ZMYND8 acetylation mediates HIF-dependent breast cancer progression and metastasis

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Altered epigenetic reprogramming contributes to breast cancer progression and metastasis. How the epigenetic reader mediates breast cancer progression remains poorly understood. Here, we showed that the epigenetic reader zinc finger MYND-type containing 8 (ZMYND8) is induced by HIF-1 and HIF-2 in breast cancer cells and also upregulated in human breast tumors, and is correlated with poor survival of patients with breast cancer. Genetic deletion of ZMYND8 decreases breast cancer cell colony formation, migration, and invasion in vitro, and inhibits breast tumor growth and metastasis to the lungs in mice. The ZMYND8’s oncogenic effect in breast cancer requires HIF-1 and HIF-2. We further showed that ZMYND8 interacts with HIF-1α and HIF-2α and enhances elongation of the global HIF-induced oncogenic genes by increasing recruitment of BRD4 and subsequent release of paused RNA polymerase II in breast cancer cells. ZMYND8 acetylation at lysines 1007 and 1034 by p300 is required for HIF activation and breast cancer progression and metastasis. These findings uncover a primary epigenetic mechanism of HIF activation and HIF-mediated breast cancer progression, and discover a possible molecular target for the diagnosis and treatment of breast cancer.

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ZMYND8 acetylation mediates HIF-dependent breast cancer progression and metastasis

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Introduction

Metastasis is a major clinical obstacle to curative cancer therapy and is responsible for about 90% of breast cancer–related deaths in women. The development of a benign and nonmalignant ductal carcinoma in situ to the advanced and metastatic breast cancer requires many dysregulated oncogenes and tumor suppressor genes, each of which provides a unique growth advantage to breast cancer cells and controls a specific phenotypic trait of tumor progression and metastasis (1, 2). Epigenetic reprogramming plays a crucial role in dysregulation of these genes and represents an important mechanism of breast tumor progression and metastasis (2). Several epigenetic writers and erasers, including DOT1L, G9a, histone deacetylase 3 (HDAC3), WDR5, and Jumonji domain-containing protein 3 (JMJD3), have been shown to induce repression of the CDH1 gene (encoding E-cadherin) in breast cancer cells and modulate the epithelial-mesenchymal transition, a key cellular program in the initiation of metastasis, thereby triggering breast tumor metastasis to distant organs (3–6). Our previous work showed that JMJD2C promotes triple-negative breast tumor growth and metastasis to the lungs in mice through inducing glycolytic and metastasis genes (7). Similarly, EZH2, JMJD2B, MLL4, and UTX also regulate invasiveness of breast tumors (8–10). Recent studies have uncovered that the epigenetic readers also emerge to influence breast tumor growth. BRD4 inhibition by its shRNA or a pharmacological inhibitor JQ1 dramatically blocks triple-negative breast tumor growth in xenograft mice (11). Conversely, another epigenetic reader, zinc finger MYND-type containing 11 (ZMYND11), suppresses triple-negative breast tumorigenesis (12). However, how the epigenetic readers control breast tumor progression and metastasis remains poorly understood.

The tumor microenvironment is increasingly recognized as a critical factor that regulates epigenetic reprogramming. A notable feature of the microenvironment of human breast tumors is reduced O2 availability (hypoxia) with median partial pressure of oxygen (PO2) values of 10 mmHg, which is markedly lower than 65 mmHg in normal breast tissues (13). The HIFs are the master transcriptional regulators mediating the adaptive responses to intratumoral hypoxia to drive breast tumor progression (14). HIFs have 3 family members, HIF-1, HIF-2, and HIF-3, each of which consists of an O2-regulated α subunit and a constitutively expressed β subunit (15–17). In well-oxygenated cells, HIF-α protein is subjected to proteasomal degradation, which is mediated by the von Hippel-Lindau protein–dependent ubiquitin system, after it is hydroxylated by prolyl hydroxylases (18). Under hypoxia, HIF-α escapes from proteasomal degradation and is translocated into the nucleus, where it dimerizes with HIF-β (16). The heterodimer binds to the hypoxia response elements (HREs; 5′-A/GCGTG-3′) in the genome, leading to transcriptional activation of hundreds of oncogenic genes (19), whose protein products regulate angiogenesis, epigenetic reprogramming, metabolism, cell migration and invasion, cell survival, and stem cell maintenance, leading to tumor growth and metastasis (14). For example, HIF-1 and HIF-2 directly activate the transcription of the proangiogenesis factor VEGFA to increase tumor angiogenesis (20). Other HIF-1 target genes — ANGPTL4, AQP1, and AGR2 — are also known to induce angiogenesis and cell migration (21–23). Lysyl oxidase (LOX) regulates collagen crosslinking and is essential for premetastatic niche formation. HIF-1 and HIF-2

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are required for this important premetastatic phenotype in breast cancer by inducing expression of the members of the LOX family, including LOX, LOXL2, and LOXL4 (24, 25). Therefore, these phenotypic traits controlled by the specific genes mediate hypoxia-driven breast tumor growth and metastasis.

Epigenetic regulators are essential for HIF-mediated transactivation (26). The histone acetyltransferases p300, CBP, and TIP60 induce acetylation of histones H3 and H4 to increase activation (26). The histone acetyltransferases p300, CBP, and DRD5 are required for this important premetastatic phenotype in breast cancer by inducing expression of the members of the LOX family, including LOX, LOXL2, and LOXL4 (24, 25). Therefore, these phenotypic traits controlled by the specific genes mediate hypoxia-driven breast tumor growth and metastasis.

To validate the microarray data, a panel of human breast cancer cell lines, including MCF-7, MDA-MB-231, SUM159, T47D, HCC1954, and MDA-MB-468, was exposed to 20% or 1% O2 for 24 hours. Quantitative reverse transcription–polymerase chain reaction (RT-qPCR) assays showed that ZMYND8 mRNA was significantly upregulated by hypoxia in all breast cancer cell lines we tested (Figure 1A). ZMYND8 protein levels were also elevated, along with induced HIF-1α and HIF-2α proteins, in these cell lines under hypoxia (Figure 1B). Knockout (KO) of HIF-1α, HIF-2α, or both by the CRISPR/Cas9 technique significantly decreased levels of ZMYND8 mRNA and protein in nonhypoxic and hypoxic MDA-MB-231 cells (Figure 1, C and D). These data indicate that ZMYND8 is induced by hypoxia in a HIF-1- and HIF-2-dependent manner in breast cancer cells.

To determine whether ZMYND8 is a direct HIF target gene in breast cancer cells, we analyzed the HIF-1α and HIF-2α chromatin immunoprecipitation–sequencing (ChIP-seq) data sets from T47D cells (36), and found a robust HIF-1α- and HIF-2α-binding peak cluster at the promoter of the ZMYND8 gene (Supplemental Figure 1C), which annotated an HRE containing a consensus core HIF binding sequence (5′-ACGTG-3′) followed by a 5′-CACAG-3′ sequence (Figure 1E). To validate the ChIP-seq data, T47D cells were exposed to 20% or 1% O2 for 24 hours. ChIP-qPCR assays showed that occupancy of HIF-1α or HIF-2α on this HRE was significantly increased by hypoxia in T47D cells (Figure 1F, left and middle), similar to HIF-1α or HIF-2α occupancy on the HRE of a known HIF-1 and HIF-2 target gene VEGFA (Supplemental Figure 1D). Increased HIF-1α enrichment was also detected on the HRE of the ZMYND8 gene in hypoxic T47D cells (Figure 1F, right). These data indicate that HIF-1 and HIF-2 directly bind to the promoter of the ZMYND8 gene.

Next, we studied whether the HRE at the promoter of the ZMYND8 gene is able to enhance gene transcription by a dual-luciferase reporter assay. A 54-bp oligonucleotide sequence containing the ZMYND8 HRE was inserted upstream of the firefly luciferase (FLuc) coding sequence in a pGL2-promoter reporter plasmid. HEK293T cells were cotransfected with WT ZMYND8 HRE reporter plasmid or empty vector (EV) and a control reporter pSV40-Renilla (in which Renilla luciferase [RLuc] is constitutively expressed), and exposed to 20% or 1% O2 for 24 hours. The WT ZMYND8 HRE significantly increased the FLuc activity in hypoxic HEK293T cells (Figure 1G). Mutation of the HRE binding sequence 5′-CGT-3′ to 5′-AAA-3′ within the HRE completely abolished hypoxia-induced FLuc activity (Figure 1G). Overexpression of HIF-1α or HIF-2α significantly enhanced WT but not mutant ZMYND8 HRE-driven FLuc activity in nonhypoxic and hypoxic HEK293T cells (Figure 1H). In contrast, CRISPR/Cas9-based KO of HIF-1α, HIF-2α, or both significantly decreased hypoxia-induced ZMYND8 HRE activity in another reporter cell line, HeLa (Figure 1I). Taken together, these data indicate that HIF-1 and HIF-2 directly bind to the HRE at the ZMYND8 gene promoter to activate its transcription in breast cancer cells.

ZMYND8 is highly expressed in human breast tumors and is associated with poor clinical outcomes in patients. Hypoxia is a hallmark of the microenvironment of breast tumors (13). Thus, we studied whether ZMYND8 is also induced in human breast tumors. Analysis of The Cancer Genome Atlas (TCGA) breast invasive carcino-
data set revealed that ZMYND8 mRNA was significantly elevated in primary human breast tumors as compared with adjacent normal breast tissues (Figure 2A). The highest levels of ZMYND8 mRNA were found in metastatic breast tumors (Figure 2A). Similar findings were also observed in another GEO data set of human breast tumors (Supplemental Figure 2A). ZMYND8 mRNA was upregulated in luminal A, luminal B, and HER2+ subtypes, and stages 1–4 and histological grades 1–4 of human breast tumors (Figure 2, B–D). Next, we performed immunohistochemistry assays to confirm ZMYND8 induction at protein levels in human breast tumors. The specificity of ZMYND8 antibody was validated in control and ZMYND8-KO MDA-MB-231 tumors harvested from an orthotopic human breast cancer xenograft model in SCID mice (Supplemental Figure 2B). ZMYND8 protein was highly expressed in estrogen receptor-positive (ER+) breast tumors as compared with adjacent nontumor cells (Supplemental Figure 2C). Notably, much higher levels of ZMYND8 protein were observed in triple-negative breast cancer (TNBC) (Supplemental Figure 2D). Thus, we focused on TNBC and further analyzed ZMYND8 protein levels in a TNBC tissue microarray (TMA) containing 160 human TNBC specimens and 91 paired adjacent normal breast tissues. Of 160 TNBC specimens, 50% and 30% of tumors expressed moderate and high levels of ZMYND8 protein, respectively (Figure 2, E and F). In contrast, moderate ZMYND8 expression was found in only a small population of adjacent normal breast tissues (18%) and no high ZMYND8 protein levels were detected in normal breast tissues (Figure 2, E and F). Negative and weak expression of ZMYND8 was predominant in normal breast tissues (Figure 2, E and F).
significantly associated with high mortality of patients with different subtypes and stages of breast cancer, but not different tumor grades (Figure 2, H–J). Together, these data indicate that ZMYND8 is highly expressed in breast tumors and is associated with poor clinical outcomes in patients with breast cancer.

ZMYND8 increases colony formation, migration, and invasion of breast cancer cells in vitro. To determine an oncogenic role of

Figure 2. ZMYND8 is highly expressed in breast tumors and predicts poor clinical outcomes in patients with breast cancer. (A–D) Analysis of ZMYND8 mRNA levels in human breast tumors and normal breast tissues. *P < 0.05, ***P < 0.001, ****P < 0.0001 versus normal breast, by 1-way ANOVA with Tukey’s t test. (E) Representative ZMYND8 immunohistochemical staining for each score (0–3) in a human TNBC tissue microarray. Scale bar, 200 μm. ND, not detected. (F) Quantification of low, moderate, and high ZMYND8 expression in normal breast and TNBC by χ² test. (G–J) Kaplan-Meier survival analysis for patients with breast cancer by log-rank test. Patients were divided by median expression levels of ZMYND8 mRNA. HR, hazard ratio.

and F). Kaplan-Meier analysis of the TCGA data set revealed that high levels of ZMYND8 mRNA were associated with poor overall survival of patients with breast cancer (Figure 2G). A similar negative correlation was found in the gene expression–based outcome (GOBO) data set (Supplemental Figure 2E). ZMYND8 was also inversely correlated with metastasis-free survival of patients with breast cancer (Supplemental Figure 2F). Moreover, ZMYND8 was significantly associated with high mortality of patients with different subtypes and stages of breast cancer, but not different tumor grades (Figure 2, H–J). Together, these data indicate that ZMYND8 is highly expressed in breast tumors and is associated with poor clinical outcomes in patients with breast cancer.

ZMYND8 increases colony formation, migration, and invasion of breast cancer cells in vitro. To determine an oncogenic role of
ZMYND8 knockdown (KD) by its shRNA also decreased the colony numbers of MCF-7 cells (Supplemental Figure 3, B–D). These data indicate that ZMYND8 increases breast cancer cell survival and colony formation in vitro.

Next, we studied the effect of ZMYND8 on breast cancer cell migration and invasion in vitro. The Boyden chamber assays demonstrated that hypoxia increased migration of scrambled control (SC) MDA-MB-231 cells across the transwell membrane by 2-fold, whereas migration was significantly decreased by ZMYND8 knockdown (KD) by its shRNA also decreased the colony numbers of MCF-7 cells (Supplemental Figure 3, B–D). These data indicate that ZMYND8 increases breast cancer cell survival and colony formation in vitro.
Figure 4. Loss of ZMYND8 suppresses breast tumor growth and metastasis to the lungs in mice. (A–C) Growth of parental, ZMYND8-KO1 (A) and -KO2 (B) MDA-MB-231, and ZMYND8-KO1 MCF-7 (C) tumors in mice (mean ± SEM, n = 4). ****P < 0.0001 versus parental by 2-way ANOVA with Sidak’s t test. The image of parental and ZMYND8-KO1 tumors harvested at the end time point is shown in the insets. ZMYND8-KO in tumors was confirmed by immunoblot assays (bottom). (D–F) Representative H&E and immunohistochemical staining of CC3 and endomucin in parental or ZMYND8-KO1 MDA-MB-231 tumors (D). Magnified images of the boxed area are shown in the insets. Scale bar, 200 μm. CC3-positive cell numbers (E) and endomucin-positive areas (F) in tumors are quantified (mean ± SEM, n = 4). ***P < 0.001, ****P < 0.0001 versus parental by 2-tailed Student’s t test. (G–J) Lung metastasis in mice bearing parental or ZMYND8-KO1 or -KO2 MDA-MB-231 tumors by H&E staining (G and I) and qPCR assays (H and J, mean ± SEM, n = 4). Magnified images of the boxed area are shown in the insets (G and I). *P < 0.05, **P < 0.01 versus parental by 2-tailed Student’s t test. Scale bar, 200 μm. (K) Lung colonization of parental and ZMYND8-KO MDA-MB-231 cells by qPCR assays (mean ± SEM, n = 11). ****P < 0.0001 versus parental by 1-way ANOVA with Dunnett’s test. (L) Circulating tumor cells in blood from mice bearing parental or ZMYND8-KO1 MDA-MB-231 tumors by qPCR assays (mean ± SEM, n = 4). ***P < 0.001 versus parental by 2-tailed Student’s t test.
ZMYND8-KD1 or -KD2 (Figure 3, C and D). Similarly, the number of MDA-MB-231 cells invaded through Matrigel in a transwell insert was also significantly decreased by ZMYND8-KD1 or -KD2 under 20% or 1% O2 (Figure 3, E and F). ZMYND8-KD did not alter the rate of in vitro cell proliferation (Supplemental Figure 3, E and F), which rules out the possibility that reduced cell migration and invasion by ZMYND8-KD is due to decreased cell proliferation. Together, these data indicate that ZMYND8 increases breast cancer cell motility in vitro.

ZMYND8 promotes breast tumor growth and metastasis to the lungs in mice in a HIF-1– and HIF-2–dependent manner. To determine whether ZMYND8 regulates breast tumor growth in vivo, we performed the orthotopic implantation of parental or ZMYND8-KO1 MDA-MB-231 cells into the mammary fat pad of SCID mice, and found that genetic deletion of ZMYND8 gene robustly blocked spontaneous breast tumor growth in mice (Figure 4A). Immunoblot assays confirmed reduced ZMYND8 protein levels in ZMYND8-KO1 tumors as compared with parental control tumors (Figure 4A). ZMYND8 protein weakly expressed in ZMYND8-KO1 tumors may be the murine form in tumor stromal cells. Similarly, ZMYND8-KO2 or -KD2 also significantly impaired MDA-MB-231 tumor growth in xenograft mice (Figure 4B and Supplemental Figure 4A). We further found that ZMYND8-KO1 or -KD2 significantly inhibited MCF-7 tumor growth in xenograft SCID mice subcutaneously administered slow-release 17β-estradiol pellets (Figure 4C, and Supplemental Figure 4, B and C). Immunohistochemistry assays showed that the number of cleaved caspase 3–positive (CC3-positive) cells was significantly increased in ZMYND8-KO1 and -KD2 MDA-MB-231 tumors and ZMYND8-KO1 MCF-7 tumors as compared with their respective control tumors (Figure 4, D and E, and Supplemental Figure 4, D, E, G, and H), suggesting increased cell death in tumors by ZMYND8-KO or -KD. Conversely, the microvessel density shown by endomucin immunohistochemical staining was significantly decreased in these tumors (Figure 4, D and F, and Supplemental Figure 4, D, F, G, and I), suggesting decreased tumor angiogenesis by ZMYND8-KO or -KD. However, expression of Ki-67, a cell proliferation marker, was not significantly different between SC and ZMYND8-KD2 MDA-MB-231 tumors (Supplemental Figure 4D), consistent with our in vitro cell growth data above (Supplemental Figure 3F). These data indicate that ZMYND8 promotes breast tumor growth in mice by increasing tumor angiogenesis and cell survival.

Figure 5. HIF-1 and HIF-2 are required for ZMYND8-mediated breast tumor growth and metastasis in mice. (A) Growth of Parental+EV, Parental+ZMYND8, HIF-1/2α–DKO+EV, and HIF-1/2α–DKO+ZMYND8 MDA-MB-231 tumors in mice (mean ± SEM, n = 5). **P < 0.01, ****P < 0.0001 by 2-way ANOVA with Tukey’s t test. NS, not significant. (B–D) Representative H&E and immunohistochemical staining of CC3 and endomucin in primary tumors. Magnified images of the boxed area are shown in the insets. Scale bar, 200 μm. CC3-positive cell numbers (C) and endomucin-positive areas (D) in tumors are quantified (mean ± SEM, n = 5). *P < 0.05, ****P < 0.0001 by 1-way ANOVA with Sidak’s t test. (E) Lung metastasis are quantified by qPCR (mean ± SEM, n = 5). *P < 0.05, ****P < 0.0001 by 1-way ANOVA with Dunnett’s test.
tion in the lungs, we injected parental, or ZMYND8-KO1 or -KO2 MDA-MB-231 cells into the tail vein of female SCID mice. Three weeks after injection, mouse lungs were harvested for detection of human genomic DNA by qPCR. We found that less human genomic DNA was present in the lungs of mice injected with ZMYND8-KO1 or -KO2 cells as compared with mice bearing parental MDA-MB-231 cells (Figure 4K). We also studied the effect of ZMYND8-KO on circulating tumor cells in the orthotopic xenograft mouse model, and detected a fair number of human tumor cells in the circulating blood of mice bearing parental MDA-MB-231 tumors (Figure 4L). However, the number of circulating tumor cells was significantly decreased in mice bearing ZMYND8-KO1 tumors (Figure 4L). The reduced number of circulating tumor cells was even found in mice bearing ZMYND8-KO2 tumors (Supplemental Figure 4L) whose volume was comparable to that of control tumors (Figure 4B), indicating that reduction in circulating tumor cells by ZMYND8-KO is not due to its inhibitory effect on tumor growth. Taken together, these data indicate that ZMYND8 increases circulating tumor cell populations to promote their extravasation and colonization, leading to metastasis of breast cancer cells to the lungs.

Next, we studied the effect of ZMYND8 on spontaneous breast cancer metastasis to the lungs. H&E staining detected extensive metastatic burden in the lungs of mice 37 days after MDA-MB-231 cell implantation (Figure 4G). In contrast, no lung metastases were found in mice bearing ZMYND8-KO1 or -KD2 tumors (Figure 4G and Supplemental Figure 4J). Analysis of human genomic DNA by qPCR further confirmed that no human cancer cells were detected in the lungs of these mice (Figure 4H and Supplemental Figure 4K). To rule out the possibility that the loss of metastatic ability in mice bearing ZMYND8-KO1 or -KD2 tumors is due to the small primary tumors, mice orthotopically implanted with ZMYND8-KO2 MDA-MB-231 cells were euthanized 12 days later than control mice when the volume of ZMYND8-KO2 tumors matched that of control MDA-MB-231 tumors at day 40 (Figure 4B). Again, little lung metastasis burden was detected in mice bearing ZMYND8-KO2 tumors as compared with mice bearing control tumors (Figure 4, I and J), even though the tumor volume was comparable in these 2 groups of mice (Figure 4B). These data indicate that ZMYND8 mediates spontaneous metastasis of breast cancer cells to the lungs in mice.

Cancer cell extravasation and colonization at distant organs is critical for metastatic tumor outgrowth. To determine whether ZMYND8 regulates breast cancer cell extravasation and coloniza-
with HIF-1α or HIF-2α. HEK293T cells were cotransfected with V5-tagged ZMYND8 vector and vector encoding FLAG-tagged HIF-1α, FLAG-tagged HIF-2α, or EV, and exposed to 1% O2 for 6 hours. Coimmunoprecipitation (co-IP) assays using anti-FLAG antibody showed that FLAG–HIF-1α or FLAG–HIF-2α, but not EV, pulled down ZMYND8-V5 in HEK293T cells (Supplemental Figure 5A). We also performed a reciprocal co-IP assay using anti–V5 antibody and found that ZMYND8-V5 precipitated FLAG–HIF-1α or FLAG–HIF-2α in hypoxic HEK293T cells (Supplemental Figure 5B). Further, anti-ZMYND8 antibody coprecipitated endogenous ZMYND8, HIF-1α, and HIF-2α in MDA-MB-231 cells exposed to 1% O2 for 6 hours (Figure 6A). These data indicate that ZMYND8 physically interacts with HIF-1α and HIF-2α in human breast cancer cells.

Next, we mapped the ZMYND8 domain binding to HIF-1α. HEK293T cells were transfected with vector encoding full-length (FL) or domain-deleted FLAG-ZMYND8 (Figure 6B), and exposed to 1% O2 for 6 hours. Co-IP assays using anti–HIF-1α antibody showed that FL and PB (APBP, inter (ΔInt), or C3 (ΔC3) fragment-deleted FLAG-ZMYND8 interacted with HIF-1α (Figure 6C). In contrast, deletion of the MYND domain (ΔMYND) or 2 C-terminal fragments (ΔC1 and ΔC2) abolished ZMYND8 binding to HIF-1α in hypoxic HEK293T cells (Figure 6C). These data indicate that the MYND domain of ZMYND8 is required and sufficient for interaction with HIF-1α.

**ZMYND8 activates the global HIF target genes in breast cancer cells.** To determine whether ZMYND8 regulates HIF transcriptional activity, we first performed HIF luciferase reporter assays. HeLa cells were cotransfected with a HIF reporter (containing 2 copies of HREs from the human VEGFA gene upstream of SV40 promoter and Fluc coding sequence), pSV-Renilla, and ZMYND8-V5 vector or EV, and exposed to 20% or 1% O2 for 24 hours. Overexpression of ZMYND8-V5 significantly increased HIF transcriptional activity in HeLa cells, as compared with EV (Supplemental Figure 6A).

We next set out to analyze HIF transcriptome profiling controlled by ZMYND8 in breast cancer cells. To this end, parental, HIF-1/2α–DKO, and ZMYND8-KO1 MDA-MB-231 cells were exposed to 20% or 1% O2 for 24 hours and subjected to RNA sequencing (RNA-seq). RNA-seq analysis identified 1,124 hypoxia-inducible genes in MDA-MB-231 cells, of which about one-third (367, 32.7%) were induced by HIF-1 and HIF-2 and about half (603, 53.6%) were induced by ZMYND8 (Figure 7A). Notably, 230 genes were overlapped between HIF-upregulated and ZMYND8-upregulated hypoxia-inducible genes, which represented 62.7% of HIF target genes (Figure 7A). Those genes were involved in classic HIF-dependent biological functions, including glycolysis, apoptosis, and angiogenesis (Figure 7B). Consistent with the RNA-seq data, RT-qPCR assays confirmed that hypoxia induced transcription of the HIF target genes VEGFA, ANGPTL4, AGR2, AQPI, and LOX in MDA-MB-231 cells, and induction of these genes was significantly attenuated by ZMYND8-KO1 or -KO2 (Figure 7C). However, mRNA transcription of non–HIF target gene RPL13A was not altered by ZMYND8-KO1 or -KO2 in MDA-MB-231 cells (Figure 7C). Similar results were also found in ZMYND8-KD2 SUM159 cells (Supplemental Figure 6, B and C). ZMYND8-KO or -KD did not affect protein levels of HIF-1α and HIF-2α in breast cancer cells (Supplemental Figure 3A and Supplemental Figure 6B), which rules out regulation of HIF-1α and HIF-2α protein stability as the cause of ZMYND8-enhanced HIF transcriptional activity.

We further performed ChIP-seq in hypoxic MDA-MB-231 cells to determine whether ZMYND8 colocalizes with HIF-1α at the HREs. MDA-MB-231 cells were exposed to 1% O2 for 24 hours. ZMYND8 was highly enriched near the transcription start sites, similar to HIF-1α (Figure 8, A and B). Its ChIP-seq peaks

![Figure 7. ZMYND8 activates the global HIF target genes in breast cancer cells.](jci.org)
Figure 8. ZMYND8 globally colocalizes with HIF-1α at the HREs in breast cancer cells. (A and B) Metagene analysis of the genomic distribution of ZMYND8 (A) and HIF-1α (B) in MDA-MB-231 cells exposed to 1% O2 for 24 hours (n = 2). RPKM, reads per kilobase per million mapped reads; TSS, transcription start site; TTS, transcription termination site. (C and D) Venn diagram of the overlapped ChIP-seq peaks (C) and co-occupied genes (D) by ZMYND8, HIF-1α, and H3K14ac (n = 2). (E) Co-occupancy analysis of HIF-1α and ZMYND8 ChIP-seq peaks (n = 2). (F) Motif density analysis of ZMYND8 ChIP-seq peaks (n = 2). HRE is shown in top panel. (G) ZMYND8 and HIF-1β ChIP-qPCR assays in MDA-MB-231 cells exposed to 20% or 1% O2 for 24 hours (mean ± SEM, n = 3). * P < 0.05, ** P < 0.01, *** P < 0.001, by 2-way ANOVA with Sidak’s t test. (H) Genome browser snapshots of HIF-1α, ZMYND8, H3K14ac, and H3 ChIP-seq peaks. rep, replicate.
The mRNA-seq and ChIP-seq data indicate that ZMYND8 colocalizes with HIF-1 and activates its global target genes in breast cancer cells. ZMYND8 is associated with H3K14ac and H4K16ac at the HREs and induces release of paused RNA polymerase II in breast cancer cells. To determine whether ZMYND8 binds to the HREs through modified histones, we studied occupancy of H3K14ac, H3K36me2, H3K36me3, and H4K16ac on the HREs in MDA-MB-231 cells exposed to 20% or 1% O2 for 24 hours. ChIP-seq showed that H3K14ac peaks were markedly overlapped with ZMYND8 peaks and co-occupied at the HREs (Figure 8, C and H). H3K14ac occupancy at the LOX and ANGPTL4 HREs was significantly increased by hypoxia in MDA-MB-231 cells (Figure 9, A and E). Likewise, H4K16ac was strongly enriched on the HREs of LOX and ANGPTL4 as compared with control IgG in nonhypoxic MDA-MB-231 cells, and hypoxia increased its occupancy on both HREs by 2-fold and 1.7-fold, respectively (Figure 9, B and F). In contrast, enrichments

were mainly located at the promoter (29.7%), intergenic (36.5%), and intron (30.9%) regions (Figure 8A). Strikingly, about 85.4% of HIF-1α peaks overlapped with ZMYND8 peaks (Figure 8C), and 92% of HIF-1 target genes were co-occupied by HIF-1α and ZMYND8 (Figure 8D). The metagene analysis confirmed strong colocalization of HIF-1α and ZMYND8 (Figure 8E). The motif analysis of ZMYND8 peaks further revealed marked enrichment of the HRE (Figure 8F). These data indicate genome-wide colocalization of HIF-1α and ZMYND8 at the HREs.

To validate the ChIP-seq data, ChIP-qPCR assays were performed in MDA-MB-231 cells exposed to 20% or 1% O2 for 24 hours. ZMYND8 was highly enriched at the HREs of LOX and ANGPTL4, but weakly enriched on RPL13A, as compared with control IgG in nonhypoxic MDA-MB-231 cells. ZMYND8 occupancy on the LOX and ANGPTL4 HREs was significantly increased 2.6- and 1.8-fold, respectively, by hypoxia (Figure 8, G and H). HIF-1β occupancy was similarly detected on the HREs of LOX and ANGPTL4, but not RPL13A (Figure 8, G and H). Taken together, the mRNA-seq and ChIP-seq data indicate that ZMYND8 colocalizes with HIF-1 and activates its global target genes in breast cancer cells.

ZMYND8 is associated with H3K14ac and H4K16ac at the HREs and induces release of paused RNA polymerase II in breast cancer cells. To determine whether ZMYND8 binds to the HREs through modified histones, we studied occupancy of H3K14ac, H3K36me2, H3K36me3, and H4K16ac on the HREs in MDA-MB-231 cells exposed to 20% or 1% O2 for 24 hours. ChIP-seq showed that H3K14ac peaks were markedly overlapped with ZMYND8 peaks and co-occupied at the HREs (Figure 8, C and H). H3K14ac occupancy at the LOX and ANGPTL4 HREs was significantly increased by hypoxia in MDA-MB-231 cells (Figure 9, A and E). Likewise, H4K16ac was strongly enriched on the HREs of LOX and ANGPTL4 as compared with control IgG in nonhypoxic MDA-MB-231 cells, and hypoxia increased its occupancy on both HREs by 2-fold and 1.7-fold, respectively (Figure 9, B and F). In contrast, enrichments
ment of H3K14ac and H4K16ac on RPL13A was not regulated by hypoxia in MDA-MB-231 cells (Figure 9, I and J). Occupancy of H3K36me2, H3K36me3, histone H3, or histone H4 on LOX, ANGPTL4, and RPL13A was also not altered by hypoxia in MDA-MB-231 cells (Figure 9, C, D, G, H, K, and L). The global levels of H4K16ac, but not H3K14ac, H3K36me2, H3K36me3, histone H3, and histone H4, were increased by hypoxia in MDA-MB-231 cells (Figure 9M). These data indicate that H3K14ac and H4K16ac, but not H3K36me2 and H3K36me3, mediate ZMYND8 binding to the HREs of the HIF target genes in breast cancer cells.

Previous studies have shown that RNA polymerase II is preloaded and paused at the promoter of the HIF target genes and that paused RNA polymerase II release is essential for HIF-mediated transactivation (37). Paused RNA polymerase II is phosphorylated at serine 5 (SSP), whereas serine 2 phosphorylation (S2P) of RNA polymerase II controls release of paused RNA polymerase II (38). To determine whether ZMYND8 regulates paused RNA polymerase II release to promote HIF-mediated transactivation (37). Paused RNA polymerase II is phosphorylated at serine 5 (SSP), whereas serine 2 phosphorylation (S2P) of RNA polymerase II controls release of paused RNA polymerase II (38). To determine whether ZMYND8 regulates paused RNA polymerase II release to promote HIF-mediated transactivation, we established 2 independent Tet-inducible BRD4-FLAG-BRD4 vector or EV. Analysis of FLAG immunoprecipitates of HDAC but independent of hypoxia.

Next, we mapped the ZMYND8-BRD4 binding domains in HEK293T cells by co-IP assays. HEK293T cells were transiently cotransfected with ZMYND8-V5 vector and FLAG-BRD4 vector. Anti–FLAG antibody, but not IgG, precipitated both FLAG-BRD4 and ZMYND8-V5 (Supplemental Figure 7A). Similarly, anti-BRD4 antibody coprecipitated endogenous BRD4 and ZMYND8 in MDA-MB-231 cells, but control IgG failed to do so (Figure 11A). We further performed a reciprocal co-IP assay and found that ZMYND8-V5 pulled down FLAG-BRD4 in HEK293T cells treated with HDAC inhibitor trichostatin A (TSA) (Supplemental Figure 7B). Precipitation of endogenous ZMYND8 by anti–ZMYND8 antibody also pulled down endogenous BRD4 in MDA-MB-231 cells treated with TSA (Figure 11B). Hypoxia did not influence physical interaction of ZMYND8 with BRD4 in MDA-MB-231 cells (Supplemental Figure 7C). These data indicate that ZMYND8 physically interacts with BRD4 in breast cancer cells, and that ZMYND8-BRD4 interaction is enhanced upon inhibition of HDAC but independent of hypoxia.

To determine whether BRD4 regulates HIF-mediated transactivation, we established 2 independent Tet-inducible BRD4-
Figure 11. BRD4 binds to ZMYND8 and is required for ZMYND8-mediated HIF activation in breast cancer cells. (A and B) Co-IP assays of endogenous BRD4 and ZMYND8 in MDA-MB-231 cells (n = 3). (C) Mapping the BRD4 domain binding to ZMYND8. Schematic depiction of FL and domain-deleted BRD4 (top). Co-IP assays of ZMYND8-V5 and FL or truncated FLAG-BRD4 in transfected HEK293T cells (bottom, n = 3). (D) Mapping the ZMYND8 domain binding to BRD4. Co-IP assays of endogenous BRD4 and FL or truncated FLAG-ZMYND8 in HEK293T cells treated with TSA or DMSO (–) for 6 hours (n = 3). (E) RT-qPCR analysis of indicated mRNAs in SC and BRD4-KD1 or -KD2 MDA-MB-231 cells exposed to 20% or 1% O₂ for 24 hours (mean ± SEM, n = 3). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, by 2-way ANOVA with Tukey’s t test. (F) HIF luciferase reporter assays in HeLa cells transfected with indicated plasmids and exposed to 20% or 1% O₂ for 24 hours (mean ± SEM, n = 3). ***P < 0.001, ****P < 0.0001, by 2-way ANOVA with Tukey’s t test. (G) BRD4 ChIP-qPCR assays in parental and ZMYND8-KO2 MDA-MB-231 cells exposed to 20% or 1% O₂ for 24 hours (mean ± SEM, n = 3). **P < 0.01, ***P < 0.001, ****P < 0.0001 by 2-way ANOVA with Sidak’s t test.
Figure 12. Acetylation of ZMYND8 by p300 is necessary for HIF activation and breast tumor progression. (A and B) Acetylation of ZMYND8-V5 (A) and WT or mutant FLAG-ZMYND8 (B) in HEK293T cells treated with TSA or DMSO (-) for 6 hours (n = 3). (C) Co-IP assays of BRD4 and WT or mutant FLAG-ZMYND8 in transfected HEK293T cells (n = 3). (D and E) In vitro acetylation assays of WT or K1007/1034R FLAG-ZMYND8 by purified FLAG-p300, FLAG-PCAF, or FLAG-GCN5 (n = 3). (F) Co-IP assays of endogenous ZMYND8 and p300 in MCF-7 cells (n = 3). (G) Acetylation of endogenous ZMYND8 in SC and p300-KD MCF-7 cells (n = 3). (H) Co-IP assays of endogenous ZMYND8 and BRD4 in SC and p300-KD MCF-7 cells (n = 3). (I) RT-qPCR analysis of indicated mRNAs in ZMYND8-rescued MDA-MB-231 cells exposed to 20% or 1% O2 for 24 hours (mean ± SEM, n = 3). *P < 0.05, **P < 0.01, by 2-way ANOVA with Tukey’s t test. (J–L) Clonogenic assays (J), migration assays (K), and invasion assays (L) in ZMYND8-rescued MDA-MB-231 cells exposed to 20% or 1% O2 for 12 days (J), 16 hours (K), or 24 hours (L) (mean ± SEM, n = 3). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, by 2-way ANOVA with Tukey’s t test. (M–P) Growth of ZMYND8-rescued MDA-MB-231 tumors in mice (M, mean ± SEM, n = 5). Endomucin-positive areas (N) and CC3-positive cell numbers (O) in tumors and lung metastasis (P) were quantified (mean ± SEM, n = 5). **P < 0.01, ***P < 0.001, ****P < 0.0001, by 2-way ANOVA with Tukey’s t test.
KD MDA-MB-231 cell lines (Supplemental Figure 7E). SC and BRD4-KD1 or -KD2 MDA-MB-231 cells were exposed to 20% or 1% O2 for 24 hours in the presence of doxycycline. RT-qPCR assays showed that BRD4-KD1 or -KD2 significantly decreased hypoxia-induced transcription of the HIF target genes VEGFA, LOX, AGR2, AQPI, and ANGPTL4, but not RPL13A, in MDA-MB-231 cells (Figure 11E). This effect was not due to the protein stability of HIF-1α and HIF-2α, as their protein levels were not affected by BRD4-KD1 or -KD2 (Supplemental Figure 7E). These data indicate that BRD4 enhances HIF-mediated transactivation in breast cancer cells.

To determine whether BRD4 is required for ZMYND8-mediated HIF activation, we performed the HIF luciferase reporter assays. HeLa cells were transfected with HIF reporter plasmid, pSV-Renilla, SC or BRD4-KD1 or -KD2 vector, and ZMYND8-V5 vector or EV, and exposed to 20% or 1% O2 for 24 hours in the presence of doxycycline. Consistent with HIF target gene expression (Figure 11E), BRD4-KD1 or -KD2 significantly decreased HIF transcriptional activity in hypoxic HeLa cells (Figure 11F). Moreover, BRD4-KD1 or -KD2 dramatically blocked ZMYND8-induced HIF activation in nonhypoxic and hypoxic HeLa cells (Figure 11F). These data indicate that BRD4 promotes HIF transcriptional activity, and that it is required for ZMYND8-mediated HIF activation.

To determine whether ZMYND8 recruits BRD4 to the HREs of the HIF target genes, we performed ChIP-qPCR assays in parental and ZMYND8-KO2 MDA-MB-231 cells exposed to 20% or 1% O2 for 24 hours, and found that BRD4 occupied on the HREs of LOX and ANGPTL4 genes in nonhypoxic MDA-MB-231 cells, and hypoxia significantly increased its occupancy (Figure 11G, left and middle). ZMYND8-KO2 abolished hypoxia-induced BRD4 enrichment on the HREs of the LOX and ANGPTL4 genes but not the RPL13A gene in MDA-MB-231 cells (Figure 11G). Conversely, we found that BRD4-KD2 did not regulate ZMYND8 binding to the HREs of LOX, ANGPTL4, and RPL13A genes in nonhypoxic and hypoxic MDA-MB-231 cells (Supplemental Figure 7F). These data indicate that ZMYND8 recruits BRD4 to the HREs to induce HIF-mediated transactivation in breast cancer cells.

Previous studies showed that ZMYND8 recruits CHD4, a core helicase in the nucleosome remodeling and deacetylase complex, to the chromatin (33, 41). To determine whether CHD4 is involved in ZMYND8-mediated HIF activation, we performed co-IP assays and found that CHD4 physically interacted with HIF-1α and HIF-2α in MDA-MB-231 cells under hypoxia (Supplemental Figure 8A). However, ZMYND8-KO did not influence CHD4 binding to HIF-1α or HIF-2α in hypoxic MDA-MB-231 cells (Supplemental Figure 8A), suggesting that ZMYND8 does not mediate CHD4 recruitment to the HIF transactivation complex. Further, we found that CHD4 knockdown significantly inhibited expression of the HIF target genes VEGFA, LOX, and AGR2, but not AQPI, in MDA-MB-231 cells under hypoxia (Supplemental Figure 8, B and C), indicating that CHD4 does not fully phenocopy ZMYND8’s effect on HIF activation in breast cancer cells.

ZMYND8 acetylation at lysines 1007 and 1034 by p300 is required for interaction with BRD4 and HIF activation in breast cancer cells. The BRD1/2 of BRD4 specifically recognizes acetylated lysine residues of its interacting protein (40). Our binding domain mapping studies (Figure 11, C and D) suggest that ZMYND8 may interact with BRD4/2 via lysine residues of its interacting protein. Previous studies showed that ZMYND8 recruits CHD4 with HIF-1α and HIF-2α in breast cancer cells. However, the ZMYND8-mediated HIF activation is not further increased in breast cancer cells. These data indicate that ZMYND8 recruits BRD4 to the HREs to induce HIF-mediated transactivation in breast cancer cells.
To identify the lysine acetyltransferase that acetylates ZMYND8, we screened acetyltransferases by in vitro acetylation assays. FLAG-ZMYND8 was expressed in HEK293T cells and purified by anti–FLAG antibody. Purified FLAG-ZMYND8 was then incubated in the presence of acetyl-CoA with the acetyltransferase FLAG-p300, FLAG-PCAF, or FLAG-GCN5 that was expressed and purified from Sf9 cells (42), and analyzed by immunoblot assays with anti-acetyllysine antibody. FLAG-p300 strongly induced acetylation of FLAG-ZMYND8 in vitro (Figure 12D). In contrast, no detectable acetylation of FLAG-ZMYND8 was found after incubation with FLAG-PCAF or FLAG-GCN5 (Figure 12D). Moreover, K1007/1034R abolished FLAG-p300–induced acetylation of FLAG-ZMYND8 (Figure 12E). We next studied whether p300 catalyzes ZMYND8 acetylation in vivo. Co-IP assays showed that endogenous p300 interacted with endogenous ZMYND8 in MCF-7 cells (Figure 12F), and their interaction was not regulated by hypoxia (Supplemental Figure 9F). p300-KD by 2 independent shRNAs completely abolished lysine acetylation of endogenous ZMYND8 in MCF-7 cells (Figure 12G). Pharmacological inhibition of p300 by its inhibitor L002 also reduced acetylation of ZMYND8 in MCF-7 cells (Supplemental Figure 9G). Further, we found that p300-KD blocked ZMYND8-BRD4 interaction in MCF-7 cells (Figure 12H). Together, these findings indicate that p300 is a lysine acetyltransferase for ZMYND8.

To determine whether ZMYND8 acetylation is required for HIF-mediated transactivation, we generated the rescued cell lines by transducing ZMYND8-KD2 MDA-MB-231 cells with lentivirus encoding WT or K1007/1034R FLAG-ZMYND8 or EV. WT or K1007/1034R FLAG-ZMYND8 protein was expressed at comparable levels to endogenous ZMYND8 protein in SC MDA-MB-231 cells (Figure 12A). These rescued cell lines and SC MDA-MB-231 cells were exposed to 20% or 1% O2 for 24 hours. RT-qPCR (Supplemental Figure 10A). These rescued cell lines and SC MDA-MB-231 cells were exposed to 20% or 1% O2 for 24 hours. RT-qPCR assays showed that the reduced transcription of LOX mRNA in ZMYND8-KD2 MDA-MB-231 cells was restored by overexpression of WT FLAG-ZMYND8 to that in SC MDA-MB-231 cells under hypoxia (Figure 12I). In contrast, overexpression of FLAG-ZMYND8 (K1007/1034R) failed to restore reduced transcription of LOX mRNA in ZMYND8-KD2 MDA-MB-231 cells (Figure 12I). Neither WT FLAG-ZMYND8 nor FLAG-ZMYND8 (K1007/1034R) influenced the RPL13A mRNA levels in nonhypoxic and hypoxic MDA-MB-231 cells (Figure 12I). These data indicate that acetylation of lysines 1007 and 1034 is necessary for ZMYND8-mediated HIF activation in breast cancer cells.

Acetylated ZMYND8 mediates breast tumor growth and lung metastasis. Next, we studied whether ZMYND8 acetylation mediates the oncogenic effects in vitro and in vivo. Overexpression of WT FLAG-ZMYND8, but not FLAG-ZMYND8 (K1007/1034R), restored the colony formation, migration, and invasion abilities of ZMYND8-KD2 MDA-MB-231 cells (Figure 12, J–L, and Supplemental Figure 10A). Reduced MDA-MB-231 tumor growth conferred by ZMYND8-KD2 was partially rescued by WT but not K1007/1034R FLAG-ZMYND8 in xenograft mice (Figure 12M, and Supplemental Figure 10D). Notably, overexpression of WT but not K1007/1034R FLAG-ZMYND8 completely restored microvessel density and CC3-positive cell numbers in ZMYND8-KD2 tumors (Figure 12, N and O, and Supplemental Figure 10E). Further, lung metastasis burden shown by H&E staining and qPCR assays was also partially but significantly rescued by overexpression of WT, but not K1007/1034R, FLAG-ZMYND8 in xenograft mice (Figure 12P and Supplemental Figure 10E). These data indicate that ZMYND8 acetylation is required for breast cancer progression and metastasis to the lungs.

Discussion
In the present study, we delineate an epigenetic mechanism by which the epigenetic reader ZMYND8 promotes HIF-mediated transactivation and breast cancer progression and metastasis (Figure 13). ZMYND8 physically interacts with HIF-1α and HIF-2α and also binds to the HREs possibly through H13K14ac and H4K16ac, where ZMYND8 is acetylated at lysines 1007 and 1034 by the HIF coactivator p300. Acetylated ZMYND8 recognizes the bromodomains of BRD4 and recruits the latter to the HREs, leading to increased RNA polymerase II phosphorylation at serine 2 and subsequent transcriptional elongation of the HIF target genes in breast cancer cells. Consequently, the ZMYND8/p300/BRD4/HIF axis increases angiogenesis and cell motility and decreases cancer cell death to promote breast tumor progression and metastasis. ZMYND8 itself is a HIF-1 and HIF-2 target gene, and thus provides a positive feedback mechanism that amplifies HIF-mediated transactivation and subsequent breast cancer progression and metastasis (Figure 13).

Several epigenetic regulators have been shown to coactivate a subset of HIF target genes in cancer cells (7, 26, 27, 43). Our RNA-seq and ChiP-seq data indicate that ZMYND8 co-occupies more than 85% of the genome-wide HIF-1 binding sites and activates more than 60% of the global HIF-dependent coding genes in breast cancer cells, indicating that ZMYND8 also regulates HIF-dependent long noncoding RNAs as they are the direct HIF targets under hypoxia (37). These findings reveal that ZMYND8 is a primary HIF regulator that plays a dominant role in HIF activation in breast cancer. In line with its dominant role in transcription of the HIF target genes in breast cancer cells, ZMYND8 phenocopies HIF’s oncogenic effects in breast cancer progression and metastasis. Strikingly, ZMYND8 acetylation at lysines 1007 and 1034 by p300 is the key to switch on HIF activation and breast cancer progression and metastasis. Our data indicate that this novel posttranslational modification mediates assembly of the HIF transcription machinery to promote HIF activation in breast cancer cells. ZMYND8 acetylation may be reversible in breast cancer cells, as treatment of an HDAC inhibitor TSA dramatically increases ZMYND8 acetylation. HDAC1 and HDAC2 have been shown to interact with ZMYND8 (33), and are also known to regulate HIF-1 transcriptional activity (26). Further studies are needed to determine which HDAC deacetylates ZMYND8.

Recent studies suggest that RNA polymerase II pausing and release finely switch HIF activation on and off (37, 43). The HIF transcription machinery, including RNA polymerase II, is preassembled and preloaded at the promoter of the target genes under nonhypoxic conditions, and release of paused RNA polymerase II triggers gene elongation once HIF binds to the HREs under hypoxia (37). We found that BRD4 is recruited by ZMYND8 to the HREs of the HIF target genes. Their interaction is constitutive and not regulated by O2 tension. BRD4 is known to interact with positive
transcription elongation factor b (P-TEFb) and regulates P-TEFb-induced phosphorylation of RNA polymerase II at serine 2, thereby promoting release of paused RNA polymerase II at the promoter (39). Thus, the ZMYND8/BRD4 axis well supports the current model of HIF activation and represents an important molecular mechanism underlying release of paused RNA polymerase II and elongation of the HIF target genes.

Apart from BRD4, P-TEFb–mediated release of paused RNA polymerase II is also regulated by the super elongation complex (SEC) via a distinct mechanism (44). A previous report demonstrated a role of the SEC in release of paused RNA polymerase II at the promoter of a subset of HIF-1 target genes in HCT116 cells (43). CDK8 controls the recruitment of AFF4, a key component of the SEC, to the HIF-1 target gene ANKRD37, but has no effect on BRD4 recruitment. Notably, ZMYND8-controlled HIF target genes such as AQPI, AGR2, and LOX are not regulated by the CDK8/AFF4 axis (43). Therefore, ZMYND8/BRD4 and CDK8/AFF4 selectively regulate P-TEFb–induced release of paused RNA polymerase II on their respective HIF target genes and subsequent gene elongation.

Our extensive functional studies in multiple ER+ and TNBC cell lines in vitro and breast cancer mouse models indicate that ZMYND8 is an oncoprotein in breast cancer. This concept may be extended to prostate cancer because a previous study showed that ZMYND8 increases VEGFA expression and angiogenesis in prostate DU145 xenograft tumors from zebrafish (45). Previous genetic studies identified ZMYND8 fusion proteins in patients with acute erythroid leukemia and breast cancer and they may be pathogenic for the diseases (46, 47), also supporting an oncogenic role of ZMYND8 in human cancers. However, recent studies showed that ZMYND8 suppresses growth of ZR-75-30 or DU145 xenograft tumors by inducing gene silencing (48, 49). It is unclear whether these discrepancies are due to the experimental context or cell types. Nevertheless, we showed that DKO of HIF-1α and HIF-2α abolished ZMYND8-mediated breast cancer progression and metastasis in mice, suggesting that ZMYND8’s oncogenic functions in breast cancer require HIF-1 and HIF-2. ZMYND8 is known to bind to and enhance ER α transcriptional activity in MCF-7 cells (50), and also regulates the transcription of all-trans retinoic acid–dependent genes in SH-SYSY cells (34). Whether or not these HIF-independent ZMYND8 functions contribute to breast tumorigenesis needs to be investigated.

In conclusion, our studies in human breast cancer cells, breast cancer mouse models, and human breast cancer patients provide a strong rationale for ZMYND8 as a potential biomarker and therapeutic target for the diagnosis and treatment of breast cancer.

Methods

Cell culture and transfection. MDA-MB-231 (gift from Rolf Brekken, UT Southwestern, Dallas, Texas, USA), MDA-MB-468 (ATCC), HEK-293FT (Invitrogen), HeLa, H5K293T, MCF-7, HCC1954, T47D, and SUM159 (gifts from Cheng-Ming Chiang, UT Southwestern, Dallas, Texas, USA) cells were cultured in DMEM, RPMI1640, or DMEM/SEC, to the HIF-1 target gene ANKRD37, and exposed to 20% or 1% O2, or cell types. Nevertheless, we showed that DKO of HIF-1 and HIF-2. ZMYND8...
ing to the formula: volume = 0.52 × length × height × width. Lungs were perfused with PBS and analyzed by H&E staining and qPCR assays with primers for human HK2 gene and mouse and human 18S rRNA.

For the tail vein injection model, 1 × 10⁶ cells in 100 μl PBS were injected into the tail vein of female SCID mice. Three weeks later, lungs were perfused with PBS and subjected to qPCR assays with primers for human HK2 gene and mouse and human 18S 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), and quantified by qPCR assays with primers for the human HK2 gene and mouse and human 18S rRNA. MDA-MB-231 cells were mixed with blood from tumor-free SCID mice to generate a standard curve. The number of circulating tumor cells in mouse blood was calculated according to the standard curve.

Immunohistochemistry assays. Immunohistochemistry assays were performed by the Dako Autostainer Link 48 system. Briefly, the slides were baked, deparaffinized, and hydrated, followed by antigen retrieval in a Dako PT Link. The tissues were incubated with a peroxidase block and then a following primary antibody: 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).

Human breast tumor studies. Human ER+ breast tumor and TNBC tissues and a TNBC TMA were obtained from surgical breast cancer patients from the UT Southwestern Tissue Resource, and analyzed by immunohistochemistry assays. Each staining was scored by Yan Chen and Yan Peng using 4 grades (range 0–3) according to the percentage of immunopositive cells and immunostaining intensity.

Statistics. Statistical analysis was performed by 2-tailed Student’s t test between 2 groups, and 1-way or 2-way ANOVA with multiple testing correction within multiple groups. Quantification of ZMYND8 protein levels between normal breast tissues and human TNBC tissues was determined by y² test. Kaplan-Meier survival curve was analyzed by log-rank test. RNA-seq and ChIP-seq were repeated twice, and other experiments were repeated at least 3 times. Data were expressed as mean ± SEM. P < 0.05 was considered significant.

Accession number. The RNA-seq and ChIP-seq data were deposited at the GEO database with accession number GSE108833.

Study approval. Animal experiments were approved by the Animal Care and Use Committee at UT Southwestern Medical Center. The de-identified human ER+ breast tumor, TNBC, and adjacent normal breast tissues were used and the study was approved by the institutional review board at UT Southwestern Medical Center with informed consent.

Author contributions
WL and YW conceived the study, analyzed the data, and wrote the paper; YC performed most experiments, analyzed the data, and wrote the paper; BZ generated mutant ZMYND8 plasmids; LB generated the HIF reporter plasmid, HIF-1/2α sgRNA plasmids, and HIF-1/2α–KO cell lines; LJ assisted in animal studies; MY performed tail vein injection experiments; YP provided and analyzed TMA; AK and CX analyzed RNA-seq and ChIP-seq data; CW prepared cell lysates; JW performed mouse breeding and lentivirus production; XZ generated p300-KD cells.

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