AWARD NUMBER: W81XWH-19-1-0361

TITLE: Inhibiting Lysine-Specific Demethylase 1 Activity as a Potential Therapeutic Treatment for Castration-Resistant Prostate Cancer

PRINCIPAL INVESTIGATOR: Changmeng Cai

CONTRACTING ORGANIZATION: University of Massachusetts Boston

REPORT DATE: October 2022

TYPE OF REPORT: Annual Report

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2022	Annual Report	09/01/2021-08/31/2022

4. TITLE AND SUBTITLE

Inhibiting Lysine-Specific Demethylase 1 Activity as a Potential Therapeutic Treatment for Castration-Resistant Prostate Cancer

5a. CONTRACT NUMBER
W81XWH-19-1-0361

5b. GRANT NUMBER
PC180163

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Changmeng Cai

E-Mail: changmeng.cai@umb.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Massachusetts
Boston
100 Morrissey Blvd
Boston, MA 02125

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Epigenetic reprogramming induced by aberrant expression and activity of histone modifying proteins has emerged as a critical mechanism for prostate cancer resistance to AR signaling inhibition treatments. LSD1 functions as a transcriptional corepressor through demethylation of histone 3 lysine 4 but also has a coactivator function on AR. In this project, we have shown that LSD1 broadly enhances AR chromatin binding by increasing enhancer accessibility prior to androgen stimulation through demethylating FOXA1 and stabilizing FOXA1 chromatin binding. Our comprehensive RNA-seq analyses confirmed AR and FOXA1 as major targets of LSD1 *in vivo*. In addition to regulating AR signaling, LSD1 and BRD4 can form nuclear condensates and are co-enriched at the super-enhancers that are associate with oncogenic transcription factor genes, such as MYC. We show that the combination treatment of LSD1 inhibitor and BET inhibitors can act in synergy in treating CRPC models.

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			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	10	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

1. INTRODUCTION

While PCa tumors respond to the androgen deprivation therapies (ADTs), including more aggressive AR signaling inhibition (ARSi) agents, tumor cells eventually escape from these therapies and develop resistance to become castration-resistant PCa (CRPC). One of the major mechanisms is epigenetic reprogramming driven by the altered expression and activity of histone modifier proteins. Lysine-specific demethylase 1 (LSD1, KDM1A) is well known for its function to demethylate histone 3 lysine 4 and repress gene transcription. However, LSD1 can also function as a coactivator of AR in PCa cells with an unclear mechanism. We reported previously that LSD1 is associated with FOXA1 and activates AR-dependent enhancers to facilitate the transcription of androgen-regulated genes. Our recent data suggest that LSD1 functions to maintain the accessibility of AR-regulated enhancers through promoting the chromatin binding of FOXA1, a critical pioneer factor of AR. Inhibition of LSD1 globally impairs the chromatin binding of FOXA1 and thus disrupts FOXA1-dependent AR cistrome, resulting in the suppression of PCa growth *in vitro* and *in vivo*. Mechanistically, we found that LSD1 can directly demethylate FOXA1 at K270 and this demethylation stabilizes FOXA1 chromatin binding and opens the chromatin structure at the enhancers. Our new data indicate that LSD1-mediated demethylation of FOXA1 may play an important role in recruiting BRD4 and supporting super-enhancers that are associated with the activation of oncogenic transcriptional programs, such as MYC signaling. Mechanistically, LSD1 and BRD4 can form nuclear condensates that potently regulate transcription. Therefore, our current progress supports the hypothesis that LSD1 inhibitors can synergize with BET inhibitors in treating CRPC.

2. KEYWORDS

LSD1, KDM1A, FOXA1, androgen receptor, AR, CRPC, lysine demethylation, BRD4, super-enhancers

3. ACCOMPLISHMENTS

- **What were the major goals of the project?**

Specific Aim 1*: Identify mechanisms of action by which LSD1 maintains FOXA1 chromatin binding and subsequent AR recruitment.*

Major Task 1: Determine the role of LSD1 on chromatin structure and enhancer distributions

We have established LSD1 knock-out cell lines using the CRISPR/CAS9 method and used small molecular inhibitors that are currently in clinical trials to inhibit LSD1 activity. The downregulation of LSD1 significantly impaired FOXA1 chromatin binding and the subsequent AR recruitment, and resulted in the repression of FOXA1 targets and AR signaling (including full-length AR and its PCa-specific splice variant AR-V7).

Month 1-12, Percentage of completion: 100%

Major Task 2: Determine the role of FOXA1 as a critical LSD1 substrate

We have developed stable cell lines overexpressing wild-type FOXA1 and K270R mutant. Our data have demonstrated that the K270R mutant has enhanced chromatin binding and is not responding to LSD1 inhibition.

Month 6-30, Percentage of completion: 100%

Major Task 3: Identify the additional components of the AR enhancer-associated LSD1/FOXA1 complex

We have identified BRD4 as a major component of the LSD1/FOXA1 complex by directly interacting with FOXA1. LSD1 and BRD4 can form nuclear condensates.

Month 24-36, Percentage of completion: 100%

Specific Aim 2*: Assess the therapeutic potential of LSD1 inhibitors in CRPC models.*

Major Task 4: Determine the therapeutic efficacy of LSD1 inhibitor treatment alone and in combination with enzalutamide or bromodomain inhibitor in CRPC patient-derived xenograft models.

We have shown that LSD1 inhibitor treatment can repress tumor growth in multiple CRPC PDX models. A

comprehensive RNA-seq analysis using tumor samples from these xenograft studies has been done. We have identified MYC, E2F, FOXA1, and AR as major targets of LSD1 activation function, and interferon pathway and intrinsic immune response as major targets of LSD1 repression function. Moreover, we have demonstrated that the combination treatment of LSD1 inhibitor and BET inhibitor can synergistically repress CRPC tumor growth *in vivo*.

Month 1-36, Percentage of completion: 90%

- **What was accomplished under these goals?**

Major Activities: Through the support of this grant, I have been able to continue my proposed research at University of Massachusetts Boston. We have made significant progress for both specific aims. A manuscript summarizing critical targets of LSD1 in CRPC and demonstrating its activity in supporting super-enhancers is currently in revision for publication. For research networking, I have continued a monthly joint lab meeting with collaborators - Drs. Steven P. Balk (Beth Israel Deaconess Medical Center) and Housheng Hansen He (University of Toronto) to discuss the project progress and plan the experiments. In addition, I have actively participated in various seminars and meetings within the Harvard Cancer Center Program, such as the Dana-Farber Cancer Institute prostate cancer SPORE seminars. I have also attended the SBUR (Society for basic Urological Research) annual meeting (Nov 4-7, 2021, remote) and AACR annual meeting (April 8-13, 2022, remote) and supported graduate students to present the progress of this project in those meetings.

Specific Objectives: Specific aim 1 is to identify mechanisms of action by which LSD1 maintains FOXA1 chromatin binding and subsequent AR recruitment. Specific aim 2 is to assess the therapeutic potential of LSD1 inhibitors in CRPC models.

Significant Results: For **aim 1**, we have completed the proposed studies. We identified BRD4 as a binding partner of FOXA1 and demonstrated that LSD1 may form nuclear condensates with BRD4 to regulate super-enhancers. For **aim 2**, we tested LSD1 inhibitors in two CRPC cell lines-derived xenograft models and four CRPC PDX models. The RNA-seq analysis was performed in the xenograft tumor samples to identify LSD1 inhibition-targeted pathways. We also demonstrated that co-targeting LSD1 and BRD4 can synergistically repress tumor growth *in vivo*. Important results were summarized below.

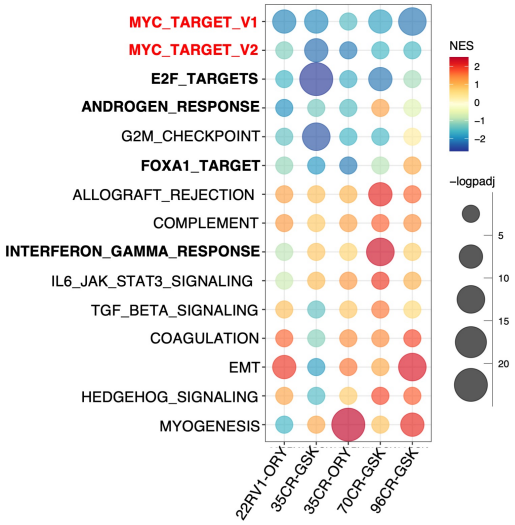

Figure 1. LSD1 inhibition represses MYC signaling in various CRPC models. Tumor samples from each xenograft model receiving LSD1 inhibition were subjected to RNA-seq studies. Gene set enrichment analysis (GSEA) was done by comparing the LSD1 inhibitor treatment with vehicle treatment.

LSD1 inhibition targets MYC signaling. We performed a large-scale RNA-seq analysis in all the xenograft responders (a total of 34 tumor samples collected from CRPC xenografts treated with LSD1-i: GSK2879551 or ORY-1001) to further determine the molecular underpinnings of how LSD1-i represses tumor growth. Using Gene Set Enrichment Analysis (GSEA) on the RNA-seq data, we found that expressions of MYC and E2F transcriptional targets were consistently downregulated by LSD1-i (**Fig. 1**). Decreased expression of E2F targets by LSD1-i is consistent with our recent finding that LSD1 can increase E2F1 activity by enhancing its chromatin binding. AR activity and G2/M cell cycle-related genes were also inhibited in most of the models, consistent with previous reports. Since our recent study has suggested that a major function of LSD1 is to stabilize FOXA1 chromatin binding and thus increase enhancer accessibility, we next examined whether LSD1-i targets the FOXA1 activity in these CRPC models. To test that, we developed a FOXA1-target signature (upregulated genes) that was derived from FOXA1 silencing in LNCaP and 22Rv1 cells and LSD1-i treatment decreased the FOXA1 activity in all models but LuCaP96CR. In addition, we also observed upregulated immune response hallmark genes which were activated in all the models, consistent with the previous finding showing that LSD1 suppresses tumor immunogenicity.

BRD4 binding at super-enhancers is dependent on FOXA1. MYC gene expression is well-known for its regulation by Super-Enhancers (SEs) occupied by master regulators such as BRD4. Since LSD1 can function to increase chromatin accessibility by promoting the binding of pioneer factor FOXA1, we hypothesize that LSD1 may be critical for maintaining SEs in PCa cells. At the region ~250kb upstream of the MYC gene, we identified a SE which is marked by a cluster of H3K27ac and H3K4me2 peaks and co-occupied by BRD4, LSD1, and FOXA1 (**Fig. 2A**). The motif enrichment analysis of published BRD4 ChIP-seq peaks revealed strong enrichment of Forkhead DNA binding motifs (**Fig. 2B**). To determine whether LSD1-mediated FOXA1 chromatin binding affects BRD4 recruitment, we performed ChIP-V5 and ChIP-BRD4 in previously established 22Rv1 stable cell lines expressing doxycycline-inducible V5-tagged wildtype (WT) or mutant (K270R) FOXA1, the latter of which binds to chromatin more tightly and is resistant to LSD1 inhibition (**Fig. 2C**). BRD4 binding was markedly decreased by LSD1-i in the WT cell line but was not affected in the K270R mutant cell line. These results suggest that unmethylated FOXA1 may recruit BRD4 to the chromatin. Next, we sought to investigate the interaction between FOXA1 and BRD4. In 22Rv1 cells cultured under hormone-depleted conditions, LSD1 interacted with FOXA1, consistent with the previous study (**Fig. 2D**). However, LSD1 did not seem to directly interact with BRD4, whereas FOXA1 strongly interacted with BRD4. Moreover, we can also detect the interaction between BRD4 and FOXA1 in the tumor samples of LuCaP35CR and 96CR PDXs (**Fig. 2E**). To further examine whether BRD4 binding is globally associated with LSD1 and FOXA1, we performed BRD4 ChIP-seq in LNCaP cells under hormone-depleted conditions, and BRD4 binding was associated with LSD1, FOXA1, and active enhancer marks (**Fig. 2F**). Together, these results suggest a framework of LSD1, FOXA1, and BRD4, in which LSD1-demethylated FOXA1 can recruit BRD4 to chromatin, and the association between FOXA1 and BRD4 may be important for enhancer activation.

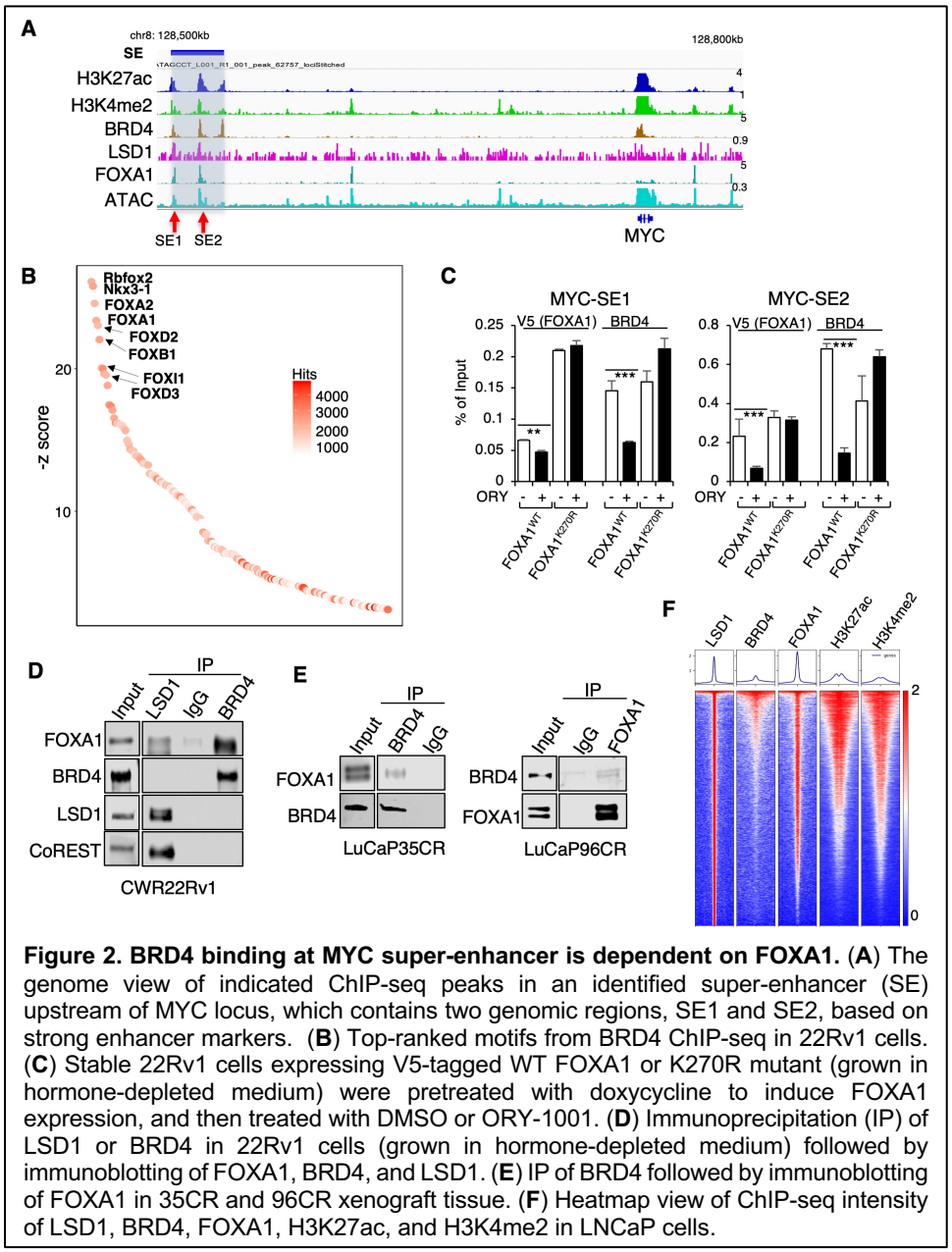

Figure 2. BRD4 binding at MYC super-enhancer is dependent on FOXA1. (**A**) The genome view of indicated ChIP-seq peaks in an identified super-enhancer (SE) upstream of MYC locus, which contains two genomic regions, SE1 and SE2, based on strong enhancer markers. (**B**) Top-ranked motifs from BRD4 ChIP-seq in 22Rv1 cells. (**C**) Stable 22Rv1 cells expressing V5-tagged WT FOXA1 or K270R mutant (grown in hormone-depleted medium) were pretreated with doxycycline to induce FOXA1 expression, and then treated with DMSO or ORY-1001. (**D**) Immunoprecipitation (IP) of LSD1 or BRD4 in 22Rv1 cells (grown in hormone-depleted medium) followed by immunoblotting of FOXA1, BRD4, and LSD1. (**E**) IP of BRD4 followed by immunoblotting of FOXA1 in 35CR and 96CR xenograft tissue. (**F**) Heatmap view of ChIP-seq intensity of LSD1, BRD4, FOXA1, H3K27ac, and H3K4me2 in LNCaP cells.

LSD1 and BRD4 form nuclear condensates. It has been well characterized that phase separation, via recruiting a high density of transcriptional apparatus, occurs on the SEs to drive robust transcription, and the intrinsically disordered regions of proteins are attributed to the formation of biomolecular condensates. Indeed,

the N-terminus of LSD1 (aa 1-165) was predicted to be highly disordered and may contribute to the condensate formation (**Fig. 3A**). Under high-resolution confocal microscopy in CWR22Rv1 cells, we observed that LSD1 exhibited similar puncta-like staining as BRD4, which is a characteristic of LLPS (**Fig. 3B**). Treating cells with 1,6-hexanediol, which is a commonly used compound for disrupting biomolecular condensates, reduced the puncta-like formation of LSD1 and BRD4. More importantly, LSD1 inhibition in CWR22Rv1 cells disrupted the puncta-like formation of BRD4 (**Fig. 3C**), suggesting that LSD1 may function to stabilize the BRD4-enriched nuclear condensates which potentially interact with super-enhancers.

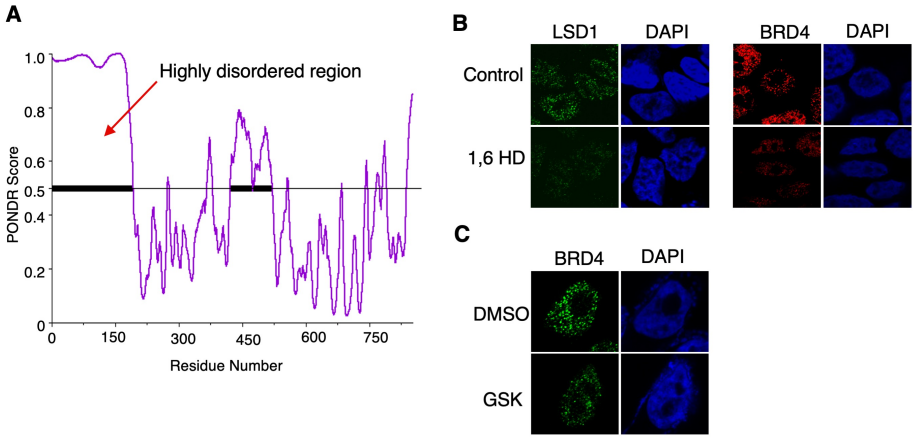

Figure 3. LSD1 is enriched in super-enhancers. (**A**) PONDR (VSL2) scores of LSD1 proteins for disordered regions. (**B**) Representative immunofluorescent images of LSD1 or BRD4 in 22Rv1 cells, before and after treatment with 3% hexanediol. (**C**) Representative immunofluorescent images of BRD4 in 22Rv1 cells treated with DMSO or GSK2879552.

LSD1-i and BET-i synergistically suppress CRPC tumor growth. To further determine whether the combination treatment can effectively target CRPC, we assessed the effects of the combination treatment in two CRPC PDX models, LuCaP77CR and LuCaP35CR. Significantly, the combo treatment achieved almost a complete blockade of tumor growth in the 35CR model (**Fig. 4**, left). Even though 77CR tumors grew much more robustly, the combination treatment still showed a significant advantage in repressing the tumor growth than any single agent, although tumors may seem to eventually relapse (**Fig. 4**, right).

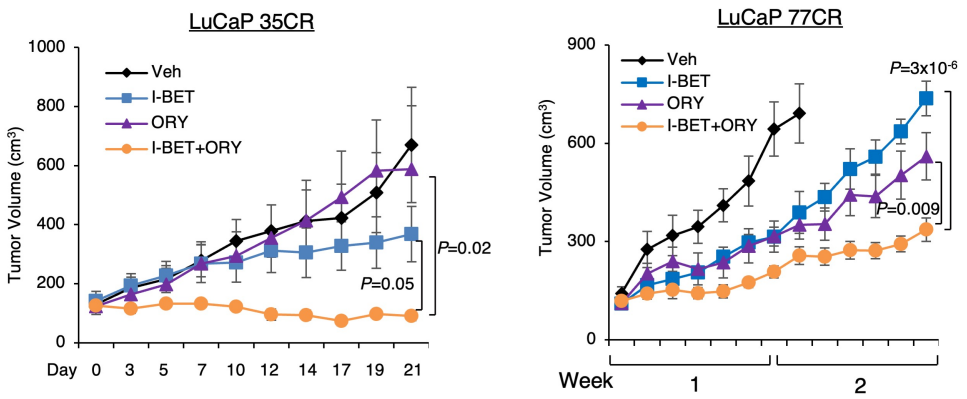

Figure 4. LSD1 inhibitor can synergize with BRD4 inhibitor to treat CRPC tumors. (Castrated SCID mice bearing 35CR or 77CR xenografts were treated with vehicle, I-BET762 (16mg/kg, daily), ORY-1001 (0.03mg/kg, every two days), or the combination. Tumor volume was measured by caliper.

Summary of results: These findings present a global view of LSD1 targeted pathways in CRPC *in vivo* and suggest that LSD1 inhibition can suppress multiple oncogenic transcription programs, including MYC. The findings further suggest that BRD4 can be recruited to chromatin by unmethylated FOXA1 and that LSD1 and BRD4 may form nuclear condensates and are co-enriched at the super-enhancers. Moreover, LSD1 inhibitors can act in synergy with BET inhibitors in treating CRPC tumors.

- **What opportunities for training and professional development has the project provided?**

  Nothing to Report

- **How were the results disseminated to communities of interest?**

Nothing to Report

- **What do you plan to do during the next reporting period to accomplish the goals?**

  For Aim 1, we have met our goals. For Aim 2, we will focus on analyzing the RNA-seq analyses using the tumor samples obtained from LSD1 inhibitor-treated PDX models and will perform single-cell RNA sequencing on a selected model. Moreover, we will also perform RNA-seq analyses on the CRPC tumor samples receiving the combination treatment of LSD1 inhibitor and BRD4 inhibitor.

4. IMPACT

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

  Our data have identified major targets of LSD1 inhibition *in vivo*, which will provide a foundation for the future development of LSD1-targeted therapies. Moreover, our data also suggest a novel function of LSD1 in regulating the formation of BRD4-enriched nuclear condensates and the activity of super-enhancers. These findings provide a strong rationale for combining LSD1 inhibitor with BET inhibitor to target MYC signaling and super-enhancer-driven oncogenic transcription networks.

- **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

Nothing to Report

6. PRODUCTS

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

Han W, Liu M, Han D, Toure AA, Li M, Besschetnova A, Wang Z, Patalano S, Macoska JA, Lam HM, Corey E, He HH, Gao S, Balk SP, and ***Cai C**. (**2022**) Exploiting the tumor-suppressive activity of the androgen receptor by CDK4/6 inhibition in castration-resistant prostate cancer. ***Molecular Therapy**.* 30(4):1628-1644.

Han W, Liu M, Han D, Li M, Toure AA, Wang Z, Besschetnova A, Patalano S, Macoska JA, Gao S, He HH, and ***Cai C**. (**2022**) RB1 loss in castration-resistant prostate cancer confers vulnerability to LSD1 inhibition. ***Oncogene***. 41(6):852-864.

Liao Y, Chen CH, Yang M, Xiao T, **Cai C**, Gao S, Xue P, Liu Z, Xu H, Lee J, Li W, Mei S, McKeown M, Pierre R, Shu S, Fei T, Duarte MS, Zhao J, Bradner JE, Polyak K, Kantoff PW, Long H, Balk SP, Liu XS, Brown M, and Xu K. (**2021**) Pharmacological EZH2 inhibition enhances cancer cell sensitivity to genotoxic insults through suppressing DNA damage repair. ***PNAS***. 119(3): e2105898119.

Zifeng Wang (**2021**) FOXA1 chromatin binding is repressed by MLL1/SETD7-mediated lysine methylation. ***SBUR Annual Meeting***. Poster Presentation

- **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 ORGANIZATION

- **What individuals have worked on the project?**

Name:	*Changmeng Cai*
Project Role	*Principle Investigator*
Research Identifier (e.g. ORCID ID):	*0000-0002-8701-2586*
Nearest person month worked:	*3*
Contribution to Project:	*Dr. Cai has been in charge of the overall administration and execution of this project, supervising the graduate student, and coordinating the preparation of manuscripts describing the work.*
Funding Support:	NIH R01CA211350

Name:	*Muqing Li*
Project Role	*Graduate Student*
Research Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*4.5*
Contribution to Project:	*Muqing has been working on running molecular biology assays related to this project and on animal studies*
Funding Support:	*N/A*

Name:	*Zifeng Wang*
Project Role	*Graduate Student*
Research Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*3*
Contribution to Project:	*Zifeng has been working on running high-throughput sequencing*
Funding Support:	*CSM PhD Fellowship*
Name:	*Mingyu Liu*
Project Role	*Graduate Student*

Research Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*3*
Contribution to Project:	*Mingyu has been working on analyzing high-throughput sequencing data.*
Funding Support:	*N/A*

Name:	*Shuai Gao*
Project Role	*Research Faculty/Postdoc*
Research Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*3*
Contribution to Project:	*Shuai has been working on designing experiments, running molecular biological assays, and supervising graduate students.*
Funding Support:	*N/A*

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

  One additional grant has been awarded since the last reporting period.

  U54 CA156734 (PI: Colon-Carmona) 09/01/2021-08/31/2024
  NCI/NIH
  University of Massachusetts Boston and Dana-Farber Cancer Institution partnership program
  Regular Project (co-PIs: Cai and Balk)
  Title: Targeting androgen receptor signaling in prostate cancer in men with African ancestry

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

  Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

Nothing to Report

9. APPENDICES

N/A