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TITLE: Androgen Deprivation Enhances PLZF-Repressed Cistrome that Promotes the Castration-Resistant Phenotype

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14. ABSTRACT Androgen deprivation therapy (ADT) is an important therapeutic strategy which has a fundamental impact on prostate cancer biology. However, the mechanism by which ADT influences this negative androgenic regulation in CRPC development is unclear. Here, I identified an androgen-inducible tumor suppressor, Promyelocytic leukemia zinc finger protein (PLZF) which plays different roles in growth control, senescence, self-renewal, and tumor suppression in various cancer types. Interestingly, PLZF was reported as an androgen-responsive gene with anti-proliferative activity in prostate cancer cells. Moreover, decreased PLZF gene expression was observed in CRPC as compared to primary tumors, suggesting that loss of PLZF expression may have a role on CRPC development.					
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Introduction:

A long-standing clinical challenge in the management of prostate cancer is its heterogeneous response to androgen deprivation therapy (ADT), a standard treatment to disrupt the androgen receptor (AR) signaling pathway, since AR has a profound effect on prostate cancer development through the regulation of not only transcriptional networks but also genomic stability, and the development of gene fusions[1]. While ADT is effective in most patients with prostate cancer, prostate cancers inevitably become resistant to ADT and castration-resistant prostate cancer (CRPC) emerges [2]. Since multiple molecular mechanisms modulating resistance to ADT have been proposed [3], the recent surge in prostate cancer genomic information may permit molecular classification of prostate cancer [4]. Whole exome sequencing of metastatic castration resistant prostate tumors (mCRPC) from the Michigan cohort [5] and the East Coast Stand Up to Cancer/Prostate Cancer Foundation (SU2C/PCF) study recently revealed that ~10% of tumors harbor promyelocytic leukemia zinc finger (PLZF) genomic alternations [6]. PLZF also known as the zinc finger and BTB-containing protein 16 (ZBTB16) was originally identified as a gene fused to RAR α in acute promyelocytic leukemia (APL) patients [7] and was reported as an androgen-responsive tumor suppressor gene[8]. However, the role of PLZF in prostate cancer progression is unclear.

Keyword:

Whole exome sequencing, CRPC, ADT, PLZF, homozygous deletion

Accomplishment:

I interrogated the biological consequences of the loss of PLZF expression and performed a bioinformatic analysis of PLZF ChIP seq and PLZF-regulated gene expression profiling to define the PLZF transcriptional program. In addition, I examined the biological function of *PLZF* in CRPC development *in vitro* and *in vivo*. Collectively, our data strongly support that PLZF functions as a tumor suppressor in prostate cancer and demonstrate that PLZF inactivation as an important molecular event for CRPC development [9].

What were the major goals of the project?

Task 1. Identify *PLZF* transcription program mediating castration resistance in prostate cancer cells. (Months 1-12)

- a. Determine the *PLZF*-regulated genes in LNCaP cells by using the microarray gene profiling technique (Months 1-4) and compare their gene expression in LNCaP-Abl, a castration resistant counterpart. (Months 5-6)
Verify the expression of PLZF and its targets in LAPC4-PR/CR and VCaP/VCS2, two other pairs of hormone naïve and castration resistant cells. (Months 7-8)
- b. Integrated analysis of *PLZF* cistrome and *PLZF* binding regions by using the chromatin immunoprecipitation sequencing (ChIP-seq) technique. Perform PLZF ChIP-seq in the presence and absence of DHT in LNCaP. (Months 9-10) Bioinformatic integration of PLZF ChIP-seq and microarray data. (Months 11-12)

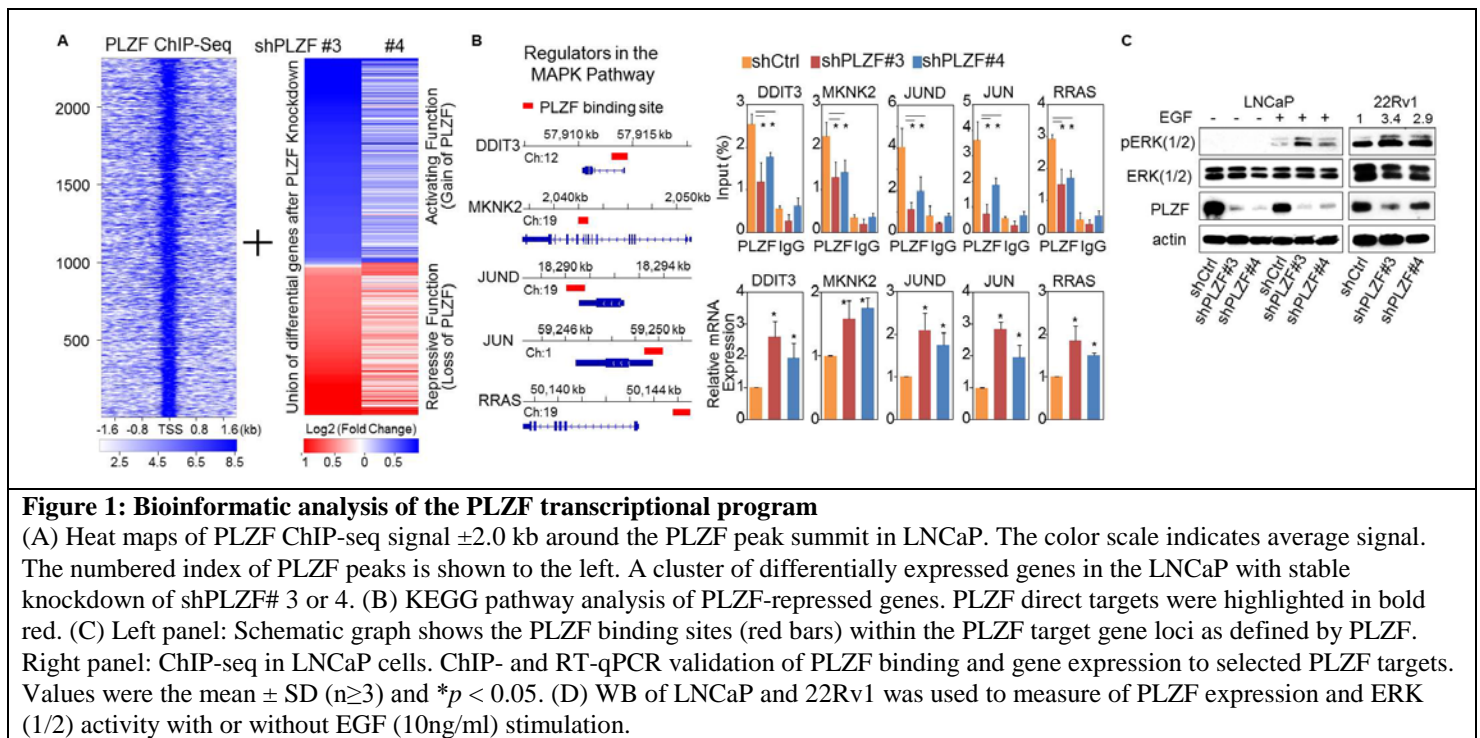
Task 2. Investigate the biological function of *PLZF* in CRPC development. (Months 13-24)

- a. Determine whether *PLZF*-depletion confers prostate cancer cell castration resistant growth *in vitro*. Perform cell proliferation assay in reduced medium (C-FBS) using transient RNAi silencing of PLZF in LNCaP (Months 13-14)
- b. Determine whether *PLZF*-depletion promotes CRPC tumor growth *in vivo*. Generate PLZF stable shRNA knockdown cells and conduct mouse xenograft studies. (Month 19-24)

What was accomplished under these goals?

Task 1. Identify *PLZF* transcription program mediating castration resistance in prostate cancer cells

To interrogate the biological consequences of the loss of *PLZF* expression, we performed a bioinformatic analysis of *PLZF* ChIP seq and *PLZF*-regulated gene expression profiling to define the *PLZF* transcriptional program (Figure 1A). Since AR exerts both growth-promoting and growth-suppressing functions in maintaining the equilibrium between cell differentiation and proliferation, we hypothesized that ADT designed to disrupt AR signaling may stimulate an AR-repressed oncogenic program through *PLZF* in adaption to ADT, contributing to the castration resistant phenotype [11]. Interestingly, we uncovered that the genes whose expression were up-regulated in *PLZF*-depleted cells were highly associated with the MAPK pathway, including 5 *PLZF* direct targets, *RRAS*, *MKNK2*, *DDIT3*, *JUND* and *JUN* (Figure 1B, C, and D), suggesting that *PLZF* might be regulating MAPK signaling pathway. Taken together, our data suggest that ADT results in down-regulation of *PLZF*, which, in turn, activates MAPK activity. These findings are consistent with the findings that MAPK signaling is up-regulated in CRPC patients and murine models [12, 13]. In summary, we propose an intrinsic resistance mechanism through *PLZF* down regulation or loss, wherein an AR-repressed oncogenic program allows residual prostate tumor cells to adapt to castrate levels of androgens.



Task 2. Investigate the biological function of *PLZF* in CRPC development.

We examined the growth of LNCaP cells using 2 additional shRNAs directed at *PLZF*. These shRNAs also induced androgen-independent growth in LNCaP cells; whereas non-specific silenced (shCtrl) cells did not grow, supporting the findings of the screen [9]. Interestingly, while androgen depletion alone (cultured in CSS) lowers *PLZF* expression, it is actually the further reduction of *PLZF* by the shRNAs that promotes growth. As a complementary approach, we also addressed whether *PLZF* has tumor suppressing effects on cell viability. Importantly, overexpression of *PLZF* resulted in a profound growth inhibitory effect on *PLZF*-depleted cells as early as at the first week (Figure 1C). It should be noted that the pictures of crystal violet (CV) staining were taken after 3 weeks of CSS incubation which allows sh*PLZF* (#7816)/plx-GFP colonies to accumulate. Taken

together, our *in vitro* experiments functionally show that PLZF is an androgen-responsive gene involved in growth suppression (Figure 2 A-D).

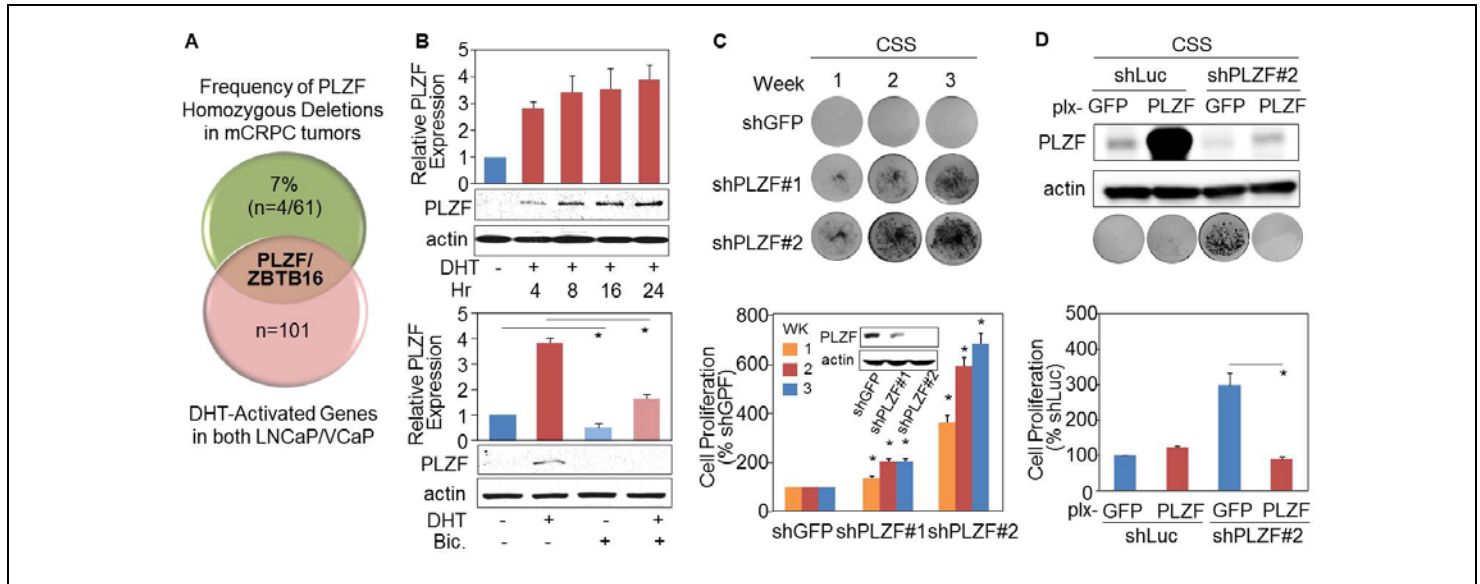


Figure 2: PLZF is an androgen-regulated gene involved in growth suppression

(A) Venn diagram showing the frequency (%) of PLZF homozygous deletions (n=homozygous deletions/total mCRPC tumors) (11) and PLZF as a putative tumor suppressor gene with strongest AR binding merged from two AR cistrome datasets. RT-qPCR and Western blotting were used to measure PLZF mRNA and protein expression of LNCaP cells which were cultured in charcoal-stripped serum (CSS), followed by (B) 10nM of DHT and/or 10μM of bicalutamide (Bic.) treatment. The colonies were stained by crystal violet (CV) and photographed. The efficiency and efficacy of (C) PLZF shRNA knockdown and (D) ectopic re-expression of PLZF was measured by Western blot. Each column was relative to the corresponding the first column and shown as mean ± SD (n ≥ 3), and *p < 0.05.

To block potential AR action, we used enzalutamide to examine the cell growth. Significantly, PLZF-depleted cells initially responded to enzalutamide but rapidly developed resistance, regardless of culturing conditions, indicating that LNCaP cells with PLZF inactivation is growth promoting even in the absence of androgen and further when blocked by enzalutamide as shown below (Figure 3 A-C).

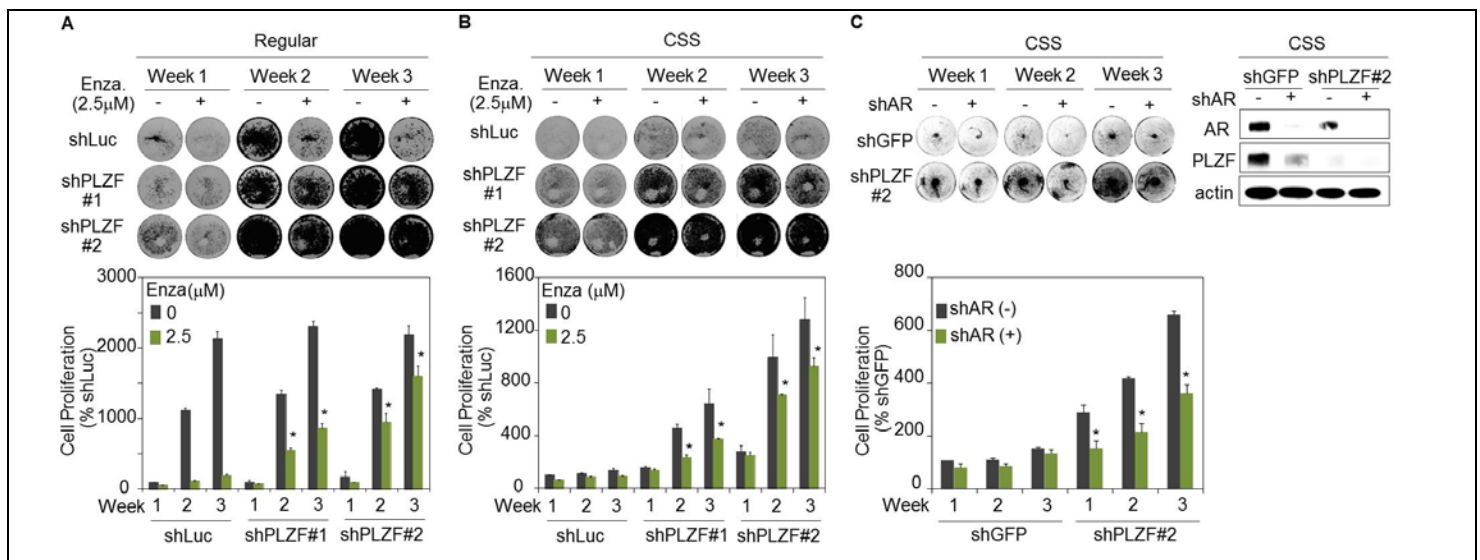


Figure 3: PLZF depletion alters the growth inhibitory effect of enzalutamide

LNCaP cells with or without PLZF knockdown were cultured in (A) 5% FBS or (B) CSS medium and treated with or without 2.5 μM of enzalutamide or (C) IPTG-inducible shAR knockdown, followed by CV staining at the time as indicated. Each column was relative to the corresponding the first column and shown as mean ± SD (n ≥ 3), and *p < 0.05.

To determine the requirement of PLZF loss for androgen-independent growth *in vivo*, we conducted a mouse xenograft study and showed that tumors with PLZF depletion had a substantial growth advantage as compared to the control (Ctrl) arm in castrated nude mice (Figure 4 A). To broaden our *in vitro* and *in vivo* findings, we investigated PLZF gene expression in different stages of human prostate tumors. The gene expression of PLZF is significantly decreased in mCRPC compared to its expression in primary prostate tumors (Figure 4 B), indicating that PLZF functions as a tumor suppressor in prostate cancer and its gene expression is decreased in mCRPC. Collectively, our *in vitro* and *in vivo* together with human genomic sequencing data strongly support that PLZF functions as a tumor suppressor in prostate cancer and demonstrate that PLZF inactivation as an important molecular event for CRPC development.

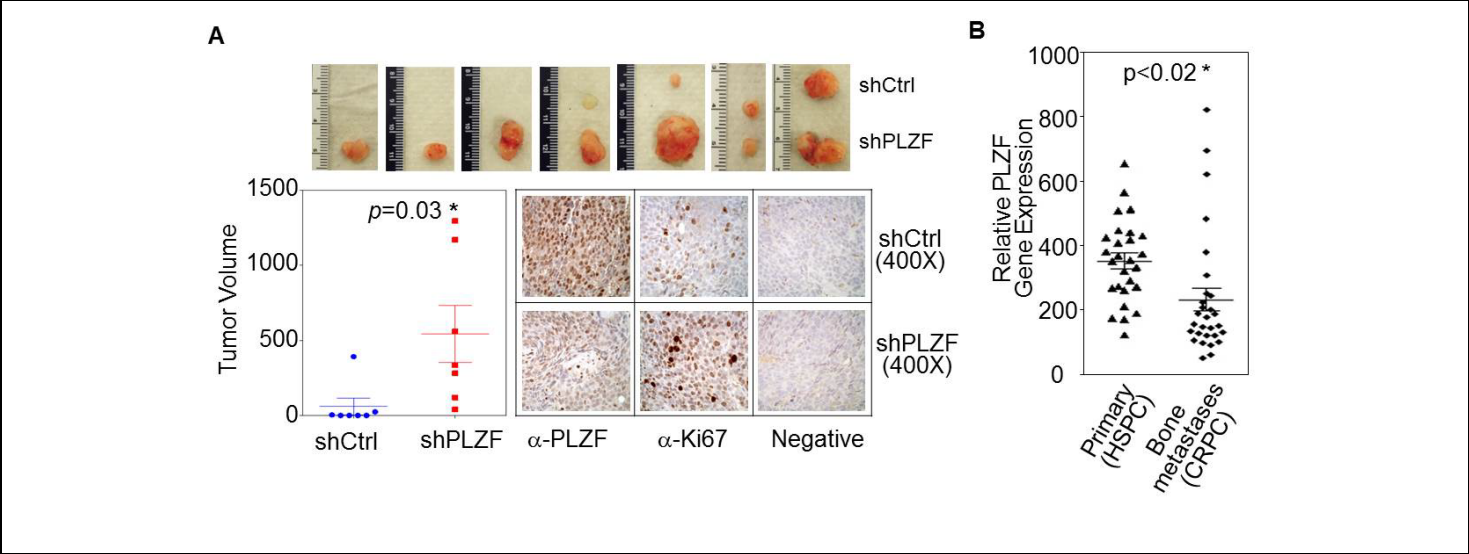


Figure 4: PLZF functions as a tumor suppressor *in vivo*
(A) Tumor formation assays of castrated male nude mice injected with shCtrl and PLZF stable silencing 22Rv1 cells. Bottom right: averaged xenograft tumors (mean±SEM); left: PLZF and Ki-67 immunohistochemistry (IHC) were used to monitor the efficacy of PLZF knockdown and cell proliferation in 22Rv1 xenografts. (B) PLZF gene expression from 27 hormone-sensitive prostate cancer (HSPC) and 29 bone metastatic castration-resistant prostate cancer (mCRPC).

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?

"Nothing to Report"

IMPACT:

Promyelocytic Zinc Finger Protein (PLZF) is genetically lost in 5-10% of tumors from metastatic castration resistant prostate cancer (mCRPC) patients who previously were treated with androgen deprivation therapy (ADT) and developed resistance. In addition, PLZF expression can be down-regulated by ADT, since PLZF is a well-known androgen receptor (AR)-stimulated gene. Our data demonstrated that prostate cancer cells in which

PLZF loss is mimicked by short hairpin RNA-mediated knockdown exhibited a CRPC and enzalutamide (a second-generation antiandrogen) resistant phenotype *in vitro* and *in vivo*. The hope is that this approach to define that patients with PLZF somatic deletions represent a distinct molecular subtype in genetically heterogeneous CRPC, serving as a potential predictive biomarker of drug response to AR-directed therapies, as well as providing a basis for clinical development of personalized therapy. As such, this proposal addresses the Overarching Challenge of “Developing effective treatments and addressing mechanisms of resistance for men with high-risk or metastatic prostate cancer”. In so doing, our proposal also addresses the Focus Areas of “Genetics”, “Mechanisms of Resistance” and “Therapy”.

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

Whole exome sequencing of metastatic castration resistant prostate tumors (mCRPC) from the Michigan cohort and the East Coast Stand Up to Cancer/Prostate Cancer Foundation (SU2C/PCF) study recently revealed that 5~10% of tumors harbor promyelocytic leukemia zinc finger (PLZF) focal homozygous deletions. This cohort of mCRPC patients who were previously treated with androgen deprivation therapy (ADT) developed castrate resistance. Intriguingly, from a “Genome-Wide RNAi Suppressor Screen”, aiming to uncover genes whose silencing were crucial for androgen-independent growth global RNAi screen, we found that PLZF was the only gene that was also induced by androgens, suggesting that PLZF is both androgen-responsive and growth suppressive and may be a part of an AR-repressed oncogenic program essential for developing resistance to ADT.

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:

1. Selected poster “Intensive androgen deprivation therapy compensatorily activates PLZF-repressed oncogenic circuitry that reprograms the residual prostate cancer “ for the AACR/PCF Conference: Advances in prostate cancer research (January, 2014, San Diego, CA)
2. AACR-Alfac, Incorporated Scholar-in-Training Award by AACR (January, 2014)

3. Invited short talk “Androgen deprivation therapy activates PLZF-repressed oncogenic circuitry that reprograms the residual prostate cancer” at 7th Annual Prostate Cancer Program Retreat (March, 2014, Fort Lauderdale, FL)
4. Selected poster “PLZF, a Tumor Suppressor Genetically Lost in Metastatic Castration Resistant Prostate Cancer, is a Mediator of Resistance to Androgen Deprivation Therapy “ for the AACR Conference: Translation to the Cancer Genome (February, 2015, San Francisco, CA)
5. Selected poster “PLZF, a Tumor Suppressor Genetically Lost in Metastatic Castration Resistant Prostate Cancer, is a Mediator of Resistance to Androgen Deprivation Therapy “ at 8th Annual Prostate Cancer Program Retreat (March, 2015, Fort Lauderdale, FL)
6. Publication in a peer-reviewed journal:
 - **Hsieh CL**, Teng Fei, Yiwen Chen, Tiantian Li, Yanfei Gao, Xiaodong Wang, Tong Sun, Christopher L. Sweeney, Gwo-Shu Mary Lee, X. Shirley Liu, Myles Brown and Philip W. Kantoff. Enhancer RNAs participate the androgen receptor-driven looping that selectively enhances gene activation. *Proc Natl Acad Sci U S A*. 2014 May 20;111(20):7319-24.
 - **Hsieh CL***, Botta G*, Gao S, Li T, Van Allen EM, Treacy DJ, Cai C, He HH, Sweeney CJ, Brown M, Balk SP, Nelson PS, Garraway LA and Kantoff PW. PLZF, a Tumor Suppressor Genetically Lost in Metastatic Castration Resistant Prostate Cancer, is a Mediator of Resistance to Androgen Deprivation Therapy. *Cancer Res*. 2015 May 15;75(10):1944-8

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

"Nothing to Report"

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"

APPENDICES:

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