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| 14. ABSTRACT<br>Lysine specific demethylase 1 (LSD1) has been well-characterized as a transcriptional repressor through demethylation of histone 3 at lysine 4, but it can also act to stimulate the transcriptional activity of several transcription factors including androgen receptor (AR) by unclear mechanisms. The data we have generated show that LSD1 broadly stimulates the transcriptional activity of the AR in prostate cancer (PCa) cells. Moreover, we have identified the transcription factor FOXA1 as a potential substrate for LSD1. These results suggest that FOXA1 demethylation by LSD1 may be important for FOXA1 binding to chromatin and recruitment of AR, and provide new insight into the actions of both LSD1 and FOXA1 that may be relevant in a number of cancers. We further found that short-term treatment with LSD1 inhibitors can markedly suppress PCa growth, while stable knockdown of LSD1 had no clear effect on growth. Further studies are needed to understand how PCa cells adapt to LSD1 inhibition or loss, and to determine whether effective therapies targeting LSD1 can be developed for PCa. |                                 |                                  |                                                |                                              |                                            |
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## INTRODUCTION

Lysine specific demethylase 1 (LSD1) forms a complex with CoREST and has been well-characterized as an epigenetic regulator that mediates transcriptional repression through demethylation of mono- or dimethylated histone 3 lysine 4 marks (H3K4me1,2). In prostate cancer (PCa) cells, the ability of androgen receptor (AR) to directly repress a subset of genes is dependent on LSD1. However, LSD1 has also been found increase the transcriptional activity of several transcription factors including AR, and the mechanistic basis for this coactivator function has not been established. LSD1 inhibitors that are currently in the clinic for relapsed acute myeloid leukemia, and we proposed that LSD1 may have activity in PCa, and particularly in advanced castration-resistant prostate cancer (CRPC), where AR activity persists and its function may be altered by epigenetic mechanisms. Specifically, we hypothesized that LSD1 activity in PCa may allow tumor cells to epigenetically reprogram the AR cistrome by closing AR binding sites through which AR stimulates expression of genes driving differentiated functions, and sites through which AR represses expression of other genes mediating G1/S cell cycle progression. Moreover, on the AR regulated genes that drive tumor growth, we hypothesized that LSD1 can then function as a coactivator to stimulate AR transcriptional activity. Finally, we hypothesized that the H3K4me1,2 demethylation activity of LSD1 contributes to the epigenetic silencing of multiple AR-independent growth-inhibitory genes in advanced CRPC, and that these genes may be reactivated by LSD1 inhibition. Our objectives in this proposal were to test these hypotheses and validate LSD1 as a therapeutic target in PCa, and to determine whether there are molecular features in a subset of PCa that may predict responses.

## KEYWORDS

lysine specific demethylase 1, CoREST, epigenetics, prostate cancer, androgen receptor, castration-resistant prostate cancer, histone, demethylation

## ACCOMPLISHMENTS

### Major Goals

**Aim 1. Assess epigenetic reprogramming of AR functions in response to LSD1 inhibition in CRPC cell line models.**

- a. Generate PCa cell lines grown through multiple passages in the presence of LSD1 inhibitor versus control.
- b. Molecular characterization of cell lines generated in 1a.

**Aim 2. Assess LSD1 as a therapeutic target in PCa xenograft models with regulated LSD1 downregulation or loss.**

- a. Establish xenografts with regulated LSD1 expression.
- b. Assess responses to LSD1 depletion.

### Major Activities

Our major focus over the past year has been to assess responses of PCa cell lines to suppression of LSD1 activity, and determine the underlying mechanisms. As our data have begun to indicate that LSD1 is acting through FOXA1, much of our mechanistic work has focused on FOXA1.

### Specific Objectives

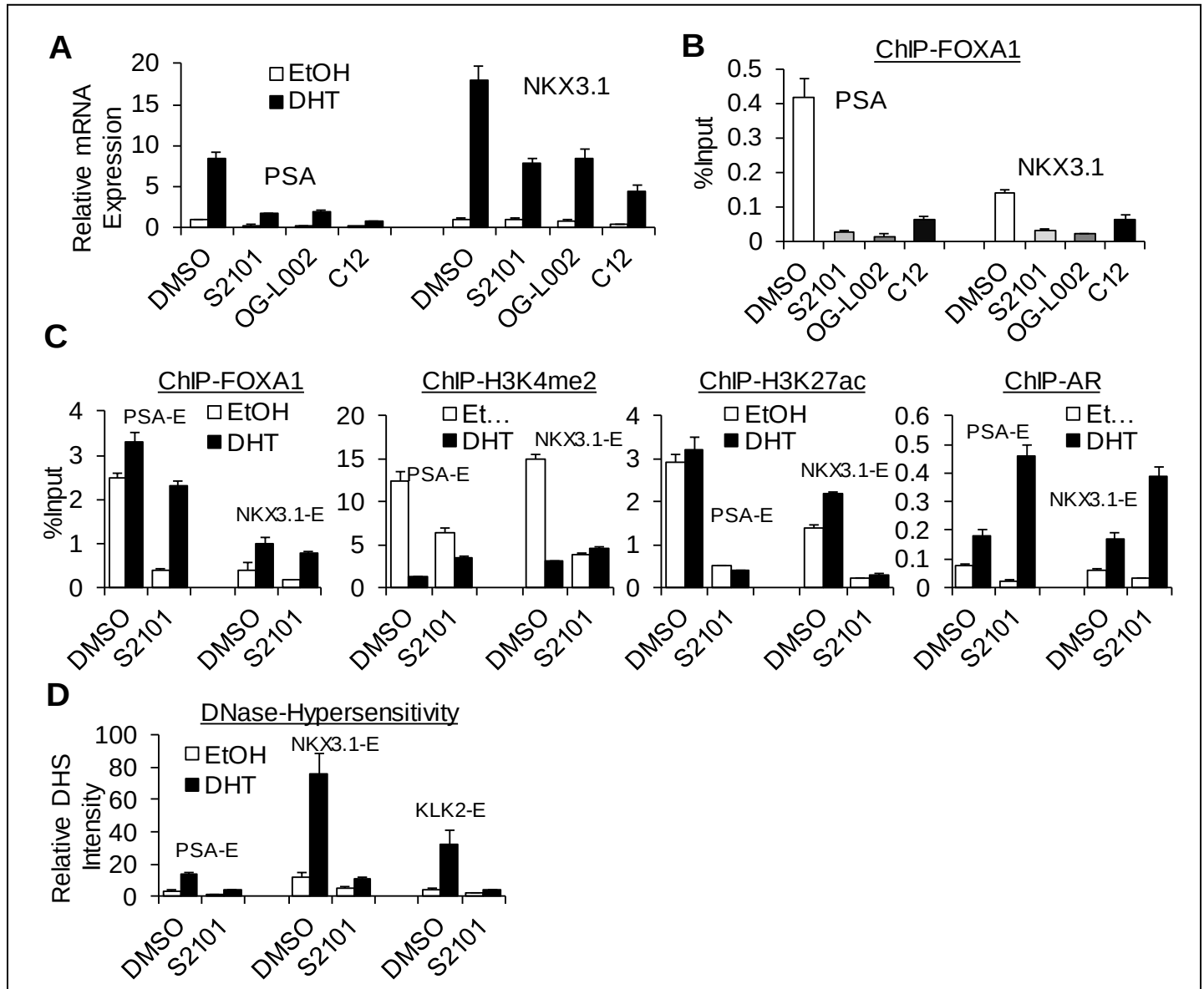
Our overall objectives are to determine precisely how LSD1 modulates AR activity, and to determine if LSD1 inhibition may be efficacious in PCa.

### Significant Results or Key Outcomes

#### Major Findings Related to Aim 1

Our previous data indicated that LSD1 inhibition could suppress AR transcriptional activity. This was further confirmed by examining expression on AR regulated genes (PSA, NKX3.1) in response to a series of LSD1

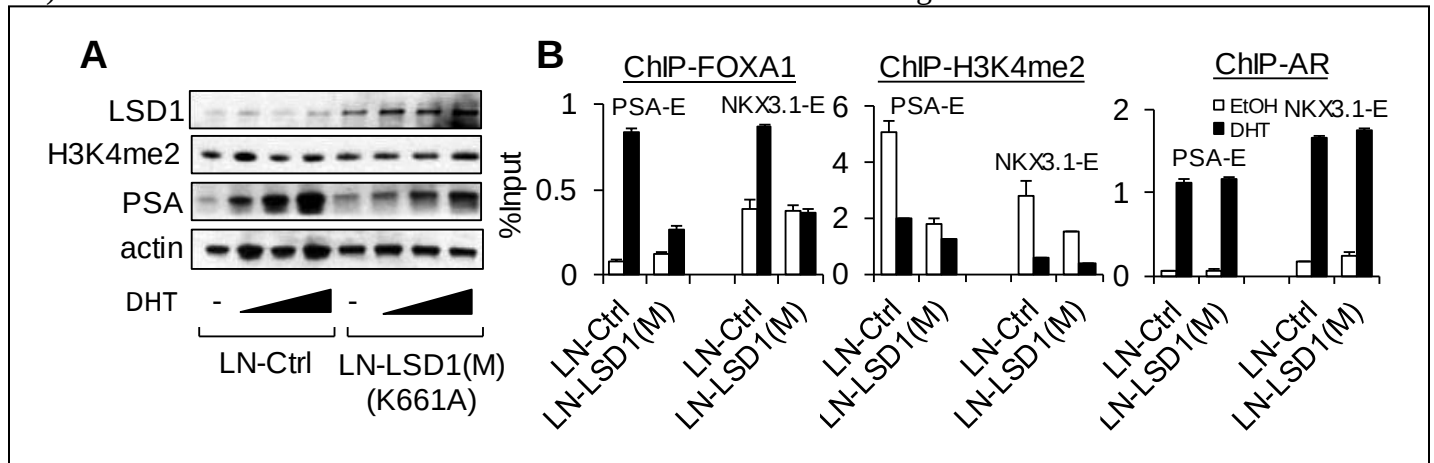
inhibitors (Fig. 1A). A possible mechanism for this inhibition suggested by our previous work was decreased binding of the transcription factor FOXA1, which is required for AR binding at many gene. Indeed, by ChIP-PCR we found that each LSD1 inhibitor markedly decreased FOXA1 binding to the AR binding sites in the PSA and NKX3.1 genes prior to DHT stimulation (Fig. 1B). Levels of H3K4me2 and H3K27ac, associated with active transcription were also decreased, as were DHT stimulated binding of FOXA1 and AR (Fig. 1C). Consistent with decreased FOXA1, DNase-hypersensitivity was also decreased at these sites, indicative of decreased chromatin accessibility (Fig. 1D). Together these results indicate that LSD1 inhibition directly or indirectly decreases FOXA1 binding and thereby suppresses AR activity.



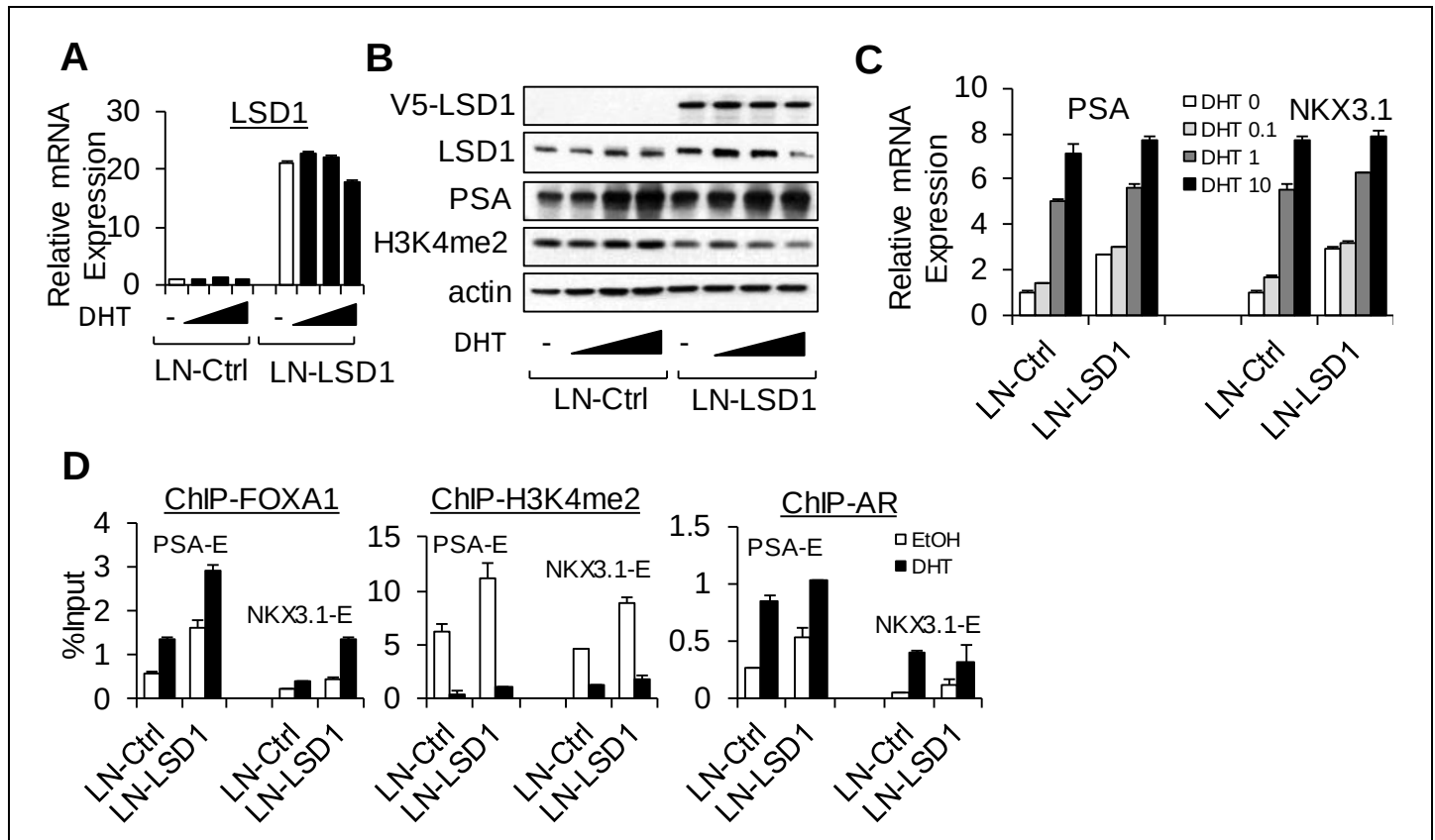
**Figure 1. LSD1 inhibition impairs AR activity, and decreases FOXA1 binding and enhancer availability.** (A) LSD1 inhibitors impair expression of AR-regulated genes in LNCaP PCa cells. (B) LSD1 inhibitors substantially decrease FOXA1 binding to AR-regulated enhancers prior to androgen treatment. (C) LSD1 inhibitor S2101 decreases active enhancer markers H3K4me2 and H3K27ac. (D) DNase-Hypersensitivity assay showed that LSD1 inhibitor S2101 decreases DNase-HS at AR regulated enhancers.

To validate the results with LSD1 inhibitors, we generated stable LNCaP cells that were stably overexpressing a catalytically inactive LSD1 (K661A mutant that abrogates demethylation activity), which can act in a dominant negative fashion. This mutant decreased basal H3K4me2 and suppressed expression of the AR regulated PSA

gene in response to DHT (Fig. 2A and B). It also decreased FOXA1 binding to chromatin (Fig. 2B). Finally, we similarly examined the effects of overexpression the wild-type LSD1 (Fig. 3A,B). Conversely to the LSD1 inhibition studies, increasing LSD1 resulted in an increase sensitivity to lower DHT levels (Fig. 3B,C). Basal and DHT stimulated FOXA1 binding were also increased, and there was a small increase in AR binding (Fig. 3D). Overall these results are consistent with the LSD1 inhibitor findings.

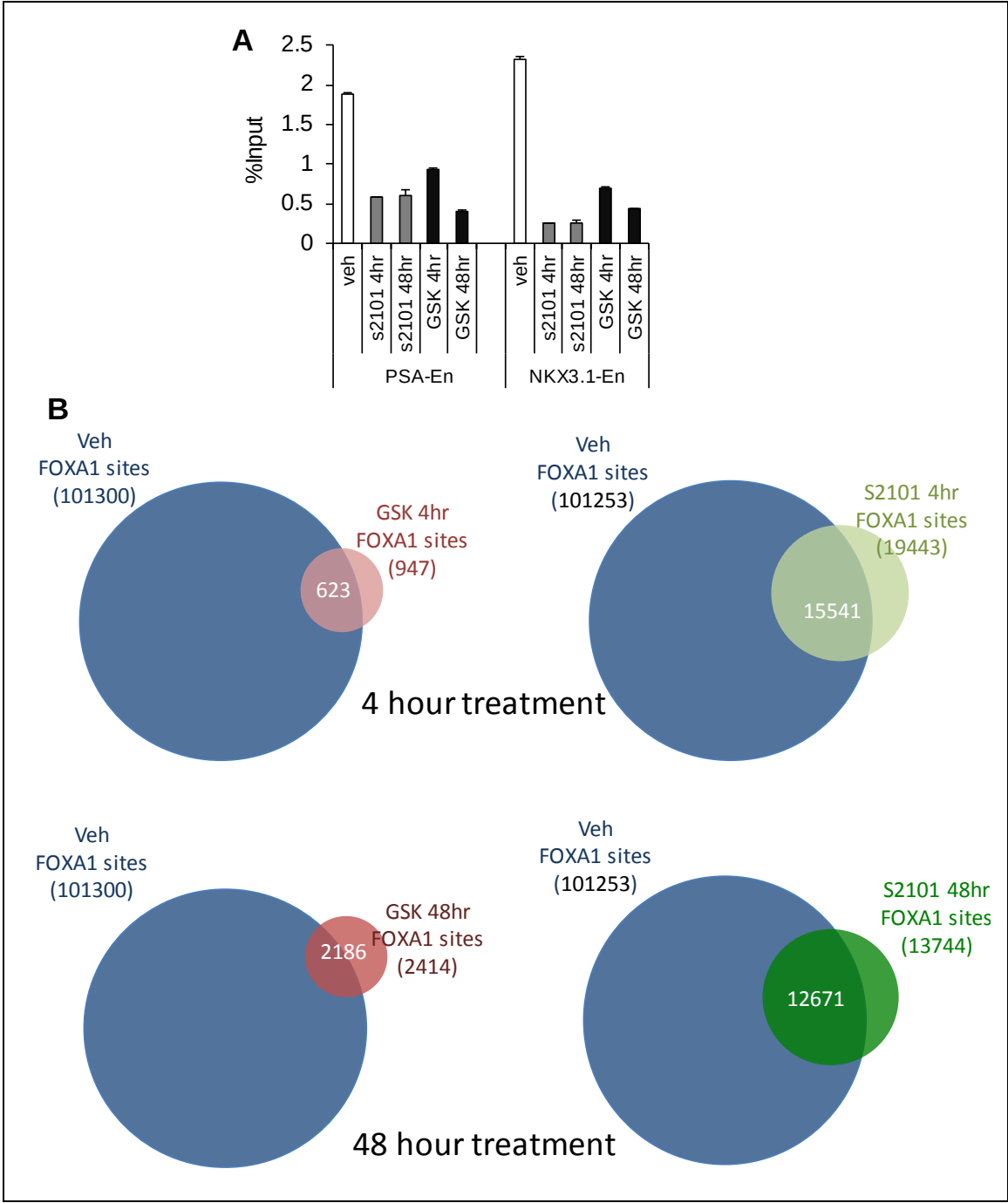


**Figure 2. Catalytically dead LSD1 decreases FOXA1 binding and enhancer availability.** (A) A catalytically dead LSD1 (K661A mutant) cell line was generated and mutant LSD1 impaired AR activity. (B) Overexpressing LSD1-K661A decreased FOXA1 binding and H3K4me2 histone mark.



**Figure 3. LSD1 overexpression increases FOXA1 binding and enhancer availability.** (A) LSD1 overexpressing cell line was generated. (B and C) Overexpressing wild-type LSD1 sensitized AR to lower androgen level. (D) Overexpressing wild-type LSD1 increased FOXA1 binding, H3K4me2 histone mark, and basal AR binding.

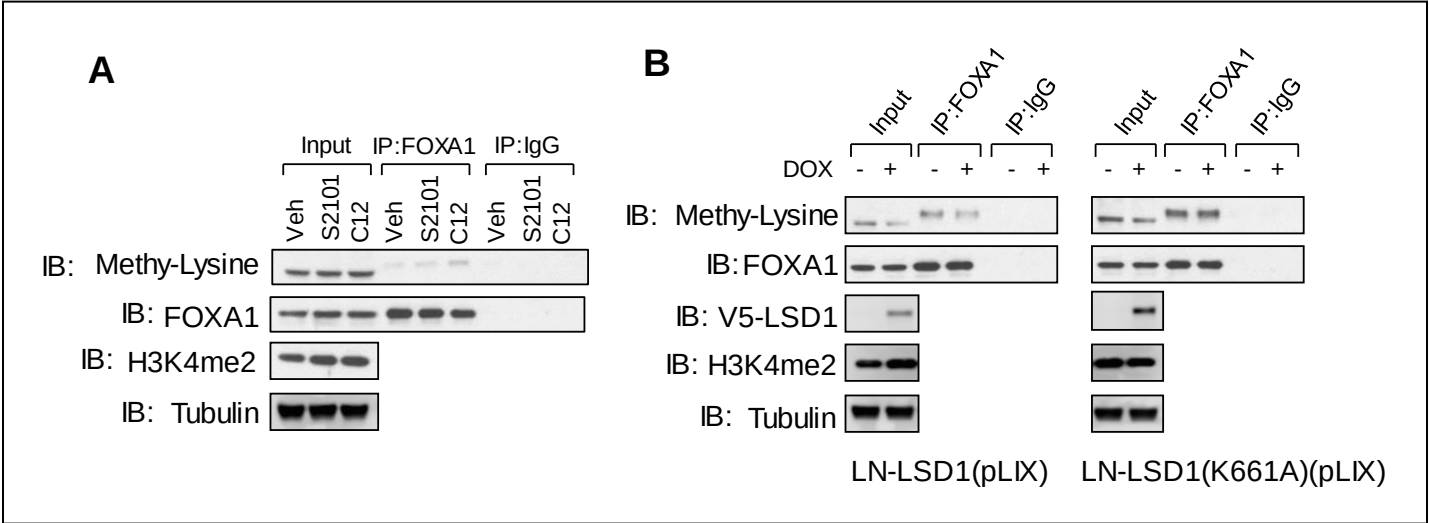
To determine whether LSD1 inhibition was broadly decreasing FOXA1 binding, we next carried out FOXA1 ChIP-seq studies. We initially determined that treatment with 2 different LSD1 inhibitors (S2101 and GSK-LSD1) for 4 or 48 hours markedly decreased FOXA1 binding at the AR regulated enhancers in the PSA and NKX3.1 genes (Fig. 4A). Remarkably, ChIP-seq under each of these conditions then showed a dramatic decrease in FOXA1 binding sites (Fig. 4B). These findings show that LSD1 inhibition causes a global decrease in FOXA1 binding to chromatin.



**Figure 4. LSD1 inhibition globally abolished FOXA1 chromatin binding.** (A) ChIP FOXA1 in LNCaP cells treated with LSD1 inhibitors for 4 hours or 48 hours. (B) ChIP-seq FOXA1 in LNCaP cells treated for 4h or 48h with LSD1 inhibitors

Based on these findings with respect to FOXA1, we have begun to examine whether FOXA1 may be a direct substrate for LSD1 mediated demethylation. Using a pan-anti-methyl-lysine antibody, we initially found that treatment with LSD1 inhibitors appeared to increase FOXA1 methylation (Fig. 5A). For the converse experiment we generated LNCaP cells stably transduced with doxycycline inducible LSD1 transgenes (V5-

epitope tagged, either wild-type or catalytically inactive). Consistent with the LSD1 inhibitor result, doxycycline induction to increase wild-type LSD1 decreased FOXA1 methylation, although induction of the mutant LSD1 (which can have dominant negative effects) did not have a clear effect (Fig. 5B).



**Figure 5. LSD1 decreases the level of lysine-methylated FOXA1 protein. (A)** LNCaP cells were treated with indicated LSD1 inhibitors, followed by immunoprecipitation of rFOXA1 and immunoblotting with a pan-anti-methyl-lysine antibody. The bands reacting with anti-methyl-lysine in the input wells are bovine immunoglobulin from the culture medium. **(B)** LNCaP cells with a stable inducible wildtype LSD1 transgene (left panel), or catalytically inactive LSD1 transgene (right panel) were treated +/- doxycycline, followed by FOXA1 immunoprecipitation and immunoblotting as indicated. As above, the input methyl-lysine bands are bovine immunoglobulin. LSD1 overexpression appeared to slightly decrease FOXA1 methylation, induction of the mutant LSD1 did not appear to increase methylation.

As these results suggested that LSD1 may function by demethylating FOXA1, we next carried out a series of mass spectrometry studies. These studies have identified methylation at K270, K288, K316, and K350 (Fig. 6). Although there were no clear increases in methylation at these sites in response to an LSD1 inhibitor (GSK-LSD1), the overall stoichiometry is low and these studies are not quantitative. Therefore, we are currently examining the functional consequences of mutating each site. These studies also revealed a series of FOXA1 phosphorylation sites (Fig. 6).

| *Tri-methylation of FOXA1                  |                 | Phosphorylation of FOXA1 |        |
|--------------------------------------------|-----------------|--------------------------|--------|
| Veh                                        | GSK             | Veh                      | GSK    |
| <u>me-K270</u>                             | <u>me3-K270</u> | p-S234                   | p-S234 |
| me3-K288                                   | me3-K288        | P-S280                   | ---    |
| me3-K316                                   | ---             | P-S282                   | ---    |
| *Tri-methylation could also be acetylation |                 | P-S301                   | ---    |
|                                            |                 | P-S304                   | P-S304 |
|                                            |                 | P-S307                   | P-S307 |
|                                            |                 | P-S331                   | P-S331 |
|                                            |                 | P-T334                   | P-T334 |
|                                            |                 | P-S347                   | ---    |
|                                            |                 | P-S431                   | ---    |

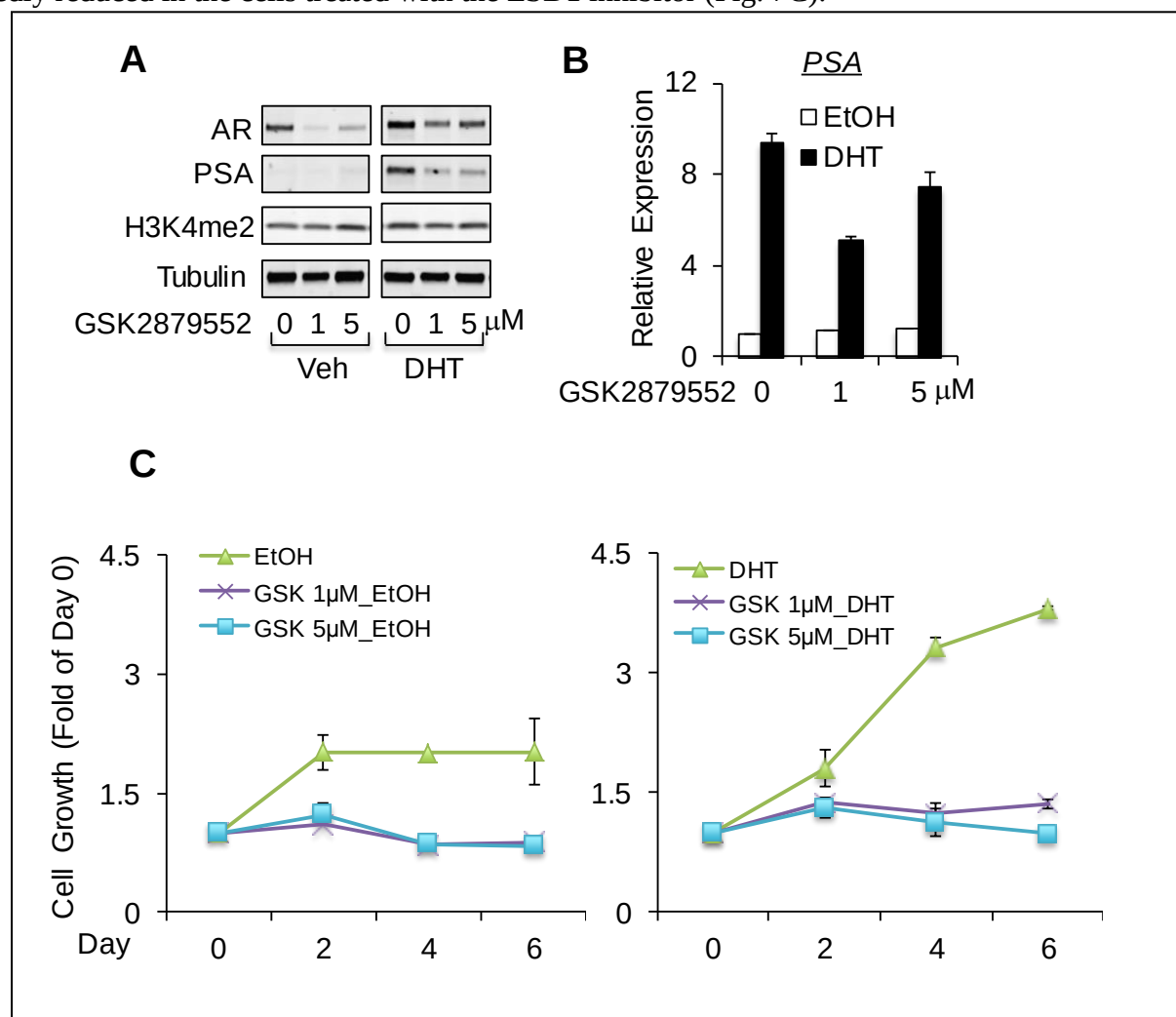
  

| Mono-methylation of FOXA1 |         |
|---------------------------|---------|
| Veh                       | GSK     |
| me-K270                   | me-K270 |
| me-K350                   | ---     |

**Figure 6. Identification of FOXA1 posttranslational modifications by LC-MS/MS.** FOXA1 was immunopurified from LNCaP cells treated with vehicle or LSD1 inhibitor (GSK-LSD1). The band corresponding to the mass of FOXA1 was cut out from SDS-PAGE, subjected to in-gel trypsinization, and analyzed by LC-MS/MS. Although LSD1 can't demethylate tri-methyl lysine, we presume that loss of LSD1 may provide increase substrate for trimethylation.



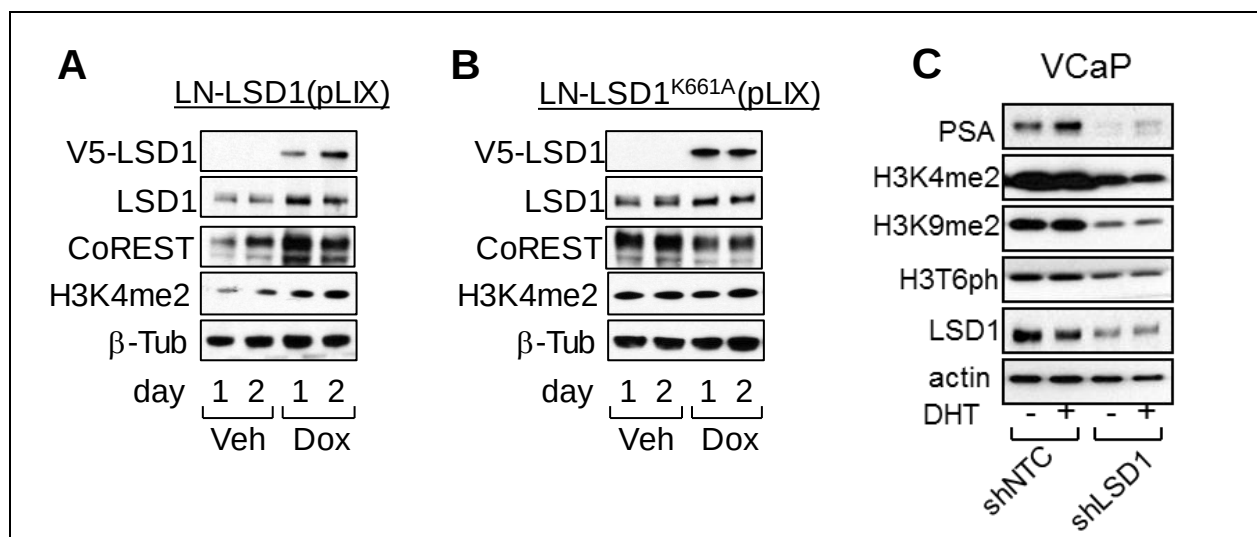
In parallel with these studies, we have examined effects of longer term in vitro LSD1 inhibitor treatment. LNCaP cells treated for 2 weeks with LSD1 inhibitor (GSK-2879552) had markedly reduced levels of AR protein, and PSA production (protein and mRNA) in response to DHT treatment was similarly decreased (Fig. 7A,B). The basis for this decrease in AR protein is not yet clear and is being explored. Proliferation was also markedly reduced in the cells treated with the LSD1 inhibitor (Fig. 7C).



**Figure 7. Long-term treatment with LSD1 inhibitor decreases AR and cell proliferation.** (A, B) Immunoblotting or qRT-PCR in LNCaP cells pre-treated with GSK2879552 for 2 weeks. (C) Cell proliferation assay for LNCaP cells pre-treated with GSK2879552 for 2 weeks.

### Major Findings Related to Aim 2

In order to carry out in vivo studies, we have generated cells with doxycycline regulated expression of LSD1 or catalytically inactive LSD1 (which can act as a dominant negative) (Fig. 8A,B). Growth of these cells in vivo as xenografts is currently being examined. We have also generated VCaP cells stably expressing an LSD1 shRNA, which have reduced levels of LSD1 (Fig. 8C). These cells cultured in vitro for ~7 weeks showed decreased AR activity. Interestingly, H3K4me2 and H3K4me9 were also decreased, presumably through an indirect mechanism. Surprisingly, preliminary results show that the LSD1 suppression in these cells does not markedly impair growth in vitro, or in vivo as subcutaneous xenografts (not shown). More detailed analyses of these xenografts to understand how they have adapted to LSD1 downregulation are underway.



**Figure 8. Establishment of stable cells expressing inducible LSD1, catalytic dead mutant, or shRNA.** (A) Doxycycline induced overexpression of V5-LSD1 in LNCaP cells. (B) Doxycycline induced overexpression of LSD1-K661A mutant in LNCaP cells. (C) VCaP cells stably transduced with lentiviral vector expressing LSD1 or nontargeting control shRNA.

### Developments

As noted above, a major development has been the accumulating evidence that LSD1 may be acting, at least in part, through FOXA1.

### Conclusions

Based on these studies, our working hypothesis is that FOXA1 is an LSD1 substrate, and that loss of LSD1 impairs AR activity by increasing FOXA1 methylation and thereby decreasing its binding to chromatin. Significantly, previous studies have shown that FOXA1 loss can result in loss of many AR binding sites, but some sites are not FOXA1 dependent while other sites may be gained in response to FOXA1 loss. Therefore, the effects of LSD1 inhibition on AR function and PCa growth will likely be complex. Indeed, as outlined above, short term treatment with LSD1 inhibitors suppresses PCa cells growth, but longer term depletion of LSD1 by shRNA does not have a marked growth suppressive effect. Further studies are needed to understand how PCa cells adapt to LSD1 inhibition or loss, and to determine whether effective therapies targeting LSD1 can be developed for PCa.

### Other Achievements

Nothing in addition to those outlined above.

### Stated Goals Not Met

We have largely met our goals, although we clearly have not yet fully elucidated the functions of LSD1 and further in vitro and in vivo studies are warranted.

### **Opportunities for Training and Professional Development Provided by the Project**

Nothing to Report

### **Disseminated To Communities of Interest**

Nothing to Report

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

Nothing to Report

## **IMPACT**

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

We believe these studies, when reported, will reveal novel actions of LSD1 and shed new light on mechanisms that regulate AR activity in PCa.

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.

Lysine specific demethylase 1 (LSD1) has been well-characterized as a transcriptional repressor through demethylation of histone 3 at lysine 4, but it can also act to stimulate the transcriptional activity of several transcription factors including androgen receptor (AR). One mechanism that may contribute to this latter activity is demethylation of histone 3 at lysine 9, but it is likely that LSD1 functions through additional substrates. As LSD1 inhibitors are entering the clinic, it is critical to understand precisely how it functions and whether LSD1 inhibitors may be efficacious in particular cancers. The data we have generated show that LSD1 broadly stimulates the transcriptional activity of the AR in prostate cancer (PCa) cells. Moreover, we have identified the transcription factor FOXA1 as a potential substrate for LSD1. These results suggest that FOXA1 demethylation by LSD1 may be important for FOXA1 binding to chromatin and recruitment of AR, and provide new insight into the actions of both LSD1 and FOXA1 that may be relevant in a number of cancers. We further found that short-term treatment with LSD1 inhibitors can markedly suppress PCa growth, while stable knockdown of LSD1 had no clear effect on growth. Further studies are needed to understand how PCa cells adapt to LSD1 inhibition or loss, and to determine whether effective therapies targeting LSD1 can be developed for PCa.

What was the impact on other disciplines?

LSD1 appears to modulate the function of many additional transcription factors, and LSD1 inhibitors are in trials for several other cancers. Therefore, the results will be of interest beyond the PCa field.

What was the impact on technology transfer?

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.

Nothing to Report

What was the impact on society beyond science and technology?

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.

Nothing to Report

## **CHANGES/PROBLEMS**

Nothing to Report

## **PRODUCTS**

Nothing to Report (manuscripts are anticipated over the next year)

## **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

What individuals have worked on the project?

Name: Steven Balk

Project Role: PI  
Researcher Identifier (e.g. ORCID ID): I'm going to use eRA user name: STEVENBALK  
Nearest person month worked: 1  
Contribution to Project:  
Funding Support: NIH, DFCI/HCC

Name: Sen Chen  
Project Role: Post-doctoral associate  
Researcher Identifier (e.g. ORCID ID): eRA Commons username: schen2  
Nearest person month worked: 8  
Contribution to Project:  
Funding Support: NIH, DFCI/HCC

Name: Olga Voznesensky  
Project Role: Lab manager  
Researcher Identifier (e.g. ORCID ID): eRA Commons username: N/A  
Nearest person month worked: 1  
Contribution to Project:  
Funding Support: NIH, DFCI/HCC

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

The PI received a new DOD PCRP Impact Award: DOD Prostate Cancer Research Program Impact Award W81XWH-16-1-0431; Title: Development of Precision Neoadjuvant-Adjuvant Therapies; Dates: 09/30/16–09/29/19

What other organizations were involved as partners?

Nothing to Report

#### **SPECIAL REPORTING REQUIREMENTS**

None

#### **APPENDICES**

None