KDM4A promotes NEPC progression through regulation of MYC expression

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Translational Relevance

Neuroendocrine prostate cancer (NEPC) represents a highly lethal subtype of prostate cancer that lacks effective treatment options. Since epigenetic dysregulation is a hallmark of NEPC, we aim to identify novel epigenetic drivers in NEPC progression and test the therapeutic efficacy of epigenetic inhibitors. By combining RNA sequencing (RNA-seq) analysis of publicly available datasets and immunohistochemical validation, we identify KDM4A as a highly expressed histone lysine demethylase in NEPC. Our preclinical results using genetic and pharmacological approaches demonstrate that KDM4A plays a critical role in NEPC growth *in vitro* and *in vivo*. The present work suggests that targeting KDM4A with pan-KDM4 inhibitors is a highly effective therapeutic approach in NEPC.

ABSTRACT

Purpose: Despite advancement in treatment, prostate cancer (PCa) remains the second leading cause of death among men. Neuroendocrine prostate cancer (NEPC) represents one of the most lethal forms of PCa and lacks life-prolonging treatment. Here we identified histone lysine demethylase KDM4A as a driver in NEPC progression and an effective therapeutic target.

Experimental Design: The expression of KDM4A in human and mouse NEPC was examined by analyzing publicly available RNA-seq datasets and immunohistochemistry (IHC). KDM4A was knocked down (KD) using lentiviral RNA-interference or knocked out (KO) using CRISPR/Cas9 genome editing. Western blotting, cell proliferation, soft-agar colony formation assay, subcutaneous xenograft, RNA-seq, ChIP-seq, and small molecule inhibitors treatment in mice were used to determine the functions of KDM4A, KDM4A's downstream signaling pathways, and the therapeutic response of KDM4 inhibitors.

Results: We found that KDM4A mRNA and protein are overexpressed in human and mouse NEPC compared to adenocarcinoma. Also, we showed that knockdown or knockout of KDM4A in NEPC cell lines suppressed cancer cell growth *in vitro* and *in vivo*. Mechanistically, we found that *KDM4A* KD led to suppression of MYC signaling through the direct transcriptional regulation of *MYC*. Importantly, MYC signaling is hyper-activated in human and mouse NEPC. Furthermore, a potent pan-KDM4 inhibitor QC6352 significantly reduced NEPC cell growth *in vitro* and *in vivo*.

Conclusions: We demonstrated that KDM4A drives NEPC progression through regulation of MYC and targeting KDM4A can potentially be an effective therapeutic strategy for NEPC.

INTRODUCTION

Neuroendocrine prostate cancer (NEPC) is a highly lethal subtype of castration-resistant prostate cancer (CRPC) with a median survival of seven months after initial diagnosis,[1-3] as compared to a median survival of 13 to 31 months in castration-resistant prostate adenocarcinoma, the more common subtype of CRPC.[4] NEPC is characterized by attenuated androgen receptor (AR) signaling, the expression of neuroendocrine lineage markers (e.g., synaptophysin), uncontrolled hyperproliferation, and widespread metastasis (e.g., bone, liver, and lung). De novo NEPCs are rare (2%-5%); the majority arise as a mechanism of resistance from prostate adenocarcinoma treated with potent AR pathway inhibitors (ARPIs).[5] The widespread use of ARPIs in non-metastatic CRPC and hormone-sensitive metastatic tumors has led to an increase in the incidence NEPC. Due to the lack of life-prolonging systemic therapies, there is an *urgent need* to better understand the mechanisms underlying the pathogenesis of NEPC.

Recent evidence suggests that epigenetic dysregulation is a hallmark of NEPC.[5] Aberrantly expressed transcription factors (TFs) and regulators of DNA methylation and histone modification were found in NEPC preclinical models and patients.[5] Extensive studies have led to the discovery of key regulators driving the transition from adenocarcinoma to NEPC.[5] Among the various epigenetic regulatory mechanisms, histone lysine methylation, which is balanced by writers (histone lysine methyltransferase [KMT]) and erasers (histone lysine demethylases [KDM]), plays an important role in development and cancer, including prostate.[6] Multiple lysine methylation modifiers (e.g., EZH2, WHSC1, DOT1L, LSD1/KDM1A, KDM4A, KDM4B)[7-11] have been implicated in prostate cancer progression. However, their roles in NEPC are just

started to emerge. For example, EZH2 has been recently implicated as a key player in NEPC.[5,12-17] Whether KDMs play any role in NEPC progression is unknown.

KDM4A regulates cell cycle, replication, and DNA damage response in cancers and is amplified or overexpressed in multiple cancer types, including breast, lung, and prostate.[18] In prostate cancer, KDM4A overexpression (OE) is not sufficient to drive the formation of prostate adenocarcinoma in a genetically engineered mouse model (GEMM).[19] However, KDM4A cooperates with loss of tumor suppressor PTEN and OE of oncogenic ETV1 to enhance the development of primary prostate cancer.[19] Although OE or shRNA KD of KDM4A affects prostate cancer cell proliferation,[19] whether KDM4A plays a role in NEPC is unknown. Here, we identified histone lysine demethylase KDM4A as a potential driver of NEPC progression and established alternative epigenetic pathways driving NEPC progression. We also showed that KDM4A is a promising therapeutic target, which will contribute to novel therapies to treat NEPC.

MATERIALS and METHODS

Mice strains

Pb-Cre4; Pten $^{loxP/loxP}$ (*Pten* $^{pc-/-}$), *Pb-Cre4; Pten* $^{loxP/loxP}$*;Smad4* $^{loxP/loxP}$ (aka ***P****ten*$^{pc-/-}$***S****mad4*$^{pc-/-}$, **PS**), and *Pb-Cre4;Pten*$^{loxP/loxP}$*;Trp53*$^{loxP/loxP}$ (aka *Pten*$^{pc-/-}$ *Trp53*$^{pc-/-}$, **PT**) models were developed previously[20]. *Rb1*$^{loxP/loxP}$ strain (FVB;129-Rb1tm2Brn/Nci) was obtained from NCI Mouse Repository. *Pten*$^{pc-/-}$*Smad4*$^{pc-/-}$ and *Pten*$^{pc-/-}$*Trp53*$^{pc-/-}$ mice were crossed with *Rb1*$^{loxP/loxP}$ mice to generate *Pb-Cre4;Pten* $^{loxP/loxP}$*;Smad4* $^{loxP/loxP}$*;Trp53*$^{loxP/loxP}$*;Rb1*$^{loxP/loxP}$ (***P****ten*$^{pc-/-}$***S****mad4*$^{pc-/-}$***T****rp53*$^{pc-/-}$***R****b1*$^{pc-/-}$, **PSTR**) and *Pb-Cre4;Pten*$^{loxP/loxP}$*;Trp53*$^{loxP/loxP}$*;Rb1*$^{loxP/loxP}$ mice (***P****ten*$^{pc-/-}$***T****rp53*$^{pc-/-}$***R****b1*$^{pc-/-}$, **PTR**). The genotyping of the Pb-Cre4 transgene and all the conditional alleles were performed using conventional PCR as described previously.[20-23] The copy number of TRAMP

transgene will be determined by quantitative PCR[24] and only mice that are homozygous for the transgene were used in this study. Mice were maintained in pathogen-free conditions at M.D. Anderson Cancer Center. All manipulations were approved under MD Anderson Cancer Center (MDACC) Institutional Animal Care and Use Committee (IACUC) under protocol number 00001713-RN01.

Human prostate tumor tissues and patient-derived xenografts (PDX)

FFPE Primary castration-resistant prostate tumor (CRPC) tissues, including prostate adenocarcinoma and NEPC, were obtained from Dr. Patricia Troncoso and Dr. Miao Zhang (MD Anderson Tissue Bank). FFPE and fresh tumor tissues from PDXs were obtained from Dr. Nora Navone (GU PDX core). The Movember PDX TMA has been described previously.[25]

Cell lines and cell cultures

PTR and PSTR cell lines were isolated from *Pten*$^{pc-/-}$*Trp53*$^{pc-/}$ *Rb1*$^{pc-/-}$ and isolated from *Pten*$^{pc-/-}$ *Smad4*$^{c-/-}$*Trp53*$^{pc-/-}$ *Rb1*$^{pc-/-}$ mice. All cell lines tested for mycoplasma were negative within 6 months of performing the experiments. Cell line authentication was not performed. 293T, NCI-H660, LASCPC-01, and MYC-CaP cells were obtained from ATCC. 144-13 was generated from PDX MDA PCa 144-13.[26] 293FT cells were obtained from Thermo Fisher Scientific Inc. 144-13, LASCPC-01 and NCI-H660 cells were cultured in HITES medium supplemented with 5% fetal bovine serum using ATCC-formulated RPMI-1640 Medium (Catalog No.30-2001) with the following components to the base medium: 0.005 mg/ml Insulin, 0.01 mg/ml Transferrin, 30 nM Sodium selenite (final conc.), 10 nM Hydrocortisone (final conc.), 10 nM beta-estradiol (final conc.), extra 2mM L-glutamine (final conc. of 4 mM).

Small interfering RNAs (siRNAs)/shRNA knockdown and CRISPR/Cas9 genome editing

siRNAs were ordered from Dharmacon Inc (human KDM4A: GUAUGAUCUUCCAGACUUA;

GUGCGGAGUCUACCAAUUU; mouse Kdm4a: GAACAUCCUACGACGAUAU; GUUCGUGAGUUCCGCAAGA; human MYC: AACGUUAGCUUCACCAACA; AACGUUAGCUUCACCAACA; mouse Myc: GGACACACAACGUCUUYGGA; UCGAAACUCUGGUGCAUAA). Lentiviral shRNA plasmids were obtained from Sigma Aldrich (Human KDM4A: shKDM4A#1 TRCN0000013493, shKDM4A#2 TRCN0000013494, shKDM4A#3 TRCN0000013495; Mouse Kdm4a: shKdm4a#2 TRCN0000103526). Mouse shKdm4a#4 plasmid was obtained from VectorBuilder of which sequence of siKdm4a#4 (GUUCGUGAGUUCCGCAAGA) was cloned into the lentiviral vector (VB210619-1017gjm). Lentiviruses were packaged in 293FT cells using second-generation packaging vectors, psPAX2 (Addgene plasmid 12260) and pMD2.G (Addgene plasmid 12259). Synthetic guided RNA (sgRNA) was ordered from Synthego Inc. Recombinant cas9 was obtained from Thermo Fisher Scientific Inc. sgRNAs and recombinant cas9 were transfected into cells according to the protocol from the manufacturer. Single clones were selected, and *Kdm4a* KO clones were confirmed by western blot analysis.

Chemicals and inhibitors

QC6352 (HY-104048), NCGC00247743 (HY-112308), Enzalutamide (HY-70002), and MYCi975 (HY-129601) were ordered from MedChemExpress Inc. Incucyte Caspase 3/7 Dye for Apoptosis (Cat#4440) was ordered from Sartorius.

Western Blot Analysis

Cells were lysed on ice using RIPA buffer (Boston BioProducts) supplemented with Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). Proteins with loading buffer (20 μg) were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. Membranes were blocked in 2% non-fat dry milk for an hour before being incubated with primary antibodies

prepared in Tris- buffered saline (TBS) containing 0.1% Tween 20 (TBST) consisting of 0.5% BSA overnight at 4°C. Next, the membrane was washed 3 times with TBS containing 0.1% Tween 20 (TBST) and was incubated with HRP- conjugated secondary antibody for 2 h at room temperature. After washing 3 times with TBST, the membrane was exposed to Clarity Western ECL Substrate (Bio-Rad) according to the protocol and imaged with Azure Biosystems c600. The following antibodies were used in this study: KDM4A (ab191433, Abcam), beta-Actin (MA5-15739, Invitrogen), cleaved-PARP (Mouse specific #9544; human specific #9546, Cell Signaling Technology) cleaved-caspase 3 (#9662, Cell Signaling Technology), cMyc (ab32072, Abcam), c-Anti-Rabbit IgG HRP- linked antibody (#7074, Cell Signaling Technology), Anti-Mouse IgG HRP-linked antibody (#7076, Cell Signaling Technology).

Immunohistochemistry analyses

Tissues were fixed in 10% formalin overnight and embedded in paraffin. Immunohistochemical (IHC) analysis was performed as described earlier (Aguirre et al., 2003). Primary antibodies used for immunohistochemistry are as follows: KDM4A (ab191433, Abcam), KDM4A (3393S, CST), Ki-67 (GTX16667, GeneTex), Synaptophysin (M7315, Dako), AR (ab133273, Abcam), Chromogranin A (20086, ImmunoStar). Secondary antibodies used are as follows: anti-rabbit (RMR622L, Biocare), anti-mouse (8125S, CST). The immunohistochemistry signals were developed with DAB Quanto (TA-125-QHDX, Epredia).

Cell proliferation, foci-formation, soft-agar colony forming, and apoptosis assays

For cell proliferation assay, $5x10^2$-$2x10^3$ cells per well were seeded in 96-well plates with or without corresponding treatment. After 5 days, absorbance was measured using Cell Counting Kit-8 (B34034, Bimake) according to the manufacturer's manual. For foci-formation assay, 1×10^3 cells were seeded in 6-well plates and cultured for 5 to 7 days before they were fixed and

stained with crystal violet as described.[27] For soft-agar colony forming assay, $2.5x10^3$ cells were seeded in 6-well. Cells were cultured for 3 weeks. Colony sizes and numbers were measured as described.[28] Apoptosis was detected by Western blot analysis of cleaved caspase 3 and cleaved PARP or by Cytation 5 using Caspase3/7 dye for apoptosis (Cat#4440, Sartorius).

Xenograft Studies

PSTR ($1x10^5$ cells/100ul per mouse) and 144-13 ($2x10^6$/100ul per mouse) cells were prepared in 1:1 PBS and Matrigel (BD Biosciences) and injected into flanks of 6-week-old nude mice (n = 5-10 per group). Mice were treated with 50 mg/kg QC6352 (once a day) when tumors reached $100mm^3$. Tumor growth was monitored by measuring tumor sizes twice a week. Tumor Volume was calculated according to the formula: Volume = length × $width^2/2$. All xenograft experiments were approved by the MD Anderson IACUC under protocol number #00001713-RN01.

Quantitative RT-PCR and RNA-sequencing

Cell mRNA was isolated by Direct-zol RNA MiniPrep (50ug) (#11-331, ZymoResearch). iScript cDNA Synthesis Kit (Bio-Rad) was used to generate cDNA for quantitative PCR (qPCR) analysis using PerfeCTa reg SYBR reg Green SuperMix Reaction Mixes (QuantaBio). The primers used for qPCR analysis were designed using Primer3 and ordered from Sigma Aldrich. The primers used were listed in Suppl. Table 4. RNA-seq of PTR cells with shRNA control and shKdm4a#2 and shKdm4a#4 was performed on total RNA (3 replicates) using NEBNext® Ultra II Directional RNA Library Prep Kit for Illumina NextSeq at the Cancer Genomics Center, The University of Texas Health Science Center at Houston. The raw data will be submitted to the NCBI Gene Expression Omnibus (GEO). Processed human RNA-seq datasets from previous studies (Grasso et al.,[29] Abida et al.,[30] and Beltran et al.[31]) were downloaded from cBioportal.[32,33] Publicly available RNA-seq datasets (GSE158467,[34] GSE90891,[13] GSE95293,[35] and

GSE137953[36]) were downloaded from NCBI GEO. DeSeq2 was used to identify differentially expressed genes (DEGs). Gene Set Enrichment Analysis (GSEA) was performed as described.[21]

Chromatin-immunoprecipitation (ChIP)-sequencing

ChIP-seq was performed in 144-13 cells as described[27] using the following antibodies at the MD Anderson Epigenomics Profiling Core: anti-KDM4A antibody ((#5766, lot 021110, Schuele Laboratory), H3K27ac (ab4729, Abcam), H3K9me3 (C15410056, Diagenode), H3K36me3 (ab9050, Abcam). Libraries were prepared using NEBNext Ultra DNA Library Kit (E7370). Sequencing was performed using an Illumina HiSeq 2500 instrument. Reads were aligned to a reference genome Hg19 as described.[37] The raw data will be submitted to the NCBI Gene Expression Omnibus (GEO). The publicly available ChIP-seq dataset GSE95190[35] was downloaded from NCBI GEO.

Statistical analysis

All the experiments were replicated at least twice in the laboratory except for microarray and ChIP. Data are presented as mean ± SD unless indicated otherwise. Student's t-test assuming two-tailed distributions was used to calculate statistical significance between groups.

**** $P < 0.0001$, *** $P < 0.001$; ** $P < 0.005$; * $P < 0.05$.

Data Availability Statement

The datasets generated during the current study are available from the corresponding author on reasonable request. The data generated in this study will be deposited into the publicly available Gene Expression Omnibus (GEO).

RESULTS

KDM4A is overexpressed in neuroendocrine prostate cancer (NEPC)

To examine whether histone lysine demethylases play a role in NEPC, we analyzed a well-annotated RNA-seq dataset,[31] which includes five pathologically defined subtypes based on a proposed classification approach:[38] adenocarcinoma (n=34), NEPC (n=7), mixed NEPC-adenocarcinoma (N=2), small cell prostate carcinoma (SCPC) (n=4), and mixed SCPC-adenocarcinoma (n=2). Due to the small sample size of the non-adenocarcinoma samples, we combined NEPC and mixed NEPC-adenocarcinoma as one group (NEPC), and SCPC and mixed SCPC-adenocarcinoma as another group (SCPC). We compared the genome-wide gene expression of NEPC (NEPC and mixed NEPC-adenocarcinoma) to adenocarcinoma and SCPC (SCPC and mixed SCPC-adenocarcinoma), respectively, to identify differentially expressed genes (DEGs). We identified 4669 and 1834 upregulated genes in NEPC compared to adenocarcinoma and SCPC, respectively (**Supple. Table 1-2**). Also, we identified 1525 and 33 downregulated genes in NEPC compared to adenocarcinoma and SCPC, respectively (**Supple. Table 1-2**). KDM4A and KDM5D were uniquely upregulated in NEPC compared to adeno-CRPC and SCPC among the histone lysine demethylases (**Figure 1A-B**). Because KDM4A, but not KDM5D (also named JARID1D),[39] was shown to play an oncogenic role in prostate adenocarcinoma,[19] we decided to focus on the functions of KDM4A in NEPC progression. To determine whether KDM4A mRNA is also upregulated in mouse NEPC, we took advantage of a publicly available RNA-seq dataset from Pb-Cre+;**P**ten$^{f/f}$;**N**MYC/NMYC;**R**b1$^{f/f}$ NEPC model (referred to as **PNR** hereafter), we found that KDM4A, but not KDM5D, is upregulated in advanced tumors from older mice (16 weeks) compared to tumors from younger mice (12-13 weeks) (**Figure 1C**), suggesting KDM4A overexpression may play a role in NEPC progression. Also, KDM4A is overexpressed in the more aggressive NEPCs from PNR mice compared to tumors from Pb-Cre+;**P**ten$^{f/f}$;**N**MYC/NMYC (**PN**) and Pb-Cre+;**P**ten$^{f/f}$;NMYC/+;**R**b1$^{f/+}$ (**PN^{het}**

R$^{\mathbf{het}}$) mice with comparable age (16-17 weeks) (**Figure 1C**), which develop prostate adenocarcinoma.

To confirm the findings from the human and mouse RNA-seq data, we performed IHC staining of KDM4A in human and mouse primary prostate adenocarcinoma and NEPC. We found that KDM4A is overexpressed in human NEPC but not prostate adenocarcinoma (**Figure 1D**). Also, we examined KDM4A protein expression in a well-characterized prostate cancer patient-derived xenograft (PDX) tissue microarray (TMA).[25] We found that KDM4A protein is overexpressed in NEPC PDXs compared to adenocarcinoma PDXs (**Figure 1E**). To determine the expression of KDM4A protein in murine NEPC, we examined its expression in three genetically engineered mouse models (GEMMs) that generate NEPC: TRAMP,[40] prostate specific conditional knockout of *Pten*/*Trp53*/*Rb1* (PTR),[13] and a recently generated prostate-specific cKO of *Pten*/*Smad4*/*Trp53*/*Rb1* (PSTR). We found that KDM4A protein is highly expressed in the primary tumors from all three NEPC GEMMs compared to two well-established prostate adenocarcinoma models, i.e., the prostate specific cKO of *Pten* and *Pten*/*Smad4* (PS model) (**Figure 1F & Suppl. Figure 1A**), consistent with our findings in human prostate tumor tissues. Of note, the specificity of KDM4A antibody was validated using *Kdm4a*-KO PSTR cells, a cell line derived from PSTR primary tumors (**Suppl. Figure 1B**). We also determined whether KDM4A overexpression is associated with neuroendocrine markers. Using the Beltran et al. RNA-seq dataset,[31] we observed a strong positive correlation between *KDM4A* mRNA and multiple neuroendocrine (NE) markers, including *CHGA*, *CHGB*, *ENO2*, and *DLL3* (**Figure 1G & Suppl. Figure 1C**). We also examined the expression of KDM4A in the Abida et al. RNA-seq data set,[30] which also contains a small subset of NEPC. We found that *KDM4A* mRNA is significantly higher in mCRPC tumor samples with neuroendocrine features compared to those

without NE features (**Suppl. Figure 1D**). Collectively, our data strongly suggested that KDM4A may play a role in NEPC progression.

KDM4A KD or KO impairs the growth of NEPC *in vitro* and *in vivo*

To examine the functions of KDM4A in NEPC progression, we used shRNA and siRNA to generate KDM4A knockdown (KD) and knockout (KO) cells. Mouse prostate cancer cell lines derived from PSTR and PTR primary tumors, which were confirmed to have upregulated expression of NE markers such as *Ncam1*, *Chga*, *Syp*, *Foxa2*, *Prox1*, and *Sox2* compared to Myc-CaP adenocarcinoma using qRT-PCR (**Suppl. Figure 2A-B**), were used in this study. We confirmed the efficient KD of *Kdm4a* using Western blot in PSTR cells (**Figure 2A**). Given the highly proliferative nature of NEPC cells, we first examined whether KDM4A is required for the proliferation of NEPC cell lines using *Kdm4a*- KD cells and control cells. We found that *Kdm4a* KD using shRNA or siRNA significantly reduced cell proliferation in PSTR cells as shown by foci-formation assay (**Figure 2B & Suppl. Figure 2C**). *Kdm4a* KD in PTR cells also reduced cell proliferation (**Figure 2C-D**). To confirm the findings using shRNA, we also used the CRISPR/Cas9 system to generate *Kdm4a* KO and control PSTR cells. We confirmed the complete KO of *Kdm4a* using Western blot (**Figure 2E**). We found that *Kdm4a* KO in PSTR cells dramatically reduced cell proliferation (**Figure 2F**). To determine the effect of KDM4A KD on human NEPC cells, we generated KDM4A KD and control human NEPC cells using the 144-13 and LASCPC-01 models (**Figure 2G & Suppl. Figure 2D**). Human NEPC cell line 144-13 was derived from an NEPC PDX MDA PCa 144-13)[26] and LASCPC-01 was derived from Myr-AKT/N-MYC transformed PCa.[16] We found that *KDM4A* KD in 144-13 and LASCPC-1 cells also significantly reduced cell proliferation (**Figure 2H & Suppl. Figure 2E**), consistent with

our findings using mouse NEPC cell lines. Together, our data suggest that KDM4A is required for the growth of NEPC cells *in vitro*.

We also tested the impact of *KDM4A* KD and KO in NEPC cells on the anchorage independence growth, an assay that measured the tumorigenicity of cancer cells *in vitro*, using soft agar colony formation assay. We found that *KDM4A* KD in 144-13 cells led to reduced colony numbers and sizes compared to control siRNA (**Figure 2I**). Similar results were observed in PSTR *Kdm4a*-KO cells (**Figure 2J**). To examine the effect of *Kdm4a* KO on the NEPC cell growth *in vivo*, we implanted *Kdm4a-KO* PSTR cells and control cells subcutaneously in nude mice. We found that *Kdm4a* KO significantly reduced tumor growth *in vivo* (**Figure 2K**).

Collectively, our data suggest that KDM4A plays an important role in NEPC growth *in vitro* and *in vivo*.

KDM4A regulates MYC expression to promote tumor progression in NEPC cells.

To determine the mechanisms by which KDM4A promotes NEPC progression, we performed RNA-seq of *Kdm4a*-KD and -control PTR cells. We identified 1270 genes that are downregulated and 2448 genes that are upregulated in *Kdm4a*-KD cells compared to control PTR cells (FDR≤0.05, fold change≥1.5) (**Suppl. Table 3**). We performed Gene Set Enrichment Analysis (GSEA) to identify pathways that are activated or suppressed in *Kdm4a* KD cells. We found that *Kdm4a* KD led to reduced activation of E2F signaling (**Suppl. Figure 3A**), which is consistent with previous findings that KDM4A interacts with E2F1 to regulate transcription.[41] Interestingly, we identified MYC signatures (HALLMARK MYC TARGETS V1 and HALLMARK MYC TARGETS V2) as the top downregulated pathways in *Kdm4a* KD cells (**Figure 3A**). Multiple MYC target genes (e.g., *Ldha*, *Nme1*, and *Srsf2*, etc.) were significantly

downregulated in *Kmd4a*-KD cells (**Figure 3B**). Interestingly, MYC has been implicated to play a functional role in NEPC, as overexpression of MYC, AKT1, BCL2, or RB1-KD/p53-KD transformed normal prostate epithelial cells into NEPC.[42] We also found that MYC is the top hallmark pathway activated in both human and mouse NEPC as shown by GSEA analyses of publicly available RNA-seq data of adenocarcinoma and NEPC from patients[31] and mice[13] (**Figure 3C-D**). These data suggest that KDM4A may promote NEPC progression through the regulation of MYC signaling.

To further delineate the mechanisms by which KDM4A regulates MYC signaling, we first determined whether KDM4A directly regulates MYC transcription, given the well-characterized function of KDM4A in epigenetic regulation. We performed qRT-PCR and Western blot analyses to examine the effect of *KDM4A* KD on MYC expression in PSTR, PTR, and 144-13 cell lines. We found that *KDM4A* KD reduced *MYC* mRNA and protein expression in all three cell lines (**Figure 3E-G & Suppl. Figure 3B**). To determine whether MYC is a direct target gene of KDM4A, we performed ChIP-seq in 144-13 cells using anti-KDM4A, anti-H3K9me3, anti-H3K36me3, anti-H3K27ac, and anti-CTCF. We found that KDM4A binds directly to the MYC promoter as shown by ChIP-seq analysis (**Figure 3H**), suggesting an active MYC transcription in NEPC cells. We also found that H3K9me3 is barely detectable at the MYC locus whereas H3K36me3 and H3K27ac were present at MYC locus (**Figure 3H**), suggesting active transcription at the MYC locus. As a positive control for anti-H3K9me3, ZNF114, ZNF333, and ZNF175, genes that are constitutively silent due to the large H3K9me3 domain, showed a robust H3K9me3 signal at its promoter region (**Suppl. Figure 3C-E**). Lastly, we determined the effect of *MYC* KD or MYC inhibitor on NEPC cell proliferation in 144-13 cells. We found that *MYC* KD or MYC inhibitor MYCi975 significantly reduced cell proliferation in

144-13 (**Fig. 3I-J**). Collectively, our data suggests that KDM4A promotes NEPC progression through direct regulation of MYC transcription.

KDM4 inhibitor suppresses cell proliferation in NEPC *in vitro*

Because our genetic data showed that KDM4A plays an important role in NEPC progression, we examined whether targeting KDM4A can be an effective therapeutic strategy for NEPC. Although there are no KDM4A-specific inhibitors, several pan-KDM4 inhibitors (KDM4i) [e.g.,QC6352,[35,43] NSC636819,[44] NCGC00244536,[45] IOX1,[46] SD70[47]] have been shown to suppress the growth of cancer cells. For example, QC6352 was shown to suppress the growth of colorectal cancer (CRC) and triple-negative breast cancer (TNBC), and NCGC00244536 was shown to suppress the growth of prostate cancer cell lines PC3 and LNCaP. Among them, QC6352 is a highly selective inhibitor against the KDM4 family and rigorously characterized (e.g., cocrystal structure with KDM4A) with potent anti-tumor activities, favorable pharmacokinetics, and low toxicity in mice.[35,43] We found that treatment of PSTR cells with QC6352 resulted in a significant increase in the global level of H3K9me3 and H3K36me3 expression, two of KDM4's substrates **(Figure 4A**). We then examined the effect of QC6352 on the growth of PSTR cells *in vitro*, which are largely resistant to AR inhibitor enzalutamide (ENZ) (**Suppl. Figure 4A**). We found that QC6352 treatment led to a dramatic decrease in the proliferation of PSTR cells in a dose-dependent manner (**Figure 4B**). QC6352 treatment also dramatically suppressed the proliferation of PTR cells (**Figure 4C**) and 144-13 cells (**Figure 4D**). Furthermore, NCGC00244536, another potent inhibitor for KDM4 histone lysine demethylase,[45] suppressed the proliferation of 144-13 and PSTR cells (**Figure 4D & Suppl. Figure 4B**). To determine whether KDM4A inhibition is responsible for the effect of QC6352 on

cell proliferation, we compared the response of *Kdm4a*-WT and -KO PSTR cells to QC6352 treatment. We found that *Kdm4a* KO largely abrogated the response of PSTR cells to QC6352 (**Figure 4E**), supporting the notion that KDM4A is the critical target of QC6352 in NEPC cells. Importantly, we found that MYC expression was downregulated in QC6352-treated NEPC cells compared to control cells (**Suppl. Figure 4C**), consistent with the role of KDM4A in regulating MYC expression.

KDM4 inhibitor suppresses NEPC progression *in vivo*

To determine whether QC6352 could suppress NEPC growth *in vivo*, we treated mice bearing PSTR tumors and 144-13 tumors subcutaneously in immune-deficient mice. We found that QC6352 treatment dramatically suppressed the growth of PSTR cells (**Figure 5A-B**) and 144-13 cells (**Figure 5C-D**), as shown by reduced tumor sizes and tumor weights. To ensure QC6352 has similar effects on tumor progression in immune-competent hosts, we treated PSTR GEMM with QC6352. Similarly, QC6352 treatment in PSTR GEMM model led to a significant reduction in tumor burden as shown by tumor weights (**Figure 5E).** We then examined the effects of QC6352 treatment on tumor cell proliferation. We found that QC6352 treatment led to a significantly reduced proliferation in PSTR GEMM as shown by Ki67 IHC staining (**Figure 5F**). We did not observe noticeable toxicity as shown by the measurement of peripheral blood count and the measurement of weight of major organs **(Suppl. Figure 5A-B)**, consistent with the findings in the original study.[43] Together, our data suggest that KDM4 inhibitor QC6352 can significantly delay NEPC progression in vivo.

DISCUSSIONS

Neuroendocrine prostate cancer (NEPC) has been increasingly observed in the clinic, and most of these NEPCs occur in patients who develop therapeutic resistance to potent androgen pathway inhibitors. Despite a high response rate to platinum-based chemotherapy that is of transient nature, there are no life-prolonging therapeutic options for NEPC patients. In recent years, epigenetic dysregulation has emerged as a hallmark for NEPC, and targeting epigenetic regulators has become a promising approach. Here we identified KDM4A as an important regulator of NEPC progression and demonstrated that targeting KDM4A can be a promising therapeutic approach.

KDM4A is an epigenetic modifier, and its dysregulation may alter the expression of many genes. Our findings that KDM4A regulates MYC expression in NEPC provide a novel mechanism for how KDM4A exerts its effects on prostate cancer progression. MYC has previously been shown to be a key player in prostate cancer progression and prostate-specific expression of MYC was sufficient to induce prostate cancer and promote tumor progression in mouse models.[48-50] Since Myc plays an important role in NEPC,[42] our study reveals a mechanism by which Myc is activated and provides a novel insight into how an epigenetic modifier regulates oncogene expression. This novel finding also promoted us to examine whether such regulation occurs in other cancer types. We analyzed a publicly available RNA-seq dataset[36] and found *Myc* mRNA was downregulated in mouse squamous cell carcinoma cell lines upon *Kdm4a* KO compared to control cells (**Suppl. Figure 6A**). Also, QC6352 treatment led to reduced expression of MYC mRNA in triple-negative breast cancer (TNBC) (**Suppl. Figure 6A**)using a publicly available RNA-seq dataset.[35] Furthermore, we also examined a publicly available ChIP-seq dataset (KDM4A) in TNBC[35] and found that KDM4A binds to MYC promoter/gene body in TNBC (**Suppl. Figure 6B**). These findings suggest that the regulation of

MYC expression by KDM4A may also contribute to tumor progression in other cancer types with KDM4A overexpression.

We showed that *KDM4A* KD or KO suppresses NEPC proliferation, at least in part mediated through MYC. Interestingly, GSEA analysis identified apoptosis signature in *Kdm4a*-KD PTR cells compared to control cells (**Suppl. Figure 6C**), although it does not rank as the top pathway. Thus, we examined whether *Kdm4a* KD led to apoptosis. We did not observe any noticeable increase in apoptosis in *Kdm4a* KD cells as shown by Western blot for cleaved caspase 3 and cleaved PARP1 (**Suppl. Figure 6D**). Interestingly, KDM4 inhibitor QC6352 not only suppresses cell proliferation but also induces apoptosis in PTR, PSTR, and 144-13 cells as shown by Western blot analysis for cleaved caspase 3 and cleaved PARP1 and positivity of green fluorescent signal from IncuCyte Caspase-3/7 Green Apoptosis Assay Reagent (**Suppl. Figure 4E-H**). Since QC6352 inhibits multiple KDM4 family members,[35,43] the increase in apoptosis observed in QC6352-treated cells may be attributed to inhibition of other KDM4 family members in NEPC cells. This notion is supported by the previous reports that KDM4 family members, such as KDM4B and KDM4D, play a role in DNA damage response.[51,52]

In summary, our study established KDM4A as an important epigenetic regulator that drives NEPC progression and can serve as an effective therapeutic target.

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Author Contributions

G.W. contributed to the study's conception and design of this study. G.W., C.M., M.Z., X.L., F.W., A.G.H., X.S., D.L., J.P., M.Z., P.T., and J.Z. performed the experiments and acquired, analyzed, and interpreted the data (e.g., statistical analysis, biostatistics, computational analysis). P.S., N.N., E.M., B.A.F., and R.S. provided key reagents. A.K.J., M.G.L, P.C., C.J.L., A.A., provided scientific inputs for the development of the project. C.M., M.Z., S.H.L and G.W. contributed to the writing and editing the manuscript.

Competing Interests statement

CJL reports receiving commercial research grants from Bayer, Sanofi, Janssen, Astellas Pharma, Pfizer; and honoraria from Bayer, Janssen, Sanofi, Astellas Pharma. No potential conflicts of interest were disclosed by the other authors.

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FIGURE LEGENDS

Figure 1. KDM4A is overexpressed in human and mouse NEPC. (**A**) Venn Diagram showing KDM4A and KDM5D as the two histone lysine demethylases that are overexpressed in NEPC using the Beltran et al. RNA-seq dataset. (**B**) The expression of *KDM4A* and *KDM5D* in NEPC compared to adeno-CRPC and SCPC. (**C**) *Kdm4a* mRNA is upregulated in prostate tumors from older PNR mice than tumors from young PNR mice, PN mice, and $PN^{het}R^{het}$ mice. (**D**) KDM4A IHC in primary NEPC and adenocarcinoma-CRPC. (**E**) KDM4A IHC staining in prostate cancer PDXs. (**F**) KDM4A IHC in primary tumors from PS, PTR, PSTR and TRAMP mice. (**G**) Correlation of KDM4A mRNA expression with NE markers *CHGA* and *CHGB* in the Beltran et al. RNA-seq dataset.

Figure 2. KDM4A knockdown or knockout suppresses NEPC growth in vitro. (**A-B**) *Kdm4a* KD in PSTR cells, as confirmed by Western blot (A), led to significantly reduced growth in foci-forming assay *in vitro* (B). (**C-D**). *Kdm4a* KD in PTR cells, as confirmed by Western blot (C), led to significantly reduced growth *in vitro* (D). (**E-F**) *Kdm4a* KO in PTR cells, as confirmed by Western blot (E), led to significantly reduced growth in foci-forming assay *in vitro* (F). (**G-H**)

KDM4A KD in 144-13 cells, as confirmed by Western blot (G), led to significantly reduced cell number (H). (**I-J**) *KDM4A* KD in 144-13 cells (I) or *Kdm4a* KO in PSTR cells (J) led to significantly reduced number of colonies and reduced colony sizes using soft agar colony formation assay. (**K**) *Kdm4a* KO delayed the growth of PSTR cells in subQ implantation model.

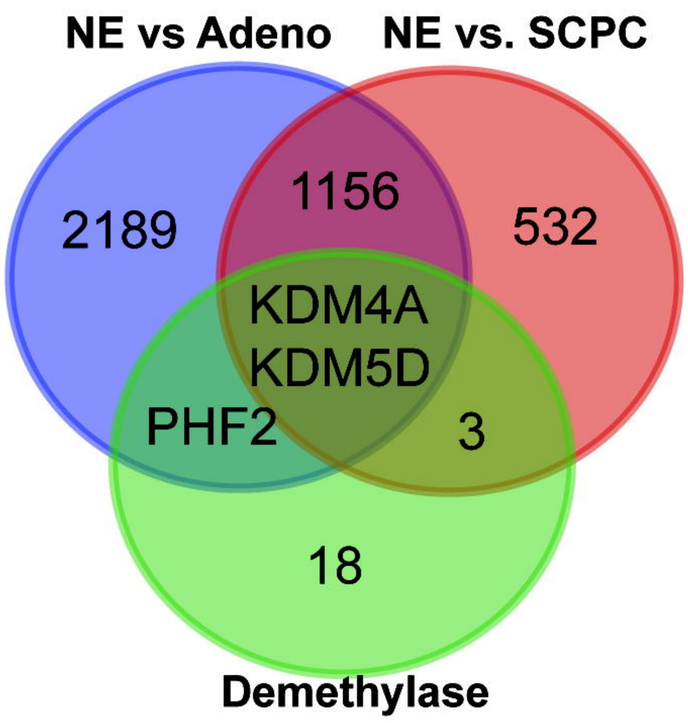
Figure 3. KDM4A regulates MYC transcription in NEPC. (**A**) GSEA analysis of RNA-seq on *Kdm4a*-KD and control PTR cells showed that MYC signaling is suppressed in *Kdm4a*-KD cells. (**B**) Fold changes in the expression of selective MYC-target genes comparing *Kdm4a*-KD cells to control cells. (**C-D**) GSEA analysis of Beltran et al. RNA-seq data (C) and Ku et al. RNA-seq data showed that MYC signaling is activated in NEPC compared to prostate adenocarcinoma. (**E-F**) Western blot showed that KDM4A KD led to reduced MYC expression in PSTR (E) and PTR (F) cells. (**G**) qRT-PCR showed that *Kdm4a* KD led to reduced *Myc* mRNA expression in PTR cells. (**H**) ChIP-seq analyses identified *MYC* as a KDM4A-target gene in 144-13 cells. (**I**) *MYC* KD led to reduced cell proliferation in 144-13 cells. (**J**) MYC inhibitor MYCi975 led to reduced cell proliferation in 144-13 cells.

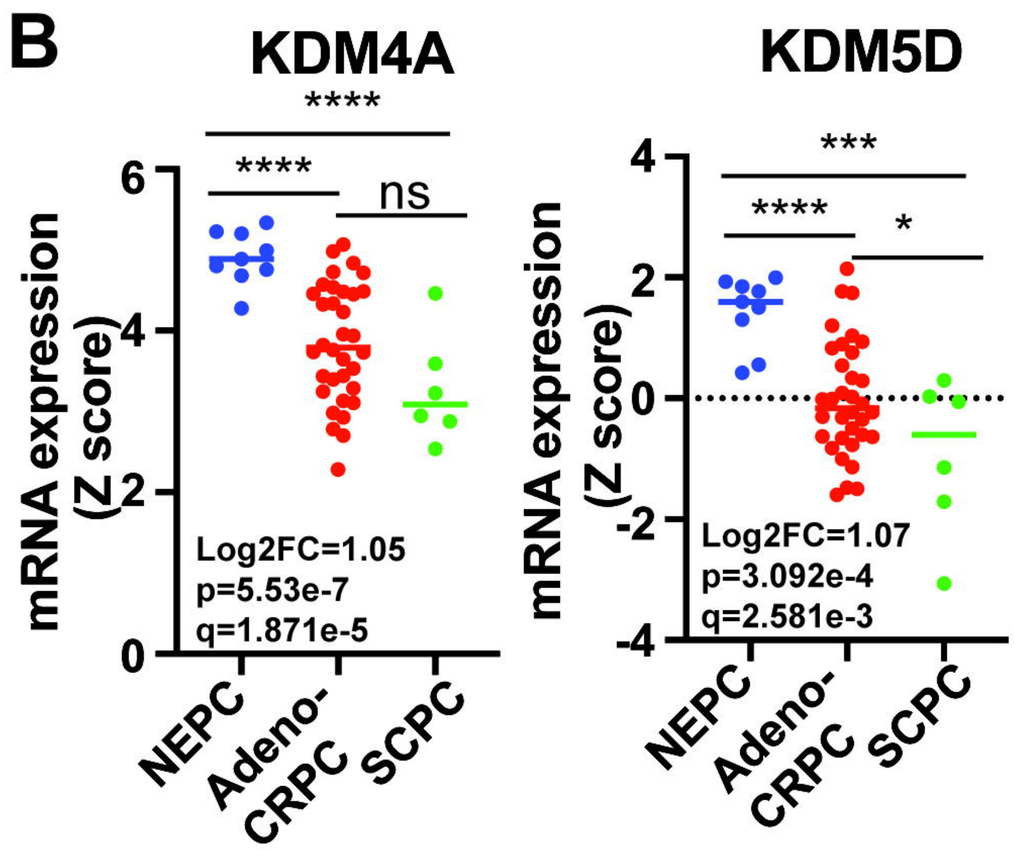
Figure 4. KDM4 inhibitors suppress NEPC cell growth and induce apoptosis in vitro. (**A**) KDM4 inhibitor QC6352 treatment led to increased total H3K9me3 and H3K36me3 in PSTR cells. (**B**) QC6352 suppressed PSTR growth *in vitro* as shown by foci-forming assay. (**C**) QC6352 suppressed the growth of PTR cells. (**D**) QC6352 and NCGC00244536 suppressed the growth of 144-13 cells *in vitro*. (**E**) *Kdm4a*-KO blunts the effect of QC6352 treatment on cell growth in *vitro*.

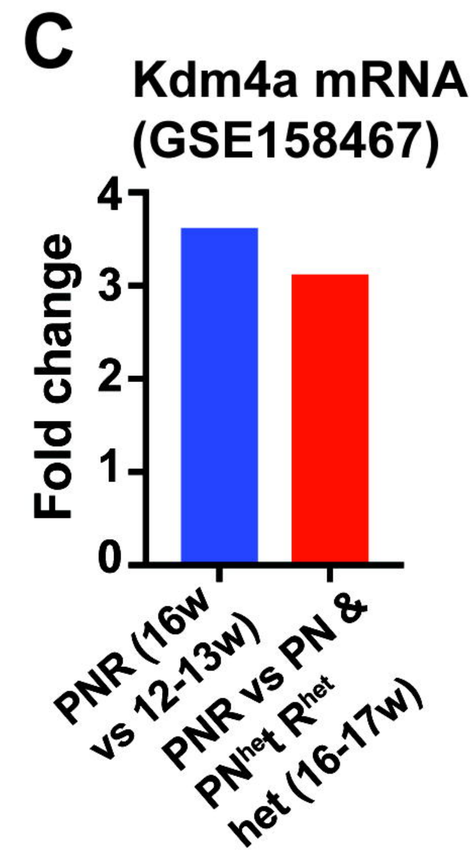
Figure 5. KDM4 inhibitor suppresses NEPC cell growth in vivo. (**A-B**) QC6352 treatment (50 mg/kg daily) suppressed the growth of subQ implanted PSTR cells as shown by weekly measurement of tumor volumes (A) and measurement of tumor weights at endpoint (B). (**C-D**)

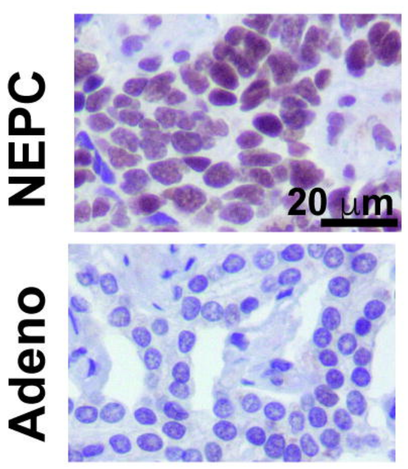
QC6352 treatment suppressed the growth of subQ implanted 144-13 cells as shown by weekly measurement of tumor volumes (C) and measurement of tumor volume and tumor weights at the endpoint (D). (**E**) QC6352 treatment suppressed the growth of primary tumors in PSTR GEMM as shown by tumor weights at the endpoint. (**F**) QC6352 treatment reduced NEPC proliferation in vivo as shown by IHC staining of Ki66 in tumors from PSTR mice.

Figure 1









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	++/+++	+/-
Adeno	2	8
NEPC	7	1
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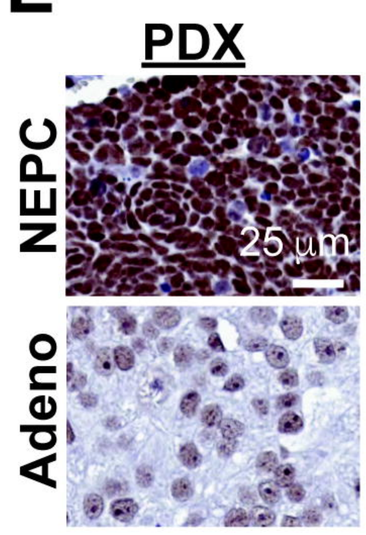
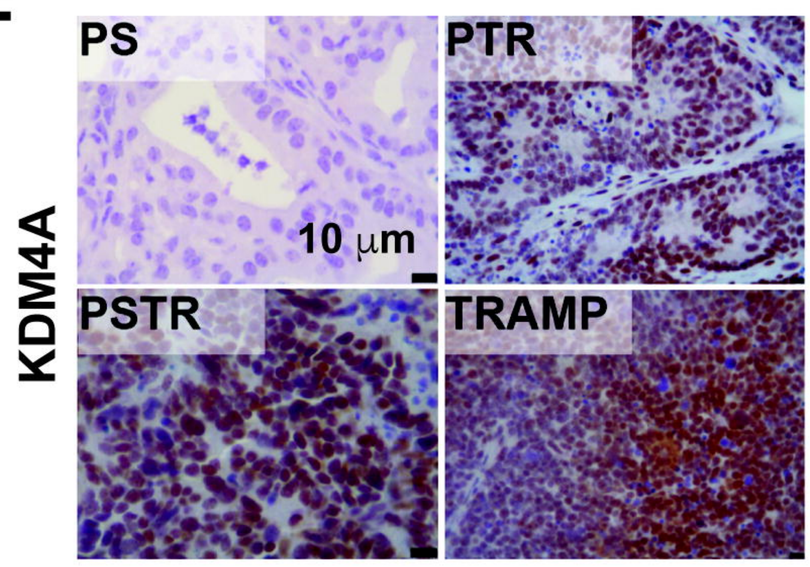


KDM4A		
	+++/+++	++/+
Adeno	4	16
NEPC	10	1
	p=0.0002	



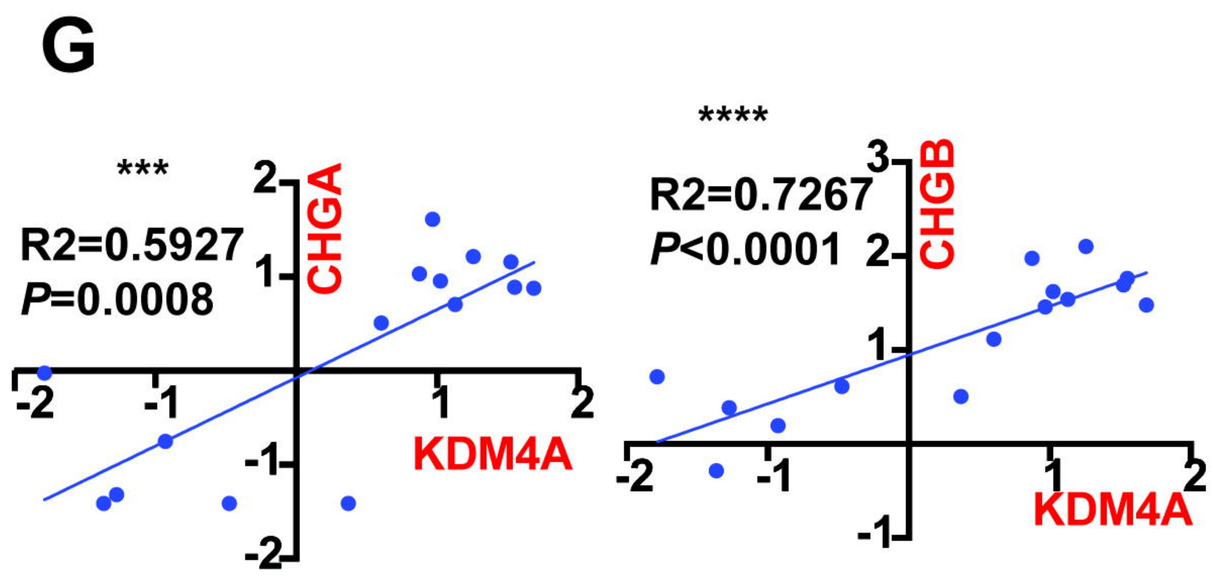
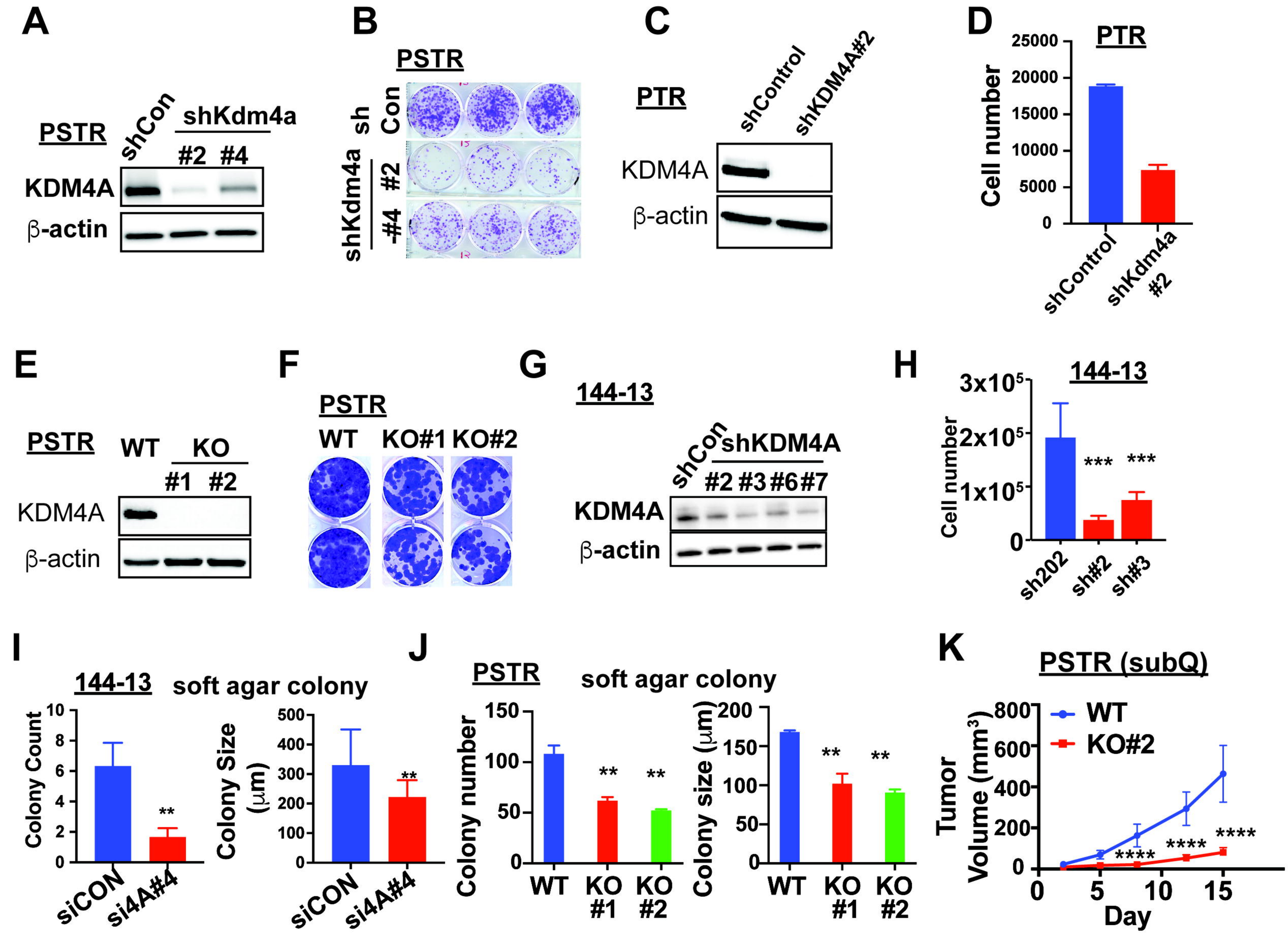


Figure 2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shKdm4a
#2 #4
KDM4A
β-actin
B
PSTR
sh Con
shKdm4a #2
#4
C
PTR
shControl
shKDM4A#2
KDM4A
β-actin
D
PTR
Cell number
25000
20000
15000
10000
5000
0
shControl
shKdm4a #2
E
PSTR
WT
KO
#1 #2
KDM4A
β-actin
F
PSTR
WT KO#1 KO#2
G
144-13
shCon
shKDM4A
#2 #3 #6 #7
KDM4A
β-actin
H
144-13
Cell number
3x10^5
2x10^5
1x10^5
0
***
***
sh202
sh#2
sh#3
I
144-13 soft agar colony
Colony Count
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8
6
4
2
0
**
siCON
si4A#4
Colony Size (μm)
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400
300
200
100
0
**
siCON
si4A#4
J
PSTR soft agar colony
Colony number
150
100
50
0
**
**
WT
KO #1
KO #2
Colony size (μm)
200
150
100
50
0
**
**
WT
KO #1
KO #2
K
PSTR (subQ)
Tumor Volume (mm³)
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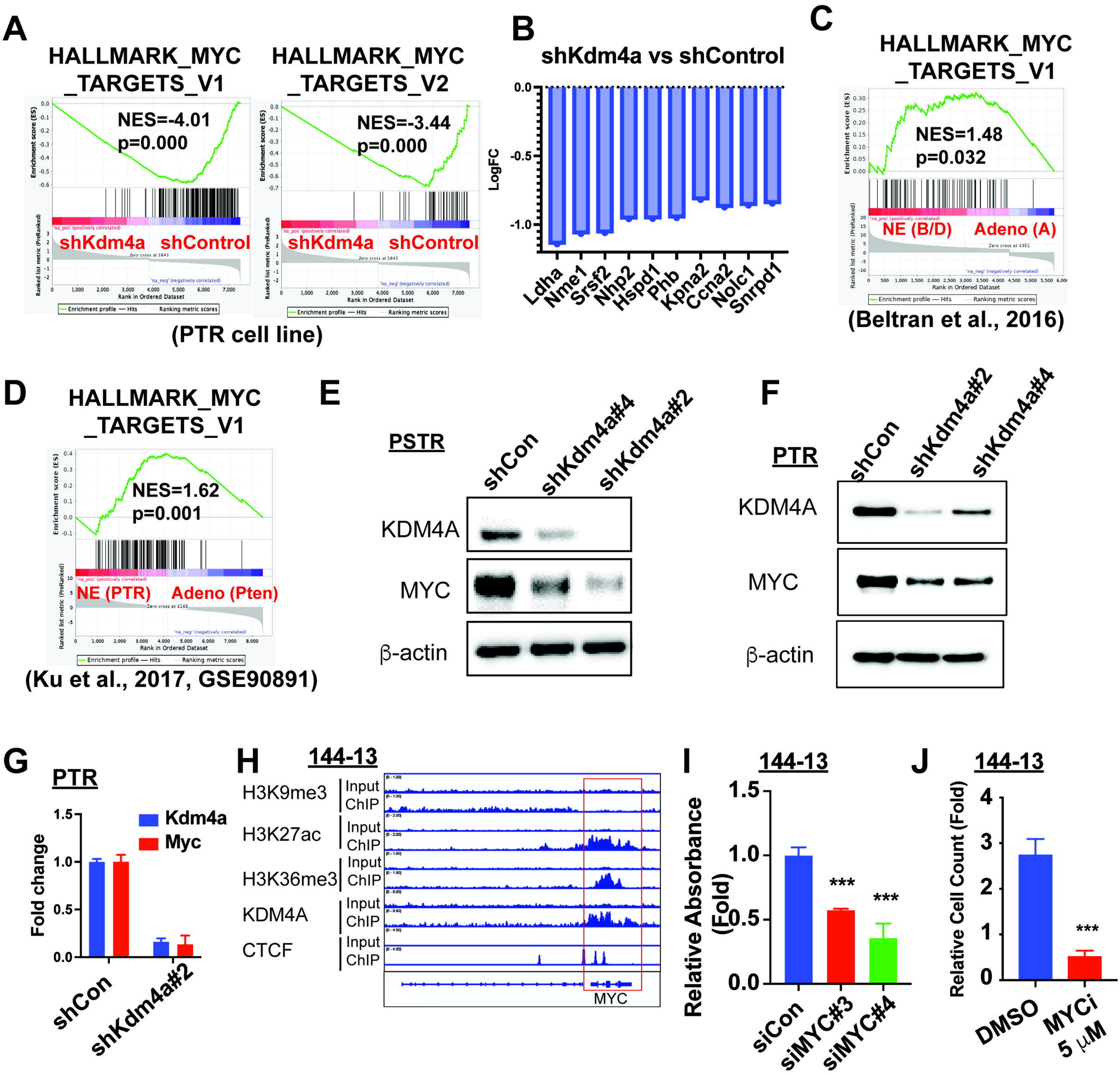
600
400
200
0
WT
KO#2
****
****
****
0
5
10
15
Day

Figure 3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p=0.000
shKdm4a
shControl
(PTR cell line)
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shKdm4a vs shControl
LogFC
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-0.5
-1.0
Ldha
Nme1
Srsf2
Nhp2
Hspd1
Phb
Kpna2
Ccna2
Nolc1
Snrpd1
C
HALLMARK_MYC _TARGETS_V1
NES=1.48
p=0.032
NE (B/D)
Adeno (A)
(Beltran et al., 2016)
D
HALLMARK_MYC _TARGETS_V1
NES=1.62
p=0.001
NE (PTR)
Adeno (Pten)
(Ku et al., 2017, GSE90891)
E
PSTR
shCon
shKdm4a#4
shKdm4a#2
KDM4A
MYC
β-actin
F
PTR
shCon
shKdm4a#2
shKdm4a#4
KDM4A
MYC
β-actin
G
PTR
Fold change
Kdm4a
Myc
shCon
shKdm4a#2
H
144-13
H3K9me3
H3K27ac
H3K36me3
KDM4A
CTCF
Input
ChIP
MYC
I
144-13
Relative Absorbance (Fold)
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siMYC#3
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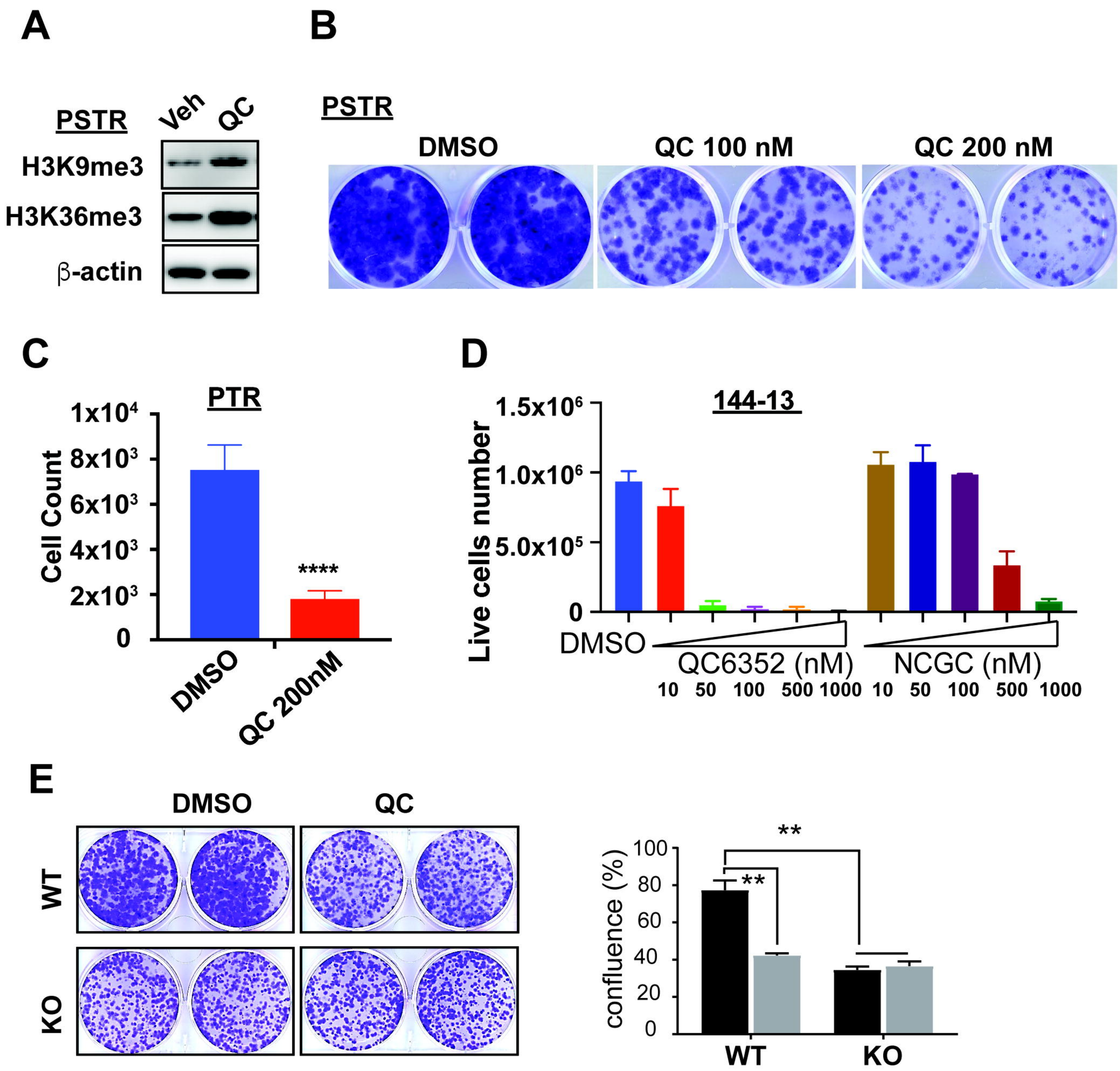
J
144-13
Relative Cell Count (Fold)
DMSO
MYCi 5 μM

Figure 4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QC 200 nM
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PTR
Cell Count
1x10⁴
8x10³
6x10³
4x10³
2x10³
0
****
DMSO
QC 200nM
D
144-13
Live cells number
1.5x10⁶
1.0x10⁶
5.0x10⁵
0
DMSO
QC6352 (nM)
10 50 100 500 1000
NCGC (nM)
10 50 100 500 1000
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DMSO
QC
WT
KO
confluence (%)
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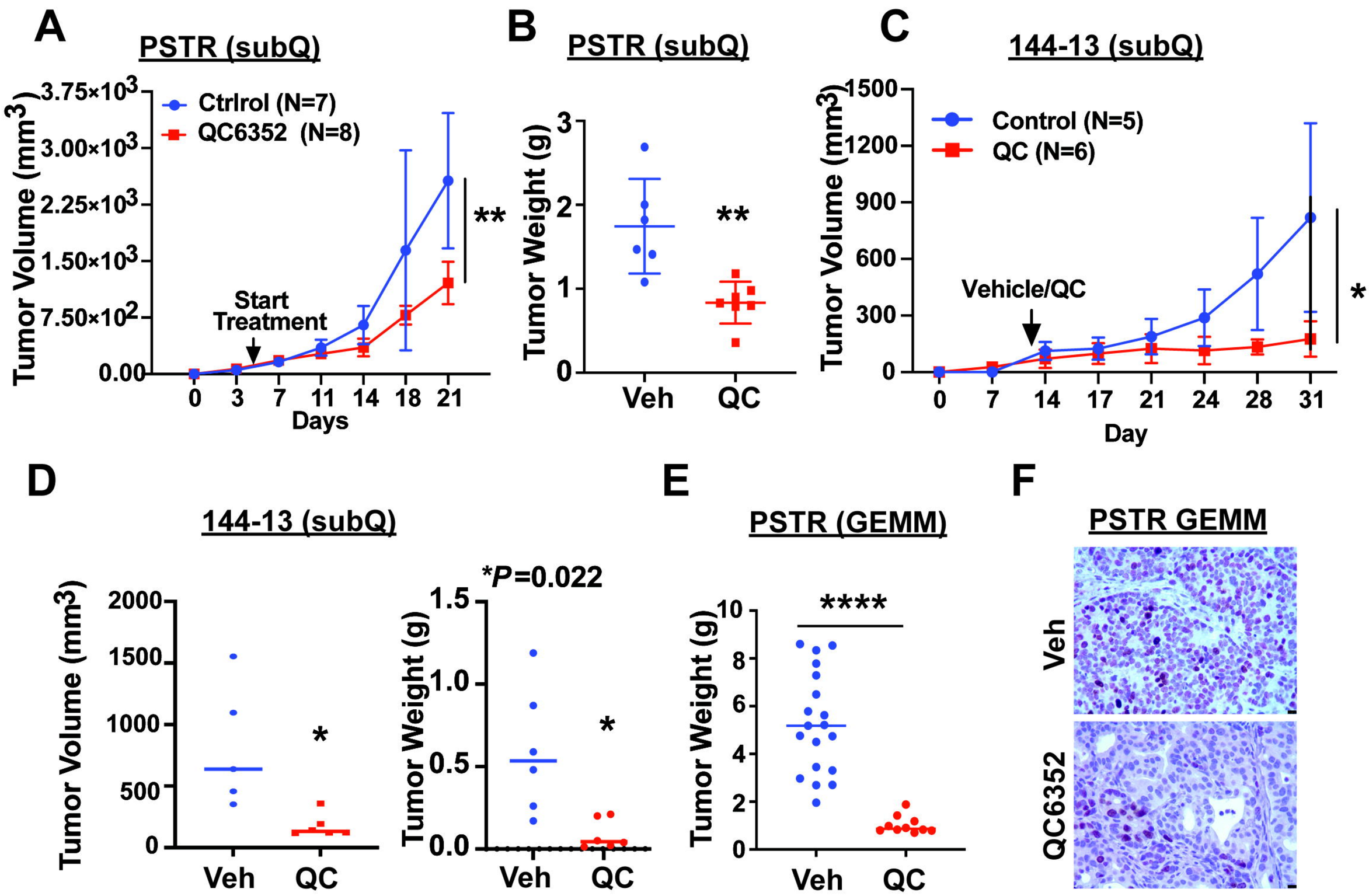
80
60
40
20
0
**
**
WT
KO

Figure 5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PSTR (subQ)
Tumor Volume (mm3)
3.75×10^3
3.00×10^3
2.25×10^3
1.50×10^3
7.50×10^2
0.00
Ctrlrol (N=7)
QC6352 (N=8)
Start Treatment
**
0 3 7 11 14 18 21
Days
B
PSTR (subQ)
Tumor Weight (g)
3
2
1
0
**
Veh
QC
C
144-13 (subQ)
Tumor Volume (mm3)
1500
1200
900
600
300
0
Control (N=5)
QC (N=6)
Vehicle/QC
*
0 7 14 17 21 24 28 31
Day
D
144-13 (subQ)
Tumor Volume (mm3)
2000
1500
1000
500
0
*
Veh
QC
*P=0.022
Tumor Weight (g)
1.5
1.0
0.5
0.0
*
Veh
QC
E
PSTR (GEMM)
Tumor Weight (g)
10
8
6
4
2
0
****
Veh
QC
F
PSTR GEMM
Veh
QC6352