Pantothenate Kinases as Collateral Lethality Targets in Glioblastoma Multiforme

Specific Aims

Genomic deletion of tumor suppressor genes (TSGs) is a hallmark of tumorigenesis. When TSGs are deleted, there can be concomitant loss of neighboring housekeeping genes as passengers due to their proximity to the TSG locus. The Muller lab has previously identified and validated the framework of *collateral lethality*, where cancer cells tolerate the loss of critical housekeeping genes due to functional redundancy imparted by expression of the redundant paralogs of the genes [1,2]. Glioblastoma Multiforme (GBM) is an intractable tumor of the central nervous system, which is also the most common and aggressive of all gliomas [3-5]. Despite extensive research and clinical trials to develop new and effective therapies against this debilitating malignancy, the currently available therapies can extend the survival by only 15 months [3,4]. Considering the aggressive nature of the tumor, the dismal survival rate and the lack of treatments, the identification of new and effective therapeutic targets has never been so urgent. Approximately 40% of GBM cases are characterized by the loss of *PTEN*, a critical tumor suppressor gene, commonly deleted in a variety of cancers [6]. Most TSGs including the *PTEN* homozygous deletions are not currently considered therapeutically targetable. However, by using the framework of collateral lethality, the redundant isoforms of important housekeeping genes that are co-deleted with the TSGs can be targeted to specifically kill cancer cells [1,2]. One such co-deleted gene in the *PTEN* locus (10q23) is Pantothenate Kinase 1 (*PANK1*) (Figure 1). *PANK1* passenger deletions occur along with the tumor suppressor *PTEN* in many cancer types, and most prominently in GBM (1.1%) and prostate adenocarcinoma (~8%). The family of pantothenate kinases constitutes four catalytically active isoforms encoded by three distinct genes: PANK1 with two splice variants: nuclear PANK1 α and cytosolic PANK1 β, mitochondrial PANK2 and cytosolic PANK3. These enzymes catalyze the first committed and the rate limiting step of coenzyme A (CoA) biosynthesis [7] . CoA is a critical cofactor with cardinal functions in the tricarboxylic (TCA) cycle, lipid metabolism, fatty acid oxidation, biosynthesis as well as protein acetylation [8]. Based on the cell essential function of PANK in lower organisms, as well knock out phenotype in mice, I have identified PANK as a promising collateral lethality candidate in GBM. **Therefore, I hypothesize that targeting the redundant isoforms of PANK in tumors with *PANK1* homozygous deletion can selectively kill cancer cells.** I will test my hypothesis in the following aims.

Aim 1: To determine the cell-essentiality of PANK and identify the isoforms redundant with *PANK1*

In this aim, I will determine the dependence of cancer cells on PANK for their survival and establish if each isoform is essential and/or redundant with *PANK1* in cancer cells. PANK activity is essential in *E. coli* and yeast which have only one *PANK* gene [9]. Mammals have four catalytically active isozymes with distinct regulatory properties and encoded by three individual genes. Although individual knockout of each isoform is tolerated at the organism level [10,11], double knockout (dko) of *Pank* isoforms are either embryonic or post-natal lethal in mice [12,13]. This indicates some redundancy in Pank function and reinforces that Pank activity is imperative for organism viability, but whether the same is true for cancer cells remains to be validated. I hypothesize that PANK3 is redundant with PANK1 due to their cytosolic co-localization, facilitating compensation when one isoform is absent**.** The preliminary data I generated suggests that *PANK1* deleted cancer cells are selectively toxic to *PANK3* inhibition by antisense oligonucleotides (ASOs). I will verify this by CRISPR knock out and shRNA knock down of PANK isoforms in wild type (WT) and *PANK1* homozygously deleted cell lines, with both constitutive and inducible systems. Using the inducible systems, I will assess the effect on cell viability and proliferation of eliminating *PANK2* or *PANK3* in *PANK1* deleted and intact cells.

Aim 2: To determine the biochemical consequences of *PANK* elimination

In this aim, I will test the hypothesis that CoA depletion will severely impact metabolic pathways in the cells. Acetyl-CoA is integral to diverse biochemical reactions such as lipid metabolism, fatty acid synthesis and oxidation, as acetyl CoA in TCA cycle [10,11,13] and protein acetylation including epigenetic regulation via histone acetylation [14]. I performed metabolomics on *PANK1* CRISPR knock out HeLa cells generated in Aim 1. Loss of *PANK1* does not alter metabolites in the CoA synthesis pathway suggesting that loss of *PANK1* is compensated in HeLa cells. I will also use *PANK2* and *PANK3* CRISPR knock out HeLa cells as well inducible shRNA system developed in Aim 1 to assess the biochemical changes in the acute phase of PANK depletion by metabolomics. In order to gain an insight on the mechanism of toxicity, I will analyze changes in gene expression by transcriptomics and key-signaling pathways by Reverse Phase Protein Array (RPPA).

Aim 3: To validate *PANK* inhibition as a targeted therapy in PANK1 deleted tumors *in vivo*.

In this aim, I will test the efficacy of PANK activity inhibition in tumors *in vivo*. I will perform intracranial injection of *PANK1* homozygously deleted and *PANK1* reconstituted cell lines with doxycycline inducible shRNA against *PANK2/3* in mice. I will monitor tumor growth by non-invasive imaging using T2 MRI, and induce the shRNA knockdown of PANK isoforms by doxycycline administration. I will then monitor growth/regression of tumors as a result of PANK2/3 ablation. I will perform similar experiments with ASOs against *PANK2/3* injected intra-tumorally. I will also perform histopathological analyses as well as metabolomics on tumor samples obtained from the mice to elucidate the mechanism of toxicity

Overall, this project seeks to validate the collateral lethality paradigm in *PANK1* homozygously deleted GBM tumors. Considering the frequency of *PTEN* deletion, as well as critical function of PANK in cells, the collateral lethality framework on *PANK1* may be clinically relevant for a wide variety of malignancies.

Background and Significance

Glioblastoma multiforme is the most common and aggressive brain tumor. It is highly infiltrative, and neurologically destructive with extremely poor prognosis [3-5]. Surgical resection followed by adjuvant chemotherapy with temozolomide or radiotherapy is the current standard of care, but these treatment modalities only yield median survival of 15-23 months and an incredibly low 5-year survival rate of 6% [3]. This underscores the urgent need for the identification of novel and effective therapeutic targets for a complete eradication of this malignancy. Continued efforts for genomic characterizations of glioblastoma tumors have led to sophisticated understanding of GBM pathogenesis as well as identification of several molecular alterations that are potential candidate for therapeutic targets. However, despite encouraging progress on this front, the clinical success of these therapeutic targets has been very dismal in GBM. Most of the clinical trials on GBM have focused on the profound driver mutations of activated oncogenes such as *EGFR* and *PDGFR*, but the clinical outcome of these studies is invariably negative due to recurrence of the disease [5]. Similarly, while immunotherapy has proven to be effective in other cancers such as melanoma, the curative potential of immunotherapy is still bleak in GBM [15]. Therefore, it is critical to identify additional targetable vulnerabilities in GBM. To this end, I am proposing a novel idea to target vulnerabilities exposed by the deletion of tumor suppressor genes (TSGs), rather than targeting amplified oncogenes. In my study, I have incorporated the framework of collateral lethality, where cancer cells tolerate the deletion of integral housekeeping genes lost along with TSGs, due to functional redundancy of these housekeeping genes. As a result, targeting these redundant isoforms will deprive the cancer cells of the essential housekeeping proteins and render cancer cells vulnerable while leaving normal cells unaffected. The Muller lab has successfully validated this novel paradigm of collateral lethality in preclinical models of glioma [1,2]. More specifically, I have demonstrated that deletion of the 1p36 locus also results in passenger deletion of important metabolic genes such as *ENO1* and *NMNAT2*, and inhibition of the redundant paralogs *ENO2* and *NMNAT1,* respectively, can specifically kill the cancer cells. Relying on the techniques employed for validation of *ENO1/ENO2*[1,2], as well as *NMNAT1/NMNAT2* (unpublished data) for their collateral lethal interactions, we aim to demonstrate similar results in *PANK1* deleted tumors.

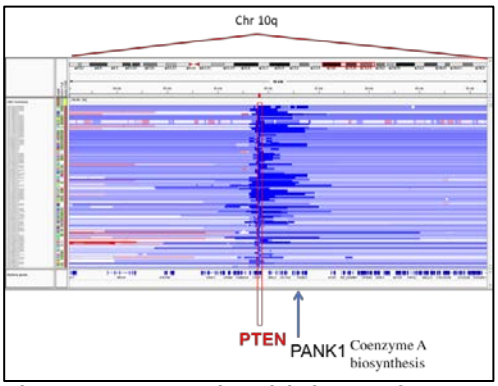

Figure 1: Genomic deletions of tumor suppressor genes can encompass critical metabolic housekeeping genes as passengers. The genomic landscape of the 10q23 region from selected TCGA primary tumors (y-axis) as a function of chromosomal position (x-axis) is shown. Homozygous genomic deletions (dark blue) eliminate tumor suppressor genes (e.g. *PTEN*, in red) as well as chromosomal neighbors or "passenger" genes (black, e.g. ***PANK1***) playing important roles in metabolism and expose a vulnerability that can be selectively targeted in

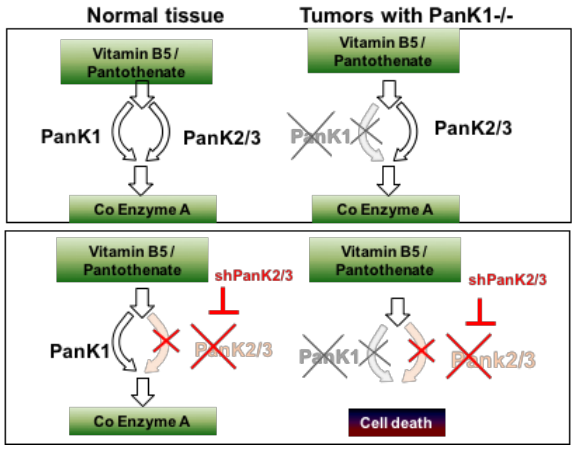

Figure 2: Collateral Lethality Framework for PANK1 co-deleted with PTEN Homozygous deletions of PTEN (10q.23) can also carry passenger deletion of PANK1. PANK1 catalyzes the conversion of pantothenate to co-Enzyme A, a cofactor critical for many essential cellular functions. Loss of PANK1 may be tolerated owing to the functional redundancy imparted by PANK isoforms (PANK2 and PANK3). By exogenously inactivating the redundant isoforms PANK2 and PANK3, with small molecule inhibitors such as shRNA, it is possible to selectively target cancer cells, without compromising normal cell functions.

The 10q 23 locus contains *PTEN*, one of the frequently deleted TSG in a variety of cancers, which accounts for up to 40% of all GBM cases [6], 39% of castration resistant prostate cancer [16], as well as invasive breast carcinoma[17,18] , head and neck carcinoma[19] and melanoma [20] . Additionally, *PTEN* deletion has been associated with poor outcome in GBM[21] and resistance to immune checkpoint inhibitors[22]. By identifying a metabolic vulnerability that coincides with *PTEN* deletion, I am proposing an efficient and unique way to kill *PTEN* null cancer cells specifically. *PANK1*, a neighboring gene in the *PTEN* locus, is the most critical regulatory enzyme in the CoA biosynthesis pathway. *PANK1* has two other catalytically active isoforms, *PANK2* and *PANK3* [7,13] (Figure 3). Acetyl Co-A, a derivative of Co-A is a central molecule situated at the intersection of many critical metabolic pathways involving fatty acids, proteins, carbohydrates and ketone bodies [13]. Obliteration of PANK activity in the cells does not permit viability [10-13,23]. Therefore, by exploiting this metabolic vulnerability, we can induce *PANK1* deleted cancer cell specific death, without perturbing normal *PANK1* intact cells by targeting the redundant isoforms of PANK1 (Figure 2). A successful implementation of this proposal can benefit thousands of patients with *PTEN-PANK1*

deletion in a variety of cancers. More importantly, this proposal seeks to target a metabolic vulnerability that is indispensable for cancer cell survival, and is therefore indiscriminate to the degree of cancer as well as site of cancer origin, making this applicable for a multitude of malignancies.

Innovation

TSG deletions are prototypic events for tumor initiation, but they cannot be therapeutically targeted. Different studies have circumvented this limitation by identifying compensatory synthetic lethal partners of the deleted TSG. The proposed study focuses on vulnerability exposed by deletion of metabolic housekeeping genes in the PTEN locus, instead of identifying the synthetic lethal partners of the gene itself. Deletion of TSGs can accompany neighboring housekeeping genes that are redundant in expression and critical for the cells. By targeting the redundant paralogs, it is possible to deprive the cancer cells of these essential proteins, which can selectively kill cancer cells. Therefore, **a key innovation of this proposal rests on myapproach of targeting essential, but redundant passenger deleted metabolic genes rather than driver tumor suppressor genes for collateral lethality**. Similarly, the roles of pantothenate kinases and co enzyme A has been extensively studied in normal cells, but the dependence of cancer cells on co enzyme A remains to be elucidated, which also reinforces the ingenuity of my study.

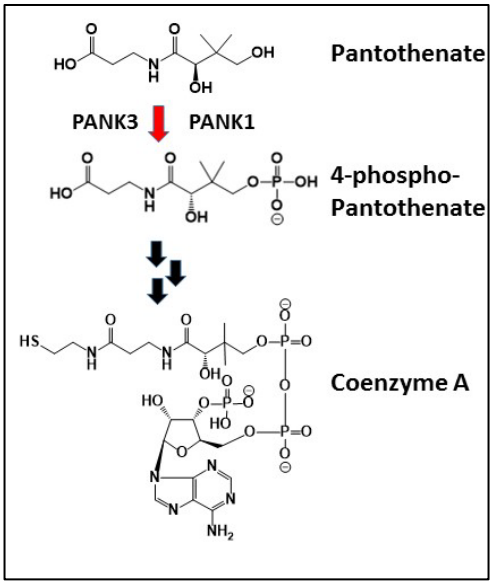

Figure 3: PANK activity is an essential enzyme for the synthesis of Coenzyme A. Pantothenic acid (Vitamin B5) is phosphorylated by PANK to 4-phosphopantethonate, and in a series of enzymatic steps is converted to Coenzyme A. Pantothenic acid is readily cell permeable but once phosphorylated, is trapped intracellularly by virtue of the negatively charged phosphate. Because of this charge, mitochondrial matrix and cytosolic Coenzyme A pools are segregated.

Preliminary Data

I have identified multiple cell lines and patient derived xenografts with *PTEN-PANK1* homozygous deletions and validated the loss by western blot **(Figure 4)**. Furthermore, I have successfully generated CRISPR knockouts of *PANK1* in *PANK1* intact cell lines (**Figure 4)**. Similarly, in order to identify the redundant isoforms of PANK1, I also tested anti-sense oligonucleotides against *PANK3* in *PANK1* deleted 537 Mel cells as well as *PANK1* intact melanoma cells. I demonstrated that inhibition of *PANK3* with anti-sense oligonucleotides in *PANK1* homozygously deleted 537 MEL spheres selectively kills 537 Mel cells, indicative of a potential collateral lethal interactions between *PANK1* and *PANK3* **(Figure 5).**

Methodology

Aim 1: **Determine the cell essentiality of *PANK* and identify the paralogs redundant with *PANK1* in cancer cells.**

Rationale/Hypothesis: The knock out studies of PANK isoforms in lower organisms and mice indicate that complete ablation of *PANK* activity is not compatible with viability [9,13]. However, the essentiality of PANK isoforms in the context of cancer cells has not been studied. In mammals, the pantothenate kinase family consists of three catalytically active isoforms: nuclear PANK1 α and cytosolic PANK1 β (splice variants of *PANK1*), mitochondrial *PANK2* and cytosolic *PANK3* [7,13]. Isoform-specific subcellular localization indicates that the PANK isoforms may have site specific function. Based on the cytosolic co-localization of PANK1 β and PANK3 and embryonic lethal phenotype in *PANK1/PANK3* dko mice, **I hypothesize that PANK3 is redundant with PANK1 β and therefore compensates for the loss of PANK1**. To elucidate this, we propose the following experiments.

Generation of cell lines with individual PANK isoform knock out: I will use CRISPR-Cas9 based system to knock out PANK isoforms individually. I will design two guide RNAs (gRNAs) targeted against the conserved catalytic domain or exon 6 of all three genes using online algorithms on http://chopchop.cbu.uib.no. The gRNAs will be cloned on px260 vector based on the protocol established by the Zhang lab. The plasmid expressing the gRNAs will be introduced to HeLa cells by lentiviral infection. I will use transient transfection of 293 T cells to produce recombinant lentiviral particles. I will use third generation lentiviral delivery system, with 10 μg of sgRNAs, 2.5 μg psPAX2 and 2.5 μg of pMD2.G and transfect using polyethylenimine (1ug/ul Polysciences #23966-2) into 293 T cells plated on a 100-mm dish. I will collect viral particles after 48 hours and centrifuge and filter the supernatant to remove residual 293T cells. I will infect HeLa cells plated on 6-well plates with the viral solution with 4ug/ml

polybrene, followed by positive selection using puromycin after 48 hrs. Using FACS sorting, I will sort single cells in 96-well plates, grow colonies and screen individual clones for knock out of each gene by RT qPCR and immunoblotting. Testing anti sense oligo nucleotides against *PANK2* and *PANK3*: The Muller lab collaborating with IONIS pharmaceuticals to generate druglike antisense oligonucleotides that cells freely take up. IONIS ASOs are 15-20 nucleotides long, and upon binding to the target mRNA activate RNAse H which degrades the target mRNA. As a proof of concept, I have already tested a set of 13 oligonucleotides targeting *PANK3* mRNA in *PANK1*-deleted 537 Mel cells as well as *PANK1* intact control melanoma cell lines. *PANK1* deleted 537 MEL spheres are selectively sensitive to *PANK3* inhibition, as assessed by TMRE intensity **(Figure 5)**. TMRE is a cell permeant and cationic fluorescent dye that is sequestered by active mitochondria, indicative of live, viable cells. *PANK2* ASOs are also currently being synthesized and will be tested as explained above. Testing a putative Pantothenate Kinase inhibitor: Recently, Sharma *et al*. (2015) reported a putative pan-PANK inhibitor with propensity to inhibit all PANK isoforms, and more strongly PANK3 (IC50 25 nM) [24]. I will subject *PANK1*-null and *PANK1* intact cells to PANK inhibitor to determine if the inhibitor shows selective and adverse toxicity in *PANK1*-null context. I will perform these experiments with both pantothenate supplemented and pantothenate depleted medium. Generation of *PANK1* reconstituted cell line: In order to rescue the effects of *PANK1* deletion/knock out, I will reconstitute PANK1 expression in G59 and 537 Mel cells using an ectopic plasmid (pCMV-*PANK1*). I will obtain sequence verified Gateway compatible *PANK1* cDNA from the Harvard Plasmid Repository and clone them into the expression vector pHAGE-CMV. I will use lentiviral delivery method as explained above to transduce the pCMV-PANK1 into *PANK1-null* cells. A successful reconstitution of *PANK1* will be verified by immunoblotting. Generation of cell lines carrying inducible PANK2/3 CRISPR and shRNA: I will use shRNA and CRISPR systems to inhibit *PANK2* and *PANK3* in cell lines that have harbored *PANK1* homozygous deletions. I will use G59 and 537 MEL cell lines for these experiments since they have been validated to have spontaneous *PANK1* and *PTEN* homozygous deletion (Figure 4). G59 is a glioma cell line derived from anaplastic astrocytoma, while 537 Mel is a human melanoma cell line. Additionally, I have also successfully created *PANK1* knock out in HeLa cells using CRISPR (Figure 4). I will screen ten shRNA sequences targeting different regions of *PANK2* and PANK3 mRNA and identify four independent hairpins that confer >60% knock down to make an inducible shRNA system. I will use doxycycline inducible pTRIPZ vector from Dharmacon to create inducible PANK2 and PANK3 using the sequences from the initial screen in PANK1-null and PANK1 intact cells. The hairpins will be introduced into the cells by lentiviral delivery as explained above. Doxycycline (DOX, 1µg/mL) will be administered to the cells to induce shRNA expression and the knockdown of *PANK2/3* will be verified by western blot and RT qPCR. Additionally, I will also verify the knock down of PANK2/3 by pantothenate kinase activity assay. I will utilize a TLC ^{14}C labeled assay for this purpose. Lysates of various cell lines will be incubated with media containing D-[1-^{14}C]pantothenate and ATP. After stopping the reaction with acetic acid, the mixture will be separated on a Whatman DE81 ion-exchange filter disk, which only retains phosphorylate species including 4′-Phosphopantothenate. 4′-[1-^{14}C] Phosphopantothenate will be quantified by scintillation counting [25]. Cell viability experiments using inducible system: After we establish the inducible system, I will perform cell viability experiments to assess the toxicity elicited by *PANK2* or *PANK3* inhibition in *PANK1*-null and PANK1 reconstituted cells. I will use crystal violet staining as well as the Promega Cell Titer-Glo proliferation kit (Roche) to measure the cell growth and viability in response to PANK ablation. Furthermore, I will also assess the changes in colony forming and migration capacity of these cells using the established assays. Additionally, I will also determine the live cell confluence measurement of wild type and *PANK1* deleted cells upon *shPANK 2/3* induction using the IncuCyte live cell imaging system (Essen Bioscience). I will generate growth curves by confluence imaging every 4 hours for 7 days. Time-lapse images of the cells using the IncuCyte will allow morphological analysis of cells in response to treatment (proliferation, senescence, apoptosis, and necrosis).

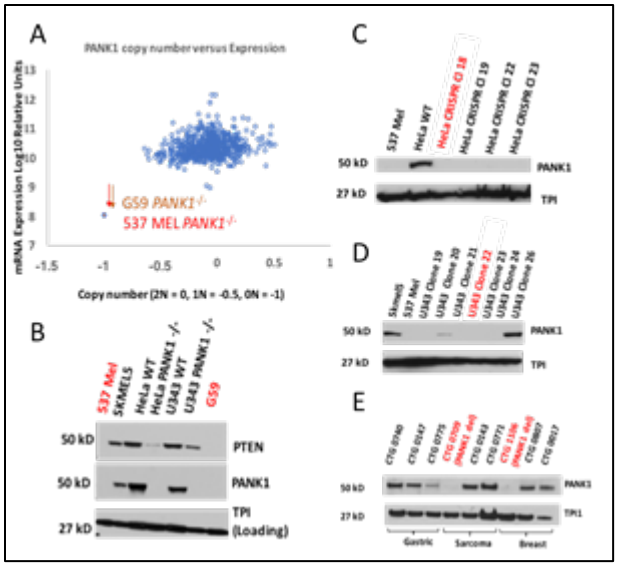

Figure 4: Identification of *PANK1*-homozygous deleted cell lines and xenografts. A: RNA-seq expression data (y-axis) and normalized genomic copy number (x-axis) for *PANK1* in cell lines from the Genenetch Cell line Panel are plotted. G59 and 537 MEL have lowest mRNA expression and show N = 0 copy number. **B**: PTEN and PANK1 protein (expected size: 50kD) are readily detected in a panel of diverse cancer cell lines but is undetectable in the 537 MEL and G59 *PTEN*/*PANK1*-homozygous deleted lines. **C and D**: PANK1 was successfully deleted by CRISPR in HeLa cells and U343 cells. **E**: PANK1 is undetectable in Champion's Oncology *PANK1*-deleted patient derived xenografts.

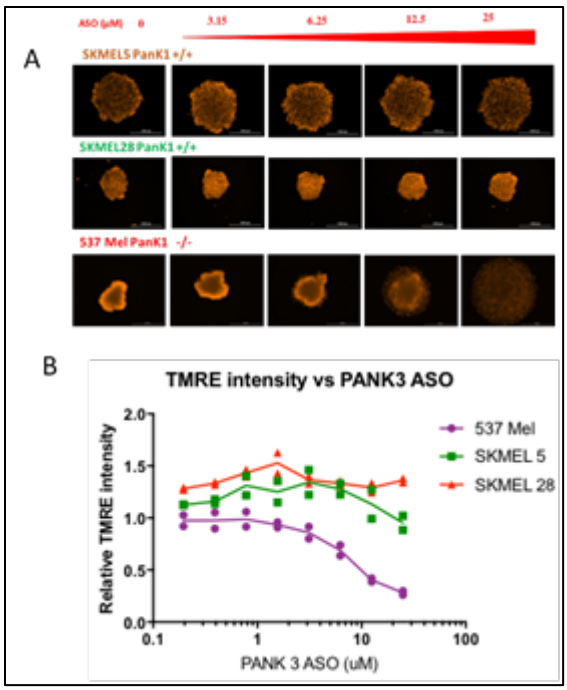

Figure 5: PANK3 ASOs selectively kill PANK1 deleted 537 mel cells in spheres.
A and B : *PANK1*-deleted 537 Mel cells, *PANK1* intact SKMEL5 and SKMEL28 cells were seeded in spheres and treated with antisense oligonucleotides against *PANK3* along with TMRE which stains active mitochondria, a reporter of cell viability. TMRE intensity decreases in 537 Mel cells, in a dosage dependent manner, but the *PANK1* intact control cells are unaffected.

Additionally, I will conduct cell cycle analysis and apoptosis induction by flow cytometry using annexin V-PE and 7-AAD.

Anticipated results: I anticipate that *PANK3* knock down will confer the strongest collateral lethality. This is based on the double knock out phenotype of *PANK1* and *PANK3* in mice which is embryonic lethal and the fact that *PANK1* and *PANK3* are both cytosolic whereas *PANK2* is in the mitochondrial matrix. As a result of *PANK3* knock down in *PANK1*-null cells, I anticipate the cells to be slower in their growth, as well as have reduced migration and invasion ability, compared to the *PANK3* knock down in *PANK1* intact cells. This is also corroborated by the observation in Figure 5 where targeting *PANK3* by ASOs in *PANK1* deleted spheres lead to a decrease in TMRE signal, indicative of a collateral lethal relationship between *PANK3* and *PANK1*.

Potential problems and resolution

1. The CRISPR knock out of individual isoforms may not be possible if the isoforms are essential, so we may not be able to achieve a complete knock out. However, the newly released AVANA CRISPR whole genome screen data set suggests that none of the PANK genes are essential on their own, indicating that the genes are individually dispensable.
2. One potential problem could be that targeting *PANK2* or *PANK3* in *PANK1*-null cells can lead to complete cell death. This will support our hypothesis, but I will not be able to determine the differences in cell proliferation as well as biochemical consequences of PANK ablation and understand the mechanism of toxicity. To resolve this issue, we can modulate doxycycline concentration and assess the effect of acute loss of PANK.
3. On the contrary, the knock down of either *PANK2* or *PANK3* may not impact the cancer cell survival. This could be because of inefficient knock down of the genes by shRNA. To alleviate this problem, I will perform a quick siRNA mediated knockdown using the smartpool-onTarget siRNA against PANK2 and PANK3 from Dharmacon. Additionally, I will use an inducible CRISPR system to knock out PANK2 and PANK3 genes. We can also address this problem by employing a combination of shRNA and ASOs or the pan-PANK inhibitor. Similarly, it is also likely that CRISPR knock out of *PANK2* or *PANK3* or both in PANK1-null cells may have no discernible effect on cancer. This will be an interesting discovery in itself, as it will provide groundbreaking insight into our current understanding of CoA metabolism, and dependence of cancer cells on CoA for their survival. However, this is unlikely because the AVANA CRISPR screen identified all the downstream enzymes in the CoA synthesis pathway as highly essential. This implies that CoA synthesis pathway is indispensable in cancer cells. Panthothnate kinase regulates the rate limiting step of CoA synthesis pathway, therefore its complete ablation cannot permit cell proliferation.

Specific Aim 2: **To determine the biochemical consequences of *PANK* inhibition**

Rationale/Hypothesis*:* Pantothenate Kinase regulates the first and committed step of CoA biosynthesis. CoA is required for essential biochemical reactions such as fatty acid oxidation and synthesis, regulation of lipid metabolism, and pyruvate oxidation in TCA cycle. Additionally, acetyl CoA regulates all acetylation reactions, and has the propensity to impact critical processes such as gene transcription through histone acetylation, splicing, cell cycle, chromatin remodeling, DNA replication, nuclear transport of proteins as well as cell signaling [26]. **Therefore, I hypothesize that the loss of PANK activity will deplete CoA pool from the cells which can impact critical metabolic and transcriptional profile of the cells**. I will demonstrate this with the following experiments.

Metabolome Profiling: I will perform metabolomics profiling on wild type G59 cells and dox inducible shPANK2/3 G59 as well as its isogenic rescue cells and assess comprehensive changes in the level of small molecule metabolites. In order to assess temporal changes in the level of metabolites, I will perform metabolomics on both the cell pellet and the supernatant media in which the cells were cultured in both pantothenate supplemented

and depleted medium at different time points. To recover polar metabolites, I will extract the samples with 80% methanol at -80 °C, and analyze it by LC/MS. I will outsource our samples to Dr John Asara's mass spectrometry core at the Beth Israel Deaconess Medical Center. Lipidomics: I will also assess how loss of PANK activity affects lipid levels in the cells. I will trypsonize and harvest the cells at RT and extract non-polar lipids with methanol and methyl-tert-butyl ether. The samples will be dried on a speed vac and submitted to the John Asara platform for analysis. Transcriptome Profiling**:** I hypothesize that PANK ablation will also severely compromise acetylation status of different proteins. To test this, I will perform western blot on cell lysates of G59 WT and shPANK 2/3 cells, and probe acetylated proteins using the antibody against acetylated lysine and acetyl histone H3. Additionally, I will also employ SILAC technology to identify differentially acetylated proteins. G59 WT and G59 shPANK 2/3 cells and the isogenic rescue cells will be grown in medium containing either light or heavy isotopes of L-arginine and L-Lysine. The cells lysates will be prepared in -20 C acetone and submitted to the proteomics core at MD Anderson Cancer Center to quantify differences in acetylation levels of proteins as a result of PANK activity ablation. If we observe discernable differences in the acetylated protein levels, I will perform RNA-seq at the MD Anderson core facility to identify genes that are differentially regulated as a result of PANK depletion from the cells. Proteomics**:** To complement the RNA-seq data, I will use reverse phase protein array (RPPA) at the functional Proteomics core at MD Anderson Cancer Center to evaluate protein activities in the signaling networks. RPPA assay can quantify approximately 300 proteins in major cellular signaling pathways with pivotal roles in proliferation, stress signaling, etc. Furthermore, PANK ablation should lead to a reduction of the downstream TCA cycle intermediates, and increase the upstream glycolysis intermediates. I will assess these changes using the XF96 Seahorse analyzer which will quantify oxygen consumption rate (OCAR) and extracellular acidification rate (ECAR), as a measure of lactic acid production.

Anticipated results: From the metabolomics profile, I anticipate that *PANK* inhibition will lead to decreased levels of metabolites downstream of pantothenate kinase in the co-enzyme A biosynthesis pathway. Additionally, as a result of Co-A depletion, we also expect the lipid biosynthesis pathway to be severely disrupted. Similarly, we also expect to see an increase in glycolytic intermediates. Likewise, we also anticipate an increase in ECAR and decrease in OCR in as a result of PANK depletion from the cells. Similarly, acetyl CoA being the only acetyl group donor in the cells, I expect that protein acetylation will decrease globally, affecting the transcriptional activation status of genes globally, which will be reflected in the RNA-seq and RPPA data.

Potential Problems and Resolution:

1. One of the potential problems that we may encounter in this aim is that in both shRNA and CRISPR mediated elimination of *PANK3* in *PANK1* deleted cells, I will see no considerable effect in CoA levels, and the subsequent effects on metabolites as well as acetylated proteins. This could suggest two possibilities. a. CoA pool from mitochondria can move to the cytosol when *PANK1*/*PANK3* activity is eliminated. Although there is precedence of cytosolic co-enzyme A to move into the mitochondria, there is no evidence of mitochondrial CoA movement into the cytosol. This is because the highly negative phosphate groups on CoA limits its movement across the membrane. But if this were to occur, we would be able to detect these differences through the metabolomics data. b. Alternatively, it is also possible that there are other sources of CoA that have not been studied and identified before. Both RNA-seq and metabolomics data will be informative in addressing this possibility.

Specific Aim 3**: To validate *PANK* ablation as a targeted therapy in PANK1 deleted tumors in *vivo*.**

Rationale/Hypothesis: Individual knock out of *PANK* in mice is tolerated at the organism level. This warrants the possibility of developing specific inhibitors against *PANK* isoforms to target cancer cells without compromising normal cell function. Additionally, the structures of the PANK isoforms have sufficient differences and therefore have a window for differential and isoform-specific druggability. Additionally, the crystal structures of these proteins are available on Protein Data Bank, which indicate multiple sites within the protein that allow allosteric regulation of enzymatic activity. In this aim I will test the hypothesis that *PANK1* deletion is a targetable vulnerability *in vivo*. Our preliminary *in vitro* data with the ASOs against *PANK3* suggest that inhibiting *PANK3* selectively kills *PANK1* deleted cells in spheres (Figure 5). On this premise, I will test the hypothesis that *PANK3* inhibition will selectively kill intracranial *PANK1* deleted glioma tumors *in vivo* but not its isogenic rescued tumors. To this end, we propose the following experiments.

Generation of orthotopic intracranial glioma: I will use the MD Anderson Intracranial Injection Core to perform intracranial implantation of G59 cells. A total of 200,000 G59 and G59 *PANK1* rescued cells with inducible sh*PANK2/3* re-suspended in 10 uL PBS will be injected into the brain of athymic nude mice through a plastic bolt. I will include a total of ten mice for this experiment, five of which will be injected with G59, and the remaining five with the isogenic rescued cells G59 *PANK1*. I will validate the tumor establishment with T2-weighed MRI at

the M.D. Anderson small animal imaging facility (SAIF) on a weekly basis. After confirming the tumor establishment, I will monitor the growth or regression of tumor following doxycycline administration on a weekly basis with the T2 MRI. shRNA induction of *PANK2/3* will be performed via doxycycline containing water. I will verify the knock down via RFP reporter under dox in the pTRIPZ vector, and visualize the fluorescent tumors using the IVIS at the Department of Cancer Systems Imaging at MD Anderson Cancer Center. Additionally, I will use immunohistochemistry with antibodies against PANK2 and PANK3 in the fixed brain section as a post mortem analysis to confirm the knock down of *PANK2* and *3* in control and dox administered mice. For a comparative analysis, I will use a set of tumor-bearing mice with the current standard of care, temozolomide and evaluate the efficacy of two different treatment modalities. Furthermore, I will also use 537 Mel cells to generate subcutaneous tumors in mice and induce shRNA mediated knock down *PANK2/3* and monitor tumor growth by IVIS imaging as explained above. Metabolomics on tumor sample: To investigate the effects of PANK activity elimination *in vivo*, I will obtain tumors from G59 and G59 *PANK1* cells injected mice, and subject the tissues to metabolomics profiling as explained above. The result will be critical to corroborate the metabolomics data obtained from *in vitro* experiments. Post Mortem Histopathological Analysis: If tumor regression is observed upon dox administration, I will monitor the mice for 3 months. I will also continue to monitor the tumor bearing mice in the control group if they show no sign of morbidity. At morbidity, the mice will be sacrificed and perfused with paraformaldehyde. The brain will be fixed *in situ*, paraffin embedded and sectioned at the M.D. Anderson Pathology Core. Critical parameters evaluated in H&E stained slides will include tumor cellularity, edema, hemorrhage, and necrosis (pseudopalissade). I will perform Immunohistochemistry (IHC) on paraffin sections to quantify apoptosis with cleaved caspase 3 and cell cycle with Ki67. Additionally, we can also examine cancer stem cell niches to evaluate cell death in the quiescent cell compartment. BrdU (Bromodeoxyuridine) pulse chase lineage tracing will be performed in mice. I will administer 150 mg/kg BrdU by intra-peritoneal injection or through drinking water (1 mg/ml) one week, followed by a week of no BrdU administration. I will detect the cancer stem cells with the anti-mouse Brdu antibodies [27]. If the *in vivo* study with the shRNA is warranted, I will perform similar experiments with ASOs injected intra-tumorally. I will monitor tumor growth and perform histopathological analysis as explained above.

Testing the putative pan-PANK inhibitor in tumors *in vivo* : If the PANK inhibitor is effective in inducing PANK1-deleted tumor cell death *in vitro*, I will also test the inhibitor in the tumors *in vivo*. The inhibitor will be administered intra-venously in mice injected with G59 or its isogenic rescue G59 *PANK1* cells, and the tumor response to drug measured by T2 weighed MRI as explained above. Additionally, I will also record the body weight of the mice as a measure of the drug induced toxicity. At morbidity, the mice will be sacrificed and histopathological and metabolomics analysis performed as explained above.

Anticipated Results: I expect that upon *shPANK3* induction in G59 cells, the tumors will regress significantly compared to the *PANK1* reconstituted G59 cells. Additionally, I expect the *PANK1* reconstituted tumors to reflect the histopathology features characteristic of glioma, such as edema, pseudopalissading cells, and hemorrhage, and increased expression of Ki-67, indicative of actively proliferating cells within the tumor. In *PANK1* deleted tumors, induction of sh*PANK3* should lead to tumor a substantial tumor regression due to apoptosis, which would be reflected by an increased cleaved caspase-3 staining, compared to the control.

Potential problems and resolution

1. One of the potential problems with the ectopic expression of *PANK1* in G59 or 537 MEL cells is the use of the CMV promoter in the pHAGE-CMV plasmid. Different studies have shown that the CMV promoter gets methylated in subsequent passages *in vitro* as well as in mice, which could potentially reduce the expression of PANK1. I haven't encountered such problems in the experiments performed previously in our lab. However, if needed, we can alleviate this problem by using alternative plasmids such as pWPXL or pWPT which contain the EF-1 alpha promoter, and can allow constitutive expression of the transgene.
2. It is possible that I will be faced with a situation similar in Aim 1 where upon induction of sh*PANK2/3* it is possible that the tumors will be completely eliminated. Although this is unlikely, because the knock down efficiency of shRNA is not 100%, we propose to alleviate this problem by providing mice doxycycline water at a lower dosage and only for 72 hours, so that loss of *PANK2/3* will be acute. If no discernable differences are present between the *PANK1* null and *PANK1* reconstituted G59 cells due to a compromise in knock down efficiency by the shRNA, we can intra-tumorally inject *PANK2/3* ASOs in the mice and assess the effect. Alternatively, we can use the inducible CRISPR against *PANK2/3* in G59 cells and determine the effect of PANK ablation in tumors *in vivo*.

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