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CONTRACTING ORGANIZATION:
Catalan Institute of Oncology

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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 1st 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

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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 pree-established age	4-12	Postdoc/ technician	10/2019 (50%)
Subtask 4: Sacrifice and necropsy and tissue processing.	6-12	Postdoc/ technician	10/2019 (50%)
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	7/2019 (50%)
Milestone(s) Achieved: *Preclinical characterization of the NPp53 and NPp53N mice upon enzalutamide treatment.*	12	Postdoc/PI	10/2019 (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	NA (0%)
Subtask 2: Produce CRISPRi/CRISPRa Nsd2, Ezh2 and Sox2 virues and transduce and select primary cultured cells.	1-6	Postdoc	5/2019 (25%)
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	10/2019 (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	10/2019 (25%)
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	NA
Milestone(s) Achieved: *Generate the first chromatin accessibility map for enzalutamide treated GEM prostate cancer models*	20	Posdoc/PI	10/2019 (50%)
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	10/2019 (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	10/2019 (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	NA (0%)
Subtask 2: Immune scoring for all markers in all speciments done blinded by two independent pathologists	24-27	PI/ collaborators	NA (0%)
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	NA (0%)
Milestone(s) Achieved: *Assessment of the utility of Nsd2 as prognostic/ predictive biomarker for prostate cancer patients*	30	Posdoc/PI/ collaborators	NA (0%)
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)

- Establishment of the DKO and TKO cohorts (Subtask 2, Major Task 1, Aim1). Crossing of the DKO mice with the Nsd2$^{floxed/floxed}$ mice to generate the TKO cohort was accomplished as planned
- Castration and randomization to treatment arms (Subtask 3, Major Task 1, Aim1): This subtask has been slightly delayed due to inefficient deletion of the Nsd2 allele in the TKO mice, which made us established an alternative approach to obtain TKO mouse models (see section 5 for details). We have so far established pilot experiments for castrated and anti-AR treatment in this new models. Briefly, castrated TKO mice show significant enhanced response to anti-AR treatment in vivo (Fig 1A) and in vitro (Fig 1B).

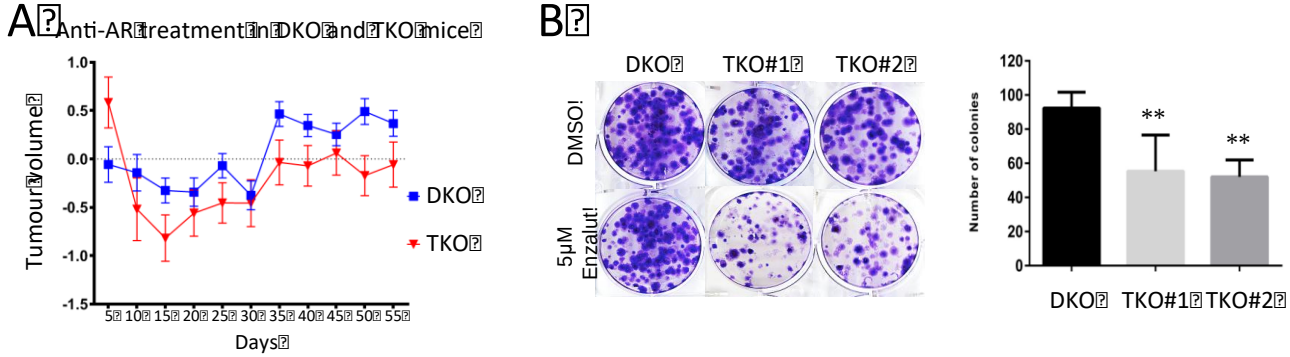

Figure 1. Role of Nsd2 in response to AR inhibition. **(A)** DKO and TKO mice (N=20) were treated with Abiraterone Acetate for the indicated time and tumor growth measured using MRI. **(B)** Primary cultures of DKO and TKO mice were treated with Enzalutamide at the indicated concentration and durg sensitivity assessed by clonogenic assays.

- Regarding Subtask 5, Major Task 1, Aim 1, the phenotype characterization of this new mice as also been delayed and is being currently carried out. We have however successfully carried out the molecular characterization as planned, which included transcriptome analysis from the control and treated DKO and TKO mice. Importantly, data shows that while anti-AR treatment with in the partially silences the AR cistrome defined in our previously published prostate cancer interactome, and that deletion of Nsd2 in the TKO model enhances AR cistrome downregulation (Fig 2A-B).

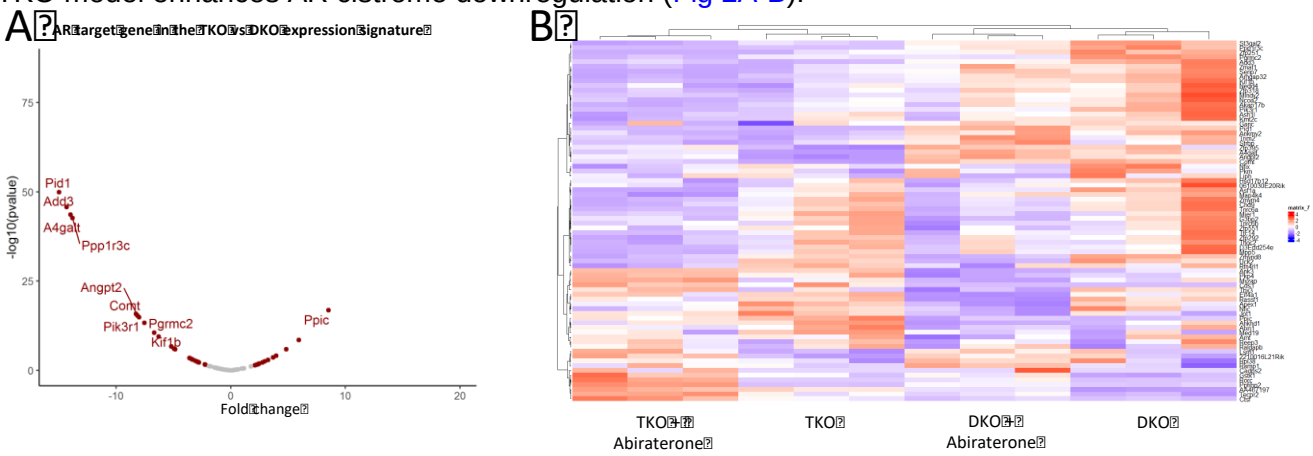

Figure 2. Transcriptome analysis of AR inhibition in DKO and TKO tumors. RNAseq was performed in vehicle or Enzalutamide treated DKO and TKO mice. **(A)** Volcano plot showing AR cistrome fold change between DKO and TKO mice. Note that there is a shift towards downregulation of AR targets in TKO tumors. **(B)** Heatmap representing the expression levels of the AR regulon in vehicle or Enzalutamide treated DKO and TKO tumors. Note that the combonation of Nsd2 KO and Enzalutamide entirely shifts gene expression while this reversion is only partially achieved with either Enzalutamide or Nsd2 alone.

- Establishment of DKO and TKO derived organoids (Subtask 1, Major Task 2, Aim1) has not been achieved yet in part due to the delayed caused by the inefficient deletion of Nsd2 in the floxed allele. As such this task will be carried out during the next reporting period. We have however established primary cultures from DKO and TKO models and partially carry out the functional validation tasks detailed in Subtask 2 and 3, Major Task 2, Aim 1. In particular, TKO primary cultures display significantly increased sensitivity to Enzalutamide and Abiraterone compared to DKO models measured in proliferation (Fig. 3A) and gene reporter assays (Fig 3B).

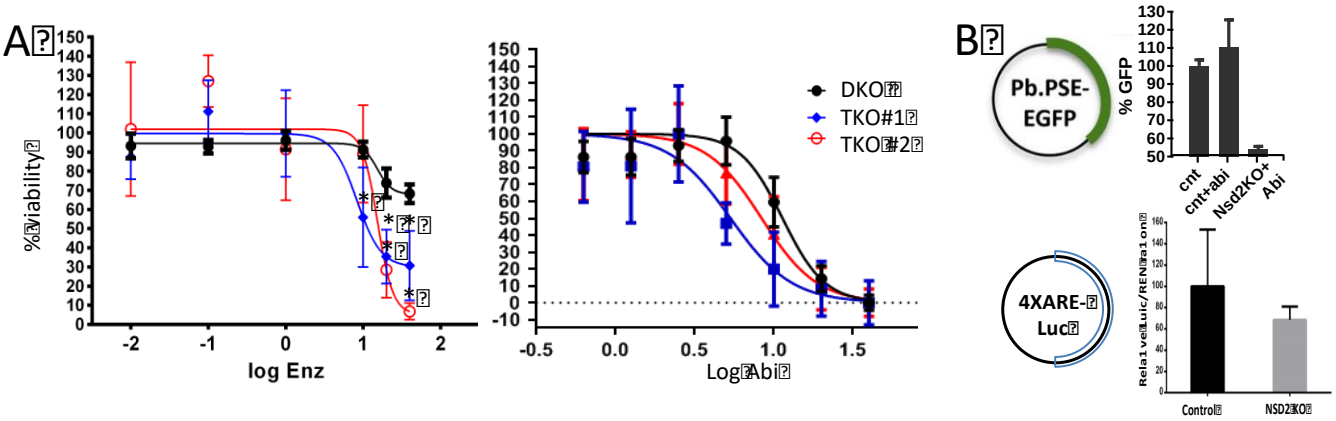

Figure 3 Nsd2 silencing sensitizes PCa cells to AR signaling inhibition. (A) Primary cultures from DKO or TKO tumors were subjected to Enzalutamide (left) or Abiraterone (right) treatment and the dose response assessed by MTT assays. In bith cases, silencing of Nsd2 sensitizes PCa cells to anti-AR treatment. **(B)** Gene reporter assays using a Probasin-Prostate Specific Enhancer promoter to express a GFP construct (Top) or a 4X Androgen Responsive Element to express a luciferase construct (bottom) demonstrate a decrese in AR transcriptional activity in Abiraterone treated cells upon Nsd3 deletion.

- The unexpected delayed in some of the taks assigned to this first reporting period as a result of problems encountered with the Nsd2$^{floxed/floxed}$ allele has on the other hand allowed faster progress in tasks mostly assigned to the 2nd reporting period. In particular, ATACseq in anti-AR treated DKO and TKO models (Subtask 1 and 2, Major Task 1, Aim 2) has been carried out and the results are currently being analyzed. Preliminary data clearly shows a pattern of differential chromatin accessibility in DKO compared to TKO PCa models (Fig. 4A) and a good correlation between chromatin accessibility and gene expression (Fig. 4B). We have also finalized the RIME experiments (Subtask 1 and 2, Major Tak 2, Aim 2). The results of the mass spectrometry analysis on the AR interactome on DKO compared to TKO PCa models shows an overrepresentation of AR-bound peptides related to subunits of the SWI/SNF chromatin remodeling complex (Fig. 4C) which will be further validated during the next reporting period. In addition, ChIPseq experiments have been initiated to assess the contribution of Nsd2 to AR cistrome remodeling (Subtask 3, Major Tak 2, Aim 2). So far, ChIP-qPCR has been used to initially assessed whether AR binding to canonical AR target gene promoters is affected by Nsd2. Data indicates that Nsd2 deletion results in a significant enrichment of AR binding to the TMPRSS2 and AR promoters (Fig. 4D), hence suggesting that Nsd2 in fact alters the AR cistrome, which will be confirmed once the ChIPseq data is analyzed during the next reporting period.

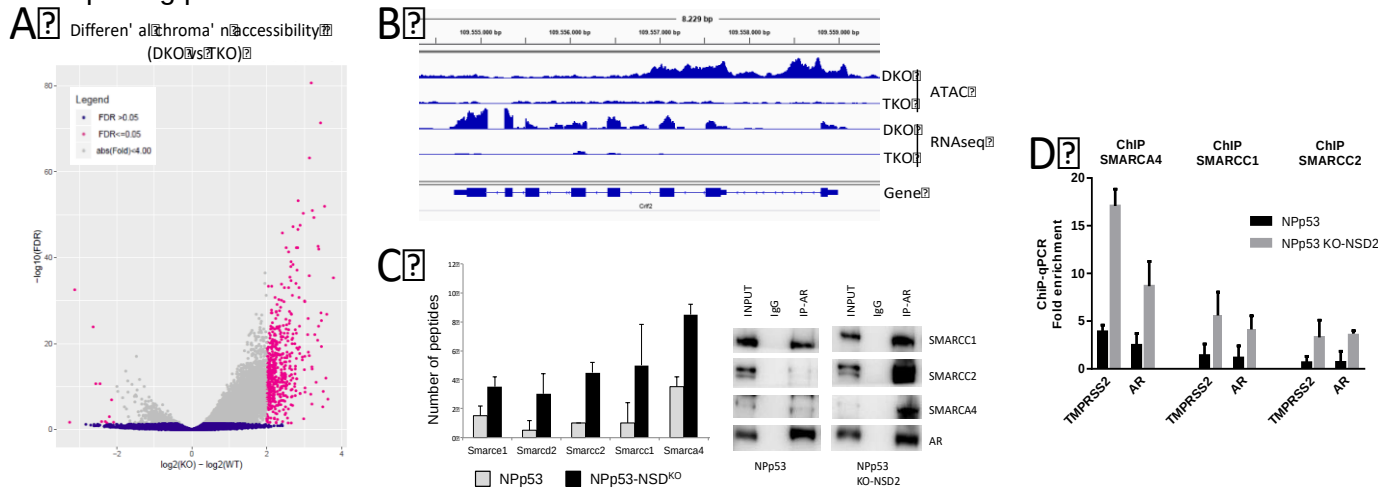

Figure 4. Role of Nsd2 in chromatin accessibility and AR interactome. (A) Differentially accessible chromatin loci in DKO vs TKO tumors. **(B)** Example of a genomic locus comparing integratin chromatin accessibility by ATACseq with gene expression by RNAseq. **(C)** Enrichment of SWI/SNF subunits peptides in the Mass Spectometry analyisis (RIME) comparing DKO vs TKO (left) and co-IP with AR Ab to show enrichment of the indicated SWI/SNF subunits in the TKO tumors. **(D)** ChIP-qPCR assays validating the enrichment of AR bound to AR and TMPRSS2 promoters in the TKO compared to DKO tumors.

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?
As detailed above, about 50% of the work planned for months 12 to 24, the 2nd reporting period, has been carried out during the 1st reporting period. This will allow carrying out the delayed tasks from the 1st reporting period described above. In particular, we are now ready to finalize the preclinical assessment of the response to anti-AR treatment in the TKO vs DKO mice described in Subtask 3, Major Task 1, Aim 1. We will also finalize the establishment of DKO and TKO derived organoids (Subtask 1, Major Task 2, Aim 1) and the gain and loss of function studies with Nsd2, Ezh2 and Sox2 (Subtask 2 and 3, Major Task 2, Aim 1).
On the other hand substantial efforts during the next reporting period will be dedicated to finalize the analyses f the ATACseq (Subtask 1 and 2, Major Task 1, Aim 2) and ChIPseq data (Subtask 3, Major Task 2, Aim 2) to determined the contribution of Nsd2 to AR cistrome remodeling, which is the objective of Aim 2, scheduled for year 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.
As mentioned in section 3.2., we unexpectedly encounter a problem in the efficiency of Nsd2 deletion in the $Nsd2^{floxed/floxed}$ allele. In particular, upon tamoxifen administration to the DKO and TKO mice to induce recombination and deletion of the $Pten^{floxed/floxed}$, $p53^{floxed/floxed}$ and $Nsd2^{floxed/floxed}$ alleles, we were unable to detect a significant change in the expression levels of Nsd2, even in YFP FACS sorted cells (thanks to the R26-YFP reporter allele also crossed in these mice), which indicated that the Nsd2 allele was not properly deleted. To overcome this, we established a protocol for CRISPR/cas9-induced Nsd2 deletion ex vivo in DKO cells to generate TKO prostate tumors and engraftment in syngenic mice. This has proven successful and we are currently using this new TKO models to accomplish the objectives and goals associated to the use of the DKO and TKO mice. As mentioned in section 3.5., the tasks and subtasks affected by this unexpected delay are in progress and will be accomplished during the next reporting period. To ensure that this is feasible, some of the tasks scheduled to year 2 (next reporting period) have already been accomplished (see section 3.2 and 3.5).

5.3. Changes that had a significant impact on expenditures
By the time of the Grant Agreement and first installment was paid, my postdoc Katia Ruggero had obtained alternative funding from the European Association of Urology. Hence a search for a new postdoc was initiated and finally in April 2019 Dr. Rana El Bizri was hired as a postdoctoral research scientist funded under this project. While this has not impact the progress on the project it resulted in a delay in the expenditure of personnel costs.

6. Products

Nothing to report

7. Participants & Other Collaborating Organizations

7.1. What individuals have worked on the project?

Name	Katia Ruggero
Project role	Postdoc
Researcher Identifier	NA
Nearest person month worked	12
Contribution	Carried out ex vivo work, including primary cultures, CRISPR silencing, ATACseq, RIME and ChIPseq assays as well as in vitro drug treatment
Funding Support	European Association of Urology

Name	Xieng Wan Chen
Project role	Technician
Researcher Identifier	NA
Nearest person month worked	6
Contribution	Carried out all the mouse work including breeding, colony maintenance, genotyping as well as necropsy and tissue processing
Funding Support	Dr. Aytes start up funds

Name	Rana El Biizri
Project role	postdoc
Researcher Identifier	NA
Nearest person month worked	5
Contribution	Carried out preclinical work in vivo and in vitro.
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