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14. ABSTRACT

Deletion of the PTEN locus, an early and frequent event in prostate cancer (PCa), invariably results in co-deletion of neighboring genes. While many of these co-deleted genes encode cell-essential functions, these cancer cells can survive due to expression of functionally redundant paralogs elsewhere in the genome. We have established that pharmacological or genetic extinction of such sustaining paralogs results in cancer cell death while not impacting normal cells, providing a cancer-specific vulnerability that we termed collateral lethality. In this proposal, collateral lethal relationships of each deleted bystander gene residing in the PTEN locus will be assessed and validated systematically. Hypothesis: We hypothesize that collateral or synthetic lethality can result from deletion of tumor suppressor loci that sustain co-deletion of neighboring genes encoding cell essential functions.

15. SUBJECT TERMS
None listed.

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TABLE OF CONTENTS

INTRODUCTION:

Deletion of the PTEN locus, an early and frequent event in prostate cancer (PCa), invariably results in co-deletion of neighboring genes. While many of these co-deleted genes encode cell-essential functions, these cancer cells can survive due to expression of functionally redundant paralogs elsewhere in the genome. We have established that pharmacological or genetic extinction of such sustaining paralogs results in cancer cell death while not impacting normal cells, providing a cancer-specific vulnerability that we termed collateral lethality. Collateral lethality identifies therapeutic targets in cancers with homozygous tumor suppressor deletions. In the context of prostate cancer, approximately 20% of patients exhibit PTEN loss. Interestingly, within this subset of patients, a subgroup experiences the additional loss of PAPSS2, a gene responsible for producing sulfate donors crucial for sulfation processes. Moreover, across other PTEN-null cancer types, PAPSS2 loss also frequently coincides with PTEN loss, and it appears to be well-tolerated and potentially compensated by a functionally redundant paralog, PAPSS1, situated on chromosome 4q24. Thus, we hypothesized that targeting this remaining paralog, PAPSS1, in PTEN/PAPSS2-null cancers would result in cancer cell death. Our *in vitro* results did not reveal any notable proliferation impairment when depleting or deleting PAPSS1 in PTEN/PAPSS2-null prostate cancer cells cultured in either complete or sulfate-free media. In contrast, our preliminary *in vivo* investigation unveiled a substantial tumor growth delay solely attributed to the loss of PAPSS1 only in chronic leukemia cells. Additionally, consistent with these results, we observed delayed tumor growth and reduced tumor formation in cells lacking both PAPSS1 and PAPSS2 synthases *in vivo*. In summary, our comprehensive investigation into the effects of PAPSS1 depletion or deletion in PTEN/PAPSS2-null prostate cancer cells underscore the remarkable adaptability of these cells in response to these genetic alterations. The lack of discernible proliferation impairment in the *in vitro* setting suggests the existence of compensatory mechanisms or alternative pathways that mitigate the impact of PAPSS1 loss. However, the importance of the synthases becomes evident in our *in vivo* studies, where the loss of PAPSS1 alone demonstrates a profound delay in tumor growth within the context of chronic leukemia cells. This pivotal role of PAPSS1 in tumor progression highlights its potential as a significant therapeutic target. Moreover, the cumulative effects observed in cells lacking both PAPSS1 and PAPSS2 synthases further emphasize their importance in tumor formation. These findings collectively contribute to our understanding of the essentiality of sulfation in cancer development and provide valuable insights into the potential avenues for targeted therapeutic interventions.

KEYWORDS:

Collateral Lethality, PTEN, tumor suppressor, PAPSS1, PAPSS2, sulfation, post-translational modification, gene essentiality, prostate cancer

ACCOMPLISHMENTS:

I. What were the major goals of the project?

TASKS	MONTHS	COMPLETION
Aim 1: *In vivo* validation of PAPSS1 as a therapeutic target for *PAPSS2*-null prostate cancer.		
Aim 1 - Major Task 1: Validate PAPSS1 as a collateral lethal target across multiple genetic models.		
Subtask 1: Seek IACUC/ACURO regulatory approval for the mouse experments proposed in this project.	1-6	100%
Subtask 2: Deplete PAPSS1 in human PCa cell lines, with or without enforced expression of WT and/or enzyme dead mutant form of PAPSS1. The cells will be assayed for changes in sulfation, proliferation and survival. Cell lines used: PC-3, DU145 and LNCaP (in lab; obtained from ATCC)	1-18	100%

Subtask 3: Deplete PAPSS1 in patient-derived organoids (PDOs), with or without enforced expression of WT and/or enzyme dead mutant form of PAPSS1. The cells will be assayed for changes in sulfation, proliferation and survival. PDOs used: MSKPCa1, 2, 15 and 16 (in lab; obtained from MSKCC)	1-18	20%
Subtask 4: Engineer isogenic B6 mouse PCa cell lines null for PAPSS2 (via CRISPR deletion) and express inducible shRNA to deplete PAPSS1. To test shRNA specificity, we will rescue with enforced expression of shRNA-resistant PAPSS1 ORF encoding WT and or enzyme-dead PAPSS1. The cells will be tested for changes in sulfation, proliferation, and survival. Mouse PCa cell lines will be generated (our own lab)	1-18	80%
Subtask 5: Engineer a GEM model to assess the impact of somatic deletion of PAPSS1, gaining insights into mechanism-based toxicity, if any. C57BL/6 mice (250, both genders); Swiss Webster foster (20, female)	6-36	25%
Subtask 6: Orthotopically implant PCa cell lines and PDOs into appropriate mouse model and monitor tumor growth and overall survival. Serial phenotypic and molecular analyses will be performed. nude / SCID mice (420, male); C57BL/6 mice (300, male)	13-36	70%
Aim1 - Major Task 2: Identify specific inhibitors for PAPSS1 that selectively target PAPSS2-null cancer cells.		
Subtask 1: Mutate the predicted critical residues of PAPSS1. WT and mutant PAPSS1 will be purified and tested for their enzymatic activities and substrate binding.	1-18	80%
Subtask 2: Virtual screening to identify PAPSS1 selective inhibitors.	1-18	50%
Subtask 3: Top inhibitors will be tested for their inhibitory activity on PAPSS1 and PAPSS2 enzymatic activity.	6-24	10%
Subtask 4: Top inhibitors will be tested for their differential growth inhibitory effects on PAPSS2 null versus WT prostate cancer cell lines.	18-36	10%
Subtask 5: Final top inhibitors emerging from cell culture assays will be tested for anti-tumor activity in mice.	18-36	10%
Aim 2: Comprehensive high-resolution discovery of collateral and synthetic lethal targets of deleted bystander genes in the PTEN locus.		
Aim 2 - Major Task 1: Test collateral lethality of *PTEN* passenger deletions that have clear paralogs.		
Subtask 1: Collateral lethality between PAPSS1 and PAPSS2 will be confirmed using enCas12a/CRISPR system.	6-24	80%
Subtask 2: Collateral lethality between GLUD1 and GLUD2 will be tested using enCas12a/CRISPR system.	13-36	20%
Subtask 3: Collateral lethality between PANK1 and PANK 2,3,4 will be tested using enCas12a/CRISPR system.	13-36	100%
Aim 2 - Major Task 2: Genome-wide screening for synthetic lethality of PTEN passenger deletions that do not have paralogs.		
Subtask 1: Synthetic lethal targets of ATAD1 will be screened with library of prostate-expressed genes via enCas12a/CRISPR system.	6-36	20%
Subtask 2: Synthetic lethal targets of MINPP1 will be screened with library of prostate-expressed genes via enCas12a/CRISPR system.	6-36	20%

II. What was accomplished under these goals?

AIM 1: *In vivo* validation of PAPSS1 as a therapeutic target for *PAPSS2*-null prostate cancer.

AIM 1 - Major Task 1 - Subtask 2 - Validate PAPSS1 as a collateral lethal target across multiple genetic models.

1) Specific objectives:

Assess changes in sulfation, proliferation and survival of cells following PAPSS1 depletion in cancer cells, especially those that are PTEN and PAPSS2-null.

2) Significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative):

Previously, we utilized the CCLE database to identify PTEN and PAPSS2-null prostate cancer cell lines, specifically LNCaP and C4-2. After this, we engineered doxycycline-induced PAPSS1 knockdowns across the two cell-lines to observe whether cell proliferation will be affected. However, minimal growth perturbations were observed following PAPSS1 knockdown, suggesting residual PAPSS1 enzymes may still be sufficient for supporting cell growth. Thus, PAPSS1 KO LNCaP and C4-2 cells were generated via CRISPR-cas9 and western blots confirmed its knockout efficacy (**Fig 1A**). Across both complete and sulfate-free media conditions, discernible differences in proliferation rates were not observed between the PAPSS1 wildtype and knockout groups. However, it is worth noting that a slight reduction in proliferation was detected in cells cultured in sulfate free media initially. This minor slowdown in growth appeared to be transient, as the cells subsequently adapted and restored their proliferation rates to levels comparable to those in normal media (**Fig 1B**).

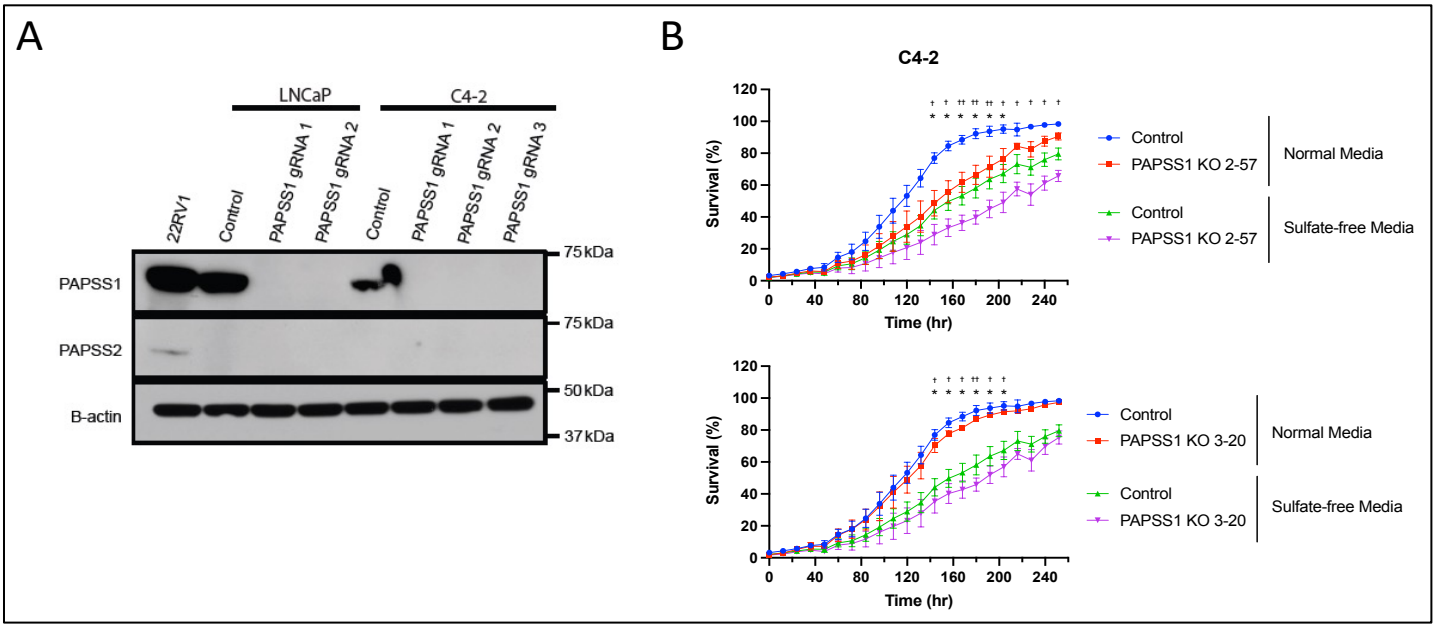


Figure 1. PAPSS1 Knockout in LNCaP and C4-2 Cells. (A) Western blot analysis illustrates the effective PAPSS1 knockout efficacy in LNCaP and C4-2 cells. The designations sgRNA1, sgRNA2, and sgRNA3 correspond to the specific guide RNAs targeting exons 1, 2, and 3 of PAPSS1, respectively. **(B)** No significant proliferation impairment was detected between PAPSS1 wildtype and knockout groups cultured in both complete and sulfate-free media. But a marginal reduction in proliferation was observed in C4-2 cells cultured with sulfate-free media compared to normal media. Results represents the mean ±SEM. *Asterisks denote the comparison between the control group cultured in sulfate-free media and the control group in normal media. † Cross symbols represent the comparison between the PAPSS1 KO group cultured in sulfate-free media and the control group in normal media. * $p < 0.05$; ** $p < 0.01$; † $p < 0.05$; †† $p < 0.01$

Considering our understanding that membrane receptor proteins are prominent targets for tyrosine sulfation, we deemed it appropriate to delve into the examination of PAPSS1 within an *in vivo* context. This approach offers a dynamic milieu where diverse factors, encompassing immune reactions and tissue interactions, can collectively shape the response to tumor progression. Subsequently, we utilized a C4-2 control group along with three distinct guide RNAs (gRNAs) targeting exon 1, 2, and 3 of PAPSS1. Subcutaneous injections were administered in

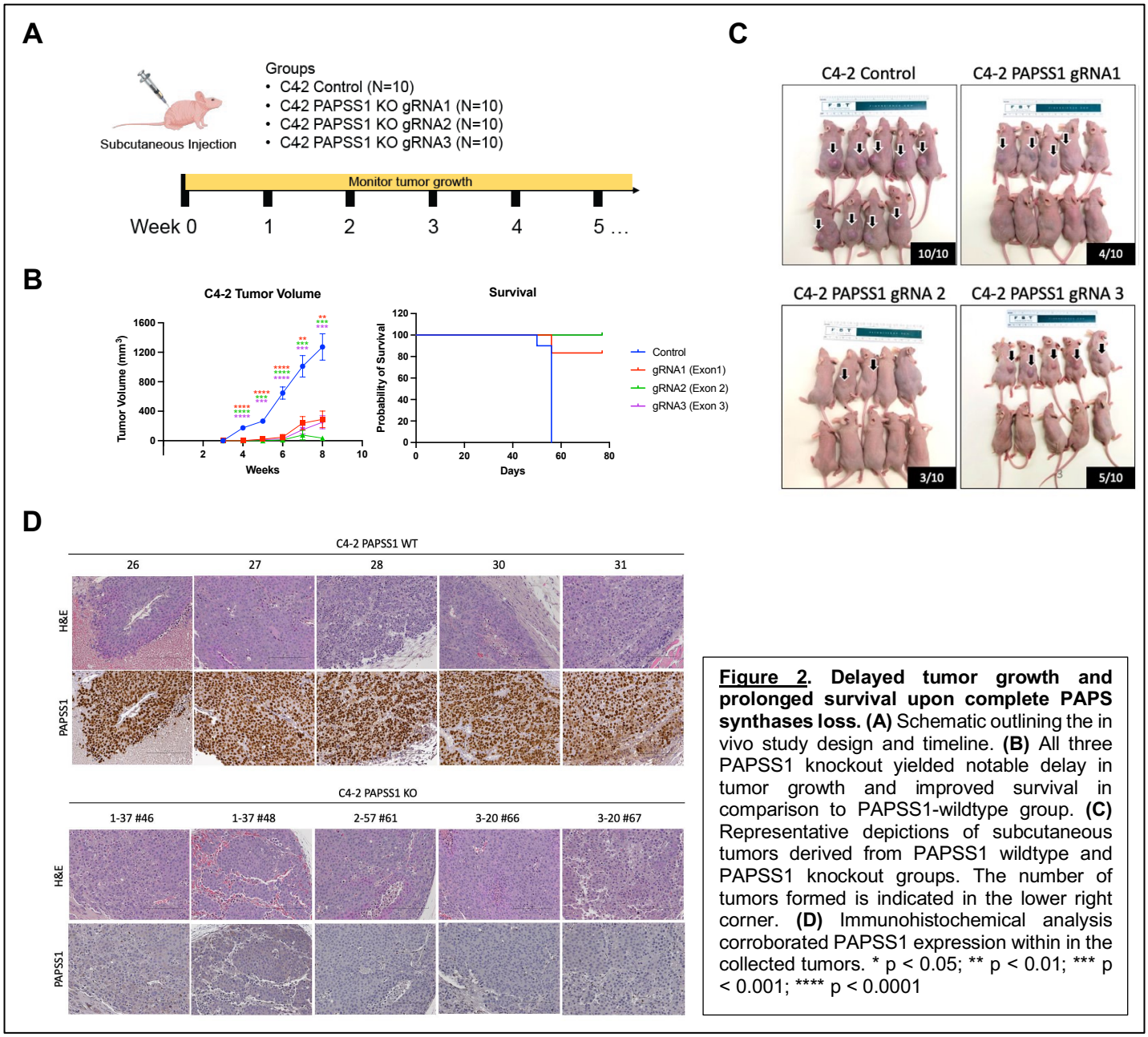


Figure 2. Delayed tumor growth and prolonged survival upon complete PAPS synthases loss. (A) Schematic outlining the in vivo study design and timeline. **(B)** All three PAPSS1 knockout yielded notable delay in tumor growth and improved survival in comparison to PAPSS1-wildtype group. **(C)** Representative depictions of subcutaneous tumors derived from PAPSS1 wildtype and PAPSS1 knockout groups. The number of tumors formed is indicated in the lower right corner. **(D)** Immunohistochemical analysis corroborated PAPSS1 expression within in the collected tumors. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

immunodeficient nude mice and tumor growth were monitored over time (**Fig 2A**). Our *in vivo* experimental outcomes revealed delayed tumor growth and prolonged survival were observed in the PAPSS1 knockout groups (**Fig 2B**). Moreover, approximately 50% reduction in number of tumors formed across all three PAPSS1 knockout groups compared to the control (**Fig 2C**). Immunohistochemical analyses further verified the presence and absence of PAPSS1 expression within the collected tumors (**Fig 2D**).

Of the three PAPSS1 KO groups, PAPSS1 gRNA2 group demonstrated strongest difference in terms of tumor growth rate and number of tumor formation in comparison to control. Thus, we used this cell-line to generate a doxycycline-inducible PAPSS1 rescue line. These cells were then administered via subcutaneous injections into nude mice and treated with or without doxycycline water. While the experiment is ongoing, initial observations corresponding to previous in vivo studies, indicating that:

- PAPSS1 KO cells exhibit a slower rate of tumor development and form fewer tumors compared to the control.
- Similarly, the PAPSS1 Rescue group without doxycycline treatment shows a comparable outcome to the PAPSS1 KO group.

- Notably, the PAPSS1 Rescue group, with doxycycline treatment, demonstrates a similar pattern of tumor formation over time as compared to the control.

In summary, our investigation into the indispensability of PAPS synthases in cancer progression has revealed a notable contrast between findings from *in vitro* and *in vivo* studies. While our *in vitro* experiments indicate a possible transient hindrance in cell proliferation when sulfate levels are greatly restricted in culture, this effect is rapidly compensated over time. In contrast, our transition to an *in vivo* context unveils a more pronounced anti-tumor influence upon complete PAPS synthase depletion in cancer cells. This outcome underscores the necessity of studying sulfation within an *in vivo* framework, particularly considering its pivotal role in catalyzing tyrosine sulfation of membrane receptor proteins. The thorough elimination of PAPS synthases yields crucial insights into the impact of PAPSS1 on tumor growth and formation. The consistent trend of delayed tumor growth and enhanced survival across diverse PAPSS1 KO groups reinforces the significance of sulfation in driving tumor progression. The subsequent generation of a doxycycline inducible PAPSS1 rescue line reaffirms these observations, suggesting that PAPSS1's presence or absence significantly influences tumor growth dynamics. As our *in vivo* experiment continues to unfold, the compelling initial findings bolster the notion that PAPSS1 may serve as a potential therapeutic target warranting further investigation.

(4) Other achievements.
A significant hurdle in sulfation research is the limited availability of reagents essential for evaluating cellular sulfation levels, a crucial aspect in assessing the significance of PAPSS1/2 in this post-translational modification. To tackle this challenge, we collaborated with experts in the field to create and validate antibodies pertinent to our project. Through partnership with Cell Signaling Technology, we successfully developed both polyclonal and monoclonal antibodies targeting PAPSS1 and PAPSS2, which have undergone validation within the DePinho lab. Additionally, in conjunction with the MD Anderson Antibody Core, we engineered and verified monoclonal antibodies capable of detecting sulfotyrosine, marking a significant advancement for the field.

AIM 1 - Major Task 1 - Subtask 3
We initially planned to utilize the organoid system, but it may no longer be necessary, considering the extensive in vitro and in vivo studies conducted with human cell lines.

AIM 1 - Major Task 1 - Subtask 4
We obtained the DX1 cell line from our prostate cancer mouse model (GEMM), which includes a GFP reporter, probasin-Cre transgene, and conditional alleles of Pten, Trp53, and Smad4 (CPPSML). Subsequently, we employed the CRISPR-Cas12 system to introduce various genetic modifications related to PAPS synthases. Specifically, we generated the following cell lines: cas12_Con, PAPSS1 KO, PAPSS2 KO, and a double KO of both PAPSS1 and PAPSS2. These cell lines have undergone assessments for proliferation and *sulfation in vitro.* We plan to carry out *in vivo* studies monitoring tumor growth in an immune-competent environment.

AIM 1 - Major Task 1 - Subtask 5
The lab is currently generating a Frt/Flpase based conditional knockout allele for Papss1. We are in the final stages of cloning the targeting construct. The allele will have Frt sites flanking exon 2 of the gene. Flpase expression will remove the second exon and produce a frameshift downstream. Concomitantly, we are generating a Papss2 LoxP/Cre based allele to elucidate the role of each gene separately and together. Completion of the Papss1 targeting construct will represent 25% of the total effort for generation of the mouse line.

AIM 1 - Major Task 1 - Subtask 6
A primary murine prostate cancer cell line (DX1) has been established from our prostate cancer GEMM model, which comprises a GFP reporter, probasin-Cre transgene, and conditional alleles of Pten, Trp53, and Smad4 (CPPSML). The DX1 cell line exhibits expression of both PAPSS1 and PAPSS2 proteins. We utilized the cas12 system from Hart Lab to insert multiple gRNAs into a single vector, enabling simultaneous delivery of gRNAs and cas12 proteins. This approach yielded the creation of the following cell lines: (1) Control, (2) PAPSS1 Knockout (KO) Only, (3) PAPSS2 KO Only, (4) PAPSS1 and PAPSS2 Double Knockout (DKO). Additionally, with the generated DKO cell line, we plan to generate inducible PAPSS1 and PAPSS2 rescue lines. These

diverse cell lines will be introduced into B6 mice to facilitate the study of sulfation within an immunocompetent background.

AIM 1 - Major Task 2: Identify specific inhibitors for PAPSS1 that selectively target PAPSS2-null cancer cells. We have curated the genomics profile of over 1156 cell lines, including their 19177 gene expression levels, mutation status, copy numbers, etc. Further genomics analysis showed that different cell lines have distinct PAPSS1/2 expression profiles. As demonstrated in (**Fig 3**), LNCaP shows reasonable level of PAPSS1 but low on PAPSS2, while PC3 cell line has comparable level of both PAPSS1 and 2. This provides use selective strategy of cell-line based screening for inhibitors, as summarized Task 3. In addition, structural analysis revealed pivotal residues such as Phe101, Lys171, Cys207, and Cys212 essential for substrate binding and enzymatic reactions. Leveraging this structural understanding, we curated varied datasets and performed virtual screening to pinpoint selective small molecule inhibitors of PAPSS1. Promising top hit/lead candidates have been chosen and are presently undergoing comprehensive experimental evaluation encompassing biochemical assays and cellular models.

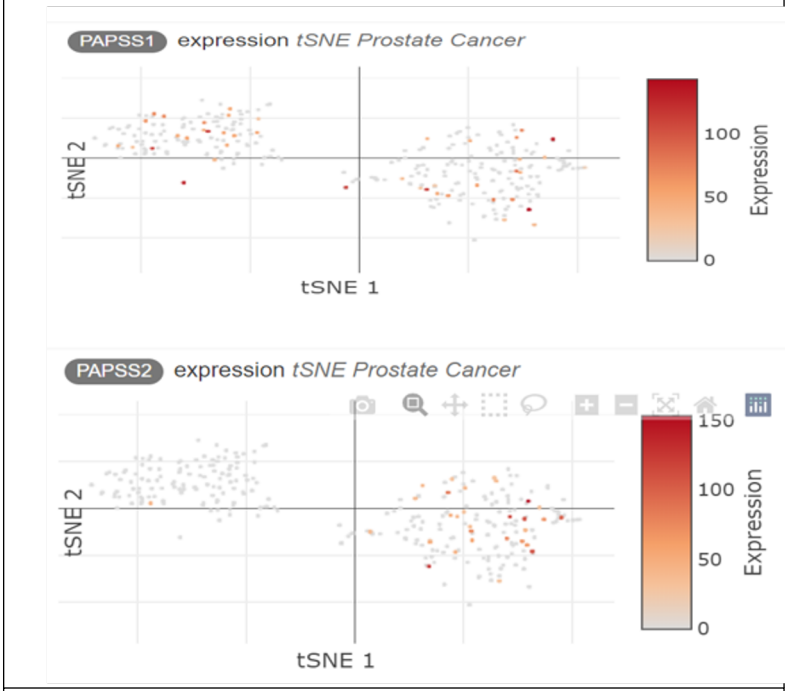


Figure 3: Genomic analysis shows two distinct clusters for LNCaP (left) and PC3 (right). The upper panel shows PAPSS1 is highly expressed in both cell lines while PAPSS2 only shows in PC3.

AIM 1 - Major Task 2 – Subtask 2: Screen inhibitors for PAPSS1.

In addition to the cell line genomics data, we also curated a large dataset including 130K compounds along with their experimentally tested IC_{50} in all 1156 cell lines, totaling ~1.3 million data points. Utilizing our internally developed artificial intelligence (AI) program, which was built upon our collection of big data of the cell line genomics, chemical structures and their affiliated bioactivities, we screened and identified 3920 compounds with activities (IC_{50}) in μM or stronger in LNCaP but not PC3 cell line.

To enhance the precision of our inhibitor selection process, we conducted an exhaustive analysis of the interactions between these compounds and the PAPSS1 protein. The protein's structural data were obtained from PDB (entry code 2OFX). This process employed an active docking technique rooted in physics-based computational methods and active learning algorithm as implemented in the Glide SP docking program from Schrödinger Suite. Initially, we conducted a pilot task, screening 1000 compounds, yielding promising predictions as shown in (**Fig. 4**). Furthermore, we extended our analysis to a test dataset consisting of 393 compounds using the best model along with associated uncertainty, as illustrated in (**Fig 5A**). Additionally, (**Fig 5B**) showcases the docking results for a compound noted as smi_35731.

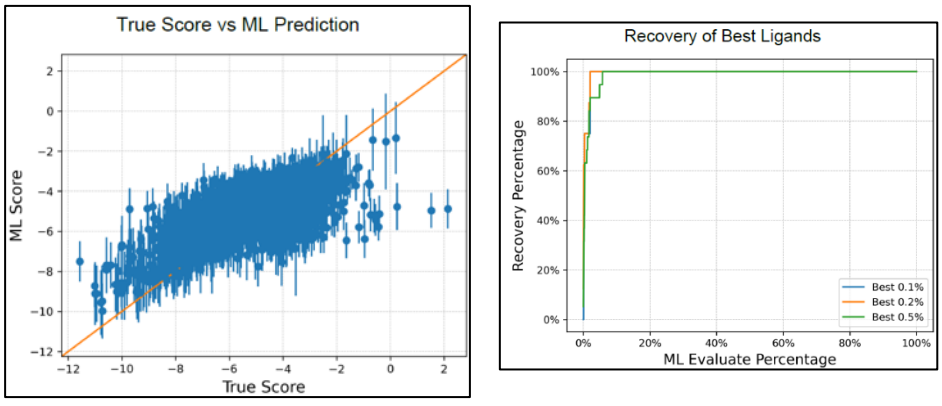


Figure 4. (A) This plot shows the true scores vs machine learning model predictions of all the pilot ligands. The correlation R^2 is 0.47. The root-mean-square error (RMSE) is 1.16. **(B)** The plot shows how effectively the model can recover the top ligands by scoring early in the list of ML predictions. The production run on the full library should be expected to out-perform the pilot study.

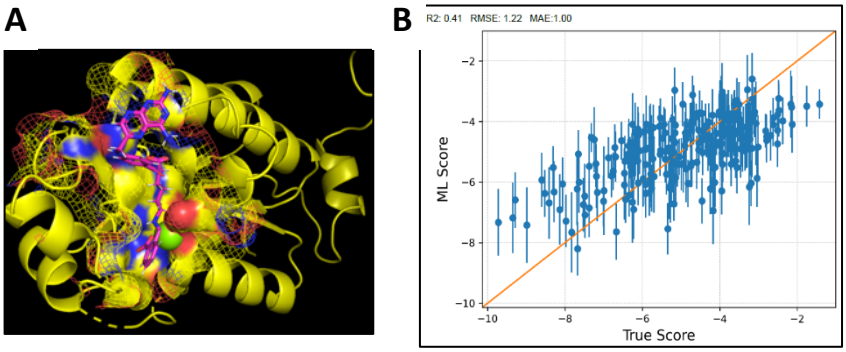
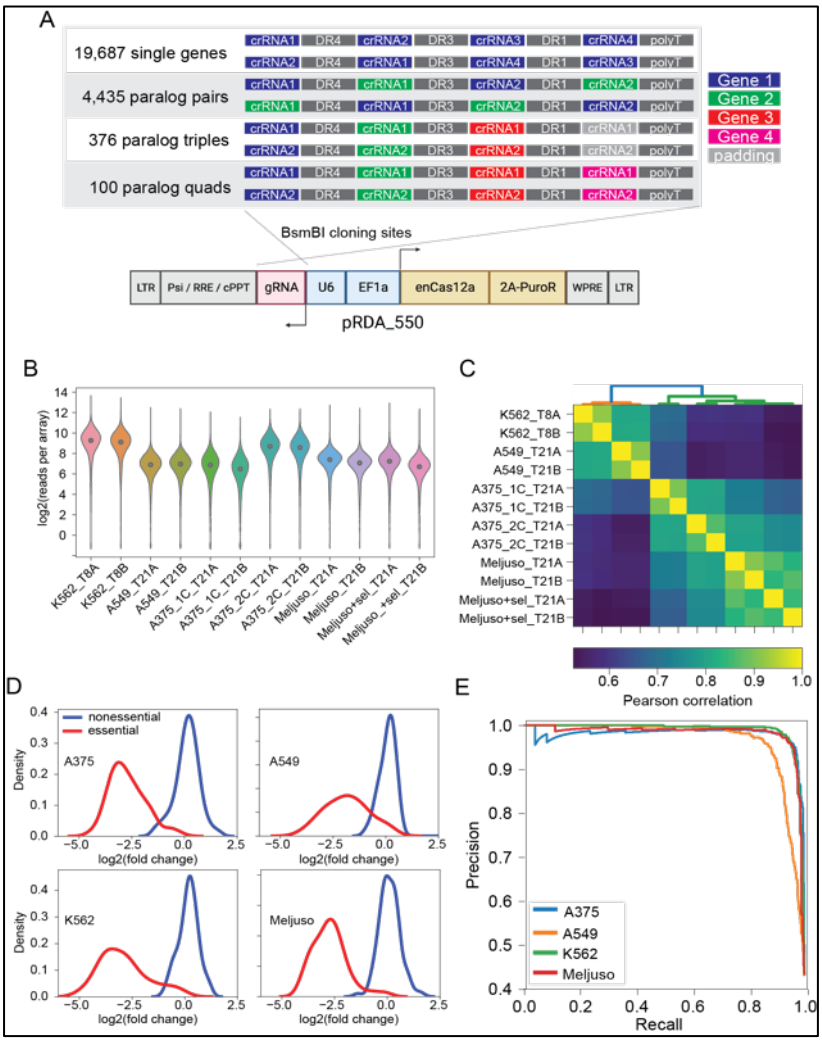


Figure 5: **(A)** Prediction of 393 compounds using the refined model with uncertainty. **(B)** Compound smi_35731 docked to protein PAPSS1 with predicted binding score as -7.50.

AIM 2: Comprehensive high-resolution discovery of collateral and synthetic lethal targets of deleted bystander genes in the PTEN locus.

AIM 2 - Major Task 1: Test collateral lethality of *PTEN* passenger deletions that have clear paralogs.



Building on the success of the prototype library we previously described, we designed a second human genome library with several modifications. The new library targets roughly twice as many paralogs (4,435 pairs, 376 triples, and 100 quads; **Fig 6**), includes nontargeting arrays to facilitate the estimation of fitness effects arising from multiplex locus-nonspecific DNA double strand breaks, and other minor technical changes such as using nontargeting sequences to extend 3mer paralog constructs into 4mer guide arrays, instead of random selection of nonessential guides as used in the

Figure 6. In4mer platform for whole-genome screening. (**A**) Inzolia human whole-genome library targets single genes and paralog pairs, triples, and quads with arrays of 4 Cas12a gRNA. Each gene or gene family is targeted by two arrays encoding the same gRNA in different order. Commercially synthesized oligo pools are cloned into the one component pRDA_550 lentiviral vector; schematic created in Biorender. (B-F) Screening in K562 CML cells and A459 lung cancer cells. (**B**) Read counts from the plasmid and experimental timepoints after lentiviral transduction. (**C**) Correlation of sample read counts. Endpoint replicates are highly correlated. (**D**) Fold change distributions of arrays targeting reference essential (red) and nonessential genes (blue) in four cell lines. (**E**) Precision/recall analysis from ranked mean fold change of arrays targeting each gene, calculated against reference essential and nonessential genes.

prototype. This library, which we call *Inzolia*, contains ~49k unique arrays (**Fig 6A**), and was cloned into both the pRDA_550 one-component vector and pRDA_052 guide-only expression vector for two-component CRISPR/Cas12a systems.

We screened the Inzolia library in A549 lung cancer cells and K562 leukemia cells with the one-component library, Meljuso melanoma cells with the two-component (split-vector) library and A375 melanoma cells with both the one- and two-component systems. Both cell lines effectively identified essential and nonessential genes (**Fig 6D,E**) and the screens showed results consistent with previous Cas12a screens using the Humagne library. Further, the one-component (pRDA_550) and two-component (pRDA_174 + pRDA_052) libraries yielded equivalent results.

The whole-genome libraries target small paralog families as well as single genes. To evaluate paralog genetic interactions, we used the multiplicative model to calculate the expected fitness of pairwise knockouts by summing the log fold change of the single gene knockouts. We then compared the observed mean fold change of guide arrays targeting gene pairs with the expected fold change under the multiplicative null model to calculate a dLFC that represents the magnitude of the genetic interaction (**Fig 7A**). Gene pairs with strongly negative dLFC are highly concordant with the gold standard paralog synthetic lethals we previously determined. The Inzolia library targets 24 of the 26 gene pairs that are hits in >1 of 5 previously published paralog synthetic lethality screens, and 12 of the 13 candidate gold standard paralog synthetic lethals. Of those 12, 9 have dLFC < -1 in Meljuso cells, for an estimated sensitivity of 75% (**Fig 7B**). Moreover, all 12 pairs (100%) with high paralog score are essential, regardless of synthetic lethality, as are 10 of 12 pairs (83%) with lower paralog scores (**Fig 7C**), consistent with either synthetic lethality or one paralog being essential. Many other paralogs show genetic interactions as strong as these positive controls (see **Fig 7D** for selected examples), with sequence

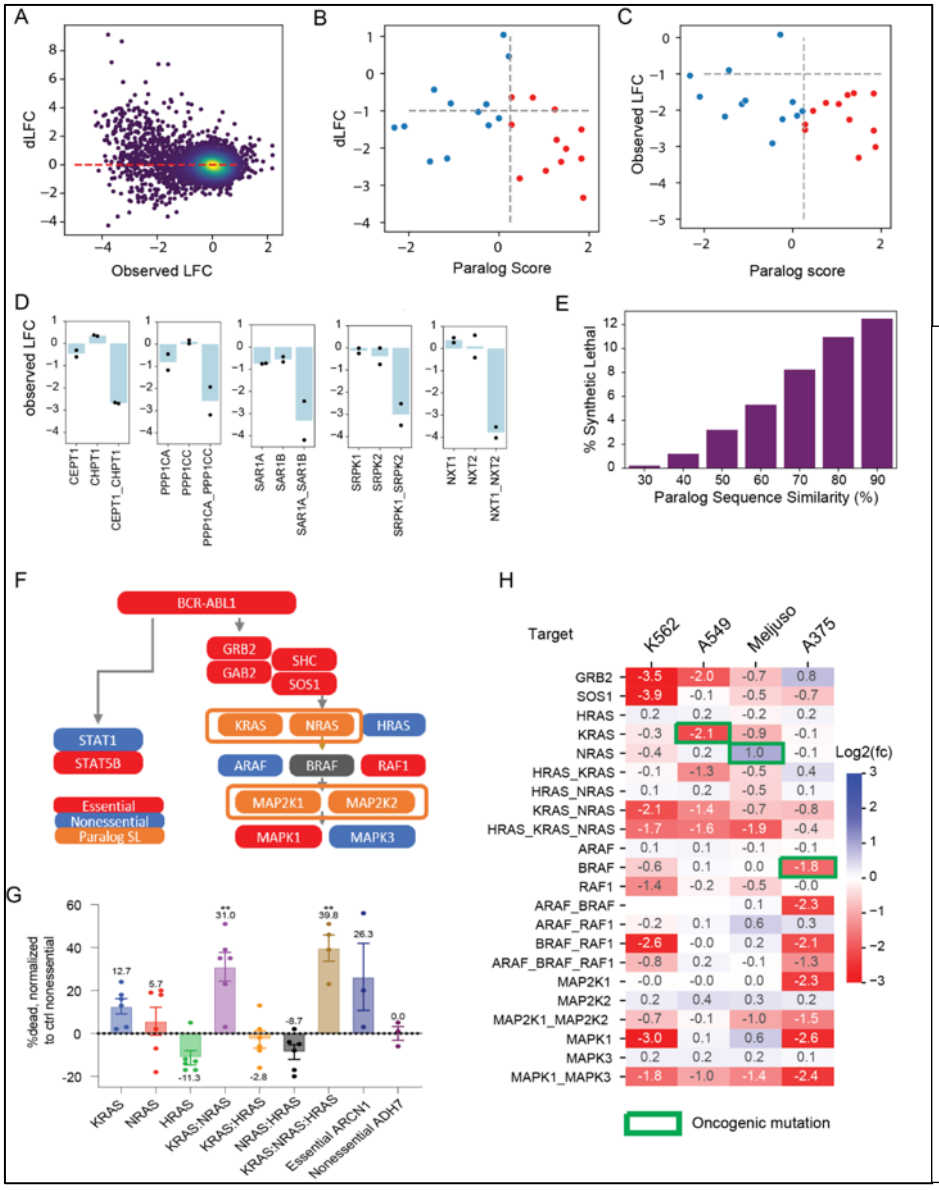


Figure 7. Paralog synthetic lethality with Inzolia. (**A**) Fold change vs. dLFC for >4,000 paralog families in Meljuso cells. (**B**) dLFC vs. Paralog Score from meta analysis of published paralog screens. Of 12 paralogs with score > 0.25 (red), 9 show dLFC < -1 in Meljuso cells. (**C**) Fold change vs. paralog score in Meljuso cells. Most scored paralogs are essential, regardless of synthetic lethality. (**D**) Selected synthetic lethals in Meljuso cells showing single and double knockout fitness phenotype. Bar chart, mean fold change. Points indicate fold change of single array of gRNA (mean of 2 replicates). (**E**) Fraction of synthetic lethal paralogs by amino acid sequence similarity in Meljuso cells. (**F**) Pathway activation by BCR-ABL1 fusion in K562 cells. Red, essential gene in in4mer screen; blue, nonessential; orange, synthetic lethal paralog pair. (**G**) Fraction of dead cells, normalized to controls, for single, double, and triple knockouts of RAS genes in K562. KRAS-NRAS joint knockout shows increased cell death. ARCN1, control essential gene. ADH7, control nonessential gene. (**H**) Single, double, and triple knockout phenotype of RTK/MAP kinase pathway genes in all four cell lines. White, target not in library.

similarity between paralogs being a strong predictor of GI (**Fig 7E**), in keeping with prior observations by De Kegel & Ryan.

In total, the Inzolia library includes ~50k unique guide arrays, with ~40k targeting single genes and 9,822 arrays targeting paralog doubles, triples, and quads. Inzolia is therefore on par with latest-generation genome-scale CRISPR/Cas knockout libraries (**Fig 8**) and is unique among such libraries in including thousands of reagents targeting paralogs. Moreover, the efficiency gain realized by having two guides targeting each of two genes in a paralog pair makes detection of genetic interactions tractable with only six reagents per gene pair, a fivefold improvement over the prior state of the art (**Fig 8**).

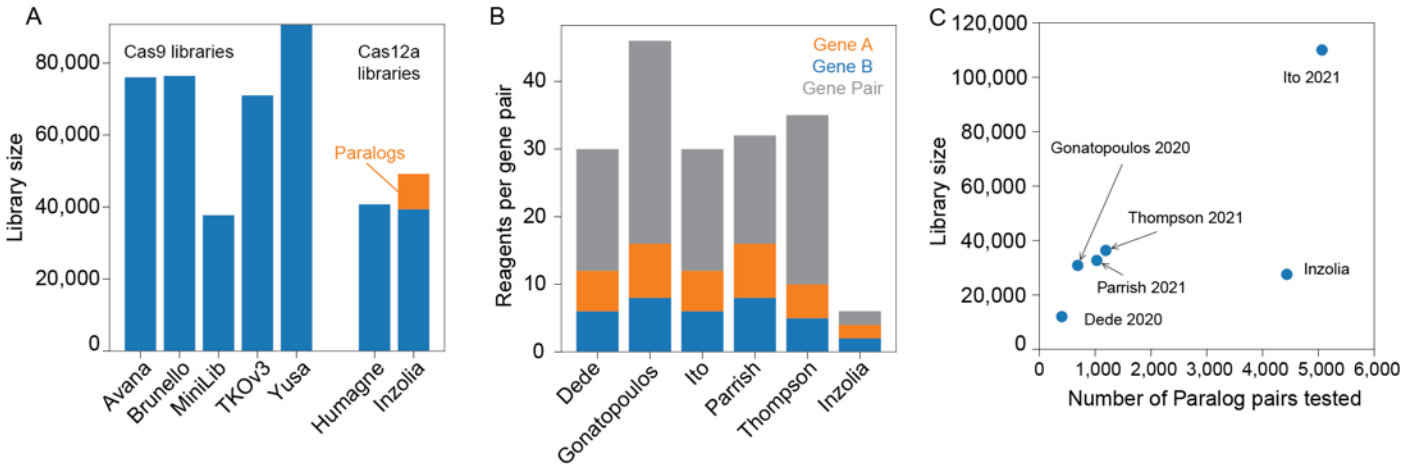


Figure 8. Library size comparison. (A) Representative Cas9 and Cas12a whole-genome libraries. Inzolia library targets 19k protein coding genes and additionally includes 9,822 guide arrays targeting paralog doubles, triples, and quads. (B) Five recent publications screening for genetic interactions between paralogs. Bar plot shows number of reagents per paralog pair tested, including single and double knockouts. (C) Comparison of library efficiency. Number of paralog pairs tested vs. library size for recent publications. For this plot, Inzolia library only includes paralog doubles (4,435), triples (376), quads (100), plus corresponding single gene knockouts (8,870).

AIM 2 – Major Task 1 - Subtask 3:
We interrogated the collateral lethal relationship of PANK proteins using CRISPR and inducible shRNA systems. We assessed the effect of PANK abrogation on cancer cell viability in vitro using crystal violet assay. Additionally, we also tested our hypothesis in vivo using genetically engineered cell lines expressing inducible shRNA against PANK2 or PANK3 in PANK1/2 double knockout or PANK 2/3 double knockout cells in orthotopic subcutaneous tumors. We confirmed the collateral lethality among PANK proteins in both in vitro and in vivo tumor models. (**Fig. 9**)

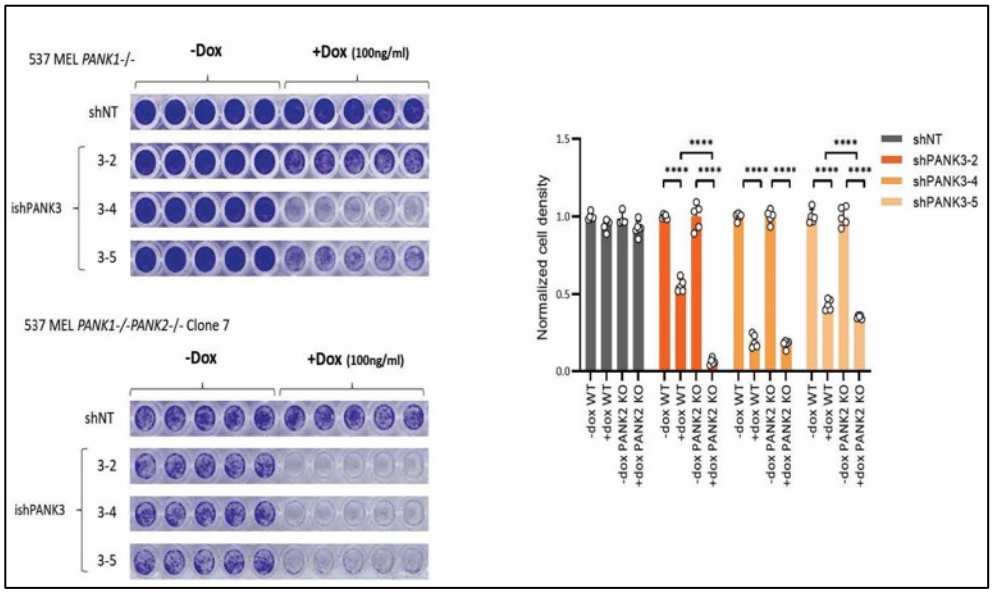


Figure 9. Knockdown of the PANK3 significantly impairs cell viability of PANK1-/- PANK2-/- cancer cells. Three independents inducible PANK3 shRNAs were stably expressed in 537 MEL (PANK1-/-) and 537 MEL (PANK1 -/- and PANK2-/-) cells. Following doxycycline administration, effects on cell viability because of PANK3 knockdown was assayed by crystal violet assay and quantified by measuring absorbance of the extracted dye. Knockdown of PANK3 significantly exacerbates killing of 537 MEL PANK1-/- and PANK2-/- cells compared to 537 MEL wild type cells.

AIM 2 - Major Task 2: Genome-wide screening for synthetic lethality of PTEN passenger deletions that do not have paralogs.

AIM 2 – Major Task 2 - Subtask 1:
PTEN-null prostate cancers often sustain loss of the neighboring gene *ATAD1,* which is important for mitochondrial function. We conducted genome-wide screening which identified *BIRC6* as a specific "partner" target for *ATAD1*. However, extensive validation of BIR6 as well as metabolism pathways failed to identify a robust and actionable synthetic lethal relationship. Given the significant and exciting results of our PAPSS efforts, we are focusing our efforts and resources on advancing this program as noted above.

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

N/A

IV. How were the results disseminated to communities of interest?

Nothing to Report

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

AIM 1 - Major Task 1

- Analyzing Tumor Volume Results: We will thoroughly analyze the tumor volume data obtained from orthotopic injections of C4-2 Control, PAPSS1 KO, doxycycline-induced PAPSS1 rescue, and doxycycline-induced PAPSS2 rescue cell lines. This analysis will help us understand the impact of PAPSS1 and PAPSS2 on tumor growth and progression in the context of prostate cancer.
- *In Vivo* Studies in an Immunocompetent Background: We will continue our in vivo studies to investigate the effects of the loss of PAPS synthases in an immunocompetent background. This research aims to provide valuable insights into how the immune system interacts with and responds to alterations in PAPS synthase expression, which is critical for understanding the broader implications of our findings.
- Additionally, we will conduct data analysis, interpretation, and synthesis to draw meaningful conclusions from the gathered results. These efforts will contribute to achieving our Aim1/Task1 research goals and objectives for the upcoming reporting period.

AIM 1 - Major Task 2

- For inhibitor identification, we will further investigate the difference of genomic profiles of all available prostate cancer cell lines, in particular those with distinct difference of PAPSS1/2 expression. Based on such data, we will obtain consensus prediction to identify a manageable number of compounds (e.g., 20-50) that are confidently predicted to have promising bioactivity (IC_{50}<1uM) in cells due to inhibition of PAPSS1. Also based on the structural difference of PAPSS1/2, we will select and design more potent, selective inhibitors towards PAPSS1, not PAPSS2. These compounds will be further validated with cellular and animal models as studied in other Aims, and such experimental validation will provide us feedback to further refine our computational models and guide our rational design.

AIM 2

- The previous reporting period focused largely on developing the technology necessary to conduct the screens necessary to meet program goals. With this accomplished, our focus turns to applying these tools to the cell lines and contexts specified in the proposal.

IMPACT:

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

Major cellular signaling in cancer biology heavily relies on post-translational protein modifications. While phosphorylation, methylation, and acetylation have been extensively studied, the role of sulfation in cellular processes remains vastly unexplored, especially in the context of cancer. Existing literature primarily identifies PAPS synthases as the exclusive sulfate donor generators for downstream sulfation pathways. Additionally, sulfation plays a pivotal role in shaping glycosaminoglycan structures and protein functionality, particularly cytokines. Among the 42 known sulfotransferases catalyzing sulfation reactions post PAPS synthesis, previous research has mainly targeted one sulfotransferase in cancer cells with diverse genetic profiles to understand sulfation's implication in cancer, yielding inconclusive results. The field has encountered limited progress over the past two decades, likely due to scarce reagents available for sulfation studies.

Also, for the first time, we employed our internally developed AI program to screen and identify genomics-based cell line-specific inhibitors based on the difference of PAPSS1/2 expression. Then the result will be further studied through molecular docking to model interactions between compounds and PAPSS1/2. Such integrated approaches robustly ensure the active inhibitors in cells are hitting the desired target. It addresses previous challenges that when a compound is active in cell line, it is not necessarily due to the desired mechanism of action, while some true target inhibitors are not active in cells (e.g., cannot enter the cells).

Our approach is distinctive in its focus on genetically manipulating the upstream PAPSS1 and PAPSS2 genes within the sulfation assimilation pathway. Furthermore, we have identified specific vulnerabilities within unique genetic contexts in various cancer types. This perspective enhances the creation of more effective model systems for assessing the pivotal role of sulfation in cancer cells. In the event that sulfation proves to be a collateral lethal target, this project holds the potential to expedite drug discovery, expediting the translation of laboratory findings into clinical applications for treating prostate cancer patients.

As CRISPR perturbation technology has advanced into genetic interactions, it has become clear that a similar gold standard for synthetic lethals is needed. We reasoned that paralogs that showed synthetic lethality within and across screening platforms are likely to be globally synthetic lethal, analogous to core essential genes, and the fact that 12 of our 13 candidate reference paralogs show more than 70% identity (and all are constitutively expressed) is consistent with this interpretation.

We developed the in4mer platform for arrays encoding four independent gRNA, each with an optimized spacer sequence from the CRISPick algorithm and with diverse but proven DR sequences to minimize the chance of recombination. By targeting single genes with four independent CRISPR guide RNAs, we lower the odds that any single reagent fails to induce the desired phenotype, and having multiple independent gRNA on each array reduces the total number of reagents required to induce reliable gene perturbation.

To construct our Inzolia genome-scale human library, we began with reagents targeting single protein-coding genes and added arrays targeting more than four thousand paralog pairs, triples, and quads, with the in4mer arrays encoding two guides targeting each of the two genes in a pair or one guide per gene in a triple or quad family. Inzolia screens show high (at least 75%) sensitivity to detect synthetic lethals with just two reagents targeting each single knockout and two reagents targeting the double knockout and offer the potential for novel biology arising from three- and four-way paralog synthetic lethals. The Inzolia library is thus a smaller and more efficient whole-genome library that addresses one of the major gaps of monogenic perturbation libraries -- functional buffering by paralogs – that is one of the major areas of focus of this project.

II. What was the impact on other disciplines?

The reagents that the DePinho lab have been created and rigorously validated possess significant potential to greatly enhance the effectiveness and applicability of sulfation assays across a wide spectrum of scientific disciplines and research domains. In addition, the rapid ascendancy of CRISPR-mediated genetic perturbation technologies over RNA interference methods was driven by major advances in assay sensitivity and specificity, with the absence of established gold standards arguably contributing to the shortcomings of RNAi-based studies

of mammalian gene function. We and others have created widely used reference sets of essential and nonessential genes for use in quality control of monogenic loss of fitness screens.

III. What was the impact on technology transfer?
The DePinho Lab has established various cell-line systems for precise assessment of sulfation in the cancer contexts. Notably, they have created reliable tools to investigate crucial upstream genes, namely PAPSS1 and PAPSS2, governing sulfation regulation. In collaboration with the MD Anderson Antibody Core, they've pioneered the development of an exclusive antibody capable of detecting sulfotyrosine in cell lysate extracts via western blotting. The newly developed anti-sulfotyrosine antibody surpasses the commercially accessible counterpart, facilitating the identification of tyrosine sulfation in purified proteins as well as cell lysates. Furthermore, upon successful experimental validation of newly identified PAPSS1 inhibitors, the potential for patenting arises, with prospects for translating this intellectual property into advancements in patient care.

IV. What was the impact on society beyond science and technology?
- Improved Cancer Treatments: Discoveries from the project could lead to more effective cancer treatments, improving the lives of patients and their families.

- Enhanced Public Awareness: Findings may increase public awareness of the importance of certain biological pathways in cancer, potentially leading to earlier detection and prevention efforts.

CHANGES/PROBLEMS:

I. Changes in approach and reasons for change
Nothing to Report

II. Actual or anticipated problems or delays and actions or plans to resolve them
Nothing to Report

III. Changes that had a significant impact on expenditures
Nothing to Report

IV. Significant changes in use or care of human subjects
Nothing to Report

V. Significant changes in use or care of vertebrate animals
Nothing to Report

VI. Significant changes in use of biohazards and/or select agents
Nothing to Report

PRODUCTS:

I. **Publications, conference papers, and presentations**
Nothing to Report

II. Books or other non-periodical, one-time publications.
Nothing to Report

III. Other publications, conference papers, and presentations.
- Invited Talk: Ronald A. DePinho – “Precision Oncology and Tumor Biology”, Champalimaud Foundation, Lisboa, Portugal, 6/15/2022

- Invited Talk: Ronald A. DePinho – “Targeting the Tumor Microenvironment”, University of Kansas Cancer Center Seminar Series, Kansas City, KS, 9/13/2022
- Invited Talk: Ronald A. DePinho – “Targeting the Tumor Microenvironment”, Keck School of Medicine of USC, Translational Genomics, Houston, TX, 10/5/2022
- Invited Talk: Ronald A. DePinho – “Precision Tumor Biology”, Norwegian Oncology Forum, Norway, 11/14/2022
- Invited Talk: Ronald A. DePinho – “Precision Genomics and Tumor Biology”, Connect Science Virtual Seminar Series, Harvard Medical School Dana-Farber Cancer Institute, Houston, TX, 2/14/2023
- Invited Talk: Ronald A. DePinho – “Prostate Cancer Genetics and Immunity”, AACR Special Conference on Advances in Prostate Cancer Research, Denver, CO, 3/15/2023
- Invited Talk: Ronald A. DePinho – “Targeting the Tumor Microenvironment”, Weill Cornell Medicine Pathology and Laboratory Medicine, New York, NY, 4/3/2023
- Invited Talk: Ronald A. DePinho – “The Genetics and Biology of Prostate Cancer”, Gallick Lab Seminar, The University of Texas MD Anderson Cancer Center, Department of Genitourinary Medical Oncology-Research, Houston, TX, 4/24/2023
- Invited Talk: Ronald A. DePinho – “Synthetic Essentiality: Linking Stemness Pathways and Tumor Immunity”, National Cancer Institute, Rockville, MD, 5/1/2023
- Invited Talk: Ronald A. DePinho – “Cancer Genomes and Tumor Biology”, Montefiore Einstein Cancer Center, New York, NY, 5/10/2023
- Invited Talk: Ronald A. DePinho – “Making Cancer History: Advances in Cancer Prevention and Treatment”, Evo Medical, International Cooperation Department, Suzhou, China, 6/3/2023
- Chen, KC, Liu YH, Lin, CC…Muller F, DePinho R. Sulfation is required for prostate cancer xenograft tumor formation but is dispensable for cell viability *in vitro*. Poster presented at The American Association for Cancer Research Annual Meeting; April 4th, 2023; Orlando, FL.

IV. Website(s) or other Internet site(s)
Nothing to Report

V. Technologies or techniques
We have produced an advanced antibody capable of detecting tyrosine sulfation via western blotting and ELISA. Although it's not currently available for commercial purchase, we are open to sharing reagents and protocols upon request. Additionally, we have curated large datasets which have been used to develop machine learning programs employed here for inhibitor discovery. They will be shared through publications and potentially made available online to the public.

VI. Inventions, patent applications, and/or licenses
Nothing to Report

VII. Other Products
Nothing to Report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

I. What individuals have worked on the project?

1. Ronald DePinho, Ph.D. (PI)
2. Traver Hart, Ph.D. (Co-I)
3. Shuxing Zhang, Ph.D. (Co-I)
4. Yonghong Liu, Ph.D. (Postdoctoral Fellow)
5. Beibei Huang, Ph.D. (Postdoctoral Fellow)

6. Ko-Chien Chen (Graduate Student)
7. Lori Bertolet (Research Investigator)
8. Yan Xia (Co-I)