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14. ABSTRACT
Using a panel of patient-derived models, we are identifying the factors affecting response of glioblastoma to novel and specific small molecule inhibitors of cyclin-dependent kinase 4 and 6 (CDK4/6) and mouse-double minute homologue 2 (MDM2), which are oncogenic negative regulators of tumor suppressors retinoblastoma protein (Rb) and tumor protein 53 (p53), respectively. The activation of CDK4 and MDM2 by gene amplification, frequently in extrachromosomal DNA (ecDNA), is considered in our study. CDK4/6 and MDM2 inhibitors, selected based on our previous studies showing specificity and potency, were tested as single agents and in combination with radiation therapy in patient derived cancer stem cells and orthotopic mouse xenografts. Our data show that MDM2 antagonist pre-treatment has the potential to sensitize resistant wildtype p53 glioblastoma tumors to radiation therapy, and to CDK4/6 inhibitors. Taking advantage of the heterogeneous ecDNA amplification of PDGFRA in glioblastoma, we report that single cell clones which are PDGFRA ecDNA negative present a significantly reduced tumor growth rate in mouse brains, relative to the ecDNA positive populations from the same tumor. Further, we identify pathways that are differentially active in the ecDNA positive and negative models, with significant implications for the treatment PDGFRA activated glioblastomas.

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.

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1. **INTRODUCTION**

Glioblastoma, the most aggressive primary brain tumor, is driven by genomic abnormalities leading to deregulation in the retinoblastoma protein (Rb), p53, and receptor tyrosine kinase - AKT 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 PDGFRA receptor tyrosine kinases. 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. Employing this panel, we are studying the genomic and molecular correlates of efficacy of pharmacological agents targeting CDK4/6 or MDM2. The standard of care for glioblastoma patients comprises surgical resection followed by treatment with fractionated radiation therapy (RT) and the DNA-alkylating agent temozolomide (TMZ), both modalities leading to DNA damage. Non-homologous end joining (NHEJ) is responsible for the repair of most DNA double-stranded breaks. DNA-dependent protein kinase catalytic subunit (DNA-PKcs) has an essential role in NHEJ, and therefore has been implicated in tumor cell resistance to RT. There is currently not enough evidence to support the optimization of targeted therapy in combination with the standard of care. Here we show results from combination of targeted agents with radiation therapy in glioblastoma CSCs and PDXs.

We previously reported that we found a strong selection for cells carrying CDK4 and/or MDM2 ecDNA amplification, such that the frequency of ecDNA(+) cells in vitro and in vivo is 100% (2020 Technical Report, also see https://pubmed.ncbi.nlm.nih.gov/29686388/). Conversely, we observed that ecDNA amplification of receptor tyrosine kinases (RTK) is more heterogeneous, and we were able to isolate ecDNA(-) and ecDNA(+) cell populations from two patient derived models carrying PDGFRA and MET amplification, respectively. Analyzing the tumorigenicity and transcriptome of the ecDNA(+) and ecDNA(-) subpopulations from the same tumor represents a powerful tool to explore intra-tumoral heterogeneity to gain insights on tumor biology and adaptation to the absence of the amplification-activated oncogene.

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 (DNAPK).

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): 75% 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): ongoing, 50% complete

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

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

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)

- 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): 70% complete

Milestone (year 2): efficacy of MDM2 and CDK4 inhibition as monotherapy or in combination in a glioblastoma PDX panel and effect on ecDNA (Mar/21). Treatments were completed for 3 lines.

- Major task 4: Adaptive response by RNAseq analysis:

Transcriptome analysis of ecDNA(+) and ecDNA(-) CSCs and PDXs has been completed.

Brains samples from treated and control PDX have been alternately frozen or processed for FFPE. Samples are currently being analyzed by IHC for selection for RNA sequencing.

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): 75% complete

5.2. Verify specificity of inhibitors in engaging the target (Feb/20 – Mar/20): 10% completed

5.3. Short-term combination therapy (Apr/20 – Jun/20): 25% complete

5.4. Long term treatment (Jun/20 – Oct/20): not yet started

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): partially achieved (M3814 dose-response curves).

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% completed

6.2. Impact of KD on oncogene expression and ecDNA copy number (Mar/20 – Dec/21): stable CSC lines presenting shRNA-mediated downregulation of PRKDC have been obtained and these experiments are planned for the fall 2021.

- Major task 7: PDX treatment

7.1. Pilot brain penetrance and target engagement study for two DNAPK inhibitors (Mar/20 – Aug/20): target engagement results were unconclusive and are being repeated in the Fall 2021.

7.2. PDX treatment of control and DNAPK knockdown cells (Oct/21 – May/22): scheduled

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) Comparison of ecDNA(+) and ecDNA(-) cell populations isolated from the same tumor in relation to tumorigenic potential and transcriptional programs.

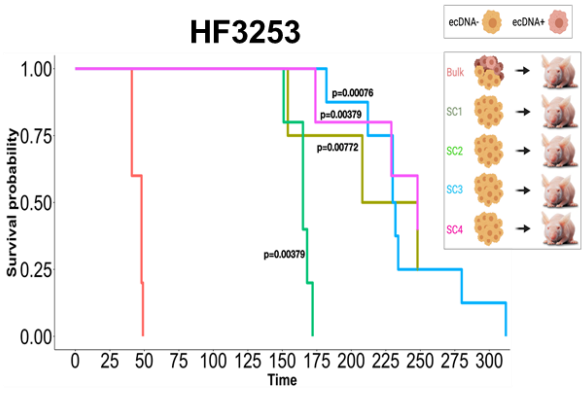
As mentioned on the 2020 Technical Report, PDGFRA amplification in ecDNA present in a glioblastoma tumor (HF3253) was maintained in the low passage neurospheres (CSC) cultures, at a subclonal frequency (https://pubmed.ncbi.nlm.nih.gov/29686388/). PDGFRA ecDNA(+) and ecDNA(-) cell subpopulations were cultured from these CSCs, and ecDNA(-) clones were tumorigenic in nude mice, but tumors grew at a considerably slower rate (Fig. 1A). Similarly MET ecDNA(+) and ecDNA(-) CSCs were obtained from another glioblastoma sample (HF3035). MET ecDNA(-) CSCs were also tumorigenic. For both lines ecDNA(+) and ecDNA(-) PDX tumors could be compared. RNA was isolated from the samples listed

Table 1. Samples for RNA sequencing

Sample	Biological replicates
HF3253 CSC PDGFRA ecDNA(+)	4
HF3253 CSC PDGFRA ecDNA(-)	4
HF3253 PDX PDGFRA ecDNA(+)	5
HF3253 PDX PDGFRA ecDNA(-)	6
HF3035 PDX MET ecDNA(+)	6
HF3035 PDX MET ecDNA(-)	5

on Table 1. RNAseq libraries were prepared using TruSeq Stranded Total RNA with Ribo-Zero Gold (Illumina) and sequenced at 60M paired-end reads depth (Psomagen). FASTQ files were aligned, batch corrected, low count filtered, and RPKM normalization performed. Differential gene expression analysis was performed using NOISeqBIO. Differentially expressed genes were further filtered by <-2 or >2 fold-change, and enrichment analysis was performed using Metascape. Whole transcriptome PCA show clustering by sample type (CSC vs PDX) and by ecDNA status (Fig. 1B). Results for the HF3253 models are listed on Figure 1. We verified that PDGFRA was indeed downregulated in ecDNA(-) cells and PDXs (Fig. 1C). Consistent with proposed roles for PDGFRα, ecDNA(+) CSCs presented enrichment in regulation of oligodendrocyte differentiation, while ecDNA(-) CSCs were enriched in oxidative phosphorylation, pro-apoptotic pathways and negative regulation of proliferation. Also consistent with roles previously proposed for PDGFRA, extra-cellular matrix components and organization was also enriched in ecDNA(+) CSCs and PDXs. We observed a robust enrichment in eukaryotic translation elongation function in ecDNA(-) CSCs for which we do not yet have an explanation. Findings that potentially explain the dramatically decreased tumor growth rate in ecDNA(-) PDX genes include down

A



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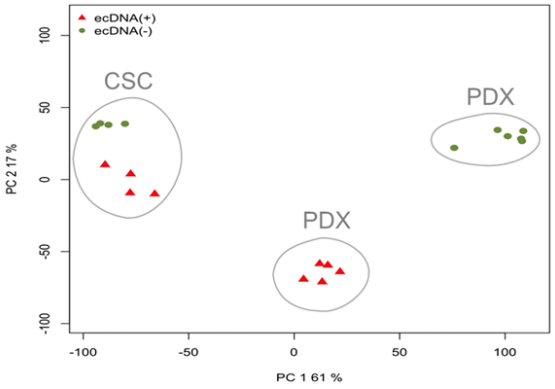


C

CSC

Up in ecDNA(+) — 235 DEGs

Top protein coding DEGs

Gene	log2FC
PDGFRA	-13.067
PMP2	-12.127
VCAN	-9.364
MAGEB2	-8.873
TAFA5	-8.466
IFI44	-7.964

Enrichment		LogP
hsa04612	Antigen processing and presentation	-8.037
M2867	TBK1.DF UP	-5.411
GO:0048713	regulation of oligodendrocyte differentiation	-4.282
GO:0005201	extracellular matrix structural constituent	-4.365

Up in ecDNA(-) — 737 DEGs

Top protein coding DEGs

Gene	log2FC
TRDD3	7.291
TRDJ1	6.665
GABRQ	5.507
TMEM74	4.673
NEFL	4.652
LIN7A	4.589

Enrichment		LogP
R-HSA-156842	eukaryotic translation elongation	-49.969
GO:0006119	oxidative phosphorylation	-8.427
GO:0043065	positive regulation of apoptotic process	-5.441
GO:0008285	negative regulation of cell population proliferation	-4.289

PDX

Up in ecDNA(+) — 298 DEGs

Top protein coding DEGs

Gene	log2FC
COL2A1	-8.43637
DACT2	-7.740695
SPP1	-7.602954
IGF2	-7.16584
COL9A3	-6.879349
PDGFRA	-5.137986

Enrichment		LogP
GO:0006323	DNA packaging	-38.058
R-HSA-1474244	Extracellular matrix organization	-11.854
WP4553	FBXL10 enhancement of MAP/ERK signaling in diffuse large B-cell lymphoma	-13.380
M5930	hallmark epithelial mesenchymal transition	-9.054

Up in ecDNA(-) — 887 DEGs

Top protein coding DEGs

Gene	log2FC
SPON1	10.033498
MSTN	8.9897479
NELL2	8.9851535
DPP6	8.6796816
ERBB4	8.4109046
TAL1	8.2785995

Enrichment		LogP
GO:0050804	modulation of chemical synaptic transmission	-12.198
GO:0034330	cell junction organization	-11.114
GO:0010001	glial cell differentiation	-9.139
M2698	P53 DN.V1 UP	-7.684

Figure 1. PDGFRA ecDNA(+) and ecDNA(-) cell populations derived from the same glioblastoma tumor present different tumorigenic potential and gene expression enrichment. A) Kaplan-Meier survival curves for mice implanted with HF3253 CSCs classified as ecDNA(+) (bulk) and 3 ecDNA(-) single cell clones. The ecDNA(-) PDX curves were compared to the ecDNA(+) curve by Log Rank, and p-values are shown. B) PCA plot for the HF3253 RNAseq data (Table 1), ecDNA status is indicated. C) RNAseq analysis. Number of differentially expressed genes (DEG) between ecDNA(+) and ecDNA(-) CSCs are shown on the left panels, and between ecDNA(+) and ecDNA(-) PDX are shown on the right panels. DEG were determined using NOISeq, and enrichment analyses were performed using Metascape.

upregulation of gene set “P53_DN.V1_UP”, comprise of “genes up-regulated in NCI-60 panel of cell lines with mutated TP53”, given that HF3253 has genomic loss of TP53, and the upregulation of glial cell differentiation,

indicating a loss of stemness in vivo. These results indicate that for this patient, PDGFRα is a promising therapeutic target, represent an incentive for the development of PDGFRα inhibitors with better pharmacological properties for brain tumors. We are now actively using this analysis to identify and test targetable vulnerabilities specific to the ecDNA(-) cells.

B) Efficacy of RT combined with CDK4/6, MDM2 or DNA-PK pharmacological inhibitors against glioblastoma cancer stem cells.

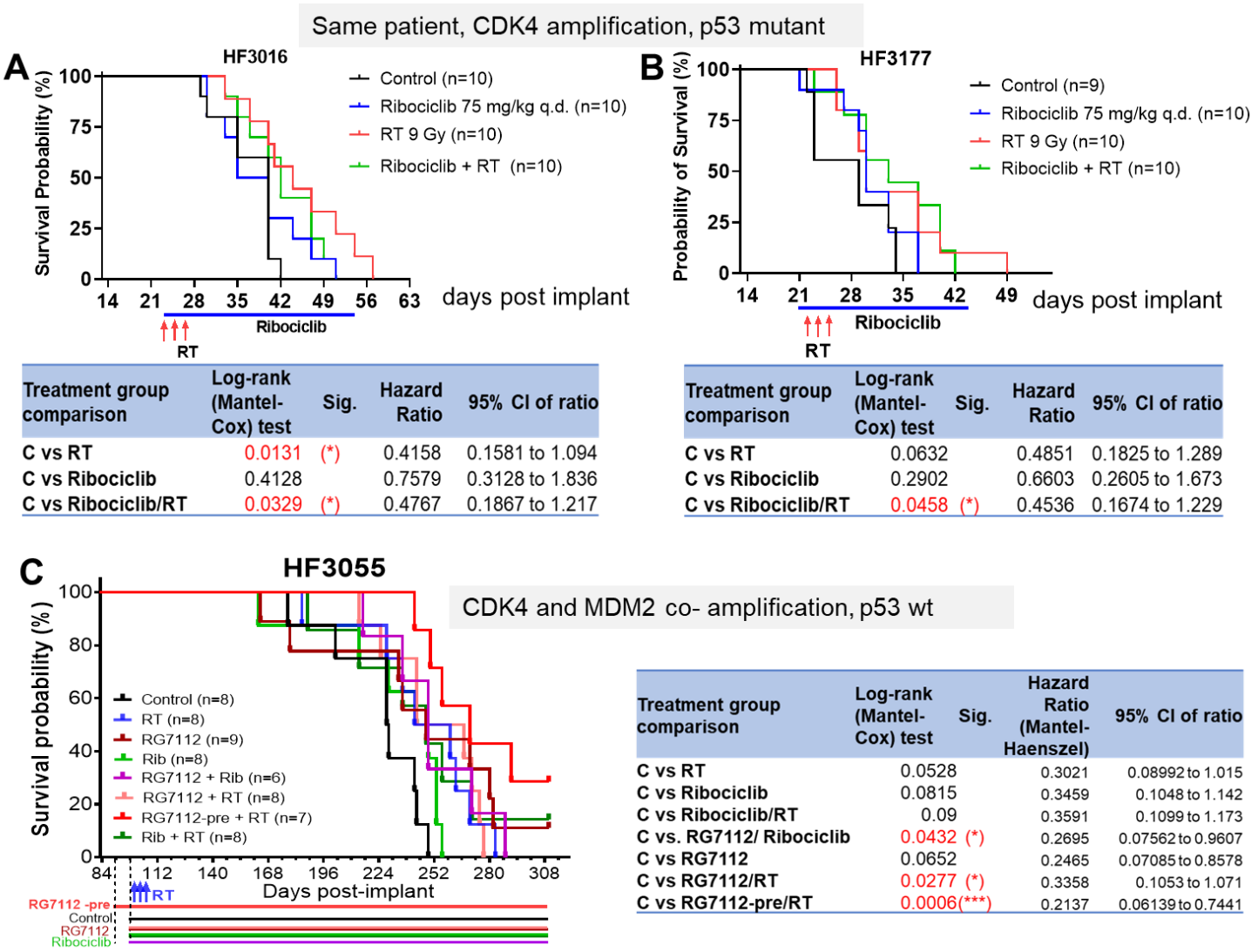
We have reported 2020 Annual report the sensitivity glioblastoma CSCs to CDK4/6 inhibitors Abemaciclib mesylate (Selleck # S7158) and Ribociclib (MedChemexpress # HY-15777); MDM2 antagonists RG7112 (SelleckChem # S7030) and AMG232 (MedChemExpress # HY-12296); DNA-PK inhibitors M3814, (Nedisertib, MedChemExpress # HY-101570) and AZD7648 (SelleckChem # S8843). Here we subjected CSCs to 0 – 5 Gy single dose radiation (Varian Edge linear accelerator), as a monotherapy or in combination with the IC30 concentration of each inhibitor. Control cells were treated with mock radiation and DMSO, all treatment groups had 5 replicates. Cells were incubated for 7 days after radiation in the presence of the inhibitors and cell viability was evaluated with CellTiterGlo (Promega). The surviving fractions (SF) and concentration for the inhibitors are shown in Appendix 1. For the radiation monotherapy groups, the mean lethal dose (Do) was calculated by the single hit multi-target model in R (Appendix 2). The combination therapy curves did not fit this model. To evaluate the response to combinations, we calculated the ratio of the SF(RT+Rx) combination to the SF(RT) monotherapy, for each RT dose and inhibitor (Table 2). The SF(RT +Rx)/SF(RT) for each radiation dose was compared to SF(Rx),RT=0 Gy, by unpaired t test with Welch correction, with False Discovery Rate (FDR), calculated by Two-stage step-up (Benjamini, Krieger, and Yekutieli), set to 1.00%. The statistically significant combinations were classified as antagonistic or sensitizing (Table 2). A human astrocyte line (HF3726) was used as control. This line presented resistance to RT (Appendix 2), and all four MDM2 and CDK4/6 inhibitors were protective at higher doses of radiation (Table 2). At all radiation doses, the DNAPK inhibitor M3814 sensitized two CSC lines, HF3177 and HF3253 to RT. For a few lines, the CDK4/6 inhibitors were antagonistic, when treatment with inhibitor was initiated simultaneously with RT. For most CSC lines and targeted agents, the inhibitor and radiation treatment each contributed to reduce cell viability without observed antagonism or synergism (Table 2, Appendix 1). We are currently integrating this data with the known genomic and molecular feature of each CSC line, results from the clonogenic assay, the CSC specific sensitivity to RT alone (Appendix 2), and the single agent treatment with the inhibitors (Annual Report 2020), to identify patterns of correlation. Based on these results, we are also testing different schedules, such as pre-treatment, other inhibitor concentrations, and fractionated radiation for selected CSC lines.

C) Efficacy of CDK4/6 inhibitor and MDM2 antagonist to treat glioblastoma PDXs.

In the pilot studies reported in the 2020 Annual Report, we determined that 3 sequential doses of 3 Gy delivered to the mice brain using the Small Animal Radiation Research Platform (SARRP, Xstrahl) was well tolerated by the mice and resulted in a significant increase in PDX survival for the test line (HF3016). HF3016 model was derived from a newly diagnosed glioblastoma, and the HF3177 model is derived from a recurrence from the same patient, after treatment with temozolomide and radiation. ecDNA amplification of CDK4 has been confirmed in both models. Here we have treated the newly diagnosed and recurrent models with the maximum tolerated dose of CDK4/6 inhibitor Ribociclib, shown to reach the brain tumors and engage the target in PDX (2020 Annual Report). The daily oral treatment started simultaneously with RT and continued for up to 7 weeks, control group received mock treatment, and mice were sacrificed when symptomatic. Brains were harvested and prepared for histology and molecular analysis. The HF3016 model was sensitive to 9 Gy RT alone ($p=0.0131$), but HF3177 was resistant ($p=0.0632$) CDK4/6i was not effective as a monotherapy in either model, while the combination of RT and ribociclib resulted in a modest but significant increase in survival for both PDX models (Fig. 2A, B). HF3055 glioblastoma model carries CDK4 and MDM2 co-amplification in ecDNA, and being wildtype (wt) TP53 is a candidate for treatment with the MDM2 antagonist RG7112, in addition to ribociclib. HF3055 PDX did not respond to RT, ribociclib or RG7112 single agent treatment (Fig. 2C). However, when RG7112 treatment was combined with RT or with ribociclib, a significant increase in survival in relation to control treatment was observed. Further, pre-treatment with RG7112 for 1 week prior to radiation, followed by continued treatment, resulted in the largest survival advantage, with subjects asymptomatic at the end of the study (310 days post implant). We conclude that pre-treatment with MDM2 antagonists in combination therapy can potentiate radiation therapy in wt TP53 resistant glioblastoma tumors and are seeking additional funds to expand these studies.

TABLE 2. Combination Therapy

		SF(Rx+RT)/SF(RT)												
CSC line	Rx	RT=0 Gy	SE (*)	RT=1Gy	SE (*)	RT=2Gy	SE (*)	RT=3Gy	SE (*)	RT=4Gy	SE (*)	RT=5Gy	SE (*)	Significant
HF2354	Abemaciclib	0.500	0.027	0.472	0.025	0.548	0.025	0.454	0.018	0.921	0.049	0.687	0.043	antagonistic
	Ribociclib	0.618	0.039	0.710	0.049	0.608	0.041	0.581	0.056	0.804	0.047	0.751	0.053	
	M3814	0.560	0.068	0.293	0.019	0.233	0.016	0.161	0.007	0.226	0.016	0.240	0.020	sensitizing
HF2381	AMG232	0.337	0.030	0.236	0.021	0.161	0.017	0.124	0.011	0.111	0.012	0.104	0.007	
	RG7112	0.630	0.054	0.543	0.042	0.520	0.033	0.500	0.025	0.521	0.027	0.525	0.032	
HF2381 (b)	Abemaciclib	0.428	0.059	0.610	0.095	0.429	0.028	0.675	0.092	0.657	0.039	0.551	0.054	
	Abemaciclib	0.745	0.141	1.017	0.197	0.657	0.097	0.931	0.129	0.736	0.058	0.709	0.066	
	Ribociclib	0.633	0.112	0.859	0.136	0.603	0.051	0.734	0.101	0.787	0.050	0.671	0.071	
	Ribociclib	0.656	0.150	0.806	0.200	0.836	0.068	0.884	0.150	0.851	0.065	0.808	0.096	
	RG7112	1.132	0.188	1.201	0.195	0.912	0.055	0.831	0.116	0.877	0.092	0.542	0.066	
	M3814	0.634	0.140	0.688	0.140	0.512	0.051	0.339	0.068	0.506	0.055	0.311	0.058	
	AZD7648	0.990	0.187	1.052	0.194	0.563	0.055	0.649	0.120	0.546	0.035	0.478	0.082	
	Ribociclib/RG7112	1.063	0.152	1.374	0.221	0.959	0.083	0.878	0.182	0.891	0.074	0.567	0.051	
HF2587	Abemaciclib	0.621	0.096	0.359	0.149	0.641	0.321	1.592	0.944	1.638	0.248	1.436	0.556	
	Ribociclib	0.338	0.077	0.441	0.258	1.263	0.245	2.192	0.894	1.194	0.219	2.223	1.030	
	AMG232	0.216	0.053	0.094	0.035	0.022	0.005	0.034	0.020	0.036	0.021	0.086	0.034	
	RG7112	0.554	0.097	0.323	0.104	0.421	0.149	0.827	0.344	1.185	0.415	0.925	0.318	
HF2927	Abemaciclib	0.529	0.036	0.787	0.087	0.799	0.047	0.923	0.068	0.950	0.057	1.046	0.058	
	Ribociclib	0.390	0.035	0.899	0.099	0.706	0.044	0.818	0.059	0.973	0.035	0.968	0.050	
	AMG232	0.473	0.034	0.602	0.064	0.496	0.034	0.528	0.050	0.625	0.025	0.555	0.035	
	RG7112	0.652	0.052	0.678	0.080	0.669	0.032	0.722	0.055	0.810	0.061	0.871	0.046	
HF3016	Abemaciclib	0.569	0.047	0.619	0.042	0.698	0.064	0.568	0.043	0.678	0.099	1.228	0.083	
	Ribociclib	0.979	0.089	0.770	0.082	0.976	0.084	0.721	0.084	0.888	0.110	1.042	0.120	
	AMG232	1.142	0.086	1.056	0.070	1.145	0.101	0.858	0.042	0.981	0.090	1.045	0.082	
	RG7112	0.974	0.069	0.923	0.074	1.045	0.082	0.727	0.051	0.834	0.063	0.942	0.081	
	M3814	0.630	0.048	0.514	0.044	0.493	0.058	0.508	0.059	0.552	0.063	0.771	0.107	
HF3016_b	M3814	0.538	0.078	0.240	0.034	0.295	0.042	0.555	0.086	0.269	0.061	0.669	0.089	
	Ribociclib	0.578	0.112	0.447	0.079	0.417	0.073	0.632	0.066	0.523	0.061	0.916	0.116	
HF3016_c	Abemaciclib	0.511	0.019	0.555	0.017	0.498	0.016	0.776	0.046	0.933	0.033	1.198	0.026	
	Ribociclib	0.573	0.023	0.604	0.024	0.540	0.024	0.798	0.049	0.902	0.060	1.085	0.039	
	AZD7648	0.802	0.031	0.725	0.028	0.668	0.020	0.689	0.044	0.730	0.026	0.763	0.033	
	M3814	0.711	0.041	0.633	0.021	0.545	0.022	0.543	0.037	0.646	0.028	0.736	0.053	
HF3035	Abemaciclib	1.040	0.043	1.448	0.088	1.211	0.030	1.153	0.114	1.291	0.099	1.202	0.133	
	Ribociclib	1.000	0.135	0.977	0.062	0.864	0.059	0.840	0.079	1.024	0.068	0.992	0.098	
	AMG232	0.692	0.056	0.776	0.074	0.568	0.020	0.704	0.071	0.812	0.074	0.741	0.087	
	RG7112	0.665	0.035	0.684	0.104	0.583	0.024	0.749	0.077	0.810	0.076	0.678	0.086	
	M3814	0.933	0.047	0.939	0.144	0.701	0.026	0.640	0.052	0.820	0.043	0.720	0.086	
HF3035 _b	M3814	1.252	0.113	1.156	0.044	1.085	0.049	1.033	0.037	1.060	0.026	0.957	0.043	
	AZD7648	1.346	0.122	1.296	0.060	1.166	0.053	1.187	0.041	1.155	0.037	1.093	0.055	
HF3055	Abemaciclib	1.118	0.150	0.489	0.043	0.485	0.042	0.472	0.057	0.899	0.113	0.466	0.041	
	Ribociclib	0.927	0.132	0.688	0.144	0.373	0.042	0.383	0.052	0.827	0.103	0.418	0.032	
	AMG232	0.357	0.039	0.691	0.070	0.664	0.093	0.801	0.102	0.712	0.063	0.607	0.109	
	RG7112	0.339	0.072	0.354	0.045	0.378	0.038	0.423	0.062	0.432	0.046	0.431	0.044	
	M3814	0.372	0.047	0.447	0.066	0.425	0.028	0.362	0.033	0.470	0.068	0.416	0.060	
	AZD7648	0.312	0.031	1.003	0.135	1.016	0.111	1.062	0.152	0.551	0.081	0.847	0.081	
HF3077	Abemaciclib	1.031	0.112	1.058	0.111	1.024	0.079	1.089	0.080	1.138	0.093	1.970	0.151	
	Ribociclib	1.106	0.126	1.125	0.121	0.986	0.082	1.119	0.092	1.150	0.097	2.153	0.163	
	AZD7648	0.267	0.039	0.273	0.029	0.298	0.028	0.265	0.032	0.319	0.026	0.432	0.034	
	M3814	0.211	0.025	0.228	0.024	0.247	0.020	0.271	0.024	0.336	0.026	0.411	0.031	
HF3160	Abemaciclib	0.805	0.071	0.745	0.032	0.929	0.055	1.005	0.066	1.053	0.091	1.397	0.151	
	Ribociclib	0.888	0.151	0.726	0.040	0.971	0.074	1.173	0.119	1.275	0.097	1.547	0.172	
	M3814	0.987	0.087	0.807	0.076	0.905	0.044	0.961	0.069	0.868	0.094	1.008	0.121	
	AZD7648	1.489	0.136	1.231	0.077	1.252	0.088	1.174	0.072	1.020	0.085	1.210	0.137	
HF3177	Abemaciclib	0.841	0.039	0.782	0.025	0.893	0.021	0.926	0.038	0.965	0.020	1.275	0.047	
	Ribociclib	1.089	0.058	1.038	0.041	1.086	0.035	1.149	0.049	1.029	0.021	1.258	0.037	
	M3814	0.835	0.037	0.260	0.010	0.272	0.017	0.317	0.015	0.335	0.013	0.512	0.018	
HF3178	Abemaciclib	1.023	0.148	1.145	0.049	1.154	0.120	1.195	0.075	1.095	0.075	0.679	0.065	
	Ribociclib	0.910	0.114	0.974	0.059	0.995	0.082	1.049	0.064	1.012	0.079	0.629	0.070	
	AMG232	0.928	0.085	0.918	0.045	0.787	0.076	0.877	0.066	0.806	0.045	0.486	0.044	
	RG7112	0.889	0.100	0.819	0.039	0.782	0.050	0.764	0.052	0.813	0.051	0.538	0.050	
HF3203	Abemaciclib	1.179	0.114	1.271	0.239	1.289	0.206	1.108	0.143	1.546	0.195	1.852	0.179	
	Ribociclib	1.026	0.120	1.338	0.191	1.433	0.172	1.203	0.158	1.478	0.207	1.343	0.101	
HF3203 (b)	Abemaciclib	0.984	0.108	1.164	0.125	0.987	0.078	0.926	0.065	0.878	0.060	0.836	0.052	
	Ribociclib	1.272	0.082	1.458	0.152	1.052	0.059	0.753	0.028	0.812	0.052	0.793	0.040	
	M3814	1.660	0.139	1.875	0.162	1.684	0.106	1.231	0.054	1.327	0.073	1.192	0.057	
	AZD7648	1.578	0.155	1.562	0.152	1.622	0.115	1.323	0.062	1.307	0.061	1.236	0.082	
HF3253	Abemaciclib	0.474	0.027	0.614	0.039	0.908	0.056	1.387	0.094	2.276	0.162	3.270	0.205	
	Ribociclib	0.479	0.023	0.617	0.031	0.865	0.067	1.088	0.064	1.356	0.116	1.855	0.126	
	AMG232	0.760	0.040	0.956	0.041	1.039	0.083	1.063	0.078	1.022	0.083	1.335	0.083	
	RG7112	0.829	0.120	1.070	0.058	1.021	0.072	1.094	0.073	1.180	0.095	1.330	0.131	
	M3814	0.474	0.026	0.081	0.007	0.062	0.004	0.062	0.004	0.101	0.009	0.147	0.010	
HF3451	AMG232	0.550	0.076	0.604	0.054	0.586	0.057	0.606	0.064	0.731	0.137	0.753	0.088	
	RG7112	0.693	0.200	0.701	0.068	0.686	0.082	0.636	0.101	0.960	0.113	1.067	0.088	
HF3726	Abemaciclib	0.652	0.014	0.769	0.025	0.794	0.026	0.900	0.047	0.936	0.050	1.054	0.033	
	Ribociclib	0.655	0.009	0.745	0.034	0.735	0.030	0.839	0.049	0.982	0.034	0.969	0.032	
	AMG232	0.634	0.016	0.648	0.015	0.691	0.021	0.726	0.036	0.778	0.026	0.814	0.022	
	RG7112	0.657	0.020	0.658	0.016	0.675	0.017	0.687	0.043	0.743	0.020	0.846	0.033	



Treatment group comparison	Log-rank (Mantel-Cox) test	Sig.	Hazard Ratio	95% CI of ratio
C vs RT	0.0131	(*)	0.4158	0.1581 to 1.094
C vs Ribociclib	0.4128		0.7579	0.3128 to 1.836
C vs Ribociclib/RT	0.0329	(*)	0.4767	0.1867 to 1.217

Treatment group comparison	Log-rank (Mantel-Cox) test	Sig.	Hazard Ratio	95% CI of ratio
C vs RT	0.0632		0.4851	0.1825 to 1.289
C vs Ribociclib	0.2902		0.6603	0.2605 to 1.673
C vs Ribociclib/RT	0.0458	(*)	0.4536	0.1674 to 1.229

Treatment group comparison	Log-rank (Mantel-Cox) test	Sig.	Hazard Ratio (Mantel-Haenszel)	95% CI of ratio
C vs RT	0.0528		0.3021	0.08992 to 1.015
C vs Ribociclib	0.0815		0.3459	0.1048 to 1.142
C vs Ribociclib/RT	0.09		0.3591	0.1099 to 1.173
C vs. RG7112/ Ribociclib	0.0432	(*)	0.2695	0.07562 to 0.9607
C vs RG7112	0.0652		0.2465	0.07085 to 0.8578
C vs RG7112/RT	0.0277	(*)	0.3358	0.1053 to 1.071
C vs RG7112-pre/RT	0.0006	(***)	0.2137	0.06139 to 0.7441

Figure 2. Effect of targeted and radiation therapy on CDK4 amplified glioblastoma PDX. Kaplan-Meier survival curves for HF3016 (A), HF3177 (recurrent tumor matched to HF3016) (B), and HF3055 (C). CSC implanted nude mice were randomized to the indicated treatment groups. Schedule for radiation and targeted therapy, and the number of mice in each study are shown. Survival curves for the treatments were compared to control mice by Log-rank (Mantel-Cox) test, and significant differences are indicated in red.

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. She also received a AACR Minority Scholar in Cancer Research award to present the results of her research funded by this award at the Evolutionary Dynamics in Carcinogenesis and Response to Therapy meeting, originally scheduled to take place in Denver (CO), March 12-15, 2020, postponed to March 2022. Both students have presented their work at national and local meetings (listed below).

Two Wayne State University medical students joined the lab to contribute to this project after the introductory training. Jacob Gluski (4th year) participated in a 2021 summer research rotation, programing the Keyence

Imaging System to quantify images of clonogenic assays to evaluated cancer stem cell response to treatment. Annie Tonnu (1st year) has joined the lab in August 2021, for a multi-year part-time volunteer research position to participate in brain tumor pre-clinical research. She has received the appropriate training and is already contributing to this project, by assessing target engagement in PDX in response to treatment through quantitative analysis of immunohistochemistry, and real-time PCR.

How were the results disseminated to communities of interest?
The results from this project were presented to:
A) Local clinicians and scientists, through multiple internal presentation at Research retreat, Research Symposium, and focused work 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 on Aug 12, 2021.

What do you plan to do during the next reporting period to accomplish the goals?
As stated in the approved no cost extension (NCE), during the Sep 2021 – Aug 2022 period, we plan on accomplishing the following:

- Analysis of samples collected during long-term treatment of glioblastoma CSC with MDM2i and CDK4/6i (SA1.2, exp. 3).
- The glioblastoma PDX study testing MDM2i and CDK4/5i (SA1.3) has been partially completed, and two additional lines will be tested in the second semester of 2021.
- Sample collection and RNA sequencing have been completed for 24 samples generated in SA 1.1, leading to novel insights in the adaptation of glioblastoma stem cells to the loss of extrachromosomal amplification of oncogenes. The next batch of 48 samples for RNA sequencing analysis will be submitted in the second semester, 2021 (SA1.4), and the last batch of 24 samples in the first semester 2022. We anticipate the results from these analyses will greatly contribute to the understanding of mechanisms of resistance and identify new targets for combination therapies for gliomas.
- Long term treatment of glioblastoma CSCs with DNAPKi (SA2.1, experiment 4) will be accomplished by December 2021. We expect the functional analysis of the consequences of DNAPK knockdown in glioblastoma cancer stem cells described in SA2.2 experiment 2 and SA2.3, experiment 2 will be completed by February 2022. We are eager to complete these experiments, which we anticipate will lead to supporting evidence for the pharmacological inhibition of this important kinase in the treatment of glioblastoma.
- Manuscripts reporting the findings presented here are in preparation and will be listed on the final report.

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 in this study 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.

What was the impact on other disciplines?
This study has encouraged other departments in our institution to build similar pre-clinical programs. I am listed as a collaborator in two proposals submitted in 2021 to the Department of Defense for the development of patient-derived models for testing novel therapeutics for non-small cell lung cancer and prostate cancer.

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. We believe this parallel pre-clinical trial will contribute to the understanding of why after decades of clinical trials, no targeted agent has shown effectivity against this devastating malignancy, as an essential step to propose more sophisticated patient selection and therapeutic strategies.

5. **CHANGES/PROBLEMS:**
Changes in approach and reasons for change
No major change to report.

Actual or anticipated problems or delays and actions or plans to resolve them
Pandemic associated delays affecting research in general: As mentioned on the NCE request, we had challenges created by the adoption of safety measures in response to the COVID19 pandemic at our institution, and by third party vendors providing products and services for this project. What affected this project the most were: A) Lower capacity in the animal facilities leading to the need to stagger the animal work to avoid having more than a few cages of mice at a time. B) Difficulty in acquiring certain laboratory supplies, such as RNA extraction kits and even pipets. These shortages have now been resolved. C) Two graduate students who are named on this award (no salary) were prevented from working in the lab for 5 months in 2020, and at reduced effort for another 4 months. The students are now back full time in the lab and will continue to complete experiments associated with this project. D) Third parties providing RNA sequencing service for this project were closed for business for most of 2020 and backlogged upon re-opening. The vendors have now returned to the normal turnaround time.

Changes that had a significant impact on expenditures
The PDX mice represented in Figure 2C, presented a longer symptom-free survival time than predicted by our historical data. Because our experimental design demanded continuous treatment with the pharmacological inhibitors until symptom development or end of the study, set to 310 days, we used considerably more inhibitors than anticipated. The MDM2 antagonist we had selected which presented the best blood-brain barrier penetration (RG7112, Roche), is no longer in clinical trials due to side effects, and relatively high doses for clinical efficacy. Another improved nutlin derivative, RG7388 (idasanutlin), which has demonstrated higher efficacy and lower toxicity, is progressing in clinical trials, including an umbrella phase I/IIa clinical trial for glioblastoma (NCT03158389). Although we have not observed signs of toxicity in mice with the prolonged RG7112 treatment, to maintain the translational value of our research, we will use RG7388 in the remaining mice cohort to be tested.

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
Nothing to report.

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

Other publications, conference papers, and presentations

Invited oral presentation

"Pharmaco-omics studies using patient-derived models support precision medicine for glioblastoma". 2nd Annual Neuro-Oncology Symposium (Pakistan Society for Neuro-Oncology). Session 7, Precision Medicine & Innovations in Neuro-Oncology. September 4, 2021.

Virtual Oral presentation by graduate students

Berezovsky AD, Datta I, She R, Hasselbach LA, Poisson L, **deCarvalho AC**, Assessing Adaptive Responses to Loss of Extrachromosomal DNA Amplification (OTEH-12) Neuro-Oncology Advances, Volume 3, Issue Supplement_2, July 2021, Page ii13, https://doi.org/10.1093/noajnl/vdab070.051; SNO Basic and Translational Omics of Brain Tumors and Their Microenvironment Conference, Virtual, July 15-16, 2021.

Virtual Poster presentation and published abstract by graduate students

Nuga O, Meng Y, **deCarvalho AC.** Pharmacological Inhibition of Cyclin Dependent Kinase 4/6 (CDK4/6) in Glioblastoma (EXTH-36) 25TH ANNUAL MEETING. Society for Neuro-Oncology Virtual meeting November 19-21, 2020.

Berezovsky AD, Irtenkauf SM, Transou AD, Hasselbach LA, Mikkelsen T, **deCarvalho AC**. Platelet derived growth factor receptor alpha as an oncogenic driver in glioblastoma (CSIG-06) 25TH ANNUAL MEETING. Society for Neuro-Oncology Virtual meeting November 19-21, 2020.

Website(s) or other Internet site(s)

This Award has contributed to increased awareness of the importance of pre-clinical research in oncology, resulting in a video production highlighting our program: https://www.youtube.com/watch?v=Z4-Wj1HypMg .

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
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	0000-0003-4266-0931
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 until June 2021, when she transitioned to another position.
Funding Support:	

Name	Thais Sabedot
Project Role	Bioinformatician
ORCID ID	0000-0003-4266-0931
Nearest person month worked:	0.28
Contribution to Project:	Ms. Sabedot has replaced Ms. Datta for the bioinformatics work from June to August 2021.
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. Surviving fractions for radiation and targeted therapy combination

CSC line/ Passage	Rx	[conc] uM	SF0		SF1		SF2		SF3		SF4		SF5	
			Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
HF2354	vehicle		1.000	0.035	0.960	0.046	0.806	0.010	0.758	0.013	0.339	0.016	0.114	0.006
P26	Abemaciclib	1.000	0.500	0.021	0.453	0.010	0.441	0.019	0.344	0.013	0.312	0.008	0.078	0.002
	Ribociclib	1.000	0.618	0.032	0.682	0.034	0.490	0.032	0.440	0.042	0.273	0.009	0.086	0.004
	M3814	0.360	0.560	0.065	0.281	0.012	0.188	0.013	0.122	0.005	0.077	0.004	0.027	0.002
HF2381	vehicle		1.000	0.051	0.990	0.044	0.845	0.028	0.597	0.016	0.473	0.014	0.361	0.017
P27	AMG232	0.003	0.337	0.025	0.234	0.018	0.136	0.013	0.074	0.006	0.052	0.005	0.037	0.002
	RG7112	0.009	0.630	0.043	0.538	0.034	0.439	0.023	0.299	0.013	0.246	0.011	0.190	0.008
HF2381_b	vehicle		1.000	0.129	0.674	0.101	0.611	0.010	0.415	0.056	0.365	0.017	0.299	0.024
P29	Abemaciclib	0.100	0.428	0.022	0.411	0.017	0.262	0.016	0.281	0.008	0.240	0.009	0.165	0.010
	Abemaciclib	0.010	0.745	0.103	0.685	0.083	0.401	0.059	0.387	0.015	0.269	0.017	0.212	0.011
	Ribociclib	0.100	0.633	0.077	0.579	0.029	0.368	0.031	0.305	0.011	0.287	0.012	0.201	0.014
	Ribociclib	0.010	0.657	0.123	0.543	0.107	0.510	0.040	0.367	0.038	0.311	0.019	0.242	0.021
	RG7112	0.009	1.132	0.118	0.810	0.050	0.557	0.032	0.345	0.014	0.320	0.030	0.162	0.015
	M3814	0.100	0.634	0.113	0.464	0.064	0.312	0.031	0.141	0.021	0.185	0.018	0.093	0.016
	AZD7648	0.100	0.990	0.136	0.709	0.076	0.344	0.033	0.269	0.034	0.199	0.009	0.143	0.022
	Ribociclib / RG7112	0.1 / 0.009	1.063	0.066	0.926	0.052	0.586	0.050	0.365	0.058	0.325	0.022	0.169	0.007
HF2587	vehicle		1.000	0.107	0.483	0.146	0.212	0.024	0.103	0.041	0.092	0.011	0.064	0.020
P21	Abemaciclib	0.060	0.621	0.070	0.173	0.049	0.136	0.066	0.164	0.072	0.150	0.014	0.092	0.021
	Ribociclib	0.800	0.338	0.068	0.213	0.107	0.267	0.042	0.226	0.017	0.109	0.015	0.142	0.049
	AMG232	0.006	0.216	0.048	0.045	0.010	0.005	0.001	0.003	0.002	0.003	0.002	0.005	0.001
	RG7112	0.012	0.554	0.077	0.156	0.018	0.089	0.030	0.085	0.009	0.108	0.036	0.059	0.008
HF2927	vehicle		1.000	0.055	0.902	0.090	0.357	0.016	0.294	0.018	0.240	0.008	0.224	0.009
P16	Abemaciclib	0.100	0.529	0.021	0.709	0.034	0.285	0.011	0.272	0.010	0.228	0.012	0.235	0.009
	Ribociclib	0.100	0.390	0.028	0.810	0.039	0.252	0.010	0.241	0.009	0.234	0.004	0.217	0.007
	AMG232	0.005	0.473	0.022	0.543	0.020	0.177	0.009	0.155	0.011	0.150	0.003	0.124	0.006
	RG7112	0.027	0.652	0.038	0.612	0.039	0.238	0.003	0.212	0.010	0.195	0.013	0.195	0.006
HF3016	vehicle		1.000	0.049	0.991	0.059	0.848	0.045	0.895	0.041	0.812	0.051	0.681	0.032
P17	Abemaciclib	1.000	0.569	0.039	0.614	0.019	0.592	0.044	0.508	0.031	0.550	0.073	0.836	0.040
	Ribociclib	1.000	0.979	0.076	0.763	0.067	0.828	0.057	0.645	0.069	0.721	0.077	0.709	0.074
	AMG232	0.007	1.142	0.065	1.046	0.031	0.971	0.069	0.768	0.015	0.797	0.053	0.711	0.044
	RG7112	0.156	0.974	0.050	0.914	0.049	0.886	0.051	0.650	0.034	0.677	0.028	0.641	0.046
	M3814	0.810	0.630	0.037	0.509	0.031	0.418	0.044	0.455	0.049	0.448	0.042	0.525	0.069
HF3016_b	vehicle		1.000	0.141	1.147	0.133	0.909	0.119	0.615	0.032	0.941	0.060	0.416	0.046
P19	M3814	0.400	0.538	0.018	0.275	0.023	0.268	0.015	0.341	0.050	0.253	0.055	0.278	0.021
	Ribociclib	2.000	0.578	0.076	0.512	0.068	0.379	0.044	0.388	0.036	0.492	0.048	0.381	0.024
HF3016_c			1.000	0.022	0.900	0.026	0.853	0.023	0.621	0.036	0.486	0.015	0.365	0.007
P16	Abemaciclib	1.000	0.511	0.016	0.500	0.005	0.425	0.007	0.482	0.005	0.454	0.007	0.437	0.005
	Ribociclib	2.000	0.573	0.019	0.543	0.015	0.460	0.016	0.495	0.009	0.439	0.025	0.396	0.012
	AZD7648	0.400	0.802	0.025	0.653	0.017	0.569	0.006	0.428	0.011	0.355	0.006	0.278	0.011
	M3814	0.400	0.711	0.038	0.570	0.009	0.465	0.014	0.337	0.011	0.314	0.009	0.268	0.019
HF3035	vehicle		1.000	0.032	0.652	0.032	0.747	0.015	0.673	0.054	0.560	0.035	0.622	0.060
P21	Abemaciclib	1.000	1.040	0.027	0.944	0.034	0.904	0.013	0.776	0.044	0.723	0.033	0.747	0.040
	Ribociclib	2.000	1.000	0.132	0.637	0.025	0.645	0.042	0.566	0.028	0.574	0.015	0.616	0.011
	AMG232	0.005	0.692	0.051	0.506	0.041	0.424	0.012	0.474	0.029	0.455	0.031	0.461	0.030
	RG7112	0.042	0.665	0.028	0.446	0.064	0.435	0.016	0.504	0.033	0.454	0.032	0.422	0.035
	M3814	0.150	0.933	0.040	0.612	0.091	0.523	0.018	0.431	0.025	0.459	0.011	0.448	0.048
HF3035_b			1.000	0.076	0.782	0.020	0.604	0.024	0.611	0.012	0.567	0.010	0.580	0.025
P22	M3814	0.100	1.252	0.060	0.904	0.025	0.655	0.013	0.632	0.018	0.601	0.010	0.556	0.006
	AZD7648	0.100	1.346	0.066	1.014	0.039	0.704	0.014	0.726	0.020	0.655	0.018	0.634	0.016
	vehicle		1.000	0.097	1.035	0.086	1.124	0.055	0.886	0.076	0.857	0.069	1.016	0.044
HF3055	Abemaciclib	1.000	1.118	0.104	0.506	0.013	0.545	0.039	0.418	0.035	0.770	0.075	0.473	0.037
P28	Ribociclib	1.000	0.927	0.097	0.712	0.137	0.419	0.043	0.339	0.036	0.709	0.067	0.425	0.027
	AMG232	0.006	0.357	0.017	0.715	0.041	0.746	0.098	0.710	0.066	0.610	0.023	0.616	0.108
	RG7112	0.156	0.339	0.064	0.366	0.035	0.425	0.037	0.375	0.045	0.370	0.026	0.438	0.041
	M3814	2.000	0.372	0.030	0.463	0.057	0.478	0.020	0.321	0.009	0.402	0.049	0.422	0.058
	AZD7648	2.000	0.349	0.008	0.508	0.053	0.553	0.054	0.444	0.051	0.425	0.052	0.401	0.034
HF3077 (a) I	vehicle		1.000	0.010	0.894	0.025	0.781	0.016	0.695	0.017	0.489	0.045	0.577	0.023
HF3077	vehicle		1.000	0.108	0.890	0.091	0.809	0.060	0.751	0.055	0.684	0.050	0.584	0.043
P35	Abemaciclib	0.060	1.031	0.014	0.942	0.024	0.829	0.018	0.818	0.009	0.778	0.029	1.150	0.023
	Ribociclib	0.400	1.106	0.041	1.001	0.033	0.798	0.030	0.841	0.033	0.786	0.034	1.256	0.020
	AZD7648	0.700	0.267	0.027	0.243	0.007	0.242	0.014	0.199	0.020	0.218	0.008	0.252	0.006
	M3814	0.700	0.211	0.010	0.203	0.006	0.200	0.007	0.204	0.010	0.230	0.007	0.240	0.003
HF3160	vehicle		1.000	0.064	1.103	0.025	0.827	0.030	0.692	0.027	0.605	0.041	0.455	0.047
P20	Abemaciclib	0.100	0.805	0.049	0.822	0.030	0.768	0.036	0.695	0.037	0.637	0.033	0.636	0.020
	Ribociclib	0.200	0.887	0.139	0.801	0.040	0.803	0.054	0.811	0.076	0.772	0.025	0.704	0.029
	M3814	0.400	0.986	0.060	0.891	0.081	0.748	0.024	0.665	0.041	0.525	0.044	0.459	0.028
	AZD7648	0.400	1.489	0.098	1.359	0.079	1.035	0.062	0.812	0.039	0.617	0.029	0.551	0.025
HF3177	vehicle		1.000	0.038	1.069	0.024	0.879	0.018	0.744	0.026	0.697	0.012	0.437	0.009
P29	Abemaciclib	1.000	0.841	0.023	0.836	0.020	0.785	0.008	0.689	0.014	0.673	0.007	0.557	0.016
	Ribociclib	1.000	1.089	0.040	1.110	0.036	0.954	0.024	0.855	0.020	0.718	0.007	0.549	0.011
	M3814	0.300	0.835	0.019	0.277	0.008	0.239	0.014	0.236	0.008	0.234	0.008	0.223	0.006
HF3178	vehicle		1.000	0.089	0.723	0.027	0.657	0.028	0.608	0.030	0.620	0.033	1.000	0.089
P19	Abemaciclib	1.000	1.023	0.116	0.827	0.017	0.758	0.072	0.726	0.029	0.680	0.030	0.679	0.023
	Ribociclib	2.000	0.910	0.080	0.704	0.034	0.653	0.046	0.638	0.023	0.628	0.036	0.629	0.042
	AMG232	0.004	0.928	0.020	0.664	0.021	0.517	0.045	0.533	0.030	0.500	0.009	0.486	0.008
	RG7112	0.040	0.889	0.061	0.592	0.017	0.514	0.024	0.465	0.022	0.504	0.017	0.538	0.014
HF3203	vehicle		1.000	0.084	0.509	0.059	0.397	0.042	0.315	0.034	0.182	0.017	0.172	0.007
P24	Abemaciclib	1.000	1.179	0.057	0.647	0.095	0.511	0.061	0.349	0.024	0.281	0.024	0.319	0.028
	Ribociclib	1.000	1.026	0.084	0.681	0.056	0.569	0.032	0.379	0.028	0.269	0.028	0.231	0.014
HF3203_b	vehicle		1.000	0.060	0.751	0.059	0.704	0.031	0.739	0.013	0.679	0.029	0.711	0.031
P26	Abemaciclib	1.000	0.984	0.091	0.874	0.064	0.695	0.045	0.685	0.046	0.596	0.032	0.594	0.026
	Ribociclib	2.000	1.272	0.029	1.095	0.074	0.741	0.025	0.557	0.018	0.551	0.026	0.564	0.015
	M3814	0.010	1.660	0.097	1.408	0.049	1.186	0.053	0.910	0.037	0.900	0.031	0.847	0.018
	AZD7648	0.010	1.578	0.123	1.173	0.067	1.143	0.063	0.978	0.043	0.887	0.017	0.879	0.044
HF3253	vehicle		1.000	0.045	0.546	0.020	0.270	0.015	0.145	0.006	0.083	0.006	0.048	0.002
P21	Abemaciclib	1.000	0.474	0.016	0.335	0.017	0.245	0.006	0.201	0.010	0.189	0.003	0.156	0.007
	Ribociclib	1.000	0.479	0.008	0.337	0.011	0.233	0.012	0.158	0.006	0.112	0.006	0.088	0.004
	AMG232	0.007	0.760	0.020	0.521	0.011	0.280	0.016	0.154	0.009	0.085	0.004	0.064	0.003
	RG7112	0.156	0.829	0.114	0.584	0.023	0.276	0.011	0.159	0.008	0.098	0.004	0.063	0.006
	M3814	0.010	0.474	0.015	0.044	0.004	0.017	0.001	0.009	0.000	0.008	0.001	0.007	0.000
HF3451	vehicle		1.000	0.052	0.767	0.054	0.695	0.045	0.641	0.065	0.634	0.067	0.482	0.031
P18	AMG232	0.007	0.550	0.070	0.463	0.025	0.407	0.029	0.388	0.013	0.463	0.072	0.363	0.035
	RG7112	0.030	0.693	0.196	0.538	0.036	0.476	0.047	0.408	0.050	0.608	0.033	0.515	0.026
HF3726 (As	vehicle		1.000	0.005	0.702	0.010	0.601	0.012	0.577	0.025	0.518	0.007	0.481	0.010
P9	Abemaciclib	1.000	0.652	0.014	0.539	0.016	0.477	0.013	0.520	0.016	0.485	0.025	0.507	0.012
2% FBS	Ribociclib	2.000	0.655	0.008	0.523	0.023	0.442	0.016	0.484	0.019	0.509	0.016	0.466	0.011
	AMG232	0.006	0.634	0.016	0.455	0.008	0.415	0.010	0.419	0.010	0.403	0.012	0.391	0.007
	RG7112	0.022	0.657	0.020	0.462	0.009	0.406	0.007	0.397	0.018	0.385	0.009	0.407	0.013

Appendix 2. Radiation dose-response curve analysis by the single hit multi-target model

	Do	SE (CV%)	95% CI		R
HF2354	0.793	5.2	0.708	0.877	0.945
HF2381	2.655	11.4	2.036	3.274	0.928
HF2587	1.695	21.1	0.960	2.430	0.827
HF2927	2.790	12.8	2.057	3.523	0.836
HF3016	2.718	9.8	2.172	3.265	0.947
HF3077	7.612	22.4	4.109	11.110	0.850
HF3160	3.380	21.0	1.923	4.837	0.828
HF3177	2.755	10.7	2.150	3.361	0.899
HF3203	4.334	22.6	2.329	6.339	0.890
HF3253	1.602	4.9	1.441	1.762	0.983
HF3726 (Ast)	31.560	24.8	15.490	47.630	0.974
HF3451	res				
HF3035	res				
HF3055	res				
HF3178	res				