AWARD NUMBER: W81XWH-21-1-0231

TITLE: The Therapeutic Role of lncRNA-Mediated DNA Repair in Lung Cancer

PRINCIPAL INVESTIGATOR: Marconett, Crystal N.

CONTRACTING ORGANIZATION: University of Southern California, Los Angeles, CA

REPORT DATE: June 2022

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE
June 2022

2. REPORT TYPE
Annual

3. DATES COVERED
15May2021-14May2022

4. TITLE AND SUBTITLE
The Therapeutic Role of lncRNA-Mediated DNA Repair in Lung Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-21-1-0231

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Marconett, CN; Correa, Michele Ramos; Mihalakakos, Evan; Castillo Jonathan.

E-Mail:cmarcone@usc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Southern California
1441 Eastlake Ave
NTT 6418A
Los Angeles, CA 90089

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
The purpose of the proposal was to determine the role LINC00261 plays in mediating cellular response to DNA damage in lung cancer. Scope of work was designed to test the mechanistic role of LINC00261 in effecting DNA repair through NER pathway, the effect LINC00261 has on total mutational burden and subsequent presentation of neoantigens, and whether the presence of LINC00261 affects sensitivity of lung cancer to small molecule therapeutics targeting DNA repair (olaparib) or activity of pembrolizumab (anti-PD-L1) to optimize application of neoadjuvant therapy. Interim results for Aim 2 indicate that LINC00261 expression levels are elevated under interferon gamma-mediated immune stimulation, and that the presence of LINC00261 enhances activation of MHC Class II presentation markers; indicating that LINC00261 may serve as a surrogate for high neoantigen load and a complementary biomarker to PD-L1 expression for effective immunotherapy. Less progress has been made on Aims 1 and 3; Aim 1 progress demonstrated that the S1m strategy for identifying LINC00261 binding partners has failed. Approved alt. strategies are being pursued. Aim 3 regulatory approvals are obtained.

15. SUBJECT TERMS None listed.

16. SECURITY CLASSIFICATION OF: UNCLASSIFIED			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRDC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified	14	

TABLE OF CONTENTS

	Page
1. Introduction	4
2. Keywords	5
3. Accomplishments	6-9
4. Impact	10
5. Changes/Problems	10
6. Products	11
7. Participants & Other Collaborating Organizations	12
8. Special Reporting Requirements	14
9. Appendices	NONE

INTRODUCTION:

The purpose of the proposal was to determine the role LINC00261 plays in mediating cellular response to DNA damage in lung cancer. Scope of work was designed to test the mechanistic role of LINC00261 in effecting DNA repair through NER pathway, the effect LINC00261 has on total mutational burden and subsequent presentation of neoantigens, and whether the presence of LINC00261 affects sensitivity of lung cancer to small molecule therapeutics targeting DNA repair (olaparib) or activity of pembrolizumab (anti-PD-L1) to optimize application of neoadjuvant therapy. Significant progress has been made in Aim 2, which indicate that LINC00261 expression levels are elevated under interferon gamma-mediated immune stimulation, and that the presence of LINC00261 enhances activation of MHC Class II presentation markers; indicating that LINC00261 may serve as a surrogate for high neoantigen load and a complementary biomarker to PD-L1 expression for effective immunotherapy. Analysis of large-scale data indicates that LINC00261 is associated with total mutational burden across a variety of cancer and conditions; and is associated with genomic instability. Less progress has been made on Aims 1 and 3; Aim 1 progress has demonstrated that the S1m strategy for identifying LINC00261 binding partners yielded negative results and no identifiable binding partners. Approved alternative strategies are being pursued but have not yet yielded significant results. In discussion with Co-Investigators we are planning to obtain approval for alternative strategy (ChIRPseq) that utilizes the same technology as the approved plan (biotin/strepdavidin binding), but has direct biotin binding instead of the failed S1m moiety. Aim 1 immunoflourescence assays have begun, we have optimized UV exposure to induce DNA damage breaks and are currently determining LINC00261 localization patterns upon UV exposure. We have also ordered XPA and XPC shRNA knockdown constructs and are optimizing the amount of doxycycline needed to induce activation and/or repression of endogenous LINC00261. An additional issue has arisen with TRE-dCas9-VPR in that optimal DOX needed to induce dCas9 expression was unable to activate LINC00261. We believe this is due to previously described epigenetic repression at the LINC00261 locus and found that 5-aza-CdR can alleviate this problem. Aim 3 regulatory approvals are obtained, mouse work has not yet started pending completion of the in vitro pembrolizumab experiments.

KEYWORDS:
Lung Cancer
Long non-coding RNAs
DNA damage repair
CRISPR-mediated gene manipulation
Epigenetic toggle switch
Tumor mutational burden
Neoantigen presentation
Neoadjuvant therapy
LINC00261

ACCOMPLISHMENTS:

MAJOR GOALS AND PROGRESS DURING THE PERFORMANCE PERIOD
:

MAJOR GOALS OF THE PROJECT	TARGET COMPLETION DATE	COMPLETION DATE	% COMPLETE
1. Epistasis analysis NER (XPA/XPC) +/- LINC00261	10/1/2022 (18 months)	NA	30%
2. LINC00261 colocalization w/ NER cofactors	5/14/2023 (24 months)	NA	10%
3. Binding partners using S1m adapter a. *Negative result: Alternative strategy devised, needs approval to proceed*	5/14/2022 (12 months)	3/15/2022	100%
4. LINC00261 connection to MHC Class II factor expression	2/15/2022 (9 months)	11/30/2021	100%
5. In vitro efficacy of DDR and anti-PD-L1 +/- LINC00261	5/14/2022 (12 months)	NA	50%
6. In vivo testing of olaparib & pembrolizumab +/- LINC00261	5/14/2023	NA	10% (ACURO approval)

Task 1: Epistasis NER+/- LINC

We aimed to determine if LINC00261 interacted with NER factors to affect DNA fidelity. To do so, stable doxycycline-inducible H522 TRE-*LINC00261*-VPR cell line utilizing the CRISPR-dCas9 system was created but it was a polyclonal system so we FACS sorted for single cells to create monoclonal lines for consistent dcas9 activity and subsequent *LINC00261* expression for future experiments:

Fig 1: dCas9 stable integration of H522 TRE-*LINC00261*-VPR monoclonal cell lines

Genomic DNA was extracted from stably transfected H522 TRE-*LINC00261*-VPR monoclonal cell lines: H9, D11, A2, and C1 and was assessed for the presence of dCas9; the H522 polyclonal cell line served as a positive control for dCas9 expression and H522 was a negative control; dCas9 expected bp size: 241 bp

H522 TRE-*LINC00261*-VPR monoclonal cell lines: H9 and D11 were induced with a doxycycline range of 100 and 1000 ng/ml for 48 hours to check for dCas9 expression and subsequent *LINC00261* expression (inconclusive). Fold change expression of dCas9 is relative to GAPDH.
We found that 100 ng/ml was a sufficient dose to get induction of dCas9, however, the expression of *LINC00261* remained the same.

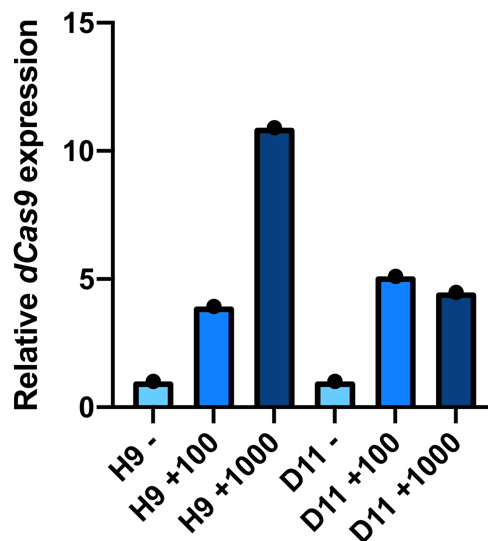
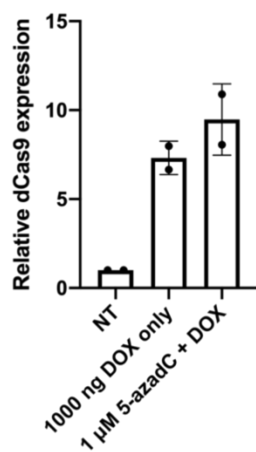


Fig 2: dCas9 doxycycline-inducible H522 TRE-*LINC00261*-VPR monoclonal expression

We checked the most consistent H9 monoclonal line for dCas9 efficiency of transcription as well as demethylating the *LINC00261* region to allow euchromatin formation for the efficient VPR function as *LINC00261* expression is regulated by DNA methylation.



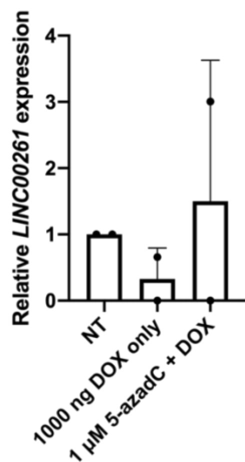


Fig 3: qPCR of treated monoclonal H9 with doxycycline to induce dCas9-VPR expression and a combination of doxycycline and 5-AZA-2-deoxycytidine, a DNA demethylating drug, to

Task 2: Colocalization NER with LINC

We also set out to determine if LINC00261 allowed NER to localize to sites of DNA damage and NER via RNA FISH. Pilot RNA FISH conducted on hAEC-FT-C7 and A549 cell lines (low and endogenous levels of *LINC00261*) in the presence and absence of UV light damage were used as planned. MALAT1 was used as a positive control for nuclear localization, and LINC00261 specific probes. methanol was used to decrease background fluorescence. We found that MeOH fixation over 48 hours had reduced background autoflourescence. Preliminary results indicate we can visualize LINC00261 in nuclear and cytoplasmic fractions with reduced background. No progress yet on UV damage conditions or co-staining with known NER members..

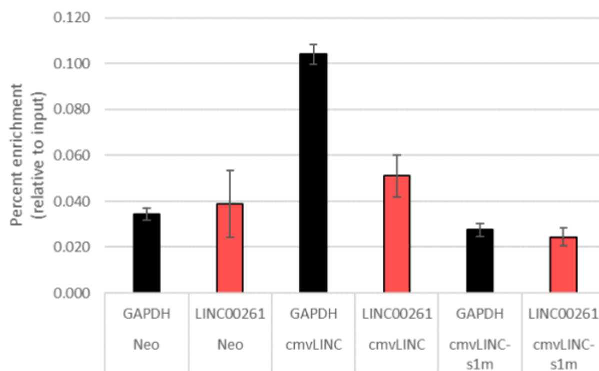


Figure 4: RNA eluted and isolated from streptavidin beads via Trizol, measured via qPCR. GAPDH primers were used to measure non-specific RNA binding.

Task 3: S1m-binding

In order to identify proteins, bound to *LINC00261*, we performed column purification where *LINC00261* is bound to a streptavidin matrix via an S1m RNA inserted onto the 3' end of the *LINC00261*. The S1m aptamer, once transcribed, folds into a secondary structure that can bind to streptavidin. This allows for affinity purification of tagged RNA, where interacting proteins can separate and identified.

We utilized the LUAD cell line, H522 tested whether the *LINC00261*-S1m transcript from the lysate could successfully bind to the streptavidin beads. Followed by incubation, beads were subjected to Trizol reagent to isolate bound RNA. qPCR was performed on the isolated RNA to measure the amount of *LINC00261* RNA enriched from the pulldown, compared to input. GAPDH primers were used to measure amount of non-specific RNA bound to beads. Figure 1 shows that the S1m aptamer failed to bind onto the streptavidin beads in a meaningful amount. The S1m-tagged *LINC00261* failed to enrich at a greater amount compared to non-specific RNA (GAPDH) and untagged *LINC00261*. Our results indicate that the S1m aptamer falls to interact with high enough affinity to the streptavidin beads to perform a proper pulldown.

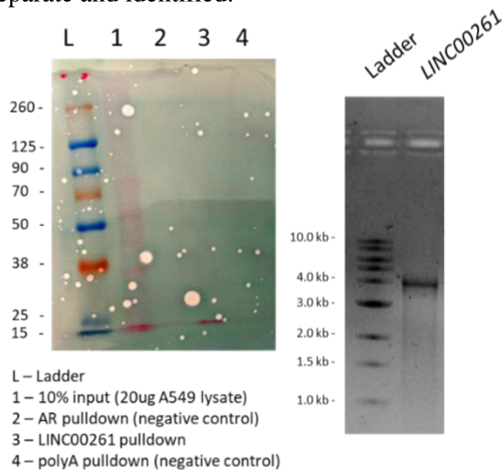


Figure 5: (A) Agarose gel LINC00261 binding partners. SDS-PAGE gel stained with Ponceau S of eluted proteins from the respective RNA-pulldown. AR 3´-UTR and polyA transcript RNA was used as a negative control.

We also attempted to identify if there were any specific bands identified in the LINC00261 pulldown. Here, *LINC00261* was bound to streptavidin beads to pull down co-bound factors in A549 lysate. Bound proteins were then eluted and subjected to SDS-PAGE to identify unique bands present in the LINC00261 pulldown. Figure 2B shows a band around 15kb, which may be part of the column, as the only identified protein component. The strategy of utilizing S1m to isolate LINC00261 binding partners was a negative result. An alternative utilizing CHIRP-MS in vivo is being pursued. Additional approvals will be sought.

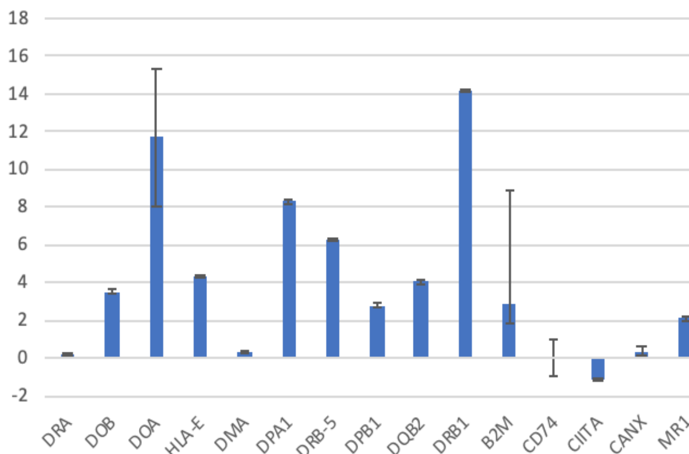
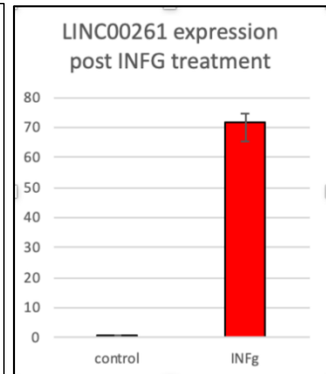


Figure 6: H522 cells expressing with manipulated LINC00261 expression had RNA isolated and qRT-PCR quantification of MHC Class II gene expression. Expression is normalized to GAPDH and H522 control cells (no LINC).

Task 4: LINC00261 connection to MHC Class II

In order to test if LINC00261 expression affects MHC class II gene expression we tested the expression of multiple MHC Class II genes in the presence or absence of LINC00261 in H522 cells. The selected genes were highly correlated to LINC00261 expression in the TCGA LUAD dataset. We observed that MHC Class II genes were highly upregulated in both conditions, but the presence of LINC00261 increased expression of multiple MHC Class II molecules had on target gene expression (right panel, blue). We observed that LINC significantly affected DOA, DOB, HLA-E, DPA1, DRB-5, DRB1 and B2M. In contrast, DRA, DMA, CD74, CANX, and MR1 levels of stimulation were not different between H522-LINC and H522-control conditions. We are performing additional repeats this to validate observations.

Figure 7: H522 cells expressing with manipulated LINC00261 expression had RNA isolated and qRT-PCR quantification of MHC Class II gene expression.

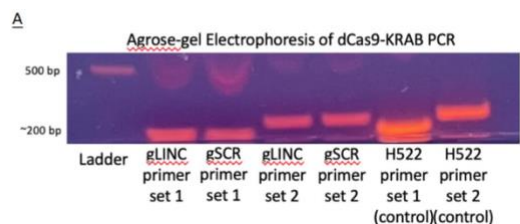


We also tested the converse, where we determined if LINC00261 expression was affected by interferon gamma expression. We observed that INFg significantly increased LINC00261 expression by ~70fold (Right panel, red).

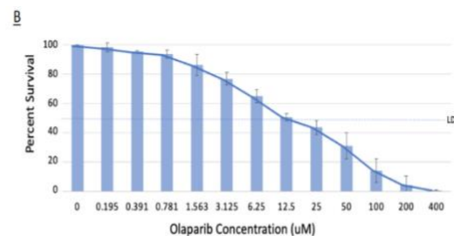
In sum, both conditions were deemed to be true. Namely, activation of immune signaling increased LINC00261 expression, and LINC00261 presence increased the expression of MHC class II markers.

Task6: In vitro efficacy of DDR and anti-PDL1 treatment

We hypothesized that since LINC00261 affected activation of DNA damage components, it may affect the sensitivity of cells to small molecule inhibitors that work by affecting DNA damage signaling. First, we confirmed that sgRNA targeting of LINC00261 in the TRE-dCas9-KRAB system was effectively being activated with DOX induction (Figure X). Subsequently, The PARP-targeting olaparib was therefore tested for (1) efficacy in lung cancer cells and (2) if LINC00261 affected the sensitivity of cells to olaparib. We observed that the optimal concentration for olaparib to observe LD_{50} in A549 cells was 12uM (Blue bars, left figure middle). Additionally, our initial observation was that LINC00261 expression was able to block cellular sensitivity to olaparib (bottom figure, Blue+orange bars). However, subsequent repeats of the experiment did not show a noticeable effect of LINC00261 on olaparib sensitivity (grey and red bars below). As a control LINC00261 levels were measured and it was determined that the DOX failed to repress LINC00261 in the repeats that showed no effect. We are therefore testing why DOX was no longer able to repress LINC00261 levels, and will repeat once this has been optimized.



A) PCR and electrophoresis verification of dCAS/KRAB system transfection



B) LD50 of olaparib in WT A549 ~12uM. Identified ~3.125 uM at ~80% survival for optimal dose to use in combination treatment.

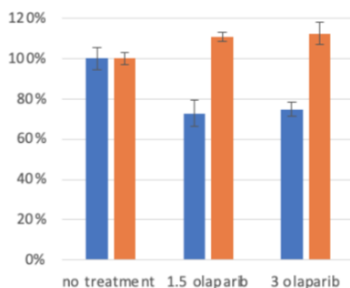
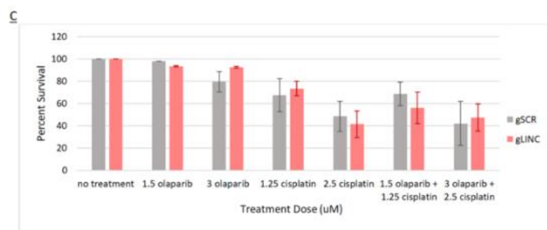
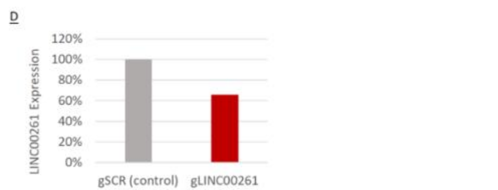


Figure 8: A549 cells with LINC00261 repression have differential sensitivity to olaparib. (Blue = LINC present, orange = LINC repressed).



C) Combinations of sublethal olaparib and cisplatin treatments.



D) qPCR of *LINC00261* expression compared between transfected gSCR and gLINC cells

Figure 9: in subsequent repeats DOX failed to repress LINC00261 expression and there was no effect of olaparib. Troubleshooting is ongoing.

Task7: In vivo efficacy of DDR and anti-PDL1 treatment

ACURO approval obtained. Mouse experiments anticipated in Year 2 once in vitro experiments are validated.

ACCOMPLISHMENTS:

OPPORTUNITIES FOR TRAINING & PROFESSIONAL DEVELOPMENT:

Students have had several opportunities for training during the performance period. MCR has undertaken responsible conduct of research, Castillo has completed his dissertation and is in the process of transitioning into a postdoctoral position in the lab. MCR has learned electroporation, immunofluorescence, and lentiviral transduction.

Dr. Marconett has had numerous avenues of professional development as a result of this grant. Successful extramural funding allowed Dr. Marconett to obtain a dual promotion to the tenure-track as well as Associate level position in the Department of Translational Genomics at USC, anticipated start date 7.1.2022. Dr. Marconett was asked and served as a reviewer for the P2RMIS DOD concept award reviewer 2021 cycle. Dr. Marconett has also been afforded several speaking engagements to disseminate research stemming from this grant, including the American Cancer Society Relay for Life and LA Best 2022. As part of LA BEST 2022 she received professional training in speaking and engagement. Dr. Marconett was also named to the Scientific Advisory Board for the Hastings Center for Pulmonary Research. Dr. Marconett was not able to participate in off-site conferences this year, and she was erroneously diagnosed with Stage IA lung cancer and underwent lower lobe wedge resection in January 2022, which required extended recovery. Thankfully, it was diagnosed post resection as tuberculosis and she has been under the auspices of LA county public health since March 2022, when she was allowed to physically return to lab.

DISSEMINATION OF INFORMATION TO COMMUNITIES OF INTEREST

Dr. Marconett currently serves as the genetics and epigenomics program representative to the Community Outreach & Engagement Internal Oversight Committee for the Norris Comprehensive Cancer Center. She participated in community speaking events to disseminate information to the Norris catchment area as part of the Relay for Life as the invited scientific expert. In addition, Dr. Marconett invited a BRAVO magnet HS student, Christell Pelico, into her lab. BRAVO high school is the local HS within walking distance to the USC Health sciences campus and has a 95% Latino student population. Dr. Marconett invited 5 students into her lab during year 1, including 3 from underrepresented minorities in higher education.

PLANS TO COMPLETE AIMS OVER THE PROJECT PERIOD

The PhD student being supported by this grant, Jonathan Castillo, will continue to on this project as he transitions into his postdoctoral research and complete Aims 2 and 3. Michele Ramos Correa, a 2nd year PhD student, will focus on accomplishment of Aim 1. She is primarily supported by an NSF fellowship. Additionally, a MS student will be recruited in the Fall to complete bioinformatic and assist with mouse studies in Aim 3/Task 7.

IMPACT:

The major impact of the proposal (so far) was validating mechanistically that LINC00261 and expression of MHC ClassII factors are linked in LUAD. This has implications for application of immunotherapy, as the primary cause for anti-PDL1 treatment is a "cold" tumor with low neoantigen burden. Recognizing who will benefit based on expression of biomarkers, of which LINC00261 is a promising target, will dramatically improve determination of which patients should receive therapy. In line with this, our bioinformatic results have shown that TMB is connected to LINC00261 expression, may provide a mechanistic link between antigen presentation and DNA fidelity in lung cancer. We also show that the presence of LINC00261 affects cellular response to olaparib. Additional impact is pending completion of the aims.

Noting to Report on impact on technology transfer.

Nothing to Report on impact on society beyond science and technology.

CHANGES/PROBLEMS TO REPORT:

S1m was unable to bind strepdavidin and pull down LINC00261 associated factors (negative result). We plan to submit in writing a change of procedure to allow testing using ChIRP-seq, which utilizes an array of biotinylated probes to pull down LINC00261 and identify binding targets.

Problems with DOX-induced efficiency of epigenetic toggle switch were also identified. Problem of VPR activation was relieved when 5-aza-CdR was added to mitigate epigenetic repression of the locus. Problem of lack of KRAB silencing in olaparib treatments is ongoing as a dose response is being optimized.

No changes to Vertebrate Animal(s)
No changes to Human Subjects

PRODUCTS:

Publications:

The evolutionarily conserved long non-coding RNA LINC00261 drives neuroendocrine prostate cancer proliferation and metastasis via distinct nuclear and cytoplasmic mechanisms.
Mather RL, Parolia A, Carson SE, Venalainen E, Roig-Carles D, Jaber M, Chu SC, Alborelli I, Wu R, Lin D, Nabavi N, Jachetti E, Colombo MP, Xue H, Pucci P, Ci X, Hawkes C, Li Y, Pandha H, Ulitsky I, **Marconett C**, Quagliata L, Jiang W, Romero I, Wang Y, Crea F. Mol Oncol. 2021 Jul;15(7):1921-1941. doi: 10.1002/1878-0261.12954. Epub 2021 Apr 26. PMID: 33793068

Presentations:

1. *Student Presentation*: Jonathan Castillo "*LINC00261* moderates cisplatin response in lung adenocarcinoma." Hastings Center for Pulmonary Research, 2022 Los Angeles CA.
2. *Second Place Poster Award:* Mihalakakos E, Castillo J, Marconett CN. Assessing the combinatorial effects of olaparib on LINC00261-mediated cisplatin resistance in lung cancer. BMM retreat, Los Angeles CA
3. Castillo J, Marconett CN. LINC00261 is a tumor suppressor essential for activation of the DNA damage response. BMM retreat, 2022.
4. *Student Presentation*: Castillo J, Xue T, Miao L, Bellerez S, Johnson S, **Marconett CN**: The role of *LINC00261* in the DNA damage response. Genomic Integrity Joint meeting, UPC/main campus, USC, September 2021.

Press Releases:

1. AUGUST 2021: COMMUNITY FOR A CURE NEWSLETTER, USC NORRIS COMPREHENSIVE CANCER CENTER.

WEBSITES:

https://sites.google.com/view/marconettlab

NO INVENTIONS, PATENTS, OR LISCENCES

PARTICIPANTS:

Name:	Crystal N. Marconett, PhD
Project Role:	PI
Research Identifier:	0000-0002-8463-7126
Nearest Person month worked:	15% (1.8 months)
Contribution to Project:	PI, master of ceremonies
Funding Support:	Other FTE comes from co-Investigator responsibilities from Cystic Fibrosis Foundation, NIH NHLBI, and TRDRP as well as American Cancer Society.

Name:	Jonathan Castillo, MS
Project Role:	Graduate Student (PhD)
Research Identifier:	
Nearest Person month worked:	6 months (50% for full-time work due to classes)
Contribution to Project:	Portions of results for Aim 1 (S1m), oversight for MCR and EM
Funding Support:	

Name:	Michele Ramos Correa
Project Role:	Graduate Student (PhD)
Research Identifier:	
Nearest Person month worked:	6 months (50% for full-time work due to classes)
Contribution to Project:	Portions of Aim 1 (NER and IF)
Funding Support:	NSF fellowship supports her salary. Materials only on DOD

Name:	Evan Mihalakakos
Project Role:	Graduate Student (MS)
Research Identifier:	
Nearest Person month worked:	3 (non-salaried)
Contribution to Project:	Olaparib results (Aim 3)
Funding Support:	

Name:	Christell Pelico
Project Role:	BRAVO HS student
Research Identifier:	
Nearest Person month worked:	6 (volunteer)
Contribution to Project:	Assist MCR with Aim 1 progress
Funding Support:	

Name:	John Carpten, PhD
Project Role:	Co-I
Research Identifier:	0000-0002-6862-2821
Nearest Person month worked:	0.6
Contribution to Project:	Assistance with TMB interpretation/molecular mechanisms
Funding Support:	

Name:	Matt Michael, PhD
Project Role:	Co-I
Research Identifier:	
Nearest Person month worked:	0.6
Contribution to Project:	Tons of advice on LINC00261 binding assays (Aim 1)
Funding Support:	

Name:	Alan Epstein, MD
Project Role:	Co-I
Research Identifier:	
Nearest Person month worked:	0.6
Contribution to Project:	Interpretation of Aim 2 (LINC-immune) results
Funding Support:	

NO CHANGE IN SUPPORT RELATED TO LINC00261 AMONG ALL PIs OR SENIOR PERSONNEL

MARCONETT CHANGE IN SUPPORT OVERALL:

NEW ACTIVE GRANT:
<u>NIH NHLBI 2R01HL114959</u>

Active Dates: 01/01/2022-12/31/2025
Total Direct costs: (/year for 3 years) Agency: NIH NHLBI
Role: Co-I (0.25 cal months, 3% effort)
Focus: Stress-induced fibrosis modulated by GRP78
Co-Investigator(s): Beiyun Zhou (PI), Amy Lee (Co-I), David Ann (Co-I).

OTHER ORGANIZATIONS:

NOTHING TO REPORT

NO SPECIAL REPORTING REQUIREMENTS