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14. ABSTRACT

Due to inevitable therapeutic resistance, prostate cancer remains a lethal disease, so is critical to delineate mechanisms underlying therapeutic resistance to implement novel treatments that will extend patient survival and quality of life. We hypothesize MYBL2 over-expression facilitates resistance through a mechanism involving induction of lineage plasticity. We will test this hypothesis and determine novel treatment strategies for MYBL2 high prostate cancers and validate the power of MYBL2 expression/activity to predict ADT resistance in prostate cancer samples.

15. SUBJECT TERMS
MYBL2, castrate resistant prostate cancer, aggressive variant prostate cancer, tumor stemness, mouse models, chromatin

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INTRODUCTION

Prostate cancer (PCa) is the second most frequently diagnosed cancer type among men in America. Currently, the cumulative five-year survival rate for patients with prostate cancer is over 98%. Unfortunately, the average survival for men with metastatic or recurrent prostate cancer drops considerably. Second-generation androgen deprivation therapies (ADT) have provided significant life-extending therapies for recurrent, or metastatic castration resistant prostate cancer (mCRPC) patients. A particular resistant, aggressive subset of these mCRPC tumors is independent of AR activity (CRPC-AI). CRPC-AI primarily adapt to ADT via lineage plasticity rather than a result of resistant mutations, adopting a phenotype no longer reliant on AR expression and signaling. These tumors may display neuroendocrine features, a stem or basal cell-like phenotype, altered kinase signaling, and characteristic epigenetic alterations, including upregulation of EZH2. Currently there is no therapeutic option to provide long term durable response to CRPC-AI patients. Therefore, a deeper knowledge of molecular mechanisms driving CRPC-AI will significantly move the field forward to provide discoveries for therapeutic vulnerabilities to successfully inhibit progression or treat CRPC-AI. From this, we have a novel candidate, MYBL2, that may act as a master-regulator transcription factor that upon RB1 LOF drives linage plasticity and resistance to ADT.

KEYWORDS

MYBL2, castrate resistant prostate cancer, aggressive variant prostate cancer, tumor stemness, mouse models, chromatin

ACCOMPLISHMENTS

What were the major goals of the project?

To date the Ellis has accomplished the following major goals:

Major Task #1

1. Breed Mybl2 floxed alleles into DKO mice to generate models of heterozygous (DKO+Mybl2F/+) and homozygous (DKO+Mybl2F/F) Mybl2 loss.
2. Determine phenotype of Mybl2 knockout molecularly and pathologically.
3. Determine the dependence of MYBL2 in human models of CRPC-AI.

Major Task #2

1. Perform in vitro drug screening.

Major Task #3

1. Conduct gene-expression profiling in collaboration with Genome-Dx.
2. Perform gene-expression analysis based on MYBL2 quartiles.
3. Perform Kaplan Meier analysis towards MYBL2 predictive power towards therapy response.

What was accomplished under these goals?

Major Task #1

1. We successfully generated all required mouse genotypes (DKO, DKO:Mybl2+/-, and DKO:Mybl2-/-) and conducted both our 38-40 week endpoint and overall survival studies (Fig. 1).

Upon conclusion of the endpoint study and during our survival study we had performed regular validation of mouse genotypes that had been sacked prior to further analysis. During this, we had noticed that use of recombination PCR to ensure our target genes have been targeted correctly, we unfortunately observed in some of our mice that desired genes, especially Mybl2, had not been genetically knocked out completely (Fig.2). Due to this we will not move forward with collected samples and all mouse colonies have been ended and their generation has started again.

To compensate for this loss, we have been generating Mybl2 KO cell lines using our murine cell lines and Crispr technology. While the DOD award focuses of Mybl2 KO within the DKO cells/tumors we are expanding the generation of Mybl2 KO in multiple genotypes from our mouse cell lines. These include from the application SKO (Pten KO) and DKO (Pten:Rb1 KO). In addition we will use DKO-CR (Castrate Resistance

Pten:Rb1:Trp53^{mut}), and TKO (Pten:Rb1:Trp53 KO). We are also currently generating MYBL2 KO in our described human cell lines from the application. Currently, we have successfully generated and validated DKO:Mybl2 KO cell lines and have determined the dependence of Mybl2 for anchorage independent growth and self-renewal (stemness) of DKO cells (Fig. 3). This work continues and will be finalized in the very near future.

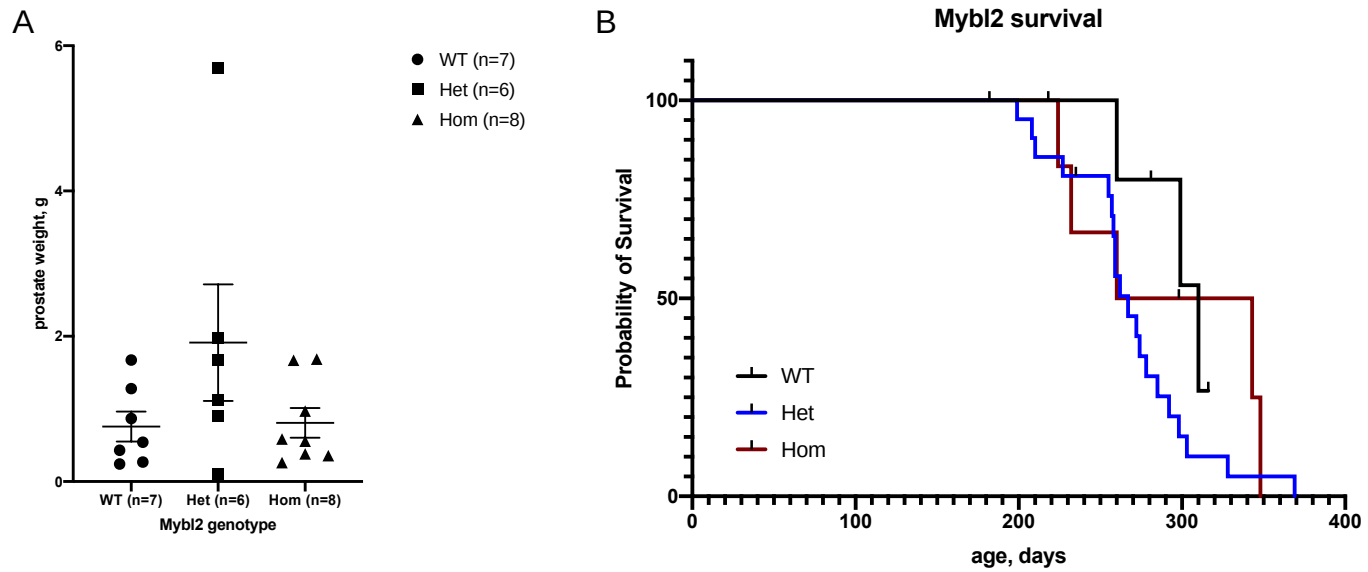

Figure 1: (A) Genetically engineered mouse models representing PbCre:Pten$^{L/L}$:Rb1$^{L/L}$ (DKO, wt for Mybl2), PbCre:Pten$^{L/L}$:Rb1$^{L/L}$:Mybl2$^{L/+}$ (Mybl2 heterozygous deletion) and PbCre:Pten$^{L/L}$:Rb1$^{L/L}$:Mybl2$^{L/L}$ (Mybl2 homozygous deletion) were aged to 30 weeks and genitourinary tracts were weighed. (B) Same genotypes per (A) were generated and placed into a survival analysis. All experiments and data collection were discontinued due to Cre functional issues demonstrated in Fig. 2.

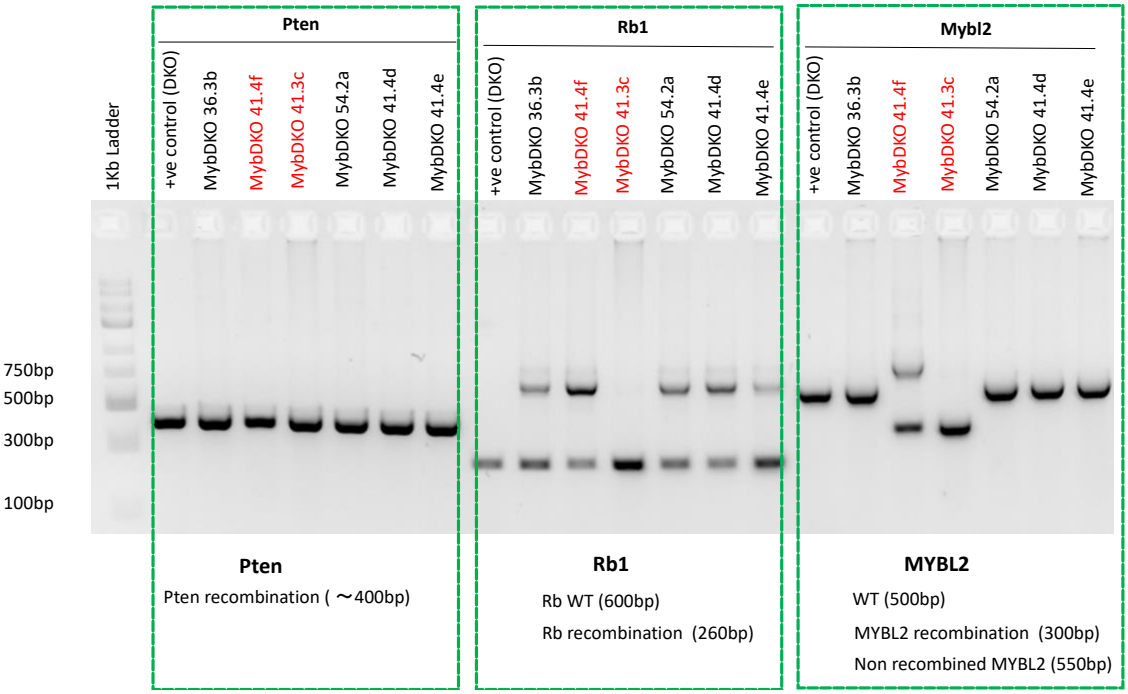

Figure 2: (A) Genetically engineered mouse models representing the genotype PbCre:Pten$^{L/L}$:Rb1$^{L/L}$:Mybl2$^{L/L}$. Using recombination PCR, we demonstrate all tumors show complete recombination of Pten (homozygous Pten deletion). However, only 1 tumor demonstrates homozygous recombination of Rb1 (mouse 41.3c). The same mouse – 41.3 – is the only mouse that also resulted in homozygous recombination of Mybl2.

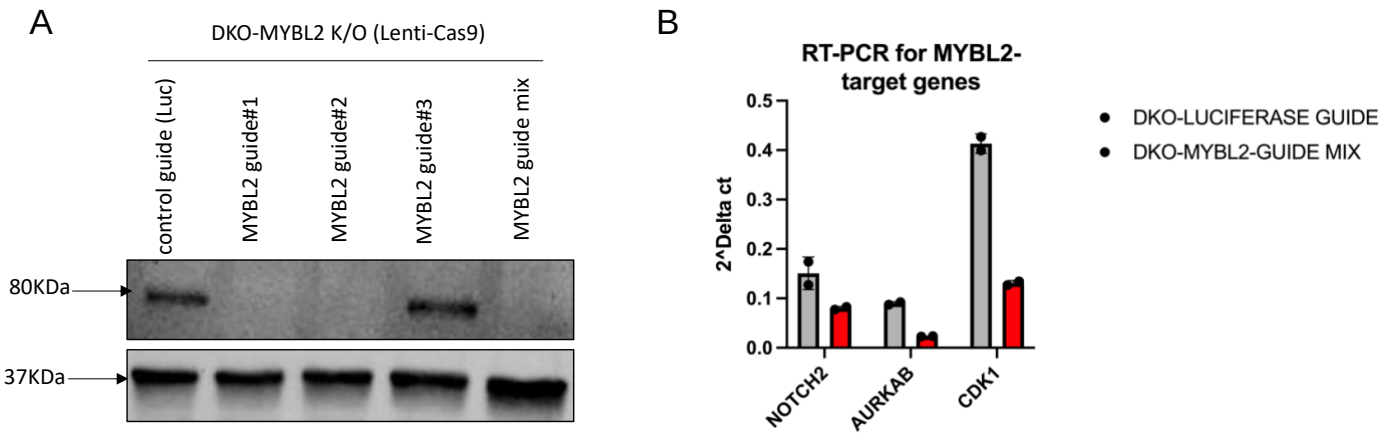

Figure 3: (A) Western blot analysis of Pten:Rb1 KO 2D cell lines (DKO) for Mybl2 expression. We show that we have successful KO of Mybl2 using the above sgRNA's designed towards Mybl2. Note – only Mybl2 guide mix DKO cells maintained stable KO of Mybl2, therefor these cells have been chosen for future experiments, and the guide mix will be used to generate all remaining Mybl2 KO cell lines. (B) Real-time PCR analysis for Mybl2 target genes providing functional validation of Mybl2 KO in DKO cells.

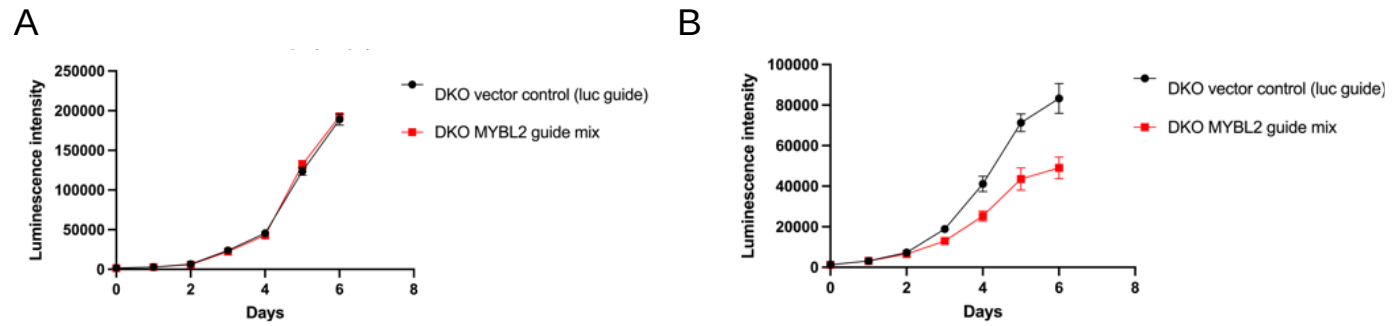

Figure 4: (A) Two-dimensional assays show that Mybl2 KO does not negatively affect growth of DKO cells. (B) Three-dimensional spheroid assays indicate the Mybl2 KO does significantly inhibit anchorage independent growth and self-renewal (stemness) of DKO cells.

We have completed the generation of DKO:control and DKO:Mybl2 KO cell lines. We further generated a second independent model using Pten:Tp53 KO (PPKO) cell lines – PPKO:control and PPKO:Mybl2 KO cells. We first repeated our 3D GILA assay to validate Mybl2 dependence in DKO cells and compare this dependence in PPKO cells. The GILA assay which assesses anchorage independent growth and regenerative/stemness potential of a cell validated the need for Mybl2 by both genotypes indicating that Mybl2 largely contributes to these attributes of these cells (Fig 5A). However, we don't observe exhaustion of growth, instead we only slow it down. This observation is consistent with loss of overall spheroid size (Fig 5B) and DNA synthesis (Fig 5C). Most importantly, transplant of these cells to mice demonstrated that significant inhibition of tumor initiation and growth occurred (Fig 5D. Overall, these data validate the dependence of Mybl2 expression and function towards the aggressive phenotypic plasticity emerging in therapy resistant prostate cancer. Because Mybl2 is a transcription factor that drives gene expression, we carried out RNA sequencing from our genetically defined cell lines. Because significant differences were noticed in 3D culture, we generated spheroids for RNA extraction. Current analysis from our genomics core facility of control verse KO for both models using Euclidean Hierarchical Clustering and Principal Component Analysis demonstrates significant difference at the gene expression level when Mybl2 is KO from DKO and PPKO cells (Fig 5E). This again demonstrates the important function of Mybl2 towards this aggressive phenotype. We are continuing our RNA sequencing to further delineate exact gene targets important for Mybl2.

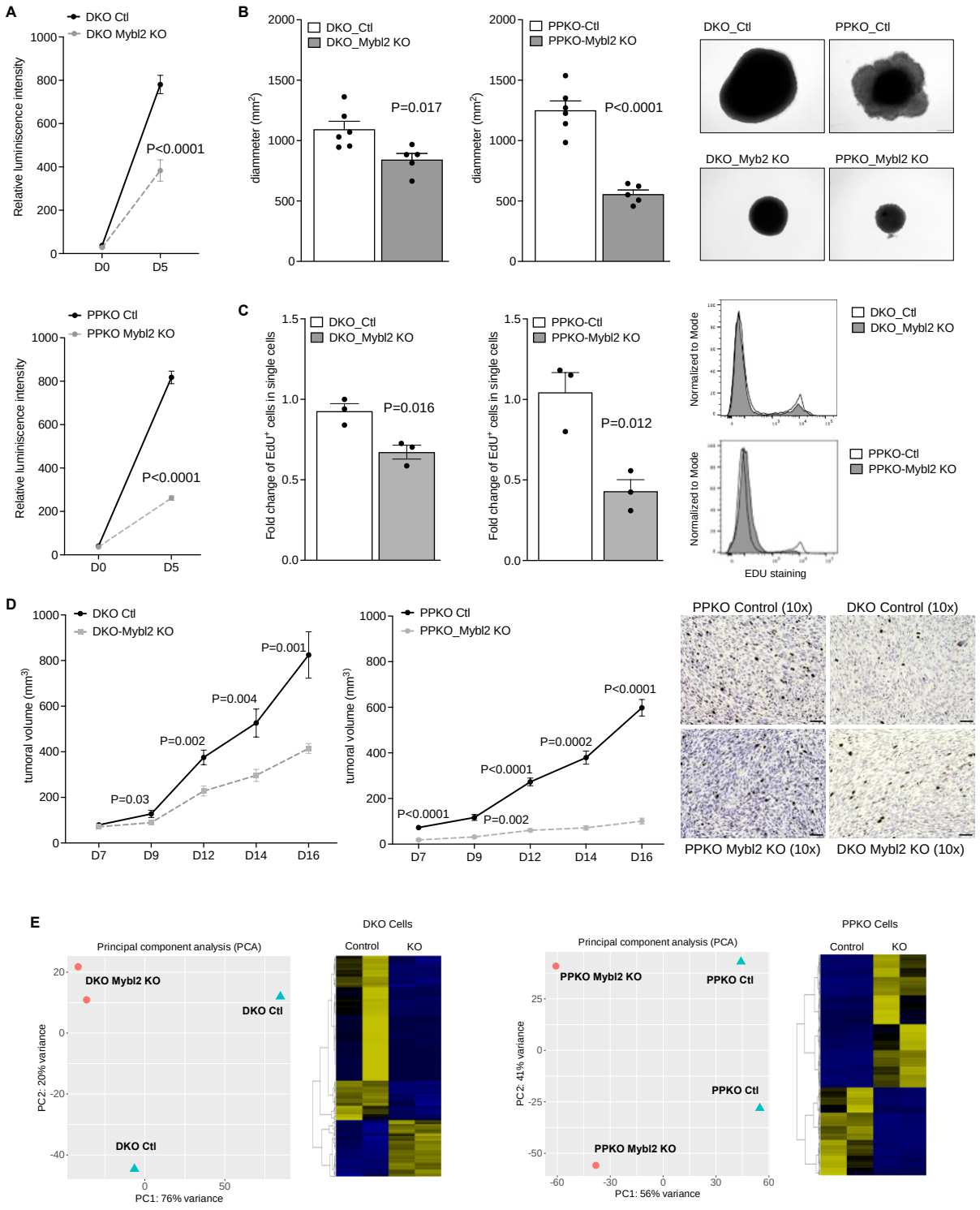

Figure 5: (A) Representation of the gila assay of murine DKO and PPKO control cells and Mybl2 KO cells at day 5. (B) Diameter quantitation of spheroids from A. Representative pictures of the generated spheroids in control cells (DKO and PPKO) and Mybl2 KO cells. (C) Flow cytometry analysis of the incorporation of EdU in control and Mybl2 KO cells. Analysis performed 1 hour after EdU addition. (D) Control and Mybl2 KO tumoral growth curves and Ki67 staining (n = 5 mice per treatment group, +/-1SEM). (E) Initial bulk RNAseq analysis from control and Mybl2 KO spheroids including principal component analysis (PCA) and the Euclidean Hierarchal Clustering analysis of the top 500 genes.

Major Task #2

1. Perform in vitro drug screening.

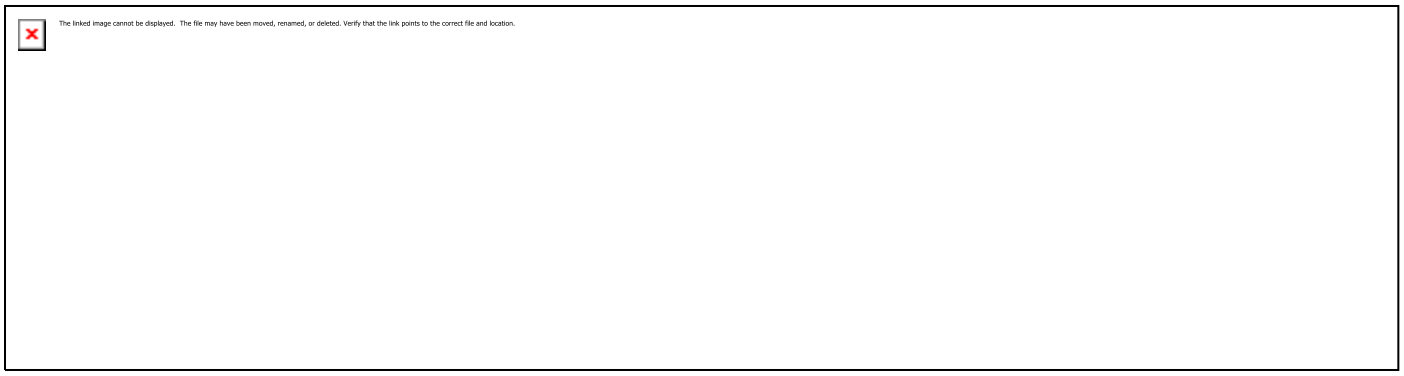

Figure 6: A Mybl2 target gene signature was uploaded to the Broad Institute Cancer Dependency Map to identify current therapeutic targets to be tested in NEPC models.

We had initially proposed to test response to numerous drug target families based off our data gained from the Broad Cancer Dependency Map database following the upload of a Mybl2 target gene signature (Fig. 6). It was our initial plan to test therapies targeting PI3K/mTORC, HSP90, DNA replication, and CDKs. With lost time due to the lab move and time to hire new staff, it was decided that our therapy focus would be towards CDKs. With a past of negative data in CRPC clinical trials including PI3K/mTORC, HSP90, and DNA replication, it appeared targeting CDKs would be the most novel and innovative direction to follow. As in Fig. 6 we proceeded to test our mouse cell line model response to the pan-CDK inhibitor PHA-793887.

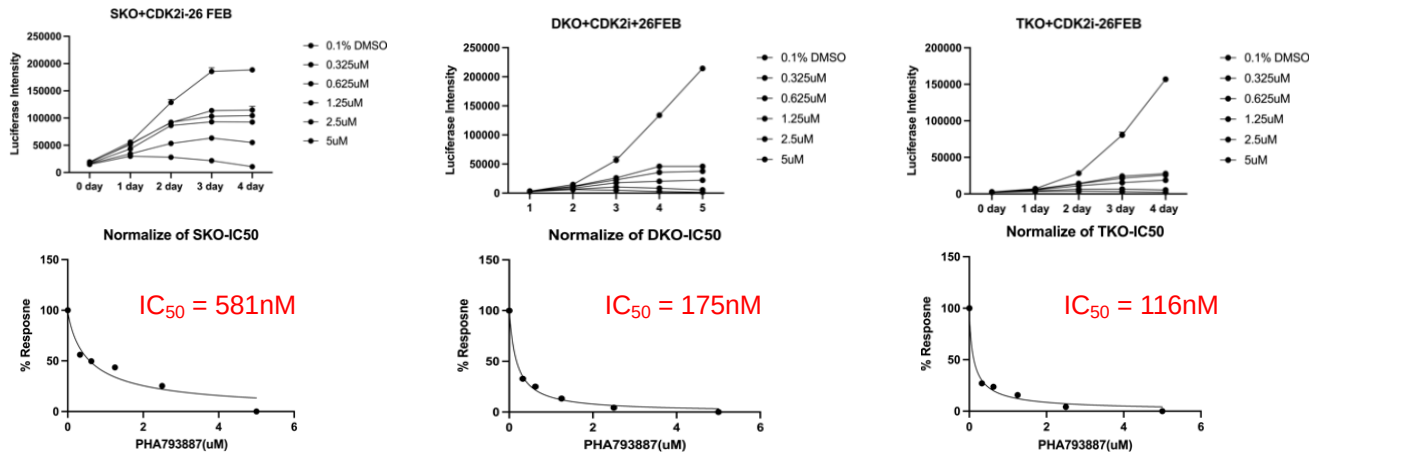

Figure 7: Dose response analysis of SKO (Pten KO), DKO (Pten:Rb1 KO), TKO (Pten:Rb1:Tp53 KO) cell lines to the pan CDK inhibitor PHA793887.

Data in Fig. 7 clearly shows that all cell lines to have a robust response to pan CDK inhibition, as expected for any cycling cell. However, from these initial results it was clear that cell lines with a NEPC genotype (Rb1 and/or Tp53 KO) displayed a more significant response to this inhibition. To investigate if all proposed CDKs from Fig. 6 were necessary for therapeutic targeting, we further used the Broad's Dependency Map. Upon a specific look at all human prostate cancer cell lines available, it was clear that only CDK2 was a possible synthetic lethal dependency in the human NEPC cell lines – H660 (Fig. 8A). These data further encouraged us to focus on targeting CDK2 in our mouse cell lines. We tested 2 independent CDK2 inhibitors – AUZ454 and BGG463. First, AUZ454 did show inhibition in all cell lines indicating a potential low specificity for CDK2 and potential off target effects (Fig. 8B). Second, BGG463 exclusive sensitivity towards DKO and TKO cell lines compared to SKO. With this, we are continuing to generate both single agent and combination therapy results using our proposed mouse and human cell lines.

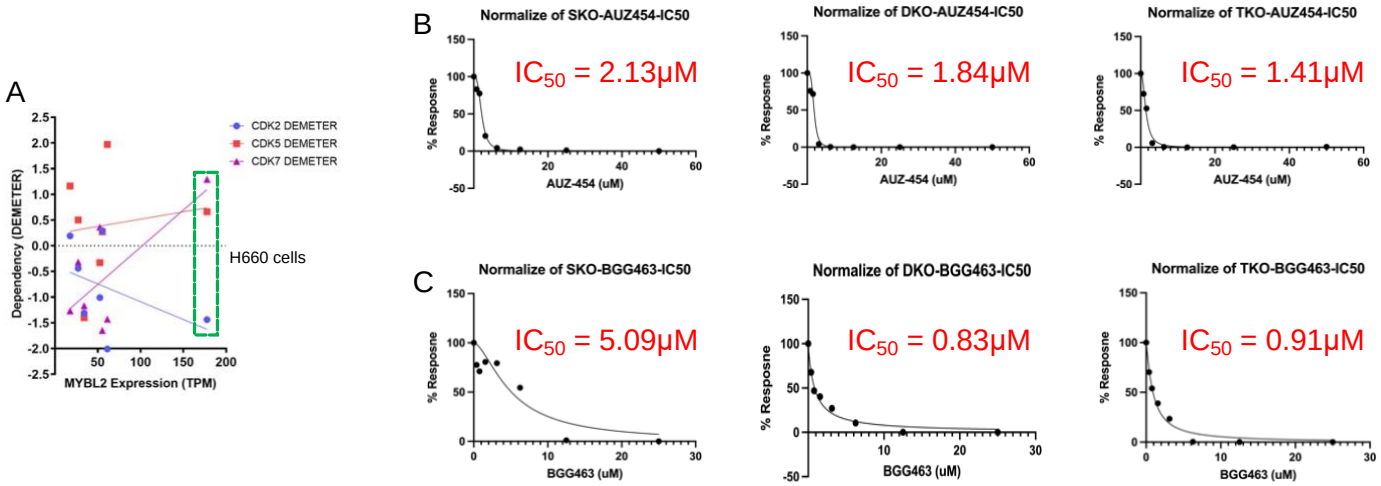

Figure 8: Using the Broad Institute's Cancer Dependency website, it was determined that CDK2 was the only CDK2 indicating dependency for the human NEPC cell line – H660.

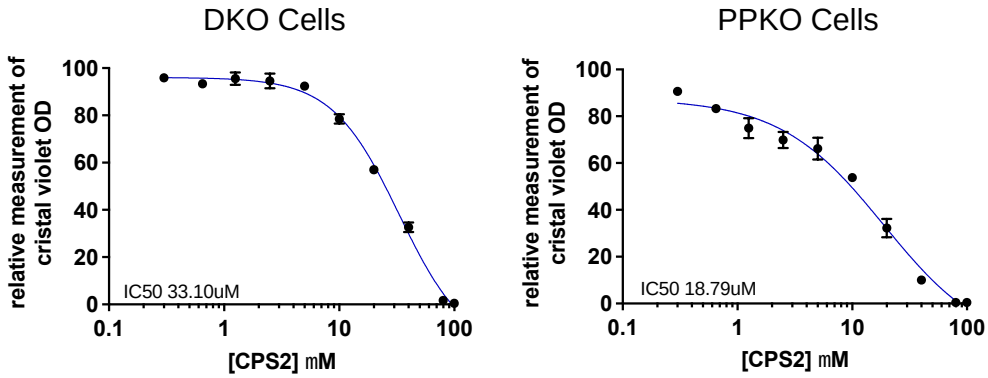

Figure 9: DKO (left) and PPKO (right) cells were treated with the CDK2 degrader – CPS2 at indicated doses for 72h.

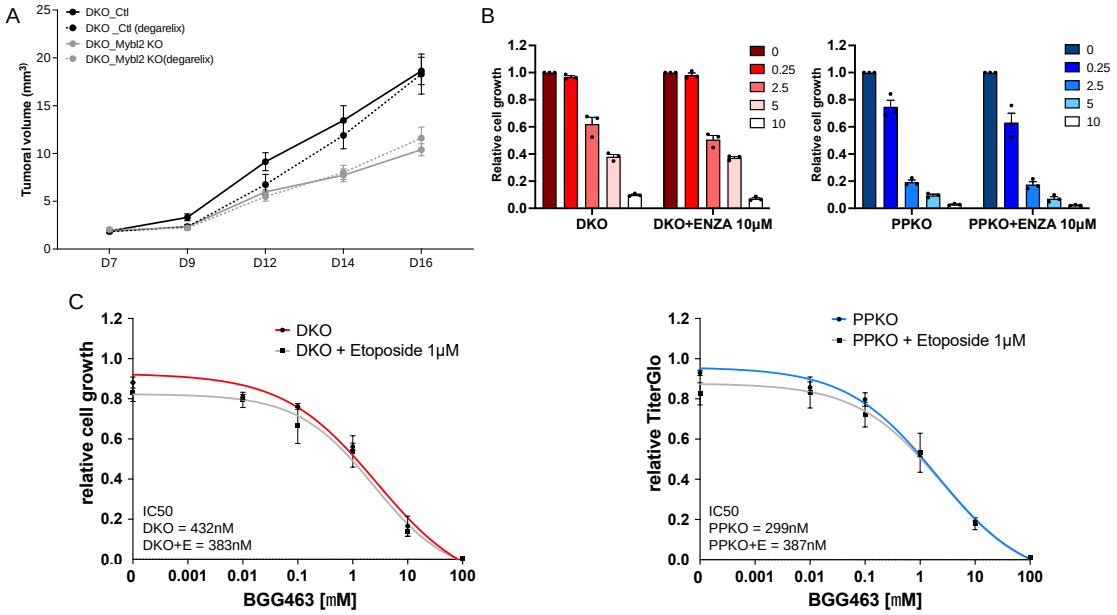

Figure 10: (A) C57BL/6 male mice were had DKO cells injected subcutaneously. Tumors were treated with either vehicle or degarelix to chemically castrate mice. Loss of Mybl2 expression/function does not reverse resistance to castration. (B) DKO (left) or PPKO (right) cells were treated with indicated doses of the CD2i – BGG463 with or without 10μM enzalutamide for 72h. (C) DKO (left) or PPKO (right) cells were treated with the CDK2i BGG463 with (grey lines) or without (red-DKO, blue-PPKO) the DNA damaging chemotherapy etoposide for 72h.

In addition to the 3 independent CD2Ki trialed previously, we also tested response of DKO and PPKO cells to the CDK2 degrader – CPS2 (Fig. 9). While the CDK2i, especially BGG463 resulted in low nanomolar IC50 concentrations within lineage plasticity models (DKO, TKO) (Fig. 8), the lineage plasticity models (DKO, PPKO) were less sensitive to the CDK2 degrader CPS2 with IC50 concentrations of 33.1μM and 18.79μM respectively (Fig. 9). Deciding that the use of a CDK2 degrader would be more novel/innovative, we have gone ahead and purchased a larger quantity of CPS2 to conductor in vivo preclinical experiments.

Further, we tested the ability if genetic loss of Mybl2 or CDK2 inhibition would sensitize lineage plasticity models DKO and PPKO to the androgen receptor antagonist enzalutamide or etoposide chemotherapy. In both situations we were unable to show additive or synergistic combination effect (Fig. 10). Overall, we will now pursue full in vivo studies focused on the ability of CPS2 (CDK2 degradation) to act as a monotherapy for phenotypic plasticity prostate cancer. With success, we will be able to then pursue molecular studies to understand/identify molecular pathways of resistance to CPS2 that will be tested and validated as viable combination approaches. In addition, this also highlights that a MYBL2-CDK2 axis does not drive phenotypic plasticity via done regulation of androgen receptor expression/function, but rather via alternate signaling like stemness/pluripotency-based mechanisms. Towards this, we performed RNA sequencing in DKO and PPKO spheroid models with or without Mybl2 to determine exact pathways driven by Mybl2. These data have just been received and is currently with our computational staff for full analysis (Fig. 5).

Major Task #3

1. Conduct gene-expression profiling in collaboration with Genome-Dx.
2. Perform gene-expression analysis based on MYBL2 quartiles.
3. Perform Kaplan Meier analysis towards MYBL2 predictive power towards therapy response.

Using gene expression analysis performed from prostate cancer patient samples from the CHAARTED trial led by Dr. Christopher Sweeney – MYBL2 gene expression was determined, and patients were ranked by quartile distribution. Kaplan-Meier analysis was performed based on patient overall survival and time to development of CRPC. There was no significant predictive value for determination of patients who would progress to CRPC more rapidly. However, for overall survival analysis, MYBL2 gene expression were a significant indicator of patients with a survival advantage in both treatment arms (low verse high). Even within the treatment groups, MYBL2 expression levels indicated patients had an overall survival advantage with ADT + docetaxel combination therapy (Fig. 11).

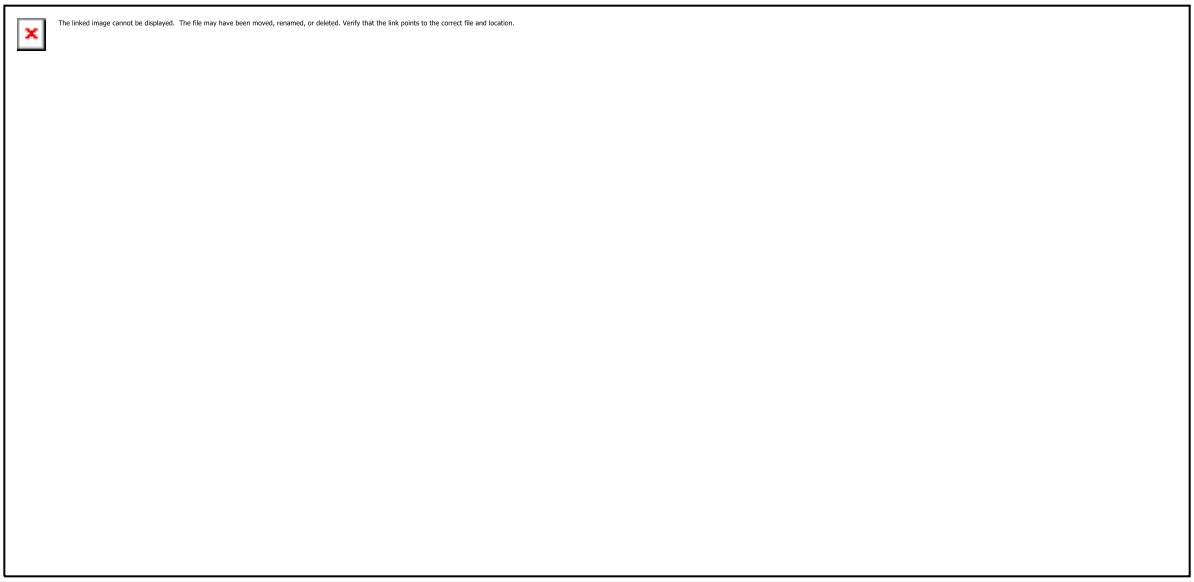

Figure 11: Data represented by lowest quartile for MYBL2 (low) verse all remaining quartiles (high) from CHAARTED patients. (Left) MYBL2 gene expression significantly determines overall survival benefit in both patient cohorts. (Right) MYBL2 gene expression does not significantly determine patients who will progress to castration resistance more rapidly.

This data analysis is now complete in collaboration with Dr. Christopher Sweeney. From a separate DOD application led by Dr. Sweeney (and publication) it was reported that high MYBL2 expression was associated with higher rate of relapse in localized disease and poorer overall survival in patients with metastatic disease in CHAARTED. Because of this we will use a MYBL2 gene signature that does outperform analysis using just MYBL2 expression as demonstrated by using SU2C and TCGA datasets (Fig 12).

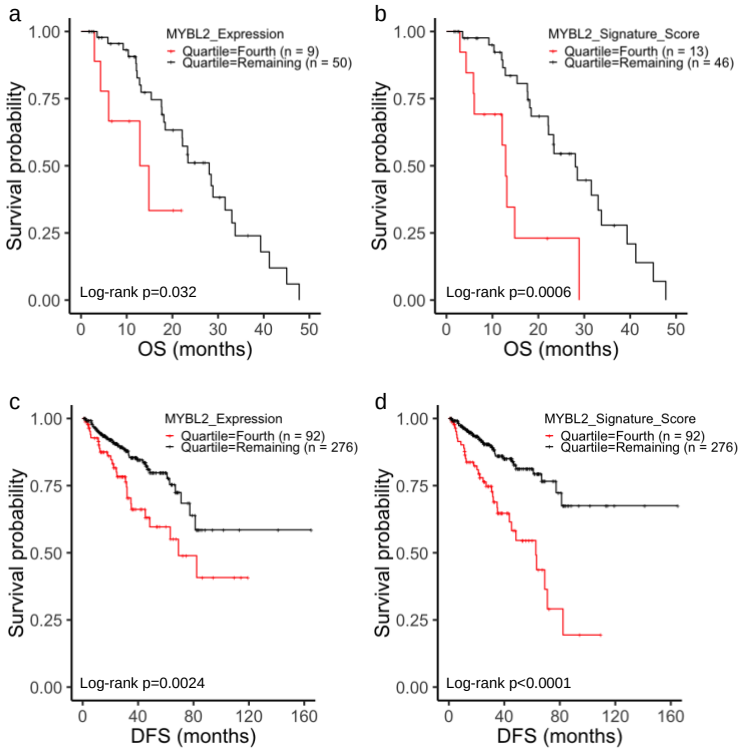

Figure 12: (a-b) Kaplan-Meier survival analysis indicating SU2C CRPC with increased MYBL2 expression/activity have significantly shorter overall survival. (c-d) Kaplan-Meier survival analysis indicating TCGA primary prostate cancer with increased MYBL2 expression/activity have significantly shorter disease-free survival.

What opportunities for training and professional development has the project provided?
The project has provided opportunities for postdoctoral scientists to currently present this data at internal meetings. Further, learning the design robust/rigorous experimental design and analysis and thinking about next steps as been invaluable for them. This will be converted to fellowship applications by the postdocs.

How were the results disseminated to communities of interest?
The data has been disseminated by the postdocs and PI at internal institute meetings. Also, the PI has included selected data in invited national and international talks which have consisted to scientific communities conducting prostate and non-prostate research and patient care.

What do you plan to do during the next reporting period to accomplish the goals?
We will continue to conduct in vitro and in vivo experiments to determine the role of Mybl2 in driving tumor cell aggressiveness and resistance to ADT. We will continue to finalize the data and prepare a manuscript for submission to BioRxiv and a peer reviewed journal. The postdocs will also continue to present data internally and also begin to present this work and local and national level meetings.

IMPACT
What was the impact on the development of the principal discipline(s) of the project?
Experimental design, data analysis and interpretation, integration of data with current literature to determine next steps, presenting data.

What was the impact on other disciplines?
Nothing to report.

What was the impact on technology transfer?
Nothing to report.

What was the impact on society beyond science and technology?
Nothing to report.

CHANGES/PROBLEMS
Changes in approach and reason for change.
Because of our loss of GEMMs with our move to LA we aim to compensate these experiments using in vivo transplant experiments and in vitro 3D assays using already established 2D lines from our GEMMs.

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

Changes that had 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.

PRODUCTS
Publications, conference papers, and presentations
Journal publications
Nothing to report.

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

Other publications, conference papers, and presentations
Nothing to report.

Website(s) or other internet site(s)
Nothing to report.

Technologies or techniques
Nothing to report.

Inventions, patent applications, and/or licenses
Nothing to report.

Other products
Nothing to report.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS
What individuals have worked on the project?

Name:	Dr. Leigh Ellis
Project Role:	Co-PI
Researcher Identifier:	0000-0003-4739-5049
Nearest person month worked:	3
Contribution to project:	Co-PI
Funding Support:	NCI, DOD

Name:	Dr. Christopher Sweeney
Project Role:	Co-PI
Researcher Identifier:	
Nearest person month worked:	3
Contribution to project:	Co-PI
Funding Support:	NCI, DOD

Name:	Dr. Jagpreet Nanda
Project Role:	Postdoc Scientist
Researcher Identifier:	
Nearest person month worked:	6
Contribution to project:	Postdoc Scientist
Funding Support:	

Name:	Dr. Beatriz German
Project Role:	Postdoc Scientist
Researcher Identifier:	
Nearest person month worked:	6
Contribution to project:	Postdoc Scientist
Funding Support:	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to report.

What other organizations were involved as partners?
Nothing to report.

SPECIAL REPORTING REQUIRMENTS
Nothing to report.

APPENDICES
Nothing to report.