PNAS

Supporting Information for

CBX5 loss drives EGFR inhibitor resistance and results in therapeutically actionable vulnerabilities in lung cancer

Suresh Bugide, Yvonne J. K. Edwards, Romi Gupta, Michael R. Green, and Narendra Wajapeyee

Correspondence to:

Narendra Wajapeyee, **Email:** nwajapey@uab.edu

Michael R. Green, **Email:** Michael.Green@umassmed.edu

This PDF file includes:

SI Materials and Methods
Figures S1 to S12
Tables S1 to S3
Legends for Datasets S1 to S3
SI References

Other supporting materials for this manuscript include the following:

Datasets S1 to S3

SI Materials and Methods

MTT assays. For MTT assays, 5×10^3 LUAD cells were plated in triplicate in a volume of 100 µl in 96-well plates. After 24 h, erlotinib was administered at various concentrations, as indicated in the figures and figure legends. After 5 days of treatment, cell viability was evaluated by adding 20 µl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution, dissolved in 1X PBS, to each well of the 96-well plate and incubating for 1 h at 37ºC. MTT solution was then gently removed, and 100 µl of DMSO was added to each well. After mixing well by pipetting, absorbance was measured at 590 and 630 nm, using the BioTek Synergy MX Multi Format Microplate Reader (Winooski, VT, USA). The average measurement at 630 nm was subtracted from the average at 590 nm, and the relative growth rate was plotted with respect to vehicle control-treated cells. *P*-values were calculated using GraphPad Prism software.

Immunoblot analysis. Cells were lysed using Pierce IP Lysis Buffer (Thermo Fisher Scientific), containing Roche Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland) and Phosphatase Inhibitor Cocktail (Sigma-Aldrich). Cell lysates were centrifuged at 10,000 rpm for 10 min, and protein concentrations were estimated using the Bradford Assay (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose members. These were blocked with 5% non-fat milk and incubated with primary antibodies at 4°C overnight. Membranes were then washed three times for 15 min each with 1X Tris-buffered saline plus Tween 20 (TBS-T) and incubated with a secondary antibody for 2 h at room temperature. Protein signals were detected using SuperSignal West Pico Chemiluminescent Substrate Kit (Thermo Fisher Scientific) or SuperSignal West Femto Chemiluminescent Substrate Kit. All antibodies and the dilutions used for this study are listed in **SI Appendix, Table S3**. Quantification of the western blot bands in Figure 1C was carried out by optical densitometry and analyzed using the ImageJ software. The expression of CBX5 protein analyzed was normalized with ACTINB. Normalized band intensity values were converted into fold changes and plotted as bar graphs.

RNA isolation and qRT-PCR analysis. Total RNA for mRNA expression analyses was extracted with TRIzol Reagent (Invitrogen, Thermo Fisher Scientific) and purified using RNeasy mini columns (QIAGEN, Hilden, Germany). Total mRNA was reverse transcribed into cDNA using the M-MuLV First-Strand cDNA Synthesis Kit (New England Biolabs, Ipswich, MA, USA), according to the manufacturer's instructions, and qPCR reactions were performed using the Power SYBR® Green Kit (Applied Biosystems, Thermo Fisher Scientific), according to the manufacturer's instructions. Expression data were normalized to *ACTINB* expression as an internal control. Primer sequences used in this study are listed in **SI Appendix, Table S3**.

CUT&RUN assays. CUT&RUN assays were performed in HCC827 or PC9 cells using the CUT&RUN Assay Kit (Cat #86652; Cell Signaling Technology, Danvers, MA, USA), according to the manufacturer's instructions. Briefly, 2×10^5 cells were harvested, washed, bound to activated Concanavalin A-coated Beads, and permeabilized. Bead–cell complexes were incubated with the respective antibody overnight at 4°C. Cells were then washed three times, resuspended in 100 µl pAG/MNase (Protein A and Protein G fused to micrococcal nuclease), and incubated for 1 h at room temperature. Samples were washed three times with Digitonin Buffer plus Protease Inhibitor Cocktail, resuspended in 150 µl Digitonin Buffer, and incubated for 5 min on ice. MNase was activated by adding calcium chloride, and samples were incubated at 4ºC for 30 min. The reaction was stopped by adding 150 µl of 1X Stop Buffer, and samples were incubated at 37ºC for 10 min to release the DNA fragments. The DNA was extracted using the included DNA Purification Spin Columns, and qPCR was performed with *E2F1* or *BIRC5* promoter-specific primers. Relative fold-change was calculated as the ratio of immunoprecipitated DNA to IgG-precipitated DNA. Primer sequences and antibodies used for CUT&RUN assays are listed in **SI Appendix, Table S3**.

RNA-seq. RNA-seq was performed for nine samples representing three biological replicates of the following three groups: *CBX5* shRNA1 (G7), *CBX5* shRNA1 (G1), and NS shRNA. Single-end 76-bp reads were sequenced using the Illumina HiSeq 2500 sequencing instrument. Pre-alignment

quality assessments of the raw fastq sequences were performed using FastQC, v0.11.9. The number of reads for the nine samples ranged from 20 M to 34 M. The raw fastq sequences were aligned to the human hg38 reference genome (GenBank assembly accession: GCA_000001405.28), and alignments were performed using STAR, v2.7.1a (1), with default parameters. Post-alignment quality assessments were performed with RSeQC, v2.6.3 (2), and MultiQC, v1.4 (3). SAMtools, v0.0.19 (4), and IGV, v2.6.2 (5) were used for indexing and viewing the alignments, respectively. Gene expression was quantified at the gene level using the htseq-count function in HTSeq, v.0.12.3, with default parameters, and the University of California, Santa Cruz (UCSC) gene annotations for the human genome (Hg38). Genes for which there were less than three samples with normalized counts greater than or equal to four were filtered out. Differentially expressed genes (DEGs) were identified using DESeq2, v1.28 (6), with default parameters (Love et al 2014). Genes were considered differentially expressed if *P*adj-value <0.05 and absolute log2 fold-change >1. Normalized gene expression data were used for all downstream analyses. Venn diagrams were generated using InteractiVenn (7), and heatmaps were generated with the ComplexHeatmap package, v.1.12.0 in Bioconducter. The volcano plot was produced with the R Bioconductor package EnhancedVolcano, and DEGs were analyzed by the Ingenuity Pathway Analysis (IPA) tool (QIAGEN). RNA-sequencing data have been deposited in NCBI's Gene Expression Ominibus (GEO accession number GSE114563).

Annexin V assays. The binding of annexin V to cells was measured using the PE-Annexin V Apoptosis Detection Kit I (Cat #559763; Pharmingen™, BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's protocol. In brief, PC9 and HCC827 cells expressing either an NS or *CBX5* shRNA, or PC9 *BIRC5/CBX5* double-knockdown cells, were treated with 50 nM erlotinib for 24 h or HCC827-R1, HCC827-R2, HCC2935-R1, HCC2935-R2 (20 nM), PC9-R1 and PC9-R2 (50 nM) treated with BIRC5 inhibitor YM155. Post-treatment, cells were collected, washed two times with 1X PBS, and resuspended in 1x Binding Buffer. Cells were then stained with 5 µl PE-Annexin V and 5 µl 7-amino-actinomycin D (7-AAD) and incubated for 15 min in the dark. Flow cytometry analysis was performed using a BD LSRFortessa Flow Cytometer (BD Biosciences, San Jose, CA, USA), and data were analyzed using FlowJo software (Ashland, OR, USA).

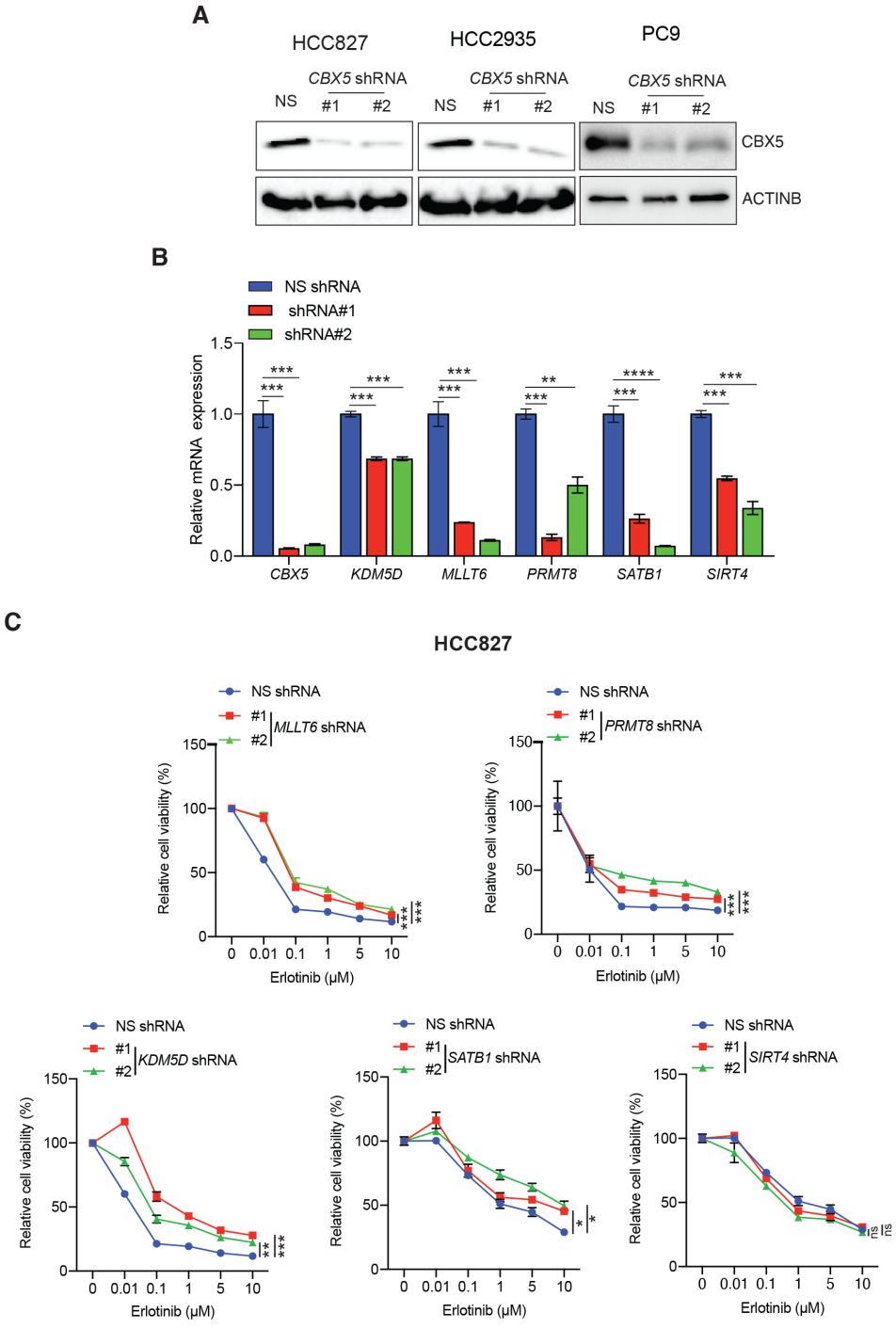

Fig. S1. Identification of epigenetic regulators of EGFRi resistance. *(A)* Immunoblot analysis measuring CBX5 levels in the indicated LUAD cell lines. ACTINB was used as a loading control. *(B)* Expression of the indicated genes in HCC827 cells expressing shRNAs targeting those genes analyzed by qRT-PCR; mRNA expression of indicated genes in knockdown cells relative to expression in NS shRNA-expressing cells is plotted. Data were normalized to *ACTINB*. *(C)* Relative survival (%) of HCC827 cells expressing an NS shRNA or shRNA targeting the indicated gene treated for 5 days with the indicated concentrations of erlotinib or dimethyl sulfoxide (DMSO) (0), as measured by MTT assay. Data are presented as the mean ±SEM. ns = not significant; * P<0.05, ** P<0.01, *** P<0.001, and **** P<0.0001.

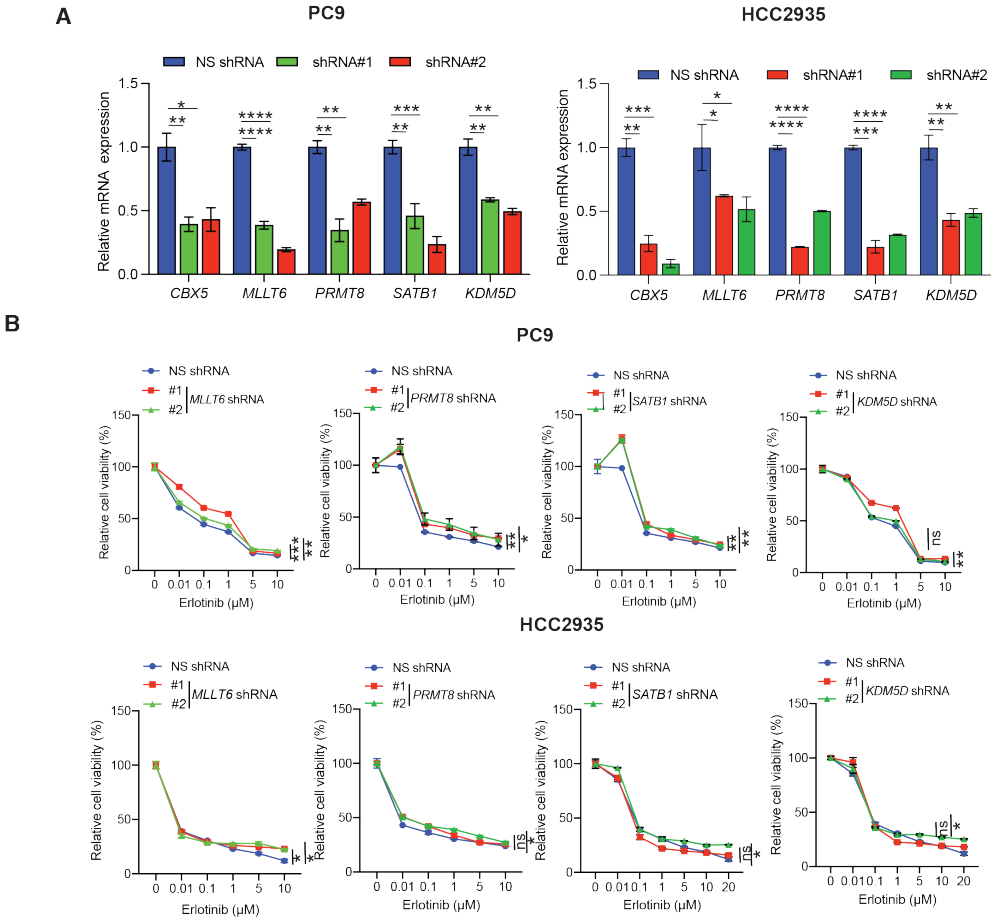

Fig. S2. Knockdown validation, cell survival assays, and expression of candidates identified from the large-scale epigenome-wide shRNA screen. *(A)* Expression of the indicated genes in PC9 and HCC2935 cells expressing shRNAs targeting those genes analyzed by qRT-PCR; mRNA expression of the indicated genes in knockdown cells relative to NS shRNA-expressing cells is plotted. *(B)* Relative cell viability (%) of PC9 and HCC2935 cells expressing an NS shRNA or shRNAs targeting the indicated genes treated for 5 days with the indicated concentrations of erlotinib or DMSO (0), as measured by MTT assay. Data are presented as the mean ± SEM. ns = not significant; * P<0.05, ** P<0.01, *** P<0.001, and **** P<0.0001.

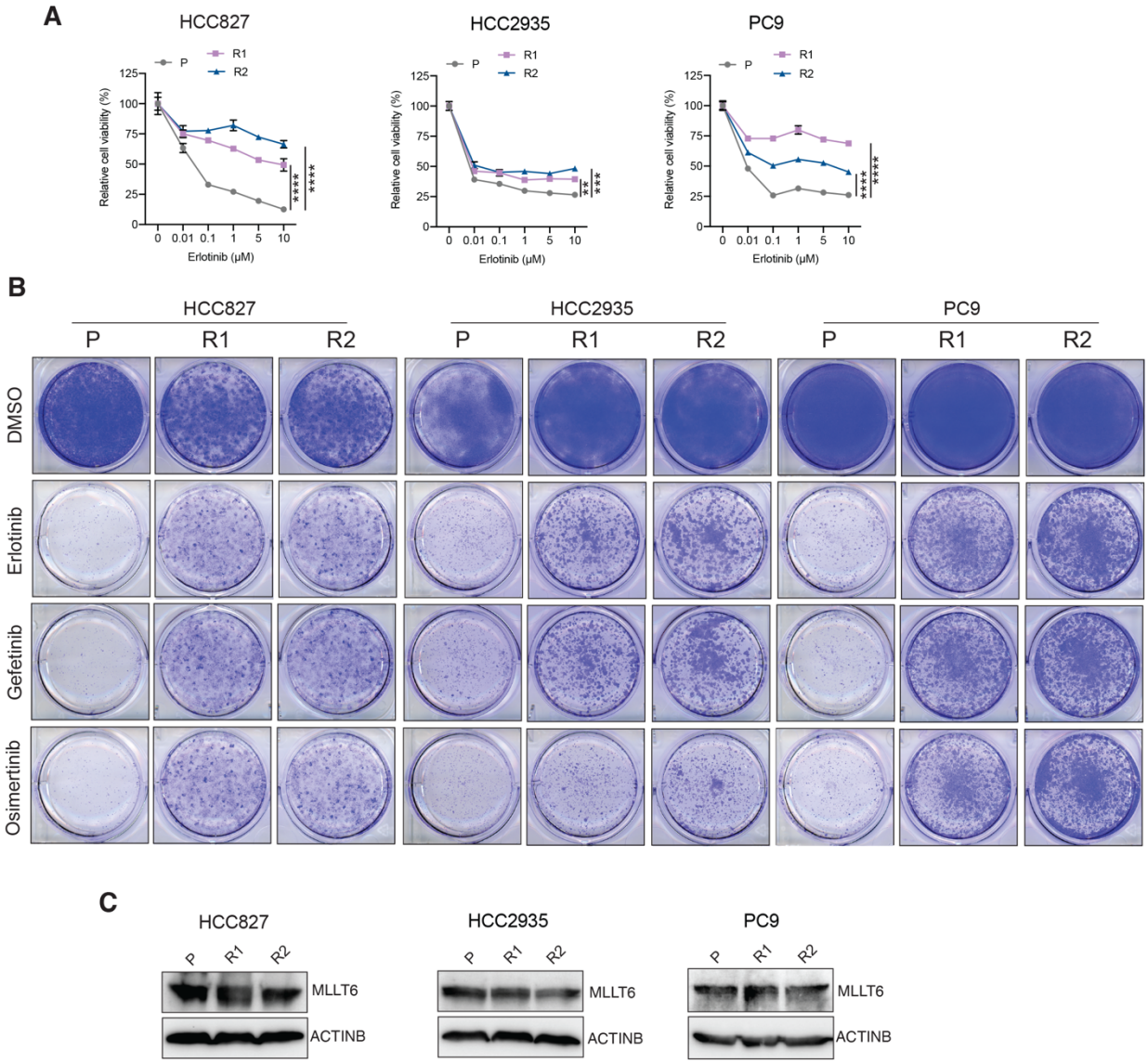

Fig. S3. Validation of EGFRi-resistant LAUD cell lines. *(A)* Relative cell viability (%) of parental (P) and EGFRi-resistant HCC827, HCC2935, and PC9 cell lines (R1, R2) treated with erlotinib or DMSO at indicated concentrations for 5 days, as measured by MTT assay. *(B)* The indicated EGFRi-resistant LUAD cell lines were treated with erlotinib, gefitinib or osimertinib (250 nM for erlotinib and gefitinib, 100 nM for osimertinib) or DMSO, and survival was measured in clonogenic assays. Representative wells for cells grown under the indicated conditions are shown. *(C)* Immunoblot analysis measuring MLLT6 expression in the indicated EGFRi-sensitive or EGFRi-resistant EGFR-mutant LUAD cell lines. Data are presented as the mean ± SEM. ***P*<0.01, ****P*<0.001, and *****P*<0.0001.

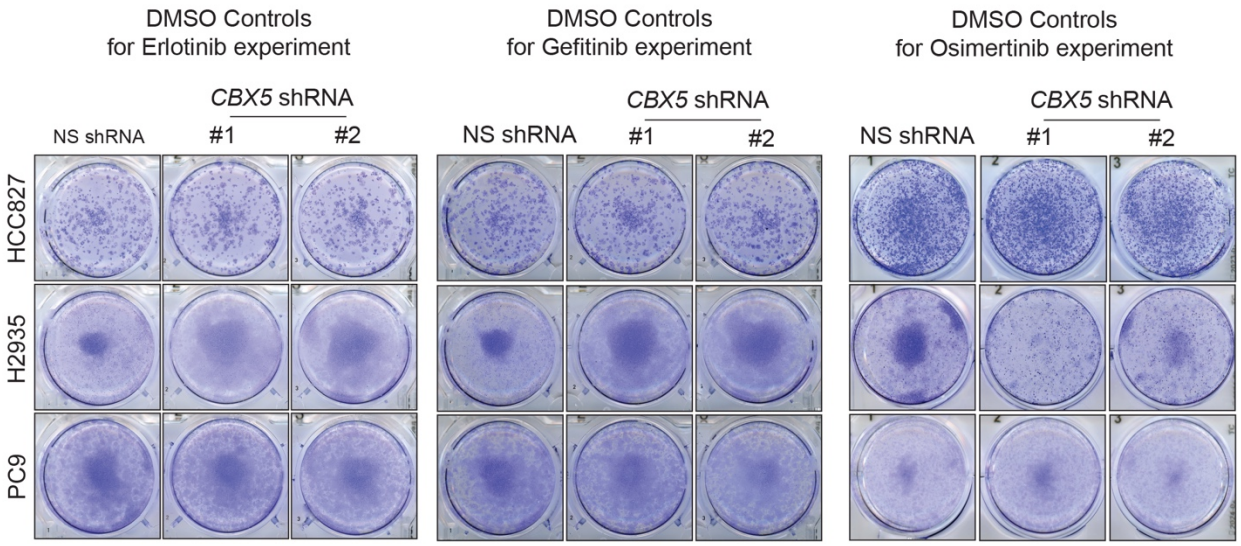

Fig. S4. Control wells from clonogenic assays. Representative DMSO-treated control wells from clonogenic assays with the indicated LUAD cell lines expressing an NS or *CBX5* shRNA are shown (related to Fig. 1*D*).

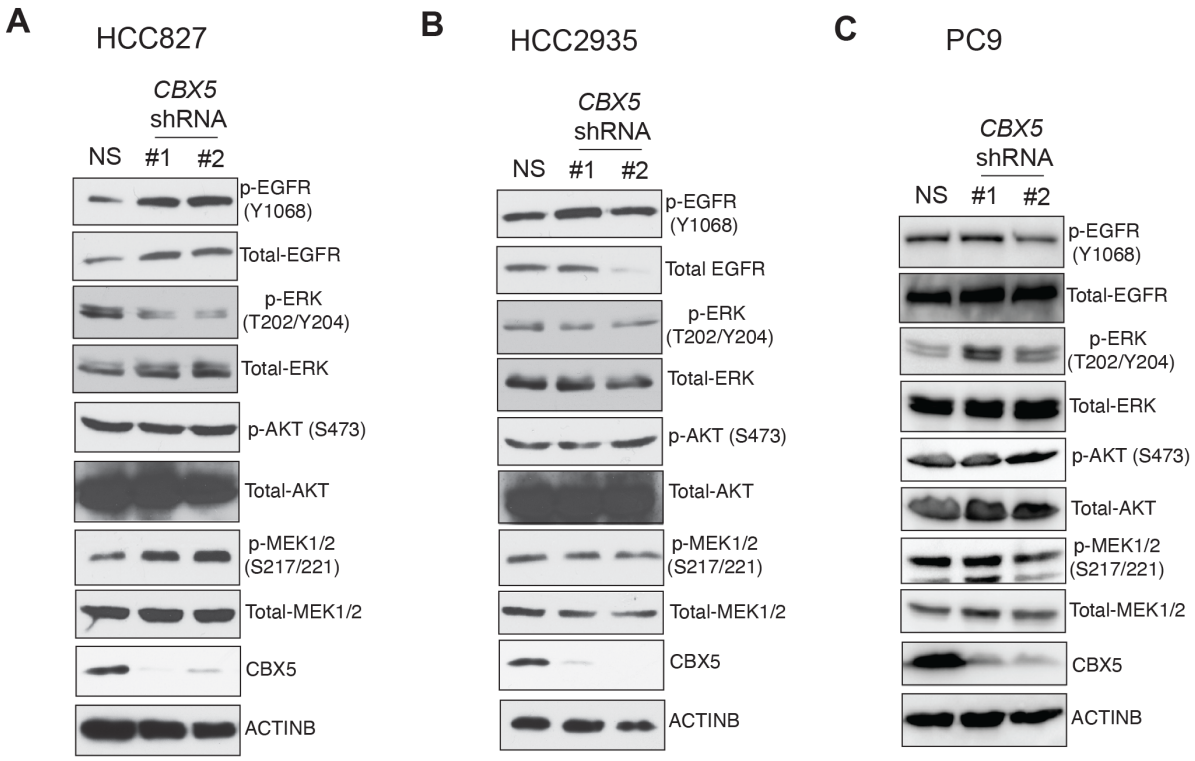

Fig. S5. Measurement of EGFR and other downstream signaling pathways in *CBX5*-knockdown EGFR-mutant LUAD cells. Immunoblot analysis measuring expression of the indicated proteins in EGFR-mutant LUAD cell lines expressing an NS or *CBX5* shRNA. ACTINB was used as a loading control.

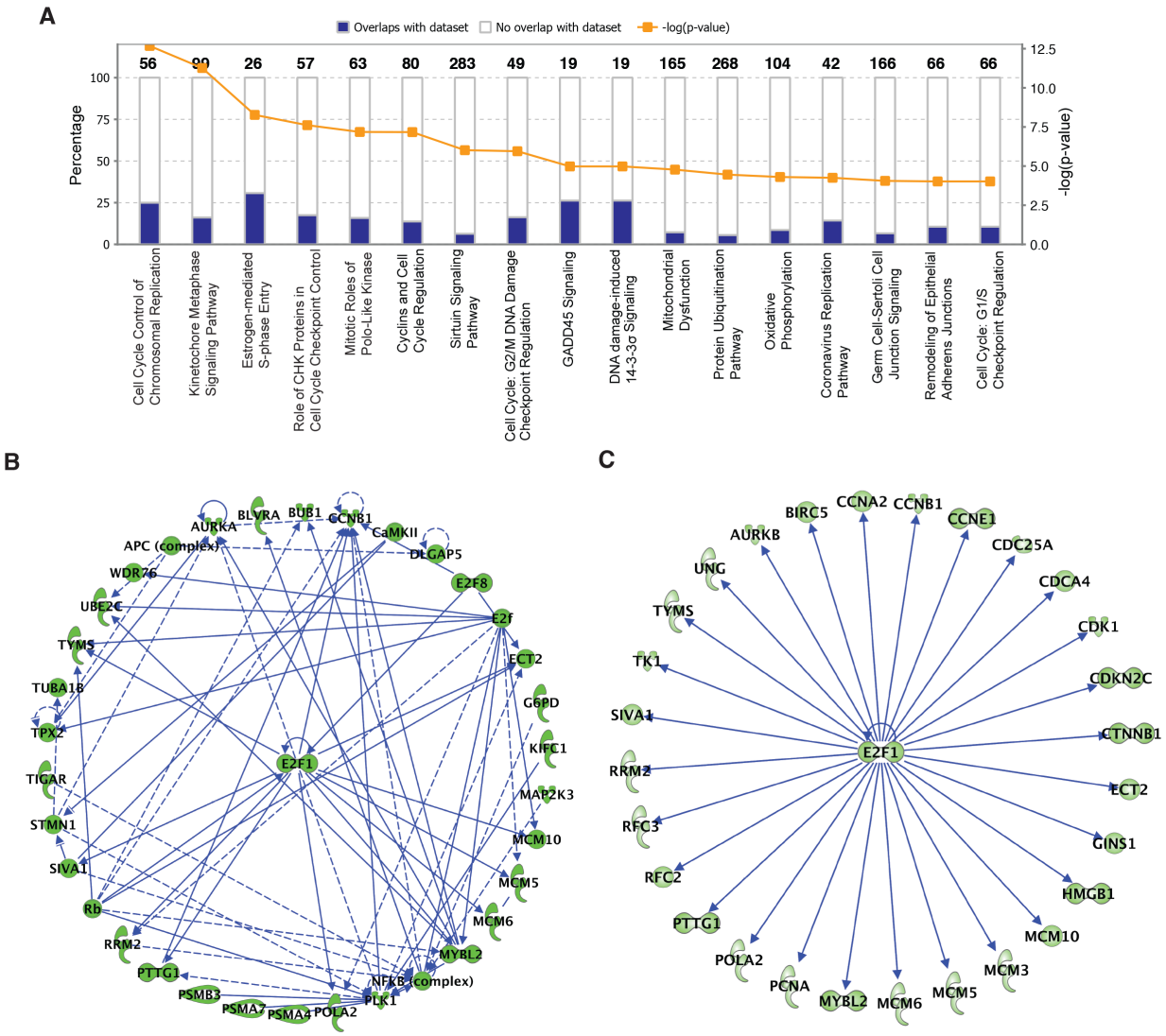

Fig. S6. Ingenuity pathway analysis identifies E2F1 as a major upstream regulator of genes upregulated in *CBX5*-knockdown cells. *(A)* Key biological pathways enriched among genes upregulated in *CBX5*-knockdown HCC827 cells identified by RNA-seq. *(B, C)* Pathway map for E2F1-regulated genes identified from RNA-seq analysis of *CBX5*-knockdown HCC827 cells.

Figure S7

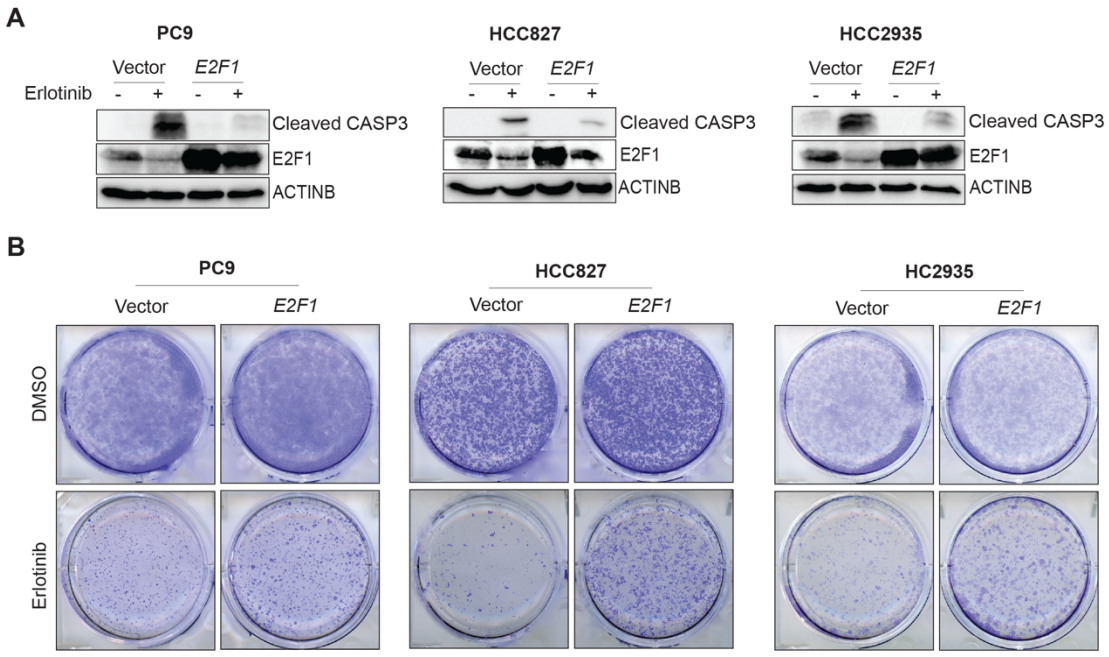

Fig. S7. E2F1 is a downstream mediator of CBX5-loss-driven EGFRi resistance in EGFR-mutant LUAD. *(A)* Immunoblot analysis measuring expression of the indicated proteins in LAUD cell lines overexpressing E2F1 or empty vector, treated with DMSO or erlotinib (50 nM) for 24 h. *(B)* The indicated LAUD cancer cell lines overexpressing E2F1 or vector were treated with erlotinib (20 nM for HCC827 and PC9; 100 nM for HCC2935) or DMSO, and survival was measured in clonogenic assays.

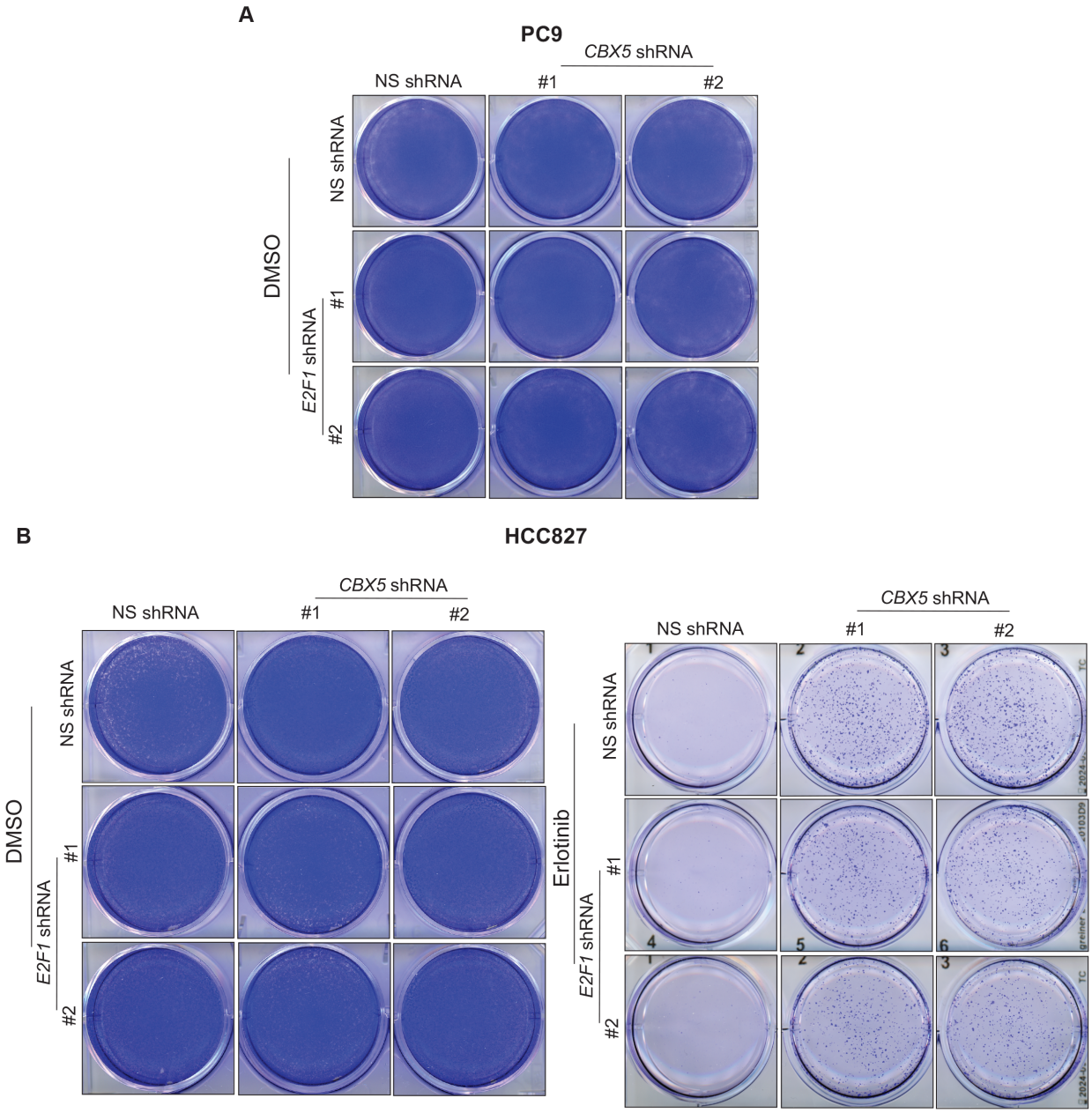

Fig. S8. E2F1 is necessary for CBX5-loss-induced resistance to EGFRi. *(A)* Representative DMSO-treated control wells from clonogenic assays with PC9 cells expressing an NS or *CBX5* shRNA alone, or in combination with an *E2F1* shRNA, are shown (related to Figure 2E). *(B)* Representative wells from clonogenic assays with HCC827 cells expressing an NS or *CBX5* shRNA alone, or in combination with an *E2F1* shRNA, were treated with DMSO (left) or erlotinib (50 nM, right), and survival was measured in clonogenic assays.

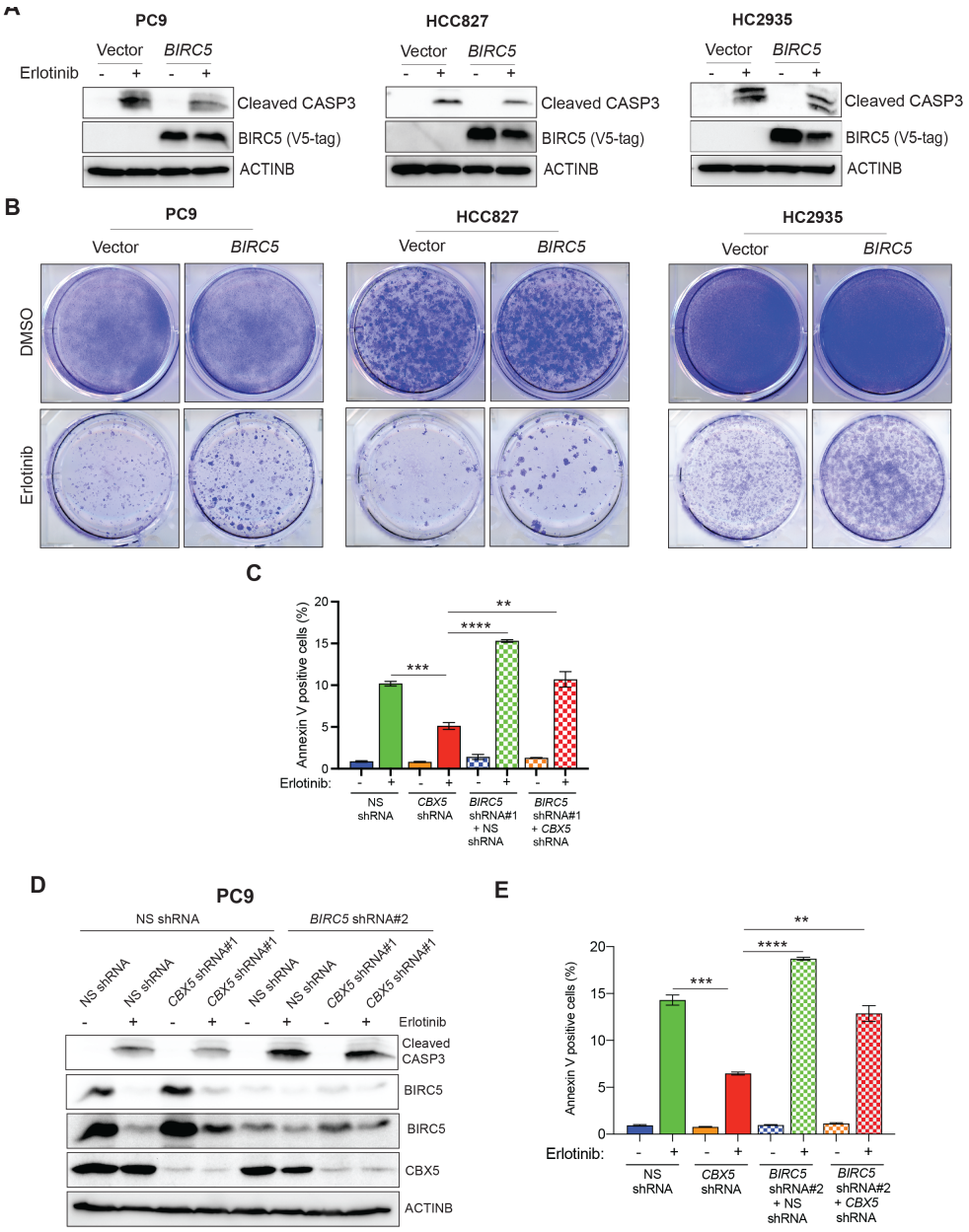

Fig. S9. BIRC5 is a downstream mediator of CBX5-loss-driven EGFRi resistance in EGFR-mutant LUAD. *(A)* Immunoblot analysis measuring expression of the indicated proteins in LAUD cell lines overexpressing BIRC5 or vector, treated with DMSO or erlotinib (50 nM) for 24 h. *(B)* The indicated LAUD cancer cell lines overexpressing BIRC5 or vector were treated with erlotinib (20 nM for HCC827 and PC9; 100 nM for HCC2935) or DMSO, and survival was measured in clonogenic assays. *(C)* PC9 cells expressing an NS or *CBX5* shRNA alone, or in combination with an NS or *BIRC5* shRNA#1, were treated with DMSO or erlotinib (50 nM) for 24 h and then analyzed by FACS-based annexin V-PE staining. % of Annexin V positive cells at indicated conditions is shown. *(D)* PC9 cells expressing an NS or *CBX5* shRNA alone, or in conjunction with *BIRC5* shRNA#2, were treated with DMSO or erlotinib (50 nM) for 24 h, and expression of the indicated proteins was measured by immunoblot analysis. *(E)* PC9 cells expressing an NS or *CBX5* shRNA alone, or in combination with an NS shRNA or *BIRC5* shRNA#2, were treated with DMSO or erlotinib (50 nM) for 24 h and then analyzed by FACS-based annexin V-PE staining. % of Annexin V positive cells at indicated conditions is shown. Data are presented as the mean ± SEM. ***P*<0.01, ****P*<0.001, and *****P*<0.0001.

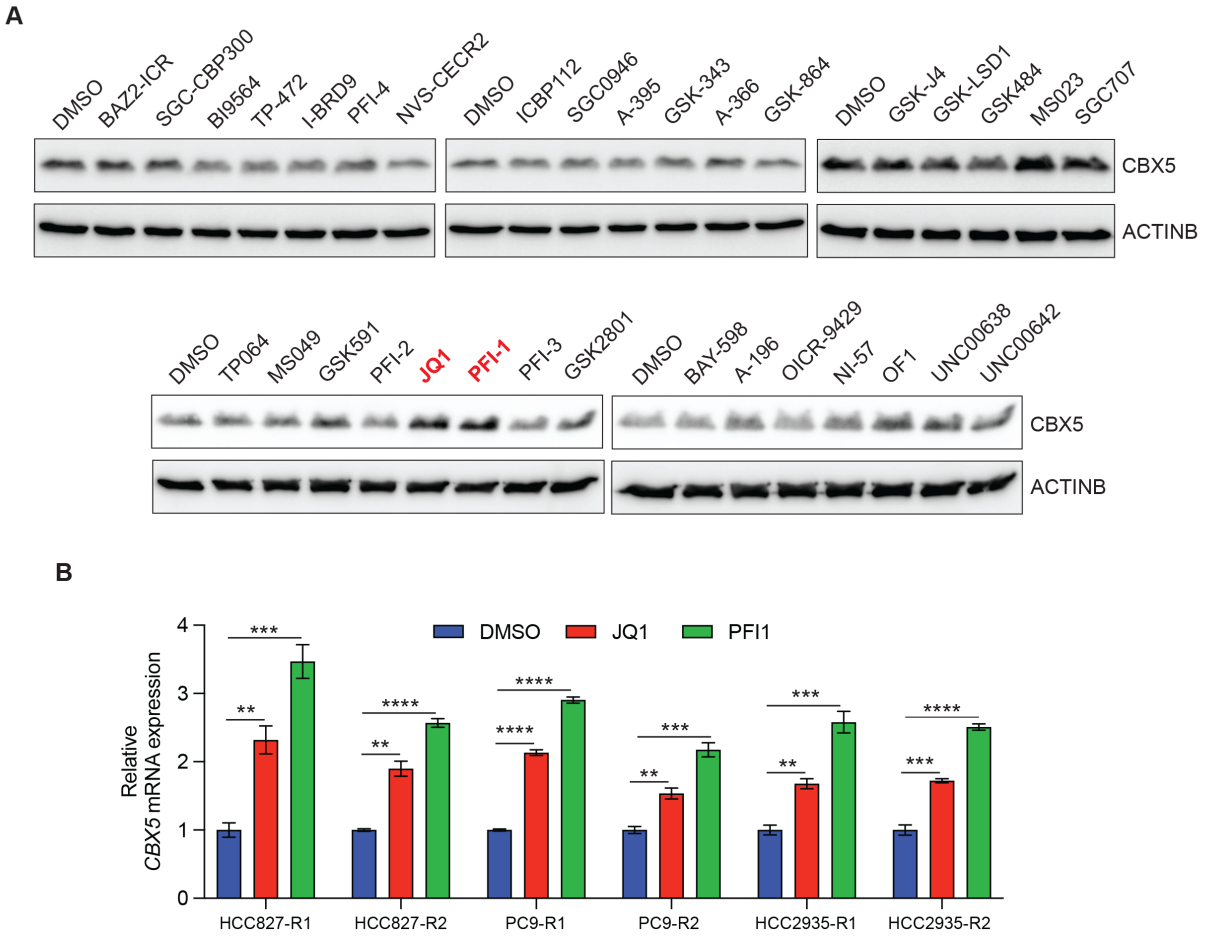

Fig. S10. Epigenetic regulation of CBX5 expression in EGFRi-resistant cell lines. *(A)* EGFRi-resistant PC9 cells (PC9–R2) were treated with DMSO or the indicated small-molecule inhibitors at the concentrations shown in Table S2 for 48 h, and expression of CBX5 was measured by immunoblot analysis. *(B)* The indicated EGFRi-resistant cell lines were treated with DMSO or BETi JQ1 (2 μM) or PFI-1 (2 μM) for 48 h, and expression of *CBX5* was measured by qRT-PCR. Relative *CBX5* mRNA expression in BETi-treated cells relative to DMSO-treated cells is plotted. Data are presented as the mean ± SEM. ***P*<0.01, ****P*<0.001, and *****P*<0.0001.

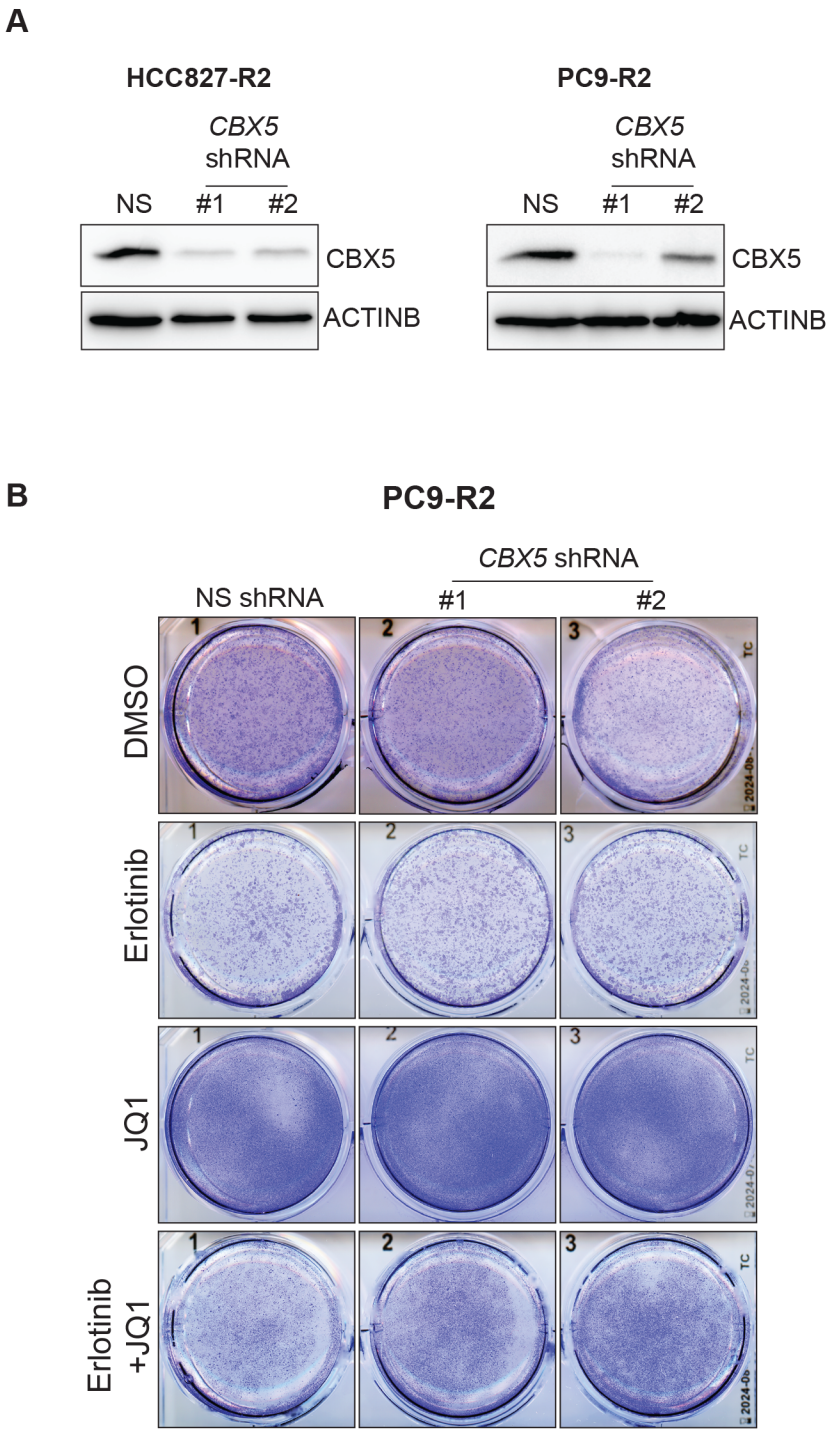

Fig. S11. BETi treatment sensitizes EGFRi-resistant cells to EGFRi. *(A)* Immunoblot analysis measuring CBX5 expression in the indicated EGFRI resistant LUAD cell lines (HCC827-R2 and PC9-R2). *(B)* PC9–R2 cells expressing an NS or *CBX5* shRNA were treated with DMSO, erlotinib (25 nM), JQ1 (0.125 μM), or a combination of erlotinib (25 nM) + JQ1 (0.125 μM), and survival was measured in clonogenic assays. Representative wells for cells grown under the indicated conditions are shown.

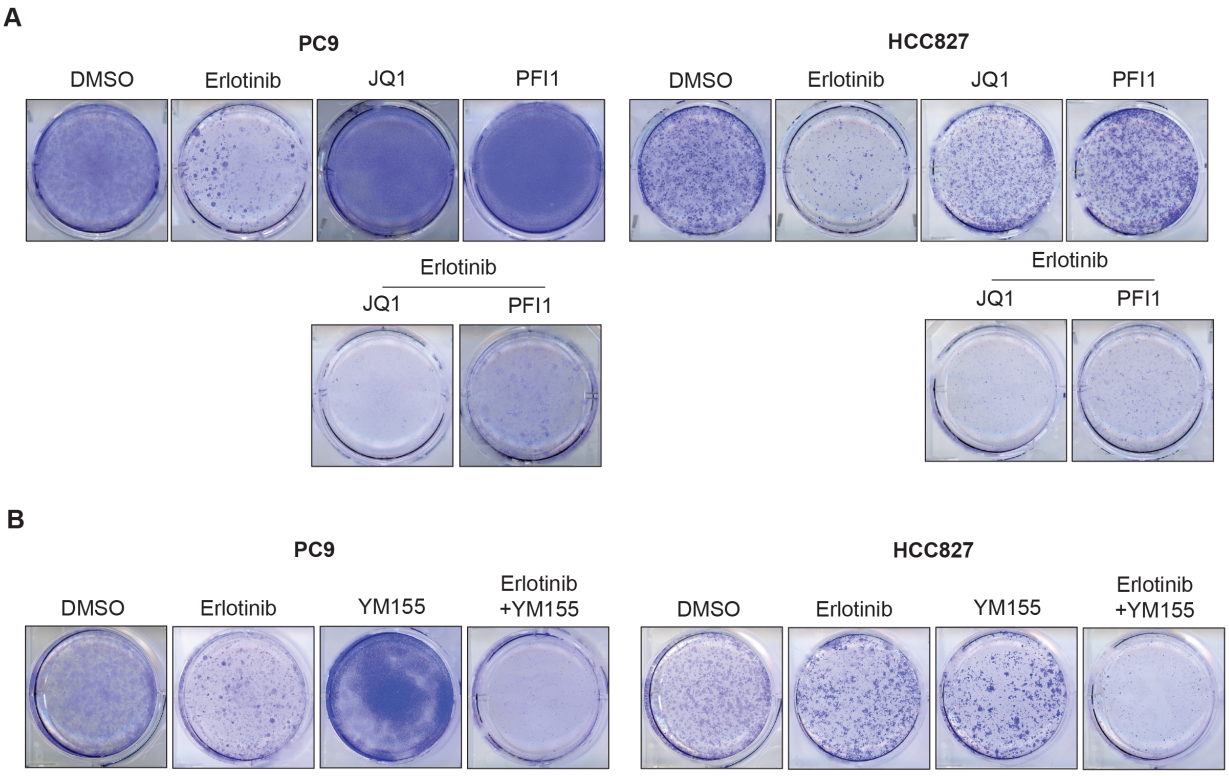

Fig. S12. BETi and BIRC5 inhibitors forestall development of EGFRi resistance in EGFR-mutant LUAD cells. *(A)* The indicated LUAD cell lines were treated with DMSO, erlotinib (25 nM), JQ1 (250 nM for PC9; 500 nM for HCC827), or PFI1 (250 nM for PC9; 500 nM for HCC827) alone, or with a combination of erlotinib (25 nM) + JQ1 (250 nM for PC9; 500 nM for HCC827) or erlotinib (25 nM) + PFI (250 nM for PC9; 500 nM for HCC827), and survival was measured in clonogenic assays. Representative wells for the indicated cell lines under the indicated treatment conditions are shown. *(B)* The indicated LUAD cell lines were treated with DMSO, erlotinib (25 nM), or YM155 (40 nM for PC9; 2.5 nM for HCC827) alone, or with a combination of erlotinib (25 nM) + YM155 (40 nM for PC9; 2.5 nM for HCC827), and survival was measured in clonogenic assays. Representative wells for the indicated cell lines under the indicated treatment conditions are shown.

Table S1. Genes identified as drivers of resistance to the EGFRi erlotininb in EGFR-mutant HCC827 LUAD cells.

S.No.	Gene Symbol	Gene Name	Accession number	Gene ID
1	CXB5	Chromobox 5	NM_001127322.1	23468
2	KDM5D	Lysine-Specific Demethylase 5D	NM_001146705.1	8284
3	MLLT6	Mixed-Lineage Leukemia, Translocated To, 6	NM_005937.3	4302
4	PRMT8	Protein Arginine Methyltransferase 8	NM_019854.4	56341
5	SATB1	Special AT-rich Sequence Binding Protein 1	NM_001195470.2	6304
6	SIRT4	Sirtuin 4	NM_012240.2	23409

Table S2. List of inhibitors targeting indicated chromatin modifiers and the concentrations at which they were used in the chemical genetic screen.

S.No.	Target protein	Inhibitor	Inhibitor concentration (μm)
1	BAZ2A/2B	BAZ2-ICR	1
2	BAZ2A/2B	GSK2801	1
3	BET family	JQ1	1
4	BET family	PFI1	1
5	BRD9/7	BI-9564	1
6	BRD9/7	TP-472	1
7	BRD9	I-BRD9	1
8	BRPF1/2/3; BRPF1B	NI-57	1
9	BRPF1/2/3; BRPF1B	OF1	1
10	BRPF1/2/3; BRPF1B	PFI-4	1
11	CECR2	NVS-CECR2-1	1
12	CREBBP, EP300	I-CBP112	1
13	CREBBP, EP300	SGC-CBP30	1
14	DOT1L	SGC0946	1
15	EED	A-395	1
16	EZH2/H1	GSK343	3
17	G9a (EHMT2)/GLP	A-366	1
18	G9a (EHMT2)/GLP	UNC0638	1
19	G9a (EHMT2)/GLP	UNCO642	1
20	IDH1 mutant	GSK864	1
21	JMJD3/UTX (KDM6A/B)	GSK-J4	5
22	LSD1 (KDM1A)	GSK-LSD1	1
23	PAD4 (PADI4)	GSK484	10
24	PRMT Type I	MS023	1
25	PRMT3	SGC707	1
26	PRMT4	TP-064	1
27	PRMT4/6	MS049	5
28	PRMT5	GSK591	1
29	SETD7	®-PFI-2	1
30	SMARCA2/4, PB1	PFI-3	1
31	SMYD2	BAY-598	1
32	SUV420H1/H2 (KMT5B/C)	A-196	1
33	WDR5	OICR-9429	3

Table S3. List of reagents and software used in this study with source and identifiers.

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CBX5	Cell Signaling Technology	Cat# 2616; RRID:AB_2070987
Cleaved Caspase 3	Cell Signaling Technology	Cat# 9664S; RRID:AB_2070042
P-EGFR(Y1068)	Cell Signaling Technology	Cat# 8543S; RRID:AB_10828604
P-Akt(S473)	Cell Signaling Technology	Cat#4060; RRID:AB_2315049
Akt	Cell Signaling Technology	Cat#9272; RRID:AB_329827
p-ERK(T202/Y204)	Cell Signaling Technology	Cat# 4376S; RRID:AB_331772
ERK	Cell Signaling Technology	Cat# 4695S; RRID:AB_390779
P-MEK(S217/221)	Cell Signaling Technology	Cat# 9121S; RRID:AB_331648
MEK	Cell Signaling Technology	Cat# 9122S; RRID:AB_823567
V5-Tag (D3H8Q)	Cell Signaling Technology	Cat# 13202S; RRID:AB_2687461
MLLT6	Bethyl Laboratories	Cat# A302-198A; RRID:AB_1659777
Survivin (BIRC5)	Cell Signaling Technology	Cat#2808; RRID:AB_2063948
E2F1	Cell Signaling Technology	Cat# 3742; RRID:AB_2096936
β-Actin (D6A8)	Cell Signaling Technology	Cat#4970; RRID:AB_2223172
Chemicals, peptides, and recombinant proteins		
Erlotinib	Selleck Chemical LLC	Cat# S1023
Gefitinib	Selleck Chemical LLC	Cat# S1025
Osimeritinb	Selleck Chemical LLC	Cat# S7297
JQ1	Selleck Chemical LLC	Cat# S7110
PFI1	Selleck Chemical LLC	Cat# S1216
YM155	Selleck Chemical LLC	Cat# S1130
SGC- Epigenitic Probe Library	Cayman Chemicals	Cat# 17525
DMEM	GIBCO	Cat# 11965-092
RPMI	GIBCO	Cat# 11875-093
Fetal Bovine Serum	GIBCO	Cat# 10437-028
Trypsin-EDTA	GIBCO	Cat# 25200-056
Penicillin-Streptomycin	GIBCO	Cat# 15140-122
Effectene Transfection Reagent	QIAGEN	Cat# 301427
Critical commercial assays		
CUT&RUN Assay Kit	Cell Signaling Technology	Cat# 86652
Annexin V assay kit	BD Pharmingen	Cat#559763
Deposited data		
RNA-Seq performed with HCC827CBX5 knockdown cells	This paper	GEO: GSE114563
Experimental models: Cell lines		
HEK-293T	ATCC	CRL-11268
HCC827	ATCC	CRL-2868

HCC2935	ATCC	CRL-2869
PC9	Sigma-Aldrich	Cat# 90071810
Experimental models: Organisms/strains		
Mouse: NSG	Jackson Laboratory	Stock No. 005557
Oligonucleotides		
BIRC5	CGTCCGGTTGCGCTTTCCTT	ATGGCACGGCGCACTTTCTC
CBX5	TCCTGAGGATGCGGAAAACA	AGGAGAGGAGGCAGGGAGGT
KAT6A	CCAGAATGTTTGCGCTGGAC	CTGGCACTGTGGCTCTTCGT
KDM5D	CCAGTGTGCAGGGGAGTTTG	AGCCGGCGGTAGTGTTCAAT
MLLT6	AATCCCCTCCTCTCCCAAGC	GCAGAGGCTGTCTGGTCGAA
PRMT8	TGCTCCCTACACCCACTGGA	TTTGGCATTTGGCTTCATGG
SATB1	CACCACCCTTGGGAAAGGAG	TTGCTGCTGCTGTTGCTGAG
SIRT4	TGCCAGCAAGTCCTCCTCTG	TCCGGACAAAATCACCATGC
E2F1	TCCAAGAACCACATCCAGTG	CTGGGTCAACCCCTCAAG
ACTINB	GCATGGAGTCCTGTGGCATC	TTCTGCATCCTGTCGGCAAT
E2F1 (CUT&RUN Primer)	GCCTTTCCGGTTTCCCACAGC	TCTGCAAACTGGGTGCACAT
BIRC5 (CUT&RUN primer)	TCTCAGCTACTCGGGAGGCT	AGCTCACTGCAACCTCCTCCC
ACTINB (CUT&RUN Primer)	TCTTGGCTGGGCGTGACTGT	AAGGTGGGCTCTACAGGGCA
Plasmids, ORFs and shRNAs		
pLKO.1	Addgene	Cat No# 10878
pMD2.G	Addgene	Cat No# 12259
psPAX2	Addgene	Cat No# 12260
pZIPZ NS shRNA	Open Biosystems	Cat No# RHS4346
TRC NS shRNA	Open Biosystems	Cat No# RHS6848
pLX304-BIRC5	Open Biosystems	Cat No# OHS6085-213578374
KDM5D	RHS4430-101102580	
MLLT6	RHS4430-101161520	
PRMT8	RHS4430-101070759	
STAB1	RHS4430-101165442	RHS4430-101169043
SIRT4	RHS4430-101103028	RHS4430-101102122
E2F1	TRCN0000000250	TRCN0000000251
BIRC5	TRCN0000073718	TRCN0000073721
Software and algorithms		
Prism 8.0	GraphPad	www.graphpad.com/scientific software/prism
ImageJ	https://imagej.nih.gov/ij	N/A
Reactome pathway analysis from RNA sequencing data	Reactome Pathway Database	https://reactome.org/
Flowjo	https://www.flowjo.com/	N/A

Dataset S1. List of genes targeted by the human epigenome-wide shRNA library and shRNA IDs.

Dataset S2. List of genes identified from the RNA-seq analysis measuring changes in mRNA expression following *CBX5* knockdown.

Dataset S3. Ingenuity Pathway Analysis.

SI References

1. A. Dobin A *et al.,* STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29**,15-21 (2013).
2. L. Wang, S. Wang, W. Li, RSeQC: quality control of RNA-seq experiments. *Bioinformatics* **28**, 2184-2185 (2012).
3. P. Ewels, M. Magnusson, S. Lundin, M. Kaller, MultiQC: summarize analysis results for multiple tools and samples in a single report. *Bioinformatics* **32**, 3047-3048 (2016).
4. H. Li *et al.*,The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25**, 2078-2079 (2009).
5. H. Thorvaldsdottir, J.T. Robinson, J.P. Mesirov, Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. *Brief Bioinform* **14**, 178-192 (2013).
6. M.I. Love, W. Huber W, S. Anders, Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* **15**, 550 (2014).
7. H. Heberle, G.V. Meirelles, F.R. da Silva, G.P. Telles, R. Minghim, InteractiVenn: a web-based tool for the analysis of sets through Venn diagrams. *BMC Bioinformatics* **16**, 169 (2015).