Supplemental Data

Western Blotting

For western blotting, samples were mixed with equal volume of 2 X sample buffer (1M TRIS/HCl, 10% SDS, 0.1% bromophenol-blue, 10% b-mercaptoethanol, 10% glycerol), and heated for 10 minutes at 95°C. Proteins were resolved on SDS-polyacrylamide gels (SDS-PAGE) and transferred onto PVDF membrane (Immobilon-P, Millipore). After being incubated with the primary antibody, horseradish peroxidase-(HRP) conjugated secondary antibody (Dako, Ely, UK) at 1: 5000 was used for 1 hour incubation. The signals were detected by chemiluminescence (ECL, Amersham).

Cell Viability and Colony Formation Assays

A consecutive siRNA transfection was performed to maximally deplete the expression of target genes. After 24 hours of the second transfection, cells were split into a 96 well plate for cell viability assay or into a 10 cm dish for colony formation assay. Cells were allowed for 24 hour incubation in normal conditions and then incubated in hypoxia and harvest at different time points. For cell viability assay, CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) (Promega) or CellTiter Glo (Promega) was used according the manufacture's instructions. For the colony formation assay, cells were incubated in normoxia or hypoxia (1% O_2) for 6-12 days. The medium was removed and colonies washed twice with PBS, fixed in ice cold methanol for 20 min at -20^{o}C, and then incubated with 0.5% crystal violet solution for 30 minutes after aspiration of methanol. The plates were subsequently washed with distilled water and allowed to air dry. Stained colonies were scored and counted. For cell number counting, BECKMAN COULTER Z Series system was used to measure cell numbers.

qRT-PCR

Quantitative RT-PCR was performed as previously described (1). Detailed methods and primers were described in supplemental data. Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen). cDNA was synthesized by reverse transcription using the High Capacity cDNA Archive Kit (Applied Biosystems,

Warrington, UK) according to the manufacturer's instruction. Real-time quantitative PCR (qPCR) reactions were performed in triplicate using the Corbett Research Rotor Gene RG-3000 (Sydney, Australia). Each reaction contained 5 μl of cDNA, 12.5 μl of TaqMan PCR Master Mix (Abgene, Epsom, UK), 0.25 μl of probe, 1μl of forward and reverse primer and 5.25 μl of H_2O. Conditions for the PCR reaction were 2min at 50°C, 10min at 95°C and then 40 cycles, each consisting of 15 s at 95°C, and 1min at 60°C. Information for probes can be requested.

Patients and Tissue Samples

Formalin-fixed paraffin-embedded tissue blocks and corresponding pathology reports were obtained for 239 primary breast cancers (John Radcliffe Hospital, Oxford, UK). Tissue microarrays were assembled as described previously (Ref. Bubendorf et al. J Pathol 2001;195:72-9.). An additional 10 whole sections of primary breast cancer and 10 whole sections of glioblastomas were also included in this study. Ethical approval was obtained for the use of all human tissue.

The primary breast cancer series comprised surgical resection specimens from 188 ductal, 28 lobular, 15 mixed ductal/lobular and 15 other types of breast cancers (definitive surgery was performed from 1989 to 1998 at the John Radcliffe Hospital, Oxford, UK). The mean follow-up time was 9.0 years (range, 65 days to 16.3 years). Post-operatively 191 patients (85%) received radiotherapy, 52 (23%) patients received chemotherapy and 163 (72%) patients received hormonal therapy. The mean age at diagnosis was 57 years (range, 26 to 90 years) and all patients were female. Treatment data were available for 236 patients. Fifty-three patients (23%) received chemotherapy, 199 patients (84%) received radiotherapy and 164 patients (70%) received hormonal therapy. Relpase-free survival data were available for 236 patients: 127 (54%) patients were censored and 109 (46%) experienced events during the follow-up period. Overall survival data were available for 235 patients: 131 (56%) patients were censored and 104 (44%) died during the follow-up period.

Statistics

The χ^{2-}test was used to evaluate associations between categorical variables. The Benjamini and Hochberg false discovery rate controlling procedure was used to eliminate spurious statistical associations as a result of multiple testing (Ref. Benjamini Y, Hochberg Y: Controlling the false discovery rate: a practical and powerful approach

to multiple testing. J R Statist Soc B. 1995;57:289-300). Median survival times within each subgroup were estimated from Kaplan-Meier curves. Patients were censored in survival analyses according to the date last seen by a doctor. The log-rank (Mantel-Cox) test was used to assess the significance of univariate survival analyses. For multivariate survival models, a Cox regression analysis was used to identify independent prognostic factors. All statistical analyses were carried out using SPSS Statistics (version 16.0; SPSS, Chicago, IL). Two-sided p values <0.05 were considered statistically significant.

Supplemental Figure Legends

Figure S1. Molecular concept analysis of interaction between HIF-1α and ERα pathways

A, We used the molecular concept map (www.oncomine.com) to analyze the connections of ERα-positivity signature (a set of genes that are highly expressed in ER-positive breast cancer patients) to those significantly linked concepts. To simplify our findings, here we used Venn diagram to show the overlap of the ERα signature from Minn et al (2) studies consisting of the top 1% of genes most overexpressed in ER-positive breast cancers and the one that was treated with hypoxic mimetics CoCl2 in MCF7 cells from Lamb et al (www.cmap.com)(3). *JMJD2B* is one of the 56 overlapped genes that were regulated by ERα and hypoxia/HIF-1α.

B, Gene Set Enrichment Analysis (GSEA) (4) was used to analyze the enrichment of ERα genes in a HIF-1α-regulated gene set. We used microarray gene expression data from HIF-1α knocking down in MCF7 cells (5) to search the gene signature database in GSEA and found that HIF-1a target gene is significantly associated with ERα-positivity gene signature (6).

C, The GSEA enrichment plot is shown. $p < 0.001$.

D, JMJD2B expression data derived from our microarray analysis of breast cancer patients were re-analyzed by t-test in Prism program.

Figure S2. ER-dependent regulation of JMJD2B in MCF7 cells

A, After 3 days hormonal starvation, MCF7 cells were treated with 10 nM of E2 for 16 hours. Quantitative RT-PCR was performed to assess the expression of the indicated genes.

B, Immunoblot was performed to assess the expression of the JMJD2B from treatment of (*A*).

C, MCF7 cells were treated with 1 μM of ICI182780 for 16 hours. Quantitative RT-PCR was performed to assess the expression of the indicated genes.

D, Immunoblot was used to assess JMJD2B expression from treatment of (*C*).

(**P<0.01)

Figure S3. ER-dependent regulation of JMJD2B

A. T47D cells were treated with 1 μM of ICI182780 for 16 hours. Immunoblot was used to assess JMJD2B expression.

B. Bioinformatics analysis reveals that JMJD2B gene contains an estrogen response element (ERE) in the first intron.

C. Quantitative ChIP-PCR was used to show ERa binding on ERE of JMJD2B gene.

Figure S4. HIF-1α-dependent regulation of JMJD2B in ERα-positive breast cancer cells

A, MCF7 and T47D cells were treated in hypoxia (1% O_2) for 16 hours. JMJD2B mRNA expression was assessed by quantitative RT-PCR.

B, Immunoblot was used to assess hypoxic induction JMJD2B in T47D cells.

C, T47D cells were transfected with siRNA oligonucleotides against HIF-1α and HIF-2α, JMJD2B mRNA expression was assessed by quantitative RT-PCR.

D, MCF7 cells were transfected with siRNA olionucleotidess against HIF-1α and HIF-2αJMJD2B mRNA expression was assessed by quantitative RT-PCR.

E, Immunoblot was used to assess the JMJD2B expression after knocking down HIFα in hypoxic MCF7 cells.

F, ChIP-PCR was used to show the binding of HIF-1α to JMJD2B promoter.

Figure S5. Cell viability assay after JMJD2B depletion

After depletion of JMJD2B, the MCF7 cell viability over a 3-day time course was measured by CellTiter-Glo. The luminescent signal that indicates the amount of ATP is directly proportional to the number of cells present in culture. Error bars show variation of the triplicates. (**P<0.01)

Figure S6. Immunoblot analysis of cell cycle markers after depletion of JMJD2B.

After 48 hour depletion of JMJD2B with two different siRNAs, the whole cell lysates were assessed with indicated antibodies by western blotting.

Table S1: Association of JMJD2B score with clinical and pathological variables in breast cancer.						
Variable	**JMJD2B Nuclear**		**p value**	**JMJD2B Cytoplasmic**		**p value**
Score	**Negative**	**Positive**		**Negative**	**Positive**	
n	116	123		133	106	
Age (years)						
<50	38	29	0.18	41	26	0.39
≥50	78	92		92	78	
Histology						
Ductal	93	95		104	84	
Lobular	15	13	0.84*	18	10	0.49*
Mixed	6	9	0.66*	8	7	0.92*
Other	2	4	0.72*	3	3	0.86*
Tumour size						
≤2 cm	43	55	0.22	63	35	0.046†
>2 cm	73	65		70	69	
Nodal status						
Negative	62	71	0.49	74	59	1
Positive	51	47		55	43	
Grade						
1	7	15	0.02†	15	7	0.04†
2	38	58		58	38	
3	46	33		35	44	
Radiotherapy						
No	18	19	0.92	22	15	0.82
Yes	98	101		111	88	
Chemotherapy						
No	81	101	0.01†	99	83	0.34
Yes	35	19		34	20	
Hormonal Therapy						
No	37	33	0.55	45	25	0.15
Yes	79	87		88	78	
ER						
Negative	43	17	**<0.001**	32	28	0.81
Positive	68	97		93	72	
Her2						
Negative	84	110	0.03†	113	81	1
Positive	16	7		13	10	
CA9						
Negative	56	51	0.92	72	35	0.71
Positive	28	26		34	20	

*versus ductal carcinoma. †These p values were not deemed significant following correction for multiple testing by the false discovery rate controlling procedure with a cut off = 0.05. Abbreviations: ER, estrogen receptor.

Table S2. Multivariate relapse-free survival analysis in breast cancer (n=171).				
Variable	Hazard Ratio	95% Confidence Intervals	Association with Longer Survival	p value
Tumor size	1.08	0.93 – 1.25		0.34
Nodal Involvement	1.95	1.21 – 3.16	Negative	**0.006**
Grade	1.51	1.02 – 2.21	Low	**0.037**
Estrogen Receptor	0.51	0.31 – 0.83	Positive	**0.006**
HER2	1.81	0.97 – 3.38		0.06
JMJD2B Nuclear	1.46	0.89 – 2.40		0.13
JMJD2B Cytoplasmic	0.98	0.61 – 1.60		0.95

Table S3. Multivariate overall survival analysis in breast cancer (n=217).				
Variable	Hazard Ratio	95% Confidence Intervals	Association with Longer Survival	p value
Age	1.03	1.00 – 1.06	Young	**0.037**
Menopause	1.57	0.73 – 3.37		0.24
Tumor Size	1.17	1.02 – 1.33	Smaller	**0.027**
Nodal Involvement	1.64	1.04 – 2.57	Negative	**0.033**
Estrogen Receptor	0.57	0.36 – 0.90	Positive	**0.016**
Radiotherapy	0.75	0.41 – 1.37		0.35
JMJD2B Nuclear	1.06	0.68 – 1.65		0.78
JMJD2B Cytoplasmic	1.05	0.68 – 1.63		0.81

Table S4 Antibodies

Protein	Ref.	Provider	WB	IP	ChIP	IF	IHC
ERa (HC-20)	SC-543	Santa Cruz	1: 500	1:100			
ERa (MC-20)	SC-542	Santa Cruz		1:100	1:50		
HIF-1a	610958	BD Transduction Laboratories	1:1000				
HIF-1a	NB100-134	Novus Biologicals			1:400		
HIF-2a	NB100-122	Novus Biologicals	1:500				
HA (3F10)	11867423001	Roche				1:1000	
JMJD2B	A301-478A	Bethyl Laboratories	1:2000		2	1:200	1:500
JMJD2B	A301-477A	Bethyl Laboratories				1:500	
H3K9me3	Ab8898	Abcam			1:400		
H3K9me3	07-523	UPSTATE	1:1000			1:1000	

H3K9me2	07-441	UPSTATE	1:1000			1:1000	
H3K9me1	07-450	UPSTATE	1:1000			1:1000	
H3	06-775	UPSTATE	1:500				
CA9		In-house	1:2000				
Cyclin B1	554179	BD Pharmingen	1;1000				
Cyclin D1	CC12	Calbiochem	1:200				
Wee1	4936	Cell Signaling	1;1000				
pS10H3	9706	Cell Signaling	1:1000			1:200	
b-actin	A-1978	Sigma	1:5000				
HDAC1	SC-6298	Santa Cruz					
GATA3	SC-269	Santa Cruz	1:500				

Table S5 ChIP PCR primers

GENE	FORWARD	REVERSE
CCNA1	5-CCTAAATCCCCACATCTCCTCT-3	5-CCGCTAACAACCCCCTCTAAC-3
CCND1	5-CTTTCCATTCAGAGGTGTGTTTCT-3	5-GCGACTGCATCTTCTTTCATTT-3
WEE1	5-AACATGGAGAAGGAAGCATATACAC-3	5-GCCTTTAGAGTTCAAGCGATTCT-3
PGR	5-AGGCTTACCCCGATTAGTGAC-3	5-GCCAAATGTCTTCCTCTGTGTT-3
PMP22	5-GTCCCCGCTATTACTGTCTGC-3	5-GCTGATTTCCTTGGCTGCTC-3
SOCS2	5-CAGAGGTCCAAAGAGGGTAAGTG-3	5-GCAGATGATAAGCAGATGAGTCG-3
HRE	5-TCCTGTGTTTCAGCCAATGA-3	5-CACCTTTCTCTCCCCTCTCC-3
ERE	5-TCACAGCTGGAATGGTGGT-3	5-CACCTCAGGCCCTCAACA-3

Table S6 qRT-PCR primers

GENE	PROBE	FORWARD	REVERSE
CCNA1	78	5-AATGGGCAGTACAGGAGGAC-3	5-CCACAGTCAGGGAGTGCTTT-3
WEE1	2	5-GGCGATAGTCGTTTTCTTGC-3	5-CGCAAAAATATCTGCTTTTGGT-3
SOCS2	65	5-CAGTCACCAAGCCCCTTC-3	5-AAGGGATGGGGCTCTTTCT-3
PMP22	9	5-CCTCAGGAAATGTCCACCAC-3	5-TGGTGGCCTGGACAGACT-3
PGR	89	5-GGCATGGTCCTTGGAGGT-3	5-CACTGGCTGTGGGAGAGC-3
JMJD2B	33	5-TCACCAGCCACATCTACCAG-3	5-GATGTCCCCACGCTTCAC-3

ACTIN	64	5-CCAACCGCGAGAAGATGA-3	5-CCAGAGGCGTACAGGGATAG-3
TGFBR2	7	5-GGGAAATGACATCTCGCTGTA-3	5-CACCTTGGAACCAAATGGAG-3
CCND1	67	GAAGATCGTCGCCACCTG	GACCTCCTCCTCGCACTTCT
MYC	75	TTTTTCGGGTAGTGGAAAACC	TTCCTGTTGGTGAAGCTAACG
pS2	35	5-CCCCTGGTGCTTCTATCCTAA-3	5-GATCCCTGCAGAAGTGTCTAAAA-3

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