Supplementary Information

ABCB7 simultaneously regulates apoptotic and non-apoptotic cell death by modulating mitochondrial ROS and HIF1α-driven NFκB signaling

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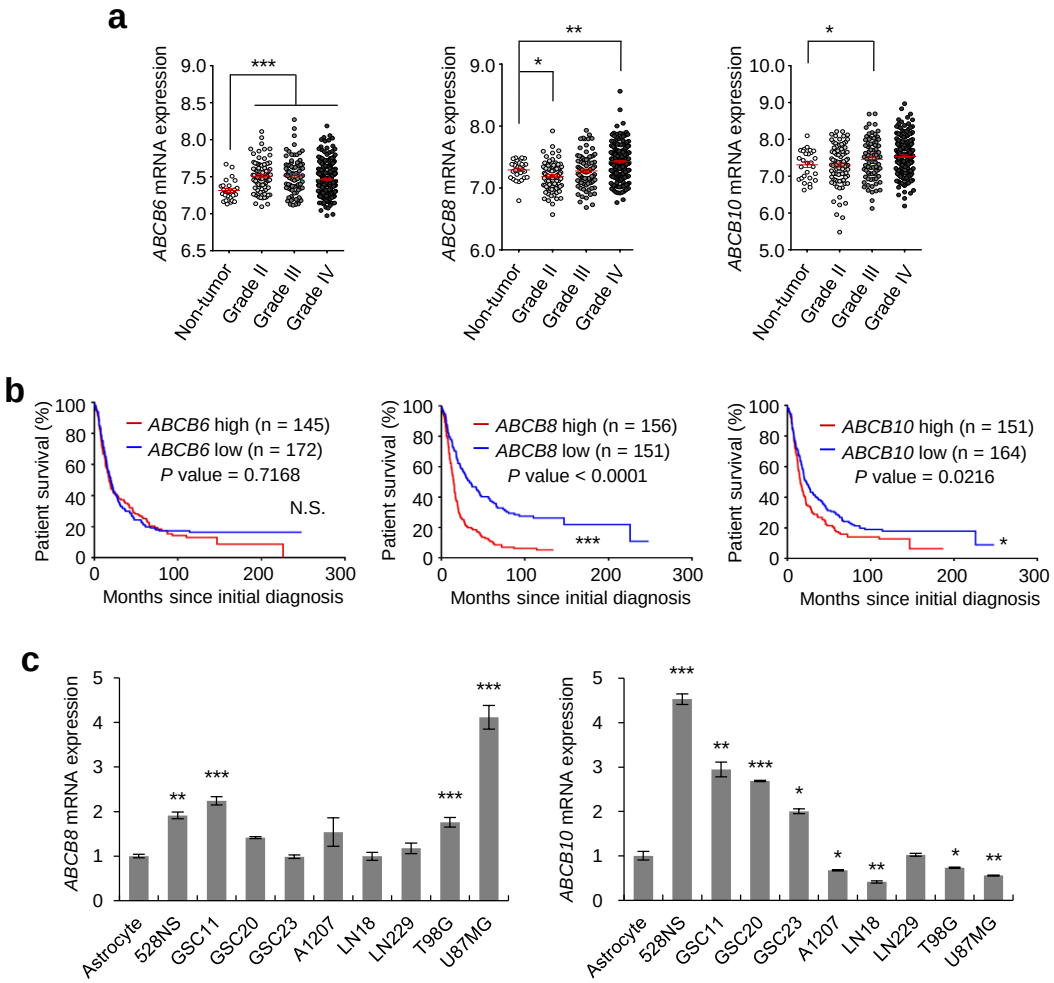


Figure S1. Expression of mitochondrial ABC transporters in the REMBRANDT database and human glioma cells, and the survival of glioma patients with mitochondrial ABC transporter expression.

(a) Analysis of mRNA levels of mitochondrial ABC transporters according to the WHO grade of brain tumors in the REMBRANDT database. The data consist of the WHO grade II (n = 94), III (n = 81), IV (n = 219), and non-tumor (n = 28). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

(b) The survival time of glioma patients according to the expression levels of mitochondrial ABC transporters. The patients were divided into two groups (high and low) based on their expression (mean ± S.E.M). *p*-value with log-rank (Mantel-Cox) test. N.S.; Non-significant, *$p < 0.05$, ***$p < 0.001$.

(c) *ABCB8* (right) and *ABCB10* (left) mRNA levels in human GBM cells and GSCs. Their expression levels in the glioma cells are normalized using those in normal astrocytes. Data were obtained from three independent experiments (mean ± S.E.M). Aa asterisk (*) indicates a statistical significance based on the two-tailed unpaired *t*-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

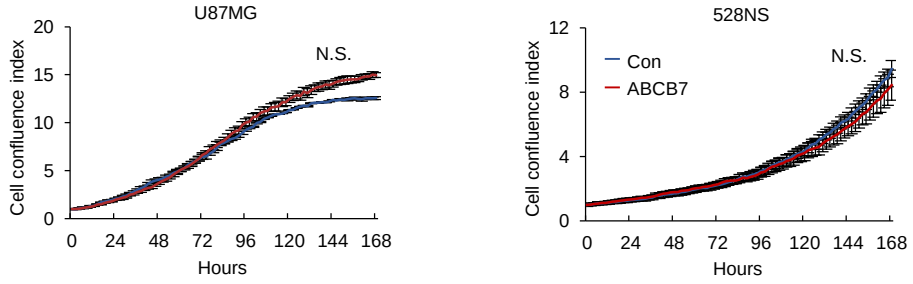


Figure S2. Cell proliferation of U87MG-ABCB7 and 528NS-ABCB7 cells *in vitro*. Cell confluence analysis was conducted using IncuCyte™. N.S.; Non-significant.

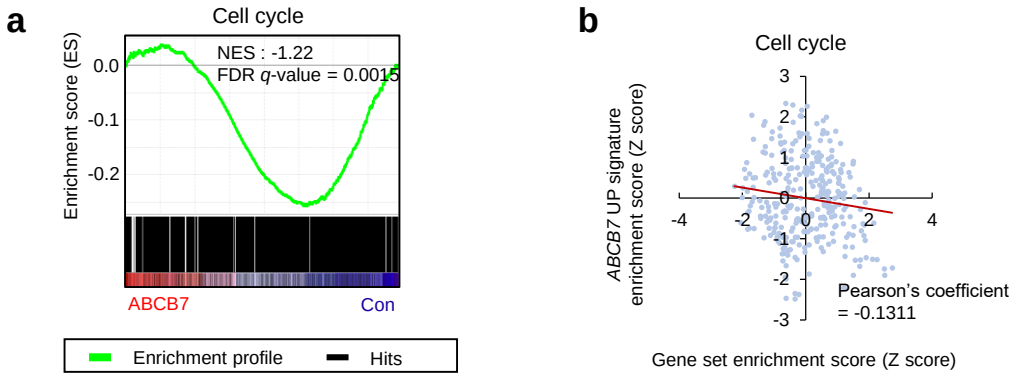


Figure S3. The ABCB7 UP signature negatively correlates with the cell cycle gene set.

(a) Gene set enrichment analysis using the cell cycle gene set (Broad MsigDB hallmark) in U87MG-ABCB7 cells.

(b) Correlation of the enrichment score of the ABCB7 UP signature and the cell cycle gene set in the REMBRANDT dataset.

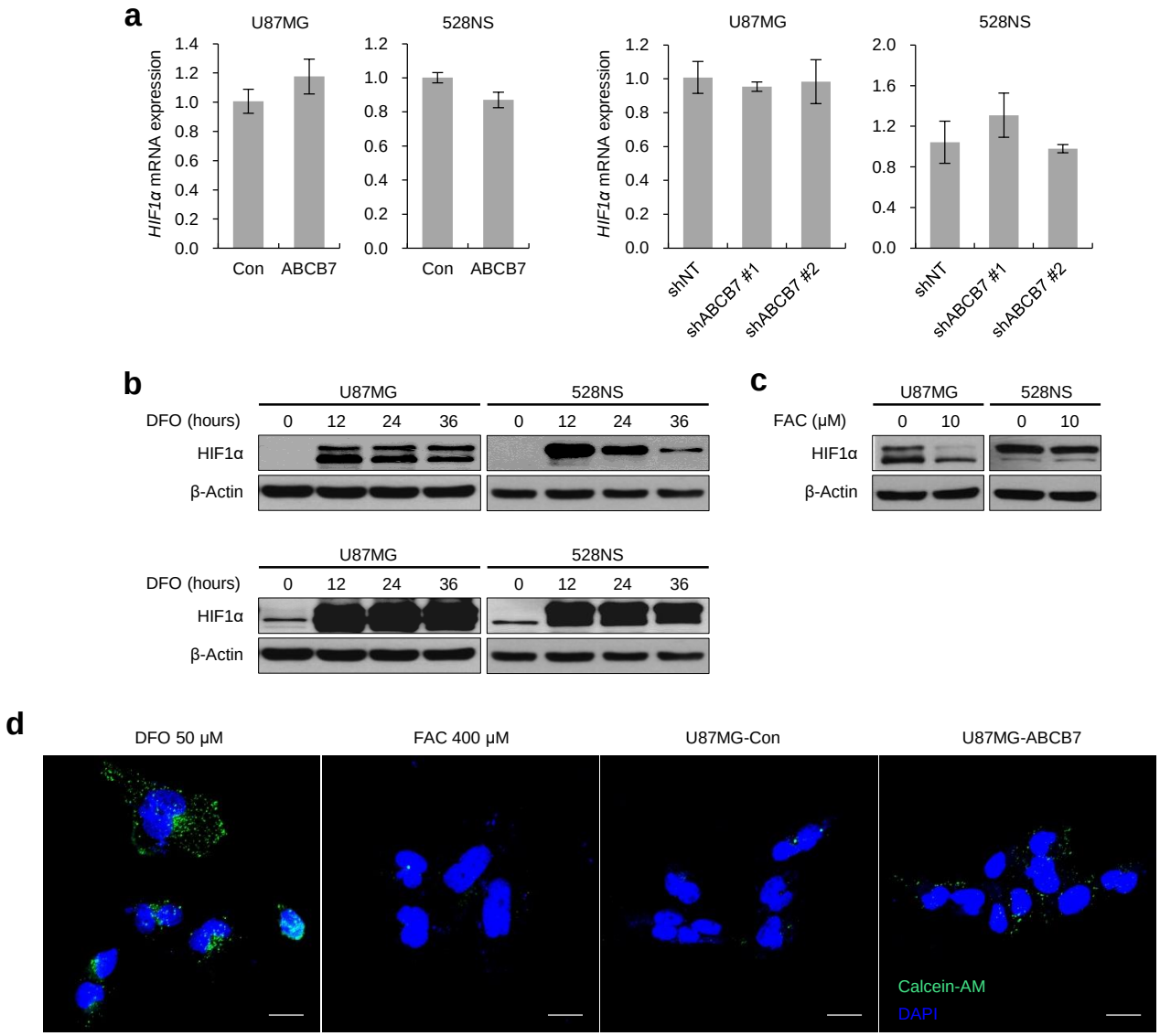


Figure S4. Change of *HIF1α* mRNA level and effect of iron chelator and supplier on HIF1α expression.

(a) The mRNA expression of *HIF1α* in ABCB7-overexpressing and -depleted cells.

(b-c) HIF1α protein expression was examined in U87MG and 528NS cells treated with deferoxamine (DFO) **(b)**, an intracellular iron chelator, or ferric ammonium citrate (FAC) **(c)**, a free iron supplier. β-actin was used as a control. The exposure time of each blot was different (b; upper - 10 sec, lower – 30 sec, c; 1 min). The long exposure plot shows the level of HIF1α.

(d) Images showing intracellular iron concentration (Calcein-AM fluorescence signal) in iron-chelated (DFO), iron efficient (FAC), and control, and ABCB7-overexpressing cells. Magnification 200×. Scale bar = 20 μm.

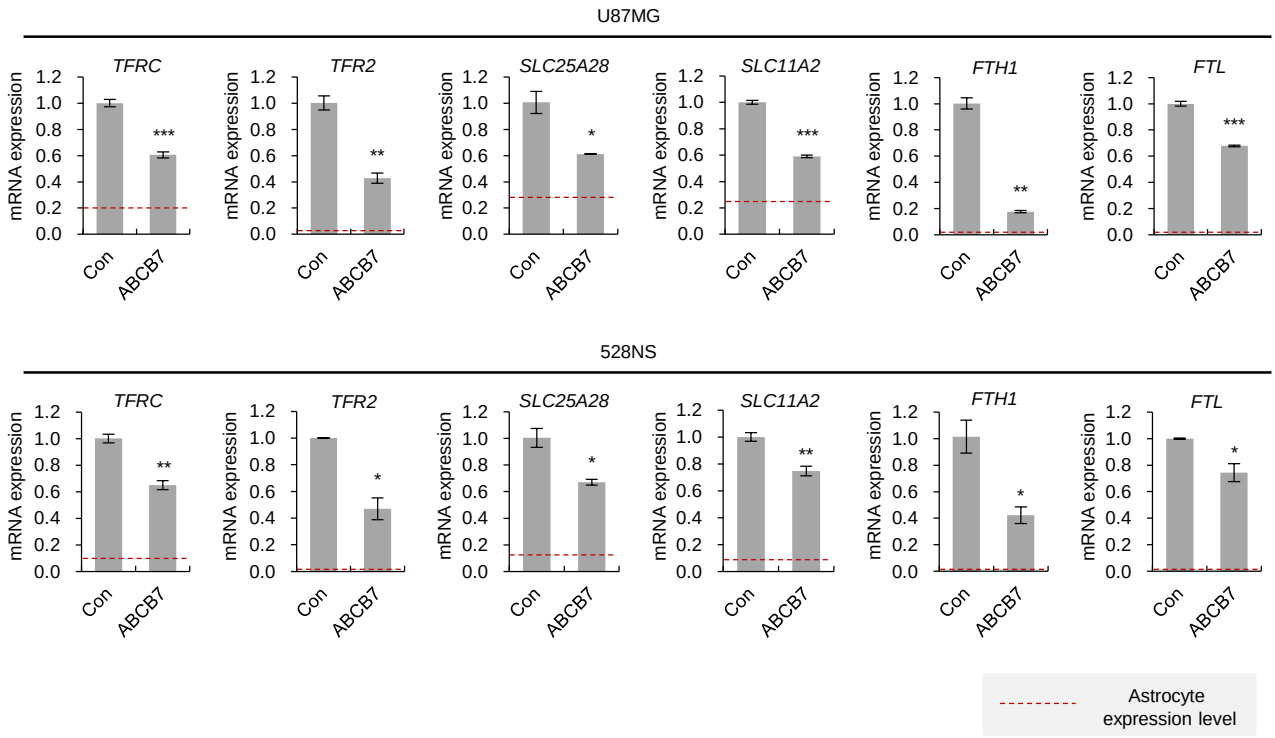


Figure S5. Expression of iron transporters in ABCB7-overexpressing cells.

(a) The mRNA levels of iron transporters in U87MG-Con and U87MG-ABCB7 cells. *p < 0.05, **p < 0.01, ***p < 0.001.

(b) The mRNA levels of iron transporters in 528NS-Con and 528NS-ABCB7 cells. *p < 0.05, **p < 0.01.

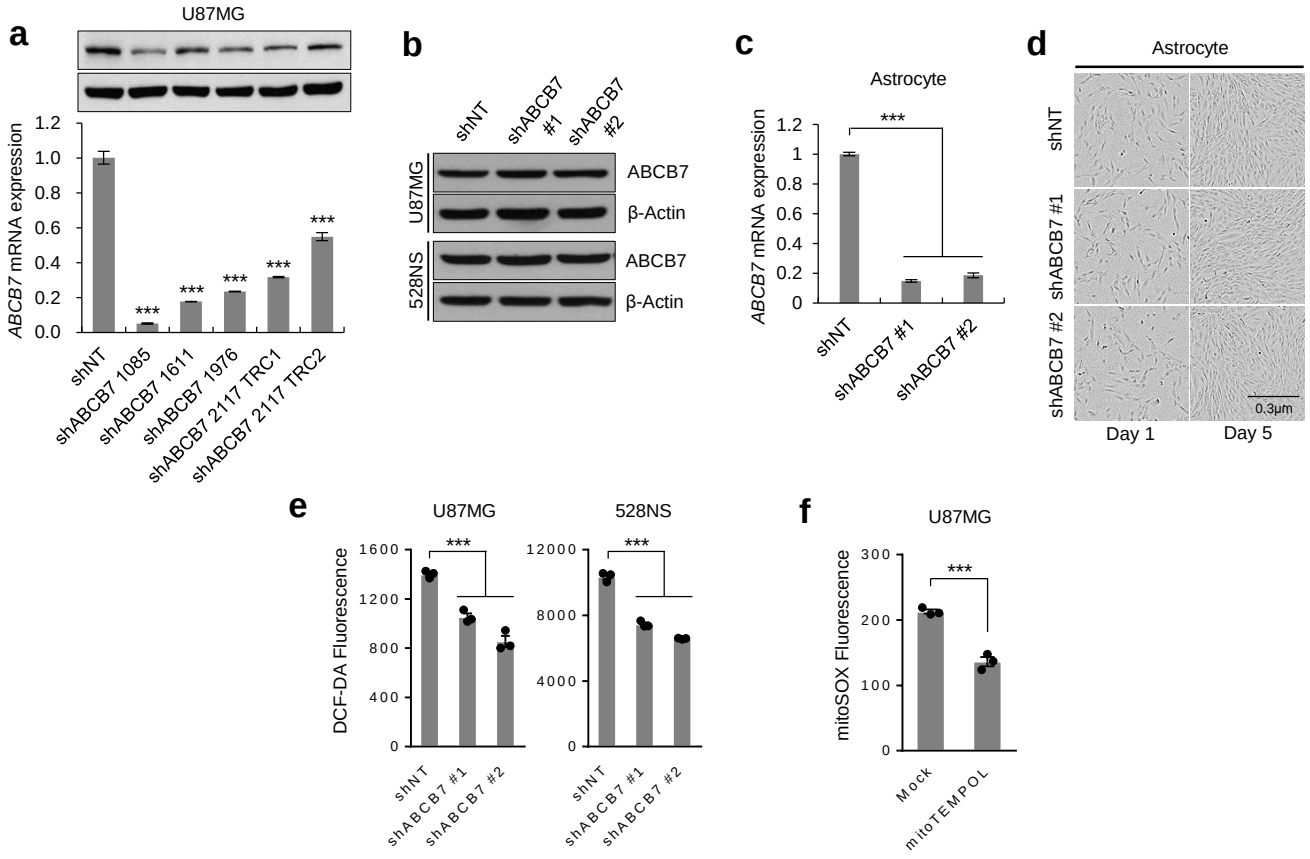


Figure S6. ABCB7 depletion induces cell death in glioma cells.

(a) ABCB7 protein and mRNA levels in U87MG cells infected with five different shABCB7 lentiviruses. ***p < 0.001.

(b) ABCB7 protein levels in live cells after shABCB7 lentivirus infection. β-actin was used as a control.

(c) *ABCB7* mRNA levels in normal human astrocytes deficient in ABCB7 due to shABCB7 lentivirus infection. ***p < 0.001.

(d) Microscopic images showing the morphology of normal human astrocytes deficient in ABCB7. The scale bar represents 0.3 μm.

(e) Cytosolic ROS levels in U87MG-shABCB7 and 528NS-shABCB7 cells. Cytosol ROS was detected using 2′,7′-dichlorofluorescein diacetate (DCF-DA). ***p < 0.001.

(f) Mitochondrial ROS (MitoSOX™ Red) was detected in cells by treatment with mitoTEMPOL. ***p < 0.001.

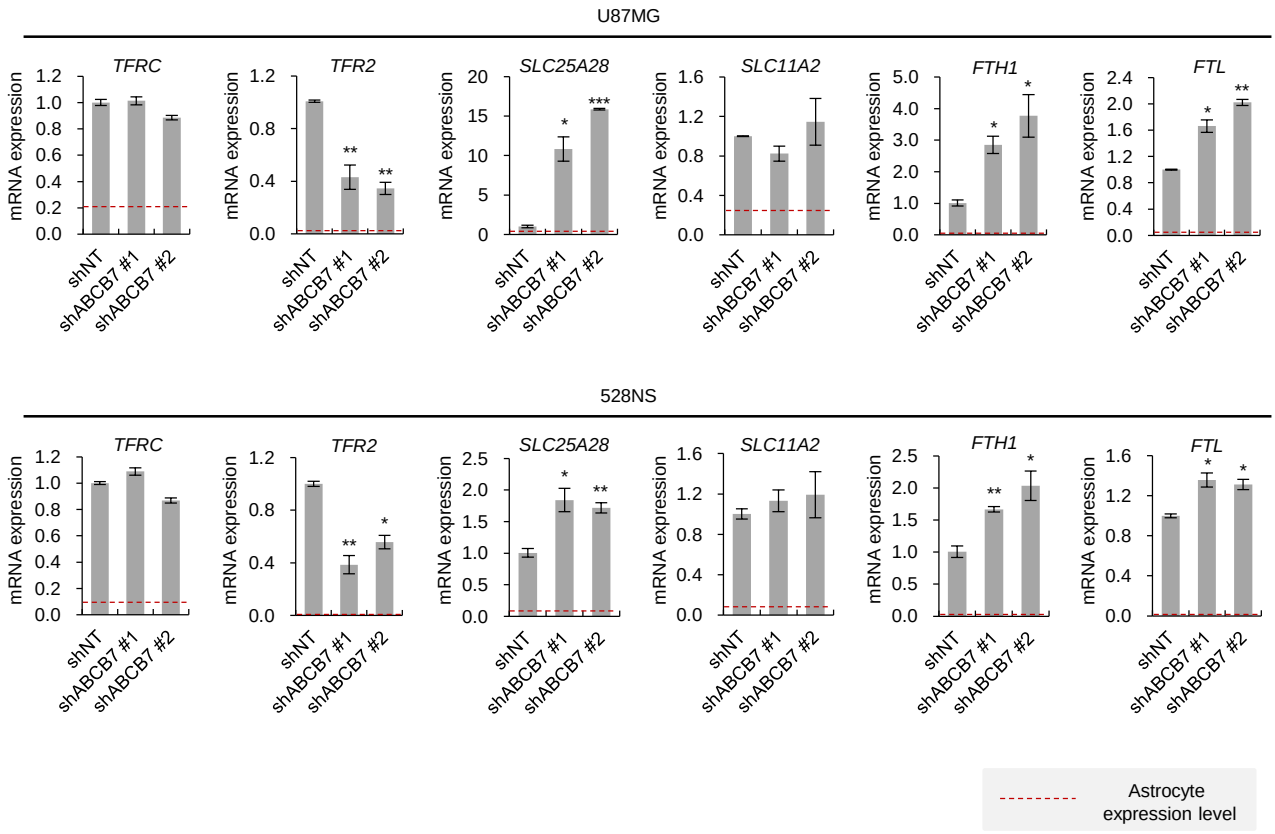


Figure S7. Expression of iron transporters in ABCB7-depleted cells.

(a) The mRNA levels of iron transporters in U87MG-shNT (Control) and two U87MG-shABCB7 cells. *p < 0.05, **p < 0.01, ***p < 0.001.

(b) The mRNA levels of iron transporters in 528NS-shNT (Control) and two 528NS-shABCB7 cells. *p < 0.05, **p < 0.01.

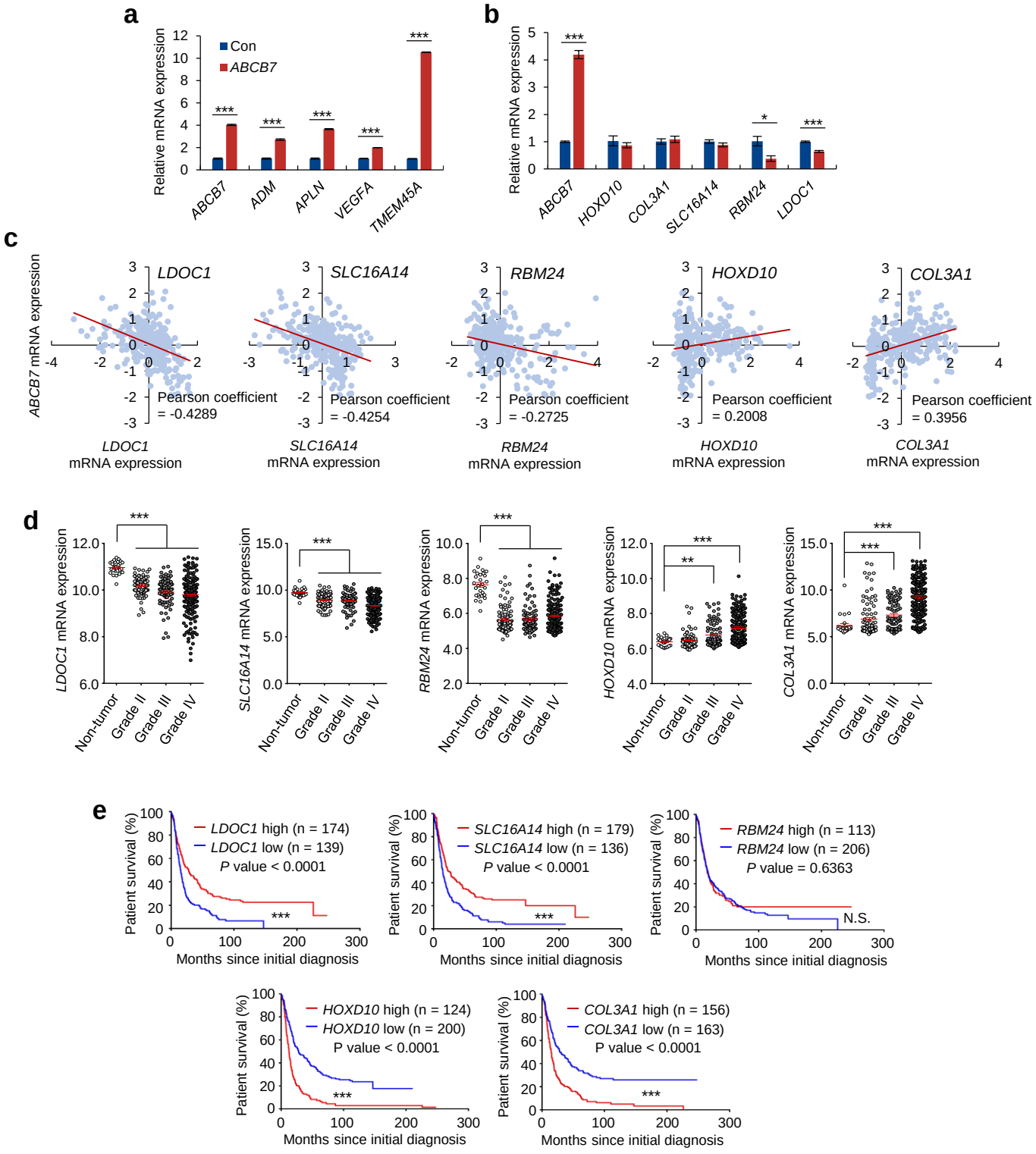


Figure S8. See next page for caption

Figure S8. LDOC1, SLC16A14, RBM24, HOXD10, and COL3A1 are putative target genes regulated by hypoxia-independent HIF1α in glioma cells overexpressing ABCB7.

(a) The mRNA expression levels of HIF1α target genes in U87MG-Con and U87MG-ABCB7 cells. ***$p < 0.001$.

(b) The mRNA expression levels of ABCB7 target genes in U87MG-Con and U87MG-ABCB7 cells. *$p < 0.05$, ***$p < 0.001$.

(c) The correlation between *ABCB7* and its predicted target genes (*LDOC1, SLC16A14, RBM24, HOXD10,* and *COL3A1*) in the REMBRANDT database.

(d) The mRNA expression of the predicted target genes of ABCB7 according to the WHO grades in the REMBRANDT database. **$p < 0.01$, ***$p < 0.001$.

(e) The patient survival times according to the expression levels of the predicted target genes of ABCB7. p-value with log-rank (Mantel-Cox) test. N.S.; non-significant, ***$p < 0.001$.

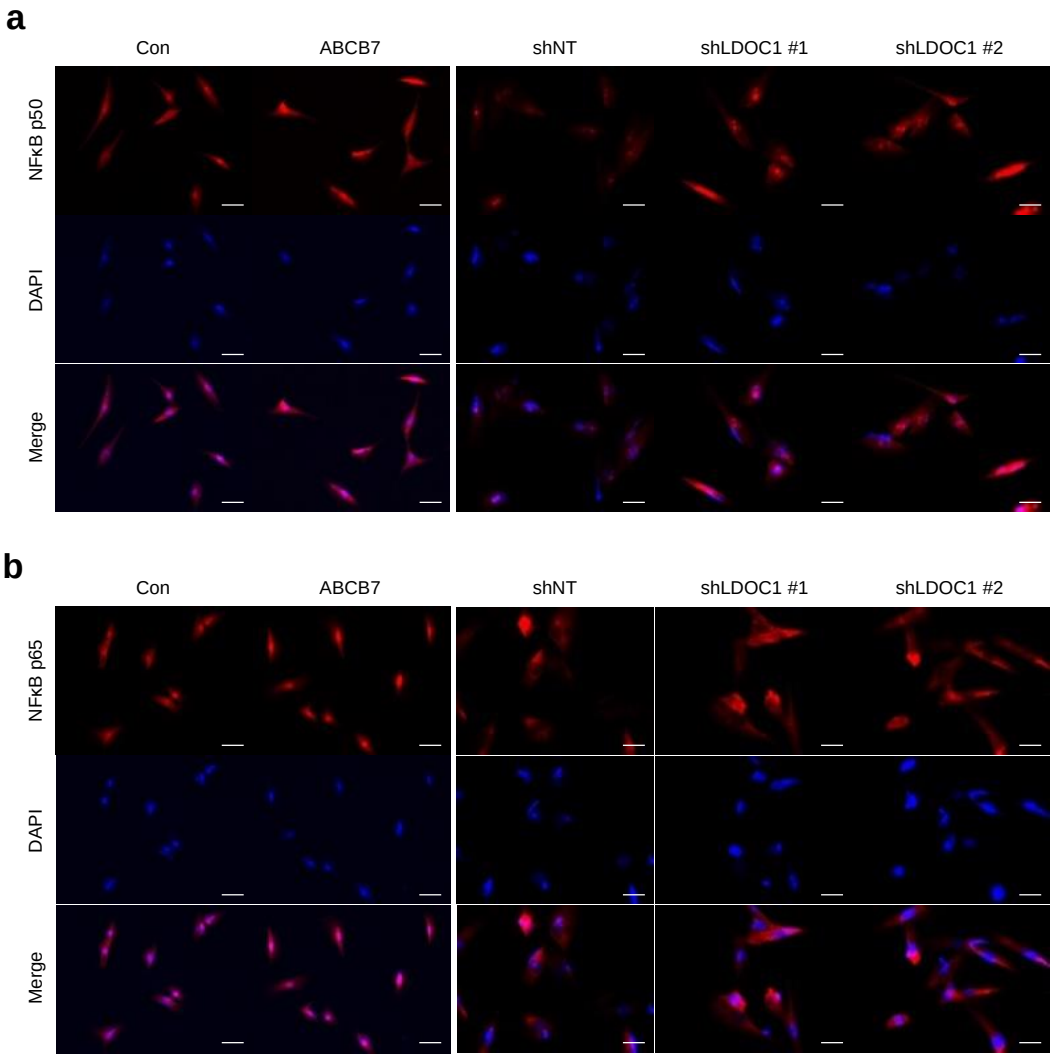


Figure S9. Immunofluorescence showing the expression levels and cellular distribution of NFκB subunits p50 and p65 proteins.

(a) Expression of the NFκB subunit p50 protein (red) in U87MG-Con vs. U87MG-ABCB7 cells and U87MG-shNT vs. two U87MG-shLDOC1 cells.

(b) Expression of the NFκB subunit p65 protein (red) in U87MG-Con vs. U87MG-ABCB7 cells and U87MG-shNT vs. two U87MG-shLDOC1 cells.

DAPI (blue) was used to stain nuclei. Magnification 200×. Scale bar = 50 μm.

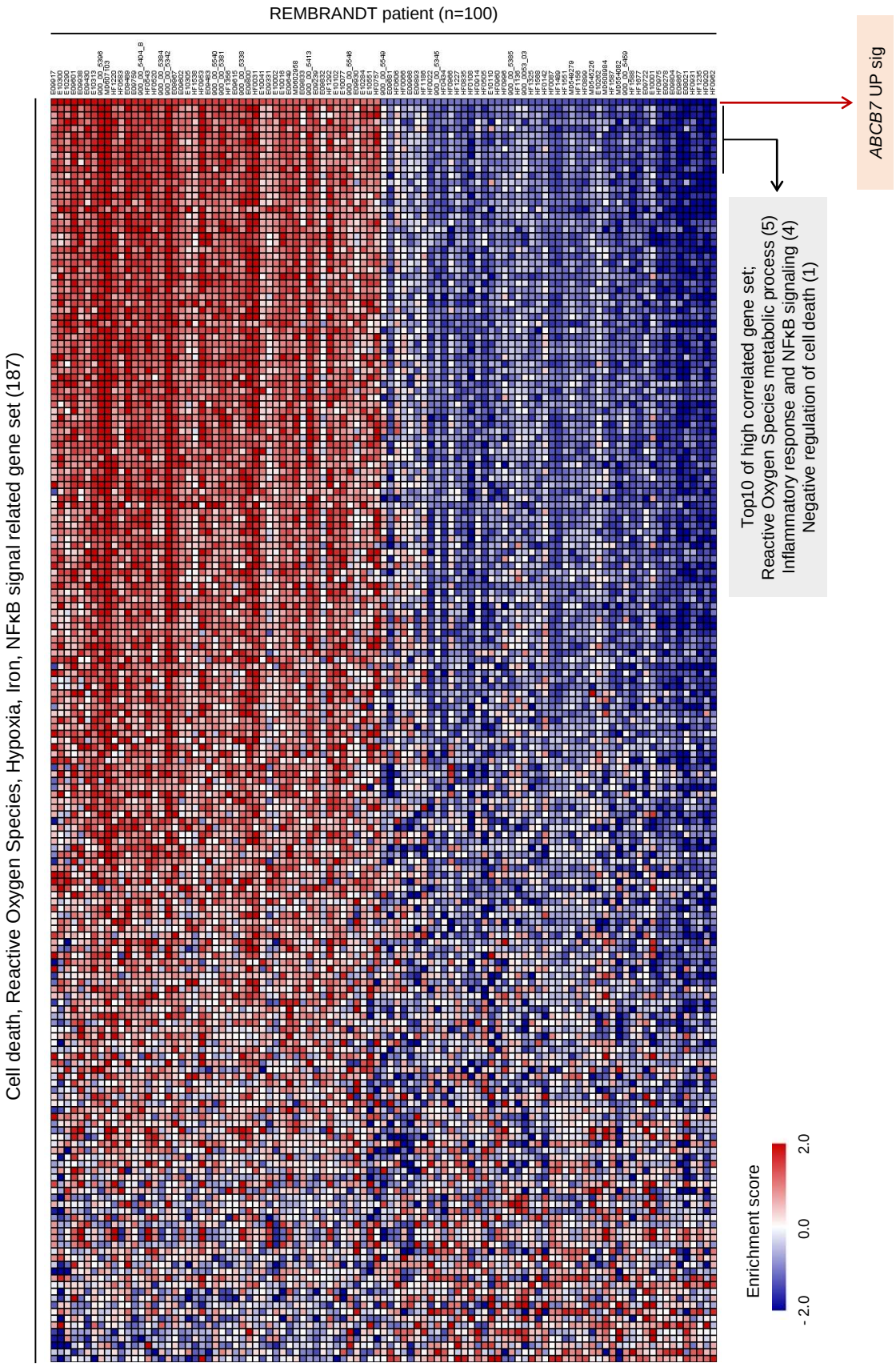


Figure S10. See next page for caption

Figure S10. Clinical relevance of ABCB7-regulated phenotypic changes (cell death, ROS, hypoxia, iron, and NFκB signal gene sets). The enrichment scores between the ABCB7 UP signature and various gene sets related to the ABCB7-regulated phenotypic changes were calculated by ssGSEA.

Figure. 1d

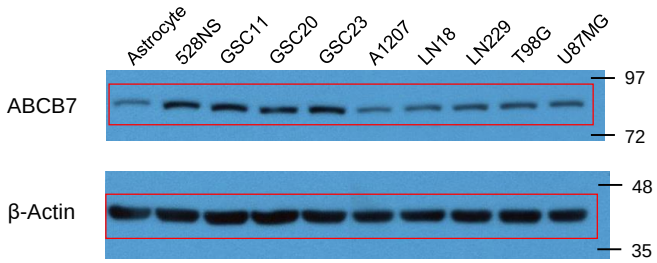


Figure. 3a

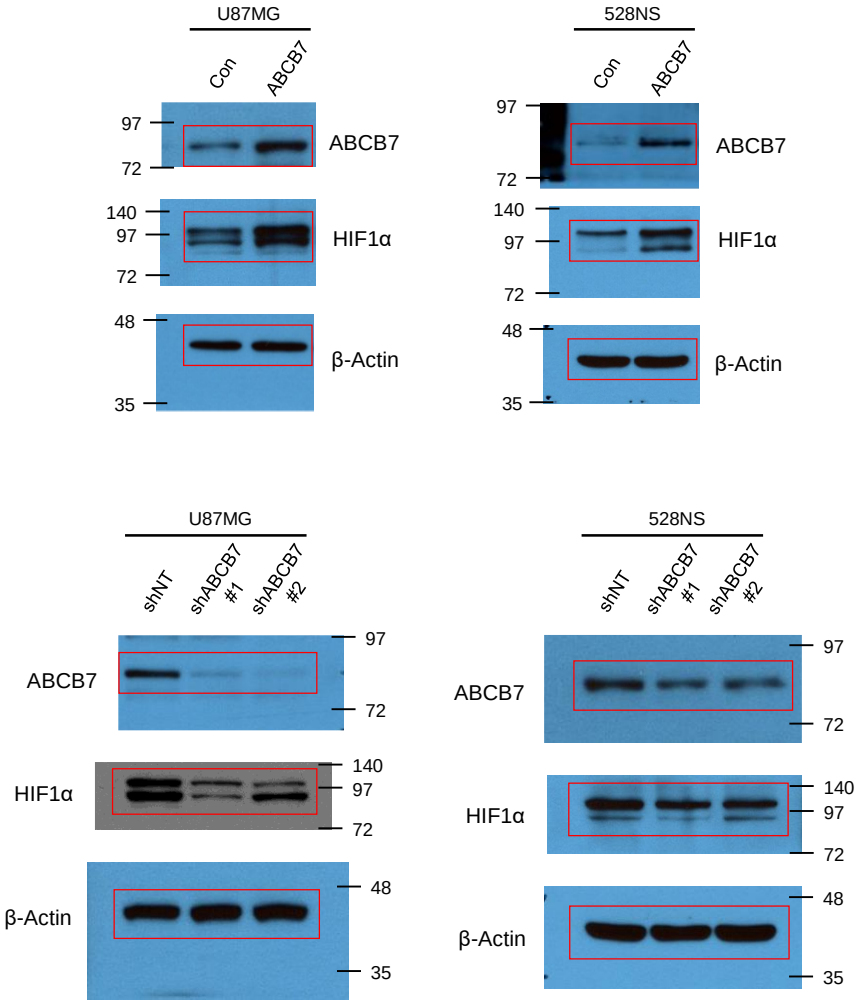


Figure S11. Unprocessed images of immunoblots.

Figure. 3d

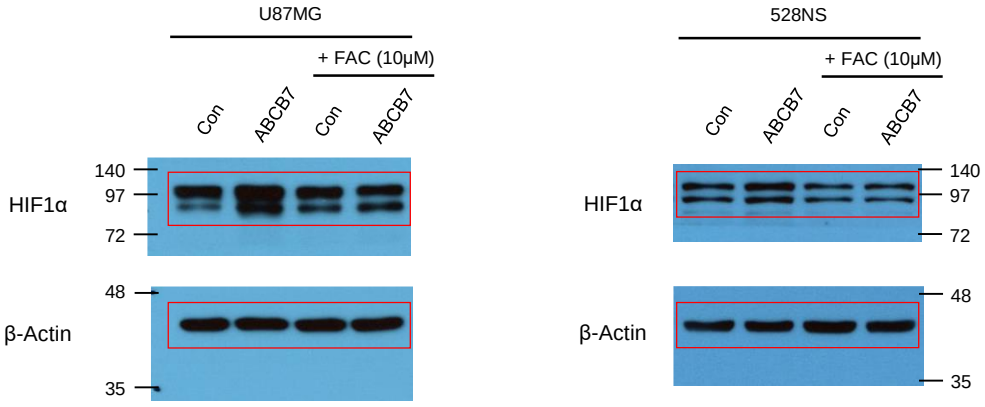


Figure S4b

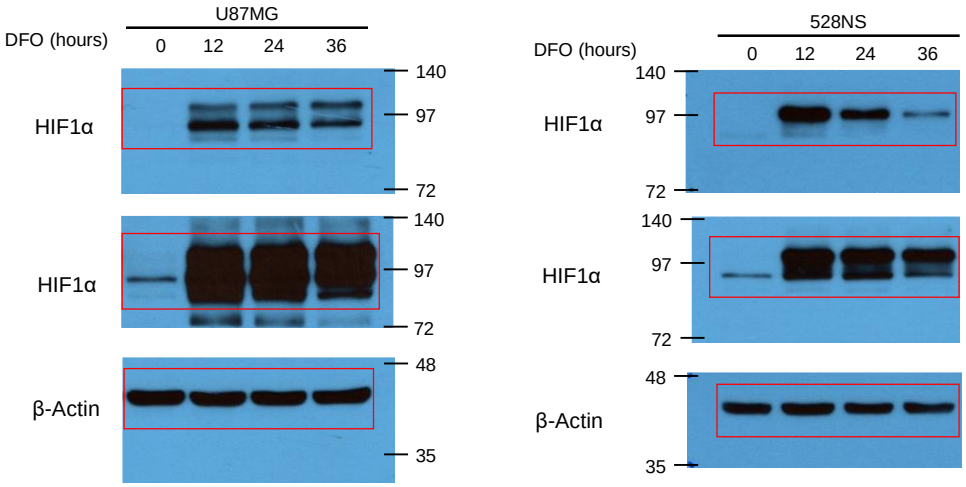


Figure S4c

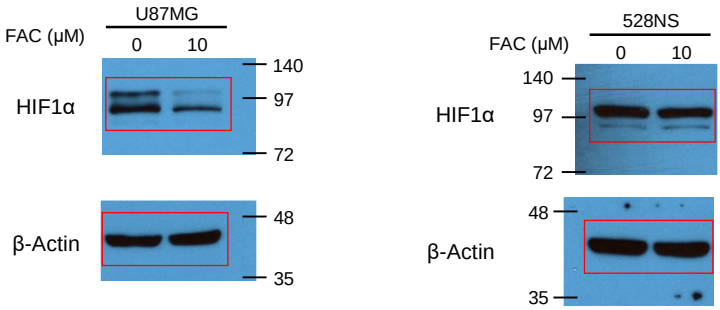


Figure S11. Unprocessed images of immunoblots.

Figure. 4a

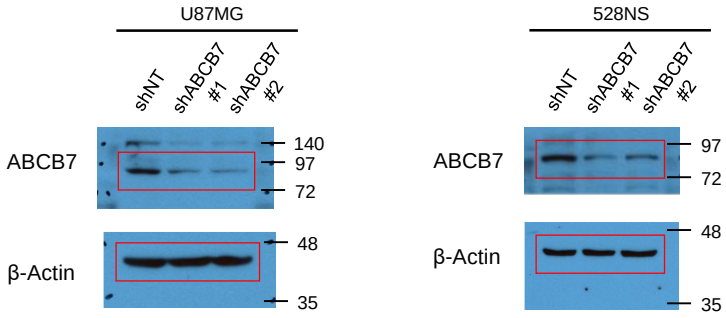
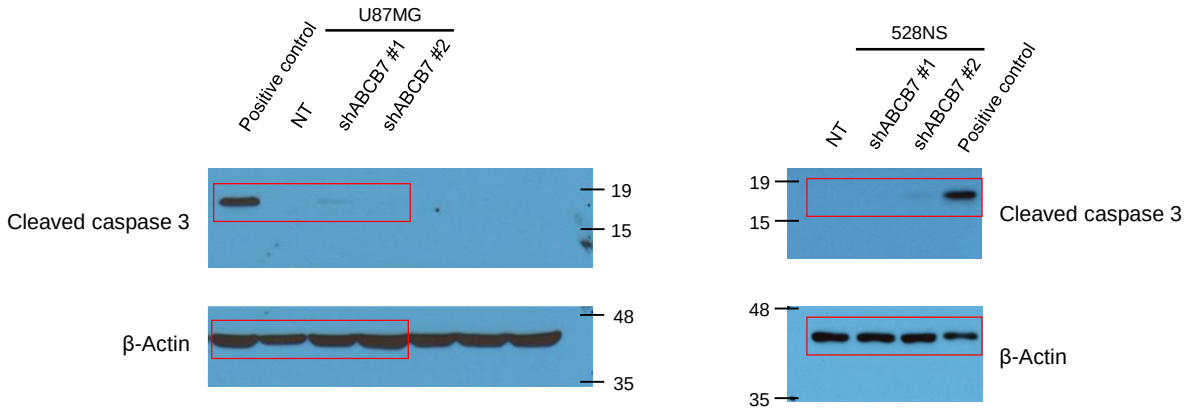


Figure. 4d



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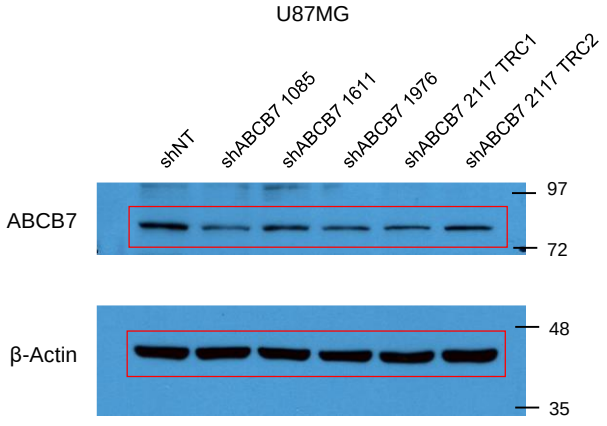

Figure S11. Unprocessed images of immunoblots.

Figure S6b

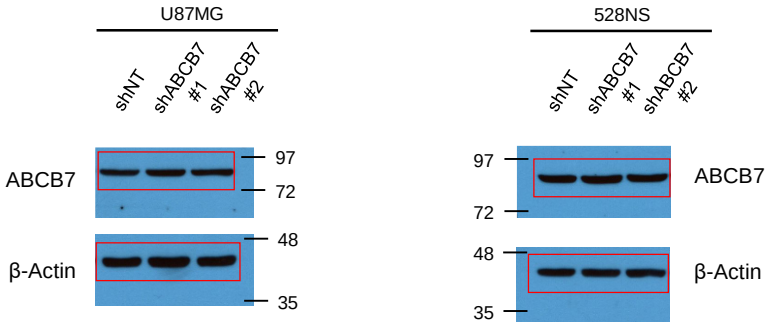

Figure. 5f

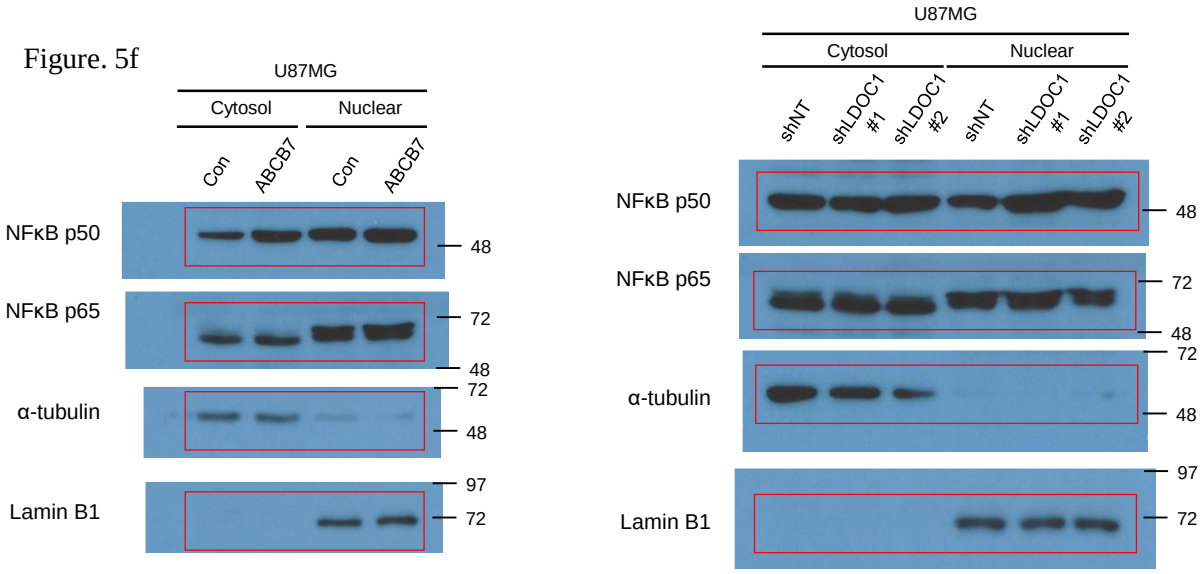

Figure. 5g

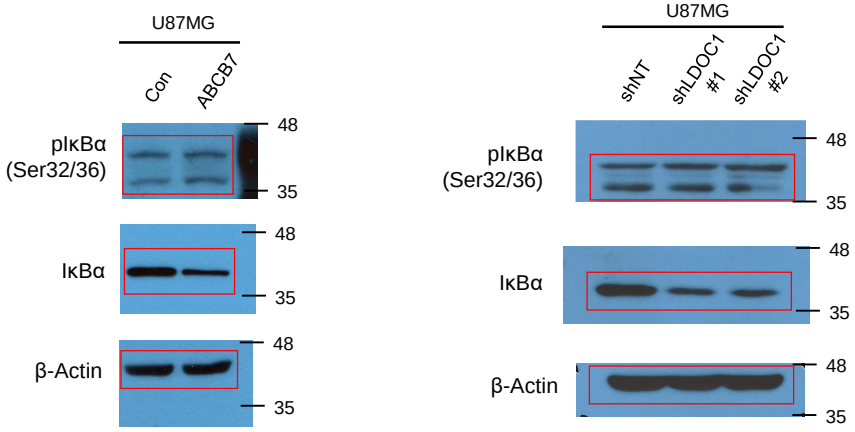

Figure S11. Unprocessed images of immunoblots.

Table S1. Statistical significance of cell death rates on the indicated day after shABCB7 lentivirus infection

Non-apoptotic cell death

		Day 1			Day 2			Day 3			Day 4			Day 5		
		shNT	shABCB7 #1	shABCB7 #2	shNT	shABCB7 #1	shABCB7 #2	shNT	shABCB7 #1	shABCB7 #2	shNT	shABCB7 #1	shABCB7 #2	shNT	shABCB7 #1	shABCB7 #2
Day 1	shNT	1.000	0.011	0.004	0.006	0.014	0.001	0.398	0.001	0.000	0.009	0.000	0.003	0.083	0.000	0.000
	shABCB7 #1	0.011	1.000	0.018	0.137	0.712	0.007	0.929	0.001	0.000	0.034	0.000	0.003	0.003	0.000	0.000
	shABCB7 #2	0.004	0.018	1.000	0.002	0.001	0.088	0.464	0.001	0.000	0.341	0.000	0.003	0.000	0.000	0.000
Day 2	shNT	1.000	1.000	0.001	1.000	0.012	0.001	0.828	0.001	0.000	0.080	0.000	0.003	0.000	0.000	0.000
	shABCB7 #1	0.012	0.012	0.000	0.012	1.000	0.000	0.880	0.001	0.000	0.041	0.000	0.003	0.000	0.000	0.000
	shABCB7 #2	0.001	0.001	1.000	0.001	0.000	1.000	0.387	0.001	0.000	0.594	0.000	0.003	0.000	0.000	0.000
Day 3	shNT	1.000	1.000	0.003	0.828	0.880	0.387	1.000	0.001	0.003	0.321	0.002	0.000	0.272	0.002	0.001
	shABCB7 #1	0.001	0.001	0.049	0.001	0.001	0.001	0.001	1.000	0.049	0.000	0.004	0.029	0.001	0.008	0.002
	shABCB7 #2	0.003	0.003	1.000	0.000	0.000	0.000	0.003	0.049	1.000	0.000	0.000	0.122	0.000	0.000	0.000
Day 4	shNT	1.000	1.000	0.001	0.080	0.041	0.594	0.321	0.000	0.000	1.000	0.000	0.001	0.010	0.000	0.000
	shABCB7 #1	0.000	0.000	0.642	0.000	0.000	0.000	0.002	0.004	0.000	0.000	1.000	0.642	0.000	0.313	0.000
	shABCB7 #2	0.001	0.001	1.000	0.003	0.003	0.003	0.000	0.029	0.122	0.001	0.642	1.000	0.003	0.785	0.074
Day 5	shNT	1.000	1.000	0.000	0.000	0.000	0.000	0.272	0.001	0.000	0.010	0.000	0.003	1.000	0.000	0.000
	shABCB7 #1	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.008	0.000	0.000	0.313	0.785	0.000	1.000	0.000
	shABCB7 #2	0.000	0.000	1.000	0.000	0.000	0.000	0.001	0.002	0.000	0.000	0.000	0.074	0.000	0.000	1.000

Early apoptosis

		Day 1			Day 2			Day 3			Day 4			Day 5		
		shNT	shABCB7 #1	shABCB7 #2	shNT	shABCB7 #1	shABCB7 #2	shNT	shABCB7 #1	shABCB7 #2	shNT	shABCB7 #1	shABCB7 #2	shNT	shABCB7 #1	shABCB7 #2
Day 1	shNT	1.000	0.004	0.000	0.000	0.001	0.001	0.105	0.064	0.021	0.141	0.005	0.018	0.036	0.000	0.000
	shABCB7 #1	0.004	1.000	0.144	0.000	0.001	0.000	0.152	0.081	0.027	0.241	0.006	0.029	0.099	0.000	0.000
	shABCB7 #2	0.000	0.144	1.000	0.000	0.002	0.001	0.143	0.078	0.026	0.220	0.007	0.028	0.084	0.000	0.001
Day 2	shNT	1.000	1.000	0.000	1.000	0.052	0.000	0.155	0.809	0.303	0.055	0.014	0.016	0.003	0.000	0.002
	shABCB7 #1	0.052	0.052	0.002	0.052	1.000	0.002	0.244	0.859	0.733	0.076	0.029	0.019	0.001	0.002	0.001
	shABCB7 #2	0.000	0.000	1.000	0.000	0.002	1.000	0.554	0.175	0.075	0.773	0.027	0.345	0.081	0.000	0.000
Day 3	shNT	1.000	1.000	0.317	0.155	0.244	0.554	1.000	0.310	0.317	0.493	0.620	0.818	0.266	0.031	0.060
	shABCB7 #1	0.310	0.310	0.746	0.809	0.859	0.175	0.310	1.000	0.746	0.141	0.413	0.224	0.108	0.100	0.266
	shABCB7 #2	0.317	0.317	1.000	0.303	0.733	0.075	0.317	0.746	1.000	0.083	0.328	0.097	0.028	0.021	0.053
Day 4	shNT	1.000	1.000	0.485	0.055	0.076	0.773	0.493	0.141	0.083	1.000	0.180	0.485	0.563	0.014	0.023
	shABCB7 #1	0.180	0.180	0.125	0.014	0.029	0.027	0.620	0.413	0.328	0.180	1.000	0.125	0.004	0.002	0.001
	shABCB7 #2	0.485	0.485	1.000	0.016	0.019	0.345	0.818	0.224	0.097	0.485	0.125	1.000	0.052	0.004	0.003
Day 5	shNT	1.000	1.000	0.000	0.003	0.001	0.081	0.266	0.108	0.028	0.563	0.004	0.052	1.000	0.001	0.000
	shABCB7 #1	0.001	0.001	0.007	0.000	0.002	0.000	0.031	0.100	0.021	0.014	0.002	0.004	0.001	1.000	0.007
	shABCB7 #2	0.000	0.000	1.000	0.002	0.001	0.000	0.060	0.266	0.053	0.023	0.001	0.003	0.000	0.007	1.000

Late apoptosis

		Day 1			Day 2			Day 3			Day 4			Day 5		
		shNT	shABCB7 #1	shABCB7 #2	shNT	shABCB7 #1	shABCB7 #2	shNT	shABCB7 #1	shABCB7 #2	shNT	shABCB7 #1	shABCB7 #2	shNT	shABCB7 #1	shABCB7 #2
Day 1	shNT	1.000	0.111	0.017	0.003	0.001	0.006	0.070	0.001	0.069	0.005	0.004	0.007	0.002	0.000	0.000
	shABCB7 #1	0.111	1.000	0.313	0.001	0.000	0.005	0.076	0.000	0.075	0.005	0.004	0.007	0.001	0.000	0.000
	shABCB7 #2	0.017	0.313	1.000	0.004	0.002	0.010	0.073	0.001	0.072	0.005	0.004	0.007	0.002	0.000	0.000
Day 2	shNT	1.000	1.000	0.003	1.000	0.041	0.003	0.176	0.000	0.172	0.007	0.005	0.010	0.001	0.000	0.000
	shABCB7 #1	0.041	0.041	0.004	0.041	1.000	0.004	0.137	0.001	0.135	0.007	0.005	0.010	0.002	0.000	0.000
	shABCB7 #2	0.003	0.003	1.000	0.003	0.004	1.000	0.101	0.001	0.099	0.006	0.005	0.008	0.001	0.000	0.000
Day 3	shNT	1.000	1.000	0.983	0.176	0.137	0.101	1.000	0.277	0.983	0.101	0.016	0.028	0.686	0.051	0.068
	shABCB7 #1	0.277	0.277	0.285	0.000	0.001	0.001	0.277	1.000	0.285	0.103	0.015	0.043	0.015	0.001	0.004
	shABCB7 #2	0.983	0.983	1.000	0.172	0.135	0.099	0.983	0.285	1.000	0.104	0.017	0.028	0.706	0.052	0.070
Day 4	shNT	1.000	1.000	0.112	0.007	0.007	0.006	0.101	0.103	0.104	1.000	0.023	0.112	0.027	0.068	0.162
	shABCB7 #1	0.023	0.023	0.341	0.005	0.005	0.005	0.016	0.015	0.017	0.023	1.000	0.341	0.009	0.178	0.094
	shABCB7 #2	0.112	0.112	1.000	0.010	0.010	0.008	0.028	0.043	0.028	0.112	0.341	1.000	0.024	0.799	0.376
Day 5	shNT	1.000	1.000	0.001	0.001	0.002	0.001	0.686	0.015	0.706	0.027	0.009	0.024	1.000	0.000	0.001
	shABCB7 #1	0.000	0.000	0.017	0.000	0.000	0.000	0.051	0.001	0.052	0.068	0.178	0.799	0.000	1.000	0.017
	shABCB7 #2	0.001	0.001	1.000	0.000	0.000	0.000	0.068	0.004	0.070	0.162	0.094	0.376	0.001	0.017	1.000

Annotation

Non significant	p < 0.05	p < 0.01	p <0.001

Table S2. List of genes of TNFα signaling via NFκB gene set according to the enrichment score in U87MG-ABCB7 cells

NAME	PROBE	RANK IN GENE LIST	RANK METRIC SCORE	RUNNING ES	CORE ENRICHMENT
row_0	**ID2**	179	1.604516	0.004358	Yes
row_1	**PFKFB3**	182	1.599821	0.019201	Yes
row_2	**CXCL1**	203	1.561345	0.032613	Yes
row_3	**JAG1**	242	1.466984	0.044072	Yes
row_4	BTG1	283	1.366999	0.054477	Yes
row_5	SDC4	287	1.363451	0.06705	Yes
row_6	SAT1	337	1.295213	0.076248	Yes
row_7	TUBB2A	390	1.219383	0.084559	Yes
row_8	TNFRSF9	418	1.189536	0.094078	Yes
row_9	MARCKS	435	1.170595	0.104074	Yes
row_10	SOCS3	440	1.160828	0.114692	Yes
row_11	RHOB	507	1.112643	0.121172	Yes
row_12	NFKBIA	526	1.100148	0.13039	Yes
row_13	F3	530	1.095602	0.140457	Yes
row_14	ICAM1	564	1.066838	0.148472	Yes
row_15	CXCL3	568	1.065594	0.158259	Yes
row_16	LAMB3	714	0.963847	0.158648	Yes
row_17	SOD2	788	0.907155	0.162789	Yes
row_18	ZC3H12A	845	0.876546	0.167656	Yes
row_19	CXCL2	873	0.867549	0.174163	Yes
row_20	BHLHE40	881	0.864626	0.181833	Yes
row_21	JUNB	960	0.828398	0.18494	Yes
row_22	CYR61	974	0.825724	0.191889	Yes
row_23	KLF4	1032	0.808414	0.196059	Yes
row_24	RCAN1	1111	0.775299	0.19867	Yes
row_25	SERPINB2	1289	0.712596	0.194806	Yes
row_26	EGR1	1308	0.704801	0.200326	Yes
row_27	TNIP1	1309	0.704789	0.206918	Yes
row_28	MSC	1371	0.685561	0.209701	Yes
row_29	RELB	1393	0.68145	0.214824	Yes
row_30	PHLDA1	1413	0.676161	0.220018	Yes
row_31	GADD45A	1469	0.661381	0.222931	Yes
row_32	BCL3	1500	0.653716	0.227261	Yes
row_33	PTGER4	1503	0.652879	0.233247	Yes
row_34	HES1	1536	0.646167	0.237387	Yes
row_35	INHBA	1540	0.645429	0.243245	Yes
row_36	PPAP2B	1570	0.638807	0.247494	Yes
row_37	CEBPD	1587	0.635285	0.252483	Yes
row_38	KLF6	1603	0.630469	0.257487	Yes
row_39	TAP1	1613	0.628897	0.262834	Yes
row_40	DUSP1	1621	0.626435	0.268276	Yes

Table S3. List of primers used in this study

qRT-PCR		
Gene name	Forward	Reverse
ABCB7	AAGATGTGAGCCTGGAAAGC	AGAGGACAGCATCCTGAGGT
ABCB8	GTACCTGCAATGCACCTTCA	AGCTGCTCCATCAATGCAC
ABCB10	GCCATGACATCCGTCAGCTA	TGGATTTCCTCAGCGGTCAC
LDOC1	GGCTCCCCGAGTTTATCGTG	CGGTGAGGAGGCTGATTAGG
ID2	ATTCAGAAGCCTGCAAGGAC	TGGACTCGCATCCCACTATT
PFKFB3	CAGAGCCGCATCGTGTACTA	TTCTGCTCCTCCACGAACTT
CXCL1	CTCTTCCGCTCCTCTCACAG	GGGGACTTCACGTTCACACT
JAG1	TCTGAGGACAACACCACCAA	TGGGCACTTTCCAAGTCTCT
ADM	GGGATGAAGCTGGTTTCCGT	AGTTCCCTCTTCCCACGACT
APLN	AAGACAAGAGCCTGATGCCC	GGGGAGGTGGATAGGCAAAC
VEGFA	CATGAACTTTCTGCTGTCTT	CATTTGTTGTGCTGTAGGAA
TMEM45A	CTCCTGGGCTGGCATCATTTC	CGGCCATGAGTGTGGTTGTAG
HIF1α	CACTTCCACATAATGTGAGTTCGG	GGTTCACAAATCAGCACCAAGCAGG
TFRC	AAAATCCGGTGTAGGCA	TTAAATGCAGGGACGAAAGG
TFR2	GGGCTCAAGGAGTGCTCATAT	CTGGAGGGAACTGGGTTTGAT
SLC25A37	TAGCCAACGGGATAGCTGG	GTGGTGTAGCTCCGGTAGAAG
SLC25A28	CTGCGTGATGTACCCCATCG	CCTGTTGCTGTGACGTTCAG
SLC40A1	GCGGTGTCTGTGTTTCTGGTA	GCATTCTTGTCCACCCAGTCA
SLC11A2	CTAGACTGGGAGTGGTTACTGG	AGGATGACTCGTGGGACCTT
FTMT	AGCTCTATGCGTCCTACGTG	TTTCCCAGTCGTCCTGTTCC
FTH1	GCTTCAACAGTGCTTGGACG	GTCCTGGTGGTAGTTCTGGC
FTL	CCTACCTCTCTCTGGGCTTCTA	TTCATGGCGTCTGGGGTTTTA
18s	TGCATGGCCGTTCTTAGTTG	AGTTAGCATGCCAGAGTCTCGTT

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Cell culture conditions

Human GBM cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Cat. #17-605E; Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS, Cat. #7101; Biotechnics Research, Inc., Canton, OH, USA), 1% penicillin and streptomycin (P/S, Cat. #17-602E; Lonza), 1% L-glutamine (Cat. #17-605E; Lonza), and gentamicin sulfate (50 μg/ml, Cat. #MT-61-098-RF; Mediatech, Mooresville, NC, USA). Human astrocytes were maintained in Astrocyte Medium (Cat. #1801; ScienCell, Carlsbad, CA, USA) supplemented with 2% FBS (Cat. #0010; ScienCell), 1% Astrocyte Growth Supplement (AGS, Cat. #1852; ScienCell) and 1% P/S (Cat. #0503; ScienCell). All glioblastoma stem cells (GSCs) were cultured using DMEM and Ham's F-12 (DMEM/F12, Cat. #12-719Q; Lonza) supplemented with 1% P/S (Cat. #17-602E; Lonza), 1% L-glutamine, 0.2% B27 (Cat. #17504-044; Invitrogen, Carlsbad, CA, USA), epidermal growth factor (EGF, 20 ng/ml, Cat. #236-EG; R&D Systems, Minneapolis, MN, USA), basic fibroblast growth factor (bFGF, 20 ng/ml, Cat. #4114-TC; R&D Systems), and gentamicin sulfate (50 μg/ml). A hypoxic culture was performed by plating cells on a culture dish maintained using high purity grade 85% N_2, 10% H_2, and 5% CO_2 in an anaerobic chamber (ThermoFisher Scientific, Waltham, MA, USA). Conditions that mimicked hypoxia were induced using deferoxamine (DFO, 100 μM, Cat. #D9533; Sigma-Aldrich, St. Louis, MO, USA) and cobalt chloride ($CoCl_2$, 300 μM, Cat. #232696; Sigma-Aldrich). The iron-free culture condition was maintained using the DFO iron chelator. The iron supply was maintained using ferric ammonium citrate (FAC, 10 μM, Cat. #F5879; Sigma-Aldrich).

Vector construction and lentivirus infection

Transient U87MG- hypoxia-inducible factor 1α (HIF1α) was transfected with the HIF1α construct (pcDNA3.1(+)-HIF1α-puro) using the PolyExpress™ in vitro DNA transfection reagent (Cat. #EG-1072; Excellgen, Rockvie, MD, USA). To produce the lentivirus, the HEK293FT packaging cell line (Cat. #R70007; Invitrogen) was transfected with lentiviral vectors (pCDH-ABCB7-puro, Cat. #hMU008618; Korea Human Gene Bank (KHGB) and pCDH-LDOC1-HA-Dsred, Cat. #HG13384-CY; Sino Biological Inc., Beijing, China) and short hairpin (sh)RNAs for knockdown of the ABCB7 (NM_004299.3-1085s1c1, NM_004299.3-1611s1c1, NM_004299.3-1976s1c1,

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NM_004299.3-2117s1c1, NM_004299.3-2117s21c1; all from Sigma-Aldrich) and LDOC1 genes (NM_012317.2-931s21c1, NM_012317.2-1170s1c1; both from Sigma-Aldrich) using the PolyExpress^{TM} in vitro DNA transfection reagent. The lentivirus was filtered through a 0.45 μm syringe filter and concentrated with Lenti-XTM Concentrator (Cat. #631231; Clontech, Mountain View, CA, USA). Cells plated 24 h earlier using 3.0×10^5 cells in a 6 cm dish (U87MG) or 6.0×10^5 cells in a 6 cm dish (528NS) were infected with supernatant medium containing lentivirus and 6 μg/ml polybrene (Cat. #107689; Sigma-Aldrich). Stable U87MG-ABCB7 and U87MG-shLDOC1 cells were selected in DMEM supplemented with 10% FBS and 3 μg/ml puromycin (Cat. #631306 Clontech) for seven days. 528NS-ABCB7 cells were selected in DMEM/F12 supplemented with B27, EGF, bFGF, and 1 μg/ml puromycin for seven days. The phenotype of the cells (U87MG and 528NS) infected with shABCB7, and the LDOC1 gene was confirmed on days one to five.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated from the cells using QIAzol lysis reagent (Cat. #79306; Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNase-free, DNase-treated RNA (1 μg) was used as the template to synthesize cDNA using the RevertAid First-Strand cDNA Synthesis Kit (Cat. #K1621; ThermoFisher Scientific). The qRT-PCR analysis was performed on an iCycler IQ real-time detection system (Bio-Rad) using the IQ Supermix with SYBR-Green (Cat. #RR420A; TaKaRa Bio, Shiga, Japan). The expression levels of target genes were normalized to that of 18S rRNA. The primers for qRT-PCR amplification are listed in Table S3.

Western blot analysis

For Western blotting, whole-cell extracts were prepared with the radioimmunoprecipitation assay (RIPA) lysis buffer comprised of 150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), and 50 mM Tris (pH 7.4) containing 1 mM NaF, 1 mM Na3VO4, 1 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate, and protease inhibitor (Cat. #11836-153-001; Roche, Basel, Switzerland). Nuclear and cytosolic proteins were prepared using a Nuclear/Cytosol Fractionation Kit (Cat. #K266-100; BioVision, Milpitas, CA, USA), according to the manufacturer's instructions.

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The proteins were quantified via the Bradford assay reagent (Cat. #500-0006, Bio-Rad) according to the manufacturer's instructions. Protein (30-60 μg) was separated by 8% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride membrane (Cat. #BSP0161; Pall Corporation, New York, NY, USA) overnight. The membranes were blocked with 5% non-fat milk and incubated with the following antibodies: anti-ABCB7 (1:500, Cat. #SC-20968; Santa Cruz Biotechnology, Dallas, TX, USA), anti-HIF1α (1:1,000, Cat. #ab51608; Abcam, Cambridge, UK), anti-β-actin (1:10,000, Cat. #SC-47778; Santa Cruz Biotechnology), anti-cleaved caspase-3 (1:1,000, Cat. #9661 Cell Signaling Technology, Beverly, MA, USA), anti-nuclear factor-kappa B (NFκB) p50 (1:1,000, Cat. #sc-114; Santa Cruz Biotechnology), anti-NFκB p65 (1:1,000, Cat. #ab16502, Abcam), anti-pIκBα (Ser32/36, 1:1,000, Cat. #9246S; Cell Signaling Technology), and anti-IκBα (1:1,000, Cat. #sc-371; Santa Cruz Biotechnology). Each membrane was incubated with a horseradish peroxidase-conjugated anti-secondary IgG antibody (Pierce, Rockford, IL, USA) and visualized with SuperSignal West Pico Chemiluminescent Substrate (Cat. #EBP-1073; Elpis Bio, Seoul, South Korea). β-actin was used as a loading control. All unprocessed images of the Western blots are presented in Fig. S11.

Cell proliferation assay

U87MG cells were plated at a density of 1.0×10^3 cells/well, and 528NS were seeded at a density of 5.0×10^3 cells/well in 96-well plates and were monitored using IncuCyte ZOOM™ (Essen BioScience Inc., Ann Arbor. MI, USA). Time-lapse phase-contrast images were obtained every 2 h. Cell confluence was analyzed by the software provided with IncuCyte ZOOM™.

Cell invasion assay

An invasion assay was performed using 24-well Transwell units coated with matrigel that were allowed to settle for 4 h at 37℃. Next, 5.0×10^4 cells suspended in 100 μl serum-free medium DMEM were seeded into the upper chamber, and 500 μl serum-containing medium DMEM was added to the lower chamber. The cells invaded for 72 h at 37℃. After this, the upper chamber was fixed and stained with crystal violet. Images of the entire upper chamber were taken and the invasion area was qualified using ImageJ (https://imagej.nih.gov).

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Spheroid sprouting assay

To generate spheroids, 5.0×10^3 U87M or 1.0×10^4 528NS cells were seeded and incubated in an ultra-low attached 96-well plate for 72 h. After incubation, the spheroids were collected and immediately seeded on a collagen-coated 6-well plate. Each well was monitored at 1 h intervals, and the sprouting area and longest sprout length from the center of the spheroid was quantified using IncuCyte ZOOM™.

Cell viability assay

The viability of the cells was determined using the 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST) based assay (CytoX, Cat. #CYT3000; LPS solution, Daejeon, South Korea), according to the manufacturer's instructions. Cells (1-6×103) were added to each well in a 96-well plate and were incubated at 37℃ for 48 and 72 h. CytoX reagent was added to each well, and the cells were incubated at 37℃ for 2-3 h. The relative viability of the cells was determined by measuring the absorbance at 450 nm. Experiments were repeated three times, and data represent the mean of replicate wells ± standard error of the mean (S.E.M.).

Immunofluorescence

The cells (1×10^4 cells per well) were seeded on cover slips in a 24-well plate. The next day, cells were rinsed with ice-cold phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 20 min at 25℃, followed by permeabilization with PBS plus 0.3% Triton X100, 10% serum and 0.1% bovine serum albumin (BSA). The cells were stained with NFκB p50 antibody (1:200, Cat. #sc-114; Santa Cruz Biotechnology) or NFκB p65 antibody (1:200, Cat. #ab16502; Abcam) overnight at 4℃. Cells were washed with ice-cold PBS three times for 5 min each and incubated with Alexa 594-conjugated secondary antibody at 25℃ for 2 h. After staining with 4,6-diamidino-2-phenylindole (DAPI, 1:1,000, Cat. #D9542; Sigma Aldrich) for 5 min, slides were mounted in mounting solution (Cat. #P36930, Invitrogen), and stored at 4℃ in the dark. Fluorescence was detected using microscopy.

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Luciferase reporter gene activity assay

To determine the HIF1α binding activity to hypoxia response element (HRE) sequences, pcDNA3.1-3×HRE-puro plasmids and pRL-TK were transiently co-transfected into U87MG-Con and U87MG-ABCB7 cells using the PolyExpress™ in vitro DNA transfection reagent. Promoter activity was analyzed by the Dual-Glo Luciferase Assay System (Cat. #E2920; Promega, Madison, WI, USA). Transfection efficiency was normalized to the activity of Renilla luciferase according to the manufacturer's instructions.

Iron staining

Cells (2×10^4 per well) were seeded on coverslips in a 24-well plate. After 48 h, the cells were rinsed with ice-cold PBS and treated with 500 nM Calcein-AM (Cat. #C1430; ThermoFisher Scientific) in the dark at 37℃ for 30 min. The cells were washed with ice-cold PBS three times for 5 min each time and fixed with 4% paraformaldehyde for 20 min at 25℃. After DAPI (1:1,000, Cat. #D9542; Sigma Aldrich) staining for 5 min, slides were mounted in mounting solution (Cat. #P36930, Invitrogen) and stored at 4℃ in the dark. Fluorescence was detected using an LSM 800 confocal microscope (Carl Zeiss, Jena, Germany).

In silico analysis

Gene expression and patient prognosis were analyzed in microarray datasets from the REpository for Molecular BRAin Neoplasia DaTa (REMBRANDT) database of the National Cancer Institute (https://caintergator.nci.nih.gov/rembrandt/). The REMBRANDT database was classified based on the expression of mitochondrial ABC transporters and further based on the high/low ABCB7 UP signature according to the mean ± S.E.M. value of the enrichment score developed using single sample Gene Set Enrichment Analysis (ssGSEA; http://software.broadinstitute.org/cancer/software/genepattern) [1]. The Enrichment Map, a Cytoscape plugin for functional enrichment visualization, was used to visualize the enriched function, as described previously [2]. To identify upstream regulators and networks of altered genes in ABCB7 overexpressing cells, Ingenuity Pathway Analysis (IPA; https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis) was performed [3].

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Upstream regulators were ranked according to activation Z-score. Gene Set Enrichment Analysis (GSEA) was performed using the hypoxia signature derived from GSE73556 [4] and MsigDB Hallmark (https://www.broadinstitute.org/gsea) [5, 6]. Gene ontology (GO) terms representing the phenotype associated with ABCB7 were extracted in AmiGO (http://amigo.geneontology.org/amigo) and the enrichment score of these GO terms was calculated using ssGSEA. The enrichment score of each patient was represented in a heatmap using MeV software. The fitness score was analyzed using CRISPR-Cas9 fitness screening data from the project Score database (https://score.depmap.sanger.ac.uk) [7]. This screening was performed in 324 cell lines from 13 different cancer types. These screening data contain two main gene fitness matrixes (loss of fitness score and corrected fold-change of targeting sgRNAs). Loss of fitness scores are calculated from scaled Bayesian factors using the BACEL method to verify significantly depleted genes. Loss of fitness score < 0 was considered indicative of a statistically significant effect on cell fitness.

Supplemental References

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