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14. ABSTRACT The goal of this award is to determine whether inhibition of ATM is synergistic with the FDA-approved drug fenofibrate and the mechanism underlying the synergy. In Year 1, we have mechanistically uncovered a role for glucose uptake and consumption through the glycolytic pathway in the combination. We have established that altered glucose metabolism in ATM inhibitor-treated cells is a therapeutic vulnerability of chemoresistant high grade serous ovarian cancer cells. Our ability to target this pathway using the FDA-approved drug fenofibrate may have tremendous benefit for the 75% of high grade serous ovarian cancer patients with chemoresistant disease.					
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1. INTRODUCTION

Epithelial ovarian cancer (EOC) is the deadliest gynecological malignancy in the US. Over 60% of EOC patients are diagnosed at advanced stages, and the 5-year survival rate for these patients is only ~25%. Platinum-based therapies have been the standard-of-care for EOC patients for decades, but these regimens are largely ineffective due to resistance and high toxicity. EOC cells display characteristic changes in metabolism such as high rates of aerobic glycolysis (the Warburg Effect). This suggests that dependence of EOC cells on aerobic glycolysis could be therapeutically exploited. We previously showed that Ataxia Telangiectasia Mutated (ATM) is a negative regulator of tumor-associated aerobic glycolysis. Indeed, loss or inhibition of ATM increases glucose consumption and lactate production, markers of increased aerobic glycolysis. Our published data indicate this is in part due to increased MYC expression. Using an unbiased, high-throughput approach to determine inhibitors that specifically target metabolic vulnerabilities induced by inhibition of ATM, the FDA-approved drug fenofibrate was identified to induce a conditional synthetic lethality when combined with ATM inhibition in EOC cells. Fenofibrate is a potent agonist of peroxisome proliferator activated receptor alpha (PPAR α), a nuclear receptor transcription factor that plays a key role in repression of genes that positively regulate aerobic glycolysis. We hypothesize that increased dependence on aerobic glycolysis by inhibition of ATM provides a metabolic vulnerability to induce a synthetic lethality using fenofibrate.

Accordingly, the objectives of the proposed studies are:

- 1) To investigate the mechanism by which inhibition of ATM selectively sensitizes EOC cells to fenofibrate.
- 2) To determine whether this can lead to a long-term, durable response in platinum-resistant EOC models.

2. KEYWORDS

Ovarian cancer; therapeutic resistance; cisplatin; metabolism; Ataxia Telangiectasia Mutated; MYC; peroxisome proliferator activated receptor alpha; glucose

3. ACCOMPLISHMENTS

What were the major goals of the project?

These are described below, broken down by Specific Aim, Major Task, and Subtask from the approved Statement of Work (SOW).

Specific Aim 1: Dissect the mechanistic basis of ATM inhibition and fenofibrate synthetic lethality.

- **Major Task 1:** Determine the mechanism of how inhibition of ATM increases glycolysis of chemoresistant EOC cells.
 - *Milestone #1:* To have determined the direct role of MYC in the transcriptional regulation of metabolic genes and functional metabolic pathways in ATMi-treated EOC cell lines. Subtasks 3-4 not initiated due to negative results from Subtask 2.
 - Subtask 1: Validate MYC shRNA, engineer MYC knockout cells using CRISPR, and determine dose for MYC inhibitor.
Results: For reasons of cost and time efficiency, we first validated MYC shRNA and performed Subtask 2 (below). This proved to be a well-founded decision as knockdown of MYC using 2 independent shRNAs did not affect glucose uptake by ATMi. These results indicate that additional experiments in Subtask 1 are irrelevant; therefore, they were not performed.
 - Subtask 2: Determine effects of ATMi in the absence of MYC on glucose consumption.

Results: Two different HGSOC cell lines (Ovcar3 and Ovcar10) were transduced with 2 independent hairpins targeting MYC and then treated with ATMi. Figure 1 demonstrates that knockdown of MYC (**Fig. 1A & 1D**) does not affect uptake of the fluorescent glucose analog 2NBDG (**Fig. 1B-C & 1E-F**).

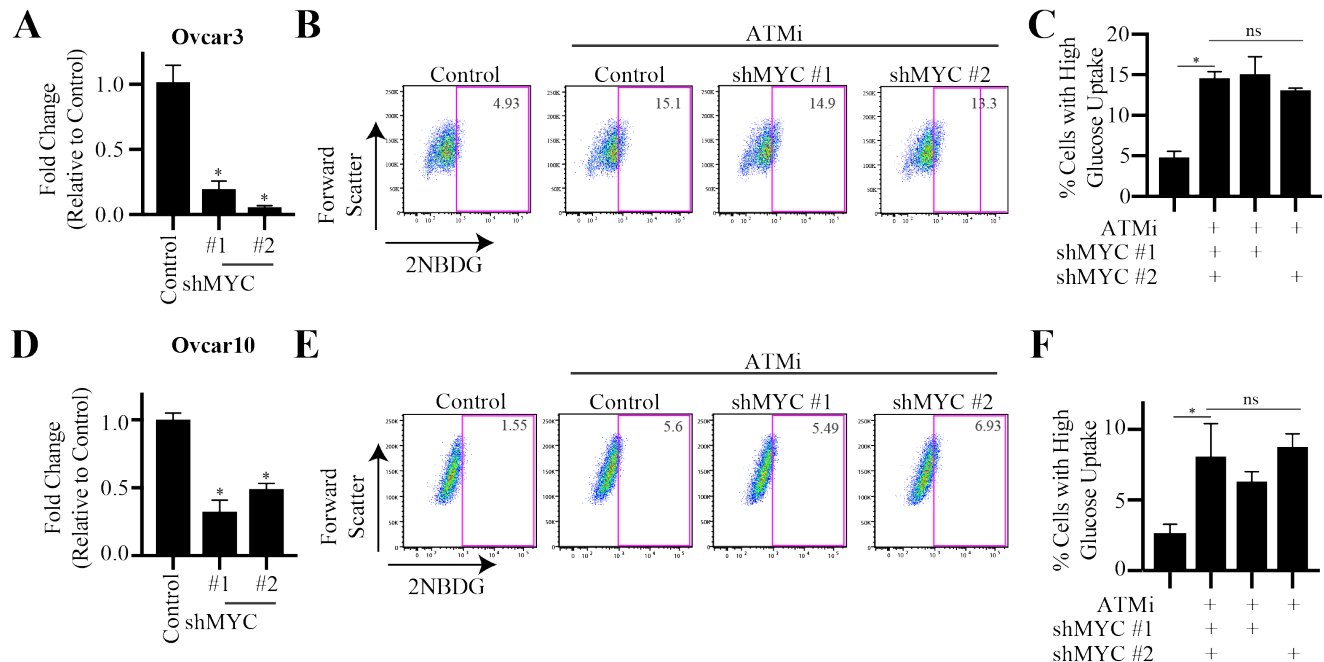


Figure 1. Knockdown of MYC does not affect glucose uptake by inhibition of ATM. (A) Ovcar3 chemoresistant HGSOC cells were infected with lentivirus expressing two independent hairpins targeting MYC (shMYC). shGFP was used as a control. MYC mRNA expression was determined by RT-qPCR. (B) Same as (A), but cells were treated with 5uM ATM inhibitor (ATMi; KU60019) for 72h. (C) Same as (A), but Ovcar10 chemoresistant HGSOC cells were used. (D) Same as (B), Ovcar10 chemoresistant HGSOC cells were used. *p<0.05 compared to control. ns = not significant.

- Subtask 3: Determine effects of ATMi in the absence of MYC on transcription of metabolic genes.
Results: Because the results of Subtask 2 were negative, we did not perform Subtask 3 and directed our efforts towards more productive award-relevant activities.
- Subtask 4: MYC chromatin immunoprecipitation studies
Results: Because the results of Subtask 2 were negative, we did not perform Subtask 3 and directed our efforts towards more productive award-relevant activities.
- **Major Task 2:** Determine whether fenofibrate suppresses glycolysis of chemoresistant EOC cells treated with ATMi
 - *Milestone #2: Characterization of the effects of fenofibrate on metabolic gene expression and metabolism after ATMi treatment.*
- Subtask 1: Determine effects of fenofibrate on glucose uptake and consumption in ATMi treated cells using 2-NBDG and the YSI Bioanalyzer.
Results: We used two different chemoresistant HGSOC cell lines (Ovcar3 and Ovcar10) and analyzed glucose uptake by the fluorescent glucose analog 2NBDG and consumption by analyzing the amount of glucose in the media using the YSI Bioanalyzer. We determined that

fenofibrate decreases glucose uptake/consumption by 2NBDG staining (Fig. 2A-B & 2D-E) and analysis of the media using the YSI bioanalyzer (Fig. 2C & 2F).

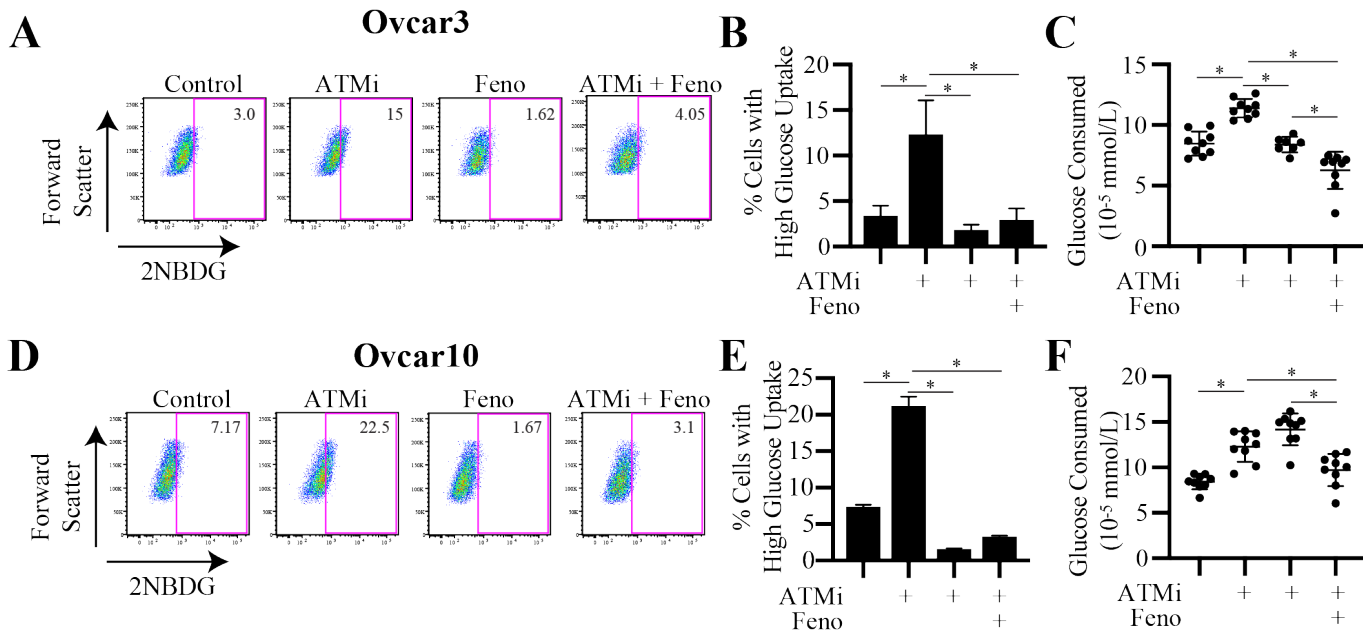


Figure 2. Fenofibrate decreases ATMi-mediated glucose uptake. (A-B) Ovar3 chemoresistant HGSOC cells were treated with 5uM ATM inhibitor (ATMi; KU60019), 10uM fenofibrate (Feno), or the combination for 72h. Glucose uptake was determined using the fluorescent glucose analog 2-NBDG followed by flow cytometry. **(A)** Scatter plots. **(B)** Quantification of glucose uptake. **(C)** Same as **(A)**, but media was used to determine glucose consumption using the YSI Bioanalyzer. **(D-F)** Same as **(A-C)**, but Ovar10 chemoresistant HGSOC cells were used. *p<0.05

- Subtask 2: Determine effects of fenofibrate on glycolytic functionality and metabolic phenotype using the Seahorse instrument.

Results: To determine the effects of ATM inhibition and fenofibrate on glycolysis, we performed the Seahorse glycolytic stress test in chemoresistant Ovar3 cells. This measures both basal glycolysis and the capacity of cells to perform glycolysis, and subsequent ATP production, after inhibition of mitochondrial respiration (Fig. 3A). We found that inhibition of ATM increased basal extracellular acidification rate (ECAR), glycolysis, glycolytic reserve, and non-glycolytic acidification (Fig. 3B-F). Moreover, fenofibrate or the combination of fenofibrate plus ATM inhibitor completely abolished glycolysis (Fig. 3A-F). These data are consistent with the original hypothesis that inhibition of ATM leads to increased glycolysis, and the combination of ATMi + fenofibrate is synergistic because glycolysis is inhibited. Interestingly, we also observed an increase in non-glycolytic acidification, suggesting that other metabolic pathways may also be affected.

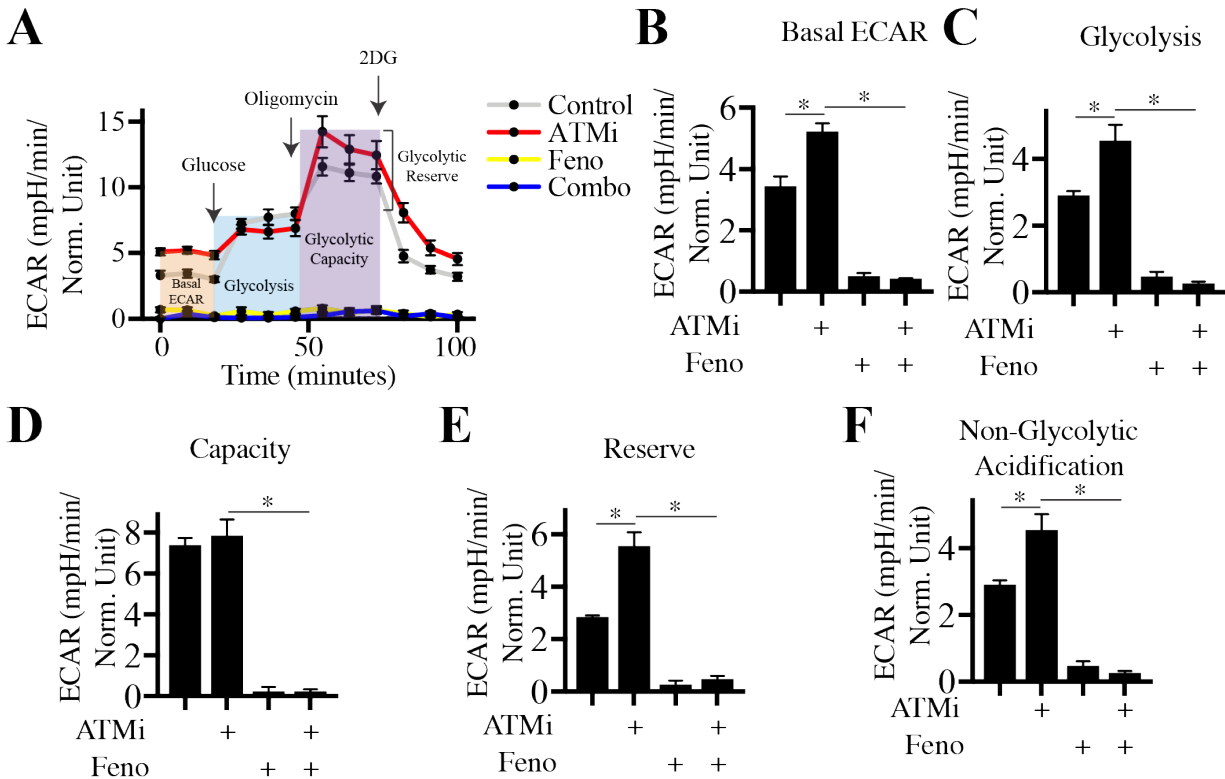


Figure 3. Glycolytic function is increased by inhibition of ATM and decreased by the combination of ATM inhibitor and fenofibrate. Ovar3 chemoresistant HGSOC cells were treated with 5uM ATM inhibitor (ATMi; KU60019), 10uM fenofibrate (Feno), or the combination for 72h. Glycolysis was functionally assessed using the Glycolytic Stress Test. **(A)** Extracellular acidification rate (ECAR) as a function of time. **(B)** Basal ECAR. **(C)** Glycolysis levels. **(D)** Glycolytic capacity. **(E)** Glycolytic reserve. **(F)** Non-glycolytic acidification. * $p < 0.05$

- Subtask 3: Determine effects on fenofibrate on transcription of metabolic genes.

Results: Using two different chemoresistant HGSOC cell lines (Ovar3 and Ovar10), we determined the expression of genes related to glycolysis (*HK2*, *PKM*, *PKM2*) and glucose transporters (*SLC2A1/2/4*). Inhibition of ATM alone did not increase expression of any glycolytic genes or glucose transporters (**Fig. 4A-B**). Our initial hypothesis was that ATM inhibition increases MYC to affect transcription of these genes. Therefore, these data are consistent with data in Figure 1, showing that MYC knockdown does not affect glucose uptake upon ATM inhibition in HGSOC cells. Fenofibrate alone also had very little effect on the glycolytic genes analyzed (**Fig. 4A**), although it robustly increased both *SLC2A2* and *SLC2A4* (encoding for GLUT2 and GLUT4, respectively). The combination of ATMi + fenofibrate decreased *PKM2* in both cell lines (**Fig. 4A**). These data suggest that the increased glucose consumption after inhibition of ATM is not linked to transcription of these genes, and therefore, the combination of ATMi + fenofibrate is likely working through an alternative mechanism.

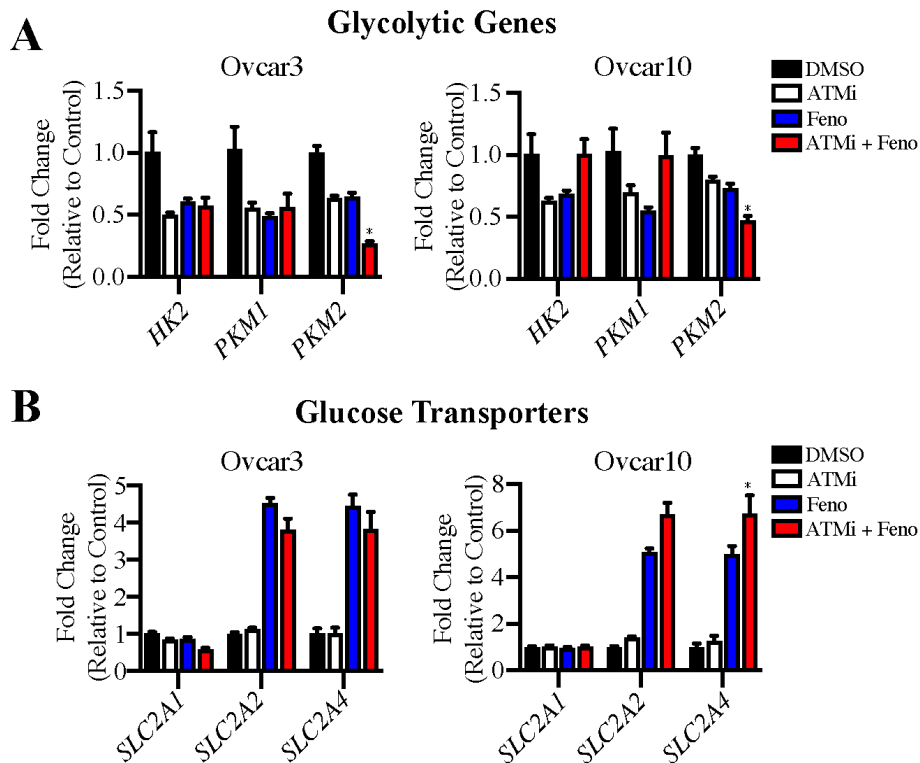


Figure 4. The combination of ATM inhibition and fenofibrate affects *PKM2* transcription. Ovarcar3 (left) or Ovarcar10 (right) chemoresistant HGSOC cells were treated with 5uM ATM inhibitor (ATMi; KU60019), 10uM fenofibrate (Feno), or the combination for 72h. **(A)** The indicated glycolytic genes were analyzed by RT-qPCR. **(B)** The indicated glucose transporter genes were analyzed by RT-qPCR. * $p < 0.05$ compared to both ATMi and Feno alone.

- Major Task 3:** Investigate the role of PPAR α -mediated transcriptional regulation of glycolytic genes to the observed synthetic lethality and whether PPAR α is necessary for the observed synthetic lethality
 - Milestone #3:** To have determined whether PPAR α transcriptionally regulates metabolic genes downstream of fenofibrate and if this is necessary for the observed synthetic lethality. Subtasks 1-4 not initiated due to negative results from Major Task #2, Subtask #3.
 - Subtask 1:** Validate PPAR α shRNA, engineer PPAR α knockout cells using CRISPR, and determine dose for PPAR α antagonist.

Results: Based on negative results in Major Task #2, Subtask #3, which demonstrated that glycolytic genes are not transcriptionally regulated in the context of ATM inhibition, we did not perform this task and directed our efforts towards more productive award-relevant activities.
 - Subtask 2:** Determine effects of ATMi in the absence of PPAR α on transcription of metabolic genes.

Results: Based on negative results in Major Task #2, Subtask #3, which demonstrated that glycolytic genes are not transcriptionally regulated in the context of ATM inhibition, we did not perform this task and directed our efforts towards more productive award-relevant activities.
 - Subtask 3:** PPAR α chromatin immunoprecipitation studies

Results: Based on negative results in Major Task #2, Subtask #3, which demonstrated that glycolytic genes are not transcriptionally regulated in the context of ATM inhibition, we did not perform this task and directed our efforts towards more productive award-relevant activities.

- Subtask 4: Determine effects of ATMi + fenofibrate in the absence of PPAR α on proliferation and cell death.

Results: Based on negative results in Major Task #2, Subtask #3, which demonstrated that glycolytic genes are not transcriptionally regulated in the context of ATM inhibition, we did not perform this task and directed our efforts towards more productive award-relevant activities.

Specific Aim 2: Development of novel EOC therapeutic strategies by combining ATM inhibition and fenofibrate in platinum-resistant models.

- **Major Task 1:** Determine whether inhibition of ATM in combination with fenofibrate induces synthetic lethality in platinum-resistant, but not sensitive, EOC cell lines and patient-derived ovarian cancer specimens
 - *Milestone #4: Correlation of chemoresistance and response to the combination in EOC cell lines and normal fallopian tube cells.*

- Subtask 1: Dose response curves to ATMi, fenofibrate, combination.

Results: Using 2 different chemoresistant HGSOC cell lines, we found that the combination of ATMi and fenofibrate is synergistic (**Fig. 6**). Using immortalized fallopian tube cells, we

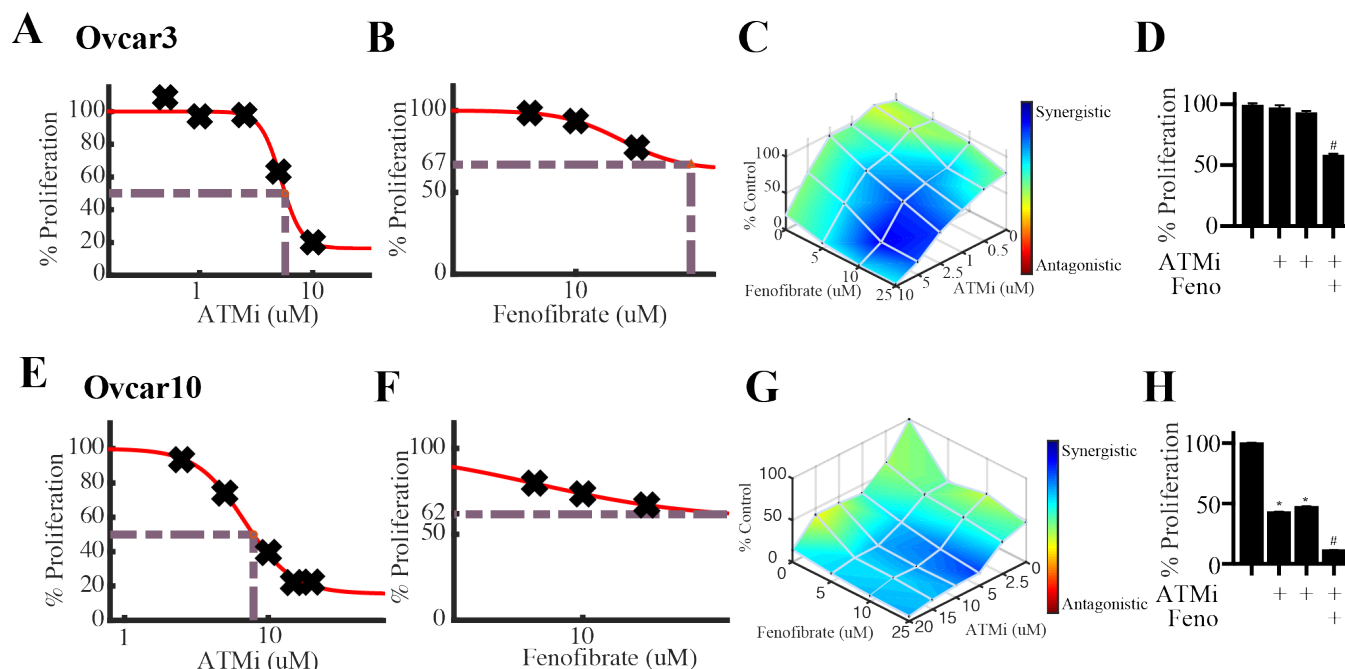


Figure 6. ATM inhibition and fenofibrate are synergistic in chemoresistant HGSOC cell lines. Ovar3 (A-D) or Ovar10 (E-H) chemoresistant HGSOC cells were treated with 5uM ATM inhibitor (ATMi; KU60019), 10uM fenofibrate (Feno), or the combination for 72h. (A, E) Dose response curves to the ATM inhibitor. (B, F) Dose response curves to fenofibrate. (C, G) Synergy analysis using the Combeneft software. (D, H) Percent proliferation of the indicated treatments. *p<0.05 vs. control; #p<0.05 vs. ATMi or Feno alone.

determined that the combination is not synergistic; however, the ATM inhibitor is toxic in these cells at the dose used (**Fig. 7**).

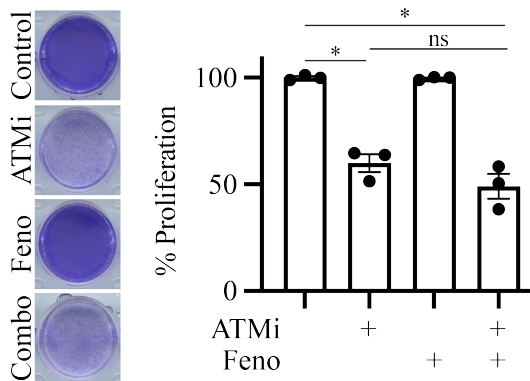


Figure 7. ATM inhibition and fenofibrate are not synergistic in fallopian tube cells. FT282 fallopian tube cells were treated with 5uM ATM inhibitor (ATMi; KU60019), 10uM fenofibrate (Feno), or the combination for 72h. Percent proliferation of the indicated treatments. * $p < 0.05$ vs. control; ns = not significant.

- Subtask 2: Assessment of proliferation and apoptosis markers in response to ATMi, fenofibrate, combination.

Results: Chemoresistant HGSOc cell lines have decreased proliferation upon treatment with the combination of ATM inhibitor and fenofibrate (**Fig. 6D & H**). To determine the mechanism underlying the change in proliferative rate, we first analyzed cell death by 7-AAD staining in two different chemoresistant HGSOc cell lines. We did not observe any increase in 7-AAD staining (**Fig. 8A**), suggesting that the combination does not affect cell death or apoptosis pathways. Based on these results we did not perform additional experiments for apoptotic markers. We next assessed the cell cycle profile using propidium iodide (PI) staining. We observed a small, but consistent, decrease in the percentage of cells in S-phase in the combination compared to either treatment alone. Together, these data suggest that the difference in cell proliferation is likely not due to cell death but some other mechanism.

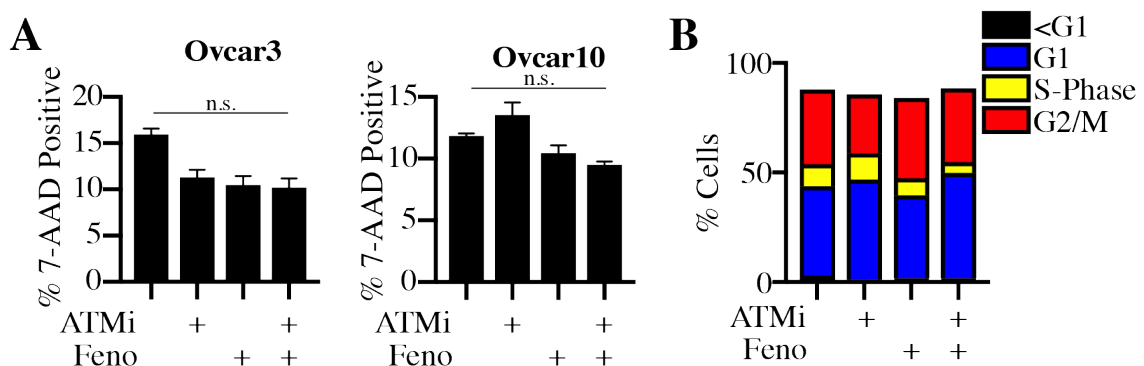


Figure 8. ATM inhibition and fenofibrate induce cell cycle arrest and not cell death. (A) Ovarcar3 (left) and Ovarcar10 (right) chemoresistant HGSOc cells were treated with 5uM ATM inhibitor (ATMi; KU60019), 10uM fenofibrate (Feno), or the combination for 72h. Cell death was assessed using 7-AAD staining followed by flow cytometry. n.s.=not significant. (B) Same as (A) but Ovarcar3 cells were fixed and stained with propidium iodide to assess cell cycle distribution.

- Milestone #5: IRB and HRPO approval received- COMPLETED August 2018.

- Subtask 3: Amend IRB approval & submit necessary documents for HRPO review.
Results: IRB approval (STUDY00009225) was attained 04/11/2018 from the Penn State College of Medicine. HRPO approval was received 08/06/2018.
- Milestone #6: Correlation of chemoresistance and response to the combination in patient-derived cells. We were unable to characterize the combination in multiple patient-derived cells due to poor growth conditions after prolonged culturing.
- Subtask 4: Identification of platinum-resistant cells from patient ascites.
Results: We have been able to culture 3 independent isolates from patient ascites and found that all 3 are resistant to cisplatin similar to Ovar3 cells (**Fig. 9**). Unfortunately, these cells senesce after prolonged culturing.

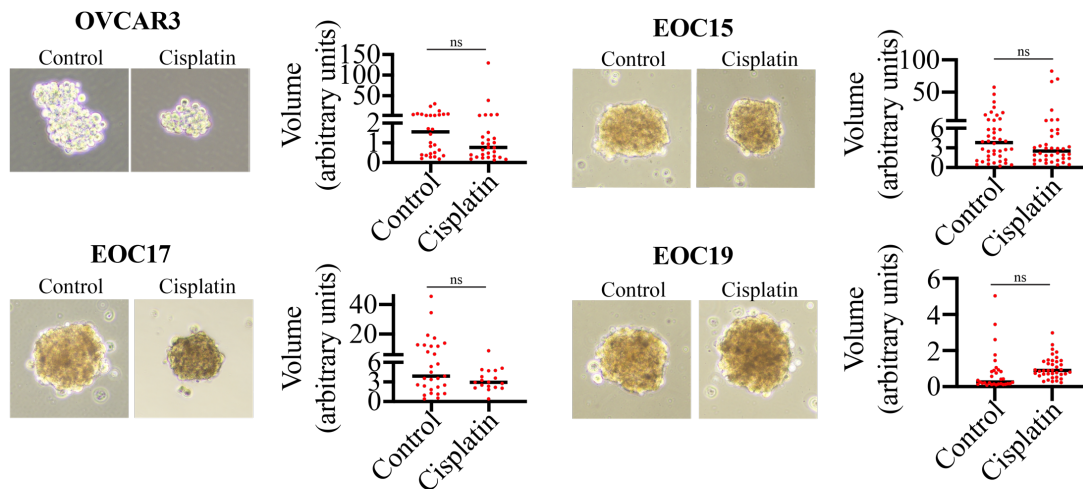


Figure 9. Patient-derived ovarian cancer cells are resistant to cisplatin. Three individual patient-derived ovarian cancer cells were cultured in ultra-low attachment plates and treated with 2μM cisplatin for 48h. Spheroid volume was assessed using ImageJ. ns = not significant

- Subtask 5: Dose response curves to ATMi, fenofibrate, combination
Results: Using the independent isolates from patient ascites (**Fig. 9**), we found that the combination of ATMi and fenofibrate is synergistic in EOC15 (**Fig. 10**). We were unable to test the combination in the other patient isolates due to senescence of the cells after prolonged culturing.
- Subtask 6: Assessment of proliferation and apoptosis markers in response to ATMi, fenofibrate, combination
Results: These experiments were not initiated due to not being able to culture the cells over multiple passages. Only EOC15 survived in vitro for long enough to perform experiments.

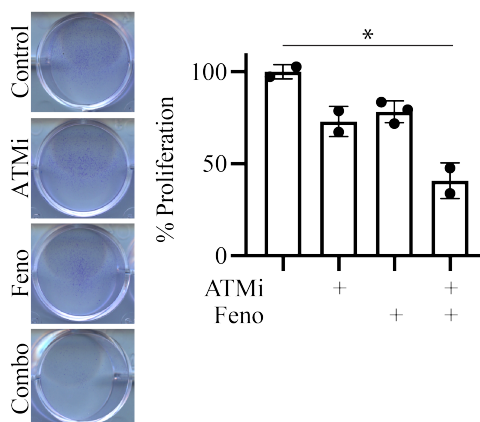


Figure 10. The combination of ATM inhibitor and fenofibrate is synergistic in patient-derived ovarian cancer cells that are resistant to cisplatin. EOC15 chemoresistant patient-derived EOC cells were treated with 5uM ATM inhibitor (ATMi; KU60019), 10uM fenofibrate (Feno), or the combination for 72h. Proliferation was assessed by crystal violet staining. * $p < 0.001$ vs. ATMi or Feno alone.

- **Major Task 2:** Determine whether inhibition of ATM in combination with fenofibrate induces synthetic lethality *in vivo*.
 - Milestone #7: IACUC approval received- COMPLETED May 2018.
 - Subtask 1: Obtain IACUC approval.
Results: IACUC approval (PROTO201800050) was attained 03/29/2018 from the Penn State College of Medicine. ACURO approval was received 05/10/2018.
 - Milestone #8: To have determined tumor progression and survival in mice treated with the combination and correlated with molecular markers of metabolism, proliferation, and cell death. Subtasks 3&4 not initiated due to negative results from Major Task #2, Subtask #2.
 - Subtask 2: Determine the effects of ATMi, fenofibrate, combination *in vivo*.
Results: We injected Ovar8 ovarian cancer cells intraperitoneally and treated mice with the ATM inhibitor alone, fenofibrate alone, and the combination. We did not observe a significant difference in tumor burden *in vivo*, suggesting that the observed responses are an *in vitro* phenomenon (**Fig. 11**). Alternatively, the doses used were not high enough to observe synergy *in vivo*. As shown in **Fig. 11**, there is a slightly trend towards decreased tumor burden in the combination group, although large intragroup variability exists.
 - Subtask 3: Investigate whether the combination results in a sustained response by following mouse survival after discontinuing drug treatment.
Results: Because the results of Subtask 2 were negative, we did not perform Subtask 3 and directed our efforts towards more productive award-relevant activities.
 - Subtask 4: Determine molecular markers of glycolysis, proliferation, and apoptosis of tumors.
Results: Because the results of Subtask 2 were negative, we did not perform Subtask 4 and directed our efforts towards more productive award-relevant activities.
 - Subtask 5: Statistical analysis of *in vivo* studies.

Results: There was no statistically significant difference in tumor burden, either longitudinally or at the end of the experiment (**Fig. 11**).

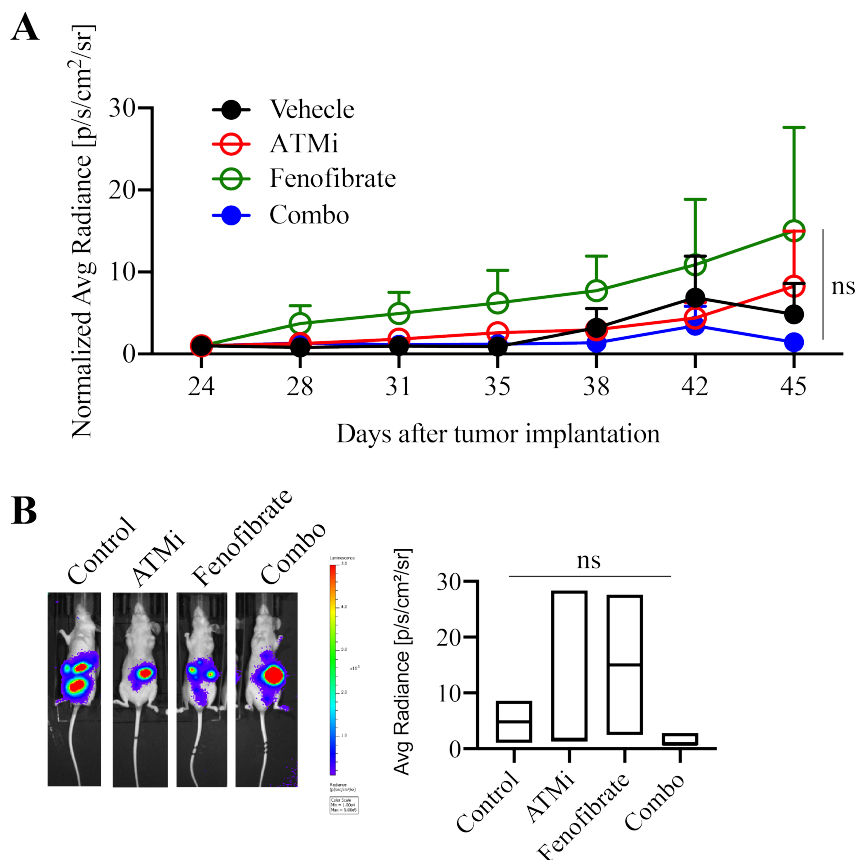


Figure 11. Treatment of mice with ATM inhibitor and fenofibrate combination at the concentrations used in does not significantly decrease tumor burden. Ovar3 human ovarian cancer cells expressing luciferase were injected intraperitoneally. After allowing tumors to establish, mice were treated every 3 days with the ATM inhibitor AZD0156 alone, fenofibrate alone, or the combination. **(A)** Tumor growth curve overtime. **(B)** Representative images of tumors (left) and quantification (right) from day 45 of the study. ns = not significant

- *Milestone #9: Manuscript submission describing the mechanistic basis of the synthetic lethality between ATMi and fenofibrate and its use in platinum-resistant EOC models.*

A manuscript describing the *in vitro* findings of our work was preprinted to bioRxiv May 2020 and is currently under review at a peer-review journal (**see Appendix I**).

- *Milestone #10: Submit grant application to NCI R01 for follow up work*

A DoD OCRP Teal Expansion Award has been submitted based on data generated from these studies (July 2020).

What opportunities for training and professional development has the project provided?

Nothing to Report

How were the results disseminated to communities of interest?

Results were disseminated to the scientific community in the form of multiple poster presentations and two preprints that have been submitted to peer-review journals (see Section 6).

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

Nothing to report

4. IMPACT

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

This project demonstrated that ATM inhibition alters nutrient consumption downstream of ATM inhibition in high grade serous ovarian cancer (HGSOC). HGSOC therapy remains an important clinical problem as relapse and chemoresistance occurs in the majority of patients. Our lab has focused efforts on a novel combination of ATM inhibitor and an FDA-approved drug fenofibrate as a potential combination for chemoresistant HGSOC patients. An important feature of our work is understanding the molecular mechanisms underlying cellular metabolism in ovarian cancer cells to determine whether exploitation of these pathways will ultimately result in new therapies. We determined that ATM expression correlates with a metabolic signature and that the combination of ATM inhibition and treatment with fenofibrate is synergistic *in vitro* through induction of senescence (**Appendix I**).

The mechanism of how ATM inhibition affects metabolism in HGSOC cells is an important and impactful question since ATM inhibitors have recently entered clinical trials in ovarian cancer. We have found that ATM inhibition increases nutrient uptake at least in part in a transporter-independent manner via macropinocytosis (**Appendix II**). Further, while glucose is taken up by macropinocytosis, we determined that branched chain amino acid (BCAA) uptake is likely more important in the context of ATM inhibition. These mechanistic studies may give us a new understanding of how cells may become resistant to ATM inhibitors through modulating metabolism. Therefore, expansion of these studies may lead to novel combinatorial interventions for patients treated with ATM inhibitors.

What was the impact on other disciplines?

ATM inhibitors have recently entered clinical trials for multiple solid tumors (clinicaltrials.gov). Therefore, these studies may have a broad impact not only for HGSOC patient therapy, but also for other cancer types. Moreover, the understanding of how ATM physiologically suppresses cellular metabolism is an important cell biology finding and may give rise to a new understanding of A-T patient pathologies.

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

Changes in approach and reasons for change

We have had to reconsider two of our initial hypotheses. First is the relationship between ATM and MYC. Using two independent shRNAs targeting MYC in two different chemoresistant HGSOC cell lines, we did not observe a difference in glucose uptake upon treatment with the ATM inhibitor (**Fig.**

1). Moreover, we hypothesized that MYC induced by ATM inhibition would upregulate glycolytic genes and/or glucose transporters. Our results indicate that inhibition of ATM does not upregulate *HK2*, *PKM*, or the glucose transporters *SLC2A1/2/4* (**Fig. 4**). This is consistent with results from Figure 1. As glucose uptake is markedly upregulated by ATM inhibition and suppressed by fenofibrate, we decided to determine whether other glucose transporters may play a role in this process. Interestingly, knockdown of glucose transporters (*SLC2A1*/Glut1 or *SLC2A4*/Glut4) did not inhibit glucose uptake due to ATM inhibition (**Fig. 12**). We explored this transporter-independent uptake of glucose further, and discovered that ATM inhibition induces uptake of nutrients by a non-specific endocytic process called macropinocytosis. These findings along with follow up mechanistic studies were preprinted to bioRxiv and are currently in revisions at a peer-review journal (**Appendix II**).

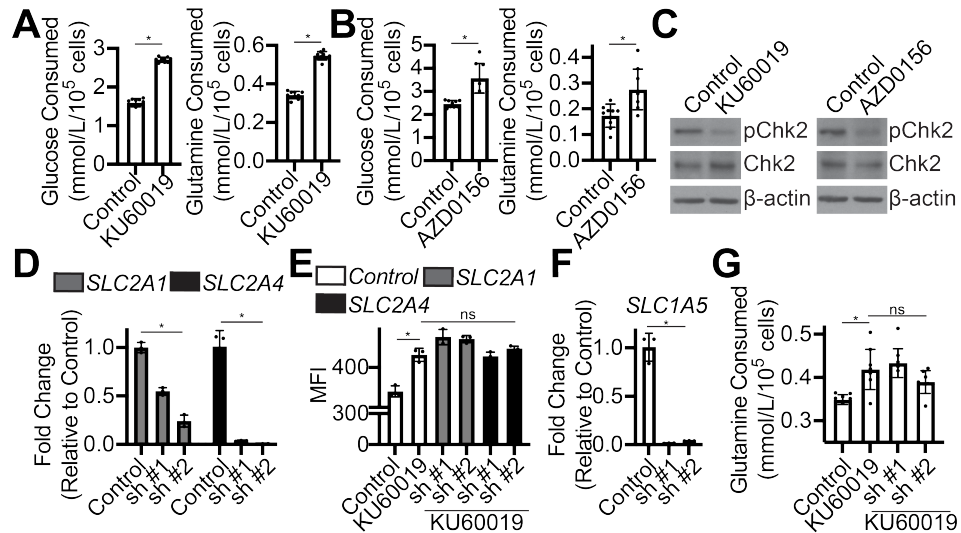


Figure 12. Knockdown of glucose and glutamine transporters in ATM inhibited cells does not affect glucose or glutamine consumption. (A-C) Ovar8 cells were treated with the ATM inhibitors (KU60019 or AZD0156) for 24h. (A-B) Glucose and glutamine uptake were determined. n=9/group, one of 3 experiments is shown. Data represent mean $\pm$ SD. *p<0.005; Student's t-test. (C) pChk2 and total Chk2 expression were determined by immunoblotting. β -actin was used as a loading control. One of 3 experiments is shown. (D) Ovar3 cells were infected with lentivirus expressing short hairpin RNAs (shRNAs) targeting *SLC2A1* (GLUT1) or *SLC2A4* (GLUT4). shGFP was used as a control. mRNA expression was determined by RT-qPCR. n=3/group, one of 2 experiments is shown. Data represent mean $\pm$ SD. *p<0.01 vs. control; one-way ANOVA. (E) Same as (D), but cells were treated with KU60019 for 24h, and glucose uptake using the fluorescent glucose analog 2NBDG was determined by flow cytometry. MFI=median fluorescence intensity. n=3/group, one of 3 experiments is shown. Data represent mean $\pm$ SD. *p<0.0001 vs. KU60019; ns=not significant; one-way ANOVA with Tukey's multiple comparisons. (F) Ovar8 cells were infected with lentivirus expressing short hairpin RNAs (shRNAs) targeting *SLC1A5*. shGFP was used as a control. mRNA expression was determined by RT-qPCR. n=3/group, one of 2 experiments is shown. Data represent mean $\pm$ SD. *p<0.0001 vs. control; one-way ANOVA. (G) Same as (F), but cells were treated with KU60019 for 24h, and glutamine consumption was determined. n=3/group, one of 3 experiments is shown. Data represent mean $\pm$ SD. *p<0.005 vs. KU60019; ns=not significant; one-way ANOVA with Tukey's multiple comparisons.

The second hypothesis is the mechanism of decreased cell proliferation. We observed no change in cell death in the combination treatment compared to either drug alone (Fig. 7). Instead, there is a slight but consistent decrease in S-phase cells (Fig. 7B). Therefore, we assessed the senescence marker Lamin B1 in the combination treated cells. *LMNB1* (encoding Lamin B1) was significantly downregulated upon treatment with the ATM inhibitor in combination with fenofibrate compared to either drug alone (Fig. 13). Further studies showed that in multiple cell lines, the combination treatment was synergistic through induction of senescence (Fig. 14).

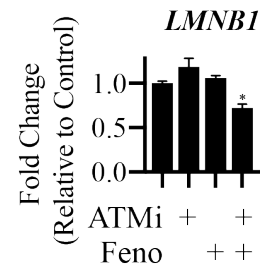


Figure 13. The combination of ATM inhibition and fenofibrate induces senescence. Ovar3 chemoresistant HGSOC cells were treated with 5uM ATM inhibitor (ATMi; KU60019), 10uM fenofibrate (Feno), or the combination for 72h. *LMNB1* expression was determined using RT-qPCR. *p<0.05 vs. ATMi or Feno alone.

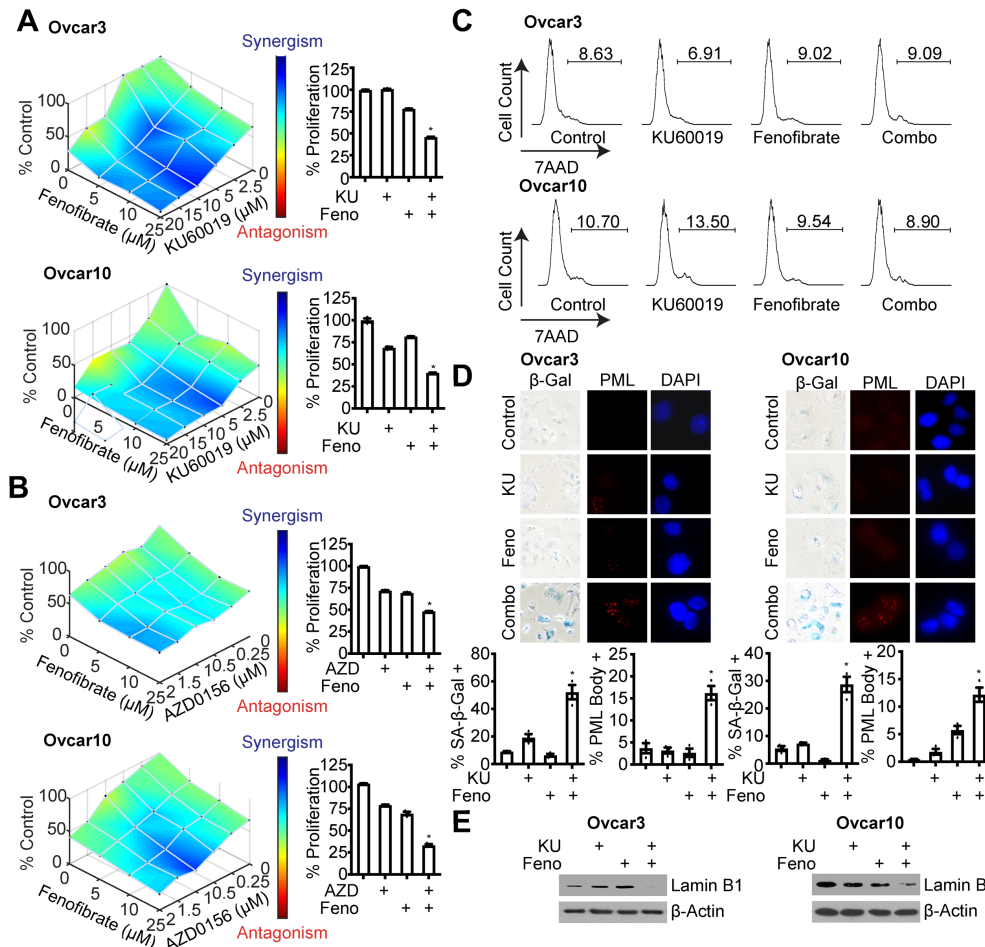


Figure 14. Combined inhibition of ATM and treatment with fenofibrate is synergistic in HGSOC cell lines. (A-B) Ovar3 and Ovar10 cells were treated with the ATM inhibitors KU60019 (A) or AZD0156 (B) or the PPARα agonist fenofibrate alone or in combination for 3 days. Proliferation was determined by crystal violet staining. n=3/group, one of 3 experiments is shown. *p<0.001 (C) Cell death was assessed by 7AAD staining. n=3/group, one of 3 experiments is shown. Data represent mean ± SEM. (D) SA-β-Gal and PML body staining. n=3/group, one of 3 experiments is shown. Data represent mean ± SEM. *p<0.001. (E) Immunoblot analysis of lamin B1. β-actin was used as a loading control. One of 3 experiments is shown.

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

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals.

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS

- Publications, conference papers, and presentations

Preprints (submitted to peer-review journals)

- Chen CW, Buj R, Dahl ES, Leon KE, Varner EL, von Krusenstiern E, Snyder NW, and Aird KM. ATM inhibition drives metabolic adaptation via induction of macropinocytosis. bioRxiv. doi: /10.1101/2020.04.06.027565
- Chen CW, Buj R, Dahl ES, Leon KE, and Aird KM. ATM Inhibition Synergizes with Fenofibrate in High Grade Serous Ovarian Cancer Cells. bioRxiv. doi.org/10.1101/2020.05.29.123919

Poster Presentations

- PSU Gynecological Malignancies Ovarian Cancer Symposium, Hershey, PA (2019), “ATM Inhibitor Synergizes with Glycolysis Inhibition in Ovarian Cancer Cells”
- Penn State Cancer Institute Retreat, Hershey, PA (2019), “ATM Inhibitor Synergizes with Glycolysis Inhibition in Ovarian Cancer Cells”
- AACR Advances in Ovarian Cancer Research Meeting, Atlanta, GA (2019), “ATM Inhibitor Synergizes with Glycolysis Inhibition in Ovarian Cancer Cells.”
- Midwest Metabolism Meeting, Van Andel Institute, Grand Rapids, MI (2019), “Suppression of ATM Induces Macropinocytosis to Influence Cancer Cell Metabolism.”
- Data & Dine Postdoctoral Research Day, Hershey, PA (2020), “ATM inhibition drives metabolic adaptation via induction of macropinocytosis.”

- Website(s) or other Internet site(s)
N/A
- Technologies or techniques
N/A
- Inventions, patent applications, and/or licenses
N/A
- Other Products
N/A

1. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name: Katherine M. Aird
 Project Role: PI
 Researcher Identifier ORCID ID: 0000-0002-5828-2325
 (e.g. ORCID ID):
 Nearest person month worked: 2mo
 Contribution to Project: Dr. Aird has designed, performed, and analyzed experiments proposed in both Specific Aims of this proposal.
 Funding Support: This award, W. W. Smith Charitable Trust Award, R00CA194309, R37CA240625, American Cancer Society RSG CCG 134157, Sandy Rollman Ovarian Cancer Research Foundation Award, Penn State Pathways to Partnerships Stage 2 Award (see below)

Name: Erika S. Dahl
 Project Role: Graduate Student
 Researcher Identifier ORCID ID: 0000-0001-6506-9103
 (e.g. ORCID ID):
 Nearest person month worked: 7mo
 Contribution to Project: Ms. Dahl has designed, performed, and analyzed experiments proposed in Specific Aim 1 of this proposal.
 Funding Support: N/A

Name: Maithili Navaratnarajah
 Project Role: Technician
 Researcher Identifier n/a
 (e.g. ORCID ID):
 Nearest person month worked: 1mo
 Contribution to Project: Ms. Navaratnarajah assisted Ms. Dahl with experiment proposed in Specific Aim 1.
 Funding Support: N/A

Name: Chi-Wei Chen
 Project Role: Postdoctoral Scholar
 Researcher Identifier ORCID ID: 0000-0001-8658-055X
 (e.g. ORCID ID):

Nearest person month worked: 16mo

Contribution to Project: Dr. Chen has designed, performed, and analyzed experiments proposed in Specific Aims 1 & 2 of this proposal.

Funding Support: N/A

Name: Junjia Zhu

Project Role: Collaborator (Biostatistician)

Researcher Identifier (e.g. ORCID ID): n/a

Nearest person month worked: 1mo

Contribution to Project: Dr. Zhu has assisted with biostatistics for experiments in Specific Aims 1 & 2 of this proposal.

Funding Support: 5P50DA036107-05; CTA152161; R21DE028650-01; P01CA171983-03 (see below)

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

Katherine M. Aird (PI)

- The W. W. Smith Charitable Trust Award (PI: Aird) has closed.
- The Sandy Rollman Ovarian Cancer Research Foundation Award (PI: Aird) has closed.
- PSU Pathways to Partnerships Stage 2 Grant (PI: Moldovan) is now active
Grants Officer: John Anthony, jtal1@psu.edu
Title: Precision Oncology Therapy by Targeting the Replication Stress Response
Goals: The goal of this proposal is to determine whether targeting Chk1 inhibits ovarian cancers with increased replication stress.
Specific Aims: Project 1: Specific Aim 1: Targeting a replication vulnerability in p16-low ovarian cancers. Specific Aim 2: Impact of POLH on ATR/Chk1 inhibitor efficacy.
Role: PI Project 1
Overlap: None
- R37CA240625 (PI: Aird) is now active
NIH 4.0 Calendar
Grants Officer: Chelsea Daly, chelsea.daly@nih.gov
Title: Investigating p16 Loss in Pro-tumorigenic Metabolism
Goals: The goal of this study is to assess the role of p16-loss-mediated nucleotide metabolism in senescence bypass and response to melanoma standard-of-care therapy.
Specific Aims: Specific Aim 1: To dissect the ATR-mediated nucleotide metabolic reprogramming via mTORC1 upon p16 loss and determine the contribution of this pathway to replication stress tolerance. Specific Aim 2: To determine whether depletion of nucleotides by targeting the ATR-mTORC1 axis is a novel therapeutic strategy for p16-low melanomas.
Role: PI
Overlap: None
- American Cancer Society Research Scholar Grant RSG CCG 134157 (PI: Aird) is now active
American Cancer Society 3.0 Calendar \$660,000 total costs 01/01/20-12/31/23
Grants Officer: Greta McShan, greta.mcshan@cancer.org
Title: Cyclin E-dependent Metabolic Vulnerabilities in Ovarian Cancer

Goals: The goals of this study are to: 1) determine how IDH1 regulates the replication stress response through the KDM4D-BRCA2 axis; and 2) to determine whether the combination of PARP inhibitors and IDH1 inhibitors lead to a synthetic lethality of ovarian cancer cells in vitro and in vivo.

Specific Aims: Specific Aim 1: To investigate the mechanism underlying IDH1-mediated increased *BRCA2* expression and whether this allows for tolerance of cyclin E-induced replication stress. Specific Aim 2: To determine whether IDH1 inhibition in combination with PARPi is synthetic lethal by inducing HR-deficiency.

Role: PI

Overlap: None

Junjia Zhu (Biostatistician)

- R01DA026815-05 (PI: Muscat) has closed.
- R03CA201951-02 (PI: Muscat/Zhu) has closed.
- 1 R21 DE028650-01 (PI: Hu) is now active

NIH 0.24 Calendar \$125,000 04/01/2019-03/31/2021

Grants Officer: Paul Newgen, paul.newgen@nih.gov

Title: The role of chronic cannabis and its two major psychoactive ingredients in papillomavirus-associated oropharyngeal disease

Goals: At the successful completion of the study, we expect to better understand the role of marijuana and its two major psychoactive ingredients in the local immune cell landscape and their association with oropharyngeal mucosal papillomavirus infection in both males and females, a key knowledge gap in the field.

Specific Aims: Specific Aim 1: Determine the impact of chronic marijuana smoke (up to nine month post viral infection) on the immune cell landscape of the oropharyngeal mucosae of both male and female mice. Specific Aim 2: Determine the impact of chronic marijuana smoke on viral infection and persistence in the CVP and associated VE glands in male and female mice.

Role: Biostatistician

Overlap: None

- R37CA240625 (PI: Aird) is now active

NIH 4.0 Calendar

Grants Officer: Chelsea Daly, chelsea.daly@nih.gov

Title: Investigating p16 Loss in Pro-tumorigenic Metabolism

Goals: The goal of this study is to assess the role of p16-loss-mediated nucleotide metabolism in senescence bypass and response to melanoma standard-of-care therapy.

Specific Aims: Specific Aim 1: To dissect the ATR-mediated nucleotide metabolic reprogramming via mTORC1 upon p16 loss and determine the contribution of this pathway to replication stress tolerance. Specific Aim 2: To determine whether depletion of nucleotides by targeting the ATR-mTORC1 axis is a novel therapeutic strategy for p16-low melanomas.

Role: Biostatistician

Overlap: None

- American Cancer Society Research Scholar Grant RSG CCG 134157 (PI: Aird) is now active

American Cancer Society 3.0 Calendar \$660,000 total costs 01/01/20-12/31/23

Grants Officer: Greta McShan, greta.mcshan@cancer.org

Title: Cyclin E-dependent Metabolic Vulnerabilities in Ovarian Cancer

Goals: The goals of this study are to: 1) determine how IDH1 regulates the replication stress response through the KDM4D-BRCA2 axis; and 2) to determine whether the combination of

PARP inhibitors and IDH1 inhibitors lead to a synthetic lethality of ovarian cancer cells in vitro and in vivo.

Specific Aims: Specific Aim 1: To investigate the mechanism underlying IDH1-mediated increased *BRCA2* expression and whether this allows for tolerance of cyclin E-induced replication stress. Specific Aim 2: To determine whether IDH1 inhibition in combination with PARPi is synthetic lethal by inducing HR-deficiency.

Role: Biostatistician

Overlap: None

- **What other organizations were involved as partners?**

Nothing to report

2. SPECIAL REPORTING REQUIREMENTS

Not applicable

3. APPENDICES

- Appendix 1: Manuscript #1

- Chen CW, Buj R, Dahl ES, Leon KE, and Aird KM. ATM Inhibition Synergizes with Fenofibrate in High Grade Serous Ovarian Cancer Cells. bioRxiv. doi.org/10.1101/2020.05.29.123919

- Appendix 1: Manuscript #2

- Chen CW, Buj R, Dahl ES, Leon KE, Varner EL, von Krusenstiern E, Snyder NW, and Aird KM. ATM inhibition drives metabolic adaptation via induction of macropinocytosis. bioRxiv. doi: /10.1101/2020.04.06.027565

Appendix 1: Manuscript #1

Chen CW, Buj R, Dahl ES, Leon KE, and Aird KM. ATM Inhibition Synergizes with Fenofibrate in High Grade Serous Ovarian Cancer Cells. bioRxiv. doi.org/10.1101/2020.05.29.123919

ATM Inhibition Synergizes with Fenofibrate in High Grade Serous Ovarian Cancer Cells

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Abstract

While therapies targeting deficiencies in the homologous recombination (HR) pathway are emerging as the standard treatment for high grade serous ovarian cancer (HGSOC) patients, this strategy is limited to the 50% of patients with a deficiency in this pathway. Therefore, patients with HR-proficient tumors are likely to be resistant to these therapies and require alternative strategies. We found that the HR gene Ataxia Telangiectasia Mutated (ATM) is wildtype and its activity is upregulated in HGSOC compared to normal fallopian tube tissue. Interestingly, multiple pathways related to metabolism are inversely correlated with ATM expression in HGSOC specimens, suggesting that combining ATM inhibition with metabolic drugs would be effective. Analysis of FDA-approved drugs from the Dependency Map demonstrated that ATM-low cells are more sensitive to fenofibrate, a PPAR α agonist that affects multiple cellular metabolic pathways. Consistently, PPAR α signaling is associated with ATM expression. We validated that combined inhibition of ATM and treatment with fenofibrate is synergistic in multiple HGSOC cell lines by inducing senescence. Together, our results suggest that metabolic changes induced by ATM inhibitors are a potential target for the treatment for HGSOC.

Introduction

Epithelial ovarian cancer (EOC) remains the most lethal gynecological malignancy (1). EOCs are divided into multiple subtypes with high grade serous ovarian cancer (HGSOC) as the most common. Most HGSOC patients are diagnosed at advanced stages (III-IV), and the 5-year survival rate for these patients is <30%. Current standard-of-care for HGSOC is debulking surgery followed by platinum-based chemotherapy (2). While the majority of patients initially respond to therapy, relapse with chemoresistant disease occurs in a significant number of patients (3). Recurrent disease is treated with poly(ADP)ribose polymerase (PARP) inhibitors if the patient is BRCA mutant or previously responded to platinum-based therapy (4). Response to platinum and PARP inhibitors occurs due to deficiencies in the DNA damage repair pathway homologous recombination (HR) (5). Homologous recombination deficiency (HRD) occurs in ~50% of patients and includes loss-of-function mutations in multiple proteins related to HR-mediated DNA repair (4). Unfortunately, the 50% of HGSOC patients with HR-proficient disease typically do not respond well to current therapies and have worse overall survival (6). Therefore, identification of therapies to treat this subset of HGSOC patients is urgently needed.

Ataxia Telangiectasia Mutated (ATM) is a serine/threonine kinase that is critical for HR-mediated repair of DNA double strand breaks (DSBs) (7,8). Upon sensing of DSBs by the MRN complex, ATM is recruited to the damage site, where it is first activated by autophosphorylation of S1981 (9). Upon activation, ATM phosphorylates a number of downstream substrates, including Chk2 and H2AX, that promote cell cycle arrest/delay and DNA repair (8). Germline mutations in ATM lead to the disorder Ataxia Telangiectasia (A-T), which has a number of pathological consequences, including predisposition to cancer and metabolic dysfunction (7,8). Given that A-T patients have a higher risk of cancer and *Atm*^{-/-} mice develop malignancies (7,8,10), ATM has been thought to be a tumor suppressor. However, many tumors rely on elevated DNA repair pathways, and a recent publication demonstrates that ATM is required for tumorigenesis (11). Additionally, a previous study in HGSOC showed that patients with high nuclear ATM expression have worse survival (12). Together, these

studies suggest that ATM may be an actionable target in the subset of tumors where it is wildtype and elevated. Indeed, ATM inhibitors have been in clinical development for the past two decades (13,14). Multiple pre-clinical studies have indicated that ATM inhibitor monotherapy is not likely to be effective (14-18). However, combined inhibition of ATM and DNA damaging agents such as PARP inhibitors and irradiation is synergistic (18), and recently a Phase I clinical trial using the ATM inhibitor AZD0156 in combination with a variety of DNA damaging agents has commenced (clinicaltrials.gov). As ATM inhibition also affects metabolic pathways (19-21), identifying targets beyond DNA damaging agents for combinatorial therapy with ATM inhibitors may therefore open up a new paradigm for treatment.

Peroxisome Proliferator Activated Receptors (PPARs: PPAR α , PPAR δ , PPAR γ) are nuclear receptors and ligand-inducible transcription factors (22,23). Upon ligand induction, PPARs heterodimerize with retinoid X receptor (RXR) and drive transcription of a variety of downstream targets. Downstream targets of the PPARs differ greatly, likely due to dissimilarity in endogenous and exogenous ligands. Ligands for PPAR α include endogenous ones like long chain unsaturated fatty acids and exogenous ones such as fibrates (23). Activation of PPAR α leads to transcription of multiple metabolic genes, such as those related to fatty acid oxidation or inhibition of glycolysis (24). This has been therapeutically exploited in patients with dyslipidemia by using fenofibrate or clofibrate, exogenous ligands for PPAR α (25). The role of PPAR α in cancer is not fully defined, as it is tumor-promoting in rodents but not humans and tumor suppressive in a context- and cancer-type dependent manner (26). Activation of PPAR α by treating cancer cells with fenofibrate or clofibrate alone or in combination with other drugs has been shown to decrease proliferation and survival through a variety of mechanisms (27). However, the combination of fenofibrate and ATM inhibitors has never been explored.

Here we show that ATM is wildtype and upregulated in HGSOC. Analysis of HGSOC TCGA datasets found that multiple metabolic pathways are associated with low ATM, suggesting that these pathways could be targeted in combination with ATM inhibitors. Using data from Dependency Map, we identified

the PPAR α agonist fenofibrate as an FDA-approved drug that inhibits ATM-low cell survival to a greater extent than ATM-high cancer cells. Indeed, PPAR α correlates with ATM expression in HGSOC patient specimens. Consistent with high throughput data, combined inhibition of ATM and treatment with fenofibrate is synergistic in HGSOC cells by inducing senescence. These results provide a proof-of-principle study to used combined inhibition of ATM and treatment with a metabolic drug for HGSOC therapy.

Results

ATM is wildtype and upregulated in HGSOC

ATM is thought to be a tumor suppressor as mutations in ATM predispose patients to tumorigenesis (7,8). However, recent data suggest that ATM expression is required for malignant transformation in response to oncogenic stress (11), suggesting that ATM plays a context-dependent role in tumorigenesis. Analysis of TCGA data demonstrate that the proportion of patients with ATM mutations varies widely among different tumor types (**Fig. 1A**). Interestingly, although HGSOC is known to have defects in HR (4,28), ATM is only mutated in ~0.5-2% of these patients (**Fig. 1B**). Compared to normal fallopian tube, the likely cell-of-origin for HGSOC (29,30), HGSOC specimens do not have markedly increased ATM protein expression (**Fig. 1C**) (31). However, phosphoproteomics analysis demonstrates that ATM kinase activity is significantly upregulated in HGSOC compared to normal fallopian tube, as indicated by increased S1981 autophosphorylation (**Fig. 1D**). Previous reports have shown that high ATM expression at both the protein and mRNA level is associated with worse overall and progression-free survival (12). Together, these data suggest that ATM is wildtype and its signaling pathway is upregulated in HGSOC, indicating that it may be an actionable target for these patients.

ATM low HGSOC tumors display a metabolic gene signature that is targetable

ATM may be an actionable target in HGSOC. However, many preclinical studies have demonstrated that inhibition of ATM as a single agent is not likely to be effective (14-18). Combined inhibition of ATM and other therapeutic agents has shown potential to inhibit cancer cell survival both *in vitro* and *in vivo* (13,14,18,32). We reasoned that genes and pathways that are inversely correlated with ATM expression may lead to the identification of novel targets for combinatorial therapeutics. Gene set enrichment analysis (GSEA) using HGSOC patient data from TCGA suggested that terms related to both cell cycle and DNA damage are enriched in ATM-low tumors as expected (**Table S1**). Interestingly, we also found that metabolic pathways such as oxidative phosphorylation, MYC signaling, mTORC1

signaling, fatty acid metabolism, glycolysis, and TCA cycle metabolism are enriched in ATM-low HGSOCs, suggesting that target these pathways may be synergistic with ATM inhibitors (**Fig. 2A and Table S1**). This is consistent with previous data demonstrating that suppression of ATM, either through mutation, small molecule inhibition, or knockdown, affects multiple metabolic processes (8,19-21,33-36). To further investigate potential drug combinations, we took advantage of the Dependency Map database (depmap.org) PRISM drug repurposing screen (37). We divided the cell lines into ATM high and ATM low based on protein expression, and looked for FDA-approved drugs that kill ATM-low cells to a greater degree ($\log_2FC < 0$; $FDR < 25\%$). We found 17 “hits” that met these criteria (**Fig. 2B and Table 1**). Of these, the majority were inhibitors of EGFR, which have already been shown to have synergistic effects with ATM inhibitors (38). Interestingly, fenofibrate was found as one of the hits (**Fig. 2B and Table 1**). Fenofibrate is a PPAR α agonist that has previously been shown to inhibit multiple tumor-promoting metabolic pathways including oxidative phosphorylation and glycolysis (27,39). These data indicate that inhibition of metabolic pathways using the FDA-approved drug fenofibrate may be a novel therapy to use in combination with ATM inhibitors.

PPAR α signaling is associated with ATM expression in HGSOC

ATM-low cancer cells are slightly, although significantly more sensitive to fenofibrate (**Fig. 2**), suggesting that these cells may have low PPAR α signaling. Indeed, there is a positive correlation between ATM and PPAR α in both cell lines and HGSOC patient samples (**Fig. 3A-B**), suggesting that ATM-low cancers have low PPAR α , which may be therapeutically exploited using fenofibrate.

Combination of ATM inhibitor and fenofibrate is synergistic in HGSOC cells

We found that ATM-low cancer cells are more sensitive to fenofibrate (**Fig. 2**) and HGSOC cells with low ATM expression have low PPAR α signaling (**Fig. 3**), suggesting that the combination of fenofibrate with ATM inhibitors may be synergistic in HGSOC cells. Indeed, inhibition of ATM using two small molecule inhibitors (KU60019 and AZD0156) synergized with fenofibrate in multiple HGSOC cell lines

to decrease cell proliferation (**Fig. 4A-B**). While cell death, as determined by 7AAD staining, was not affected (**Fig. 4C**), we observed multiple senescence markers, including increased SA- β -Gal and PML bodies and decreased lamin B1 expression in the combination treated cells (**Fig. 4D-E**). Together, these data demonstrate that the combination of ATM inhibition and fenofibrate treatment is synergistic in HGSOC through induction of senescence.

Discussion

Many tumors are addicted to DNA repair signaling (40), which has led to investigation of ATM inhibitors as cancer therapies (13,14). These inhibitors are not effective as a monotherapy, demonstrating the need to identify new targets for combinatorial therapeutic strategies. Here, we identified fenofibrate as a potential drug combination for use with ATM inhibitors. This may be due in part to upregulation of multiple metabolic pathways in ATM-low cancers. The combination induced senescence, a stable cell cycle arrest that is considered a positive patient outcome (41-43). Together, our results provide rationale for exploration of drugs that modify metabolism as combinatorial therapies with ATM inhibitors.

ATM is an critical mediator of DNA DSB repair through HR (7,8,14). Based on this, and the fact that both A-T patients and *Atm* knockout mice are predisposed to cancer, it has been well-appreciated that ATM is a tumor suppressor (7,8,10). However, many cancer cells are addicted to DNA damage repair and ATM signaling, and a recent study demonstrated that ATM is required for breast tumorigenesis (11). This suggests that in some contexts, ATM may act as an oncogene. Indeed, we found that HGSOC patients overwhelmingly harbor wildtype *ATM* alleles, and ATM kinase activity is upregulated in HGSOC samples compared to fallopian tube (**Fig. 1**). This is consistent with another paper that demonstrated increased ATM nuclear expression in serous ovarian cancer, which was associated with worse survival (12). Therefore, in the context of the ~50% of HGSOCs with HR-proficient disease, ATM can be considered to act more like an oncogene than tumor suppressor. This suggests that inhibition of ATM may be relevant for a large subset of HGSOC patients. As these patients often have worse survival than HR-deficient patients, identification of novel therapies is a clinical need. Indeed, ATM inhibitors, while not effective on their own, have shown promise in multiple cancer cell types in combination with irradiation or other DNA damage agents (13,14,16,18).

ATM has multiple functions outside of its role in DNA repair (8,19-21,44). We found that ATM-low HGSOC specimens showed multiple terms related to metabolism, including oxidative phosphorylation, TCA cycle metabolism, fatty acid metabolism, glycolysis, and signaling related to both MYC and mTORC1 (**Fig. 2**). This is consistent with previous reports from us and others that have shown suppression of ATM alters metabolic functions in multiple ways (8,19-21,33-36,45). For instance, we found that inhibition of ATM increases consumption of multiple metabolites, including glucose, glutamine, and branched chain amino acids (19,20,44). Similarly, A-T patients and A-T patient cells display multiple metabolic phenotypes, including mitochondrial dysfunction, insulin resistance, and an increased susceptibility to both diabetes and cardiovascular disease (7,8). Considering ATM inhibitors are not effective as a monotherapy, exploring metabolic vulnerabilities of ATM inhibited cancer cells may lead to additional combinatorial therapeutic strategies.

PPAR α has not been well-studied in ovarian cancer. One study found that PPAR α is expressed at a higher level in pleural effusions than in primary or metastatic tumors (46). Interestingly, they also found PPAR α to be expressed at a much lower level than either PPAR δ or PPAR γ , suggesting that PPAR α signaling while present may be low in ovarian cancers. Consistently, multiple studies have found that PPAR α agonists inhibit ovarian cancer proliferation and growth both *in vitro* and *in vivo* through a variety of mechanisms (47-49). We also found that at the dose and timing used in this study, the PPAR α agonist fenofibrate moderately inhibits HGSOC cell proliferation (**Fig. 4**). Fenofibrate is an FDA-approved with limited toxicity, suggesting that further studies are warranted to determine whether PPAR α agonists hold promise for HGSOC therapy.

Here, we found that the combination of ATM inhibition and fenofibrate is synergistic by inducing senescence (**Fig. 4**). Senescence is a tumor suppressive mechanism due to its inhibition of cancer cell proliferation (42,43,50). Recent data from HGSOC patients suggest that senescence occurs *in vivo* after therapy and is associated with a better outcome (41). Indeed, other publications have also

indicated that senescence is a beneficial therapeutic response (42,43,51-54). The mechanism of senescence induction by the combination of ATM inhibition and fenofibrate remains to be explored. We found that PPAR α expression is associated with ATM expression both in HGSOC patient samples (**Fig. 3**), and ATM-low patients have altered metabolic pathways (**Fig. 2**). Thus, is possible that enhanced PPAR α signaling using an agonist competes with metabolic pathways that are altered in ATM-low/inhibited cells. For instance, we previously published that ATM suppression increased glucose uptake and utilization (19), whereas fenofibrate decreases this process (27). Similarly, others have reported that *Atm* knockout cells display mitochondrial dysfunction (34). This may be further exacerbated by fenofibrate, which decreases mitochondrial metabolism and oxidative phosphorylation through a variety of mechanisms (27,39). Regardless of the mechanism, given that fenofibrate is FDA-approved and has an excellent safety profile, our results provide a proof-of-principle study to combine fenofibrate or other PPAR α agonists with ATM inhibitors. Our studies may also suggest that cancers with a high prevalence of ATM mutations (for instance melanoma) may be especially sensitive to PPAR α agonists.

Conclusions

The present study shows that ATM is wildtype and upregulated in HGSOC, which corresponds to low PPAR α expression. ATM-low cells display changes in multiple metabolic pathways that reveal a therapeutic vulnerability to use for combinatorial treatment with ATM inhibitors. The combined inhibition of ATM and treatment with the PPAR α agonist fenofibrate was synergistic in HGSOC cell lines. Thus, our study provides a new potential combination therapy for HGSOCs that are HR-proficient. As multiple metabolic terms were associated with ATM-low HGSOC specimens, we predict that additional metabolic drugs may have synergistic effects with ATM inhibitors.

Material and Methods

TCGA database and GSEA analysis

Spearman's correlation in HGSOC (TCGA, PanCancer Atlas) was calculated using cBioportal (55,56) for each gene in the RNA-Seq. Genes were ranked according to the Spearman correlation coefficient and the p-value of the correlation as follows: $-\log_{10}(\text{p value}) * \text{sign}(\log_2 \text{ correlation coefficient})$ (57). Pre-ranked files were used to run pre-ranked GSEA (MSigDB collection KEGG and Reactome) (58) under predefined parameters. Following GSEA documentation: https://software.broadinstitute.org/cancer/software/gsea/wiki/index.php/FAQ#Why_does_GSEA_use_a_false_discovery_rate_.28FDR.29_of_0.25_rather_than_the_more_classic_0.05.3F. Terms with a q-value < 0.25 were considered significant.

Dependency Map data analysis

Raw data from the PRISM drug repurposing screen (37) and reverse phase protein array (RPPA) (37,59) were downloaded from depmap.org. Cell lines were divided in half based on ATM protein expression. Only "launched" drugs were considered. Drugs were considered "hits" if $\log_2 \text{foldchange} < 0$ (ATM low vs. ATM high) and $\text{FDR} < 0.25$. Other analyses were performed using the depmap.org online tool.

Cell lines and culture conditions

Ovcar3 and Ovcar10 HGSOC cells were cultured in RPMI-1640 (Corning, Cat# 10-040-CV) supplemented with 5% FBS. All cell lines were cultured in MycoZap and were routinely tested for mycoplasma using a highly sensitive PCR-based method (60). Tumor cell lines were authenticated using STR Profiling using Genetica DNA Laboratories.

Colony Formation

Cells (5×10^6 /well in 12-well plates) were seeded, allowed to adhere overnight, and washed twice with 1x PBS the next day. Cells were cultured with serum-free RPMI-1640 for another 24 hours and then treated with 2.5-20 μ M KU60019 (A8336, ApexBio) or 0.25-2 μ M AZD0156 (B7822, ApexBio) and a combination of 5-20 μ M fenofibrate (F6020-5G, Sigma) in RPMI-1640 supplemented with 0.1% FBS for 3 days. KU60019 and AZD0156 were administered 5 hours prior to fenofibrate. All drugs were supplied to the media daily. Colony formation was visualized by fixing cells in 1% paraformaldehyde for 5 min and staining with 0.05% crystal violet for 20 min. Wells were destained for 5 min in 500 mL 10% acetic acid. Absorbance (590nm) was measured using a spectrophotometer (Spectra Max 190). Each sample was assessed in triplicate. The synergy studies were further analyzed using Combenefit (61).

Flow cytometry

Cells (5×10^6 /well in 12-well plates) were seeded, allowed to adhere overnight, and washed twice with 1x PBS the next day. Cells were cultured cell with serum-free RPMI-1640 for another 24 hours. Ovar3 cells were then treated 1 μ M KU60019 and 10 μ M fenofibrate; Ovar10 cells were treated 10 μ M KU60019 and 25 μ M fenofibrate in RPMI-1640 + 0.1% FBS for 3 days. KU60019 was administered 5 hours prior to fenofibrate. KU60019 and fenofibrate were supplied to the media daily. Cells were harvested by trypsin and washed twice with PBS. Cells were suspended in 0.5 μ g/ml 7-AAD (13-6993-T500, Tonbo Biosciences) in 1mL staining solution [900 μ L H₂O + 100 μ L NaCitrate (380mM)] for 15 minutes at room temperature. Cells were run on a 10-color FACSCanto flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (Ashland, OR).

Western blotting

Cell lysates were collected in 1X sample buffer (2% SDS, 10% glycerol, 0.01% bromophenol blue, 62.5mM Tris, pH 6.8, 0.1M DTT) and boiled for 10 min at 95°C. Protein concentration was determined using the Bradford assay. Proteins were resolved using SDS-PAGE gels and transferred to nitrocellulose membranes (Fisher Scientific) (110mA for 2 h at 4°C). Membranes were blocked with 5%

nonfat milk or 4% BSA in TBS containing 0.1% Tween-20 (TBS-T) for 1 h at room temperature. Membranes were incubated overnight at 4°C in primary antibodies (anti-lamin B1, ab16048, Abcam, 1:5000; anti- β -Actin, A1978, Sigma, 1:10,000) in 4% BSA/TBS + 0.025% sodium azide. Membranes were washed 4 times in TBS-T for 5 min at room temperature after which they were incubated with HRP-conjugated secondary antibodies (Cell Signaling, Danvers, MA) for 1 h at room temperature. After washing 4 times in TBS-T for 5 min at room temperature, proteins were visualized on film after incubation with SuperSignal West Pico PLUS Chemiluminescent Substrate (ThermoFisher, Waltham, MA).

Immunofluorescence

Cells (5×10^6 /well in 12-well plates) were seeded overnight and washed with 1x PBS twice on next day. Cells were cultured with serum-free RPMI for another 24 hours. Ovar3 cells were then treated 1 μ M KU60019 and 10 μ M fenofibrate; Ovar10 cells were treated 10 μ M KU60019 and 25 μ M fenofibrate in RPMI + 0.1% FBS for 3 days. KU60019 was administered 5 hours prior to fenofibrate. KU60019 and fenofibrate were supplied daily. Cells were fixed in 4% paraformaldehyde for 10min and permeabilized in 0.2% Triton X-100 for 5 min. Cells then were stained with PML (1:200, Santa Cruz, Cat# sc-966) in 3% BSA/PBS at room temperature for 1 h. Cells were further incubated with 0.15 μ g/ml DAPI in PBS (1 min), mounted, and sealed. Cells were washed three times and then incubated in Cy3 anti-mouse secondary antibody (1:5000, Jackson ImmunoResearch Labs, Cat# 715-165-150) in 3% BSA/PBS at room temperature for 1 h. Finally, cells were incubated with 0.15 μ g/ml DAPI in PBS for 1min, washed three times with PBS, mounted, and sealed. Images were acquired at room temperature using a Nikon Eclipse 90i microscope with a 20x/0.17 objective (Nikon DIC N2 Plan Apo) equipped with a CoolSNAP Photometrics camera.

Senescence-associated beta-galactosidase staining

SA- β -Gal staining was performed as previously described (62). Cells were fixed in 2% formaldehyde/0.2% glutaraldehyde in PBS for 5 min and stained (40 mM Na₂HPO₄, 150 mM NaCl, 2 mM MgCl₂, 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, and 1 mg/ml X-gal) overnight at 37 °C in a non-CO₂ incubator. Images were acquired at room temperature using an inverted microscope (Nikon Eclipse Ts2) with a 20X/0.40 objective (Nikon LWD) equipped with a camera (Nikon DS-Fi3). Each sample was assessed in triplicate and at least 100 cells per well were counted (> 300 cells per experiment).

Quantification and Statistical Analysis

GraphPad Prism version 8.0 was used to perform statistical analysis. One-way ANOVA or t-test were used as appropriate to determine p-values of raw data. p-values < 0.05 were considered significant.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

- 1) Conceived and designed experiments: Chi-Wei Chen and Katherine M. Aird
- 2) Performed the experiments: Chi-Wei Chen, Raquel Buj, Erika S. Dahl, Kelly E. Leon
- 3) Analyzed and interpreted the data: Chi-Wei Chen, Raquel Buj, Katherine M. Aird
- 4) Contributed reagents, materials, analysis tools or data: n/a
- 5) Wrote the paper: Chi-Wei Chen and Katherine M. Aird

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Figure Legends

Figure 1. ATM is wildtype and upregulated in high grade serous ovarian cancer patients.

(A) Analysis of ATM alterations in TCGA PanCancer Atlas Studies.

(B) Analysis of ATM alterations in TCGA HGSOC studies.

(C) Total ATM protein expression in normal fallopian tube and HGSOC specimens. ns = not significant.

(D) Phosphorylated ATM (S1981) protein expression in normal fallopian tube and HGSOC specimens.

*p<0.001

Figure 2. ATM-low specimens have increased metabolic gene signature and are more sensitive to fenofibrate.

(A) GSEA enrichment analysis in ATM-high vs. ATM-low HGSOC specimens from TCGA. Negative NES means enrichment of expression in ATM-low HGSOC specimens.

(B) Volcano plot of FDA-approved drugs in ATM-low vs. ATM-high cell lines. Line represents p-value<0.05.

Figure 3. PPAR α expression is associated with ATM expression in cell lines and HGSOC patient specimens.

(A) Correlation between *ATM* and *PPARA* (encoding PPAR α) expression in cell lines from Dependency Map.

(B) Correlation between of *ATM* and *PPARA* expression in HGSOC patient specimens from TCGA.

Figure 4. Combined inhibition of ATM and treatment with fenofibrate is synergistic in HGSOC cell lines.

(A-B) Ovc3 and Ovc10 cells were treated with the ATM inhibitors KU60019 (A) or AZD0156 (B) or the PPAR α agonist fenofibrate alone or in combination for 3 days. Proliferation was determined by crystal violet staining. n=3/group, one of 3 experiments is shown. *p<0.001

(C) Cell death was assessed by 7AAD staining. n=3/group, one of 3 experiments is shown. Data represent mean $\pm$ SEM.

(D) SA- β -Gal and PML body staining. n=3/group, one of 3 experiments is shown. Data represent mean $\pm$ SEM . *p<0.001.

(E) Immunoblot analysis of lamin B1. β -actin was used as a loading control. One of 3 experiments is shown.

Table Legends

Table 1: FDA-approved drugs that sensitize ATM-low cells to a greater extent than ATM-high cells.

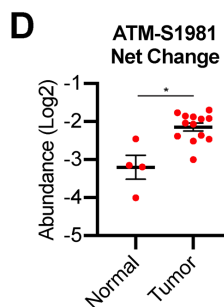
Drug Name	Target	Log ₂ FC (ATM-low vs. ATM-high)	FDR
lapatinib	EGFR, ERBB2	-0.1431668	4.54E-07
osimertinib	EGFR	-0.1575673	6.22E-06
ibrutinib	BTk	-0.1669926	1.95E-05
gefitinib	EGFR	-0.139171	0.00012
afatinib	EGFR, ERBB2, ERBB4	-0.1432169	0.000225
icotinib	EGFR	-0.1019151	0.001532
olmutinib	EGFR	-0.0559589	0.003793
vandetanib	EGFR, VEGFR, RET	-0.1091835	0.007295
fenofibrate	PPARA	-0.0821997	0.012972
spironolactone	Various	-0.0930345	0.067266
erlotinib	EGFR	-0.0937315	0.075598
alfacalcidol	VDR	-0.0450776	0.177706
bosutinib	Bcr-Abl, Src	-0.0724218	0.189293
maxacalcitol	VDR	-0.0500369	0.189293
paricalcitol	VDR	-0.0490078	0.191315
loperamide	Various	-0.0341769	0.19634
dequalinium	Various	-0.1146281	0.219346

Table 1: FDA-approved drugs that sensitize ATM-low cells to a greater extent than ATM-high cells.

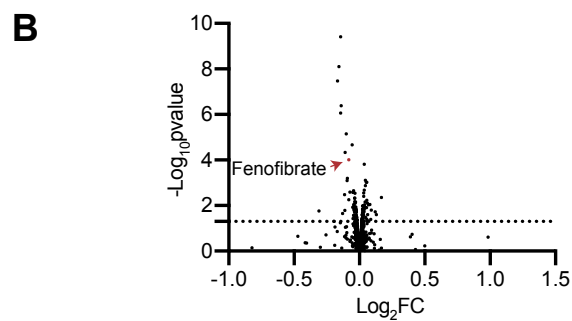
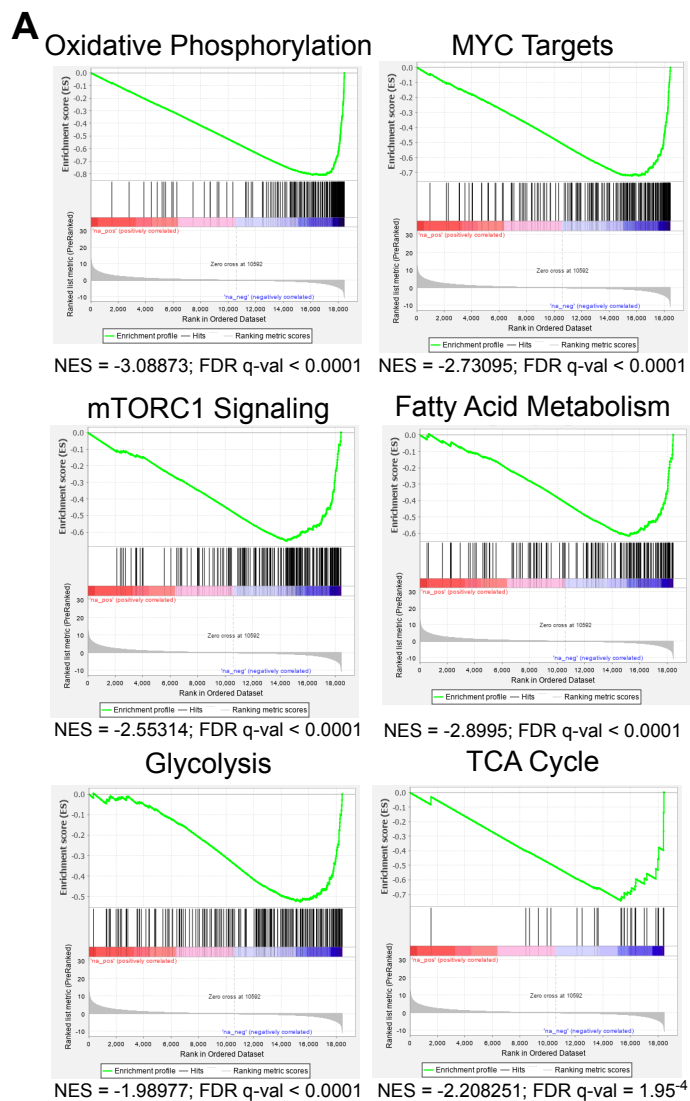
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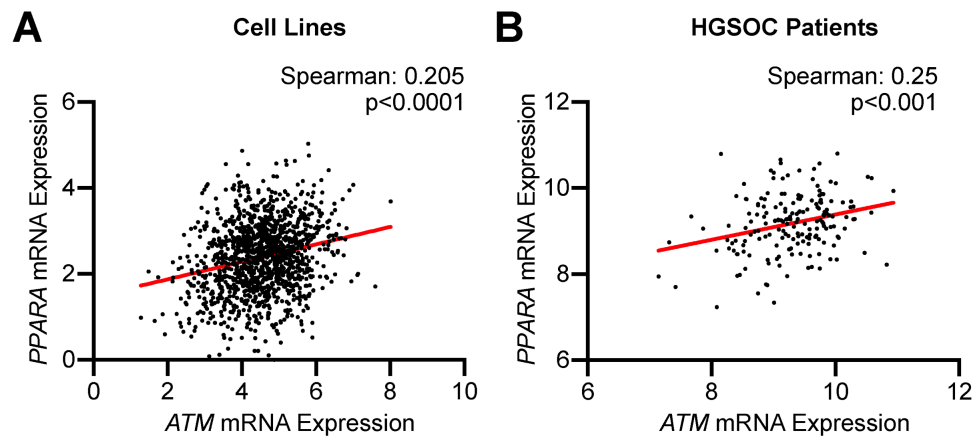
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 ■ Truncating Mutation (putative driver)
 ■ Amplification
 ■ mRNA High
 ■ mRNA Low
 ■ Protein High
 ■ Protein Low
 ■ No Alterations



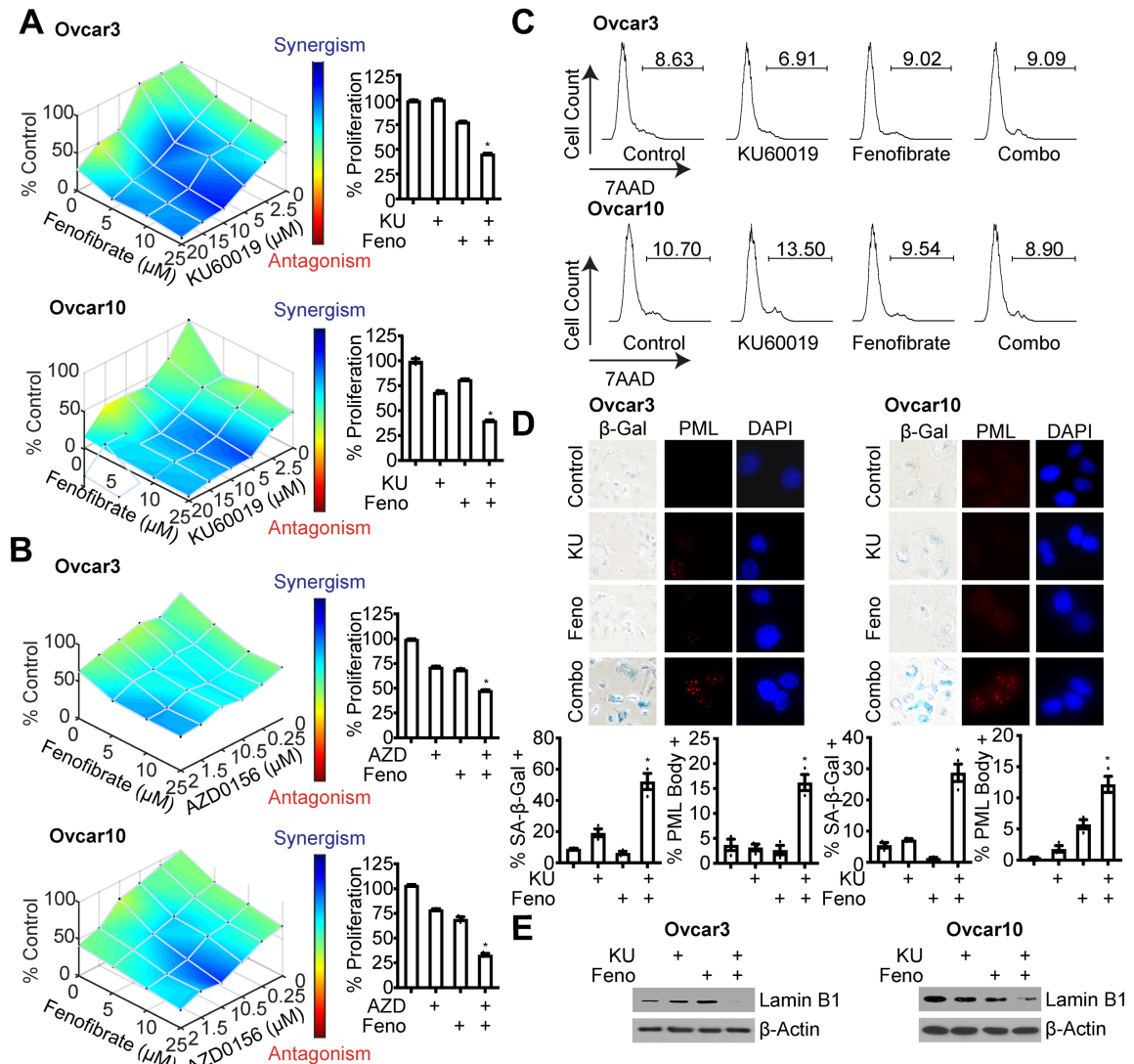
Chen et al.- Figure 2



Chen et al.- Figure 3



Chen et al.- Figure 4



Appendix 2: Manuscript #2

Chen CW, Buj R, Dahl ES, Leon KE, Varner EL, von Krusenstiern E, Snyder NW, and Aird KM. ATM inhibition drives metabolic adaptation via induction of macropinocytosis. bioRxiv. doi: /10.1101/2020.04.06.027565

ATM inhibition drives metabolic adaptation via induction of macropinocytosis

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Running Title: ATM inhibition induces macropinocytosis

Keywords: AMPK; branched chain amino acids; nutrient stress; TCA cycle; EIPA; Pak1

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Summary

Macropinocytosis is a nonspecific endocytic process that enhances cancer cell survival under nutrient-poor conditions. Ataxia-Telangiectasia mutated (ATM) is a tumor suppressor that plays a role in cellular metabolic reprogramming. We report that suppression of ATM increases macropinocytosis in an AMPK-dependent manner to promote cancer cell survival in nutrient-poor conditions. Combined inhibition of ATM and macropinocytosis suppressed proliferation and induced cell death both *in vitro* and *in vivo*. Metabolite analysis of the ascites and interstitial fluid from tumors indicated decreased branched chain amino acids (BCAAs) in the microenvironment of ATM-inhibited tumors. Supplementation of ATM inhibitor-treated cells with BCAAs abrogated AMPK phosphorylation and macropinocytosis and rescued the cell death that occurs due to combined inhibition of ATM and macropinocytosis. These data reveal a novel molecular basis of ATM-mediated tumor suppression whereby loss of ATM promotes pro-tumorigenic uptake of nutrients to promote cancer cell survival and reveal a metabolic vulnerability of ATM-inhibited cells.

Introduction

Macropinocytosis is an endocytic process whereby cells take up fluid, macromolecules, metabolites, and other cargo from the surrounding microenvironment (King and Kay, 2019; Palm, 2019; Zhang and Commisso, 2019). Many studies have evaluated the critical role of macropinocytosis as a nutrient scavenging mechanism in cancers under nutrient-deprived conditions (Commisso et al., 2013; Davidson et al., 2017; Hodakoski et al., 2019; Kamphorst et al., 2015; Kim et al., 2018; Lee et al.; Redelman-Sidi et al., 2018; Tejeda-Munoz et al.; Yao et al., 2019). To date, these studies have focused on cancers with high PI3K activity, such as those with mutant RAS or PTEN loss, which act upstream of the Rac1-Pak1 actin remodeling pathway to promote macropinosome formation (Zhang and Commisso, 2019). As macropinocytosis is a process that is driven by multiple nutrient sensing pathways, including AMPK and mTOR (Kim et al., 2018; Palm et al., 2015), it is likely that other tumor-associated pathways also induce macropinocytosis as a means to provide nutrients.

Ataxia-Telangiectasia mutated (ATM) is a tumor suppressor, and mutation or loss of ATM expression promotes genomic instability and predisposes cells to tumorigenesis (McKinnon, 2004, 2012). While ATM is known to be important for the response to DNA double strand breaks (Shiloh, 2003), it is also known to play a role in cellular metabolism (Dahl and Aird, 2017; Guleria and Chandna, 2016). We and others have shown that inhibition of ATM increases uptake of glucose and glutamine to provide nutrients for cell growth and proliferation (Aird et al., 2015; Dahl and Aird, 2017). Our previous results suggest that inhibition of ATM reprograms metabolism through suppression of p53 signaling and enhanced c-MYC protein stability (Aird et al., 2015). Indeed, these and other models where ATM suppression or mutation has been shown to alter cellular metabolism are in cells with wildtype p53 and normal c-MYC expression. Whether ATM similarly alters nutrient uptake and cellular metabolism in the context of cancers with mutated p53 and high c-MYC expression is unclear. Moreover, ATM inhibitors are currently undergoing clinical trials (Jin and Oh, 2019); thus, understanding how inhibition of ATM

drives metabolic reprogramming may be important towards identifying potential resistance mechanisms or other targets that could be used in combination with these inhibitors in ATM-wildtype tumors.

We found that suppression of ATM increases nutrient uptake in p53 mutant and c-MYC amplified cancer cells independently of transporters. This non-specific nutrient uptake under nutrient poor conditions occurred via AMPK-dependent macropinocytosis. Underscoring the importance of macropinocytosis in ATM-inhibited cells, suppression of macropinocytosis using 5-(N-ethyl-N-isopropyl) amiloride (EIPA) (Commisso et al., 2014; Ivanov, 2008) significantly inhibited proliferation of cancer cells both *in vitro* and *in vivo*. Analysis of tumor metabolites suggested increased uptake of branched chain amino acids (BCAAs) in ATM inhibited tumors, and supplementing cells *in vitro* with exogenous BCAAs decreased macropinocytosis and AMPK phosphorylation and suppressed the synergy between ATM and macropinocytosis inhibition. Together, these data demonstrate that inhibition of ATM reprograms cellular metabolism through AMPK-mediated induction of macropinocytosis and reveal that macropinocytosis is a vulnerability of ATM inhibited tumors.

Results and Discussion

Inhibition of ATM kinase activity increases glucose and glutamine consumption in a transporter-independent manner

ATM loss or inhibition alters whole body and cellular metabolism (Aird et al., 2015; Cosentino et al., 2011; Dahl and Aird, 2017; Guleria and Chandna, 2016; Valentin-Vega et al., 2012). We previously published that increased uptake and consumption of glucose and glutamine, two carbon sources that are critically important for cancer cell metabolism, is in part due to inactivation of p53 and increased c-MYC stability downstream of ATM knockdown (Aird et al., 2015) and may be due to the regulation of glucose and glutamine transporters by these transcription factors (Schwartzberg-Bar-Yoseph et al., 2004; Zhao et al., 2019). However, the transporter-dependence of nutrient uptake in ATM inhibited cells

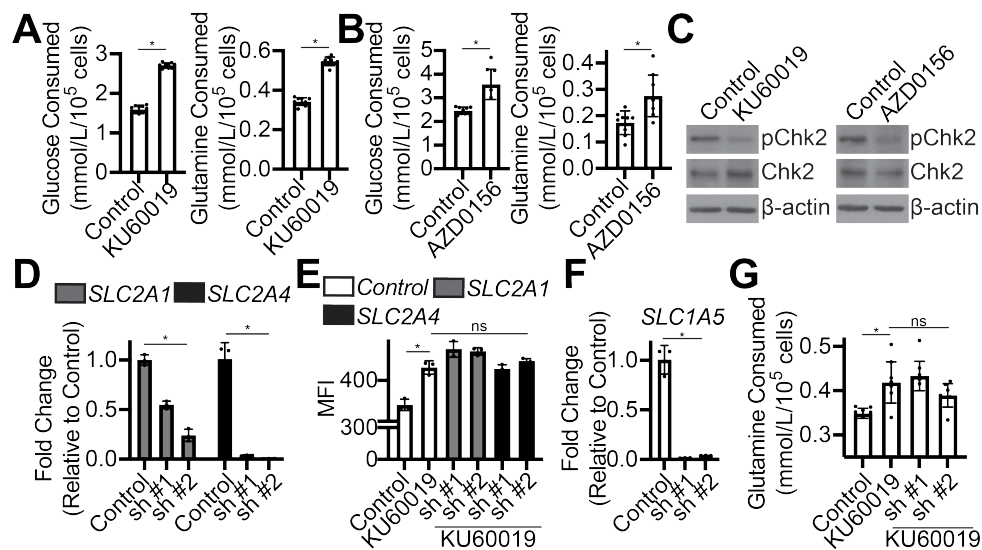


Figure 1. Knockdown of glucose and glutamine transporters in ATM inhibited cells does not affect glucose or glutamine consumption

(A-C) Ovar8 cells were treated with the ATM inhibitors (KU60019 or AZD0156) for 24h. (A-B) Glucose and glutamine uptake were determined. n=9/group, one of 3 experiments is shown. Data represent mean $\pm$ SD. *p<0.005; Student's t-test. (C) pChk2 and total Chk2 expression were determined by immunoblotting. β-actin was used as a loading control. One of 3 experiments is shown. (D) Ovar3 cells were infected with lentivirus expressing short hairpin RNAs (shRNAs) targeting *SLC2A1* (GLUT1) or *SLC2A4* (GLUT4). shGFP was used as a control. mRNA expression was determined by RT-qPCR. n=3/group, one of 2 experiments is shown. Data represent mean $\pm$ SD. *p<0.01 vs. control; one-way ANOVA. (E) Same as (D), but cells were treated with KU60019 for 24h, and glucose uptake using the fluorescent glucose analog 2NBDG was determined by flow cytometry. MFI=median fluorescence intensity. n=3/group, one of 3 experiments is shown. Data represent mean $\pm$ SD. *p<0.0001 vs. KU60019; ns=not significant; one-way ANOVA with Tukey's multiple comparisons. (F) Ovar8 cells were infected with lentivirus expressing short hairpin RNAs (shRNAs) targeting *SLC1A5*. shGFP was used as a control. mRNA expression was determined by RT-qPCR. n=3/group, one of 2 experiments is shown. Data represent mean $\pm$ SD. *p<0.0001 vs. control; one-way ANOVA. (G) Same as (F), but cells were treated with KU60019 for 24h, and glutamine consumption was determined. n=3/group, one of 3 experiments is shown. Data represent mean $\pm$ SD. *p<0.005 vs. KU60019; ns=not significant; one-way ANOVA with Tukey's multiple comparisons.

has never been directly tested. To answer this question, we used 3 ovarian cancer cell lines with functional/wildtype ATM and differential status of p53 and c-MYC (Ovcar8: mutant p53, c-MYC amplification; Ovcar3: mutant p53; Ovcar10: het mutant p53) and tested glucose and glutamine uptake upon inhibition of ATM. We found that pharmacological inhibition of ATM kinase activity using the small molecule inhibitor KU60019 increased glucose and glutamine consumption in all three cell lines (**Fig. 1A and Fig. S1A-C**). Consistently, a second ATM inhibitor AZD0156 also increased glucose and glutamine consumption (**Fig. 1B**). Suppression of ATM kinase activity was confirmed by decreased Chk2 phosphorylation (pChk2) (**Fig. 1C and Fig. S1D**). Interestingly, knockdown of the glucose transporters SLC2A1/GLUT1 and SLC2A4/GLUT4 or the glutamine transporter SLC1A5/ACST2 did not respectively alter the glucose or glutamine uptake induced by ATM inhibition (**Fig. 1D-G**), suggesting another route of metabolite uptake in these cells. Active ATM is known to signal through AKT to promote GLUT4 translocation (Halaby et al., 2008). Since we are inhibiting ATM, GLUT4 is not likely induced or translocated in our model. Together, these data suggest that inhibition of ATM increases uptake and consumption of multiple nutrients in a transporter-independent manner.

Inhibition of ATM kinase activity induces macropinocytosis through activation of AMPK

Previous studies have shown that macropinocytosis is one mechanism whereby cancer cells scavenge nutrients from the microenvironment to support survival and proliferation (Commisso et al., 2013; Davidson et al., 2017; Hodakoski et al., 2019; Kamphorst et al., 2015; Kim et al., 2018; Lee et al., 2019; Palm, 2019; Palm et al., 2017; Recouvreux and Commisso, 2017; Redelman-Sidi et al., 2018; Yao et al., 2019; Zhang and Commisso, 2019). Due to its large size, fluorescently-labeled dextran is used as a surrogate for macropinocytosis as it cannot be taken up through other endocytic pathways (Commisso et al., 2014; Ivanov, 2008). Excitingly, suppression of ATM using multiple inhibitors or shRNA-mediated knockdown increased uptake of fluorescently-labeled dextran in multiple cell lines, indicating increased macropinocytosis in these cells (**Fig. 2A-D and Fig. S2A-C**). Inhibition of ATM kinase activity was confirmed by decreased pChk2 (**Fig. S2D**). Ataxia telangiectasia and Rad3-related protein (ATR) is

another kinase that is associated with the DNA damage response and has similar and often overlapping roles with ATM (Shiloh, 2003). Inhibition or knockdown of ATR, which decreases pChk1, did not increase dextran uptake, suggesting this is specific for ATM (**Fig. S2E-H**). This is consistent with our previous report demonstrating that knockdown of ATM, but not ATR, rescues proliferation defects due to metabolic deficiencies (Aird et al., 2015). To further confirm the increase in dextran uptake is due to macropinocytosis, we treated cells with EIPA, a Na⁺/H⁺ exchanger that is the gold standard inhibitor of macropinocytosis (Commisso et al., 2014; Ivanov, 2008). EIPA suppressed the increased dextran uptake

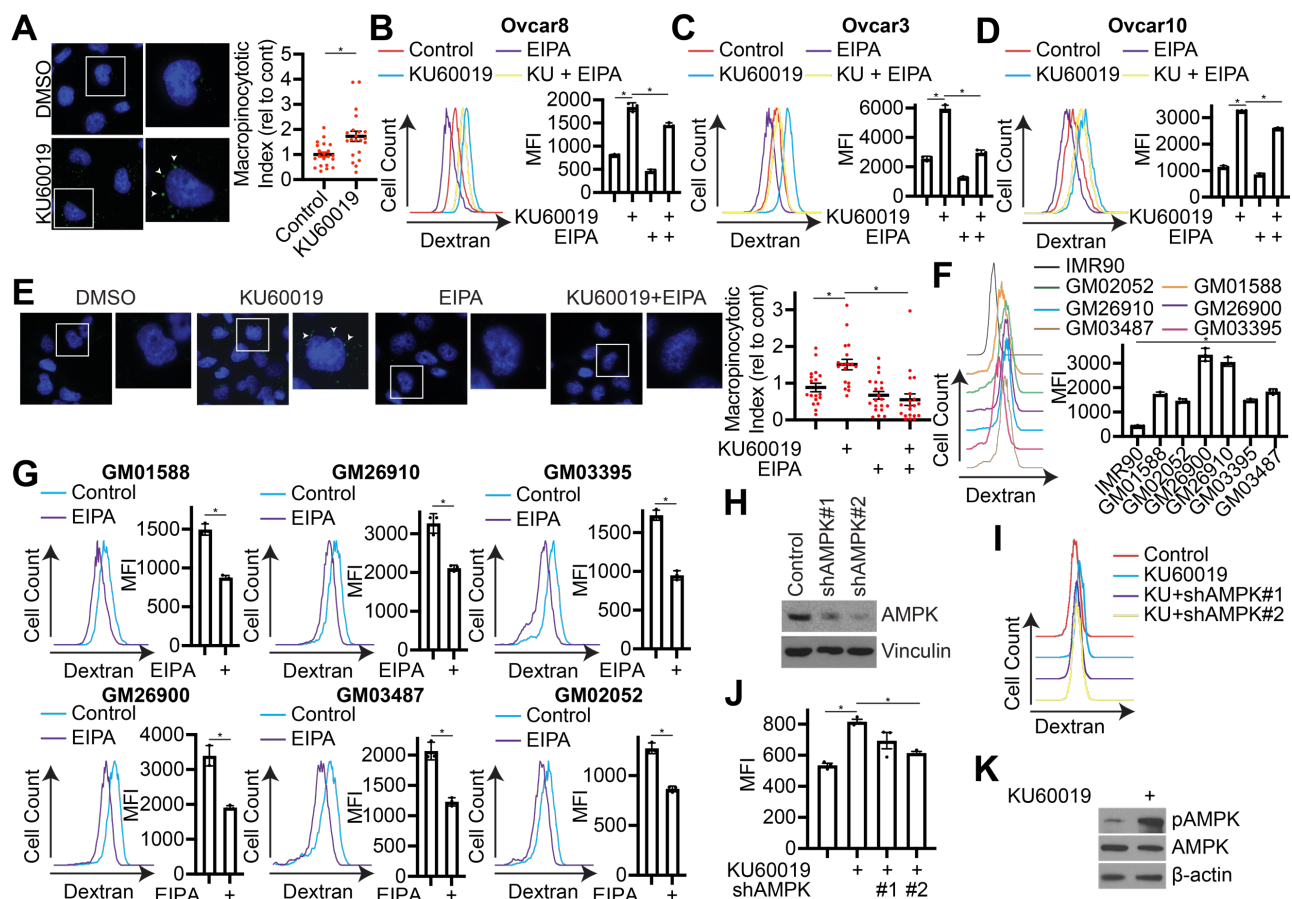


Figure 2. Inhibition of ATM kinase activity induces macropinocytosis through activation of AMPK.

(A) Ovar8 cells were treated with the ATM inhibitor KU60019 for 2h, and dextran uptake was determined by immunofluorescence. Arrowheads point to dextran-FITC. N>20/group, one of 3 experiments is shown. Data represent mean $\pm$ SD. * p <0.005; Student's t-test. (B-E) Ovar8 (B, E), Ovar3 (C), and Ovar10 (D) cells were treated with the ATM inhibitor KU60019 alone or in combination with the macropinocytosis inhibitor EIPA for 2h, and dextran uptake was determined by flow cytometry. MFI=median fluorescence intensity. n=3/group, one of at least 3 experiments is shown. Data represent mean $\pm$ SD. * p <0.0001 vs. KU60019; one-way ANOVA with Tukey's multiple comparisons. (E) Dextran uptake was determined by immunofluorescence. Arrowheads point to dextran-FITC. N>18/group, one of 2 experiments is shown. Data represent mean $\pm$ SD. * p <0.005 vs. KU60019; one-way ANOVA with Tukey's multiple comparisons. (F) Dextran uptake in IMR90 (ATM WT) and A-T fibroblasts was determined by flow cytometry. MFI=median fluorescence intensity. n=3/group, one of 3 experiments is shown. Data represent mean $\pm$ SD. * p <0.0001 vs. control; one-way ANOVA. (G) Dextran uptake in A-T patient fibroblasts treated with the macropinocytosis inhibitor EIPA for 2h was determined by flow cytometry. MFI=median fluorescence intensity. n=3/group, one of 3 experiments is shown. Data represent mean $\pm$ SD. * p <0.005 (H-J) Ovar8 cells were infected with lentivirus expressing short hairpin RNAs (shRNAs) targeting AMPK. shGFP was used as a control. (H) Immunoblot analysis of AMPK. Vinculin was used as a loading control. One of 2 experiments is shown. (I) Dextran uptake was determined by flow cytometry. (J) Quantification of (I). MFI=median fluorescence intensity. n=3/group, one of 2 experiments is shown. Data represent mean $\pm$ SD. * p <0.05 vs. KU60019; one-way ANOVA with Tukey's multiple comparisons. (K) Ovar8 cells were treated with the ATM inhibitor KU60019 for 2h. Immunoblot analysis of pAMPK and total AMPK. β -actin was used as a loading control. One of 5 experiments is shown.

in cells treated with the ATM inhibitor (Fig. 2B-E). EIPA in combination with the ATM inhibitor did not increase pChk2 (Fig. S2I), suggesting that increased ATM activity is not the cause of decreased macropinocytosis. EIPA alone decreased pChk2, although to varying degrees in the cell lines tested. This indicates that the signaling downstream of ATM to regulate macropinocytosis is likely not through

Chk2, and pChk2 is only a surrogate for confirming activity of the ATM inhibitors. To validate our findings in a different model, we used fibroblasts from patients with ATM mutations who have a disorder termed ataxia-telangiectasia (A-T), who are predisposed to cancer and also exhibit metabolic dysfunction (McKinnon, 2012). A-T patient fibroblasts with various ATM mutations displayed increased dextran uptake compared to ATM wildtype IMR90 fibroblasts (**Fig. 2F**). Treatment of A-T patient fibroblasts or ATM inhibited IMR90 cells with EIPA decreased dextran uptake (**Fig. 2G and S2J-K**). Together, these data indicate that suppression of ATM kinase activity induces macropinocytosis.

Next we aimed to determine the mechanism of macropinocytosis induction downstream of ATM inhibition. Towards this goal, we used pharmacological or genetic inhibition of PI3K/AKT, WNT, and AMPK, three known inducers of macropinocytosis (Commisso et al., 2013; Kim et al., 2018; Redelman-Sidi et al., 2018; Tejeda-Munoz et al.). While inhibition of PI3K, AKT, or WNT had no effect (**Fig. S2L-M**), suppression of AMPK using shRNA decreased dextran uptake in ATM inhibited cells in a dose-dependent fashion (**Fig. 2H-J**). Consistently, we observed an increase in phosphorylation of AMPK upon inhibition of ATM in multiple cell lines (**Fig. 2K and S2N**). AMPK is phosphorylated by multiple kinases in response to nutrient stress and other stimuli (Herzig and Shaw, 2018). Previous reports have demonstrated that AMPK is both directly phosphorylated by ATM or its phosphorylation by LKB1 occurs in an ATM-dependent manner (Alexander et al., 2010; Luo et al., 2013; Sanli et al., 2010; Sanli et al., 2014; Sun et al., 2007; Suzuki et al., 2004; Tripathi et al., 2013), suggesting that direct or LKB1-mediated activation of AMPK is not occurring in ATM inhibited cells. Loss of ATM induces mitochondrial reactive oxygen species (ROS) (Valentin-Vega et al., 2012), and AMPK may be activated by mitochondrial ROS (Rabinovitch et al., 2017). This suggests that inhibition of ATM may increase macropinocytosis via mitochondrial ROS-mediated AMPK phosphorylation, although further experiments are needed to directly test this mechanism. Finally, we aimed to determine whether ATM inhibition-mediated macropinocytosis occurs through actin remodeling. AMPK can affect actin remodeling and macropinocytosis through the Pak1 pathway (Kim et al., 2018). We found that

knockdown of Pak1 decreased macropinocytosis due to ATM inhibition (**Fig. S2O-P**), indicating this is the actin remodeling pathway that is likely induced downstream of ATM inhibition and AMPK phosphorylation. Together, these data suggest that AMPK activates macropinocytosis downstream of ATM inhibition in a Pak1-dependent manner.

Suppression of macropinocytosis limits survival only in ATM inhibited cells

We next aimed to determine whether macropinocytosis is required for survival of ATM inhibited cells. We treated cells with the ATM inhibitor KU60019 and inhibited macropinocytosis using EIPA. The combination significantly decreased proliferation and increased apoptosis compared to single treatment controls in multiple cell lines (**Fig. 3A-B and S3A-B**). Similar results were observed *in vivo* where EIPA only affected ovarian tumor growth in combination with the ATM inhibitor KU60019 (**Fig. 3C-E**). *In vivo* the combination also induced apoptosis as we observed increased cleaved caspase 3 staining by IHC in the tumors (**Fig. 3F-G**). We also validated that KU60019 inhibited ATM by blotting for pChk2 in the tumors (**Fig. 3H and Fig. S3C**). Similar to our *in vitro* studies, we noted that pChk2 was decreased in the EIPA alone treated mice. To confirm inhibition of ATM induces macropinocytosis *in vivo*, mice were injected with fluorescently-labeled dextran 30min prior to harvesting the tumor. Consistent with our *in vitro* data (**Fig. 2**), KU60019 induced macropinocytosis, which was suppressed by EIPA (**Fig. 3I-J**). We

observed co-localization of dextran with the lysosomal marker LAMP2, suggesting that the nutrients taken up by macropinocytosis are degraded within the lysosome. Together, these data demonstrate that inhibition of macropinocytosis limits proliferation/survival only of cells where ATM is inhibited and identify a metabolic vulnerability of tumors with low or mutated ATM.

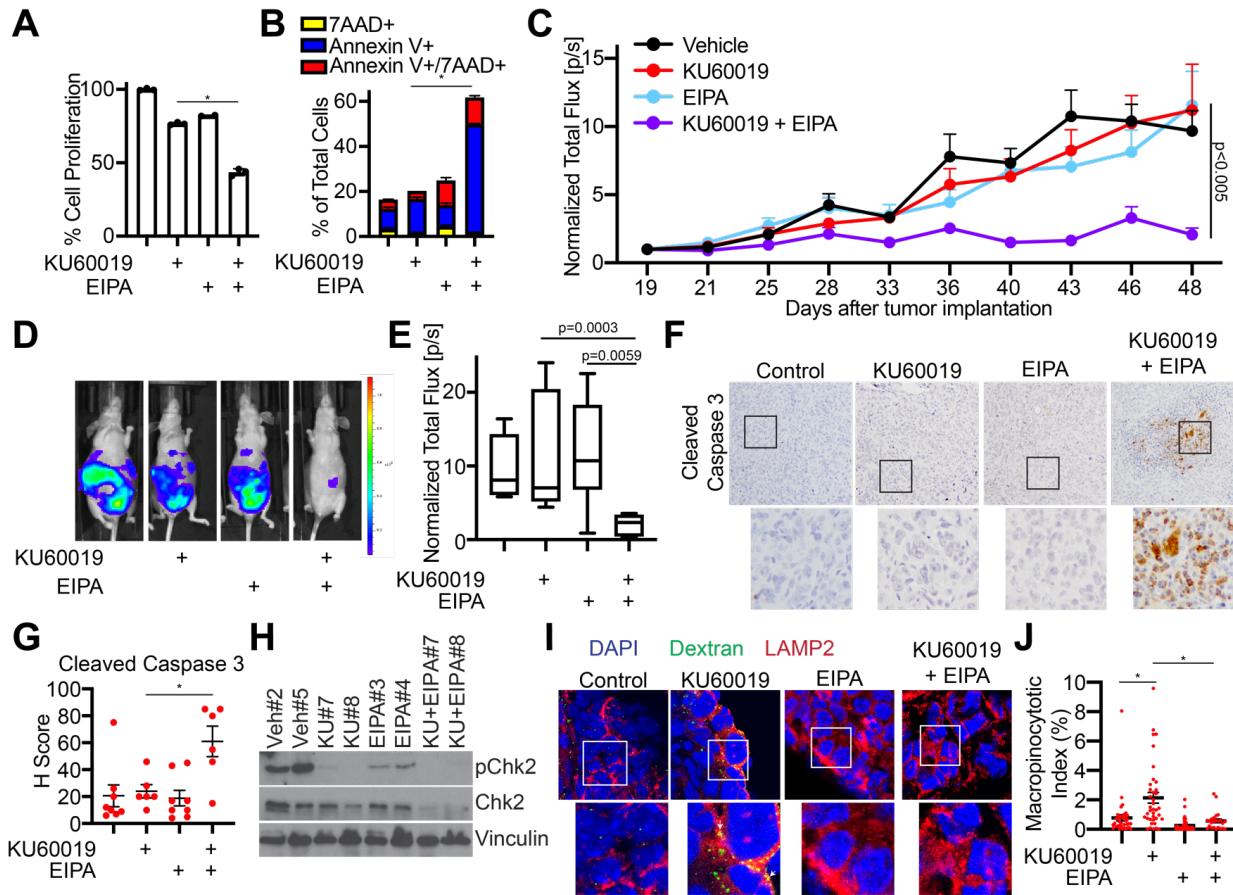


Figure 3. Inhibition of macropinocytosis is a vulnerability of ATM inhibited cells both *in vitro* and *in vivo*.

(A-B) Ovar8 cells were treated with the ATM inhibitor KU60019 or the macropinocytosis inhibitor EIPA alone or in combination for 4 days. (A) Proliferation was assessed by colony formation. n=3/group, one of 3 experiments is shown. Data represent mean $\pm$ SD. *p<0.001; one-way ANOVA. (B) Apoptosis was assessed by Annexin V/7AAD staining. n=3/group, one of 3 experiments is shown. Data represent mean $\pm$ SD. *p<0.001; one-way ANOVA. (C) Ovar8 cells expressing luciferase were injected intraperitoneally into nude mice (6-8 mice/group). At day 19, mice were randomized and thereafter treated daily with the ATM inhibitor KU60019 or the macropinocytosis inhibitor EIPA alone and in combination. Shown is the tumor growth curve over 30 days normalized to Day 19 tumors. Longitudinal analysis: Combination vs. Vehicle, p=0.00141; Combination vs. KU60019, p=0.002334; Combination vs. EIPA, p=0.003603; Linear mixed model fit REML. (D) Representative luciferase images of mice from each group at Day 48. (E) Quantification of (D) normalized to Day 19 tumors. Cross-sectional analysis: ANOVA with Wilcoxon rank sum test. (F) Representative cleaved caspase 3 immunohistochemistry in the indicated tumors. (G) H-Score of cleaved caspase 3 IHC. Data represent mean $\pm$ SEM. *p<0.01; one-way ANOVA. (H) Representative pChk2 and total Chk2 immunoblot analysis of the indicated tumors. Vinculin was used as a loading control. (I) Representative confocal images of dextran-FITC and the lysosomal marker LAMP2 in the indicated tumors. Arrows point to dextran-FITC puncta co-localized with LAMP2. (J) Macropinocytotic index of dextran-FITC uptake in the tumors was assessed. Data represent mean $\pm$ SEM. *p<0.0005 vs. KU60019; one-way ANOVA with Tukey's multiple comparisons.

Macropinocytosis induced by ATM inhibition increases BCAA uptake to promote proliferation and survival

Finally, we aimed to determine the metabolic consequences of increased macropinocytosis due to inhibition of ATM. Previous studies have demonstrated that diverse nutrients including proteins, lipids, glucose, and others are taken up by macropinocytosis to promote cancer cell survival under nutrient

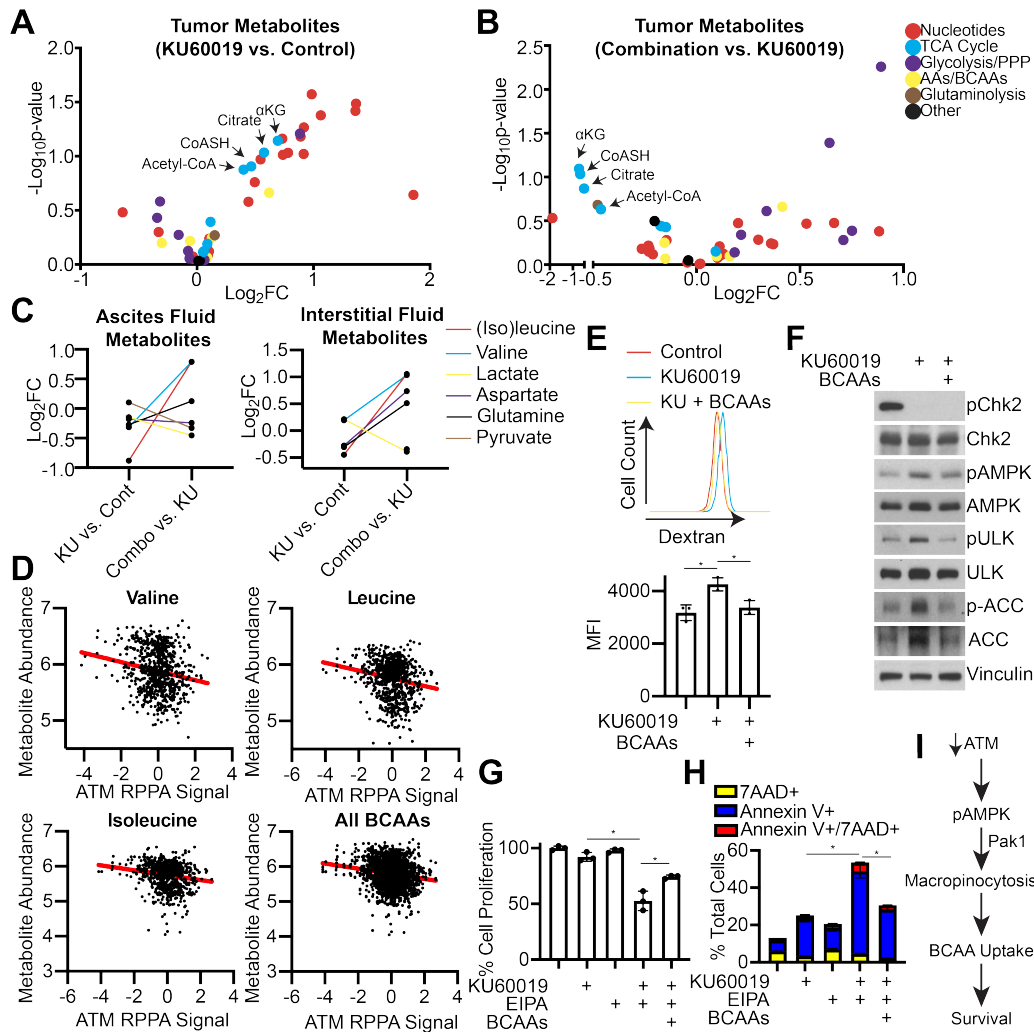


Figure 4. ATM inhibitor-induced macropinocytosis leads to increased branched chain amino acid uptake to promote cell survival.

(A) Volcano plot of fold change (Log_2FC) of metabolites in ATM inhibitor KU60019-treated vs. control tumors. (B) Volcano plot of fold change (Log_2FC) of metabolites in combination-treated (KU60019+EIPA) vs. KU60019-treated tumors. (C) Fold change (Log_2FC) of metabolites in the ascites fluid and interstitial fluid in the indicated treatment groups. (D) BCAA metabolite abundance vs. ATM protein expression from DepMap.org. (E) Ovcar8 cells were treated for 2h with the ATM inhibitor KU60019 alone or in combination with BCAAs (mixture of valine, isoleucine, leucine). Dextran uptake was determined by flow cytometry. MFI=median fluorescence intensity. $n=3/\text{group}$, one of 3 experiments is shown. Data represent mean $\pm$ SD. $*p<0.001$ vs. KU60019; one-way ANOVA with Tukey's multiple comparisons. (F) Ovcar8 cells were treated for 2h with the ATM inhibitor KU60019 and a combination of BCAAs (mixture of valine, isoleucine, leucine), and expression of the indicated proteins was determined. Vinculin was used as a loading control. (G) Ovcar8 cells were treated with the ATM inhibitor KU60019 and a combination of EIPA or BCAAs (mixture of valine, isoleucine, leucine) for 4 days. Cell proliferation was assessed by colony formation. $n=3/\text{group}$, one of 3 experiments is shown. Data represent mean $\pm$ SD. $*p<0.05$; one-way ANOVA with Tukey's multiple comparisons.

stress (Commisso et al., 2013; Hodakoski et al., 2019; Kamphorst et al., 2015; Kim et al., 2018; Lee et al.; Palm, 2019; Palm et al., 2017; Recouvreux and Commisso, 2017; Redelman-Sidi et al., 2018; Tejeda-Munoz et al.; Zhang and Commisso, 2019).

To determine the metabolites that may be important for tumors in the ATM inhibitor treated mice, we performed metabolomics on the tumor tissue, interstitial fluid, and the ascites fluid, which commonly occurs in ovarian cancer models. Interstitial fluid and ascites fluid can be used to assess microenvironment metabolite abundance. We observed an overall increase in multiple nucleotides within the tumors treated with the ATM inhibitor (**Fig. 4A and Table S1**), which is consistent with our previous report (Aird et al., 2015). We also observed an increase in metabolites related to the TCA cycle. In the combination group, TCA cycle metabolites and nucleotides were decreased, although nucleotides to a much lesser extent (**Fig. 4B**). This suggests that while ATM inhibition increases nucleotide metabolism, the TCA cycle and metabolites feeding into the TCA cycle are likely more important for survival and proliferation of ATM inhibited tumors. To investigate metabolite uptake from the microenvironment, we assessed metabolite abundance in the ascites and interstitial fluids. Valine and (iso)leucine, both branched chain amino acids (BCAAs), were decreased upon treatment with the ATM inhibitor and increased in the combination group (**Fig. 4C and Table S1**), suggesting that these carbon sources are consumed by ATM inhibited tumors to maintain proliferation and survival. Interestingly, BCAAs were not increased in the tumors, potentially because they have been catabolized for downstream pathways, such as the TCA cycle, or for protein synthesis. Finally, we assessed AMPK activation in the tumors. pAMPK was increased slightly in the ATM inhibitor only-treated group and further increased in the combination treated tumors (**Fig. S4A**). This suggests that the combination treatment is effective at least in part due to depletion of nutrients, leading to sustained AMPK activation.

Next, we aimed to determine whether uptake of BCAAs in ATM inhibitor treated cells are critical for macropinocytosis and cancer cell survival *in vitro*. Interestingly, using publicly- available datasets from cell lines (depmap.org), we found protein expression of ATM or pChk2 negatively correlated with

abundance of BCAAs and the TCA cycle metabolite citrate identified in our *in vivo* studies (**Fig. 4D and S4B-C**), suggesting this is a universal phenomenon of ATM low or inhibited cells. Notably, these studies were not done under nutrient-limiting conditions (Li et al., 2019), suggesting that there is basal macropinocytosis, which is likely further amplified during nutrient stress. If macropinocytosis increases BCAA uptake, we reasoned that supplementing cells with exogenous BCAAs would abrogate macropinocytosis in ATM inhibited cells. Indeed, supplementation of ATM inhibited cells with exogenous BCAAs suppressed macropinocytosis (**Fig. 4E**). Supplementation with BCAAs also abrogated the increase in AMPK phosphorylation and downstream signaling through ACC and ULK (**Fig. 4F**), suggesting that BCAA depletion is in part the cause of activated AMPK signaling. Consistent with the idea that BCAA uptake in ATM inhibited cells promotes proliferation and survival, supplementation of cells with BCAAs partially rescued the inhibition of proliferation and apoptosis observed in ATM inhibitor plus EIPA-treated cells (**Fig. 4G-H**). Together, these data suggest that inhibition of ATM increases macropinocytosis to promote cell survival at least in part through uptake and consumption of BCAAs from the microenvironment. The downstream mechanism of how BCAAs are affecting cell survival in ATM inhibited cells remains unknown. The irreversible step of BCAA metabolism via the branched chain alpha-keto acid dehydrogenase (BCKDH) complex generates mitochondrial NADH in addition to providing acetyl- and succinyl-CoA to the TCA cycle, and BCAAs have been shown to inhibit ROS (Sivanand and Vander Heiden, 2020). Since ATM inhibition increases ROS (Valentin-Vega et al., 2012), which in turn can promote AMPK activation (Rabinovitch et al., 2017), it is possible that BCAAs are consumed to promote redox homeostasis. Future studies are warranted to dissect this mechanism.

In summary, we identified a new mechanism of macropinocytosis through inhibition of ATM, which increases uptake of BCAAs to promote cancer cell proliferation and survival under nutrient stress (**Fig. 4I**). This study provides a novel mechanism of metabolism controlled by ATM inhibition and suggests that macropinocytosis or BCAA metabolism represents a novel metabolic vulnerability that can be

targeted in combination with ATM inhibitors, which have recently entered clinical trials (clinicaltrials.gov). Moreover, this pathway may also be a vulnerability of ATM mutated or low expressing tumors. Since A-T patients with ATM mutations also have metabolic disorders, our data may have implications beyond cancer for controlling metabolism in the context of low or mutated ATM.

Chen et al.- Figure S1

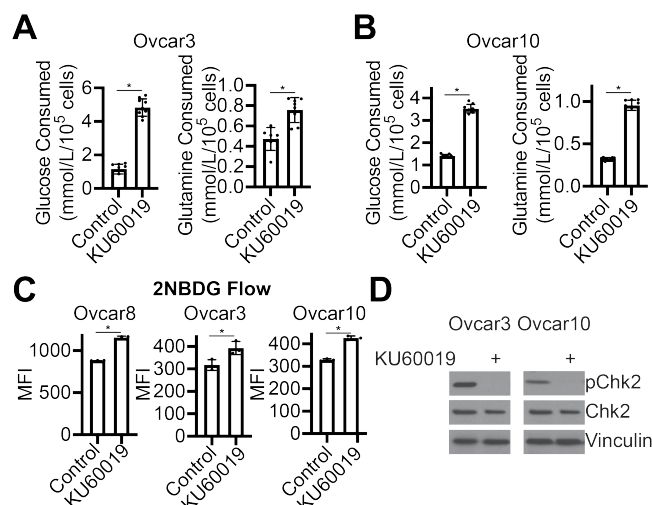


Figure S1. Inhibition of ATM increases glucose and glutamine consumption in multiple cell lines; related to Figure 1.

(A-B) Ovar3 (A) and Ovar10 (B) cells were treated with the ATM inhibitor KU60019 for 24h. Glucose and glutamine consumption was determined. n=9/group, one of 2 experiments is shown. Data represent mean $\pm$ SD. *p<0.005; Student's t-test. **(C)** Glucose uptake in the indicated cells by 2NBDG was determined by flow cytometry after treatment with the ATM inhibitor KU60019 for 24h. MFI=median fluorescence intensity. n=3/group, one of at least 2 experiments is shown. Data represent mean $\pm$ SD. *p<0.05; Student's t-test. **(D)** The indicated cells were treated with KU60019 for 24h, and pChk2 and total Chk2 expression were determined by immunoblotting. Vinculin was used as a loading control. One of 3 experiments is shown

Chen et al.- Figure S2

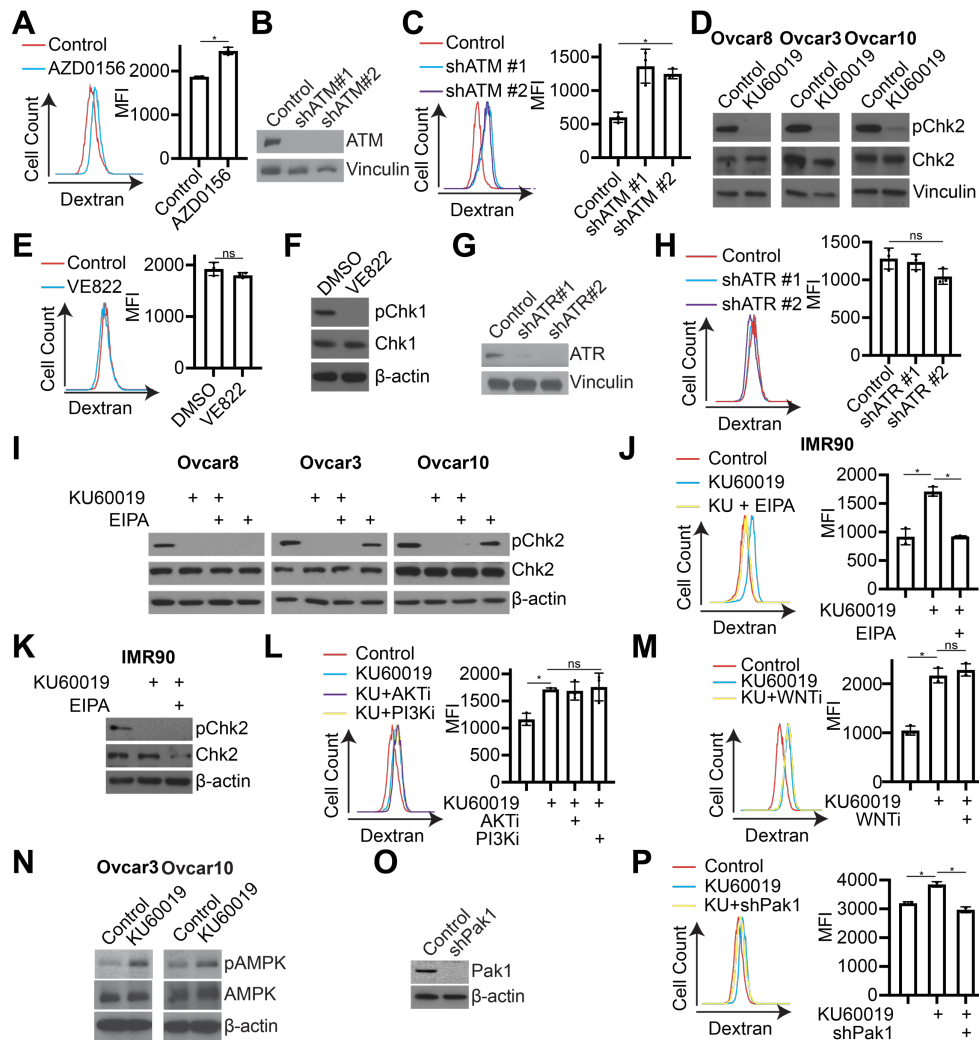


Figure S2. Inhibition of ATM, but not ATR, induces macropinocytosis and is independent of PI3K and WNT; related to Figure 2.

(A) Ovar8 cells were treated with the ATM inhibitor AZD0156 for 2h, and dextran uptake was determined by flow cytometry. MFI=median fluorescence intensity. n=3/group, one of 3 experiments is shown. Data represent mean $\pm$ SD. *p<0.005; Student's t-test. (B-C) Ovar8 cells were infected with lentivirus expressing short hairpin RNAs (shRNAs) targeting ATM. shGFP was used as a control. (B) Immunoblot analysis of ATM. Vinculin was used as a loading control. One of 3 experiments is shown. (C) Dextran uptake was determined by flow cytometry. MFI=median fluorescence intensity. n=3/group, one of 3 experiments is shown. Data represent mean $\pm$ SD. *p<0.0001 vs. control; one-way ANOVA. (D) Ovar8, Ovar3, and Ovar10 cells were treated with the ATM inhibitor KU60019 for 2h. Immunoblot analysis of p-Chk2 and total Chk2. Vinculin was used as a loading control. One of 3 experiments is shown. (E-F) Ovar8 cells were treated with the ATR inhibitor VE822. (E) Dextran uptake was determined by flow cytometry. MFI=median fluorescence intensity. n=3/group, one of 3 experiments is shown. Data represent mean $\pm$ SD. ns=not significant; Student's t-test. (F) Immunoblot analysis of p-Chk1 and total Chk1. β-actin was used as a loading control. One of 3 experiments is shown. (G-H) Ovar8 cells were infected with lentivirus expressing short hairpin RNAs (shRNAs) targeting ATR. shGFP was used as a control. (G) Immunoblot analysis of ATR. Vinculin was used as a loading control. One of 3 experiments is shown. (H) Dextran uptake was determined by flow cytometry. MFI=median fluorescence intensity. n=3/group, one of 3 experiments is shown. Data represent mean $\pm$ SD. ns=not significant; one-way ANOVA. (I) Ovar8, Ovar3, and Ovar10 cells were treated with the PI3K inhibitor KU60019 and the WNT inhibitor EIPA. Immunoblot analysis of p-Chk2 and total Chk2. Vinculin was used as a loading control. One of 3 experiments is shown. (J) IMR90 cells were treated with the PI3K inhibitor KU60019 and the WNT inhibitor EIPA. Dextran uptake was determined by flow cytometry. MFI=median fluorescence intensity. n=3/group, one of 3 experiments is shown. Data represent mean $\pm$ SD. *p<0.005; Student's t-test. (K) IMR90 cells were treated with the PI3K inhibitor KU60019 and the WNT inhibitor EIPA. Immunoblot analysis of p-Chk2 and total Chk2. Vinculin was used as a loading control. One of 3 experiments is shown. (L) IMR90 cells were treated with the PI3K inhibitor KU60019 and the WNT inhibitor EIPA. Dextran uptake was determined by flow cytometry. MFI=median fluorescence intensity. n=3/group, one of 3 experiments is shown. Data represent mean $\pm$ SD. ns=not significant; one-way ANOVA. (M) IMR90 cells were treated with the PI3K inhibitor KU60019 and the WNT inhibitor EIPA. Dextran uptake was determined by flow cytometry. MFI=median fluorescence intensity. n=3/group, one of 3 experiments is shown. Data represent mean $\pm$ SD. *p<0.005; Student's t-test. (N) Ovar3 and Ovar10 cells were treated with the PI3K inhibitor KU60019. Immunoblot analysis of pAMPK and total AMPK. Vinculin was used as a loading control. One of 3 experiments is shown. (O) Ovar3 and Ovar10 cells were infected with lentivirus expressing short hairpin RNAs (shRNAs) targeting Pak1. shGFP was used as a control. Immunoblot analysis of Pak1. Vinculin was used as a loading control. One of 3 experiments is shown. (P) Ovar3 and Ovar10 cells were infected with lentivirus expressing short hairpin RNAs (shRNAs) targeting Pak1. shGFP was used as a control. Dextran uptake was determined by flow cytometry. MFI=median fluorescence intensity. n=3/group, one of 3 experiments is shown. Data represent mean $\pm$ SD. *p<0.005; Student's t-test.

(I) Ovar8, Ovar3, and Ovar10 cells were treated with the ATM inhibitor KU60019 alone or in combination with EIPA for 2h. Immunoblot analysis of pChk2 and total Chk2. β -actin was used as a loading control. One of 3 experiments is shown. **(J-K)** IMR90 fibroblasts (ATM WT) were treated with the ATM inhibitor KU60019 or in combination with EIPA for 2h. **(J)** Dextran uptake was determined by flow cytometry. MFI=median fluorescence intensity. n=3/group, one of 3 experiments is shown. Data represent mean $\pm$ SD. *p<0.0001; one-way ANOVA with Tukey's multiple comparisons. **(K)** Immunoblot analysis of pChk2 and total Chk2. β -actin was used as a loading control. One of 3 experiments is shown. **(L)** Ovar8 cells were treated with the ATM inhibitor KU60019 alone or in combination with the AKT inhibitor AZD5363 or the PI3K inhibitor buparlisib for 2h. Dextran uptake was determined by flow cytometry. MFI=median fluorescence intensity. n=3/group, one of 2 experiments is shown. Data represent mean $\pm$ SD. *p<0.05; one-way ANOVA with Tukey's multiple comparisons. **(M)** Ovar8 cells were treated with the ATM inhibitor KU60019 alone or in combination with the WNT inhibitor ICG-001 for 2h. Dextran uptake was determined by flow cytometry. MFI = median fluorescence intensity. n=3/group, one of 2 experiments is shown. Data represent mean $\pm$ SD. *p<0.0001, ns=not significant; one-way ANOVA with Tukey's multiple comparisons. **(N)** Ovar3 or Ovar10 cells were treated with the ATM inhibitor KU60019 for 2h. Immunoblot analysis of pAMPK and AMPK. β -actin was used as a loading control. One of 3 experiments is shown. **(O-P)** Ovar8 cells were infected with lentivirus expressing a short hairpin RNA (shRNA) Pak1. shGFP was used as a control. **(O)** Immunoblot analysis of Pak1. β -actin was used as a loading control. One of 2 experiments is shown. **(P)** Dextran uptake was determined by flow cytometry. MFI=median fluorescence intensity. n=2-3/group, one of 2 experiments is shown. Data represent mean $\pm$ SD. *p<0.01; one-way ANOVA with Tukey's multiple comparisons.

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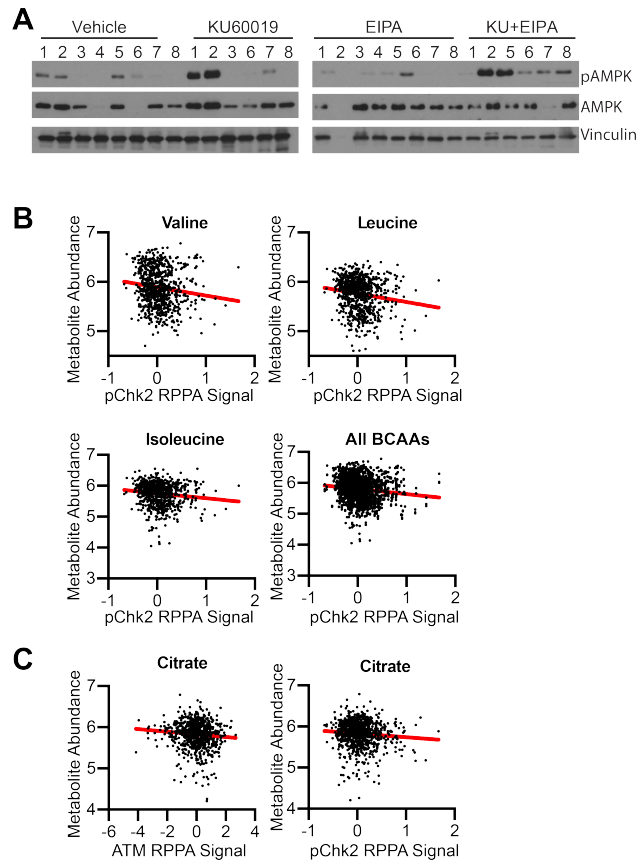


Figure S4. ATM and pChk2 protein expression negatively correlate with branched chain amino acids and the TCA cycle metabolite citrate. Related to Figure 4.

(A) AMPK and total AMPK immunoblot analysis in all of the tumors. Vinculin was used as a loading control. **(B)** BCAAs metabolite abundance vs. pChk2 protein expression from DepMap.org. **(C)** Citrate metabolite abundance vs. ATM or pChk2 protein expression from DepMap.org.

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Author Contributions

Conceptualization, C.W.C. and K.M.A.; Methodology, E.L.V., E.V.K., N.W.S.; Investigation, C.W.C, R.B., E.S.D., K.E.L., E.L.V., E.V.K, N.W.S., K.M.A.; Writing, C.W.C., N.W.S, and K.M.A.; Visualization, C.W.C, N.S.W., K.M.A.; Supervision, N.W.S., K.M.A.; Funding Acquisition, N.W.S., and K.M.A.

Declaration of Interests

The authors declare no competing interests.

Materials and Methods

Cell lines and culture conditions

Ovcar8, Ovcar3, and Ovcar10 cells were cultured in RPMI (Corning, Cat# 10-040-CV) with 5% FBS. 293FT cells were cultured in DMEM (Corning, Cat# 10-013-CV) with 5% FBS. Normal diploid IMR90 (purchased from ATCC) and A-T human fibroblasts (GM01588, GM02052, GM03995, GM04387, GM22690, and GM22691 purchased from Coriell Institute) were cultured in low oxygen (2% O₂) in DMEM (Corning, Cat# 10-017-CV) with 15% FBS supplemented with L-glutamine, non-essential amino acids, sodium pyruvate, and sodium bicarbonate. Experiments were performed on IMR90 between population doubling #25-35 and A-T fibroblasts below passage 10. All cell lines were cultured in MycoZap and were routinely tested for mycoplasma using a highly sensitive PCR-based method (Uphoff and Drexler, 2005). Tumor cell lines were authenticated using STR Profiling using Genetica DNA Laboratories.

Glucose uptake by flow cytometry

Cells (10⁵/well in 12-well plates) were incubated with 5μM of the fluorescent glucose analog 2NBDG (N13195, Thermo Fisher Scientific) in Opti-MEM™ I Reduced Serum Medium (31985088, Thermo Fisher Scientific) for 2h. Cells were run on a 10-color FACSCanto flow cytometer (BD Biosciences). Data were analyzed with FlowJo Software.

YSI Bioanalyzer Analysis

Glucose and glutamine consumption were measured using a YSI 2950 Bioanalyzer (Yellow Springs, OH). Briefly, the same number of cells (10⁵/well in 12-well plates) were seeded in complete media. One day later, the media was changed to RPMI+0.1% FBS and cells were incubated in 10μM KU60019 or 1μM AZD0156 for 24h. Media was collected, and the number of cells per well was counted to normalize for cell number.

Western blotting

Cell lysates were collected in 1X sample buffer (2% SDS, 10% glycerol, 0.01% bromophenol blue, 62.5mM Tris, pH 6.8, 0.1M DTT) and boiled (10 min at 95°C). Protein concentration was determined using the Bradford assay. Proteins were resolved using SDS-PAGE gels and transferred to nitrocellulose membranes (Fisher Scientific) (110mA for 2 h or overnight at 4°C). Membranes were blocked with 5% nonfat milk or 4% BSA in TBS containing 0.1% Tween-20 (TBS-T) for 1 h at room temperature. Membranes were incubated overnight at 4°C in primary antibodies (**Table S2**) in 4% BSA/TBS + 0.025% sodium azide. Membranes were washed 4 times in TBS-T for 5 min at room temperature after which they were incubated with HRP-conjugated secondary antibodies (Cell Signaling, Danvers, MA) for 1 h at room temperature. After washing 4 times in TBS-T for 5 min at room temperature, proteins were visualized on film after incubation with SuperSignal West Pico PLUS Chemiluminescent Substrate (ThermoFisher, Waltham, MA).

Lentiviral packaging and infection

Lentiviral constructs were transfected into 293FT cells using polyethylenimine (PEI). Lentivirus was packaged using the ViraPower Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Cells were infected overnight with lentivirus targeting the gene of interest or control shGFP and selected with 1µg/ml puromycin for 3 days. The following shRNAs were used: shATM#1: TRCN0000038658; shATM#2: TRCN0000010299; shATR#1: TCRN0000039615; shATR#2: TCRN0000039616; shSLC2A1#1: TRCN0000043585; shSLC2A1#2: TRCN0000043584; shSLC2A4#1: TRCN0000043628; shSLC2A4#2: TRCN0000043629; shSLC1A5#1: TRCN0000043118; shSLC1A5#2: TRCN0000043119; shPAK1#1: TRCN0000002226; shAMPK#1: TRCN0000000857; shAMPK#2: TRCN0000000859.

RT-qPCR

Total RNA was extracted from cells with Trizol, DNase treated, cleaned, and concentrated using Zymo columns (Zymo Research, Cat# R1013) following the manufacturer's instructions. Optical density values of RNA were measured using NanoDrop One (Thermo Scientific) to confirm an A260 and A280 ratio above 1.9. Relative expression of target genes (**Table S3**) were analyzed using the QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific) with clear 96 well plates (Greiner Bio-One). Primers were designed using the Integrated DNA Technologies (IDT) tool (<http://eu.idtdna.com/scitools/Applications/RealTimePCR/>). A total of 25ng of RNA was used for One-Step qPCR (Quanta BioSciences) following the manufacturer's instructions in a final volume of 10 μ l. Conditions for amplification were: 10 min at 48°C, 5 min at 95°C, 40 cycles of 10 s at 95°C and 7 s at the corresponding annealing temperature (**Table S3**). The assay ended with a melting curve program: 15 s at 95°C, 1 min at 70°C, then ramping to 95°C while continuously monitoring fluorescence. Each sample was assessed in triplicate. Relative quantification was determined to multiple reference genes (*B2M*, *MRPL9*, *PSMC4*, and *PUM1*) using the delta-delta Ct method.

Flow cytometry

For Dextran-FITC experiments: cells were incubated with 1mg/mL dextran-FITC (Sigma-Aldrich, Cat # 90718-1G) in base media (Sigma-Aldrich, Cat# D5030) for 2h. Where indicated, cells were incubated with 10 μ M KU60019, 25 μ M EIPA, 4mM BCAAs (mixture of valine, isoleucine, and leucine), 10 μ M AZD5363, 10 μ M Buparib, or 25 μ M ICG001 for 2h. After washing twice with 1X PBS, cells were trypsinized and run on a 10-color FACSCanto flow cytometer (BD Biosciences). For Annexin V/7AAD experiments: Cells (2x10⁴/well in 12-well plates) were treated for 4 days with 2 μ M KU60019, 2 μ M EIPA, and BCAAs (mixture of 4mM valine, isoleucine, leucine) in RPMI + 5% FBS. Cells were stained with 2x10⁶ cells/ml Annexin V (R37176, Thermo Fisher Scientific) and 0.5 μ g/ml 7AAD (13-6993-T200, Tonbo Biosciences) in 2.5 mM Ca²⁺-containing RPMI for 15 minutes at room temperature. Data were analyzed using FlowJo software (Ashland, OR).

Dextran uptake by immunofluorescence

Cells were incubated with 5mg/ml dextran-FITC (Sigma-Aldrich, Cat# 90718-1G) in base media (D5030, Sigma) for 2h. A low pH buffer (0.1 M Sodium acetate + 0.05 NaCl) was used to wash away non-specific binding of dextran to the cell membrane. Cells were fixed in 4% paraformaldehyde (10min) and permeabilized in 0.2% Triton X-100 (5 min). Cells were further incubated with 0.15 μ g/ml DAPI in PBS (1 min), mounted, and sealed. For macropinocytotic index, images were acquired at room temperature using a Nikon Eclipse 90i microscope with a 20x/0.17 objective (Nikon DIC N2 Plan Apo) equipped with a CoolSNAP Photometrics camera. Macropinocytotic index was determined using the method described in (Lee et al., 2019). For the tumor model: 0.2mg/mice Dextran, Oregon Green™ (D7173, Thermo Fisher Scientific) in 200 μ l Evans Blue dye (1% in sterile water; E2129, Sigma) was injected intraperitoneally 30min prior to harvesting tumors. Tumors were frozen at -80°C in OCT (Tissue Tek) and sectioned using a cryostat. Sections were stained with LAMP2 (1:100, Santa Cruz, Cat# sc-18822) in 3% BSA/PBS at room temperature for 1 h. Tissues were washed three times and then incubated in Cy3 anti-mouse secondary antibody (1:5000, Jackson ImmunoResearch Labs, Cat# 715-165-150) in 3% BSA/PBS at room temperature for 1 h. Finally, tissues were incubated with 0.15 μ g/ml DAPI in PBS for 1min, washed three times with PBS, mounted and sealed. Images were acquired at room temperature using a confocal microscope (Leica SP8) with a 64X oil objective. Macropinocytotic index was determined using the method described in (Commisso et al., 2014).

Colony Formation

Cells (10^5 /well in 12-well plates) were treated with the 2 μ M KU60019 and a combination of 2 μ M EIPA or 4mM BCAAs (mixture of valine, isoleucine, and leucine) in 5% FBS RPMI for 4 days. KU60019, EIPA, and BCAAs were supplied daily. Colony formation was visualized by fixing cells in 1% paraformaldehyde (5 min) and staining with 0.05% crystal violet (20 min). Wells were destained for 5 min in 500 mL 10% acetic acid. Absorbance (590nm) was measured using a spectrophotometer (Spectra Max 190). Each sample was assessed in triplicate.

Murine tumor model

Two-month old female nude mice were purchased from Jackson Labs. All mice were maintained in a HEPA-filtered ventilated rack system at the Milton S. Hershey Medical Center animal facility. Mice were housed up to 5 mice per cage and in a 12-hour light/dark cycle. This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All experiments with animals were performed in accordance with institutional guidelines approved by the Institutional Animal Care and Use Committee (IACUC) at the Penn State College of Medicine (PROTO201800050). Ovarcar8 ovarian cancer cells (5×10^6 in 200 μ l PBS) were injected intraperitoneally into mice (8 mice per group based on pilot experiments). Mice were monitored every 3-4 days by non-invasive luciferase imaging by intraperitoneal injection of 150 mg/kg Luciferin (PerkinElmer) and quantification of luciferase activity using Imaging Systems (IVIS Spectrum System; Xenogen Corporation). On day 19, mice were randomized and thereafter treated daily with 10mg/kg KU60019, 10mg/kg EIPA both alone and in combination via intraperitoneal injection daily. All animals were euthanized at day 48 post tumor implantation, and ascites fluid, interstitial fluid (Sullivan et al., 2019), and tumor tissues were collected.

Immunohistochemistry

Immunohistochemistry (IHC) was conducted on formalin fixed paraffin embedded tissues using cleaved caspase 3 antibody (1:300, Cell Signaling, Cat# 9661), EnVision+ Dual Link HRP secondary (Agilent, Cat#K406311-2), and the ImmPACT DAB Peroxidase (HRP) Substrate (Vector Laboratories, Cat# SK-4105) following the manufacturer's instructions. Briefly, the tissue was rehydrated by incubating in a series of zylenes/ethanol baths. Antigen retrieval was performed by steaming in citrate buffer (Thermo Fisher Scientific, Cat# 005000) for 40min. Endogenous peroxidase was quenched by incubating in 3% H_2O_2 /MeOH for 20min after which slides were washed and blocked in 1% BSA/PBS at room temperature for 30min. Slides were incubated overnight at 4°C in primary antibody (1:300), washed,

and incubated in secondary-HRP for 45min. DAB was used to develop, and Mayer's Hemotoxylin (Sigma-Aldrich, Cat# MHS16) was used to counterstain the tissue. Finally, slides were dehydrated and mounted. H score was determined on blinded samples, where H score = $[1 \times (\% \text{ cells } 1+) + 2 \times (\% \text{ cells } 2+) + 3 \times (\% \text{ cells } 3+)]$.

Metabolomics

Metabolomic profiling was conducted using ion pairing reversed phase liquid chromatography- high resolution mass spectrometry (LC-HRMS) modified from previous methods for polar analytes (Guo et al., 2016) and nucleotides (Kuskovsky et al., 2019). Samples were spiked with a stable isotope mix containing AMP- $^{13}\text{C}_{10}, ^{15}\text{N}_5$, dAMP- $^{13}\text{C}_{10}, ^{15}\text{N}_5$, ATP- $^{13}\text{C}_{10}, ^{15}\text{N}_5$, dATP- $^{13}\text{C}_{10}, ^{15}\text{N}_5$, dTMP- $^{13}\text{C}_{10}, ^{15}\text{N}_2$, dTTP- $^{13}\text{C}_{10}, ^{15}\text{N}_2$, dCMP- $^{13}\text{C}_9, ^{15}\text{N}_3$, CTP- $^{13}\text{C}_9, ^{15}\text{N}_3$, dCTP- $^{13}\text{C}_9, ^{15}\text{N}_3$, $^{13}\text{C}_3$ -Sodium Pyruvate, $^{13}\text{C}_3$ -Lactate, $^{13}\text{C}_4$ -Fumaric Acid, $^{13}\text{C}_4$ -Succinic acid, $^{13}\text{C}_4^{15}\text{N}_1$ -Aspartic Acid, $^{13}\text{C}_6$ -Citric Acid, $^{13}\text{C}_6$ -Glucose-6-phosphate, $^{13}\text{C}_2$ -AcetylCoA, $^{13}\text{C}_5$ -D- α -Hydroxyglutaric acid, $^{13}\text{C}_5^{15}\text{N}_2$ -Glutamine from Sigma-Aldrich or Cambridge Isotope Laboratories. 1 mL of Optima LC-MS grade 80:20 methanol:water prechilled to -80°C was then added to each sample, followed by a 30 second vortex mixing, 15 second pulse sonication with a probe tip sonicator then samples were returned to the -80 °C freezer for 30 min. Insoluble debris was precipitated by centrifugation for 10 min, 17,000 x g at 4°C. Supernatant was evaporated to dryness under nitrogen, resuspended in 100 μL 5% 5-sulfosalicylic acid in water, and 5 μL was injected for analysis. LC-HRMS was conducted on an Ultimate 3000 UHPLC equipped with a refrigerated autosampler (at 6 °C) and a column heater (at 55 °C) with a HSS C18 column (2.1 $\times$ 100 mm i.d., 3.5 μm ; Waters, Milford, MA) used for separations. Solvent A was 5 mM DIPEA and 200 mM HFIP and solvent B was methanol with 5 mM DIPEA 200 mM HFIP. The gradient was as follows: 100 % A for 3 min at 0.18 mL/min, 100 % A at 6 min with 0.2 mL/min, 98 % A at 8 min with 0.2 mL/min, 86 % A at 12 min with 0.2 mL/min, 40 % A at 16 min and 1 % A at 17.9 min-18.5 min with 0.3 mL/min then increased to 0.4 mL/min until 20 min. Flow was ramped down to 0.18 mL/min back to 100 % A over a 5 min re-equilibration. For MS analysis, the UHPLC was coupled to a Q Exactive HF mass spectrometer

(Thermo Scientific, San Jose, CA, USA) equipped with a HESI II source operating in negative mode. The operating conditions were as follows: spray voltage 4000 V; vaporizer temperature 200 °C; capillary temperature 350 °C; S-lens 60; in-source CID 1.0 eV, resolution 60,000. The sheath gas (nitrogen) and auxiliary gas (nitrogen) pressures were 45 and 10 (arbitrary units), respectively. Single ion monitoring (SIM) windows were acquired around the $[M-H]^-$ of each analyte with a 20 m/z isolation window, 4 m/z isolation window offset, $1e^6$ ACG target and 80 ms IT, alternating in a Full MS scan from 70-950 m/z with $1e6$ ACG, and 100 ms IT. Data was analyzed in XCalibur v4.0 and/or Tracefinder v4.1 (Thermo) using a 5 ppm window for integration of the peak area of all analytes and internal standards used for normalization.

Quantification and Statistical Analysis

GraphPad Prism version 8.0 was used to perform statistical analysis. One-way ANOVA or t-test were used as appropriate to determine p values of raw data. P-values < 0.05 were considered significant. Longitudinal and cross-sectional analysis of tumor volume were calculated using TumorGrowth tool using default parameters (Enot et al., 2018).

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