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**AWARD NUMBER:** W81XWH-16-1-0532

**TITLE: Epigenetic Machinery Regulates Alternative Splicing of Androgen Receptor (AR) Gene in Castration-Resistant Prostate Cancer (CRPC)**

**PRINCIPAL INVESTIGATOR:** Jer-Tsong Hsieh

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**PREPARED FOR:** U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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Epigenetic Machinery Regulates Alternative Splicing of Androgen Receptor (AR) Gene in Castration-Resistant Prostate Cancer (CRPC)

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**14. ABSTRACT**
Androgen deprivation therapy (ADT) is the primary treatment for metastatic prostate cancer (PCa) since PCa depends on androgen for growth. Although initially responsive, most tumors progress into androgen-independent/castration-resistant PCa (CRPC). No curative therapy is available. One of the reasons for the resistance to ADT and newer anti-androgen drugs is the emergence of constitutively active AR variants (AR-Vs) such as AR-V7 that are induced under ADT conditions. Our research goal is to test the hypothesis that the epigenetic regulator KDM4B, a histone lysine demethylase, promotes AR-V7 via alternative splicing, leading to CPRC. A multi-disciplinary approach including molecular biology, tumor biology, cell biology, and biochemical method is used to test this hypothesis. In collaboration with a partnering principal investigator we are also testing the efficacy of our newly identified KDM4B inhibitor(s) as a monotherapy or combined with approved anti-androgen agents in AR-V7-expressing CRPC in preclinical mouse models.

**15. SUBJECT TERMS**

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | USAMRMC |
| Unclassified | Unclassified | Unclassified | Unclassified | | 19b. TELEPHONE NUMBER *(include area code)* |

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1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.
The subject of our research is to identify the molecular mechanism of the drug resistance in castration-resistant prostate cancer (CRPC). Our preliminary data suggest that one of the mechanisms of the resistance is the emergence of constitutively active androgen-receptor variants such as AR-V7. Our goals are to demonstrate that histone lysine demethylase KDM4B regulates AR-V7 via alternative splicing and to test the efficacy of our newly identified KDM4B inhibitor(s) as a monotherapy or combined with approved anti-androgen agents in AR-V7-expressing CRPC in pre-clinical animal models of CRPC.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).
Histone lysine demethylase, castration-resistant prostate cancer, alternative splicing, AR-V7, KDM4B, small molecule inhibitors.

3. **ACCOMPLISHMENTS:**
**What were the major goals of the project?**
*List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.*
There are two specific aims in this proposal. We have made significant progresses for both aims in the past year. One manuscript for publication is written and is currently under review.

**Aim 1. To establish that KDM4B promotes AR-V7 expression and identify the regulatory mechanisms.**

**Major Task 1:** Determine the role of KDM4B in promoting AR-V7 expression in various PCa cell lines, including those resistant to enzalutamide.—**completed (6/30/2017).**

**Major Task 2:** Determine how KDM4B binds to the spliceosome associated with pre-mRNA.—**completed (6/30/2017).**

***Milestone #1:*** *Co-author manuscript on KDM4B-RNA interaction.—We have met this milestone. The manuscript was submitted to cancer cell and is currently under review (6/30/2018).*

**Major Task 3:** Map RNAPII, H3K9/K36me3 occupancy around AR locus using ChIP-qPCR in several CRPC cells.—in progress, 60% completed.

**Major Task 4:** To identify potential KDM4B-regulated alternative splice gene(s) using RNA-seq and map KDM4B-RNA interactions with CLIP-seq.—in progress, 60% completed.

***Milestone #2:*** *Co-author manuscript on mechanism by which KDm4B regulates AR-V7 at chromatin level---We have met this milestone. The manuscript is under 2$^{nd}$ revision for Cancer Cell (7/30/2018).*

**Aim 2. To evaluate the clinical application of KDM4B inhibitors on CRPC tumors expressing AR-Vs.**

**Major Task 5:** Identify two lead compounds using CPRC cell lines and optimizing their dosage and schedule in xenograft models.

**What was accomplished under these goals?**

1) **Major activities;**

Aim 1 Major Task 1 and 2: using xenograft model, we demonstrated KDM4B is critical for the in vivo growth of PCa tumor in castrated host because KDM4B knockdown 22RV1 cells (i.e., cl4 and cl7) glow slower than parental cells in vivo (Figure 1A). Based on immunohistochemical staining and western blot analyses, AR-V7 expression is significantly decreased in cl4 and cl7 tumors compared with parental tumors (Figure 1B). These results support our hypothesis that KDM 4B is critical for the growth of prostate cancer cells in androgen-deprived condition because KDAM4B can increase AR-V7 expression.

Aim 2, Major task 5: we first optimized treatment dosage of B3 using 22RV1 subcutaneous tumor model. After tumor becoming palpable (about 50 mm$^3$), we used minipump containing three different dosages of B3 (5, 20, and 50 mg/kg), which can last one week. As shown in Figure 2A, only 50 mg/kg dosage exhibita

significant tumor inhibition after 10 days after treatment. Indeed, reduced KDM4B protein levels can be detected in treated tumors, which support the drug targeting effect.

Furthermore, we compared the therapeutic efficacy of B3 (50 mg/kg) and Enzalutamide (50 mg/kg, daily orally administration for 1 week) using 22RV1 subcutaneous tumor model. Since 22RV1 is known as androgen-independent tumor, Enzalutamide failed to suppress tumor growth (Figure 3A). In contrast, B3 was able to suppress 22RV1 tumor growth (Figure 3A), which could be due to its inhibitory effect on AR-V7 Protein expression (Figure 3B).

**Figure 1**. **KDM4B knockdown inhibits CRPC growth in vivo.** (A) Representative tumor xenografts derived from ctl, cl4, and cl7 cells (upper panel). The lower panel shows tumor weight and take-up rate (n=10-18, mean±SEM **, *p<0.01*). (B) Representative IHC staining of AR-V7 and AR in tumors of (upper panel). The lower panel shows western blot of indicated proteins from tumors.

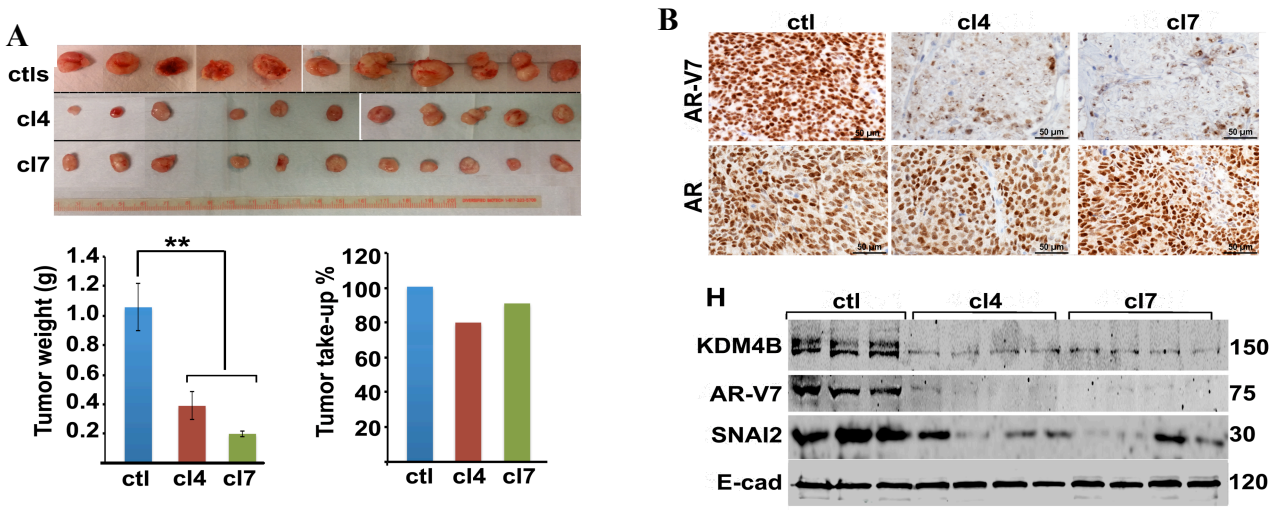



**Figure 2**. **The dosage effect of KDM4B inhibitor B3 on CRPC growth in vivo.** (A) Tumor growth curves of 22Rv1-xenografts treated with veh (n=4), or different dosages of B3 (n=4 for each dose). Mean ± SEM, *, *p<0.05*. (B) Representative IHC staining of AR-V7 and AR in tumors of (upper panel). Western blot of indicated proteins from tumors.

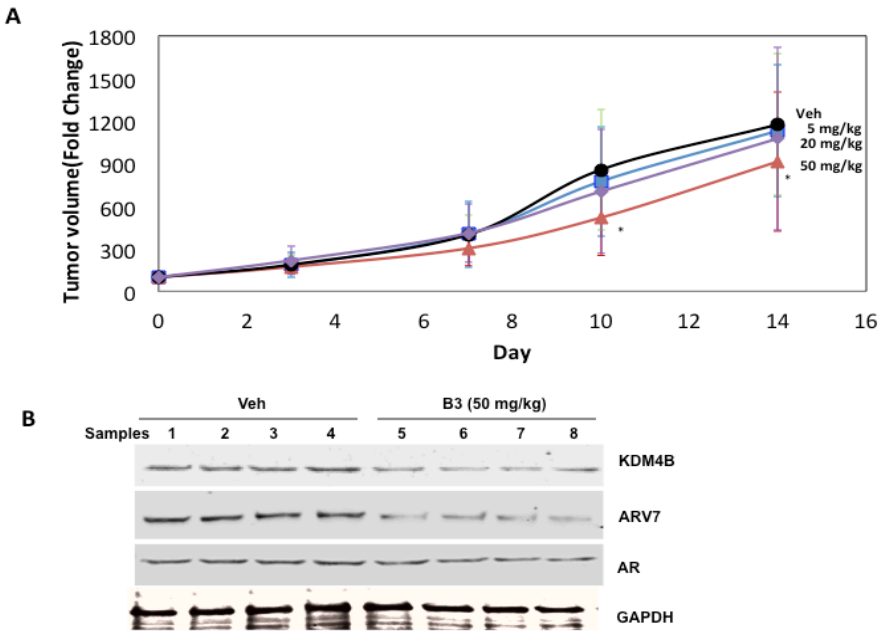
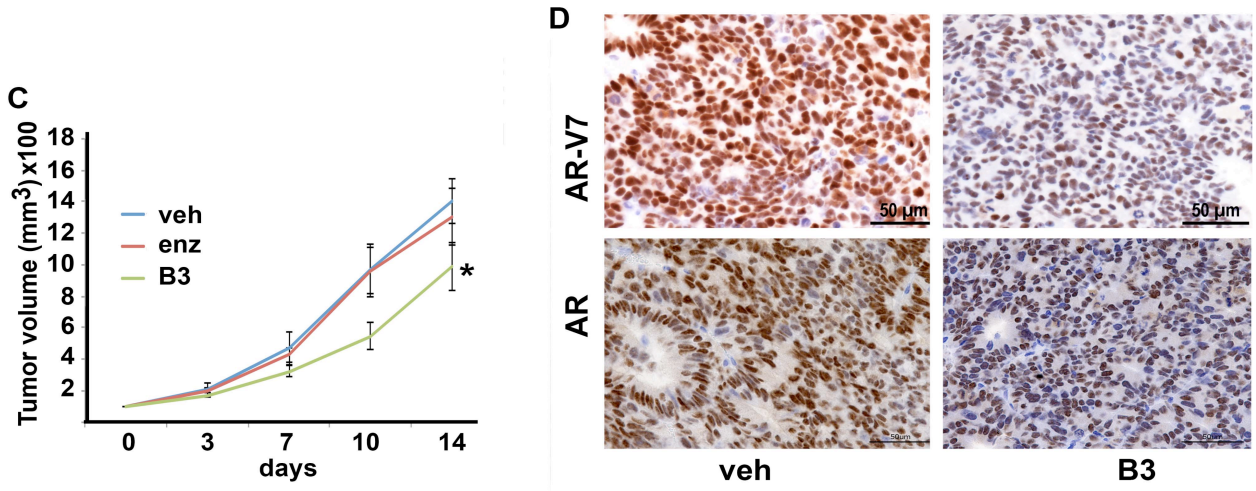


**Figure 3. The therapeutic efficacy of B3 or Enzalutamide on CRPC growth in vivo. (**A) Tumor growth curves of 22Rv1-xenografts treated with veh (n=6), Enzalutamide (n=8), or B3 (n=11). Mean ± SEM, *, *p<0.05*. (B) IHC of AR and AR-V7 in tumors.




2) **Specific objective;**
We completed the majority part of the specific aim 1. We are exploring clinical applicability of KDM4B small molecule inhibitor on specifically targeting CRPC patients-expressing AR variant.

3) **Significant results or key outcomes;**
**Significant results** Our animal model provides additional evidence that KDM4B promotes CPRC, at least partially via AR-V7. Pharmacological inhibition of CRPC xenograft with the KDM4 inhibitor B3 suppressed tumor growth, diminished AR-V7 expression.
**Key outcomes** Successfully targeting KDM4B with a specific small molecule inhibitor offers a new therapeutic strategy in CRPC patients.

**What opportunities for training and professional development has the project provided?**
Nothing to report

**How were the results disseminated to communities of interest?**
We have submitted a manuscript to Cancer cell under review right now.

**What do you plan to do during the next reporting period to accomplish the goals?**
We will follow the proposed plan in SOW to complete our experimental therapy of using KDM4B small molecule inhibitor (B3). We will expand the xenograft model to use other CRPC cell lines to ensure the tumor heterogeneity will not alter the drug potency of B3. In addition, we will examine any synergistic effect of B3 by combining Enzalutamide.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

**What was the impact on the development of the principal discipline(s) of the project? What was the impact on other disciplines?**
Alternative splicing not only increases protein variety but the resultant gene products might have considerably different functions. In fact, more than 90% of genes are subjected to alternative splicing in humans. Obviously, alternative splicing greatly contributes to biological complexity, alternation in splicing machinery has been correlated with oncogenesis and drug resistance. Thus targeting this machinery represents a new avenue of cancer therapy. Since splicing is ubiquitously present in every cell, agents directly targeting this machinery are often very toxic. Thus, designing agents targeting regulator of gene splicing machinery could circumvent this problem. Here, we identified a novel mechanistic link between epigenetic factor (i.e., histone lysine demethylase KDM4B) and alternative splicing of AR gene. KDM4B is overexpressed in prostate cancer and

many other cancer types; we have shown the potential efficacy of KDM4B small molecule inhibitor in prostate cancer. The outcome of this project is expected to impact on therapeutic strategy of prostate cancer but also other cancer types as well.

**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:** The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

**Changes in approach and reasons for change**
Nothing to report

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

**Changes that had a significant impact on expenditures**
Nothing to report

**Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

**Significant changes in use or care of human subjects**
Nothing to report

**Significant changes in use or care of vertebrate animals**
Nothing to report

**Significant changes in use of biohazards and/or select agents**
Nothing to report

6. **PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Nothing to report
- **Books or other non-periodical, one-time publications.**
  Nothing to report
- **Other publications, conference papers and presentations**.
  Nothing to report
- **Website(s) or other Internet site(s)**
  Nothing to report
- **Technologies or techniques**
  Nothing to report
- **Inventions, patent applications, and/or licenses**
  Nothing to report
- **Other Products**
  Nothing to report

## 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:* | *Zhi-Ping Liu* |
| *Project Role:* | *PI* |
| | *No change* |
| *Name:* | *LingLing Duan* |
| *Project Role:* | *Research associate* |
| | *No change* |
| *Name:* | *Qing-Jun Zhang* |
| *Project Role:* | *Research Associate* |
| | *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?**

Nothing to Report

**What other organizations were involved as partners?**

Nothing to Report

## 8. SPECIAL REPORTING REQUIREMENTS

**COLLABORATIVE AWARDS:** please see Initiating PI Zhi-Ping Liu's progress report

## 9. Appendix

N/A