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14. ABSTRACT

Objective: This proposal will determine how m^{6}A -metabolism modulates the cell's capacity to maintain genome integrity. We hypothesize that changes in the epitranscriptome contribute to the cancer state by altering RNA functions that directly and/or indirectly affect genome integrity, and ultimately, and malignant transformation.

Impact: This proposal is expected to uncover m^{6}A metabolism as a novel mediator of genome maintenance, which may at least in part account for the impact of this RNA modification on tumorigenesis. In light of recent efforts to develop small molecules targeting m^{6}A writers and erasers, we anticipate to identify putative targets for cancer therapy, particularly in the context of existing, genotoxic strategies.

15. SUBJECT TERMS
Breast Cancer, Cancer, Oncology, Women's health

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1. **INTRODUCTION:** Accurate genome maintenance is critical to prevent the accumulation of genomic and epigenomic aberrations, which can result in defective cell function and disease [1-3]. DNA double-strand breaks (DSBs) are perhaps the most severe threat to genome integrity. If unrepaired, DSBs cause cell cycle arrest, cell death or chromosomal translocations. DSB repair is equally essential for tumor growth, e.g. by counteracting replication stress resulting from excessive tumor cell division. <u>The targeted manipulation of repair pathways is thus emerging as an important means not only to prevent malignant transformation but also to interfere with tumor growth.</u> The resolution of DSBs generally involves one of three major DSB repair pathways – HR), non-homologous end joining (NHEJ) or alternative NHEJ (alt-NHEJ). Recent work points to RNA as an important modulator of repair outcome[1]. In particular, pathways relying on RNA modifications have been shown to regulate repair of UV-induced single-stranded DNA lesions as well as experimentally induced DSBs [2,3]. Supporting a broader role for m^{6}A metabolism in the cellular response to DNA damage, our preliminary data show that (i) nuclear m^{6}A-modified RNA accumulates both at laser-induced DSBs and across the transcriptome. (ii) IR-induced m^{6}A accumulation is at least in part dependent on the predominant m^{6}A RNA methyltransferase METTL3, (iii) loss of METTL3 causes increased DNA break-associated chromosomal aberrations and reduced efficiency of non-homologous end joining (NHEJ), (iv) mRNAs with IR-induced increase in m6A are significantly enriched for DNA damage response genes. <u>We, thus, hypothesize that m^{6}A-modified RNA not only controls the temporal and spatial control of repair factor function in response to DSBs, but also acts to modulate the "repair transcriptome".</u>

1. Zong, D., Oberdoerffer, P., Batista, P. J. & Nussenzweig, A. RNA: a double-edged sword in genome maintenance. Nat. Rev. Genet. (2020) doi:10.1038/s41576-020-0263-7
2. Xiang, Y. et al. RNA m6A methylation regulates the ultraviolet-induced DNA damage response. Nature 543, 573–576 (2017).
3. Zhang, C. et al. METTL3 and N6-Methyladenosine Promote Homologous Recombination-Mediated Repair of DSBs by Modulating DNA-RNA Hybrid Accumulation. Mol. Cell 79, 425-442.e7 (2020).

2. **KEYWORDS:** DNA double-strand breaks (DSBs); RNA; N6-methyladenosine (m^{6}A)

3. **ACCOMPLISHMENTS**

Specific Aim 1. Characterization of m6A writers and erasers as novel modulators of DSB repair
Specific Aim 2. Identification of DSB-Induced m6A-modified RNAs and interactors
Specific Aim 3. Impact of m6A-metabolism on breast cancer malignancy

- What was accomplished under these goals

Summary of the key research accomplishments:

Aim 1: To determine how m^{6}A metabolism impacts DSB repair choice we generated CRISPR gRNA libraries targeting genes related to m^{6}A-dependent pathways (Site 2 – Batista Lab). gRNA libraries were designed for knock-out, inactivation and activation screens. Each library targets 370 genes,

including methyltransferases, demethylases and RNA binding proteins that interact with RNAs in a m^6A-dependent manner. These libraries also target genes involved in other RNA modifications. Each gene is targeted by 10 gRNAs. Sequencing of each pooled library shows good coverage of gRNA sequences. Infection of two independent cell lines with lentiviral particles generated from each library shows that gRNA sequence diversity is maintained upon infection. These results indicate that our libraries are appropriate for screening. This resource will be made readily available through public plasmid repositories. These libraries will allow us to perform pooled screens to dissect the role of RNA modification pathways in DNA damage repair.

Aim2: To identify DSB-Induced m6A-modified RNAs, we performed m6A-IP-sequencing in U2OS cells irradiated with 10 Gy (Site 1 – Nussenzweig Lab). We identified 3004 peaks in 1545 mRNAs in irradiated cells and 1687 peaks in 942 mRNAs in non-irradiated control cells. Of the 3004 peaks identified upon DNA damage, 791 are present in irradiated cells only. This experiment suggests that DNA damage can induce new and / or enhance existing m^6A modification events. Notably, DNA damage is the top gene ontology cluster among transcripts with IR-induced m^6A. mRNA with DNA damage induced sites of m^6A modification include a range of DNA repair factors, many of which have known roles in homologous recombination or replication stress (e.g. SMARCAD1, FANCM, FANCA, BRCA2, BRCA1, ATRIP, SETX, MCM10). These findings suggest that m6A RNA modifications may modulate mRNA stability, translation or location of HR—relevant repair factors.

Aim3: Work for Aim 3 was supposed to be initiated 18 months into the award (Site 1). Due to COVID-19, work on Aim 3 had to be delayed and will be initiated in the second award year.

What opportunities for training and professional development did the project provide?

Work on this project required members of both the Batista and Nussenzweig laboratories to work with the NCI genome editing core to learn new bioinformatic skills for RNA sequencing data analysis and CRISPR library design.

How were the results disseminated to communities of interest?

This data is still preliminary, and it has been presented exclusively within the NCI intramural research program.

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

Aim 1. We will use our validated CRISPR libraries to perform CRISPR screens for m6A modulators of DNA repair (Site 2). We will explore two complementary strategies in our CRISPR screens: (1) cells lines expressing reporter assays for HR, NHEJ and alt-NHEJ to provide a general assessment of potential repair function of the m6A pathway, and (2) synthetic viability screens in cells that are either proficient for or deficient in either of these repair pathways. Using a combination of live cell imaging, IF and chromatin immunoprecipitation approaches, we will assess recruitment kinetics of key mediators of HR or NHEJ when m6A metabolism is altered (Site 1). M6A modulators that alter DSB repair will similarly be tested for their recruitment to sites of DNA damage using appropriate cell-based and biochemical assays.

Aim 2. We will perform m^6A-IP-sequencing in cells exposed to other DNA damage conditions (Site 2). The combined results from the multiple m^6A-IP-sequencing experiments will be used to identify sequences that contain DNA damage induced m^6A sites. These sequences will be used to create oligonucleotide baits for in vitro capture of m6A readers. Lysates from treated and untreated cells will be used to identify m6A readers that function specifically during DNA damage. Having identified a subset of HR factors with IR-induced de novo m6A modifications, we will further design probes specific for DNA damage-specific m^6A modification in BRCA1 and BRCA2 mRNA.

Aim 3. Sensitivity to drugs used in cancer therapy will be measured upon inactivation or activation of m6A writers and erasers contained in our sgRNA library (Site 1). Viability screens will be performed in cells that are either proficient for or deficient in the BRCA1 or BRCA2 breast cancer genes to specifically address a role in modulating the survival of cells with breast-cancer-associated genetic defects in the presence or absence of genotoxic agents. In addition to genetically manipulated U2OS or RPE cell lines, we will use isogenic breast cancer cell lines that are either proficient (MDA-MB-175) or deficient for BRCA1 (MDA-MB-175-VII). Cells will be exposed to varying doses of S phase poisons, including PARP- and topoisomerase-inhibitors, and assayed for changes in growth and/or viability as well as metastatic potential via anchorage-dependent and -independent growth assays.

To assay for the consequences of m6A writers and erasers beyond BRCA1-dependent repair processes, we will expose tumor cells to a range of genotoxic agents that induce DSBs at all phases of the cell cycle, including doxorubicin, etoposide, bleomycin and IR.

4. IMPACT:

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

In accordance with recent publications[1], our preliminary data shows that m6A-dependent pathways play a significant role in DNA damage repair, not restricted to UV damage. Going forward we will be able to dissect the contribution of this pathway.

What was the impact on other disciplines?

By characterizing DNA damage as a cause for altered m6A metabolism, progress in aim 1 and aim 2 will provide valuable information to the field of RNA post-transcriptional modifications, beyond the immediate interest to the DNA repair community.

What was the impact on technology transfer?

We have generated CRISPR gRNA libraries that will be shared with the scientific community.

What was the impact on society beyond science and technology?

Completion of this study is anticipated to pave the way for the identification of new cancer therapies.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change: In aim 1 we decided to use pooled CRISPR screen as opposed to a gene by gene targeted approach. This approach allows us to test a larger group of genes and include other RNA modification pathways. Validation studies will use the gene by gene targeted approach.

Actual or anticipated problems or delays and actions or plans to resolve them: Work on this project was significantly impacted by restrictions adopted by the NIH to minimize spread of COVID-19. Work has resumed on campus, albeit at limited capacity.

Changes that had a significant impact on expenditures: Restrictions adopted by the NIH to minimize spread of COVID-19 impacted our ability to perform experiments and delayed the ability to recruit staff to work on this project.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents:

No changes

Significant changes in use or care of human subjects:

No changes in use or care of human subjects

Significant changes in use or care of vertebrate animals:

No changes in use or care of vertebrate animals

Significant changes in use of biohazards and/or select agents:

No changes in use of biohazards and/or selected agents

6. PRODUCTS:

Publication: RNA: a double-edged sword in genome maintenance (2020) Dali Zong, Philipp Oberdoerffer, Pedro J. Batista and Andre Nussenzweig. Nature Reviews Genetics, https://doi.org/10.1038/s41576-020-0263-7

Books or other non-periodical, one-time publications.

None

Other publications, conference papers, and presentations.
None

Website(s) or other Internet site(s)
None

Technologies or techniques
None

Inventions, patent applications, and/or licenses

None

Other Products
None

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

Name:	Dali Zong
Project Role:	
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	Dr. Zong has been a major contributor to the laboratory's research efforts in understanding the mechanisms of DNA repair pathway choice as it relates to BRCA-deficient breast cancers. He has been responsible for conducting fundamental studies on the molecular mechanisms of DNA damage/repair and has developed a number of sophisticated genetic, biological and biochemical assays that will impact our understanding of m6A-metabolism on breast cancer malignancy
Funding Support:	Dr. Zong is supported by the NIH Intramural Research Program

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

No changes in active other support of the PD/PI(s)

What other organizations were involved as partners?

No other organizations were involved as partners

8. APPENDICES

None