Oncogenic Deregulation of EZH2 as an Opportunity for Targeted Therapy in Lung Cancer

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ABSTRACT
As a master regulator of chromatin function, the lysine methyltransferase EZH2 orchestrates transcriptional silencing of developmental gene networks. Overexpression of EZH2 is commonly observed in human epithelial cancers, such as non–small cell lung carcinoma (NSCLC), yet definitive demonstration of malignant transformation by deregulated EZH2 remains elusive. Here, we demonstrate the causal role of EZH2 overexpression in NSCLC with new genetically engineered mouse models of lung adenocarcinoma. Deregulated EZH2 silences normal developmental pathways, leading to epigenetic transformation independent of canonical growth factor pathway activation. As such, tumors feature a transcriptional program distinct from KRA5- and EGFR-mutant mouse lung cancers, but shared with human lung adenocarcinomas exhibiting high EZH2 expression. To target EZH2-dependent cancers, we developed a potent open-source EZH2 inhibitor, JQEZ5, that promoted the regression of EZH2-driven tumors in vivo, confirming oncogenic addiction to EZH2 in established tumors and providing the rationale for epigenetic therapy in a subset of lung cancer.

SIGNIFICANCE: EZH2 overexpression induces murine lung cancers that are similar to human NSCLC with high EZH2 expression and low levels of phosphorylated AKT and ERK, implicating biomarkers for EZH2 inhibitor sensitivity. Our EZH2 inhibitor, JQEZ5, promotes regression of these tumors, revealing a potential role for anti-EZH2 therapy in lung cancer. Cancer Discov; 6(9); 1–16. ©2016 AACR. See related commentary by Frankel et al., p. 949.

INTRODUCTION
Lung cancer is the most common cancer and one of the most deadly cancers worldwide (1). Non–small cell lung cancers (NSCLC) are the most prevalent type of lung cancer, comprising a heterogeneous set of diseases (2). The identification of recurrent mutations and amplifications in many targetable oncoproteins has significantly improved overall survival of subsets of patients with NSCLC. Activating mutations in BRAF, KRAS, and EGFR, as well as fusions involving ALK, have been associated with response to kinase inhibition (3–6). Furthermore, with the advent of improved genomic profiling and next-generation sequencing, recurrent mutations and amplifications have been identified in HER2, MET, FGFR1, FGFR2, the ROS1 receptor tyrosine kinase, NRG1, NTRK1, and RET (2). Although together these alterations account for many cases of lung adenocarcinoma, a considerable population of patients with NSCLC lacks identifiable genetic lesions in therapeutically tractable targets.

Beyond growth factor signaling pathways, chromatin-associated complexes have recently been identified as recurrently altered or transcriptionally deregulated in NSCLC, including TET methylcytosine dioxygenase 2 (TET2), DNA methyltransferase 3A (DNMT3A), and enhancer of zeste homologue 2 (EZH2; ref. 7). Notably, each of these factors influences heterochromatin structure and has been linked to coordinated regulation of normal developmental transcriptional pathways (8–11). These data establish the hypothesis that disruption of chromatin architecture is a common event in lung cancer pathogenesis, either permissive with or distinct from oncogenic signaling pathways, functioning to deregulate transcriptional programs associated with cellular differentiation.

The dynamic structure of chromatin is influenced by posttranslational modifications (PTM) to DNA and to the unstructured amineterminal tails of histone proteins within nucleosomal particles. Control of gene expression pathways by DNA-binding transcriptional activators and repressors influences the recruitment of chromatin-associated enzyme complexes that confer covalent PTMs to chromatin. In general, side-chain acetylation of lysine residues on histone tails is associated with active euchromatin, notably at histone 3 lysine 27 as associated with active cis-regulatory enhancer elements (H3K27ac; ref. 12). Modification of H3K27...
EZH2 overexpression induces murine lung cancer. A, schematic depiction of LSL-EZH2 GEMM utilizing three different strategies to express Cre recombinase to induce EZH2 overexpression. B, Kaplan-Meier lung cancer–free survival summary plot for Actin-Cre;LSL-EZH2 transgenic mice (EZH2) versus WT mice. C, histology of WT lung (top) and EZH2-induced lung adenocarcinomas (bottom). Sections stained with hematoxylin and eosin (H&E) or immunostained for Ki67. Scale bar, 50 μm. D, H&E staining or immunostaining for EZH2, pAKT, and pERK1/2 in EZH2-induced mouse lung tumors (top) and KRAS-induced mouse lung tumors (bottom). Scale bar, 50 μm. E, relative protein expression levels of pAKT, pERK1,2, and EZH2 in normal, EZH2-induced, and KRAS-induced tumor lung tissues measured by Western blot and quantified with ImageJ. n = 3 for normal lung and EZH2-induced tumor, n = 2 for KRAS-induced tumor. F, indicated mice were sacrificed 6 weeks after Cre induction for immunostaining for EZH2. G, Kaplan-Meier lung cancer–free survival summary plot for the indicated mice.
An Oncogenic Role for EZH2 in Lung Cancer

A. Schematic diagram of the experimental design:
- LSL-EZH2 GEMM
- Actin-Cre;LSL-EZH2
  - Constitutive expression
- UBC;LSL-EZH2
  - Induced by tamoxifen at 6 wks
- Adeno-Cre;LSL-EZH2
  - Lung-specific induction at 6 wks

B. Lung cancer–free survival (%)

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C. Representative images of Ki67 staining:
- WT normal lung
- EZH2 lung cancer
- KRAS lung cancer

D. Representative images of H&E and pAKT, pERK staining:
- EZH2 lung cancer
- KRAS lung cancer

E. Graph showing relative levels of pAKT, pERK, and EZH2:
- Normal lung
- EZH2 lung tumor
- KRAS lung tumor

F. Representative images of LSL-EZH2, LSL-KRAS, LSL-KRAS;LSL-EZH2

G. Lung cancer–free survival (%)

- LSL-KRAS
- LSL-KRAS;LSL-EZH2
- LSL-EZH2

H&E: Hematoxylin and Eosin
pAKT: Phosphorylated Akt
pERK: Phosphorylated ERK

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mice had no evident phenotype and all harvested lungs were normal at 80 weeks, suggesting a causal role of EZH2 overexpression in lung tumorigenesis. To extend these findings, we restricted EZH2 overexpression to lung in a third GEMM using inhaled Adeno-Cre virus to direct Cre expression to the pulmonary epithelium of LSL-EZH2 mice (Fig. 1A; ref. 31). Adeno-Cre was administered to animals at 6 weeks of age and 42% of these animals developed lung adenocarcinoma, demonstrating that EZH2 overexpression in lung epithelial cells is sufficient to induce cancer. In sum, our data demonstrate that 45% of EZH2-overexpressing mice develop lung adenocarcinomas with an average survival to 73.6 weeks of age (Fig. 1B).

The histology of mouse lung tumors demonstrated features of human grade 1–2 lung adenoma/adenocarcinoma. As compared to normal mouse lung, EZH2-overexpressing lung adenocarcinomas showed high cellularity and less differentiation, all consistent with low- and intermediate-grade adenocarcinoma (Fig. 1C). IHC analysis of tumors arising from EZH2 overexpression demonstrated an increase in the proliferative marker Ki67 as compared to normal lung tissue (Fig. 1C). In a comparison to murine lungs cancers driven by expression of activated KRAS (KRASG12D), EZH2 expression was markedly higher in the EZH2-driven lung tumors (Fig. 1D). Analysis for the expression of pathway markers typically identified in KRAS-driven lung cancer, such as phosphorylated AKT (pAKT) and phosphorylated ERK (pERK), revealed low pAKT and pERK expression in EZH2-induced mouse lungs tumors (Fig. 1D). Western blot analysis further confirmed that EZH2 mouse lung tumors have significantly less pAKT and pERK than both KRAS-induced mouse lung tumors and normal mouse lung (Fig. 1E and Supplementary Fig. S1E). Taken together, these data suggest that lung tumors driven by EZH2-mediated epigenomic deregulation are histologically similar but molecularly distinct from lung tumors driven by KRAS-dependent oncogenic signaling.

As KRAS and EZH2 expression are not mutually exclusive (Fig. 1D), we wanted to explore possible synergy between KRAS and EZH2 in lung tumorigenesis. KRASG12D (LSL-KRAS) was introduced into the lung epithelium with or without EZH2 overexpression (±LSL-EZH2) via inhalation of viral Adeno-Cre. Six weeks after induction, LSL-KRAS and LSL-KRAS;LSL-EZH2 mice had comparable tumors with comparable morphology, even though EZH2 overexpression was evident only in the LSL-KRAS;LSL-EZH2 mice (Fig. 1F). Significantly, we did not observe an increase in tumorigenesis or a decrease in survival in the presence of EZH2 overexpression (Fig. 1G), suggesting that EZH2 overexpression does not modify tumorigenesis driven by the KRAS oncogene.

**EZH2-Driven Lung Cancer as a Molecularly Distinct Entity**

To dissect the molecular features of EZH2-induced mouse lung cancer, we first performed RNA sequencing (RNA-seq) to compare the gene expression profiles of EZH2-overexpressing precancerous normal lung tissue and EZH2-overexpressing lung adenocarcinoma tumors from mice (Supplementary Table S3). Using unsupervised hierarchical clustering, gene expression profiles from these samples were compared to expression profiles of EGFR-mutated and KRAS-mutated lung adenocarcinoma mouse tumors (Fig. 2A). EZH2-overexpressing tumors segregated as transcriptionally distinct from EZH2-overexpressing normal lung and EGFR-mutated and KRAS-mutated lung tumors. These data suggest that EZH2 modulation of chromatin leads to alteration of transcriptional pathways distinct from canonical lung adenocarcinomas driven by EGFR and KRAS hyperactivating mutations. Additionally, analysis of RNA-seq data for secondary driver mutations revealed that there are no recurrent somatic alterations in the coding portion of expressed known driver oncogenes or tumor repressors in murine EZH2-overexpressing tumors (data not shown).

Having defined an EZH2-dependent, tumor-specific transcriptional state in murine lung adenocarcinoma, we next assessed whether a comparable subset of human NSCLC exists. Using publicly available data from The Cancer Genome Atlas (TCGA), we identified a cohort of patients with lung adenocarcinoma with elevated tumor EZH2 expression (Supplementary Fig. S2A). Nearly all lung tumors overexpressed EZH2 as compared to normal tissue. EZH2 expression in human lung cancer was found to be broadly distributed over a >50-fold range, and high EZH2 levels were not mutually exclusive with KRAS or EGFR mutations. In tumors with the highest EZH2 expression (mean + 2 SD, 28 tumors), 21% harbored canonical “driver mutations” (e.g., EGFR and KRAS) whereas 43% of tumors harbored these mutations in the overall dataset (230 tumors). We further selected highly EZH2-overexpressing tumors (top 20%) with WT KRAS and EGFR to emulate the genetics of our murine models. Pathway enrichment was assessed by Gene Set Enrichment Analysis (GSEA; refs. 32, 33). Transcriptional signatures associated with MEK and mTOR activation were repressed in EZH2-overexpressing tumors as compared with tumors with low EZH2 expression regardless of the presence or absence of oncogenic KRAS or EGFR mutations (Fig. 2B), corroborating again that EZH2-driven tumors are molecularly distinct from tumors driven by canonical signaling pathways.

**Influence of EZH2 Overexpression on Chromatin Structure in Murine Lung Cancer**

To understand the dynamic effects of EZH2 overexpression on chromatin structure in the context of malignant transformation, we performed comparative epigenomic analysis of normal and malignant murine lung tissues. We and others have used genome-wide assessment of enhancer–promoter activity, measured by H3K27ac chromatin immunoprecipitation with massively parallel DNA sequencing (ChIP-seq), to comparatively study malignant and inflammatory cell states (34, 35). Focusing epigenomic analysis on regions of massive H3K27ac enrichment, so-called super enhancers (SEs), has afforded inferences into oncogenic signaling (36) and the subclassification of human tumors (34). Therefore, we first mapped active enhancers across the three tissue types by H3K27ac ChIP-seq, and identified regions of differential hyperacetylation (SEs), as we previously reported (34, 37). Unsupervised hierarchical clustering segregated murine tumor (KRAS- and EZH2-driven tumors) and pulmonary tissue (WT), demonstrating a distinct euchromatin epigenome structure (Supplementary Fig. S2B). Differential analysis of
Figure 2. EZH2-driven lung cancer as a molecularly distinct entity. 

A, heatmap of log2 fold-change (LFC) gene expression in murine EZH2-overexpressing (OE) normal lungs (green), KRAS-mutant lung tumors (black), EGFR-mutant lung tumors (blue), and EZH2-OE lung tumors (red). All genes were selected across all samples for clustering. B, box plot of ssGSEA comparing the enrichment of MEK (left) and mTOR (right) gene sets in human TCGA lung adenocarcinomas with specific driver mutations (KRAS, EGFR, unknown) or high EZH2 levels. C, waterfall plot showing rank-ordered change in H3K27ac signal at SE-containing regions between mouse WT lung and EZH2_OE (left), and KRAS and EZH2_OE (right). The x-axis depicts the LFC in H3K27ac signal. SEs are ranked by LFC in signal with regions gaining the most H3K27ac in tumor at the top. D, core transcriptional regulatory circuitry in murine WT and tumor lung tissues (EZH2- or KRAS-driven) as defined by ChIP-seq for H3K27ac. Nodes are transcription factors (TF) that are associated with an SE. Edges indicate a regulatory interaction between two TFs as defined by an enrichment of TF binding motifs in the respective SE.

highly occupied H3K27ac regions in EZH2-overexpressing tumors and normal lung revealed global redistribution of H3K27ac, with 771 individual loci exhibiting a greater than log2 1.5-fold change in H3K27ac (Fig. 2C, left). The same analysis between EZH2-overexpressing and KRAS-driven tumors also revealed a distinct redistribution of H3K27ac, with 317 individual loci exhibiting a greater than log2 1.5-fold change in H3K27ac (Fig. 2C, right).

Differences in gene expression and the SE signatures upon EZH2 overexpression versus KRAS-driven transformation in mice may be due to changes in the activated signaling molecules leading to modulation of different transcription...
factor (TF) networks. To identify TFs responsible for the gene expression changes and SE signatures upon EZH2 versus KRAS transformation, we identified TF binding sites enriched at SEs activated upon transformation. We constructed an SE-defined transcriptional network for normal and tumorigenic cell states by integrating maps of SE-associated master TFs with TF binding site data to reconstruct the core transcriptional regulatory circuitry (38). Our analysis defines three independent core transcriptional regulatory circuitries, which further underscores the mechanistically unique tumorigenic activity of EZH2 versus KRAS (Fig. 2D). Thus, we have further established a unique signature of EZH2-overexpressing murine lung tumors, which exhibit divergent core circuitries where deregulated master TFs are enforced by a unique network of super-enhancer–associated TFs.

**Influence of Super-Enhancers on Gene Expression in EZH2-Overexpressing Lung Cancer**

To determine whether changes in H3K27ac at SEs resulted in reciprocal changes in gene expression at adjacent expressed genes, we analyzed RNA-seq data from WT and EZH2-overexpressing murine tumors and found that the modulation of chromatin affected gene expression in these tumors (Fig. 3A). Unbiased leading-edge analysis of genes proximal to regions of lost H3K27ac in murine tumors identified polycomb repressive target gene signatures, implicating PRC2-mediated repression of these regulatory elements (Fig. 3B and C).

To link EZH2 function to lost SEs, we next performed H3K27me3 ChIP-seq on WT and EZH2-overexpressing mouse samples. Among regions of lost H3K27ac SEs, we identified a distinct subgroup of 33 cis-regulatory regions where loss of H3K27ac was accompanied by strong gain of the polycomb H3K27me3 mark (Fig. 3D). Functionally, 32 genes associated with these 33 regulatory regions showed decreased gene expression in murine tumors by RNA-seq (Fig. 3E), and were comprised of numerous developmental transcriptional regulators, including Foxa1, Irf8, Hoxa9, and Meis1, as well as other chromatin factors such as Afl3 (Fig. 3F). Repression of Hoxa9, Meis1, Irf8, and Foxa1 has been observed in NSCLC, and their activity has been functionally linked to decreased tumorigenesis, suggesting a tumor-suppressive role for these gene regulators (39, 40). Visual inspection of master developmental TFs repressed in tumor samples confirmed an epigenomic switch from large hyperacetylated enhancer elements to broad regions of H3K27 trimethylation (Supplementary Fig. S2C–S2E).

Among the gained SE-associated regions in murine tumor samples, notably, were genes encoding well-characterized, trimethylation (Supplementary Fig. S2C–S2E).

**Human NSCLC with High EZH2 Expression Is Sensitive to EZH2 Depletion**

To examine the oncogenic potential of EZH2 in human cells, we overexpressed EZH2 in an immortalized normal human lung epithelial cell line (hTBE) and monitored oncogenic potential (Supplementary Fig. S3A). EZH2-overexpressing hTBE cells (oEZH2) formed colonies on soft agar and acquired higher transformation capacity over time (serial passages) as compared to control cells (Supplementary Fig. S3B and S3C). Differential SE landscape analysis from hTBE and hTBE-oEZH2 cells illustrates a significant gain and loss of SEs when EZH2 is overexpressed (Supplementary Fig. S3D). These data demonstrate that overexpressing EZH2 modifies the SE landscape as a primary effect of its overexpression in human cells.

The observation that EZH2 overexpression produces lung cancer in mice and transforms normal human lung epithelia cells suggests that EZH2 may play an essential role in a subset of human NSCLCs. Using two previously verified EZH2 shRNAs, we knocked down EZH2 expression in a panel of rare, human oncogene WT NSCLC cell lines (41–43). Both shRNAs showed nearly complete inhibition of EZH2 protein expression as compared to cells with a nontargeting shRNA control (Fig. 4A). Cell proliferation assays revealed that H522 and H661 cells, which express high levels of EZH2 with no other known oncogenic mutations, displayed more than 50% growth inhibition in response to EZH2 knockdown compared to control cells (Fig. 4B and C; Supplementary Fig. S3E). Growth of human NSCLC cell lines expressing slightly lower levels of EZH2, namely, H292, H969, H2250, and H2258 cells, were largely unaffected by knockdown of EZH2 (Fig. 4B and D; Supplementary Fig. S3E and S3F). Finally, we tested whether EZH2 expression was required for tumor formation in mouse xenograft models of human NSCLC. H661 and H292 cells expressing either control or shEZH2 vectors were injected subcutaneously into mice, and tumor formation was monitored biweekly. EZH2 knockdown significantly inhibited growth of H661 tumors in vivo while having no effect on H292 tumor formation (Fig. 4E and F). Therefore, we identified human lung cancer cell lines that are oncogene WT and highly dependent on EZH2 overexpression.

**Dependency of EZH2-Overexpressing Lung Cancer on Catalytically Active EZH2**

To assess the dependency of EZH2-overexpressing lung tumors on sustained EZH2 enzymatic activity, we used a chemical genetic approach. Recently, several pyridinone-based small-molecule inhibitors of EZH2 were reported as chemical probes (e.g., GSK126, UNC1999, and EPZ-6438; Supplementary...
Figure 3. EZH2 overexpression establishes a unique and conserved super-enhancer–associated transcriptional landscape. A, box plot of RNA-seq expression in units of FPKM of murine genes associated with SEs that are gained (1,812 genes), unchanged (4,421 genes), or lost (432 genes) in tumor versus WT lung tissues. Significance was calculated using a two-tailed t test. **, \( P < 2 \times 10^{-4} \); ***, \( P < 2 \times 10^{-6} \). B, scatter plot of normalized enrichment score (NES) versus false discovery rate (FDR) q-value comparing MSigDB curated gene set enrichment in murine tumor versus WT SE-associated genes. The x-axis shows NES for evaluated gene sets. The y-axis shows false FDR q-value for each gene set. Gene sets upregulated in tumors have a high positive NES, whereas downregulated gene sets have a negative NES. Dotted line indicates significance cutoff q-value of 0.05. Red dots indicate PRC2-associated signatures, \( n = 8 \). C, SE-associated gene set enrichment analysis showing downregulation of EED targets in murine tumor versus WT tissues from RNA-seq analysis. The y-axis shows false FDR q-value for each gene set. Gene sets upregulated in tumors have a high positive NES, whereas downregulated gene sets have a negative NES. Dotted line indicates significance cutoff q-value of 0.05. Red dots indicate PRC2-associated signatures, \( n = 8 \). D, heat map of LFC in H3K27ac over H3K27me3 signals at SE-containing regions. Blue regions indicate SEs with strong gains of H3K27me3 in tumors \( n = 33 \). E, dot plot of RNA-seq expression in units of log10 FPKM for genes proximal to SE regions with a strong gain of H3K27me3 in murine tumor versus WT. Significance was calculated with a two-tailed t test. **, \( P < 1 \times 10^{-5} \). F, 32 mouse genes proximal to SE regions with strong H3K27me3 gain. G, gene tracks of ChIP-seq signals in units of rpm/bp for H3K27ac and H3K27me3 at the DUSP4 locus in either murine WT or tumor lung tissues. H, Western blot analysis of lysates prepared from murine normal lung (N-1, N-2, and N-3) and lung tumor (T-1, T-2, and T-3) samples. I, box plot of ssGSEA comparing the enrichment of our mouse H3K27me3 gene set in human TCGA lung adenocarcinomas with high EZH2 levels and normal lung tissue. The H3K27me3 gene set is comprised of the 32 mouse genes proximal to SE regions with strong H3K27me3 gain in murine EZH2-overexpressing tumors.
Fig. S4A; refs. 28, 44, 45). Both of these near-structural analogues are potent and selective inhibitors of EZH2; however, the broader utility of these probes in biological research in vivo may be limited by low potency (high dose administration), limited bioavailability (twice-daily dosing), and uncertain availability (cost of synthesis, pharmaceutical material transfer, undisclosed structure of EPZ-6438 while we conducted these studies). We therefore undertook to develop an EZH2 inhibitor as an open-source chemical probe for the scientific community. Lacking the guidance of crystallographic data, structure–activity relationships were deduced empirically from iterative analogue synthesis and biochemical testing.

Emerging from follow-up chemistry is JQEZ5, which features a pyrazolopyridine core displaying a 6-substituted solubilizing feature and a preserved pyridinone warhead (Fig. 5A; highlighted in red). The synthesis of JQEZ5 is nine linear steps, high-yielding and scalable (see Supplementary Methods), to support broad distribution. As a paired control, we developed JQEZ23 with the substitution of the active pyridinone to a predicted inactive pyridinium ring (Fig. 5A; highlighted in blue). Both compounds were evaluated in enzymatic assays with a five-component PRC2 complex with radiometric labeled S-adenosyl methionine (SAM). JQEZ5 inhibited enzymatic functionality of PRC2 with a biochemical IC₅₀ of 80 nmol/L, similar to GSK126 and UNC1999, whereas JQEZ23 had little inhibitory activity toward purified PRC2 (Fig. 5B; Supplementary Fig. S4B). JQEZ5 exhibited SAM-competitive inhibition of PRC2, as determined by

Figure 4. A subset of human NSCLC cells are dependent on EZH2 overexpression. A, human NSCLC H661 (left) and H292 (right) cells expressing nontargeting control shRNA (NT) or two different shRNAs targeting EZH2 (shEZH2-A and shEZH2-B) were analyzed for EZH2 expression by Western blotting. B, Western blots comparing EZH2 expression levels between human NSCLC cell lines H661 and H292. C and D, relative cell growth of H661, H522 (C), or H292 (D) cells expressing nontargeting control shRNA (NT) or two different shRNAs targeting EZH2 (shEZH2-A and shEZH2-B) was measured by MTS assay. Error bars, SEM; n = 3. **, P < 0.001. E and F, human NSCLC cell lines, H661 and H292, infected with lentivirus containing control (NT) or shEZH2 (shEZH2-A and shEZH2-B) were s.c. injected into the flank of nude mice. When the biggest tumor reached approximately 150 mm³, mice were euthanized and tumors were quantified relative to shNT tumor size (E) and documented (F). Mean ± SEM, n = 3/treatment.
biochemical inhibition assessed in the presence of escalating unlabeled SAM cofactor concentration (Fig. 5C; Supplementary Fig. S4C). To understand the putative mode of molecular recognition of EZH2 by our inhibitor, we modeled binding of JQEZ5 to EZH2 using a recently reported computational model (Fig. 5D; ref. 46). The binding model we established indicates that the d-pyridinone ring of JQEZ5 binds to Asn78 on EZH2, and that the pyrazolopyridine ring is deeply buried in the SAM-binding pocket of EZH2. The ligand interaction diagram (LID) of JQEZ5 and EZH2 (Supplementary Fig. S4D) also predicts that the piperazine ring on JQEZ5 extends out of the SAM-binding pocket of EZH2 and is thus amenable to further modification. Based on this prediction, we appended a biotin tag to the piperazine ring of JQEZ5 to create JQEZ6 (Supplementary Fig. S4E). Using surface plasma resonance (SPR), we measured the binding affinity of the five-component PRC2 complex for JQEZ6 immobilized on a streptavidin chip surface. The binding affinity measured by SPR (87.0 nmol/L) is similar to other biochemical assays that we performed (Fig. 5E). Negative control compounds (ex. biotinylated-JQ1) did not exhibit binding to the PRC2 complex (data not shown), further demonstrating the specificity of JQEZ6 for the PRC2 complex. Finally, the specificity of JQEZ5 for EZH2 was confirmed via an orthogonal study of a panel of 22 recombinant, purified lysine methyltransferases (Reaction Biology; Supplementary Fig. S4F; ref. 47).

After biochemically validating the specificity and potency of JQEZ5, we treated human oncogene WT NSCLC cells in...
dose-ranging biochemical and cellular studies. H661 cells treated with increasing concentrations of JQEZ5 demonstrated acutely reduced levels of H3K27me3 without affecting H3K27 mono- or dimethylation, as assessed by Western blotting (Fig. 6A). Treatment had no effect on H3K27me3 levels in the nonsensitive H292 human NSCLC cell line. Treatment with the negative control compound, JQEZ23, did not have any effect on H3K27 methylation or acetylation status (Fig. 6B). H3K27me3 reduction correlated with compound concentration as well as length of treatment (Fig. 6C). Similar to our EZH2 shRNA studies, JQEZ5 suppressed the proliferation of EZH2-overexpressing H661 and H522 cells after treatment for 7 days without affecting the proliferation of cell lines that were deemed insensitive to EZH2 knockdown (Fig. 6D and E; Supplementary Fig. S5A and S5B). In a direct comparison to alternate SAM-competitive EZH2 inhibitors, JQEZ5 inhibited cell growth more effectively than GSK126 and GSK343 in the H522 cell line (Supplementary Fig. S5B). Finally, shRNA knockdown of EZH2 in H661 cells rendered cells insensitive to JQEZ5-induced cell growth arrest (Fig. 6F). Together, these data demonstrate the limited off-target activity of the chemical probe and support the notion that JQEZ5 is effectively targeting EZH2 in human lung cancer cell lines.

**JQEZ5 Inhibits Tumor Growth in EZH2-Overexpressed Lung Cancer**

To further explore the translational relevance of this research, we undertook therapeutic trials of JQEZ5 in tumor-bearing GEMMs. JQEZ5 was formulated for intraperitoneal (i.p.) administration, and repeat dosing studies established 75 mg/kg i.p. daily as a tolerated dose and schedule with no observed toxicities (Supplementary Fig. S5C). Pharmacokinetic studies confirmed excellent exposure to JQEZ5 without the need for twice-daily dosing (Supplementary Fig. S5D).
To prepare for therapeutic studies, Actin-Cre;LSL-EZH2 mice and UBC;LSL-EZH2 mice (treated with tamoxifen at 6 weeks of age) were monitored weekly for the onset of symptoms of lung adenocarcinoma (breath distress). At that time (t = 0), lung cancers were visualized and confirmed by MRI (Fig. 7A and B). Tumor-bearing mice were then treated with JQEZ5 for 3 weeks (75 mg/kg i.p. daily), and the tumor volume of the lungs was comparatively visualized by MRI. Animals treated with JQEZ5 exhibited rapid and pronounced tumor regression over the 3-week treatment course, as demonstrated by two-dimensional MRI and volumetric measurements (Fig. 7C). Additionally, H3K27me3 levels were largely reduced with treatment, further confirming the on-target effect of JQEZ5 in mice (Fig. 7D). To model the therapeutic opportunity of EZH2 inhibition in vivo, we evaluated the tolerability and antitumor efficacy of JQEZ5 in a murine xenograft model of human NSCLC cells (H661 cells). Tumor-bearing mice were treated with JQEZ5 administered by i.p. injection (75 mg/kg daily) or vehicle control for 18 days. Administration of JQEZ5 was well tolerated. The level of H3K27me3 was largely reduced with the treatment without effect on EZH2 levels in both the tumor (Fig. 7E) and the lung tissue of treated animals (Fig. 7F). More importantly, JQEZ5 treatment attenuated tumor progression as determined by serial volumetric measurement (Fig. 7G) to further confirm the therapeutic potential of EZH2 inhibitors in this subset of human lung cancer.

**DISCUSSION**

As the leading cause of death from cancer, lung cancer comprises a profound unmet medical need. A subset of patients with lung cancer have benefited from targeted therapies in the past decade, yet the majority of patients will not benefit from these approaches, and all metastatic disease remains incurable. There exist a large number of patients who cannot be effectively treated due to the lack of druggable oncogenic drivers (e.g., KRAS). As such, discovering new actionable drivers and tumor dependencies in these remaining tumors is an urgent and important endeavor.

Though not affected by somatic alteration, deregulated overexpression of EZH2 is observed in a subset of human lung cancers (22–24). To date, a causal role in lung tumor development has not been established. In this study, we explored the oncogenic potential of EZH2 deregulation by generating a series of GEMMs with conditional EZH2 overexpression. These GEMMs establish evident oncogenic activity for EZH2 in NSCLC. Indeed, 45% of mice engineered to overexpress human and murine lung cancer models on EZH2. Toward pharmacologic target validation, we have demonstrated the dependency of EZH2-overexpressing human and murine lung cancer models on EZH2. Using functional genetic (shRNA) and chemical genetic approaches, we have demonstrated the dependency of EZH2-overexpressing human and murine lung cancer models on EZH2. Toward pharmacologic target validation, we have created a SAM-competitive inhibitor that establishes an evident therapeutic index for targeting EZH2-overexpressing tumors in vivo. JQEZ5 demonstrates consistent antitumor activity at 75 mg/kg versus reported studies of GSK126 at doses as high as 150 mg/kg (28). We expect the JQEZ5 chemical probe to be useful to the broader research community, in a manner similar to our experience openly distributing probes for BET bromodomain (51). Successful clinical translation of targeted lung cancer therapeutics has been facilitated by genomic or immunohistochemical biomarkers. Here, we show that EZH2 is essential for the growth of human lung cancer cell lines that express high levels of EZH2, while being dispensable for cell lines with lower levels of EZH2. As such, EZH2 expression may be a useful biomarker for patient selection or planned stratification in downstream clinical trials. These findings support prior work in prostate cancer, where EZH2 was found to be dispensable for cell growth in LNCaP-ABL cells with higher EZH2 levels but not in LNCaP cells with lower EZH2 levels (43).
**Figure 7.** JQEZ5 inhibits lung cancer growth in vivo. **A** and **B**, MRI scans of individual tumor-bearing Actin-Cre;LSL-EZH2 mice (t = 0) and after 1 to 3 weeks of treatment with JQEZ5 at 75 mpk, daily. Lung tumor is indicated by the red circle. H, heart. **C**, quantification of relative tumor volume of mouse lungs based on MRIs using 3D Slicer. Relative tumor volume was compared before and after 3 weeks of JQEZ5 treatment (mean ± SEM, n = 2). **D**, tumor-bearing Actin-Cre;LSL-EZH2 mice were untreated or treated with JQEZ5 at 75 mpk for 3 weeks. Lung sections were prepared and immunostained for H3K27me3. Scale bar, 50 μm. **E**, nude mice were injected s.c. with 2×10⁶ H661 human NSCLC cells. When tumors reached ~200 mm³, mice were randomized and treated with vehicle or JQEZ5 (75 mg/kg/d, i.p.) for 18 days. Western blot analysis was performed on tumors following either vehicle or JQEZ5 treatment for 18 days. **F**, Western blot analyses of lung tissue from mice after 18 days of treatment with vehicle or JQEZ5 at 75 mpk. **G**, tumor volume from the mouse xenograft model of human lung cancer was measured by caliper (mean ± SEM; n = 3/vehicle, n = 6/JQEZ5).
EZH2 inhibition has previously been proposed as a therapeutic strategy in NSCLC in the context of BRG1 or EGFR mutations (41). This study found that EZH2 inhibitors can sensitize NSCLC cells with EGFR or BRG1 mutations to chemotherapy, and a combination of EZH2 inhibition with Topoisomerase II inhibition was proposed. Here, we establish EZH2 inhibition as an effective single-agent therapy in the defined subset of NSCLC that overexpresses EZH2 without other known concurrent oncogenic mutations. Still, we anticipate that the genetic complexity and tumor heterogeneity that is a common feature of lung adenocarcinoma will require pairing EZH2 inhibition with other effective therapeutic strategies. Pairing epigenomic EZH2 inhibition with oncogene-directed therapy or immune checkpoint therapies is an appealing concept.

In sum, this work establishes an oncogenic role for EZH2 deregulation in lung adenocarcinoma, creates faithful models of a unique subset of human disease, describes and characterizes a chemical probe for studying EZH2 function in both xenograft and GEM models, and provides the rationale for human clinical investigation.

**METHODS**

**Cell Culture**

H661 (ATCC HTB-183), H292 (ATCC CRL-1848), and H522 (ATCC CRL-5810) human lung carcinoma cell lines were purchased from and authenticated through routine short tandem repeat analysis and cytogenetic studies by ATCC. H969, H2250, and H2258 cell lines were a kind gift from Dr. William C. Hahn at Dana-Farber Cancer Institute. All other cell lines were received and last authenticated in 2011; all other cells were received and last authenticated by standard methods in 2014. Human lung carcinoma cell lines were cultured in RPMI-1640/10% FBS/1% penicillin-streptomycin. The 293ft cells (Invitrogen, #R40048) and sequenced by SE75 Next-Seq.

**Western Blot**

Tissues were lysed in 1X lysis buffer (Cell Signaling Technology, #9803) with protease inhibitor cocktail (EMD Biosciences, 539131). Relative quantifications were performed using ImageJ software.

**Chromatin Preparation**

Mouse lung tissue was pulverized using a Covaris Tissue Smasher (model #CP02) following the CryoPrep Dry Pulverization Manual. Lung tissue was smashed 1 to 2 times on setting 4 in the tissueTUBE (Covaris, #S20071). Approximately 50 mg of pulverized lung tissue was cross-linked with prewarmed 1% formaldehyde (ThermoScientific, #28906, diluted in PBS) for 20 minutes at 37°C. The tissue was spun down at 1,000 rpm for 2 minutes and quenched with 0.125 mol/L glycine in PBS + 0.5% BSA for 20 minutes at room temperature. It was spun down at 1,000 rpm for 2 minutes and washed with PBS + 2X Protease Inhibitor Cocktail (PIC, Roche, #1187358001) + 5 mmol/L Sodium Butyrate (Millipore, #19-137) and then spun down at 1,000 rpm for 2 minutes. The cross-linked tissue was lysed with 390 μL ChIP Lysis Buffer (1% SDS, 10 mmol/L EDTA pH 8.0, 50 mmol/L Tris-HCl pH 8.0, 2X PIC and 5 mmol/L Sodium Butyrate) on ice for 1 hour. The lysate was split into 3 microTUBE (Covaris, #520045) and sheared on the Covaris E210 Series with 5% Duty Cycle, 5 Intensity, 200 Cycles per Burst for a total of 27 minutes. The sheared chromatin was spun down at 14,000 rpm for 15 minutes at 4°C. An aliquot of input was saved and the remaining chromatin was snap-frozen and stored at −80°C. Input was brought up to 100 μL with Tris-EDTA (TE), 10 μg of RNaseA (Roche) added and incubated for 30 minutes at 37°C followed by the addition of 100 μg of Proteinase K (Roche) and incubation at 65°C overnight. Input was purified with Qagen PCR Purification Kit (#28104) and quantified.

**Library Preparation and ChiP**

The prepared chromatin was thawed on ice while 10 μg of antibodies against either H3K27ac (Abcam, #Ab4729) or H3K27me3 (Cell Signaling Technology, #CS9733S) was conjugated to a mix of magnetic Protein A- and Protein G-coupled beads (Invitrogen, #100.02D and #100.04D, respectively) in the presence of 0.5% BSA in PBS with rotation at 4°C for 2 hours. Beads were washed 3 times with 0.5% BSA in PBS and either 5 μg of chromatin was added to the H3K27ac ChiP or 10 μg of chromatin was added to the H3K27me3 ChiP and rotated overnight at 4°C. The beads were washed twice with Tris-based RIPA buffer (0.1% SDS, 1% Triton X-100, 10 mmol/L Tris-HCl pH 7.4, 1 mmol/L EDTA pH 8.0, 0.1% sodium deoxycholate), 2 times with 0.3 mol/L NaCl RIPA (0.1% SDS, 1% Triton X-100, 10 mmol/L Tris-HCl pH 7.4, 1 mmol/L EDTA pH 8.0, 0.1% sodium deoxycholate, 0.3 mol/L NaCl), 2 times with LiCl Buffer (250 mmol/L LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mmol/L EDTA pH 8.0, 10 mmol/L Tris-HCl pH 8.0), and 2 times with TE buffer pH 7.6 (Fisher Scientific, cat. no. BP2474-1). The beads were resuspended in 100 μL of TE and RNaseA and proteinase K digested/reverse cross-linked and purified as described in the Chromatin Preparation section. Chromatin libraries were prepared with 10 to 20 ng of Input or ChiP'd DNA according to the ThruPLEX-FD Prep Kit (Rubicon, #R40048) and sequenced by SE75 Next-Seq.

**PRC2 Methylltransferase Assay**

Recombinant five-component PRC2 (EZH2/EED/SUZ12/RBBP4/AEBP2) was coexpressed in S9P cells and purified as described (52). PRC2 activity was measured using a radiometric Scintillation Proximity Assay (SPA) performed in 384-well OptiPlates (Perkin Elmer). For IC50 determination, 2.3 pmol/L PRC2 was incubated for 90 minutes at room temperature with 1 μmol/L histone H3 (21–44)-lys(biotin) (Anaspec), 1.5 μmol/L SAM (NEB), and 500 nmol/L H-SAM in 20 μL reaction buffer (50 mmol/L Tris pH 8.5, 5 μmol/L DTT, and 0.01% Tween-20) containing compound or DMSO. Reactions were quenched with trichloroacetic acid and, following the addition of PVT streptavidin-coated SPA beads (Perkin Elmer; 40 μL of 140 ng digested in PBS), incubated for 1 hour at room temperature. Continuous peptide methylation values were measured using the TopCount NXT plate reader. Percent activity values were calculated by setting the average background (no-enzyme wells) to 0% and the average DMSO wells to 100% activity. Standard deviations were determined from four replicate measurements for each compound concentration. Data were analyzed and plotted using GraphPad Prism v6, using the “log(inhibitor) vs. normalized response – variable slope” analysis module to calculate IC50.

For determination of JQ1E25 mechanism of action and Ki values, reactions were carried out as described above in the presence of
varying concentrations SAM/γH-SAM (at a 1:20 ratio) with a fixed concentration of 1 μmol/L histone H3 peptide. Data were analyzed and plotted using “Enzyme Kinetics – inhibition” and “Enzyme Kinetics – substrate versus velocity” analysis modules in GraphPad PRISM v6.

In Vivo Studies with JQEZ5

JQEZ5 was dissolved in DMSO and then diluted 1:10 in 10% (2-Hydroxypropyl)-β-cyclodextrin (Sigma-Aldrich). DMSO (vehicle) was diluted 1:10 in 10% (2-Hydroxypropyl)-β-cyclodextrin. Tumor-bearing GEMMs were monitored for onset of symptoms (breath distress) and then treated with JQEZ5 for 3 weeks (75 mg/kg, i.p. daily). Tumors were visualized by MRI, and tumor volume of the lungs was calculated using 3D Slicer (S3). For xenograft experiments, H661 cells were dissociated into single cells, counted and resuspended at 2 × 10^6 cells per 250 μL of 1:1 media/Matrigel (BD). Eight-to-12-week-old female FOXN1+/−/FOXN1−/− (Nude) mice (Harlan) were injected s.c. with 2 × 10^6 cells in two to three spots on the flanks. Tumors were allowed to grow to an approximate size of 200 mm^3 (~10 weeks), and the mice were randomized for vehicle (n = 3) or JQEZ5 administration (n = 6, 75 mg/kg/d, i.p.) for 18 days. Tumor growth was measured by caliper measurements, and tumor volume was calculated by standard methods. All mice were housed in pathogen-free animal facilities, and all experiments were performed with the approval of the Animal Care and Use Committee at Harvard Medical School and Dana-Farber Cancer Institute.

Sequencing Data

Sequencing data reported in this article have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE70047.

Synthesis of Small-Molecule Inhibitors

GSK126, GSK343, and UNC1999 were purchased directly from Sigma-Aldrich, Inc. The structure and purity of these two compounds were further confirmed by nuclear magnetic resonance and LC/MS. The detailed syntheses of compounds JQEZ5, JQEZ6, and JQEZ23 are described in Supplementary Methods.

Disclosure of Potential Conflicts of Interest

M. Brown reports receiving a commercial research grant from Novartis and is a consultant/advisory board member for the same. J.E. Bradner is President, NIBR, at Novartis. No potential conflicts of interest were disclosed by the other authors.

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