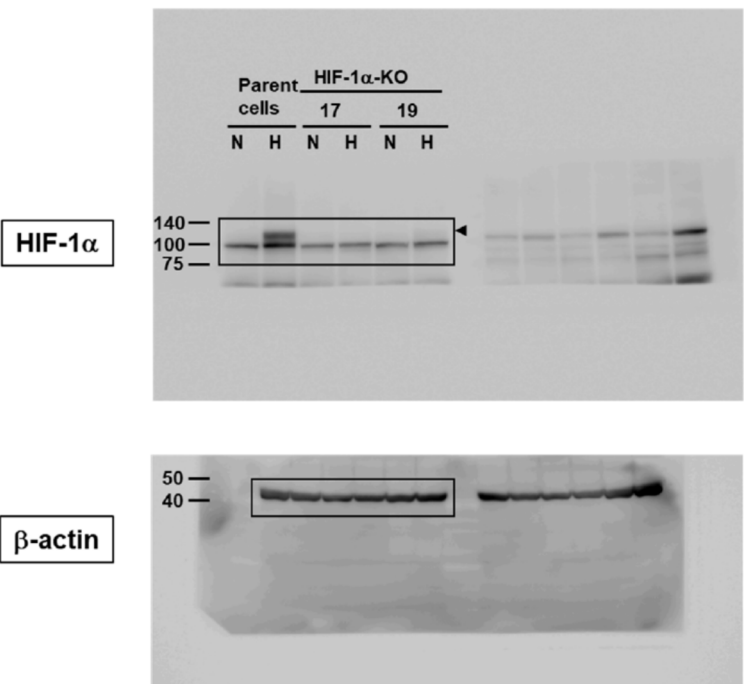


Fig. 4g

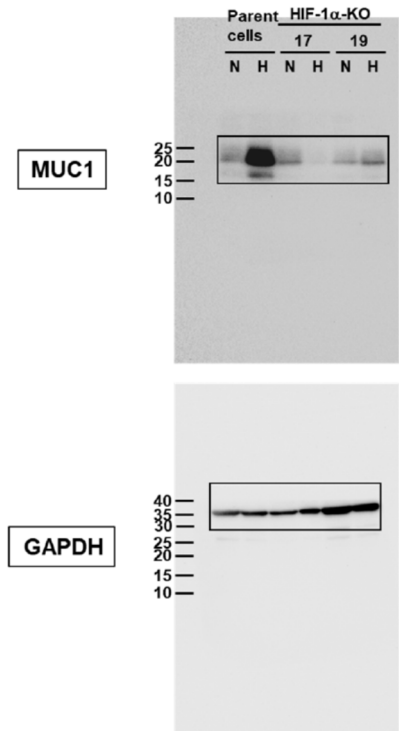


Fig. 4h

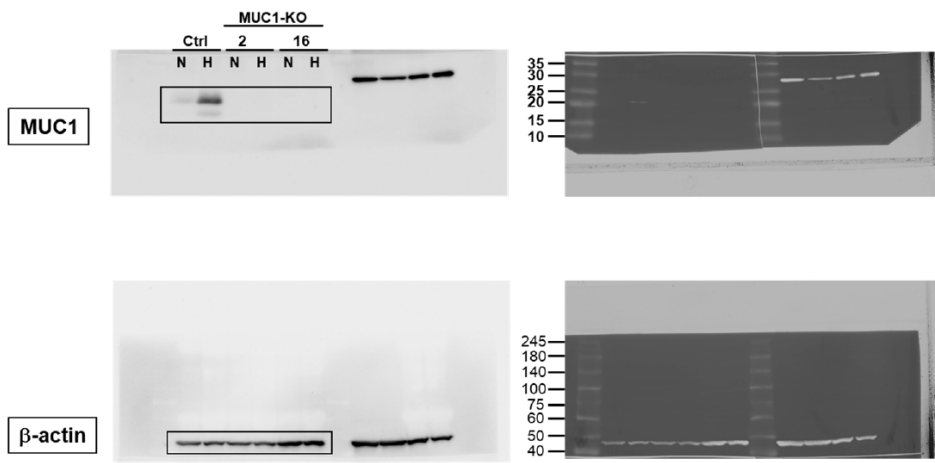


Fig. 5c

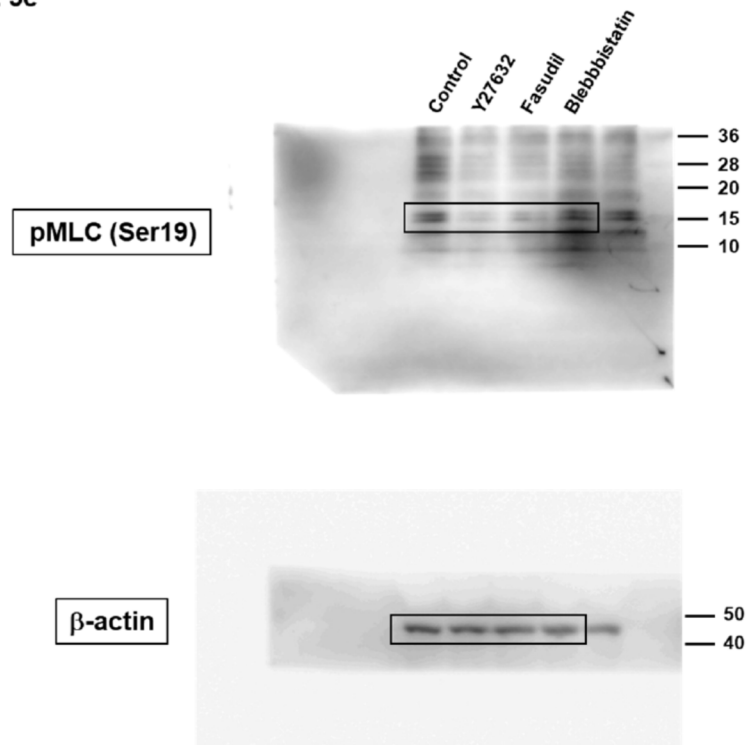


Fig. 5d

