Pantothenate kinase isoforms as collateral lethality targets in Glioblastoma Multiforme

NAME OF STUDENT

On-Topic Candidacy Exam

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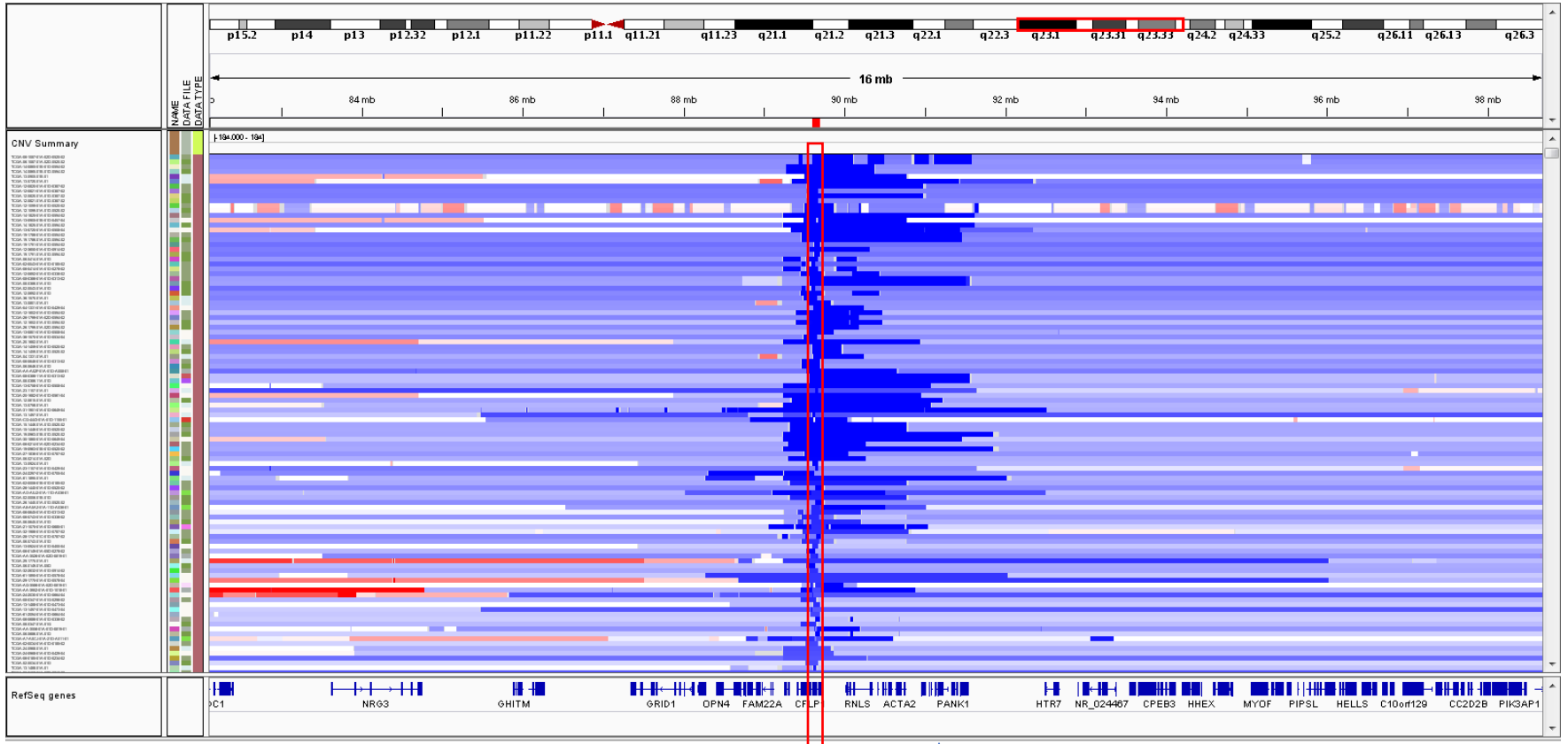
THE UNIVERSITY OF TEXAS MD Anderson Cancer Center | UTHealth The University of Texas Health Science Center at Houston

Graduate School of Biomedical Sciences

Concomitant loss of passenger genes occur with genomic deletion of tumor suppressor genes

TCGA Pan-Cancer Dataset



DNA Copy Number
- =0
- =1
- =2
- >2

PTEN

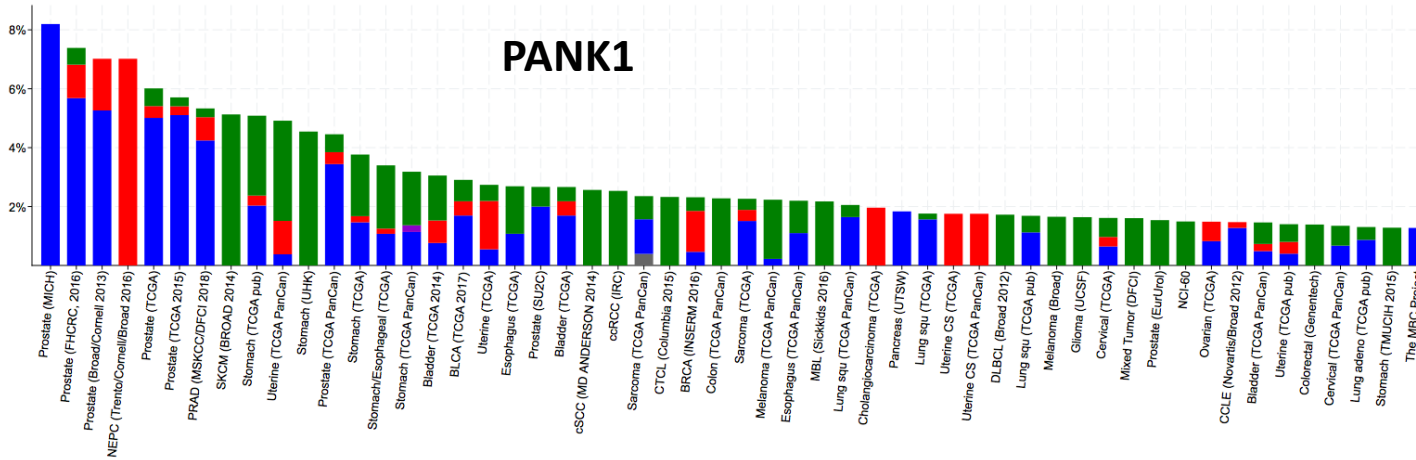
PANK1

Coenzyme A Biosynthesis

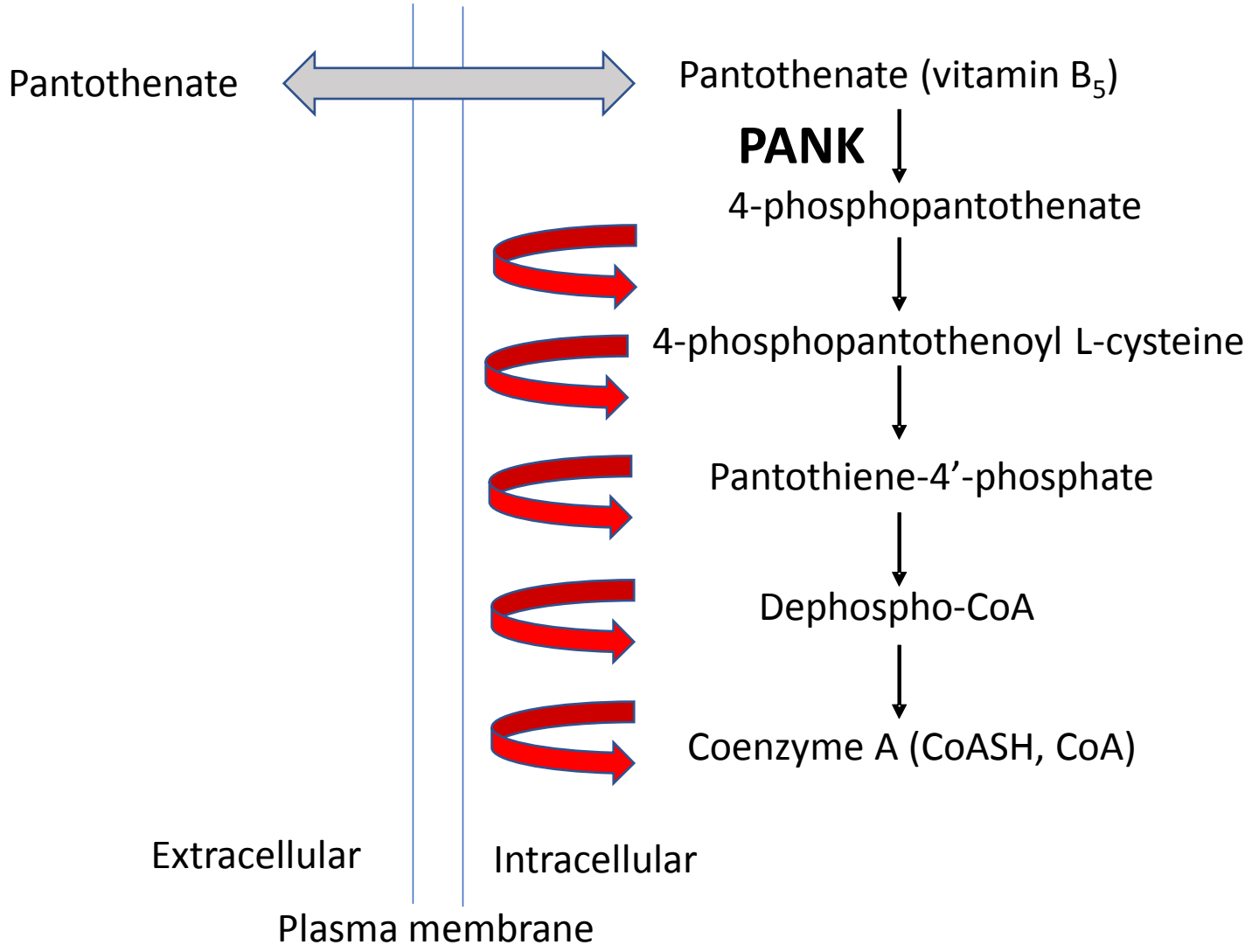
Prevalence of *PTEN-PANK1* homozygous deletions across cancer types

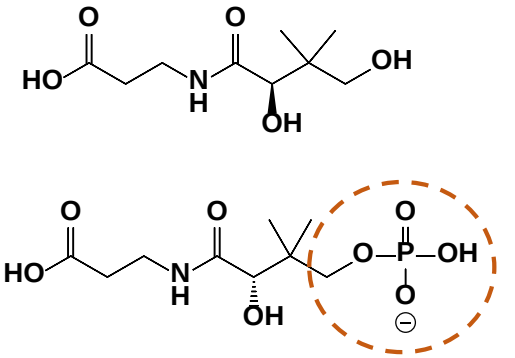
PANK1 homozygous deletions are found in cancers with *PTEN* homozygous deletions

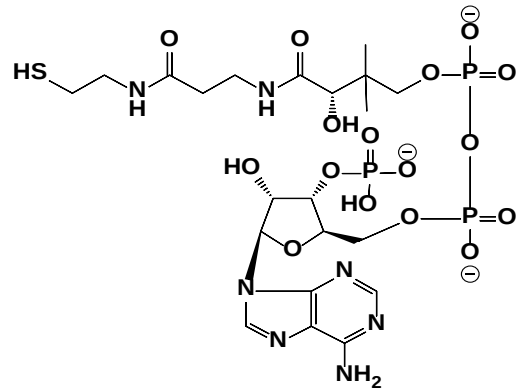
- Prostate Adenocarcinoma
- Glioblastoma
- Ovarian Cancer
- Melanoma
- Cervical Carcinoma



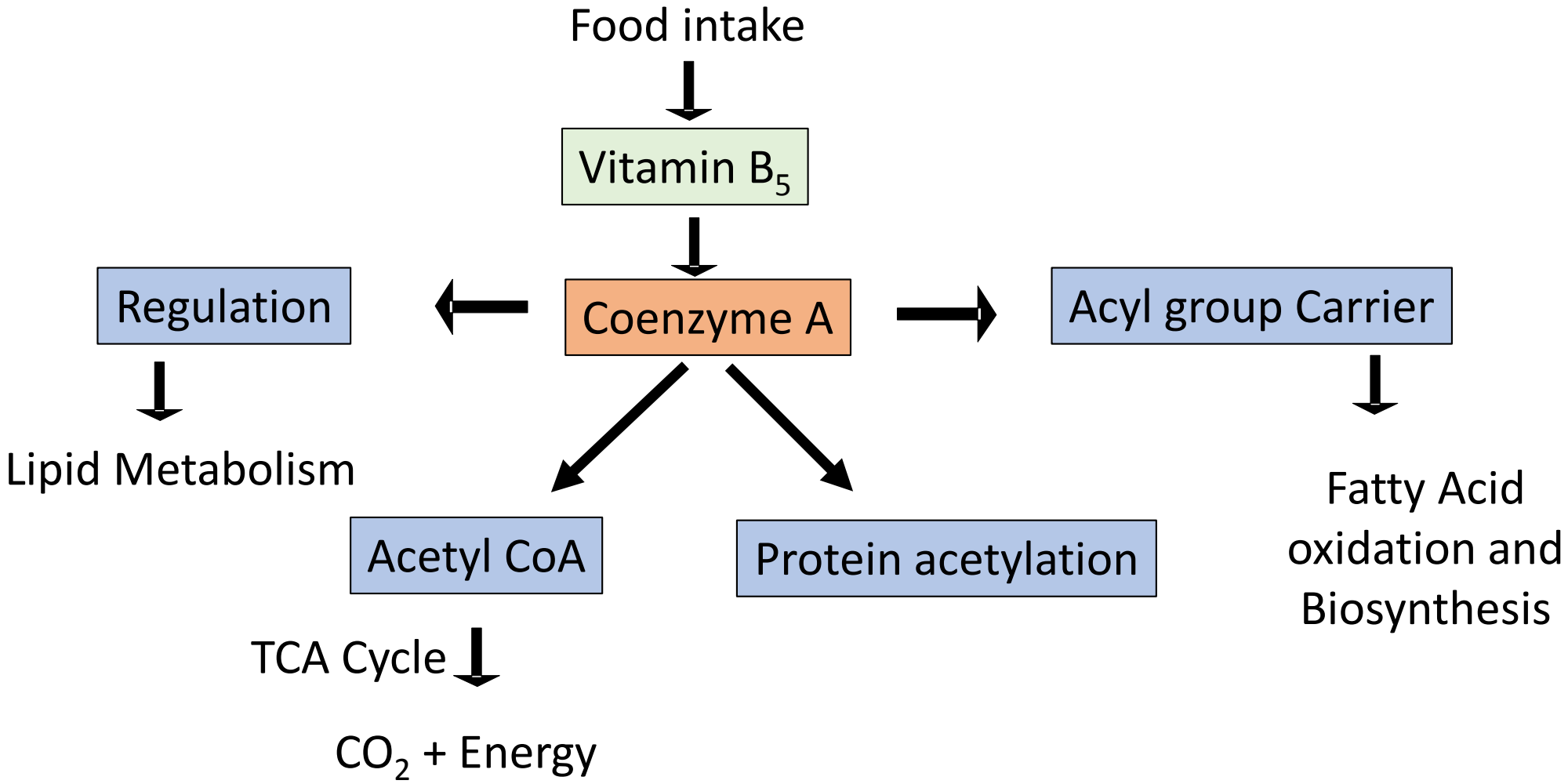
Pantothenate kinases are rate limiting factors in coenzyme A production





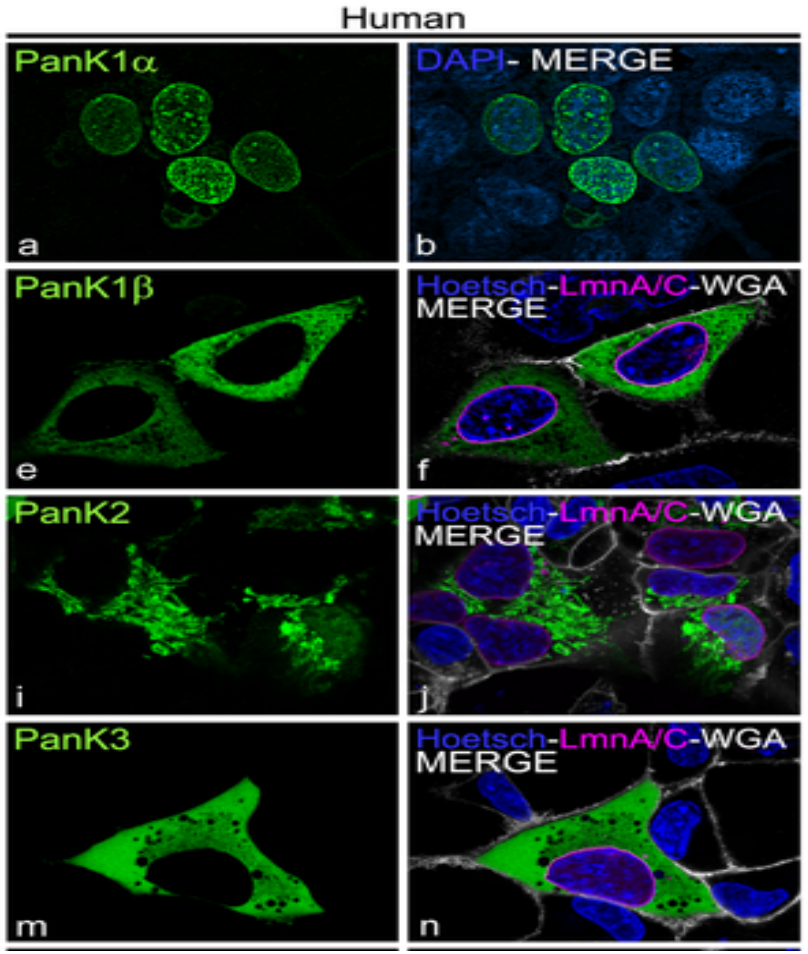


Coenzyme A regulates a multitude of essential cellular functions



PANK isoforms have distinct cellular localization and tissue specific distribution

- PANK1
  - ➢PANK1α (nuclear; Liver and kidneys)
  - ➢PANK1β (cytosolic; Liver)
- PANK2 (mitochondrial; neuronal tissue)
- PANK3 (cytosolic; all tissue types)
- PANK4 (catalytically inactive)



Alfonso-Pecchio A *et al* (2012) PLOS One, 7: 11, Figure 2

Cell essentiality of PANK is evidenced by PANK knockout phenotype in lower organisms

- *E. coli*, *S. cerevisiae and D. melanogaster* only have one PANK isoform
- *PANK* knockout is lethal in all unicellular organisms

Organismal essentiality of PANK is evidenced by PANK isoform knockout phenotypes in mice

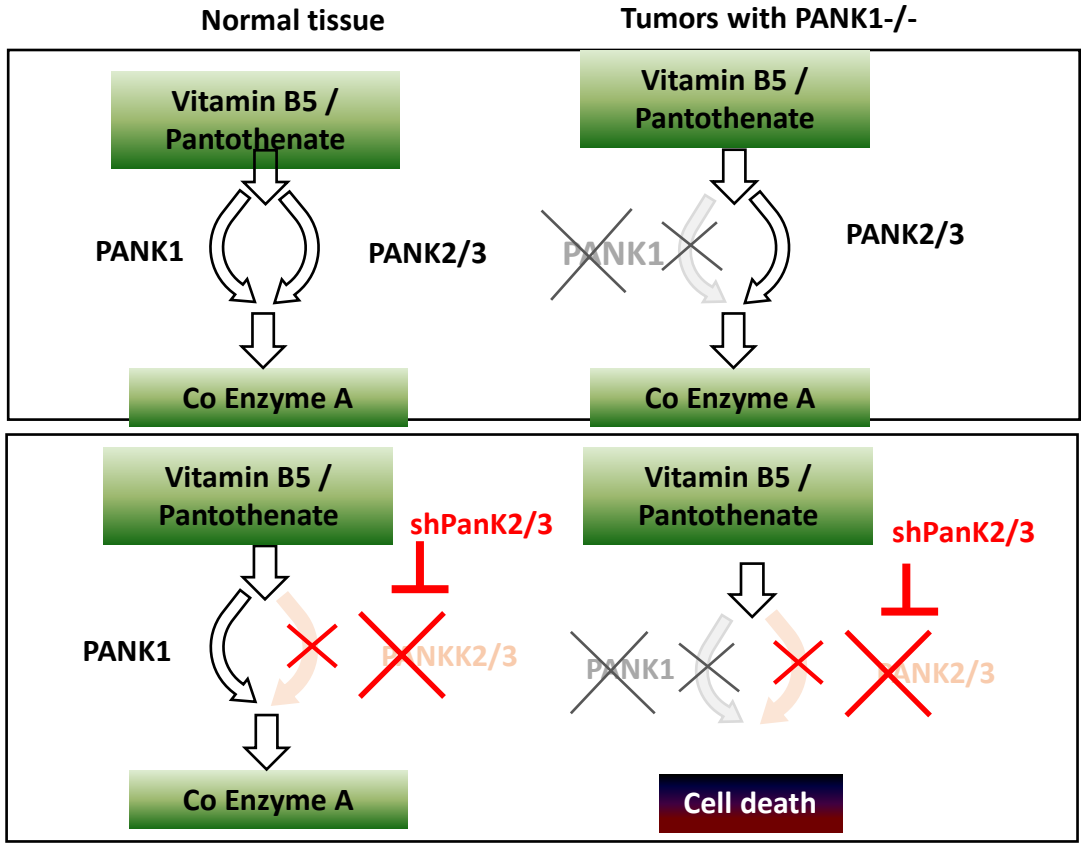
- **Individual PANK paralog knockout mice are viable**
  - *Pank1* -/- : Hepatic fatty acid oxidation and gluconeogenesis impaired in fasted state
  - *Pank2* -/-: Pantothenate Kinase Associated Neurodegeneration in human; retinal degeneration and impaired spermatogenesis in mice
  - *Pank3 -/- :* No known phenotype

- **Double knockout mice are embryonic or post natal lethal**
  - *Pank1*$^{-/-}$*Pank3*$^{-/-}$ and *Pank2*$^{-/-}$*Pank3*$^{-/-}$ dko mice are embryonic lethal
  - *Pank1*$^{-/-}$*Pank2*$^{-/-}$ dko are late post-natal lethal

Conclusion: At least two PANK isoforms are required for organism viability

CENTRAL HYPOTHESIS

Targeting the redundant isoforms of PANK in tumors with PANK1 homozygous deletion can selectively kill cancer cells.



Specific Aim 1

To determine if PANK activity is cell essential and identify the paralog redundant with PANK1 in cancer cells

Hypothesis: Based on the cytosolic co-localization of PANK1 β and PANK3, I hypothesize that PANK3 is redundant with PANK1 β and therefore compensates for loss of PANK1.

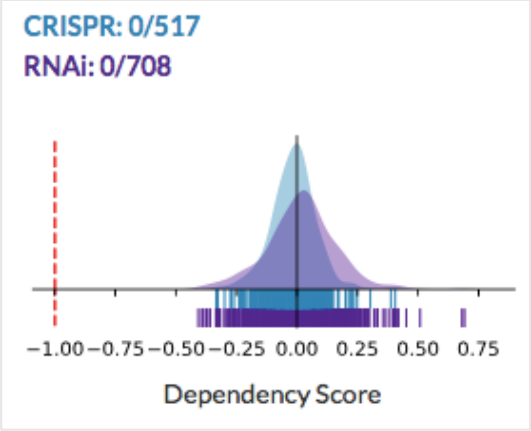
Specific Aim 1

To determine if PANK activity is cell essential and identify the paralog redundant with PANK1 in cancer cells

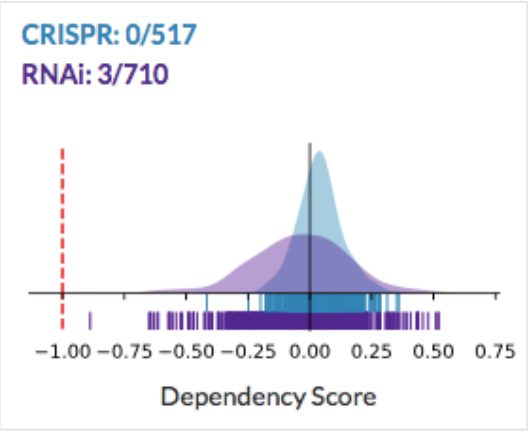
- Identification and generation of cancer cell lines with *PANK1* homozygous deletion
- Test the effect of a pan-PANK inhibitor and antisense oligonucleotides against PANK isoforms
- CRISPR KO of PANK isoforms alone or in the context of *PANK1* homozygous deletion
- Generation of *PANK1* isogenic rescued cell lines
- Constitutive and inducible knock down of *PANK2* and *PANK3* in *PANK1* deleted and intact cells
- Cell viability assays

AVANA CRISPR data set identifies PANK isoforms as non-essential genes

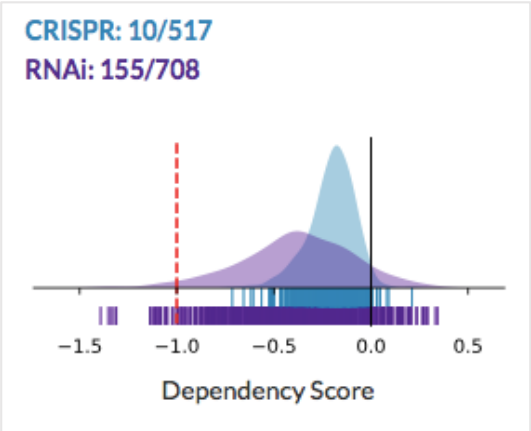
PANK1 (53354)



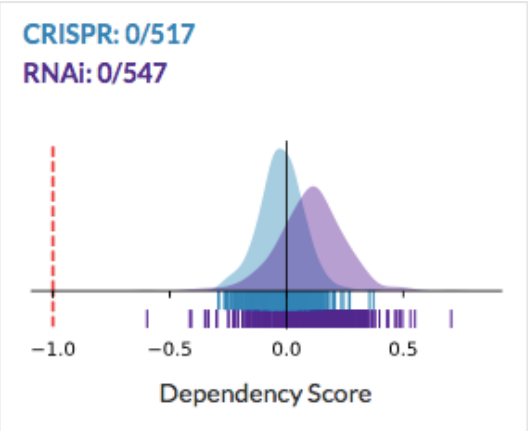
PANK2 (80025)



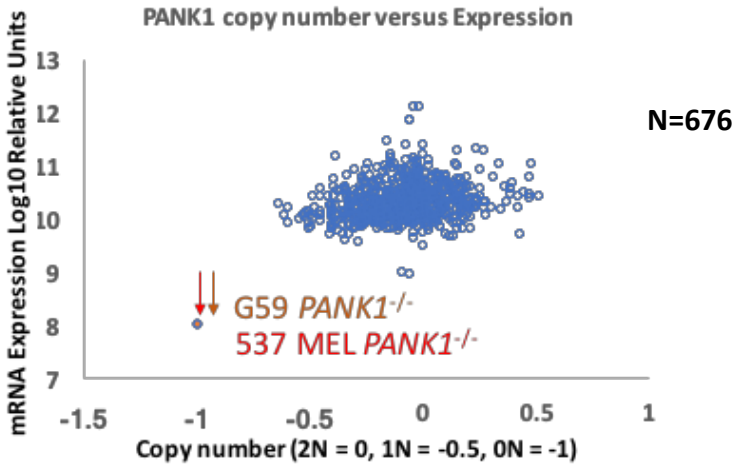
PANK3 (79646)



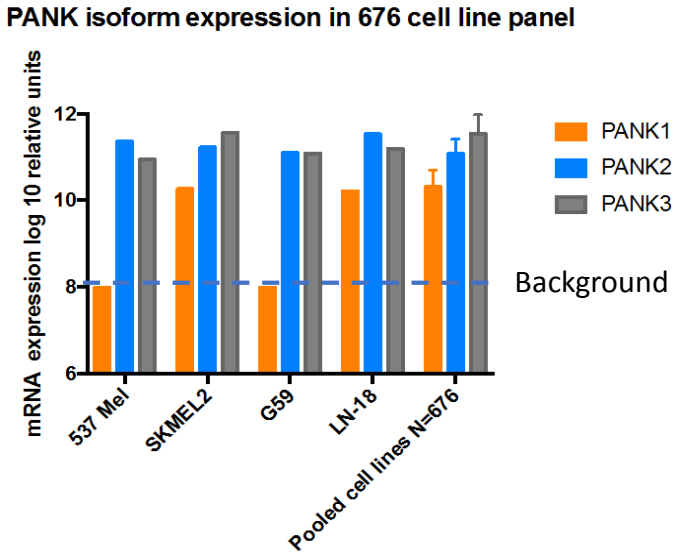
PANK4 (55229)

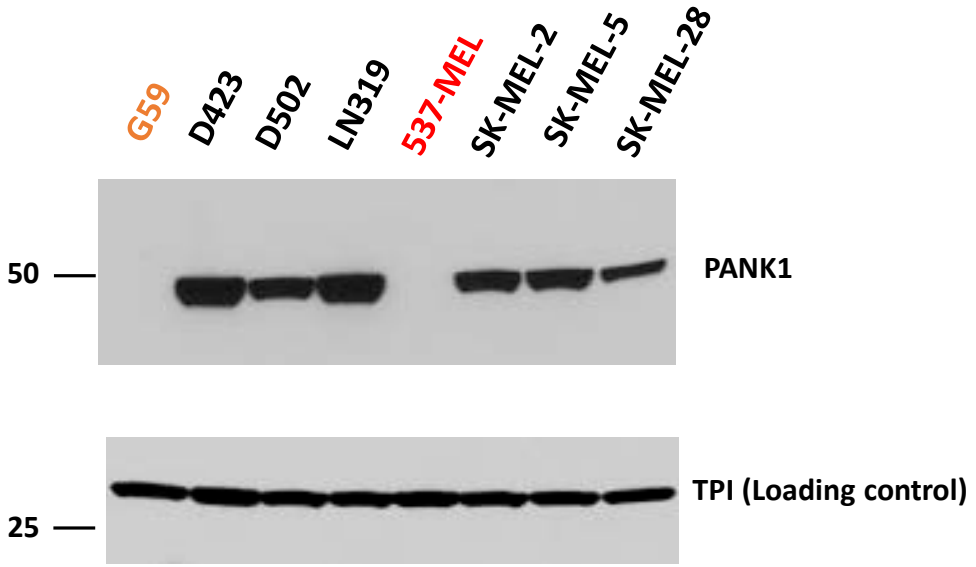


Selection of *in vitro* model with *PANK1* homozygous deletion



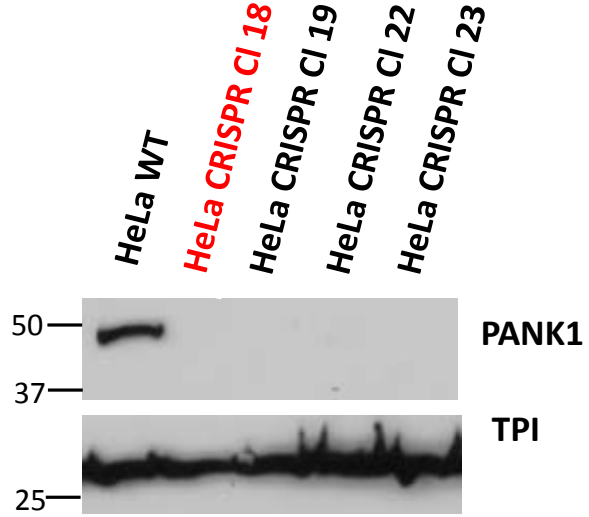
N=676

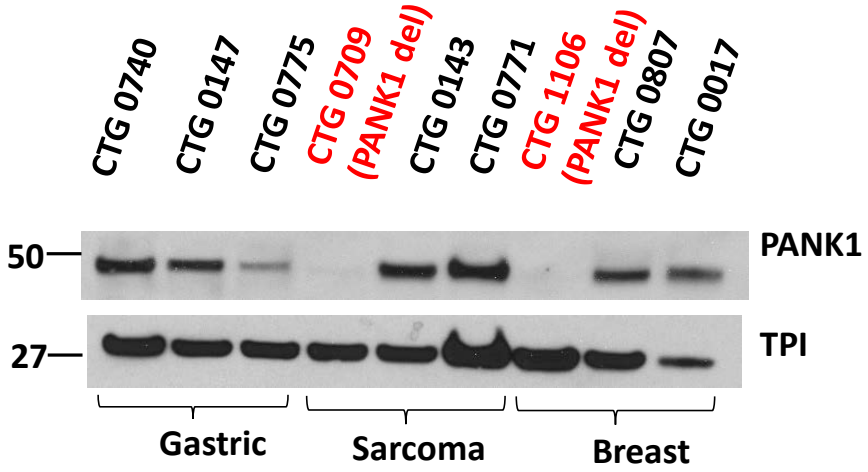


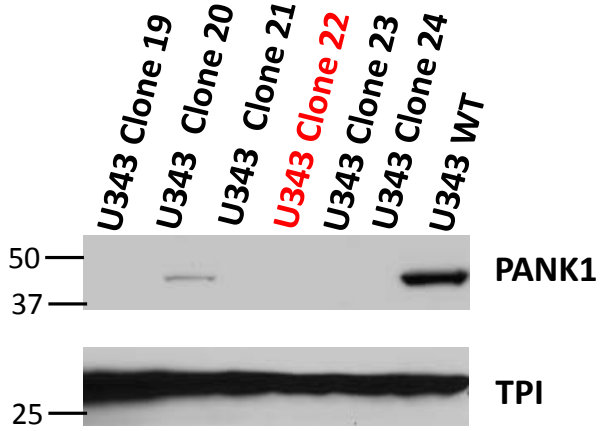


Analyzed based on data from : Klijn, C et al. 2015, Nature Biotechnology 33: 306–312, Supplemental Data 1

Generation of *PANK1* CRISPR KO cancer cell lines

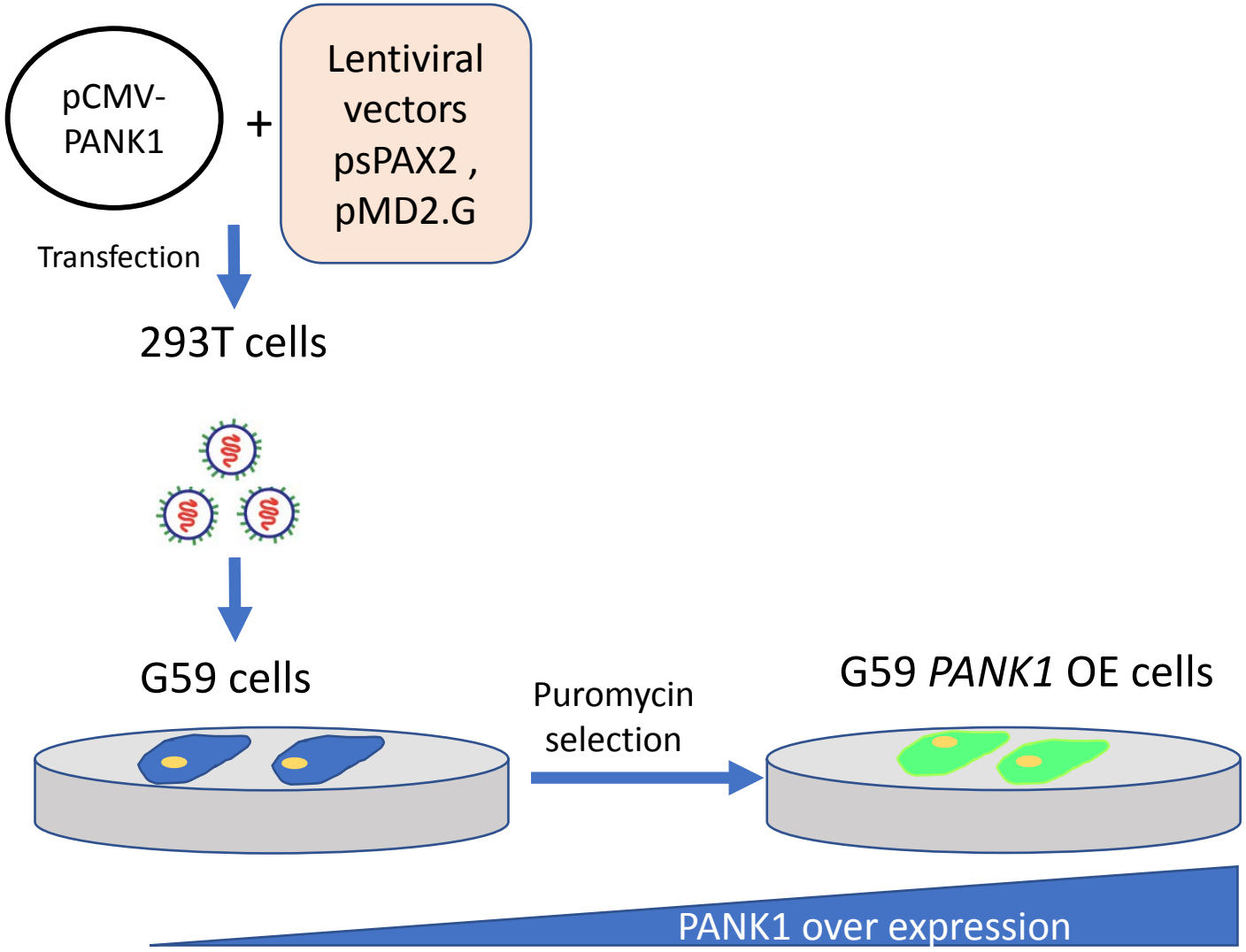




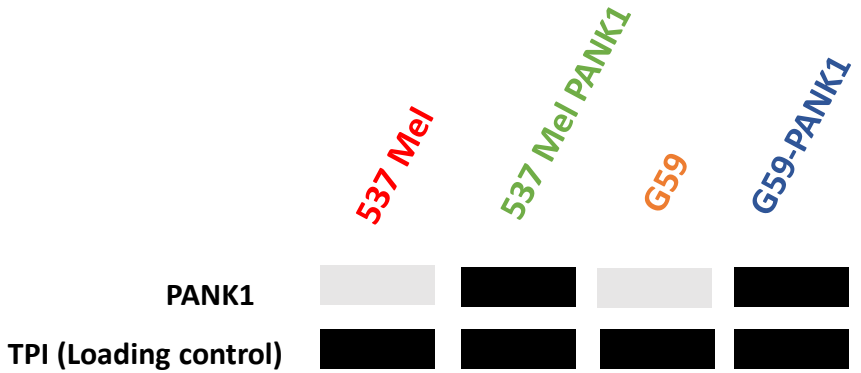


Conclusion: PANK1 is dispensable in cancer cells

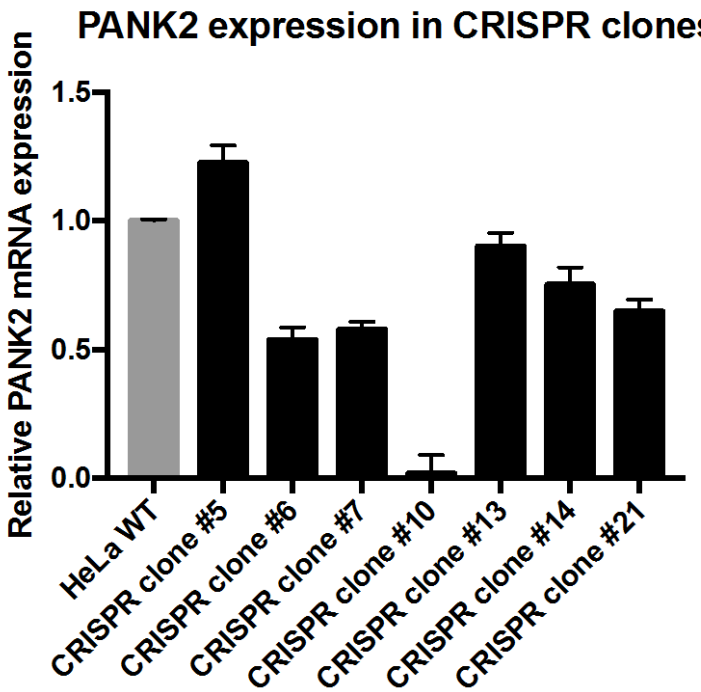
Generation of *PANK1* isogenic rescued cell lines

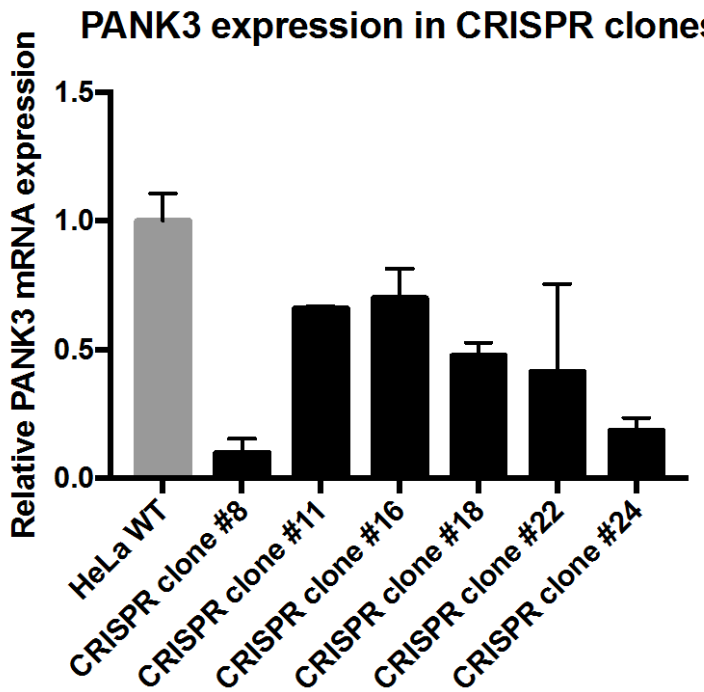


Expected Western Blot result



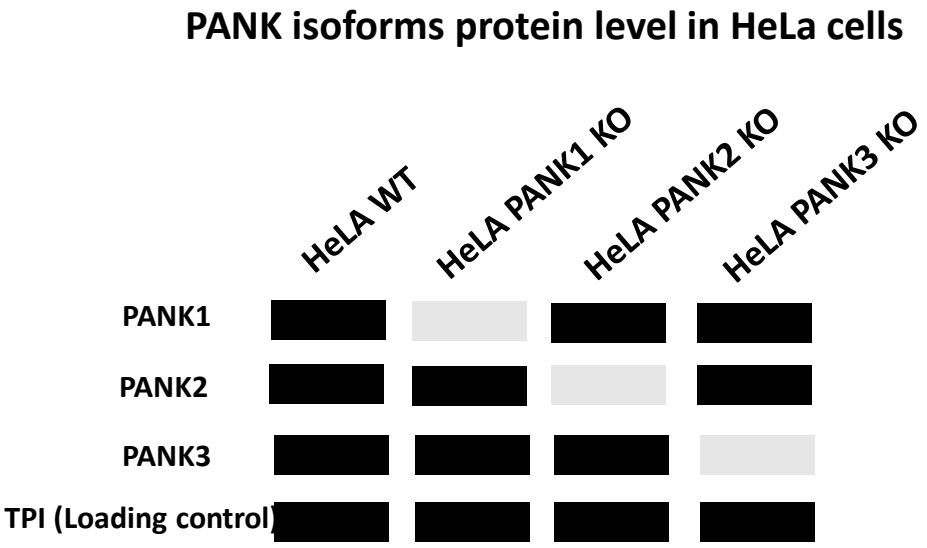
Generation of HeLa *PANK2* and *PANK3* CRISPR KO line

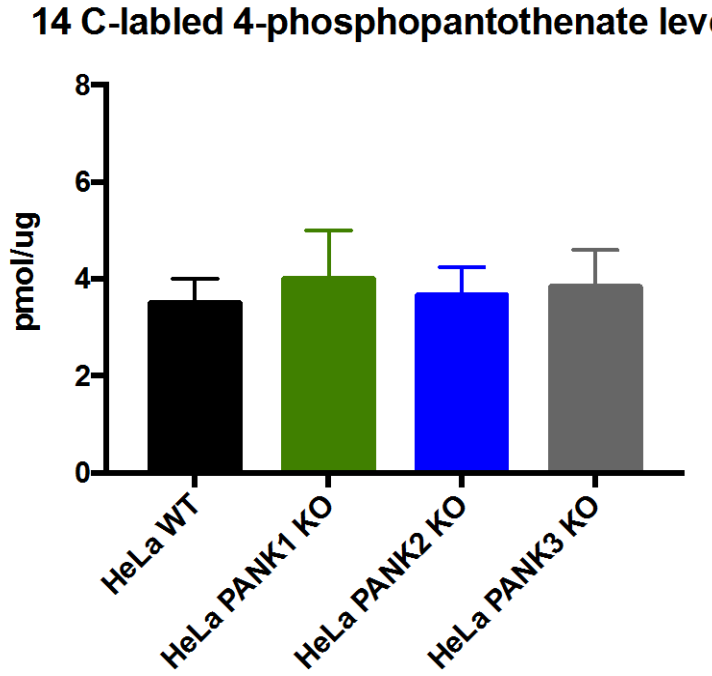


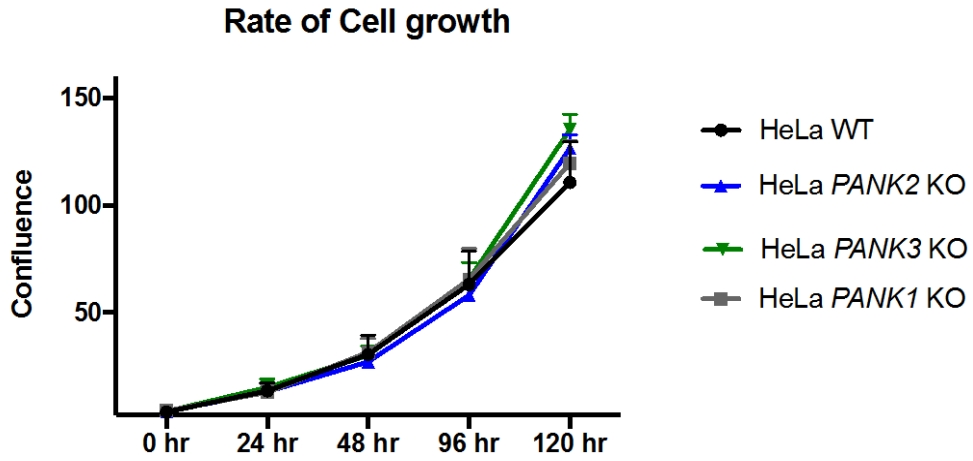


Anti-PANK2 and anti-PANK3 antibody development in progress

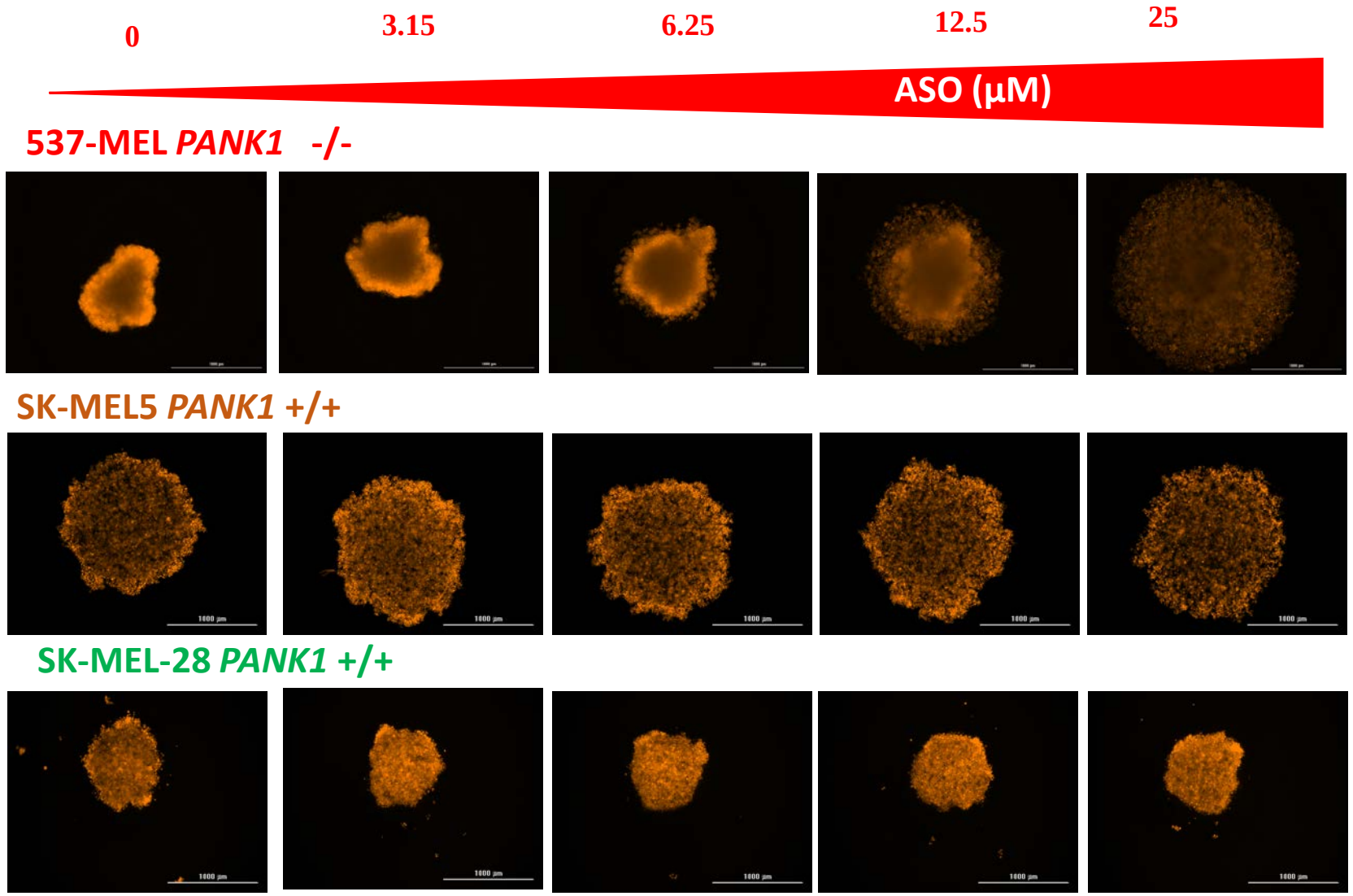
Expected outcome:
PANK isoforms are individually dispensable

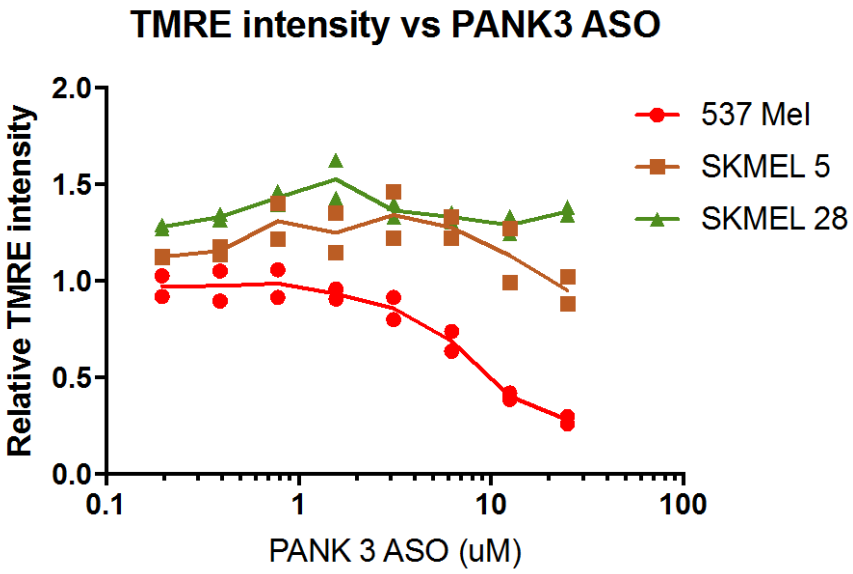






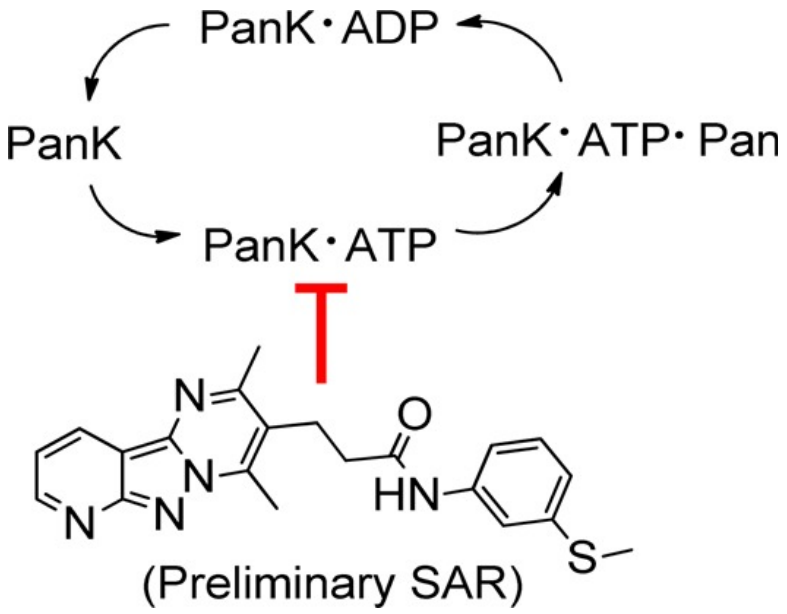
PANK1 homozygously deleted 537-MEL cells respond to *PANK3* inhibition

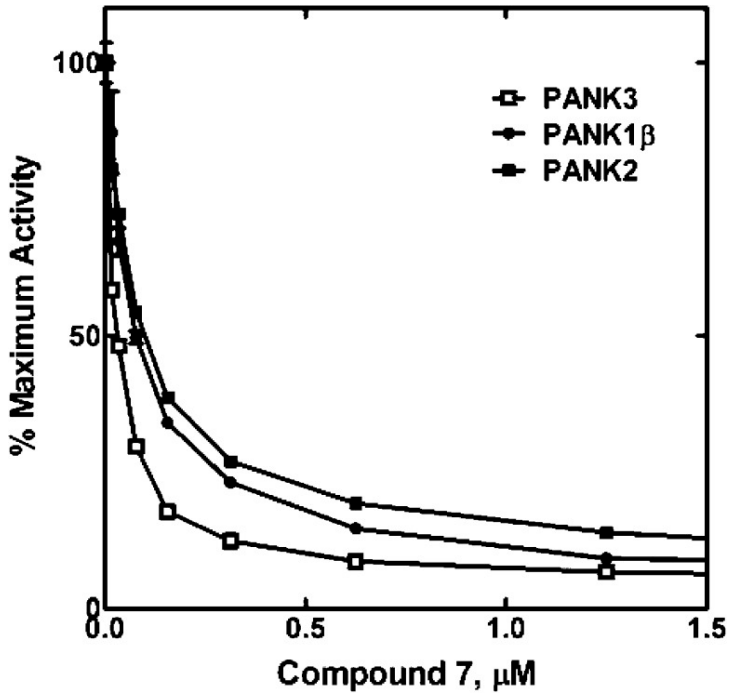




Day 4

Compound 7, a Pan-PANK inhibitor has modest selectivity for PANK3





PANK Isoforms	IC50
PANK1 β	70 ± 1.1 nM
PANK2	92 ± 2.0 nM
PANK3	**25 ± 1.8 nM**

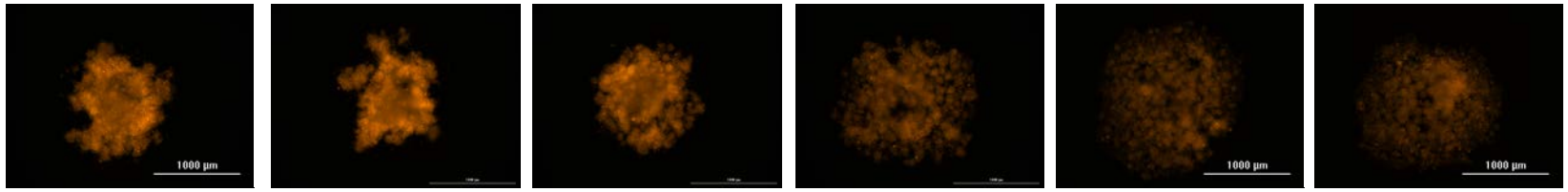
Sharma et *al* J Med Chem. 2015 Feb 12; 58(3): 1563–1568.

Compound 7 shows selective toxicity to PANK1 deleted melanoma tumor-spheres

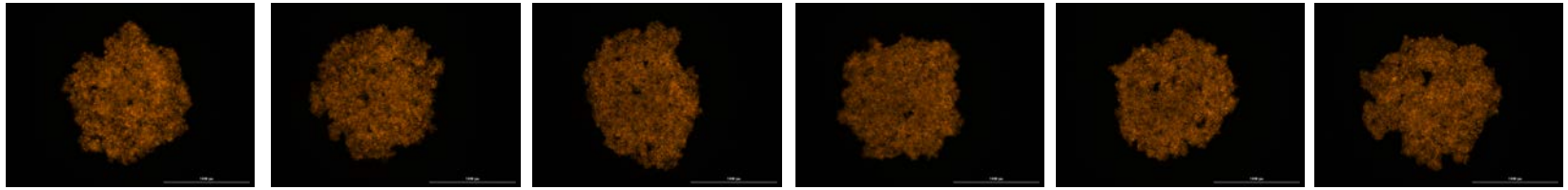
Compound 7 PANK inhibitor

0 uM | 3.12 uM | 6.25uM | 12.5 uM | 25uM | 50uM

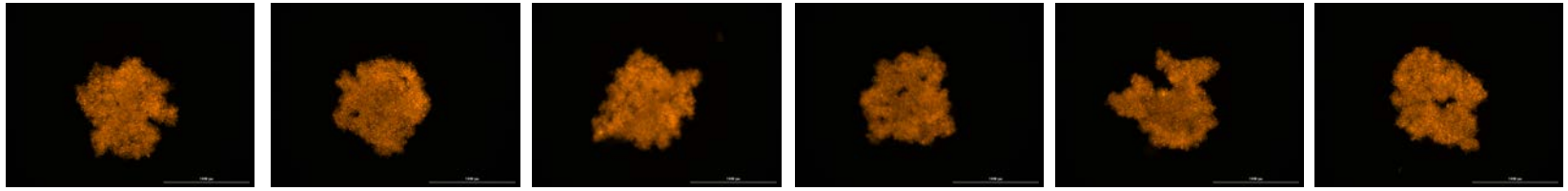
537-MEL *PANK1* -/-

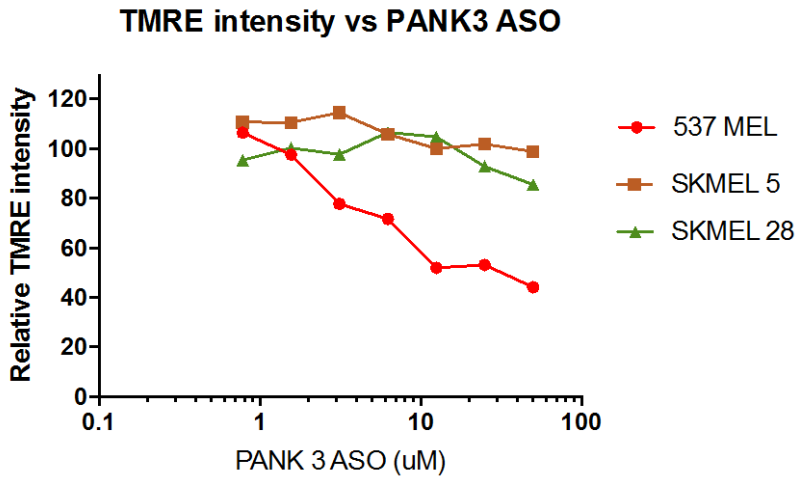


SK-MEL5 *PANK1* +/+



SK-MEL-28 *PANK1* +/+





Day 6

shRNA mediated knockdown of *PANK2* and *PANK3* in G59 cells

10 individual short hairpins

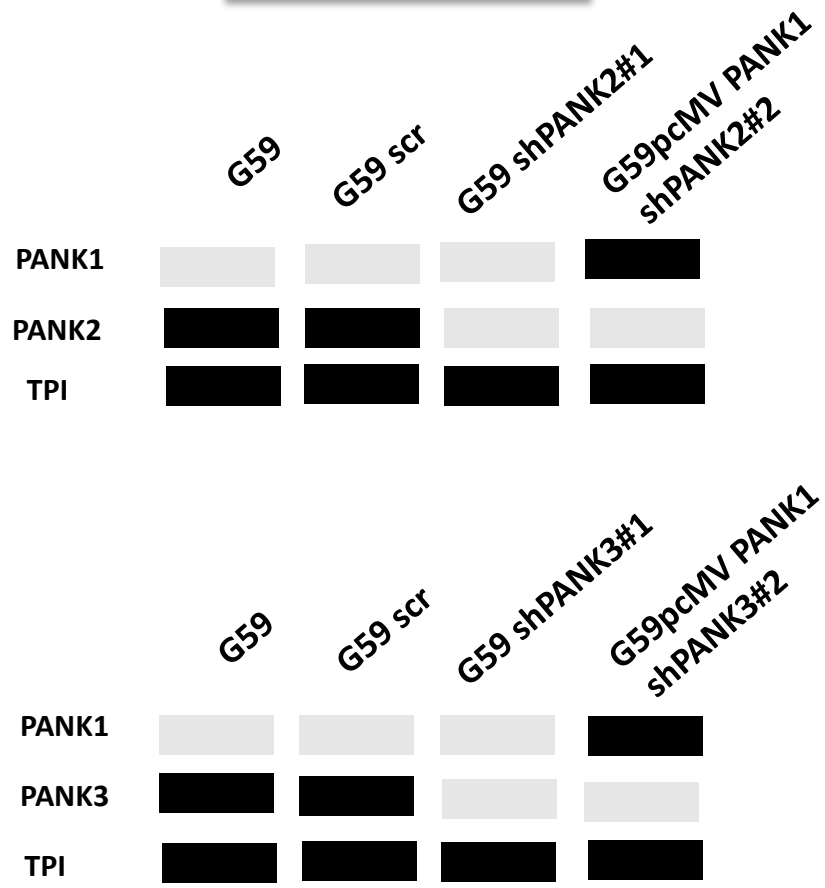
pLKo-shPANK 2/3

+

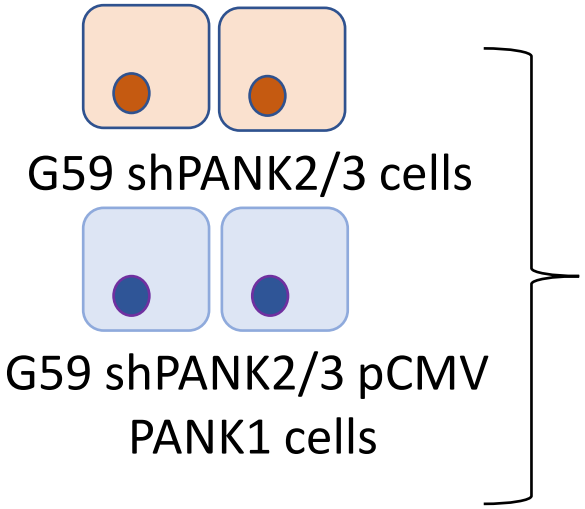
2nd generation lentiviral packaging system psPAX2 , pMD2.G

G59 and G59 PANK1 OE cells
(Puromycin selection)
Identify shRNA clones with KD>60%
To clone into inducible pTRIPZ vector

Expected Result



Examining the effect of loss of PANK activity on cancer cell viability

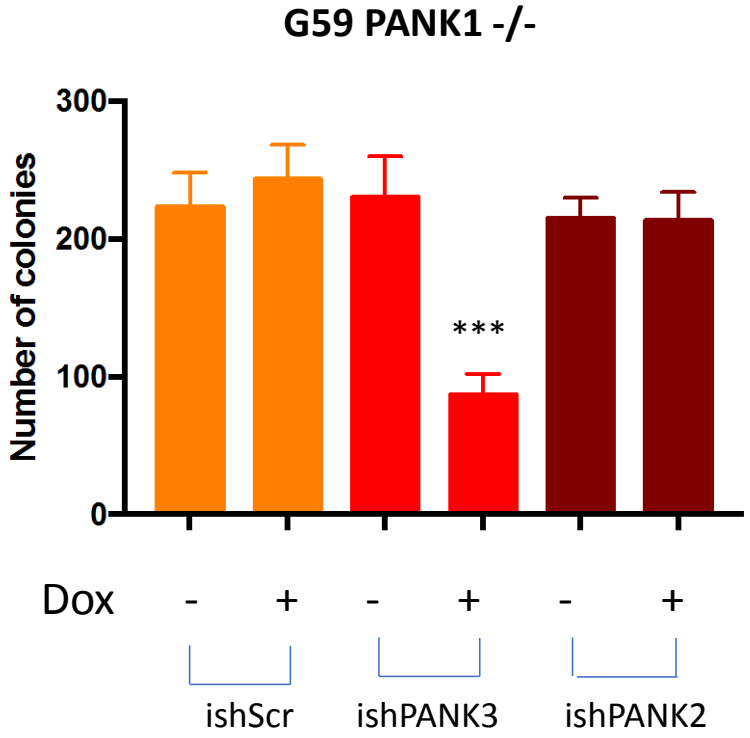


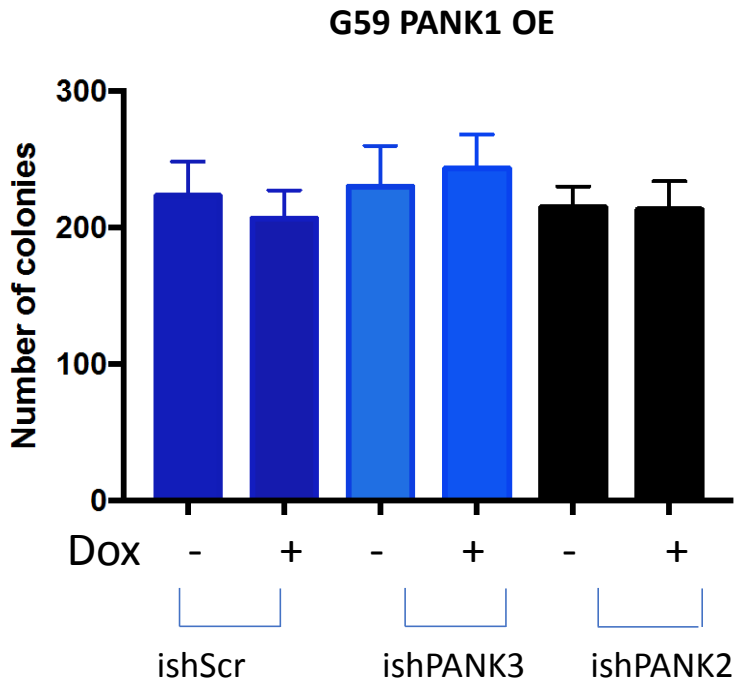
G59 shPANK2/3 cells

G59 shPANK2/3 pCMV PANK1 cells

- Cell viability Assay with Crystal violet staining/Cell-titre-glo assay
- Colony formation Assay
- Cell growth Analysis by IncuCyte
- FACS with annexin V-PE and 7-AAD to detect apoptosis

Expected Result: PANK3 inhibition will selectively impede PANK1 null tumor cell growth in vitro





- Slow rate of cell growth in G59 shPANK3
- Increased annexin V-PE and and reduced 7-AAD

AIM1- Pitfalls/Alternative Approach

- Individual isoform may be essential and CRISPR KO may not be feasible.
  - Recently released AVANA dependency map identifies PANK isoforms as non essential

- shRNA mediated knock down of PANK may lead to a complete cell death
  - Modulation of dox concentration to measure the effect of acute loss of PANK activity.

- If shRNA mediated knock down is not informative in identification of the redundant isoform
  - Generate inducible CRISPR KO of PANK isoform.

- If PANK activity is dispensable in cancer cells, it will contradict my hypothesis, but also suggest that cancer cells can survive without Co-enzyme A.
  - Unlikely, because all other downstream enzymes in the CoA biosynthesis pathway are essential based on the dependency score, suggesting that this pathway is essential.

Specific Aim 2

To determine the biochemical consequences of PANK ablation

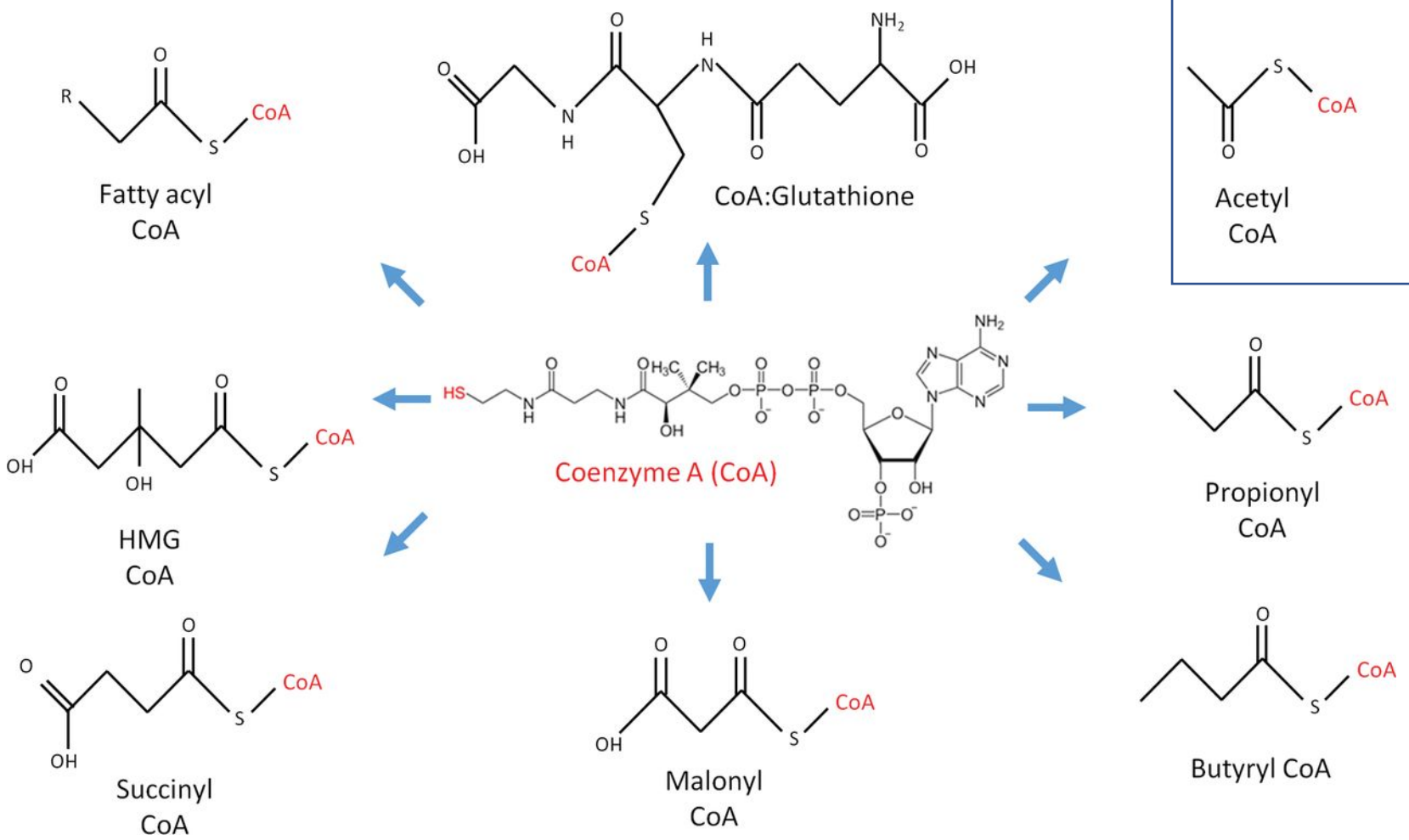
Hypothesis: I hypothesize that the loss of PANK activity will deplete CoA level from the cells which can impact critical metabolic and transcriptional profile of the cells.

Specific Aim 2

To determine the biochemical consequences of PANK ablation

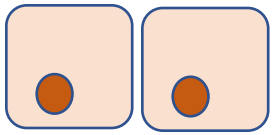
- Profile small molecule metabolites by metabolomics
- Determine the effects of protein acetylation
  - Identify key signaling pathways by RPPA
  - Transcriptomics following PANK ablation in PANK1 homozygously deleted and PANK1 intact cells.

Co-enzyme A plays critical role in a multitude of biochemical reactions

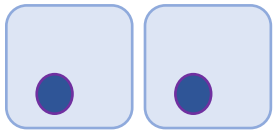


AcetylCoA is required for all protein acetylation reaction!

Metabolomics to identify key metabolites altered by co-enzyme A depletion



G59 shPANK3 cells



G59 PANK1 OE shPANK3

ther PANK1 intact Glioma Cells:
D423, D502, LN319

Extraction of polar metabolites with 80% methanol at -80 C

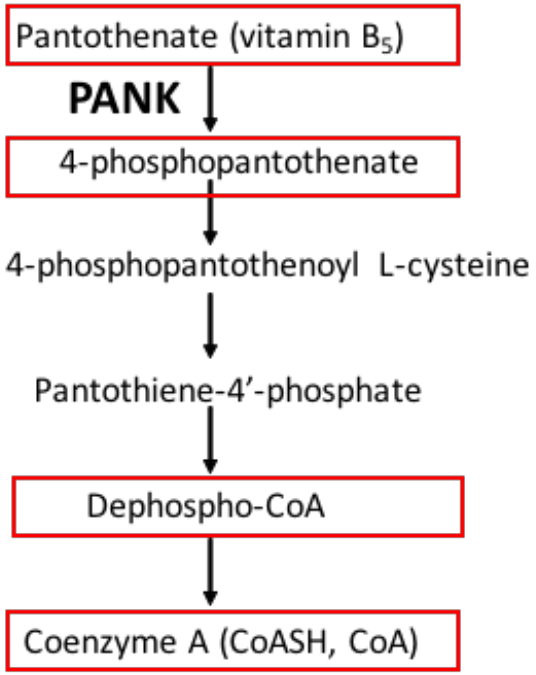
Mass spectrometry (LC/MS)
ASARA MS core at BIDMC

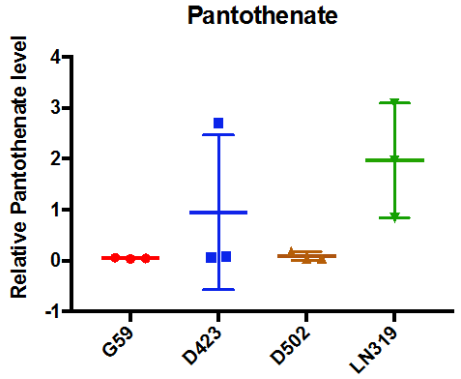
Analysis of metabolites

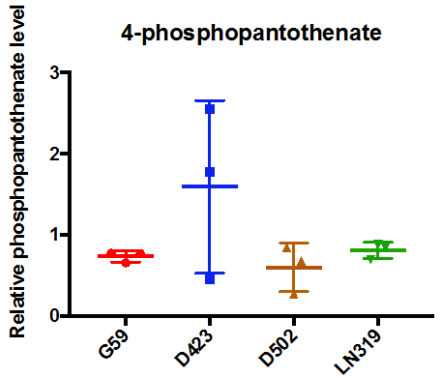
Expected Outcome:

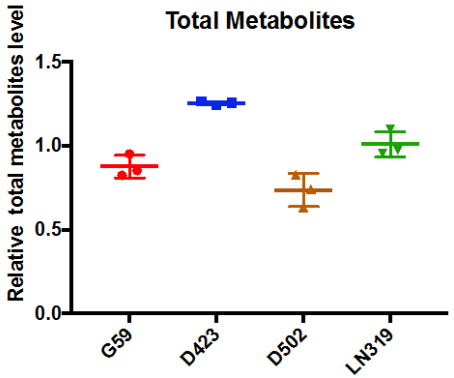
- Massive disruption of Fatty Acid Biosynthesis and oxidation pathway
- Decreased metabolites in the CoA synthesis pathway
- Decrease in OCR and increase in glycolytic intermediates, as well as ECAR

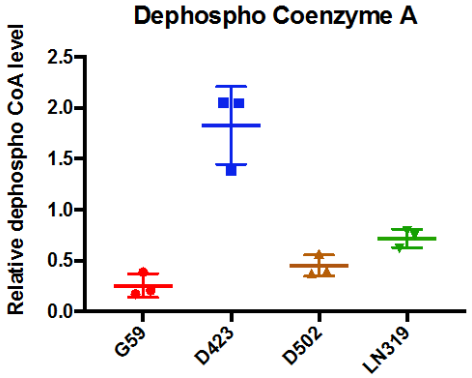
Status of metabolites in *PANK1* deleted G59 cells

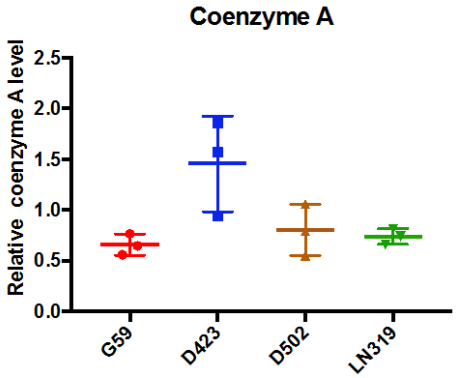




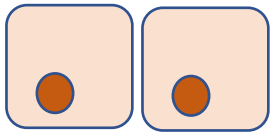




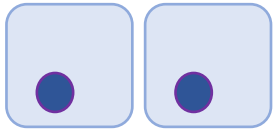




Assessment of global changes in protein acetylation and gene expression in G59 cells



G59 shPANK3 cells



G59 PANK1 OE shPANK3 cells
Other PANK1 intact Glioma Cells:
D423, LN319, D502

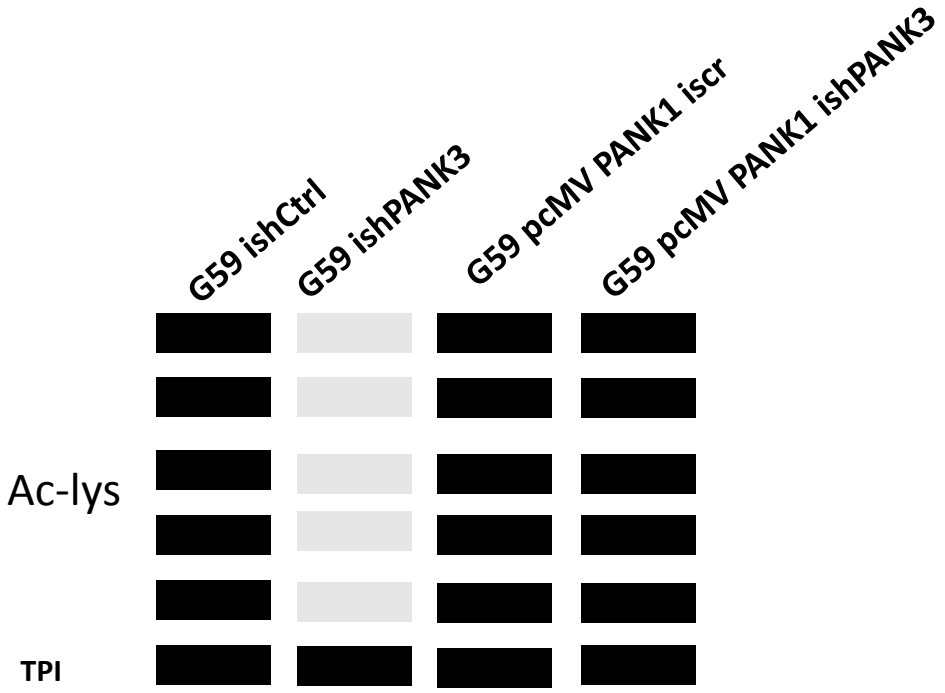
Global Acetylation levels detected by western blot

RNA extraction from the cells or tumor and submitted to the MD Anderson Core for an unbiased RNA seq analysis

Preparation of tumor/cell lysates in the RPPA buffer and samples submitted to the proteomics core at MD Anderson

Expected Outcome

A global decrease in protein acetylation



RNA seq and RPPA

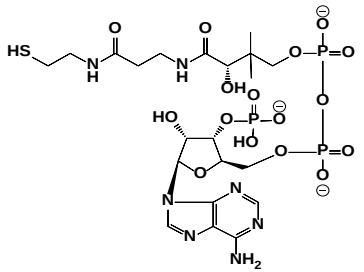
Expected changes

- Changes in protein stability and localization
- **Increase in p53/caspase mediated apoptosis**
- **Increased sensitivity to DNA damage**
- Disruption in protein synthesis machinery in ribosomes
- **Decreased structural integrity of tubulins**

AIM2- Pitfalls/Alternative Approach

- If there is no considerable effect in co-enzyme A levels or 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.
    a. CoA is highly charged and therefore cannot cross mitochondrial membrane



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 RPPA will be informative in addressing this possibility.

Specific Aim 3

To validate PANK paralog inhibition as targeted therapy for PANK1-homozygously deleted tumors in vivo

Hypothesis: *PANK3* inhibition will selectively kill intracranial *PANK1* deleted glioma tumors *in vivo* but not its isogenic rescued tumors .

Specific Aim 3

To validate PANK paralog inhibition as targeted therapy for PANK1-homozygously deleted tumors in vivo

- Generate xenografted tumors with *PANK1* homozygously deleted and intact cells in mice, with an inducible CRISPR or shRNA against PANK isoforms
- Test the efficacy of the PANK inhibitor in vivo
- Monitor tumor growth by IVIS and T2 MRI
- Identify target engagement markers with the metabolomics results and test the oligonucleotides for anti neoplastic activity

Orthotopic tumor implantation in nude mice

Intracranial injection

N=5

G59 shPANK3

N=5

G59- pCMV PANK1 shPANK3 (PANK1 rescued)

Week 1

Confirmation of tumor establishment by T2 weighed MRI and IVIS and doxycycline administration

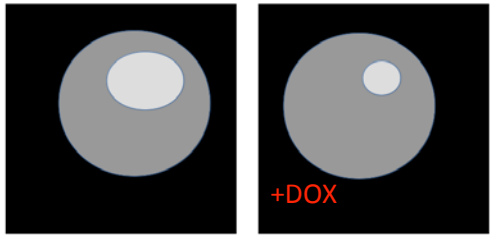
Weekly Tumor monitoring

Tumor regression: Follow mice for 2 months

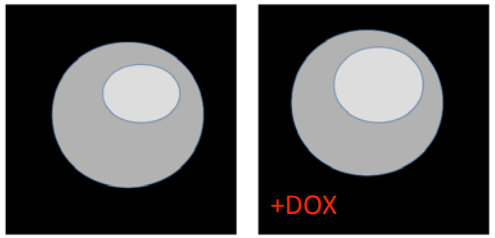
Euthanize at morbidity Post Mortem Metabolomics and Histopathological Analysis

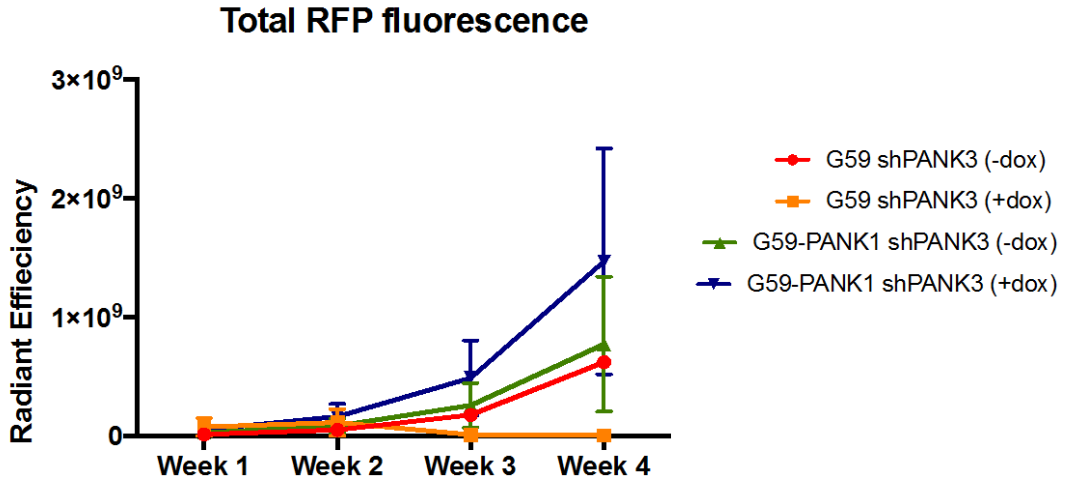
Expected Result: PANK3 inhibition leads to PANK1 null tumor regression.

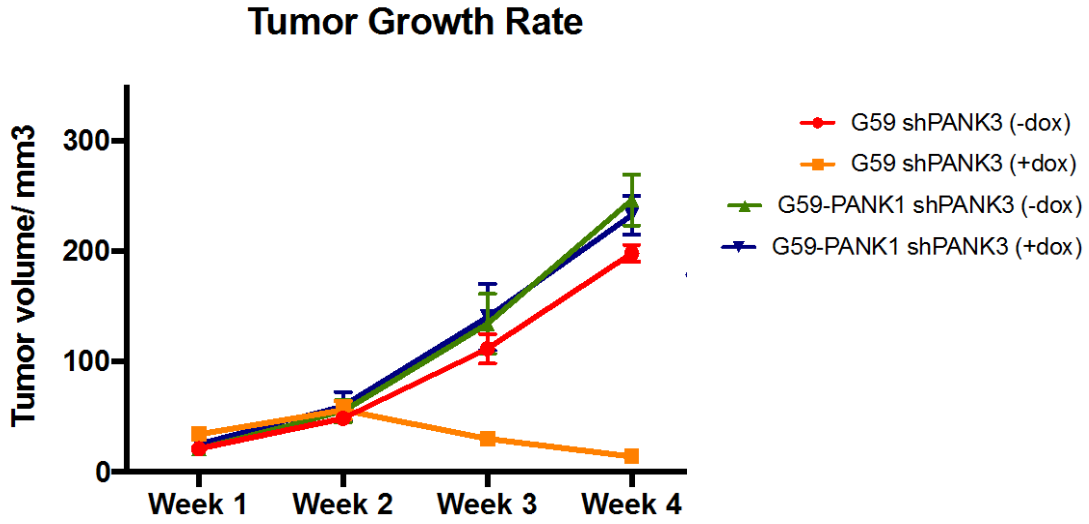
G59 shPANK3



G59- pCMV PANK1 shPANK3 (PANK1 rescued)







Histopathological Analysis: Expected Outcome

- Reduced Ki67+ in G59 shPANK3 (+dox) cells
- Increased Cleaved Caspase 3
- Reduction in phenotype characteristic of GBM tumors such as pseudopallisading cells, necrosis, hemorrhage

AIM3- Pitfalls/Alternative Approach

- pHAGE-CMV promoter might get methylated in mice, which can reduce PANK1 expression.
  - I will alleviate this problem by using alternative plasmids such as pWPXL or pWPT which contains the EF-1 alpha promoter, and can allow constitutive expression of the transgene.

- If there is a complete reduction of tumor growth, I will administer doxycycline water to mice at a lower dosage so that loss of *PANK2/3* will be acute.

- If no discernable differences are present between the *PANK1* deleted and *PANK1* reconstituted G59 cells due to a compromise in knock down efficiency by the shRNA,
  - I will intra-tumorally inject PANK2/3 ASOs alone or in combination with the pan PANK inhibitor 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.

Summary

- Hypothesis: *Targeting the redundant isoforms of PANK in tumors with PANK1 homozygous deletion can selectively kill cancer cells.*

  - AIM1: To determine the cell essentiality of PANK isoforms and identify the isoform redundant with PANK1 in cancer cells

  - AIM2: To determine the biochemical consequences of PANK elimination

  - AIM3: To validate PANK paralog inhibition as a targeted therapy in *PANK1* homozygously deleted tumors *in vivo*.