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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, MA

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Reactivation of androgen receptor (AR) signaling are found in the majority of castration-resistant prostate cancer (CRPC) and CRPC resistant to enzalutamide, indicating a pressing need for further development of novel AR-targeted therapies. Lysine-Specific Demethylase 1 (LSD1) functions as a transcriptional corepressor through demethylation of histone 3 lysine 4 (H3K4) but also has a coactivator function on AR. In this project, we have shown that LSD1 broadly enhances AR chromatin binding and activity by increasing enhancer accessibility prior to androgen stimulation through demethylating the pioneer factor FOXA1 and stabilizing FOXA1 chromatin binding. Moreover, in addition to regulating AR signaling, LSD1 and BRD4 are co-enriched at the super-enhancers that are associate with oncogenic transcription factor genes, such as MYC, and regulate their expressions. We are currently testing the combination treatment of LSD1 inhibitor and BET inhibitors in CRPC models. Our findings provide novel therapeutic insights on developing LSD1 inhibitor treatment in CRPC.

15. SUBJECT TERMS
None listed.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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INTRODUCTION

Androgen receptor (AR) signaling is critical for prostate cancer (PCa) development. While PCa tumor initially responds to the first-generation androgen deprivation therapies (ADTs), it can develop resistance to ADTs within a few years and progresses to a more aggressive stage called castration-resistant PCa (CRPC). CRPC can be further treated with more aggressive ADTs, such as enzalutamide and abiraterone. However, tumors still relapse through multiple mechanisms and most of tumors have at least partially restored AR signaling. Therefore, there is a pressing need to develop novel strategies targeting AR signaling in CRPC. 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 current 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 data also indicate that LSD1-mediated demethylation of FOXA1 may play an important role in supporting super-enhancers that are associated with the activation of oncogenic transcriptional programs, such as Myc signaling. Therefore, our current progress supports the hypothesis that LSD1 inhibitors may synergize with BET inhibitors in treating CRPC.

KEYWORDS

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

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: 95%

  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: 95%

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

  We have identified BRD4 as a possible component of the LSD1/FOXA1 complex by directly interacting with FOXA1.

  Month 24-36, Percentage of completion: 50%

  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. 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: 70%

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

Major Activities: Through the support of this grant, I have been able to continue my proposed research in the Center for Personalized Cancer Therapy, at University of Massachusetts Boston. For research networking, in addition to the established collaborations with labs of Drs. Steven P. Balk (Beth Israel Deaconess Medical Center), Timothy Rebbeck (Harvard TH Chan School of Public Health), Housheng Hansen He (University of Toronto), and Eva Corey (University of Washington), I have initiated new collaborations with Drs. Xiaohong Li (University of Toledo), Leigh Ellis (Cedars-Sinai Medical Center), and Kourosh Zarringhalam (University of Massachusetts Boston). 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 12-14, 2020, remote) and AACR annual meeting (April 9-14, 2021, remote). Members from my lab have given poster presentations related to this funded project during these meetings. I was also invited by Prostate Cancer Foundation to orally present data related to this project (Jan 6, 2021, remote). A second DoD Idea Expansion Grant that further studies the regulation of FOXA1 methylations was awarded (W81XWH-21-1-0267, PI: Cai, 7/15/2021-7/14/2024).

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 successfully determined that FOXA1 can be directly demethylated by LSD1 and that the enhancing effect of LSD1 on FOXA1 chromatin binding is mediated through this demethylation. In addition, we have also performed a RIME assay to identify components of the FOXA1 chromatin complex. For **aim 2**, we have tested LSD1 inhibitors in a total of four CRPC PDX models. The RNA-seq analysis was performed in the xenograft tumor samples to identify LSD1 inhibition-targeted pathways. We have also found that LSD1 and BRD4 have enriched co-occupation at super-enhancers and co-targeting LSD1 and BRD4 can synergistically repress tumor growth *in vitro* and *in vivo*. Important results were summarized below.

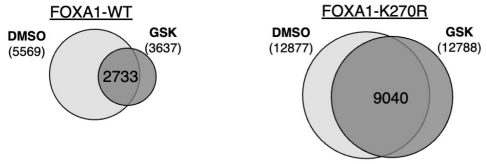

Figure 1. FOXA1 chromatin binding is less affected by LSD1 inhibition when K270 was mutated. CWR22RV1-tetFOXA1^{WT} or CWR22RV1-tetFOXA1^{K270R} cells treated with LSD1 inhibitor (GSK2879552) were subjected to V5 ChIP-seq analyses.

The chromatin binding of FOXA1-K270R mutant is not disrupted by LSD1 inhibition. We have established CWR22-RV1 stable cell lines expressing doxycycline-regulated V5-tagged FOXA1-K270R mutant or FOXA1-WT (wildtype control). The ChIP-seq analysis of V5 was performed to examine the global response for FOXA1 binding to LSD1 inhibition. As shown in **Fig. 1**, the total binding peaks for FOXA1-K270R mutant were 2.3-fold higher than FOXA1-WT, indicating enhanced binding. While over 50% of FOXA1-WT binding peaks were disrupted by LSD1 inhibition, only less than 30% of FOXA1-K270R binding was diminished by LSD1 inhibition, suggesting that LSD1-mediated

A

Histone:		**Histone associated:**		
Histone H3		SETD2	**KDM1A**	BRD4
Histone H4		SET	HDAC1	
Histone H2B		SETSIP	HDAC2	
Histone H1		WDR18	BAZ2A	
Histone H2A		RBBP4	CBX3/5	
DNA replication/repair:		**Transcription:**		
PARP1	TOP2B	**AR**	NF1A/C	HMGB3
PARP2	TOP1	ILF2/3	ZMYM2/4	MED13
DNAPKc	RAD52	ZBTB10	ZC3H8	NCOA5
XRCC6	MCM2/7	FOXA1	TRIM24/28	CDK9/12
DEK		FOXA3	TLE1/2/3/5	FBXO3

B

Figure 2. Identification of FOXA1-interacting proteins. A, V5-FOXA1 was immunoprecipitated in CWR22RV1-tetFOXA1^{WT} cells (crosslinked) and the co-immunoprecipitated proteins were identified from the mass spectrometry analysis (top candidates). **B**, Validation of LSD1 and BRD4 interaction with endogenous FOXA1.

K270-demethylation is a major mechanism regulating the FOXA1 chromatin binding.

Identification of FOXA1-interacting proteins at chromatin. To identify potential components of FOXA1 chromatin complex, we performed RIME (Rapid Immunoprecipitation Mass Spectrometry) in CWR22RV1-tetFOXA1^{WT} cells (V5-IP) to identify potential FOXA1-interacting proteins. As shown in **Fig. 2A**, we have found previously known FOXA1-interacting proteins, including AR and LSD1 (KDM1A), and also identified many novel interacting proteins, such as BRD4, SET, SETD2, MED13, and TLEs. The interactions between endogenous FOXA1 and LSD1 or BRD4 were validated using a Co-IP assay in CWR22-RV1 cells fixed by formaldehyde to stabilize chromatin complex (**Fig. 2B**).

LSD1 inhibitor treatment suppresses CRPC tumor growth in multiple PDX models. To further determine the efficacy of LSD1 inhibitors *in vivo*, we have tested two additional CRPC PDX (patient-derived xenograft) models, LuCaP70CR and LuCaP96CR, both of which have high expression levels of AR and FOXA1. As shown in **Fig. 3A-B**, the tumor growth of both models was significantly repressed by the LSD1 inhibitor, GSK2879552 (currently in phase I clinical trials). This treatment increased H3K4me2 levels in both models (**Fig. 3C-D**). We also examined the expression of AR-FL/V7-regulated genes in LuCaP70CR models. As shown in **Fig. 3E**, the classic AR target (*ZBTB16*) and two AR/AR-V7 regulated lipid synthesis genes (*ELOVL5*, *MBOAT2*) were significantly repressed by LSD1 inhibitor treatment. Together with previously tested LuCaP35CR and 77CR models, these *in vivo* animal studies suggest that the efficacy of LSD1 inhibitor treatment in CRPC may be correlated with the expression levels of FOXA1.

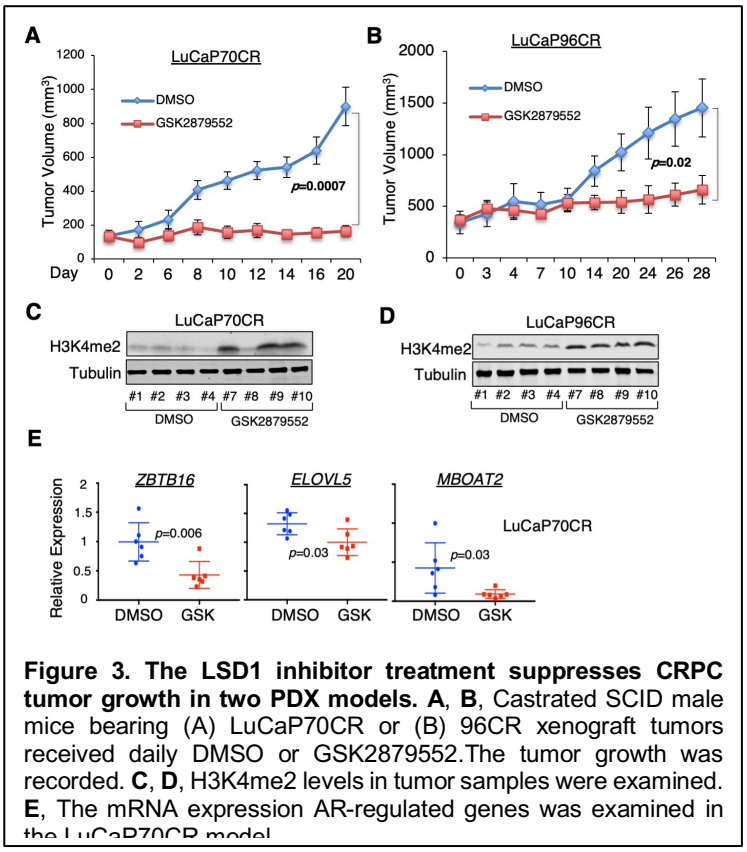

Figure 3. The LSD1 inhibitor treatment suppresses CRPC tumor growth in two PDX models. A, **B**, Castrated SCID male mice bearing (A) LuCaP70CR or (B) 96CR xenograft tumors received daily DMSO or GSK2879552.The tumor growth was recorded. **C**, **D**, H3K4me2 levels in tumor samples were examined. **E**, The mRNA expression AR-regulated genes was examined in the LuCaP70CR model

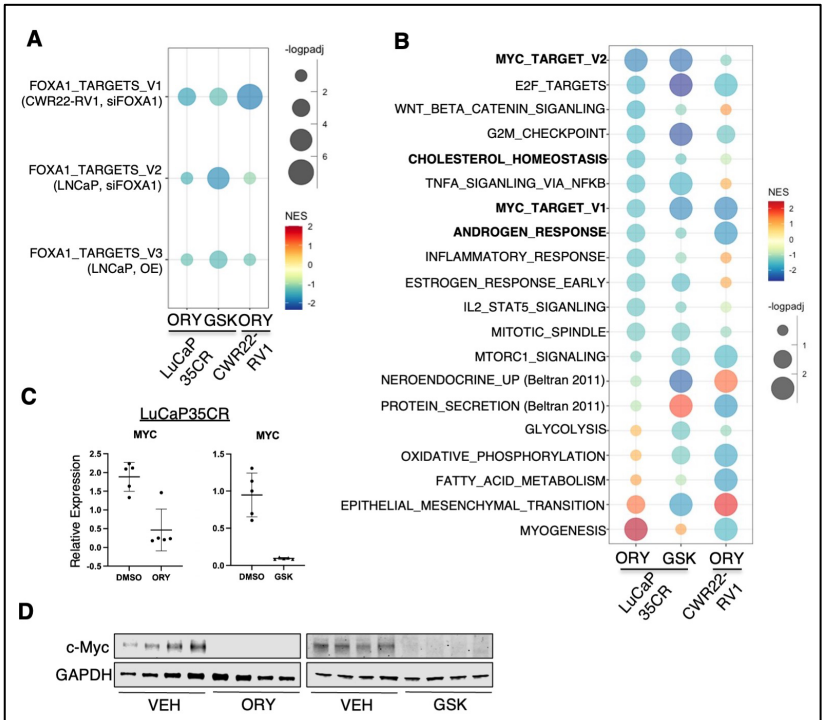

Figure 4. LSD1 inhibition suppresses MYC signaling. A, **B**, RNA-seq analyses were performed using tumor samples from LuCaP35CR and CWR22RV1 studies and GSEA analyses were conducted. **C**, MYC mRNA expression was shown. **D**. c-Myc protein expression was examined in the tumor samples.

LSD1 inhibition impairs MYC signaling through repressing MYC expression. We next performed RNA-seq analysis using tumor samples from LuCaP35CR treated with GSK2879552 or ORY1001 (another LSD1 inhibitor, currently in phase II trials) and from CWR22-RV1 xenografts treated with GSK2879552. To determine whether FOXA1 activity was broadly repressed by LSD1 inhibition, we developed three FOXA1-targets gene sets from public databases and then performed the Gene

Set Enrichment Analysis (GSEA). As shown in **Fig. 4A**, LSD1 inhibitor-repressed genes were significantly enriched for FOXA1-targets, indicating that FOXA1 transcriptional pioneering activity was broadly impaired by both inhibitors. Next, we examined the effect of LSD1 inhibition on other signaling pathways using hallmark genesets. As shown in **Fig. 4B**, LSD1 inhibition repressed androgen response and cholesterol homeostasis pathways, the latter of which is also a well-known AR target pathway, indicating that LSD1 inhibition represses AR activity *in vivo*. Moreover, the cell cycle-related pathways, including E2F targets and G2/M checkpoint, were also repressed by LSD1 inhibition, supporting the oncogenic activity of LSD1 in driving tumor growth. Significantly, we also found that MYC signaling (V1 and V2) was highly enriched in LSD1 inhibitor-repressed genes, suggesting that LSD1 may function to enhance MYC activity. Examining the expression of MYC mRNA and c-Myc protein in the tumor samples, we found that MYC expression was dramatically decreased by LSD1 inhibitor treatments (**Fig. 4C-D**).

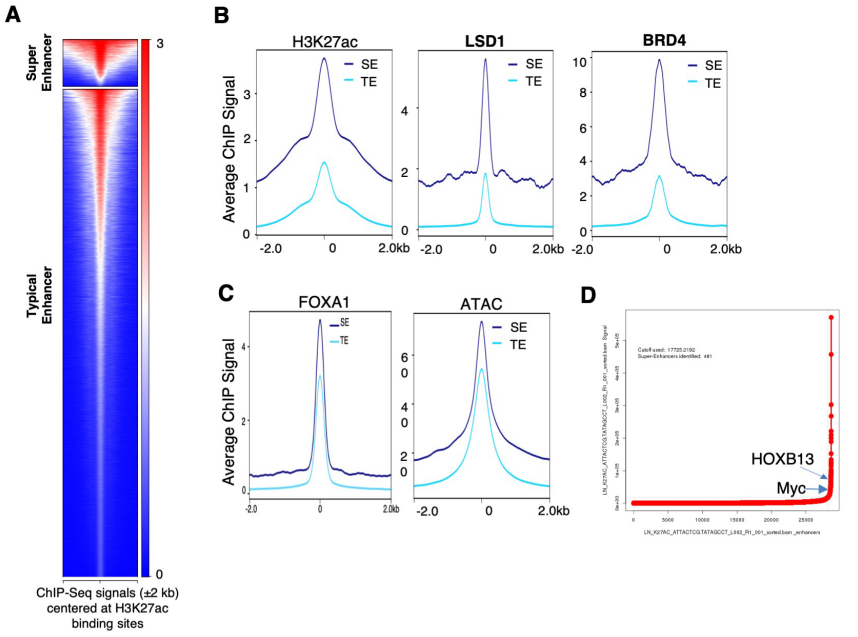

Figure 5. LSD1 is enriched in super-enhancers. A, Super-enhancers were identified using H3K27ac ChIP-seq (LNCaP). **B**, **C**, The average levels of (B) LSD1/BRD4 and (C) FOXA1/chromatin accessibility on super enhancers. **D**, Super-enhancers were identified at MYC and HOXB13 gene loci.

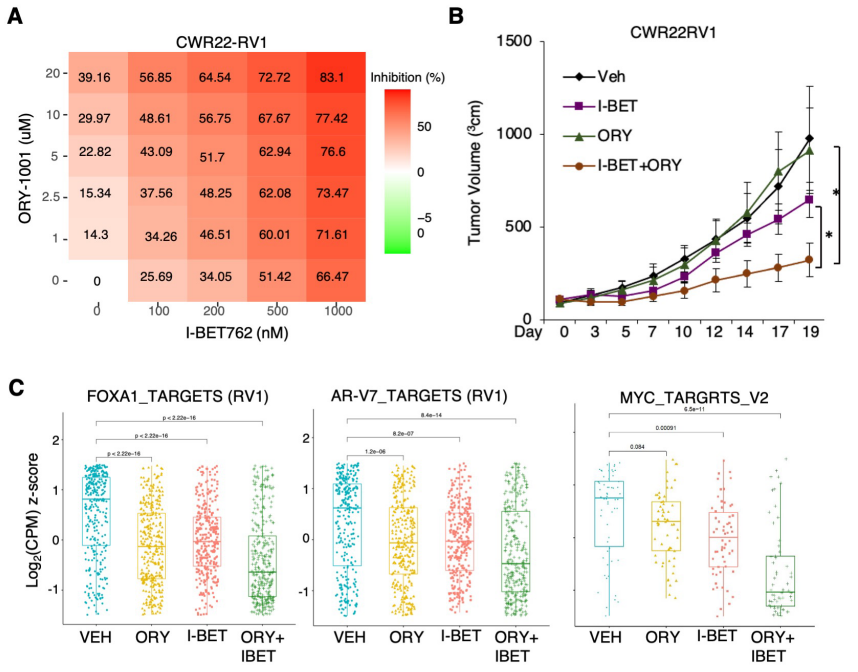

Figure 6. LSD1 inhibitor can synergize with BRD4 inhibitor to treat CRPC tumors. A, The effect of the combination of ORY-1001 and I-BET762 on CWR22-RV1 cell proliferation. **B**, Castrated SCID male mice bearing CWR22-RV1 xenograft tumors received daily ORY-1001, I-BET762, or the combination, and the tumor growth was measured. **C**. The effect of the combination treatment on FOXA1 targets, AR-V7 targets, and MYC targets.

LSD1 and BRD4 were co-enriched at super-enhancers. MYC mRNA and protein are overexpressed in many cancer types and this overexpression is primarily driven by MYC gene-associated super-enhancers (SE). Given the critical role of LSD1 in regulating chromatin accessibility, we hypothesize that LSD1 may be enriched at super-enhancers to maintain the opening of chromatin which is critical for bindings of multiple transcription factors. We first identified super-enhancers using the previously reported algorithm on the H3K27ac ChIP-seq data and identified ~800 super-enhancers in LNCaP PCa cells (**Fig. 5A**). The average levels of H3K27ac and BRD4, which is a chromatin marker for super-enhancers, were markedly higher in super-enhancers than the typical-enhancers (TE) (**Fig. 5B**). Notably, we found that LSD1 was also

highly enriched in super-enhancers. Consistent with higher LSD1 level, FOXA1 chromatin binding and the chromatin accessibility (measure by ATAC-seq) were also higher in super-enhancers than typical-enhancers (**Fig. 5C**). Examining the super-enhancer-associated genes, we validated that the MYC gene contains several nearby super-enhancers (**Fig. 5D**). Interestingly, we have also identified HOXB13, another important prostate cancer-specific pioneer factor, as a candidate of the super-enhancer-associated genes.

The combination treatment of LSD1 inhibitor and BRD4 inhibitor. Given the important functions of BRD4 in meditating super-enhancer activities and AR signaling (BRD4 can function as an AR coactivator), we next assessed whether LSD1 inhibitor can be combined with BRD4 inhibitor to repress AR signaling and super-enhancer associated oncogenic transcription programs in CRPC cells. We first tested this strategy *in vitro*. As shown in **Fig. 6A**, an additive effect on suppressing CWR22-RV1 cell growth was clearly observed when two inhibitors were combined. More important, we have also observed a strong synergetic effect of LSD1 inhibitor with BET inhibitor (both inhibitors using lower dosages) using xenograft tumor models (**Fig. 6B**). The combination treatment also more strongly repressed FOXA1 and AR-V7 targets, and MYC signaling (**Fig. 6C**). Overall, these data clearly demonstrate a synergistic effect of combining LSD1 inhibition and BRD4 inhibition in CRPC models.

Summary of results: These findings indicate that LSD1 maintains the enhancer accessibility to AR or its splice variant AR-V7 by stabilizing FOXA1 chromatin binding. Therefore, LSD1 inhibition is a promising therapeutic strategy to target FOXA1-AR signaling in CRPC. Our recent findings further suggest that LSD1 and BRD4 are co-enriched at the super-enhancer region and thus regulate super-enhancer-driven oncogenic transcription programs, such as MYC signaling. Therefore, 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 finished most experiments proposed for this aim. In the next funding period, we will focus on analyzing the high-throughput sequencing results obtained from current studies and also further validate the identified components of the FOXA1 complex. For Aim 2, we will perform additional RNA-seq analyses using the tumor samples obtained from LSD1 inhibitor-treated PDX models. We will also assess the combination treatment of LSD1 inhibitor and BRD4 inhibitor in CRPC PDX models.

IMPACT

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

  Our current data clearly indicate a critical role of LSD1 in mediating chromatin accessibility by stabilizing the FOXA1 chromatin binding at AR-dependent and -independent enhancers, and oncogene-associated super-enhancers. Our study provides a strong rationale for combining LSD1 inhibitor with BET inhibitor to target bot AR signaling and MYC signaling. Overall, the current development of the project has provided a solid foundation for the future clinical trials of LSD1 inhibitors in treating FOXA1-high CRPC.

- **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

CHANGES/PROBLEMS

Nothing to Report

PRODUCTS

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

  Han D, Owiredu JN, Healy BM, Li M, Labaf M, Steinfeld JS, Patalano S, Gao S, Liu M, Macoska JA, Zarringhalam K, Siegfried KR, Yuan X, Rebbeck TR, and ***Cai C**. (2021) Susceptibility-associated genetic variation in *NEDD9* contributes to prostate cancer initiation and progression. *Cancer Research*. 81(14): 3766-3776.

- **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

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:	*Zifeng Wang*
Project Role	*Graduate Student*

Research Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*6*
Contribution to Project:	*Zifeng has been working on running molecular biology assays related to this project, including RT-PCR, immunoblotting, ChIP, flow cytometry, and high-throughput sequencing*
Funding Support:	*N/A*

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:	*Dong Han*
Project Role	*Postdoc*
Research Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*4*
Contribution to Project:	*Dong 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?**

  Two additional grants have been awarded since the last reporting period.

  W81XWH-21-1-0267 (PI: Cai)
  US Army Prostate Cancer Research
  Idea Expansion Award 07/15/2021-07/14/2024
  Title: Targeting FOXA1 methylation in castration-resistant prostate cancer

  Proposal Development Grant (PI: Cai)
  University of Massachusetts Boston
  Proposal Development Grant (internal) 06/27/2021-12/31/2022
  Title: A pilot study for molecular functions of MLL1 and SETD7 in mediating FOXA1 activity in prostate cancer

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

  Nothing to Report

SPECIAL REPORTING REQUIREMENTS

Nothing to Report

APPENDICES

N/A