Lysine-Specific Demethylase 1 Mediates AKT Activity and Promotes Epithelial-to-Mesenchymal Transition in PIK3CA-Mutant Colorectal Cancer

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ABSTRACT

Activation of the epithelial-to-mesenchymal transition (EMT) program is a critical mechanism for initiating cancer progression and migration. Colorectal cancers contain many genetic and epigenetic alterations that can contribute to EMT. Mutations activating the PI3K/AKT signaling pathway are observed in >40% of patients with colorectal cancer contributing to increased invasion and metastasis. Little is known about how oncogenic signaling pathways such as PI3K/AKT synergize with chromatin modifiers to activate the EMT program. Lysine-specific demethylase 1 (LSD1) is a chromatin-modifying enzyme that is overexpressed in colorectal cancer and enhances cell migration. In this study, we determined that LSD1 expression is significantly elevated in patients with colorectal cancer with mutation of the catalytic subunit of PI3K, PIK3CA, compared with patients with colorectal cancer with WT PIK3CA. LSD1 enhances activation of the AKT kinase in colorectal cancer cells through a noncatalytic mechanism, acting as a scaffolding protein for the transcription-repressing CoREST complex. In addition, growth of PIK3CA-mutant colorectal cancer cells is uniquely dependent on LSD1. Knockdown or CRISPR knockout of LSD1 blocks AKT-mediated stabilization of the EMT-promoting transcription factor Snail and effectively blocks AKT-mediated EMT and migration. Overall, we uniquely demonstrate that LSD1 mediates AKT activation in response to growth factors and oxidative stress, and LSD1-regulated AKT activity promotes EMT-like characteristics in a subset of PIK3CA-mutant cells.

Implications: Our data support the hypothesis that inhibitors targeting the CoREST complex may be clinically effective in patients with colorectal cancer harboring PIK3CA mutations.

Introduction

Cancer cells have numerous genetic and epigenetic alterations that contribute to tumor formation, progression, and therapy resistance. One role of chromatin-modifying complexes is to maintain cellular identity by chemically modifying amino acid residues on histone and nonhistone substrates. Lineage-specific transcriptional networks maintained by these complexes provide context for specific mutations that determines the phenotypic outcome of the mutation (1). This information can be leveraged to identify how chromatin modifiers synergize with specific driver mutations in tissue-specific tumor models and discover synthetic-lethal relationships.

Mutations in the PI3K/AKT pathway are critical for invasive properties and malignant transformation in colorectal cancer (2). The two most common genetic PI3K/AKT pathway alterations in colorectal cancer are activating mutations in the PI3K catalytic subunit gene PIK3CA or loss of the pathway suppressor PTEN (3). Mutations in PIK3CA occur in roughly 25% of patients with colorectal cancer (4) and have been functionally implicated in epithelial-to-mesenchymal transition (EMT), migration, and chemoresistance (5). While aberrant activation of the PI3K/AKT pathway has been implicated in colorectal cancer progression, single-nucleotide PIK3CA mutations that activate the PI3K/AKT pathway are not significantly associated with alterations in patient survival (6). These findings indicate that PI3K pathway-activating mutations may require additional factors for full activation of the pathway. Recently, the lysine demethylase JMJD2A was found to be critical for steps involved in activation of AKT, including the recruitment of AKT to the cell membrane and phosphorylation of AKT at threonine 308 (7, 8). These studies suggest that aberrant overexpression of chromatin-modifying proteins can further activate the PI3K/AKT pathway and therefore may work synergistically with PIK3CA mutations. Little is known with regard to how chromatin modifiers function in the context of PIK3CA mutation to mediate tumorigenic processes in the gut.

The chromatin modifier lysine-specific demethylase 1 (LSD1) is overexpressed in colorectal cancer and positively correlates with advanced tumor staging (9). LSD1 is functionally linked to EMT-like changes and invasion in colorectal cancer (10–12). LSD1 is a member of the RE1 silencing transcription factor corepressor (CoREST) complex (13), which also contains the scaffolding protein RCoR1 and other chromatin-modifying subunits, including histone deacetylase 1...
and 2 (HDAC1/2; refs. 14, 15). LSD1 and HDAC1/2 within CoREST demethylate and deacetylate active chromatin, respectively, to maintain a repressive chromatin state. In some cellular contexts, LSD1, as a member of CoREST, demethylates di-methyl Histone H3 Lysine 4 (H3K4me2) at the promoter of epithelial genes to drive colorectal cancer (10–12). Recent studies, however, have highlighted catalysis-independent functions for LSD1, where it instead acts as a scaffold for the CoREST complex to maintain transcriptional repression of lineage-specific genes (16, 17). For example, CoREST can confine expression of neuronal genes to neuronal cells by mediating their silencing in nonneuronal cell types through the recruitment of CoREST (14, 15, 18). Furthermore, mechanistic studies of LSD1 catalytic inhibitors in SCLC (19), AML (20, 21), and erythroleukemia (22) demonstrate that these inhibitors reactivate gene expression and alter processes such as survival, proliferation, and differentiation by disrupting the recruitment of CoREST to chromatin by SNAG domain transcription factors as opposed to inhibiting LSD1 demethylase activity. These studies further support the notion that noncatalytic LSD1 functions are critical for tumorigenesis.

We hypothesize that LSD1 overexpression synergizes with PIK3CA mutation to enhance invasive phenotypes in colorectal cancer. In this study, we demonstrate that LSD1 is significantly overexpressed in patients harboring PIK3CA mutations in the gut, but not in cancers arising from other tissues. This observation is functionally significant as we demonstrate that PIK3CA-mutant colorectal and stomach cancer cells exhibit reduced growth after perturbation of LSD1. We further find that LSD1 regulates activation of AKT at the level of phosphorylation at serine 473 and EMT characteristics downstream of active AKT through a noncatalytic scaffolding role in the CoREST complex. Altogether, we illustrate a paradigm wherein LSD1 synergizes with a specific PIK3CA mutation to enhance EMT characteristics and migration.

Materials and Methods
Cell culture and treatments
All cell lines were maintained in a humidified atmosphere with 5% CO2. Our study included five colon cell lines (HT29, SW480, HCT116, LoVo, and RKO) and one stomach cell line (AGS). HT29, SW480, HCT116, and LoVo cells were cultured in McCoy 5A media (Corning) and RKO and AGS were cultured in RPMI1640 media (Corning) supplemented with 10% FBS (Gibco). All cell lines were purchased from the ATCC and authenticated and tested for Mycoplasma by IDEXX on June 20, 2019. All cells used in experiments were passaged fewer than 15 times with most being passaged fewer than 10 times. For hydrogen peroxide (H2O2) treatments, 30% H2O2 (Sigma) was diluted using 4% paraformaldehyde in PBS for 15 minutes at room temperature. Cells were then incubated with anti-LSD1 (1:100) and anti-pAKT (1:100) in 1% BSA in PBST for 1 hour. This was followed by incubation with Alexa Fluor anti-mouse 488 (1:1,000) for 1 hour at room temperature. Coverslips were mounted using Prolong Gold Antifade with DAPI (1:100) in 1% BSA in PBST for 1 hour at room temperature. Cells were permeabilized with 0.5% Triton-X in PBS for 10 minutes at room temperature and then fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature. Cells were permeabized with 0.5% Triton-X in PBS for 10 minutes at room temperature and then fixed with 1% BSA in PBST (PBS + 0.2% Tween 20) for 30 minutes. They were then incubated with anti-LSD1 (1:100) and anti-pAKT (1:100) in 1% BSA in PBST for 1 hour at room temperature. This was followed by incubation with Alexa Fluor anti-rabbit 594 (1:500) and Alexa Fluor anti-mouse 488 (1:1,000) for 1 hour at room temperature. Coverslips were mounted using Prolong Gold Antifade with DAPI molecular probes (Cell Signaling Technology #8961). Images were acquired using Leica SP8 confocal microscope at a magnification of 63 ×. The NA of 63 × objective used is 1.4. Images were processed using ImageJ.

Knockdown, knockin, and transient transfections
LSD1 (KDM1A; TRCN0000327856), RCON1 (TRCN0000128570), and HDAC1 (TRCN0000195467, TRCN0000195103) knockdown (KD) constructs were purchased from Sigma-Aldrich mission shRNA; empty plasmid was used as a vector control. Lentiviral-mediated KDs were performed as described previously (23). CRISPR/Cas9 LSD1 KO plasmid (sc-430289) and LSD1 HDR plasmid (sc-430289-HDR) were purchased from Santa Cruz Biotechnology and knockout was performed according to manufacturer’s protocol. Individual LSD1 KO clones were isolated for experiments, or mixed population KO’s were used, as defined in the figure legends. Cells were transfected with Lipofectamine 3000 (Invitrogen) per the manufacturer’s protocol. N-terminus HA-tagged LSD1 plasmid was purchased from Sino Biologicals (HG13721-NY) with pCMV3-N-HA used as a negative control vector.

Site-directed mutagenesis
Mutagenesis was performed according to manufacturer’s protocol (NEB, E0554) using HA-LSD1 plasmid as template, and confirmed via Sanger sequencing by Eurofins Scientific. LSD1 K661A substitution primers were generated using NEBaseChanger.

Primer sequences
The following primer sequences were used: forward, CAACCT-TAAACgGTTGGTtTGG; reverse, CCAAATCCCATCCttTGG. Annealing temperature was 58°C.

Chromatin immunoprecipitation sequencing
Chromatin immunoprecipitation (ChIP) was performed using Diagenode iDeal ChiP-seq Kit (C01010055, for transcription factors; C01010057, for histone modifications) as per the manufacturer’s protocol. Libraries were generated for sequencing using the NEBNext Ultra DNA Library Prep Kit for Illumina (NEB) as per the manufacturer’s protocol.

Chromatin-bound fraction and whole-cell isolation
Chromatin-bound (or tight chromatin) fractions were isolated as described previously (23). For protein isolation, cell pellets were lysed in 4% SDS buffer using a Qiashredder (Qiagen). Relative densitometry for Western blots was determined using ImageJ software and normalized to density of loading controls Lamin-B, β-actin, or Histone H3. All antibodies used in this study are included in the Supplementary Materials and Methods.

Immunofluorescence and imaging
A total of 2 × 105 HT29 cells were grown on coverslips at 37°C. After 48 hours, they were treated with 250 μmol/L H2O2 for 1 hour. The cells were then fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature. Cells were permeabized with 0.5% Triton-X in PBS for 10 minutes at room temperature and then fixed with 1% BSA in PBST (PBS + 0.2% Tween 20) for 30 minutes. They were then incubated with anti-LSD1 (1:100) and anti-pAKT (1:100) in 1% BSA in PBST for 1 hour at room temperature. This was followed by incubation with Alexa Fluor anti-rabbit 594 (1:500) and Alexa Fluor anti-mouse 488 (1:1,000) for 1 hour at room temperature. Coverslips were mounted using Prolong Gold Antifade with DAPI molecular probes (Cell Signaling Technology #8961). Images were acquired using Leica SP8 confocal microscope at a magnification of 63 ×. The NA of 63 × objective used is 1.4. Images were processed using ImageJ.

Proliferation assays
Assays were performed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega #G7572) as per manufacturer’s protocol. Briefly, 1 × 104 cells were plated in 96-well plates and allowed to incubate under standard growth conditions. Luminescent signals were detected on a SYNERGY H1 microplate reader (BioTek) using Gen 5
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software (v2.09). All plate readings were normalized to a control plate to account for variance in plating density.

**Clonogenic growth and migration assays**

Five-hundred (HT29) or 1,500 (SW480) single cells were plated and allowed to culture at 37°C. After 10 days, cells were fixed with 10% formalin and stained with crystal violet. Crystal violet–stained cells were imaged using a SYNGENE G:BOX and quantified using the GeneSys and GeneTools programs. For migration, 7.5 × 10^4 cells in serum-free media were plated into transwell in 24-well plates (Corning #40578) for 48 hours with media containing 10% FBS at the bottom. Transwell inserts were stained using Hema 3 Stat Pack (Thermo Fisher Scientific #123-869). Migration inserts were randomized prior to manual quantification and the outer 5% of the inserts were not included during quantification to reduce edge-effect bias. All images were taken on an EVOS FL Auto microscope.

**RNA isolation for RNA sequencing and quantitative PCR**

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, 74104). For RNA sequencing of empty vector and LSD1 KD cells, libraries were generated using Illumina TruSeq Stranded mRNA (Illumina, 20020594), or as previously described for RNA sequencing of DMSO and GSK-LSD1–treated cells (24). For qPCR, RNA was used to generate cDNA via reverse transcription (Thermo Fisher Scientific, K1642). cDNA was amplified using gene-specific primers and FastStart Essential DNA Green Master (Roche, 06402712001). Cq values of nonhousekeeping genes were normalized to GAPDH expression. qPCR primer sequences listed below:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>LSD1</td>
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<tr>
<td>SNAI1</td>
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<tr>
<td>GADPH</td>
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**Sequencing, The Cancer Genome Atlas, and statistical analyses**

Detailed description included in the Supplementary Materials and Methods. All experiments were performed in biological triplicate unless otherwise specified, with representative results displayed. All quantitative plots depict mean ± SD unless otherwise stated.

**Data availability**

RNA-sequencing and ChIP-sequencing (ChIP-seq) data have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE139927.

**Results**

**Knockdown of LSD1 reduces phosphorylation of AKT S473**

To initially determine whether LSD1 works synergistically with PIK3CA, we analyzed The Cancer Genome Atlas (TCGA) patient data from seven different cancer types where PIK3CA mutations are common. LSD1 expression was significantly higher in PIK3CA-mutant versus wild-type (WT) tumors for gastrointestinal cancers colon adenocarcinoma (COAD) and stomach adenocarcinoma (STAD; Fig. 1A). There was a trend toward increased LSD1 expression in PIK3CA-mutant rectal adenocarcinomas (READ) in the modest number of cases analyzed. All other tumor types either had no significant change (BLCA, LUSC) or a significant decrease (BRCA, HNSC) in LSD1 expression in the presence of a PIK3CA mutation compared with PIK3CA WT tumors.

The PI3K pathway has been extensively shown to mediate signaling through the activation of AKT. AKT is synergistically activated (25) by phosphorylation of threonine 308 by PDK1 (26), and serine 473 mediated by mTORC2 as part of the mTORC/Rictor complex (27). Chromatin modifiers, specifically histone-demethylating enzymes, have recently been linked to the regulation of AKT phosphorylation (7, 8). Therefore, we wanted to test the hypothesis that LSD1 may be a mediator of complete AKT activation. shRNA-mediated KD of LSD1 resulted in a significant reduction in pS473-AKT relative to empty vector (EV) cells in both HT29 and SW480 colorectal cancer cell lines (Fig. 1B and C). There was no significant change in pT308-AKT in LSD1 KD SW480 cells and we were unable to detect pT308-AKT in HT29 cells, potentially due to low sensitivity. To confirm the effect of LSD1 on pS473-AKT, we used CRISPR to generate two LSD1 knock-out (KO) clones each in the SW480 and HT29 cell lines. KO of LSD1 also resulted in reduction of pS473-AKT (Fig. 1D and E). Importantly, reintroduction of LSD1 was sufficient to rescue pS473-AKT levels in both cell lines (Fig. 1D and E). To test the function of LSD1 in de novo activation of AKT, we stimulated pS473-AKT using H2O2 (28). LSD1 KD blocked H2O2-dependent pS473-AKT and, conversely, overexpression of LSD1 enhanced H2O2-dependent pS473-AKT (Fig. 1F).

Overexpression of LSD1 in combination with H2O2 reduced total AKT, potentially due to a feedback mechanism associated with hyperphosphorylation of AKT, as has been shown with some AKT inhibitors (29). LSD1-mediated regulation of pS473-AKT observed by Western blot was confirmed by immunofluorescence under both basal and H2O2-treated conditions (Fig. 1G). pS473-AKT was reduced in LSD1 KD cells compared with EV. LSD1-deficient cells exhibited no change in pS473-AKT after H2O2 treatment, while LSD1-proficient cells exhibited H2O2-induced pS473-AKT. To test the alternative hypothesis that mutant PIK3CA was directly causing increased LSD1 expression, we inhibited PI3K in PIK3CA-mutant colorectal cancer cells, but saw no change in LSD1 levels (Supplementary Fig. S1). Together, these results demonstrate that LSD1 is more highly expressed in PIK3CA-mutant colorectal cancer compared with WT PIK3CA colorectal cancer and that LSD1 levels positively correlate with pS473-AKT, highlighting a molecular connection between LSD1 levels and activation of the PI3K/AKT pathway.

**LSD1 catalytic activity is not required for its regulation of pS473-AKT**

LSD1 catalyzes demethylation of mono- and dimethylated lysine residues on histone (13) and nonhistone substrates (30). The oncogenic function of LSD1 in colorectal cancer has previously been attributed to its ability to demethylate H3K4me2 at the promoter of epithelial genes, causing their repression and promoting more aggressive cellular phenotypes via enhanced EMT (11, 12). In contrast, newly emerging research has documented noncatalytic oncogenic functions of LSD1 (16, 17, 19–22) and suggests we need to consider LSD1 enzymatic activity in the regulation of AKT activation. To address this, we first performed ChIP-seq of LSD1 in SW480 cells to identify direct binding targets genome-wide, generating a catalog of potential catalytic target loci. Consistent with other studies, LSD1 enrichment at genes was primarily detected near transcription start sites (TSS; ref. 31; Fig. 2A). To generate a list of potential LSD1 targets de novo and confirm the specificity of our antibody, LSD1 peaks were called by normalizing LSD1 enrichment in parental cells to our LSD1 CRISPR KO. We detected 6,221 LSD1 peaks shared between replicates. Using DNase sequencing data from the SW480 cell line, we observed depletion of DNase cleavage centered at LSD1 peaks genome-wide (Fig. 2B), confirming occupancy of these regions. This DNase
footprint is not an artifact of LSD1 enrichment at TSSs, as we do not observe an overall depletion of DNase cleavages over TSSs (Supplementary Fig. S2A). To determine whether LSD1 KD alters genome-wide histone methylation status, we performed ChIP-seq for the LSD1 substrate H3K4me2 in control and LSD1 KD SW480 cells. There was a slight decrease in H3K4me2 enrichment genome-wide after LSD1 KD,
in contrast to the increase that would be expected on the basis of LSD1's known histone demethylase activity (Fig. 2C). There was no significant change in bulk H3K4me2 after LSD1 KD suggesting the slight change in ChIP-seq may not be biologically relevant (Supplementary Fig. S2B). To identify potential direct transcriptional targets of LSD1, we performed RNA-seq after LSD1 KD in SW480 cells. We combined these transcriptional data with our ChIP-seq datasets to assess H3K4me2 levels at genes with LSD1 enrichment near their promoters and significantly increased expression after LSD1 KD. The promoter regions of genes FBN3, TRPM6, and RASD2 are depicted as

Figure 2.
LSD1 catalytic activity is dispensable for regulation of gene expression and activation of AKT. A, Metageneplot and heatmap depicting ChIP-seq of LSD1 in WT (n = 2) or LSD1KO (n = 1) SW480 cells at gene enrichment sites genome-wide. Average plots and heatmaps depicting LSD1 enrichment peak overlap with DNase-seq peaks in SW480 (B) and H3K4me2 ChIP-seq signal at TSS enrichment sites genome-wide in shEV and shLSD1 SW480 cells (N = 3). C–E, Values are derived from CPM (counts per million) normalized reads. E, Differentially expressed genes (DEG) from RNA-seq (log2FC/21 and FDR/20 = 0.05 = purple) after 40 nmol/L GSK-LSD1 for 48 hours versus DMSO or shLSD1 versus shEV in SW480 cells (N = 3). D, ChIP-seq gene tracks of representative DEGs in LSD1 versus EV KD SW480 cells with or without LSD1 promoter enrichment. F, Cells pretreated with DMSO or 40 nmol/L GSK-LSD1 for 48 hours then treated with 250 μmol/L H2O2 for 1 hour. Western blots were quantified by densitometric analysis and normalized to loading control and DMSO. Graph represents mean ± SD; ns, not significant. Significance determined using one-way ANOVA with Tukey multiple comparisons test (N = 3). G, Mixed population LSD1 KO cells were transfected with vector control, HA-LSD1, or HA-LSD1 (K661A) for 48 hours. Whole-cell extract from untreated and cells treated with 250 μmol/L H2O2 for 1 hour were analyzed by Western blot analysis.
representative loci for this analysis. There were no significant changes in H3K4me2 enrichment at the promoter of FBXN3, TRPM6, or RASD2 (Fig. 2D), nor at the promoter of any gene following LSD1 KD (data not shown). CDH1, a published target of LSD1, showed significantly increased gene expression (log2 FC = 1.070, FDR = 0.035) after LSD1 KD. However, there was no change in H3K4me2 at the CDH1 promoter after LSD1 KD, nor did we detect enrichment of LSD1. A recent study in PC3 prostate cancer cells demonstrated similar results, wherein levels of H3K4me2 following LSD1 KD only changed at the promoter of 2 genes (16). Together, these data suggest that LSD1 impacts gene expression in SW480 colon cells without demethylating H3K4me2.

To further characterize LSD1's role in SW480 cells, we treated these cells with a highly potent and selective inhibitor, GSK-LSD1 (31) and performed RNA-sequencing. Treatment with this small molecule had a minimal effect on gene expression (0 genes with expression significantly altered >Log2FC = 1), whereas LSD1 KD caused robust activation of gene expression, consistent with the role of LSD1 as a transcriptional repressor (Fig. 2E). In addition, while H2O2 treatment induced the expected increases in pS473-AKT, inhibiting LSD1 did not significantly alter basal or H2O2-induced pS473-AKT levels (Fig. 2F), with similar results in HT29 cells (Supplementary Fig. S2C). Moreover, catalytically defective K661A-mutant LSD1 was able to rescue pS473-AKT levels in LSD1 KO HT29 cells similarly to WT LSD1, both basally and in response to H2O2 (Fig. 2G). Together, these studies strongly support the concept that LSD1-mediated transcriptional repression and regulation of AKT activation are independent of LSD1 catalytic activity in these colorectal cancer cell lines.

**LSD1 regulates pS473-AKT by scaffolding the CoREST complex on chromatin**

Our RNA- and ChIP-seq data suggest that LSD1-mediated regulation of AKT activation is independent of demethylase activity; we therefore hypothesized that this regulation may instead occur through LSD1's stabilizing role in the repressive CoREST complex. To address this, we performed a chromatin fractionation assay. KD of LSD1 in SW480 cells led to a significant decrease in whole-cell and chromatin-bound levels of the CoREST scaffold protein RCOR1 (Fig. 3A). HDAC1 and HDAC2 can be recruited to assist in the repressive functions of CoREST complexes (32). While whole-cell levels of HDAC1/HDAC2 remain unchanged after LSD1 KD, there is a significant reduction in the chromatin binding of HDAC1 (Fig. 3A). This result supports the notion that loss of LSD1 reduces formation of the CoREST complex and therefore reduces HDAC1 recruitment to chromatin. This is consistent with a recent study, where loss of LSD1 decreased HDAC1 binding at LSD1-enriched enhancers in leukemia cells (20). There was a slight but insignificant decrease in the recruitment of HDAC2 to chromatin (Fig. 3A). It is possible that a smaller proportion of cellular HDAC2 is recruited to CoREST complexes in comparison with HDAC1. RCOR1 is one of three CoREST isoforms that interact with LSD1 (33). RCOR contains a SANT domain that enhances the complex's interaction with histones and therefore plays an important scaffolding role for the CoREST complex (34). To determine whether the CoREST complex functions in the regulation of AKT activation, we performed KD of RCOR1 using shRNA and induced pS473-AKT using H2O2. KD of RCOR1 in HT29 cells reduced basal levels of pS473-AKT and blocked H2O2-induced pS473-AKT (Fig. 3B). In addition, there was a decrease in whole-cell levels of LSD1 protein, consistent with the scaffolding function ascribed to RCOR1 (Fig. 3B).

To our knowledge, HDAC1 does not function as a scaffold in the CoREST complex, but instead contributes to the formation of a repressive chromatin environment by catalyzing the removal of acetyl marks from histone lysine residues. To determine whether loss of HDAC1 alone is sufficient to block AKT activation, we knocked down HDAC1 using two independent shRNAs and stimulated AKT using H2O2. HDAC1 KD had no effect on H2O2-induced pS473-AKT or whole-cell levels of LSD1 in HT29 (Fig. 3C) or SW480 cells (Supplementary Fig. S3A). Overall, KDs of proteins that stabilize the CoREST complex (LSD1 or RCOR1) were sufficient to perturb AKT activation while KD of a protein (HDAC1) that interacts with the core CoREST complex had no effect on AKT activation, indicating that an intact, chromatin-associated core CoREST complex is required for full activation of AKT.

Recently, a single-molecule hybrid inhibitor targeting multiple chromatin modifiers within the CoREST complex was synthesized. The compound corin contains an LSD1 inhibitor (tranylcypromine analogue) fused to a class 1 HDAC inhibitor (MS-275) and exhibits near irreversible inhibition of the CoREST complex (ref. 35; Fig. 3D). In vitro and cellular studies demonstrated this compound is selective for complexes containing HDAC1 and LSD1 over complexes that only contain HDAC1. Corin treatment led to a dose-dependent reduction in pS473-AKT, with the effect increasing over time (Fig. 3E). Consistent with HDAC1 inhibition, global H3K9Ac levels increased after treatment with corin. At similar concentrations, MS-275 alone does not reduce pS473-AKT (36).

To test whether these observations mirror the patient data available through the TCGA, we investigated RCOR1 expression in PIK3CA WT and mutant tumors. Like LSD1, expression levels showed significantly higher levels of expression in PIK3CA mutant compared with WT in gastrointestinal cancer types COAD, READ, and STAD (Fig. 3F). Generally, there was no significant association between RCOR1 expression and PIK3CA mutation in nongastrointestinal cancer types (Supplementary Fig. S3B). However, there was no significant correlation observed between LSD1 and RCOR1 expression in COAD and READ datasets, but there was a significant correlation in the STAD dataset (Supplementary Fig. S3C). Consistent with the idea that only LSD1 or RCOR1 need to be overexpressed to stabilize the other, high expression of LSD1 or RCOR1 in COADREAD patient data was significantly associated with PIK3CA mutation compared with patients with low expression of both LSD1 and RCOR1, and the magnitude of change was significantly greater than expected by random chance from permutation testing (Fig. 3G). Altogether these data suggest that LSD1 regulates AKT activation through a scaffolding function for the CoREST complex. An intact chromatin-bound CoREST complex is required to mediate positive regulation of AKT activation via regulation of gene expression. Furthermore, high expression of CoREST core members LSD1 or RCOR1 is significantly associated with the presence of PIK3CA mutation.

**LSD1 regulates EMT-associated gene programs in PIK3CA-mutant cells**

On the basis of our observations from clinical datasets (Fig. 1A), we tested a phenotypic link between PIK3CA mutational status and cellular levels of LSD1. We selected six cell lines for this analysis: five colon cancer lines (SW480, HT29, LoVo, HCT116, and RKO) and one stomach cancer line (AGS). SW480 and LoVo are PIK3CA WT, whereas HT29, AGS, HCT116, and RKO all carry PI3K-activating PIK3CA mutation(s). The cell lines selected have several other common colorectal cancer mutations, but none are unique to the PIK3CA-mutant or WT cells (Supplementary Table S1; ref. 37). LSD1 KD had
no effect on the growth of PIK3CA WT cell lines (Fig. 4A). Interestingly, LSD1 KD caused a significant reduction in growth in all PIK3CA-mutant cell lines. Similar results were obtained with LSD1 CRISPR knockout, where LSD1 KO significantly reduced growth in PIK3CA-mutant HT29 cells, but had no effect on the growth of PIK3CA WT SW480 cells (Supplementary Fig. S4A–S4D). These data suggest that PIK3CA-mutant cancers may be sensitive to targeting of LSD1, and raise the unique possibility that LSD1 may be important for the tumorigenic activity of PIK3CA mutations.

Because perturbing LSD1 abrogates activation of AKT in both HT29 (PIK3CA mutant) and SW480 (PIK3CA WT) cells, but only reduces the viability of HT29 cells, we hypothesized that AKT may be uniquely
Figure 4.
Gastrointestinal cell lines with mutant PIK3CA are sensitive to LSD1 KD. A, SW480, LoVo, HT29, AGS, HCT116, and RKO cell growth over a 5-day time course determined by the CellTiter-Glo Luminescent Cell Viability Assay (n = 4). Graph depicts mean ± SD. Statistical analyses are performed using two-way ANOVA and Sidak multiple comparisons test with all statistically significant comparisons shown. **, Padj < 0.01; ****, Padj < 0.0001. B, Correlation plot of RNA-seq data from LSD1 versus EV KD in SW480 and HT29 cells. Significant data points are defined as abs(Log2FC) ≥ 1 and FDR ≤ 0.05 for LSD1 compared with EV KD for each cell line, including those unique to HT29 (purple), unique to SW480 (red), and shared between HT29 and SW480 (black; n = 3). C, Ridge plot depicts LSD1 versus EV KD expression changes of genes contributing to max enrichment score of Hallmark and curated gene sets accessed from the molecular Signatures Database (v6.2). GSEA Ratio shows the ratio of genes contributing to max enrichment score, to the total number of genes in the gene set. Heatmap on right shows Log2FC value for each gene enriched in HALLMARK_PI3K_AKT_MTOR_SIGNALING in either HT29 or SW480 cells sorted by HT29 Log2FC. D, Network analysis of uniquely upregulated genes in HT29 after LSD1 KD with significantly enriched processes from the Reactome database. Similar terms were manually grouped and size and color of circles were set to indicate number of genes and the P value, respectively. E, Clonogenic growth assay. Significance determined by two-way ANOVA with Sidak multiple comparisons test (n = 3). Results are represented as mean ± SD (**, Padj < 0.01; ns, not significant).
targeting pathways associated with cell survival in HT29 but not SW480 cells. To test this hypothesis, we performed RNA-seq in the EV and LSD1 KD HT29 cells and generated datasets that consisted of genes with significantly altered expression in LSD1 KD versus EV for each cell line. When comparing these two datasets, there was very little overlap between genes upregulated by LSD1 KD, exemplified by a weak positive correlation \( R^2 = 0.121 \), suggesting that the majority of significant changes were unique between the two cell lines (Fig. 4B). The largest set of genes with significantly altered expression after LSD KD compared with EV by \( \geq 3 \)-fold was genes uniquely upregulated in HT29 cells. The only commonly downregulated gene between the datasets was LSD1, further validating the specificity of our KD. To identify conserved and divergent characteristics between the two datasets, we performed gene-set enrichment analysis (Fig. 4C). As validation, genes upregulated after LSD1 KD in an independent dataset were enriched and upregulated by LSD1 KD in both HT29 and SW480 cells as determined by RNA-seq. In agreement with our finding that LSD1 KD reduces recruitment of HDACs to chromatin, genes downregulated after HDAC1 and HDAC2 overexpression were also significantly enriched and upregulated in our data. In support of our finding that LSD1 is critical for AKT activation, genes upregulated during active PI3K/AKT signaling were significantly enriched in our HT29 dataset, and trended toward downregulated after LSD1 KD in both SW480 and HT29 RNA-seq datasets, indicating reduced active AKT signaling. Sixty-five PI3K/AKT signaling genes were downregulated between the two cell lines. While 51% were commonly downregulated, 69% of the remaining genes were uniquely downregulated in HT29 cells indicating LSD1 KD may have a more significant effect on gene expression in this pathway in HT29 cells. Furthermore, by gene ontology analysis of genes uniquely upregulated after LSD1 KD in SW480 cells, no enriched genesets were associated with proliferation indicating that there is likely not a compensatory pathway activated in SW480 cells to buffer loss of AKT activation (Supplementary Fig. S4E). In addition, genes downregulated by TGFβ1 were enriched and upregulated in HT29 cells after LSD1 KD (Fig. 4C). This is interesting because cross-talk between the PI3K/AKT and TGFβ signaling networks plays a critical role in tumor progression (38).

We next narrowed our focus to genes that were uniquely upregulated in the HT29 cells after LSD1 KD (N = 523) and performed hypergeometric enrichment analysis using the Reactome database. In agreement with another study in colorectal cancer cells, ontologies associated with immune response were enriched (39). We additionally identified pathways associated with extracellular matrix organization and cellular junction organization, one example being the formation of hemidesmosomes, which play a role in epithelial cell adherence to extracellular matrices and are lost during EMT (ref. 40; Fig. 4D). Among genes uniquely downregulated after LSD1 KD in HT29 cells, were mediators of EMT-associated elastic fibre formation, TGFβ3 and LOXL1 (data not shown). Together, these data suggest that LSD1 regulates EMT characteristics in HT29 cells. Clonogenic assays test the ability of a tumor cell to survive and grow in isolation, an important characteristic of EMT and stem-like cells, which may be a critical attribute for metastasis (41, 42). HT29 cells exhibited \( \geq 7 \)-fold reduction in clonogenic survival after LSD1 KD, while there was no significant loss of clonogenic survival in PIK3CA WT SW480 cells (Fig. 4E). Together, these data demonstrate that LSD1 KD causes a significant reduction in growth and survival as well as alterations in gene expression programs related to EMT in PIK3CA-mutant, but not PIK3CA WT colorectal cancer cell lines. LSD1 regulates protein stability of Snail through regulation of AKT

AKT has been extensively implicated in the EMT process through protein stabilization of the EMT-promoting transcription factor Snail family Transcriptional Repressor 1 (SNAI1 or Snail). Snail can be phosphorylated by GSK3β leading to its ubiquitination and subsequent proteasomal degradation (43). Active AKT can phosphorylate GSK3β at serine 9 (44), inhibiting its kinase activity toward Snail, and increasing the protein half-life of Snail. However, the cellular contexts that support the AKT–GSK3β–Snail axis are not well understood. While the downstream functional outcomes of PIK3CA mutations in different domains are poorly characterized, one study suggests they may alter different downstream pathways, and require unique cofactors to exert their oncogenic effects (45). Furthermore, PIK3CA mutations in the p85, C2, helical, and kinase domains all increase PIK3CA lipid kinase activity, but only mutations in C2, helical, and kinase domains are sufficient to robustly increase pS473-AKT and transform cells (46). The exact functions of C2 domain mutations in driving tumorigenesis have remained largely uncharacterized, as researchers have focused primarily on more commonly occurring kinase and helical domain mutations. We hypothesize that the activity of the AKT–GSK3β–Snail axis in colorectal cancer cells is dependent on the PIK3CA mutational status.

To test for the effect of PIK3CA mutational status on AKT-mediated stabilization of Snail protein, we inhibited AKT across our cell line panel using a potent and selective competitive pan-AKT inhibitor, GSK690693 (29). Treating cells with this inhibitor generates a feedback loop. Inhibiting AKT causes hyperphosphorylation of S473-AKT, reduction of AKT protein levels, and loss of pS9-GSK3β. Inhibiting AKT did not change or caused an increase in Snail protein levels in PIK3CA WT (SW480/LoVo) or PIK3CA kinase–mutant (HCT116/RKO) cells, respectively (Supplementary Fig. S5A–S5D). Inhibiting AKT in PIK3CA C2 (HT29) or C2 and helical (AGS) mutant cells led to a marked decrease in the protein levels of Snail (Fig. 5A and B) independent of significant changes in the mRNA levels of Snail (Fig. 5C). Using the TCGA pancancer datasets, we stratified seven different cancer types based on the domain frequency of PIK3CA mutations (Fig. 5D). While mutations in the C2 domain represented on average 10.6% of PIK3CA mutations across the different cancer types, mutations in gastrointestinal cancers of the stomach and colon/rectum were disproportionately higher at 22.2% and 14%, respectively. Together, these results suggest that activation of the AKT–GSK3β–Snail axis may depend on PIK3CA mutational status and correlates with mutations in the C2 domain which are more prevalent in some gastrointestinal cancers.

We next sought to determine whether LSD1 enhances EMT-like changes in HT29 cells through positive regulation of the AKT–GSK3β–Snail axis. LSD1 KD resulted in a strong reduction in Snail protein levels (Fig. 5E) that was independent of significant changes in SNAI1 mRNA (Fig. 5F). As expected, changes in Snail protein level were accompanied by decreases in pS473-AKT and pS9-GSK3β. Treatment of cells with proteasome inhibitor MG-132 was sufficient to rescue Snail protein levels after LSD1 KD (Fig. 5E). While reexpression of LSD1 in LSD1 KD cells was sufficient to rescue protein levels of Snail, reexpressing LSD1 in the context of AKT inhibition was not sufficient to rescue Snail protein levels, further supporting our hypothesis that LSD1 is acting upstream of AKT in the stabilization of Snail (Fig. 5G). LSD1 did not significantly effect AKT activation in AGS cells nor did LSD1 KD significantly reduce Snail protein level, supporting the notion that LSD1-mediated changes in Snail protein rely on LSD1.
regulation of AKT (Supplementary Fig. S5E). While LSD1 KD reduced pS473-AKT in SW480 cells, LSD1 KD had no significant effect on Snail protein level (Supplementary Fig. S5F). This result is consistent with our finding that inhibiting AKT has no effect on Snail protein in this cell line (Supplementary Fig. S5A). In additional cell lines with WT (LoVo) or kinase domain (RKO, HCT116) mutant PIK3CA where AKT inhibition did not alter Snail levels (Supplementary Fig. S5B–S5D), LSD1 KD did not alter pS473-AKT levels, further suggesting this axis is mutation-dependent (Supplementary Fig. S5G–S5I). Together, these data indicate PIK3CA C2 domain mutations may function in the activation of the AKT–GSK3β–Snail axis. Furthermore, our molecular studies establish LSD1 as a context-dependent upstream regulator of this pathway.

The CRC Subtyping Consortium has previously developed a classifier that can be used to subtype cancers into four groups CMS1 (MSI/immune), CMS2 (canonical), CMS3 (metabolic), and CMS4 (mesenchymal; ref. 47). While there was no significant difference in LSD1 expression between CMS1-CMS4 subtyped COADREAD datasets, LSD1 was uniquely upregulated (P < 0.05) in PIK3CA-mutant CMS4 mesenchymal subtype tumors compared with WT (Supplementary Fig. S6A and S6B). This finding suggests our observation that LSD1 is overexpressed in PIK3CA-mutant versus WT colorectal cancer tumors (Fig. 1A) is at least in part explained by significant differences in CMS4 subtyped tumors. Furthermore, we found that PIK3CA C2 domain mutations were most common in CMS4 subtyped tumors (Supplementary Fig. S6C). This observation, in addition to our AKT inhibitor studies, established a connection between PIK3CA C2 domain mutations and mesenchymal phenotypes.

**LSD1 is required for EGF-induced migration in HT29 cells**

EGF has previously been used to enhance migratory phenotypes through the AKT–GSK3β–Snail axis (48). HT29 cells exhibited little to no basal migration, but upon stimulation with EGF exhibited a significant >30-fold increase in migration (Fig. 6A and B). Importantly, loss of LSD1, inhibition of AKT, or inhibition of the CoREST complex completely blocked EGF-induced migration (Fig. 6A and B). Treatment with EGF altered HT29 cellular morphology, with cells becoming elongated and scattered;
however, LSD1 KO abrogated EGF-mediated changes in cellular morphology (Supplementary Fig. S7). Treatment of cells with EGF led to an increase in levels of pS473-AKT, pS9-GSK3β, and Snail at 24 and 48 hours (Fig. 6C). LSD1 KD completely blocked EGF-induced activation of pS473-AKT, pS9-GSK3β, and increase in Snail protein. LSD1 KD also increased levels of the epithelial marker Claudin-1. Together, these data suggest that LSD1 is required for EGF-induced migration mediated by the AKT–GSK3β–Snail pathway.

Discussion

In this study, we implicate LSD1 in the regulation of AKT activity. We find that PIK3CA-mutant colorectal cancer cell lines are sensitive to LSD1 KD, whereas WT cells are not. Using TCGA data, we provide evidence that this connection between LSD1 and PIK3CA mutation status may be unique to gastrointestinal tumor types. PI3K inhibitors, which have been successful in gynecologic and breast cancers with mutant PIK3CA, have failed in colorectal cancer clinical trials,
suggested that additional factors influence the PI3K pathway in colorectal cancer. We demonstrate for the first time that LSD1 regulates basal AKT activation as well as activation in response to both exogenous oxidative stress and growth factors and, in a subset of PIK3CA-mutant cells, LSD1-mediated AKT activity promotes EMT-like characteristics, including migration.

Our findings suggest that LSD1 may play a role in the full activation of AKT. We highlight, for the first time, an oncogenic function for LSD1 in the regulation of PI3K/AKT signaling via catalytically independent regulation of gene expression. We hypothesize that the CoREST complex represses gene(s) that normally function to regulate S473-AKT phosphorylation such as regulators of cell receptors, negative regulators of upstream kinases, and/or phosphatases. When the complex is disrupted, these genes are expressed and perturb AKT phosphorylation. Our RNA-seq data in HT29 cells confirmed LSD1 is recruited to promoter of E-cadherin (CDH1–SNAI1 interactions in the C2 domain, RBD and ABD appeared more cancer specific. Mutations in the C2 domain were disproportionately more common in gastrointestinal cancers of the stomach and colon/rectum. The exact nature of the relationship between PIK3CA mutation and LSD1 expression is currently unclear, and further studies are required. It is possible that LSD1 and PI3K signaling may cooperate during specification of EMT-promoting factors. Our hypothesis may also explain why there is a positive association between LSD1 expression in PIK3CA-mutant gastrointestinal cancers but not in other cancer types, suggesting that this interaction may be lineage dependent. It is also consistent with our observation of EMT-associated gene expression in colorectal cancer cell line harboring PIK3CA mutation within the C2 domain.

In our TCGA analyses of PIK3CA mutation frequencies, mutations in the helical and kinase domains were most frequent across the different cancer types while less common mutations such as those in the C2 domain, RBD and ABD appeared more cancer specific. Mutations in the C2 domain were disproportionately more common in gastrointestinal cancers of the stomach and colon/rectum. The exact nature of the relationship between PIK3CA mutation and LSD1 expression is currently unclear, and further studies are required. It is possible that LSD1 expression is more closely linked to specific PIK3CA mutations, and that the prevalence of the specific mutation in a given cancer type is driving the significance of the association between LSD1 expression and PIK3CA mutational status in the TCGA data. Our study also suggests that activation of the AKT–GSK3β–Snail axis to promote EMT may be dependent on PIK3CA C2 domain mutation. This mechanism for upregulation of Snail is different from a previously described mechanism by which PIK3CA with the H1047R kinase mutation upregulates SNAI2 mRNA expression (52). Furthermore, our finding of AKT signaling–dependent Snail stabilization by LSD1 is a distinct mechanism from stabilization of Snail through interaction with the CoREST complex (10). An important caveat to our PIK3CA mutational studies is that AGS cells contain mutations in both the C2 and helical domains of PIK3CA. Our study does not disprove whether helical domain mutations alone are associated with the AKT–GSK3β–Snail axis. Future studies are required to untangle the functional role of different PIK3CA mutations and to establish a mechanistic understanding of their differential roles in mediating the AKT–GSK3β–Snail axis. While we focus on the interaction between LSD1 and PIK3CA C2 domain mutation, we also demonstrated that LSD1 KD reduced proliferation of cell lines with PIK3CA kinase domain mutations. It will be important to study LSD1 under the context of different PIK3CA mutations to potentially identify a
synthetic lethal relationship and establish prognostic value in gastrointestinal cancers.

Herein, we used EGF as tool to induce migration of HT29 cells. The tumor microenvironment plays a critical role in promoting EMT via autocrine and paracrine signaling by growth factors such as EGF, which stimulates EMT and migratory phenotypes in tumor cells (53). Further in vivo studies are required to understand how LSD1 may regulate signaling events between tumors and the surrounding microenvironment.

Overall, we propose a model where the CoREST complex can act synergistically with C2 domain PIK3CA mutations and growth factors to fully active the PI3K/AKT pathway and stabilize Snail protein to enhance cell migration and survival (Fig. 6D). Genetic or therapeutic perturbation of the CoREST complex is sufficient to block cancer cell migration and reduce survival. This work suggests that PIK3CA-mutant colorectal cancer may be particularly sensitive to LSD1 inhibitors that block the interaction of CoREST with transcription factors.

Disclosure of Potential Conflicts of Interest

H.P. Mohammad is a senior scientific director at GlassmanSmithKline. No potential conflicts of interest were disclosed by the other authors.

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Lysine-Specific Demethylase 1 Mediates AKT Activity and Promotes Epithelial-to-Mesenchymal Transition in PIK3CA-Mutant Colorectal Cancer

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