AWARD NUMBER: W81XWH-18-1-0193

TITLE: Mechanisms and Therapeutic Targeting of NSD2 in Advanced Prostate Cancer

PRINCIPAL INVESTIGATOR: Alvaro Aytes

CONTRACTING ORGANIZATION: Bellvitge Institute for Biomedical Research (IDIBELL) Gran via de L'Hospitalet 199
08907 – Hospitalet de Llobregat
Barcelona - SPAIN

REPORT DATE: OCTOBER 2020

TYPE OF REPORT: ANNUAL

PREPARED FOR: U.S. Army Medical Research and Development Command

Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
OCTOBER 2020	Annual	30SEPT2019 - 29SEPT2020

4. TITLE AND SUBTITLE

Mechanisms and Therapeutic Targeting of NSD2 in Advanced Prostate Cancer

5a. CONTRACT NUMBER
W81XWH-18-1-0193

5b. GRANT NUMBER
W81XWH-18-1-0193

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Alvaro Aytes

E-Mail: aaytes@idibell.cat

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Bellvitge Institute for Biomedical Research (IDIBELL)
Gran via de L'Hospitalet 199
08907 – Hospitalet de Llobregat
Barcelona - SPAIN

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

This project aims at elucidating the causal role of Nsd2 in in aggressive prostate cancer progression and its contribution to lineage plasticity, AR cistrome remodeling and anti-AR treatment resistance. It also wants to explore the potential use of Nsd2 as a therapeutic target and response biomarker. To do so a series of objectives and tasks were defined in the Statement of Work (see below) and this 2nd year progress report will summarize de accomplishments and reached milestones as well as the deviations from the original methodology and the justification for such changes.

15. SUBJECT TERMS

NONE LISTED

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
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Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Specific Aim 1: To investigate the causal role of NSD2 in aggressive prostate cancer lineage plasticity.			
Major Task 1: Preclinical assays with Enzalutamide in NPp53N TKO mice.	Months	Responsible	Completion date and %
Subtask 1: Obtaining approval from the USAMRMC ORP HRPO and the USAMRMC ORP Animal Care and Use Review Office (ACURO) for the use of human anatomical substances and the use of animals for research	1-3	PI	06/2019 (100%)
Subtask 2: Setting up matings, genotyping and enrolling mice in tamoxifen for tumor induction. Mice will be enrolled as litters come to guarantee that data starts as soon as possible. We will continue to enroll mice until we reach the adecuate N=20 per arm as determined by power analysis.	4-12	Postdoc/ technician	10/2019 (100%)
Subtask 3: Castration and randomization to treatment arms. Mice will be assigned at treatment arms at random within their group as tumor bearing mice reach the pre-established age	4-12	Postdoc/ technician	10/2020 (75%)
Subtask 4: Sacrifice and necropsy and tissue processing.	6-12	Postdoc/ technician	10/2020 (75%)
Subtask 5: Phenotypic and molecular characterization: will be processed for RNAseq and subsequent bioinformatics analysis to elidate differentially expressed genes as well as to infer regulatory programs distinctly activated using our human and mouse interactomes as we´ve done before. Tissue specimens from primary prostate tumors and metastasis will be stained for prostate specific markers (AR, Nkx3.1), lineage markers (CK8, CK5), neuroendocrine markers (Synapthophysin) and pluripotency markers (Sox2, Oct4) as well as for Nsd2 and Ezh2 to fully characterize the phenotype.	6-9	Postdoc	10/2020 (75%)
Milestone(s) Achieved: *Preclinical characterization of the NPp53 and NPp53N mice upon enzalutamide treatment.*	12	Postdoc/PI	10/2020 (80%)
Major Task 2: Functional validation of the dependency for NSD2.	Months	Responsible	Completion date and %
Subtask 1: Establish organoids from NP and NPp53 mice	3-6	Postdoc	10/2020 (75%)
Subtask 2: Produce CRISPRi/CRISPRa Nsd2, Ezh2 and Sox2 virues and transduce and select primary cultured cells.	1-6	Postdoc	10/2020 (100%)
Subtask 3: Perform functional assays, gather data and interpret results. Self-renewal capacity in normal and charcoal-stripped serum will be assessed by serial passaging after single cell suspension. Organoids will also be fixed and embedded to characterize the differentiation state as a result of gene manipulation as described above. For the most stringent functional assays, organoids will be engrafted back into recipient immunocompromised mice to assess the cross-talk between Nsd2, Ezh2 and Sox2 in vivo	6-12	Postdoc/PI	10/2020 (25%)

Milestone(s) Achieved: *To have defined the causal role of Nsd2 in NEPC as well as the hierarchical relationship between Nsd2, Ezh2 and the pluripotency transcription factor Sox2*	12	Posdoc/PI	10/2020 (50%)
Specific Aim 2: To investigate the changes in chromatin accessibility and AR interactome			
Major task 1: ATACseq on Enzalutamide treated DKO and TKO tumor mice	Months	Responsible	Completion date and %
Subtask 1: FACS isolate cells from DKO and TKO mice	10-14	Postdoc	10/2019 (100%)
Subtask 2: Prepare ATACseq libraries and perform deep sequencing	14-15	Postdoc	10/2019 (100%)
Subtask 3: Computational analysis. Define the differentially accessible chromatin regions. Next we will compared to our RNAseq data generated in Aim 1 and publicly available to predict transcriptional programs and master regulators that are activated or repressed as a result of the changes in chromatin remodeling	15-20	Posdoc/PI	10/2020 (50%)
Milestone(s) Achieved: *Generate the first chromatin accessibility map for enzalutamide treated GEM prostate cancer models*	20	Posdoc/PI	10/2020 (75%)
Major Task 2: RIME to define the AR interactome in Nsd2 wild type and null PCa cells	Months	Responsible	Completion date and %
Subtask 1: AR antibody optimization and pull-down validation on DKO and TKO primary cultures	18-22	Postdoc	10/2019 (100%)
Subtask 2: Mass spectrometry and computational analysis to generate the AR-protein interactome as externalized service.	22-24	N/A	10/2019 (100%)
Subtask 3: ChIPseq for AR on the same primary cultures to be able to infer how changes in the AR interactome induced by Nsd2 affect the AR cistrome	20-24	Postdoc	10/2020 (10%)
Milestone(s) Achieved*:* Provide an accurate picture regarding how Nsd2 interaction with AR shapes the AR cistrome and what are the co-factors implicated	24	Posdoc/PI	10/2020 (50%)
Specific Aim 3: Preclinical validation of Nsd2 as a therapeutic target in NEPC			
Major task 1: Investigate Nsd2 as potential biomarker in aggressive PCa.	Months	Responsible	Completion date and %
Subtask 1: Staining of human prostate cancer specimens with Nsd2, AR, ki-67 and Synaptophysin/Chromogranin	23-25	Immune scoring	10/2020 (100%)
Subtask 2: Immune scoring for all markers in all speciments done blinded by two independent pathologists	24-27	PI/ collaborators	10/2020 (50%%)
Subtask 3: Statistical analysis. Univariate and multivariate analysis using the Cox proportional hazard-ration model as well as c-Statistics will be employed and survival analysis will be carried out using the follow up data from the different cohorts	26-30	Posdoc/PI	10/2020 (25%)
Milestone(s) Achieved: *Assessment of the utility of Nsd2 as prognostic/ predictive biomarker for prostate cancer patients*	30	Posdoc/PI/ collaborators	10/2020 (50%)
Major task 2: To carry on preclinical combination treatments in vivo	Months	Responsible	Completion date and %
Subtask 1: Set up cohorts of allografted or GEM DKO mice as well as PDX mice. Induced tumors with tamoxifen or implant allografts/xenografts as required and randomize to the different treatments arms, namely castration, MCTP-39, castration+ Enzalutamide, Castration+MCTP-39 and Castration+Enzalutamide+MCTP39	28-31	Postdoc/ technician	NA (0%)
Subtask 2: Preclinical assay in the “Short-term cohort” as described in the narrative annex, section D	30-32	Postdoc/ technician	NA (0%)

Subtask 3: Preclinical assay in the "Long-term cohort" as described in the narrative annex, section D	29-36	Postdoc/ technician	NA (0%)
Milestone(s) Achieved: *Assessment of whether pharmacological inhibition of Nsd2 with small molecule inhibitors has potential clinical implications for the treatment of anti-AR resistant CRPC and/or NEPC patients*	36	Postdoc/ PI	NA (0%)

3.2 What was accomplished under these goals?

(See in the table above the accomplished subtasks, the accomplishment date and percent)

- Preclinical evaluation of anti-AR Enzalutamide in the TKO model. (Major Task 1; Subtask 3)
  As mentioned in the previous report, this task was delayed to to the low recombination rate of the NSd2 allele and the need for an alternative approach to establish the TKO model. We have now finalized a randomized preclinical assay testing the efficacy of three different anti-AR drugs, namely Enzalutamide, Abiraterone Acetate and Apalutamide. Importantly, deletion of Nsd2 significantly sensitizes these prostate cancer models to AR signaling inhibitors. Pending is the cohort for castrated counter parts that have longer latencies in tumor growth.
- Necropsy and tissue processing (Major Task 1; Subtask 4)
  Full necropsies and tissue processing have been performed for the above-mentioned preclinical assays
- Phenotypic and molecular characterization (Major Task 1; Subtask 5)
  Pathological characterization is ongoing. IHC and multiplex IF are optimized for AR, Nkx3.1, lineage markers (CK8, CK5), neuroendocrine markers (Synapthophysin) and pluripotency markers (Sox2, Oct4) as well as for Nsd2 and Ezh2. Interestingly, preliminary data suggest that Chromogranin is a better marker for neuroendocrine differentiation than synaptophysin in this model and the analisis of the RNAseq and ATACseq data suggest that SOX11 might have a more prominent role in Nsd2 driven PCa tumors in this model.
- Organoid models from the TKO and DKO models are developed in collaboration with the Kruithoff-de Julio laboratory at University of Bern (Aim 1 Major task 2, subtask 1). Procedures have been optimized to knockout Ezh2 or Sox11 in these organoid models and the functional impact of such manipulation is underway (Aim 1 Major task 2, subtask 2). The implantation of these genetically engineered organoids in tumnor growth assayts in vivo is pending ((Aim 1 Major task 2, subtask 3)
- Androgen Receptor ChIp seq continues to prove challenging. Despite good quality ChIP-qPCR results have been obtained, subsequent library preparation and sequencing has not yet passed QC requierements. We continue optimizing this procedures and in parallel we are engaging in collaboration with experts in the field to troubleshoot this. (Aim 2, Major task 2, subtask 3)
- The EMPaCT TMa has been stained for Nsd2, AR, ki-67 so far and for candidate regulators BAF155, BAF170 and BRG1 of the SWI/SNF complex and the analysis for correlations with clinicopathological features and patient outcome is ongoing (Aim 3, Major task 1 subtask 1, 2 and 3) and the statistical anslyis of the pathological characterization of the preclinical assays in Aims 1 and 2 and in

3.3 What opportunities for training and professional development has the project provided?

Nothing to report

3.4 How were the results disseminated to communities of interest?

Nothing to report

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

- The Focus of the next reporting period will be on finalizing the experimental work. We will finalize the preclinical assays including the randomization and AR targeting of the castrated TKO and DKo models (Aim1, Majorn task 1, subtask 3) as well as the assessment of the potential clinical benefit of anti-AR ansd MCTP39 treatment (Aim 3, major task 2).
- Another major focus will be in finalizing the characterization of the AR cistrome by ChIPseq (Aim 2, Major task 2, Subtask 3).
- Regarding the final analysis of the gathered data throughout the different aims, we will finalize the ATACseq and RIME data analysis (Aim2, major task 1 and Aim 2 major task 2)

4. Impact

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

Nothing to report

4.2 What was the impact on other disciplines?
Nothing to report

4.3 What was the impact on technology transfer?
Nothing to report

4.4 What was the impact on society beyond science and technology?
Nothing to report

5. Changes/Problems
5.1 Changes in approach and reasons for change
Nothing to report

5.2 Actual or anticipated problems or delays and actions or plans to resolve them.
Nothing to report

5.3 Changes that had a significant impact on expenditures
Dr. Rana El Bizri was hired as a postdoctoral research scientist funded under this project. She has recently been awarded a postdoctoral fellowship and so Personnel expenditures have been adjusted by

6. Products
Nothing to report

7. Participants & Other Collaborating Organizations
7.1 What individuals have worked on the project?

Name	Rana El Biizri
Project role	postdoc
Researcher Identifier	NA
Nearest person month worked	6
Contribution	Carried out preclinical work in vivo and in vitro. Has carried out the preclinical assays and assessment of the clinical impact off Nsd2 as a biomarker in human PCa specimens
Funding Support	This award

Name	Adrian Martinez Tebar
Project role	Predoc
Researcher Identifier	NA
Nearest person month worked	5
Contribution	Carried out preclinical work in vivo and in vitro. Has carried out the optimization of the ChIPseq and studies
Funding Support	Other funds

Name	Sonia Ferran-matas
Project role	Predoc
Researcher Identifier	NA
Nearest person month worked	1
Contribution	Data analysis
Funding Support	This award

7.2 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

7.3 What other organizations were involved as partners?

Nothing to report

8. **Special Reporting Requirements**

9. **Appendices**