Supplemental data

ZMYND8 acetylation mediates HIF-dependent breast cancer progression and metastasis

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Supplemental Methods

Plasmid constructs. Human *ZMYND8* cDNA was generated by PCR using pcDNA6.2-V5-ZMYND8-EmGFP (Addgene, #65401) as a template and cloned into BamHI/EcoRI-linearized pcFUGW-3XFLAG vector. ZMYND8 K1006R, K1007R, K1034R, K1006/1034R and K1007/1034R mutants were generated by the site-directed mutagenesis strategy. Human *EPAS1* cDNA was generated by PCR using pcDNA-HIF-2α as a template and cloned into HindIII/XbaI-linearized p3XFLAG-CMV-7 vector (Sigma). HIF luciferase reporter plasmid was generated by inserting an annealed DNA oligonucleotide containing 2 x HREs from human *VEGFA* gene into KpnI/BglII-linearized pGL2-promoter vector (Promega). DNA oligonucleotides containing wild-type (WT) or mutant *ZMYND8* HRE were annealed and ligated into MluI/BglII-linearized pGL2-promoter vector (Promega). DNA oligonucleotides of the short hairpin RNAs (shRNAs) targeting ZMYND8, p300, or CHD4 were annealed and ligated into AgeI/EcoRI-linearized pLKO.1 vector (Addgene, #8435). DNA oligonucleotides of the shRNAs targeting BRD4 were annealed and ligated into AgeI/EcoRI-linearized Teton-pLKO.1 vector (Addgene, #21915). DNA oligonucleotides of the single guide RNA (sgRNA) targeting ZMYND8, HIF-1α, or HIF-2α, were annealed and ligated into BsmBI-linearized lentiCRISPRv2 vector (Addgene, #52961). Full-length or truncated SFB (S-protein/2XFlag/Streptavidin binding peptide) tagged ZMYND8 plasmids were provided by Dr. Kyle M. Miller (UT at Austin, Austin) (33). Full-length or truncated FLAG-BRD4 plasmids were gifts from Dr. Cheng-Ming Chiang (UT Southwestern, Dallas) (42). Other constructs have been described previously (7). The DNA sequences of all recombinant plasmids were confirmed by nucleotide sequence analysis. The cloning primers are listed in Supplemental Table 4.

Virus production. The lentiviruses encoding SC, ZMYND8, p300, BRD4, or CHD4 shRNA were generated by transfection of HEK293FT cells with transducing vector and packaging vectors pMD2.G and psPAX2. After 48 h, virus particles in the medium were harvested, filtered, and transduced into cancer cells.

Generation of knockout (KO) and knockdown (KD) cell lines. ZMYND8, HIF-1α, HIF-2α, and HIF-1α+HIF-2α KO cell lines were generated using the CRISPR (Clustered regularly interspaced short palindromic repeat)/Cas9 technique. Briefly, cells were transiently transfected with sgRNA vector using PolyJet (SignaGen). 36 hours post transfection, cells were treated with puromycin for three days, and a single cell was selected and verified by genotyping and immunoblot assays.

ZMYND8, p300, BRD4, or CHD4 KD cell lines were generated by infecting cells with lentivirus encoding their shRNA and subsequent puromycin selection. Protein KD efficiency was verified by immunoblot assays.

Cell proliferation assays. 2 x 10^5 of SC and ZMYND8 KD MDA-MB-231 cells were exposed to 20% or 1% O_2 for 24, 48, or 72 hours. The viable cells were counted at each time point by trypan blue assays.

In vitro acetylation assays. HEK293T cells were transiently transfected with WT FLAG-ZMYND8 or FLAG-ZMYND8 (K1007/1034R) vector, and subjected to purification of WT and K1007/1034R FLAG-ZMYND8 proteins by anti-FLAG antibody. Purified WT or K1007/1034R FLAG-ZMYND8 protein was incubated for one hour with purified FLAG-p300, FLAG-PCAF, or FLAG-GCN5, which were gifts from Cheng-Ming Chiang (UT Southwestern, Dallas) (42), in reaction buffer (50 mM Tris-HCl, pH 8.0, 7% glycerol, 0.1 mM EDTA, 5 mM DTT) in the

presence or absence of acetyl-CoA at 30 °C, resolved by SDS-PAGE, and analyzed by immunoblot assays using antibodies against acetyl lysine (1: 2,000) or FLAG epitope (1: 5,000).

RT-qPCR assays. Total RNA was isolated from cultured cells using Trizol reagent (Thermo Fisher), treated with DNase I (Ambion), and reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad). Real-time PCR was performed by a CFX-96 Real time System (Bio-Rad) using iTaq Universal SYBR Green Supermix (Bio-Rad) with primers listed in Supplemental Table 2. The fold change in target mRNA expression was calculated based on the threshold cycle (Ct) as $2^{-\Delta(\Delta Ct)}$, where $\Delta Ct = Ct_{target} - Ct_{18S\ rRNA}$ and $\Delta(\Delta Ct) = \Delta Ct_{1\%\ O2} - \Delta Ct_{20\%\ O2}$.

RNA-seq assays. Parental, ZMYND8 KO1, HIF-1/2α DKO MDA-MB-231 cells were exposed to 20% or 1% O_2 for 24 hours. Total RNA was isolated using the RNeasy Mini Kit and treated with DNase (Qiagen). The quality of total RNA was confirmed with a RIN score 8.5 or higher by the Agilent Tapestation 4200. Four micrograms of DNA-free total RNA were used for library preparation with the TruSeq Stranded mRNA Library Prep Kit (Illumina). Briefly, mRNA with polyA was purified and fragmented before strand specific cDNA synthesis. cDNAs were end repaired and A-tailed, and the indexed sequencing adapters were ligated. After the cDNAs were amplified by PCR and purified with AmpureXP beads, the samples were sequenced on the Illumina NextSeq 500 with the read configuration as 75 bp, single end. Each sample had approximately 25 million to 35 million reads.

The Fastq files were subjected to quality check using fastqc (version 0.11.2, http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and fastq_screen (version 0.4.4, http://www.bioinformatics.babraham.ac.uk/projects/ fastq_screen), and trimmed using fastq-mcf (ea-utils/version 1.1.2-806, https://github.com/ExpressionAnalysis/ea-utils). Trimmed fastq files were mapped to hg19 (UCSC version from igenomes) using Tophat (51). Duplicates were marked

using picard-tools (version 1.127, https://broadinstitute.github.io/picard/). Read counts were generated using featureCounts (52) and the differential expression analysis was performed using edgeR (53). The differential expression results with a false discovery rate (FDR) < 0.05 and mRNA fold change > 1.5 as a cutoff were used for further downstream analysis. Gene ontology was analyzed using DAVID (version 6.8).

ChIP-qPCR assays. Parental, SC, ZMYND8 KO2, or BRD4 KD2 MDA-MB-231, or T47D cells were exposed to 20% or 1% O_2 for 24 hours, crosslinked with 1% formaldehyde for 20 minutes at room temperature, and quenched in 0.125 M glycine. Cells were lyzed in lysis buffer (50 mM Tris-HCl, 10 mM EDTA, 1% SDS, protease inhibitor cocktail), sonicated, and subjected to immunoprecipitation overnight in the presence of Salmon sperm DNA/protein A beads with antibodies against ZMYND8, H3K14ac, H3K36me2, H3K36me3, H4K16ac, histone H3, histone H4, HIF-1α, HIF-2α, HIF-1β, RNA polymerase II, RNA polymerase II (S2P), RNA polymerase II (S5P), BRD4, or IgG at 4 °C. Precipitated chromatin DNA was extensively washed, eluted with freshly prepared elution buffer (0.1 M $NaHCO_3$, 1% SDS), decrosslinked at 65°C for 4 hours followed by treatment with proteinase K at 45°C for 45 minutes, purified with phenol/chloroform/isoamyl alcohol (25:24:1, v/v), and quantified by real-time qPCR assays. The primers used for ChIP-qPCR are listed in Supplemental Table 3. Fold enrichment was calculated based on Ct as $2^{-\Delta(\Delta Ct)}$, where $\Delta Ct = Ct_{IP} - Ct_{Input}$ and $\Delta(\Delta Ct) = \Delta Ct_{antibody} - \Delta Ct_{IgG}$.

ChIP-seq assays. MDA-MB-231 cells were exposed to 1% O_2 for 24 hours, crosslinked with 1% formaldehyde for 20 minutes at room temperature, and quenched in 0.125 M glycine. Chromatin was isolated using the SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology), sonicated to 200-300 bp in length, and subjected to immunoprecipitation overnight with antibodies against ZMYND8, HIF-1α, H3K14ac, or histone H3 at 4 °C. Precipitated

chromatin DNA was extensively washed and eluted with the freshly prepared elution buffer (0.1 M NaHCO3, 1% SDS). Reverse crosslinking was performed at 65°C for 4 hours followed by treatment with proteinase K and RNase A. ChIP DNA was purified with phenol/chloroform/isoamyl alcohol (25:24:1, v/v). 5-10 ng ChIP DNA was used for library preparation with the KAPA Hyper Prep Kit. Briefly, the ChIP DNA was quantified by a Qubit® 2.0 Fluorometer (Invitrogen), end repaired, adenylated at 3' ends, and barcoded with multiplex adapters. After DNA was amplified by PCR and purified with Ampure XP beads, the samples were sequenced on the Illumina NextSeq 500. 25-35 million single end reads with read length of 75 bp were generated for each sample.

The Fastq files were subjected to quality check using fastqc (version 0.11.2, http://www.bioinformatics.babraham.ac.uk/projects/fastqc), fastq_screen (version 0.4.4, http://www.bioinformatics.babraham.ac.uk/projects/fastq_screen), and mapped to human genome (hg19) using bowtie2 (version 2.2.3) (54), filtering out alignments with mapping quality less than 10. Duplicate alignments were removed using picard-tools (version 1.127, https://broadinstitute.github.io/picard/). The ChIP-seq peaks were called using MACS2 (version 2.0.10) (55), with a q-value threshold of 0.05 and using the random background of ChIP samples as controls.

The RPKM normalized bigwig files were generated for each replicates of the library using the bamCoverage module of the deepTools (version 2.5.3) (56). The Gencode Genes (version 19) was downloaded from UCSC table browser and computeMatrix and plotProfile module of deepTools was used to find the enrichment of peaks on the merged exons within 3 kb upstream of transcription start site and 3 kb downstream of transcription termination site. The corresponding heatmaps were generated using plotHeatmap module in deepTools. The co-occupancy analysis of ChIP-seq peaks

of HIF-1α and ZMYND8 was performed on the peak called regions of HIF-1α and ZMYND8 using deepTools. Motif analysis was performed using HOMER (version 4.7.2) (57).

The narrow peak file of the replicates of each library was sorted using BEDOPS (version 2.4.2) (58) and merged using mergeBed command of BEDTools (version 2.24.0) (59). We used the merged peaks to plot overlap of HIF-1α, ZMYND8, and H3K14ac peaks using Intervene (version 0.5.8) (60). ChIPseeker (61) was used to annotate the genomic landscape of HIF-1α, ZMYND8, and H3K14ac peaks, and the overlap of the HIF-1α, ZMYND8, and H3K14ac target genes was plotted using Intervene.

Luciferase reporter assays. HeLa or HEK293T cells were plated on 48-well plates (for HeLa cells) or poly-L-lysine-coated 48-well plates (for HEK293T cells), and transiently transfected with HIF luciferase reporter plasmid (containing 2 x HREs from *VEGFA* gene) or wild-type or mutant *ZMYND8* HRE luciferase reporter plasmid; control reporter plasmid pSV-Renilla; shSC or shBRD4 vector; and pcDNA6.2-V5-ZMYND8-EmGFP-DEST, p3XFLAG-HIF-1α, p3XFLAG-HIF-2α, or empty vector. 24 hours later, cells were exposed to 20% or 1% O_2 for 24 hours in the presence or absence of doxycycline (0.5 μg/ml). The firefly and Renilla luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega).

Animal studies. For the orthotopic breast cancer mouse model (7), 2 x 10^6 of cells in 100 μl PBS/Matrigel (1:1, Corning) were injected into the second left mammary fat pad of female SCID mice (6-8 weeks old, Envigo). Mice that were implanted with MCF-7 cells were administrated subcutaneously with a slow-release 17β-estradiol pellet (0.72 mg/60-day release/pellet, Innovative Research of America) one day before cell implantation. Tumor volume was measured with a caliper every three days beginning on day 11-16 after cell implantation, and calculated according to the formula: V = 0.52 x L x H x W (V: volume, L: length, H: height, W: width). Primary tumors

were harvested, photographed, and weighted when their volume reached to about 1500 mm^3. At the same time, the peripheral blood was also collected for detecting circulating tumor cells. Half of fresh tumor tissues were analyzed by immunoblot assays. Half of the tumors were fixed in formalin, embedded paraffin, and analyzed by immunohistochemistry assays. Lungs were perfused with PBS. The left lung was inflated with 0.5% agarose, fixed in formalin, embedded in paraffin, and analyzed by hematoxylin-eosin (H&E) staining. The right lung was subjected to genomic DNA extraction and subsequent qPCR assays with primers for human *HK2* gene and mouse and human 18*S* rRNA.

For the tail vein injection model, 1 x 10^6 of cells in 100 μl PBS were slowly injected into the tail vein of female SCID mice. Three weeks later, lungs were perfused with PBS and harvested. Genomic DNA were extracted from lungs and subjected to qPCR assays with primers for human *HK2* gene and mouse and human 18*S* rRNA.

Measurement of circulating tumor cells. Genomic DNA was extracted from peripheral blood in SCID mice bearing parental or ZMYND8 KO MDA-MB-231 tumors using the QIAamp DNA Blood Mini Kit (Qiagen). To make a standard curve, 0, 10, 20, 40, 80 and 160 of MDA-MB-231 cells were mixed with 1 ml blood from tumor-free SCID mice, and genomic DNA was isolated. Genomic DNA was quantified by qPCR assays with primers for human *HK2* gene and mouse and human 18*S* rRNA. The number of circulating tumor cells in mouse blood was calculated according to the standard curve.

Human TNBC TMA studies. The TMA consisting of 160 human TNBC tissues and 91 adjacent normal breast tissues was provided by a surgical breast cancer pathologist Dr. Yan Peng and purchased from UT Southwestern Tissue Resource, and analyzed by immunohistochemistry assays. The anti-ZMYND8 antibody (1:1,000) was optimized for immunohistochemical staining

using parental and ZMYND8 KO MDA-MB-231 xenograft tumor tissues. Each staining was scored by Drs. Yan Chen and Yan Peng as four grades (0-3) according to the percentage of immunopositive cells and immunostaining intensity. Grades 0-3 represent no expression (0), weak expression (1), moderate expression (2), and strong expression (3), respectively. The slides were scanned by an Aperio slide scanner.

Immunohistochemistry assays. Immunohistochemistry assays were performed by the Dako Autostainer Link 48 system. Briefly, the slides were baked at 60 °C for 20 minutes, deparaffinized and hydrated before the antigen retrieval. Heat-induced antigen retrieval was performed at optimized pH for 20 minutes in a Dako PT Link. The tissues were incubated with a peroxidase block and then a primary antibody for 20 minutes. The following primary antibodies were used: ZMYND8 (1: 1,000), cleaved caspase-3 (1: 1,500), Ki-67 (ready to use), or Endomucin (1: 50). The staining was visualized using the EnVision FLEX visualization system (Dako). The number of cleaved caspase-3-positive cells per field was counted manually. Endomucin-positive areas in tumors were quantified by ImageJ and normalized to the total area of the image. 3-5 images each tumor were randomly selected for quantification.

TCGA breast cancer data analysis. Gene expression data in human breast tumors in the TCGA dataset were downloaded from the UCSC Cancer Browser (https://genome-cancer.ucsc.edu). *ZMYND8* mRNA expression was queried in adjacent normal breast tissues, and primary and metastatic breast tumors with different subtypes, stages, or grades. Kaplan-Meier survival analysis was performed in breast cancer patients who were divided by median expression levels of *ZMYND8* mRNA.

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Supplemental Table 1. The list of antibodies used in this paper.

Antibodies	Source	Catalog/Lot#
anti-ZMYND8, (1: 8,000)	Bethyl Laboratories	Cat#A302-089A Lot#1
anti-BRD4, (1: 5,000)	Bethyl Laboratories	Cat#A301-985A-M Lot#6
anti-p300 (N-15), (1: 2,000)	Santa Cruz Biotechnology	Cat#sc-584 Lot#F1036
anti-HIF-1α (H-206), (1: 1,000)	Santa Cruz Biotechnology	Cat#sc-10790x Lot#B1308
anti-Endomucin, (1: 50)	Santa Cruz Biotechnology	Cat#sc-65495 Lot#D1117
anti-actin, (1: 2,000)	Santa Cruz Biotechnology	Cat#sc-1616 Lot#J1615
Normal rabbit IgG antibody	Santa Cruz Biotechnology	Cat#sc-2027 Lot#G2516
Normal mouse IgG antibody	Santa Cruz Biotechnology	Cat#sc-2025 Lot#H0615
anti-cleaved caspase-3, (1: 1,500)	Cell Signaling Technology	Cat#9661 Lot#33
anti-Acetylated lysine, (1: 2,000)	Cell Signaling Technology	Cat#9441S Lot#12
anti-Histone H3, (1: 5,000)	Novus Biologicals	Cat#NB500-267 Lot#A-1
anti-Histone H4, (1: 5,000)	Novus Biologicals	Cat#NBP1-45622 Lot#50924
anti-RNA polymerase II (phosphor S5), (1: 5,000)	Novus Biologicals	Cat#NB200-598 Lot#A-3
anti-HIF-2α, (1: 1,000)	Novus Biologicals	Cat#NB100-122 Lot#CD6
anti-HIF-1β, (1: 2,000)	Novus Biologicals	Cat#NB100-110 Lot#C-8
anti-Ki-67 (ready to use)	Agilent Technologies	Cat#IR62661-2 Lot#20029886
anti-HIF-1α, (1: 1,000)	BD Bioscience	Cat#610958 Lot#6214690
anti-FLAG, (1: 5,000)	Sigma-Aldrich	Cat#F3165 Lot#SLBQ7119
anti-V5, (1: 5,000)	Proteintech	Cat#66007-1-Ig Lot#10003378
anti-Histone H4K16ac, (1: 5,000)	Active Motif	Cat#39167 Lot#33311003
anti-Histone H3K14ac (1: 5,000)	Abcam	Cat#ab52946 Lot#GR302893-4
anti-Histone H3K36me2, (1: 5,000)	Abcam	Cat#ab9049 Lot#GR298584-1

anti-Histone H3K36me3 (1: 5,000)	Abcam	Cat#ab9050 Lot#GR288636-1
anti-RNA polymerase II CTD repeat YSPTSPS (phosphor S2), (1: 5,000)	Abcam	Cat#ab5095 Lot#GR295145-1
anti-RNA polymerase II CTD repeat YSPTSPS, (1: 5,000)	Abcam	Cat#ab817 Lot#GR283948-2
anti-CHD4 (1: 2,000)	Proteintech	Cat#66222-1-Ig Lot#10003351

Supplemental Table 2. Oligonucleotide sequence used for qPCR primers

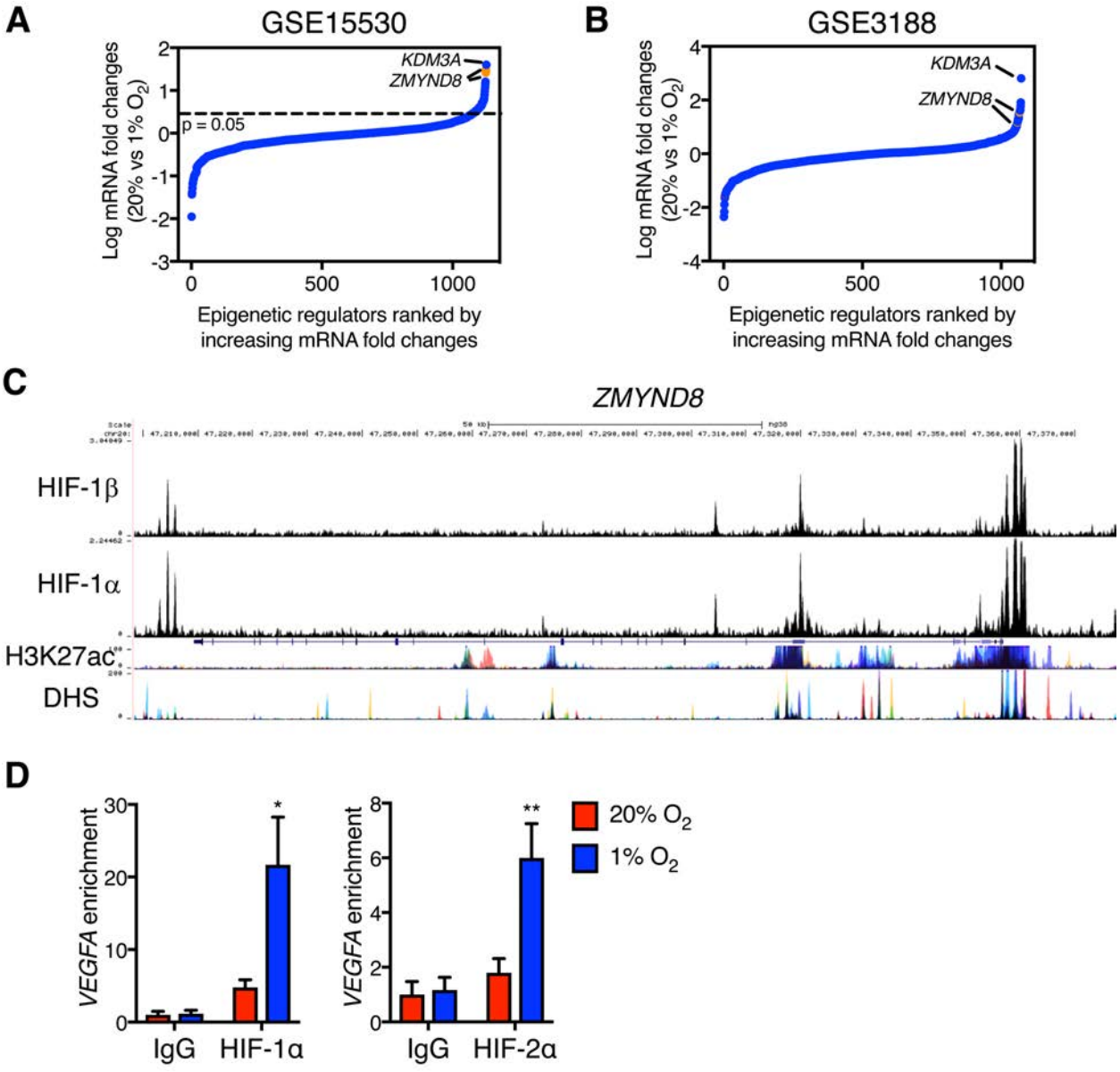
VEGFA	Forward: 5'-CTTGCCTTGCTGCTCTAC-3'
	Reverse: 5'-TGGCTTGAAGATGTACTCG-3'
AGR2	Forward: 5'-GTCAGCATTCTTGCTCCTTGT-3'
	Reverse: 5'-GGGTCGAGAGTCCTTTGTGTC-3'
AQP1	Forward: 5'-CTGGGCATCGAGATCATCGG-3'
	Reverse: 5'-ATCCCACAGCCAGTGTAGTCA-3'
LOX	Forward: 5'-CGGCGGAGGAAAACTGTCT-3'
	Reverse: 5'-TCGGCTGGGTAAGAAATCTGA-3'
ZMYND8	Forward: 5'-GGGTTTATCACGCTAAGTGTCTG-3'
	Reverse: 5'-GGCTTTACTCTGGGTCTCGATG-3'
HK2	Forward: 5'-CCAGTTCATTCACATCATCAG-3'
	Reverse: 5'-CTTACACGAGGTCACATAGC-3'
18S rRNA	Forward: 5'-CGGCGACGACCCATTCGAAC-3'
	Reverse: 5'-GAATCGAACCCTGATTCCCCGTC-3'
ANGPTL4	Forward: 5'-GTCCACCGACCTCCCGTTA-3'
	Reverse: 5'-CCTCATGGTCTAGGTGCTTGT-3'
CHD4	Forward: 5'-ACCGAATCCTCAACCACAGTG-3'
	Reverse: 5'-CACTCTCCCAAGAAGCCTGATC-3'
RPL13A	Forward: 5'-CTCAAGGTCGTGCGTCTG-3'
	Reverse: 5'-TGGCTTTCTCTTTCCTCTTCTC-3'

Supplemental Table 3. Oligonucleotide sequence used for ChIP-qPCR primers

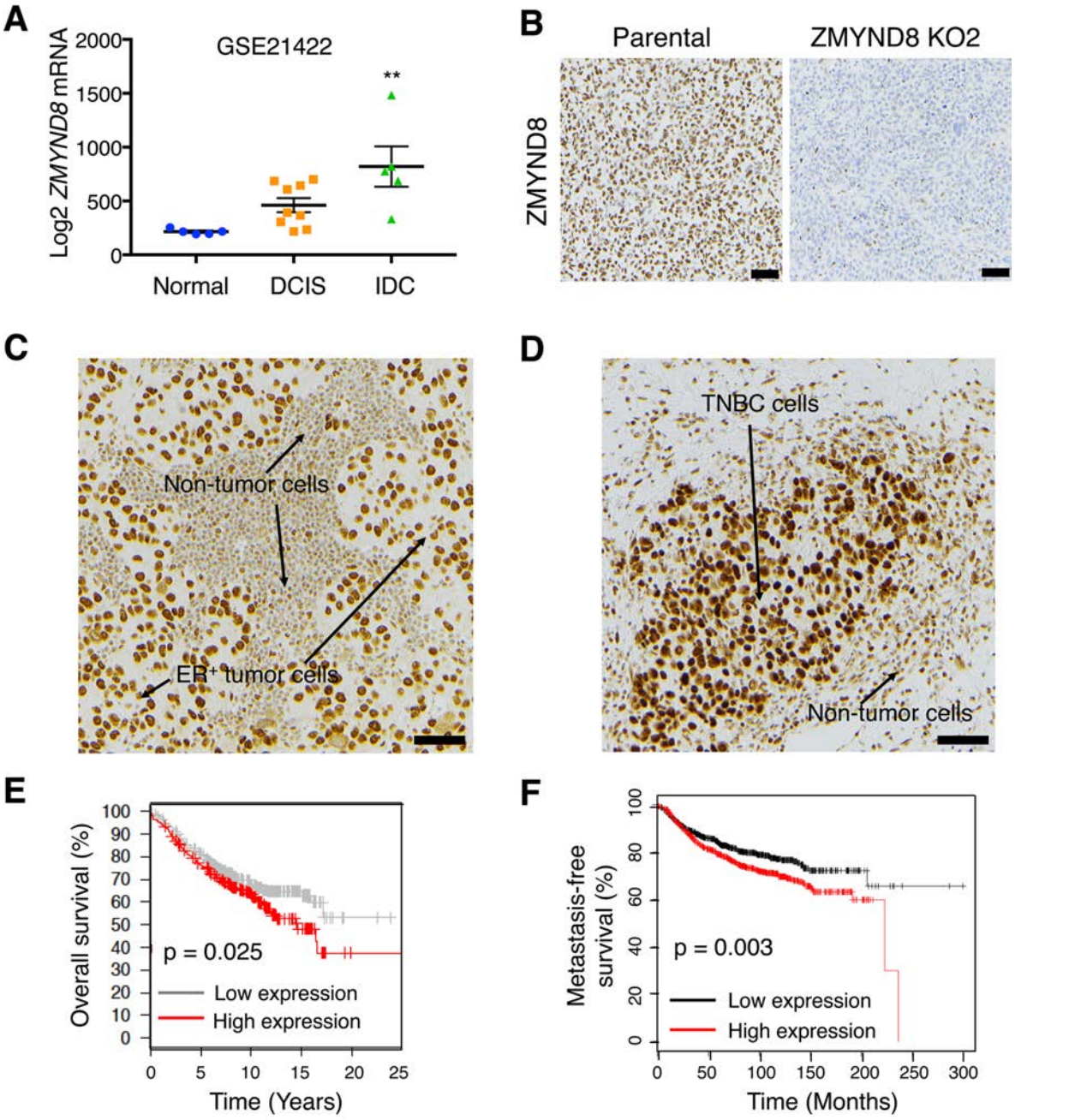
ZMYND8	Forward: 5'-GAAACCAGTGGTTCAACCAT-3'
	Reverse: 5'-GGCTGGTCTTGTGTACTTA-3'
LOX	Forward: 5'-GAAGATTTCTCCTTCCCTCAC-3'
	Reverse: 5'- GAAGCGCATCACTCCTTT-3'
VEGFA	Forward: 5'-CAGACTCCACAGTGCATAC-3'
	Reverse: 5'-AGTTTGTGGAGCTGAGAAC-3'
ANGPTL4	Forward: 5'-ATTTGCTGTCCTGGCATC-3'
	Reverse: 5'-CCAGCTCATTCTCTGGAATC-3'
RPL13A	Forward: 5'-GAGGCGAGGGTGATAGAG-3'
	Reverse: 5'-ACACACAAGGGTCCAATTC-3'

Supplemental Table 4. Oligonucleotide sequence used for shRNAs, sgRNAs, and cloning primers

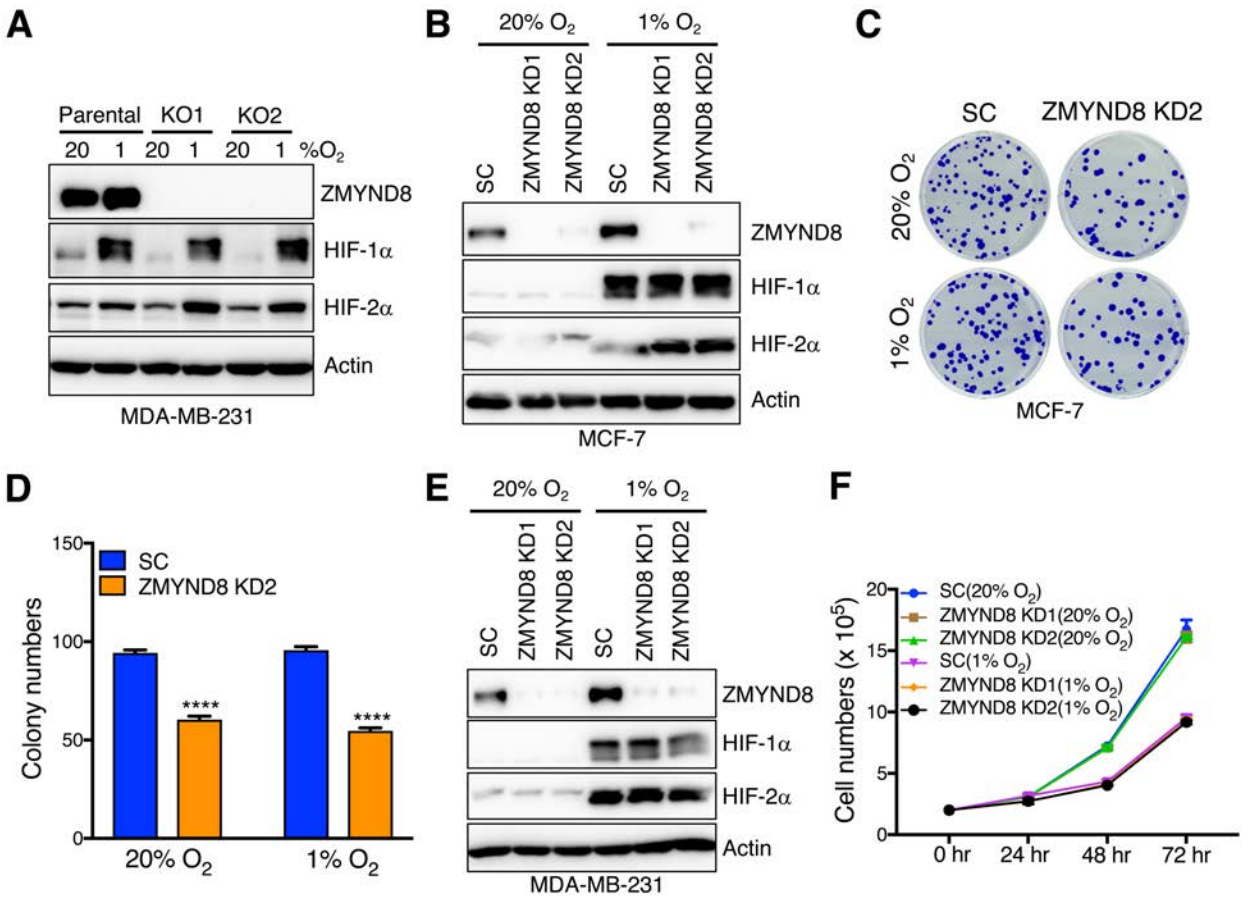
shZMYND8#1	5'-CCGGATTTCCTTGTCGGATAT-3'
shZMYND8#2	5'-TTGCCAATACTTCCTCAGTTT-3'
shp300#1	5'-ATACTCAGCCGGAGGATATTT-3'
shp300#2	5'-AGTAGCCGTTTGTGGTTTAAA-3'
shBRD4#1	5'-CCTGGAGATGACATAGTCTTA-3'
shBRD4#2	5'-CAGTGACAGTTCGACTGATGA-3'
shCHD4	5'-GCGGGAGTTCAGTACCAATAA-3'
ZMYND8 sgRNA1	5'-GACTTAGCGTGATAAACCCG-3'
ZMYND8 sgRNA2	5'-GGAGCGCGGCATATCCGACA-3'
HIF-1α sgRNA	5'-CCATCAGCTATTTGCGTGTG-3'
HIF-2α sgRNA	5'-GCTGATTGCCAGTCGCATGA-3'
ZMYND8	Forward: 5'-AAGGATCCATGGACTACAAAGACGATGACGACA AGCATCCACAGAGCTTGGCTGAAG-3'
	Reverse: 5'-AAGAATTCCTAGTCCCAGAAGGTGTCCAG-3'
ZMYND8 K1006R	Forward: 5'-AAGATGGCCTCCTTCCTGCAGTTGGCGCAC-3'
	Reverse: 5'-GCAGGAAGGAGGCCATCTTTTACTGCTG-3'
ZMYND8 K1007R	Forward: 5'-AAGATGGCCTCCCTCTTGCAGTTGGCGCAC-3'
	Reverse: 5'-GCAAGAGGGAGGCCATCTTTTACTGCTG-3'
ZMYND8 K1034R	Forward: 5'-CAGGACCTCATGTGCTCAGGCCAGTGGGC-3'
	Reverse: 5'-TGAGGTCCTGCACCCAGTCAGCTAC-3'
HIF-2α	Forward: 5'-GCCGGAAGCTTACAGCTGACAAGGAGAAGAA-3'
	Reverse: 5'-GCGTCTAGATCAGGTGGCCTGGTCCAGG-3'
ZMYND8 WT HRE	Forward: 5'-CGCGTTTGGCAATGCTTGGAGACGTGTGGGTT GTCACAGTTAGGGAGGTTGTATTACC-3'
	Reverse: 5'-GATCGGTAATACAACCTCCCTAACTGTGACAACC CACACGTCTCCAAGCATTGCCAAA-3'
ZMYND8 Mutant HRE	Forward: 5'-CGCGTTTGGCAATGCTTGGAGAAAAGTGGGTTGT CACAGTTAGGGAGGTTGTATTACC-3'
	Reverse: 5'-GATCGGTAATACAACCTCCCTAACTGTGACAACC CACTTTTCTCCAAGCATTGCCAAA-3'
VEGFA HRE	Forward:5'-CCCACAGTGCATACGTGGGCTCCAACAGGTCCTC TTGTCGAGCCACAGTGCATACGTGGGCTCCAACAGGTCCTCT T-3'
	Reverse: 5'- CTAGAAGAGGACCTGTTGGAGCCCACGTATGCA CTGTGGCTCGACAAGAGGACCTGTTGGAGCCCACGTATGCA CTGTGGGGTAC-3'



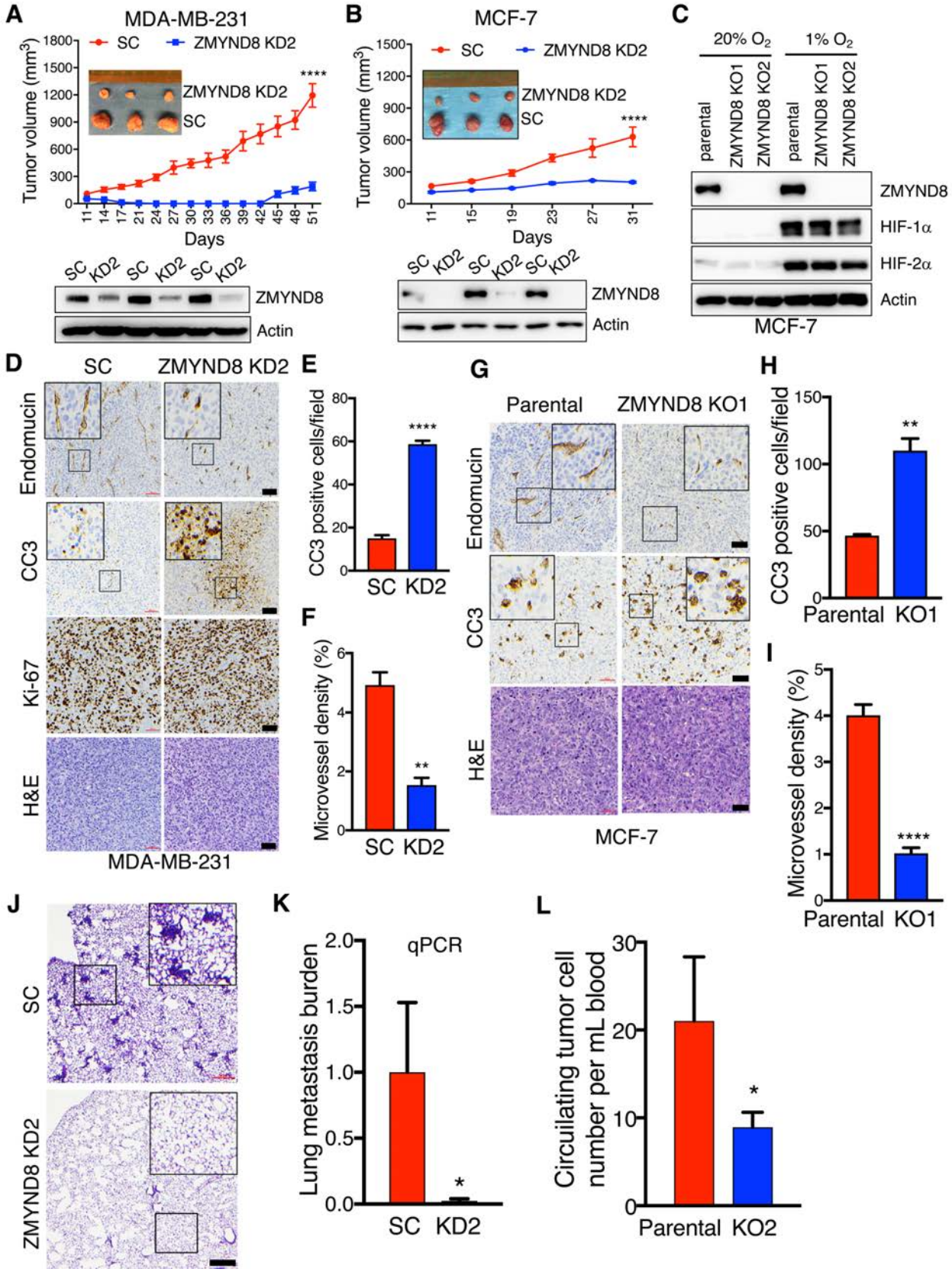
Supplemental Figure 1. *ZMYND8* is a direct HIF-1 and HIF-2 target gene. (**A** and **B**) Identification of ZMYND8 as a hypoxia-induced epigenetic regulator in MCF-7 cells. The microarray data were retrieved from the Gene Expression Omnibus database. (**C**) Analysis of the published HIF-1α and HIF-1β ChIP-seq data in T47D cells exposed to 1% O_2 for 16 hrs. ChIP-seq profiles of HIF-1α and HIF-1β at the *ZMYND8* locus are shown. The acetylated lysine 27 of histone H3 (H3K27ac) and DNase I hypersensitive site (DHS) profiles at the *ZMYND8* locus from the ENCODE project are also included. (**D**) T47D cells were exposed to 20% or 1% O_2 for 24 hrs. ChIP assays were performed with antibodies against HIF-1α, HIF-2α, or IgG, followed by qPCR assays with primers flanking the hypoxia response element of *VEGFA* gene (mean ± SEM, n = 3). $^{*}p<0.05$; $^{**}p<0.01$ versus 20% O_2, by two-way ANOVA with Sidak's test.



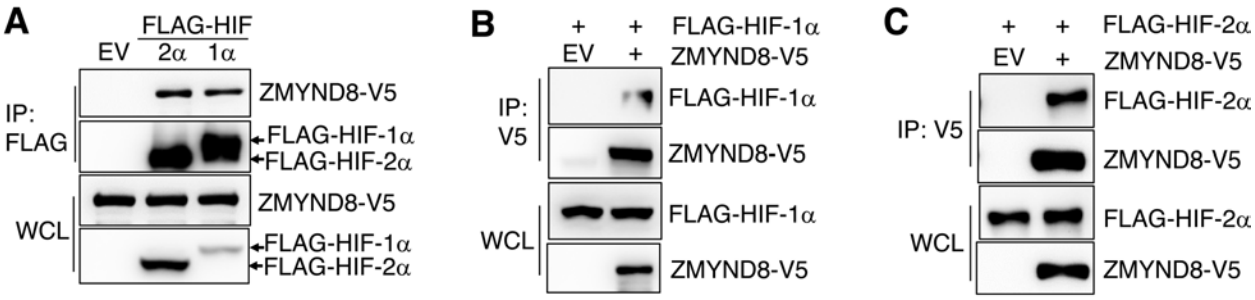
Supplemental Figure 2. ZMYND8 is highly expressed in human breast tumors and predicts poor outcome in breast cancer patients. (**A**) Analysis of *ZMYND8* mRNA levels in human breast tumors and normal breast tissues. The microarray data were retrieved from the Gene Expression Omnibus database. $^{**}p<0.01$ versus normal breast by one-way ANOVA with Tukey's test. DCIS, ductal carcinoma in situ. IDC, invasive ductal carcinoma. (**B**) Validation of the ZMYND8 antibody for immunohistochemistry assays in parental or ZMYND8 KO2 MDA-MB-231 tumor tissues. Scale bar, 200 μm. (**C** and **D**) ZMYND8 immunohistochemical staining in human ER^+ (**C**) and TNBC (**D**) tumors (n = 3). Scale bar, 200 μm. (**E** and **F**) Kaplan-Meier survival analysis for patients with breast cancer by log-rank test. Patients were divided by quartile (**E**) or median (**F**) expression levels of *ZMYND8* mRNA. The data were obtained from the GOBO (**E**) (62) and Kaplan-Meier Plotter (**F**) (63).



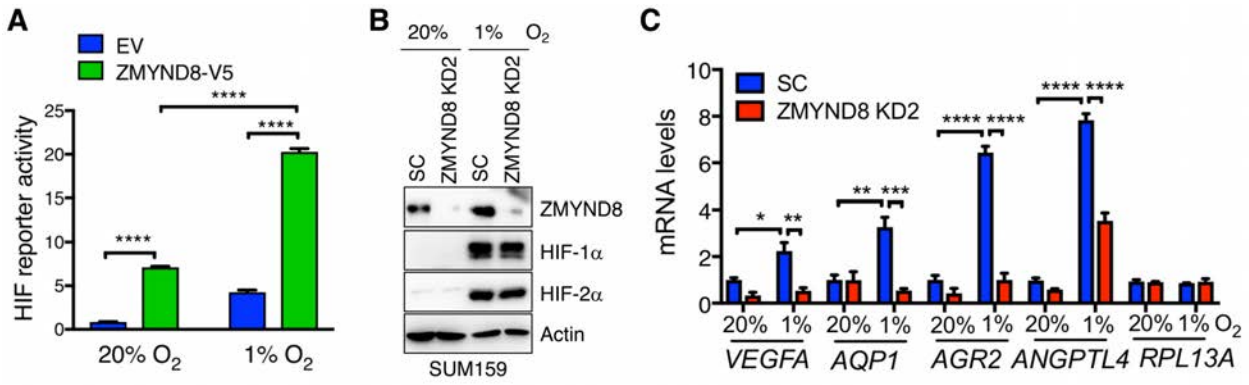
Supplemental Figure 3. ZMYND8 knockdown suppresses colony formation but has no effect on cell proliferation. (**A**) Parental and ZMYND8 KO1 or KO2 MDA-MB-231 cells were exposed to 20% or 1% O_2 for 24 hrs. Immunoblot assays were performed with antibodies against ZMYND8, HIF-1α, HIF-2α, or actin (n = 3). (**B**) SC and ZMYND8 KD1 or KD2 MCF-7 cells were exposed to 20% or 1% O_2 for 24 hrs. Immunoblot assays were performed with antibodies against ZMYND8, HIF-1α, HIF-2α, or actin (n = 3). (**C** and **D**) Colony formation of SC and ZMYND8 KD2 MCF-7 cells cultured under 20% or 1% O_2 for 18 days. Representative images from three experiments are shown in **C**. Colony numbers are quantified in **D** (mean ± SEM, n = 3). $^{****}p<0.0001$ versus SC by two-way ANOVA with Tukey's test. (**E**) SC and ZMYND8 KD1 or KD2 MDA-MB-231 cells were exposed to 20% or 1% O_2 for 24 hrs. Immunoblot assays were performed with antibodies against ZMYND8, HIF-1α, HIF-2α, or actin (n = 3). (**F**) SC and ZMYND8 KD1 or KD2 MDA-MB-231 cells were exposed to 20% or 1% O_2 for 24, 48, or 72 hrs. Cell viability was determined by trypan blue exclusion assays (mean ± SEM, n = 3).


A
MDA-MB-231
SC
ZMYND8 KD2
Tumor volume (mm3)
Days
ZMYND8
Actin
B
MCF-7
C
20% O2
1% O2
parental
ZMYND8 KO1
ZMYND8 KO2
HIF-1α
HIF-2α
D
Endomucin
CC3
Ki-67
H&E
E
CC3 positive cells/field
F
Microvessel density (%)
G
Parental
ZMYND8 KO1
H
I
J
K
qPCR
Lung metastasis burden
L
Circulating tumor cell number per mL blood
KO1
KO2

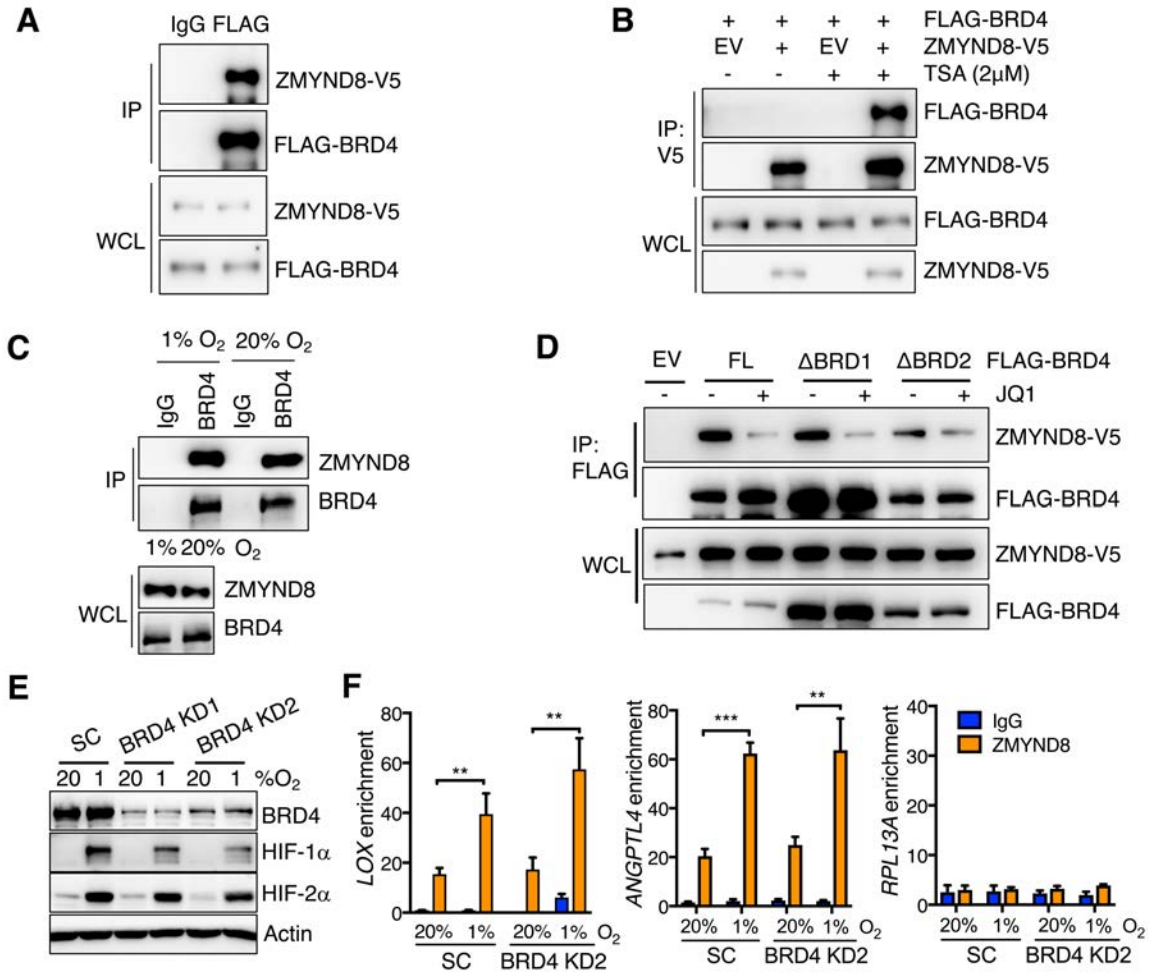
Supplemental Figure 4. ZMYND8 knockdown suppresses breast tumor growth and metastasis. (**A** and **B**) Growth of SC or ZMYND8 KD2 MDA-MB-231 or MCF-7 tumors in mice (mean ± SEM, n = 3). $^{****}p<0.0001$ versus SC by two-way ANOVA with Sidak's test. The image of primary tumors harvested at the end time point is shown in the inset. ZMYND8 KD in the tumors was confirmed by immunoblot assays (bottom). (**C**) Immunoblot analysis of indicated proteins in parental and ZMYND8 KO1 or KO2 MCF-7 cells exposed to 20% or 1% O_2 for 24 hrs (n = 3). (**D**-**F**) Representative H&E and immunohistochemical staining of Endomucin, CC3, and Ki-67 in parental or ZMYND8 KD2 MDA-MB-231 tumors (**D**). Magnified images of the boxed area are shown in the insets (**D**). Scale bar, 200 μm. CC3-positive cell numbers (**E**) and Endomucin-positive areas (**F**) in tumors are quantified (mean ± SEM, n = 3). $^{**}p<0.001$; $^{****}p<0.0001$ versus SC by two-tailed Student's *t* test. (**G**-**I**) Representative H&E staining and immunohistochemical staining of Endomucin and CC3 in parental or ZMYND8 KO1 MCF-7 tumors (**G**). Magnified images of the boxed area are shown in the insets (**G**). Scale bar, 200 μm. CC3-positive cell numbers (**H**) and Endomucin-positive areas (**I**) in tumors are quantified (mean ± SEM, n = 3). $^{**}p<0.001$; $^{****}p<0.0001$ versus SC by two-tailed Student's *t* test. (**J** and **K**) Lung metastasis in mice bearing SC or ZMYND8 KD2 MDA-MB-231 tumors by H&E staining (**J)** and qPCR assays (**K**, mean ± SEM, n = 3). Magnified images of the boxed area are shown in the insets. Scale bar, 200 μm. $^{*}p<0.05$ versus SC by two-tailed Student's *t* test. (**L**) Circulating tumor cells in blood from mice bearing parental or ZMYND8 KO2 MDA-MB-231 tumors by qPCR assays (mean ± SEM, n = 4). $^{*}p<0.05$ versus parental by two-tailed Student's *t* test.



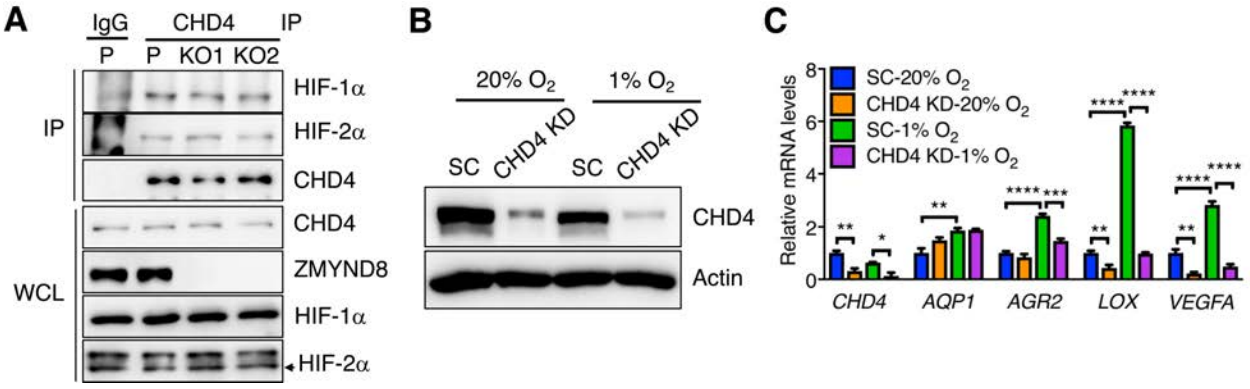
Supplemental Figure 5. ZMYND8 interacts with HIF-1α and HIF-2α in breast cancer cells. (**A**) HEK293T cells were transfected with ZMYND8-V5 vector and FLAG-HIF-1α, FLAG-HIF-2α, or EV, and exposed to 1% O_2 for 6 hrs. Co-immunoprecipitation (IP) assays were performed with an anti-FLAG antibody, followed by immunoblot assays with antibodies against FLAG or V5 (n = 3). WCL, whole cell lysate. (**B**) HEK293T cells were transfected with FLAG-HIF-1α vector and ZMYND8-V5 or EV, and exposed to 1% O_2 for 6 hrs. Co-IP assays were performed with an anti-V5 antibody, followed by immunoblot assays with antibodies against FLAG or V5 (n = 3). (**C**) HEK293T cells were transfected with FLAG-HIF-2α vector and ZMYND8-V5 or EV, and exposed to 1% O_2 for 6 hrs. Co-IP assays were performed with an anti-V5 antibody, followed by immunoblot assays with antibodies against FLAG or V5 (n = 3).



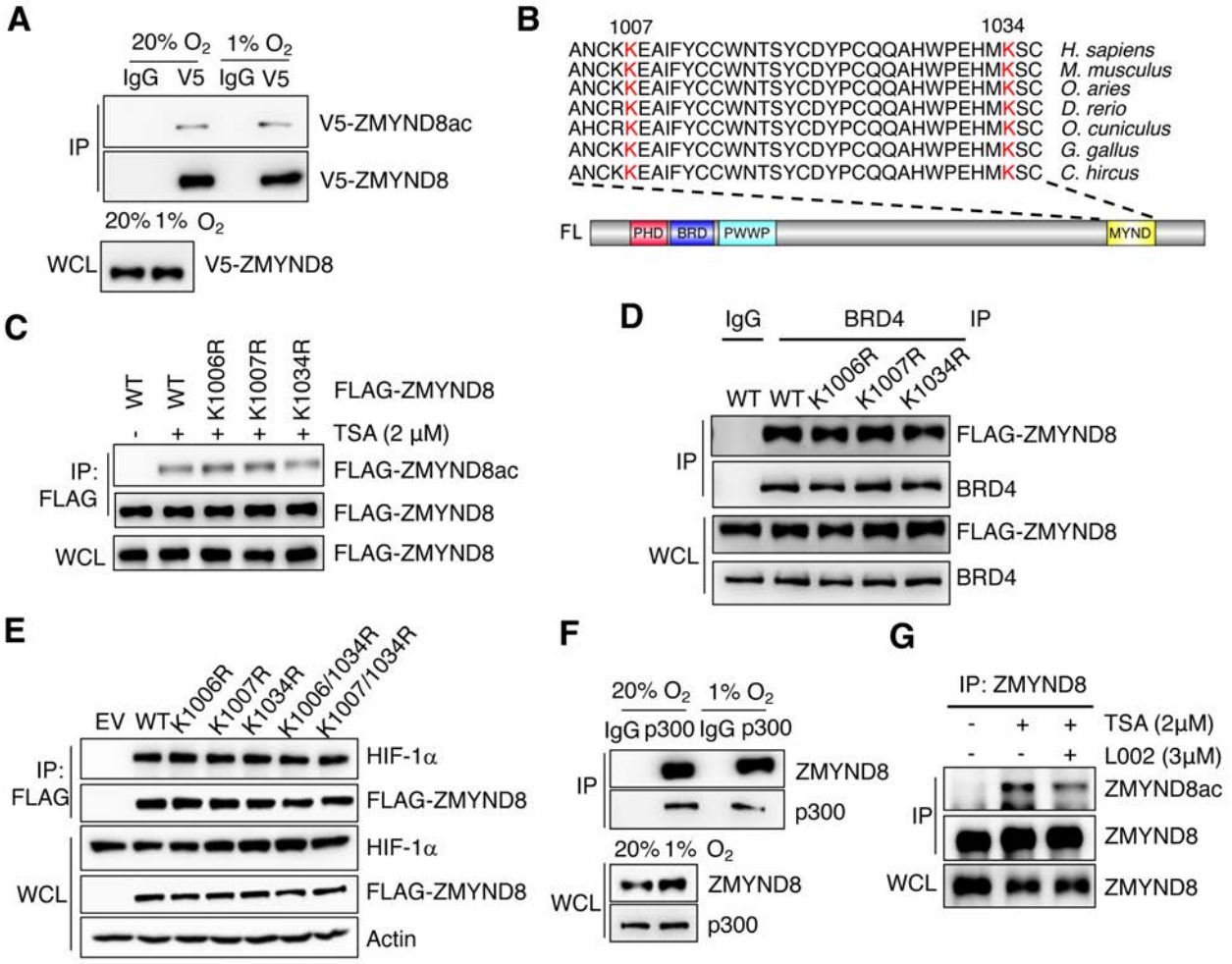
Supplemental Figure 6. ZMYND8 enhances HIF transcriptional activity in breast cancer cells. (**A**) HeLa cells were transfected with HIF luciferase reporter plasmid, pSV-Renilla, and EV or ZMYND8-V5 vector, and exposed to 20% or 1% O_2 for 24 hrs. The FLuc/RLuc activity was determined (mean ± SEM, n = 3). $^{****}p<0.0001$ by two-way ANOVA with Tukey's test. (**B** and **C**) SC and ZMYND8 KD2 SUM159 cells were exposed to 20% or 1% O_2 for 24 hrs. Immunoblot assays were performed with antibodies against ZMYND8, HIF-1α, HIF-2α, or actin (n = 3). (**B**). RT-qPCR analysis of indicated mRNAs was performed (**C**, mean ± SEM, n = 3). $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; $^{****}p<0.0001$, by two-way ANOVA with Tukey's test.



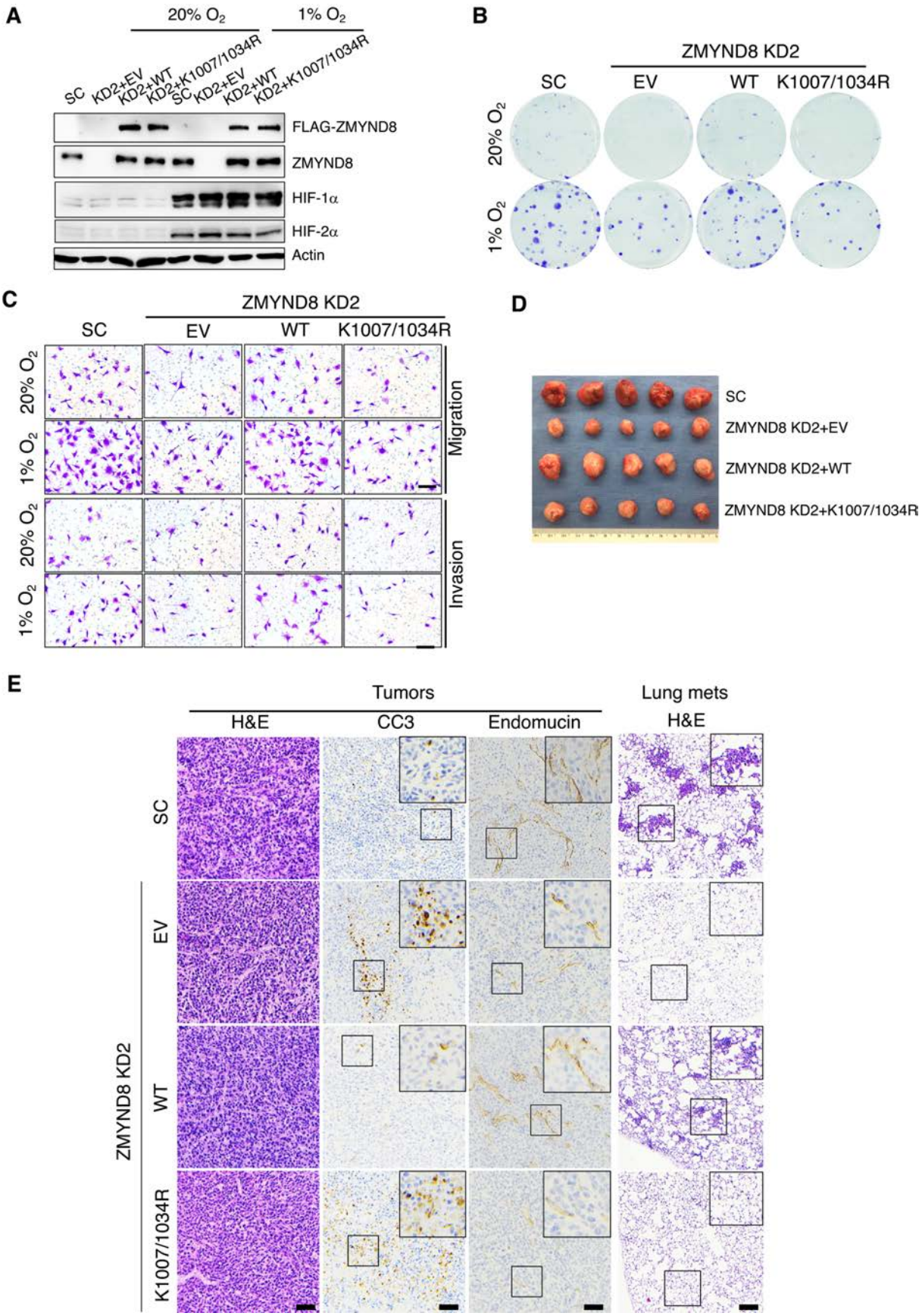
Supplemental Figure 7. BRD4 interacts with ZMYND8 in breast cancer cells. (**A**) HEK293T cells were co-transfected with ZMYND8-V5 vector and FLAG-BRD4 vector. Co-immunoprecipitation (IP) assays were performed with anti-FLAG antibody, followed by immunoblot assays with antibodies against V5 or FLAG (n = 3). WCL, whole cell lysate. (**B**) HEK293T cells were co-transfected with FLAG-BRD4 vector and ZMYND8-V5 vector or EV, and treated with TSA or DMSO (-) for 6 hrs. Co-IP assays were performed with anti-V5 antibody, followed by immunoblot assays with antibodies against V5 or FLAG (n = 3). (**C**) MDA-MB-231 cells were exposed to 20% or 1% O_2 for 24 hrs. Co-IP assays were performed with anti-BRD4 antibody or IgG, followed by immunoblot assays with antibodies against BRD4 or ZMYND8 (n = 3). (**D**) HEK293T cells were co-transfected with ZMYND8-V5 vector and full-length (FL) or bromodomain (BRD)-deleted FLAG-BRD4 vector or EV, and treated with a BET inhibitor JQ1 (1 μm) or DMSO (-) for 6 hrs. Co-IP assays were performed with anti-FLAG antibody, followed by immunoblot assays with antibodies against V5 or FLAG (n = 3). (**E**) Tet-inducible SC and BRD4 KD1 or KD2 MDA-MB-231 cells were exposed to 20% or 1% O_2 for 24 hrs in the presence of doxycycline (0.5 μg/ml). Immunoblot assays were performed with antibodies against BRD4, HIF-1α, HIF-2α, or actin (n = 3). (**F**) SC and BRD4 KD2 MDA-MB-231 cells were exposed to 20% or 1% O_2 for 24 hrs in the presence of doxycycline (0.5 μg/ml). ChIP assays were performed with anti-ZMYND8 antibody or IgG, followed by quantitative PCR assays with primers flanking the hypoxia response element of *LOX* or *ANGPTL4*, or *RPL13A* (mean ± SEM, n = 3). $^{**}p<0.01$; $^{***}p<0.001$, by two-way ANOVA with Sidak's test.



Supplemental Figure 8. ZMYND8 and CHD4 independently increase HIF transcriptional activity in breast cancer cells. (**A**) Parental (P) and ZMYND8 KO1 or KO2 MDA-MB-231 cells were subjected to immunoprecipitation (IP) assays with anti-CHD4 antibody or IgG, followed by immunoblot assays with antibodies against HIF-1α, HIF-2α, ZMYND8, or CHD4 (n = 3). (**B**) SC and CDH4 KD MDA-MB-231 cells were exposed to 20% or 1% O_2 for 24 hrs. Immunoblot assays were performed with antibodies against CHD4 or actin (n = 3). (**C**) SC and CDH4 KD MDA-MB-231 cells were exposed to 20% or 1% O_2 for 24 hrs. RT-qPCR analysis of indicated mRNAs was performed (mean ± SEM, n = 3). $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; $^{****}p<0.0001$, by two-way ANOVA with Tukey's test.



Supplemental Figure 9. ZMYND8 is acetylated by p300 in breast cancer cells. (A) HEK293T cells were transfected with ZMYND8-V5 vector and exposed to 20% or 1% O_2 for 24 hrs. Immunoprecipitation (IP) assays were performed with anti-V5 antibody or IgG, followed by immunoblot assays with antibodies against acetyl lysine or V5 (n = 3). WCL, whole cell lysate. (**B**) Amino acid sequence alignment of the MYND domain of ZMYND8 in different species. The conserved lysine residues in vertebrates are marked in red. (**C**) HEK293T cells were transfected with WT or mutant FLAG-ZMYND8 vector, and treated with TSA or DMSO (-) for 6 hrs. IP assays were performed with an anti-FLAG antibody, followed by immunoblot assays with antibodies against acetyl lysine or FLAG (n = 3). (**D**) HEK293T cells were transfected with WT or mutant FLAG-ZMYND8 vector. Co-IP assays were performed with anti-BRD4 antibody or IgG, followed by immunoblot assays with antibodies against FLAG or BRD4 (n = 3). (**E**) HEK293T cells were transfected with WT or mutant FLAG-ZMYND8 vector, or EV. Co-IP assays were performed with anti-FLAG antibody, followed by immunoblot assays with antibodies against HIF-1α, FLAG, or actin (n = 3). (**F**) MDA-MB-231 cells were exposed to 20% or 1% O_2 for 24 hrs and subjected to IP assays with anti-p300 antibody or IgG, followed by immunoblot assays with antibodies against p300 or ZMYND8 (n = 3). (**G**) MCF-7 cells were treated with TSA or DMSO (-) for 6 hrs, followed with a p300 inhibitor L002 or DMSO (-) for additional 4 hrs. IP assays were performed with anti-ZMYND8 antibody, followed by immunoblot assays with antibodies against acetyl lysine or ZMYND8 (n = 3).


A
20% O2
1% O2
SC
KD2+EV
KD2+WT
KD2+K1007/1034R
SC
KD2+EV
KD2+WT
KD2+K1007/1034R
FLAG-ZMYND8
ZMYND8
HIF-1α
HIF-2α
Actin
B
ZMYND8 KD2
SC
EV
WT
K1007/1034R
20% O2
1% O2
C
ZMYND8 KD2
SC
EV
WT
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20% O2
1% O2
Migration
20% O2
1% O2
Invasion
D
SC
ZMYND8 KD2+EV
ZMYND8 KD2+WT
ZMYND8 KD2+K1007/1034R
E
Tumors
Lung mets
H&E
CC3
Endomucin
H&E
SC
ZMYND8 KD2
EV
WT
K1007/1034R

Supplemental Figure 10. Acetylated ZMYND8 is necessary for breast tumor growth and metastasis. (**A**) SC, ZMYND8 KD2+EV, ZMYND8 KD2+WT FLAG-ZMYND8, and ZMYND8 KD2+FLAG-ZMYND8 (K1007/1034R) MDA-MB-231 cells were exposed to 20% or 1% O_2 for 24 hrs. Immunoblot assays were performed with antibodies against FLAG, ZMYND8, HIF-1α, HIF-2α, or actin (n = 3). (**B** and **C**) Clonogenic assays (**B**), migration assays (**C**), and invasion assays (**C**) of SC, ZMYND8 KD2+EV, ZMYND8 KD2+WT FLAG-ZMYND8, and ZMYND8 KD2+FLAG-ZMYND8 (K1007/1034R) MDA-MB-231 cells exposed to 20% or 1% O_2 for 12 days (**B**), 16 hrs (**C**, migration), or 24 hrs (**C**, invasion). Representative images from three independent experiments are shown. Scale bar, 100 μm. (**D** and **E**) SC, ZMYND8 KD2+EV, ZMYND8 KD2+WT FLAG-ZMYND8, or ZMYND8 KD2+FLAG-ZMYND8 (K1007/1034R) MDA-MB-231 cells were orthotopically implanted into the mammary fat pad of female SCID mice (n = 5). The image of primary tumors harvested on day 45 is shown in **D**. Representative H&E staining and immunohistochemical staining of Endomucin and CC3 in tumors are shown in **E**. Metastases in the lungs were determined by H&E staining (**E**). Magnified images of the boxed area are shown in the insets (**E**). Scale bar, 200 μm.