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CONTRACTING ORGANIZATION: University of Massachusetts, Boston, MA

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14. ABSTRACT

Metastatic prostate cancer inevitably relapses to the castration-resistant stage (CRPC) after standard or more aggressive androgen deprivation therapies, with restored AR signaling, indicating a pressing need for the development of novel therapies to target AR reactivation. FOXA1 functions as a pioneer factor and its chromatin binding is required for AR access to enhancers. We have recently discovered that FOXA1 is methylated at lysine 270 (K270), which is demethylated by LSD1, and that the demethylation of K270 is critical to stabilize FOXA1 chromatin binding in prostate cancer cells. In this report, we have shown that the K270 methylation is dramatically decreased in CRPC adapted to enzalutamide treatment and that increased levels of unmethylated K270 may lead to AR cistrome reprogramming. Moreover, we have also identified and validated SETD7 and MLL1 as methyltransferases of K270 and demonstrated that they function to oppose the effect of LSD1 on the chromatin binding of FOXA1.

15. SUBJECT TERMS
None listed.

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1. INTRODUCTION

Androgen receptor (AR), a ligand-dependent nuclear receptor transcription factor, plays a pivotal role in prostate cancer (PCa) development. However, PCa inevitably relapses to the castration-resistant stage (CRPC) after standard or more aggressive androgen deprivation therapies, with restored AR signaling, indicating a pressing need for the development of novel therapies to target AR reactivation. FOXA1 functions as a pioneer factor and its chromatin binding is required for AR access to enhancers. Recent studies revealed that FOXA1 is frequently mutated or altered in over 40% CRPC, resulting in overexpression or gained activity of FOXA1. These studies strongly suggest FOXA1 as a critical target in CRPC, but therapeutic strategies targeting FOXA1 are lacking. In a recently published study, we have discovered that FOXA1 is methylated at lysine 270 (K270), which is demethylated by LSD1. LSD1 inhibition in PCa cells globally disrupts FOXA1 binding and thus impairs AR chromatin binding and transcriptional activity. These data suggest that FOXA1 chromatin binding may be directly targeted by treatments enhancing K270 methylation.

Our objective of this grant is to determine the precise role of FOXA1 methylation in regulating its chromatin binding, enhancer accessibility, and subsequent recruitment of transcription factors, and assess the effect of FOXA1 methylation on CRPC tumor growth. Moreover, we will attempt to identify the lysine methyltransferases and additional demethylases (if any) mediating FOXA1 methylation and assess the treatments enhancing FOXA1 methylation in CRPC models.

2. KEYWORDS

LSD1, KDM1A, FOXA1, lysine 270, androgen receptor, AR, CRPC, lysine demethylation, BRD4, super-enhancers

3. ACCOMPLISHMENTS

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

  Specific Aim 1*: Determine the molecular function of FOXA1 methylation and its role in PCa resistance to enzalutamide*

  Major Task 1: Assess the effects of FOXA1 methylation on its pioneer factor activity

  Month 1-24, Percentage of completion: 50%

  Major Task 2: Assess the effect of FOXA1 methylation on CRPC resistance to enzalutamide

  Month 1-24, Percentage of completion: 30%

  Specific Aim 2*: Identify lysine methyltransferases (KMTs) and demethylases (KDMs) mediating FOXA1 methylation in PCa*

  Major Task 3: Identify the lysine methyltransferase(s) responsible for FOXA1 methylation in PCa cells

  Month 6-24, Percentage of completion: 60%

  Major Task 4: Identify additional KDMs responsible for FOXA1 demethylation in PCa cells

  Month 12-24, Percentage of completion: 0%

  Major Task 5: Assess the therapeutic effect of treatments enhancing FOXA1 methylation on enzalutamide-resistant CRPC models

  Month 18-36, Percentage of completion: 0%

- **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. We have made significant progress for both specific aims. For research networking, I have established 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). Zifeng Wang, a graduate student from my lab, has given a poster presentation related to this funded project during the SBUR meeting.

Specific Objectives: Specific aim 1 is to determine the molecular function of FOXA1 methylation and its role in PCa resistance to enzalutamide. Specific aim 2 is to Identify lysine methyltransferases (KMTs) and demethylases (KDMs) mediating FOXA1 methylation in PCa.

Significant Results: For **Aim 1**, we attempted to establish isogenic cell lines with K270R mutation in FOXA1 using the CRISPR/Cas9 approach. Multiple attempts have been done and we have screened over 100 clones. While we identified a few clones with heterozygous FOXA1-K270R, we did not find any clones with homozygous K270R mutations. Although we are still continuing to work on the CRISPR/Cas9 method, we also took our alternative approach – stably overexpressing doxycycline-inducible V5-tagged FOXA1 K270R mutant, to study how K270 methylation on PCa cell response to enzalutamide.

For **Aim 2**, we have primarily focused on identifying lysine methyltransferases of FOXA1-K270. Our screening studies have provided two candidates, MLL1 and SETD7. We have confirmed that these two proteins can methylate FOXA1 in prostate cancer cells and performed additional analyses to examine whether they function to antagonize LSD1 activity. Important results were summarized below.

The E269 and Q271 amino acids do not significantly affect K270 substrate recognition by LSD1. We have altered the -1/+1 sequences of K270 (**EKQ**) to generate two mutant peptides containing **AKQ** or **EKA** sequence. In vitro demethylase assay was then performed to determine how LSD1 catalyzes the demethylation of these peptides. Surprisingly, as shown in **Fig. 1**, alterations of the nearby amino acid sequence of K270 did not significantly affect the activity of LSD1 on the K270me-FOXA1 peptides. This result suggests that LSD1 may recognize its substrate through a more complicated pattern that may involve a larger region of amino acids or domains.

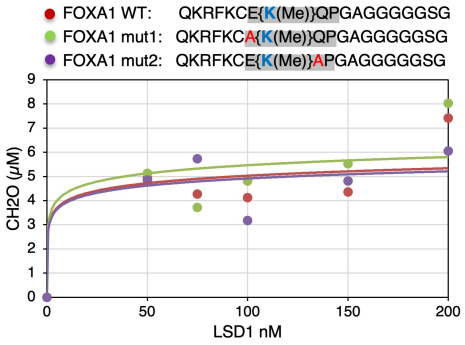

Figure 1. The -1/+1 amino acids (E and Q) near K270 does not significantly affect substrate recognition by LSD1
Three K270me-FOXA1 peptides were incubated with recombinant LSD1 proteins. The byproduct, formaldehyde, was measured using in vitro demethylation assay.

K270R mutation in FOXA1 alters AR chromatin binding in response to enzalutamide. Our published data have already indicated that K270R mutant can increase FOXA1 chromatin binding (not shown). To further determine how K270 demethylation affects AR cistrome and its response to enzalutamide, we have performed AR ChIP-seq in 22Rv1 cells overexpressing FOXA1-WT or FOXA1-K270R stimulated with DHT and treated with vehicle or enzalutamide (**Fig. 2**). First, we have detected substantially more AR binding sites in K270R cells, which is consistent with increased FOXA1 chromatin binding. Second, enzalutamide treatment led to the differential response of AR cistrome. In WT cells, AR binding peaks were significantly decreased from 7064 to 1648 (~4.3 fold decrease). However, AR binding peaks were barely changed in K270R cells, from 53895 to 47699 (~1.1 fold decrease). Third, a clear redistribution of AR binding occurred in K270R cells. These results reveal a more complicated model than what we originally expected and suggest that the K270R mutation of FOXA1 may cause an AR cistrome reprogramming to allow cells to adapt

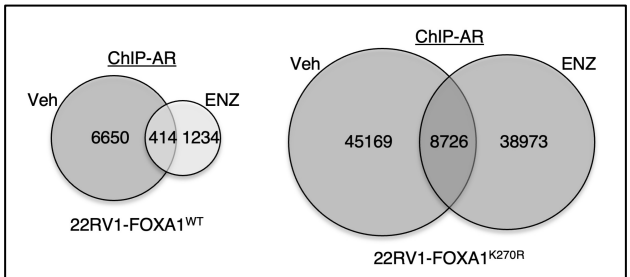

Figure 2. K270R mutation of FOXA1 leads to reprograming of AR cistrome in response to enzalutamide treatment.
22Rv1 cells stably overexpressing FOXA1-WT or FOXA1-K270R were pretreated with DMSO or enzalutamide (16h), and then stimulated by 10nM DHT (4h), followed by ChIP-seq of AR. Venn diagrams showed the binding peaks in each condition.

to enzalutamide treatment.

FOXA1 is less methylated in CRPC cells adapted to enzalutamide treatment. To examine the K270 methylation levels of FOXA1 in PCa cells adapted to enzalutamide treatment, our collaborator, Dr. Steven Balk, has developed an enzalutamide-resistant VCaP CRPC cell line (called VCaP16), which was generated by continuously culturing VCaP cells under enzalutamide treatment for over 6 months. These cells show significantly increased AR-V7 expression and activity (not shown). Therefore, we used this model to determine whether FOXA1 methylation status may be changed when CRPC cells adapte to enzalutamide. We enriched the nuclear fraction of parental VCaP cells, VCaP cells with short-term enzalutamide, and VCaP16 cells, and then immunoblotted K270-methylated FOXA1 using our established specific antibody. As shown in **Fig. 3**, K270me levels were slightly decreased in response to the short-term enzalutamide treatment but were dramatically diminished in the VCaP16 cells. These results suggest that CRPC cells may elevate the activity of K270-demethylases or decrease the activity of K270-methyltransferases to make FOXA1 less methylated, which can stabilize FOXA1 chromatin binding and thus restore AR signaling in response to AR signaling inhibition (ARSi) treatments.

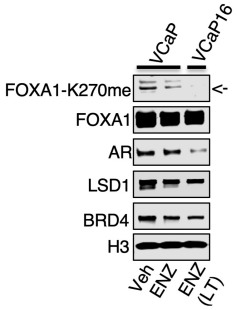

Figure 3. FOXA1-K270 methylation was significantly decreased in CRPC cells adapted to enzalutamide treatment.
Immunoblotting for FOXA1-K270me and other indicated proteins in VCaP and VCaP16 cells.

SETD7 and MLL1 are methyltransferases of K270. We next sought to identify the corresponding lysine methyltransferases (KMTs) by performing screening for a library of available recombinant KMTs using *in vitro* methylation assay (scintillation proximity assay) on a short FOXA1 peptide. As seen in **Fig. 4A-C**, the H3K4 methyltransferases SETD7 (KMT7) and MLL1 (KMT2A) showed significant enzymatic activity in methylating the FOXA1 peptide. The enzyme kinetics (K_m and k_{cat}) of both enzymes are comparable to the previously reported kinetics on methylation of H3K4 (**Fig. 4D** and **E**). The methylation levels of FOXA1-K270 were markedly decreased by gene silencing of MLL1 or SETD7 (**Fig. 4F**), further confirming that both these proteins can methylate K270 *in vivo*. SETD7 has been previously reported to methylate E2F1 at K185 in BCa cells, which is also demethylated by LSD1. The MLL gene family consists of six members, including MLL1-4, SETD1A, and SETD1B. MLL1-3 all function as tumor suppressors in PCa and are frequently mutated (loss-of-function mutations) or deleted in CRPC.

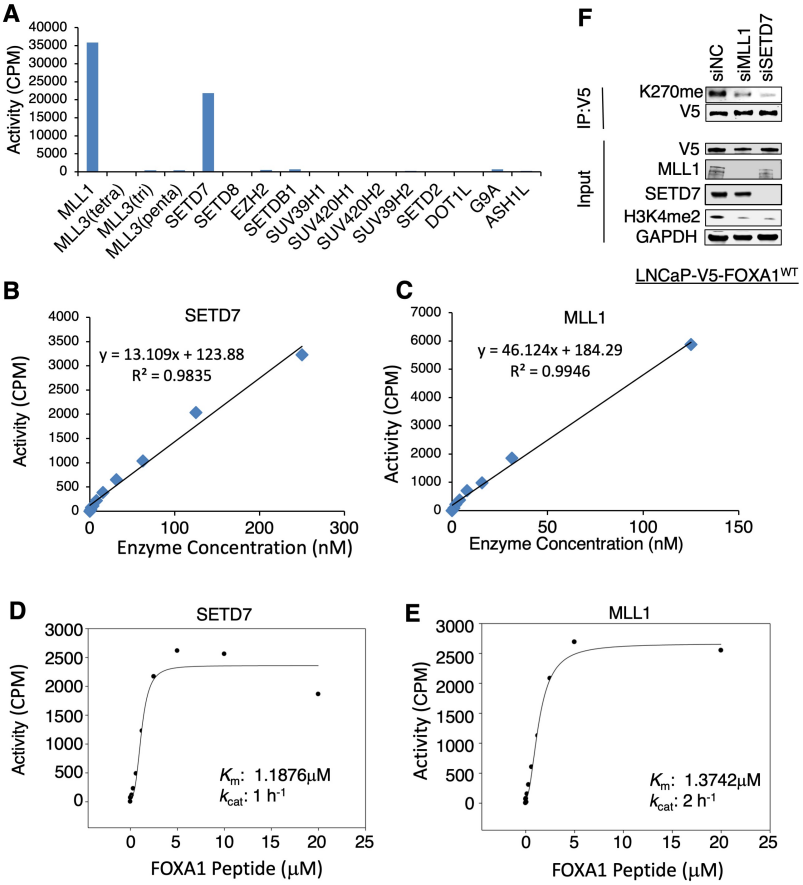

Figure 4. SETD7 and MLL1 are the methyltransferases of FOXA1-K270R
A, In vitro methylation assay using FOXA1 short peptide (263-281aa) (50μM) and recombinant lysine methyltransferase proteins (1μM). **B-C**, Validation of MLL1 and SETD7 using 1μM methyltransferase proteins titrated at 11 points and 5μM FOXA1 peptide. **D-E**, Validation of MLL1 and SETD7 using 20μM FOXA1 peptode titrated at 11 points and 100μM methyltransferase proteins. **F**, Immunoblotting for K270me in LNCaP-V5-

Since FOXA1 chromatin binding is dependent on LSD1-mediated demethylation, we next examine whether inhibition of SETD7 or MLL1 enzymatic activity can rescue the LSD1 inhibitor (LSD1-i) induced disruption of FOXA1 chromatin binding. As shown in **Fig. 5A**, consistent with our model, inhibiting MLL1 (MM102) or SETD7 (PFI) globally rescued the chromatin binding of FOXA1 from LSD1-i. Interestingly, this effect not only can be observed at AR-regulated enhancers (KLK3-enhancer and ZBTB16-enhancer) but also can be found at AR-independent enhancers (such as RET enhancer) (**Fig. 5B**).

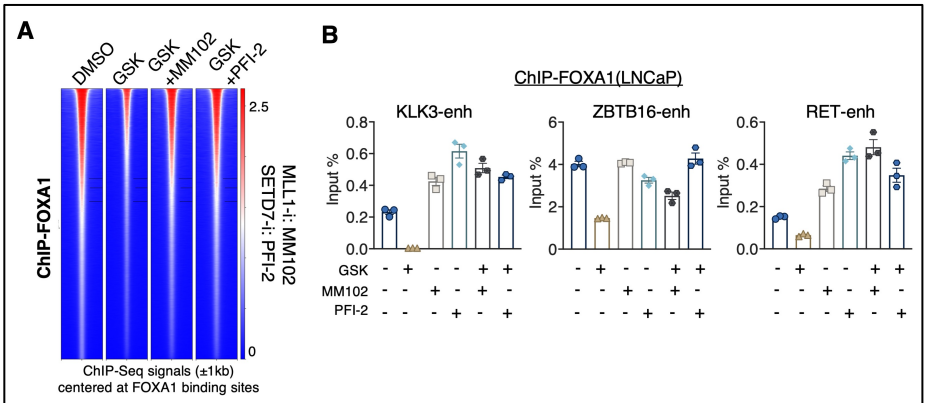

Figure 5. Inhibition of SETD7 or MLL1 can rescue the disruption of FOXA1 chromatin binding by LSD1-i. A, ChIP-seq of FOXA1 binding in LNCaP cells treated with a LSD1 inhibitor (GSK2879552), or in combination with MLL1 and SETD7 inhibitor. **B**, Validation at three FOXA1 binding sites by ChIP-qPCR.

Summary of results: The findings for specific aim 1 indicate that decreased methylation of FOXA1, which can reprogram AR cistrome, may be an important mechanism contributing to CRPC resistance to ARSi treatments. The results from specific aim 2 demonstrate that SETD7 and MLL1 are methyltransferases of FOXA1 and function to oppose the effect of LSD1 on the regulation of FOXA1 chromatin binding.

- **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 will focus on studying the AR cistrome reprogramming induced by FOXA1-K270R and attempt to examine K270 methylation levels in additional enzalutamide-resistant models. For Aim 2, we will focus on characterizing the function and molecular activity of SETD7 and MLL1 in PCa tumors.

4. 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 FOXA1 K270 methylation in mediating FOXA1 chromatin binding and AR signaling. We also successfully identified and validated SETD7 and MLL1 as the specific methyltransferases of FOXA1-K270, which may provide new molecular insights into the functions of these epigenetic factors in prostate cancer.

- **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:	*2*
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:	*4.5*
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:	*Internal CSM College Fellowship (Fall semester of 2021)*

Name:	*Mingyu Liu*
Project Role	*Graduate Student*
Research Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*7.5*
Contribution to Project:	*Mingyu has been working on conducting molecular assays and analyzing high-throughput sequencing data.*
Funding Support:	*N/A*

Name:	*Maryam Labaf*
Project Role	*Graduate Student*
Research Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*4.5*
Contribution to Project:	*Maryam has been working on analyzing high-throughput sequencing data.*
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.

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

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

8. SPECIAL REPORTING REQUIREMENTS

Nothing to Report

9. APPENDICES

Nothing to Report