Loss of KMT5C promotes EGFR inhibitor resistance in NSCLC via LINC01510-mediated upregulation of MET

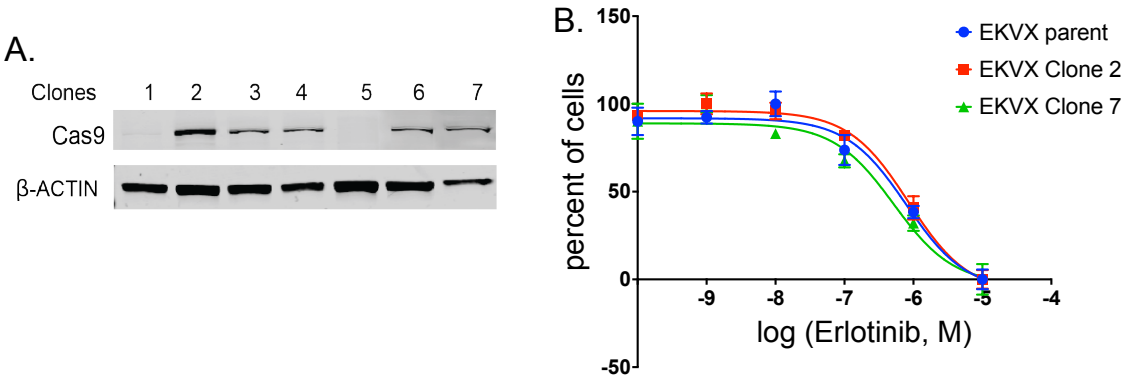
Arpita S. Pal[1,2*], Alejandra Agredo[1,2*], Nadia A. Lanman[3,4], Jihye Son[1] , Ikjot S. Sohal[1,3], Manvir Bains[1], Chennan Li[1]., Jenna Clingerman[1,2], Kayla Gates[1], Andrea L. Kasinski[1,3]

Author Affiliations: [1]Department of Biological Sciences, [2]Purdue Life Sciences Interdisciplinary Program (PULSe), [3]Purdue University Center for Cancer Research, [4]Department of Comparative Pathobiology, Purdue University, West Lafayette U.S.A
Corresponding author: akasinski@purdue.edu

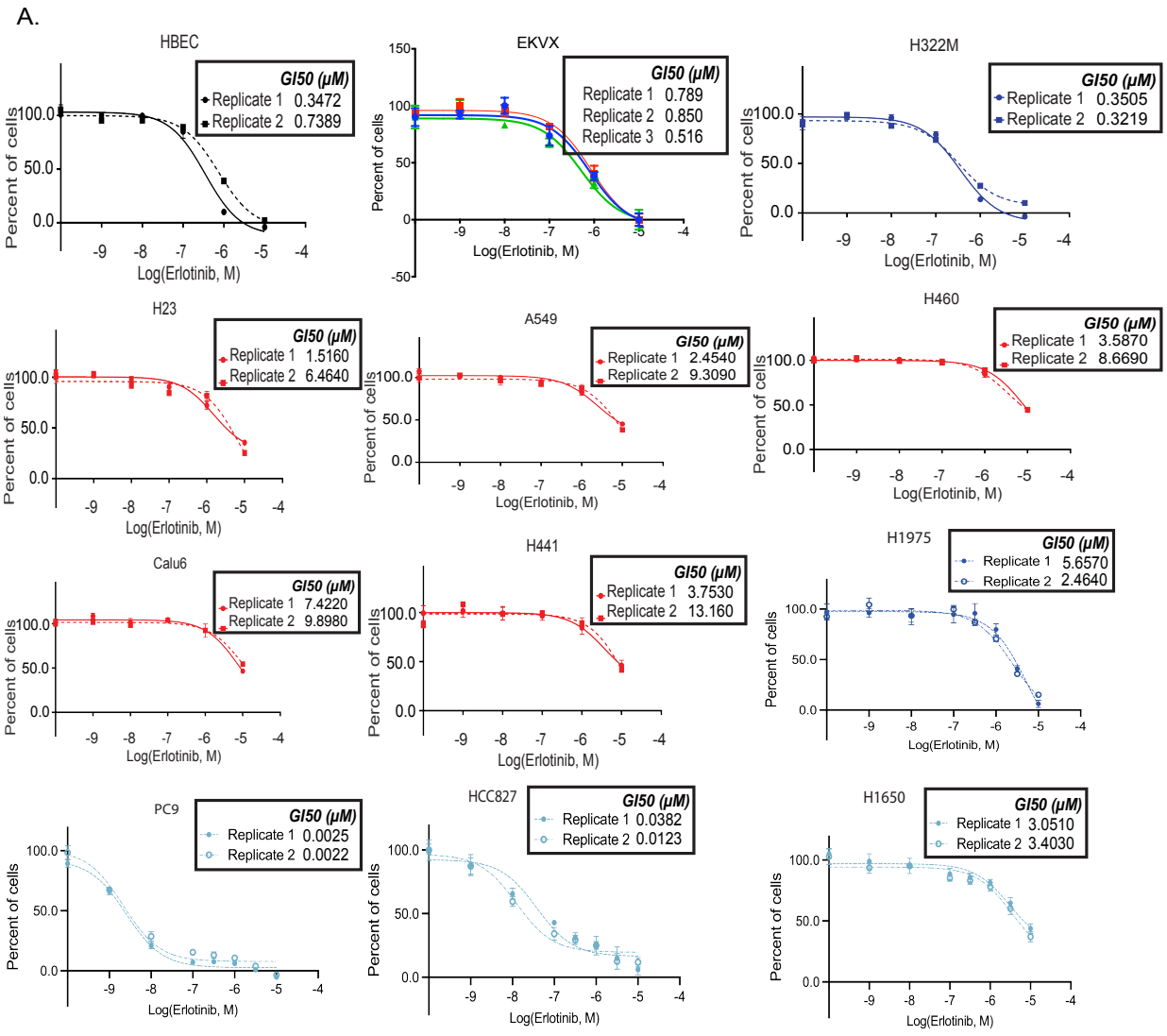
*These authors contributed equally to this work.

The authors declare no potential conflicts of interest.

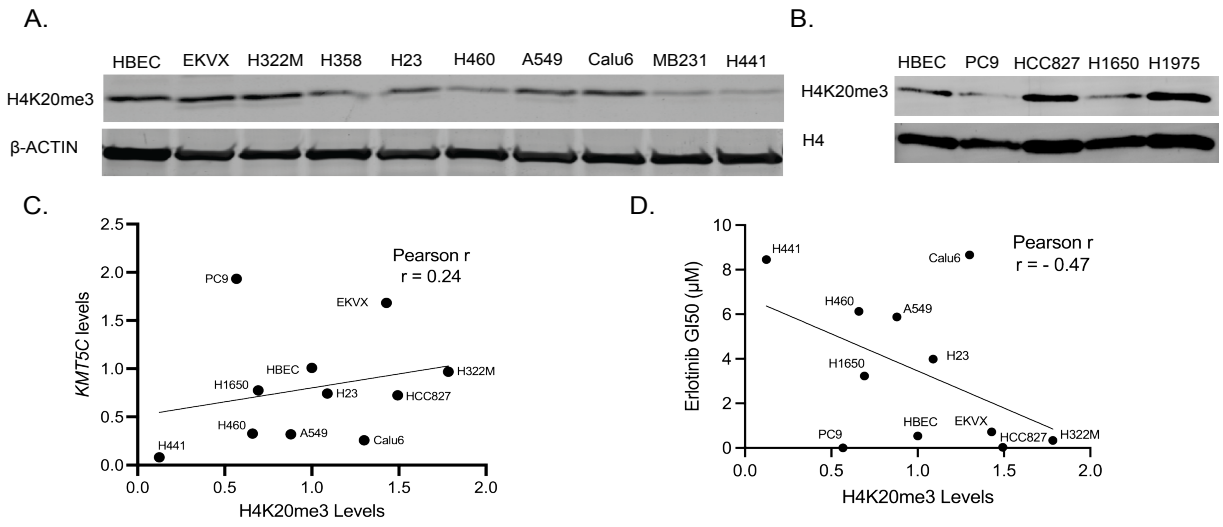
Supplemental Information:



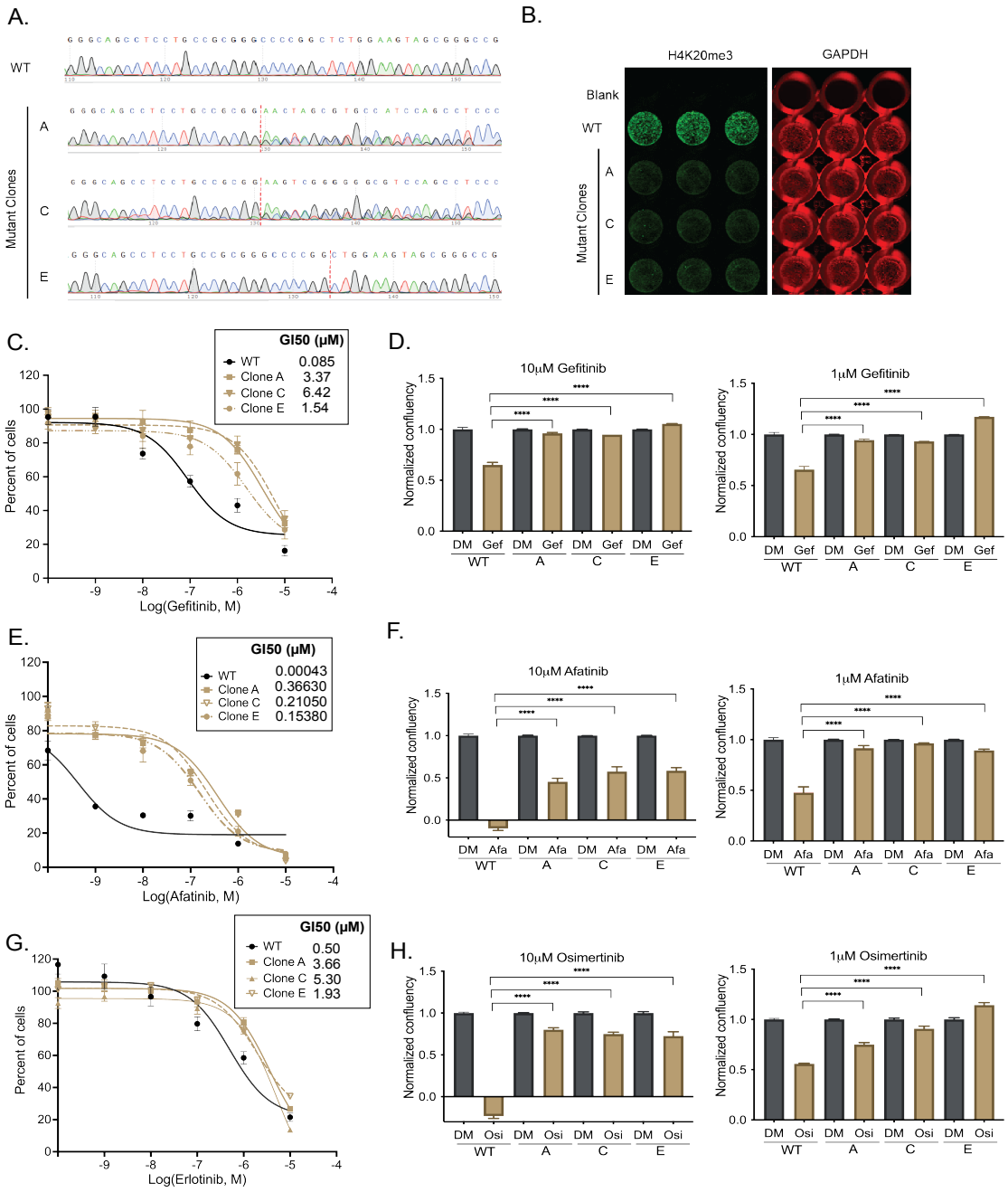
Supplementary Figure 1: Characterization of Cas9 expressing EKVX clones. A) Western Blot analysis of Cas9 levels in EKVX clones stably expressing Cas9. β-ACTIN was used as a loading control. B) Parental EKVX cells, ECas9 clone 2, and ECas9 clone 7 were exposed to varying concentrations of erlotinib or the highest equivalent volume of dimethyl sulfoxide (DMSO, negative control) containing media for 72 hours. Erlotinib dose response was evaluated using the SRB assay.



Supplementary Figure 2: Growth inhibition for a panel of NSCLC cell lines following exposure to increasing doses of erlotinib. A panel of NSCLC cell lines were exposed to varying concentrations of erlotinib or the highest equivalent volume of dimethyl sulfoxide (DMSO, negative control) containing media for 72 hours. Erlotinib dose response was evaluated using the SRB assay. Post-normalization, the GI50 concentration of erlotinib was calculated from the respective dose curve for each cell line, two replicates were performed for each cell line. GI50 values in Figure 2C are the average of the replicates indicated here.

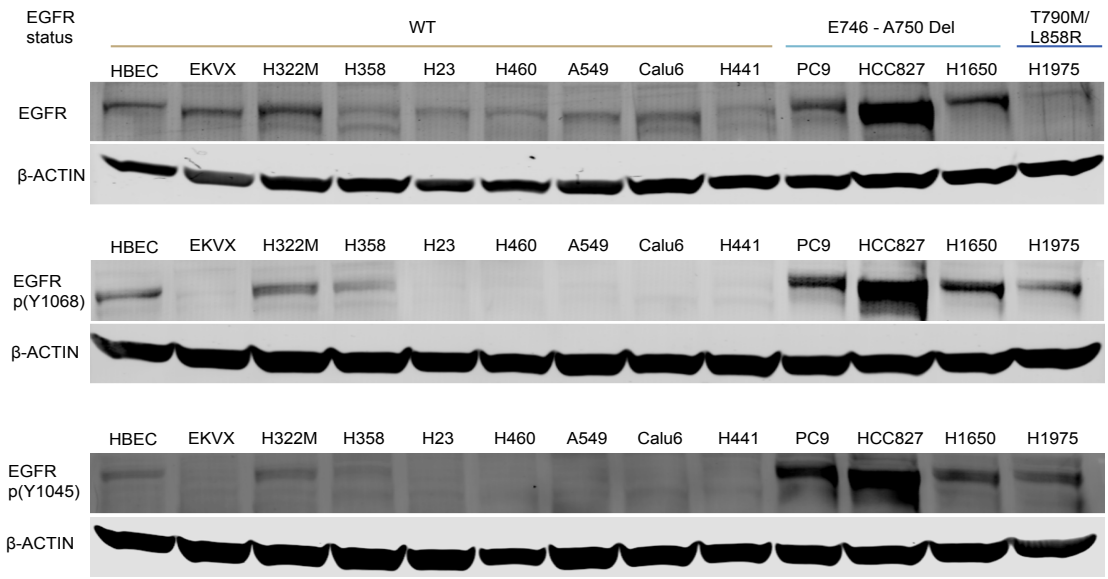


Supplementary Figure 3: Reduced H4K20me3 correlates with erlotinib resistance in NSCLC cells. Representative western blot of H4K20me3 in a panel of NSCLC cells that include A) cell lines from the NCI-60 DTP program and B) EGFR mutant cell lines. HBEC serves as a control on each blot. β-ACTIN was used as a loading control. MB231, a breast cancer cell line was included as a control cell line reported to have low levels of KMT5C (Shinchi et al., 2015). C) Correlation analysis between quantified H4K20me3 levels from panel A/B and *KMT5C* from Figure 2A/B. D) Correlation analysis between quantified H4K20me3 levels from panel A/B and GI50 erlotinib values from Figure 2C. Evaluations in C and D were conducted using the Pearson correlation test.

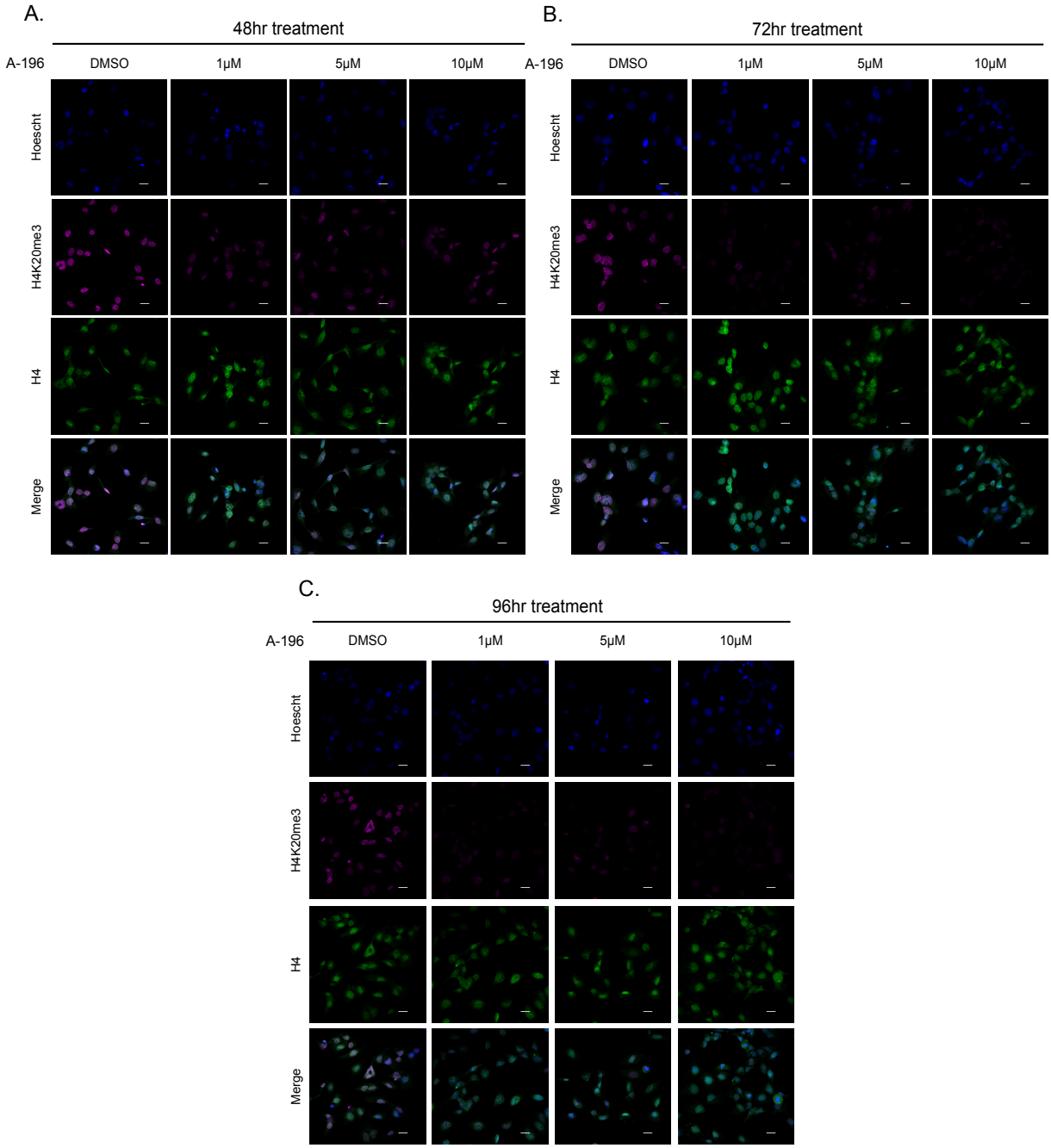


Supplementary Figure 4: KMT5C mutation confers resistance to various EGFRi. A) Genomic DNA of EKVX WT cells or mutant clones A, C, E was isolated, the region targeted by CRISPR-Cas9 sgRNA targeting KMT5C was PCR amplified, purified and sequenced. Representative chromatograms of the wildtype KMT5C (WT) cells, and the specific mutations identified in mutant clones A, C, E. B) In-cell western of H4K20me3 levels in EKVX WT cells and mutant clones A, C, E. GAPDH serves as an endogenous control. C) Gefitinib, E) Afatinib, or G) Osimertinib dose response curves. Cells were exposed to the indicated concentration of drug or to the highest equivalent volume of vehicle control containing media for 72 hours. Following normalization, the GI50 concentration of each inhibitor was calculated from the respective dose

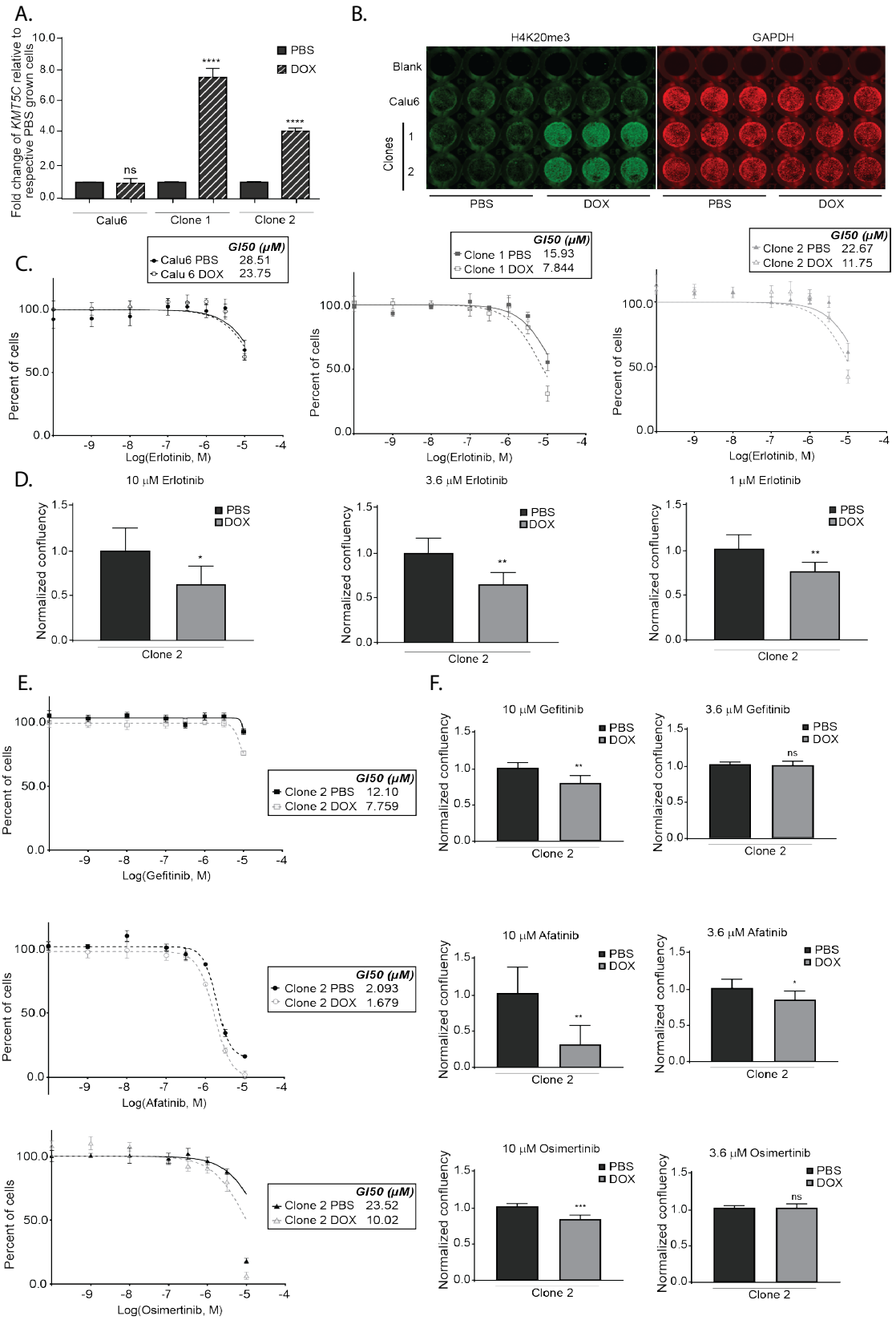
curve for each cell line. Proliferation of EKVX WT cells or mutant clones A, C, E was evaluated using the Incucyte. Cells were exposed to varying concentrations of D) Gefitinib (Gef) F) Afatinib (Afa) or H) Osimertinib (Osi) or the highest equivalent volume of DMSO (DM) containing media for 72 hours. Data relative to respective normalized DMSO control treatments is represented. One-way ANOVA followed by Dunnett's Multiple Comparison test was utilized to evaluate statistical significance of normalized confluency of clones A, C, E in the presence of 10 or 1μM of gefitinib, afatinib or osimertinib compared to WT cells.



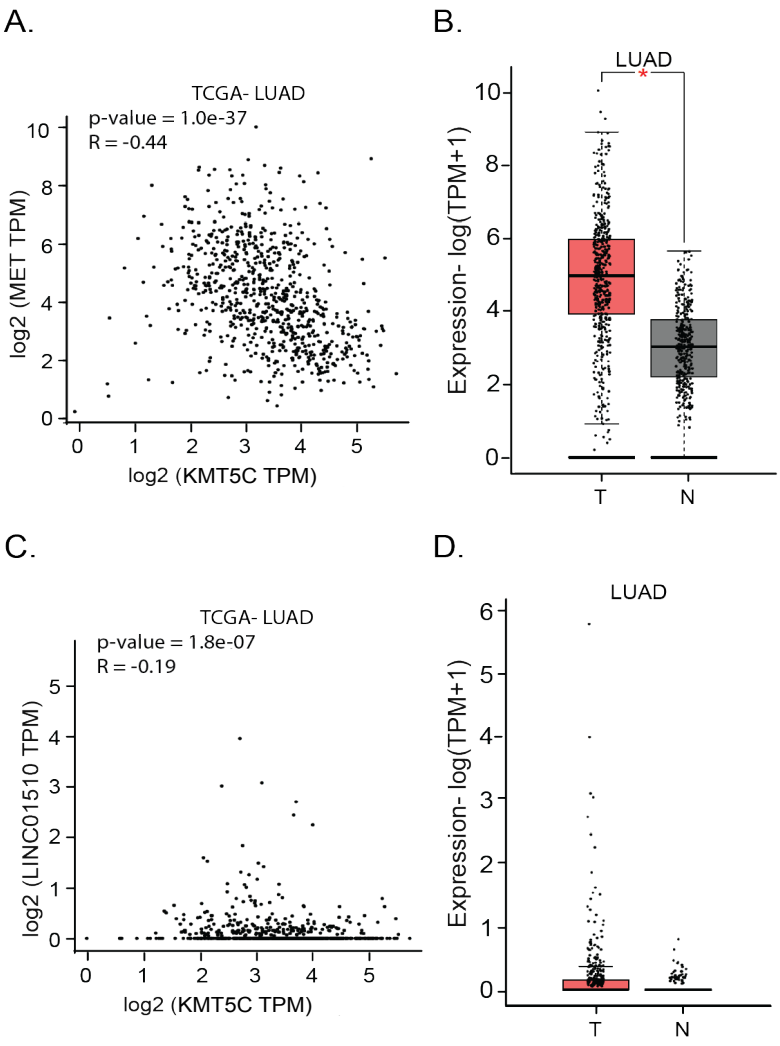
Supplemental Figure 5: EGFR status of cell lines used in this study. Western Blots of EGFR, EGFR p(Y0168) and EGFR p(Y1045) in a panel of EGFR WT and mutant NSCLC cell lines. PC9, HCC827 and H1650 harbor E746-A750 deletion and H1975 harbors the mutation T790M/L858R in EGFR. β-ACTIN was used as a loading control.



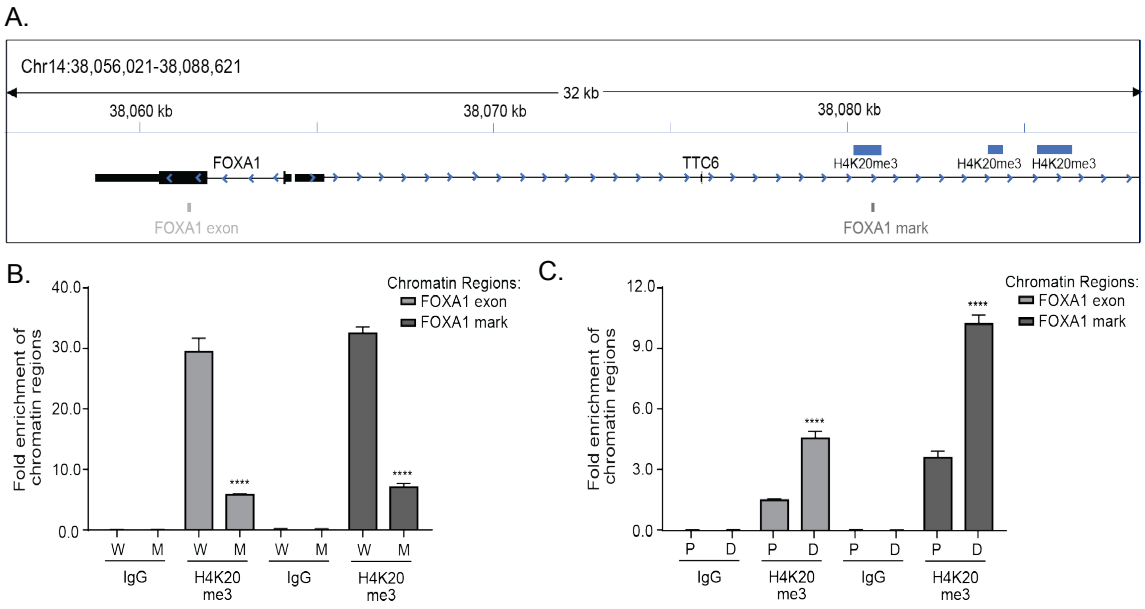
Supplementary Figure 6: Chemical inhibition of KMT5B/C induces global decrease in H4K20me3 with little to no effect on overall H4 levels. Immunofluorescence of H4K20me3 (cyan) and H4 (green) in HCC827 cells after treatment with the indicated doses of A-196 for A) 48h, B) 72h and C) 96h. Hoechst was used as a nuclear stain. Data for 120h timepoint is included in Figure 5.


A.
Fold change of KMT5C relative to respective PBS grown cells
PBS
DOX
ns
****
****
Calu6
Clone 1
Clone 2
B.
H4K20me3
GAPDH
Blank
Calu6
Clones
1
2
PBS
DOX
PBS
DOX
C.
GI50 (μM)
Calu6 PBS 28.51
Calu 6 DOX 23.75
Clone 1 PBS 15.93
Clone 1 DOX 7.844
Clone 2 PBS 22.67
Clone 2 DOX 11.75
Percent of cells
Log(Erlotinib, M)
D.
10 μM Erlotinib
3.6 μM Erlotinib
1 μM Erlotinib
Normalized confluency
PBS
DOX
Clone 2
E.
F.
GI50 (μM)
Clone 2 PBS 12.10
Clone 2 DOX 7.759
Log(Gefitinib, M)
10 μM Gefitinib
3.6 μM Gefitinib
Normlaized confluency
ns
GI50 (μM)
Clone 2 PBS 2.093
Clone 2 DOX 1.679
Log(Afatinib, M)
10 μM Afatinib
3.6 μM Afatinib
GI50 (μM)
Clone 2 PBS 23.52
Clone 2 DOX 10.02
Log(Osimertinib, M)
10 μM Osimertinib
3.6 μM Osimertinib

Supplementary Figure 7: Ectopic expression of KMT5C partially sensitizes EGFRi resistant cells to EGFRi. A) KMT5C transcript levels evaluated by qRT-PCR in Calu6 cells and Calu6 clones 1, 2 stably expressing DOX-inducible KMT5C. One-way ANOVA followed by Dunnett's Multiple Comparison test was used to evaluate statistical significance of KMT5C transcript levels relative to respective PBS treated cells. B) H4K20me3 levels evaluated by in-cell western. DOX (or PBS control) treatment was for two weeks. GAPDH serves as an endogenous control. C) Erlotinib dose response measured by SRB was evaluated after a two-week exposure to PBS or DOX containing media. Cells were then exposed to varying concentrations of erlotinib or the highest equivalent volume of DMSO containing media for 72 hours following normalization, the GI50 concentration of erlotinib was calculated from the respective dose curve for each cell line. D) Proliferation of clone 2 was evaluated using the Incucyte. Cells grown in PBS or DOX containing media for two weeks were exposed to varying concentrations of erlotinib or the highest equivalent volume of DMSO containing media for 72 hours. Normalized data relative to respective normalized PBS treated samples is represented. Unpaired t-test was used to evaluate the statistical significance for each pair.E) Dose response measured by SRB was evaluated after a two week exposure to PBS or DOX containing media for Calu6 or clones 1, 2 for gefitinib, afatininb or osimertinib. EGFRi treatments lasted for 72 hours. Following normalization, the GI50 concentration of each EGFRi was calculated from the respective dose curve for each cell line. F) Proliferation of clone 2 was evaluated using the Incucyte. Cells grown in PBS or DOX containing media for two weeks, were exposed to varying concentrations of gefitinib, afatinib, osimertinib, or the highest equivalent volume of DMSO containing media for 72 hours. Unpaired t-test was used to evaluate statistical significance of normalized confluency of DOX-cultured clone 2 cells in the presence of either 10 or 3.6 μM of gefitinib, afatinib, or osimertinib compared to respective normalized confluency of PBS-treated cells.



Supplementary Figure 8: LINC01510 correlates poorly with LUAD prognosis. Correlation analysis between A) *MET* and *KMT5C* and C) *LINC01510* and *KMT5C* transcript levels in TCGA-LUAD dataset, evaluated using GEPIA. GEPIA analysis for B) *MET* and D) *LINC01510* transcript levels in normal (N, n = 347) and tumor samples (T, n = 483) form LUAD data obtained from TCGA and GTEx databases. The majority of the samples in the normal subgroup had undetectable levels of *LINC01510*. TPM= Transcripts per million



Supplementary Figure 9: H4K20me3 is enriched at the FOXA1 locus in an KMT5C dependent manner. A) ChIP-qPCR primers designed to evaluate enrichment of H4K20me3 at the FOXA1 exonic region (FOXA1 exon), and at the predicted H4K20me3 modification upstream of the FOXA1 promoter region (FOXA1 mark). ChIP was performed using either IgG or H4K20me3 primary antibodies on chromatin isolated from B) WT or KMT5C mutant clone C or C) inducible KMT5C cells (in the presence of DOX or PBS). qPCR using the immunoprecipitated chromatin was conducted using primers shown in A (Table 3). Data are represented as fold enrichment of the chromatin region pulled-down by the H4K20me3 primary antibody relative to IgG and was evaluated for significance using one-way ANOVA. W = WT cells, M = KMT5C mutant clone C cells, P = Calu6 clones grown in PBS containing media, D = Calu6 clones grown in DOX containing media.

Supplementary Table 1:

Primer sequences used to conduct the CRISPR-Cas9 screen. Multiple PCR2 primers were used, each with an independent barcode that allows for sorting of sample-specific sgRNAs post sequencing.

PCR	Sample	Primer name	Primer direction	Primer sequence
PCR 1	All samples	1st PCR primer	Forward	TCTTTCCCTACACGACGCTCTTCCGATCTNNNNAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCG
		1st PCR primer	Reverse	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCC
PCR2	EKVX-Baseline 1	UDA5050	Forward	AATGATACGGCGACCACCGAGATCTACACTGACAATGTCACACTCTTTCCCTACACGAC
		UDA7143	Reverse	CAAGCAGAAGACGGCATACGAGATAGAAGCCAATGTGACTGGAGTTCAGACGTG
	EKVX-Replicate 1	UDA5051	Forward	AATGATACGGCGACCACCGAGATCTACACCGACCTAACGACACTCTTTCCCTACACGAC
		UDA7142	Reverse	CAAGCAGAAGACGGCATACGAGATGACTCACTAAGTGACTGGAGTTCAGACGTG
	EKVX-Baseline 2	UDA5052	Forward	AATGATACGGCGACCACCGAGATCTACACTAGTTCGGTAACACTCTTTCCCTACACGAC
		UDA7141	Reverse	CAAGCAGAAGACGGCATACGAGATAGTCTGTCGGGTGACTGGAGTTCAGACGTG

	EKVX-Replicate 2	UDA5053	Forward	AATGATACGGCGACCACCGAGATCTACACGCCGCACTCTACACTCTTTCCCTACACGAC
		UDA7140	Reverse	CAAGCAGAAGACGGCATACGAGATGTATTCTCTAGTGACTGGAGTTCAGACGTG
	EKVX-Baseline 3	UDA5054	Forward	AATGATACGGCGACCACCGAGATCTACACATTATGTCTCACACTCTTTCCCTACACGAC
		UDA7139	Reverse	CAAGCAGAAGACGGCATACGAGATACGCCTCTCGGTGACTGGAGTTCAGACGTG
	EKVX-Replicate 3	UDA5055	Forward	AATGATACGGCGACCACCGAGATCTACACAGAACCGAGTACACTCTTTCCCTACACGAC
		UDA7138	Reverse	CAAGCAGAAGACGGCATACGAGATTAACCGCCGAGTGACTGGAGTTCAGACGTG

sgRNA sequences used to generate *KMT5C* mutant cell lines. Designed and purchased from Invitrogen.

sgRNA name	**sgRNA sequence**
Exon3 sgRNA (EGFR WT cell lines)	CGGCCCGCTACTTCCAGAGC
Exon7 sgRNA1 (EGFR Mutant cell lines)	GUGAAUGCCACACCUGUGAG
Exon7 sgRNA2 (EGFR Mutant cell lines)	AAGCAUGUCACCUCGUCCCC

Supplementary Table 2: Primers utilized in the study. Designed and purchased from Integrated DNA Technologies.

Primer use		**Primer direction**	**Primer sequence**
pLV-sgKMT5C		Forward	CACCGCGGCCCGCTACTTCCAGAGC
		Reverse	AAACGCTCTGGAAGTAGCGGGCCGC
pLVX-Tetone-KMT5C		Forward	TCGTAAAGAATTCACCATGGGGCCCGACAGAGTGACAGCA
		Reverse	GAGATCTGGATCCTCAGTACAGCTCTTCACCGCCGAC
pLVX-Tetone-KMT5C-puro		Forward	CCGCTACGCGTTCAGAAGAACT
		Reverse	AGCGGCGTACGATGATTGAACA
KMT5C genomic locus amplification		Forward	GAGCAGATGGGAGGTGCGGCGACAGT
		Reverse	GAGCTCAGAAGAAAGGAGACAGAT
KMT5C Exon7 locus amplification for T7 endonuclease assay		Forward	CTCAGCTGTTGCCCCATTCCAG
		Reverse	CTTGGTCTCACGCAGCTGGTA
KMT5C genomic locus sequencing		Forward	CCTCTCCTTAGCCTGGTCCT
		Reverse	CAAGGGCTAGGAAGTCAGGG
KMT5C quantification		Forward	TCGGTTTCCGCACCCATAAG
		Reverse	CGGAGGTAGCGATAGACGTG
ChIP-QPCR	FOXA1 mark	Forward	AAGGAGAGGTGCGTTGTTTG
		Reverse	CATTCTCCCACGAAAGGCAG
	FOXA1 exon	Forward	AAGACTCCAGCCTCCTCAAC
		Reverse	CGGGTGGTTGAAGGAGTAGT
		Forward	GCTTCTTGTCCCTCCCAGAT

	Linc01510 mark	Reverse	GCAGAAGTGAGAGGAAGGGT
	Up 1	Forward	CACACTGGAGTTCTTGCCAC
		Reverse	TATGCACTCCTTCACTGGGG
	Up 2	Forward	GCAGTCCAGCTAAGCAATCC
		Reverse	GACATCTTGGGAAGGGGACA
	Up 3	Forward	CCTCTTCACATCCCACAGGT
		Reverse	CTCTGCTGGCTTGATCATTG
	MET	Forward	GATCAAGGAAATGGGGCGTT
		Reverse	GGGACTAGGGCCTATTGTCA
	Down 1	Forward	CCCTGCCTCTCATCAACTGA
		Reverse	GTTGAGCCACTAAACCACCC
	Down 2	Forward	TGCCTGGTCTCCTGTTAACA
		Reverse	ATCTGTCTTCTCCCTGTGCC
	Down 3	Forward	AGTCCAAGATCAAGGCACCA
		Reverse	AGGCCTTTCTTGTACCCCTT

Supplementary Table 3: Candidate genes identified from the CRISPR-Cas9 knock out screen. Thirty-five significant hits identified by MAGeCK-VISPR analysis and β-score, p-value, and false discovery rate (FDR)

Target	β-score	p-value	FDR
KMT5C	97	8.30E-05	0.07
ADSS	91	0.00021	0.07
OPA3	89	0.00028	0.07
LEPREL4	88	0.00032	0.07
GAREM	86	0.00049	0.07
ISG15	83	0.00065	0.07
PROM2	83	0.00065	0.07
hsa-mir-602	77	0.00082	0.07
CCDC130	81	0.00088	0.07
PCSK2	80	0.00091	0.07
FAM120AOS	79	0.001	0.07
CCL23	79	0.0011	0.07
TNFSF12	76	0.0028	0.07
hsa-mir-27b	74	0.0081	0.11
SMN2	25	0.012	0.16
OR6V1	74	0.012	0.16
SYBU	72	0.012	0.17
CASP8	73	0.012	0.17
LDLRAP1	71	0.013	0.17
PFDN2	70	0.013	0.17
CPA3	68	0.013	0.17
PP2D1	68	0.013	0.17
TMEM234	68	0.013	0.17
TMEM147	67	0.013	0.17
hsa-mir-5699	62	0.016	0.21
hsa-mir-512-1	50	0.016	0.21

MLL2	22	0.016	0.21
hsa-mir-648	43	0.016	0.21
AGAP9	22	0.016	0.21
hsa-mir-4669	43	0.016	0.21
RPL41	38	0.016	0.21
hsa-mir-3183	37	0.016	0.21
hsa-mir-1268a	34	0.017	0.22
hsa-mir-147b	34	0.017	0.22
hsa-mir-148a	27	0.018	0.24