**ARID1A**-mutated ovarian cancers depend on HDAC6 activity

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**ARID1A**, encoding a subunit of the SWI/SNF chromatin-remodelling complex, is the most frequently mutated epigenetic regulator across all human cancers. **ARID1A** and **TP53** mutations are typically mutually exclusive. Therapeutic approaches that correlate with this genetic characteristic remain to be explored. Here, we show that HDAC6 activity is essential in **ARID1A**-mutated ovarian cancers. Inhibition of HDAC6 activity using a clinically applicable small-molecule inhibitor significantly improved the survival of **ARID1A**-mutated cancers. Inhibition of HDAC6 activity in mice bearing **ARID1A**-mutated tumours. This correlated with the suppression of growth and dissemination of **ARID1A**-mutated, but not wild-type, tumours. The dependence on HDAC6 activity in **ARID1A**-mutated cells correlated with a direct transcriptional repression of HDAC6 by **ARID1A**. HDAC6 inhibition selectively promoted apoptosis of **ARID1A**-mutated cells. HDAC6 directly deacetylates Lys120 of p53, a pro-apoptotic post-translational modification. Thus, **ARID1A** mutation inactivates the apoptosis-promoting function of p53 by upregulating HDAC6. Together, these results indicate that pharmacological inhibition of HDAC6 is a therapeutic strategy for **ARID1A**-mutated cancers.

SWI/SNF chromatin remodelling complexes regulate gene transcription by changing chromatin structure through hydrolysing ATP. Cancer genome sequencing found that mutations in genes encoding for the subunits of the SWI/SNF complexes collectively occur in ~20% of all human cancers. For example, saturation analysis of The Cancer Genome Atlas cancer mutational profile reveals that the **ARID1A** subunit of the SWI/SNF complex shows one of the highest mutation rates among epigenetic regulators. Notably, **ARID1A** is mutated in over 50% of ovarian clear cell carcinomas and 30% of ovarian endometrioid carcinomas. **ARID1A** mutation is a known genetic driver of ovarian cancer. **ARID1A** and **TP53** mutations are typically mutually exclusive in ovarian cancer. Consistently, **ARID1A**-mutated ovarian cancers often lack genomic instability. However, therapeutic approaches harnessing this genetic characteristic of **ARID1A**-mutated cancers remain to be explored.

Over 90% of the **ARID1A** mutations observed in ovarian cancer are frame-shift or nonsense mutations that result in loss of **ARID1A** protein expression. Loss of **ARID1A** correlates with late-stage disease and predicts early recurrence of ovarian clear cell carcinoma. Ovarian clear cell carcinoma ranks second as the cause of death from epithelial ovarian cancer and is associated with the worst prognosis amongst the major ovarian cancer subtypes when diagnosed at advanced stages. Additionally, for advanced stage disease, there is currently no effective therapy. Notably, in Japan, its prevalence is higher than in western countries, with an estimated incidence of ~25% of epithelial ovarian cancer.

Histone deacetylase 6 (HDAC6) belongs to class Ib HDACs. Unlike other HDACs, HDAC6 primarily functions in the cytoplasm. HDAC6 deacetylates various substrates to regulate protein trafficking and degradation, cell shape and migration. HDAC6 expression is increased in several cancer types including ovarian cancer. Specific small-molecule HDAC6 inhibitors have been developed and are in clinical trials for human haematopoietic malignancies. Here we show that inhibition of HDAC6 activity is selective against **ARID1A**-mutated ovarian cancer. Our findings provide scientific rationale for targeting **ARID1A** mutation in ovarian cancer using pharmacological inhibition of HDAC6 activity.

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ARID1A-inactivated cells are selectively sensitive to HDAC6 inhibition

To examine the role of specific HDACs in the context of ARID1A-mutated ovarian cancers, we performed an unbiased short hairpin RNA (shRNA) knockdown-based evaluation against 11 histone deacetylase genes. This was performed in the context of ARID1A wild-type ovarian clear cell RMG1 cells with or without ARID1A knockdown (Fig. 1a). ARID1A knockdown allows us to mimic loss of ARID1A protein expression caused by >90% of ARID1A mutations in ovarian cancer3 and ensure the same genetic background for the unbiased evaluation. We transiently transduced pooled shRNAs for each of the 11 individual HDACs in ARID1A wild-type RMG1 cells with or without ARID1A knockdown (Supplementary Table 1). We confirmed knockdown of all the HDACs by quantitative PCR with reverse transcription and found a similar degree of knockdown of the HDACs regardless of ARID1A expression (Supplementary Fig. 1a). To measure changes in cell viability, these cells were subjected to a colony-formation assay. Similarly to previous reports19, we observed no significant difference between ARID1A wild-type RMG1 cells with or without ARID1A knockdown (Fig. 1b,c). HDAC6 knockdown showed the highest selectivity against ARID1A knockdown with the least growth inhibitory effects on controls (Fig. 1b and Supplementary Table 2). Likewise, HDAC6 knockdown was selective against ARID1A knockout in ARID1A wild-type OVCA429 cells (Fig. 1d–f and Supplementary Fig. 1b). Consistently, HDAC6 knockdown was selective against ARID1A-mutated ovarian clear cell and endometrioid cancer cell lines in the Project Achilles synthetic lethality screen database (Supplementary Fig. 1c)20.

ARID1A status correlates with response to HDAC6 inhibition

We next validated the initial findings in a panel of clear cell ovarian cancer cell lines in three-dimensional (3D) cultures using Matrigel extracellular matrix that more closely mimics the tumour microenvironment. HDAC6 knockdown had no appreciable effect on the growth of ARID1A wild-type cells but significantly suppressed the growth of ARID1A-mutated cells (Fig. 1g,h and Supplementary Fig. 1d). The observed growth inhibition depends on the enzymatic activity of HDAC6 because the growth inhibition was rescued by a wild-type HDAC6 but not a catalytically inactive H216/611A mutant21 (Fig. 2a,b and Supplementary Fig. 2a,b). Notably, the pan-HDAC inhibitor vorinostat that inhibits all other class I and II HDACs22 was not selective against ARID1A knockdown in ARID1A wild-type RMG1 cells (Supplementary Fig. 2c). Given that class I and class II HDACs are not selective against ARID1A knockdown (for example, Fig. 1b), the non-selective nature of the pan-HDAC inhibitor vorinostat against both class I and class II HDACs may have masked its inhibitory effects on HDAC6. Selective and specific HDAC6 inhibitors have been developed. We tested the HDAC6 inhibitor ACY1215 (rocilinostat)23 in a panel of cell lines with or without ARID1A mutation because it was safe in clinical trials18. Compared with ARID1A wild-type cells, the IC50 of ACY1215 was significantly lower in ARID1A-mutated cells (Fig. 2c and Supplementary Table 3). Primary clear cell ovarian tumour cultures without ARID1A expression are more sensitive to ACY1215 compared with those with ARID1A expression (Fig. 2c,d). The IC50 values of ACY1215 in primary cells are comparable to those observed in cell lines (Fig. 2c–e and Supplementary Table 3). ARID1A knockout significantly increased the sensitivity of ARID1A wild-type OVCA429 cells to ACY1215 (Fig. 2f,g). Conversely, restoration of wild-type ARID1A in ARID1A-mutated TOV21G cells reduced the sensitivity of these cells to ACY1215 (Fig. 2h–j). Interestingly, knockdown of other SWI/SNF subunits such as BRG1 (ref. 1) did not increase ACY1215 sensitivity (Supplementary Fig. 2d–f). This correlates with a compensation of BRG1 loss by the mutually exclusive catalytic subunit BRM (Supplementary Fig. 2g–i). We conclude that ARID1A-inactivated cells are selectively sensitive to HDAC6 inhibition.

HDAC6 inhibition triggers apoptosis in ARID1A-inactivated cells

We next determined the mechanism whereby HDAC6 inhibition suppresses the growth of ARID1A-inactivated cells. Treatment with the HDAC6 inhibitor ACY1215 induced apoptosis of ARID1A-inactivated cells as shown by an increase in annexin V-positive cells and upregulation of cleaved caspase 3 and cleaved PARP p85 (Fig. 3a–c and Supplementary Fig. 3a). Consistent with the observed selectivity of HDAC6 inhibition in cells with ARID1A inactivation (Fig. 1), ACY1215 did not induce a significant increase in apoptosis in ARID1A wild-type cells (Fig. 3b–e), and wild-type ARID1A restoration suppressed ACY1215-induced apoptosis in ARID1A-mutated TOV21G cells (Fig. 3f,g). Compared with ARID1A wild-type controls, the HDAC6 inhibitor ACY1215 or knockdown of HDAC6 increased markers of apoptosis in ARID1A knockout cells (Fig. 3d,e). Notably, a pan-caspase inhibitor, Q-VD-Oph, or knockdown of the intrinsic apoptotic pathway initiator caspase 9 or effector caspase 3 (ref. 24) significantly suppressed the apoptosis induced by ACY1215 (Fig. 3h,i and Supplementary Fig. 3b,c). In contrast, knockdown of caspase 8, the caspase of the extrinsic apoptotic pathway24, did not affect the apoptosis induced by ACY1215 (Supplementary Fig. 3d,e). We conclude that HDAC6 inhibition promotes apoptosis in ARID1A-inactivated cells.

ARID1A directly represses HDAC6 gene transcription

We next determined whether ARID1A affects HDAC6 expression levels. We observed a significant increase in HDAC6 messenger RNA and protein expression in ARID1A wild-type cells following ARID1A knockdown (Fig. 4a,b), which correlates with an increase in HDAC6 promoter activity (Fig. 4c). Similarly, HDAC6 was expressed at a higher level in ARID1A knockout cells compared with parental ARID1A wild-type cells (Fig. 4d,e). Conversely, HDAC6 expression was significantly repressed when wild-type ARID1A was restored in ARID1A-mutated cells (Fig. 4f). BRG1 knockdown did not affect repression of HDAC6 by wild-type ARID1A restoration in ARID1A-mutated cells (Supplementary Fig. 4a), which is consistent with the observation that BRG1 knockdown did not affect HDAC6 expression in ARID1A wild-type cells (Supplementary Fig. 2f,g). Notably, HDAC6 is the only HDAC that is upregulated by ARID1A knockout in ARID1A wild-type RMG1 cells and downregulated by wild-type ARID1A restoration in ARID1A-mutated TOV21G cells (Supplementary Fig. 4b). We next determined whether ARID1A regulates HDAC6 in vivo. We compared the HDAC6 expression in genetic mouse models of ovarian carcinomas developed from...
conditional Apc<sup>−/−</sup>/Pten<sup>−/−</sup> and Apc<sup>−/−</sup>/Pten<sup>−/−</sup>/Arid1a<sup>−/−</sup> mice as previously reported<sup>8</sup>. These two mouse ovarian carcinoma models allowed us to examine Arid1a-dependent changes on a comparable genetic background. We examined HDAC6 expression by immunohistochemical (IHC) staining. Indeed, compared with ovarian tumours developed from Apc<sup>−/−</sup>/Pten<sup>−/−</sup> mice, HDAC6 was expressed at a significantly higher level in tumours developed from Apc<sup>−/−</sup>/Pten<sup>−/−</sup>/Arid1a<sup>−/−</sup> mice (Fig. 4g,h). Consistently, cells
Figure 2 The selectivity against ARID1A mutation depends on the enzymatic activity of HDAC6. (a) Expression of HDAC6, FLAG and a loading control β-actin in ARID1A-mutated TOV21G cells expressing a shHDAC and concurrent expression of FLAG-tagged shRNA-resistant wild-type HDAC6 or a catalytically inactive H216/611A mutant. (b) The indicated cells were subjected to colony-formation assay and integrated density was measured. n = 4 independent experiments. (c) The IC_{50} of the HDAC6 inhibitor ACY1215 is significantly higher in ARID1A wild type (n = 4 cell lines) than mutated (n = 4 cell lines) cells. (d) Expression of ARID1A and a loading control β-actin in the indicated primary cultures of human ovarian clear cell carcinomas determined by immunoblot. (e) HDAC6 inhibitor ACY1215 dose response curves of primary clear cell ovarian tumour cultures with (VOA4841) and without (XVOA295) ARID1A expression. n = 3 independent experiments. (f) Control and ARID1A CRISPR OVCA429 cells were treated with or without 1.25μM ACY1215 in a colony-formation assay. (g) Quantification of f. n = 4 independent experiments. (h) Immunoblot of the indicated proteins in ARID1A-mutated TOV21G cells with or without wild-type ARID1A restoration. (i) The indicated cells treated with or without ACY1215 were plated in 24-well plates in quadruplicates and subjected to colony-formation assay for 12 days, after which they were stained with 0.05% crystal violet (shown are cells treated with 625 nM ACY1215). Note that ARID1A restoration inhibits the growth of ARID1A-mutated cells^19. To limit the potential bias in colony formation, the number of cells used for ARID1A-restored cells was twice that of the control ARID1A-mutated cells. (j) Integrated density was measured with NIH ImageJ software as a surrogate for cell growth. The concentrations of ACY1215 were 312 nM and 625 nM, respectively. n = 4 independent experiments. Error bars represent mean with s.e.m. P values were calculated using two-tailed t-test. Statistical source data are provided in Supplementary Table 6. Unprocessed original scans of all blots with size markers are shown in Supplementary Fig. 9.

| Derived from Apc^-/-/Pten^-/-/Arid1a^-/- tumours are more sensitive to ACY1215 compared with those derived from Apc^-/-/Pten^-/- tumours (Supplementary Fig. 4c). HDAC6 was the only class II HDAC that is expressed at significantly higher levels in ARID1A-mutated compared with wild-type primary human clear cell ovarian carcinomas (Fig. 4i and Supplementary Fig. 4d). In addition, ARID1A expression negatively correlates with HDAC6 expression in both clear cell and endometrioid ovarian cancer cell lines and laser capture microdissected specimens on the basis of database mining (Supplementary Fig. 4e,f). We conclude that ARID1A represses HDAC6 expression, and ARID1A inactivation upregulates HDAC6 expression. |
SWI/SNF complexes contribute to both gene activation and repression in a context-dependent manner. We determined whether ARID1A directly represses HDAC6 expression on the basis of published data from chromatin immunoprecipitation followed by next-generation sequencing (ChIP-seq) of ARID1A. Indeed, there is a significant enrichment of ARID1A at HDAC6 promoter regions (Fig. 4j). Validating these findings, we observed a significant association of ARID1A with the HDAC6 gene promoter in ARID1A wild-type cells (Fig. 4k). Supporting the notion that ARID1A directly suppresses HDAC6 transcription, ARID1A knockdown reduced its association with the HDAC6 gene promoter (Fig. 4k and Supplementary Fig. 4g,h). This correlated with a decrease in BRG1, an increase in RNA polymerase II (Pol II) and an increase in the association of acetylated histone H3 with the HDAC6 gene promoter (Fig. 4lm and Supplementary Fig. 4i,j). Conversely, wild-type ARID1A restoration in ARID1A-mutated TOV21G cells correlated with an increase in ARID1A and BRG1 and a decrease in the association of Pol II with the HDAC6 gene promoter (Fig. 4n-p). Thus, we identified ARID1A as a direct repressor of HDAC6 gene transcription.

p53 acetylated at Lys120 (p53K120Ac) is a direct substrate for HDAC6-mediated deacetylation

Next-generation sequencing revealed that ARID1A and TP53 mutation are typically mutually exclusive in The Cancer Genome Atlas.
Figure 4 ARID1A represses HDAC6 expression. (a) ARID1A wild-type RMG1 cells with or without ARID1A knockdown, n = 4 independent experiments. (b) ARID1A, HDAC6 and GAPDH protein expression. (c) Human HDAC6 gene promoter activity in ARID1A wild-type RMG1 cells with or without ARID1A knockdown, n = 3 independent experiments. (d) ARID1A wild-type and knockout OVCA429 cells were examined for expression of HDAC6 mRNA; n = 3 independent experiments. (e) ARID1A, HDAC6 and β-actin protein expression. (f) ARID1A-mutated TOV21G cells with or without wild-type ARID1A restoration were examined for ARID1A and HDAC6 mRNA and protein expression. GAPDH was used as a loading control. n = 3 for ARID1A and 4 for HDAC6 independent experiments. (g) Representative images of immunohistochemical staining of ARID1A and HDAC6 on consecutive sections of endometrioid tumours developed from Apc+/−/Pten−/− or Apc+/−/Pten−/−/Arid1a+/− conditional genetic mouse models. Scale bars, 100 μm. (h) Quantification of g. Histological score (H score) was quantified (n = 3 independent tumours) for the indicated groups. (i) Relative HDAC6 mRNA expression in ARID1A wild-type (n = 12) and mutated (n = 7) human ovarian clear cell carcinoma specimens. Mann–Whitney test was used to compare the two groups and generate the P value. (j) ARID1A ChIP-seq and input tracks at the human HDAC6 gene promoter based on a ChIP-seq data set. Similar results were also obtained for ARID1A knockdown were subjected to ChIP analysis for the HDAC6 gene promoter using antibodies against ARID1A (k), n = 6 independent experiments; BRG1 (l), n = 4 independent experiments; or Pol II (m), n = 7 independent experiments. An isotype-matched IgG was used as a control. (n-p) ARID1A-mutated TOV21G cells with or without wild-type ARID1A restoration were subjected to ChIP analysis for the HDAC6 gene promoter using antibodies against ARID1A (n), n = 3 independent experiments; anti-BRG1 (o), n = 4 independent experiments; or Pol II (p), n = 4 independent experiments. An isotype-matched IgG was used as a control. Error bars represent mean with s.e.m. P values were calculated using two-tailed t-test unless otherwise specified. Statistical source data are provided in Supplementary Table 6. Unprocessed original scans of all blots with size markers are shown in Supplementary Fig. 9.

Notably, knockdown of p53 expression significantly impaired the apoptosis and growth inhibition induced by the HDAC6 inhibitor ACY1215 in ARID1A-mutated TOV21G (Fig. 5a–e) and OVISE cells (Supplementary Fig. 5a–c). Similar results were also obtained for another HDAC6 inhibitor CAY10603 (Supplementary Fig. 5d). However,
Relative p53 mRNA expression

|         | shControl | shp53 no. 1 | shp53 no. 2 |
|---------|-----------|-------------|-------------|
| p53     | 0.5       | 1.0         | 1.0         |

Figure 5 The selectivity of HDAC6 inhibition against ARID1A inactivation depends on p53 and HDAC6 deacetylase Lys120 residues on p53. (a–c) ARID1A-mutated TOV21G cells with or without p53 knockdown were examined for TP53 mRNA expression, n = 3 independent experiments (a) and p53 and GAPDH protein expression (b); or treated with the indicated doses of the HDAC6 inhibitor ACY1215 (c); shown are representative images of colonies formed. (d) Dose-responsive curves of the indicated cells. ARID1A wild-type RMG1 and OVCA429 cells were used as controls for comparison. n = 4 independent experiments. Error bars represent s.e.m. (e) ARID1A-mutated TOV21G with or without p53 knockdown treated with ACY1215 (1.25 μM) or DMSO controls for 48 h. The percentage of apoptosis was quantified by FACS analysis based on annexin V staining. n = 3 independent experiments. Error bars represent s.e.m. (f) ARID1A-mutated TOV21G treated with vehicle DMSO control or the HDAC6 inhibitor ACY1215 (1.25 μM). Expression of the indicated proteins was determined. (g) ARID1A-mutated TOV21G cells without or with shHDAC6 knockdown were examined for expression of p53K120Ac, total p53, HDAC6 and GAPDH by immunoblot. (h) ARID1A wild-type RMG1 with or without ARID1A knockdown were treated with vehicle DMSO control or the HDAC6 inhibitor ACY1215 (1.25 μM). Expression of the indicated proteins was examined. (i) In vitro deacetylase assay using a recombinant human HDAC6 construct and the p53K120Ac-based peptide substrate Ac-Leu-His-Ser-Gly-Thr-Ala-Lys(Ac)-Ser-Val-Thr-COOH. Deacetylation substrate and product were detected and quantitated using a discontinuous LC–MS assay. The negative control assay was run in the presence of the HDAC6 inhibitor ACY1215. All assays were performed in triplicate independent experimental trials and yielded a specific activity of 3.4 ± 0.4 nmol product per nmol enzyme per min. Statistical source data are provided in Supplementary Table 6. Unprocessed original scans of all blots with size markers are shown in Supplementary Fig. 9.

HDAC6 inhibition did not affect p53 expression levels (Fig. 5f), indicating that HDAC6 may regulate p53 post-translationally. Since HDAC6 is a deacetylase, we evaluated the changes of p53 acetylation status on the lysine residues that are known to regulate apoptosis such as Lys120, Lys373 and Lys382. These residues were evaluated in ARID1A-mutated TOV21G cells following treatment with the HDAC6 inhibitor ACY1215. p53K120Ac was upregulated by ACY1215 treatment (Fig. 5g), while the acetylation status of Lys373 or Lys382 was unchanged (Supplementary Fig. 5e). To confirm that the increase in p53K120Ac was specific for HDAC6 inhibition, we knocked down HDAC6 with two individual shRNAs. We observed a strong concordance of the level of HDAC6 knockdown and the observed increase of p53K120Ac (Fig. 5g). We also evaluated p53K120Ac in ARID1A wild-type RMG1 cells with or without ARID1A knockdown after ACY1215
Figure 6 Apoptosis induced by HDAC6 inhibition in ARID1A-mutated cells correlates with mitochondrial localization of p53K120Ac. (a) ARID1A-mutated TOV21G cells treated with vehicle DMSO control or the HDAC6 inhibitor ACY1215 (1.25 μM) were fixed and subjected to immunofluorescence staining using antibodies against p53K120Ac (magenta), TOM20 (turquoise, a mitochondrial marker) and DAPI (yellow, nuclei). Scale bars, 25 μm. (b) Confocal images were processed and co-localization between p53K120Ac and TOM20 was quantified using Leica Application Suite X (LASX) software. Error bars represent s.e.m. (n = 6 independent experiments). P = 0.0027. (c) ARID1A-mutated TOV21G cells treated with vehicle DMSO control or the HDAC6 inhibitor ACY1215 (1.25 μM) were fixed and subjected to immunofluorescence staining using antibodies against p53K120Ac (magenta), HDAC6 (turquoise) and DAPI (yellow, nuclei). Images were captured using confocal microscopy. Scale bars, 25 μm. (d) Confocal images were processed, and co-localization between p53K120Ac and HDAC6 was quantified using Leica Application Suite X (LASX) software. Error bars represent s.e.m. (n = 6 independent experiments). P < 0.0001.

target apoptosis-promoting genes such as BAX and PUMA and transcription-independent mechanisms through its cytoplasmic localization in mitochondria28–32. Thus, we evaluated transcriptional changes by RNA-seq after HDAC6 inhibition using two different HDAC6 inhibitors (namely ACY1215 or CAY10603 (ref. 33)) or HDAC6 knockdown. Gene expression profiling did not reveal a canonical p53-dependent apoptotic pathway by HDAC6 inhibition (GEO accession number: GSE84405). For example, known p53K120Ac target genes such as BAX and PUMA were not significantly upregulated by HDAC6 inhibition (Supplementary Fig. 6a and Supplementary Table 5). This indicates that p53 may regulate apoptosis induced by HDAC6 inhibition in a transcription-independent manner. p53K120Ac can also promote apoptosis through its mitochondrial localization30. We therefore measured the localization of p53K120Ac to the mitochondria following HDAC6 inhibition in ARID1A-mutated cells. Immunofluorescence analysis revealed that HDAC6 inhibition induced a significant increase in co-localization of p53K120Ac and the mitochondrial marker TOM20 or HDAC6 (Fig. 6a–d). Notably, NU9056, an inhibitor of TIP60 (which acetylates p53K120 (ref. 32)), suppressed apoptosis induced by ACY1215, which correlated with the reduction of the p53K120Ac levels (Supplementary Fig. 6b,c). Cellular fractionation showed an increase in p53K120Ac in the mitochondrial fraction in cells treated with the HDAC6 inhibitor ACY1215 compared with controls (Fig. 6e).
Mitochondrial p53K120Ac promotes apoptosis through decreasing mitochondrial membrane potential\(^\text{14}\). Indeed, ACY1215 significantly decreased the mitochondrial membrane potential in ARID1A-mutated cells (Fig. 7a,b and Supplementary Fig. 6d). Consistent with the observed selectivity against ARID1A inactivation by HDAC6 inhibition (Fig. 1), ARID1A knockdown in ARID1A wild-type cells significantly decreased mitochondrial membrane potential in cells treated with ACY1215 compared with controls (Fig. 7c,d). Mitochondrial membrane potential decrease by ACY1215 was both p53 and p53K120Ac dependent, because knockdown of p53 suppressed the observed decrease in mitochondrial membrane potential and this was rescued by wild-type p53 but not a p53K120R mutant (Fig. 7c,f). Indeed, wild-type p53 but not the p53K120R mutant rescued the p53 knockdown-mediated impairment of ACY1215-induced growth inhibition (Fig. 7g). We conclude that HDAC6 inhibition promotes transcription-independent apoptosis that correlates with p53K120Ac mitochondrial localization (Supplementary Fig. 7).

**HDAC6 inhibition by ACY1215 improves the survival of mice bearing ARID1A-mutated ovarian tumours**

Clinical studies show that the HDAC6 inhibitor ACY1215 is well-tolerated without a dose-limiting toxicity\(^\text{18}\). To determine the effects of HDAC6 inhibition *in vivo* on the growth of ARID1A-mutated tumours, we orthotopically transplanted luciferase-expressing ARID1A-mutated TOV21G cells into the bursa sac covering the ovary of immunocompromised nude mice to mimic the tumour microenvironment. The injected ARID1A wild-type or mutant cells were allowed to grow for 2 weeks to establish the orthotopic tumours. Mice were then randomized and treated daily with vehicle control or ACY1215 (50 mg kg\(^{-1}\)) by intraperitoneal (i.p.) injection, the same dose as previously reported\(^\text{15}\). Indeed, ACY1215 treatment significantly inhibited the growth of ARID1A-mutated tumours (Supplementary Fig. 8a,b). We next followed the survival of the treated mice after discontinuing the treatment regimen. Importantly, ACY1215 significantly improved the survival of mice bearing the orthotopically transplanted ARID1A-mutated tumours compared with controls (Fig. 8a). Specifically, the median survival was improved from 35 days in the vehicle control group to 51 days in the ACY1215-treated group. Thus, we conclude that the HDAC6 inhibitor ACY1215 significantly improves the survival of mice bearing ARID1A-mutated tumours.

We next directly examined the effects of the HDAC6 inhibitor ACY1215 on tumour burden of the transplanted ARID1A-mutated or wild-type cells. Indeed, using tumour weight as a surrogate for tumour burden, we found that ACY1215 treatment significantly reduced the burden of ARID1A-mutated orthotopically xenografted tumours (Fig. 8b,c). Likewise, ACY1215 significantly suppressed tumour growth in the conditional *Arid1a\(^{−/−}\)/Pik3ca\(^{H1047R}\) genetic clear cell ovarian tumour mouse model\(^\text{8}\) (Supplementary Fig. 8c). Ovarian cancer often progresses by disseminating to the intraperitoneal cavity\(^\text{36}\). Thus, we quantified the number of grossly visible tumour nodules in the peritoneal cavity following treatment with vehicle control or ACY1215 in the pre-established ARID1A-mutated tumours. There was a significant decrease in the number of nodules in ACY1215-treated mice compared with controls (Fig. 8d). These findings highlight the potential clinical benefit of HDAC6 inhibitors in the treatment of ARID1A-mutated ovarian cancer.
Figure 8 HDAC6 inhibition improves the survival of mice bearing ARID1A-mutated ovarian tumours. (a) ARID1A-mutated TOV21G cells were orthotopically transplanted into the ovarian bursa sac of SCID/nude female mice. Tumours were allowed to establish for 2 weeks before the mice were randomized into two different treatment groups (n = 5 mice per group). Mice were treated with vehicle control or the HDAC6 inhibitor ACY1215 (50 mg kg$^{-1}$) daily for an additional 3 weeks. After stopping the treatment, the mice from the indicated groups were followed for survival. Shown is the Kaplan–Meier survival curves for ACY1215 or vehicle control treated mice. $P$ values were calculated by log-rank test. (b) At the end of treatment, the mice were euthanized (n = 6 mice per group). Shown are representative images of reproductive tracts with tumours from control or ACY1215-treated mice. Scale bars, 2 cm. (c) Tumour weight was measured as a surrogate for tumour burden from the control and ACY1215-treated mice. (d) The mice from the indicated group were examined for disseminated tumour nodules in the peritoneal cavity. Representative images of disseminated tumour nodules in control and ACY1215 treated mice. Asterisks (*) indicate the disseminated tumour nodules in the peritoneal cavity. (e) The number of disseminated tumour nodules in the peritoneal cavity was quantified. (f) The consecutive sections of tumours dissected from the indicated treatment groups were subjected to immunohistochemical staining for HDAC6, Ki67, cleaved caspase 3 and p53K120Ac. Scale bars, 100 μm. (g) Histological score (H score) was calculated for 5 separate fields from 6 tumours from 6 individual mice from each of the indicated groups. Error bars represent mean with s.e.m. $P$ values were calculated using two-tailed t-test. Statistical source data are provided in Supplementary Table 6.

tumour nodules in ACY1215-treated mice bearing ARID1A-mutated tumours compared with controls (Fig. 8d,e). As a control, luciferase-expressing ARID1A wild-type RMG1 cells were orthotopically transplanted in parallel. In contrast to what we observed in ARID1A-mutated tumours, ACY1215 treatment did not significantly affect the growth, tumour burden or dissemination of ARID1A wild-type tumours (Supplementary Fig. 8d–g).

Finally, we sought to correlate the observed improvement of survival, suppression of tumour growth and reduction in tumour burden in vivo with the molecular pathways we have revealed for the observed dependence of ARID1A-mutated cells on HDAC6 activity in vitro. To do so, we performed IHC analysis for markers of cell proliferation (Ki67), apoptosis (cleaved caspase 3), HDAC6 and p53K120Ac in dissected ARID1A-mutated tumours treated with ACY1215 or controls.
ACY1215 significantly decreased the cell proliferation marker Ki67 and increased the apoptotic marker cleaved caspase 3 (Fig. 8f,g). As a control, HDAC6 expression was not affected by ACY1215 (Fig. 8f,g). Furthermore, p53K120Ac staining was significantly increased by ACY1215 treatment (Fig. 8h,i). In contrast, ACY1215 did not affect the expression of Ki67, cleaved caspase 3 or p53K120Ac in ARID1A wild-type tumours (Supplementary Fig. 8h,i). This is consistent with the finding that ACY1215 did not affect the growth of ARID1A wild-type tumours in vivo (Supplementary Fig. 8d–f). On the basis of these results, we conclude that the HDAC6 inhibitor ACY1215 selectively suppresses the growth and dissemination of ARID1A-mutated ovarian tumours and improves the survival of mice bearing ARID1A-mutated tumours. This correlates with a decrease in cell proliferation, an increase in apoptosis and an accumulation of apoptosis-promoting p53K120Ac in the treated ARID1A-mutated tumours.

**DISCUSSION**

Our data demonstrate a dependence of ARID1A-mutated cells on HDAC6 activity. This was due to the direct suppression of HDAC6 transcription by ARID1A. Consequently, ARID1A inactivation upregulates HDAC6 expression. Although the SWI/SNF complex mostly promotes the transcription of its target genes, it can also repress gene transcription. Previous reports established that ARID1A inactivation correlates with silencing of tumour suppressive genes such as PIK3IP1 (ref. 19). Here we showed that HDAC6 is a direct target of ARID1A-mediated transcriptional repression. ARID1A inactivation leads to upregulation of HDAC6; therefore, HDAC6 inhibition is selective against ARID1A inactivation. This suggests that both transcriptional repression of oncogenic genes and transcriptional activation of tumour suppressor genes contribute to the tumour suppressive activity of ARID1A.

Here we show that ARID1A inactivation upregulates HDAC6, and HDAC6 directly deacetylates the apoptosis-promoting p53K120Ac post-translational modification. Our biochemical experiments show that p53K120Ac is a substrate of HDAC6 and thus identify a deacetylase for p53 post-translational modification. This suggests that ARID1A mutation functionally inactivates p53 to suppress apoptosis. This, at least in part, resolves the typical mutual exclusivity of ARID1A and TP53 mutations. Notably, HDAC6 inhibitors such as ACY1215 are well-tolerated and show minimal toxicity in clinical trials. Our studies provide scientific rationale for potential translation of these findings by repurposing clinically applicable HDAC6 inhibitors for ARID1A-mutated ovarian cancers, for which no effective therapies currently exist. Given that ARID1A shows one of the highest mutation rates among epigenetic regulators and loss of expression of ARID1A also occurs in many cancer types, our findings may have far-reaching implications for improving therapy for a wide array of cancer types.

**METHODS**

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of this paper.

**Note:** Supplementary Information is available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

B.G.B., S.W., P.H.P., Y.H., K.M.A., Y.W. and A.V.-A. performed the experiments, and analysed data. B.G.B. and R.Z. designed the experiments. A.V.K. performed the bioinformatics analysis. F.J.R.III, J.R.C.-G., W.Z. and D.W.S. participated in the experimental design; K.R.C. and Y.Z. contributed key reagents. D.G.H., D.W.C. and R.Z. supervised studies. B.G.B., K.M.A., Y.W., K.R.C., D.W.C. and R.Z. wrote the manuscript. R.Z. conceived the study.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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METHODS

Cell lines and 3D culture conditions. The protocol for using primary cultures of human ovarian clear tumour cells was approved by the University of British Columbia Institutional Review Board. Informed consent was obtained from human subjects and all protocols involving human specimens have been compiled. The primary tumour cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Ovarian clear cell carcinoma cell lines (TOV21G, OVTOKO, OVISE and RMG1) were purchased from JCRB. TOV21G, OVTOKO and OVISE cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. RMG1 cells were cultured in 1:1 Dulbecco’s modified Eagle’s medium (DMEM)/F12 supplemented with 10% FBS. Viral packaging cells were cultured in DMEM supplemented with 10% FBS at 37°C supplied with 5% CO2. Cells lines are authenticated at The Wistar Institute’s Genomics Facility using short tandem repeat DNA profiling. Regular mycoplasma testing was performed using the LookOut Mycoplasma PCR detection (Sigma). 3D culture was adapted from previously published methods with growing factor-reduced Matrigel (GFP-Matrigel; BD Biosciences). Briefly, a single-cell suspension was plated in 8-well chambers covered with Matrigel. Matrigel medium with either vehicle control (dimethylsulfoxide (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. ACY1215, Q-VD-Oph and CAY10603 were obtained from Selleckchem. N9056 was purchased from Tocris. The following antibodies were obtained from Invitrogen: rabbit supplies: mouse anti-acetylated-p53 K120 (Abcam, cat. no. ab78316, 1:1,000 for western blot and 1:100 for IHC), rabbit anti-acetylated-p53 K382 (Abcam, cat. no. ab62376, 1:1,000 for western blot), rabbit anti-acetylated-p53 K38 (Abcam, cat. no. ab75754, 1:1,000 for western blot), mouse anti-ARID1A (Santa Cruz, cat. no. sc-32761, 1:1,000 for western blot and 1:100 of IgG for each blot), mouse anti-p53 (Millipore, cat. no. OP43, 1:1,000 for western blot), mouse anti-GAPDH (Millipore, cat. no. MAB374, 1:1,000 for western blot), rabbit anti-cleaved PARP (Promega, cat. no. pRL-SV40, 1:1,000 for western blot), mouse anti-Ki67 (Cell Signaling, cat. no. 9449, 1:1,000 for IHC), rabbit anti-cleaved caspase 3 (Cell Signaling, cat. no. 9661, 1:1,000 for western blot and 1:50 for IHC), rabbit anti-HDAC6 (Cell Signaling, cat. no. 7612, 1:1,000 for western blot and Santa Cruz, cat. no. sc-14920, 1:100 for IHC), rabbit anti-RNA polymerase II (Santa Cruz, cat. no. sc-899 X, 2 µg per sample for each blot), rabbit anti-TOM20 (Santa Cruz, cat. no. sc-14145, 1:1,000 for western blot), rabbit anti-HDAC (Active Motif, cat. no. 39139, 5 µl per immunoprecipitation for each blot), rabbit anti-TIP60 (Santa Cruz, cat. no. sc-166323, 1:1,000 for western blot), mouse anti-BRG1 (Santa Cruz, cat. no. sc-17796, 1:1,000 for western blot and 2 µg per immunoprecipitation for each blot) and rabbit anti-BRM (Cell Signaling, cat. no. 11966, 1:1,000 for western blot and 3 µg per immunoprecipitation for each blot). Growth factor-reduced Matrigel was purchased from Corning.

Immunoblotting. Protein was isolated as previously described. Briefly, protein was extracted with RIPA buffer (150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0 and 1 mM phenylmethylsulfonyl fluoride). Protein was separated by SDS-PAGE and transferred to PVDF membrane. For immunoblotting, standard curves generated from the mass signals of the corresponding deacetylated M substrate in 20 mM HEPES (pH 7.5), 100 mM NaCl, 5 mM KCl, 1 mM MgCl2, for 20 min at room temperature, and the reaction was quenched by the addition of acetonitrile (equal volume to the reaction solution). The deacetylation reaction mixtures were analysed by LC-MS using a Waters SQD equipped with an Acquity UPLC (Waters) and quantified using the standard curves generated from the mass signals of the corresponding deacetylated synthetic peptide (Ac-LHSGTALKa(ac)SVT-COOH). As a negative control, the assay was run in the presence of the HDAC6-specific inhibitor ACY1215 at 10 µM. All assays were performed in triplicate. The peptide was custom-synthesized by Genscript. The human HDAC6 construct was expressed and purified as previously reported.

Chromatin immunoprecipitation (ChIP). ChIP was performed as we have previously described. The following antibodies were used to perform ChIP: ARID1A (Santa Cruz), RNA polymerase II (Santa Cruz), H3Ac (Active Motif) and BRG1 (Santa Cruz). An isotype-matched IgG was used as a negative control. ChIP DNA was analysed by quantitative PCR as the promoter of the human HDAC6 gene. All primer sequences are in Supplementary Table 1. For single-site PCR, the primers for position –880 upstream of the transcription starting site were used.

Immunofluorescence and immunohistochemical staining. Immunofluorescence was performed after 48 h 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, followed by a wash and secondary antibodies (Invitrogen) for 1 h at room temperature and mounted with Prolong anti-fade reagent (Invitrogen). Immunostained cells were imaged using a Leica Confocal microscope. Immunohistochemical staining was performed as we have described previously on consecutive sections from xenografted tumours dissected from control or ACY1215-treated immunocompromised nude female mice. Expression of the stained markers was scored using a histologic score (H score) as previously described.

Mitochondrial isolation. Mitochondria were isolated using the Mitochondria Isolation Kit (Thermo Fisher Scientific). Isolation was performed according to the
Intrabursal orthotopic xenograft models in vivo. The protocols were approved by the Institutional Animal Care and Use Committee (IACUC). All mouse experiments were conducted according to ethical regulations. For in vivo experiments, the sample size of 6 mice per group was determined on the basis of the data shown from in vitro experiments. Intrabursal orthotopic xenograft was performed as described previously. Briefly, 1 × 10^6 luciferase-expressing TOV21G or RMG1 cells were unilaterally injected into the ovarian bursa sac of 6-8-week-old female immunocompromised mice (n = 6 per group). Two weeks after injection, tumours were visualized by injecting luciferin (i.p.: 4 mg per mouse) resuspended in PBS and imaged with an In Vivo Imaging System (IVIS). The mice were then randomized into two groups based on luciferase activity and treated with vehicle control (2% DMSO/30% PEG 300/ddH_2O) or ACY1215 (50 mg/kg daily) for three weeks and imaged for luciferase activity. Images were analysed using Live Imaging 4.0 software. Imaging analysis was performed blindly but not randomly. At the end of the experiments, tumours were surgically dissected and tumour burden was calculated on the basis of tumour weight. Intrapertioneally disseminated tumour nodules were quantified.

**Arid1a<sup>−/−</sup>/Pik3ca<sup>H1047R</sup>** genetic clear cell ovarian tumour model. All experiments were approved by IACUC. Transgenic mice with latent mutations in Arid1a and Pik3ca were generated by crossing Arid1a<sup>fl<sup>h94778</sup></sup> and Pik3ca<sup>H1047R</sup> mice (kindly provided by C. Wang, U. Michigan, USA) and crossed onto a C57BL/6J background for nine generations) with R26-Pik3ca<sup>H1047R</sup> mice carrying inducible Pik3ca mutations (Jackson Laboratory, Jax no. 016977). Administration of intrabursal adeno-Cre, performed as previously reported, induced ovarian clear cell carcinoma in ~45 days, which is similar to a previous report. All mice were maintained in specific pathogen-free barrier facilities. To induce tumorigenesis, 6-10-week-old Pik3ca<sup>H1047R</sup>/Arid1a<sup>fl<sup>h94778</sup></sup> female mice were intrabursally injected with adenovirus-Cre as previously described. Mice were randomized and treated with ACY1215 (50 mg/kg) or vehicle control for 21 days as previously published. Following treatment, mice were euthanized and the reproductive tracts were removed. The changes in volumes of tumours formed on the injected ovary were calculated against the contrary side non-injected ovary from the same mice.

**Statistical analysis and reproducibility.** Experiments were repeated 3 times unless otherwise stated. The representative images were shown unless otherwise stated. Statistical analysis was performed using GraphPad Prism 5 (GraphPad) for Mac OS. Quantitative data are expressed as mean ± s.e.m. unless otherwise stated. Analysis of variance (ANOVA) with Fisher’s least significant difference was used to identify significant differences in multiple comparisons. For all statistical analyses, the level of significance was set at 0.05. For correlation studies, Pearson’s correlation was used for calculating P and r values GraphPad Prism 5 (GraphPad) for Mac OS. Imaging analysis was performed blindly but not randomly. Animal experiments were randomized. There was no exclusion from the experiments.

**Data availability.** Gene expression profiling data based on RNA-seq have been deposited in the Gene Expression Ominibus (GEO) under accession code GSE84405. Previously published RNA-seq data for ARID1A wild-type or mutated human ovarian clear cell carcinoma specimens that were re-analysed here are available at the European Genome-Phenome Archive (EGAS) under accession code EGAS000000000757. For correlation between ARID1A and HDAC6 expression, gene expression data obtained from GEO (under accession code: GSE36139) for clear cell and endometrioid ovarian cancer cell lines in the Cancer Cell Line Encyclopedia and a microarray database obtained from GEO (under accession code: GSE29450) for profiling gene expression in laser capture microdissected human clear cell ovarian tumour specimens and ovarian surface epithelial cells were used. ARID1A chromatin immunoprecipitation followed by next-generation sequencing (ChIP-seq) and input tracks at the human HDAC6 gene promoter were based on ChIP-seq data (GEO accession number: GSE69568).

Source data used for statistical analyses of Figs 1b,f,h, 2b,c,e,g,j, 3b,c,gi, 4a,c,d,h,i,k,p, 5a,d,e, 6b,d, 7b,d,f,g and 8a,c,e,g,i are provided as Supplementary Table 6 (Statistics source data). All other data supporting the findings of this study are available upon request.

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Supplementary Figure 1 ARID1A-inactivated cells are more sensitive to HDAC6 inhibition. (a) ARID1A wild-type RMG1 cells with or without ARID1A knockdown were transduced with lentivirus encoding shRNA to each of the 11 individual HDACs. RNA was isolated from the indicated cells and subjected to qRT-PCR for the indicated HDACs. n=3 independent experiments. (b) Confirmation of HDAC6 knockdown by qRT-PCR in OVCA429 cells. n=6 independent experiments. (c) HDAC6 knockdown is selective against ARID1A-mutated clear cell or endometrioid ovarian cancer cell lines based on the Project Achilles database. The whiskers of the boxplot represent minima to maxima of the relative growth of cell lines with (n=5 cell lines) or without ARID1A mutation (n=11 cell lines). The box represents median bar with the first and the third quartiles. (d) Expression of HDAC6 determined by immunoblot in a panel of ovarian clear cell carcinoma cell lines with known ARID1A mutational status without or with HDAC6 knockdown using two individual shHDAC6s. GAPDH expression was used as a loading control. Error bars represent mean with S.E.M. P-value calculated via two-tailed t-test. Statistical source data are provided in Supplementary Table 6. Unprocessed original scans of all blots with size marker are shown in Supplementary Fig. 9.
Supplementary Figure 2 BRG knockdown does not affect sensitivity to HDAC6 inhibition. (a-b) Expression of HDAC6, FLAG and loading control β-actin in ARID1A-mutated OVISE cells with knocking down of endogenous HDAC6 expression using a shRNA that targets the 3’ UTR region of the human HDAC6 gene and concurrent expression of FLAG-tagged shRNA resistant wildtype HDAC6 or a catalytically inactivated H216/611A mutant (a). The indicated cells were subjected to colony formation assay and integrated density was measured with NIH Image J software as a surrogate for cell growth (b). n=4 independent experiments. (c) ARID1A wildtype RMG1 cells with or without ARID1A knockdown were determined for Vorinostat dose responsive curves in a 12-day colony formation assay (f), n=4 independent experiments. (g-i) BRM compensates for the knockdown of BRG1 at the HDAC6 gene promoter. ARID1A wildtype RMG1 cells were infected with the indicated shBRG1 or shControl. Expression of BRG1, BRM1, ARID1A and a loading control β-actin was determined by immunoblot (g). The indicated cells were subjected to ChIP analysis for the HDAC6 gene promoter using antibodies against BRG1 (h) or BRM (i). An isotype matched IgG was used as a control. n=4 independent experiments. Error bars represent mean with S.E.M. P-value calculated via two-tailed t-test. Statistical source data are provided in Supplementary Table 6. Unprocessed original scans of all blots with size marker are shown in Supplementary Fig. 9.
Supplementary Figure 3 Caspase 3, Caspase 8 and Caspase 9 knockdown in TOV21G cells. (a) The gating strategy used for determining apoptosis based on AnnexinV-FITC and propidium iodide staining. Note that total apoptotic cells are calculated based on both early and late apoptotic fractions. (b-c) Expression of CASP3 (encodes caspase 3) mRNA (b), n=4 independent experiments; and CASP9 (encodes caspase 9) mRNA (c), n=3 independent experiments; determined by qRT-PCR in ARID1A-mutated TOV21G cells with or without Caspase 3 (b) or Caspase 9 (c) knockdown. (d-e). Caspase 8 inhibition does not affect apoptosis induced by HDAC6 inhibition. ARID1A-mutated TOV21G cells were infected with the indicated shCaspase 8 or shControl. Relative expression of CASP8 (encodes caspase 8) was determined by qRT-PCR (d). n=4 independent experiments. The indicated cells were treated with HDAC6 inhibitor ACY1215 (1.25μM) or vehicle DMSO control for 72 hours and the percentages of apoptotic cells were quantified by Annexin V staining (e). n=3 independent experiments. Error bars represent mean with S.E.M. .

P-value calculated via two-tailed t-test. Statistical source data are provided in Supplementary Table 6.
ARID1A regulates HDAC6 expression. (a) Expression of HDAC6, BRG1 and a loading control β-actin in ARID1A-mutated TOV21G cells with or without wildtype ARID1A restoration and with or without concurrent BRG1 knockdown. ARID1A restoration the same as Fig. 3f. (b) Relative expression of the indicated HDACs mRNA determined by qRT-PCR in ARID1A-mutated TOV21G cells with wildtype ARID1A restoration or ARID1A wildtype RMG1 cells with ARID1A knockdown. n=3 independent experiments. * P<0.01. P-value calculated via two-tailed t-test. (c) Tumour cells derived from the indicated genetic mouse endometrioid tumours were treated with ACY1215 (1.25 μM). Tumour cells derived from the indicated genetic mouse endometrioid tumours were treated with ACY1215 (1.25 μM). (d) Relative mRNA expression of the indicated class II HDACs in ARID1A wildtype (n=12) and mutated (n=7) human ovarian clear cell carcinoma specimens. Note that none of them are significant. (e) ARID1A expression negatively correlates with HDAC6 expression at the mRNA level in clear cell and endometrioid ovarian cancer cells in the cell lines encyclopedia. Pearson correlation was used for calculating P-value. (f) ARID1A expression negatively correlates with HDAC6 expression at the mRNA level in a published dataset of laser capture microdissected (LCM) clear cell ovarian carcinomas and normal human ovarian surface epithelial cells. Pearson correlation was used for calculating P-value. (g) Schematic of primers’ positions for ChIP-PCR in the human HDAC6 gene locus. (h-i) ARID1A wildtype RMG1 cells with or without ARID1A knockdown were subjected to ChIP analysis using antibodies against ARID1A (h), anti-Pol II (i) or an IgG control. ChIP products were subjected to qPCR analysis using primers as indicated in (g) for the human HDAC6 gene locus. n=4 independent experiments. (j) ARID1A wildtype RMG1 cells with or without ARID1A knockdown were subjected to ChiP analysis using an antibody against acetylated histone H3 (H3Ac) or an IgG control for the human HDAC6 gene locus. n=4 independent experiments. Error bars represent mean with S.E.M. Statistical source data are provided in Supplementary Table 6. Unprocessed original scans of all blots with size marker are shown in Supplementary Fig. 9.
Supplementary Figure 5  p53 is required for the observed selectivity against ARID1A mutation by HDAC6 inhibitor. (a–c) ARID1A-mutated OVISE cells with or without p53 knockdown were examined for TP53 mRNA expression by qRT-PCR (a), n=4 independent experiments; or examined for p53 protein expression by immunoblot (b), GAPDH expression was used as a loading control; or determined for dose response curves with the indicated concentration of ACY1215 for 12 days in a colony formation assay (c). Growth inhibition was calculated based on integrated density as measured in NIH ImageJ, and values were normalized to vehicle control. n=4 independent experiments. (d) ARID1A-mutated TOV21G cells with or without p53 knockdown were treated with the indicated concentration of CAY10603 to generate dose response curves. ARID1A wildtype RMG1 and OVCA429 were used as controls for comparison. n=4 independent experiments. (e) ARID1A-mutated TOV21G treated with vehicle DMSO control or the HDAC6 inhibitor ACY1215 (1.25 μM). Expression of the indicated proteins was determined by immunoblot. GAPDH expression was used as a loading control. Error bars represent mean with S.E.M. P-value calculated via two-tailed t-test. Statistical source data are provided in Supplementary Table 6. Unprocessed original scans of all blots with size marker are shown in Supplementary Fig. 9.
Supplementary Figure 6 TIP60 inhibition impaired apoptosis induced by HDAC6 inhibition in ARID1A-mutated cells. (a) ARID1A-mutated TOV21G cells were treated with vehicle control, shControl, shHDAC6, ACY1215 (1.25 mM) or CAY10603 (312 nM). RNA was extracted and utilized for next generation sequencing (RNA-seq). Expression of p53 target genes known to regulate apoptosis such as BAX, PUMA and NOXA, and known to regulate cell cycle arrest such as CDKN1A were not altered by HDAC6 inhibition in RNA-seq analysis. (b-c) ARID1A-mutated TOV21G cells were treated with the HDAC6 inhibitor ACY1215 (1.25 μM), or TIP60 inhibitor NU9056 (10 μM) or a combination. Expression of p53K120Ac and a loading control β-actin was determined by immunoblot (b). Percent apoptosis was quantified by Annexin V staining in the indicated cells (c). n=3 independent experiments. Error bars represent mean with S.E.M. P-value calculated via two-tailed t-test. (d) The gating strategy in determining mitochondria membrane potential (MMP) by FACS analysis. Examples of maintenance of MMP and loss of MMP are shown. Statistical source data are provided in Supplementary Table 6. Unprocessed original scans of all blots with size marker are shown in Supplementary Fig. 9.
Supplementary Figure 7 A model for the mechanism by which HDAC6 inhibition promotes apoptosis in ARID1A-mutated cells.
Supplementary Figure 8. HDAC6 inhibition significantly inhibits tumour growth ARID1A-mutated, but not wildtype, tumours. (a-b) Luciferase-expressing ARID1A-mutated TOV21G cells were orthotopically transplanted into the ovarian bursa sac of SCID/nude female mice. Tumours were allowed to establish for 14 days before randomized into two groups (n=6 mice/group). Mice were treated with vehicle control or HDAC6 inhibitor (ACY1215, 50mg/kg) daily for 21 days. Representative images of control and ACY1215 treated mice at the end of treatment (a). Total flux (photons/sec) is graphed at the indicated time points (b). *P = 0.0313. Error bars represent S.E.M. P-value calculated via two-tailed t-test.

(c) 6-10-weeks-old Pik3caH1047R/Arid1aflox/flox female mice were intrabursally injected with adenovirus-Cre to induce clear cell ovarian carcinomas. Mice were randomized and treated with vehicle control (n=5 mice) or ACY1215 (50mg/kg, n=4 mice) daily for 21 days. The changes in volumes of tumours formed on the injected ovary were calculated against the contrary side non-injected ovary from the same mice. (d-i) Luciferase-expressing ARID1A-wildtype RMG1 cells were orthotopically transplanted into the ovarian bursa sac of SCID/nude female mouse. Tumours were allowed to establish for 14 days before randomized into two groups (n=6 mice/group). Mice were treated with vehicle control or HDAC6 inhibitor (ACY1215, 50mg/kg) daily for 21 days. Representative images of control and ACY1215 treated mice at the end of treatment (d). At the indicated time interval during treatment, mice were imaged for luciferase expression to monitor tumour growth. Total flux (photons/sec) is graphed (e). The weight of tumours dissected from control and ACY1215 treated mice was measured at the end of treatment as a surrogate for tumour burden (f). The number of disseminated tumour nodules was counted in the indicated treatment groups (g). The serial sections of tumours dissected were subjected to immunohistochemical staining for HDAC6, Ki67, cleaved caspase 3 and p53K120Ac (h). Scale bar = 100 μm. Histological score (H-score) was calculated for 5 separate fields from 6 tumours from 6 individual mice from each of the indicated groups (i). Error bars represent mean with S.E.M. P-value calculated via two-tailed t-test. Statistical source data are provided in Supplementary Table 6.
Supplementary Figure 9 Unprocessed images of blots. Unprocessed images of scanned immunoblots shown in Figures and Supplementary Figures are provided.
Supplementary Figure 9 Continued
Supplementary Figure 9 Continued
Supplementary Table Legends

Supplementary Table 1 List of shRNAs used in the pooled screen for each of the 11 individual HDACs and primer sequences for qRT-PCR or ChIP analysis.

Supplementary Table 2 HDAC6 knockdown is selective against ARID1A knockdown in ARID1A wildtype cells. ARID1A wildtype ovarian clear cell RMG1 cells with or without ARID1A knockdown were transiently transduced with pooled small-hairpin RNAs for each individual HDACs or shControls and incubated for 48hrs. Equal number of cells were plated in quadruplicate in 24-well plates. Cells were allowed to grow for 12 days and cells were assayed for colony formation using crystal violet staining. Integrated density (ID) for each well was calculated using Image J software. Integrated density was normalized to shControl (% Control). P-value was calculated using two-tailed t-test. n=4 independent experiments. Fold change= shControl/shARID1A.

Supplementary Table 3 Cell lines used in the studies with characteristics of ARID1A mutational status, ACY1215 IC50 and other known genetic alterations.

Supplementary Table 4 Mutual exclusivity between ARID1A and TP53 mutations in the TCGA database or the indicated publication.

Supplementary Table 5 Genes significantly upregulated or downregulated (>2-fold and P<0.05 calculated by EdgeR method 3) by HDAC6 inhibitors (1.25 μM ACY1215 or 312 nM CAY10603) or HDAC6 knockdown based on RNA-seq analysis

Supplementary Table 6 Statistics source data. The source data used for statistical analyses of Figures 1b, 1f, 1h, 2b-c, 2e, 2g, 2j, 3b-c, 3g-i, 4a, 4c-d, 4f, 4h-i, 4k-p, 5a, 5d-e, 6b, 6d, 7b, 7f-g, 8a, 8c, 8e, 8g, and Supplementary Figs 1a-c, 2b-c, 2e-f, 2h-i, 3b-e, 4b-f, 4h-i, 5a, 5c-d, 6c, 8b-c, 8e, 8f-g and 8i.

References

1. Barretina, J., et al. The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. Nature 483, 603-607 (2012).
2. Stany, M.P., et al. Identification of novel therapeutic targets in microdissected clear cell ovarian cancers. PLoS One 6, e21121 (2011).
3. Robinson, M.D., McCarthy, D.J. & Smyth, G.K. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26, 139-140 (2010).
### Experimental design

1. **Sample size**
   - Describe how sample size was determined. For in vivo experiments, the sample size was determined based on the data shown from in vitro experiments.

2. **Data exclusions**
   - Describe any data exclusions. There was no exclusion from the experiments.

3. **Replication**
   - Describe whether the experimental findings were reliably reproduced. All attempts at replication were successful.

4. **Randomization**
   - Describe how samples/organisms/participants were allocated into experimental groups. Experiments were all randomized.

5. **Blinding**
   - Describe whether the investigators were blinded to group allocation during data collection and/or analysis. Analysis was performed blindly.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. **Statistical parameters**
   - For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed). n/a  Confirmed

|   |   |
|---|---|
| □ | □ |

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
- A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- A statement indicating how many times each experiment was replicated
- The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
- A description of any assumptions or corrections, such as an adjustment for multiple comparisons
- The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted
- A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
- Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.
Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

Prism software was used for statistical analysis.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

ARID1A CRISPR cell lines are readily available from the authors.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

mouse anti-acetylated-p53 K120 (Abcam, Cat. No. ab78316, 1:1000 for western blot and 1:100 for IHC), rabbit anti-acetylated-p53 K373 (Abcam, Cat. No. ab62376, 1:1000 for western blot), rabbit anti-acetylated-p53 K382 (Abcam, Cat. No. ab75754, 1:1000 for western blot), mouse anti-ARID1A (Santa Cruz, Cat. No. sc-32761, 1:1000 for western blot and 10 μg per sample for ChiP), mouse anti-p53 (Millipore, Cat. No. OP43, 1:1000 for western blot), mouse anti-GAPDH (Millipore, Cat. No. MAB374, 1:10000 for western blot), rabbit anti-cleaved PARP p85 (Promega, Cat. No. 7344, 1:1000 for western blot), mouse anti-Ki67 (Cell Signaling, Cat. No. 9449, 1:1000 for IHC), rabbit anti-cleaved caspase 3 (Cell Signaling, Cat. No. 9661, 1:1000 for western blot and 1:50 for IHC), rabbit anti-HDAC6 (Cell Signaling, Cat. No. 7612, 1:1000 for western blot and Santa Cruz, Cat. No. sc-11420, 1:100 for IHC), rabbit anti-RNA polymerase II (Santa Cruz, Cat. No. sc-899 X, 2 μg per sample for ChiP), rabbit anti-TOM20 (Santa Cruz, Cat. No. sc-11415, 1:1000 for western blot), rabbit anti-H3Ac (Active Motif, Cat. No. 39139, 5 μl/ immunoprecipitation for ChiP), rabbit anti-TIP60 (Santa Cruz, Cat. No. sc-166323, 1:1000 for western blot), mouse anti-BRG1 (Santa Cruz, Cat. No. sc-17796, 1:1000 for western blot and 2 μg/immunoprecipitation for ChiP) and rabbit anti-BRM (Cell Signaling, Cat. No. 11966, 1:1000 for western blot and 3 μg/immunoprecipitation for ChiP).

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

Ovarian clear cell carcinoma cell lines (TOV21G, OVTOKO, OVISE and RMG1) were purchased from JCRB.

b. Describe the method of cell line authentication used.

Cells lines are authenticated at The Wistar Institute’s Genomics Facility using short tandem repeat DNA profiling.

c. Report whether the cell lines were tested for mycoplasma contamination.

Regular Mycoplasma testing was performed using LookOut Mycoplasma PCR detection (Sigma).

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

No commonly misidentified cell lines used.
Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

For intrabursal orthotopic xenograft, 6-8-week-old female immunocompromised mice were used. Transgenic mice with latent mutations in Arid1a and Pik3ca were generated by crossing Arid1aflox/flox mice (kindly provided by Dr. Wang, U. Michigan) and crossed onto a C57BL/6J background for 9 generations with R26-Pik3caH1047R mice carrying inducible Pik3ca mutations (Jackson Laboratory, Jax#016977). To induce tumorigenesis, 6-10 weeks old Pik3caH1047R/ Arid1aflox/flox female mice were intrabursally injected adenovirus-Cre.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

We only received de-identified primary cultures.
Flow Cytometry Reporting Summary

Data presentation

For all flow cytometry data, confirm that:

1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
2. The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
3. All plots are contour plots with outliers or pseudocolor plots.
4. A numerical value for number of cells or percentage (with statistics) is provided.

Methodological details

5. Describe the sample preparation.
   Samples preparation follows Annexin V FITC and PI kit (Thermo fisher, V13242). Briefly, cells were washed with cold PBS and resuspended in Annexin V binding buffer and stained with Annexin V-FITC and propidium iodide (PI) at room temperature and then analyzed immediately. To measure change in mitochondria membrane potential cells were treated with 200nM of tetramethylrhodamine, ethyl ester (TMRE; Abcam cat. No. ab113852) for 15min at 37 ºC.

6. Identify the instrument used for data collection.
   Becton-Dickinson LSR18

7. Describe the software used to collect and analyze the flow cytometry data.
   FlowJo version 7

8. Describe the abundance of the relevant cell populations within post-sort fractions.
   10000 cells were used to sort and at approximately 90% cells were used to analyze.

9. Describe the gating strategy used.
   Forward and side scatter gating strategy was used to eliminate the cell debris, fragments and pyknotic cells. Subsetting gating strategy (Forward scatter area and height) was used to identify single cell population.
   For AnnexinV-FITC/PI positive cells were gated for early and late apoptosis. Early and late apoptosis = Total Apoptosis.
   For TMRE mitochondria membrane potential (MMP) was examined via PI channel (549/575nm). Vehicle control (Maintainence of MMP ) and Loss of MMP were utilized to assay for changes in MMP.
   Examples of gating strategies for AnnexinV/PI and TMRE assays are demonstrated in Supplementary Fig. 3a and 6e, respectively.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.  

