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14. ABSTRACT

Prostate cancer continues to be a significant and incurable health problem that will only become more prevalent as life expectancy increases. Thus, there is a dire need for alternative approaches to prevent cancer initiation, discern indolent from aggressive tumors, and treat metastatic disease. Recent evidence of germline HOXB13 mutations within a subset of familial prostate cancers supports a key role for HOX regulation pathways in prostate initiation and progression. Moreover, the majority of HOXB13 mutations are located within the MEIS-interacting domain and thus emphasizes the importance of MEIS-HOX protein interactions in prostate tumor biology. This proposal builds upon significant published and unpublished work demonstrating a key role for the MEIS proteins as critical transcription factors and HOX protein co-factors in suppressing prostate tumor progression, blocking cell proliferation, and promoting anti-metastatic gene expression. However, there remain significant shortcomings in our ability to translate our pathologic and mechanistic discoveries into patient benefit. The work proposed here has the high potential to identify new therapeutic directions for targeting prostate cancer cells and achieving more efficacious approaches to preventing, staging, and treating prostate cancer.

15. SUBJECT TERMS
Prostate Cancer, MEIS proteins, HOXB13, metastasis, Androgen Receptor

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1. Introduction

Prostate cancer continues to be a significant and incurable health problem that will only become more prevalent as life expectancy increases. Thus, there is a dire need for alternative approaches to prevent cancer initiation, discern indolent from aggressive tumors, and treat metastatic disease. Recent evidence of germline HOXB13 mutations within a subset of familial prostate cancers supports a key role for HOX regulation pathways in prostate initiation and progression. Moreover, the majority of HOXB13 mutations are located within the MEIS-interacting domain and thus emphasizes the importance of MEIS-HOX protein interactions in prostate tumor biology. This proposal builds upon significant published and unpublished work demonstrating a key role for the MEIS proteins as critical transcription factors and HOX protein co-factors in suppressing prostate tumor progression, blocking cell proliferation, and promoting anti-metastatic gene expression. However, there remain significant shortcomings in our ability to translate our pathologic and mechanistic discoveries into patient benefit.

The goal of this study is to translate our pathologic and mechanistic discoveries of MEIS-associated tumor suppression into patient benefit. The ***objective*** of this proposal is to define how MEIS disrupts oncogenic AR-HOXB13 interactions, develop robust and reliable reagents to detect MEIS expression in tumor specimens, and to further develop compounds that can be used clinically to increase MEIS expression. Our ***central hypothesis*** is that MEIS protein expression confers and indolent tumor phenotype, MEIS inhibits AR-activity by disrupting HOXB13 interactions, and drugs that increase MEIS expression will also disrupt AR-HOXB13 interactions and block cell proliferation. Completion of this work will represent a significant leap forward in our understanding of MEIS, AR, and HOX protein function in prostate cells, and has the high potential to lead to new reagents for cancer staging and therapeutic intervention. We will accomplish our objectives via the following three Aims:

***Specific Aim 1*:** To define the functional and phenotypic impact of MEIS expression on AR signaling in prostate cancer cells

***Specific Aim 2*:** To develop robust MEIS detection reagents for formalin-fixed, paraffin-embedded (FFPE) prostate tissues.

***Specific Aim 3*:** To determine if epigenetic restoration of MEIS expression can suppress cell growth and inhibit formation of AR–HOXB13 complexes.

2. Keywords

Prostate Cancer; MEIS1, MEIS2, HOXB13, Androgen Receptor (AR)

3. Accomplishments

Research accomplishments are based upon the outlined Statement of Work. These are as follows:

Major Task 1: Determine the ability of MEIS expression to alter responses to AR agonists and antagonists in vitro.

Subtask 1: Perform growth, death, invasion, and differentiation assays of MEIS-expressing cells in the presence or absence of AR agonist and/or antagonist.

Progress: We have completed the work proposed. We engineered and characterized MEIS over-expressing CWR22Rv1 and LAPC4 cells; MEIS-expression results in decreased in vitro growth and invasion. Further, MEIS expression results in variable AR protein expression. We thus characterized how these changes in AR protein expression modify the expression of AR in response to AR antagonists. Our data demonstrates that MEIS expression does not stabilize AR expression when cells are treated with the anti-androgen Enzalutamide vitro. Data is shown in **Figure 1 and Figure 2**.

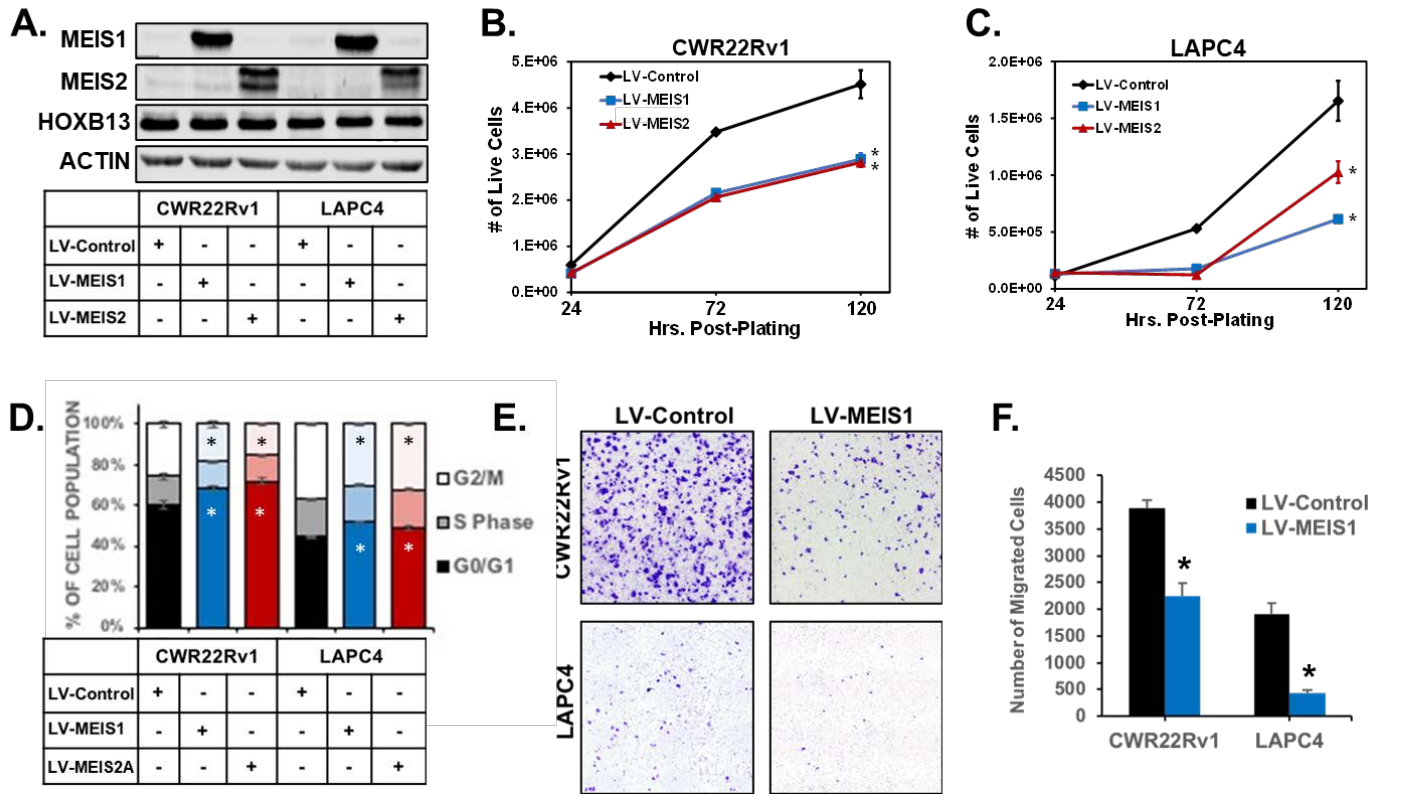

Figure 1: Expression of MEIS1 or MEIS2 in PrCa cell lines is sufficient to decrease growth and invasion *in vitro*. (A) Western blot confirmation of lentiviral overexpression of MEIS1 or MEIS2 in CWR22Rv1 and LAPC4 cell lines. LV-Control encodes an expression plasmid for constitutive Cas9 expression. Endogenous HOXB13 expression was also assessed in all lines. Actin was used as a loading control. **(B and C)** Proliferation of CWR22Rv1 and LAPC4 with exogenous expression of MEIS1 (blue), MEIS2A (red), or control (black). Cell number over time was assessed by manual counting of live cells on a hemocytometer. Data represent mean count and SEM at each time point (technical replicates, n=3). Data for LV-Control and LV-MEIS2A is the same as in Figure S2D-E **(D)** Cell cycle analysis determined by propidium iodide (PI) fluorescence intensity in CWR22Rv1 and LAPC4 cells with exogenous expression of MEIS1 (blue), MEIS2A (red), or control (black). Data represent mean (technical replicates, n=3) and SEM. **(E)** Representative images of transwell migration assays for CWR22Rv1 (top) and LAPC4 (bottom) of cells with exogenous expression of control (left) or MEIS1 (right). **(F)** Quantification of transwell migrations performed in Figure 1E. Data represent mean (technical replicates, n=4) and SEM. * indicates P<0.05.

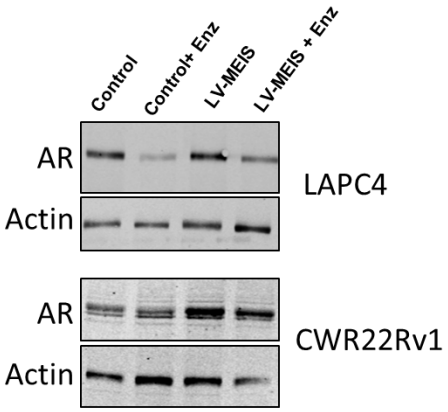

Figure 2: Ectopic MEIS expression increases AR protein expression, but does not stabilize AR in response to enzalutamide.

Major Task 2: Determine the ability of MEIS expression to alter responses to host castration in vivo.
Subtask 1: Obtain IACUC and ACURO regulatory approval
Progress (From Previous Report): We obtained IACUC and ACURO regulatory approval.

Subtask 2: Tumor xenograft growth and response to host castration.

Progress: We have completed the proposed animal experiment using both CWR22Rv1 and LAPC4 cell lines. These data demonstrated a clear and statistically-significant tumor suppression by MEIS1 in both cell lines between both intact and castrate conditions. Kaplain-Meier data of host survival is shown in **Figure 3.**

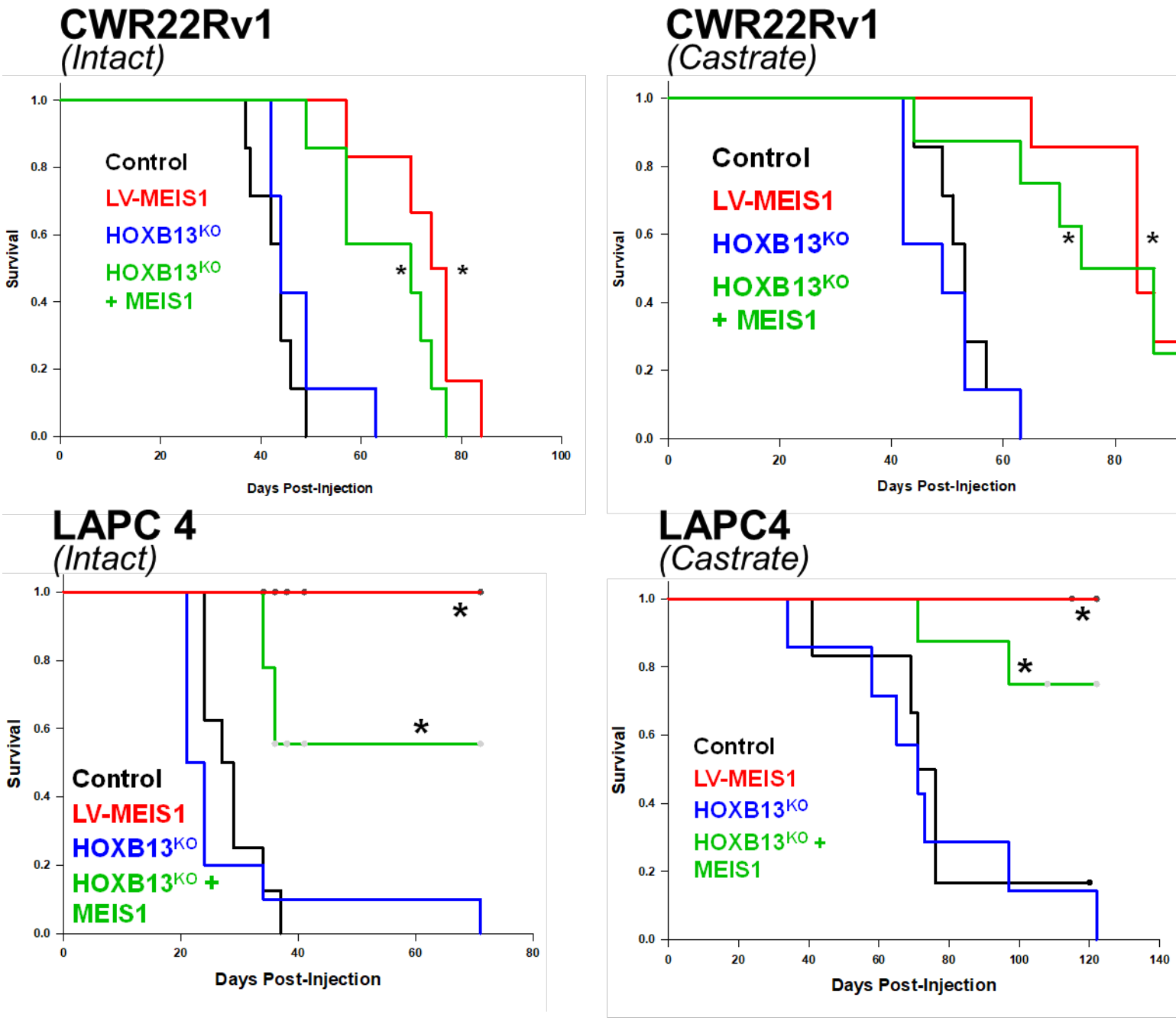

Figure 3: In Vivo Tumor Xenograft Growth of MEIS1-Expressing CWR22Rv1 and LAPC4 Prostate Cancer Cells in Hormonally Intact vs. Castrate Conditions. Stable MEIS1 expression in prostate cancer cells (CWR22Rv1 and LAPC4) was tested for it's ability to modulate tumor growth and AR-mediated tumor growth between hormonally-intact vs. castrated conditions. In addition, CRISPR-mediated deletion of HOXB13, a major MEIS1 transcriptional co-factor, was also evaluated. These data show significant suppression of tumor growth when MEIS1 is expressed, regardless of HOXB13 expression. * indicates $P<0.05$.

Major Task 3: Identify differential AR transcriptional targeting and gene regulation in the presence of MEIS1.
Subtask 1: Treat MEIS1-expressing CWR22Rv1 and LAPC4 cells with either AR agonist or antagonist. Collect RNA as various time post treatment and perform RNA-seq.
Progress: We have completed the RNA-sequencing. All 48 RNA-Sequencing libraries have been sequenced, and returned high quality QC results (**Figure 4**). Bioinformatic analyses of these datasets has also been completed (**Figure 5**). These analyses are revealing multiple gene sets of interest that are

associated with MEIS expression and modulate AR gene targets in both intact and castrated conditions. These data are being integrated with our AR-ChIP data from identical conditions to identify changes in AR-gene binding and transcriptional regulation as it relates to MEIS expression. These analyses are iterative and ongoing, and will be incorporated into a manuscript we are preparing.

Subtask 2: Treat MEIS1-expressing CWR22Rv1 and LAPC4 cells with either AR agonist or vehicle. Perform AR CHIP-seq.
Progress: AR ChIP-Seq has been completed and is undergoing bioinformatic analyses. AR ChIP-Seq has been optimized and performed, along with IgG controls. Libraries were prepared and we have recently received sequencing data. This data is currently being analyzed for QC, peak calling, and integrated with our RNA-Seq data.

Figure 4: Quality Scores of 48 RNA-Sequencing Libraries derived from the Tumor Xenografts in Figure 2. Three distinct CWR22Rv1 and LAPC4 tumor xenografts (Control, LV-MEIS1, HOXB13^{KO}, and HOXB13^{KO}+LV-MEIS1) were prepped for RNA extraction and RNA-Seq library creation. This resulted in 48 total RNA-Sequencing libraries (CWR22Rv1 and LAPC4; Control, LV-MEIS1, HOXB13^{KO}, and HOXB13^{KO}+LV-MEIS1 lines; intact vs. castrate conditions; 3 libraries per condition for a total of 48 libraries). Graphs depict satisfactory QC and per Sequence QC scores for all 48 libraries. Bioinformatic analyses is currently underway.

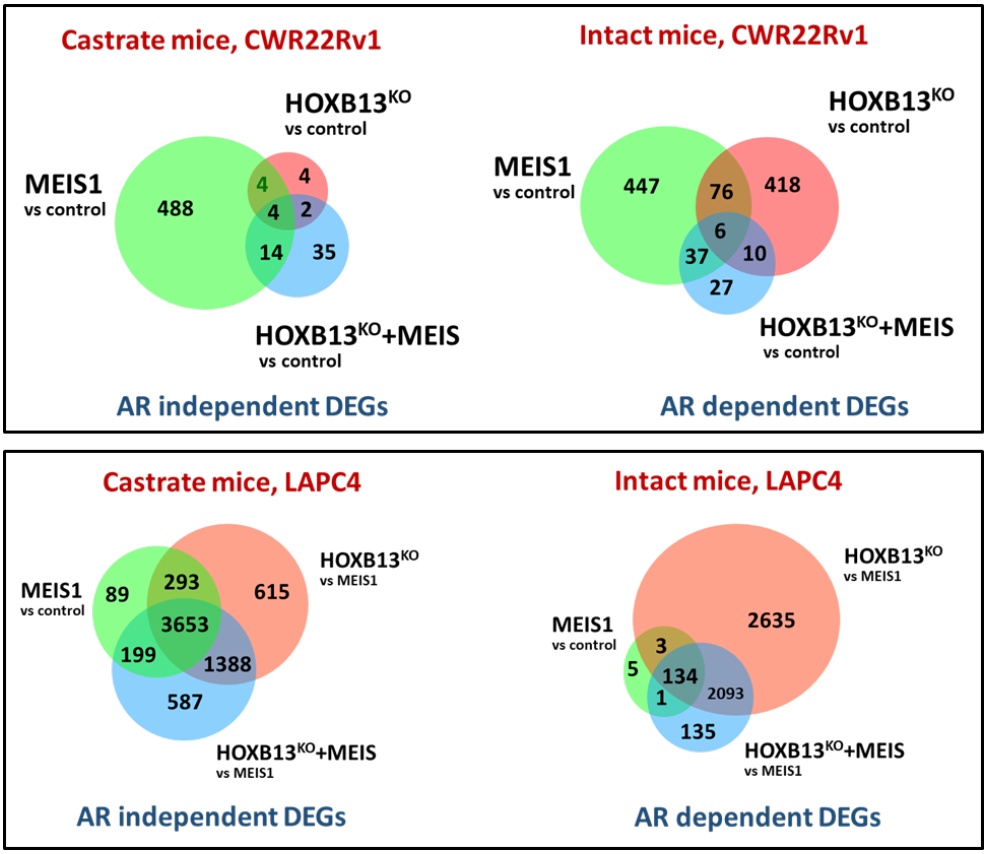

Figure 5: Initial Analyses of RNA-Seq data. Venn diagrams of differentially-expressed genes between CWR22Rv1 and LACP4 tumors under various conditions and gene expression modulations (MEIS expression, HOXB13-deletion), and host hormone status (intact vs. castrate). These data prioritize MEIS-associated genes that confer tumor suppression, and enable downstream analyses of how MEIS proteins modulate AR-associated gene targets.

Major Task 4: Develop new anti-MEIS antibodies using antibody phage display (sub-award with Dr. Aaron LeBeau)

Subtask 1: Creation of antibodies using phage display.

Progress: Purified MEIS1 and MEIS2 proteins were prepared and quality assured (**Figure 6**). These proteins were sent to our collaborator, Dr. LeBeau, for the purpose of creating antibodies specific to MEIS1, MEIS2, and a duo-MEIS1/MEIS2 antibody. His lab has created two antibodies specific for MEIS1, two that cross-react with 1 and 2, and two that are specific for MEIS2 by western blot and ELISA. He has provide a slot blot analyses demonstrating successful creation of anti-MEIS1 (**Figure 7**). These constructs were cloned into IgG expression vectors, shipped to our lab, and we are currently optimizing expression and validation using western blotting. Antibodies were shipped to our lab, but we have had challenges expressing sufficient quantities to critically evaluate. We are currently working with our collaborator to troubleshoot the expression system, and once fixed we can easily test and validate the antibodies, as all controls and staining conditions are in hand and ready to be stained.

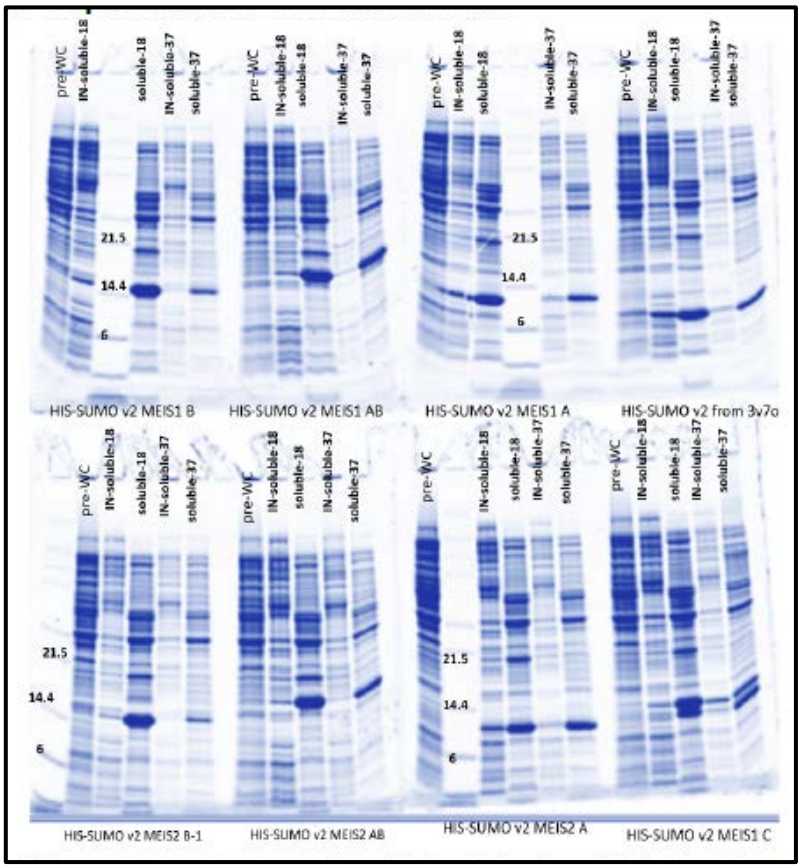

Figure 6: SDS-PAGE Results of MEIS1 and MEIS2 purified protein expression. Eight MEIS constructs were transformed into BL21-DE3 cells. 3 ml overnight cultures were inoculated with fresh colonies and grown in TB/Carb media at 37 deg. C. The next day, 3 ml of autoinduction media were inoculated with 120 µl of the overnight growth in duplicate. 1 ml of the overnight growth was harvested and labelled as pre- induction sample (PRE). Both samples were shaken at 37 deg C at 275 rpm for 2 hours. One sample was moved to 18 deg. C overnight, the second sample was left at 37 deg. C overnight. 1 ml of both 18 deg C and 37 deg. C samples were harvested the next day, lysed in 500µl of Bugbuster HT. Lysate was spun at 13K rpm for 30 mins, and the supernatant was labelled as soluble fraction and the pellet as the insoluble fraction. The PRE sample was also lysed in 500µl of Bugbuster HT but was not fractionated and labelled as whole cell, WC. The pellet from the fractionated samples, insoluble, was taken up in 500µl TBS. 7.5 µl of each sample was reduced with gel loading dye plus DTT and run on a 4-20% tris-glycine gel.

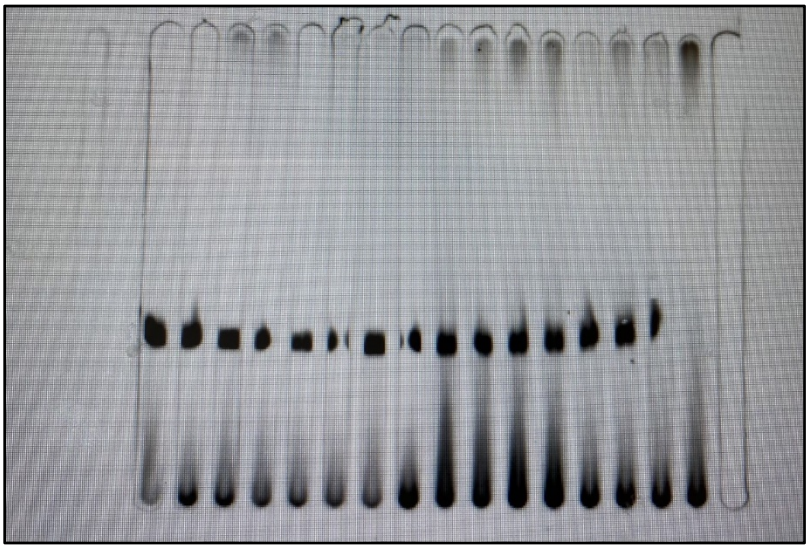

Figure 7: Binding of Novel MEIS Antibodies. Slot blot binding of new MEIS antibodies to purified MEIS antigens. Lanes are:
Wells 1-2: Empty for Standard
Wells 3-6: 4B3 (4 uM, 2 uM, 1 uM, 500 nM)
Wells 7-10: 4B8 (4 uM, 2 uM, 1 uM, 500 nM)
Wells 11-14: 2D12 (4 uM, 2 uM, 1 uM, 500 nM)
Wells 15-18: 2A3 (4 uM, 2 uM, 1 uM, 500 nM)
Well 19: Empty
Well 20: Not pictured and empty

Major Task 6: Evaluation of AR/HOXB13 and MEIS/HOXB13 interactions in prostate cancer cells after MEIS re-expression via HDAC inhibitor.
Subtask 1: *In vitro* analyses of drug-treated commercially available cell lines with panobinostat, westerns to verify protein induction, AR agonist and antagonist treatment with assays for growth, death, and AR target gene expression. PLA to quantify AR/HOXB13 and MEIS/HOXB13 interactions.
Subtask 2: CRiSPR-Cas9 KO and MEIS1/2 and engineering of MEIS-reporter lines. Design CRISPR-targeting vectors against MEIS1/2, transfection and selection of KO and reporter clones, AR agonist and antagonist treatment with assays for growth, death, and AR target gene expression. Treatment of clones with panobinostat, PLA to quantify AR/HOXB13 interactions.
Progress: We have successfully created MEIS1 and MEIS2 reporter lines using CRISPR-Cas9. This approach enabled us to splice in a bioluminescent nLuc reporter within the endogenous MEIS gene locus, thus creating an nLuc-MEIS1 and nLuc-MEIS2 fusion protein under the control of the endogenous MEIS promoters

(**Figure 8**). The nLuc reporter lines and MEIS-knockout lines will be utilized to screen and evaluate pharmacologic agents that increase MEIS expression in prostate cancer cell lines. We screened a panel of epigenetic modifier drugs, and multiple bromodomain inhibitors emerged as promising candidates. This panel identified numerous bromodomain inhibitors that increased MEIS expression at uM concentrations (**Figure 9**). However, the protein induction of MEIS was not robust enough to detect interactions with HOXB13 using PLA or Co-IP.

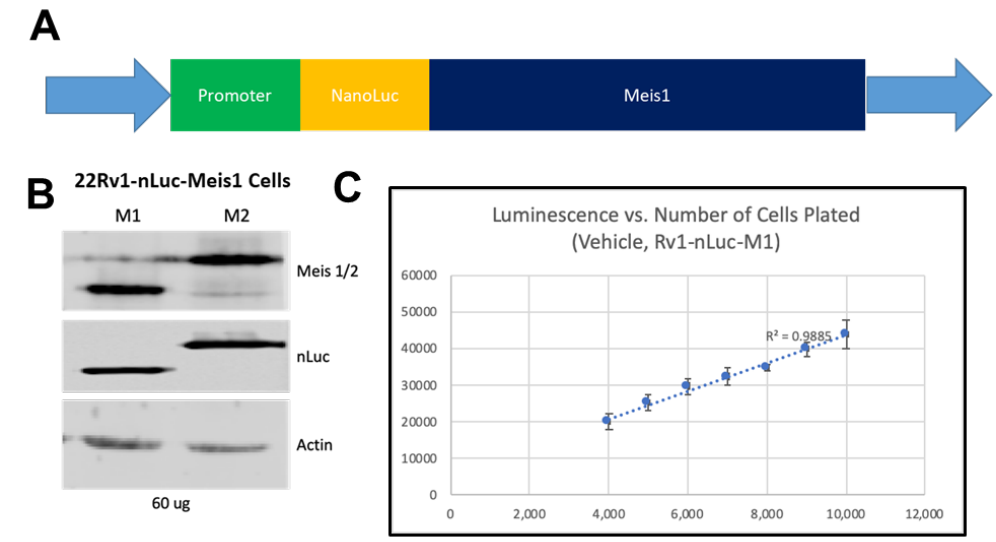

Figure 8: nLuc-Tagging of Endogenous MEIS Proteins to Create Reporter Cell Lines. A) Schematic of CRISPR-targeting strategy to create a chimeric nLuc-MEIS1 protein under the control of the endogenous MEIS1 promoter. This line, along with the corollary nLuc-MEIS2 line, will be used to evaluate compounds that modulate MEIS expression. B) Western blot demonstrating nLuc and MEIS protein expression. C) Standard curve demonstrating a linear association with nLuc-MEIS1 expression and cell number.

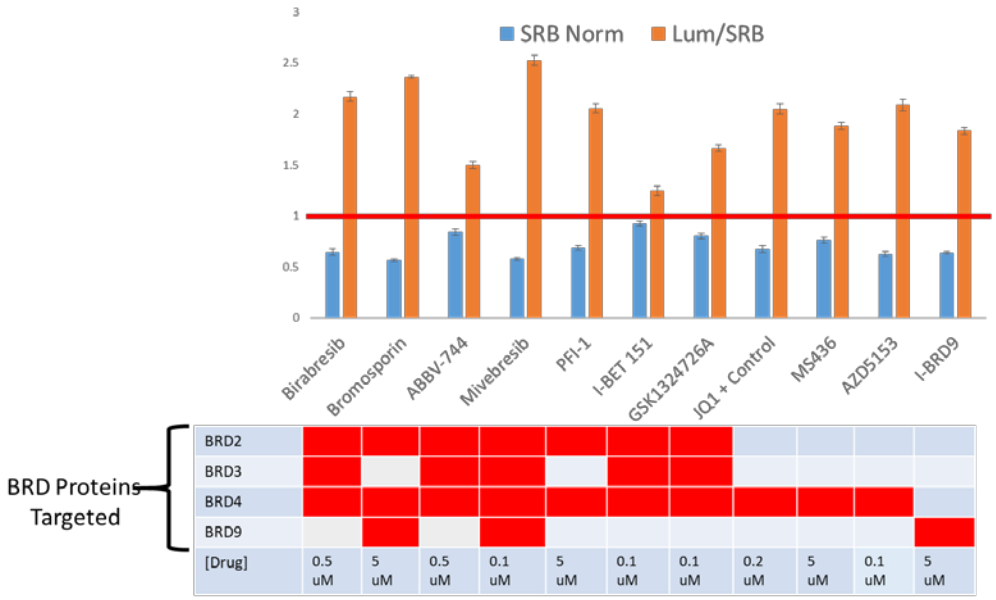

Figure 8: Induction of MEIS expression in CWR22Rv1-nLuc-MEIS prostate cancer cells by multiple bromodomains (BRD) inhibitors. Nano-luciferase-MEIS-expressing cells were treated with a cohort of epigenetic modifying compounds and increases in MEIS were quantified using bioluminescent imaging. These data demonstrated that multiple inhibitors of BRD proteins increased MEIS expression; however, expression increases were not sufficient to conduct robust PLA or Co-IP approaches, especially since sub-optimal antibodies exist.

Major Task 7: Formulation of 2 peer-reviewed manuscripts for review.

Subtask 1: Writing and formulation of figures and text, submission for peer-review, and revision and further experiments based upon review.

Progress: We are currently compiling our animal data, RNA-Seq data, and AR ChIP-Seq data into a manuscript for peer review. Our goal is that this manuscript should be published by the next progress report cycle.

4. Impact

Data from a few of our initial experiments within the first year were incorporated into our recent manuscript, entitled: **"***MEIS-mediated suppression of human prostate cancer growth and metastasis through HOXB13-dependent regulation of proteoglycans.*" This was published in eLife in June 2020.

Data from the *in vivo*, RNA-Seq, and AR ChIP-Seq data is being incorporated into a manuscript currently under preparation. We anticipate, based upon our progress, to submit this manuscript in 2023.

Data from the drug screening and nLuc-CRISPR reporter lines is being incorporated in a manuscript currently under preparation. We anticipate, based upon our progress, to submit this manuscript in 2023.

5. Changes/Problems

We have encountered no problems or hurdles which require modifications to our Statement of Work. Further, we were fortunate to have initiated our animal experiments prior to COVID-related shutdowns, and were able to maintain these experiments during lab closures.

6. Products

Nothing to report.

7. Participants & Other Collaborating Organizations

Nothing to report.

8. Special Reporting Requirements

Nothing to report.

9. Appendices

VanOpstall C, Perike S, Brechka H, Gillard M, Lamperis S, Zhu B, Brown R, Bhanvadia R, and **Vander Griend DJ**. *MEIS-Mediated Suppression of Prostate Cancer Growth and Metastasis Through HOXB13-Dependent Regulation of Proteoglycans.* eLife; June 18, 2020; 9:e53600. doi: 10.7554/eLife.53600.
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