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TITLE:
Characterizing the Role of Hep27 in Liver and Colorectal Cancer Stress Tolerance

PRINCIPAL INVESTIGATOR:
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RECIPIENT:
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14. ABSTRACT
This project seeks to characterize a potentially novel ROS responding protein that may be especially important in colorectal and liver cancer cells. This protein, Hep27, is overexpressed in hepatocellular carcinoma, and its high expression correlates with decreased survival in colon cancers. It was hypothesized that Hep27 overexpression is a mechanism of resistance to cancer therapies by increasing ROS tolerance in colorectal and liver cancers through its enzymatic activity. This project proposed to use human cell lines to test whether modulation of Hep27 expression contributes to cell ROS tolerance and survival. Thus far, the work on this project has not demonstrated that Hep27 plays a role in ROS tolerance in liver cancer cells.

15. SUBJECT TERMS
Hep27, ROS, stress, cancer

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1. INTRODUCTION:

Reactive oxygen species (ROS) are byproducts of normal cell metabolism, but at high levels they react with and damage macromolecules, causing dysfunction and cell death. Cancer cells are highly proliferative and tend to have elevated levels of ROS. In addition, many cancer treatments utilize ROS to promote cell death. However, cancer cells often upregulate antioxidant enzymes to combat ROS, and this increased antioxidant capacity contributes to resistance to traditional therapies. I am interested in characterizing a potentially novel ROS responder that may be especially important in colorectal and liver cancer cells. This protein, Hep27, is overexpressed in hepatocellular carcinoma, and its high expression correlates with decreased survival in colon cancers. I hypothesize that Hep27 overexpression is a mechanism of resistance to cancer therapies by increasing ROS tolerance in colorectal and liver cancers through its enzymatic activity. I will use human cell lines to test whether modulation of Hep27 expression contributes to cell ROS tolerance and survival.

2. KEYWORDS:

Hep27, hepatocellular carcinoma, colorectal cancer, reactive oxygen species, dicarbonyls, apoptosis, DNA damage

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Goal 1: Obtain training and educational development in translational cancer research, including colorectal and liver cancers

Goal 2: Confirm whether Hep27 deletion can sensitize colorectal and liver cancer cell lines to oxidative stress

Major Task: Test oxidative stress tolerance of Hep27 KO cells

Goal 3: Explore the mechanism through which Hep27 confers tolerance to oxidative stress

Major Tasks: Test whether Hep27 enzymatic activity or Hep27 regulation of p53 is required for oxidative stress tolerance.

Goal 4: Investigate whether Hep27 modulates therapeutic sensitivity in colorectal and liver cancers.

Major Task: Test whether knockdown or overexpression of Hep27 can sensitize colorectal and liver cancer cells to ROS-inducing cancer therapies.

What was accomplished under these goals?

Goal 1: Obtain training and educational development in translational cancer research, including colorectal and liver cancers

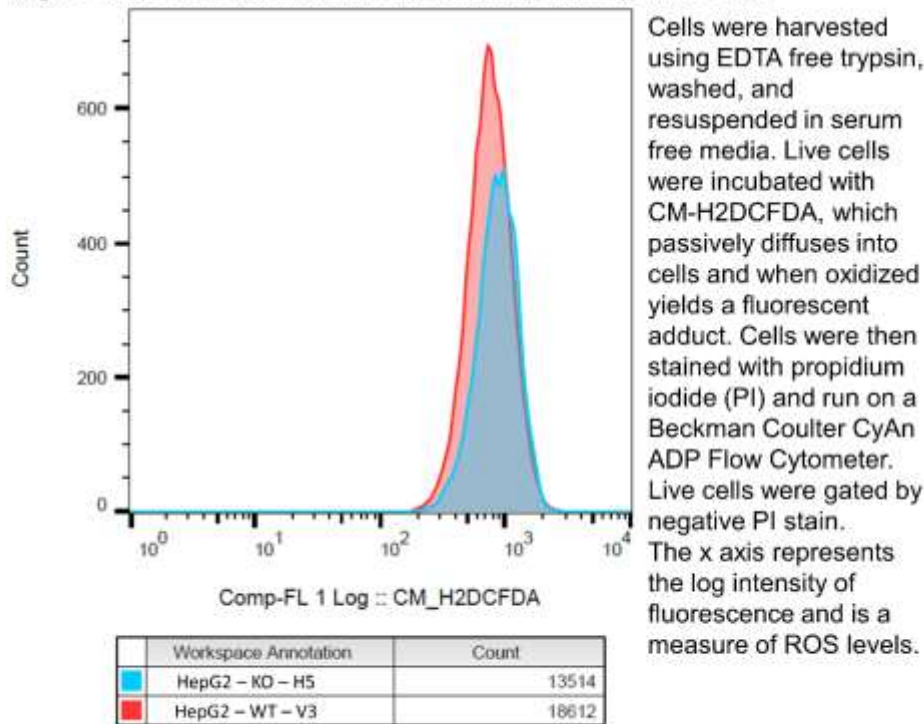
- Attended AACR Annual Meeting in Washington DC
- Presented research at student seminars for Genetics and Molecular Biology curriculum, attended seminars presented by other students
- Presented project-related and published research at weekly lab meetings
- Attended seminars sponsored by The Center for Gastrointestinal Biology and Disease
- Presented research at Genetics and Molecular Biology curriculum annual retreat

- Obtained a position facilitating clinical research
- Did not attend AACR Translational Cancer Research for Basic Scientists Workshop
- Did not participate in the UNC Academic Research conference planning committee
- Did not present data for national meeting

Goal 2: Confirm whether Hep27 deletion can sensitize colorectal and liver cancer cell lines to oxidative stress

- Major Task: Develop Hep27 knockout clones in colorectal and liver cancer cell lines.
    - o Attempted to grow RKO cells from lab stock, but were unable to culture cells consistently enough for transfection or monoclonal selection
    - o HCT116 cells had very low basal protein expression of Hep27 compared to HepG2 cells, so we have not yet developed these knockout cells and continued the majority of the work on this project with HepG2 cells
- Major Task: Test oxidative stress tolerance of Hep27 KO cells
    - o Received training in the use of Beckman Coulter CyAn ADP Flow Cytometer and Summit software from the UNC Flow Cytometry Core facilities
    - o We have tested ROS accumulation in several Hep27 knockout clones in response to H_2O_2 and Ionizing Radiation treatments. Representative data and experimental conditions are listed below.

Figure 1. ROS Accumulation in untreated WT and Hep27 KO cells



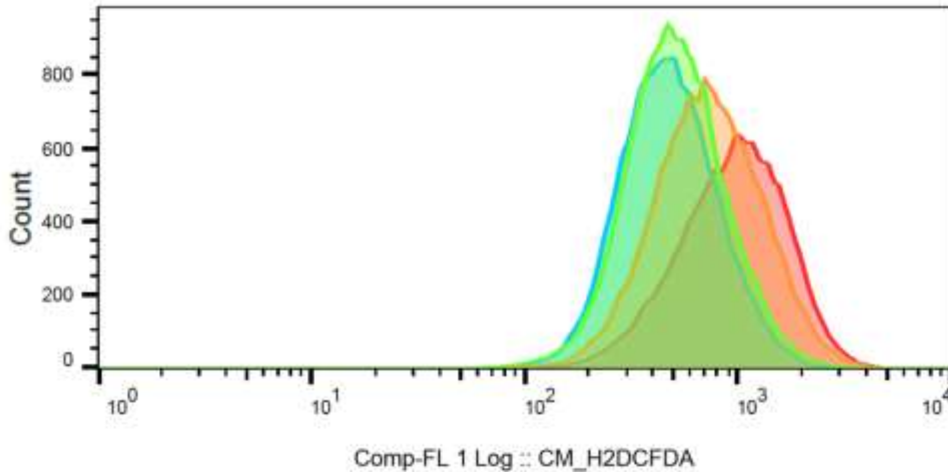
Cells were harvested using EDTA free trypsin, washed, and resuspended in serum free media. Live cells were incubated with CM-H2DCFDA, which passively diffuses into cells and when oxidized yields a fluorescent adduct. Cells were then stained with propidium iodide (PI) and run on a Beckman Coulter CyAn ADP Flow Cytometer. Live cells were gated by negative PI stain. The x axis represents the log intensity of fluorescence and is a measure of ROS levels.

To perform the majority of the work in this section, we utilized three clones derived from HepG2 cells treated with a CRISPR/Cas9 plasmid targeting the Hep27 coding region. V3 is a clone with wild type (WT) Hep27, while H4 and H5 are Hep27 knockout clones. In order to measure basal ROS content in these cells, we performed live cell staining with CM-H2DCFDA (5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester), a derivative of DCF-DA with enhanced an ability to bind to intracellular components, thereby prolonging the dye's cellular retention. We then stained the cells with propidium iodide (PI). We measured the

fluorescence of each cell with a flow cytometer and gated out cells with positive PI staining (dead or dying cells) for ROS measurement. A significant difference in ROS levels would indicate a significant difference in the abilities of the cells to cope with oxidative insults or intracellular oxidative metabolism. Figure 1 shows that Hep27 knockout did not drastically affect the ROS content of untreated cells in HepG2 Hep27 knockout (KO) clone #H5 compared to Hep27 WT clone #V3.

We further treated these WT and KO cells with ROS-inducers (described below), and then stained the cells as described above. I will perform these experiments in three cell lines with multiple independently derived clones. The first objective of this project was to confirm that the role for Hep27 in H_2O_2 stress tolerance that has been previously shown to occur in osteosarcoma cells (see Project Narrative) is recapitulated HepG2 cells. Figure 2A shows one of these experiments. At basal levels, each of these cells appeared to have similar ROS levels, but there appeared to be a large shift in ROS content in the H5 KO clone after H_2O_2 that was greater that what occurred in the V3 WT clone (Figure 2B).

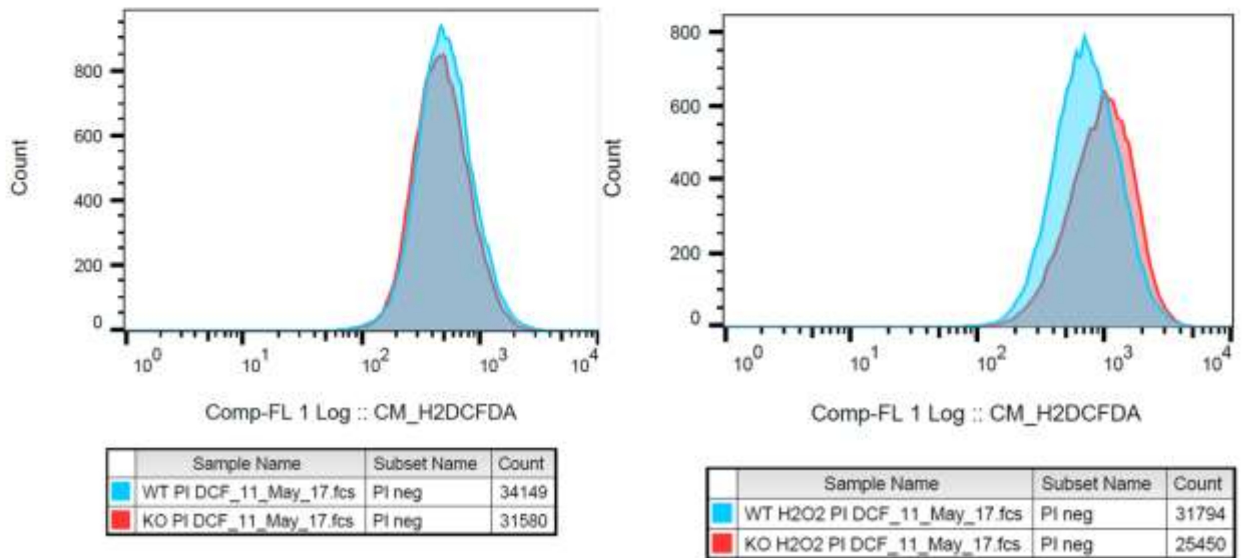
Figure 2A. ROS Accumulation after H_2O_2 treatment



	Sample Name		Subset Name	Count
(green)	WT	HepG2 – KO – H5	PI neg	34149
(orange)	WT H2O2	HepG2 – KO – H5	PI neg	31794
(blue)	KO	HepG2 – WT – V3	PI neg	31580
(red)	KO H2O2	HepG2 – WT – V3	PI neg	25450

Cells were treated with 1mM H_2O_2 for 30 mins or left untreated. Live cells were incubated with CM-H2DCFDA, stained with propidium iodide (PI) and run on a Beckman Coulter CyAn ADP Flow Cytometer. Live cells were gated by negative PI stain.

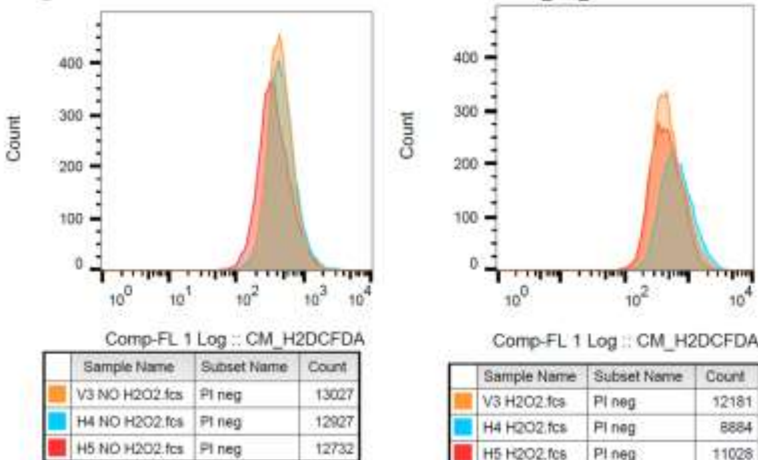
Figure 2B. ROS Accumulation after H_2O_2 treatment



Cells from Figure 2A, viewed by treatment

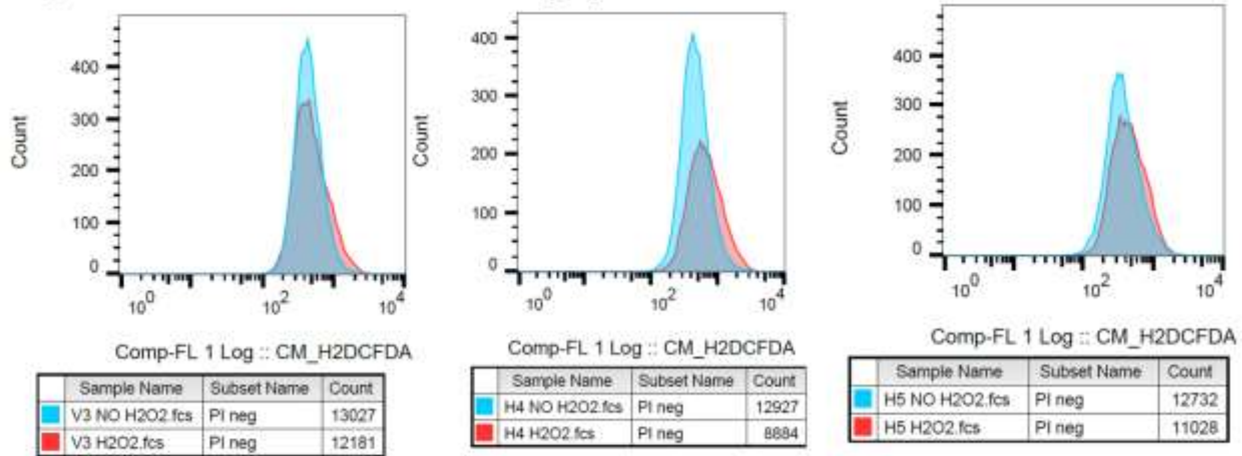
We performed this same experiment multiple times, with varying results. Sometimes, there were large differences in ROS content between the two cell types, but in other experiments, there was no discernable difference in ROS content. Figure 3A shows one such experiment. At both basal levels and after H_2O_2 treatment, the ROS content in the WT and KO cells remained similar. Further, H_2O_2 treatment did not always produce a reliable and detectable shift in ROS content (Figure 3B).

Figure 3A. ROS Accumulation after H_2O_2 treatment



Cells were treated with 1mM H_2O_2 for 30 mins or left untreated. Live cells were incubated with CM-H2DCFDA, stained with propidium iodide (PI) and run on a Beckman Coulter CyAn ADP Flow Cytometer. Live cells were gated by negative PI stain.
V3 = Hep27 WT
H4 = Hep27 KO
H5 = Hep27 KO

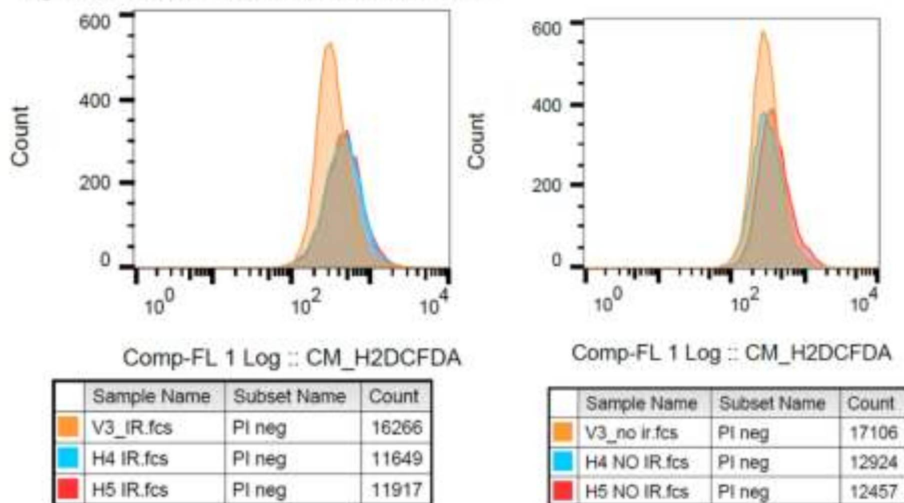
Figure 3B. ROS Accumulation after H_2O_2 treatment



Cells from Figure 3A, viewed by cell line

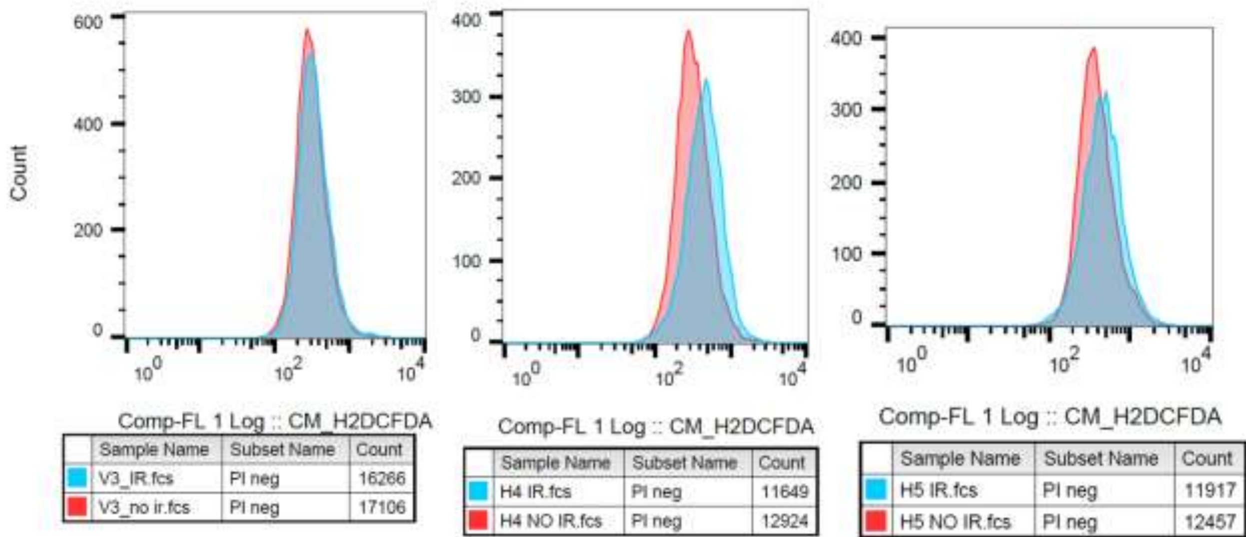
We also detected ROS content following treatment with ionizing radiation, another ROS-inducer. Here, we saw a slight shift in ROS content (compared to WT cells) that was consistent between the two KO clones (Figure 4A-B). Again, in untreated cells, ROS contents were equivalent.

Figure 4A. ROS Accumulation after IR



Cells were treated with 10 Gy IR and incubated for 24 hrs. Live cells were incubated with CM-H2DCFDA, stained with propidium iodide (PI) and run on a Beckman Coulter CyAn ADP Flow Cytometer. Live cells were gated by negative PI stain.
V3 = Hep27 WT, H4 = Hep27 KO, H5 = Hep27 KO

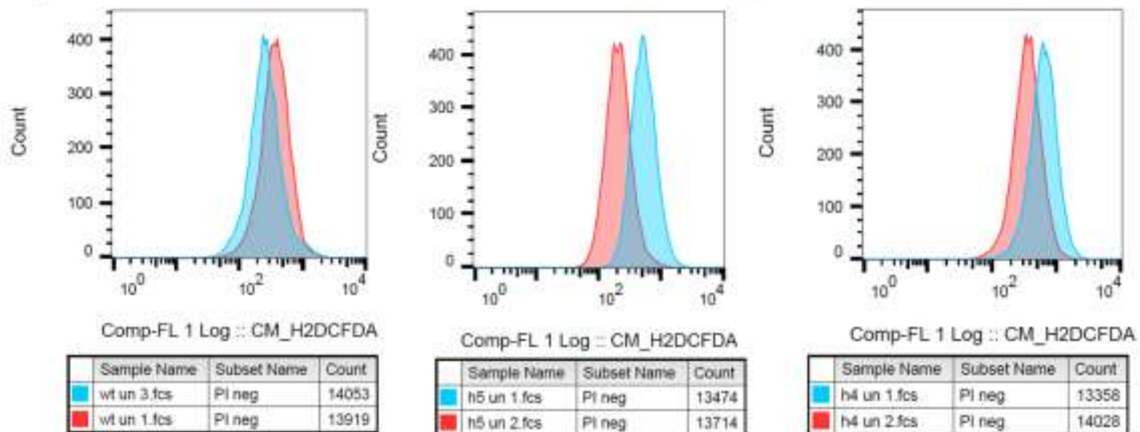
Figure 4A. ROS Accumulation after IR



Cells from Figure 4A, viewed by cell line

Overall, we have struggled to obtain consistent data while using the CM-H2DCFDA dye. One such example of this is shown in Figure 5. In this experiment, we performed each treatment using two biological replicates, and the differences that we observed in ROS content were inconsistent between the replicates, sometimes appeared to be greater than differences observed between untreated and ROS-inducer treated cells. We attempted to optimize dye concentration and incubation conditions, but inconsistencies in staining remained problematic. The results from these experiments are difficult to interpret and remain inconclusive.

Figure 5. Basal ROS levels are inconsistent between replicates



Live cells were incubated with CM-H2DCFDA, stained with propidium iodide (PI) and run on a Beckman Coulter CyAn ADP Flow Cytometer. Live cells were gated by negative PI stain.
V3 = Hep27 WT, H4 = Hep27 KO, H5 = Hep27 KO, Un= untreated

Goal 3: Explore the mechanism through which Hep27 confers tolerance to oxidative stress
- Major Tasks: Test whether Hep27 enzymatic activity or Hep27 regulation of p53 is required for oxidative stress tolerance.
    - Because we have been unable to confidently demonstrate that Hep27 knockout (KO) cells are significantly different from wild type (WT) cells in their ability to tolerate oxidative stress (see Goal 2, above), we did not complete work towards this project goal. If we are unable to demonstrate that the loss of Hep27 sensitizes colorectal and liver cancer cells to oxidative stress, then exploration of the mechanism through which Hep27 confers tolerance to oxidative stress would be unnecessary to complete.

Goal 4: Investigate whether Hep27 modulates therapeutic sensitivity in colorectal and liver cancers.
- Major Task: Test whether knockdown or overexpression of Hep27 can sensitize colorectal and liver cancer cells to ROS-inducing cancer therapies.
    - This task has not been started due to limitations on time resources, including the graduation and job search of the PI.

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

Nicole Tackmann

Training
- Received instruction and certification in the use of the Beckman Coulter CyAn ADP Flow Cytometer from the UNC Flow Cytometry Core Facility
- Participated in a 160 hour internship at Cato Research Ltd., sponsored by the ImPACT (Immersion Program to Advance Career Training) Program at UNC
    - Learned more about the conduct of clinical research and wrote regulatory documentation for clinical trials
    - Highlighted accomplishments:
        - Built clinical study report (CSR) shell for Phase I open label 4-way crossover study
        - Authored cover letters and forms for Investigational New Drug (IND) and New Drug Application (NDA) annual FDA reports
        - Generated evidence tables for Modified Risk Tobacco Product (MRTP) application
        - Developed NDA storyboard template for client interactions
        - Provided writing support for pre-IND meeting package for Phase I HIV vaccine adjuvant study
        - Wrote prevalence and incidence estimations for an orphan drug application
        - Received Good Clinical Practice (GCP) and Good Manufacturing Practice (GMP) training
        - Advised and provided quality control support for a manuscript describing a Phase 2b botulism vaccine antigen study
- Participated in the Regulatory Affairs Training Program, a 6-week course sponsored by the Duke University Office of Regulatory Affairs and Quality
    - Training program topics: preparation, and maintenance of INDs and IDEs; FDA communications; medical device regulations; human subject protections, briefing documents, clinical investigator brochures
- Completed the following additional courses:

- Project Management, UNC
- GCP for Clinical Trials with Investigational Drugs, Biologics and Devices, CITI Program
- Data Management for Clinical Research, Vanderbilt University (Coursera)
- Design and Interpretation of Clinical Trials, Johns Hopkins University (Coursera)

Professional Development

- Completed dissertation and received a Doctor of Philosophy in Genetics and Molecular Biology
- Attended the 2017 AACR Annual Meeting in Washington DC
- Participated in selecting and inviting speakers to the UNC Curriculum Genetics and Molecular Biology Seminar series
- Presented research at weekly lab meetings
- Served as a writer and editor for *The Pipettepen*, the UNC Science Writing and Communication Club Blog
  - Wrote articles on scientific topics and generated content for a broad audience
  - Edited articles written by others to improve their logic, grammar, clarity, and style

How were the results disseminated to communities of interest?
Some of the results of this project were reported to attendees of the UNC Department of Genetics Scientific Retreat 2016 and to the UNC Curriculum in Genetics and Molecular Biology student seminar series. Other than this, nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?
Nothing to report, leaving UNC for another position

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?
Nothing to report

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

5. CHANGES/PROBLEMS:

Prior to this report, I was in the process of requesting a 12 month extension for this project, without funds. However, I will not be continuing with this project because I have accepted a position outside of UNC.

Changes in approach and reasons for change

Currently, I have been unable to confidently demonstrate that Hep27 knockout (KO) cells are significantly different from wild type (WT) cells in their ability to tolerate oxidative stress (Aim 1, Major task 3 from the SOW). If I am unable to definitively show that the loss of Hep27 sensitizes colorectal and liver cancer cells to oxidative stress, then Aim 2 (Explore the mechanism through which Hep27 confers tolerance to oxidative stress) will be unnecessary to complete. Thus, the remainder of the project would have been focused on Aim 3 (Investigate whether Hep27 modulates therapeutic sensitivity in colorectal and liver cancers) and required the completion of the following tasks:

Aim 3: Investigate whether Hep27 modulates therapeutic sensitivity in colorectal and liver cancers.

1. Major Task 6: Test whether knockdown of Hep27 can sensitize colorectal and liver cancer cells to ROS-inducing cancer therapies.
    a. Subtask 18: Treat Hep27 WT and KO cells with cancer therapies, measure DNA damage by phospho-γH2AX immunofluorescent staining
    b. Treat Hep27 WT and KO cells with cancer therapies, measure apoptosis by Annexin V staining coupled with flow cytometry
2. Major Task 7: Test whether overexpression of Hep27 can sensitize colorectal and liver cancer cells to ROS-inducing cancer therapies.
    a. Subtask 20: Overexpress Hep27 in WT cells, treat with cancer therapies, measure DNA damage by phospho-γH2AX immunofluorescent staining
    b. Subtask 21: Overexpress Hep27 in WT cells, treat with cancer therapies, measure apoptosis by Annexin V staining coupled with flow cytometry
3. Major Task 8: Submit publication to peer-reviewed journal (if data is publishable)
    a. Subtask 22: Prepare figures for manuscript
    b. Subtask 23: Write manuscript, submit for publication

Actual or anticipated problems or delays and actions or plans to resolve them

Several factors contributed to delays in project completion, chiefly including the following:

1. Time spent writing my dissertation and preparing for graduation
2. Time spent writing a review paper published in Journal of Molecular Cell Biology entitled, “Mouse modelling of the MDM2/MDMX-p53 signalling axis.”
3. Lack of consistently observed differences in oxidative stress tolerance in Hep27 KO cell lines (HepG2)
4. Problems with culturing certain cell lines (RKO)
5. Time spent writing three additional manuscripts, one submitted to Cell Reports, one submitted to Molecular Cancer, and one that has yet to be submitted.

Several of the issues contributing to the delay of the project, including my own graduation and time spent writing manuscripts, were resolved and would have allowed me to complete the project with an additional 12 months extension.

Changes that had a significant impact on expenditures

My graduation in March 2017 and transition to a Postdoctoral Research Associate position in April 2017 led to the use of additional funds for salary.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to report

Significant changes in use or care of human subjects
Nothing to report

Significant changes in use or care of vertebrate animals.
Nothing to report

Significant changes in use of biohazards and/or select agents
Nothing to report

6. **PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Nothing to report

  Books or other non-periodical, one time publications
  Nothing to report

  Other publications, conference papers, and presentations.
  - Tackmann NR. "*In Vivo* Discoveries in MDM2-Mediated p53 Regulation." Dissertation Defense. 23 Mar 2017.
  - Tackmann NR and Zhang Y. "Mouse modelling of the MDM2/MDMX-p53 signalling axis." *Journal of Molecular Cell Biology*. 2017 Feb; 9(1): 34-44.
  - Tackmann NR and Zhang Y. "Disrupting MDM2 E3 ligase activity prolongs survival in MYC-induced lymphoma." (in preparation).
  - Tian H, Tackmann NR, Jin A, Zheng J and Zhang Y. "Functional inactivation of the MDM2 RING domain augments p53 transcriptional activity." *Molecular Cancer* (submitted, under review).
  - Yang J, Tackmann NR, Jin A, Itahana Y, Macias E, Zheng J and Zhang Y. "*In vivo*, MDMX is essential for p53 degradation by facilitating MDM2-p53 binding and recruiting an E2 ubiquitin-conjugating enzyme." *Cell Reports*. (submitted, under review)
  - Tackmann NR. "In vivo regulation of p53 by MDM2 E3 ligase activity." GMB Student seminar series. 15 Nov 2016.
  - Tackmann NR, Meng X, Liu S, Yang J, Dong J, Wu C, Cox AD and Zhang Y. "RPL23 links oncogenic RAS signaling to p53-mediated tumor suppression." Poster. Genetics Annual Retreat.

- **Website(s) or other Internet site(s)**
  Nothing to report

- **Technologies or techniques**

3 Plasmids that can be transfected in cells to knockout the *Hep27* (*Dhrs2*) gene

- **Inventions, patent applications, and/or licenses**
  Nothing to report

- **Other Products**
  Collection of HepG2 cells with Hep27 knockout

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*Nicole Tackmann, PhD*
Project Role:	*Principal Investigator*
Nearest person month worked:	*10*
Contribution to Project:	*Dr. Tackmann performed all activities associated with this project.*

Name:	*Yanping Zhang, PhD*
Project Role:	*Mentor*
Nearest person month worked:	*1*
Contribution to Project:	*Dr. Zhang provided career and experimental mentorship to Dr. Tackmann.*

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

Nicole Tackmann
Was primarily supported by the current award, but participated in a UNC-funded internship program that lasted 2 days a week for 10 weeks (160 hours). This internship was approved by DOD prior to Dr. Tackmann's participation.

What other organizations were involved as partners?

- Organization Name: UNC Flow Cytometry Core Facilities
  - Location of Organization: UNC, Chapel Hill, NC
  - Partner's contribution to the project: Flow cytometry facilities and equipment

8. SPECIAL REPORTING REQUIREMENTS

Not applicable to this project

9. APPENDICES:

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Nothing to report.