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14. ABSTRACT

Epithelial ovarian cancer (EOC) is the leading cause of death among gynecological malignancies in the United States. Among all EOC subtypes, ovarian clear cell carcinoma (OCCC) carries the worst prognosis when diagnosed at an advanced stage, and there is currently no effective therapy for this disease. The gene *ARID1A*, which encodes a subunit of the epigenetic SWI/SNF chromatin-remodeling complex, is the highest mutated gene in OCCC that occurs in over 50% of the cases. In addition, *ARID1A* is also mutated in ~30% of endometrioid subtype of EOC. Since *ARID1A* is the highest mutated gene and a known driver mutation in OCCC, we performed an unbiased screen and demonstrated that in *ARID1A*-mutated OCCC the inhibition of EZH2, another epigenetic regulator, is synthetically lethal. In addition to EZH2, our unexplored data suggest that *ARID1A*-mutated ovarian cancer cells are also selectively sensitive to the inhibition of HDAC6. Our central hypothesis is that *ARID1A*-mutated ovarian cancers can be treated and ultimately eradicated based on the synthetic lethality through targeting EZH2 and HDAC6 using clinically applicable small molecule inhibitors. The objective of this proposal is to develop first effective targeted therapeutic approach for *ARID1A*-mutated ovarian cancers.

15. SUBJECT TERMS
Epithelial ovarian cancer, ovarian clear cell carcinoma, ARID1A, SWI/SNF, synthetic lethality

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1. INTRODUCTION:

Epithelial ovarian cancer (EOC) is the leading cause of death among gynecological malignancies in the United States. Among all EOC subtypes, ovarian clear cell carcinoma (OCCC) carries the worst prognosis when diagnosed at an advanced stage, and there is currently no effective therapy for this disease. The gene *ARID1A*, which encodes a subunit of the epigenetic SWI/SNF chromatin-remodeling complex, is the highest mutated gene in OCCC that occurs in over 50% of the cases. In addition, *ARID1A* is also mutated in ~30% of endometrioid subtype of EOC. Since *ARID1A* is the highest mutated gene and a known driver mutation in OCCC, we performed an unbiased screen and demonstrated that in *ARID1A*-mutated OCCC the inhibition of EZH2, another epigenetic regulator, is synthetically lethal. In addition to EZH2, our unexplored data suggest that *ARID1A*-mutated ovarian cancer cells are also selectively sensitive to the inhibition of HDAC6. Our central hypothesis is that *ARID1A*-mutated ovarian cancers can be treated and ultimately eradicated based on the synthetic lethality through targeting EZH2 and HDAC6 using clinically applicable small molecule inhibitors.

2. KEYWORDS:

Epithelial ovarian cancer, ovarian clear cell carcinoma, ARID1A, SWI/SNF, synthetic lethality.

3. ACCOMPLISHMENTS:

What were the major goals and objectives of the project?

The objective of this proposal is to develop the first effective targeted therapeutic approach for *ARID1A*-mutated ovarian cancers.

Specific Aim 1: To investigate the molecular basis underlying the observed synthetic lethality between *ARID1A* mutation and inhibition of HDAC6 activity.

Specific Aim 2: To investigate the effects of a combination therapeutic strategy for *ARID1A*-mutated ovarian cancer by simultaneously inhibiting HDAC6 and EZH2.

What was accomplished under these goals?

Since the starting of the award, substantial progress has been made toward achieving the goals as outlined in the application.

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 eleven histone deacetylase genes. This was done in the context of *ARID1A* wildtype ovarian clear cell RMG1 cells with or without ARID1A knockdown (**Fig. 1a**).

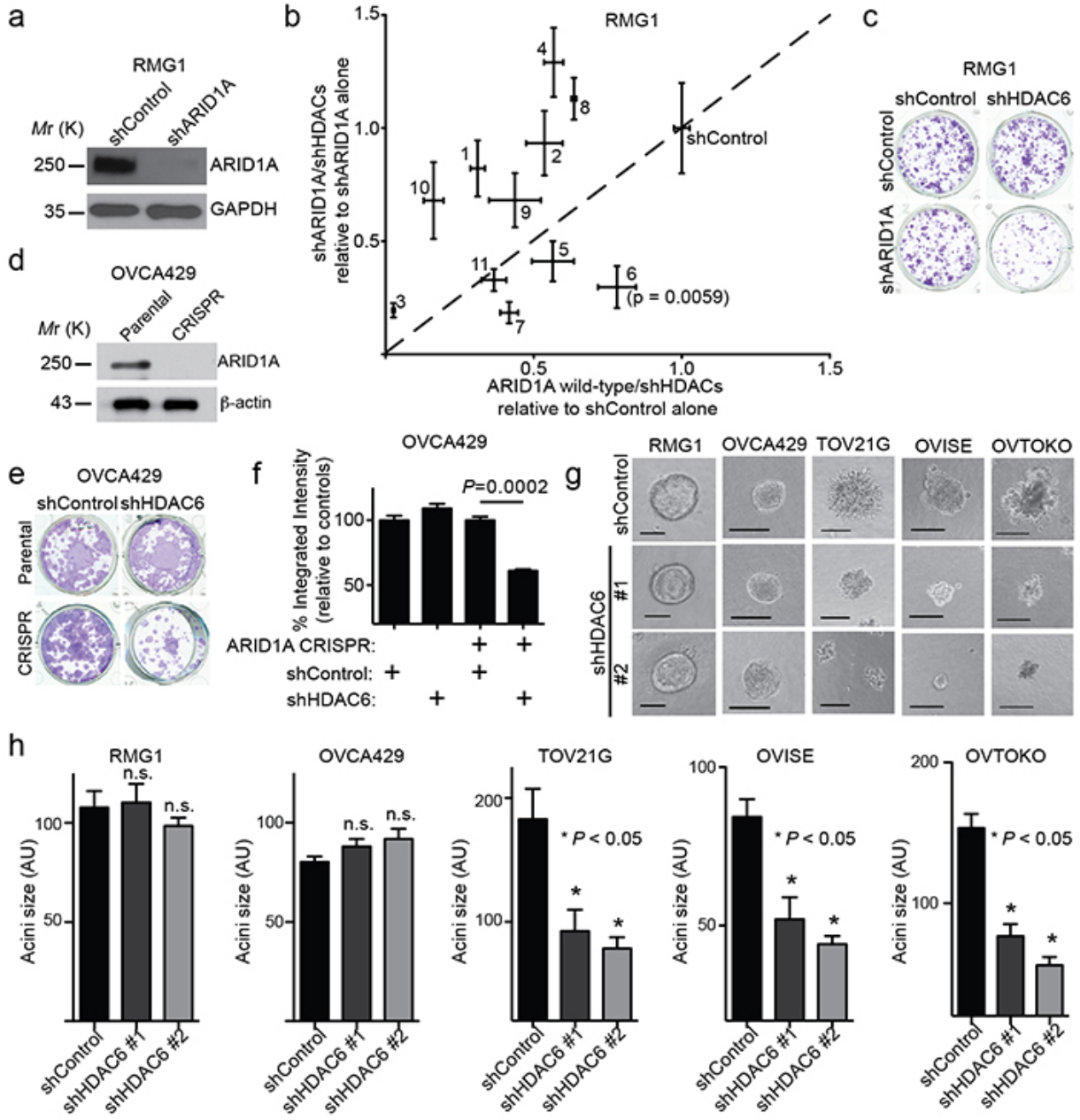

Figure 1. ARID1A-inactivated cells are selectively sensitive to HDAC6 knockdown. **(a-c)** ARID1A and a loading control GAPDH protein expression in *ARID1A* wildtype RMG1 cells with or without ARID1A knockdown (a). Cells were transduced with lentivirus encoding shRNA to each of the 11 individual HDACs (HDAC1-11) and subjected to colony formation assay. Scatterplot of the integrated density normalized to control. The x-axis indicates changes in cell growth induced by individual shHDACs in control *ARID1A* wildtype treated cells, while the y-axis indicates changes in cell growth induced by the same shHDACs in shARID1A-expressing cells. Individual HDACs are indicated with their respective numbers (b). n=4 independent experiments. Colony formation by the indicated cells (c). **(d-f)** ARID1A protein expression in parental and ARID1A CRISPR OVCA429 cells (d). Colony formation assay using the indicated OVCA429 cells with or without HDAC6 knockdown (e), which was quantified (f). n=3 independent experiments. **(g-h)** A panel of cell lines with known *ARID1A* mutational status with or without HDAC6 knockdown were grown in 3D using Matrigel. Shown are acini formed by the indicated cells (g). Scale bar = 75 AU in NIH Image J software. The diameters of acini (n=50; representative of three biological repeats) were quantified (h). n.s. indicates not significant. Error bars represent mean with S.E.M. *P*-value calculated via two-tailed *t*-test.

ARID1A knockdown allows us to mimic loss of ARID1A protein expression caused by >90% of *ARID1A* mutations in ovarian cancer [3] and to ensure the same genetic background for the unbiased evaluation. We transiently transduced pooled shRNAs for each of the 11 individual HDACs in *ARID1A* wildtype RMG1 cells with or without ARID1A knockdown. To measure changes in cell viability, these cells were subjected to a colony formation assay. Similar to previous reports [1], we observed no significant difference between *ARID1A* wildtype 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**). Likewise, HDAC6 knockdown was selective against ARID1A knockout in *ARID1A* wildtype OVCA429 cells (**Fig. 1d-f**).

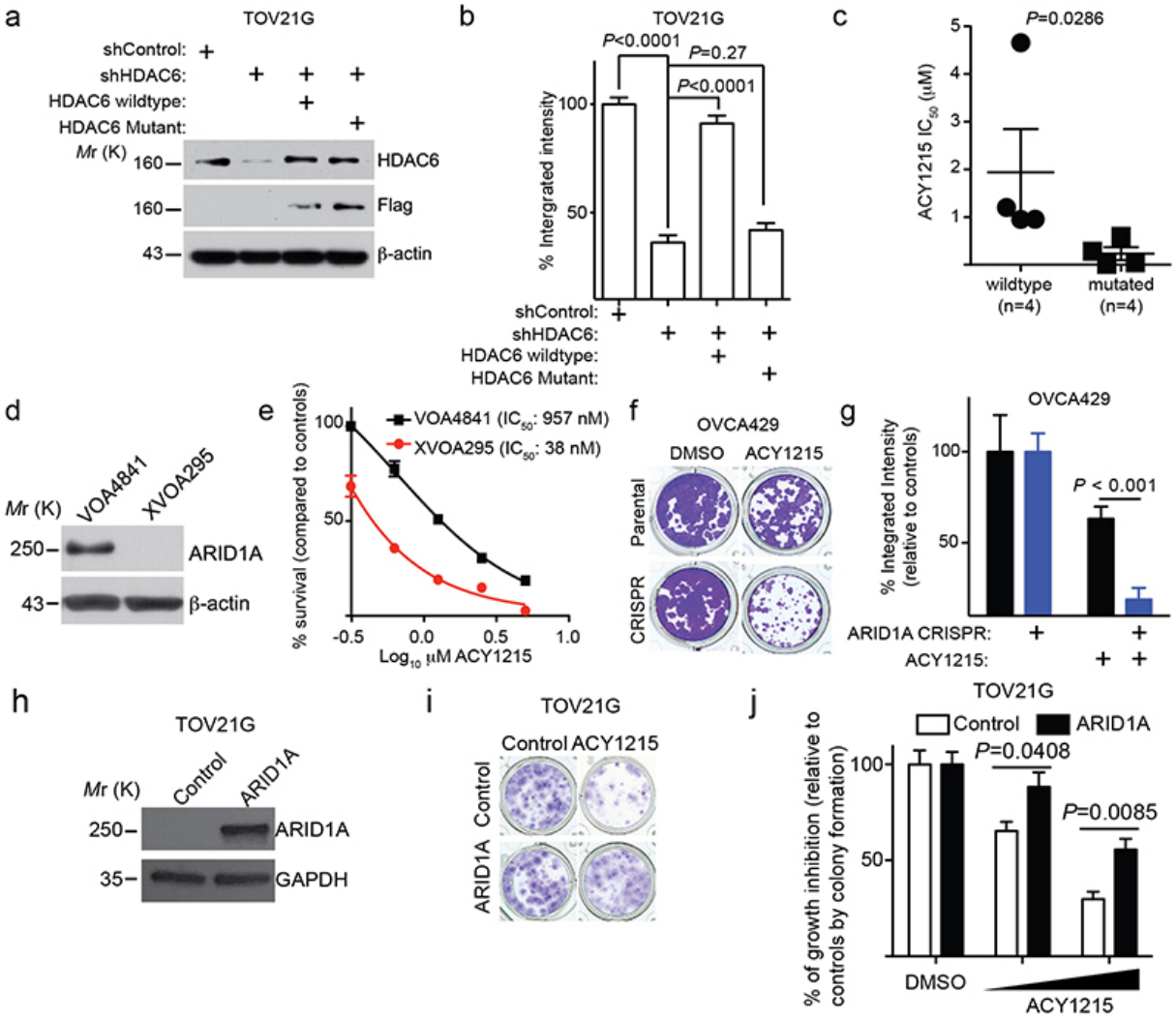

Figure 2. The selectivity against ARID1A mutation depends on the enzymatic activity of HDAC6. **(a-b)** Expression of HDAC6, FLAG and a loading control β-actin in *ARID1A*-mutated TOV21G cells expressing a shHDAC and concurrent expression of FLAG-tagged shRNA resistant wildtype HDAC6 or a catalytically inactive H216/611A mutant (a). The indicated cells were subjected to colony formation assay and integrated density was measured (b). n=4 independent experiments. **(c)** IC_{50} of HDAC6 inhibitor ACY1215 is significantly higher in *ARID1A* wildtype (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 tumor cultures with (VOA4841) and without (XVOA295) ARID1A expression. n=3 independent experiments. **(f)** Control and ARID1A CRISPR OVCA429 cells were treated with or without 1.25 μM ACY1215 in a colony formation assay. **(g)** Quantification of (f). n=4 independent experiments. **(h-j)** Immunoblots of the indicated proteins in *ARID1A*-mutated TOV21G cells with or without wildtype ARID1A restoration (h). 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 [1] [2]. To limit the potential bias in colony formation, the number of cells used for ARID1A restored cells were 2-fold of the control *ARID1A*-mutated cells (i). Integrated density was measured with NIH Image J software as a surrogate for cell growth (j). The concentrations of ACY1215 were 312 nM and 625 nM, respectively. n=4 independent experiments. Error bars represent mean with S.E.M. *P*-value calculated via two-tailed *t*-test.

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 3 dimensional (3D) cultures using Matrigel extracellular matrix that more closely mimics the tumor microenvironment. HDAC6 knockdown had no appreciable effect on the growth of *ARID1A* wildtype cells but significantly suppressed the growth of *ARID1A*-mutated cells (**Fig. 1g-h**). The observed growth inhibition depends on the enzymatic activity of HDAC6 because the growth inhibition was rescued by a wildtype HDAC6 but not a catalytically inactive H216/611A mutant [4] (**Fig. 2a-b**).

Notably, selective and specific HDAC6 inhibitors have been developed. We tested the HDAC6 inhibitor ACY1215 (Rocilinostat) [5] in a panel of cell lines with or without *ARID1A* mutation because it was safe in clinical trials [6]. Compared with *ARID1A* wildtype cells, the IC_{50} of ACY1215 was significantly lower in *ARID1A*-mutated cells (**Fig. 2c**). Primary clear cell ovarian tumor cultures without ARID1A expression are more sensitive to ACY1215 compared to those with ARID1A expression (**Fig. 2c-d**). The IC_{50} values of ACY1215 in primary cells are comparable to those observed in cell lines (**Fig. 2c-e**). ARID1A knockout significantly increased the sensitivity of *ARID1A* wildtype OVCA429 cells to ACY1215 (**Fig. 2f-g**). Conversely, restoration of wildtype ARID1A in *ARID1A*-mutated TOV21G cells reduced the sensitivity of these cells to ACY1215 (**Fig. 2h-j**). 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. HDAC6 inhibitor ACY1215 treatment 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**). 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* wildtype cells (**Fig. 3b-e**), and wildtype ARID1A restoration suppressed ACY1215 induced apoptosis in *ARID1A*-mutated TOV21G cells (**Fig. 3f-g**). Compared with *ARID1A* wildtype controls, the HDAC6 inhibitor ACY1215 or knockdown of HDAC6 increased markers of apoptosis in ARID1A knockdown cells (**Fig. 3d-e**). Notably, a pan-caspase inhibitor Q-VD-Oph or knockdown of intrinsic apoptotic pathway initiator caspase 9 or effector caspase 3 [7] significantly suppressed the apoptosis induced by ACY1215 (**Fig. 3h-i**). We conclude that HDAC6 inhibition promotes apoptosis in ARID1A-inactivated cells.

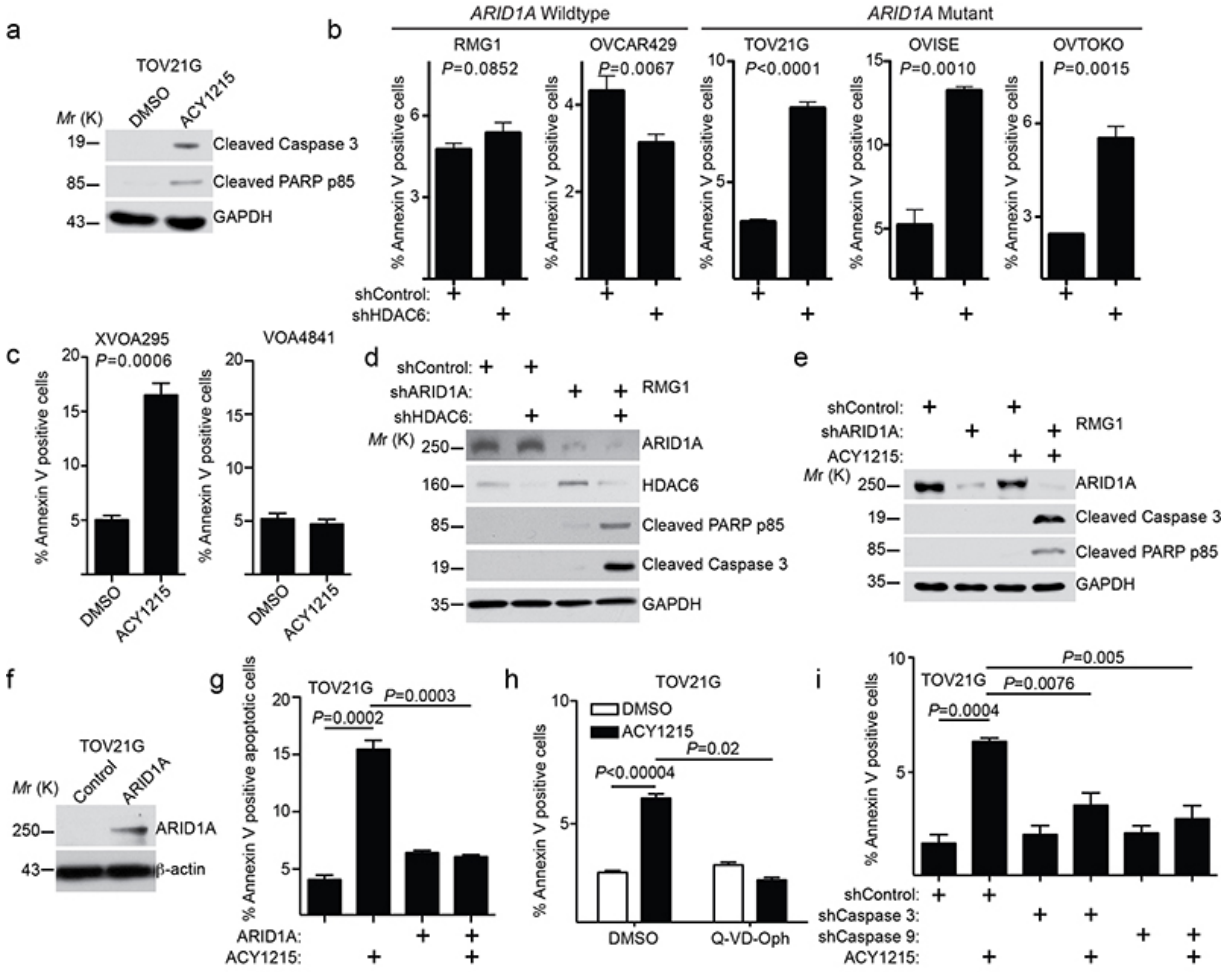

Figure 3. HDAC6 inhibition induces apoptosis in ARID1A inactivated cells. **(a)** *ARID1A*-mutated TOV21G cells treated with 1.25 μM ACY1215 or DMSO control were examined for expression of markers of apoptosis cleaved caspase 3, cleaved PARP p85 or a loading control GAPDH by immunoblot. **(b)** Percent apoptosis in the indicated cell lines was quantified by FACS based on Annexin V staining. **(c)** Percent apoptosis of in the indicated primary clear cell ovarian tumor cultures was quantified by FACS based on Annexin V staining. **(d)** *ARID1A* wildtype RMG1 cells with the indicated knockdown of ARID1A, HDAC6, or a combination was examined for expression of apoptotic markers cleaved caspase 3 and cleaved PARP p85 and a loading control GAPDH by immunoblot. **(e)** *ARID1A* wildtype RMG1 cells with or without ARID1A knockdown were treated with 1.25 μM ACY1215. Expression of apoptotic markers cleaved caspase 3 and cleaved PARP p85 and a loading control GAPDH determined by immunoblot. **(f)** Expression of ARID1A and a loading control β-actin in TOV21G cells with or without wildtype ARID1A restoration. **(g)** Percent apoptosis based on Annexin V staining in *ARID1A*-mutated TOV21G cells with or without wildtype ARID1A restoration and treated with 1.25 μM ACY1215 or DMSO controls for 96 hrs. **(h)** Percent apoptosis based on Annexin V staining in *ARID1A*-mutated TOV21G cells treated with 1.25 μM ACY1215, 20 μM pan-caspase inhibitor Q-VD-Oph or a combination for 48 hrs. **(i)** Percent apoptosis based on Annexin V staining in *ARID1A*-mutated TOV21G cells treated with 1.25 μM ACY1215 for 48 hrs with or without knockdown of caspase 3 or caspase 9. For quantifications in b, c, g, h and i, n=3 independent experiments. Error bars represent mean with S.E.M. *P*-value calculated via two-tailed *t*-test.

ARID1A directly represses *HDAC6* gene transcription.

We next determined whether ARID1A affects HDAC6 expression levels. We observed a significant increase in HDAC6 mRNA and protein expression in an *ARID1A*-wildtype cells upon 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* wildtype cells (**Fig. 4d-e**). Conversely, HDAC6 expression was significantly repressed when wildtype ARID1A was restored in *ARID1A*-mutated cells (**Fig. 4f**).

SWI/SNF complexes contribute to both gene activation and repression in a context-dependent manner [8]. We determined whether ARID1A directly represses HDAC6 expression based on published data from chromatin immunoprecipitation followed by next-generation sequencing (ChIP-seq) of ARID1A [9]. Indeed, there is a significant enrichment of ARID1A at *HDAC6* promoter regions (**Fig. 4g**). Validating these findings, we observed a significant association of ARID1A with the *HDAC6* gene promoter in *ARID1A* wildtype cells (**Fig. 4h**). Thus, we identified ARID1A as a direct repressor of *HDAC6* gene transcription.

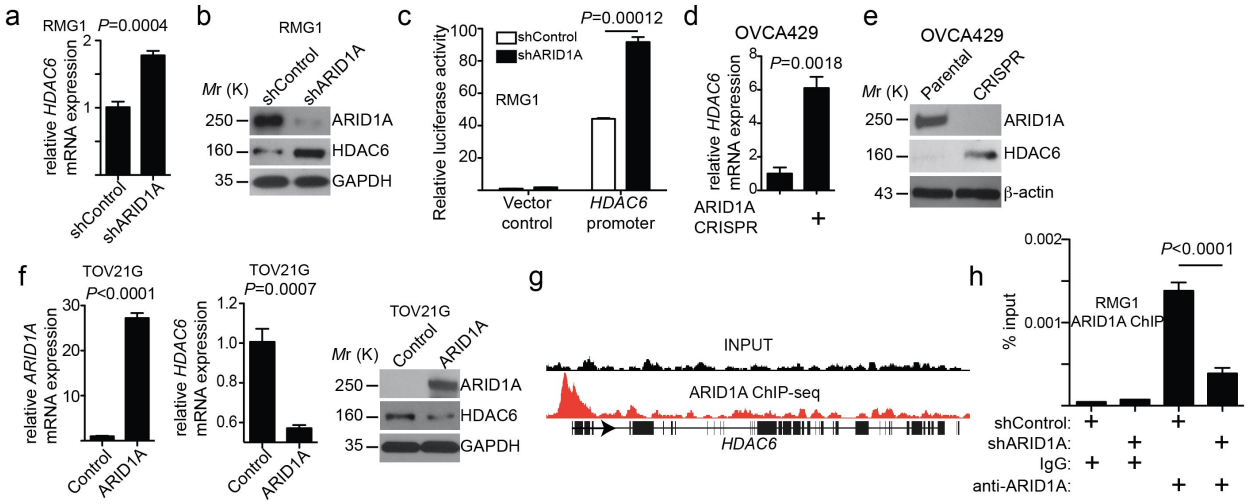

Figure 4. ARID1A represses HDAC6 expression. (a-b) *ARID1A* wildtype RMG1 cells with or without ARID1A knockdown were determined for *HDAC6* mRNA expression (a) n=4 independent experiments; or ARID1A, HDAC6 and GAPDH protein expression (b). **(c)** The human *HDAC6* gene promoter activity in *ARID1A* wildtype RMG1 cells with or without ARID1A knockdown. n=3 independent experiments. **(d-e)** *ARID1A* wildtype parental and knockout OVCA429 cells were examined for expression of *HDAC6* mRNA (d), n=3 independent experiments; or ARID1A, HDAC6 and β-actin protein expression (e). **(f)** *ARID1A*-mutated TOV21G cells with or without wildtype 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)** ARID1A ChIP-seq and input tracks at the human *HDAC6* gene promoter based on a ChIP-seq dataset [9]. **(h)** *ARID1A* wildtype RMG1 cells with or without ARID1A knockdown were subjected to ChIP analysis for the *HDAC6* gene promoter using antibodies against ARID1A. An isotype matched IgG was used as a control. Error bars represent mean with S.E.M. *P*-value calculated via two-tailed *t*-test.

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 [6]. 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* wildtype 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) by intraperitoneal (i.p.) injection, the same dose as previously reported [10]. Indeed, ACY1215 significantly improved the survival of mice bearing the orthotopically-transplanted *ARID1A*-mutated tumours compared with controls (**Fig. 5a**). 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 wildtypes 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. 5b-c**). Ovarian cancer often progresses by disseminating to the intraperitoneal cavity [11]. 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 tumour nodules in ACY1215 treated mice bearing *ARID1A*-mutated tumours compared to controls (**Fig. 5d-e**). Together, we conclude that the HDAC6 inhibitor ACY1215 selectively suppresses the growth and dissemination of *ARID1A*-mutated ovarian tumours and improves the survival of *ARID1A*-mutated tumour bearing mice.

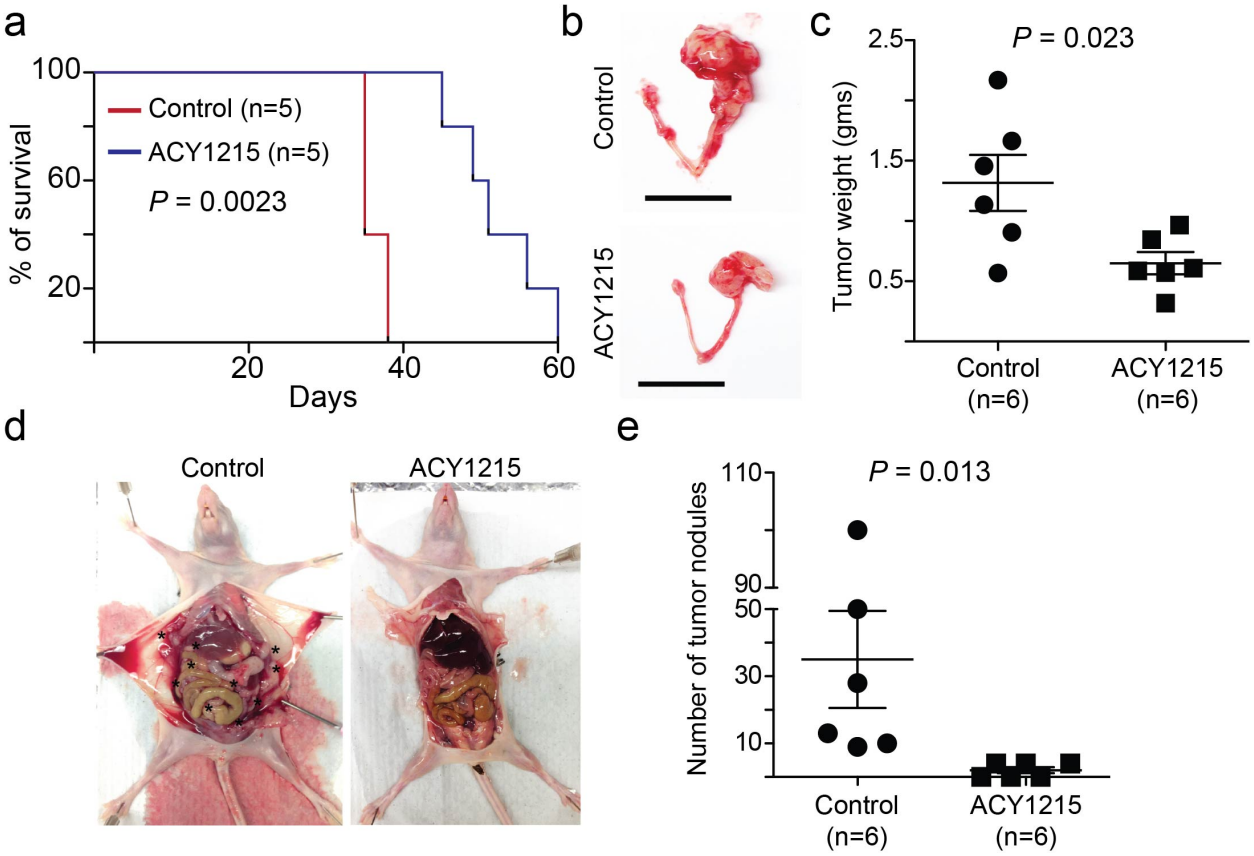

Figure 5. HDAC6 inhibition improves the survival of mice bearing *ARID1A*-mutated ovarian tumours. *ARID1A*-mutated TOV21G cells were orthotopically transplanted into the ovarian bursa sac of SCID/nude female mice. Tumours were allowed to establish for 2 weeks before the mice were randomized into two different treatment groups (n=5/group). Mice were treated with vehicle control or the HDAC6 inhibitor ACY1215 (50 mg/kg) 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 **(a)**. *P*-value was calculated by log-rank test. At the end of treatment, the mice were euthanized (n=6/group). Shown are representative images of reproductive tracts with tumours from control or ACY1215 treated mice **(b)**. Scale bar = 2 cm. Tumour weight was measured as a surrogate for tumour burden from the control and ACY1215 treated mice **(c)** or examined for disseminated tumour nodules in the peritoneal cavity. Representative images of disseminated tumour nodules in control and ACY1215 treated mice **(d)**. Asterisks (*) indicate the disseminated tumour nodules in peritoneal cavity. The number of disseminated tumour nodules in peritoneal cavity was quantified **(e)**. Error bars represent mean with S.E.M. *P*-value calculated via two-tailed *t*-test.

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What opportunities for training and professional development did the project provide?

"Nothing to Report."

How were the results disseminated to communities of interest?

"Nothing to Report."

What do you plan to do during the next reporting period to accomplish the goals and objectives?

In the next reporting period, we plan to:

1) We discovered that HDAC2 instead of HDAC6 as a co-repressor of EZH2 in regulating EZH2/ARID1A target genes. Thus, we will investigate the molecular mechanism by which EZH2 and HDAC2 cooperatively regulate EZH2/ARID1A target genes such as PIK3IP1.
2) Investigate the effects of HDAC6 and EZH2 inhibition on the growth of OCCCs in preclinical models.

4. IMPACT:

"Nothing to Report."

What was the impact on the development of the principal discipline(s) of the project?

"Nothing to Report."

What was the impact on other disciplines?

"Nothing to Report."

What was the impact on technology transfer?

"Nothing to Report."

What was the impact on society beyond science and technology?

"Nothing to Report."

5. CHANGES/PROBLEMS:

"Nothing to Report."

Changes in approach and reasons for change

"Nothing to Report."

Actual or anticipated problems or delays and actions or plans to resolve them

"Nothing to Report."

Changes that had a significant impact on expenditures

"Nothing to Report."

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

"Nothing to Report."

6. PRODUCTS:

1. Fukumoto T, Park PH, Wu S, Fatkhutdinov N, Karakashev S, Nacarelli T, Kossenkov AV, Speicher DW, Jean S, Wang TL, Shih IM, Conejo-Garcia JR, Bitler BG, Zhang R. 2018. Repurposing pan-HDAC inhibitors for *ARID1A*-mutated ovarian cancer. ***Cell Rep*** 22:3393-3400. PMCID: PMC5903572.
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Books or other non-periodical, one-time publications.

"Nothing to Report."

Other publications, conference papers, and presentations.

"Nothing to Report."

- **Website(s) or other Internet site(s)**

"Nothing to Report."

- **Technologies or techniques**

"Nothing to Report."

- **Inventions, patent applications, and/or licenses**

"Nothing to Report."

- **Other Products**

"Nothing to Report."

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

What individuals have worked on the project?

Name:	*Rugang Zhang*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*2*
Contribution to Project:	Supervised the study.
Funding Support:	This award

Name:	*Takeshi Fukumoto*
Project Role:	*Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	*9*
Contribution to Project:	Performed the study.
Funding Support:	This award

Name:	*Pingyu Liu*
Project Role:	*Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	*2*
Contribution to Project:	Performed the study.
Funding Support:	This award

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

“Nothing to Report.”

What other organizations were involved as partners?

“Nothing to Report.”

8. SPECIAL REPORTING REQUIREMENTS: None.

9. APPENDICES: None.