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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 for stabilizing FOXA1 chromatin binding. In this report, we have shown that the K270 demethylation expands FOXA1 chromatin binding and subsequently alters AR binding, particularly in response to enzalutamide treatment. Furthermore, we have also identified SETD7 as the primary methyltransferase of K270 and demonstrated that reduced expression of SETD7 in CRPC cells can reprogram FOXA1 cistrome, promoting cancer progression.

15. SUBJECT TERMS

NONE LISTED

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1. INTRODUCTION

FOXA1 serves as a pioneer transcription factor, playing a pivotal role in relaxing the densely compacted chromatin structure by displacing the repressive histone H1. The binding of FOXA1 to chromatin is indispensable for enabling the androgen receptor (AR) to access enhancer regions. Recent investigations have unveiled a frequent occurrence of mutations or alterations in FOXA1, affecting more than 40% of cases of castration-resistant prostate cancer (CRPC). These alterations often lead to FOXA1 overexpression or an increase in its functional activity. This mounting body of research strongly underscores the significance of FOXA1 as a prime target in CRPC. However, therapeutic approaches aimed at FOXA1 are currently absent.

In recently published research, our team made the discovery that FOXA1 undergoes methylation at lysine 270 (K270), a modification that is subsequently reversed by the enzyme LSD1. Notably, the inhibition of LSD1 in PCa cells results in a disruption of FOXA1 binding to chromatin. This disruption consequently impairs the chromatin binding and transcriptional activity of both the AR and its variant AR-V7. This compelling set of observations underscores the pivotal role played by protein methylation of FOXA1 in governing its oncogenic activity.

The first goal of the research grant in question is to elucidate the precise impact of FOXA1 methylation on its interaction with chromatin, its ability to access enhancers, and the subsequent recruitment of other transcription factors. Furthermore, our endeavor will encompass the identification of the specific lysine methyltransferases involved in FOXA1 methylation, along with an exploration of any additional demethylase enzymes that might be implicated.

This annual report presents some compelling findings. Notably, the introduction of the K270R mutation in FOXA1 leads to substantial alterations in its chromatin binding properties and its response to LSD1 inhibition. Intriguingly, our investigations also demonstrate a significant redistribution of the AR in cells expressing the K270R mutant, particularly in response to treatment with enzalutamide. Another accomplishment of our research is the identification of SETD7 as the methyltransferase responsible for catalyzing the methylation of FOXA1 at the K270 site, a discovery that has been successfully published in the *Proceedings of the National Academy of Sciences* very recently (PMID: 37549269).

KEYWORDS

LSD1, KDM1A, FOXA1, lysine 270, androgen receptor, AR, CRPC, lysine demethylation, BRD4, super-enhancers, SETD7

2. 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: 80%

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

  Month 1-24, Percentage of completion: 80%

  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: 100%

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

  Month 12-24, Percentage of completion: 50 %

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

resistant CRPC models

Month 18-36, Percentage of completion: 10%

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

Major Activities: Thanks to the support of this grant, I have been able to actively advance my proposed research within the Center for Personalized Cancer Therapy at the University of Massachusetts Boston. Our progress has been substantial across both specific aims. To enhance research collaboration, I had a monthly joint lab meeting involving critical collaborators: Dr. Steven P. Balk from Beth Israel Deaconess Medical Center and Dr. Housheng Hansen He from the University of Toronto. These meetings serve as a platform to discuss project advancements and strategize upcoming experiments.

Moreover, my engagement within the Harvard Cancer Center Program has been noteworthy. I've taken part in various seminars and gatherings, including the Dana-Farber Cancer Institute prostate cancer SPORE seminars. My involvement extended to external conferences such as the AACR Cancer Epigenomics Conference (10/06-10/08/2022), Prostate Cancer Foundation Annual Retreat (10/27-10/29/2022), SBUR (Society for Basic Urological Research) Annual Meeting (11/10-11/13/2022), and the AACR Annual Meeting (4/8-4/13/2023). During these events, graduate students from my lab presented posters closely related to the project funded by this grant.

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**, a series of ChIP-seq experiments was conducted on both FOXA1 and AR in PCa cells expressing either the wild-type (WT) FOXA1 or the K270R mutant variant. The primary objective was to discern the influence of the K270 modification on FOXA1's activity and its functional interaction with AR. Our gathered data demonstrates that K270 demethylation induces an expansion in FOXA1 chromatin binding. Intriguingly, this phenomenon also triggers a redistribution of AR binding, inducing alterations in its responsiveness to enzalutamide. Moreover, our investigation delved into FOXA1 Forkhead-domain mutations, which hold greater prevalence within CRPC. Interestingly, these mutations demonstrated no discernible impact on K270 methylation, suggesting that the chromatin activities of these mutants may still be governed by LSD1-mediated K270 demethylation.

For **Aim 2**, a significant breakthrough has been achieved as we successfully identified SETD7 as the primary methyltransferase responsible for FOXA1-K270 methylation. This discovery takes on a functional role that opposes the activity of LSD1 within CRPC cells. Through its distinct mechanism, SETD7 acts as a transcriptional repressor and, consequently, a tumor suppressor within the context of CRPC. Our findings reveal that the decreased expression of SETD7 in CRPC triggers a consequential redistribution of FOXA1. This phenomenon leads to the activation of a subset of cryptic enhancers, which in turn, may fuel cancer progression. These profound insights have been documented and have now been published in the esteemed journal *PNAS* and are readily accessible online.

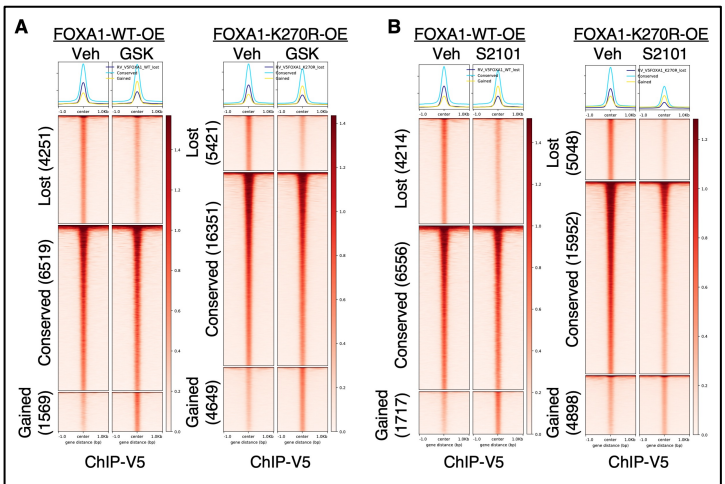

Figure 1. K270R mutation expands FOXA1 binding. A, **B**, ChIP-seq analyses of V5 in 22Rv1 cells overexpressing V5 tagged FOXA1 WT or K270R mutant and treated with GSK2879552 (A) or S2101 (B). Heatmap view illustrates the change of binding intensity of FOXA1.

Important results were summarized below.

K270R mutation expands FOXA1 chromatin binidng and impairs its reponse to LSD1 inhibtion. ChIP-seq analyses of V5 were performed in CWR-22Rv1 cells overexpressing tetracycline-regulated V5-tagged WT FOXA1 and its K270R mutant. These cells wer also treated with LSD1 inhibitors,

GSK2879552 or S2101. To gain further insights, these cellular setups were subjected to treatment with LSD1 inhibitors, GSK2879554, or S2101. The results, as shown in **Fig. 1A** and **B,** underscore a remarkable expansion in FOXA1's chromatin binding. Specifically, the binding sites were increased from ~12,000 to ~26,000 sites (2.2 fold). Importantly, all these sites exhibited a marked enrichment for Forkhead binding motifs. Of significant interest is the behavior of the expanded FOXA1 sites in response to LSD1 inhibition. Our findings show a noteworthy resilience of these sites to the inhibitory effects of LSD1. These observations provide robust validation for our initial hypothesis, affirming that K270 demethylation indeed reinforces FOXA1's chromatin binding potency within CRPC cells.

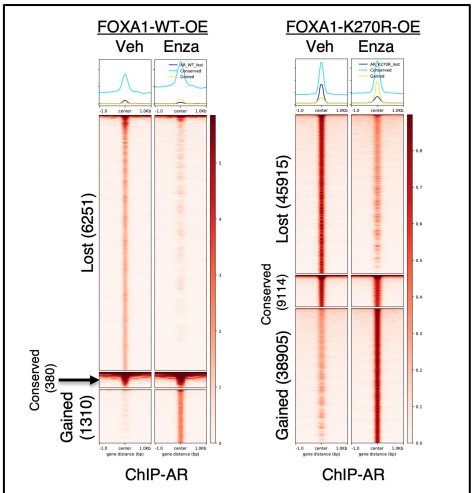

Figure 2. K270R mutation confers AR resistance to enzalutamide. ChIP-seq analyses of AR in 22Rv1 cells overexpressing V5 tagged FOXA1 WT or K270R mutant and treated with enzalutamide. Heatmap view illustrates the change of binding intensity of AR.

K270R mutation confers AR resistance to enzalutamide. Subsequently, we conducted ChIP-seq analyses on AR using the 22Rv1 stable cells as previously described, wherein these cells were treated either with or without enzalutamide (**Fig. 2**). In cells expressing FOXA1-WT, enzalutamide treatment resulted in a significant reduction in AR's chromatin binding, affecting approximately 80% of binding sites. Conversely, within FOXA1-K270R cells, the impact of enzalutamide was less pronounced, with disruption occurring in less than 50% of sites. Notably, a considerable redistribution of AR binding sites, around 40%, was evident in the latter scenario. These findings signify a substantial shift in AR behavior within PCa cells expressing the K270R mutation when exposed to agents that inhibit AR signaling, such as enzalutamide. This phenomenon may be a potential mechanism driving enzalutamide resistance in CRPC.

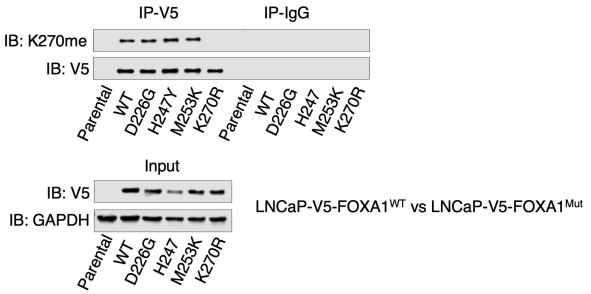

Figure 3. FOXA1 Forkhead mutations do affect K270 methylation. Immunoprecipitation of V5-FOXA1 was performed in 22Rv1 stable cells overexpressing FOXA1 mutants. Immunoblotting of methylated K270 was then conducted.

FOXA1 Forkhead-domain mutations do not affect K270 methylation. Castration resistance can emerges due to an elevated mutation rate within the Forkhead domain of FOXA1. Nevertheless, the impact of these mutations on the K270R modification remains uncertain. To address this question, we generated stable cells that overexpress a range of FOXA1 mutations, including D226G, H247Y, and M253K. We proceeded to conduct immunoblotting on immunopurified FOXA1 proteins in these cells, utilizing our specific antibody targeting methylated K270. As illustrated in **Fig. 3**, the levels of methylated K270 observed in these mutants were similarly to those detected in FOXA1-WT. This evidence strongly suggests that the chromatin binding of these mutants might still be regulated by K270 methylation and demethylation. Consequently, this infers that inhibitors targeting LSD1 might retain efficacy in treating CRPC driven by recurrent FOXA1 mutations.

SETD7 is the primary methyltransferase of K270. Our recent report demonstrated that LSD1 can demethylate the lysine 270 of FOXA1 in PCa cells, leading to the stabilization of FOXA1 chromatin binding. However, the identity of the methyltransferase responsible for FOXA1 methylation and negative regulation of the FOXA1-LSD1 oncogenic axis remains unknown. In this study proposed in **Aim 2**, we aimed to investigate the role of SETD7 as a methyltransferase of FOXA1-K270, destabilizing FOXA1 chromatin binding and competing with the activity of LSD1. Additionally, we sought to uncover the tumor suppressor function of SETD7 in preventing tumor growth and metastasis in various PCa models under castrated conditions. Through the examination of transcriptional programs in CRPC models, we also identified SETD7's ability to repress oncogenic transcriptional programs driven by FOXA1, MYC, and E2F. Importantly, our integrated transcriptomic and cistromic analyses indicated that the major activity of SETD7 in PCa involves repressing gene transcription rather than activating it. Furthermore, we found that reduced SETD7 expression in CRPC cells can lead to the redistribution of FOXA1 chromatin binding. Overall, our findings provide novel molecular insights into the

tumor suppressor function of SETD7 during the progression of CRPC, suggesting that the downregulation of SETD7 expression may play a critical role in the reprogramming of FOXA1 activity and adaptation to ARSi in CRPC cells. The proposed working model is presented in **Fig. 4** and detailed information and results can be found in our repcently published paper (PMID: 37549269).

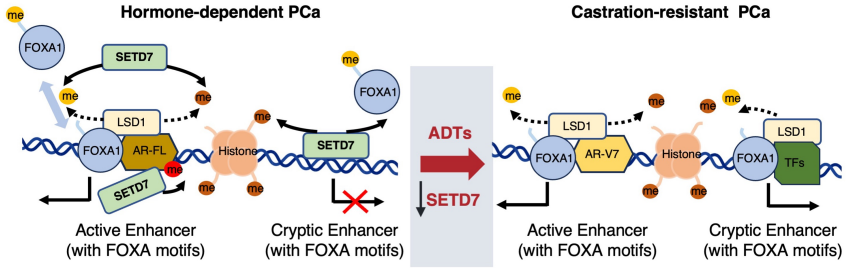

Figure 4. Graphis summary of the working model.

- **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 complete the bioinformatic analyses for the ChIP-seq/RNA-seq analyses and examine methylated K270 levels in our xenograft tumor samples. For Aim 2, we will focus on exploring the additional lysine demethylases that may contribute to K270 demethylation.

3. 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 regulating FOXA1 chromatin binding and AR signaling. We also successfully identified and validated SETD7 as the primary methyltransferases of FOXA1-K270, which may provide new molecular insights into the functions of this epigenetic factor in prostate cancer progression.

- **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

4. CHANGES/PROBLEMS

Nothing to Report

5. PRODUCTS

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

1. Besschetnova A, Han W, Liu M, Gao Y, Li M, Wang Z, Labaf M, Patalano S,Venkataramani K, Muriph RE, Macoska JA, Siegfried KR, Evans J, Balk SP, Gao S, Han D, and ***Cai C**. (**2023**) Demethylation of EHMT1/GLP protein reprograms its transcriptional activity and promotes prostate cancer progression. ***Cancer Research Communications***. [Published online].
2. Wang Z, Petricca J, Liu M, Chen S, Zhang S, Li M, Besschetnova A, Patalano S, Venkataramani K, Siegfried KR, Macoska JA, Han D, Gao S, Vedadi M, Arrowsmith C, He HH & ***Cai C**. (**2023**) SETD7 functions as a transcription repressor in prostate cancer via methylating FOXA1. ***Proceedings of the National Academy of Sciences (PNAS)***. [Published online]
3. Li M, Liu M, Han W, Wang Z, Han D, Patalano S, Macoska JA, Balk SP, He HH, Corey E, Gao S & ***Cai C**. (**2023**) LSD1 inhibition disrupts super-enhancer driven oncogenic transcription programs in castration-resistant prostate cancer. ***Cancer Research***. 83(10):1684-1698.
4. Labaf M, Li M, Ting L, Karno B, Zhang S, Gao S, Patalano S, Macoska J, Zarringhalam K, Han D, and ***Cai C**. (**2022**) Increased AR expression in castration-resistant prostate cancer rapidly induces AR signaling reprogramming with the collaboration of EZH2. ***Frontiers in Oncology***. eCollection 2022

- **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

6. 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:	*9*
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. She was also involved in the manuscript writing.*
Funding Support:	*Internal CSM College Fellowship (Fall semester of 2022)*

Name:	*Mingyu Liu*
Project Role	*Graduate Student*
Research Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*4.5*
Contribution to Project:	*Mingyu has been working on conducting molecular assays and analyzing high-throughput sequencing data.*
Funding Support:	*N/A*

Name:	*Songqi Zhang*
Project Role	*Graduate Student*
Research Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*3*
Contribution to Project:	*Songqi has been working on running molecular assays and animal studies.*
Funding Support:	*Internal IB Program Fellowship (Fall/Spring 2022/2023)*

- **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

7. SPECIAL REPORTING REQUIREMENTS

Nothing to Report

8. APPENDICES

Nothing to Report