AWARD NUMBER: W81XWH-19-1-0693

TITLE: Targeting Oncogene Amplification in Glioblastoma

PRINCIPAL INVESTIGATOR: Ana C. deCarvalho

CONTRACTING ORGANIZATION: Henry Ford Health System, Detroit, MI

REPORT DATE: December 2022

TYPE OF REPORT: Final

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
December 2022	Final	15Aug2019-14Aug2022

4. TITLE AND SUBTITLE

Targeting Oncogene Amplification in Glioblastoma

5a. CONTRACT NUMBER
W81XWH-19-1-0693

5b. GRANT NUMBER
CA180174

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
deCarvalho, Ana C.

E-Mail: adecarv1@hfhs.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

HENRY FORD HEALTH SYSTEM
HENRY FORD HOSPITAL
1 FORD PLACE
DETROIT MI 48202 -3450

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

High grade gliomas are still untreatable, in part due to co-deregulation of multiple oncogenic pathways, genetic variation within and between tumors. Using patient derived models we addressed these challenges by integrating genomic, molecular, and phenotypic data with response to pharmacological inhibitors targeting reactivation of retinoblastoma and p53 pathways. We tested the efficacy of the combination of these inhibitors with radiation treatment in vitro and in vivo. Anticipating the development of resistance, we investigated the transcriptional reprograming and cellular phenotypes in longitudinal response to treatment. Sensitivity and adaptation to the treatments was tumor specific, even in a primary-recurrent pair. We also validated therapeutic targets for which effective inhibitors were not available by using RNA-interference mediated knockdown of DNA damage regulator DNA-PK, which was effective in combination with radiation, temozolomide and CDK inhibitors; or by comparing PDGFRA ecDNA positive and negative subclones from the same tumor.

15. SUBJECT TERMS

Pre-clinical studies; patient-derived models; glioblastoma intra-tumoral heterogeneity, DNA-dependent protein kinase; retinoblastoma protein - cyclin-dependent kinase, mouse double minute homologue -p53, extra-chromosomal DNA; PDGFRA.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	23	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

1. **INTRODUCTION**

Glioblastoma, the most aggressive primary brain tumor, is driven by genomic abnormalities leading to deregulations in retinoblastoma (Rb), p53, and receptor tyrosine kinase (RTK) pathways. The most prevalent mechanism of oncogene activation in glioblastoma is through somatic gene amplification, which frequently takes place in circular extrachromosomal DNA (ecDNA). ecDNA amplification leads to heterogenous overexpression of known drivers of malignancy, many of which are suitable therapeutic targets, including: cyclin-dependent kinase 4 (CDK4), a negative regulator of Rb; mouse double minute 2 homolog (MDM2), a negative regulator of p53; and EGFR and PDGFRα RTKs. All these amplified oncogenes are represented in the panel of glioblastoma patient-derived cancer stem cells (CSC) and mouse orthotopic xenografts (PDX) we developed for preclinical studies. Importantly, we have included models from matched newly diagnosed and recurrent glioblastoma from the same patient, to determine longitudinal treatment-induced molecular and genomic changes, which have clinical relevance. Optimization of targeted therapy in combination with the standard of care for glioblastoma has been impeded by the lack of data to guide strategies. For this funding period, we report results from experiments measuring target engagement in response to the inhibitors, namely increase in p53 transcriptional activity in response to MDM2 antagonists and decrease of phosphorylated Rb in response to CDK4/6 inhibition. We have investigated sensitivity of CSCs to CDK4/6 inhibitors, and longitudinal reprograming of transcriptome and cellular functions in response long term treatment.

Non-homologous end joining (NHEJ) is responsible for the repair of the majority of DNA double strand breaks (DSB) in somatic cells. DNA-dependent protein kinase catalytic subunit (DNA-PKcs) has an essential role in NHEJ, and therefore has been implicated in tumor cell resistance to DSB-inducing treatments, in addition to interacting with other oncogenic pathways. We tested two selective small molecule inhibitors of DNA-PKcs in pre-clinical and early clinical development for cancer treatment, both of which did not achieve enough activity in the mouse PDX brain. To validate DNA-PK as a therapeutic target for glioblastoma, we used shRNA-mediated knockdown of the gene coding for DNA-PKcs. The pharmacological inhibition of DNA-PK in vitro sensitized GBM CSCs of diverse genomic background to radiation treatment, but the effect of partial shRNA-mediated knockdown sensitized GBM PDXs that are resistant to all treatments tested not only to radiation, but also to temozolomide and CDK4/6 inhibitors, showing a remarkable promise for clinical applications. We continue to work beyond this award to complement this promising study.

The combined data from this project lead us to the following general conclusion: The vulnerability of glioblastoma to single agent or combination treatment, as well as cellular responses to various treatment vary considerably among the patient-derived models, and the genomic landscape of individual tumors provide limited prediction of response to treatment. This validates our premise that the aggregate of experimental and omics data is essential to drive more effective treatments for our patients. Our work illustrates the importance of integrating multi-modality data to overcome these challenges, which truly depends on complementary expertise of the teams, represented here by the biostatistics group led by Dr. Poisson, and the clinical perspective guiding the translational value of our research offered by co-investigator and neuro-oncologist Dr. Snyder. The parallel pre-clinical studies anticipate the range of responses of MDM2 antagonists and CDK4/6 inhibitors in ongoing clinical trials for glioblastomas and offer insights into the mechanisms of resistance and strategies for combination with the standard of care. Our work provides evidence supporting the clinical development of effective and brain-penetrant inhibitors of DNA-PK and PDGFRα.

2. **KEYWORDS:** Glioblastoma; intra-tumoral heterogeneity; patient-derived models; radiation therapy (RT); cyclin-dependent kinase 4 (CDK4); mouse double minute 2 homolog (MDM2); p53 pathway; retinoblastoma (Rb) pathway; receptor tyrosine kinase (RTK), combination therapy; oncogene amplification; extra-chromosomal DNA (ecDNA); DNA-dependent protein kinase (DNA-PK); target engagement.

3. **ACCOMPLISHMENTS:**

What were the major goals of the project?

(Some timelines have been updated to reflect the NCE period):

Aim 1. Determine the efficacy of novel inhibitors targeting MDM2 and CDK4 in the treatment of glioblastoma and the role of ecDNA dynamics in resistance to therapy.

- Major task 1: Clonal analysis of MDM2 and CDK4 amplified tumors.

1.1. Isolate single cell clones from MDM2 and CDK4 ecDNA amplified glioblastoma tumors and test for levels of amplification (Sep/19 – Apr/20): 100% complete

1.2. Compare the tumorigenic potential of one ecDNA(+) and one ecDNA(-) clone (Mar/20 – Nov/20): 100% complete.

Milestone (year 2): characterization of ecDNA(+) and ecDNA(-) single cell clones (Jul/21): 100% completed

- Major task 2. Pharmacological inhibition of MDM2 and CDK4 in glioblastoma neurosphere cells

2.1. Determine dose-response curves for 12 NS to two MDM2 and two CDK4 inhibitors (Oct/19 – Apr/20): 100% complete

2.2. Verify specificity of inhibitors in engaging the target (Apr/20 – Jun/21): 100% complete

2.3. Long term treatment (Jun/20 – July/21): Long term treatment 4 CSCs with CDK4/6 inhibitors: 100% complete

2.4. Combination therapy: inhibitors and radiation (April/20 – April/21): 100% complete

Milestones (year 1): relative response of all 12 NS lines to the 4 inhibitors (achieved) and target engagement evaluation of the inhibitors to sensitize glioblastoma cells to the standard of care (RT/TMZ)

Milestones (year 2): Cytogenetics (FISH) evaluation of adaptive response to long term treatment (Dec/21): This approach has been modified, and adaptive response to long term treatment was analyzed by transcriptome and FACS.

- Major task 3: MDM2i and CDK4/6i efficacy in PDX

3.1. Pilot study to test tumor levels and target hit for MDM2i and CDK4/6i (Oct/19 – May/20): 100% complete

3.2. PDX Treatment (Oct/19 – Feb/22): 100% complete

Milestone (year 2): efficacy of MDM2 and CDK4 inhibition as monotherapy or in combination in a glioblastoma PDX panel and effect on ecDNA.

- Major task 4: Adaptive response by RNAseq analysis: 100% complete

Aim 2. Determine to what extent targeting DNA-PK activity in glioblastoma impairs ecDNA propagation, in addition to increasing susceptibility to treatment-induced and endogenous DNA damage toxicity.

- Major task 5: DNA-PK pharmacological inhibition

5.1. Determine dose-response curves for 12 NS to two DNA-PK inhibitors (Oct/19 – Feb/20): 100% complete

5.2. Verify specificity of inhibitors in engaging the target (Feb/20 – Mar/20): ongoing

5.3. Short-term combination therapy (Apr/20 – Jun/20) 100% complete

5.4. Long term treatment (Jun/20 – Oct/20): replaced by shRNA-mediated knockdown, ongoing

Milestones (Year 1): Relative sensitivity of the panel of NS lines to DNAPKi as a monotherapy and in combination to RT/TMZ, and efficacy of the inhibitors in inhibiting the target (Jun/20).

Milestone (Year 2): Long term effect of DNAPKi in ecDNA maintenance (Apr/22)

- Major task 6: DNA-PK knockdown (KD)

6.1. Transduction of 3 CSC lines with lentiviral shRNA constructs targeting PRKDC (gene encoding DNA-PKcs) and control. (Sep/19 – Mar/20): 100% complete

6.2. Impact of KD on oncogene expression and ecDNA copy number (Mar/20 – Dec/21). 50% complete

- Major task 7: PDX treatment

7.1. Pilot brain penetrance and target engagement study for two DNAPK inhibitors (Mar/20 – Aug/20): 100% complete. Target engagement results repeated in the fall 2021 demonstrated poor brain penetration and or activity in vivo.

7.2. PDX treatment of control and DNAPK knockdown cells (Oct/21 – May/22): Due to lack of activity of the pharmacological inhibitors tested, we completed this with KD of PRKDC only.

Milestones (Year 2): efficacy of DNAPK inhibition as monotherapy or in combination in a glioblastoma PDX, and effect on ecDNA (May/22)

What was accomplished under these goals?

Below are the main accomplishments during the reporting period:

A) Cellular response and transcriptional reprograming in response to long term treatment of glioblastoma cancer stem cells with CDK4/6 inhibitors.

Long term CDK4/6 inhibition can lead to senescence or development of resistance. In our experimental design, we have measured response to inhibitors at 4 and 7 days. Based on these results we have chosen inhibitor concentrations to treat select GBM CSCs for longer periods to measure CSC adaptation and cellular responses. We employed flow cytometry (FACS) to measure cell cycle, apoptosis and senescence after 7 and 14 days of treatment, and after treatment for 14 days followed by 7-day drug withdrawal ("day 21"), to measure reversibility. Treated and control samples were compared by RNAseq at longer time points, on days 14 and 42 of treatment, and after 42-day treatment followed by 14-day drug withdrawal ("day 56") (Fig. 1). Controls for each time point were treated with vehicle.

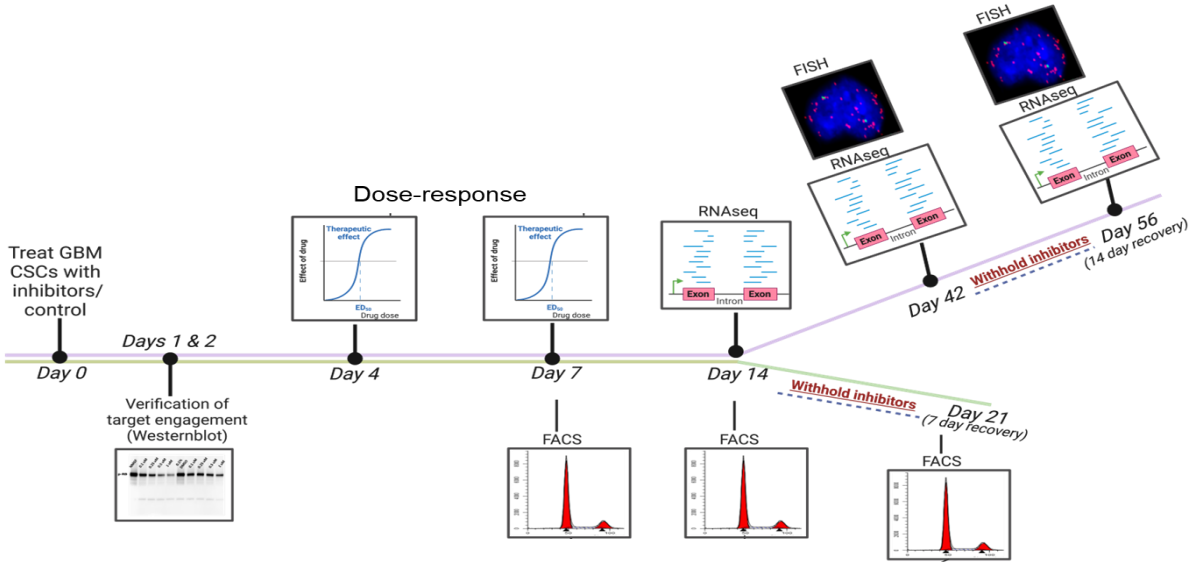


Figure 1. Experimental design to evaluate response of GBM CSCs to CDK4/6 inhibitors.

A comparative sensitivity for each of the CSC lines to CDK4/6 inhibitors ribociclib and abemaciclib suggest that key genomic features thought to predict response, such as CDK4 amplification and CDKN2A deletion, did not correlate with response in our data set. Importantly, the RB1 null line, HF3203, was highly resistant to ribociclib, but less resistant to Abemaciclib, indicating the former is more specific. Given the relative resistance of a subset of the CSC lines, we tested a new inhibitor, ebvaciclib (Pfizer), which also inhibits CDK2 in addition to CDK4/6. This triple inhibitor was highly effective in treating HF2354 and HF3253 but had no effect on the CDK4 amplified line HF3016, and a modest effect on HF3177 (recurrent tumor from the same patient as HF3016) (Fig. 2).

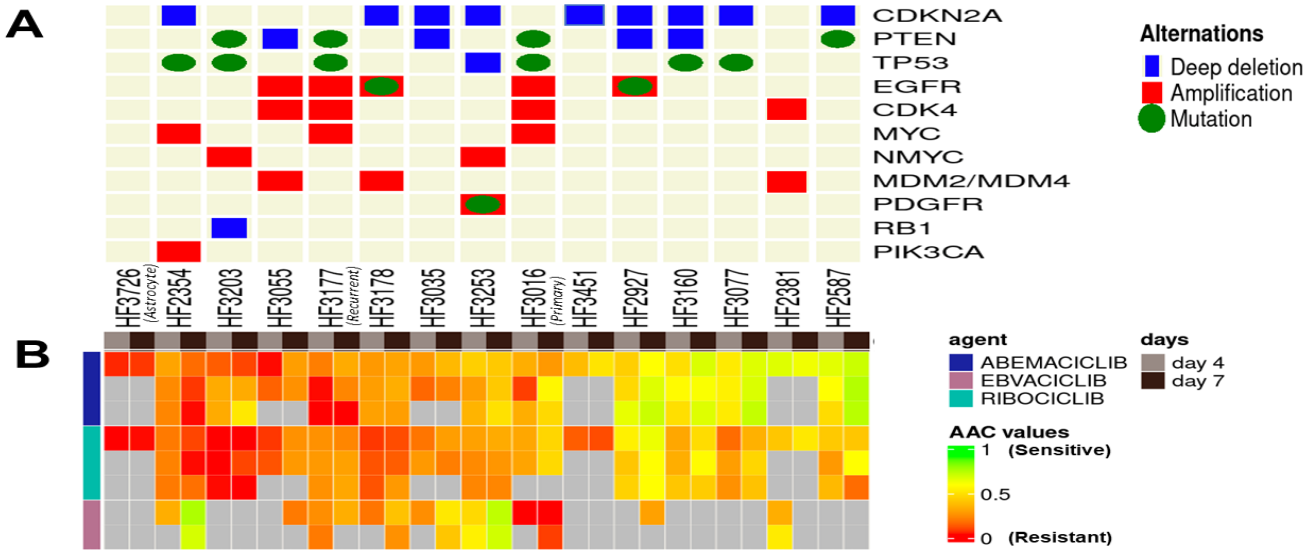


Figure 2. Comparative sensitivity of GBM CSCs to CDK4/6 inhibitors. A) Genomic landscape of 14 GBM CSCs and 1 astrocyte line (HF3726). B) Area above the curve (AAC) were calculated from dose response curves to 4 and 7-day treatment with abemaciclib, ribociclib or 7-day treatment with ebvaciclib. Results from 1 to 3 independent experiments for each group are shown.

Flow cytometry measurements of apoptosis (Fig. 3A), senescence (Fig.3B) and cell cycle (Fig. 3C) for three CSC lines, HF2354, HF3016 and HF3177 after 7 and 14-day treatment with DMSO, 2uM ribociclib and 50 nM ebvaciclib, and 14-day treatment followed by 7-day incubation in the absence of inhibitors ("Day 21") (n=3/group) is adding important insight to the longitudinal cellular responses to CDK4/6 or CDK4/6/2 inhibition.

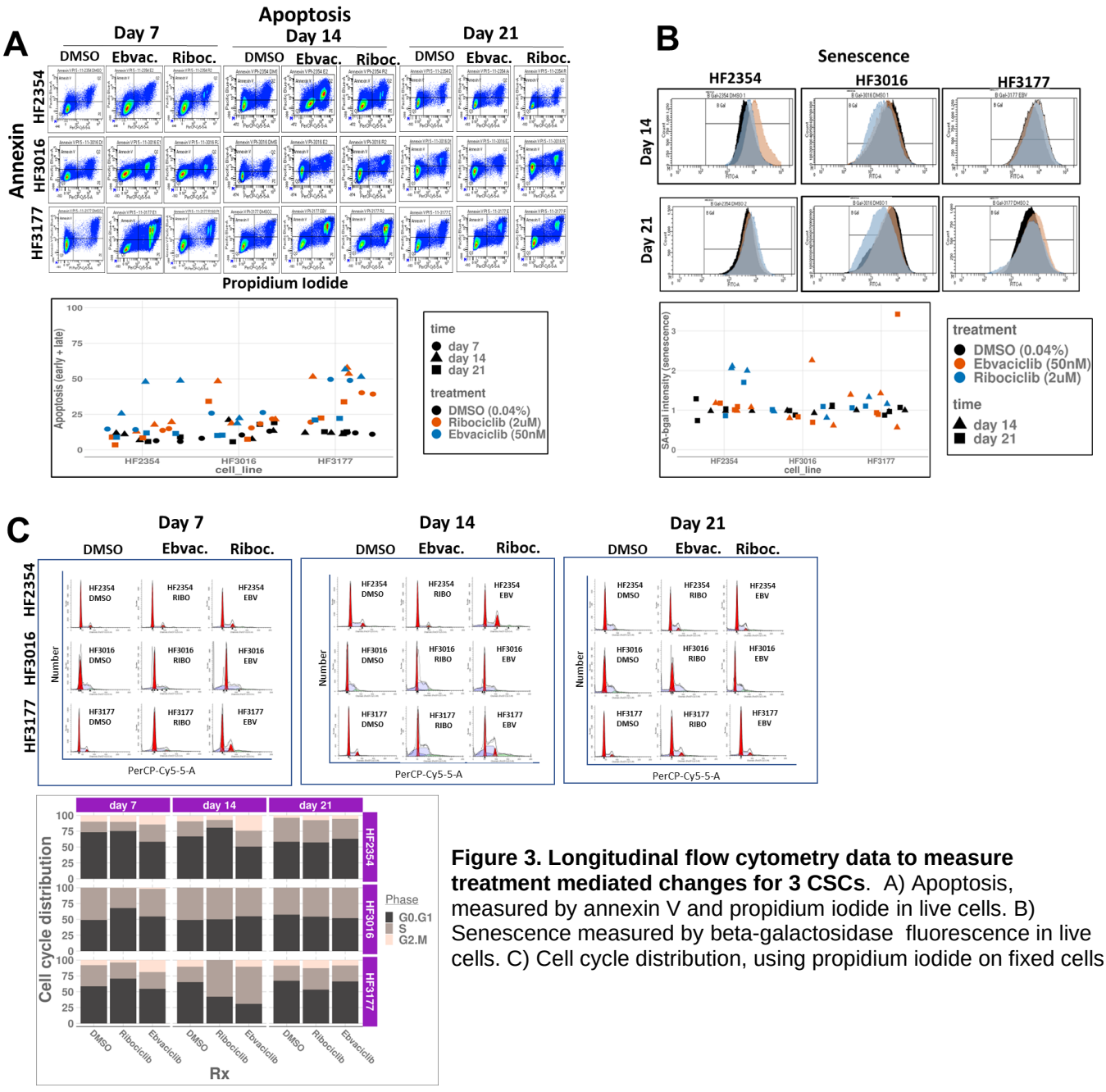


Figure 3. Longitudinal flow cytometry data to measure treatment mediated changes for 3 CSCs. A) Apoptosis, measured by annexin V and propidium iodide in live cells. B) Senescence measured by beta-galactosidase fluorescence in live cells. C) Cell cycle distribution, using propidium iodide on fixed cells.

Next, we investigated transcriptome reprograming in response to long term treatment with ribociclib, since we demonstrated greater specificity of this inhibitor. Since HF3016/HF3177 models were relatively resistant to ribociclib, although based on genomics, the patient would be expected to respond to CDK4/6 inhibitors, we collected samples treated with 1uM ribociclib and DMSO control at 14 and 42-day treatment (n=4) per group, Because the potential reversibility of cell cycle arrest after CDK4/6 inhibitor withdrawal, we also collected cells that have been treated for 42 days, and then incubated in the absence of drug for 14 days (Fig. 1). RNA was isolated for Illumina Truseq stranded mRNA libraries sequenced at 30M depth (Psomagen). Quantified raw counts were processed using NOISeq R package to determine differentially expressed genes (DEG) between control and RG7112-treated samples for each cell with q= 0.95 (NOISeq parameter) and log2FC >1.5 or <-1.5 cutoffs. The genes that are in ecDNA amplicons in these models are of particular interest (Fig. 4). Both HF3016 and HF3177, derived from the same patient and carrying CDK4

ecDNA amplification, presented a decrease in CDK4 expression relative to control, after 14-day treatment. For HF3016, the decrease persisted at 42-day treatment, but after drug withdrawal the expression of CDK4 in the previously treated cells was “overcompensated” to be higher than the control treated cells at the same timepoint. Expression of CDK4 was upregulated in treated cells at day 42 for recurrent HF3177 CSCs, and remained upregulated upon drug withdrawal. MYC, an important oncogene with potential treatment resistance implications here, is also amplified in a different ecDNA in the same cells. MYC was high upregulated after 42 days of treatment for both lines, while EGFR expression was unaltered or decreased with treatment. In Appendix 3, we show an example of gene enrichment analysis, focusing on DEGs at day 42 vs day 56 (42-day treatment and 14-day drug withdrawal), where we investigate 4 patterns of transcriptional reprograming. Analysis of this complex data is being refined for maximal biology content for a final figure for the manuscript.

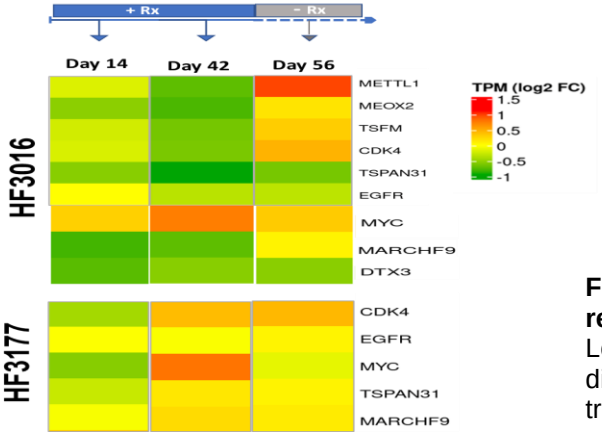


Figure 4. CDK4/6 inhibition mediated transcriptional regulation of genes located in amplified regions. Longitudinal analysis for select ecDNA-amplified genes differentially expressed between control and ribociclib treatment.

B) Acute response of glioblastoma CSCs to MDM2 antagonists

We have shown in previous reports the measurements of sensitivity of 7 wildtype p53 , 7 mutant p53 CSCs and 1 astrocyte line to two MDM2 antagonists in clinical trials for glioblastoma, AMG-232 and RG7112. Here we investigate the acute response of treatment with the IC50 concentrations of RG7112 (calculated for each line at day 4). Western blots show the expected increase in p53, MDM2 and p21 in response to 24h treatment with RG7112 (Fig. 5).

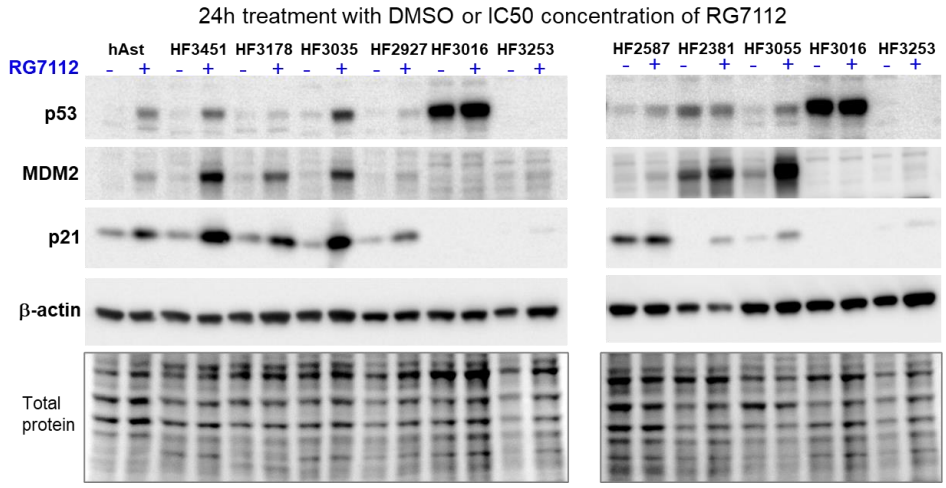


Figure 5. P53 and its targets MDM2 and p21 are upregulated in wt p53 CSCs in response to 24h MDM2 antagonist treatment. HF3016 (mutant p53) and HF3253 (null p53) were included as controls. HF3055 and HF2381 have amplified MDM2, consistent with the higher protein levels.

Based on the targeted specificity, we treated 7 wt p53 CSCs and 1 human astrocyte cell line using the same controls, RG7112 concentrations, and treatment time (24h) shown on Fig. 5. Cells were treated in triplicate and RNA was isolated for Illumina Truseq stranded mRNA libraries sequenced at 30M depth (Psomagen). Quantified raw counts were processed using NOISeq R package to determine differentially expressed genes (DEG) between control and RG7112-treated samples for each cell, with alpha=0.95 and log2FC>0.7 or <-0.7 cutoffs. Our goal was to identify

communalities in these gene lists, including direct transcriptional targets of p53, but also to identify dynamic biomarkers correlating with response to MDM2 antagonists. The number of DEGs varied considerably among the cell lines, and the intersection of the upregulated and downregulated DEG lists were significant, with high representation of p53 targets in the upregulated gene list for all cell lines (Fig. 6, top panels). From pathway enrichment analysis for each DEG list, using Comparative Pathway Integrator tool, we observed that, unsurprisingly, pathways associated with p53 activity scored high or all the upregulated DEGs. We also observed cell specific upregulation of survival pathways, such as negative regulators of programed cell death, ERK and NFkB, in less sensitive CSC lines (Fig. 6, lower left panel). E2F targets, G2-M cell cycle check point and DNA-repair pathways were highly enriched in the downregulated DEGs, except for HF2927 and HF2381 CSC lines (Fig. 6, lower right panel). E2F targets were present in the downregulated genes for these two lines, but due to the small number of genes did not achieve the statistical threshold for enrichment. It has been previously reported that most genes downregulated by p53 depend on p21 activity.

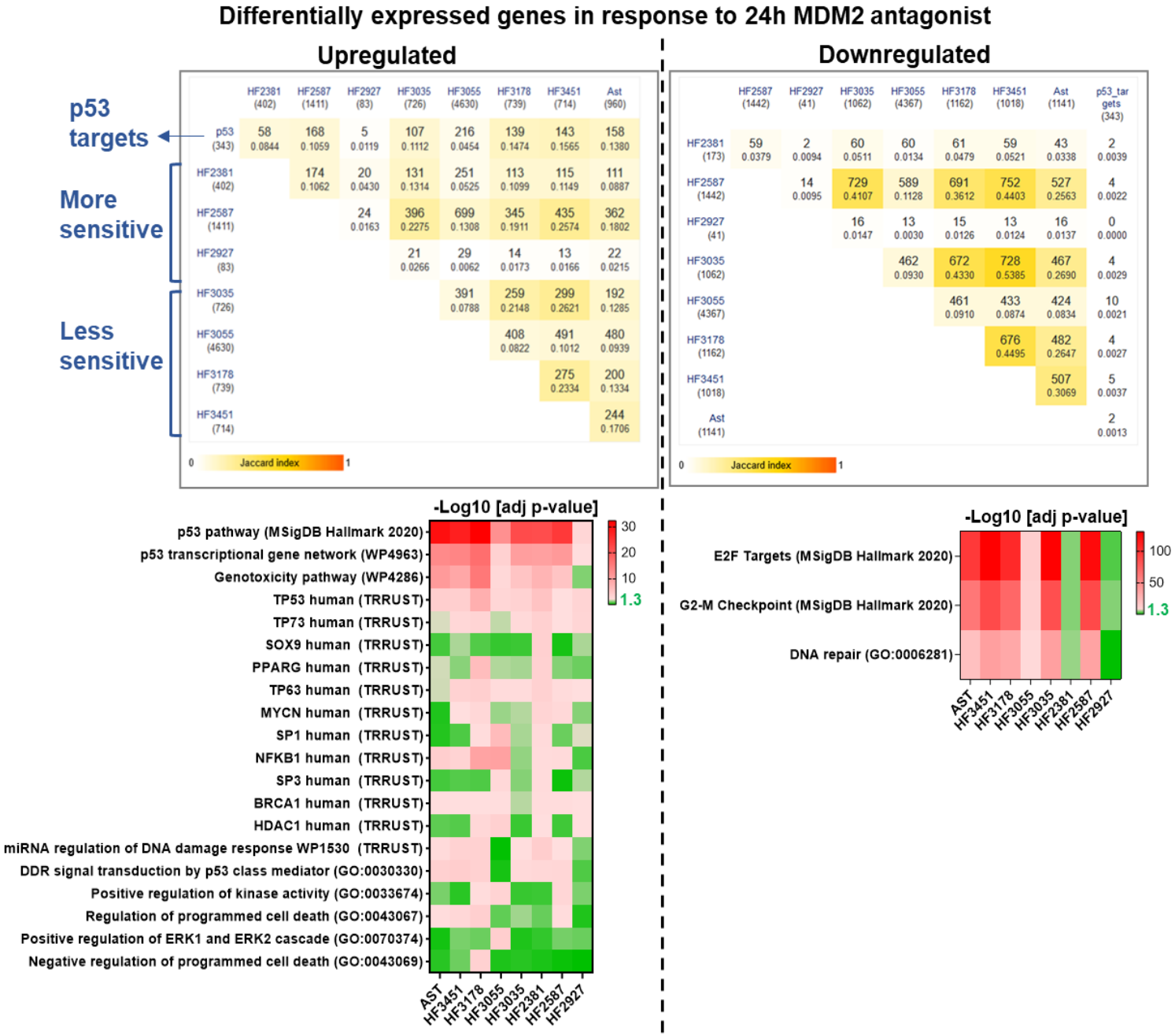


Figure 6. Transcriptome alterations in wt p53 glioblastoma CSCs and astrocyte control in acute response to RG7112 treatment. Top panels: Number of differentially expressed genes that were upregulated (left panel) or downregulated (right panel) are shown in parenthesis below the CSC ID. Heat map shows the Jaccard Index for the pairwise intersection. Validated p53 target gene list was included only in the upregulated gene analysis, as no overlap was observed for the downregulated genes. Bottom panels: enrichment analysis of the DEGs for each cell line. Red tones in the heatmap indicates adj p-value <0.05, and green adj p-values > 0.05.

C) Additional experiments testing the effect of MDM2 antagonist and CDK4/6 inhibitors in glioblastoma PDX

Abemaciclib has shown to be more potent and less specific then ribociclib in vitro (previous Annual Reports). We tested the efficacy of abemaciclib to treat the primary/recurrent PDX pair carrying CDK4 amplification. Remarkably HF3177 (recurrent) responded to the treatment while HF3016 did not (Fig. 7A). Based on the sensitivity of HF2927 CSCs to MDM2 antagonists and CDK4/6 inhibitors in vitro, we chose this PDX line to test the efficacy of these inhibitors but to our surprise, no treatment showed improvement in survival (Fig. 7B), we are analyzing the tumor tissue to determine this discrepancy.

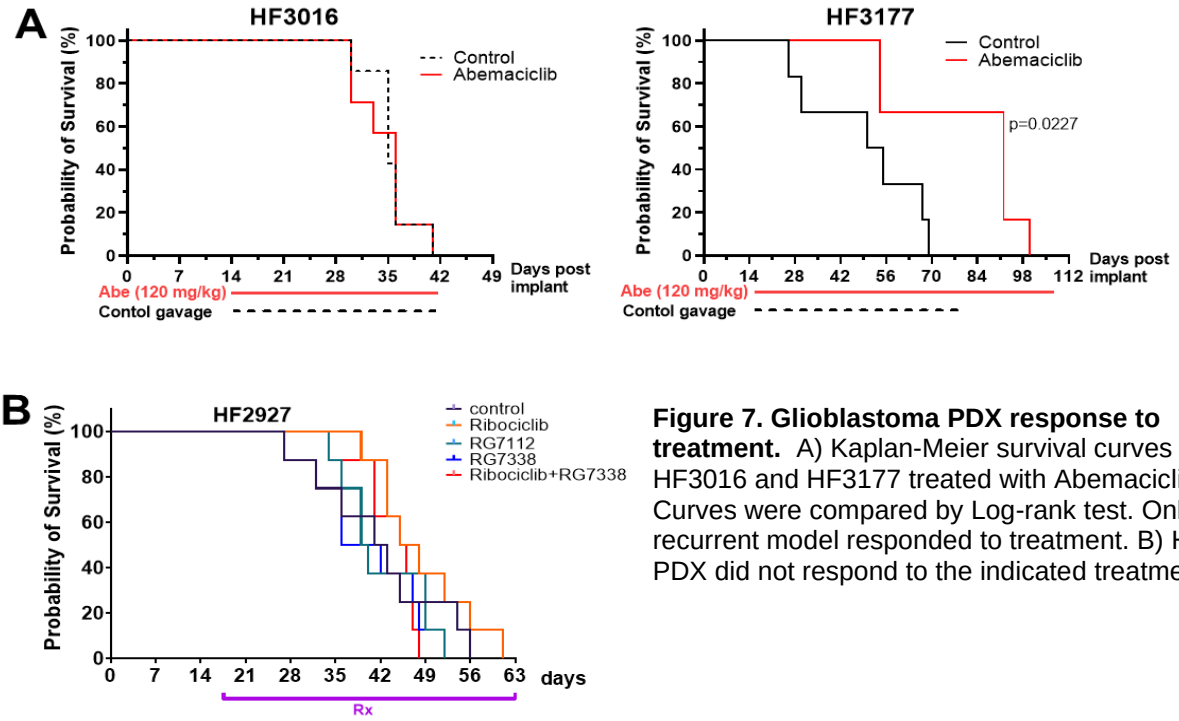
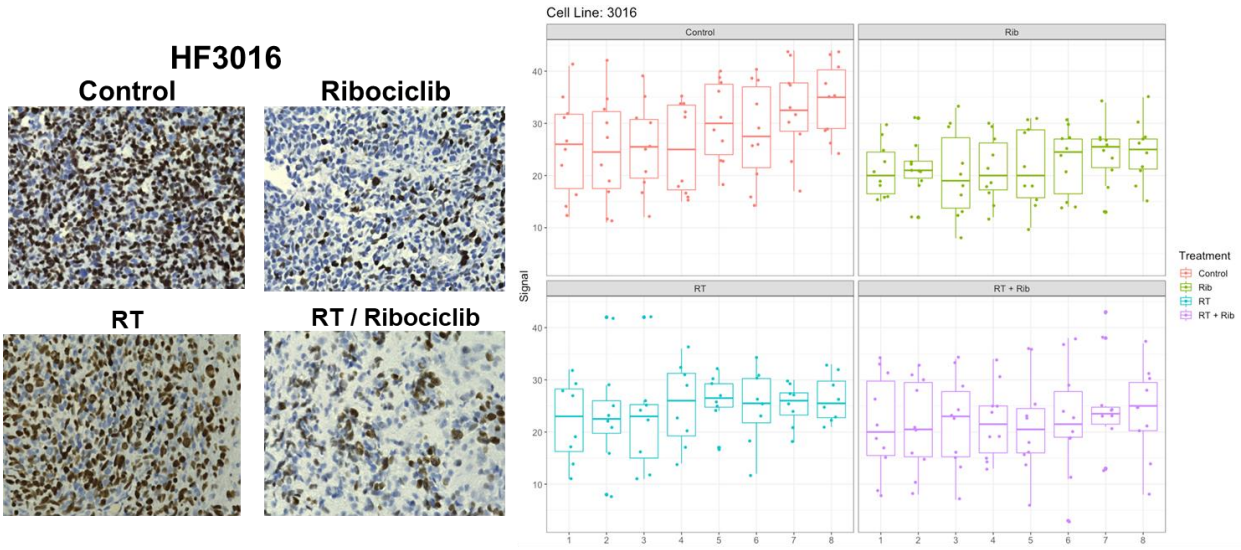


Figure 7. Glioblastoma PDX response to treatment. A) Kaplan-Meier survival curves for HF3016 and HF3177 treated with Abemaciclib. Curves were compared by Log-rank test. Only the recurrent model responded to treatment. B) HF2927 PDX did not respond to the indicated treatments.

To verify target engagement in the PDX, tumors from 8-10 mice for each treatment group were analyzed by immunohistochemistry. For CDK4/6 inhibitors, slides were stained with phospho-Rb and 8 images were captured for each mice and drug target. Phospho-Rb levels were quantified and normalized to total number of cells in the image, using a Keyence Imaging System. After testing several options for statistical analysis of this data (see also Appendix 1), Dr. Poisson's team selected a repeated measure mixed model method to compare the levels of phosphor-Rb in control vs. treated PDX tumors, followed by a Least Significant Difference post-hoc test. The results for HF3016 PDX treated with ribociclib and RT show significant effect of ribociclib treatment on the levels of phospho-Rb both as monotherapy and in combination with RT. However, ribociclib treatment as a single agent was not effective in prolonging HF3016 PDX symptom-free survival (see previous Annual Reports for survival curves, also slide 4 in Appendix 1). It is important to note that mice were treated with the inhibitors until sacrifice, so decrease in phospho-Rb in the tumors was expected.



	control	ribociclib	RT	RT + ribociclib
HF3016	30.8 [27.02, 34.59]	22.38 [18.6, 26.17] (p = 0.003 vs cont.)	24.44 [21.01, 30.33] n.s.	22.62 [18.83, 26.4] (p = 0.0038 vs. cont.)

Figure 8. Effect of treatment on HF3016 PDX phospho-Rb levels. Representative images for each treatment group, the normalized phospho-Rb signal and the comparison between treatment groups and control with mean phosphor-Rb levels and p-values from Least Significant Difference post-hoc test shown. n.s., non-significant.

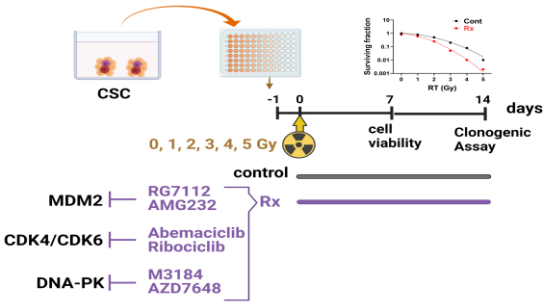
D) Further characterization of transcriptome reprograming and phenotypic changes in response to loss of PDGFRA ecDNA amplification in HF3253 model and rescue experiments

To complement the highly significant data in the previous two annual reports, we were able to perform rescue experiments for the slow tumor growth phenotype demonstrated by PDGFRA ecDNA(-) HF3253 clones. Over expression of PDGFRA partially rescued this phenotype, suggesting that indeed PDGFRA is the major driver in the ecDNA present in this tumor. Based on this data, Mr. Berezovsky was able to secure funding for single cell RNAseq analysis of ecDNA(+) and ecDNA(-) clones, which provided further insight into the intratumoral heterogeneity, which is a hallmark of glioblastoma. These data are not shown here but is featured in a manuscript in submission and in Mr. Berezovsky dissertation.

E) In vitro combination of targeted therapy and radiation

We have shown on Annual Report #2 the sensitivity of the glioblastoma CSCs to radiation. Here we show a refined statistical analysis to determine to what extent the 7-day treatment with targeted inhibitors, starting at the time of radiation (0 to 5 Gy in 1 Gy intervals), has a synergistic, antagonistic, or neutral effect on radiation. The concentrations of the inhibitors used and the surviving fractions for all treatment groups relative to control were shown in Annual Report #2 (see also dose response curves in Appendix 2). For this comprehensive experiment, testing multiple CSCs against multiple inhibitors and 6 RT doses (0 – 5 Gy), we could not introduce additional variables, leading to the following caveats when interpreting the data: the results reflect a single radiation exposure, only the IC30 concentrations of the inhibitors (cell specific), and only one schedule, that is concomitant treatment with radiation and incubation for 7 days in the presence of the inhibitors (Fig. 9). Thus, the results are meant to be a comparative overview of the adequacy of using these inhibitors in combination under these experimental conditions. Follow up experiments using different schedules and dosages for both RT and targeted inhibitors will generate further insights in the best strategies to select and optimize combinations. From these experiments we confirm what we expected, that DNA-PK inhibition tends to be synergistic, as expected from its role in DNA double strand break repair. We observed that M3814 was more effective in sensitizing the cells to radiation then AZD7648. HF3177 (mutant p53) and HF3253 (TP53 null) were remarkably sensitive to the combination treatment, while the effect was more modest on HF3016 with has an identical TP53 mutations to HF3177, as they were derived from the same patient. We plan on repeating and expanding these findings, as differential sensitivity between primary and recurrent tumor has important clinical implications. We also plan on expanding the panel of CSCs tested for Rt and M3814 combination. CDK4/6 inhibitors were antagonistic for most lines, and this effect increased with the RT doses.

HF3055, one of the lines that carry CDK4 amplification, was an exception, as CDK4/6 inhibition was synergistic with RT. MDM2 antagonist treatment presented synergy with RT for 2 wt p53 CSC lines and antagonism for 2 wt p53 CSC lines and, interestingly, 1 TP53 null CSC line (Fig. 9).



synergistic
antagonistic
additive
N/A

		RT + M3814 combination effect (q-value)					RT + AZD7648 combination effect (q-value)				
ID	TP53	1Gy	2Gy	3Gy	4Gy	5Gy	1Gy	2Gy	3Gy	4Gy	5Gy
HF2381	WT										0.046859
HF2587	WT										
HF2927	WT										
HF3178	WT										
HF3055	WT										
HF3035	WT		0.01659	0.015409		0.049323					
HF3451	WT										
HF3253	hom del	<0.000001	0.000018	0.000017	0.00004	0.000033					
HF2354	p.V216L		0.012856	0.00306	0.005642	0.004124					
HF3203	p.H179Y										
HF3016	p.M133T		0.029779					0.029779			
HF3177	p.M133T	<0.000001	0.000024	0.000037	0.00004	0.000257					
HF3077	p.G266E					0.021278				0.025431	0.002577
HF3160	p.R342*									0.045877	
Ast.	WT										

		RT + RG7112 combination effect (q-value)					RT + AMG232 combination effect (q-value)				
ID	TP53	1Gy	2Gy	3Gy	4Gy	5Gy	1Gy	2Gy	3Gy	4Gy	5Gy
HF2381	WT					0.025361		0.010038	0.001993	0.000753	0.000274
HF2587	WT							0.029779	0.041022	0.036583	
HF2927	WT					0.021278				0.021939	
HF3178	WT					0.021278					0.003772
HF3055	WT						0.040288		0.016003	0.005642	
HF3035	WT										
HF3451	WT										
HF3253	hom del					0.029688			0.030138	0.049163	0.000764
HF2354	p.V216L										
HF3203	p.H179Y										
HF3016	p.M133T										
HF3177	p.M133T										
HF3077	p.G266E										
HF3160	p.R342*										
Ast.	WT				0.040024	0.002765				0.005642	0.000549

		RT +Ribociclib combination effect (q-value)					RT + Abemaciclib combination effect (q-value)				
ID	RB1	1Gy	2Gy	3Gy	4Gy	5Gy	1Gy	2Gy	3Gy	4Gy	5Gy
HF2381	WT									0.033958	
HF2587	WT		0.029779		0.02046					0.01781	
HF2927	WT	0.03171	0.007934	0.002611	0.000049	0.000075		0.013371	0.005617	0.001479	0.000274
HF3178	WT										
HF3055	WT		0.021445	0.019635		0.010865	0.040288	0.021393	0.016003		0.006078
HF3035	WT						0.040288	0.044048			
HF3451	WT										
HF3253	WT		0.007934	0.000303	0.000561	0.000038		0.002491	0.000297	0.000051	0.00002
HF2354	WT				0.040024					0.000561	0.011444
HF3203	hom del										0.021278
HF3016	WT			0.015409	0.004524	0.000033			0.00495	0.000051	0.000001
HF3177	WT					0.049323				0.049163	0.00039
HF3077	WT					0.002577					0.002577
HF3160	WT					0.027903					0.013385
Ast.	WT		0.012467	0.022609	0.000145	0.000075	0.040288	0.012467	0.005617	0.003236	0.000033

Figure 9. Effect drugs targeting CDK4/6, MDM2 and DNA-PK in the combination with radiation. Treatment schedule schematic is shown. CSCs were treated with varying doses of RT, in the presence or absence of inhibitors (concentrations are listed in Annual Report#2) in 96 well assay plates (n=5 wells/treatment group), and cell viability assessed with CellTiterGlo (Promega) at day 7. Surviving fractions relative to untreated control were calculate, mean and SE values were used to calculate the ratio SF(RT+RX)/RT for each treatment group, and values were compared with the SF in the absence of radiation (0Gy), by unpaired multiple t-test with Welch correction, FDR was calculated by two-stage step-up (Benjamini, Krieger, and Yekutieli) and set at 0.05. q-values for the significant comparisons are shown, green shades mark synergistic effect, orange shades antagonistic, and gray additive effect.

F) Targeting DNA-PK for the treatment of glioblastoma

Sensitivity of CSCs to M3814, measured after a 7-day treatment, was not correlated to p53 status in this small dataset, in contradiction to what has been suggested by other groups, neither was it correlated with sensitivity to radiation (Table 1).

Table 1. Sensitivity of glioblastoma CSCs to DNA-PK inhibition

		RT		M3814					RT + M3814 combination effect (q-value)				
ID	TP53	Do (Gy)	(95% CI)	AAC	SE	IC50 (uM)	95% CI	R squared	1Gy	2Gy	3Gy	4Gy	5Gy
HF2381	WT	2.66	(2.04 - 3.27)	0.325	0.020	1.770	0.8565 to 3.847	0.6486					
HF2587	WT	1.70	(0.96 - 2.43)	0.748	0.131	0.052	0.03156 to nd	0.9022					
HF2927	WT	2.79	(2.06 - 3.52)	0.422	0.014	0.589	0.4914 to 0.7039	0.9725					
HF3178	WT	res		0.365	0.012	1.406	1.040 to 1.900	0.9119					
HF3055	WT	res		0.004	0.000	res							
HF3035	WT	res		0.290	0.010	3.746	2.569 to 5.778	0.8479		0.01659	0.015409		
HF3451	WT	res		0.278	0.030	2.653	1.461 to 4.970	0.6718					
HF3253	hom del	1.60	(1.44 - 1.76)	0.275	0.006	2.415	2.021 to 2.891	0.9581	<0.000001	0.000018	0.000017	0.00004	
HF2354	p.V216L	0.79	(0.71 - 0.88)	0.296	0.007	1.585	1.420 to 1.764	0.9852		0.012856	0.00306		
HF3203	p.H179Y	4.33	(2.33 - 6.339)	0.651	0.030	0.079	0.04277 to 0.1475	0.7481					
HF3016	p.M133T	2.72	(2.17 - 3.26)	0.231	0.016	2.415	2.021 to 2.891	0.9581		0.029779			
HF3177	p.M133T	2.76	(2.15 - 3.36)	0.287	0.009	1.828	1.543 to 2.153	0.9677	<0.000001	0.000024	0.000037	0.00004	
HF3077	p.G266E	7.61	(4.11 - 11.11)	0.177	0.005	14.120	9.761 to 23.63	0.8664					
HF3160	p.R342*	3.38	(1.92 - 4.84)	0.219	0.005	12.69	7.642 to 27.70	0.7728					

Do, mean lethal dose; AAC, area above the curve.

synergistic effect
neutral effect
N/D

Because M3814 did not achieve sufficient activity in the PDX brain, we resorted to knocking down the catalytic subunit to DNA-PK, coded by the gene PRKDC using lentivirus to obtain CSC derivatives stabling expressing validated shRNA targeting the gene, using a non-silencing sequence as control (Fig. 10A). We selected HF3016 and HF3177 for this experiment, as they are derived from the same patient (newly diagnosed and recurrent), carry CDK4 and MYC amplification in different ecDNA elements in 100% of the cells (deCarvalho, Nature Genetics 2018), and form tumors in the mouse brain which are resistant to temozolomide, radiation and CDK4/6 inhibitor when administered as single agents. Transduced cells expressed GFP marker (Fig. 10B), and derivative lines with decreased expression of total DNA-PK in cells transduced with lentivirus to express shRNA targeting were identified (Fig. 10C). HF3016-NS control and HF3016-KD1 cells were implanted in nude mice and each line was treated with RT, ribociclib or temozolomide (n=5 per treatment group) (Fig. 10D). RT is delivered to the mouse brain as described in previous annual reports. As expected, the HF3016-NS mice was resistant to all treatments. However, the downregulation of DNA-PK sensitized HF3016 to each of these treatments (Fig 1D). HF3177-NS, HF3177-KD1 and HF3177-KD2 were similarly implanted in nude mice and subjected to RT, n=5/group (Fig. 10E). HF3177-NS and HF3177-KD1 PDX were resistant to RT, while HF3177-KD2 PDX was greatly sensitized to RT, in fact, the 3 mice represented by the tail in the survival curve were sacrificed at the end of the study, after 250 days, with no signs of tumor. These results are in agreement with the sensitivity of HF3177 CSCs to the M3814/RT combination (Fig. 9). Because the levels of DNA-PKcs expression for HF3177-KD2 are lower than for HF3177-KD1 (Fig. 10C), we conclude that there is a dose dependence for sensitization to RT, suggesting that high efficacy and brain penetrance will be required for successful use of pharmacological DNA-PK inhibitors in combination with RT for the more resistant lines. It is important to observe that DNA-PKcs knockdown alone did not affect symptom-free survival of either HF3016 or HF3177 PDX. Based on these results, we expected that DNA-PKcs knockdown would also sensitize CSCs in vitro to other agents causing DNA damage, but surprisingly we did not observe this (Appendix 4). In conclusion, these results point towards the potential for DNA-PK inhibitors in combination treatments for glioblastoma. We are continuing this project with alternative funding, including optimizing the combination of DNA-PK loss and DNA-damaging agents to target ecDNA elements, since in the case of HF3016 and HF3177 lines, there is a strong selective pressure for the cancer cells to maintain the powerful oncogenes amplified in the ecDNA elements, MYC and CDK4.

A

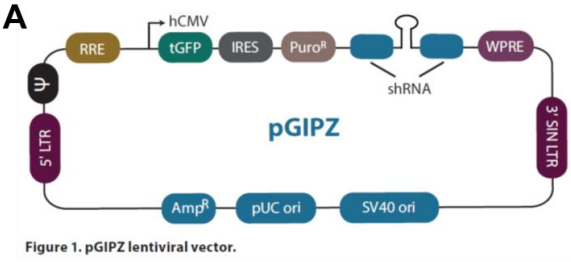
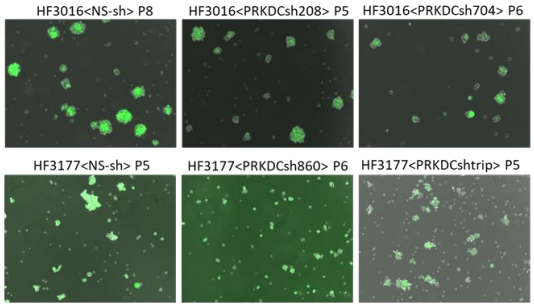


Figure 1. pGIPZ lentiviral vector.

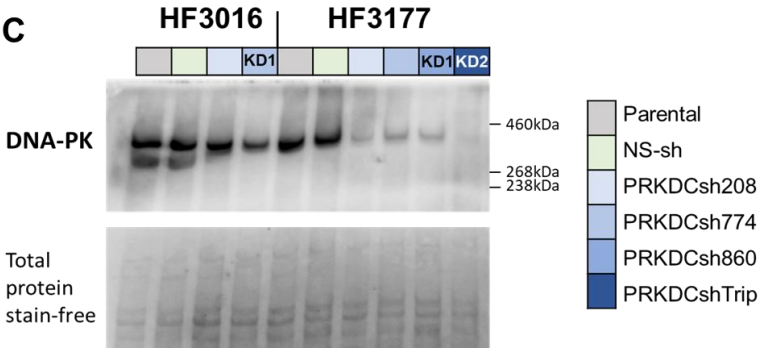
Lentivirus	Cat#	clone ID	lot	Gene target sequence	Titer (TU/uL)
PRKDCshRNA208	VGH5518-200187776	V2LHS-231208	V20022703	GGTCATGGATTCA AGAAAT	4.02 x 10^5
PRKDCshRNA774	VGH5518-200202274	V2LHS-94774	V20022703	CAGTGAAAGTCT GAATCAT	1.77 x 10^5
PRKDCshRNA860	VGH5518-200259275	V3LHS-372860	V20022704	AGACAGAATGTC GACACAA	2.57 x 10^5
Non-silencing GIPZ shRNA	RHS4348		V19090501		5.66 x 10^5

Source: Horizon Discovery

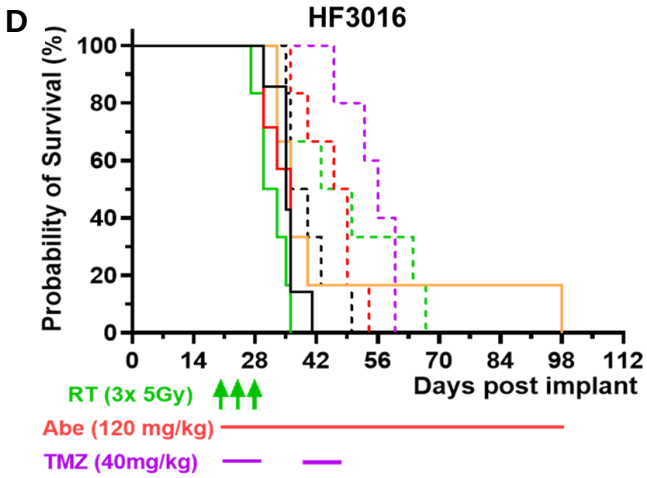
B



C



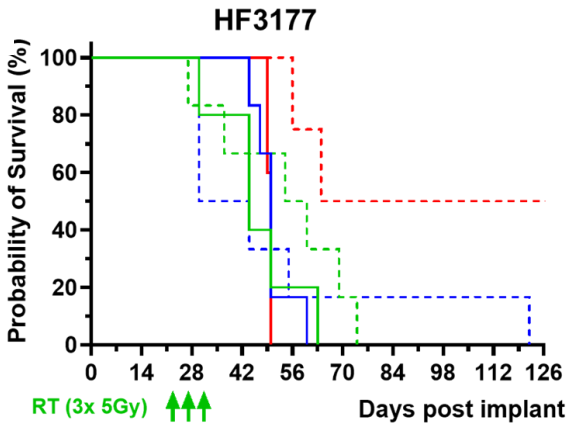
D



	PRKDC knockdown	RT	Rx	Log-rank (Mantel-Cox) test (vs. untreated NSsh)	
				P-value	HR (95% CI)
—	NSsh	0	Cont.		
—	NSsh	0	CDK4/6i	n.s.	
—	NSsh	15 Gy	Cont.	n.s.	
—	NSsh	15 Gy	CDK4/6i	n.s.	
- - -	KD1	0	Cont.	n.s.	
- - -	KD1	0	CDK4/6i	0.0053	8.708 (1903-39.85)
- - -	KD1	15 Gy	Cont.	0.0143	6.786 (1.467 – 31.38)
- - -	KD1	0	TMZ	0.0010	16.48 (3.105 – 87.48)

NSsh, non-silencing shRNA; KD, PRKDC knockdown; Rx, pharmacological agent administered orally; n.s., non-significant; HR, hazardous ratio

E



	PRKDC knockdown	RT	Log-rank (Mantel-Cox) test (vs. untreated NSsh)	
			P-value	HR (95% CI)
—	NSsh	0		
—	KD1	0	n.s.	
—	KD2	0	n.s.	
- - -	NSsh	15 Gy	n.s.	
- - -	KD1	15 Gy	n.s.	
- - -	KD2	15 Gy	0.0171	8.475 (1.463 -49.08)

NSsh, non-silencing shRNA; KD, PRKDC knockdown; n.s., non-significant; HR, hazardous ratio

Figure 10. shRNA mediated knockdown of the expression of the catalytic subunit of DNA-PK (coded by the PRKDC gene) leads to sensitization of resistant glioblastoma PDX to various treatment modalities. A) Information for the lentiviral constructs obtained from Horizon Discovery. **B)** representative images of transduced HF3016 and HF3177 cells expressing GFP. **C)** DNA-PK expression in parental cells, control cells transduced with non-silencing (NS) shRNA, and those transduced with shRNA targeting PRKDC transcript assessed by Western Blot guided the selection of cells with detectable decrease in expression, designated KD1 for HF3016 and KD1 and KD2 for HF3177. **D)** Kaplan-Meier survival curves and statistics for HF3016-NSsh and HF3016-KD1 treated with radiation, abemaciclib (abe), and temozolomide (TMZ). **E)** Kaplan-Meier survival curves and statistics for HF3177-NSsh, HF3177--KD1 and HF3177—KD2, treated with radiation.

What opportunities for training and professional development has the project provided?
Two Wayne State University PhD candidates, Ms. Nuga and Mr. Berezovsky, named on the original application and on section 7 below, have been contributing to and benefiting from this project. They have directly benefited from the training in translational research afforded by this award, and for presenting their results locally and submitting for national meetings. Mr. Berezovsky earned a competitive fellowship in May 2020, for academic year 2021-2022 https://gradschool.wayne.edu/fellowships/rumble-fellowships). Ms Nuga continues to be funded by a prestigious fellowship from the NIH for a project entitled "Cyclin Dependent Kinase 4/6 (CDK4/6) as a Therapeutic Target in Glioblastoma" (F31 CA250450-01, 04/09/2020 – 04/08/2023), which is directly related to this award.

Mr. Berezovsky has prepared his PhD dissertation for defense in the spring of 2023 and is finishing a first author manuscript directly related to this award. Based on the results of his research (accomplishment "D" above), he was awarded a small grant from Wayne State University to conduct single cell transcriptome analysis to further characterize the PDGFRA ecDNA(+) and ecDNA(-) cell populations.

PLATELET DERIVED GROWTH FACTOR RECEPTOR ALPHA (PDGFRA) ONCOGENE DEPENDENCY IN GLIOBLASTOMA

by

ARTEM BEREZOVSKY

DISSERTATION

Submitted to the Graduate School of

Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

2023

MAJOR: ONCOLOGY (CANCER BIOLOGY)

How were the results disseminated to communities of interest?
The results from this project were presented to:
A) Local scientific and clinical research communities, through multiple internal presentations at Research Symposium, and focused working groups.
B) The scientific community through presentations at national and international meetings (see Section 6)
C) Patients, their caregivers, and supporters, through an onsite presentation and lab tour for brain tumor patients held at Henry Ford Hospital at least twice a year.

What do you plan to do during the next reporting period to accomplish the goals?
This is a final report. We will update the information as the articles listed in #6 are published in peer review journals. The DNA-PK results were exiting, and we have obtained additional philanthropy funds to complete additional work needed for a publication later in 2023.

4. **IMPACT:**
What was the impact on the development of the principal discipline(s) of the project?
Before results from clinical trials testing the efficacy of the inhibitors to treat glioblastoma are available, this project is addressing molecular-based patient selection and strategies for combination therapies. Upon completion of this project, we anticipate that the results will make a significant contribution in the understanding of the molecular and genomic correlates of response to promising targeted therapy, how ecDNA impacts glioblastoma biology and response to therapy, and what are the best strategies to favor the thus far elusive clinical efficacy of targeted inhibitors, providing experimental evidence-based rationale for combination therapy. The importance of the schedule for combination of targeted therapy and radiation is exemplified in the benefits of pre-treatment with RG7112 when combining with radiation we have described in earlier annual reports. Another impactful outcome is the validation of targets for the treatment of high grade gliomas exploring the intratumor heterogeneity conferred by ecDNA amplification, such as in the case of PDGFRa, or vis RNA interference knockdown, used for DNA-PK.

What was the impact on other disciplines?
This study has encouraged other departments at our institution to build similar pre-clinical programs. Based on the pre-clinical activities funded by this award, I was invited to be a core member of the Henry Ford Health Biorepository Research core, advocating for the establishment of live biobanks institution wide.

What was the impact on technology transfer?
The dissemination of the results from this project within our institution served as a catalyst for discussions and assessment of feasibility of establishing a contract research organization (CRO) for therapeutic testing in patient-derived models of brain tumors and other cancers treated at Henry Ford Hospital. The discussions have been extended to explore commercialization by third parties.

What was the impact on society beyond science and technology?
Our research project is performed at a hospital, where clinical translation is always at the forefront. We receive significant input from oncologists and other clinicians, which have been following closely the results of our study. Our oncologist colleagues have shown hope on the evidence we have provided here that pre-clinical studies will contribute to the understanding of why after decades of clinical trials, no targeted agent has shown effectivity against this devastating disease, as an essential step to propose more sophisticated patient selection and therapeutic strategies. At our institution we have a very engaged group of brain tumor patients who follow and contribute to our research regularly.

5. **CHANGES/PROBLEMS:**
Changes in approach and reasons for change
The data-driven changes in approach were to focus more resources on experiments involving long term treatment with CDK4/6 inhibitors, including an additional phenotypic measurements and inhibitors (section 3), while for the MDM2 antagonists subproject we focused on acute response of p53 stabilization to identify dynamic biomarkers of response and therapeutic targets for combination therapy. We could not perform pharmacological inhibition of DNA-PK in mouse xenograft as explained. We identified a novel inhibitor, but the cost is prohibitive, so we focused on the knockdown approach for the in vivo work.

Actual or anticipated problems or delays and actions or plans to resolve them
This grant period ended in August of 2022. From September 2022 to March 2023, we continued to monitor long survival PDXs and collect survival data and tissue for analysis. During this time, we have invested significant effort to analyze the complex RNAseq datasets to derive the most biological meaning. We also spend significant effort analyzing FACS data for cell cycle, senescence, and apoptosis, which was included towards the end of the award to complement our earlier data.

Changes that had a significant impact on expenditures
Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Significant changes in use or care of human subjects:
Not applicable.

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

Significant changes in use of biohazards and/or select agents:
Not applicable

6. **PRODUCTS:**
Publications, conference papers, and presentations

Journal publications
The following manuscripts acknowledging this award are *in process of submission or preparation for submission* in peer reviewed journals. We anticipate each one will be in press in 2023 and will update the citations to the published articles.

Title: Genetics determines radiosensitivity of glioblastoma cancer stem cells and selection of targeted inhibitors for combination treatments with radiation
Authors: Oluwademilade Nuga, Yuling Meng, Chun-Hui Lin, Steven Brown, James Snyder, Laila Poisson, Ana C. deCarvalho

First submission: Scientific Reports

Title: Glioblastoma cell differentiation state affects response to temozolomide and radiation therapy
*Authors***:** Artem Berezovsky(*), Oluwademilade Nuga(*), Indrani Datta, Kimberly Bergman, Thais Sabedot, Kathrine Gurdziel, Susan Irtenkauf, Laura Hasselbach, Yuling Meng, Claudius Mueller Emanuel F. Petricoin III, Steven Brown, Laila Poisson, Houtan Noushmehr, Douglas Ruden, Ana C. deCarvalho
First submission: PLoS One

Title: A multiple model-system approach to studying therapeutic response in glioblastoma
*Authors***:** Laila M. Poisson, Hoon Kim, Indrani Datta, Artem Berezovsky, Tathiana Malta, Oluwademilade Nuga, Mary Winn, Claudius Mueller, Susan Irtenkauf, Kevin Nelson, Laura Hasselbach, Kimberly Bergman, Yuling Meng, Andrea Transou, Lisa Scarpace, Steven Kalkanis, Houtan Noushmehr, Tom Mikkelsen, Roel G. W. Verhaak, Ana C. deCarvalho
First submission: Clinical Cancer Research

Title: Selective response of glioblastoma patient-derived models to MDM2 antagonists
Authors: Yuling Meng, Oluwademilade Nuga, Nouman Mughal Susan Irtenkauf, Laura Hasselbach, Chun-Hui Lin, Kristyn Quenneville, James Snyder, Laila Poisson, Ana C. deCarvalho
First submission: TBD

PDGFRA subproject: title and Journal TBD
CDK4/6 inhibitors: title and journal TBD

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

Other publications, conference papers, and presentations

Invited oral presentations

"Target validation towards better therapeutic strategies for high grade glioma", Pharmacology Seminar Series, Wayne State University School of Medicine, March 25, 2022

"Pharmacogenomics using glioblastoma patient-derived models", WE-SPARK Health Institute, Windsor, Ontario, Canada. December 03, 2021

"Pre-clinical development of omics-driven therapy for high grade glioma", Translational Oncology Research Symposium, Michigan State University, East Lansing, MI. November 12, 2021

Poster presentation and published abstract by graduate student

Berezovsky A, Datta I, She R, Transou A, Irtenkauf S, Hasselbach L, Poisson L, **deCarvalho AC**. (CSIG-10) Platelet-derived growth factor receptor alpha oncogene dependency in glioblastoma. Society for Neuro-Oncology 26th Annual Meeting, Boston MA, November 18-21. 2021. Neuro-Oncology 23 (Supplement_6), vi35-vi35

Website(s) or other Internet site(s)

Technologies or techniques
Inventions, patent applications, and/or licenses
Nothing to report.

Other Products
New funding: Based on the divulgation of the results of this study, I have received a new philanthropic endowment: the "Demchik Family Fund for Glioma Patient-Derived Avatar Models", starting on 04/01/2021.

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

Participants

Name	Ana C. deCarvalho
Project Role	PI
ORCID ID	0000-0003-1183-4548
Nearest person month worked:	2.4
Contribution to Project:	Oversight of the whole project, conducting dose-response and knockdown experiments, securing resources, selecting reagents and equipment team integration: holding formal quarterly meetings with complete research team and interacting with team members daily, data analysis, writing report and manuscripts.
Funding Support:	

Name	Laila Poisson
Project Role	Biostatistician, key personnel
ORCID ID	0000-0002-3409-6536
Nearest person month worked:	0.6
Contribution to Project:	Dr. Poisson supervises all the statistics and bioinformatics analysis for the project. She meets weekly with Ms. Datta and the experimental team to go over the study design, sample size calculations, analysis of dose response curves, combination therapy and in vivo survival curves.
Funding Support:	

Name	James Snyder
Project Role	Oncologist
ORCID ID	0000-0001-9379-0491
Nearest person month worked:	0.24
Contribution to Project:	Dr. Snyder has met with our group consistently to discuss the selection of pharmacological agents for this project, giving important guidance in combination therapy selection and schedule. Dr. Snyder is also monitoring clinical trials outcomes for the pharmacological agents in this study.
Funding Support:	

Name	Yuling Meng
Project Role	Instructor Scientist
ORCID ID	0000-0001-9379-0491
Nearest person month worked:	2.64
Contribution to Project:	Dr. Meng has worked on all aspects of the in vivo work, including preparing key reagents, single cell clone isolation and screening, metaphase arrest cell preparation for FISH, MDM2 inhibitor dose-response curves and combination therapy with RT, preparation of control and treated samples for RNAseq analysis, and Western blots to verify target engagement.
Funding Support:	

Name	Susan Irtenkauf
Project Role	Research Coordinator
ORCID ID	
Nearest person month worked:	3.00
Contribution to Project:	Ms. Irtenkauf is responsible for implant of cells in the mouse brain for orthotopic xenografts, assisting with radiation treatment of the mice and cells, coordinating oral gavage with another team member, monitoring the animals, sacrificing, harvesting, and processing the tissue for downstream analysis. Additionally, she has helped culture the many cell lines necessary for the experiments.
Funding Support:	

Name	Artem Berezovsky
Project Role	Graduate student
ORCID ID	0000-0002-4925-2466
Nearest person month worked:	3.6
Contribution to Project:	Mr. Berezovsky has worked in single cell cloning, successfully isolating ecDNA(+) and ecDNA(-) clones for receptor tyrosine kinase amplified genes. He performed the differentially expressed gene analysis between ecDNA(+) and ecDNA(-) cells and PDXs and single cell RNAseq analysis.
Funding Support:	

Name	Oluwademilade Nuga
Project Role	Graduate Student
ORCID ID	0000-0003-4266-0931
Nearest person month worked:	3.6
Contribution to Project:	Ms. Nuga contributed to all the in vitro CDK4/6 inhibitor studies, and in vitro radiation treatment studies, including Western blots, long term treatment and FISH analysis.
Funding Support:	

Name	Indrani Data
Project Role	Biostatistician
ORCID ID	
Nearest person month worked:	1
Contribution to Project:	Ms. Datta has assisted with processing the FASTQ data for the RNAseq data analysis, and general statistics for the project, meeting weekly with the team August 2020 to June 2021, and June 2022 to August 2022.
Funding Support:	

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.

What other organizations were involved as partners?
Nothing to Report.

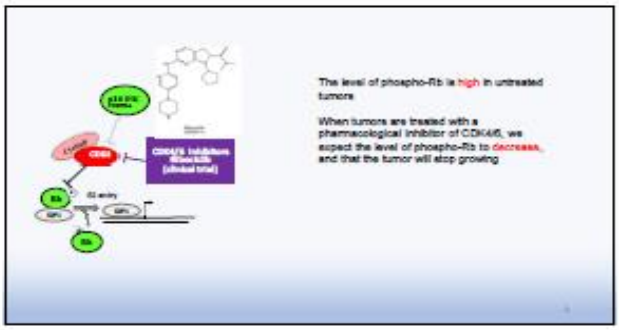
8. **SPECIAL REPORTING REQUIREMENTS**
COLLABORATIVE AWARDS:
Not applicable.

QUAD CHARTS:
Not applicable.

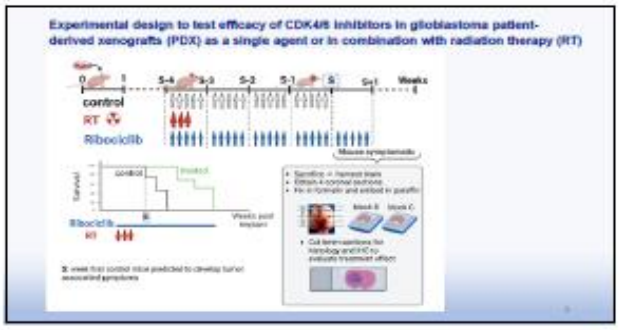
9. **APPENDICES**

Appendix 1. Statistical analysis of the phopho-Rb quantification in the different PDX treatment groups was presented as a case study at an advanced Biostatistical course at the University of Michigan. Select slides are shown.

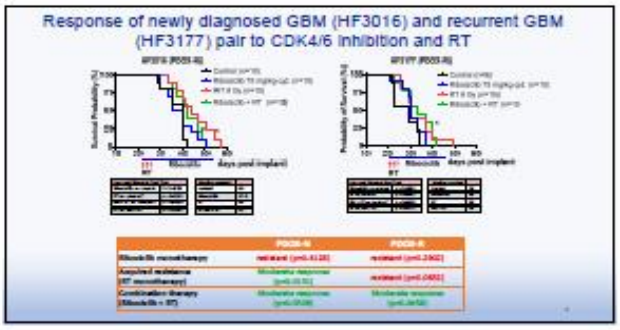
1



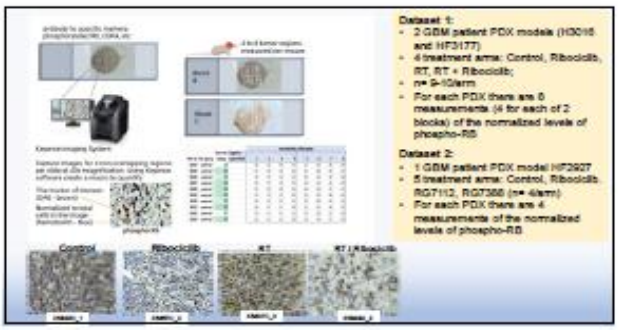
2



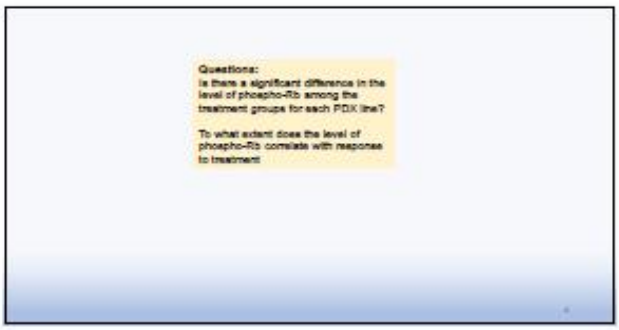
3



4

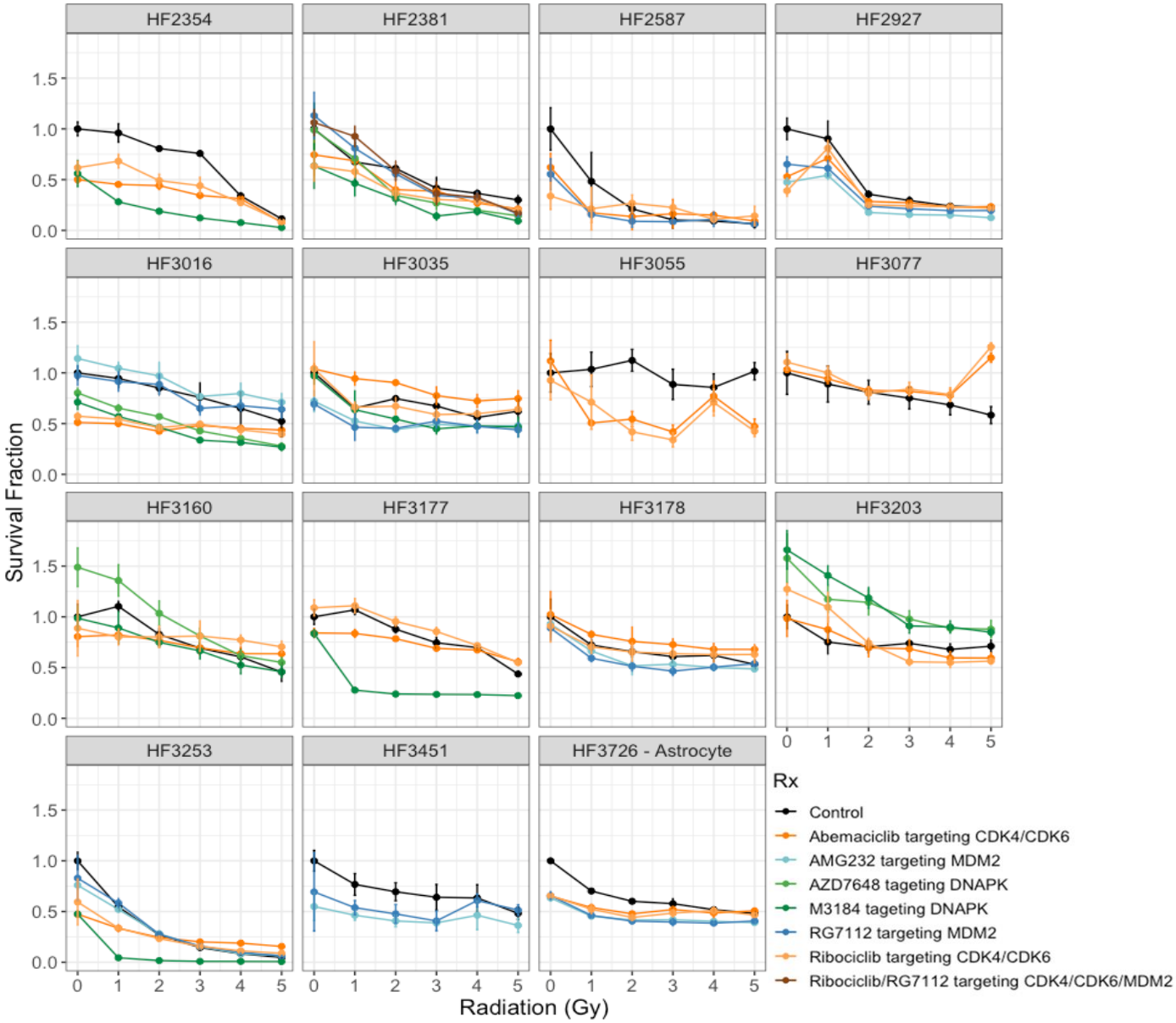


5



6

Appendix 2. Dose response curves for radiation monotherapy and in combination with targeted inhibitors.



Appendix 3. Pathway enrichment analysis of differentially expressed genes between control and treatment group after 42 days of treatment (day 42) or 42 days treatment and 14 days drug withdrawal (day 56). Four patterns of transcriptional reprograming for each of the two CSC line were observed.

Appendix 4. DNA-PK (PRKDC) knockdown did not affect response of HF3016 and HF3177 CSCs to genotoxic pharmacological agents in vitro