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14. ABSTRACT

This study aims to evaluate the effectiveness of G2/M checkpoint inhibitors as single therapeutic agent, or in combination with radiotherapy, a current standard therapy in GBM, as well as aims to identify new biomarkers for CHK1/2 sensitive GBMs. Successful completion of the aims will help us examine the effects of a new therapeutic strategy against GBM in specific molecular context.

15. SUBJECT TERMS
Glioblastoma, Targeted therapy, Synthetic lethality, Cell cycle checkpoint, *TP53, CDKN2A, CDKN2B, CDKN2C, CHK1*, *CHK2,* DNA damage response, CRISPR, Radiation

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1. INTRODUCTION:

In this study, we are investigating whether targeting the G2/M cell cycle checkpoint in glioblastoma bearing dual loss of *TP53* and *CDKN2A* can serve as a potential therapeutic strategy in inducing selective lethality. Glioblastoma (GBM) is one of the most common adult brain cancers and one of the deadliest human cancers. In United States alone, almost 10,000 new GBM cases are identified each year. The 5-year survival rate of GBM still remains strikingly low (less than 6%) with median life expectancy of 12-15 months following diagnosis. Apart from surgical resection, conventional therapies for GBM consist of systemic chemotherapy and radiotherapy. Unfortunately, all of these standard care therapies are ineffective in improving the overall survival rate. The major reasons for the lack of progress include heterogeneity of responses across subtypes of GBM and rapid development of resistance against standard therapies. Therefore, there is an urgent yet unmet need to develop effective targeted therapeutic strategies against GBM, one of which is being tested in the current proposal. Loss of both *TP53* and *CDKN2A* is a frequent event in not only GBMs, but in many other cancer types. This proposal is not only exploring the effectiveness of G2/M checkpoint inhibition in tumor cells with *TP53/CDKN2A* dual loss but will also explore the synergistic response of G2/M checkpoint inhibition with radiation, in a *TP53* mutant setting, which is also a recurrent event in multiple cancer types. Therefore, the knowledge obtained from this current study has the potential to shed light into the molecular vulnerabilities shared by a number of other cancer types, and learn how that can be targeted therapeutically, affecting a large number of patient populations.

2. KEYWORDS:

Glioblastoma, Targeted therapy, Synthetic lethality, Cell cycle checkpoint, *TP53, CDKN2A, CDKN2B, CDKN2C, CHK1*, *CHK2,* DNA damage response, CRISPR, Radiation

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Training-Specific Tasks:

Subtask 1: Present research at the weekly laboratory meetings and monthly department group meetings

Subtask 2: Attend national scientific meeting in relevant scientific field, once a year

Subtask 3: Attend Scientific Writing Retreat by Cold Spring Harbor Laboratory

Subtask 4: Prepare manuscripts based on the findings

Research-Specific Tasks:

Specific Aim 1: Test whether disruption of both *TP53* and *CDKN2A* preferentially sensitizes GBM cells to G2/M checkpoint inhibition through a synthetic lethal interaction.

Major Task 1: Test synthetic lethality in GBM model against G2/M checkpoint inhibitors
Planned period: 1-24 months
Percentages of completion: 40%

Subtask 1: Generation of the isogenic GBM cell lines of different genetic combinations (both conventional and patient-derived neurosphere lines)

Subtask 2: Test synthetic lethality induced by *CHK1/2* inhibitors and other regulators of G2/M checkpoint in cells with dual loss of *TP53/CDKN2A*

Subtask 3: Characterize the mechanism of selective lethality induced by the G2/M checkpoint inhibitors

Subtask 4: Confirm the *in vitro* finding using flank xenografts in mice *in vivo* following survival and tumor volume

Subtask 5: Validate the hypothesis from patient samples from Wee1 inhibitor trial and correlate survival and progression free survival data

Specific Aim 2: Test whether disruption of *TP53* works synergistically with loss of other genes or with genotoxic stresses, such as radiation, to increase sensitivity to G2/M checkpoint inhibition.

Major Task 2: Test synergistic response of checkpoint inhibitors with radiation, and genome-wide CRISPR analysis to identify additional dependencies with checkpoint inhibition
Planned period: 5-24 months
Percentages of completion: 20% (Majority could not be attempted due to the disruption at the research activities)

Subtask 1: Test synergistic response elicited by G2/M checkpoint inhibitor, AZD-7762, with radiation in cells with mutant *TP53* and characterize the mechanism

Subtask 2: Confirm the synergistic responses identified in 2.1 *in vivo* using flank xenografts in mice

Subtask 3: Biochemical characterization of tumors to analyze responses form 2.2

Subtask 4: Genome wide CRISPR screen in *TP53* mutant cells to identify additional dependencies following *CHK1/2* inhibition and validation

What was accomplished under these goals?

Training-Specific Tasks:
Subtask 1: Present research at the weekly laboratory meetings and monthly department group meetings

The findings and updates were presented regular intervals at the laboratory meetings, group meetings and department seminars

Subtask 2: Attend national scientific meeting in relevant scientific field, once a year

COVID-19 posed a huge challenge in attending scientific meetings in relevant scientific field as some of the most relevant meetings were canceled

Subtask 3: Attend Scientific Writing Retreat by Cold Spring Harbor Laboratory

Same reason as before – the retreat was not organized

Subtask 4: Prepare manuscripts based on the findings

Part of the findings were utilized in one of manuscript where PI is a co-author (mentioned below), and other manuscripts are in the preparations

Enache OM, Rendo V, Abdusamad M, Lam D, Davison D, Pal S, Currimjee N, Hess J, Pantel S, Nag A *et al*: **Cas9 activates the p53 pathway and selects for p53-inactivating mutations**. *Nat Genet* 2020, **52**(7):662-668.

Research-Specific Tasks:
Specific Aim 1: Test whether disruption of both *TP53* and *CDKN2A* preferentially sensitizes GBM cells to G2/M checkpoint inhibition through a synthetic lethal interaction.
Major Task 1: Test synthetic lethality in GBM model against G2/M checkpoint inhibitors

Subtask 1: Generation of the isogenic GBM cell lines of different genetic combinations (both conventional and patient-derived neurosphere lines)

Significant efforts were made first in identifying the cell line of right genotype, specifically for the patient derived lines from the living cell line bank and confirming the right genetic changes both for the classical and neurosphere GBM cultures for generating the isogenic lines. For testing our hypothesis relatively quickly, we prioritized in finding the right cell lines for the middle two

	***TP53* WT; *CDKN2A* WT**	***TP53* WT; *CDKN2A* HD**	***TP53* MUT; *CDKN2A* WT**	***TP53* MUT; *CDKN2A* HD**
Patient-derived Neurosphere lines	BT239	BT159	BT187	BT333
Classical GBM lines	CCFSTTG1	U87MG	KALS1	HS683 SNU201
	↓	↓	↓	↓
CRISPR knockout:	*TP53* *CDKN2A*	*TP53*	*CDKN2A*	-

Fig 1. Identification of the classical and neurosphere GBM cell lines. (A) Schematic of planned CRISPR knockouts in specific subgroups. Highlighted part shows the prioritized groups.

groups (highlighted) among the four group combinations (Fig.1). At the time of the cell line selection, we considered the cell line growth rate and also confirmed the Cas9 activity.

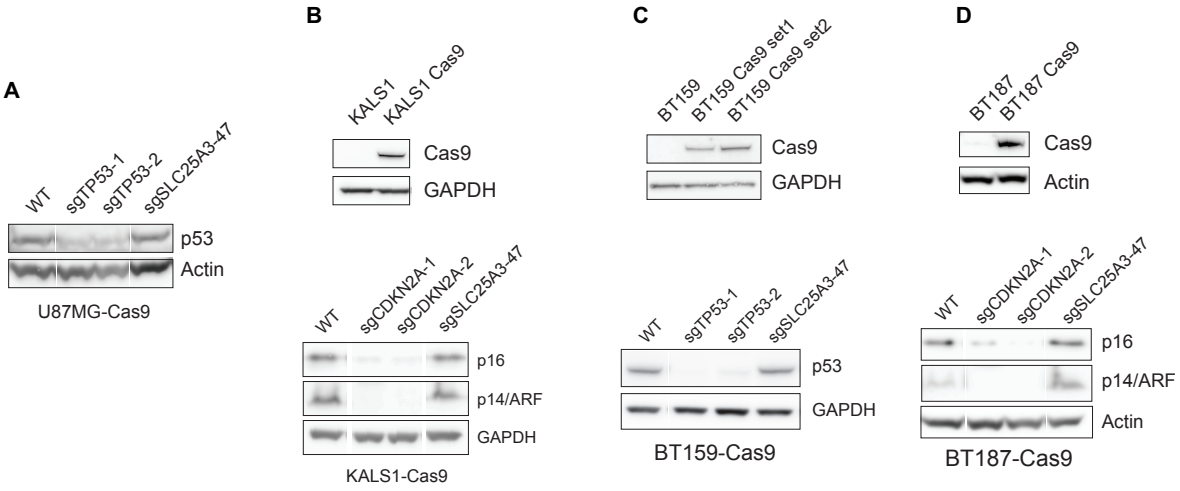


Fig 2. Generation of the isogenic GBM lines in both classical (A-B) and patient derived neurosphere lines (C-D). Western blots indicating successful generation of isogenic lines with loss of p53 (A, C), and loss of p16 and p14/ARF (B, D), the products of CDKN2A genomic locus, in both classical (U87MG, KALS1) and neurosphere (BT159, BT187) GBM culture using CRISPR/Cas9 genome editing.

Once the cell lines were confirmed, we used CRISPR/Cas9 technology to disrupt either *TP53* (in *CDKN2A* homozygous deleted background) or *CDKN2A* (in *TP53* mutant background) as needed in respective cell lines to generate isogenic $TP53^{dis}$/$CDKN2A^{dis}$ cells. We used two step lentiviral transduction system. In the first step, we infected the specific cell line as indicated above to express Cas9 and selected those cells using Blasticidin that we optimized the concentration for each of the cell lines. Then in the second step, we used this stably expressing Cas9 cells to generate the desired knockout lines using guide sequences against defined genes, which were delivered to the cells by lentiviral transduction following Puromycin selection. For both *TP53* and CDKN2A, we used two different guides to eliminate the effect of any off-target effect. Simultaneously, as a control we used a gene (SLC25A3) that is supposed to be unrelated to TP53 signaling pathway, but to normalize the effect caused by the induction of TP53 damage signaling pathway by Cas9 genome cutting (cut control), as observed by several groups including ours. We confirmed the desired knockout levels by western blot (Fig.2A-D), in each of the isogenic knockout set. Our results indicate that we can successfully generate the dual disrupted cells using CRISPR/Cas9 approach in all the cell lines that we have attempted.

Unfortunately, the only cell line that showed desired response (the data will be presented at a later section) was U87MG and at a modest level BT187, but all other cell lines showed problems/difficulties. We found that the effect of *CHK1/2* inhibition is not universally present in all of our isogenic cell line sets, and we probed further and investigated closely our preliminary data of the initial drug screen performed in our lab. What caught our attention is KALS1 and BT159 cell lines were among the cell lines showing relatively higher sensitivity to *CHK1/2* inhibitor, AZD7762, in its unaltered genetic condition already, while U87MG and BT187 were relatively more resistant to the checkpoint inhibition. There we hypothesized that it may be harder to increase the sensitivity further in a cell line background that shows an increased sensitivity to start with. Following this assumption,

we modified our cell line selection, and replaced KALS1 with SW1783 GBM classical line, and BT159 with BT232 and BT340 neurosphere line. We successfully could generate the desired knockouts in the SW1783 classical background (Fig. 3), but again failed to observe the desired effect with *CHK1/2* inhibition. The growth rate of SW1783 also wasn't great, therefore we chose another cell line M059K for this category that was of similar drug sensitivity as SW1783. Both BT232 and BT340 cell lines were significantly slow grower and the process of generating the isogenic lines were longer than the other lines, especially the challenges posed by the two-step lentiviral transduction method.

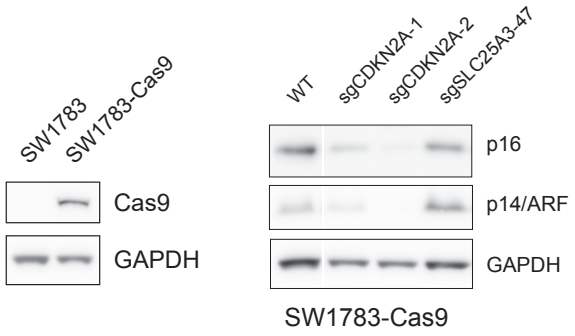


Fig 3. Generation of isogenic GBM lines without p16 and p14/ARF. Western blots indicating successful generation of isogenic lines with loss of p16 and p14/ARF in GBM classical (SW1783) culture using CRISPR/Cas9 genome editing.

To overcome this issue, we utilized an all-in-one system for generating isogenic cell lines using CRISPR-Cas9 technology. In this case, the same vector would encode for both Cas9 and the intended guide RNAs, offering a single selection cycle instead of two rounds of selection cycles. Thus, the process of generating the cell lines would shorten significantly as well as would offer greater flexibility in knocking out more than one gene if needed. We have chosen Hygromycin as our choice of antibiotic selection to render the isogenic models amenable to downstream high throughput genome wise libraries, most of which are of Puromycin selection. Our results indicate that we can successfully generate the dual disrupted cells using all-in-one CRISPR/Cas9 approach in all the cell lines that we have attempted, except one category, which is patient derived CDKN2A homoledeleted lines where TP53 would be knocked out to generate the isogenic model (Fig. 4).

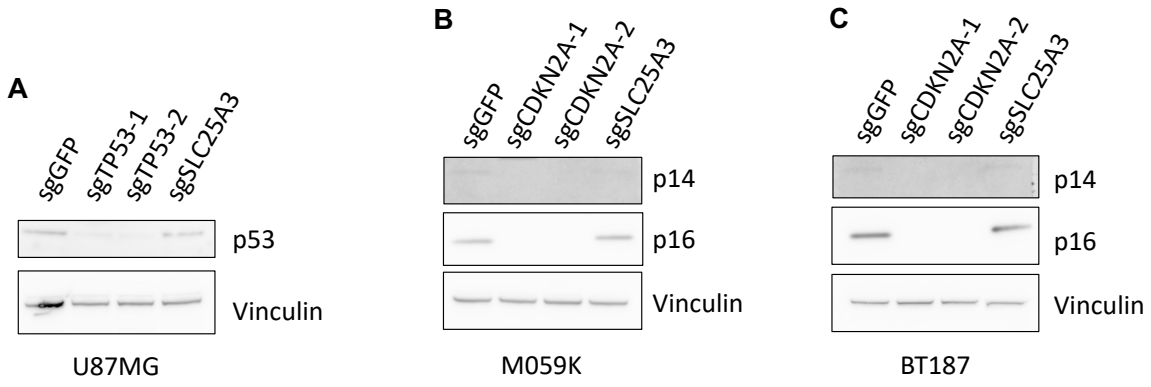


Fig 4. Generation of the isogenic GBM lines in both classical (A-B) and patient derived neurosphere lines (C) using all-in-one CRISPR-Cas9 vector. Western blots indicating successful generation of isogenic lines with loss of p53 (A), and loss of p16 and p14/ARF (B, C), the products of CDKN2A genomic locus, in both classical (U87MG, M059K) and neurosphere (BT187) GBM culture using CRISPR/Cas9 genome editing. sgGFP indicates a control non-targeting guide, where sgSLC25A3 serves as a guide that cuts in the genome, but unrelated to TP53 pathway.

Those cells are in the process of being generated. This category is particularly challenging as the cell lines we chose grow significantly slower.

One of the other alternative approaches we are trying simultaneously right now is to utilize normal astrocytes and human neural stem cells and make the isogenic models by sequential disruption of TP53 and CDKN2A. Those cells are also in the process of being generated.

Our preliminary validation experiments using the G2/M checkpoint inhibitor drugs indicated that knocking out CDKN2A alone may not be sufficient in inducing the synthetic lethality. We have not observed the intended effect of greater response in dual disrupted TP53/CDKN2A cells in comparison to controls in the presence of G2/M checkpoint inhibitor drugs. We have observed in absence of CDKN2A, there is upregulation in the levels of CDKN2B, both in the classical and patient-derived lines (Fig. 5). So, there is a possibility that CDKN2B compensates for CDKN2A in these lines, resulting in no significant differences in responses observed. More supporting evidences come from the responses observed in U87MG cell lines, where CDKN2A/B loci are homodeleted. Therefore, we hypothesize that dual disruption of CDKN2A/B is needed in absence of TP53, to create the dependency on CHK1/2. We are currently optimizing the guides for CDKN2B, and planning to generate the cell lines where both CDKN2A (encoding p16 and p14) and CDKN2B (p15) will be knocked out.

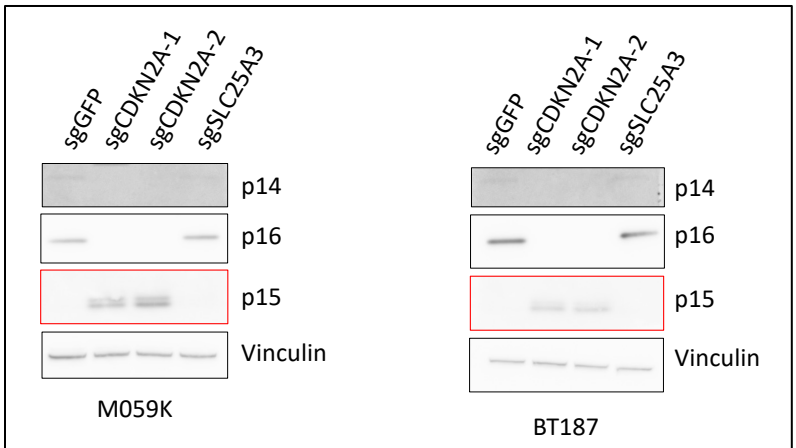


Fig 5. Upregulated levels of p15 levels in absence of p16 and p14/ARF. Western blots indicating p15 levels in isogenic lines with loss of p16 and p14/ARF in GBM classical (M059K) and patient-derived (BT187) culture.

Additionally, we will also disrupt CHK1 and CHK2 genetically using inducible shRNA systems to control for off-target LY2606368 effects and to determine whether CHK1, CHK2, or both are responsible for LY2606368 activity. We are using inducible shRNA systems as CHK1 is an essential gene. We have successfully generated the constructs and planning to confirm the dependency of the CHK1/2 on our isogenic models once we confirm the effect of the drugs reproducibly.

Subtask 2: Test synthetic lethality induced by *CHK1/2* inhibitors and other regulators of G2/M checkpoint in cells with dual loss of *TP53/CDKN2A*

Following the generation of four isogenic cell line sets by the two-step method, we tested whether the dual disrupted cells show increased sensitivity to *CHK1/2* inhibitors. We included two *CHK1/2* inhibitors, AZD7762 and LY2606368, in our analyses to confirm the effect of *CHK1/2* inhibition in these cells and measured the effect of the drug over cell proliferation using CellTiter Glo (CTG) assays. For each isogenic set, we repeated the assays at least three times and each time we used normalization against the initial seeding densities as well as the vehicle control. To illustrate the effect of the *CHK1/2* inhibition on dual $TP53^{dis}/CDKN2A^{dis}$ cells, we always compared the effect against the cut control, as mentioned above. For the assay optimization, for each isogenic set we have

determined the appropriate dose ranges and also performed the CTG assays post 72 hours and 120 hours of inhibitor treatment.

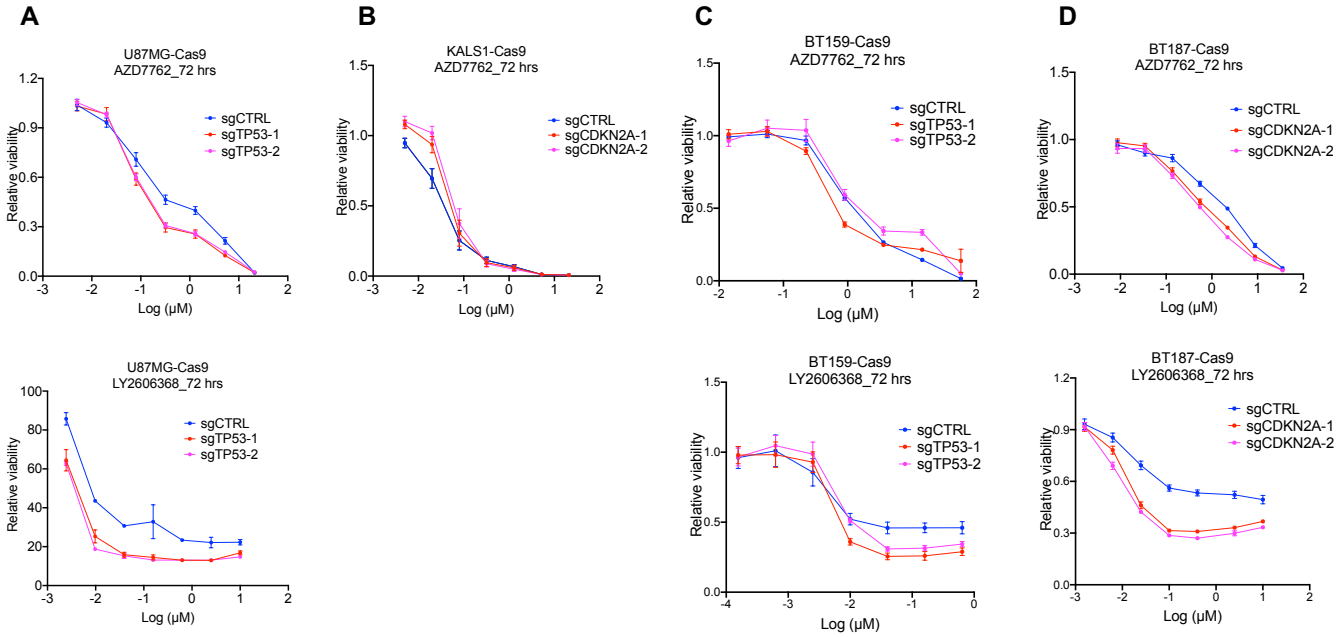


Fig 6. *TP53*^{dis}/*CDKN2A*^{dis} cells from U87MG and BT187 isogenic sets show greater sensitivity to CHK1/2 inhibitors, AZD7762 and LY2606368, compared to the isogenic control, while the other two sets (KALS1 and BT159) do not. Dose response curves in indicated GBM classical (U87MG, KALS1) and neurosphere (BT159, BT187) lines to CHK1/2 inhibitors, AZD7762 and LY2606368.

Our results indicate that 72 hours incubation period is optimal for observing the effect of the abovementioned *CHK1/2* inhibitors. Longer incubation for 120 hours in all these cells showed similar effect but the overall toxicity is increased, thereby we decided to follow the 72-hour incubation period for the remainder of the project. Our results also indicate that LY2606368 serves as a more potent inhibitor in comparison to AZD7762. So, for our mechanistic characterization we plan to utilize the *CHK1/2* inhibitor, LY2606368. When we compared our isogenic sets, we noticed that *TP53*^{dis}/*CDKN2A*^{dis} cells show increased sensitivity, although modest, to the *CHK1/2* inhibitors in comparison to the isogenic control in U87MG and BT187 background, but the effect is absent in KALS1 as well as BT159 background (Fig. 6A-D).

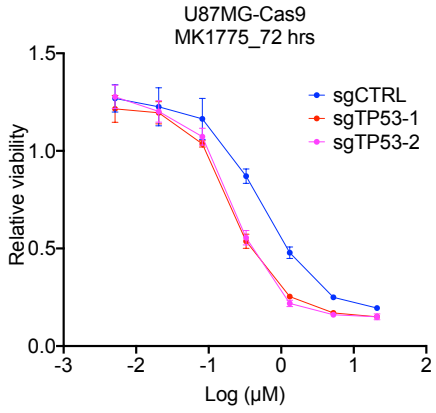


Fig 7. *TP53*^{dis}/*CDKN2A*^{dis} cells from U87MG isogenic set show greater sensitivity to WEE1 inhibitor, MK1775, compared to the isogenic control. Dose response curves in U87MG classical line to WEE1 inhibitor, MK1775.

The G2/M checkpoint is regulated by multiple proteins involved in DNA damage response, including WEE1, which is also a downstream effector of CHK1/2. WEE1 inhibitor, MK1775 is also being tested in an ongoing GBM clinical trial. Therefore, we were interested to learn whether MK1775 shows selective sensitivity towards the *TP53*^{dis}/*CDKN2A*^{dis} isogenic cells we have generated. In order to first assess the sensitivity,

we tested the effect of the inhibitor in our isogenic U87MG classical GBM line. As observed in Fig. 7, the *TP53*^{dis}/*CDKN2A*^{dis} cells show a modest but higher sensitivity towards WEE1 inhibition, when compared to the isogenic control. This result indicates that our GBM models may be effective in modeling not just the *CHK1/2* inhibition, rather the G2/M checkpoint inhibition.

Because we switched our generation of isogenic system from two-step CRISPR-Cas9 method to all-in-one CRISPR-Cas9 vector-based approach, we re-confirmed the sensitivity to *CHK1/2*

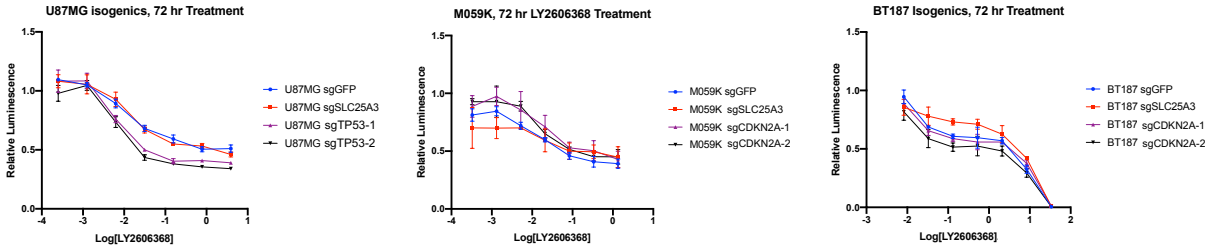


Fig 8. *TP53*^{dis}/*CDKN2A*^{dis} cells from U87MG isogenic set generated using all-in-one CRISPR-Cas9 method recapitulate the sensitivity to CHK1/2 inhibitor, LY2606368, compared to the isogenic controls, while the other two sets (M059K and BT187) do not. Dose response curves in indicated GBM classical (U87MG, KM059K) and neurosphere (BT187) lines to CHK1/2 inhibitor, LY2606368.

inhibitor, but as our previous results indicated that LY2606368 is a more potent inhibitor, we used this inhibitor only for confirming the effect following 72 hours drug treatment. Our results indicate that only dual *TP53*^{dis}/*CDKN2A*^{dis} cells from U87MG isogenic cell lines show greater sensitivity to LY2606368 compared to control guides, but the other two cell lines do not show similar effect (Fig. 8). While probing deeper, we realized that the U87MG cell lines are homodeleted for both CDKN2A and CDKN2B, as mentioned before, but while generating the isogenic lines, we only have knocked out CDKN2A from M059K and BT187 lines. In both of these cell lines, p15 levels were higher compared to controls, indicating p15 may very well compensate for p16 loss in these isogenic sets (Fig. 5). Therefore, we hypothesize currently that CDKN2B locus also needs to be knocked out along with CDKN2A locus in TP53 mutant cell lines, to show greater sensitivity to LY2606368. We are currently generating the CDKN2A/B dual knockout lines in M059K and BT187 to compare the effect in the right context.

Simultaneously, we also have tested the effect of the WEE1 inhibitor, MK1775 on these three isogenic sets, and the lines recapitulated the effect as observed with *CHK1/2* inhibitor, LY2606368

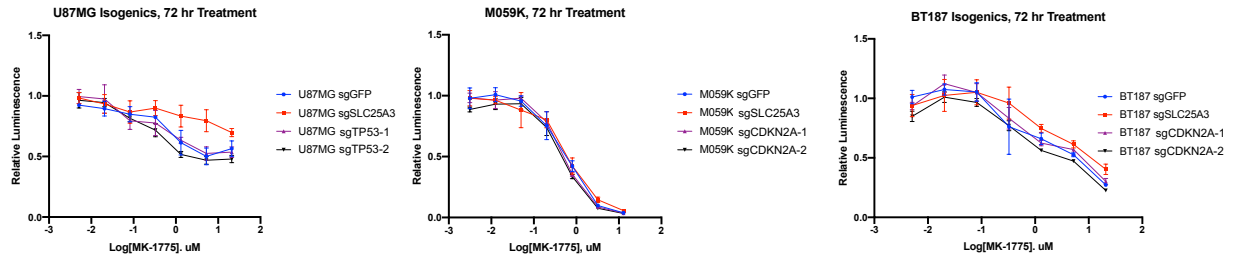


Fig 9. *TP53*^{dis}/*CDKN2A*^{dis} cells from U87MG isogenic set generated using all-in-one CRISPR-Cas9 method show the sensitivity to WEE1 inhibitor, MK1775, compared to the isogenic controls, while the other two sets (M059K and BT187) do not. Dose response curves in indicated GBM classical (U87MG, KM059K) and neurosphere (BT187) lines to WEE1 inhibitor, MK1775.

(Fig. 9), i.e., the effect was present in U87MG cell line but not in the other two isogenic cell lines. As WEE1 serves as the downstream effector of CHK1/2, this observation gives us more confidence about the effect being real, and not artifactual. We plan to re-test the sensitivity of G2/M checkpoint inhibitors on the isogenic sets where both CDKN2A and CDKN2B will be absent along with absence of WT TP53. We will also test the effect in the isogenic set of BT232 and BT340, once we have the isogenic set generated and characterized.

Subtask 3: Characterize the mechanism of selective lethality induced by the G2/M checkpoint inhibitors

Because only the U87MG cell lines show the consistent effect against both G2/M checkpoint inhibitors, CHK1/2 inhibitor LY2606368, and WEE1 inhibitor MK1775, currently we are conducting experiments to check the mechanism of selective lethality in this isogenic set only. To assess CHK1/CHK2 pathway activity, we are currently assessing total and phosphorylated levels of CHK1, CHK2, MYT1, WEE1, CDC25a-c and CDK1 using immunoblots, and also completing the analyses with cell cycle analysis and apoptosis assays. The same assays will be repeated in the isogenic lines of M059K, BT187 and BT232, when finished characterizing the initial drug responses using LY2606368 and MK1775.

Subtask 4: Confirm the *in vitro* finding using flank xenografts in mice *in vivo* following survival and tumor volume

Not attempted yet. Because the cell line verification of the drug responses are yet to be finalized, the flank xenografts have not been attempted yet.

Subtask 5: Validate the hypothesis from patient samples from Wee1 inhibitor trial and correlate survival and progression free survival data

Not attempted yet. Because the in vitro validation of the WEE1 inhibitor response is still ongoing, the rather limiting patient samples were not tested yet.

Specific Aim 2: Test whether disruption of *TP53* works synergistically with loss of other genes or with genotoxic stresses, such as radiation, to increase sensitivity to G2/M checkpoint inhibition.

Major Task 2: Test synergistic response of checkpoint inhibitors with radiation, and genome-wide CRISPR analysis to identify additional dependencies with checkpoint inhibition

Subtask 1: Test synergistic response elicited by G2/M checkpoint inhibitor, AZD-7762, with radiation in cells with mutant *TP53* and characterize the mechanism

Using the GBM isogenic lines described above (U87MG and M059K), we examined whether CHK1/2i inhibitor response synergized with the standard-of-care radiation therapy. To that end, we irradiated the cells using 2 Gy IR at the time of plating and conducted the drug treatment with CHK1/2 inhibitor, LY2606368 as before for 72 hours. Radiation didn't alter the response observed from no treated cells (Fig. 10). We are currently optimizing the radiation doses to be used and the optimal time point to be used post radiation conducting dose response and time point experiments.

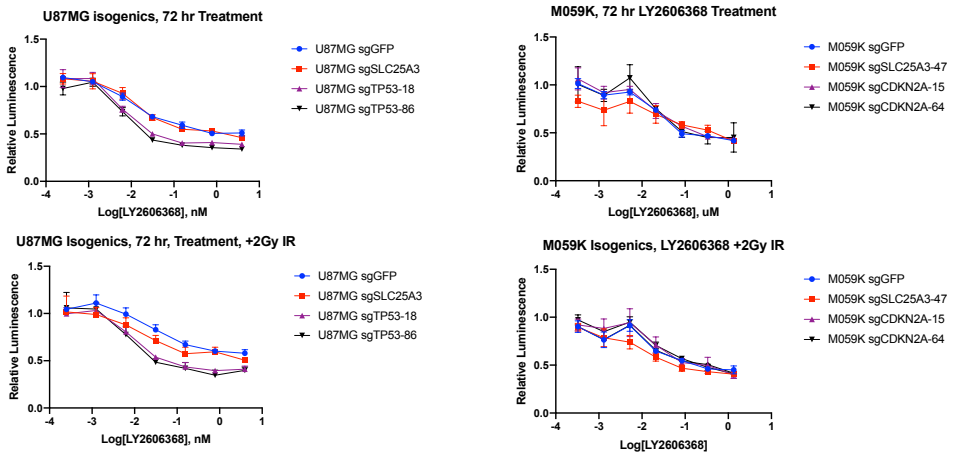


Fig 10. *TP53*^{dis}/*CDKN2A*^{dis} cells from U87MG and M059K isogenic set do not show synergistic response with radiation. Dose response curves of CHK1/2 inhibitor LY2606368 in indicated GBM classical (U87MG, KM059K) isogenic cell lines in presence and absence of radiation (2Gy IR).

Subtask 2: Confirm the synergistic responses identified in 2.1 *in vivo* using flank xenografts in mice

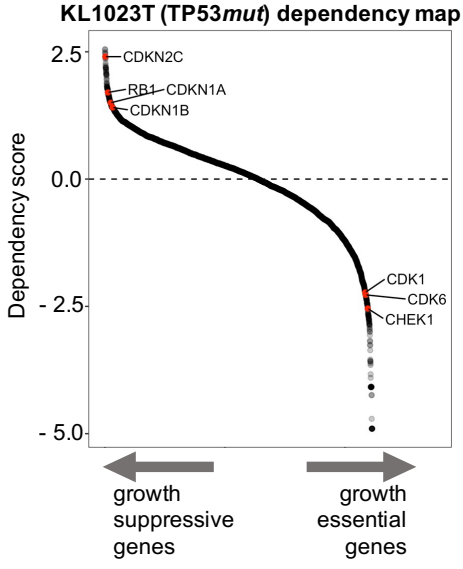
Not attempted yet, as the responses observed from checkpoint inhibitor with radiation did not seem to elicit a synergistic effect yet at the dose tried.

Subtask 3: Biochemical characterization of tumors to analyze responses form 2.2

Not attempted yet, as the animal studies are yet to be started

Subtask 4: Genome wide CRISPR screen in *TP53* mutant cells to identify additional dependencies following *CHK1/2* inhibition and validation

Because of unforeseen circumstances, we could not yet conduct the genome wide CRISPR screen as designed here yet. But we have now completed analysis of CRISPR knock-out screens of 3500 genes on our first two GBM cell lines to be analyzed as part of a Broad Institute DepMap project, one of which lines is a TP53 mutant line (Fig. 11). These results demonstrate the feasibility of using GBM patient-derived cell lines in such large scale CRISPR screens. Our initial analysis of a TP53 mutant, CDKN2A wildtype



Enriched

Pathway / function	Gene(s)
PTEN/PI3K/AKT	*TSC2, PTEN, NF2*
DNA Binding	*ETV6*
Cell cycle regulation	*CDKN2C, RB1, CDKN1A, CDKN1B*

Depleted

Pathway / function	Gene(s)
Homeobox transcription factor	*DUX4, DUXL10 --> DUXL26*
Cell cycle regulation	*PLK1, KIF23, KNL1, MASTL , PTPRR, CDK6, CCND2 , CDC16*
DNA synthesis	*RAN*
DNA replication	CDC45
TGFB signaling	SMC4
Spliceosome	*SF3B1, SRSF2, YB1 , PRPF38B, SF3B2*
EGFR signaling	EGFR, PIK3C2B

Fig 11. Results of CRISPR screen for TP53*mut* KL1023T GBM cell. Results of CRISPR screen identifying KL1023T dependencies. Right panel indicates the top genes which are most enriched and depleted at the end of the screen.

GBM line (KL1023T) indicates that knockout of members of the G1/S pathway (RB1, CDKN1A, and CDKN2C) remove constraints on growth, accelerating uncontrolled cell cycle entry. Conversely, stable loss of CHK1 was lethal as in prior experience and loss of and CDK1/6 reduced growth consistent with cell cycle checkpoint arrest (Fig. 11). Ongoing efforts are being made in optimizing the parameters for the genome wide screen.

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

1) The research strategy proposed in the current proposal allowed the PI to gain significant expertise in generating isogenic models of GBM, both in classical GBM lines and patient-derived lines, the mechanistic characterization of the CRISPR system, and in using drug-response assays. The PI is also currently gaining significant experience in optimizing genome wide CRISPR screen. So she is gaining a lot of necessary skills, and expertise to drive the project to completion.
2) For the intellectual development of the PI, the research findings were discussed in one-in-one meetings with the mentor, Dr. Beroukhim. She also worked in close collaboration with Drs. Keith Ligon and Pratiti Bandopadhayay and meet at least monthly with to go over the research progress and future plans. She presented regularly at the weekly laboratory meetings and monthly department group meetings. These include weekly Beroukhim laboratory meetings held in conjunction with Bandopadhayay laboratory and, on a monthly basis, Ligon laboratory, the Cancer Program at the Broad Institute, and the neurooncology program at DFCI. She planned to attend a national meeting but decided against it due to the pandemic situation. But she attended multiple seminar series help virtually both at Dana Farber Cancer Institute and at the Broad Institute this year. Previously, the PI attended a two-week long workshop at the Broad Institute to gain expertise on large scale data analysis and to utilize the Broad Institute resources to the fullest. She also gained practical hands-on experience in analyzing CRISPR screen results. Also, to continue her training on the large-scale data analysis, she registered for online courses offered by Harvard University (the HarvardX- Data science classes) at the time of the shutdown at DFCI and continued to learn throughout the year.
3) The PI is also part of three intellectually stimulating scientific communities: The Dana-Farber Cancer Institute, Harvard Medical School, and the Broad Institute. She utilizes all the available resources offered by these institutes to continue her training, to enrich her professional development.

How were the results disseminated to communities of interest?

As part of the Dana-Farber Cancer Institute and working closely with physician-scientists in the field of neuro-oncology, the PI receives frequent opportunities to interact with donors and patient families, even individual patients, in the Dana-Farber organized events, where often she explains her research in lay terms so that it is understandable to the non-scientists. These events help her to explain the reason why it is of utmost importance to develop targeted therapies against specific GBM sub-types. Unfortunately, during pandemic these interactions were severely disrupted, but help a few times virtually attended by the PI along with other colleagues.

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

Nothing to Report

4. IMPACT:

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

The current proposal is evaluating the effectiveness of the G2/M checkpoint ablation as a potential therapeutic strategy in inducing selective lethality in GBM cells bearing specific genetic signatures. It is also exploring whether this therapeutic strategy can work synergistically with radiotherapy as well as identify new therapeutic targets for GBM. One of the drugs being tested in this proposal is part of a clinical trial. We believe that one reason for the limited therapeutic efficacy for targeted therapies in GBM is the lack of consideration for variations across genetic background before deciding on therapeutic strategies. Identifying and validating the specific genetic context the drug is most effective against, thus, will increase the likelihood of finding a new therapy for GBM, and potentially provide us with an improved combinatorial therapy. The close collaboration of me and my mentor with other neuro-oncologists at Dana Farber Cancer Institute will allow rapid translation of our findings to future trials.

What was the impact on other disciplines?

Loss of both *TP53* and *CDKN2A* is a frequent event in not only GBMs, but in many other cancer types. This study is exploring whether G2/M checkpoint inhibition induces selective lethality in tumor cells bearing *TP53/CDKN2A* dual loss and how these cells show selective lethality in this genetic context. It is also exploring the synergistic response of G2/M checkpoint inhibition with radiation, in a *TP53* mutant setting, which is also a recurrent event in multiple cancer types. Therefore, the knowledge obtained from this current study has the potential to shed light into the molecular vulnerabilities shared by a number of other types, and learn how that can be targeted therapeutically, affecting a large number of patient populations. To our advantage, we already obtained some evidence that along with GBM, our approach may work effectively also in lung adenocarcinoma. Moreover, Dr. Adam Bass evaluated the synthetic lethal relationships between these alterations in genetically engineered mouse models of gastric cancer, again validating our result and extending its impact to other cancer types (Sethi et al., Nature Genetics, 2020)

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS: *ng, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change

Nothing to report

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

The progress of the project is delayed compared to the statement of work. The reasons are as follows-

1) The usual layers of complexity experienced in biology research, specifically the problems encountered for the model development when unanticipated additional factors influence the predicted outcome requiring further troubleshooting. Especially GBM cell lines are extremely difficult to work with and highly heterogenous, requiring additional troubleshooting than usual.
2) During the pandemic, DFCI research operations were first reduced and then shut down for all non-essential research for a few months. The developed models were frozen down as needed, but one significant problem of the GBM field is, the patient derived models take longer than usual to recover when retrieved. The unexpected interruptions caused a delay in carrying out the proposed plan as originally suggested.
3) The problems experienced due to supply chain was great for this study. Most often the supply needs can be replaced or modified. But these cell lines needed specific media or growth factors, and in absence of those cell culture could not be conducted. Another great hindrance was lack of supply of ultra-low attachment vessels of all types, which are absolutely essential for growing neurospheres. We tried to use other available products or tried to generate the prodict in-house, bit they were super low efficient and time consuming, altogether delaying the progress further.

Changes that had a significant impact on expenditure

Nothing to report

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

Nothing to report

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

  Journal publications.

Enache OM, Rendo V, Abdusamad M, Lam D, Davison D, Pal S, Currimjee N, Hess J, Pantel S, Nag A *et al*: **Cas9 activates the p53 pathway and selects for p53-inactivating mutations**. *Nat Genet* 2020, **52**(7):662-668.

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

Nothing to report

Other publications, conference papers and presentations.

Nothing to report

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

- **Technologies or techniques**

  Nothing to report

- **Inventions, patent applications, and/or licenses**

Nothing to report

- **Other Products**

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Sangita Pal, PhD
Project Role:	Research scholar
Researcher Identifier (e.g. ORCID ID):	0000-0002-4892-841X
Nearest person month worked:	24
Contribution to Project:	Dr. Pal has worked in developing the classical and patient derived GBM model to address the key questions in the study.
Funding Support:	This award

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

Yes. There have been changes to the active Other Support of Dr. Rameen Beroukhim since the last report:

PREVIOUSLY ACTIVE AWARDS, NOW CLOSED

*Title: **Center for the comprehensive analysis of somatic copy-number alterations in cancer**
Major Goals: We proposed to establish a Genomics Data Analysis Center (GDAC) that will service the GDAN with comprehensive, advanced analyses of SCNAs and the rearrangements that bound them, with the goals of identifying biologically and clinically relevant patterns of SCNA and disseminating this information to the GDAN and wider research community.
*Status of Support: Completed
Project Number: 5 U24 CA210978-05
Name of PD/PI: Beroukhim, Rameen; Cherniack, Andrew
*Source of Support: NIH/NCI
*Primary Place of Performance: The Broad Institute
Project/Proposal Start and End Date: (MM/YYYY) (if available): 09/15/16 - 08/31/21
*Total Award Amount (including Indirect Costs):

*Title: **Targeting vulnerabilities of mismatch-repair mutant cancers**
Major Goals: We proposed an integrated approach to identify synthetic vulnerabilities associated with MMR-deficient gliomas and to further characterize Werner as a potential therapeutic target for patients with hyper-mutated cancers.
*Status of Support: Completed
Project Number: None
Name of PD/PI: Beroukhim, Rameen; Bandopadhayay, Pratiti
*Source of Support: Dana-Farber/Novartis Drug Discovery Program
*Primary Place of Performance: Dana-Farber Cancer Institute
Project/Proposal Start and End Date: (MM/YYYY) (if available): 01/01/18 – 08/31/21
*Total Award Amount (including Indirect Costs): (Beroukhim award)

NEW ACTIVE AWARDS

*Title: **Expanded structural variant signatures and their role in predicting chemotherapy sensitivity**
Major Goals: To improve SV signature detection methods by including additional critical metrics, and to systematically evaluate the relationships between SV signatures and sensitivity to cancer therapeutics.
*Status of Support: Active
Project Number: None
Name of PD/PI: Beroukhim, Rameen
*Source of Support: Brown Performance Group Fund for Innovation in Cancer Informatics
*Primary Place of Performance: The Broad Institute
Project/Proposal Start and End Date: (MM/YYYY) (if available): 01/01/21 – 02/02/23
*Total Award Amount (including Indirect Costs):
*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2021	0.60 calendar
2. 2022	0.60 calendar

*Title: **Evolution of gliomas during treatment and resistance**
Major Goals: This proposal aims to determine how glioblastomas become resistant to radiation and temozolomide by determining the genetic changes they undergo during treatment, thereby enabling us to devise more effective treatment strategies.
*Status of Support: Active
Project Number: 2 R01 CA188288-06
Name of PD/PI: Beroukhim, Rameen; Chakravarti, Arnab; Ligon, Keith
*Source of Support: NIH/NCI
*Primary Place of Performance: Dana-Farber Cancer Institute
Project/Proposal Start and End Date: (MM/YYYY) (if available): 07/01/21 – 06/30/26
*Total Award Amount (including Indirect Costs):
*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2021	2.04 calendar
2. 2022	2.04 calendar
3. 2023	2.04 calendar
4. 2024	2.04 calendar
5. 2025	2.04 calendar

*Title: **Therapeutic targeting of MMR-deficient hypermutant gliomas**
Major Goals: This project will test the central hypothesis that MMR-deficient glioma cells harbor vulnerabilities that can be therapeutically targeted.
*Status of Support: Active
Project Number: 21016A
Name of PD/PI: Beroukhim, Rameen; Bandopadhayay, Pratiti
*Source of Support: DFCI-Novartis Drug Discovery and Translational Research Program
*Primary Place of Performance: Dana-Farber Cancer Institute
Project/Proposal Start and End Date: (MM/YYYY) (if available): 07/01/21 – 06/30/24
*Total Award Amount (including Indirect Costs): (Beroukhim award)

* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2021	0.30 calendar
2. 2022	0.30 calendar
3. 2023	0.30 calendar

*Title: **To develop a sophisticated approach to accurately identify deficiency in specific DNA repair pathways with a focus on homologus recombination mediated repair and the expanded use of PARP inhibitors**
Major Goals: To develop a sophisticated approach to accurately identify deficiency in specific DNA repair pathways with a focus on homologous recombination mediated repair and the expanded use of PARP inhibitors. Overall the project will identify robust biomarkers for defects in DNA repair pathways thereby defining new patient populations responsive to existing treatments including PARP inhibitors, and enable the identification of new DNA damage response-based therapeutic targets.
*Status of Support: Active
Project Number: Industry sponsored research agreement
Name of PD/PI: Beroukhim, Rameen; Chowdhury, Dipanjan
*Source of Support: Merck Sharp & Dohme Corp.
*Primary Place of Performance: Dana-Farber Cancer Institute
Project/Proposal Start and End Date: (MM/YYYY) (if available): 06/10/2021 – 06/09/2022
*Total Award Amount (including Indirect Costs):
*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2021	0.12 calendar

*Title: **Intervening in the TP53 Pathway Phosphatome in Pediatric High-Grade Gliomas**
Major Goals: This project will identify new potential phospho-protein based biomarkers and therapeutic targets. It will also establish a new technology to assess post-translational modifications essential to the activity of the TP53 or any other pathway, across both diseased (e.g. cancer) and normal cells—hence a widespread impact.
*Status of Support: Active
Project Number: CA200505
Name of PD/PI: Beroukhim, Rameen
*Source of Support: DOD CDMRP
*Primary Place of Performance: Dana-Farber Cancer Institute
Project/Proposal Start and End Date: (MM/YYYY) (if available): 09/15/21 – 09/14/24
*Total Award Amount (including Indirect Costs): total costs
*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2021	0.60 calendar
2. 2022	0.60 calendar
3. 2023	0.60 calendar

*Title: **Center for the comprehensive analysis of somatic copy-number alterations in cancer**
Major Goals: In this renewal proposal, we address two core competencies required for the

Genomics Data Analysis Center (GDAN): Copy Number/Purity Analysis (Aims 1 and 3) and Long-Read sequencing analysis (Aim 2). We also combine data across samples to determine tumor subclasses and factors shaping tumor evolution (Aim 4) and, perhaps most importantly, will integrate our personnel and SV analyses with the larger GDAN (Aim 5). This latter contribution will include contributing secondary competencies as necessary in GDAN-required analyses of Cell-Free DNA and Single-Cell RNA data.
*Status of Support: Active
Project Number: 1 U24 CA264029-01
Name of PD/PI: Beroukhim, Rameen; Cherniack, Andrew
*Source of Support: NIH/NCI
*Primary Place of Performance: The Broad Institute
Project/Proposal Start and End Date: (MM/YYYY) (if available): 09/20/21 – 08/31/26
*Total Award Amount (including Indirect Costs):
*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2021	2.40 calendar
2. 2022	2.40 calendar
3. 2023	2.40 calendar
4. 2024	2.40 calendar
5. 2025	2.40 calendar

IN-KIND SUPPORT

*Summary of In-Kind Contribution: Dr. Linda Bi is an Assistant Professor of Neurosurgery at Brigham and Women's Hospital (BWH), who participates in research in the Beroukhim lab. Her salary is supported by BWH.
*Status of Support: Active
*Primary Place of Performance: Dana-Farber Cancer Institute
Project/Proposal Start and End Date (MM/YYYY) (if available): 07/2013 – ongoing
*Person Months (Calendar/Academic/Summer) per budget period: N/A
*Estimated Dollar Value of In-Kind Information: per year (estimated salary/fringe for 1-2%time spent contributing to Dr. Beroukhim's research projects).

*Summary of In-Kind Contribution: Dr. Beroukhim is appointed as an affiliated investigator at the Centre for Cancer Biomarkers, University of Bergen, Bergen, Norway. Dr. Beroukhim receives salary and conducts collaborations through this appointment, but receives no laboratory resources of his own and does not hold any research funds. A professor from the University of Bergen plans to come to Dr. Beroukhim's lab at DFCI for a sabbatical in the upcoming year, but this visit has not yet started.
*Status of Support: Active
*Primary Place of Performance: University of Bergen, Bergen, Norway
Project/Proposal Start and End Date (MM/YYYY) (if available): 2016 – ongoing
*Person Months (Calendar/Academic/Summer) per budget period: None

*Estimated Dollar Value of In-Kind Information: per year in salary and approximately per year in support of travel costs reimbursed to Dr. Beroukhim in the years he travels to Bergen; Dr. Beroukhim has not traveled to Bergen in the last three years.

*Summary of In-Kind Contribution: Dr. Nada Jabado is a Professor of Pediatrics at McGill University, who has provided access to genomic data her laboratory has generated.
*Status of Support: Active
*Primary Place of Performance: McGill University, Montreal, QC, Canada
Project/Proposal Start and End Date (MM/YYYY) (if available): 07/2018 – ongoing
*Person Months (Calendar/Academic/Summer) per budget period: N/A
*Estimated Dollar Value of In-Kind Information:

*Summary of In-Kind Contribution: Dr. Mehdi Touat is an Assistant Professor at the Sorbonne in Paris, France, who has provided access to genomic data his laboratory has generated.
*Status of Support: Active
*Primary Place of Performance: Sorbonne, Paris, France
Project/Proposal Start and End Date (MM/YYYY) (if available): 3/2021 – ongoing
*Person Months (Calendar/Academic/Summer) per budget period: N/A
*Estimated Dollar Value of In-Kind Information:

*Summary of In-Kind Contribution: Dr. Chris Jones is a Professor at the Institute for Cancer Research (ICR) in London, who has provided access to genomic data and cancer model systems his laboratory has generated.
*Status of Support: Active
*Primary Place of Performance: ICR, London, England
Project/Proposal Start and End Date (MM/YYYY) (if available): 09/2019 – ongoing
*Person Months (Calendar/Academic/Summer) per budget period: N/A
*Estimated Dollar Value of In-Kind Information:

*Summary of In-Kind Contribution: Dr. David Jones is a Senior Researcher at DKFZ in Heidelberg, Germany, who has provided access to genomic data his laboratory has generated.
*Status of Support: Active
*Primary Place of Performance: DKFZ, Heidelberg, Germany
Project/Proposal Start and End Date (MM/YYYY) (if available): 9/2020 – ongoing
*Person Months (Calendar/Academic/Summer) per budget period: N/A
*Estimated Dollar Value of In-Kind Information:

*Summary of In-Kind Contribution: Dr. Camilla Krakstad is a Professor at the University of Bergen, Norway, who has provided access to genomic data, tissue samples, and model systems her laboratory has archived and generated.
*Status of Support: Active
*Primary Place of Performance: University of Bergen, Bergen, Norway
Project/Proposal Start and End Date (MM/YYYY) (if available): 7/2005 – ongoing
*Person Months (Calendar/Academic/Summer) per budget period: N/A
*Estimated Dollar Value of In-Kind Information:

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

W81XWH1910452: Targeting the G2/M Checkpoint in Glioblastoma With a Combined Loss of TP53 and CDKN2A

PI: Sangita Pal, Ph.D., Dana-Farber Cancer Institute, MA

Budget: $264,750

Topic Area: FY18 PRCRP (Brain cancer)

Mechanism: Horizon Award

Research Area(s): 0299 Genetics and Molecular Biology
0805 Targeted Therapies

Award Status: Completed; 01 Aug 2019 to 31 Jul 2021

Study Goals:

This study will comprehensively determine whether biomarkers present in ~10% of glioblastomas indicate sensitivity to a whole class of therapies targeting the G2/M checkpoint.

Specific Aims:

- Specific Aim 1: Test whether disruption of both *TP53* and *CDKN2A* preferentially sensitizes GBM cells to G2/M checkpoint inhibition through a synthetic lethal interaction.
- Specific Aim 2: Test whether disruption of *TP53* works synergistically with loss of other genes or with genotoxic stresses, such as radiation, to increase sensitivity to G2/M checkpoint inhibition.

Key Accomplishments and Outcomes:

- Significant progress in generating isogenic models of glioblastomas from conventional and patient derived cell lines with alterations in TP53 and CDKN2A
- Validation of the combined role of TP53 and CDKN2A in determining CHK1/2 inhibitor sensitivity
- Possible applicability in other cancer types, e.g. lung adenocarcinoma

Publications: [add any new publications]

Enache OM, Rendo V, Abdusamad M, Lam D, Davison D, Pal S, Currimjee N, Hess J, Pantel S, Nag A et al: Cas9 activates the p53 pathway and selects for p53-inactivating mutations. Nat Genet 2020, 52(7):662-668.

Patents: none

Funding Obtained: none to date

9. APPENDICES: