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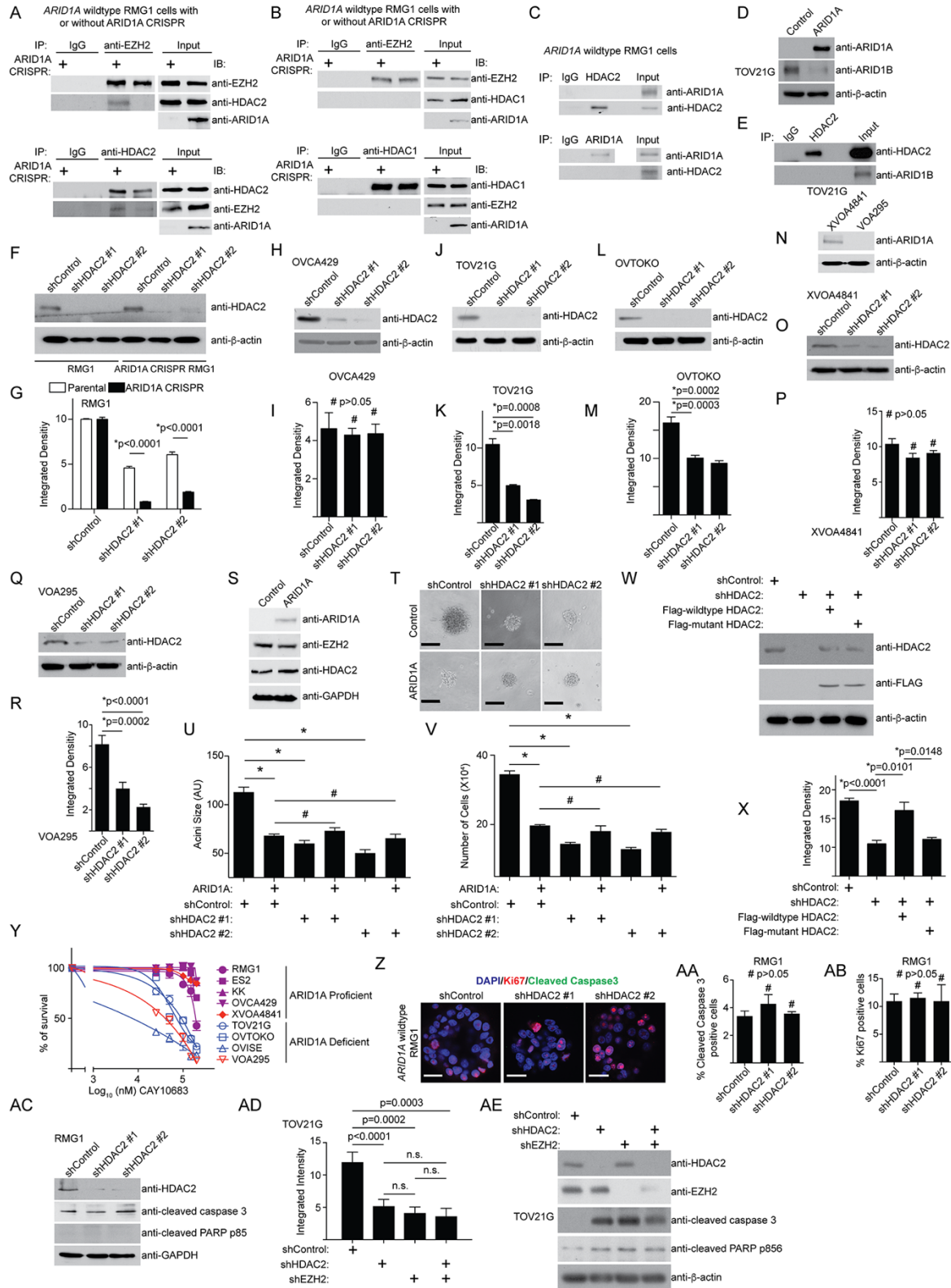
Supplemental Information

Repurposing Pan-HDAC Inhibitors for *ARID1A*-Mutated Ovarian Cancer

Takeshi Fukumoto, Pyoung Hwa Park, Shuai Wu, Nail Fatkhutdinov, Sergey Karakashev, Timothy Nacarelli, Andrew V. Kossenkov, David W. Speicher, Stephanie Jean, Lin Zhang, Tian-Li Wang, Ie-Ming Shih, Jose R. Conejo-Garcia, Benjamin G. Bitler, and Rugang Zhang

Supplemental Figures and Figure Legends

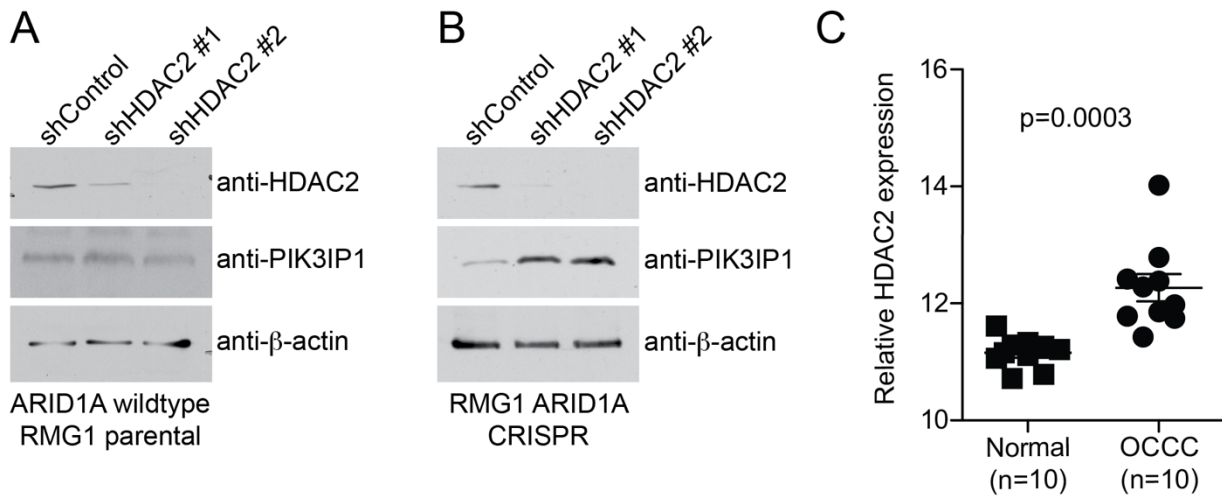
Supplemental Figure 1



Supplemental Figure 1. HDAC2 inhibition is selective against ARID1A inactivation. Related to Figure 1.

(A) Parental *ARID1A* wildtype RMG1 or *ARID1A* CRISPR knockout RMG1 cells were subjected to co-IP analysis using antibodies against EZH2 or HDAC2. An isotype matched IgG was used as a negative control. Immunoblots of the indicated proteins were performed. (B-C) Same as (A), but the cells were subjected to co-IP analysis using antibodies against EZH2 or HDAC1 (B), or using antibodies against *ARID1A* or HDAC2. An isotype matched IgG was used as a negative control. (D) Expression of *ARID1A*, *ARID1B* and a loading control β -actin was determined by immunoblot in *ARID1A*-mutated TOV21G cells with or without wildtype *ARID1A* restoration. (E) *ARID1A*-mutated TOV21G cells were subjected to co-IP analysis using antibodies against HDAC2. An isotype matched IgG was used as a negative control. Immunoblots of the indicated proteins were performed. (F-G) *ARID1A* wildtype RMG1 cells were transduced with lentivirus encoding the indicated shHDAC2s or control. Expression of HDAC2 and a loading control β -actin was determined by immunoblot (F). The indicated cells were subjected to colony formation assay. Integrated density of colonies formed by the indicated cells were quantified using the NIH Image J software (G). n=4 independent experiments. (H-M) Same as (F-G), but for *ARID1A* wildtype OVCA429 cells (H-I), *ARID1A*-mutated TOV21G (J-K) or OVTOKO (L-M) cells. n=4 independent experiments. (N) Expression of *ARID1A* and a loading control β -actin was determined by immunoblot in the indicated primary cultures of human ovarian clear cell carcinoma (OCCC) cells. (O-R) Same as (F-G), but for the indicated primary cultures of human OCCC cells. (S-V) Immunoblot of the indicated proteins in *ARID1A*-mutated TOV21G cells with or without wildtype *ARID1A* restoration (S). The indicated cells were grown in 3D Matrigel. Shown are representative images of acini formed by the indicated cells (T). Scale Bars = 75 measurable units (a.u.) using the NIH Image J software. The diameter of acini formed by the indicated cells in 3D culture was quantified using NIH Image J software (U). # $P > 0.05$, * $P < 0.05$. n=3 independent experiments. Numbers of cells recovered from 3D cultures of the indicated cells were quantified (V). # $P > 0.05$, * $P < 0.05$. n=4 independent experiments. (W-X) *ARID1A*-mutated OVISE cells with endogenous HDAC2 knockdown using a shRNA that targets the 3' UTR region of the human *HDAC2* gene were concurrently expressing a FLAG-tagged shRNA resistant wildtype HDAC2 or a catalytically inactivated H142A HDAC2 mutant. Expression of HDAC2, FLAG, and a loading control β -actin was determined by immunoblot (W). The indicated cells were subjected to growth analysis using the colony formation assay. Quantification of integrated density of colonies formed by the indicated cells using the NIH Image J software (X). n=4 independent experiments. Error bars represent SEM. P -value calculated with two-tailed t -test. (Y) Dose-responsive curves of CAY10683, a relative selective tool HDAC2 inhibitor (Pavlik et al., 2013), in the indicated *ARID1A* proficient cells and deficient cells. Error bars represent SEM. (Z) Immunofluorescence staining of Ki67 (red), cleaved caspase 3 (green), and DAPI (blue) for the acini formed by *ARID1A* wildtype RMG1 cells with or without HDAC2 knockdown in 3D culture at day 8. Images were captured using confocal microscopy. Scale bars = 20 μ m. (AA-AB) Quantification of (Z). 200 cells from each of indicated groups were examined for expression cleaved caspase 3 (AA) or Ki67 (AB). n=3 independent experiments. Error bars represent SEM. P -value calculated with two-tailed t -test. (AC) *ARID1A* wildtype RMG1 cells with or without HDAC2 knockdown were examined for expression of markers of apoptosis cleaved caspase 3 and cleaved PARP p85, HDAC2 and a loading control GAPDH by immunoblot. (AD-AE) *ARID1A*-mutated TOV21G cells were transduced with lentivirus encoding the indicated shHDAC2, shEZH2 or a combination. The indicated cells were subjected to colony formation assay. Integrated density of colonies formed by the indicated cells were quantified using the NIH Image J software (AD). n=4 independent experiments. n.s.: not significant. Expression of HDAC2, EZH2, cleaved caspase 3, cleaved PARP p85 and a loading control β -actin was determined by immunoblot (AE).

Supplemental Figure 2

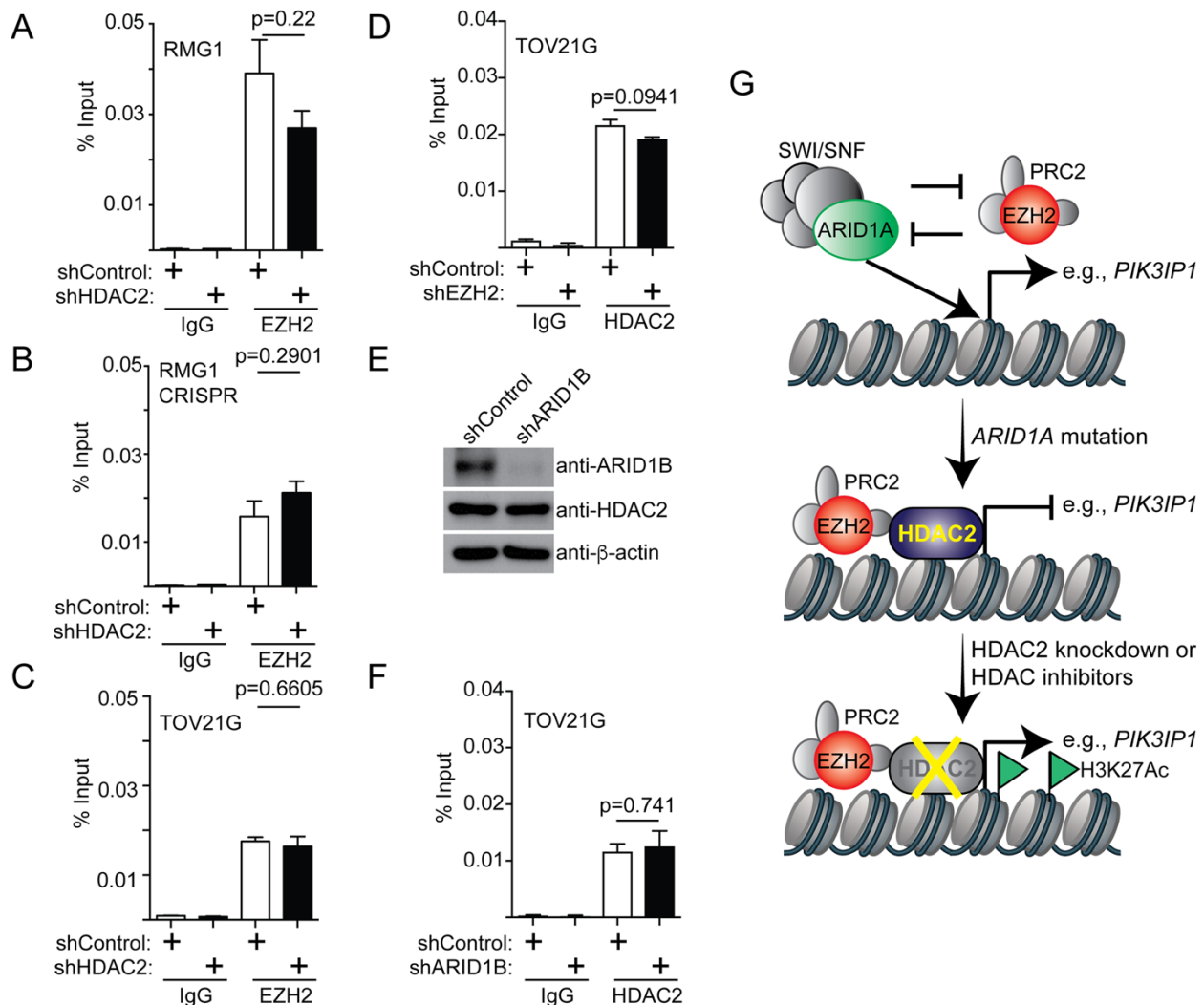


Supplemental Figure 2. HDAC2 regulates PIK3IP1 in an ARID1A-status dependent manner. Related to Figure 2.

(A-B) Immunoblot of the indicated proteins in *ARID1A* wildtype RMG1 parental cells or *ARID1A* CRISPR RMG1 cells with or without HDAC2 knockdown by the indicated shHDAC2s.

(C) Relative *HDAC2* mRNA expression in normal human ovarian surface epithelial cells (n = 10) and laser capture and microdissected OCCC tumors (n = 10) based on a published dataset (Stany et al., 2011).

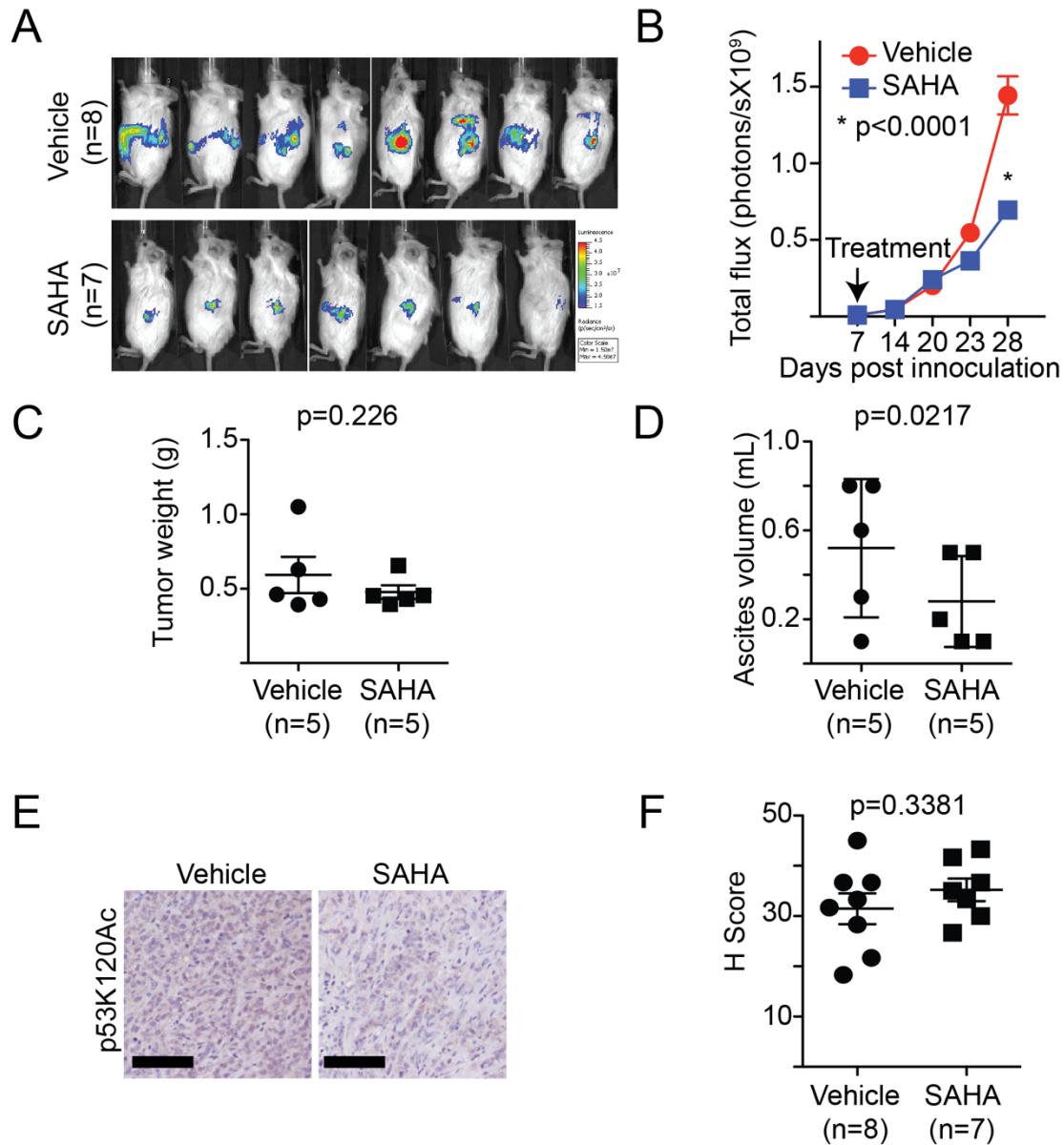
Supplemental Figure 3



Supplemental Figure 3. HDAC2 does not affect EZH2's association with the *PIK3IP1* gene promoter. Related to Figure 3.

(A-C) *ARID1A* wildtype RMG1 cells (A), *ARID1A* CRISPR RMG1 cells (B) and *ARID1A*-mutated TOV21G cells (C) with or without HDAC2 knockdown were subjected to ChIP analysis for the *PIK3IP1* gene promoter using an anti-EZH2 antibody or an isotype-matched IgG control. $n=3$ independent experiment. Error bars represent SEM. P -value calculated with two-tailed t -test. (D) *ARID1A*-mutated TOV21G cells with or without EZH2 knockdown for 48 hours were subjected to ChIP analysis for the *PIK3IP1* gene promoter using an anti-HDAC2 antibody or an isotype-matched IgG control. $n=3$ independent experiment. Error bars represent SEM. P -value calculated with two-tailed t -test. Please see Figure S1AD for EZH2 knockdown efficiency. (E-F) *ARID1A*-mutated TOV21G with or without ARID1B knockdown for 48 hours were examined for expression of ARID1B, HDAC2 and a loading control β -actin by immunoblot (E), or subjected the cells to ChIP analysis for the *PIK3IP1* gene promoter using an anti-HDAC2 antibody or an isotype-matched IgG control (F). $n=3$ independent experiment. Error bars represent SEM. P -value calculated with two-tailed t -test. (G) A proposed model for the mechanism by which HDAC2 regulates *PIK3IP1* expression in an *ARID1A* status dependent manner. In *ARID1A* wildtype cells, both ARID1A containing SWI/SNF complex and EZH2 containing PRC2 complex are present in the *PIK3IP1* gene promoter. However, ARID1A dominates over EZH2 in driving the expression of *PIK3IP1* gene in *ARID1A* wildtype cells. When ARID1A is mutated, HDAC2 is recruited to the *PIK3IP1* gene promoter and functions as a co-repressor of EZH2 to silence the expression of *PIK3IP1*. Accordingly, inhibition of HDAC2 activity by genetic knockdown or HDAC inhibitors reactivates *PIK3IP1* expression, which correlates an increase in H3K27Ac epigenetic mark in the *PIK3IP1* gene promoter.

Supplemental Figure 4



Supplemental Figure 4. SAHA reduced the burden of *ARID1A*-mutated ovarian tumors. Related to Figure 4. (A-B) Luciferase expressing *ARID1A*-mutated TOV21G cells were orthotopically injected into the ovarian bursa sac of the NSG female mice. Tumors were allowed to establish for 1 week, and the mice were randomized into two groups based on total luciferase flux. Mice were treated with vehicle control or SAHA (50 mg/kg) daily by i.p. injection for additional three weeks. Mice were imaged weekly. Shown were images taken at day 28 (A). Total flux (photons/sec) was graphed at the indicated time points (B). $n = 8$ mice in vehicle control and $n = 7$ mice in SAHA treated. Error bars represent SEM. P -value calculated with two-tailed t -test. (C-D) Mice with established *ARID1A* wildtype RMG1 tumors were randomized ($n = 5$ mice/group) and treated with vehicle control or SAHA (50 mg/kg) daily by i.p. injection for three weeks. Tumor weigh was quantified and used as a surrogate for tumor burden (C). The volume of ascites produced was quantified (D). Error bars represent SD. P -value calculated with two-tailed t -test. (E-F) Same as (A-B). Consecutive sections of the dissected xenograft tumors were subjected to immunohistochemical (IHC) staining using an anti-lysine 120 acetylated p53 (p53K120Ac) antibody (E). Scale bar = 50 μm . Expression of p53K120Ac was quantified based on H-score for the indicated groups. H-score was based on three different fields from each tumor from each of the indicated groups (F). The number of mice in each of the groups is indicated on the graph. Error bars represent SEM. P -value calculated with two-tailed t -test.

Supplemental Table Legends

	Names	IC₅₀ of SAHA (μM)
<i>ARID1A</i> wildtype	RMG1	0.57
	ES2	1.84
	KK	1.15
	OVCA429	1.97
	XVOA4841	1.42
<i>ARID1A</i> -mutated	TOV21G	0.01
	OVTOKO	0.1
	OVISE	0.03
	SKOV3	0.38
	VOA295	0.04

Supplemental Table 1. SAHA IC₅₀ of the listed cell lines and primary cultures. Related to Figure 4.

Supplemental Experimental Procedures

Cell lines and three-dimensional (3D) culture conditions.

The protocol for using primary cultures of human ovarian clear cells was approved by the University of British Columbia Institutional Review Board. The primary tumor cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin as previously described (Bitler et al., 2017). Ovarian clear cell carcinoma cell lines (OVISe, TOV21G, RMG1, and OVTOKO cell lines) were all obtained from the Japanese Collection of Research Bioresources. SKOV3 cell line was obtained from the American Type Culture Collection. OVCA429 and KK cell lines were obtained from Dr. Ie-Ming Shih. OVISe, TOV21G, SKOV3, OVCA429, and OVTOKO cells were cultured in RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin. RMG1 cells were cultured in 1:1 Dulbecco's modified Eagle's medium (DMEM): F12 supplemented with 10% FBS and 1% penicillin/streptomycin. KK cell lines were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cell lines were re-authenticated by The Wistar Institute's Genomics Facility at the end of experiments within the last three months using short tandem repeat profiling using AmpFLSTR Identifier PCR Amplification kit (Life Technologies). Mycoplasma testing was performed by LookOut Mycoplasma PCR detection (Sigma). 3D culture was adapted from previously published methods using growth factor reduced-Matrigel (GFR-Matrigel; BD Biosciences) (Bitler et al., 2015). Briefly, a single cell suspension was plated in 8-well chambers covered with Matrigel. Matrigel media with either vehicle control (DMSO) or drug was changed every 4 days, and cells were grown for 12 days. Each of the experiments was performed in duplicate in three independent experimental repeats.

Reagents and antibodies.

SAHA was obtained from ApexBio Technology LLC. The following antibodies were from the indicated suppliers: anti-HDAC1 (Cell Signaling, Cat. No. 5356, 1:1,000), anti-HDAC2 (Abcam, Cat. No. ab12169, 1:1,000), anti-HDAC2 (Cell Signaling, Cat. No. 2545, 1:1,000), anti-EZH2 (BD Bioscience, Cat. No. 612666, 1:1,000), anti-EZH2 (Cell Signaling, Cat. No. 5246, 1:1,000), anti-ARID1A (Santa Cruz, Cat. No. sc-32761, 1:500), anti-PIK3IP1 (Santa Cruz, Cat. No. sc-86785, 1:500), anti-H3K27Ac (Millipore, Cat. No. 07-360, 1:1,000), anti-Ki67 (Cell Signaling, Cat. No. 9449, 1:1,000), anti-cleaved caspase 3 (Cell Signaling, Cat. No. 9661, 1:1,000), anti-cleaved PARP p85 (Promega, Cat. No. G7341, 1:1,000), anti-pAKT (T308, Cell Signaling, Cat. No. 13038, 1:1,000), anti-AKT (Cell Signaling, Cat. No. 9272, 1:1,000), anti-RNA polymerase II (Santa Cruz, Cat. No. sc-899X, 1:500), anti- β -actin (Sigma, Cat. No. A5441, 1:10,000), anti-GAPDH (Millipore, Cat. No. MAB374, 1:10,000). Note that the antibodies against cleaved caspase 3 or cleaved PARP p85 do not recognize the non-cleaved forms of caspase 3 or PARP.

Immunoblotting and Immunoprecipitation

Protein was isolated as previously described (Bitler et al., 2015). Briefly, protein was extracted with RIPA buffer (150mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris pH 8.0, and 1mM PMSF). Protein was separated on a SDS-PAGE and transferred to PVDF membrane. For immunoprecipitation, cells were washed with ice-cold PBS and lysed in the lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.5% NP40) supplemented with Protease Inhibitor Cocktail (Sigma). The cell lysates were centrifuged at 12000 rpm for 5 min. The supernatant was incubated with indicated antibodies for 3h, followed by incubation with Protein G magnetic Dynabeads for 1 h (Invitrogen). The immunoprecipitated proteins were subsequently analyzed with Immunoblotting. An isotype-matched immunoglobulin G (IgG) was used as a negative control.

Generation of ARID1A CRISPR RMG1 cells.

RMG1 cells were transfected with CRISPR-ARID1A (pSpCas9 (BB)-2A-Puro (PX459)). The ARID1A gRNA 5'-CGGGTTGCCAGGCTGCTGGCGG-3'. The plasmid was a generous gift from Dr. Cigall Kadoch (DFCI). Fugene6 transfection reagent (Promega) was used as per manufacturer's specifications. Clonal populations for the loss of ARID1A expression were screened through immunoblot as previously described (Bitler et al., 2015).

Lentivirus infection and HDAC2 ectopic expression.

Lentivirus was packaged using the Virapower Kit from Life Technologies (Carlsbad, CA) following the manufacturer's instructions. pLenti-CMV-Puro-Luciferase was obtained from Addgene. pLKO.1-shHDAC2 (Cat. No: TRCN0000004819 and TRCN0000004823), pLKO.1-shPIK3IP1 (Cat. No. TRCN0000138560) were from Open Biosystems and obtained from the Molecular Screening Facility at The Wistar Institute. HDAC2 wildtype and a catalytically inactive H142A HDAC2 mutant were constructed by PCR-based mutagenesis using the following

primers. *HDAC2* (forward, 5'-CTCGGATCCATGGCGTACAGTCAAGGAGGCGGCAAAAAAAAAAGT-3'; reverse, 5'-GTCCTCGAGTCAGGGGTTGCTGAGCTGTTCTGATTTGG -3')
HI42A (forward, 5'-GCTGGAGGATTACATGCTGCTAAGAAATC-3'; reverse, 5'-GATTCTTAGCAGCATGTAATCCTCCAGC-3') and subcloned into lentivirus plasmid pLVX-Puro (Promega) by BamHI and XhoI sites using standard molecular cloning protocols. Cells infected with viruses encoding the puromycin resistance gene were selected 1 µg/ml puromycin.

Reverse-transcriptase quantitative PCR (RT-qPCR).

RNA was extracted from cells with RNeasy Mini Kit followed by on-column DNase digest (Qiagen). The expression of mRNA levels for *PIK3IP1* (forward, 5' –GCTAGGAGGAACTACCACCTTTG -3'; reverse, 5' –GATGGACAAGGAGCACTGTTA -3'), and *HDAC2* (forward, 5'-CATGACCCATAACTTGCTGTTAAA-3'; reverse, 5'-ATCTGGTCTTATTGACCGTAGAAA-3') was determined using SYBR green 1-step iScript (Bio-Rad) with Life Technologies QuantStudio 3. β -2-microglobulin was used as an internal control.

Colony formation assay.

Cell lines were infected with lentivirus pLKO. 1-shRNAs or pLKO. 1-control with puromycin selection marker. Infected cells were selected with 1 µg/mL of puromycin for 72 hours and the selected cells were seeded in 12-well or 24-well plates. Cell medium was changed every three days with appropriate drug doses for 12 days. Colonies were washed twice with PBS and fixed with 10% methanol and 10% acetic acid in distilled water. Fixed colonies were stained with 0.005% crystal violet. Integrated density was measured using NIH ImageJ software.

Immunofluorescence and immunohistochemical staining.

Immunofluorescence was performed after 48 hours as indicated by fixing samples in 4% paraformaldehyde and permeabilizing with 0.5% Triton-X. Samples were incubated with primary antibodies for 2 h at room temperature and with highly cross-absorbed secondary antibodies (Invitrogen) for 1 h at room temperature and mounted with ProLong antifade reagent (Invitrogen). Immuno-stained cells were then imaged using a Leica Confocal microscope. Immunohistochemical staining was performed as described previously (Bitler et al., 2017) on consecutive sections from xenografted tumors dissected from control or SAHA-treated immunocompromised female mice.

RNA-seq and bioinformatics analysis.

For RNA-seq analysis, RNA was extracted from TOV21G cells expressing two individual shHDAC2s or control by Trizol (Invitrogen) and subsequently cleaned and DNase-treated using RNeasy columns (Qiagen). Libraries for RNA-seq were prepared with ScriptSeq complete Gold kit (Epicentre) and subjected to a 75 bp paired-end sequencing run on NextSeq 500, using Illumina's NextSeq 500 high output sequencing kit following the manufacturer's instructions. RNA-seq data was deposited in GEO database (Accession number: GSE107201).

Data from RNA-seq performed on *ARID1A*-mutated TOV21G cells expressing two individual shHDAC2s or control was aligned against hg19 version of genome and Ensemble GRCh37 transcriptome using bowtie2 (Langmead and Salzberg, 2012). RSEM v1.2.12 software (Li and Dewey, 2011) was used to estimate gene level read counts and FPKM values. Only 12599 known genes (with Entrez ID) that had expression level of at least FPKM=1 were considered and EdgeR (Robinson et al., 2010) was used to estimate significance of differential expression difference between the two experimental groups. Upregulated genes with FDR<10% significance threshold were considered. Previously reported ARID1A/EZH2 target genes (Bitler et al., 2015) were overlapped with the list of significantly upregulated genes and significance of overlap was estimated using Hypergeometric test.

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