Supplementary Information

Supplementary materials and methods:

Transfection and infection. Transfections were done using Lipofectamine 2000 (Invitrogen). Retrovirus was produced by transfection of pBABE constructs into the Phoenix HEK-293 packaging cell line (RRID:CVCL_H716). Transduced A-704 cells were selected in 2 μg/mL puromycin for 10-14 days. For PBRM1 knockdown experiments, MISSION Lentiviral Transduction Particles were purchased from Sigma-Aldrich (clone IDs: TRCN0000235890 and TRCN0000015994) and used according to the manufacturer's instructions. Briefly, 1×10^5 (786-O) or 2×10^5 (ACHN) cells were transduced with the viral particles at a MOI = 3, with 12 μg/mL polybrene. 786-O cells were selected with 3 μg/mL puromycin, and ACHN cells with 1 μg/mL puromycin, for 10-14 days. Non-targeting control shRNA (Sigma, SHC016) lentiviruses were produced in HEK-293T cells (RRID:CVCL_0063). At passage 2, MEFs were infected with either control GFP adenovirus (Vector Biolabs, #1060) or Cre adenovirus (Vector Biolabs, #1045) in 6-well plates. MEFs were passaged 3 times, and then mRNA was collected for microarray and qRT-PCR analysis, and protein was collected. For siRNA knockdown experiments, siRNA sets were purchased from Qiagen: ALDH1A1 – GS216; ARID2 - GS196528; BRG1 - GS6597; SNF5 - GS6598; BRM - GS6595. The indicated siRNAs from each set were used for experiments. Sigma MISSION siRNA Universal Negative Control #1 (SIC001) was used as negative control siRNA.

Immunoblotting. Protein lysates from cell lines were collected by scraping the tissue culture plate with 2X Sample Buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 0.05% Bromophenol Blue, 8 M urea, and 10% β-mercaptoethanol added fresh) on ice, subjecting the lysates to 5x 1 second bursts of sonication, boiling for 5 minutes, and then centrifuging at 14,000 rpm for 5 min

at 4^0C. Samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on Tris-Glycine precast gels (Invitrogen) and then transferred to PVDF membrane using wet transfer methods. After probing with primary and secondary antibodies, proteins were detected using enhanced chemiluminescence (Thermo Scientific, #34080). Blots were sometimes cut based on protein molecular weight markers to probe for multiple proteins of different weights simultaneously, and the blots were sometimes stripped and reprobed for different proteins as needed. For each figure, all loading control protein bands are from the same samples and blots as the other proteins shown. A table of primary antibodies can be found below.

qRT-PCR. The primer sequences were as follows: *ALDH1A1* forward, 5′-TTGGAAGATAGGGCCTGCAC -3′, and reverse, 5′- GGAGGAAACCCTGCCTCTTTT -3′; *ARID2* forward, 5′- TACTTGCTAATGCCGGGGTG -3′, and reverse, 5′-ACGATGTCTTTCCAAAACTTAACGA -3′; *GAPDH* forward, 5′-GAAGGTGAAGGTCGGAGTCAAC-3′, and reverse, 5′-CAGAGTTAAAAGCAGCCCTGGT-3′. Samples were run in triplicate. The efficiencies of the primers were measured using standard curves and were calculated to be: ALDH1A1 – 2.03; ARID2 – 1.98; and GAPDH – 2.00. Quantitative analysis was performed based on the $\Delta\Delta Ct$ method using the housekeeping gene *GAPDH*.

Growth curve analysis. Experiments were carried out in 48-well plates at least in triplicate, unless specified (see figure legends for n-sizes for individual experiments). For 786-O and ACHN, 4×10^3 cells were plated per well, and for A-704, 6×10^3 cells were plated per well. At the indicated time intervals, cells were washed once with PBS, stained with 0.05% crystal violet in formalin at room temperature for 20–30 min, and washed again twice with PBS before extracting the dye with 10% acetic acid. Plates were shaken on a microplate shaker at medium speed for at least 1 hr, and

the absorbance (O.D.), corresponding to cell numbers attached on the plates, was measured at 595 nm on a multi-well plate reader (Bio-Rad).

Colony formation in soft-agar. 1×10^4 cells were used per plate, and experiments were performed on all samples in triplicate. Agar (Difco) was resuspended to 3% (w/v) in PBS and sterilized. 1 mL of agar diluted to 0.5% in the appropriate culture media was aliquoted into a 35 mm plate as a support layer. Healthy-looking cells were trypsinized and counted, appropriate cell numbers were spun down, and then resuspended in warm media to a single-cell suspension. When drug treatments were used (e.g., DEAB, erlotinib), they were mixed into the media at this stage. The liquid 3% agar was added to the cell suspension to a final concentration of 0.3%, the solution was thoroughly mixed, and then 1 mL of the cell:agar solution was layered onto the support layer. Colonies were allowed to form for 23 days, with careful monitoring every 3-4 days. To quantify colony formation, the cells were stained with 1 mL of 0.005% crystal violet solution in formalin for 1 hr. The staining solution was carefully removed, and then the plates were scanned and colonies quantified using ImageJ software (RRID:SCR_003070) (with a minimum colony size cut-off of 10 pixels).

Mutagenesis primers. The following primer pairs were used. Clones were sequenced to confirm the presence of the engineered mutations and the absence of secondary mutations.

Mutant	Primer
PBRM1 T232P	Forward: 5'-cctatagatctcaagcccattgcccagaggata-3' Reverse: 5'-tatcctctgggcaatgggcttgagatctataggct-3'
PBRM1 A597D	Forward: 5'-gctgatgttccggaatgacaggcactataatgagg-3' Reverse: 5'-cctcattatagtgcctgtcattccggaacatcagc-3'
PBRM1 H1204P	Forward: 5'-acatttttgtgggctcaggctctgtttcttcaggg-3'

	Reverse: 5'-ccctgaagaaacagagcctgagcccacaaaaatgt-3'
PBRM1 6AAD	Forward: 5'-cagagcatgagcccacaaaagtatttctgagtaatctgga-3'
	Reverse: 5'-tccagattactcagaaatacttttgtgggctcatgctctg-3'

Primary antibodies. The following antibodies were used for Western blotting, IP, and ChIP experiments. PBRM1 polyclonal antibodies were generated against a glutathione S-transferase fusion protein of a C-terminal PBRM1 fragment (amino acids 736–1,475).

Protein Target	Purpose	Company	Catalogue #	Type	Concentration
PBRM1	WB	in-house	--	rabbit polyclonal	1:5000
ARID2	WB, IP	Santa Cruz Biotechnology	sc-166117 (E-3) RRID:AB_2060382	mouse monoclonal	1:500 for WB, 3 µg per IP
BRG1	WB	Santa Cruz Biotechnology	sc-8749 (N-15) RRID:AB_2191848	goat polyclonal	1:500
BRG1	IP	Santa Cruz Biotechnology	sc-17796 (G-7) RRID:AB_626762	mouse monoclonal	3 µg per IP
SNF5	WB	Bethyl Laboratories	A301-087A-1 RRID:AB_2191714	rabbit polyclonal	1:5000
BRM	WB	Santa Cruz Biotechnology	sc-6450 (N-19) RRID:AB_634464	goat polyclonal	1:500
ALDH1A1	WB	Abcam	ab52492 (EP1933Y) RRID:AB_867566	rabbit monoclonal	1:1000

			A4700	mouse	
β-actin	WB	Sigma-Aldrich	RRID:AB_476730	monoclonal	1:40000
Tubulin	WB	Covance	MMS-410P (Tu27) RRID:AB_10063407	mouse monoclonal	1:50000
Vinculin	WB	Sigma-Aldrich	V9131 RRID:AB_477629	mouse monoclonal	1:50000
p-EGFR (Y1173)	WB	Cell Signaling Technology	4407 RRID:AB_331795	rabbit monoclonal	1:1000
EGFR	WB	Cell Signaling Technology	4267 (D38B1) RRID:AB_2246311	rabbit monoclonal	1:1000
p-AKT (T308)	WB	Cell Signaling Technology	4056 (244F9) RRID:AB_331163	rabbit monoclonal	1:1000
p-AKT (S473)	WB	Cell Signaling Technology	3787 RRID:AB_331170	rabbit monoclonal	1:1000
AKT	WB	Cell Signaling Technology	9272 RRID:AB_329827	rabbit polyclonal	1:1000
p-EKR	WB	Cell Signaling Technology	9101 RRID:AB_331646	rabbit polyclonal	1:1000
ERK	WB	Cell Signaling Technology	9102 RRID:AB_330744	rabbit polyclonal	1:1000
V5 agarose affinity gel	IP	Sigma-Aldrich	A7345 RRID:AB_10062721	mouse monoclonal	50 µl per IP

			ab8580	rabbit	
H3K4Me3	ChIP-seq	Abcam	RRID:AB_306649	polyclonal	3 µg/ChIP
	ChIP-seq	Abcam	ab8895	rabbit	3 µg/ChIP
H3K4Me1			RRID:AB_306847	polyclonal	
	ChIP-seq	Abcam	ab10812	rabbit	3 µg/ChIP
H3K9Ac			RRID:AB_297491	polyclonal	
H3K27Me3	ChIP-seq	Millipore	07-449	rabbit	3 µg/ChIP
			RRID:AB_310624	polyclonal	

Tumor processing. After euthanasia, tumors were harvested and weighed. For Western blot analysis, small sections were excised and placed in 500 µl of cold RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA), supplemented with fresh protease inhibitors, in pre-chilled 12x75mm borosilicate glass tubes. The excised xenografts were then homogenized for ~10 seconds, until no chunks were visible. The mixture was then sonicated 5x with 1 second bursts, and was allowed to sit on ice for 30 min, vortexing every 5 min. The solution was transferred to Eppendorf tubes, spun at 4^0C for 20 min at maximum speed, the supernatant was transferred to a new tube, and then the spin step was repeated. The supernatant was again transferred to a fresh tube and was subjected to the Bradford protein assay. 20 µg of protein was then combined with 2X sample buffer, and Western blot analysis was performed.

ALDEFLUOR assay. The ALDEFLUOR assay kit was purchased from STEMCELL Technologies (RRID:SCR_013642), and the manufacturer's instructions were followed. Briefly, cells that were ~80% confluent were trypsinized, washed in PBS, and resuspended in the provided

Assay Buffer, and then counted. 2×10^5 cells in 0.5 mL Assay Buffer were used per reaction, and 2.5 µl of substrate per reaction was used for the 786-O cells, and 5 µl per reaction for the A-704 cells. The chemical inhibitor DEAB was added to the recommended concentration of 15 µM to the negative control sample before adding the substrate to set the negative baseline of no enzyme activity. The cells were incubated with substrate for 40 min at 37^0C, centrifuged and then resuspended in 0.5 mL cold Assay Buffer, and kept on ice in the dark until fluorescence was measured using a BD LSRFortessa cell analyzer and FACSDiva software (RRID:SCR_001456). ALDH1-activity was calculated by gating on live, single cells, establishing a negative baseline using the DEAB-treated sample, and then measuring the percentage of cells that were positive for ALDH1-activity by green fluorescence levels on the x-axis. This analysis was done using Cytobank (RRID:SCR_014043).

Generation of *Pbrm1* fl/fl mice. In collaboration with the Transgenic Mouse Core at Columbia University, we designed a bacterial artificial chromosome in which a LoxP site and a Frt-Neo-Frt-LoxP cassette were engineered to flank exon 2 of the *Pbrm1* allele, such that Cre-mediated recombination would result in a frame-shifting mutation. A gene-targeting vector was then constructed with a 2kb short homology arm, a 5kb long homology arm, and a DTA negative selection marker. This vector was transfected into KV1 (129B6 hybrid) ES cells, which were then selected for recombination with G418. Two of the positively selected ES cells were then injected into C57BL/6 blastocysts to generate chimeric male mice. Mice that were positive for germline transmission of the modified allele were bred to homozygous ACTB (Flpe/Flpe) females (in a C57BL/6 background) to remove the Neo cassette. Heterozygous mice (fl/+) derived from the same initial chimeric mouse were bred to generate homozygous *Pbrm1* floxed mice (fl/fl).

Supplementary figure legends:

Fig. S1. Growth properties of ccRCC cell lines with PBRM1 knockdown or re-expression.

A) Western blot analysis of PBRM1 protein levels in ACHN cells transduced with lentiviruses containing a non-targeting shRNA (labeled as "C"), or PBRM1-targeting shRNA (labeled as "#1" or "#2"). B) 2D growth curves at normal growth conditions (10% FBS) of 786-O cells (left panel; n=3 per timepoint for each line, from independent experiments) and A-704 cells (right panel; n=3 per timepoint for each line, from independent experiments). Two-way ANOVAs with Tukey's multiple comparisons test were performed on each set of growth curves, with no significant differences. C) 2D growth curves at the indicated serum concentration of ACHN cells (n=2 per timepoint for each line, from independent experiments). Statistical testing was not performed on these growth curves. D) Colony formation in soft agar for the indicated ACHN cells. Error bars represent standard errors of the mean (SEM), n=3/line, from independent experiments. Statistical testing comparing all column means was performed using ordinary one-way ANOVA with Tukey's multiple comparisons test; NS=non-significant. E) Representative images of tumorspheres for 786-O control and PBRM1 shRNA cells, at 1st and 2nd passage. F) Cell numbers at time of passaging for 786-O control and PBRM1 shRNA #2 lines; error bars represent SEM for n=5-6, from independent experiments; an unpaired t-test was used for statistical testing. G) Excised tumor weights following mice euthanasia on day 34 post-injection with 786-O tumor cells. An unpaired t-test was performed. H) Western blot analysis of protein extracted from 786-O tumor xenografts. Western blot analysis of p21 protein levels in I) 786-O and J) ACHN cells treated with 0.4 µg/mL doxorubicin for the indicated time points.

Fig. S2. PBRM1 alters responsiveness to EGF.

A) A-704 cells were stably transduced with various retroviruses containing the indicated cancer-associated mutant versions of PBRM1. Western blot analysis of PBRM1 protein levels in the indicated A-704 lines. B) V5-immunoprecipitation experiments were performed using nuclear extracts from the indicated A-704 cell lines created in (A). Western blot analysis was performed to check for binding to SNF5, ARID2, and BRG1. C) Western blot analysis of EGF signaling in 786-O lines stimulated with the indicated concentration of EGF for 30 min under serum-free conditions. D) Western blot analysis of EGF signaling in A-704 lines stimulated with 1 ng/mL EGF for 30 min under serum-free conditions. E) and F) Quantification of protein bands from C) and D) using ImageJ software and comparing ratio of phospho-protein to total protein (blue rows). G) 2D dose-response growth curves of 786-O lines at 0.1% serum in the presence of the indicated amount of EGF (with PBS given as a control). H) 2D dose-response growth curves of A-704 lines at 2.5% serum in the presence of the indicated amount of EGF (with PBS given as a control)). For G) and H), n=6 per timepoint for each dose, technical replicates, error bars represent standard deviations. Statistical testing was not performed. I) Colony formation in soft-agar of 786-O control and PBRM1 shRNA #1 cells. Increasing doses of erlotinib were mixed in with the soft-agar at time of plating. n=3/line at each dose, error bars represent SEM, from independent experiments. Statistical testing was performed using an ordinary two-way ANOVA with the Sidak method to correct for multiple comparisons between cell lines at a given dose.

Fig. S3. Retinoic acid metabolism genes exhibit increased H3K4me3 deposition and mRNA expression following loss of PBRM1, but not more open chromatin.

A) H3K4me3 ChIP-seq tracks at the locus of *DHRS3* in PBRM1 shRNA cells (red) or control cells (blue). B) RNA-seq expression of *DHRS3* in PBRM1 shRNA cells (red) or control cells (blue). C)

H3K4me3 ChIP-seq tracks at the locus of *DHRS9.* D) RNA-seq expression of *DHRS9.* E) H3K4me3 ChIP-seq tracks at the locus of *DHRS4L2*. F) RNA-seq expression of *DHRS4L2*. G) RNA-seq expression of *UGT1A8.* H) H3K4me3 ChIP-seq tracks at the locus of *UGT1A8.* I) ATAC-seq tracks at the indicated loci comparing PBRM1 shRNA cells to control cells.

Fig. S4. With PBRM1 deficiency, ALDH1A1 expression increases at the mRNA and protein levels, in both malignant and non-transformed cells.

A) qRT-PCR analysis of *ALDH1A1* mRNA levels (relative to *GAPDH*) in the indicated cell lines. For 786-O, n=5/cell line, for A-704, n=3/cell line; from independent experiments, error bars represent SEM. The delta-delta-Ct method was used to analyze the data, with a single technical replicate 786-O control shRNA and A-704 EV sample set to 1 for each experiment. For 786-O, statistical testing comparing all column means was performed using ordinary one-way ANOVA with Tukey's multiple comparisons test; for A-704, an unpaired t-test was performed. Western blot analysis of ALDH1A1 protein levels in the indicated B) ACHN cells and C) MCF10A cells, and D) showing loss of Pbrm1 expression in the NULL MEFs. E) Microarray gene expression analysis was performed on three sets of matched *Pbrm1* conditional MEFs. A heat map of the top 50 altered genes in the MEFs is shown – *Aldh1a1* is starred. F) qRT-PCR analysis of *Aldh1a1* mRNA levels (relative to *Gapdh*) in MEFs. Errors bars represent SEM for n=3, from independent MEFs. The delta-delta-Ct method was used to analyze the data, with WT MEF lines set to 1. An unpaired t-test was performed. G-I) RNA-seq expression of *ALDH1A1* in primary ccRCC tumors from TCGA based on mutational status of G) *PBRM1,* H) *SETD2,* and I) *BAP1.* J) Gene expression microarray and K) RNA-seq expression of *ALDH1A1* in primary ccRCC tumors from two previously published cohorts based on mutational status of *PBRM1.* For all plots, the tumors were divided

into those with mutations (Mut) and those without (WT). For parts G-J, the plots represent Tukey plots with the outliers not shown. Statistical testing was performed using Mann-Whitney tests. L) Growth curve analysis of 786-O lines treated with increasing doses of DEAB or vehicle control. n=3/dose at each timepoint. Measurements represent percentage confluence as measured by the Incucyte ZOOM. M) Western blot analysis of ALDH1A1 expression levels in the same 786-O cells used for the tumorsphere assays with ALDH1A1 siRNA knockdown (#2, #5, #7) at time of plating.

Fig. S5. ARID2 expression increases with PBRM1 deficiency

A) Western blot analysis of the indicated PBAF complex subunits in ACHN cells. B) RNA-seq expression of *ARID2* in 786-O PBRM1 shRNA cells (red) or control cells (blue) at the indicated FBS concentration. ns=not significant. C) qRT-PCR analysis of *ARID2* mRNA levels (relative to *GAPDH*) in the indicated cell lines. For 786-O, n=4/cell line, for A-704, n=3/cell line; from independent experiments, error bars represent SEM. The delta-delta-Ct method was used to analyze the data, with a single technical replicate 786-O control shRNA and A-704 EV sample set to 1 for each experiment. For 786-O, statistical testing comparing all column means was performed using ordinary one-way ANOVA with Tukey's multiple comparisons test; for A-704, an unpaired t-test was performed. D) *ARID2* mRNA expression levels in ccRCC primary tumors based on data from the TCGA (left panel) or Peña-Llopis *et al* (right panel). The tumors were divided into those with *PBRM1* mutations (Mut) and those without (WT). The plots represent Tukey plots with the outliers not shown. Statistical testing was performed using Mann-Whitney tests. E) BRG1 IP experiments using A-704 cells. A matched isotype IgG was used for control IPs. Inputs are aliquots taken from pre-cleared nuclear extracts before the IPs were performed. F) Western blot analysis

of the indicated fractions (out of 24 total fractions), from glycerol gradient fractionation of nuclear extracts from 786-O cells. G) Quantification of ARID2, BRG1, and SNF5 elution patterns as percentages of total protein eluted in fractions 1-10 for each protein based on blots shown in Figure 5E for each indicated 786-O line. Bands were quantified using ImageJ.

Fig. S6. ARID2 contributes to ALDH1A1 upregulation associated with PBRM1 deficiency.
A) Western blot analysis of ALDH1A1 levels with ARID2 siRNA knockdown in the 786-O control and PBRM1 shRNA #2 lines. B) qRT-PCR analysis of *ALDH1A1* mRNA levels (relative to *GAPDH*) in 786-O PBRM1 shRNA #1 cells with ARID2 knockdown. Error bars represent standard deviations for n=3, technical replicates. The delta-delta-Ct method was used to analyze the data, with the C siRNA sample set to 1 for one replicate. Statistical testing was not performed. C) Western blot analysis of ALDH1A1 levels with ARID2 siRNA knockdown in the A-704 EV and WT lines. D) Western blot analysis of ARID2 levels in the same cells used for the tumorsphere assays with ARID2 siRNA knockdown at time of plating. E) Western blot analysis of ALDH1A1 levels with SNF5 siRNA knockdown in the A-704 EV and WT lines. F) Western blot analysis of ALDH1A1 levels with BRM siRNA knockdown in the 786-O control and PBRM1 shRNA #1 lines. G) Western blot analysis of ALDH1A1 levels with BRG1 and/or BRM siRNA knockdown in the 786-O control and PBRM1 shRNA #1 lines. BRM1 siRNA #5 and BRG1 siRNA #5 were used. H) Western blot analysis of BRG1 levels in the same cells used for the tumorsphere assays with BRG1 siRNA knockdown at time of plating.