AWARD NUMBER: W81XWH-19-1-0847

TITLE: TP53 Synthetic Lethal Screen in Organoid Avatars to Discover Novel Therapeutic Targets for Colon Cancer

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CONTRACTING ORGANIZATION: University of South Carolina, Columbia, SC

REPORT DATE: October 2020

TYPE OF REPORT: Annual Report

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	15Sep2019-14Sep2020

4. TITLE AND SUBTITLE
TP53 Synthetic Lethal Screen in Organoid Avatars to Discover Novel Therapeutic Targets for Colon Cancer

5a. CONTRACT NUMBER
W81XWH-19-1-0847

5b. GRANT NUMBER
W81XWH-19-1-0847

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

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Columbia, SC 29208-3403

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 application addresses the FY18 PRCRP topic area of Colorectal Cancer. Military relevant risk factors such as ionizing radiation often produce cancers with mutations in TP53. This proposal seeks to address a gap in colorectal cancer treatment that has profound impact on the health and well-being of military service Veterans, their beneficiaries, and extends to the American public as a whole. TP53 mutations are common in colon cancers (50%), and most often lead to resistance to chemotherapy. A systematic screen for synthetic lethal interactions between gene knockout, TP53 mutation, and chemotherapy such as Cisplatin will produce novel targets useful for treating a large fraction of colon cancers.
Hypothesis: Three-way synthetic

15. SUBJECT TERMS
None listed.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	11	19b. TELEPHONE NUMBER *(include area code)*

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Introduction.

This progress report describes our completed work during the reporting period 09012019 to 09012020. The report is organized using the Statement of Work, with comments in each section addressing successes, challenges, and plans for the next reporting period. The overall progress has been hindered by disruptions caused by the global SARS-CoV2 pandemic. This event caused a shutdown of the lab operations, loss of key personnel, and significant delays due to COVID illness among the PI and CO I. Nonetheless, in spite of these challenges, some progress has been achieved and work is continuing with remaining resources and personnel. The impacts and workarounds will be briefly mentioned in each relevant section.

Keywords: Colon Cancer. TP53. Drug Resistance. CRISPR. Gene Knockout. Synthetic Lethal Interaction.

Accomplishments.

Regulatory Review

Local IRB review and approval for non-human subjects classification of permanently de-identified, discarded surgical specimens. IRB protocol Pro00022064: "The Palmetto Health - University of South Carolina Biorepository"(continuously approved since 2004) was re-approved on April 5 2019.

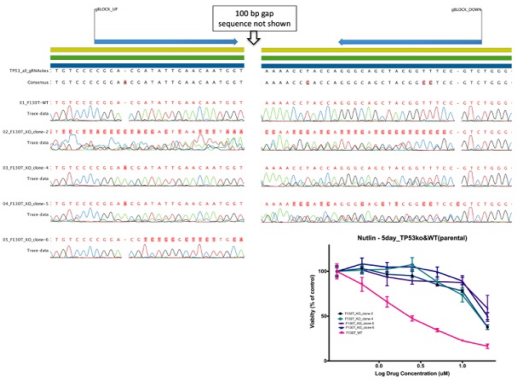
Specific Aim 1: Characterize existing and new TP53-KO derivatives of colon cancer organoids by screening the NCI Approved Oncology Drug Set IV (AOD IV, 129 drugs) and identify those drugs for which knocking out TP53 **causes** resistance. Characterize organoids by Nutlin sensitivity to determine TP53 status, and identify both TP53 WT and TP53 KO derivatives.
Major Task 1: Identify all drugs in the AOD set IV that are rendered ineffective by p53 deletion. Test on at least 10 knockout derivatives.

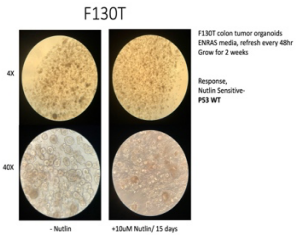
We have established over 107 viable organoids from colorectal tumor/normal pairs. These tumors were proven to be tumors (and not normal) by demonstrating their abilities to grow in the absence of WNT ligand (ENRAS media). The Normal organoids were proven to be normal by demonstrating their dependence on WNT ligand in the cell culture media (WENRAS media).

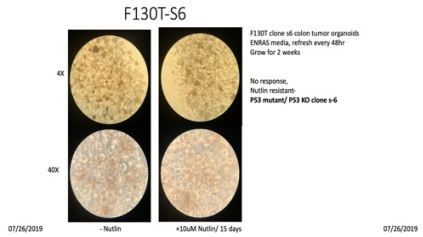
We have characterized 48 tumors for Nutlin sensitivity, and found 25 tumors that are Nutlin sensitive, indicating they have wild-type TP53 gene sequence and functional p53 dependent cell cycle arrest or apoptosis pathway. We identified 20 tumors that are Nutlin resistant, indicating the presence of inactivating mutations in TP53 or other members of the p53 pathway. Three tumors showed mixed phenotypes, indicating the presence of both wild-type and mutant sub populations of the tumors.

We have made significant progress at creating the TP53 knockout clones from 10 TP53 WT organoids.

Organoid F130T (TP53 WT, Nutlin sensitive) was transfected with two gBLOCKs and individual clones expanded and tested for Nutlin sensitivity and TP53 gene disruption. All single-clone derivatives surviving transfection, puro selection, and clonal expansion were Nutlin Resistant and harbored mutations in both alleles of TP53, at the expected cut sites targeted by the two CRISPR-Cas9 gRNAs.

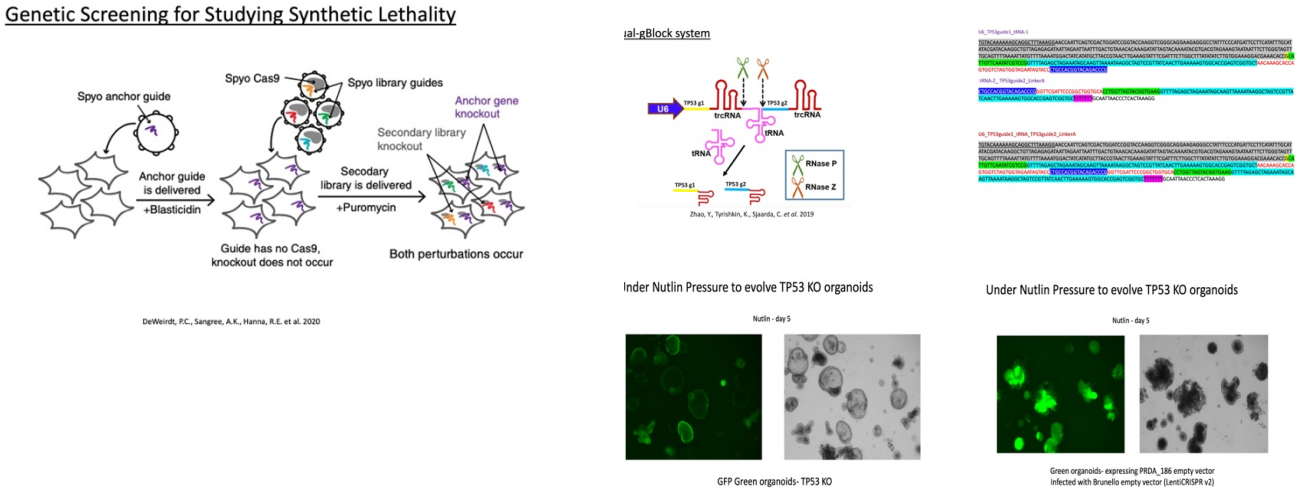






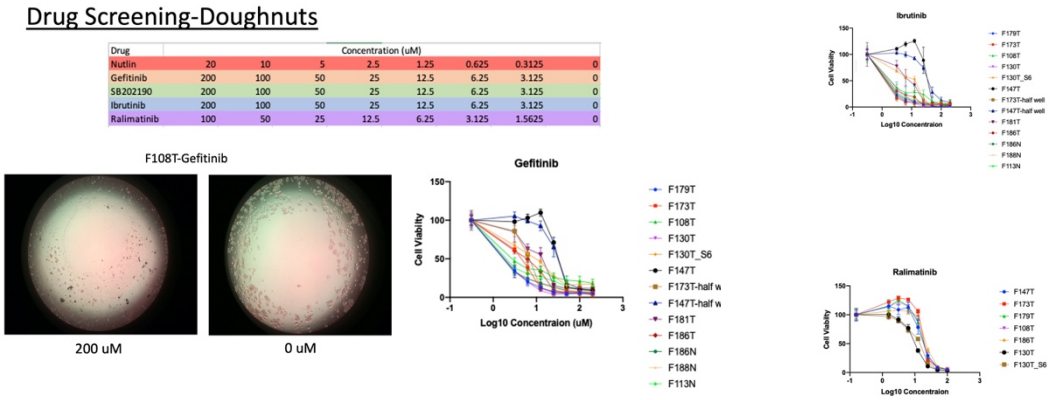
We encountered technical difficulties making additional knockout clones. Transfection of organoids with lipofectamine resulted in very small numbers of clones that often would not survive single cell clonal expansion. Different organoids have different sensitivities to single cell cloning and clonagenic survival, and this proved to be a barrier to creating sufficient numbers of TP53 knockout clones. We therefore developed an alternative approach based on the recent published work by DeWeirdt, et al that enables us to make large numbers of knockouts by viral infection. The approach pre-loads organoids with a lentiviral construct encoding guide RNAs targeting TP53, but containing no CAS9. It is phenotypically neutral to select large amounts of these lentiviral transduced cells using Blasticidin. The derivative organods are then transduced with a second lentiviral construct encoding a CAS9 nuclease, and if needed, also encoding guide RNAs against other genes to test for synthetic lethality. The pool of independent knockouts are created

in-situ, and amendable to drug and crispr library screening. We made improvements on the strategy by designing multi-cistronic guide RNAs processed by an intervening tRNA sequence (adapted from Zhao, et al, 2019). The strategy utilizes a dual- guide RNA gBlock design. A single U6 promoter driving a bi-cistronic tracer RNA encoding two different guide RNAs is in terrupted by a tRNA, which is processed by endogenous splicing machin ery, liberating the independent gRNAs in their respective scaffolds. The system was tested on cell lines and organoids and can efficiently generate large numbers of pools of knockout clones when delivered via



lentiviral vector system. (see figure). These organoids are undergoing expansion and will be tested for sensitivity/resistance to AOD IV in the coming months. The advantage of this system is that it is generalizable, it works across many different organoid avatars, and it does not require clonal expansion from single cells. This, the system by DeWirdt, et al is ideal for overcoming obstacles.

We have initiated drug screening on tumors and one TP53 knockout derivative in 96well plates. Examples shown are Gefitinib, Ibrutinib, and Ralimatinib. A total of 12 drugs have been screened and the results are show in Appendix 2. The system is established and able to utilize the tp53knockout derivatives generated in-situ with the dual vector system shown above. The viral constructs are prepared and we anticipate no further delays creating knockouts and screening the remainder of AOD set IV.

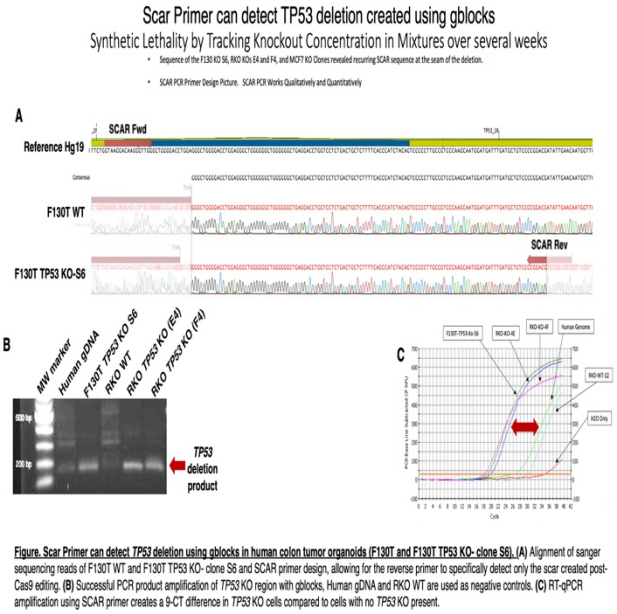


Specific Aim 2: Perform a GECKO v2.0 CRISPR library screen on TP53 WT and KO derivatives (6) of tumor organoids in the presence and absence of chemotherapies for which TP53 somatic mutations and engineered knockout render the organoids resistant (such as Cisplatin). Identify CRISPR gene knockouts that re-sensitize only TP53 knockout organoids to sub lethal concentrations of chemotherapy.

Major Task 2: Screen CRISPR library in cell fiber systems hollow fiber bioreactor.

The library screen will be done using long term culture conditions to detect slight changes in evolutionary fitness. We have tested our organoid evolution strategy by mixing together tp53 WT and Ko clones and tracking their relative abundance over time, in the

presence and absence of Nutlin. Knockout locus specific PCR primers were utilized and were sufficient to measure very slight changes in concentration of alleles, over very wide dynamic range. This system will form the basis of tracking knockout scars generated during the library screen, and quantifying knockout over wild type alleles grown as mixtures.



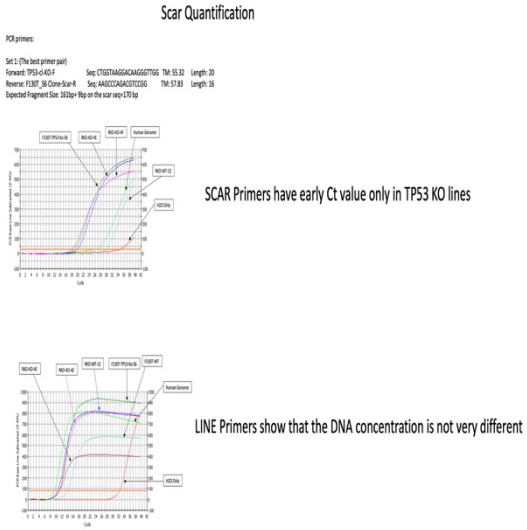
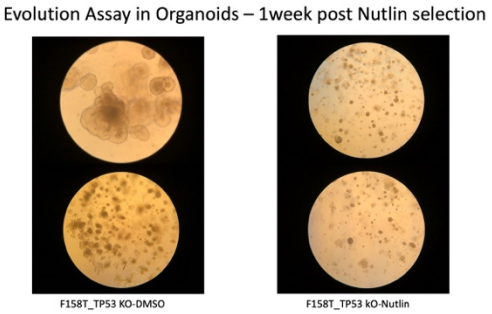
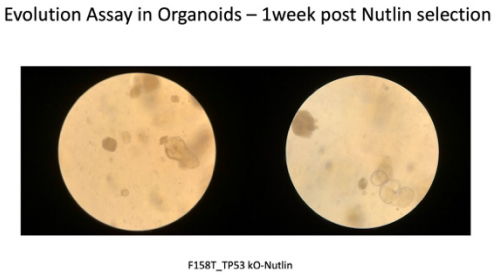
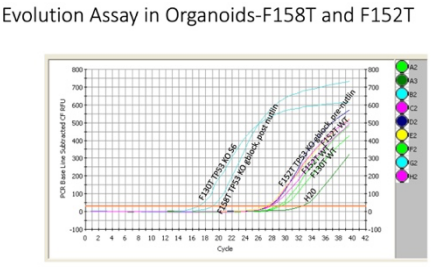
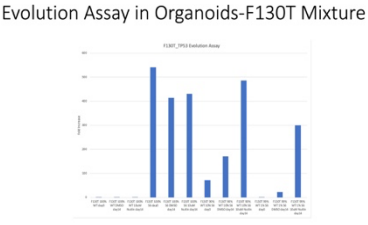


Figure. Scar Primer can detect *TP53* deletion using gblocks in human colon tumor organoids (F130T and F130T TP53 KO- clone S6). (A) Alignment of sanger sequencing reads of F130T WT and F130T TP53 KO- clone S6 and SCAR primer design, allowing for the reverse primer to specifically detect only the scar created post-Cas9 editing. **(B)** Successful PCR product amplification of *TP53* KO region with gblocks, Human gDNA and RKO WT are used as negative controls. **(C)** RT-qPCR amplification using SCAR primer creates a 9-CT difference in *TP53* KO cells compared to cells with no *TP53* KO present.









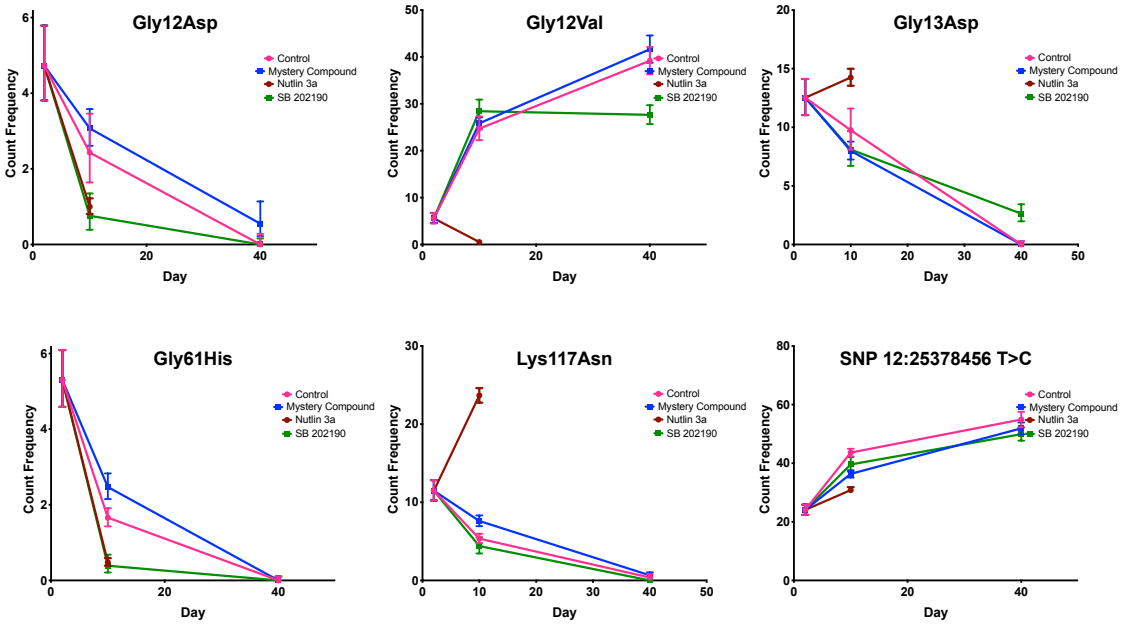
Hollow fiber bioreactor will be used for large scale mixtures of transfected organoids and tracking the evolutionarily fitness of the knockout subclones during library screening.

We anticipate screening the Brunello library using the modified strategy of DeWirdt et al over the next several months.

Major Task 3: Sequence exomes after 2 months chemotherapy selection. Identify somatic mutation evolution under pressure.

We have only begun a pilot study of whole exome sequencing of organoids. To date, 32 organoids have been sequenced, and 4,431 nonsynonymous somatic mutations identified. Somatic mutations to canonical cancer genes were identified. In vitro evolution experiments have been performed using mixtures of up to 10 organoids, as proof of principal for chemotherapy selection. We utilized SB202190 and Nutlin as selective agents and were easily able to detect Darwinian advantage of the TP53 mutant organoids over wild type organoids.

We identified private SNVs in 8 tumors and designed amplicons to cover these unique variant loci. We created a mixture of the tumors and cultured in absence and presence of select drugs for 40 days, periodically sampling and performing deep amplicon sequencing to assess the variant allele frequencies (and by proxy, their constituent organoids)…as can be seen in the figure, the organoid harboring the Gly12Val mutation (in the KRAS gene) was strongly inhibited by Nutlin but unharmed by control or other drugs. In contrast, the organoid harboring the KRAS Gly13Asp mutation was unaffected by other drugs (relative to control) but under strong positive Darwinian fitness advantage in Nutlin. This procedure is being used to track unique organoids by somatic mutations, CRISPR scars, and crispr clone bar code amplicons during library screening.



Specific Aim 3. Validate candidate genes identified in CRISPR knockout screen in the presence of chemotherapy by constructing specific clonal knockouts in colon tumor organoids that are TP53 WT, KO and somatically mutated TP53 (in diverse and relevant somatic mutation backgrounds) and determining IC50s for select chemotherapies as a function of gene disruption.

Major Task 1. Construct knockouts.

We have generated gBLOCK dual gRNA clones targeting 4 candidate genes identified in a library screen of human embryonic stem cells. Phenotyping in presence and absence of cisplatin is underway.

Impact.

Technical advances in drug screening (doughnut assay) and knockout generation (dual vector system and dual gBLOCK design) have been made and this will have substantial impact on forward progress for genetic engineering and drug testing.

Changes/Problems

The pandemic of 2020 caused by SARS-CoV-2 emerging from Wuhan China has had substantial negative impact on this project. Key personnel were lost during the shutdown and operations were harmed in the following manners.

1. Postdoctoral Fellow Candace Poole. She was unable to return to lab due to hazards of being exposed to SARS-CoV-2.
2. Organoid Biobanking / Honest Broker Gabriella Freeze. She was unable to return to the lab due to the hazards of being exposed to SARS-CoV-2.
3. CO-investigator Dr Carolyn Banister has devoted a large percentage of her time to establishing and running a COVID19 CLIA-certified testing laboratory, and her time and effort will be minimal going forward.
4. Surgeries for colon cancers slowed and the influx of new organoids stopped. We are making use of existing bio-banked specimens (about 100 patients) but are behind on our goals for recruiting new samples.
5. Lab was shut down and university closed from March to August, with limited / distanced occupancy during August to December. Research infrastructure is running on skeleton crew, with most people working from home.
6. Out of necessity to keep lab open, the Lead PI developed a novel saliva-based covid test during university lockdown, and shepherded statewide adoption of testing pipeline to allow reopening. These activities, combined with getting COVID himself, were more than a little disruptive.

Changes going forward.

A one year extension is requested in order to recruit replacement post-doctoral fellow.

Dr. Carolyn Banister will continue to contribute to the project, but will no longer be salaried by the project. She is being paid full time by the University as her role in directing a clinical diagnostic genomics laboratory (covid testing). Additional personnel (Ph.D. student) will be utilized to complete the aims of the project.

Request additional funds to cover one year salary for postdoc and lab technician/organoid biobanking person.

Products.

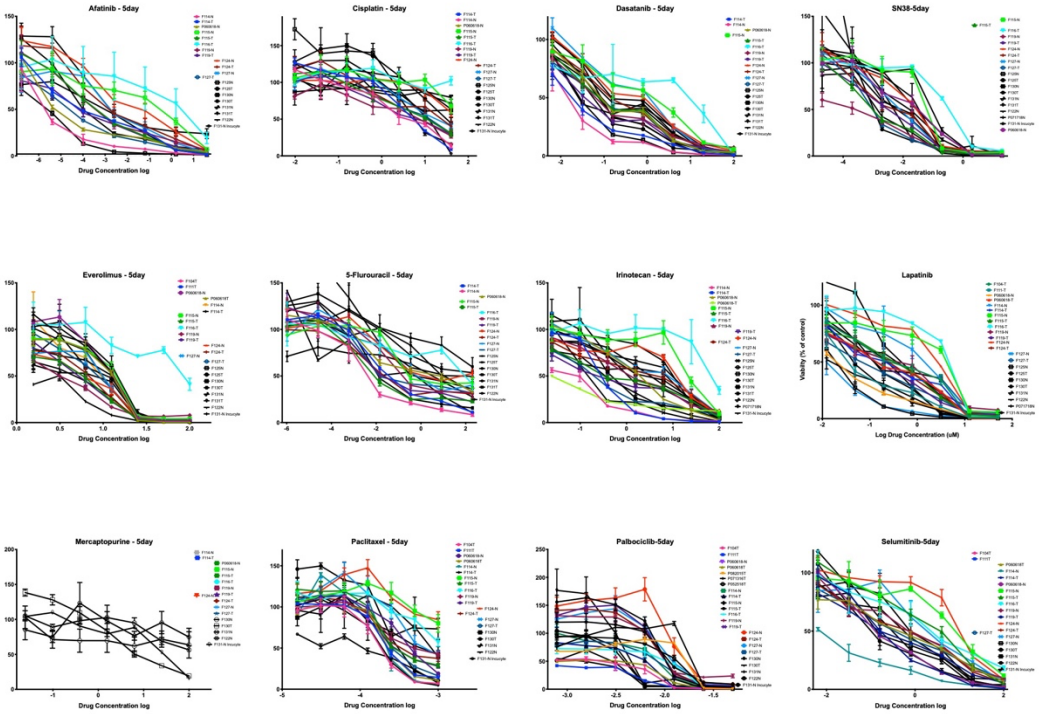
NA

Participants and other collaborating organizations
NA

Special Reporting Requirements
NA

Appendices

Appendix 1. 12 drug panel tested on organoids.



Appendix 2. Table of organoids established and used for study.

count	Tumor Organoid	Age	Ancestry	Gender	Nutlin	Num of frozen vials
1	F104	54	African American	M	Sensitive	35
2	F111	50	European American	M	Sensitive	36
3	F112	51	African American	M	Sensitive	7
4	F113	49	African American	F	Sensitive	20
5	F114	61	African American	M	Sensitive	4
6	F115	67	European American	M	Sensitive	21
7	F119	64	European American	F	Sensitive	16
8	F124	76	European American	F	Sensitive	17
9	F130	76	European American	F	Sensitive	>166
10	F152	59	African American	F	Sensitive	123
11	F158	67	African American	F	Sensitive	94
12	F173	84	European American	F	Sensitive	>29
13	F174	62	European American	M	Sensitive	5
14	F177	68	European American	M	Sensitive	18
15	F181	76	European American	F	Sensitive	14
16	F186	41	European American	M	Sensitive	>17
17	F190	94	European American	M	Sensitive	0
18	F195	64	African American	F	Sensitive	0
19	F196	68	African American	M	Sensitive	0
20	F198	58	African American	F	Sensitive	0
21	P020216	57	European American	M	Sensitive	14
22	P021916	86	European American	F	Sensitive	12
23	P050818	65	African American	M	Sensitive	4
24	P082015	85	African American	F	Sensitive	81
25	P101515A	60	African American	F	Sensitive	1
26	F131	50	African American	F	Resistant?	6
27	F138	60	European American	M	Resistant?	3
28	F108	64	European American	F	Resistant	41
29	F109	87	European American	F	Resistant	37
30	F116	56	European American	F	Resistant	97
31	F125	42	European American	M	Resistant	2
32	F127	53	European American	M	Resistant	14
33	F130T_S6 (TP53KO)	77	European American	F	Resistant	>140
34	F169	54	European American	M	Resistant	10
35	F171	50	European American	M	Resistant	
36	F176	77	European American	M	Resistant	22
37	F179	64	European American	F	Resistant	35
38	F180	70	European American	M	Resistant	8
39	F182	79	European American	F	Resistant	10
40	F191	52	European American	M	Resistant	0
41	F193	59	European American	F	Resistant	0
42	F197	41	African American	F	Resistant	0
43	P050918	72	European American	M	Resistant	7
44	P052518	77	African American	F	Resistant	13
45	P060618	45	European American	M	Resistant	8
46	P110415	67	Asian American	M	Resistant	2
47	P120417	53	African American	F	Resistant	20
48	F147	83	European American	F	Mix	>39

UNIVERSITY OF
SOUTH CAROLINA
College of Pharmacy

Stephen J. Cutler, Dean

January 19, 2021

Department of Defense
Re: Award number W81XWH1910847. Request for extension and supplemental funding.

To whom it may concern,

It is with pleasure that I'm able to write a letter of support for the extension and supplemental funding for the Department of Defense award entitled "TP53 Synthetic Lethal Screen in Organoid Avatars to Discover Novel Therapeutic Targets for Colon Cancer" that Dr. Phillip Buckhaults serves as the PI. This is a two-year research grant focused on developing novel therapies for colon cancer.

The COVID-19 pandemic caused massive disruption to operations at the University of South Carolina. In January of 2020, Dr Buckhaults had a vision to help with the pandemic by using his expertise to develop a new method for diagnosis respiratory infections, including COVID-19. This methodology allowed the University of South Carolina to open safely and ensure our campus was healthy during the fall semester. Of course, this in turn had an impact on his Department of Defense research program.

The purpose of my letter is to respectfully request a one-year extension of the timeline for the deliverables for this grant. Further, my request is for additional funds of $120,000, which would enable Dr Buckhaults' lab to replace lost research personnel and recover lost time focused on his research obligations to the DoD.

It is with great enthusiasm and without hesitation that I give my support of Dr. Buckhaults to extend his project and secure additional funding. He is a remarkable scientist and is advancing our understanding of colon cancer as well as developing new methods of testing for respiratory infections during the COVID-19 pandemic.

Please feel free to reach out to me should you require additionally information.

Sincerely,

Stephen J. Cutler, Ph.D.
Dean and Professor

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