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14. ABSTRACT
Androgen deprivation therapy (ADT) is the current standard of care for metastatic prostate cancer, however, ADT fails to provide a durable response in 90% of patients due to acquired resistance and progression to castration resistance prostate cancer. Aggressive variant prostate cancer (AVPC) is a lethal phenotype that arises upon treatment with ADT, resulting in a lineage switch to a neuroendocrine morphology and the acquisition of molecular alterations that enable the cells to survive independent of AR signalling. Our work, and the work of others, has established that combinatorial loss/mutation of tumor suppressor genes – PTEN, TP53 and RB1 – drive lineage plasticity and therapeutic resistance to ADT. Further, epigenetic regulators, including EZH2, are indicated as driver effectors of lineage plasticity downstream of these key combinatorial genetic events. With the known activation PI3K/AKT/mTOR signalling due to PTEN and the data supporting epigenetic targeting of EZH2, it remains to be determined if co-inhibition of PI3K/AKT/mTOR and EZH2 will provide a significant response in AVPC patients. This project focuses on utilizing preclinical mouse and human models of AVPC to investigate the combinatorial effect of PI3K/AKT/mTOR and EZH2 inhibition on delaying progression to, or reversing, castration resistant disease.

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1. INTRODUCTION:

Androgen deprivation therapy (ADT) is the current standard of care for metastatic prostate cancer; however, ADT fails to provide a durable response in 90% of patients due to acquired resistance and progression to castration resistance prostate cancer. Aggressive variant prostate cancer (AVPC) is a lethal phenotype that arises upon treatment with ADT, resulting in a lineage switch to a neuroendocrine morphology and the acquisition of molecular alterations that enable the cells to survive independent of AR signalling. Our work, and the work of others, has established that combinatorial loss/mutation of tumor suppressor genes – PTEN, TP53 and RB1 – drive lineage plasticity and therapeutic resistance to ADT. Further, epigenetic regulators, including EZH2, are indicated as driver effectors of lineage plasticity downstream of these key combinatorial genetic events. With the known activation PI3K/AKT/mTOR signalling due to PTEN and the data supporting epigenetic targeting of EZH2, it remains to be determined if co-inhibition of PI3K/AKT/mTOR and EZH2 will provide a significant response in AVPC patients. This project focuses on utilizing preclinicalmouse and human models of AVPC to investigate the combinatorial effect of PI3K/AKT/mTOR and EZH2 inhibition on delaying progression to, or reversing, castration resistant disease.

2. KEYWORDS:

- Prostate Cancer
- Aggressive Variant Prostate Cancer
- Castrate-Resistant Prostate Cancer
- Lineage Plasticity
- Androgen Deprivation Therapy
- Androgen Receptor
- Therapy Resistance
- PTEN
- PI3K
- AKT
- mTOR
- EZH2
- Epigenetics
- In Vivo Models

3. ACCOMPLISHMENTS:

Experimental goals:

		% completed
1	**Undertake a pilot *in vivo* study to guide tumor seeding density for major *in vivo* experiments**	
	Submit documents for Animal Care and Use Review Office (ACURO) approvals for all experiments involving animals.	*100%*

	Subcutaneous implantation of tumor cells different cell densities into the flank of NOD Scid/C57BL6J mice to establish an optimum seeding density for xenograft tumor studies.	*100%*
2	**Investigate the efficacy of PI3K/AKT/mTOR and/or EZH2 inhibition to reverse the AVPC phenotype and ADT resistance in vivo**	
	Generate Genetically Engineered Mouse Model (GEMM) and human neuroendocrine PCa-derived xenografts by subcutaneous implantation of organoids into the flank of NOD.	*100%*
	Treat newly generated xenograft models with PI3K/AKT/mTOR- i and EZH2-i as a monotherapy, and in combination with enzalutamide.	*100%*
	Process tumor samples to formalin fixed paraffin embedded (FFPE) blocks. Perform hematoxylin and eosin (H&E) and immunohistochemistry (IHC) staining of slides.	*100%*
	Send tumor samples to the Molecular Biology Core Facility (MBCF) for RNA-seq. Coordinate with the MBCF to analyze effect of combination therapy by differential gene expression, hierarchical clustering, principal component analysis, and gene set enrichment analysis.	*0%*
	Use qRT-PCR to examine expression levels of genes identified from analysis of RNA-seq, in GEMM-derived prostate cancer organoid lines.	*100%*
3	**Investigate the efficacy of PI3K/AKT/mTOR and/or EZH2 inhibition to prevent the AVPC phenotype and prolong ADT sensitivity in vivo.**	
	Generate Genetically Engineered Mouse Model (GEMM) and human neuroendocrine PCa-derived xenografts by subcutaneous implantation of organoids into the flank of NOD Scid mice.	*100%*
	Treat newly generated xenograft models with PI3K/AKT/mTOR-i and EZH2-i as a monotherapy, and in combination with enzalutamide.	*100%*
	Process tumor samples to formalin fixed paraffin embedded (FFPE) blocks. Perform hematoxylin and eosin (H&E) and immunohistochemistry (IHC) staining of slides.	*100%*
	Send tumor samples to the Molecular Biology Core Facility (MBCF) for RNA-seq. Coordinate with the MBCF to analyze effect of combination therapy by differential gene expression, hierarchical clustering, principal component analysis, and gene set enrichment analysis.	*0%*
	Use qRT-PCR to examine expression levels of genes identified from analysis of RNA-seq, in GEMM-derived prostate cancer organoid lines.	*100%*
4	**Investigate the efficacy of PI3K/AKT/mTOR and EZH2 inhibition in combination with castration in PbCre4:Ptenfl/fl:Rb1fl/fl GEMMs**	
	Generate experimental PbCre4:Ptenfl/fl:Rb1fl/fl male mice for further experiments	*100%*

	Age mice to 30 weeks of age and perform sham or surgical castration, then treat mice with PI3K/AKT/mTOR and/or EZH2 inhibitors for 28 days.	*100%*
	Send tumor samples to the MBCF for RNA-Seq. Coordinate with the MBCF to analyze effect of combination therapy by differential gene expression, hierarchical clustering, principal component analysis, and gene set enrichment analysis.	*0%*
	Process tumor samples to formalin fixed paraffin embedded (FFPE) blocks. Perform hematoxylin and eosin (H&E) and immunohistochemistry (IHC) staining of slides.	*100%*
5	**Generation of manuscript(s) outlining the efficacy of targeting PI3K/AKT/mTOR and EZH2 pathways to inhibit and/or reverse AVPC in xenograft and GEM models.**	*100%*

Accomplished experimental goals:

1. *Undertake a pilot in vivo study to guide tumor seeding density for major in vivo experiments.*

Initial pilot experiments were intended to be carried out to determine the ideal seeding density for GEMM and human-derived cell lines for this project. However, as part of another project in the laboratory several organoid and 2D cell lines were grown as xenograft tumors to test ideal seeding density for mouse studies. It was decided that these results would be used to determine ideal seedingdensity for experiments carried out for this project.

Tumor cells were evenly distributed in 100µL of 50% Matrigel in sterile PBS and injected subcutaneously into the upper flank of male mice (6-8 weeks old). Tumors were allowed to establish undisturbed for one week before measurements were started. For tumors established in castrated mice, castration occurred one week before tumor cell implantation. GEMM-derived tumor lines were implanted into C67BL/6N mice while human neuroendocrine tumor-derived WCM154 tumor cellswere implanted into immune compromised NOD.CB17-Prkdcscid/NCrCrl. Ideally, tumors would grow to 1500-2000 mm^3 within 30 days without ulceration, as planned therapy studies were to be conducted over a 30-day period.

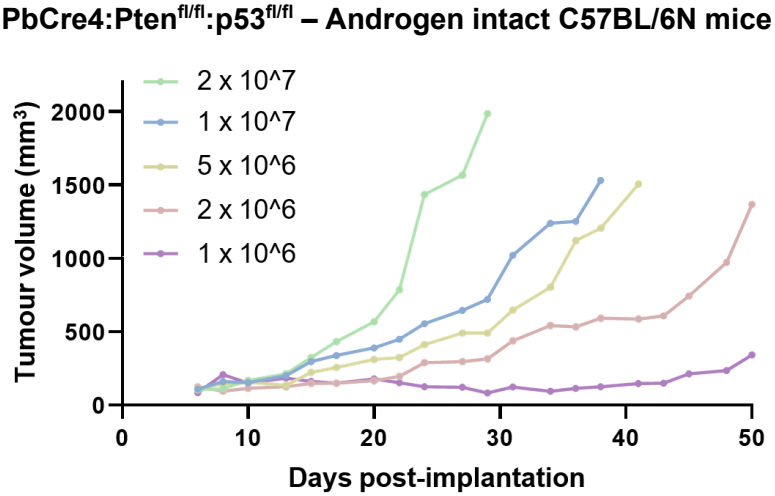


Figure 1: Tumor growth curves for PbCre4:Ptenfl/fl:p53fl/fl allograft tumors, at different seeding densities, grown in androgen intact male C57BL/6N mice.

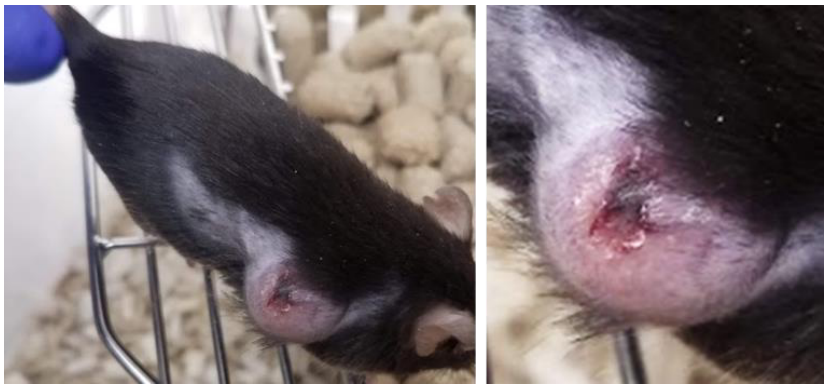


Figure 2: Ulceration of PbCre4:Pten$^{fl/fl}$:p53$^{fl/fl}$ allograft tumor seeded at 2 x 10^7 cells, due to rapid tumor growth.

The tumor seeded at 2 x 10^7 cells grew rapidly in less than 30 days. However, it was determined that tumors seeded that this cell density would grow too rapidly, resulting in ulceration of the tumor, limiting viable tissue that could be used for analysis (Figure *2*). For PbCre4:Pten$^{fl/fl}$:p53$^{fl/fl}$ tumors in androgen-intact C57BL/6N mice, it was identified that 5-10 x 10^6 cells per mouse would be the ideal seeding density used in future experiments. Viable tumor tissue was collected, resected, and banked as small frozen tumor pieces (2 x 2 mm), in the unanticipated situation that cell lines are not able to establish viable tumors, the frozen tumor pieces are able to be implanted subcutaneously to establishallograft tumors.

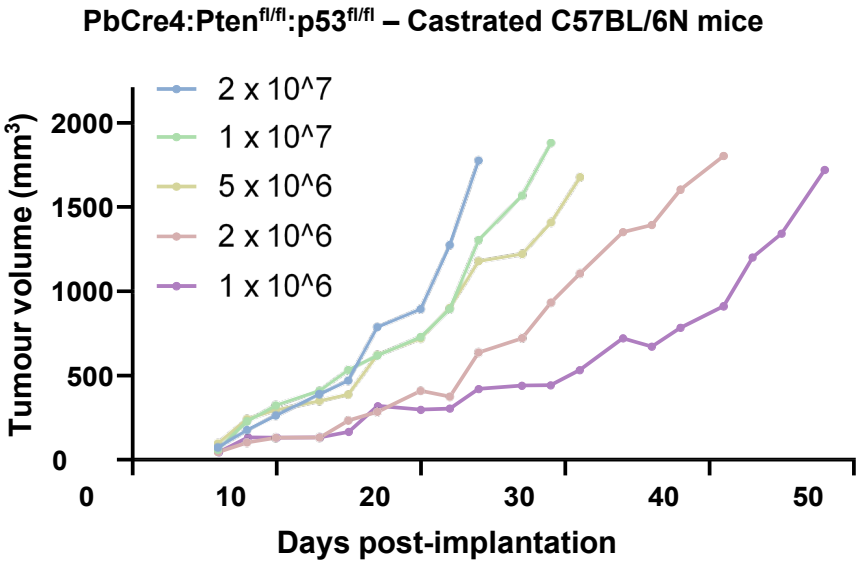


Figure 3: Tumor growth curves for PbCre4:Ptenfl/fl:p53fl/fl allograft tumors, at different seeding densities, grown in castrated male C57BL/6N mice.

In general, the castrated PbCre4:Pten$^{fl/fl}$:p53$^{fl/fl}$ allograft tumors grew at a more rapid pace compared to the intact allograft tumors (Figure *3*). This was not an unexpected result as castrate-resistant tumors are considered to be more aggressive than androgen sensitive tumors. For PbCre4:Pten$^{fl/fl}$:p53$^{fl/fl}$ tumors in castrated C57BL/6N mice, it was identified that 5-10 x 10^6 cells per mouse would be the ideal seeding density used in future experiments.

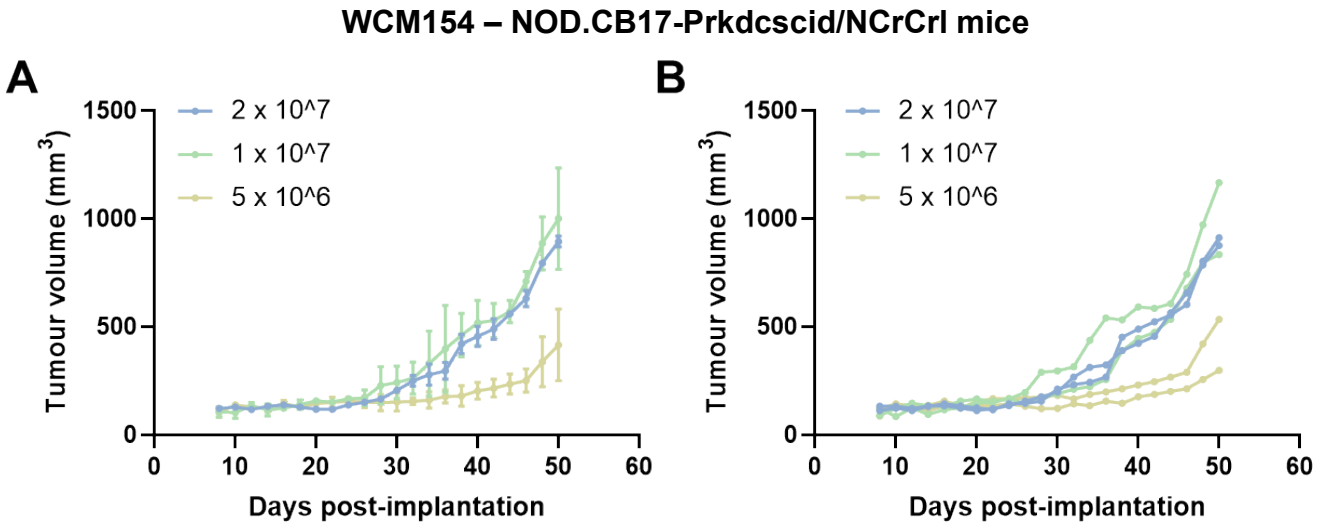


Figure 4: (A) Mean and (B) individual tumor growth curves for WCM154 xenograft tumors, at different seeding densities, grown in NOD.CB17-Prkdcscid/NCrCrl mice (n=2 mice per seeding density, +/-1SD).

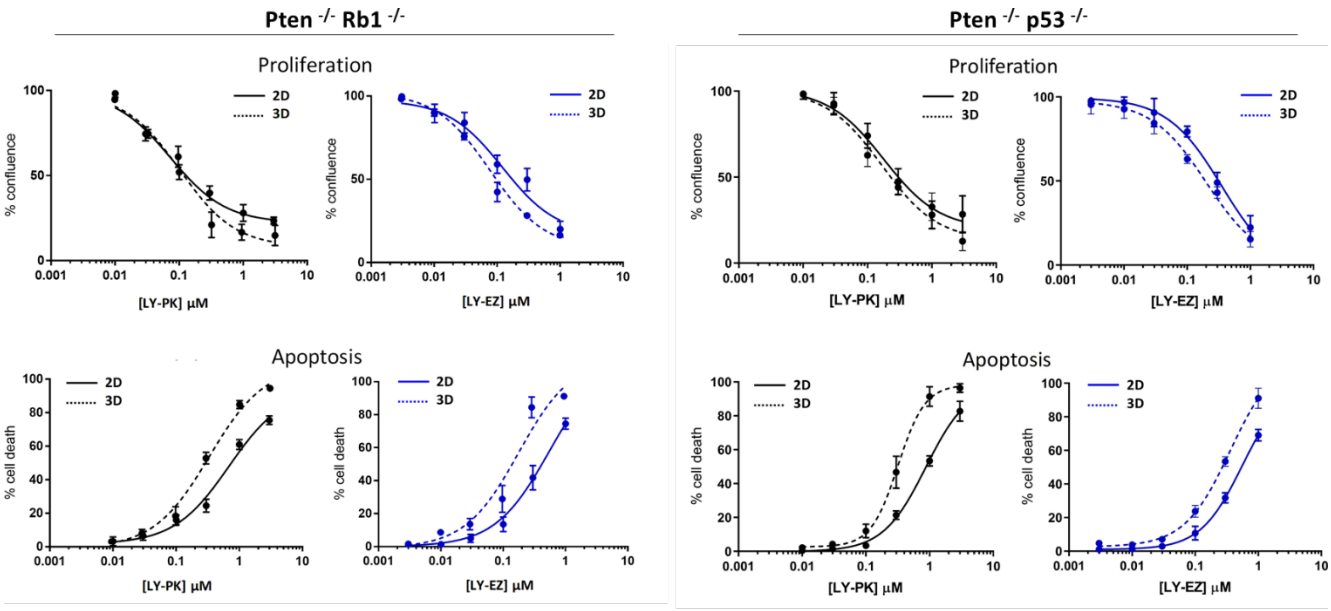
WCM154 xenograft tumors grew at a much slower rate compared to the GEMM and no seeding density grew to 1500-2000 mm^3 in less than 50 days. It was therefore determined that the xenograft tumors would be stored as small frozen tumor pieces (2 x 2 mm) and implanted as whole tumor pieces, either in donor mice or directly into experimental mice, to establish tumor bearing mice for experiments. This method allows a more rapid establishment and growth of xenograft tumors in mice.

2. *Investigate the efficacy of PI3K/AKT/mTOR and/or EZH2 inhibition to reverse the AVPC phenotype and ADT resistance in vivo.*

For chemical inhibition of EZH2 and PI3K/AKT/mTOR, it was decided that two compounds developedby Eli Lilly Pharmaceuticals would be utilized in this project. LY3023414 (LY-PK) is the PI3K/AKT/mTOR inhibitor that was used, and LY3346149 (LY-EZ) was the EZH2 inhibitor used.

Prior to starting *in vivo* experiments, several *in vitro* experiments were carried out to test the compounds in the cell lines that were to be used to generate tumors in mice. Drug dose curves and pharmacodynamic analyses by western blot were generated in PbCre4:Pten$^{fl/fl}$:Rb1$^{fl/f}$ (Pten$^{-/-}$ Rb1$^{-/-}$), PbCre4:Pten$^{fl/fl}$:p53$^{fl/f}$ (Pten$^{-/-}$ p53$^{-/-}$), and WCM154 cell lines (Figures 5-8).

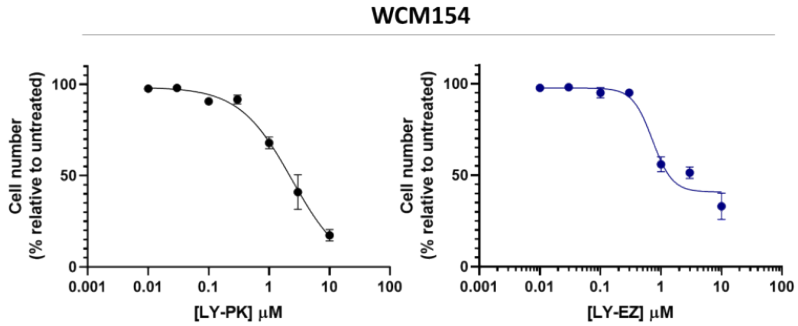
In all cell lines there was a greater anti-proliferative effect of both compounds compared to pro-apoptotic, although both compounds were pro-apoptotic at high doses (Figures 5-6). The 3D lineshad greater sensitivity to drugs compared to their 2D counterparts. This is not unexpected due to the increased surface area of the 3D lines.



	LY-PK (nM)		LY-EZ (nM)	
	IC50	LD50	IC50	LD50
2D Pten$^{-/-}$ Rb1$^{-/-}$	136	786	189	433
3D Pten$^{-/-}$ Rb1$^{-/-}$	119	251	155	321

	LY-PK (nM)		LY-EZ (nM)	
	IC50	LD50	IC50	LD50
2D Pten$^{-/-}$ p53$^{-/-}$	128	855	322	534
3D Pten$^{-/-}$ p53$^{-/-}$	143	312	210	347

Figure 5: Dose-response curves for 2D and 3D GEMM-derived cell lines (Pten-/- Rb1-/- and Pten-/- p53-/-), and related IC50 and LD50 drug concentrations. Cells with drugs were treated for 72 hours prior to analysis for dead cells (apoptosis) and total cell count (proliferation) (n=3 wells per dose, +/-1SEM).



	LY-PK	LY-EZ
IC25 (µM)	0.76	0.71
IC50 (µM)	2.35	1.23
IC75 (µM)	6.48	N/A

Figure 6: Dose-response curves for 3D human neuroendocrine PCa-derived cell lines (WCM154), and related IC25, IC50 and IC75 drug concentrations. Cells with drugs were treated for 72 hours prior to counting total remaining cell count (proliferation) (n=3 wells per dose, +/-1SEM).

Pharmacodynamic analysis of GEMM and human-derived cells treated with increasing doses of LY- PK and LY-EZ showed significant downregulation of PI3K/AKT/mTOR and EZH2 pathways at doses above 30-100nM, in GEMM-derived cells, and 100-300nM, in human-derived cells. InterestinglyEZH2 was significantly degraded in all cell lines, highlighting the LY-EZ inhibitor as particularly potent compared to other less specific LY-EZ inhibitors that often only reduce H3K27me3 expression.

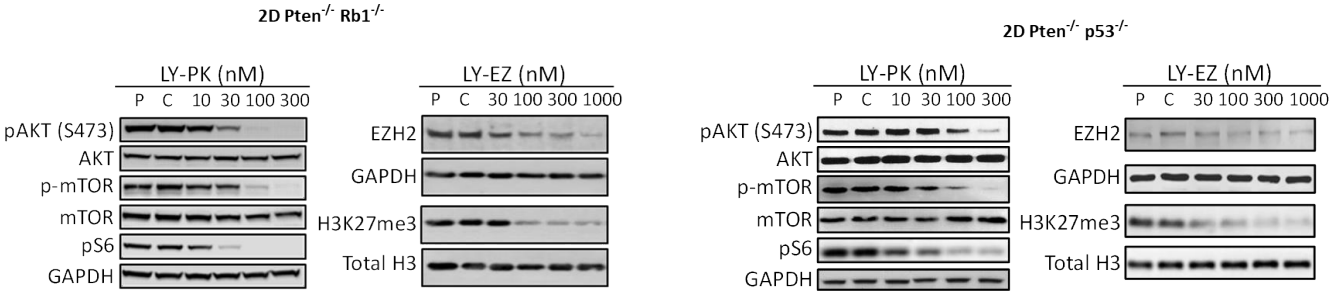


Figure 7: Pharmacodynamic protein response of GEMM-derived cell lines to treatment with increasing doses of LY-PK and LY-EZ. Cells were treated for 72 hours with drugs dissolved in equal amounts of DMSO vehicle.

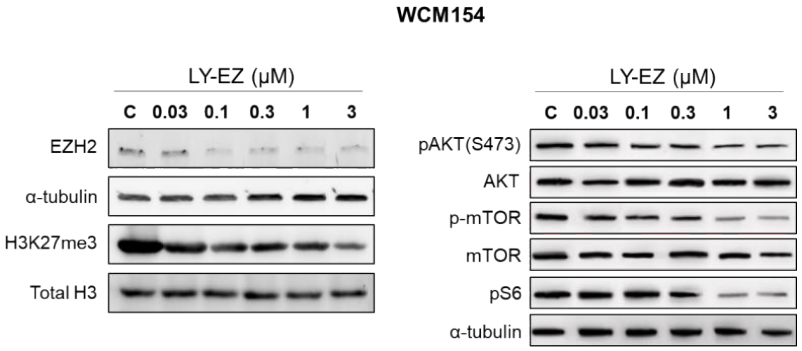


Figure 8: Pharmacodynamic protein response of 3D human neuroendocrine PCa-derived cell lines to treatment with increasing doses of LY-PK and LY-EZ. Cells were treated for 72 hours with drugs dissolved in equal amounts of DMSO vehicle.

Cells treated with LY-EZ were further probed for AR expression to confirm the ability of EZH2 inhibition to reactive AR signaling in castrate-resistant cells or cells with dampened AR signaling. Consistent with previous descriptions in the literature, AR expression increased in Pten$^{-/-}$Rb$^{-/-}$ and Pten$^{-/-}$p53$^{-/-}$ cells. This ability of EZH2 inhibitors to reactivate AR signalling makes it a perfect supplementary treatment to augment ADT.

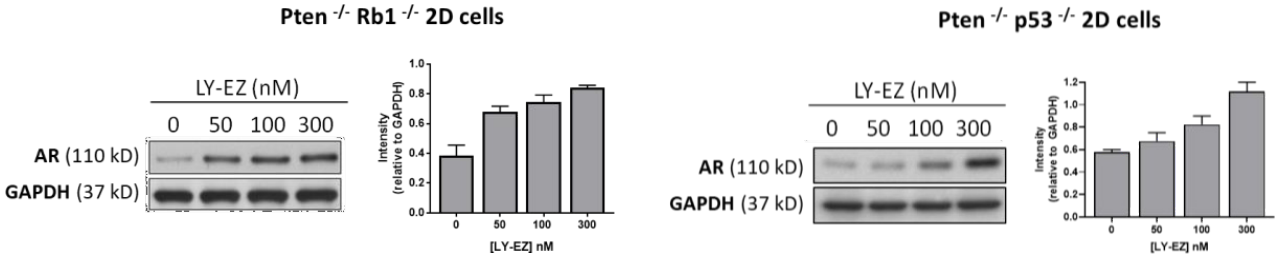


Figure 9: AR protein expression in GEMM-derived cell lines treated with increasing doses of LY-EZ. Cells were treated for 72 hours with drugs dissolved in equal amounts of DMSO (n=2, +/-1SD).

Prior to running *in vivo* experiments, a pilot experiment in 3D organoids was carried out to confirm thecombinatorial effect of LY-PK, LY-EZ and Enzalutamide. Organoid cultures were treated with drug combinations for 72 hours and then analyzed for cell death using immunofluorescent analysis (Figure 10-11). At all doses of Enzalutamide, the combination of LY-PK and LY-EZ proved significantly better at inducing cell death compared to the vehicle or single drug arms.

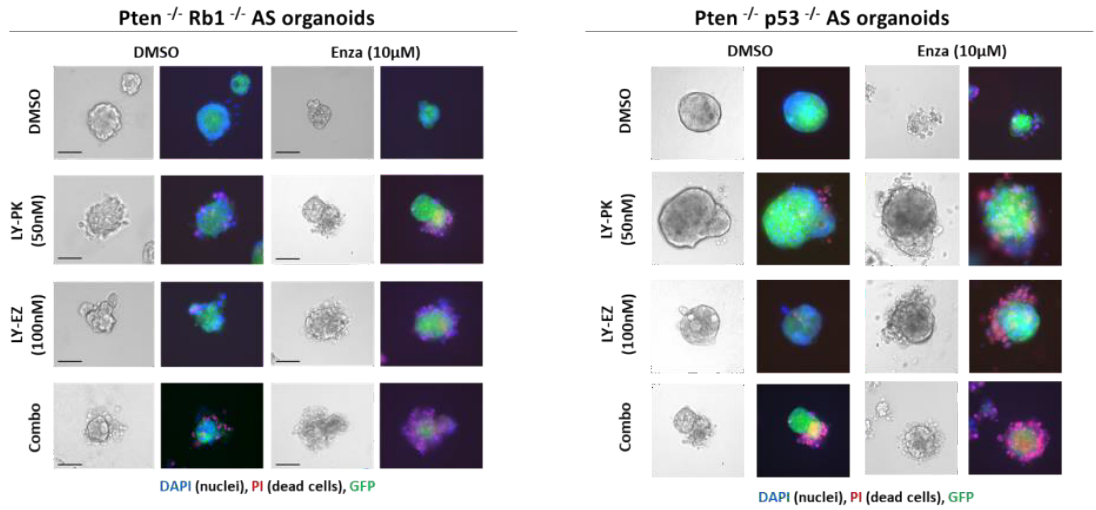


Figure 10: Brightfield and stained images of 3D GEMM-derived organoids treated +/-LY-PK (50nM), +/- LY-EZ (100nM) and Enzalutamide (10μM) for 72 hours. Organoids were specifically stained for PI (death) and DAPI (total cell nuclei).

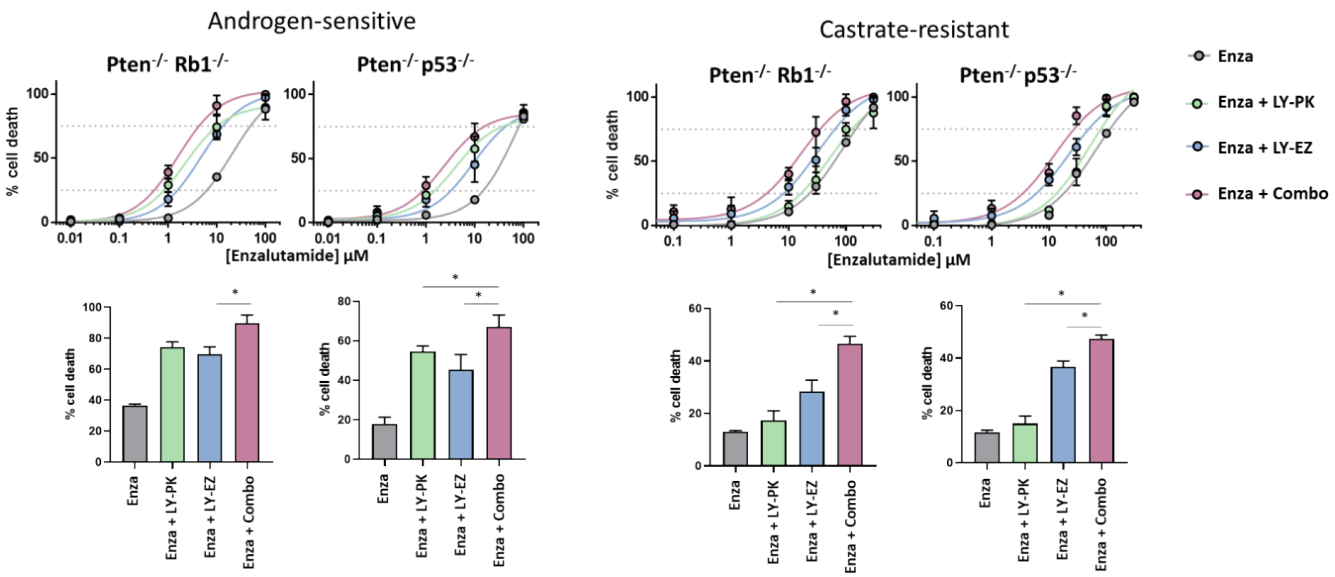


Figure 11: Dose-response curves over a range of Enzalutamide doses, and bar graphs depicting % cell death with 10 μM Enzalutamide from GEMM-derived organoid treatment experiment (n=10 organoids per treatment group, +/-1SD,

To investigate the efficacy of PI3K/AKT/mTOR and/or EZH2 inhibition to reverse the aggressive variant prostate cancer phenotype and ADT resistance in vivo, mice were prepared and treated as per Figure 12. In brief, all C57BL/6N mice were castrated at 8 weeks of age, and after 7 days castrate-resistant Pten-/-p53-/- cells were implanted into the flank of each mouse in 100μL of Matrigel (50% in PBS). Mice were treated daily with Enzalutamide (30mg/kg) by oral gavage and/or LY-PK (15 mg/kg) and/or LY-EZ (20 mg/kg) by intraperitoneal (IP) injection. During treatment tumors were measured every second day for 30 days and were then harvested and stored as fresh or fixed samples for further tissue analysis.

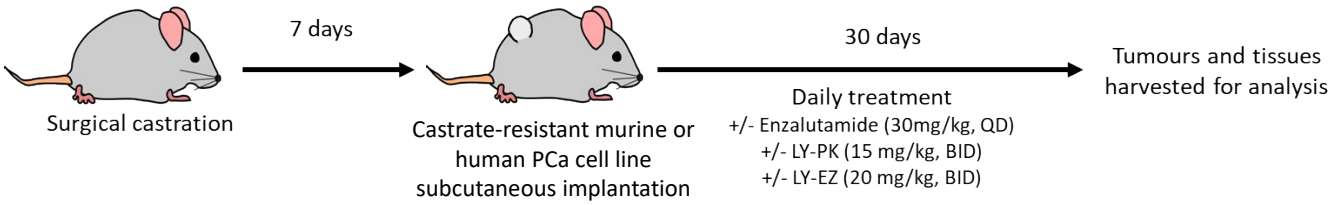


Figure 12: Schematic overview of in vivo study #1.

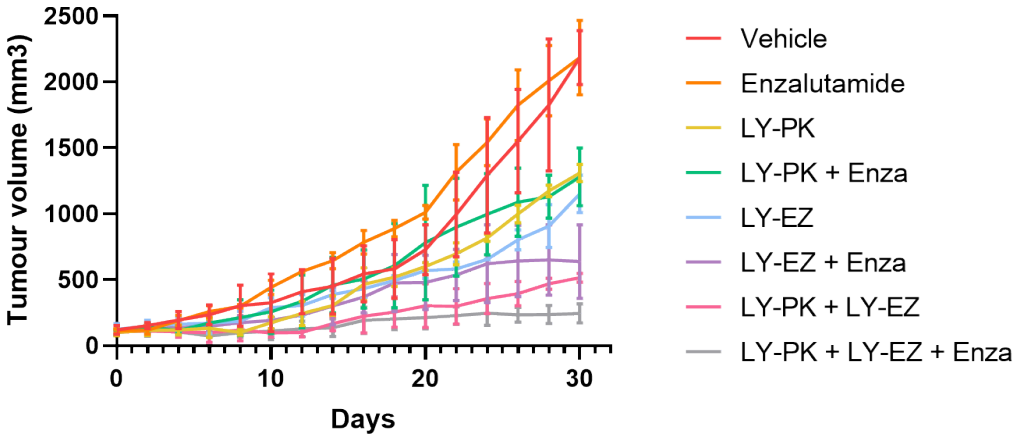


Figure 13: Complete murine Pten-/-p53-/- tumor growth curves from in vivo study #1 (n=5 mice per treatment group (+/-1SD).

Vehicle and Enzalutamide-treated tumors grew to greater than 2000mm^3 within 30 days, with the Enzalutamide-treated tumors growing slightly faster than the vehicle treatment group (Figure *13*). Thislack of response to Enzalutamide highlights the underlying castration resistance observed in the cell line. Interestingly, despite the loss of Pten in the cell line, the PI3K/AKT/mTOR inhibitor did not have a significant anti-tumor effect as a single agent, or in combination with Enzalutamide (Figure *14*). Given that androgen-sensitive cell lines responded well to LY-PK therapy (Figure *7*) it was hypothesized that the castration resistance in the cell line may have caused a dampening of this pathway. LY-EZ treatment also provided modest tumor control, although this was not unexpected, as previous studies and reports in the literature have not seen significant reduction of tumor growth with EZH2 inhibition. The combination of LY-PK and LY-EZ had significant benefit, in both the presence and absence of Enzalutamide, highlighting the benefit of combination therapy.

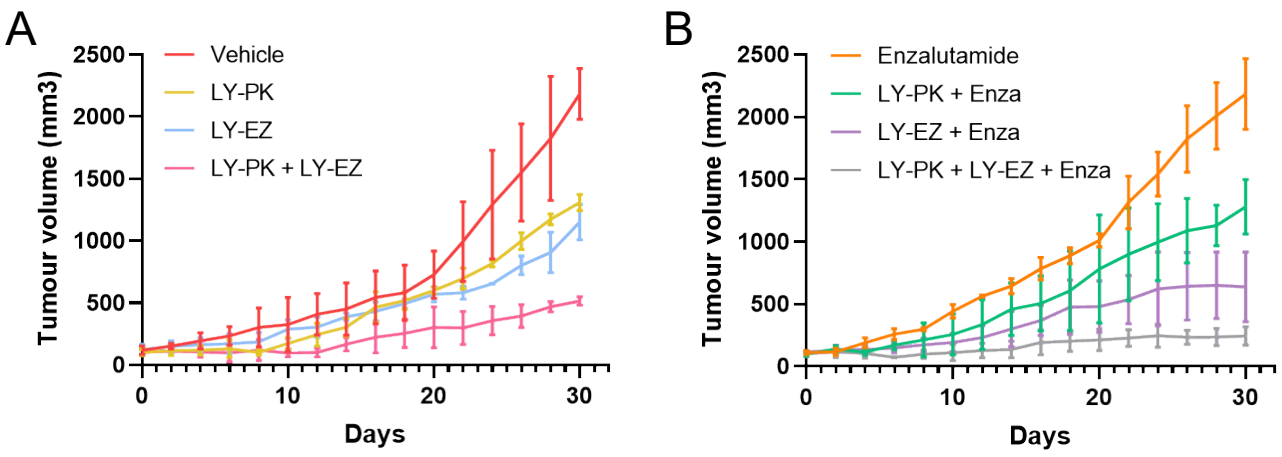


Figure 14: Murine Pten-/-p53-/- tumor growth curves from the (A) non-ADT/Enzalutamide and (B) ADT/Enzalutamide treatment arms of in vivo study #1 (n = 5 mice per treatment group, +/-1SD).

Fixed and embedded tumor sections were analyzed by IHC for key pharmacodynamic targets. As expected, treatment with H3K27me3 was downregulated in LY-EZ treatment groups (Figure *15*). Staining for γH2AX (DNA damage) was used as a marker of cell death (Figure *16*) and highlighted that there was limited cell death in any tumors, even the LY-PK and LY-EZ combination therapy arms.Conversely, the combination therapy arms showed significant reduction in proliferation via Ki-67 staining (Figure *17*) compared to vehicle and single therapy arms. AR staining (Figure *18*) showed that LY-EZ induced significant upregulation of AR, which allowed a re-sensitization to Enzalutamide. Interestingly the combination of LY-PK, LY-EZ and Enzalutamide resulted in slightly increased AR expression, compared to the combination of LY-EZ and Enzalutamide. This increased AR expression did not appear to be beneficial to tumor growth, nor did it have a pro-proliferative anti-apoptotic effect.Further RNA-expression analysis of tumor samples will give greater insight into this effect.

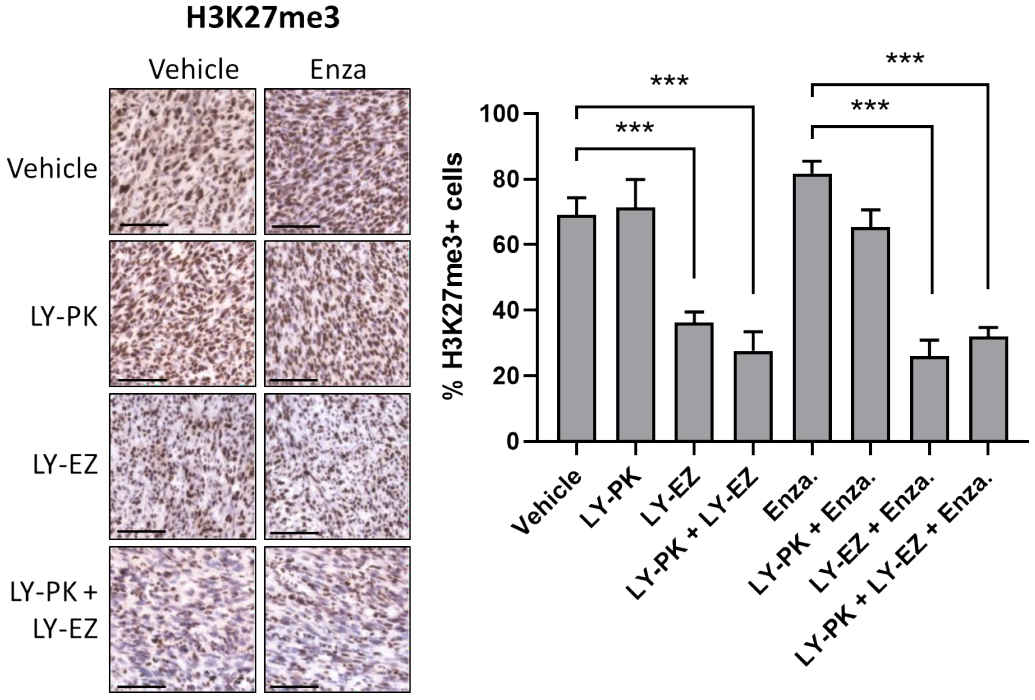


Figure 15: H3K27me3 IHC staining in murine Pten-/-p53-/- tumors from in vivo study #1, and corresponding staining analysis (n = 5 mice per treatment group, +/-1SD).

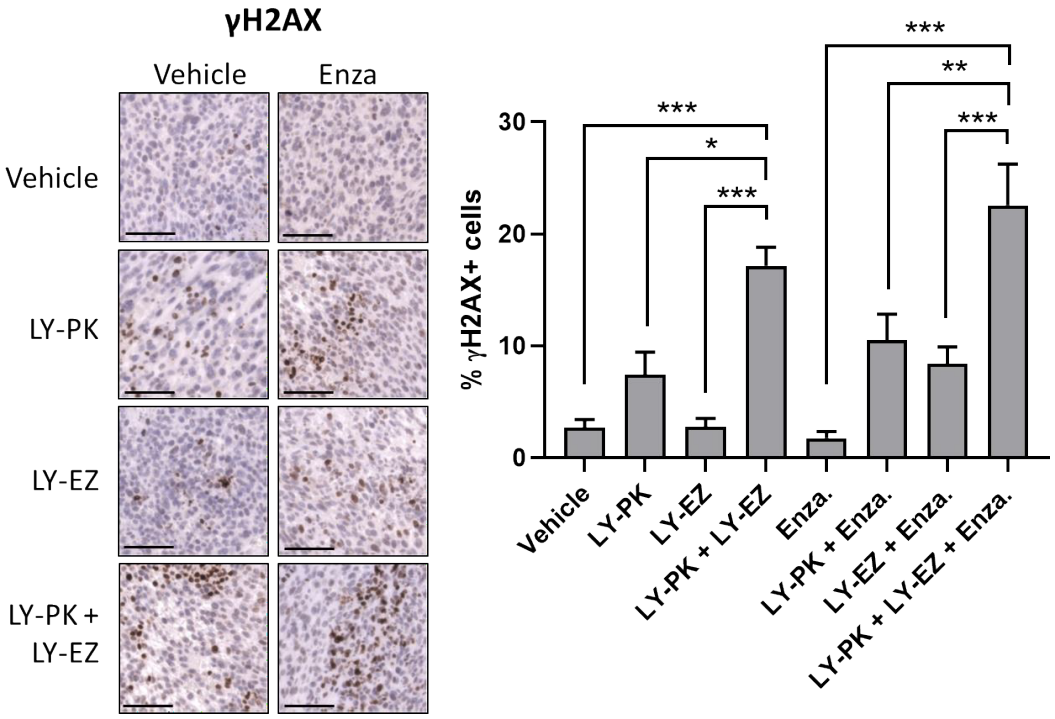


Figure 16: γH2AX IHC staining in murine Pten-/-p53-/- tumors from in vivo study #1, and corresponding staining analysis (n = 5 mice per treatment group, +/-1SD).

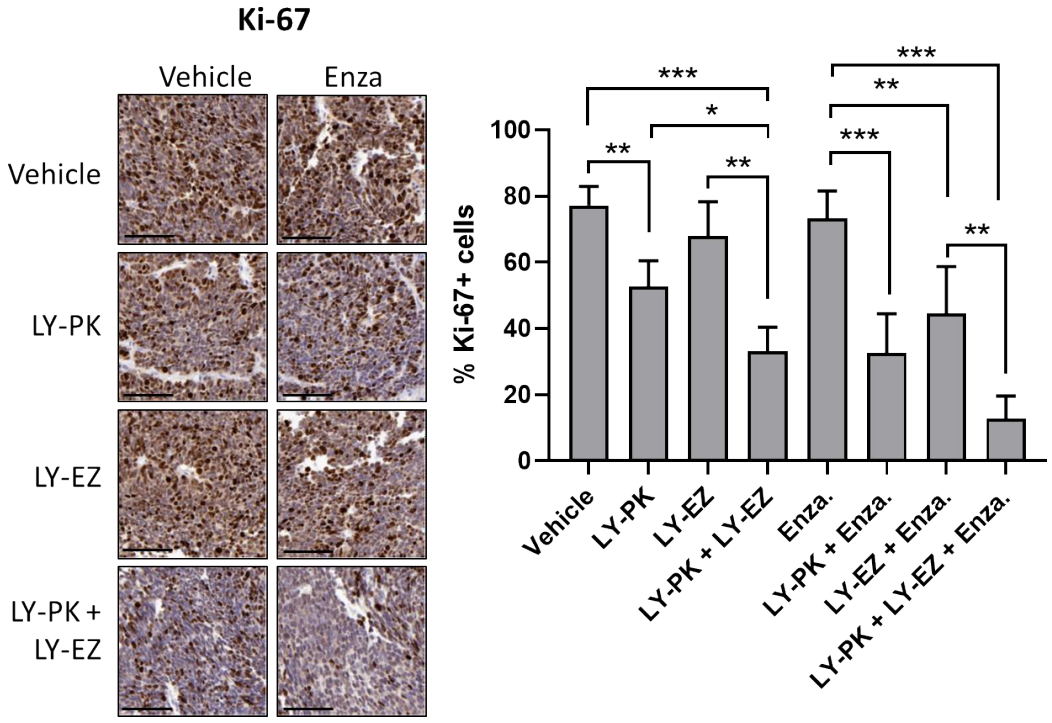


Figure 17: Ki-67 IHC staining in murine Pten-/-p53-/- tumors from in vivo study #1, and corresponding staining analysis (n = 5 mice per treatment group, +/-1SD).

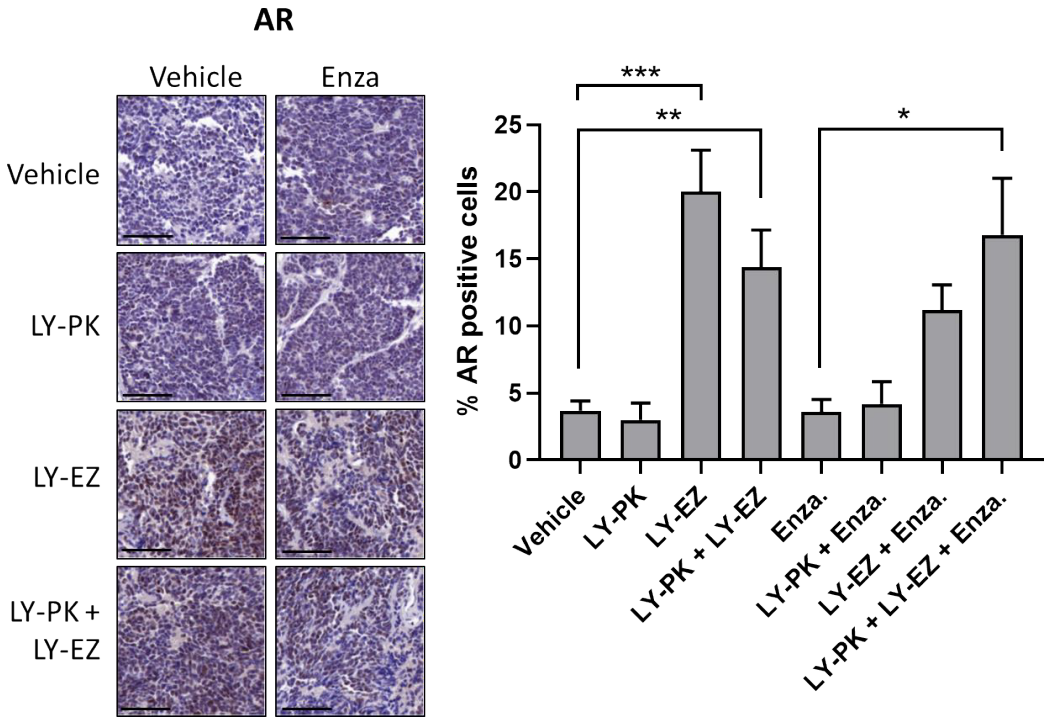


Figure 18: AR IHC staining in murine Pten-/-p53-/- tumors from in vivo study #1, and corresponding staining analysis (n = 5 mice per treatment group, +/-1SD)

In addition to murine cell line studies, in vivo xenograft therapy studies were also carried out using a human neuroendocrine PCa model (WCM154). NOD.CB17-Prkdcscid/NCrCrl mice were prepared and treated as per Figure *12*. In brief, all mice were castrated at 8 weeks of age, and after 7 days WCM154 cells were implanted into the flank of each mouse in 100µL of Matrigel (50% in PBS). Mice were treated daily with Enzalutamide (30mg/kg) by oral gavage and/or LY-PK (15 mg/kg) and/or LY-EZ (20 mg/kg) by intraperitoneal (IP) injection. During treatment tumors were measured every second day for 30 days and were then harvested and stored as fresh or fixed samples for further tissue analysis. WCM154 cells are PTEN/RB1/TP53-null and are AR-negative, therefore it was unsurprising that they had no response to ADT with Enzalutamide (*Figure 19*).

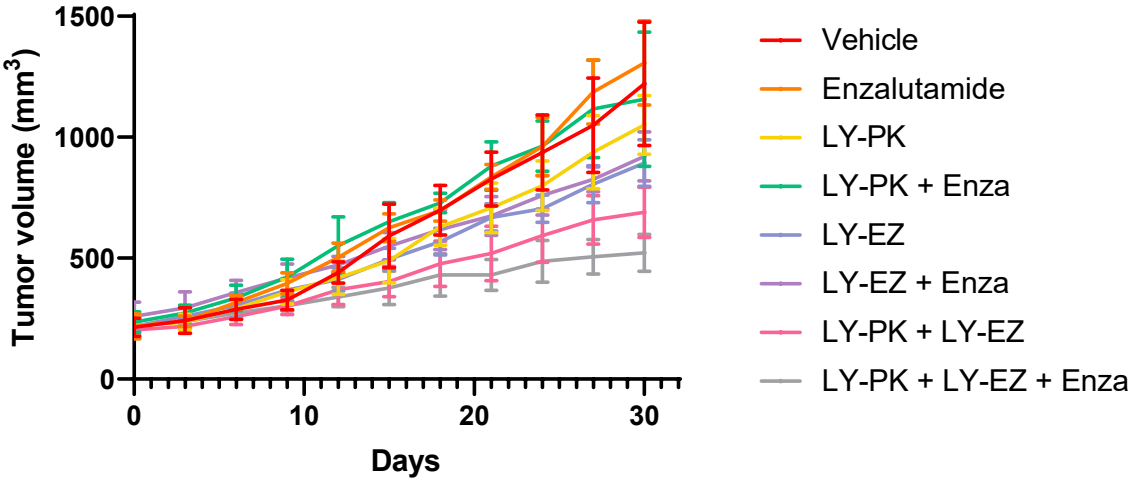


Figure 19: Complete WCM154 tumor growth curves from in vivo study #1 (n=5 mice per treatment group (+/-1SD).

WCM154 tumor response to the combinations of Enzalutamide, LY-PK and LY-EZ showed a very similar pattern to the response observed in murine Pten-/-p53-/- tumors inhibition of the PI3K/AKT/mTOR pathway by LY-PK showed limited response in the tumors and the greatest response is observed in treatment groups containing EZH2 inhibition, either alone or in combination. As with the murine tumors, the greatest tumor inhibition response was observed in the triplet combination group of Enzalutamide/ LY-PK/ LY-EZ (Figure *21*).

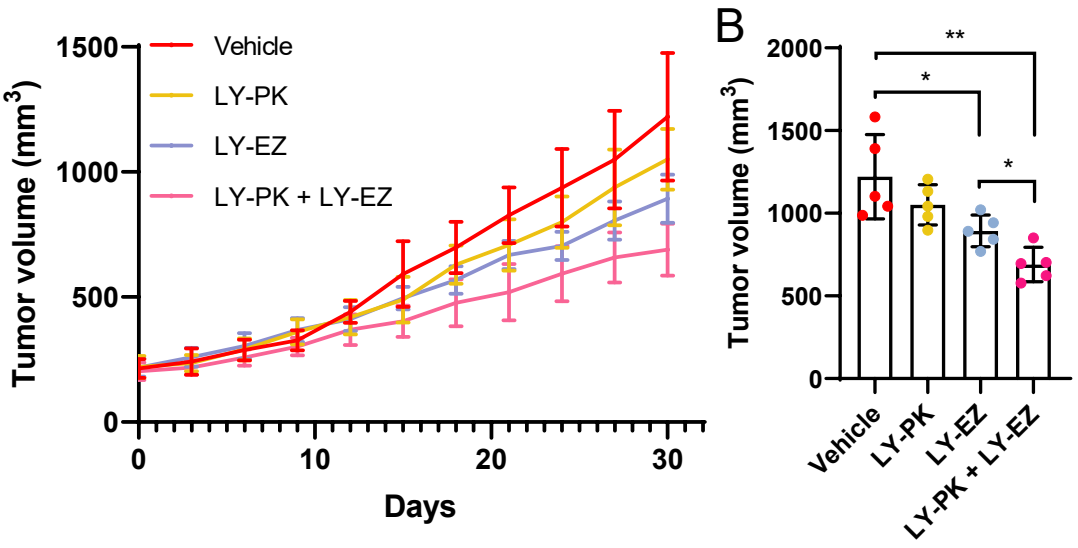


Figure 20: A) WCM154 Tumor growth curves from the non-ADT/Enzalutamide arm of in vivo study #1 (n = 5 mice per treatment group, +/-1SD).

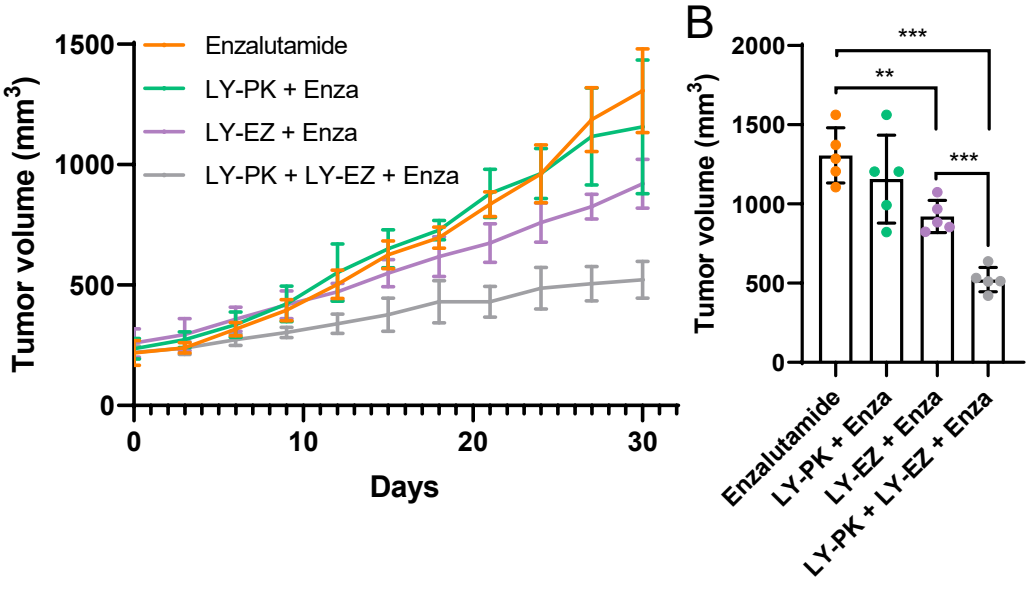


Figure 21: WCM154 Tumor growth curves from the ADT/Enzalutamide arm of in vivo study #1 (n = 5 mice per treatment group, +/-1SD).

3. *Confirmation of the efficacy of PI3K/AKT/mTOR and/or EZH2 inhibition to prevent the AVPC phenotype and prolong ADT sensitivity in vivo.*

To investigate the efficacy of PI3K/AKT/mTOR and/or EZH2 inhibition to prevent the AVPC phenotype and prolong ADT sensitivity *in vivo* mice were treated as per Figure *22*. In brief, all C57BL/6N mice were implanted with Pten-/-p53-/- cells in 100µL of Matrigel (50% in PBS), 7 days later (tumors approximately 100mm^3) mice were either surgically castrated or sham castrated. Following castration mice were treated daily with LY-PK (15 mg/kg) and/or LY-EZ (20 mg/kg) by IP injection. During treatment tumors were measured every second day for 30 days and were then harvested and stored as fresh or fixed samples for further tissue analysis.

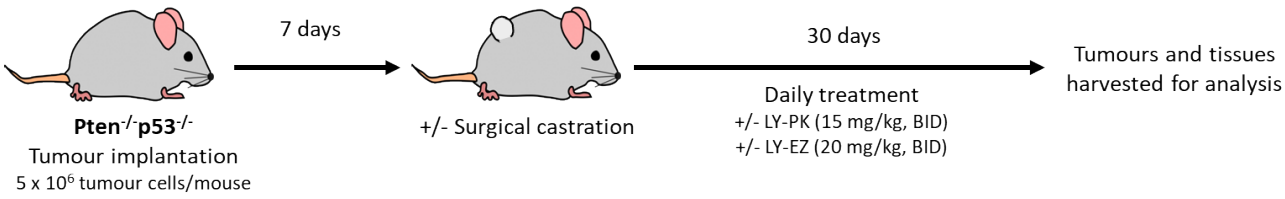


Figure 22: Schematic overview of in vivo study #2

As was observed in the previous *in vivo* study, tumors in the vehicle and castrated treatment arms grew the quickest (Figure *23*). Tumors initially responded to castration, however, began to grow again after approximately one week, after which they grew more rapidly than the intact vehicle arm.Interestingly the castrated arm of the study showed a significantly different pattern of tumor growth compared to the intact arm. In the intact arm (Figure *24*), tumors responded well to LY-PK treatment with tumors 36-40% smaller than the vehicle arm. This is a more typical response expected from Pten-null tumors, where phos-AKT and phos-s6 are increased. LY-EZ had a minimal effect on tumor control, however, the combination of LY-PK and LY-EZ had the greatest anti-tumor effect. This aligns with the previous study, where the best tumor control was observed in LY-PK and LY-EZ combinationtherapy groups. In stark contrast, in the castrated arm of the study LY-PK had very minimal anti-tumor effect compared to the castration only arm (Figure *24*). This result seems to mirror the prior experiment where all tumors were castrate resistant. This further indicates the potential that castration is somehow downregulating the PI3K/AKT/mTOR pathway and will be investigated further by RNA-sequencing. The combination of LY-PK and LY-EZ, alone or in combination with castration, resulted in significant tumor control. This was especially noticeable in the castration arm.

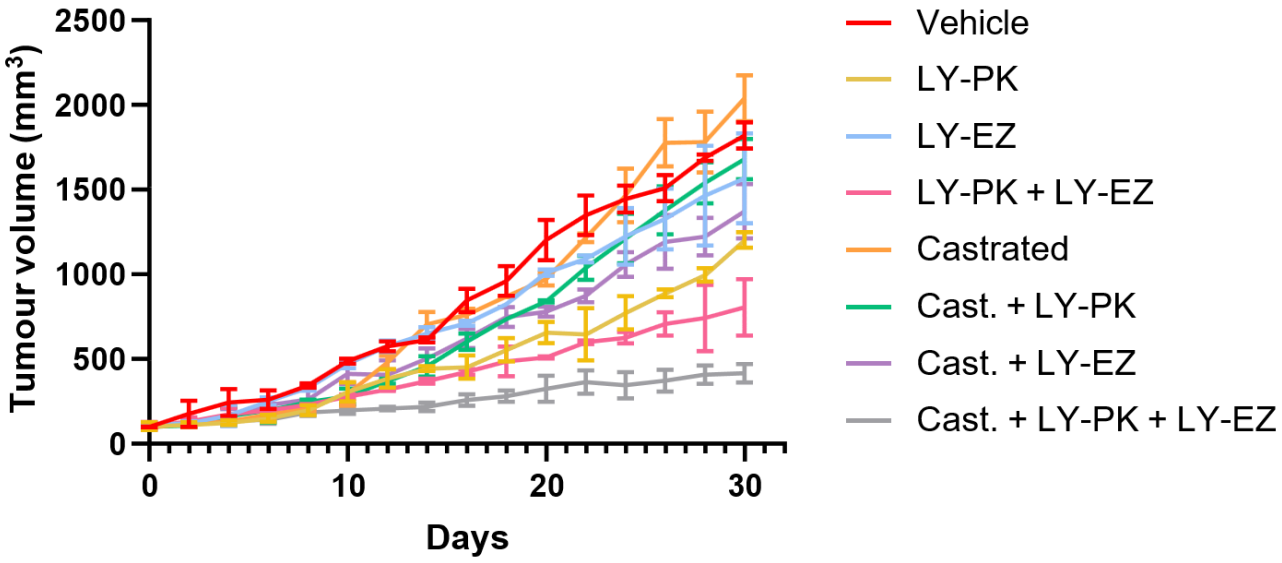


Figure 23: Complete murine Pten-/-p53-/- tumors growth curves from in vivo study #2 (n=5 mice per treatment group (+/-1SD).

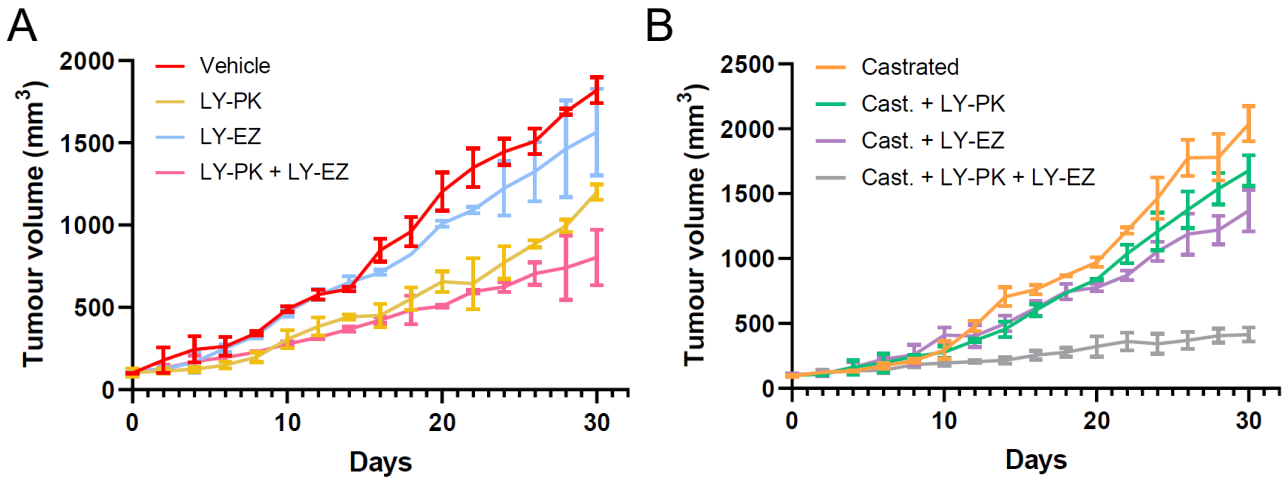


Figure 24: Murine Pten-/-p53-/- tumor growth curves from the (A) intact and (B) castrated arms of in vivo study #2 (n = 5 mice per treatment group, +/-1SD).

Matching *in vivo* studies were carried out using a Pten-/-Rb1-/- GEMM tumor transplant model and resulted in comparable therapeutic responses.

Fixed and embedded tumor sections were analyzed by IHC for key pharmacodynamic targets. As was the case in the previous *in vivo* experiment, H3K27me3 was downregulated in LY-EZ treatment groups (Figure *25*). A similar pattern of apoptosis (Figure *26*) and proliferation (Figure *27*) was observed in the tumor samples. As with previous studies, LY-EZ treatment significantly increased AR expression in tumors and help to re-sensitize tumors to castration therapy (Figure *28*).

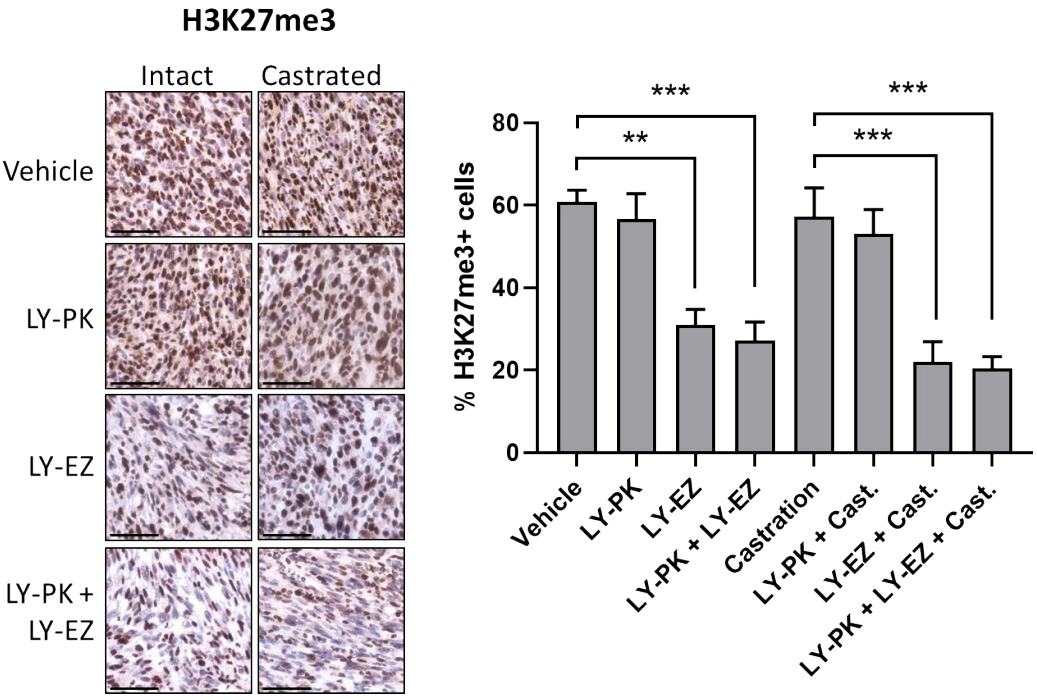


Figure 25: H3K27me3 IHC staining in murine Pten-/-p53-/- tumors from in vivo study #2, and corresponding staining analysis (n = 5 mice per treatment group, +/-1SD).

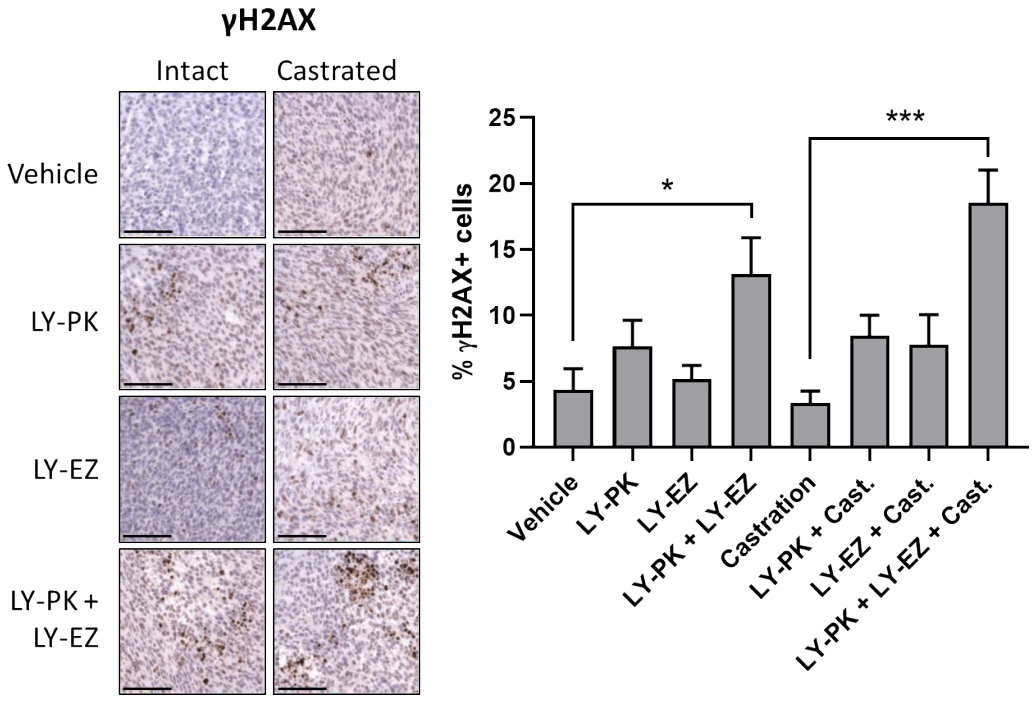


Figure 26: γH2AX IHC staining in murine Pten-/-p53-/- tumors from in vivo study #2, and corresponding staining analysis (n = 5 mice per treatment group, +/-1SD).

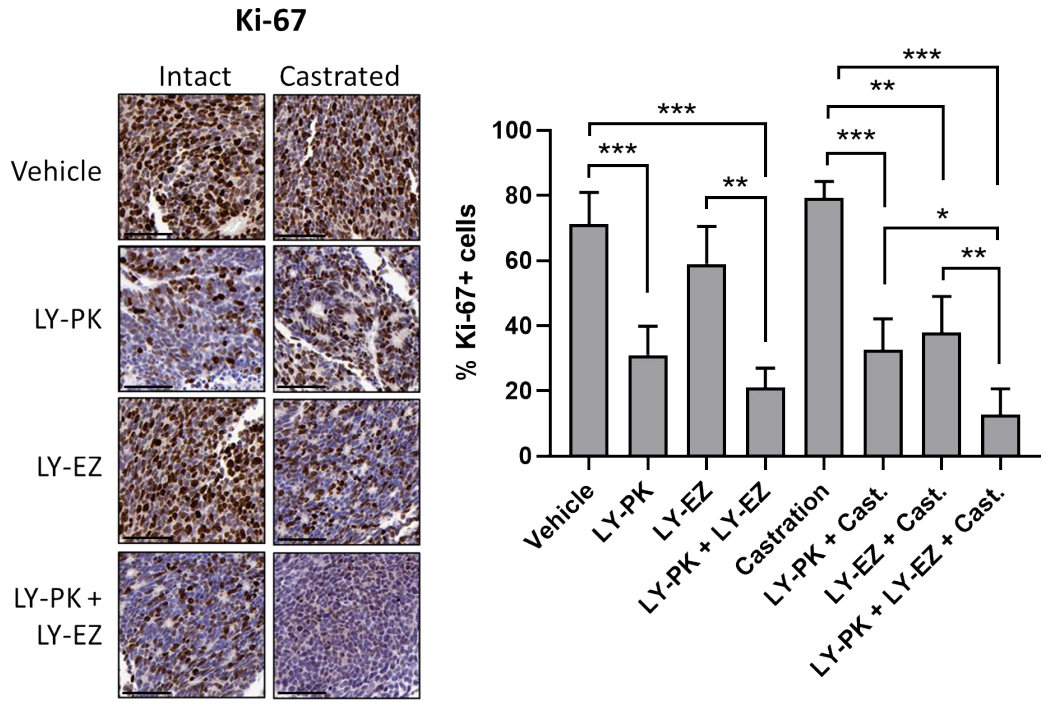


Figure 27: Ki-67 IHC staining in murine Pten-/-p53-/- tumors from in vivo study #2, and corresponding staining analysis (n = 5 mice per treatment group, +/-1SD).

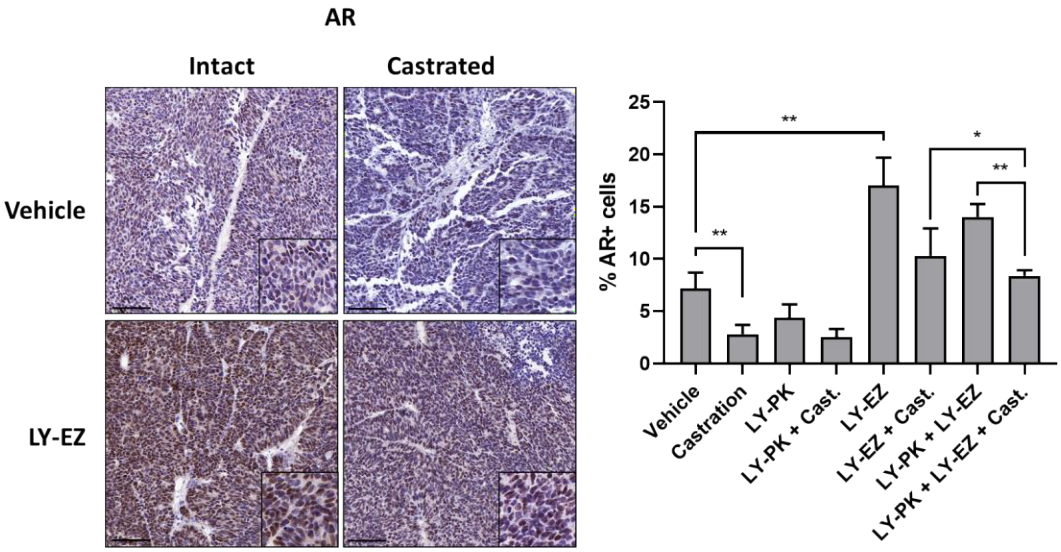


Figure 28: AR IHC staining in murine Pten-/-p53-/- tumors from in vivo study #2, and corresponding staining analysis (n = 5 mice per treatment group, +/-1SD).

Analysis of RNA expression (by qPCR) in intact and castrated tumors from in vivo therapy studies shows a clear pattern of expression on AR-related and regulated genes (*Figure 29*). In intact tumors, AR expression is slightly upregulated following EZH2 inhibition, while AR-functional genes (FKBP5 and KLK2) upregulated to a greater extent. This matches AR protein expression levels observed in the tumors. Interestingly, while castration resulted in a significant reduction in AR protein expression, mRNA is significantly upregulated. This highlights a disconnect between protein and mRNA levels, potentially due to an AR self-regulation loop. While castration results in a spike in AR mRNA expression, mRNA expression in FKBP5 and NKX3-1 is reduced, hinting

at a reduction in AR function. In all AR and AR-regulated genes examined, EZH2 inhibition in castrated tumors reduced mRNA expression.

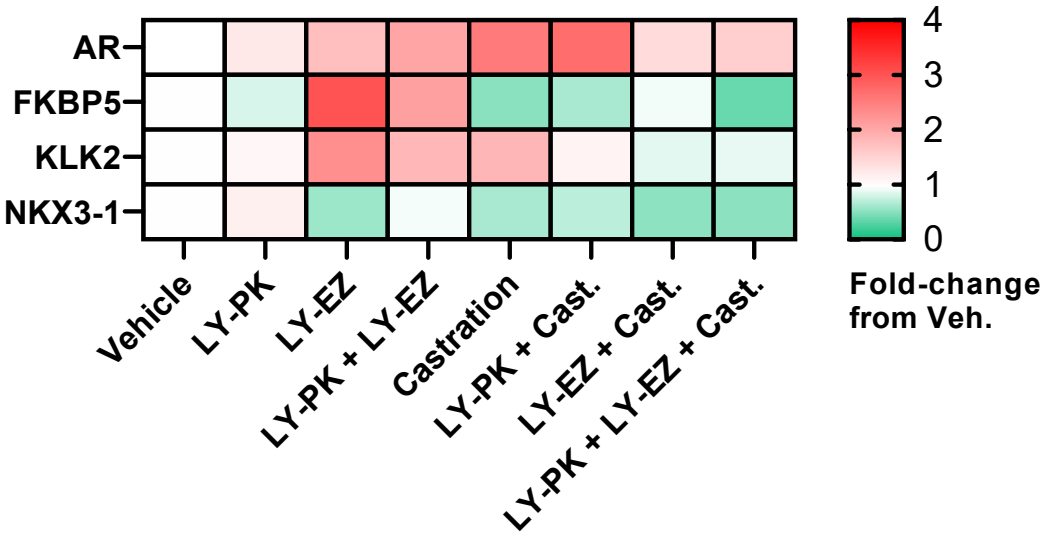


Figure 29: AR-related gene expression in GEMM tumors from in vivo study #2, quantified by qPCR, n=3 per treatment group.

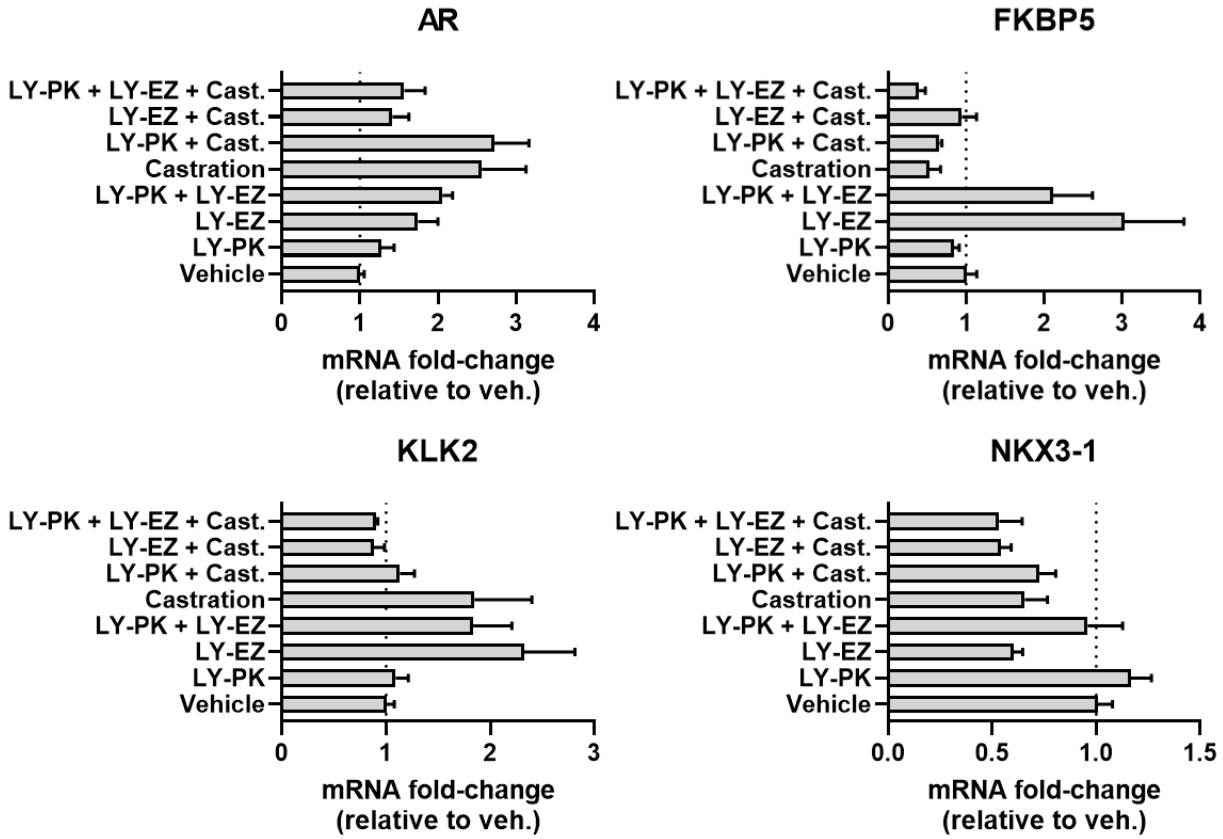


Figure 30: AR-related gene expression in GEMM tumors from in vivo study #2, quantified by qPCR, n=3 per treatment group, +/- 1SEM.

To further investigate the possibility that castration is down-regulating the PI3K/AKT/mTOR

pathway, which is commonly upregulated in PTEN-null tumors, the *in vivo* tumors were stained for phosphorylated-AKT and phosphorylated-S6 (Figure *31*). As hypothesized, the castrated samples all had significantly reduced phosphorylated-AKT and phosphorylated-S6 compared to intact samples. This pattern of downregulation was also mirrored in western blow analysis of cells *in vitro* (Figure *32*).This is a phenomenon that hasn't previously been reported in the literature and therefore may be a significant observation that requires further investigation that RNA-expression or IHC analysis alone.

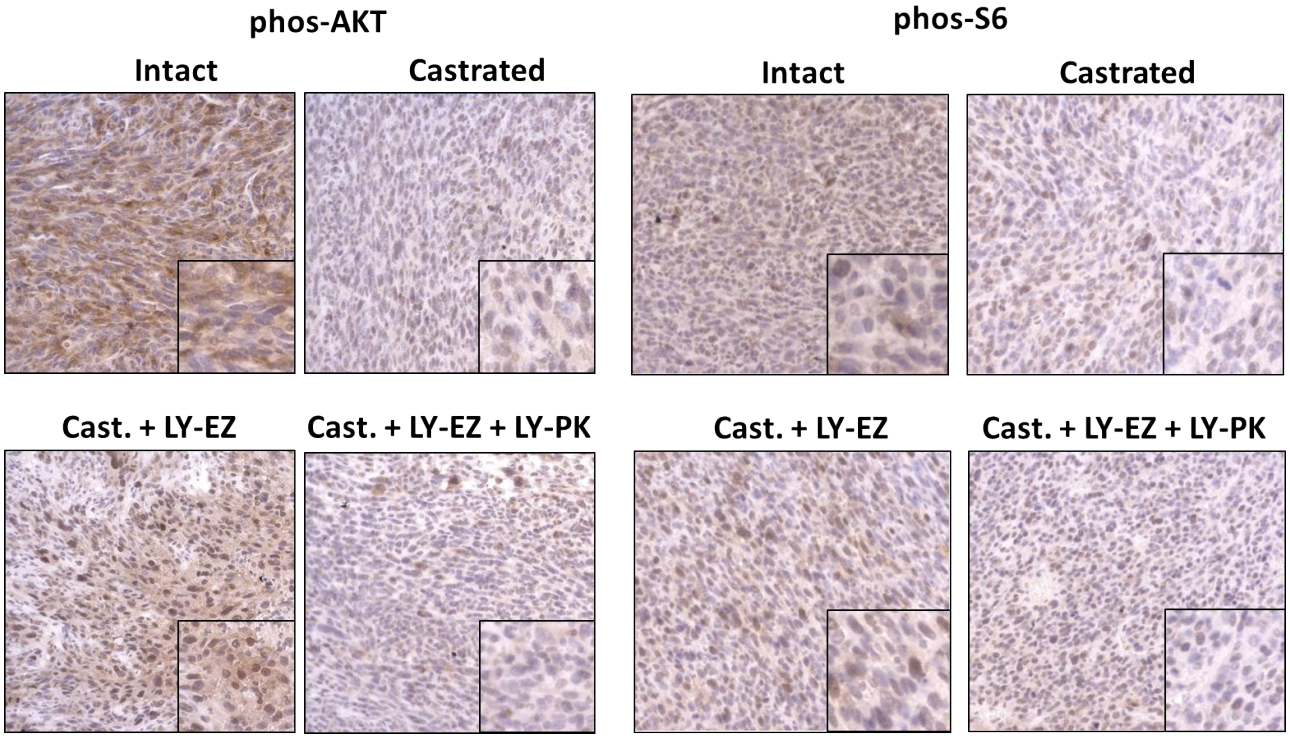


Figure 31: phos-AKT and phos-S6 IHC staining in murine tumors from in vivo study #2.

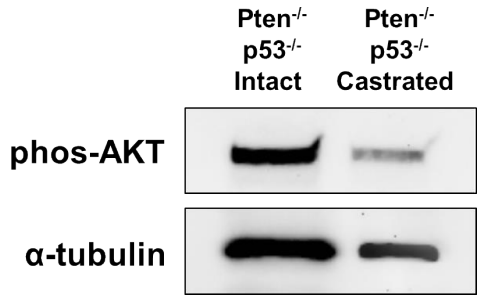


Figure 32: phos-AKT protein expression by western blot in androgen-sensitive vs castrated Pten-/-p53-/- cells.

IHC analysis of WCM154 neuroendocrine xenograft tumors revealed a similar pattern of phos-AKT and phos-S6 expression (*Figure 33*). Therapy studies in this cell line revealed baseline castration resistance in the model therefore intact and castrated tumors cannot be compared. However, IHC staining in vehicle-treated tumors revealed low expression of both phos-AKT and phos-S6. Following EZH2 inhibitor therapy expression of both protein marks was significantly increased. The combination of PI3K/AKT inhibition and EZH2 inhibition reduced expression of both proteins back to baseline levels. Taken with the fact that combinatorial inhibition of EZH2 and PI3K/AKT results in significant tumor control compared to vehicle. It is hypothesised that

EZH2 inhibition restores enough functionality of the PI3K/AKT pathway to allow PI3K/AKT inhibition to be therapeutically beneficial.

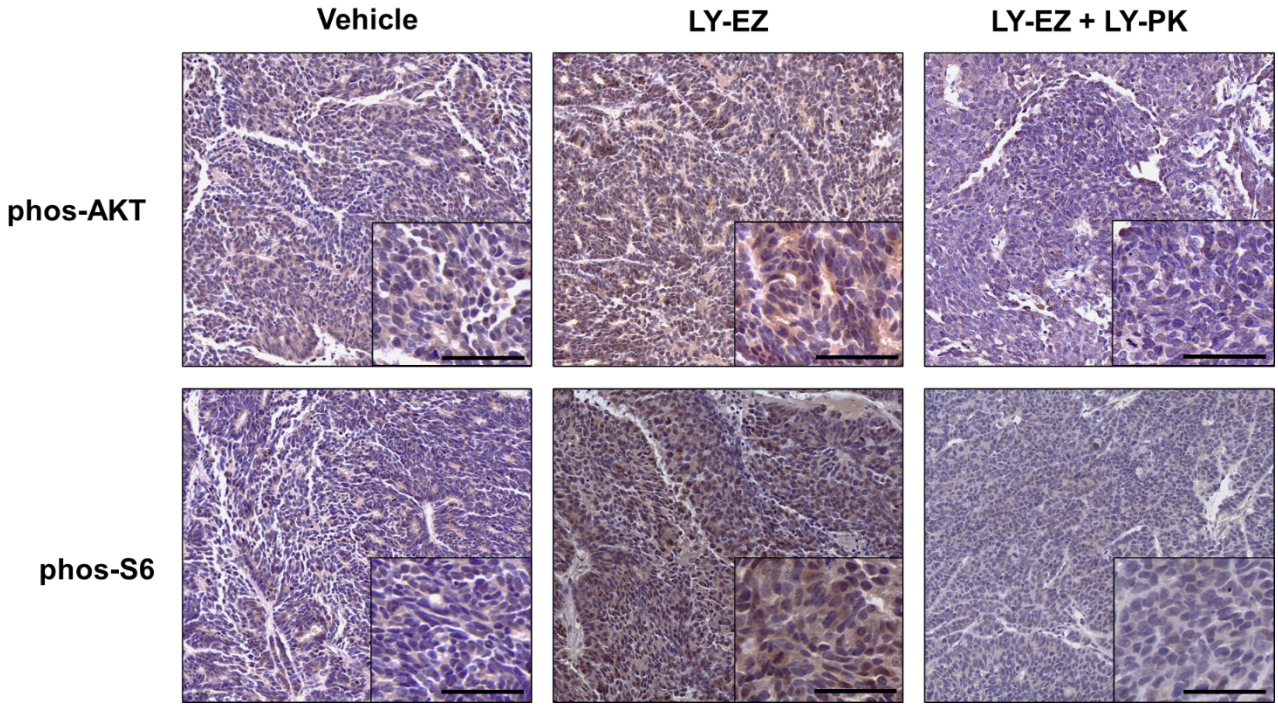


Figure 33: phos-AKT and phos-S6 IHC staining in human WMC154 xenograft tumors from in vivo study #1

Gene expression analysis of the PI3K/AKT/mTOR pathway showed a similar pattern to protein expression changes (*Figure 34*). In castrated tumors, the PI3K/AKT/mTOR genes examined were notably downregulated. In response to EZH2 inhibition in castrated cells, AKT1 and MTOR were significantly upregulated above intact baseline levels.

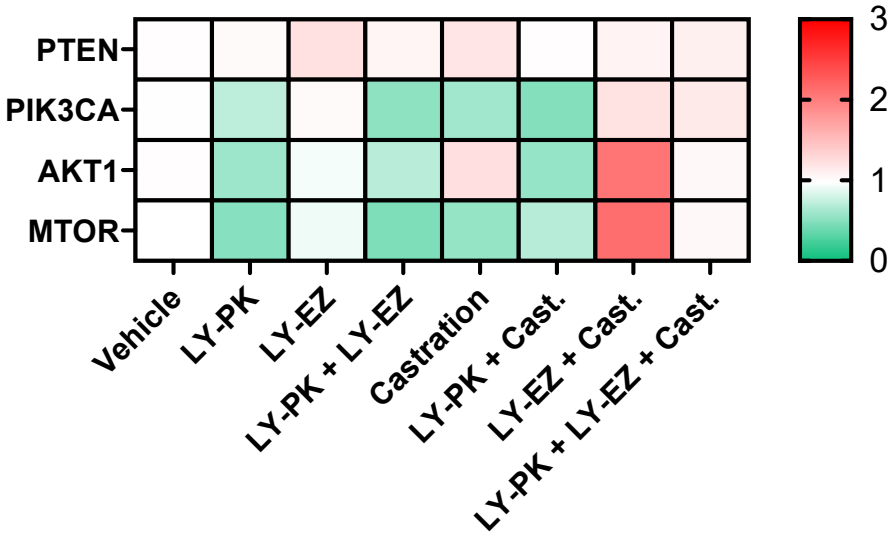


Figure 34: PI3K/AKT/mTOR pathway gene expression in GEMM tumors from in vivo study #2, quantified by qPCR, n=3 per treatment group.

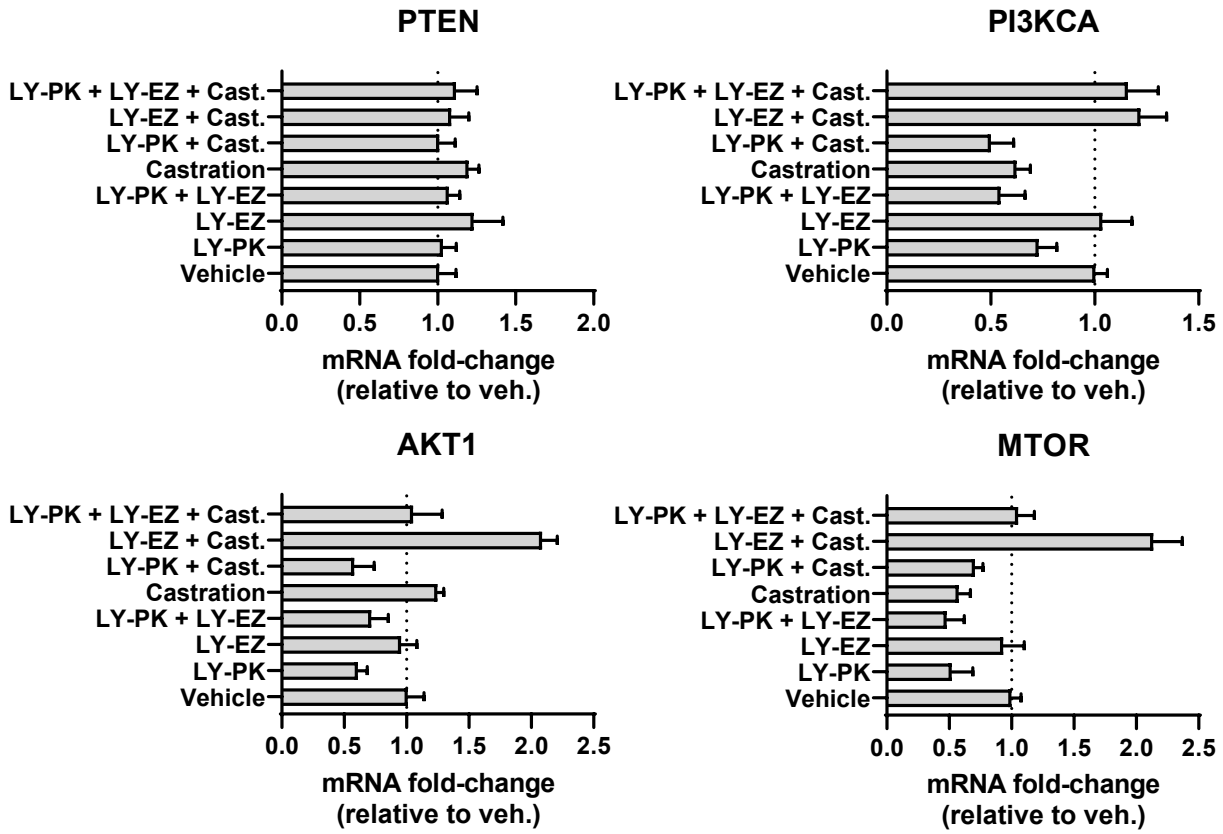


Figure 35: PI3K/AKT/mTOR pathway gene expression in GEMM tumors from in vivo study #2, quantified by qPCR, n=3 per treatment group, +/-1SEM.

Summary:

Aggressive variant prostate cancer is a lethal phenotype that arises upon treatment with ADT, resulting in a lineage switch to a neuroendocrine morphology and the acquisition of molecular alterations that enable the cells to survive independent of AR signalling. This prostate cancer phenotype is a particular stumbling block for current therapeutic strategies. Across multiple models of murine and human aggressive PCa, we showed that EZH2 inhibition was able to restore enough AR expression to restore sensitivity to ADT and delay castration resistance. In addition to restoring AR expression, there was evidence that some level of AR function was restored with transcriptional changes observed in AR target genes. The mechanism is thought to be due to direct binding of EZH2 to PSA in castrate resistant PCa cells, inhibiting its expression.

It was originally hypothesised that this ability of EZH2 inhibition to restore AR expression could be coupled with inhibition of the PI3K/Akt/mTOR pathway to target multiple key pathways in PTEN-null aggressive PCa. Interestingly, our studies showed limited therapeutic benefit of PI3K/Akt/mTOR inhibition in castrate resistant PCa cells. Further examination of these castration resistant PCa cells *in vivo* and *in vitro* revealed a reduction in Phos-AKT and Phos-S6 expression, indicating a dampening the PI3K/Akt/mTOR pathway. Loss of PTEN and subsequent activation of the PI3K/Akt/mTOR pathway is known to accelerate PCa progression and castrate resistant growth, so this observation of reduced signalling in established castration resistant PCa cells is particularly interesting. While this mechanism requires further investigation, there is evidence in the literature of cooperation between AR and mTOR signalling, suggesting progression to

castration resistance may be able to dysregulate the PI3K/Akt/mTOR pathway leading to reduced sensitivity to PI3K/Akt/mTOR inhibitors. Therapeutic studies from this project showed that inhibition of EZH2 was able to restore some amount of PI3K/Akt/mTOR signalling and re-establish sensitivity to PI3K/Akt inhibition. It is hypothesised that the dampening of PI3K/Akt/mTOR signalling in castration resistant cells may be due to an altered chromatin state, hence the ability of EZH2 inhibition to restore sensitivity and expression.

Ultimately the combinatorial inhibition of EZH2 and the PI3K/Akt/mTOR pathway was the most effective strategy to slow progression to castration resistance and restore sensitivity to ADT in castrate resistant models of PTEN-null aggressive PCa.

Training goals:

		% completed
1	**Gain an improved understanding of RNA-seq analysis at the gene and transcript level**	100%
2	**Present research at monthly chalk-talk sessions with Dr. Ellis, bi-monthly meetings with Dr. Sweeney, and multi-PI lab meetings every 4 months**	100%
3	**Present data at national scientific meetings**	100%
4	**Attend workshops and seminars offered by the DFCI Postdoc and Graduate Students Affairs Office every 2 months relevant to lab management, grant writing, and transitioning to academia**	100%
5	**Apply for sources of funding to continue prostate cancer research (eg. DoD Idea Development Award, NIH R01, PCF Young Investigator Award, NIH K99/R01)**	100%

1. *Gain an improved understanding of RNA-seq analysis at the gene and transcript level.*

In an effort to improve her ability to understand and analyse RNA-sequencing data, Dr. Morel participated in a self-directed online 8-week course, 'Data Science: R Basics', run through Harvard Medical School's edX program. The program ran from September to December 2019.

Throughout this course, Dr Morel learned the following:

- Appreciate the rationale for data analysis using R
- Define objects and perform basic arithmetic and logical operations
- Use pre-defined functions to perform operations on objects
- Distinguish between various data types
- Learn how to create, name, and access numeric and character vectors and coerce data intodifferent data types as needed
- Sort vectors in ascending and descending order and rank the elements of a vector inincreasing order
- Perform arithmetic between a vector and a single number and two vectors of same length

    - Learn to subset a vector based on properties of another vector, and use multiple logical operators to index vectors
  - Wrangle data tables using the functions in 'dplyr' package, including modifying a data table byadding or changing columns and performing a series of operations using the pipe operator
  - Plot data in scatter plots, box plots and histograms

While this course did not provide expertise in RNA-sequencing analysis, it has provided Dr. Morel with enough basic knowledge to be able to interpret and present RNA-sequencing data on a basic level, which will be a significant asset both for this project and for her career moving forward.

In addition, Dr. Morel undertook a 5-week HarvardX course, 'Case Studies in Functional Genomics', which ran from December 2020, to build on her previously gained knowledge of R-based data science. The course focused on performing RNA-Seq, ChIP-Seq, and DNA methylation data analyses, using open source software, including R and Bioconductor.

Specifically, Dr Morel learned the following:

- Use of exploratory plots to get a general overview of the shape of data and the result of an experiment.
- RNA-seq data analysis, including quality control of FASTQ files; aligning RNA-seq reads; visualizing alignments
- Analysing RNA-seq at the gene-level: counting reads in genes;
- Exploratory Data Analysis and variance stabilization for counts; count-based differential expression; normalization and batch effects.
- RNA-seq at the transcript-level: inferring expression of transcripts and differential exon usage.
- Basic steps in analysing DNA methylation data, including reading the raw data, normalization, and finding regions of differential methylation across multiple samples.
- Basic steps for analysing ChIP-seq datasets, from read alignment, to peak calling, and assessing differential binding patterns across multiple samples.

2. *Present research at monthly chalk-talk sessions with Dr. Ellis, bi-monthly meetings with Dr. Sweeney, and multi-PI lab meetings every 4 months.*

Throughout her time working on this project, Dr. Morel was able to meet regularly with Drs. Ellis and Sweeney to discuss research progress and plan her academic career. While COVID-19 restrictions limited the number of wider institutional group meetings that were available for Data from this research project has been presented on several occasions at lab meetings and seminar series at Dana-Farber Cancer Institute (DFCI), including at the multi- institutional Dana Farber/Harvard Cancer Institute monthly seminar series.

3. *Present data at national scientific meetings.*

Due to the significant restriction of national and international scientific conferences in 2020 and

2021 due to the COVID-19 pandemic, this goal was difficult to achieve. While Dr. Morel was unable to present the work from this project at a scientific meeting, she had had the opportunity to present data from additional research projects at other meetings. Dr. Morel presented a poster at the 12th Annual Multi-Institutional Prostate Cancer Program Retreat, 1-3 March 2020, and a virtual poster at the 27th Annual Scientific Retreat of the Prostate Cancer Foundation, 22- 23 October 2020. Dr. Morel also presented a talk at the Annual Society for Basic Urologic Research meeting, 11-14 November 2020.

4. *Attend workshops and seminars offered by the DFCI Postdoc and Graduate Students Affairs Office every 2 months relevant to lab management, grant writing, and transitioning to academia*

Due to the COVID-19 pandemic the DFCI Postdoc and Graduate Students Affairs Office (PGSAO) significantly reduced the number of seminars offered to trainees, however, Dr. Morel had the opportunity to attend several workshops to help further her academic career.

cBioPortal Instructional Workshops – Broad Institute trainee workshop

- Introduction to cBioPortal (April 30th, 2020)
- Mutation Details & Patient View (May 7th, 2020)
- OQL & Expression (May 14th, 2020)
- Group Comparison (May 21st, 2020)
- API & R Client (May 28th, 2020)

Advanced Manuscript Writing – DFCI four-part workshop

- Introduction (April 23rd, 2020)
- Methods (April 30th, 2020)
- Results/Illustrations (May 7th, 2020)
- Discussion/Title/Abstract (May 14th, 2020)

In addition, Dr. Morel has been actively attending and involved with monthly trainee seminars run through the DFCI PGSAO to allow postdoctoral fellows the opportunity to share research and get input from their peers.

5. *Award to fund further prostate cancer research.*

Dr. Morel has continued to apply for early career research awards to further her postdoctoral career and help start an independent career. To date this has been unsuccessful, however there has been continued feedback on these research proposals and it continues to be an excellent learning process.

4. IMPACT:

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

From this research project we have shown that murine and human models of aggressive variant prostate cancer (AVPC) are particularly sensitive to combinatorial inhibition of EZH2 and the PI3K/Akt/mTOR pathway, and that combination significantly enhances response to androgen deprivation. Moreover, we identified that as AVPC models transition to complete castration-resistance, activated Akt is significantly reduced and sensitivity to PI3K/Akt/mTOR pathway is lost, which may be rescued by inhibition of EZH2. Collectively, this data demonstrates the ability of EZH2 and PI3K/Akt/mTOR co-inhibition to significantly inhibit AR independence and maintain response to androgen deprivation therapy.

- **What was the impact on other disciplines?**

Nothing to report.

- **What was the impact on technology transfer?**

Nothing to report.

- **What was the impact on society beyond science and technology?**

Nothing to report.

5. CHANGES/PROBLEMS:

It was originally planned that tumor samples from in vivo studies would be analysed by RNA-sequencing. However, difficulties with COVID-19 laboratory restrictions and the relocation of the Ellis laboratory from Boston to Los Angeles meant that the samples were unable to be sent for sequencing analysis. As an alternative solution, tumour samples were examined by qPCR for expression of pathways of interest. While this was not as in depth as full RNA-sequencing would have been, the qPCR analysis provided valuable information about therapeutic mechanisms within the treated tumors.

It was also planned that therapy studies would be carried out on Pten-/-Rb1-/- GEMMs. A breeding strategy was put in place to generate enough GEMMs to carry out this study prior to COVID-19 laboratory restrictions; however, the COVID-19 laboratory restrictions in the Dana-Farber Cancer Institute Animal Research Facility limited the ability to generate enough mice to carry out these studies with reasonable power. As a solution to this problem, whole tumor sections were harvested from the available GEMMS, dissected into smaller tissue sections and implanted subcutaneously into the flank of C57BL/6N mice. The therapeutic response in these mice closely mirrored what was observed in the allograft murine cell line therapy studies.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Publications:
KL Morel, GP Donoho, AH Nguyen, H Beltran, CJ Sweeney, L Ellis, '**Combining Inhibition of EZH2 and PI3K/Akt/mTOR Provides Significant Anti-Tumor Activity in Models of Aggressive Variant Prostate Cancer'**
Manuscript in final draft stages.

Presentations:
K Morel, CJ Sweeney, L Ellis, **'Targeting EZH2 and PI3K/mTOR for a novel combination therapeutic strategy in aggressive variant prostate cancer'**
Data presented as a poster Dana-Farber Prostate Cancer Research Program Retreat – July 2020

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

Nothing to report.

- **Technologies or techniques**

Nothing to report.

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

Nothing to report.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

- **What individuals have worked on the project?**
    - *Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."*

Name:	*Katherine Morel*
Project Role:	*PI / Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	*0000-0002-7968-4032*
Nearest person month worked:	*12*
Contribution to Project:	*Dr. Morel has run all the in vivo and in vitro experiments and most of the analysis for this project to date.*
Funding Support:	

8. APPENDICES:

None