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14. ABSTRACT
Metastatic castration-resistant prostate cancer (mCRPC) is a lethal disease with about 30,000 estimated annual deaths in U.S, with a vast majority of CRPC driven by androgen receptor (AR) signaling. AR-signaling is critical for the development and progression of prostate cancer, and AR also the main therapeutic clinical target. AR-targeted therapies, such as AR-antagonists, provide substantial benefits in the treatment of metastatic castration-resistant prostate cancer (mCRPC); however, majority of patients fail these therapies and succumb to the disease. Therefore, there is a clear and pressing need to develop new therapeutics against the AR axis in CRPC. One such novel strategy for targeting the AR-pathway and inhibiting the growth of CRPC has been the use of bromodomain and extraterminal (BET) protein inhibitors; however, a new class of molecules that target BET bromodomain proteins through their proteasomal degradation can improve efficacy and specificity.
Based on our findings, we hypothesize that pharmacologic BET bromodomain degradation represents an important advance in the treatment CRPC, and may provide a novel therapeutic strategy for advanced prostate cancer. The overall goal of this proposal is to develop very potent small molecule that leads to the proteasomal-degradation of BET bromodomain proteins, with optimized in vivo properties, and to provide a compelling scientific rationale, including detailed mechanistic insight, to facilitate advancement of BET bromodomain degraders as a novel potential therapeutic strategy for patients with the metastatic CRPC.

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proteasomal degradation

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Effective use of BET Bromodomain Degraders to prevent resistance to AR- and BET- targeted therapies for prostate cancer.

Keywords: Androgen Receptor, BET, BRD2/3/4, Bromodomain, Castration-Resistance, Enzalutamide-Resistance, MYC, PROTAC, Protein Degradation,

Introduction: Metastatic castration-resistant prostate cancer (mCRPC) is a lethal disease with about 30,000 estimated annual deaths in U.S, with a vast majority of CRPC driven by androgen receptor (AR) signaling. AR-signaling is critical for the development and progression of prostate cancer, and AR also the main therapeutic clinical target. AR-targeted therapies, such as AR-antagonists, provide substantial benefits in the treatment of mCRPC; however, majority of patients fail these therapies and succumb to the disease. Therefore, there is a clear and pressing need to develop new therapeutics against the AR axis in CRPC. One such novel strategy for targeting the AR-pathway and inhibiting the growth of CRPC has been the use of bromodomain and extraterminal (BET) protein inhibitors; however, a new class of molecules that target BET bromodomain proteins through their proteasomal degradation can improve efficacy and specificity. Based on our findings, ***we hypothesize that pharmacologic BET bromodomain degradation represents an important advance in the treatment of CRPC, and may provide a novel therapeutic strategy for advanced prostate cancer***. The overall goal of this proposal is to develop very potent small molecule that leads to the proteasomal-degradation of BET bromodomain proteins, with optimized *in vivo* properties, and to provide a compelling scientific rationale, including detailed mechanistic insight, to facilitate advancement of BET bromodomain degraders as a novel potential therapeutic strategy for patients with the metastatic CRPC.

Accomplishments:

Training-Specific Tasks:

Major Task: Training and educational development in prostate cancer research *(only applicable to training award mechanisms)*		**UM**
Subtask 1: Attend 2017 AACR Meeting in Washington, D.C. Presented poster: **Kregel, S.,** Malik, R., Asangani, I. A., Wilder-Romans, Jiang, X., V. L., Krishnamurth, P. M., Apel, I. J., Ravi, G., Eskara-Wilke, J., Tien, J., Cao, X., Speers, C., Feng, F. Y., Wang, S., Chinnaiyan, A. M. *Functional and Mechanistic Interrogation of BET Bromodomain Degraders for the Treatment of Metastatic Castration-Resistant Prostate Cancer,* American Association for Cancer Research – Annual Meeting – Washington, D.C., April 2, 2017	Attended	Dr. Kregel
Subtask 2: Present research at the monthly department group meetings.	Ongoing	Dr. Kregel
Subtask 3: Attend Hormone Dependent Cancers Gordon Conference in Newry, Maine.	Did Not Attend	Dr. Kregel

- Will Present findings instead at 8th Great Lakes Nuclear Receptor Conference, Oral Presentation, October 18 -19, 2018	Attended and Presented	Dr. Kregel
Subtask 4: Attend 2018 AACR Meeting in Chicago, Illinois. Presented poster: **Kregel, S.,** Malik, R., Asangani, I. A., Wilder-Romans, Jiang, X., V. L., Krishnamurth, P. M., Apel, I. J., Ravi, G., Eskara-Wilke, J., Tien, J., Cao, X., Speers, C., Feng, F. Y., Wang, S., Chinnaiyan, A. M. *Functional and Mechanistic Interrogation of BET Bromodomain Degraders for the Treatment of Metastatic Castration-Resistant Prostate Cancer,* American Association for Cancer Research – Annual Meeting – Chicago, IL., April 18, 2018	Attended	Dr. Kregel
Subtask 5: Present at Michigan Center for Translational Pathology Research Summit, and at the Comprehensive Cancer Center Symposium.	Complete	Dr. Kregel
Milestone(s) Achieved: Presentation of project data at a national meeting.	24	

Research-Specific Tasks:

Specific Aim 1: To characterize and functionally assess the efficacy of BET bromodomain degraders using prostate cancer cell lines, and their effects on MYC and AR signaling.	**Status**	**UM**
Task 1: To characterize and functionally assess the efficacy of BET bromodomain degraders using prostate cancer cell lines.		
Subtask 1: Assaying cytotoxicity and calculating IC:50s of BET bromodomain degraders in normal and cancer cell lines. Cell lines and models used: AR positive (VCaP, LNCaP, 22RV1), AR negative (DU145, PC3) and normal (PNT2, RWPE1, 957E/hTERT) [ATTC and Chinnaiyan lab].	Completed See Data Below	Dr. Kregel and Chinnaiyan Lab
Subtask 2: Assaying the effects of BET bromodomain degraders on invasion and migration of normal and cancer cell lines. Cell lines and models used: AR positive (VCaP, LNCaP, 22RV1), AR negative (DU145, PC3) and normal (PNT2, RWPE1, 957E/hTERT) [ATTC and Chinnaiyan lab].	Nothing To Report	Dr. Kregel and Chinnaiyan Lab
Subtask 3: Characterization of cell death induced by BET bromodomain degraders.		Dr. Kregel

Cell lines and models used: AR positive (VCaP, LNCaP, 22RV1), AR negative (DU145, PC3) [ATTC and Chinnaiyan lab].	Completed See Data Below	
Major Task 2: Assess the effects of BET bromodomain degraders on MYC and AR signaling.		
Subtask 1: Transcript and protein analysis of AR and Myc; and their target genes. Cell lines and models used: VCaP, LNCaP, 22RV1 [ATTC and Chinnaiyan lab].	Completed See Data Below	Dr. Kregel
Subtask 2: Chromatin immunoprecipitation sequencing of AR, MYC, BRD4, and Histone H3 K27ac and analysis. Cell lines and models used: VCaP, LNCaP, 22RV1 [ATTC and Chinnaiyan lab].	Nothing to Report	Dr. Kregel and Chinnaiyan Lab
Milestone(s) Achieved: Assessed the efficacy of BET bromodomain degraders using prostate cancer cell lines, and their effects on MYC and AR signaling, prepare manuscript and submit for publication.	Manuscript In Preparation	
Aim 2: To elucidate the mechanism behind therapeutic activity of BET degraders in Prostate Cancer.		
Major Task 3: Elucidate the mechanism behind therapeutic activity of BET degraders in prostate cancer cell lines.		
Subtask 1: RNA sequencing and analysis of Prostate Cancer cells treated with BET Bromodomain Degraders and inhibitors. Cell lines and models used: VCaP, LNCaP, 22RV1 [ATTC and Chinnaiyan lab].	See Data Below for VCaP and 22Rv1 Cells	Dr. Kregel and Chinnaiyan Lab
Subtask 2: Mass Spectroscopy and analysis of Prostate Cancer cells treated with BET Bromodomain Degraders and inhibitors. Cell lines and models used: VCaP, LNCaP, 22RV1 [ATTC and Chinnaiyan lab].	See Data Below for VCaP and 22Rv1 Cells	Dr. Kregel, Chinnaiyan Lab
Subtask 3: Analysis of candidate proteins in mediating BET Bromodomain Degraders cytotoxicity.	See Data Below for	Dr. Kregel, Chinnaiyan Lab

Cell lines and models used: VCaP, LNCaP, 22RV1 [ATTC and Chinnaiyan lab].	VCaP and 22Rv1 Cells	
Milestone(s) Achieved: Elucidated the mechanism behind therapeutic activity of BET degraders in prostate cancer cell lines, prepare manuscript and submit for publication.	Manuscript Published in Clinical Cancer Research	Dr. Kregel, Chinnaiyan Lab
Aim 3: To establish in vivo efficacy of BET degraders in animal models of prostate cancer.		
Major Task 4: To establish in vivo efficacy of BET degraders in animal models of prostate cancer.		
Subtask 1: Assess *in vivo* efficacy of BET degraders as single agents using CRPC xenograft model systems of prostate cancer. Cell lines and models used: VCaP, 22RV1 [ATTC and Chinnaiyan lab]. SCID MICE [10 mice (2 xenografts/mouse) 20 per individual condition (Vehicle, ZBC-260, 20 per cell line, 60 total mice)].	See Data Below for VCaP Cells	Dr. Kregel and Chinnaiyan Lab
Subtask 2: Determine the *in vivo* efficacy of BET Bromodomain Degraders in combination with enzalutamide. Cell lines and models used: VCaP, 22RV1 [ATTC and Chinnaiyan lab]. SCID MICE [10 per individual condition (2 xenografts/mouse) (Vehicle, Enzalutamide, ZBC-260, ZBC-260 and Enzalutamide, 40 per cell line, 80 total mice)].	See Data Below for VCaP Cells	Dr. Kregel and Chinnaiyan Lab
Subtask 3: Assess in vivo efficacy of BET Bromodomain Degraders using CRPC patient-derived xenografts. Models used: Three PDX models: MDA PCa 146-12, MDA PCa 146-10 and MDA-183 (20 mice per condition, 10 vehicle and 10 treated, 60 mice total). [Dr. Nora Navone, MD Anderson, Chinnaiyan Lab].	See Data Below for MDA PCa 146-12	Dr. Kregel and Chinnaiyan Lab
Milestone(s) Achieved: Establishment of in vivo efficacy of BET degraders in animal models of prostate cancer, prepare manuscript and submit for publication.	Manuscript Published in Clinical Cancer Research	Dr. Kregel, Chinnaiyan Lab

A

IC:50 Values

	AR-Positive Prostate Cancer			AR-Negative Prostate Cancer		Benign Prostate Epithelial	
Compound	**VCaP**	**22Rv1**	**LNCaP**	**Du145**	**PC3**	**PNT2**	**RWPE1**
Thalidomide	> 3µM	>5µM	>5µM	>3µM	>3µM	>5µM	>5µM
ZBC-11	12 nM	19nM	12nM	400nM	370nM	460nM	390nM
ZBC-244	1 nM	2.3nM	9.5nM	690nM	50nM	-	-
ZBC-246	0.5 nM	1.8nM	5.6nM	250nM	20nM	23nM	5nM
OTX-015	92 nM	136nM	45nM	1.3µM	1.1µM	560nM	408nM

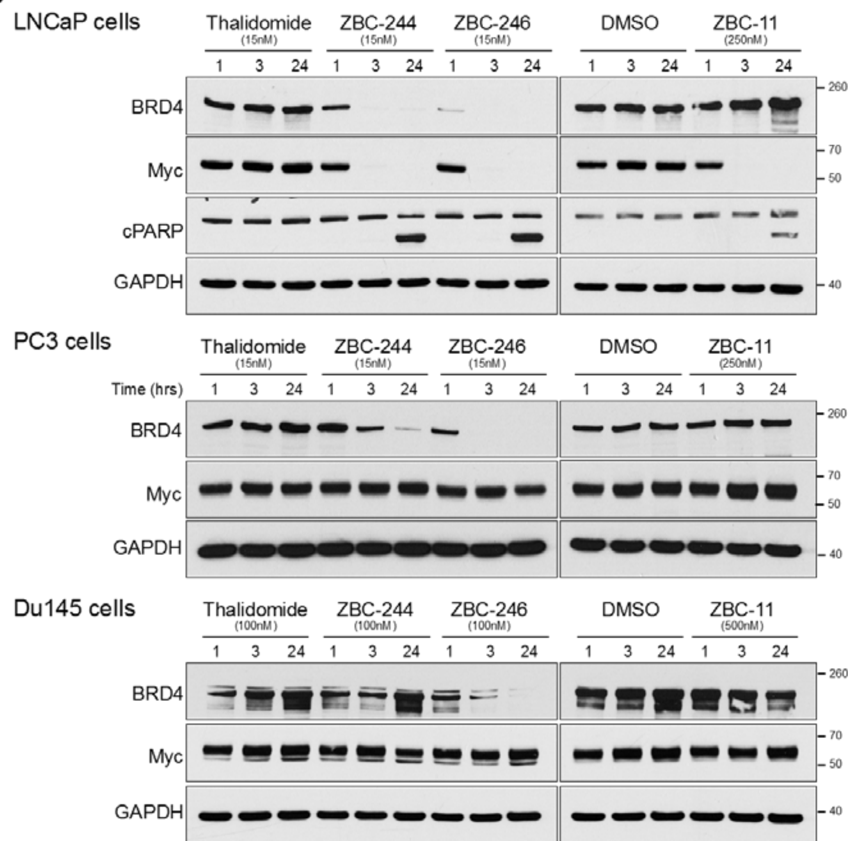

Figure 1: Efficacy of BET-degraders. A) Table of Half Maximal Inhibitory Concentration (IC_{50}) Values for BET-inhibitors and Degraders. The inhibitors ZBC-11 and OTX-015, and the BET-Degraders ZBC-244 and ZBC-246 were tested for efficacy in both AR-positive and AR-negative prostate cancer cell lines. Thalidomide control showed no toxicity. B) The effect of BET-inhibitors and Degraders on BRD-4 and MYC levels in AR-positive and AR-negative cell lines. Western blot analysis of BRD-4 and c-MYC of whole cell lysates from AR-positive LNCaP and AR-Negative Du145 and PC3 cells treated for 1, 3 and 24 hours with the degraders ZBC-244 and ZBC-246, and the inhibitor ZBC-11 compared to control (Thalidomide and DMSO, respectively). PARP cleavage (cPARP) is used as a marker of apoptosis in LNCaP cells seen as two bands at 24 hours of treatment. GAPDH serves as a loading control.

Results:

We first assessed the cellular activity and selectivity of the degraders ZBC-244 and ZBC-246 in a panel of AR-positive (VCaP, LNCaP, and 22rv1) and AR-negative (DU145 and PC3) prostate cancer cell lines. We evaluated the effect of compounds on cell proliferation and compared sensitivities, as demonstrated by the half-maximum Inhibitory Concentration (IC:50) values of the various BET inhibitors and degraders on the cell lines (Figure 1A). Thalidomide, which interacts with the E3-ubiquitin ligase Cereblon, combines with the BET inhibitor ZBC-11 and a few chemical modifications to form BET-degraders ZBC-244 and ZBC-246. For comparison, OTX-015 is a BET-inhibitor in clinical development. ZBC-244 and ZBC-246 have pico- to nano-molar IC:50 concentrations in AR-positive cancer cells with >~10-fold greater efficacy than ZBC-11 alone, and >~100-fold more effective that OTX-015 (23). However, AR-negative prostate cancer cells are much less sensitive to all treatments, similar to what we have recently identified with other BET-inhibitors (12,24). All prostate cancer cells show marked decreases in BRD4 protein after as little as 3 hours of treatment of either degrader (Figure 1B, ZBC-11 treatment as an inhibitor control). AR-positive LNCaP cells treated with ZBC-244 and ZBC-246 at 1, 3 and 24 hours show a decrease in MYC expression while AR-Negative DU145 and PC3 cells do not (Figure 1B). LNCaP cells also show PARP cleavage at 24 hours, indicating apoptosis.

BET inhibition is known to disrupt MYC and AR signaling (12,14) in prostate cancer cells. To further characterize the effects of BET-degraders on AR-positive cancer cell lines, we assayed their protein expression in 22rv1 and VCaP cell lines by western blot and quantitative mass spectroscopy. Similar to what was seen in LNCaP (Figure 1B), 22rv1 and VCaP cells treated at 1, 3 and 24 hours with either ZBC-244 or ZBC-246 show a marked a decrease in MYC expression and the degradation of all BET isoforms, BRD2/3/4, expressed in prostate cancer cell lines (Figure 2A). VCaP cells also showed decreases in expression of the canonical AR-target gene, prostate specific antigen (PSA or *KLK3*), at 24 hours after treatment (Figure 2A), thus illustrating AR-signaling disruption. Quantitative proteomic analysis of isobaric tandem mass tag (TMT) labeled peptides from whole cell lysates of VCaP and 22Rv1 cells treated with either the BET degrader ZBC-246 or thalidomide control after 12 hours confirms degradation of BET-proteins (Figure 2C, 2D). Furthermore, the AR-target gene IGFPB3 was shown to also be downregulated in VCaP cells treated with ZBC-246 when compared to control. In both cell lines analyzed, TXNIP (Thioredoxin-interacting protein), an AR- and MYC- repressed tumor suppressor protein that is commonly upregulated in response to a variety of cell stresses and promotes apoptosis (25-28), increased (Figure 2B).

BET-degraders also prevent the upregulation of BRD proteins, which is observed with inhibitor treatment. We utilized quantitative label-free mass spectroscopy of whole cell lysates from VCaP cells treated with ZBC-11 or ZBC-246 and their respective controls to assay global changes in protein levels. We identified increases of BRD2 in as little as 3 hours after treatment with ZBC-11 when compared to control (Figure 2E), and observed a five-fold increase of BRD2 at 12 hours, which was validated by increases in protein at 24 hours in both VCaP and 22Rv1 cells (Figure 2A and 2B); whereas dramatic decreases in BRD-2,-3,-4 were seen in cells treated with ZBC-246 (Figure 2E). These data suggest a potential response to BET inhibition is the upregulation of BET proteins, and which may serve as a potential mechanism for resistance to BET-inhibitors that BET-degrader treatment would prevent. Furthermore, we compared the effects of other Cereblon-based BET degraders previously shown to have efficacy in prostate cancer cell lines ARV825 (19), and ZBC-260, our ZBC-11 based degrader with the highest efficacy and tolerability *in vivo*. At the same dose, we observe greater degradation BRD4 in both VCaP and

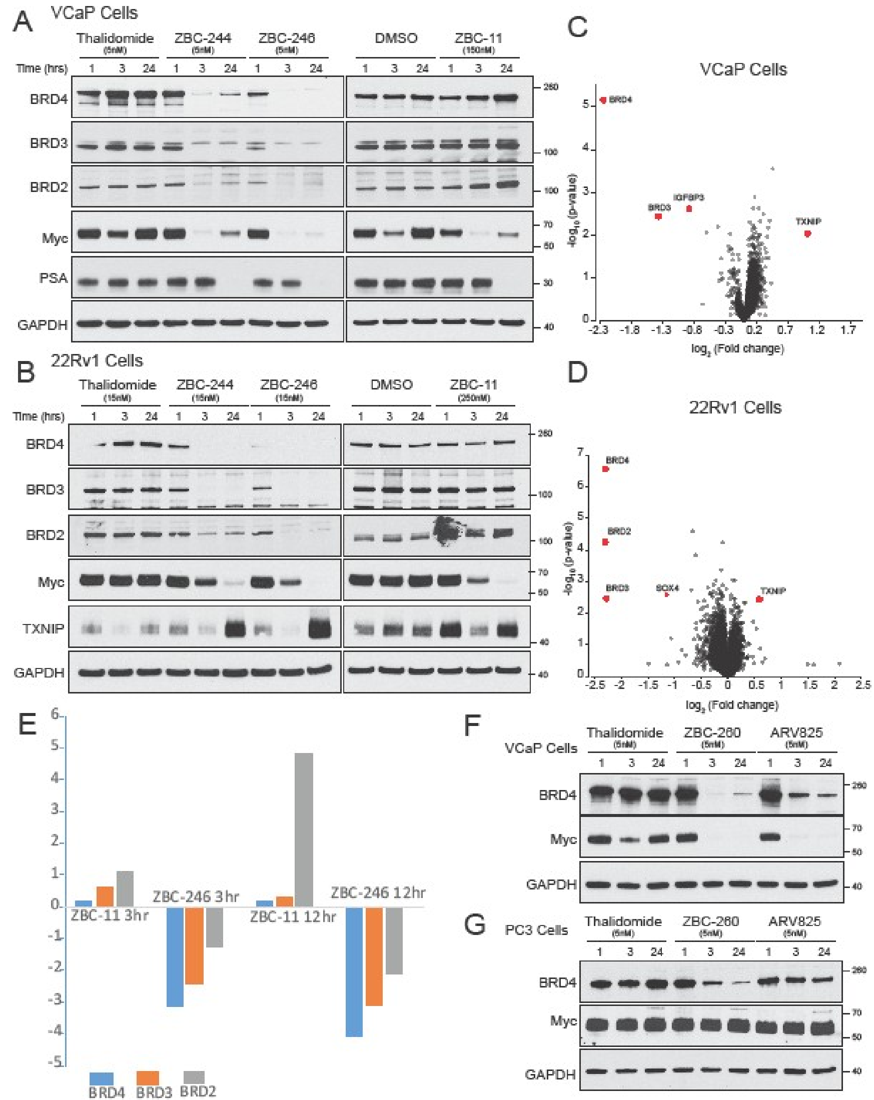

Figure 2: <u>Confirmation of on-target degradation of BET Proteins in VCaP and 22rv1 AR-positive prostate cancer cells</u>. A) Western blot analysis of BRD-2,3,4, c-MYC and the AR-target gene, PSA, of whole cell lysates from VCaP cells treated for 1, 3 and 24 hours with the degraders ZBC-244 and ZBC-246, and the inhibitor ZBC-11 compared to control (Thalidomide and DMSO, respectively). B) Western blot analysis of BRD-2,3,4, c-MYC and the MYC and AR-target gene TXNIP, of whole cell lysates from 22Rv1 cells treated for 1, 3 and 24 hours with the degraders ZBC-244 and ZBC-246, and the inhibitor ZBC-11 compared to control (Thalidomide and DMSO, respectively). C) VCaP cells were 5nM Thalidomide or 5nM ZBC-246 for 3 hours, then subjected to whole cell TMT mass spectroscopy. Relative fold changes (x-axis) between control thalidomide and ZBC-246 treatment shown. D) 22Rv1 cells were 5nM Thalidomide or 5nM ZBC-246 for 3 hours, then subjected to whole cell TMT mass spectroscopy. Relative fold changes (x-axis) between control thalidomide and ZBC-246 treatment shown. E) VCaP cells were treated with 5nM ZBC-246 or 50nM ZBC-11 for 3 or 12 hours, then subjected to whole cell label free mass spectroscopy compared to control (Thalidomide and DMSO, respectively). Comparison of ZBC-260 and ARV-825 BET-degraders in VCaP (F) and PC3 (G). Western blot analysis of protein degradation of BRD-4 and effects of c-MYC. GAPDH serves as a loading control.

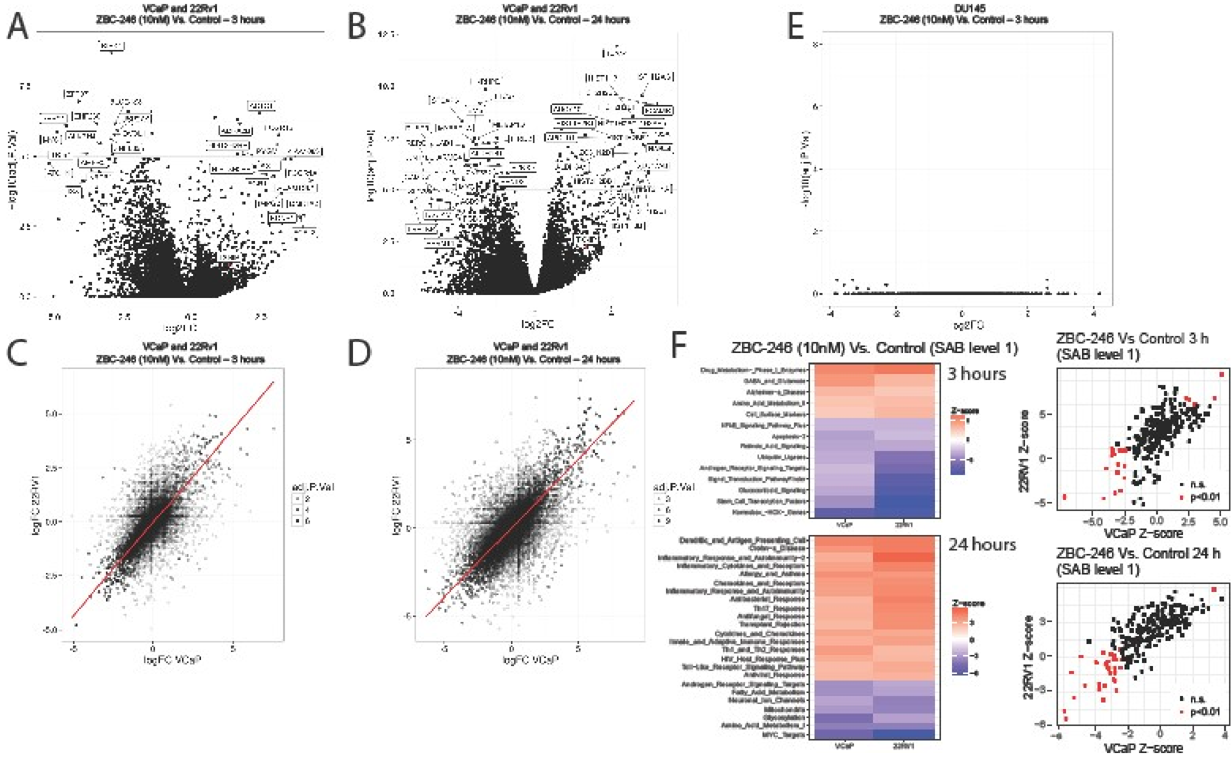

Figure 3: <u>RNA-seq confirmation of c-MYC downregulation and target genes at 3 and 24 hours in AR-positive prostate cancer cells</u>. RNA-sequencing was performed on VCaP and 22Rv1 cells treated with 10nM ZBC246 for 3 and 24 hours. Volcano plots showing the differentially expressed transcripts in both VCaP and 22rv1 cells at 3 (A) and 24 hours (B) of 10nM ZBC-246 treatment plotted as a function of average fold change verses log p-value. The most downregulated transcript in both cell lines at 3 hours was MYC, serving as a positive control. There is a significant correlation between genes differentially regulated in VCaP and 22Rv1 cells treated with ZBC-246. Correlations between the two cell lines are shown as Log Fold change of 22rv1 cells plotted against log fold change of VCaP cells of each of the differentially expressed transcripts at 3 (C) and 24 hours (D) of 10nM ZBC-246 treatment. E) RNA-seq on Du145 cells treated with 10nM ZBC-246 for 3 hours plotted as a function of average fold change verses log p-value. No expressed transcripts met significance for differential expression. F) RNA gene set pathway analysis from RNA-sequencing of VCaP and 22Rv1 cells treated with 10nM ZBC-246 degrader and thalidomide control. SA Biosciences pathway level 1 Molecular Signatures Database (MSigDB) pathway analysis in VCaP and 22rv1 cells at 3 and 24 hours of 10nM ZBC-246 treatment. Z-score of significance of individual pathways shown plotted between 22Rv1 (y-axis) and VCaP (x-axis).

PC3 cells (Figure 2F and 2G), as well as increased efficacy in inhibiting growth in 22Rv1 cells (Supplemental Figure 1) by ZBC-260 when compared to ARV825.

To fully assess the effects of the BET-degraders on downstream transcriptional regulation, we performed RNA-sequencing (RNA-seq) on VCaP, 22Rv1, and DU145 cells treated with 10nM ZBC-246 and Thalidomide control (Figure 3). Differentially expressed transcripts were identified in both VCaP and 22Rv1 cells at 3 and 24 hours of 10nM ZBC246 treatment (Figure 3A, 3C). Both VCaP and 22Rv1 cells showed a significant correlation in which transcripts were differentially expressed at both time points (Figure 3B, 3D), and MYC is downregulated at both time points and is the most downregulated transcript in both cell lines at 3 hours. As we had observed in our proteomics experiments, TXNIP is upregulated at both time points in both cell lines (Figure 3A, 3C). Overall, there is a bias towards more genes being downregulated with BET-degrader treatment, which is consistent with previous data suggesting that BET proteins function as transcriptional elongation factors that promote productive elongation of transcripts (29). We also performed RNA-seq on Du145 cells treated with ZBC-246 and compared them to thalidomide treated control cells, and we identified no changes in gene expression that met a significant p-value, thus confirming that there is little effect of BET-degradation in AR-negative cell lines.

From the differentially altered genes in VCaP and 22Rv1 cells, we performed enrichment across the Molecular Signatures Database (MSigDB) to identify altered and essential global pathways for the growth phenotype observed in AR-positive cell lines. Consistent with previous data, altered pathways in both cell lines strikingly correlate with the downregulation of pathways, primarily that of AR and MYC (Figure 3F). Curiously, immune response pathways are significantly upregulated after both 3 and 24 hours of degrader treatment in both cell lines. This result supports previous studies that have shown BET inhibition to boost anti-tumor immune responses, and suggests that targeting BET-proteins in combination with immune checkpoint inhibitors may elicit a potential synergistic response (30). Furthermore, c-MYC driven tumors are reported to have intrinsic tumor cell autonomous regulation and suppression of both innate and adaptive immune responses, and inhibition of MYC restores immune responses to tumor cells (31). We also identified that fatty acid metabolism is decreased upon treatment of BET- degraders though MSigDB at 24 hours (Figure 3F). To validate these effects and identify a lipid- based signature associated with BET-inhibition and degradation in prostate cancer cell lines, we performed lipidomic mass spectroscopy analysis on VCaP and 22Rv1 cells treated with the BET-degrader, ZBC-260 (Figure 4), and the BET-inhibitors, ZBC-11 and OTX-015 (Supplemental Figures 4-6). We find that both cell lines treated with BET-degraders have higher levels of polyunsaturated fatty acids (PUFAs) and lower levels of other lipid species including phospholipids and saturated fatty acids (Figure 4). These findings suggest that this shift in fatty acid composition reflects a decrease in de novo fatty acid synthesis as well as decreased metabolism, and corresponds to the alterations seen in the transcriptional profiles (Figure 3F). Furthermore, this correlates with the changes in MYC expression, as MYC knockout cells have been shown to have lower levels of saturated fatty acids and higher levels of PUFAs (32). Additionally, lipid metabolism is also AR-regulated in prostate cancer cells, and lipogenesis can be inhibited upon AR-inhibition in a sterol regulatory element-binding proteins (SREBP) dependent manner (33).

BET-degraders also show strong efficacy in multiple prostate cancer models *in vivo*. We first utilized a castration-resistance VCaP xenograft model. Intact CB-17 SCID mice were inoculated with tumors, and once the tumors reached 200 mm^3 in size the mice were castrated. The tumors initially regress, but start growing again as previously characterized (12). When the tumor

grew back to the pre-castration size, animals were treated with either 5mg/kg ZBC-260 or thalidomide control. Tumor volumes of the ZBC-260-treated mice were dramatically reduced when compared to control (Figure 5A). We then assayed the effects of ZBC-260 on the castration-

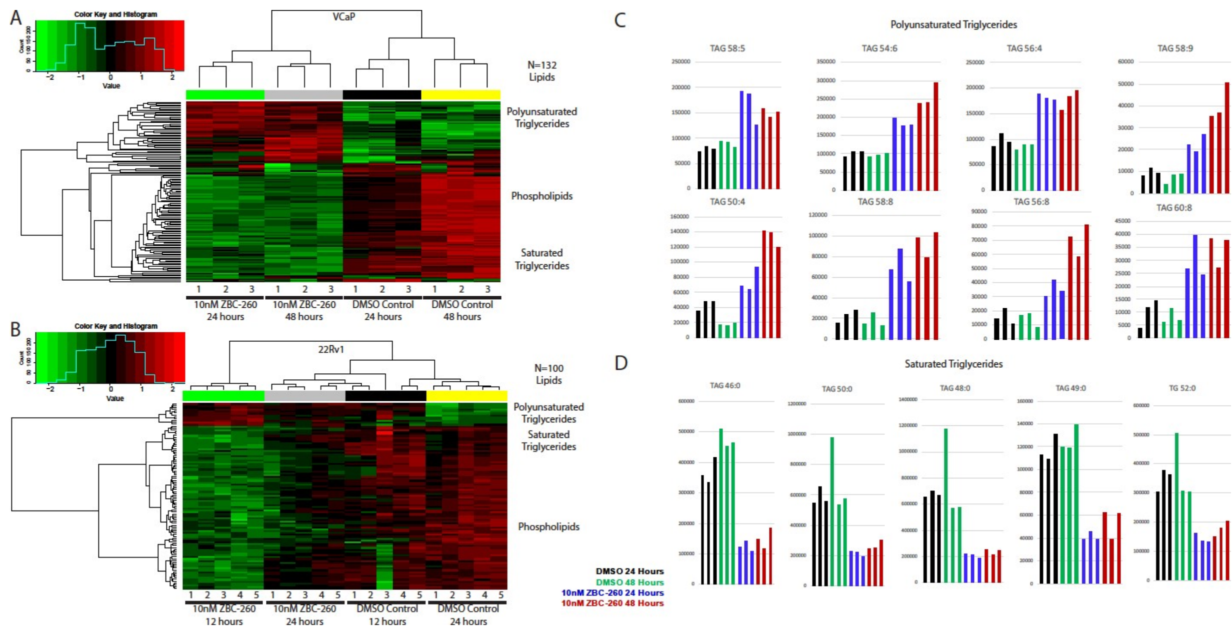

Figure 4: <u>Decreased Fatty Acid Metabolism in BET-Degrader Treated Cells.</u> Heat map visualization of significantly altered lipid species ($p<0.05$, FDR corrected) in cells treated with 10nM ZBC-260 or DMSO control for either 24 and 48 hours in VCaP (A – 132 lipid species) or 12 and 24 hours in 22Rv1 (B – 100 lipid species). Columns represent replicate samples and rows refer to distinct lipid species. Shades of red and green represent elevation and reduction of the lipids, respectively, relative to the median lipid levels. Plots of the polyunsaturated (C) and saturated (D) triglycerides altered in VCaP cells. Black = DMSO control treated cells at 24 hour time point, Green = DMSO control treated cells at 48 hour time point, Blue = 10nM ZBC-260 treated cells for 24 hours, Red = 10nM ZBC-260 treated cells 48 hours. Columns represent replicate samples. TAG = Triacylglycerol, TG = triglyceride.

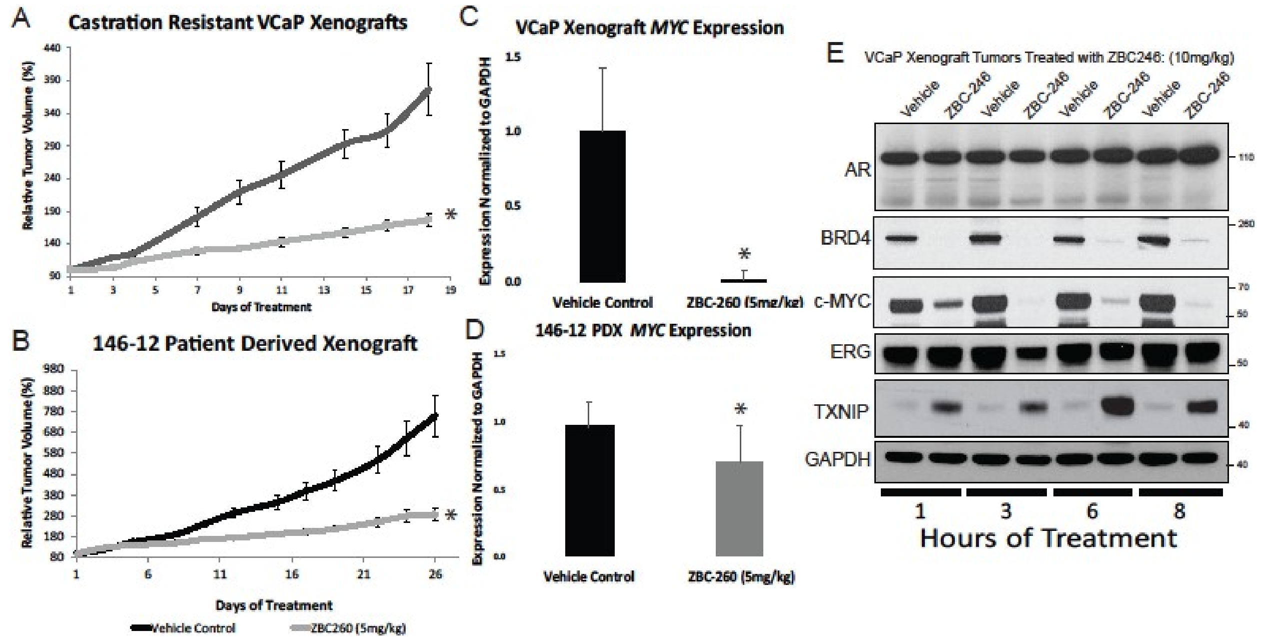

Figure 5: BET-degrader *in Vivo* Efficacy in Prostate Xenograft models. A) Growth curves of tumor volume illustrating the effects of BET-degraders on Castration-Resistant VCaP Xenografts in vivo. Mice were injected 2 × 10^6 VCaP prostate cancer cells suspended in 100 µl of PBS with 50% Matrigel (BD Biosciences) were implanted subcutaneously into the dorsal flank on both sides of the mice. Once the tumors reached 200 mm3 in size the mice were castrated, and once the tumor grew back to the pre-castration size, the animals were treated with 5mg/kg ZBC-260. B) Growth curves of tumor volume of the effects of BET-degraders on the AR-positive patient derived xenograft (PDX) 146-12. Human c-MYC mRNA expression from total RNA extracted from castration resistant VCaP xenograft (C) and PDX-146-12 tumors (D). E) Western blot of lysates were obtained from castration resistant VCaP xenograft tumors. Within as little as 1 hour, we observe degradation of BRD4, decreases of MYC and the of oncogenic, AR-driven TMPRSS2:ERG expression (ERG Western Blot), and an upregulation of TXNIP. The effects last up to at least 8 hours. GAPDH serves as a loading control.

resistant patient derived xenograft (CRPC PDX) mouse model, MDA PCa 146-12. Similar to the castration resistant VCaP model, tumor volume was significantly smaller in ZBC-260-treated CRPC PDX mice (Figure 5B). RNA extracted from tumors in both VCaP xenograft and CRPC PDX models confirmed decreases in MYC expression (Figure 5C and 5D), and rapid and sustained on-target decreases in BRD-4, MYC and ERG protein, as well as TXNIP upregulation, were observed in protein lysates of VCaP xenograft tumors from ZBC-260 treated mice (Figure 5E).

Predicting Potential Mechanisms of Resistance to BET-Degraders and Confirmation of their Mechanism of Action

Finally, instead of focusing on the redundant models proposed in the initial study, we wanted to predict resistance to our BET-degraders. We utilized a whole genome clustered regularly interspaced short palindromic repeats (CRISPR) single guide RNA (sgRNA) library screen to identify genes essential for BET-degrader-mediated growth inhibition and apoptosis. LNCaP cells were transduced with low-titer lentivirus and treated with 10 nM dBET-3 for 30 days, and surviving cells were assayed for sgRNA enrichment. As expected, cells whose sgRNAs targeted the ubiquitin ligase complex were enriched and survived treatment. The most enriched sgRNAs

targeted CRBN, Ubiquitin E2 conjugating enzyme (UBE2G1), and the COPS2 subunit of members of the constitutive photomorphogenesis 9 (COP9) signalosome complex. These proteins are essential for BET-degrader mediated ubiquitination and their loss mediates resistance to this class of drugs (Figure 6A)(32). As a control, we also assayed DU145 cells for genes essential for their growth using the same sgRNA library without drug treatment. We unsurprisingly identified genes such as the Ribonuclease (RNase)_P protein complex member RPP21 and MYC as essential for growth (33,34); however, BRD2/3/4 and BRDT (whose expression is limited to the testes and is not expressed in DU145) did not meet the significance cut off for essential for growth (Figure 6B); suggesting they are dispensable for growth in DU145. Ultimately, this illustrates the on-target effects of the BET-degraders, and further confirms the degradation of BET-proteins through the ubiquitin-proteasome pathway when treated with ZBC-260 (dBET-3).

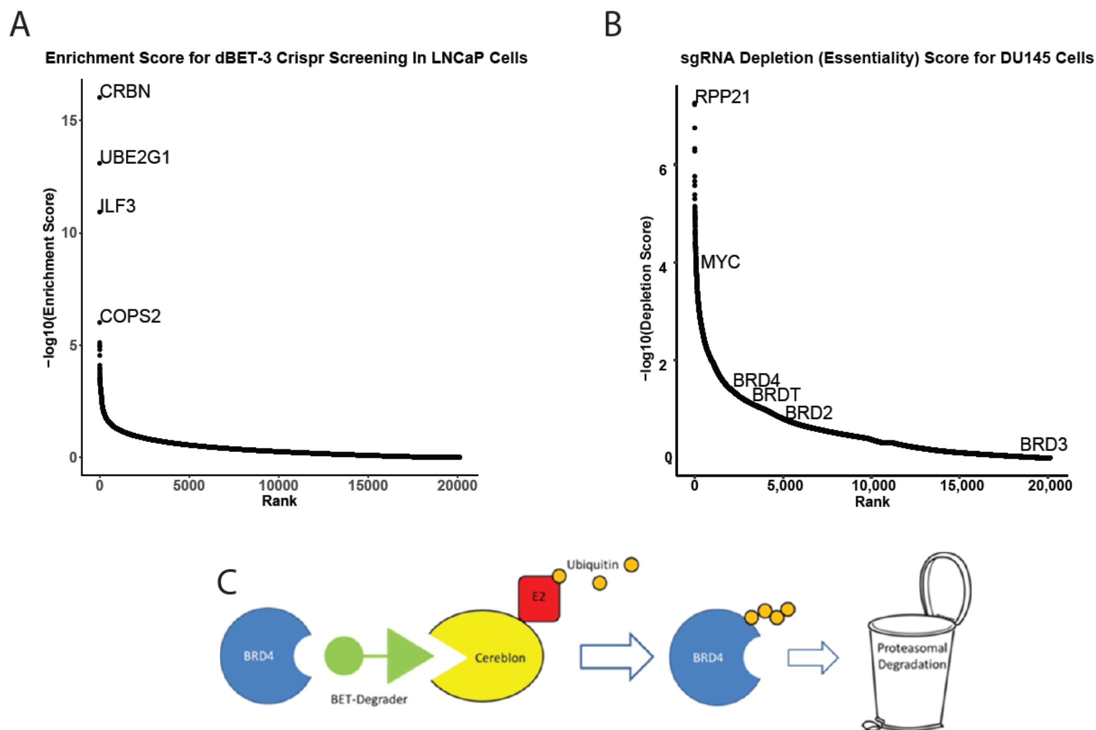

Figure 6: <u>Whole genome clustered-regularly-interspaced-short-palindromic-repeats (CRISPR) single-guide RNA (sgRNA) library screen to identify BET-degrader Resistance.</u> A) Enrichment Score of sgRNAs targeting individual protein coding genes from LNCaP cells lentivirally transduced with the Human CRISPR Knockout Pooled Library (GeCKO v2) and then for 30 days with 10nM dBET-3 (ZBC-260). Y-axis is Log_{10} enrichment score of the average of individual sgRNAs targeting the protein coding genes in the genome ranked across the x-axis. CRBN = Cereblon, UBE2G1 = Ubiquitin E2 conjugating enzyme, ILF3 = Interleukin Enhancer Binding Factor-3, COPS2 = COP9 signalosome complex subunit 2. B) Depletion Score of sgRNAs for DU145. Similar to A, DU145 cells were transduced with the Human CRISPR Knockout Pooled Library and genes essential for growth were identified as lost after 30 days of normal growth conditions. Y-axis is Log_{10} Depletion score of the average of individual sgRNAs targeting the protein coding genes in the genome ranked across the x-axis. RPP21 = Ribonuclease P subunit P21. C) Schematic of BET-degrader targeting BRD4 though Cereblon mediated poly-ubiquitin conjugation, and subsequent degradation by the proteasome complex.

Impact:

Here, with these data, we illustrate the preclinical efficacy of BET-degraders in the treatment of CRPC; bromodomain degradation disrupts AR and c-MYC signaling, and inhibits prostate cancer cell growth in vitro and in vivo. We have also identified that targeting BET-proteins in prostate cancer decreases fatty acid metabolism, most likely in a c-MYC- and AR- dependent manner, and upregulates immune-response pathways, as well as the pro-apoptotic and stress-induced protein TXNIP. Both increased PUFAs and TXNIP protein and transcript have the potential to serve as biomarkers or pharmacodynamic markers of BET-inhibition in prostate cancer cells. This will be helpful in monitoring patient's response to the drug, as well as its efficacy in treating the tumor. BET-degraders have stronger effects than BET-inhibitors, and more mechanistic work is needed to identify the difference between the function and effects of BET-degraders versus standard inhibitors. We anticipate that at the end of this proposed project, we will develop a BET bromodomain degrader as a novel potential therapeutic strategy for patients with the metastatic CRPC.

What was the impact on other disciplines?

We illustrated that PROTAC degraders indeed require the ubiquitination/Proteasome machinery to function. That is widely applicable to other diseases where PROTAC degrader molecules can be employed. We also show that PROTAC degraders are better than standard inhibitors.

What was the impact on technology transfer?

We are characterizing potential therapeutics for clinical development.

What was the impact on society beyond science and technology?

Compounds inducing the pharmacologic degradation of BET proteins effectively targets the major oncogenic drivers of prostate cancer, and ultimately present a potential advance in the treatment of mCRPC. In particular, our compound ZBC-260, is most suited for further clinical development.

4. **CHANGES/PROBLEMS:** We focused on the CRISPR screen to predict resistance instead of a few more redundant models.

5. PRODUCTS:
Publications, conference papers, and presentations:
Conferences and Presentations:

Subtask 1: Attend 2017 AACR Meeting in Washington, D.C. Presented poster: **Kregel, S.,** Malik, R., Asangani, I. A., Wilder-Romans, Jiang, X., V. L., Krishnamurth, P. M., Apel, I. J., Ravi, G., Eskara-Wilke, J., Tien, J., Cao, X., Speers, C., Feng, F. Y., Wang, S., Chinnaiyan, A. M. *Functional and Mechanistic Interrogation of BET Bromodomain Degraders for the Treatment of Metastatic Castration-Resistant Prostate Cancer,* American Association for Cancer Research – Annual Meeting – Washington, D.C., April 2, 2017	Attended	Dr. Kregel
Attend 2018 AACR Meeting in Chicago, Illinois. Presented poster: **Kregel, S.,** Malik, R., Asangani, I. A., Wilder-Romans, Jiang, X., V. L., Krishnamurth, P. M., Apel, I. J., Ravi, G., Eskara-Wilke, J., Tien, J., Cao, X., Speers, C., Feng, F. Y., Wang, S., Chinnaiyan, A. M. *Functional and Mechanistic Interrogation of BET Bromodomain Degraders for the Treatment of Metastatic Castration-Resistant Prostate Cancer,* American Association for Cancer Research – Annual Meeting – Chicago, IL., April 18, 2018	Attended	Dr. Kregel
Publication: Functional and Mechanistic Interrogation of BET Bromodomain Degraders for the Treatment of Metastatic Castration Resistant Prostate Cancer Steven Kregel, Rohit Malik, Irfan A Asangani, Kari Wilder-Romans, Thekkelnaycke Rajendiran, Lanbo Xiao, Josh Vo, Tanu Soni, Marcin Cieslik, Ester Ferna dez-Salas, Bing Zhou, Xuhong Cao, Corey Speers, Sh aomeng Wang and Arul M. Chinnaiyan Clin Cancer Res March 27 2019 **DOI:** 10.1158/1078-0432.CCR-18-3776		

Technologies or techniques
Nothing to Report.
Inventions, patent applications, and/or licenses
Nothing to Report.
Other Products
Nothing to Report.

6. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS
What individuals have worked on the project?
Name: Steven Kregel – No change
Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
- **None**

7. SPECIAL REPORTING REQUIREMENTS
- **None**

8. **APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*