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

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4. TITLE AND SUBTITLE
Epigenetic Machinery Regulates Alternative Splicing of Androgen Receptor (AR) Gene in Castration-Resistant Prostate Cancer (CRPC)

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5b. GRANT NUMBER
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5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5e. TASK NUMBER

5f. WORK UNIT NUMBER

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Alternative splicing is emerging as an oncogenic mechanism. In prostate cancer generation of constitutively active forms of androgen receptor (AR) variants including AR-V7 plays an important role in progression of castration-resistant prostate cancer (CRPC). AR-V7 is generated by alternative splicing that results in inclusion of cryptic exon CE3 and translation of truncated AR protein that lacks the ligand binding domain. Whether AR-V7 can be a driver for CRPC remains controversial as the oncogenic mechanism of AR-V7 activation remains elusive. Here, we found that KDM4B promotes AR-V7 and identified a novel regulatory mechanism. KDM4B is phosphorylated by protein kinase A in conditions that promote castration-resistance, eliciting its binding to the splicing factor SF3B3. KDM4B binds RNA specifically near the 5'-CE3, upregulates the chromatin accessibility, and couples the spliceosome to the chromatin. Our data suggest that KDM4B can function as a signal responsive trans-acting spicing factor and scaffold that recruits and stabilizes the spliceosome near the alternative exon, thus promoting its inclusion. Genome-wide profiling of KDM4B-regulated genes also identified additional alternative splicing events implicated in tumorigenesis. Our study defines KDM4B-regulated alternative splicing as a pivotal mechanism for generating AR-V7 and a contributing factor for CRPC, providing insight for mechanistic targeting of CRPC.

15. SUBJECT TERMS

NONE LISTED

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
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TABLE OF CONTENTS

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:** *The 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.*

   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 revision.

   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 Nuclear Acid Research and is currently in 3nd revision (9/15/19).
   Major Task 3: Map RNAPII, H3K9/K36me3 occupancy around AR locus using ChIP-qPCR in several CRPC cells . — completed (9/15/19)
   Major Task 4: KDM4B-regulated alternative splice gene(s) using RNA-seq. -complete (9/15/19) and map KDM4B-RNA interactions with CLIP-seq.—in progress, 50% 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 3rd revision for Nuclear Acid Research (9/15/2019).

   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 (B3 and S12) and optimizing their dosage and schedule in two xenograft models. 22Rv1 and VCaP —completed. Please see the progress report from the partner of the project Dr. JT Hsieh (PC150152P1) for details.

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

1) major activities;
a) We tested the interaction between KDM4B and splicing factor SF3B3.
b) We showed that KDM4B can be phosphorylated by PKA in response to androgen-deprivation.
c) We identified PKA-phosphorylation site on KDM4B.
d) We showed that phosphorylation of KDM4B promotes KDM4B binding to SF3B3.

2) Specific objective;
We made significant progress in specific aim 1, identifying the novel mechanism by which KDM4B interact with spliceosome. We show that androgen-deprivation activates PKA that phosphorylates KDM4B, resulting its binding to splicing factor SF3B3. We also tested B3 on additional PC xenograft growth- VCaP-cell derived tumor in vivo (please see Dr. JT Hsieh's progress report).

3) Significant results or key outcomes;
Significant results.
(1) **KDM4B binds to splicing factors in response to androgen deprivation.**
In the previous progress report, we established that KDM4B binds splicing factor. In the new reporting period, we confirmed the endogenous interactions between KDM4B and SF3B3 in VCaP cells by proximity ligation assays and found that binding of KDM4B to SF3B3 is dramatically increased under ADT conditions (CFBS+enzalutamide) (Figure 1A). ADT is known to activate PKA that promotes androgen-independent growth and neuroendocrine differentiation of PCa cells. PKA is among the KDM4B-interactive proteins identified by mass-spectrometry. Binding of KDM4B to SF3B3 is regulated by PKA as it is abolished in the presence of PKA specific inhibitor H89 (Figure 1B). PKA inhibitor H89 treatment also downregulated AR-V7 expression in cells cultured under ADT conditions (Figure 1C). We confirmed the interaction between PKA and KDM4B in Co-IP (Figure 1D) and kinase assays (Figure 1E) and identified several PKA phosphorylation sites including Ser666 on KDM4B by mass spectrometry. Mutation of Ser666 to Ala resulted in inability of KDM4B to promote AR-V7 transcription (Figure 1F-G) and to bind SF3B3 (Figure 1H-I).
(2) **B3 inhibited VCaP-derived tumor significantly in vivo** (please see partnering-PI Dr. JT Hsieh's progress report).
Key outcomes: We have identified a novel mechanism by which KDM4B regulates alternative splicing of AR; androgen-deprivation activates PKA that phosphorylate KDM4B, resulting in its binding to splicing factor.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to report

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

We submitted our findings for publication. Manuscript is now in 3rd revision for Nuclear Acid Research.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Nothing to Report.

4. **IMPACT:** D*escribe 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?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Nothing to Report.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

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
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to Report.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

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**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Nothing to report

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of*

publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

Nothing to report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to report

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

  Nothing to report

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

  Nothing to report

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

  Nothing to report

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*

- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

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
Researcher Identifier (e.g. ORCID ID): 0000-0003-1341-3878
Nearest person month worked: 3
Contribution to Project: Designed experiments, analyzed data, write progress report, and manuscript.
Funding Support: Cancer prevention and research institute of Texas (CPRIT), American heart association (AHA), DOD, NIH

Name: LingLing Duan
Project Role: Research associate
Researcher Identifier (e.g. ORCID ID): 0000-0001-7291-861X
Nearest person month worked: 8
Contribution to Project: designed and performed experiments, analyzed data, write progress report, and manuscript.
Funding Support: Cancer prevention and research institute of Texas (CPRIT), DOD

Name: Qing-Jun Zhang
Project Role: Research Associate
Researcher Identifier (e.g. ORCID ID): 0000-0002-0749-642X
Nearest person month worked: 4
Contribution to Project: performed experiments and analyzed data
Funding Support: Cancer prevention and research institute of Texas (CPRIT), American heart association (AHA), DOD, NIH

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *N/A*

QUAD CHARTS: *N/A*

9. 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.*

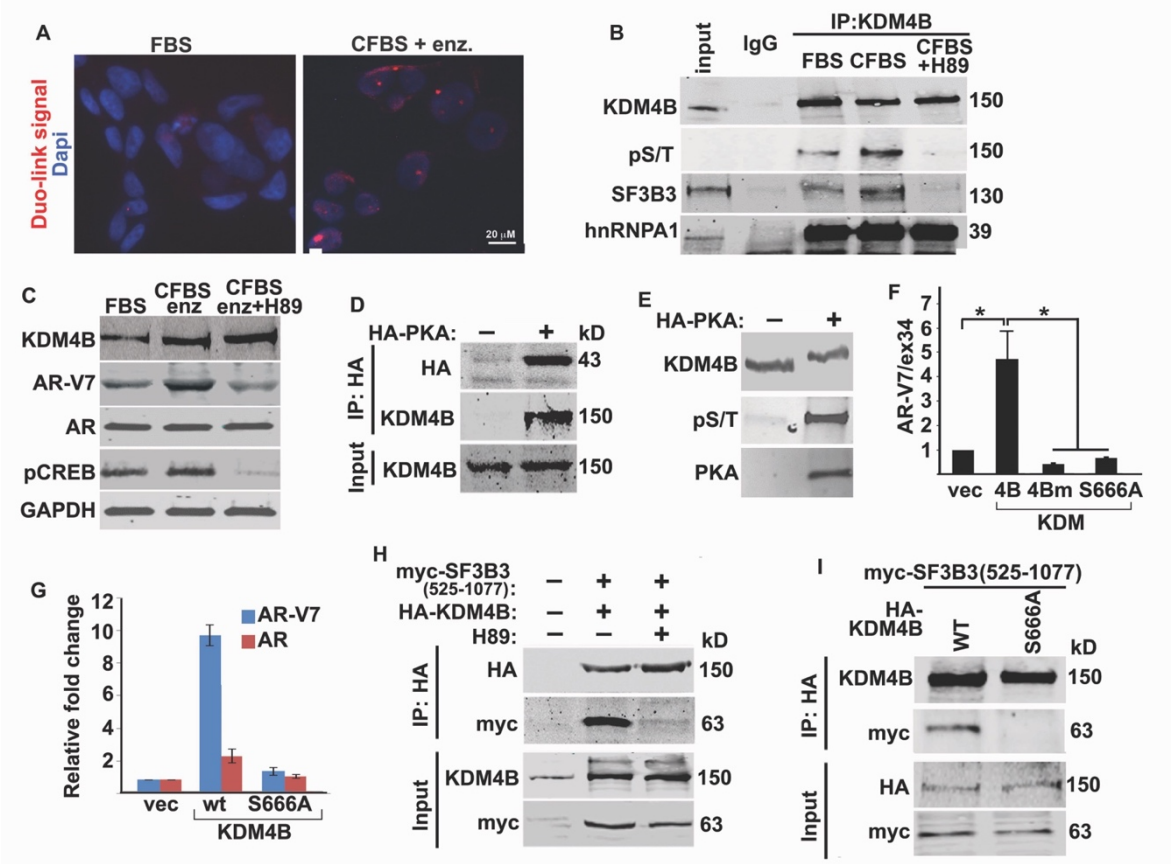

Figure 1. KDM4B binds SF3B3 in response to androgen-deprivation. (A) *In situ* PLAs were performed using antibodies against KDM4B and SF3B3 in VCaP cells cultured in FBS or CFBS plus enzalutamide (enz. 10 □M). PLA signals (red), representing interactions between KDM4B and SF3B3, were significantly upregulated in androgen-deprivation conditions. Nuclei were stained with Dapi. (B) Cell lysates from VCaP cells cultured under FBS, CFBS, CFBS +H89 were immunoprecipitated with anti-KDM4B antibody and western blotted with antibodies indicated. H89 abolished the interaction between KDM4B and SF3B3. (C) WB of indicated proteins in VCaP cells cultured under the conditions indicated. H89 downregulated AR-V7 expression that was upregulated by androgen-deprivation. (D) HEK293T cells were transfected without or with HA-PKA. Cell lysates were immunoprecipitated with anti-HA antibody and western blotted with anti-HA and anti-KDM4B antibodies. (E) Recombinant KDM4B protein was incubated without or with catalytically active PKA and analyzed by Phos-Tag gel followed by WB with antibody indicated. (F) AR-V7 minigene reporter assay with cell transfected with KDM4B, KDM4Bm, or KDM4B(S666A) (n=4, mean ± SD), *, $p<0.05$. (G) Relative mRNA of AR-V7 and AR in VCaP cells transfected with KDM4B or KDM4B(S666A). (H-I) HEK293T cells were transfected with myc-SF3B3(525-1077) together with HA-KDM4B (F) or HA-KDM4B WT or S666A (I), treated with or without PKA inhibitor H89 (H). Lysates were immunoprecipitated with anti-HA antibody and western blotted with indicated antibodies.