THE DICHOTOMOUS IMPACT OF VITAMIN B1 SUPPLEMENTATION ON MALIGNANT GROWTH

by

KELSEY HUNTER CONNELL JONUS

(Under the Direction of Jason A. Zastre)

ABSTRACT

As an essential micronutrient, vitamin B1, or thiamine, serves as an obligatory component for functional cellular metabolism. During cancer, the up-regulation of critical thiamine homeostasis genes demonstrates that tumor cells may exploit the vitamin for a malignant advantage. Preclinical evidence supports that supplemental thiamine in low-to-moderate amounts promotes tumor proliferation. In contrast, high-dose pharmacologic treatment with thiamine restricts tumor proliferation. The observed dichotomy for thiamine's impact on tumor growth suggests that thiamine and/or its derivatives may have multiple molecular impacts that are potentially unrelated to canonical metabolic cofactor function. These underlying molecular actions for thiamine in both promoting and inhibiting tumor growth remain uncharacterized. We have identified the adaptive regulation of the thiamine activating enzyme thiamine pyrophosphokinase-1 (TPK1) in response to malignant stress. TPK1 expression appears to contribute to tumor progression by maintaining production of thiamine's activated cofactor form thiamine pyrophosphate (TPP) during supplemental thiamine conditions. The molecular advantage for increasing thiamine conversion to TPP may be independent of TPP's cofactor activity. Instead, TPP

appears to serve as an intracellular antioxidant to counteract oxidative stress and confer a proliferative advantage. Alternatively, thiamine's antitumor properties at pharmacologic dosages have previously been associated with a shift in tumor cell metabolism due to activation of the mitochondrial enzyme pyruvate dehydrogenase (PDH). Increased PDH activity following high-dose thiamine therapy is presumably mediated through inhibition of pyruvate dehydrogenase kinases (PDKs), but the active thiamine species mediating this effect has not been confirmed. We have identified TPP as the thiamine moiety capable of inhibiting PDK function, supporting that TPP serves as the active species inhibiting PDK activity. Though a promising nutraceutical approach for cancer therapy, thiamine's low bioavailability may limit clinical effectiveness. Therefore, we have demonstrated that increasing thiamine bioavailability through exploiting commercially available lipophilic thiamine analogs increases thiamine's potency as an anticancer strategy. Similar to thiamine, its analogs increase intracellular TPP corresponding with decreased proliferation. Overall, we suggest that the dichotomous effects of supplemental thiamine on malignant growth are dependent on TPP maintenance, which may both promote and inhibit tumor cell proliferation.

INDEX WORDS: Cancer, Thiamine, Thiamine Pyrophosphokinase-1, Hypoxia, Hypoxia-Inducible Factor-1α, Reactive Oxygen Species, Oxidative Stress, Cell Metabolism, Pyruvate Dehydrogenase, Chemotherapy

THE DICHOTOMOUS IMPACT OF VITAMIN B1 SUPPLEMENTATION ON MALIGNANT GROWTH

by

KELSEY HUNTER CONNELL JONUS

Bachelor of Science, LaGrange College, 2014

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2018

THE DICHOTOMOUS IMPACT OF VITAMIN B1 SUPPLEMENTATION ON MALIGNANT GROWTH

by

KELSEY HUNTER CONNELL JONUS

Major Professor:	Jason A. Zastre
Committee:	Brian S. Cummings
	William N. Lanzilotta
	Jia-Sheng Wang

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
December 2018

DEDICATION

I dedicate this work to two inspiring teachers, Mrs. Kay Barnes and Mrs. Pepper Connell (Mom). Without each of your influences on my life I would be nowhere. Not only were you my teachers, you are my role models—extraordinary women who care deeply about the lives they impact on a daily basis. Regardless of where my life's journey takes me, my ultimate goal is to assimilate each of you in all that I do. If I can achieve this goal, I will be able to persevere any challenge and surpass any goal I may set for myself, all-the-while impacting countless lives along the way.

ACKNOWLEDGEMENTS

Without the countless support of so many, this achievement would not have been possible. This work is truly a belonging of each of you just as it is my own.

First and foremost, I am grateful for a heavenly Father who makes all things possible no matter how daunting or challenging they may seem.

To Dr. Jason Zastre, there are no words to describe my appreciation for you. Thank you for insisting the importance of a PhD, and more so, overcoming the challenge graduate school, when I wanted to settle and give up. As always, you were right- this experience will be invaluable going forward in life! The advice, guidance, support, and instruction you have bestowed on me will never be forgotten, and I will forever be grateful that our paths in life crossed.

To my advisory committee, thank you for the valuable time you've sacrificed out of your busy schedules to provide me with expertise and guidance for this work. Your collective efforts have made me a stronger scientist. To Joanne Mauro, you are incredible! Thank you for your support and always open-ear that helped me make it through graduate school.

To Dr. Kristy Zera, just as with Dr. Z, I will be forever grateful our paths in life crossed. Thank you for being a colleague, but also a dear friend. In hindsight, I wouldn't have wanted to share this experience with any other person.

To Julian, Shivani, and Maria, thank you for time we spent together. Graduate school would have been sad and lonely without friends like you! Good luck in each of your endeavors going forward, I can't wait to see where life takes you.

To Jasen Jonus, my very best friend that I'm privileged in sharing life with, to Clint and Pepper Connell, a dedicated father and loving mother who taught me these two attributes are the most important things in life, to Vicci Connell, my beautiful and selfless little sister who has supported me in every endeavor (sometimes by choice, other times by force), to JC and Mary Helen Connell, the grandparents who constantly remind me of their love, to Kirk and Jima Jonus, whom I blessed to call my parents-in-love, to Elnor Watkins, Jasen's grandmother who has cared and worried for me as her own, to Amanda Sanders (the Christina to my Meredith) and Dr. Ashley House (the Rory to my Paris) and last, but certainly not least Delilah and Bailey, the best pups a girl could ask for, THANK YOU. Without your support, love, and encouragement this would not have been possible. Thank you, especially Jasen, for putting up with me during the valleys and celebrating with me at the peaks of this journey. I'm so blessed to share this thing called life with each of you!

And finally, this work serves in remembrance of Jim and Jan Phillips and Ronnie Connell. I like to think that the three of you watch over me from above. I deeply believe that without Jim Phillips (Poppa Jim), this dissertation would not exist. He inspired a love of learning in an incredible woman (my mother), who passed that love along to me.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

ABBREVIATIONS

α-KG	α-Ketoglutarate
ρ^o	Mitochondrial Deficient
AA	Antimycin A
Akt	Protein Kinase B
AK1	Adenylate Kinase 1
ASC	Ascorbate
ATP	Adenosine Triphosphate
BNFO	Benfotiamine
CDDP	Cisplatin
CHX	Cycloheximide
CTL	Control
DCA	Dichloroacetate
DMOG	Dimethyloxalyglycine
DOX	Doxorubicin
EDTA	Ethylenediaminetetraacetic Acid
FBS	Fetal Bovine Serum
FHC	Fetal Human Colon Cell
GLUT1	Glucose Transporter 1
HBSS	Hank's Buffered Salt Solution

HIF-1α	Hypoxia-Inducible Factor-1α
HIF-1α CA	Hypoxia-Inducible Factor-1α Constitutive Active
HIF-2α	Hypoxia-Inducible Factor-2α
HMEC	Human Mammary Epithelial Cell
HPLC	High-performance Liquid Chromatography
HRE	Hypoxic Responsive Element
H_2O_2	Hydrogen Peroxide
IC50	Half Maximal Inhibitory Concentration
IP	Intraperitoneal
IR	Ionizing Radiation
JAK2	Janus Kinase 2
JNK	c-Jun N-terminal Kinase
LDHA	Lactate Dehydrogenase
MAPK	Mitogen-activated Protein Kinase
MDA	Malonialdehyde
miR	microRNA
mtDNA	Mitochondrial DNA
mTOR	Mammalian Target of Rapamycin
MTX	Methotrexate
N	Normoxia, 21% O_2
NAC	N-acetylcysteine
NADPH	Dihydronicotinamide-adenine Dinucleotide Phosphate
NCI	National Cancer Institute

NR	Not Reported
NRF2	Nuclear Factor Erythroid 2-Related Factor
NP40	Nonidet P40
OCT	Organic Cation Transporter
OGDC	α-Ketoglutarate Dehydrogenase Complex
OGDH	α-Ketoglutarate Dehydrogenase
O_2	Oxygen
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDC	Pyruvate Dehydrogenase Complex
PDH	Pyruvate Dehydrogenase
PDK	Pyruvate Dehydrogenase Kinase
PI3K	Phosphoinositide 3-Kinase
PMSF	Phenylmethane Sulfonyl Fluoride
PPP	Pentose Phosphate Pathway
PT	Pyrithiamine
RDA	Regular Daily Allowance, Humans
RDI	Regular Daily Intake, Mice
RFC1	Reduced Folate Carrier 1
ROS	Reactive Oxygen Species
SDS	Sodium Dodecyl Sulfate
SLBT	Sulbutiamine
SLC	Solute Carrier

T	Thiamine
TBHP	*Tert*-Butyl Hydroperoxide
TBP	Human TATA-binding Protein
TBS-T	Tris Buffered Saline-Tween 20
TCA	Tricarboxylic Acid Cycle
TDE	Thiamine Dependent Enzyme
TD 1640	Thiamine Deficient RPMI 1640
THTR1	Thiamine Transporter 1
THTR2	Thiamine Transporter 2
TKT	Transketolase
TKTL1	Transketolase-like 1 Protein
TKTL2	Transketolase-like 2 Protein
TMP	Thiamine Monophosphate
TMPase	Thiamine monophosphatase
TPC	Thiamine Pyrophosphate Carrier
TPK1	Thiamine Pyrophosphokinase-1
TPN	Total Parenteral Nutrition
TPP	Thiamine Pyrophosphate
TPPase	Thiamine Pyrophosphatase
TPPT	Thiamine Pyrophosphate Transporter
TRLX	Trolox
TTP	Thiamine Triphosphate
VEGF	Vascular Endothelial Growth Factor

WCL	Whole Cell Lysates
WE	Wernicke's Encephalopathy
4EBP1	Eukaryotic Translation Initiation Factor 4E Binding Protein 1
5-FU	5-Fluorouracil

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Vitamins are essential micronutrients required for the maintenance of all cells, but either cannot or can only partially be synthesized within the human body. Therefore, they necessitate consistent dietary intake in minimal amounts to maintain health and promote well-being. Although the Western diet contains ample vitamin sources, it has become common place for the general population to consume nutraceutical vitamin supplements in an attempt to prevent disease (Comerford 2013). Current estimates suggest that ~48% of adults in the United States consume some form of vitamin supplement (Manson & Bassuk 2018; Kantor *et al.* 2016). However, less than a quarter of all supplement products are used with the recommendation of a physician (Bailey *et al.* 2013). Contrary to popular belief, routine micronutrient supplementation among the general population is not medically advised for the prevention of chronic diseases such as cancer (Manson & Bassuk 2018). While some beneficial effects have been concluded for vitamin supplementation against the occurrence and progression of cancer, data obtained from randomized clinical trials also demonstrate a lack of evidence for vitamin supplementation in cancer prevention (Mamede *et al.* 2011; Schwingshackl *et al.* 2017). Furthermore, clinical results suggest that vitamin supplementation exceeding the recommended dietary allowance (RDA) may increase cancer-related mortality (Bjelakovic *et al.* 2007; Omenn *et al.* 1996; Schwingshackl *et al.* 2017). Recent pre-

clinical findings support that supplemental vitamin E enhances malignant progression (Le Gal *et al.* 2015). Vitamin B1, or thiamine, has also been demonstrated to directly influence *in vivo* tumor growth (Comin-Anduix *et al.* 2001; Daily *et al.* 2012). As demonstrated in **Fig 1.1**, thiamine presents a unique paradigm as it may possess the potential to be both tumor-promoting and tumor–inhibiting. At low-to-moderate dosages above the RDA, supplemental thiamine increases *in vivo* tumor growth (Comin-Anduix *et al.* 2001). While extremely high pharmacologic amounts of thiamine inhibit tumor proliferation (Comin-Anduix *et al.* 2001). Elucidating mechanisms for how thiamine exerts its dichotomous effects on tumor growth will provide further insight into the potential benefits and drawbacks of excessive thiamine consumption by cancer patients. These findings are necessary for the proper medical advisement of the patient population in regard to the safety of vitamin supplementation during malignancy.

1.2 Cancer: a disease of deregulated cellular proliferation

The National Cancer Institute (NCI) estimates that nearly 40% of men and women in the United States will be diagnosed with cancer at some point during their lifetime. This staggering statistic illustrates the prevalence of a disease predicted to claim the lives of roughly 600,000 Americans in 2018 alone. Cancer is defined by the NCI as a term for diseases in which abnormal cells proliferate without abandon and invade nearby tissues. Driving their elevated proliferation rate, tumor cells rapidly produce energy in the form of ATP, while simultaneously synthesizing biomass and maintaining a delicate redox balance (Cairns *et al.* 2011).

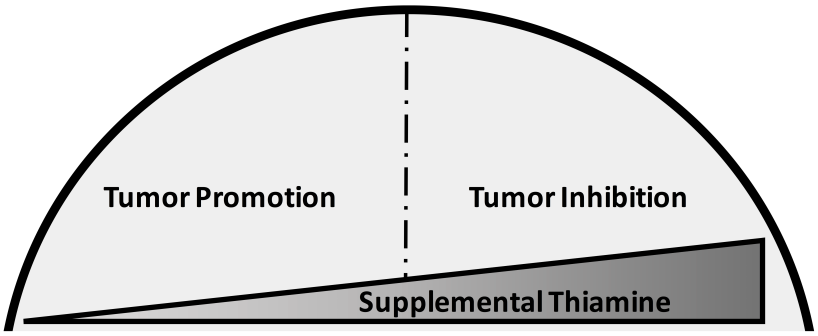


Fig 1.1. Schematic diagram depicting dose-dependent, biphasic effect of thiamine supplementation on tumor cell proliferation Pre-clinical evidence demonstrates dual effects of supplemental thiamine on tumor growth. At low doses above the RDA for thiamine, supplemental amounts of the vitamin demonstrate tumor promotion. Alternatively, pharmacologic supplementation with thiamine demonstrates inhibitive properties toward malignant growth.

1.2.1 Oncogenic-driven metabolism facilitates ATP production in cancer cells

A signature "Warburgian" metabolic phenotype fuels the elevated proliferation rate of tumor cells. Otto Warburg first identified that ascites tumor cells consume glucose at an elevated rate compared to non-proliferative, non-cancerous cells (Koppenol *et al.* 2011). Subsequently, tumor cells convert the glycolytic end product pyruvate into lactate in place of using pyruvate as fuel for mitochondrial oxidative metabolism **(Fig 1.2)** (Warburg *et al.* 1927). Though glycolysis yields significantly less ATP than oxidative phosphorylation, the rate at which cancer cells can maintain glycolytic flux makes it an efficient pathway for their energy requirements (Vander Heiden *et al.* 2009).

In non-cancerous cells, growth-factor signaling tightly regulates glucose uptake (Vander Heiden *et al.* 2009). Growth-factor stimulation results in the activation of the highly conserved, widely expressed PI3K/Akt/mTOR pathway. This activation enhances nutrient acquisition (i.e. glucose, amino acids) through the up-regulation of cell-surface transporter expression (DeBerardinis *et al.* 2008). Mutations within the PI3K/Akt/mTOR pathway are among the most prevalent alterations that occur following oncogenic transformation and result in the pathway's constitutive activation in human tumors (DeBerardinis *et al.* 2008). In so, tumor cells demonstrate enhanced glucose import as a function of aberrant activation of the oncogenic PI3K/Akt/mTOR pathway. This activation directly promotes glucose transporter (GLUT1) mRNA and protein expression, as well as cell surface localization (DeBerardinis *et al.* 2008; Barthel *et al.* 1999; Rathmell *et al.* 2003). Other oncogenes, including Ras and Src, also contribute to enhanced glucose transport during malignancy by transcriptionally driving glucose transporter expression (Macheda *et al.* 2005). Activation of the PI3K/Akt/mTOR

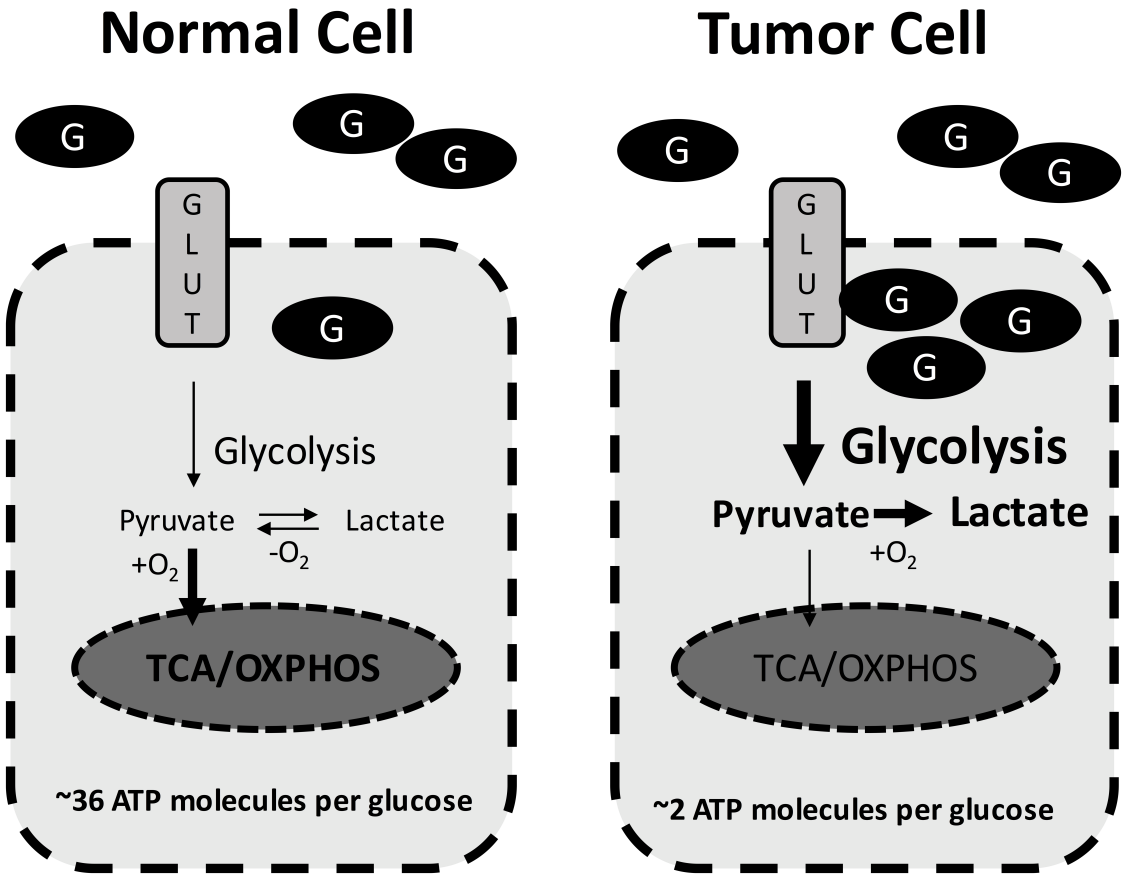


Fig 1.2. Schematic diagram depicting normal and tumor cell metabolism Compared with non-cancerous cells, tumor cells demonstrate oncogenic-driven accumulation of glucose. They subsequently rely on glycolysis as the predominant pathway for energy production, whereas normal cells generate ATP through mitochondrial dependent oxidative phosphorylation.

pathway in cancer also promotes the activity of key glycolytic enzymes including hexokinase (HK) and phosphofructokinase 1 (PFK1) (Rathmell *et al.* 2003; Ward & Thompson 2012). Together, oncogenically driven up-regulation of glucose transport and activation of glycolytic enzymes supports elevated rates of glycolytic flux within tumor cells.

Concurrent with oncogene activation, the reduced function of critical tumor suppressor proteins also contributes to the Warburgian metabolic signature of cancer cells (Vousden & Ryan 2009). Of the most well-described tumor suppressors, p53 serves as a transcriptional regulator orchestrating cellular responses to stress (Laptenko & Prives 2006). Loss-of function mutations within the p53 gene (*TP53*) occur in 5-50% of all human cancers depending on subtype and are linked with poor patient prognosis (Olivier *et al.* 2010; Kandoth *et al.* 2013). Following significant intracellular damage, activation of wild-type p53 can induce cell cycle arrest and cell death (Laptenko & Prives 2006). Therefore, the p53 signaling axis may protect against malignant transformation by eliminating cells that demonstrate genomic instability due to DNA damage (Kastenhuber & Lowe 2017). Alternatively, wild-type p53 has been suggested to halt oncognically-driven cell cycle progression, and therefore the absence of its activity may encourage tumor growth (Kastenhuber & Lowe 2017). In addition to its well defined roles in regulating cell cycle progression and DNA damage responses, p53 contributes to a multitude of other cellular functions including migration and invasion, redox homeostasis, autophagy, inflammation, and metabolism (Kastenhuber & Lowe 2017). In regard to the latter, several mechanisms have been described to explain how the loss of wild-type p53 expression promotes a glycolytic phenotype in tumor cells (Vousden &

Ryan 2009). Wild-type p53 directly represses GLUT1 expression, and mutations common to malignancy within the p53 DNA-binding domain impair this inhibition to promote tumor growth (Schwartzenberg-Bar-Yoseph *et al.* 2004). In addition to limiting glucose uptake, wild-type p53 signaling impedes glycolytic flux by reducing the expression of phosphoglycerate mutase (PGM) and increasing the expression of TP53 induced glycolysis and apoptosis regulator (TIGAR) (Vousden & Ryan 2009; Bensaad *et al.* 2006). In parallel to restricting glycolysis, wild-type p53 activity promotes mitochondrial respiration by activating the cytochrome *c* oxidase complex and maintaining the production of mitochondrial DNA (Okamura *et al.* 1999; Matoba *et al.* 2006; Bourdon *et al.* 2007). Based on its metabolic functions, p53 has been suggested to maintain aerobic respiration in normal cells, and loss of p53 function during cancer appears to support the signature metabolic shift associated with malignancy (Vousden & Ryan 2009).

1.2.2 Tumor hypoxia further drives glycolytic signature in tumor cells

As a solid tumor rapidly develops, limited vascularization deprives the delivery of oxygen (O_2) to the innermost cells resulting in hypoxic tumor microenvironments (Hockel & Vaupel 2001). Tumor hypoxia is associated with enhanced tumor aggressiveness and increased resistance to chemotherapeutic intervention and radiation therapy (Subarsky & Hill 2003; Muz *et al.* 2015). Stabilization of the oncogenic transcription factor hypoxia-inducible factor-1α (HIF-1α) in low O_2 environments facilitates adaptive responses of tumor cells to hypoxic conditions and supports survival (Semenza 2010). Recently, it has become increasingly accepted that HIF-1α accumulates

within tumor cells in response to events other than the classically defined hypoxic condition. These events include: build-up of glycolytic intermediates (coined "pseudohypoxia"); high cell density conferring pericellular hypoxia; and activation of other oncogenic signaling cascades such as the PI3K/Akt/mTOR pathway (Lu *et al.* 2002; Sheta *et al.* 2001; Zhong *et al.* 2000). Together these factors may converge to confer a more global HIF-1α signature in solid tumors than what has been previously appreciated.

Among its numerous downstream effects, HIF-1α signaling promotes a further enhancement of glycolytic metabolism in tumor cells (Majmundar *et al.* 2010). HIF-1α activation transcriptionally increases expression of GLUT1 as well as multiple glycolytic enzymes (Iyer *et al.* 1998). In addition to increasing necessary gene expression for glycolytic flux, HIF-1α activation also directly inhibits mitochondrial dependent metabolism through the upregulation of pyruvate dehydrogenase kinases (PDKs) (Kim *et al.* 2006). HIF-dependent enhancement of PDK expression results in increased phosphorylation of pyruvate dehydrogenase (PDH) (Kim *et al.* 2006). Phosphorylation of PDH subsequently impedes the conversion pyruvate into acetyl-CoA, which is the initial substrate of the tricarboxylic (TCA) cycle (Saunier *et al.* 2016). Therefore, inhibition of PDH by PDK following HIF-1α activation promotes an enhanced reliance on glycolysis for ATP production (Saunier *et al.* 2016). Silencing PDK activity, restores mitochondrial-dependent metabolism resulting in a reduction of tumor cell proliferation (Shen *et al.* 2013; Bonnet *et al.* 2007; McFate *et al.* 2008).

1.2.3 Warburgian metabolism also supports anabolic capacity during malignancy

In addition to rapid ATP production, the uncoupling of glycolysis and oxidative phosphorylation in tumor cell metabolism promotes the shunting of glycolytic intermediates into branching anabolic pathways to generate the necessary biosynthetic precursors required for replication (Thompson 2011). This supports anabolic growth during nutrient-replete conditions, but also allows glucose catabolism to support cell survival when nutrients are scarce (Boroughs & DeBerardinis 2015). One of the predominant metabolic shunts stemming from glycolysis is the pentose phosphate pathway (PPP), also known as the hexose monophosphate shunt or the phosphogluconate pathway (Patra & Hay 2014). Glycolytic-derived glucose-6-phosphate (G6P) can be directed into the oxidative-branch of the PPP through the activity of G6P dehydrogenase (G6PDH) (Riganti *et al.* 2012). Multiple oncogenic signaling cascades converge to accelerate flux through the PPP mediated by positive regulation of G6PDH activity (Patra & Hay 2014). Through the remaining irreversible reactions carried out in the oxidative PPP, NADPH and ribulose-5-phosphate (Ru5P) are formed (Riganti *et al.* 2012). Ribose-5-phosphate isomerase subsequently converts Ru5P to ribose-5-phosphate (R5P), the biosynthetic precursor required for nucleotide synthesis (Riganti *et al.* 2012). In the second branch of the PPP (non-oxidative), reversible reactions mediated by the enzymes transketolase (TKT) and transaldolase (TALD) catalyze 2 and 3 unit transfers between carbon intermediates (Riganti *et al.* 2012). During conditions of oxidative stress, this flux can be used to recycle G6P from surplus R5P and replenish the oxidative branch of the PPP to produce additional NADPH necessary for glutathione synthesis (Patra & Hay 2014). Alternatively to support rapid proliferation, the non-oxidative PPP can flow in the

opposite direction forming R5P from excess glycolytic intermediates including fructose-6-phosphate (F6P) and glyceraldehyde-3-phosphate (G3P) (Patra & Hay 2014). It is estimated that in rapidly proliferating tumor cells, 80% of ribonucleotides are derived from the activity of the non-oxidative PPP (Boros *et al.* 1997).

Warburg and his colleagues hypothesized that tumor cells preferentially exploit glycolysis because they possess damaged mitochondrial respiration. Indeed, several studies demonstrate a high rate of mutation for mitochondrial DNA (mtDNA) in multiple cancer types including renal adenocarcinoma, neuroblastoma, and oncocytoma in addition to cancers of the head and neck, astrocytes, thyroid, breast, ovary, colon, prostate, and bladder (Wallace 2012). However, emerging evidence demonstrates that an active TCA cycle within functional mitochondria promotes cancer cell proliferation (Magda *et al.* 2008; Ahn & Metallo 2015). This is evidenced by the finding that mitochondrial deficient (ρ^o) tumor cells demonstrate reduced growth rates *in vitro* and reduced tumor formation *in vivo* (Magda *et al.* 2008). In light of recent findings like this, a paradigm shift in thought has taken place regarding the importance of mitochondria in tumor cells. Now mitochondrial mutations are not considered to inhibit mitochondrial metabolism, but instead force a shift in their bioenergetic and biosynthetic properties (Wallace 2012). Instead of being the sole "power house" of tumor cells, mitochondria act as biosynthetic factories fueling the production of necessary precursors required for the biosynthesis of lipids, amino acids, glutathione, and nucleotides (Ahn & Metallo 2015). In example, acetyl-CoA (from pyruvate) is converted to citrate within the mitochondria. Citrate can be subsequently transported out of the mitochondria and converted back to acetyl-CoA through the activity of ATP citrate lyase. This series of reactions supplies

cytosolic acetyl-CoA to be used in lipogenesis for the eventual production of fatty acids. Oncogenic stimulation (i.e. Kras, Akt) drives cancer cells to primarily use glucose-derived pyruvate to fuel lipid biosynthesis (Hatzivassiliou *et al.* 2005; Ahn & Metallo 2015). Exceptions to this finding occur in specific cases such as hypoxia, where PDK stimulation inhibits PDH conversion of pyruvate to acetyl-CoA. In this situation, tumor cells are able to maintain glucose-mediated biosynthesis through alternative pathways (Ahn & Metallo 2015).

1.2.4 Maintenance of delicate redox balance contributes to tumor cell proliferation

Tumor cells must maintain a delicate redox balance to promote a proliferative state **(Fig 1.3)**. Reactive oxygen species (ROS) are formed from the intracellular decomposition of molecular oxygen (O_2) and can either be free radical molecules including the hydroxyl radical ($\bullet OH$) and superoxide anion ($O_2^{\bullet -}$) or non-radical molecules like hydrogen peroxide (H_2O_2) (Liou & Storz 2010). Enhanced levels of ROS have been detected in almost all cancers (Liou & Storz 2010). Supporting this finding, *in vitro* experiments detailing neoplastic transformation demonstrate enhanced intracellular levels of ROS in transformed cells compared with their non- cancerous counterparts (Trachootham *et al.* 2006). Sources of ROS during malignancy include both exogenous and endogenous factors (Galadari *et al.* 2017). Endogenously, enhanced metabolic activity, mitochondrial dysfunction, activated immune responses, and increased activity of ROS-producing enzymes (i.e. oxidases, cyclooxygenases, lipoxygenases, thymidine phosphorylase) result in the intracellular accumulation of ROS (Liou & Storz 2010). Exogenous factors that can induce ROS in tumor cells include tumor hypoxia as well as

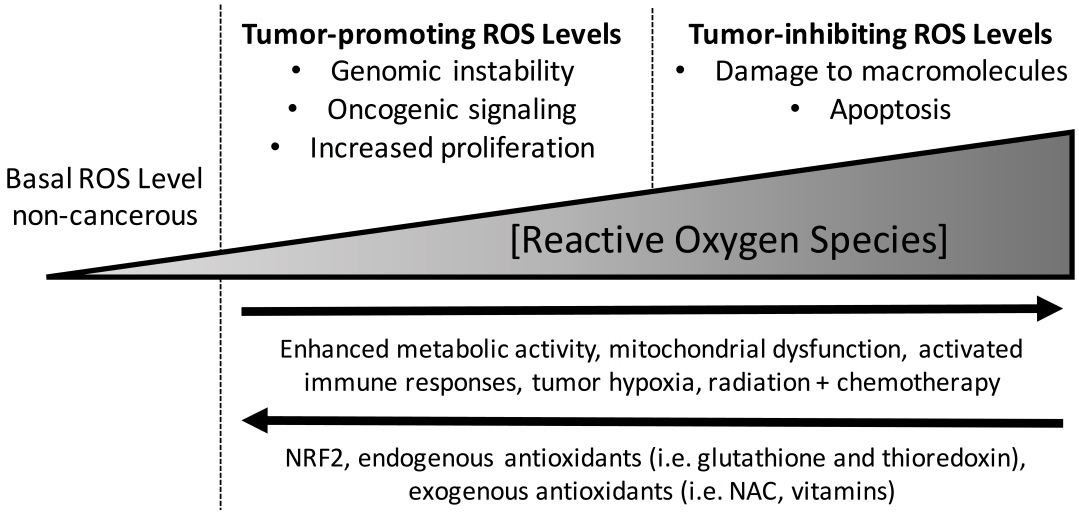


Fig 1.3. Schematic diagram depicting impact of ROS on tumor cell proliferation Compared with non-transformed cells, malignant cells demonstrate elevated levels of reactive oxygen species (ROS). Exogenous and endogenous factors contribute to both the generation and inhibition of ROS. The delicate intracellular concentration of ROS that is maintained by these factors directly impacts tumor cell proliferation. Moderate increases in ROS promote tumor cell proliferation, whereas ROS overload can inhibit tumor growth. Adapted from (Liou & Storz 2010).

therapeutic intervention with radiation and chemotherapeutics (Gorrini *et al.* 2013; Galadari *et al.* 2017).

The level of intracellular ROS directly associates with the proliferation rate of tumor cells. Modest increases in ROS are pro-proliferative evidenced by the treatment of tumor cells with low concentrations of H_2O_2 increasing intracellular ROS and promoting tumor cell proliferation (Wang *et al.* 2013; Kim *et al.* 2012). The pro-proliferative effects of ROS may be accounted for by their roles as signaling molecules and critical secondary messengers in oncogenic signaling cascades (Gorrini *et al.* 2013). H_2O_2 is produced in all mammalian cells as a signaling molecule mediating proliferation, differentiation, and migration (Rhee 2006). Therefore, the cell signaling effects of H_2O_2 may be directly exploited by tumor cells for enhanced proliferation (Rhee 2006). ROS have been demonstrated to inactivate PI3K/Akt phosphatases including phosphatase and tensin homolog (PTEN) and protein tyrosine phosphatase 1B (PTP1B) (Salmeen *et al.* 2003; Kwon *et al.* 2004). This inactivation impedes negative regulation of the PI3K/Akt pathway and therefore may activate proliferative signaling mediated by the PI3K/Akt axis (Galadari *et al.* 2017). The MAPK pathway is a second oncogenic signaling cascade critical for tumor cell proliferation. ROS have been shown to activate MAPK signaling through the inhibition of MAP kinase phosphatase (Seth & Rudolph 2006).

Contrary to the pro-proliferative effects observed for modest ROS increases, excessive ROS are toxic to tumor cells as they cause damage to DNA, proteins, and lipids resulting in cellular senescence and apoptosis (Liou & Storz 2010). Therefore, tumor cells have evolved a sophisticated antioxidant response comprised of endogenous and exogenous elements to minimize ROS-mediated toxicity (Gorrini *et al.* 2013). A key

effector in this response is nuclear factor erythroid 2-related factor (NRF2). As a transcription factor, NRF2 coordinates the expression of genes in several different antioxidant pathways critical for the detoxification of ROS including glutathione and thioredoxin maintenance as well as NADPH production (Gorrini *et al.* 2013). The oncogenes Kras and MYC have been demonstrated in the oncogenic accumulation of NRF2 in tumor cells and subsequent activation of the NRF2-mediated antioxidant response (DeNicola *et al.* 2011). In addition to oncogenic driven expression, mutations in NRF2 and its associated regulatory proteins resulting in its constitutive expression and nuclear localization have been identified in patient tumor samples during malignancy (Gorrini *et al.* 2013; Kim *et al.* 2010; Shibata *et al.* 2008). Silencing of NRF2 directly impedes tumor cell proliferation in association with deregulation of intracellular antioxidant molecules (Homma *et al.* 2009).

Based on the evidence established for the importance of endogenous antioxidant pathways in tumor cell proliferation, recent work has questioned whether exogenous antioxidants may also impact tumor growth. Sayin *et al.* demonstrated that dietary supplementation with the functionally unrelated antioxidants *N*-acetylcysteine (NAC) and vitamin E increases tumor progression and reduces survival *in vivo* (Sayin *et al.* 2014). Supplementation with NAC and vitamin E also directly enhances tumor cell proliferation, while reducing intracellular ROS levels *in vitro* (Sayin *et al.* 2014; Diao *et al.* 2016).

1.3 The role of thiamine in enhancing malignant growth

Thiamine is an essential micronutrient required for functional cellular metabolism. The activated form of thiamine, thiamine pyrophosphate (TPP), provides a

necessary cofactor for multiple enzymes within the metabolic network. In addition to its canonical role in cellular metabolism, thiamine may serve in other non-canonical capacities related to cell signaling and redox homeostasis. As described above, low-to-moderate supplemental doses of thiamine appear to enhance malignancy but it remains unclear mechanistically how these effects are exerted.

1.3.1 Pre-clinical data

To demonstrate the impact of dietary thiamine levels on the development and growth of mammary tumors, Daily *et al.* utilized the genetically predisposed MMTV^{neu} mouse model of spontaneous tumor development. In this model, consumption of lower thiamine levels (2 mg per 4,057 kcal) in conjunction with a normal fat intake, defined as 10% of total calories from fat, resulted in a significant increase of tumor latency time to an average of 295 days compared with an average of 225 days under normal thiamine (6 mg per 4,057 kcal) conditions (Daily *et al.* 2012). In addition to speeding the development of tumors, consuming a higher level of thiamine decreased overall percent survival time of mice (Daily *et al.* 2012). All protective effects of minimizing dietary thiamine were abolished by a high fat diet where 60% of total calories where derived from fat (Daily *et al.* 2012). In an alternative model, subcutaneous implants of MDA-MB-231 tumor cells demonstrated growth inhibition when mice were fed thiamine-free chow compared with normal chow (Liu *et al.* 2010). These findings support that reduced thiamine supply restricts tumor growth. In addition to the *in vivo* results documented above, *in vitro* evidence supports the importance of thiamine to tumor cell growth. The addition of thiaminase, a thiamine degrading enzyme, is cytotoxic to breast cancer cell

lines (Liu *et al.* 2010). Exposure to thiaminase also resulted in synergistic toxicity when administered alongside standard chemotherapeutics including doxorubicin and paclitaxel suggesting that thiamine may be a contributing factor in tumor cell survival following chemotherapeutic treatment (Liu *et al.* 2010).

Comin-Anduix *et* al. also provide evidence for the direct impact of supplemental thiamine on tumor growth. In this model, mice implanted with Erlich ascites tumors were administered supplemental thiamine in incremental doses of the Regular Daily Intake (RDI, suggested daily intake for mice) *via* intraperitoneal (IP) injection (Comin-Anduix *et al.* 2001). Thiamine doses ranging from 12.5-37.5 times the RDI significantly stimulated tumor cell proliferation compared to control (Comin-Anduix *et al.* 2001). Maximal enhancement of tumor growth was observed at a dose of 25 times the RDI demonstrated as an ~250% increase in cellular proliferation (Comin-Anduix *et al.* 2001).

1.3.2 Diet, nutritional questionnaires and clinical data

Cancer typically occurs less frequently in Asian and African countries than in Western countries; much speculation suggests that one factor in this difference is diet, in particularly, the abundant content of thiamine consumed by Western civilization (Boros 2000). In addition to higher levels of thiamine available in the food source, the general population of these countries consume thiamine through nutritional supplements and over-the-counter vitamins ranging from anywhere from 100% to 8000% the RDA (Yates *et al.* 1998; Zastre *et al.* 2013b). In Asian and African countries, not only does the diet include less thiamine, but it also typically includes foods that are high in thiaminase, which corresponds with reduced circulating thiamine levels (Boros 2000). Though not

tested in a clinical setting, this evidence supports the hypothesis that a diet rich in thiamine may result in a higher risk for cancer incidence. In contrast, correlative evidence obtained from nutritional questionnaires and dietary intake studies relates a higher consumption of thiamine, in conjunction with a multitude of other vitamins, with a decreased risk for bladder, cervical, gastric, and prostate cancer supporting that thiamine may play a preventative role in the context of cancer (Lu'o'ng & Nguyen 2013; Zastre *et al.* 2013b). High thiamine intake, compared to low thiamine intake, has also been demonstrated to significantly lower the risk of breast cancer (Cancarini *et al.* 2015). However, an alternative nutritional study demonstrates no significant correlation between the intake of B-vitamins, including thiamine, and the occurrence of breast, colorectal, endometrial, lung, or ovarian cancer (Kabat *et al.* 2008).

In addition to general nutritional observational studies, the overall thiamine status of cancer patients has been directly measured. Patients suffering from breast carcinoma, bronchial carcinoma, myelomonocytic leukemia, B-chronic lymphocytic leukemia and Burkett's lymphoma have been characterized as thiamine deficient (Basu & Dickerson 1976; van Zaanen & van der Lelie 1992; Seligmann *et al.* 2001). Reduced serum thiamine levels have been identified in patients suffering from colorectal carcinoma, cholangiocarcinoma, ovarian carcinoma, squamous cell carcinoma, non-small cell lung cancer, Hodgkin's lymphoma, acute myeloid leukemia, multiple myeloma and glioma (Isenberg-Grzeda *et al.* 2015; Papila *et al.* 2010; Isenberg-Grzeda *et al.* 2016a; Cefalo *et al.* 2014). Reduced serum TPP levels have also been noted in patients suffering from non-small cell lung cancer (Tsao *et al.* 2007). Wernicke's encephalopathy (WE) is an acute, neuropsychiatric syndrome characterized by a triad of symptoms including nystagmus,

ataxia, and delirium, and is the clinical manifestation of thiamine deficiency (lack of TPP) (Osiezagha *et al.* 2013; Sechi & Serra 2007). Interestingly, delirium is noted as one of the most prevalent comorbid neuropsychiatric conditions to occur in the advanced stage cancer population, with incidences estimated to affect ~40% of all patients (Lawlor *et al.* 2000; Stiefel *et al.* 1992; Centeno *et al.* 2004). A growing body of clinical case reports suggest that WE may be an under-recognized factor in the occurrence of delirium comorbid to malignancy. In 2009, Kuo *et al*. identified 24 cases of WE among cancer patients reported in the scientific literature. In addition, the authors described five new cases of WE identified from the patient population at the M.D. Anderson Cancer Center (Kuo *et al.* 2009). The authors highlight that the most common symptom associated with WE in cancer patients is delirium and concluded that all cancer patients, especially those with rapidly growing tumors, malnutrition, or those who have undergone bone marrow transplants, presenting with a confused cognitive state should be considered for thiamine deficiency (Kuo *et al.* 2009).

Since the work of Kuo *et al*., multiple other reports of WE in cancer patients have been described in the clinical literature (**Table 1.1**). Based on these reports, patients undergoing active therapeutic regimens and with reported malnutrition may be at an elevated risk for WE. A 2016 retrospective analysis performed by Isenberg-Grzeda *et. al* (not included within Table 1.1 for brevity) of patient charts from 217 in-patients with varying cancer types referred for psychiatric consultation demonstrated that 55.3% of patients were characterized as thiamine deficient (defined by serum thiamine levels <8 nmol/L) and exhibited symptoms of WE (Isenberg-Grzeda *et al.* 2016b). This study provides substantiation for the high prevalence of WE occurring comorbid to advanced

Table 1.1 Clinical case reports of Wernicke's Encephalopathy in cancer patients from 2009-2018

Gender, Age	Cancer Type	Treatment Regimen	Malnutrition Reported	Clinical Symptoms	[Serum Thiamine], Clinical Diagnosis	Reference
Female, 61	Non-Hodgkin Lymphoma	NR	Appetite Loss	Ataxia	Abnormal, NR	(Onishi *et al.* 2018b)
Female, 50	Breast, Metastatic	NR	Appetite Loss	Ataxia	Abnormal, NR	(Onishi *et al.* 2018b)
Female, 78	Lung	Nivolumab	Appetite Loss	Delirium	Abnormal, NR	(Onishi *et al.* 2018a)
Female, 13	Acute lymphoblastic leukemia	Methotrexate, purinethol	Appetite Loss	Delirium, Nystagmus	NR, Abnormal MRI	(Kizilocak *et al.* 2017)
Female, 18	Acute myeloid leukemia	FLAG-IDA	TPN w/o vitamins	Altered mental status	NR, Abnormal MRI	(Maden *et al.* 2016)
Female, 64	Lymphoma	None	Weight loss	Ataxia, Nystagmus	NR, Abnormal MRI	(Seo *et al.* 2017)
Female, 46	Nasopharyngeal	5-FU + Cisplatin	None suspected	Dizziness, Nystagmus	Normal, Abnormal MRI	(Cho *et al.* 2009)
Female, 61	Hodgkin's Lymphoma	None active at diagnosis	Bowel obstruction	Delirium	Low, Abnormal MRI	(Isenberg-Grzeda *et al.* 2015)
Male, 68	Colon, Metastatic	FOLFOX, Irinotecan	Weight loss	Delirium	Low, NR	(Isenberg-Grzeda *et al.* 2015)
Male, 80	Squamous cell carcinoma	Radiation	Decreased energy	Ataxia	NR, Abnormal MRI	(Isenberg-Grzeda *et al.* 2015)

Gender, Age	Cancer Type	Treatment Regimen	Malnutrition Reported	Symptoms	[Serum Thiamine], Clinical Diagnosis	Reference
Female, 38	Gastric	5-FU, Cisplatin	Weight loss	Delirium	Not reported, Normal MRI	(Kwon *et al.* 2010)
Female, 56	Colon, Metastatic	5-FU	None suspected	Delirium, Nystagmus	Low, Abnormal MRI	(Papila *et al.* 2010)
Female, 48	Gastric, Metastatic	Paclitaxel-S-1	Weight loss	Nystagmus, Ataxia	Not reported, Abnormal MRI	(Jung *et al.* 2010)
Female, 58	Gastric, Metastatic	FOLFOX	Weight loss	Delirium, Ataxia, Nystagmus	Not reported, Abnormal MRI	(Jung *et al.* 2010)
Male, 72	Pancreatic	GI Surgery	Weight loss	Nystagmus, Altered mental status	Not reported, Abnormal CT	(Rufa *et al.* 2011)
Female, 49	Colon	GI Surgery	Vomiting, anemia	Nystagmus, Altered mental status	Not reported, Abnormal MRI	(Rufa *et al.* 2011)
Female, 69	Pancreatic	GI Surgery	Weight loss	Altered mental status	Not reported, Abnormal MRI	(Rufa *et al.* 2011)
Female, 60	Colon	GI Surgery	Vomiting, Acute anemia	Nystagmus, Altered mental status	Not reported, Abnormal MRI	(Rufa *et al.* 2011)
Male, 70	Rectal	GI Surgery	Weight loss	Nystagmus, Altered mental status	Not reported, Abnormal MRI	(Rufa *et al.* 2011)
Male, 63	Rectal	GI Surgery	Weight loss	Nystagmus, Altered mental status	Not reported, Abnormal MRI	(Rufa *et al.* 2011)
Female, 76	Gallbladder	GI Surgery	Anemia	Nystagmus, Altered mental status	Not reported, Normal MRI	(Rufa *et al.* 2011)

Gender, Age	Cancer Type	Treatment Regimen	Malnutrition Reported	Symptoms	[Serum Thiamine], Clinical Diagnosis	Reference
Male, 63	Colon, Metastatic	GI Surgery	Weight loss	Altered mental status	Not reported, Abnormal MRI	(Rufa *et al.* 2011)
Male, 53	Gallbladder	GI Surgery	Anemia	Altered mental status	Not reported, Abnormal CT	(Rufa *et al.* 2011)
Female, 74	Gastric, Metastatic	GI Surgery	Weight loss	Nystagmus, Altered mental status	Not reported, Abnormal MRI	(Rufa *et al.* 2011)
Female, 76	Peritoneal, Metastatic	Paclitaxel, carboplatin	Nausea	Ataxia, Disorientation	Normal, Abnormal MRI	(Kim 2013)
Male, 16	Acute lymphoblastic leukemia	Methotrexate, 6-mercaptopurine	TPN	Delirium, Ataxia	Not reported, Abnormal MRI	(Muwakkit *et al.* 2009)
Female, 28	Squamous cell carcinoma	Chemoradiation	Weight loss	Delirium, Nystagmus, Ataxia	Not reported	(Fikhman *et al.* 2011)
Female, 68	Squamous cell carcinoma	Chemoradiation	Vomiting	Delirium, Ataxia	Not reported	(Fikhman *et al.* 2011)
Male, 6	Glioma	Chemoradiation	None suspected	Ataxia	Low, Abnormal MRI	(Cefalo *et al.* 2014)
Male, 12	Neuroectodermal tumor	Chemoradiation	Weight loss	Delirium	Low, Abnormal MRI	(Cefalo *et al.* 2014)

NR- Not Reported

TPN- Total parenteral nutrition

stage malignancies (Isenberg-Grzeda *et al.* 2016b). Although it remains unclear as to why WE occurs in cancer patients, the effect has been observed regardless of patient age, gender, or cancer type. Based on the reports described above, risk factors include low oral intake, nausea and vomiting, administration of corticosteroids, infection, rapidly growing tumors and chemotherapeutic administration

1.4 Potential factors contributing to altered thiamine homeostasis during malignancy

1.4.1 Nutrition

As a water-soluble micronutrient with limited storage within the body (half-life 1-12 h), thiamine must be consistently consumed in the diet (Whitfield *et al.* 2018). The RDA for thiamine consumption (**Table 1.2**) depends on energy intake (Institute of Medicine (U.S.). Standing Committee on the Scientific Evaluation of Dietary Reference Intakes. *et al.* 1998; Whitfield *et al.* 2018). Therefore, suggested consumption is higher for men compared to women and is highest for pregnant and lactating women (Institute of Medicine (U.S.). Standing Committee on the Scientific Evaluation of Dietary Reference Intakes. *et al.* 1998). Based on consumption as outlined, thiamine plasma levels in healthy individuals range between 10 and 20 nM (Gangolf *et al.* 2010). Depicted in **Table 1.3**, the standard Western diet contains ample sources of thiamine (Zastre *et al.* 2013b). The United States fortifies wheat flour with thiamine in an attempt to eliminate disease (i.e. Beriberi) due to thiamine deficiency, making bread and pasta significant sources of dietary thiamine (Whitfield *et al.* 2018). Thiamine can also be obtained from

Table 1.2 Recommended dietary allowance for thiamine by subpopulation

Subpopulation	RDA (mg/day)
Infants*, mo	
0-6	0.2
7-12	0.3
Children, y	
1-3	0.5
4-8	0.6
9-13	0.9
Males, y	
14-18	1.2
≥19	1.2
Females, y	
14-18	1.0
≥19	1.1
Pregnancy, Lactation	1.4

* RDA has not been established for infants. Values for infants represent assessed mean intakes (adequate intakes) of infants who consume only human milk from well-nourished mothers (0-6 mo) or a mixed diet of human milk and solid food (7-12 mo).

Table 1.3 Thiamine content (amount, percent daily value) found in common food sources and nutraceuticals

Dietary source	Thiamine Content (mg)*	%DV**
Natural		
Pork, fresh (3 oz.)	0.6	50%
Fish (1/2 fillet)	0.3	25%
Black beans (1 cup)	0.4	34%
Lima beans (1 cup)	0.3	25%
Potatoes (1 cup)	0.3	25%
Okra (1 cup)	0.2	17%
Chicken (1 cup)	0.2	17%
Peas (1 cup)	0.2	17%
Sunflower seeds (1 cup)	0.7	58%
Pistachios (1 oz.)	0.2	17%
Fortified		
General Mills, total raisin bran (1cup)	1.6	133%
General Mills, total corn flakes (1.3 cups)	1.5	125%
Breadcrumbs (1 cup)	1.2	100%
White rice (1 cup)	1.1	92%
Submarine sandwich (6" sandwich)	1.0	84%
Cornmeal (1 cup)	0.9	75%
Supplements		
Centrum		
Adult	1.5	125%
Child (≥4 yrs)	1.5	250%[a]
One A Day		
Women's 50+	4.5	410%[b]
Women	1.5	136%[b]
Girl Teen	2.3	230%[c]
Men's 50+	4.5	375%
Men	1.2	100%
Solaray		
Boy Teen	3.8	317%
B Complex	7.5	625%
Nature's Way		
Vitamin B1	100	8,333%
Nature Made		
Vegan B Complex	25	2,083%
B Complex	15	1250%

Table modified from (Zastre *et al.* 2013b)
*Values of thiamine in common food sources and nutraceuticals as defined by (Zastre *et al.* 2013b).
**RDA calculated based on recommended consumption of 1.2 mg/day in adult men, except:
[a]RDA for children 0.6 mg/day
[b]RDA for women 1.1 mg/day
[c]RDA for teen girl 1.0 mg/day

the diet by eating foods rich in the compound including fish, meat, eggs, legumes, and milk (Zastre *et al.* 2013b). Alternatively, thiamine may by consumed using over-the-counter nutraceuticals. Vitamin supplements often contain thiamine in significantly high amounts. Consumption of a standard multi-vitamin may exceed the RDA (1.2 mg) for thiamine by 100 to 2,083% (Zastre *et al.* 2013b). The Nature's Way Vitamin B1 supplement contains 100 mg of thiamine, which represents >8000% of the RDA for a healthy adult (Zastre *et al.* 2013b).

As the body's thiamine store is inherently linked with diet, much emphasis has been placed on linking poor nutritional status and WE in cancer. However, one report noted that few patients diagnosed with WE were actually underweight (22%), and instead many were characterized as overweight (44%). This highlights that malignancy associated malnutrition may not be the driving force in of thiamine deficiency in every case (Isenberg-Grzeda *et al.* 2016a). In the same study, thiamine deficiency occurred in the absence of other vitamin deficiencies (i.e. folate, B12), suggesting the potential for an elevated turnover rate for thiamine during malignancy when compared to other vitamins (Isenberg-Grzeda *et al.* 2016a). Alternatively, Basu *et al.* proposed that the decrease in thiamine status during cancer may be due to an increase in urinary excretion of the vitamin (Basu & Dickerson 1976)

1.4.2 Chemotherapeutic interactions with thiamine

Evidence suggests that the administration of chemotherapeutic treatments may also be a factor in thiamine deficiency among cancer patients (Aksoy *et al.* 1980; Ulusakarya *et al.* 1999; Kondo *et al.* 1996; Cho *et al.* 2009). Multiple unrelated

mechanisms through which chemotherapy may induce thiamine deficiency have been demonstrated. During a longitudinal analysis, patients treated with the chemotherapeutic 5-fluorouracil (5-FU) developed reversible thiamine deficiency, while those administered regimens lacking 5-FU showed no deficiency (Aksoy *et al.* 1980). 5-FU, an anti-metabolite and thymidylate synthase inhibitor, demonstrates no structural similarity to thiamine and does not inhibit thiamine transport in cancerous or non-cancerous cells (Longley *et al.* 2003; Zastre *et al.* 2013b; Heier & Dornish 1989). In so, WE comorbid to cancer patients treated with 5-FU does not appear to be associated with impaired intestinal absorption of thiamine. Treatment with 5-FU has actually been found to promote thiamine transport cancer cells (Heier & Dornish 1989). Increased thiamine transport following 5-FU treatment associates with an increase in its intracellular conversion to TPP (Heier & Dornish 1989). Therefore, 5-FU may mechanistically induce thiamine deficiency by increasing thiamine homeostasis in malignant cells resulting in decreased peripheral thiamine levels (Zastre *et al.* 2013b). Ifosfamide acts as an alkylating agent to treat malignancy, and, like 5-FU, demonstrates no structural similarity to thiamine (Fleming 1997). Treatment with ifosfamide induces symptoms of thiamine deficiency without altering the thiamine levels of cancer patients (Buesa *et al.* 2003). This suggests the compound may limit the production of TPP without altering thiamine availability, or alternatively, that ifosfamide (and/or an ifosfamide metabolite) may inhibit an intracellular thiamine-dependent pathway (Zastre *et al.* 2013b; Buesa *et al.* 2003).

Other chemotherapeutics exert targeted effects on thiamine transport that may account for the observation of thiamine deficiency among cancer patients. Metformin, the

most commonly prescribed drug used to treat type 2 diabetes, also demonstrates chemotherapeutic activity and has been used as a successful adjuvant therapy in cancer patients with and without diabetes (Kasznicki *et al.* 2014). Administration of metformin reduces intestinal thiamine accumulation in mice through competitive inhibition of thiamine transport (Chen *et al.* 2014). Therefore, treatment with metformin may mechanistically induce WE by reducing intestinal thiamine absorption. The clinical development of fedratinib, a Janus kinase (JAK2) inhibitor, was recently halted following increased frequency of WE reported in patients treated with the compound for the myeloproliferative neoplasm myelofibrosis (Pardanani *et al.* 2015). *In vitro* transport studies provide a molecular basis for WE in these patients revealing that fedratinib inhibits carrier-mediated thiamine uptake in the human-derived intestinal epithelial Caco-2 cell line (Zhang *et al.* 2014). These reports demonstrate the need to evaluate interactions of standard and investigational chemotherapeutics with thiamine transport when further defining causes of WE among cancer patients.

1.4.3 Redistribution of thiamine homeostasis between cancerous and non-cancerous tissue

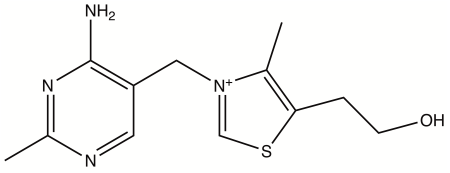
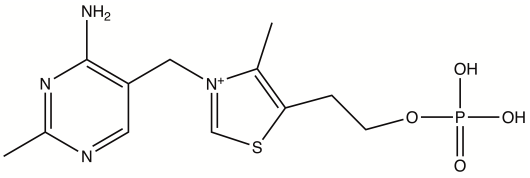
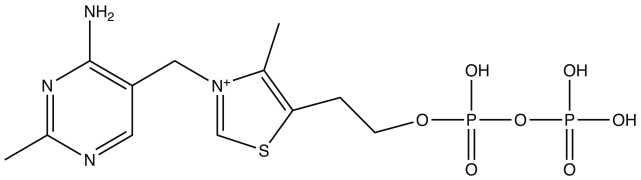
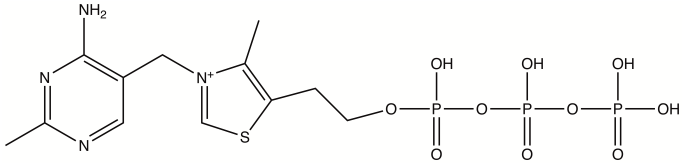
In another explanation for thiamine deficiency among cancer patients, thiamine's bioactivity may be exploited by cancer cells as a means of maintaining tumorigenic potential. In this hypothesis, the insult of a rapidly developing tumor triggers a redistribution of the body's total thiamine store resulting in thiamine accumulation within the tumor tissue and depletion of the body's non-cancerous tissues. Evidence obtained from clinical tissues provides support for this hypothesis demonstrating a 2.5-fold

increase in thiamine levels identified in colon adenocarcinoma tissue relative to uninvaded control tissue (Baker *et al.* 1981). In a similar fashion, malignant tissue maintained constant TPP levels while non-cancerous control tissue showed perpetual declines of thiamine and TPP during tumor growth (Trebukhina *et al.* 1982; Trebukhina *et al.* 1984). These findings suggest alterations in thiamine homeostasis at the cellular level.

1.4.3.1 General overview of cellular thiamine homeostasis

As depicted in **Table 1.4**, the chemical structure of thiamine consists of a substituted thiazole ring joined to an aminopyrimidine ring through a methylene bridge. The quaternary nitrogen located within thiamine's thiazole ring and overall hydrophilicity of the molecule necessitates carrier-mediated transport across the plasma membrane. Two dedicated thiamine transporters, both members of the Solute Carrier (SLC) family of active transporters, facilitate the cellular uptake of thiamine (Ganapathy *et al.* 2004). THTR1 (*SLC19A2*), a low affinity, high capacity thiamine transporter (K*m,* 2.5 μM), and THTR2, (*SLC19A3*) a high affinity, low capacity thiamine transporter (K*m*, 27 nM), share 48% sequence homology (Dutta *et al.* 1999; Eudy *et al.* 2000). Though both transporters are widely expressed, they demonstrate differential tissue manifestation suggesting distinct roles in overall thiamine homeostasis (Rajgopal *et al.* 2001). Furthermore, the differential localization of THTR1 and THTR2 in polarized cells mediates thiamine absorption into the bloodstream. THTR2, highly expressed at the brush border membrane of the intestinal tract, facilitates the absorption of thiamine in the gut (Boulware *et al.* 2003). Whereas, THTR1 demonstrates expression at both the apical

Table 1.4 Chemical structures of thiamine and its phosphate ester metabolites

Thiamine Derivative	Chemical Structure
Thiamine	
Thiamine Monophosphate (TMP)	
Thiamine Pyrophosphate (TPP)	
Thiamine Triphosphate (TTP)	

and basolateral membranes of polarized cells and facilitates plasma uptake of thiamine (Ganapathy *et al.* 2004; Boulware *et al.* 2003). In addition to THTR1 and THTR2, thiamine has recently been identified as an endogenous substrate of other SLC transporters. The organic cation transporters (OCT) mediate absorption of organic cations in the small intestine and have been shown to mediate intracellular thiamine accumulation (Lemos *et al.* 2012). Specifically, OCT1 (*SLC22A1*) serves as a high-

capacity thiamine transporter involved in hepatic thiamine homeostasis (Chen *et al.* 2014).

The phosphorylation of thiamine into TPP by the activity of thiamine pyrophosphokinase-1 (TPK1) contributes to intracellular accumulation of thiamine (Bettendorff 1995). TPK1 is ubiquitously expressed, but significantly high in tissues including testis, kidney, and small intestine (Zhao *et al.* 2001a). Two isoforms of the *TPK1* gene exist, however only variant 1 (NM_022445.3) has been shown to catalyze the phosphorylation of thiamine (Nosaka *et al.* 2001). *TPK1* splice variant 2 (NM_001042482.1) lacks exon 7, which results in a 49-amino acid deletion from a conserved portion of the full-length protein. It is unclear if this deletion alters functional activity for the phosphorylation of thiamine (Mayr *et al.* 2011). TPK1 exists as a homodimer diphosphotransferase enzyme and transfers two phosphate groups from ATP onto thiamine yielding TPP and adenosine monophosphate. TPP serves as the activated cofactor form of thiamine and maintains thiamine dependent enzyme (TDE) activity in both the cytosol and in the mitochondria following mitochondrial uptake by the thiamine pyrophosphate carrier (TPC, *SLC25A19*) (Lindhurst *et al.* 2006). Free TPP not bound as enzymatic cofactor may be further metabolized to other phosphate derivatives of thiamine including thiamine monophosphate (TMP), thiamine triphosphate (TTP), or adenylated conjugates of TPP and TTP (Gangolf *et al.* 2010). Both TMP and TPP can be effluxed out of the cell by the Reduced Folate Carrier 1 (RFC1, *SLC19A1*) (Zhao *et al.* 2002). Additionally, TPP was recently shown to be directly transported into cells by the thiamine pyrophosphate transporter (TPPT, *SLC44A4*) (Nabokina *et al.* 2014). TPPT demonstrates saturable activity for TPP transport (K*m* ~170 nM), and is highly specific

for TPP over thiamine and its other derivatives (Nabokina *et al.* 2014). Considering the high expression of TPPT, within the colon, this transport mechanism may contribute to systemic thiamine homeostasis by facilitating intestinal absorption of microbiota-generated TPP (Nabokina *et al.* 2014). As mentioned above, TPP canonically serves as a cofactor for metabolic enzymes. Non-canonical biological roles for TPP as well as thiamine and its other derivatives are under investigation. TTP has been characterized as a factor in neuronal excitotoxicity, while both thiamine and TPP have been demonstrated to possess antioxidant properties (Bettendorff *et al.* 2014; Okai *et al.* 2007).

1.4.3.2 Gene expression changes support redistribution of thiamine during malignancy

In 2013, *Zastre et al.* identified that multiple thiamine homeostasis genes are significantly upregulated during breast cancer. qRT-PCR analysis of the TissueScan Breast Cancer cDNA Array II revealed significant increases for *SLC19A2*, *SLC25A19*, and *TPK-1* transcripts in tumor tissue relative to non-tumor control tissue (Zastre *et al.* 2013a). In addition, this work demonstrated the induction of both mRNA and protein for *SLC19A2* (THTR1), *SLC25A19* (TPC), and TPK-1 in multiple breast tumor cell lines relative to control human mammary epithelial cells (HMECs) (Zastre *et al.* 2013a). The up-regulation of thiamine homeostasis genes correlated with significantly increased thiamine transport and overall thiamine accumulation in tumorigenic cell lines relative to HMECs (Zastre *et al.* 2013a). Work has since been performed aimed at defining how cancer cells achieve the adaptive up-regulation of thiamine homeostasis genes. This has resulted in evidence for the involvement of oncogenic transcription factors and microRNAs (miRs) in the regulation of thiamine homeostasis (Sweet *et al.* 2010; Zera *et*

al. 2016; Kim *et al.* 2015). **Figure 1.4** depicts an overview of intracellular thiamine homeostasis and highlights up-regulation of critical thiamine homeostasis genes noted during malignancy.

1.4.3.3 Oncogenic HIF-1α signaling regulates thiamine transport

HIF-1α has been implicated to regulate thiamine homeostasis on a conditional basis, such as when tumor cells are exposed to hypoxia (Sweet & Zastre 2013). When tumor cells experience decreased oxygen tension, HIF-1α stabilizes, resulting in its rapid accumulation and subsequent translocation into the nucleus where it activates genes that regulate angiogenesis, cell survival, migration, and metabolism (Semenza 2002; Semenza 2013). As discussed in section 1.2.1, it has been well established that activation of HIF-1α signaling confers a more glycolytic phenotype to tumor cells (Majmundar *et al.* 2010). Therefore, Zastre *et. al* hypothesized that HIF-1α may also adaptively regulate supporting nutrient and micronutrient pathways to fuel and maintain glycolysis (Zastre *et al.* 2013b). Using the breast cancer model of BT474 cells, a chromatin immunoprecipitation (ChIP) assay revealed that HIF-1α directly regulates the expression of THTR2 by transactivating the *SLC19A3* minimal promoter region (Zera *et al.* 2016). Activation of the HIF-1α signaling axis in BT474 cells following hypoxic treatment results in increased THTR2 transcript and protein expression and enhanced thiamine transport (Sweet & Zastre 2013). This defines an oncogenic signaling pathway that may enable tumor cells to accumulate thiamine under oxygen stress, and further suggests that an increased intracellular concentration of thiamine may be necessary to sustain tumor cells

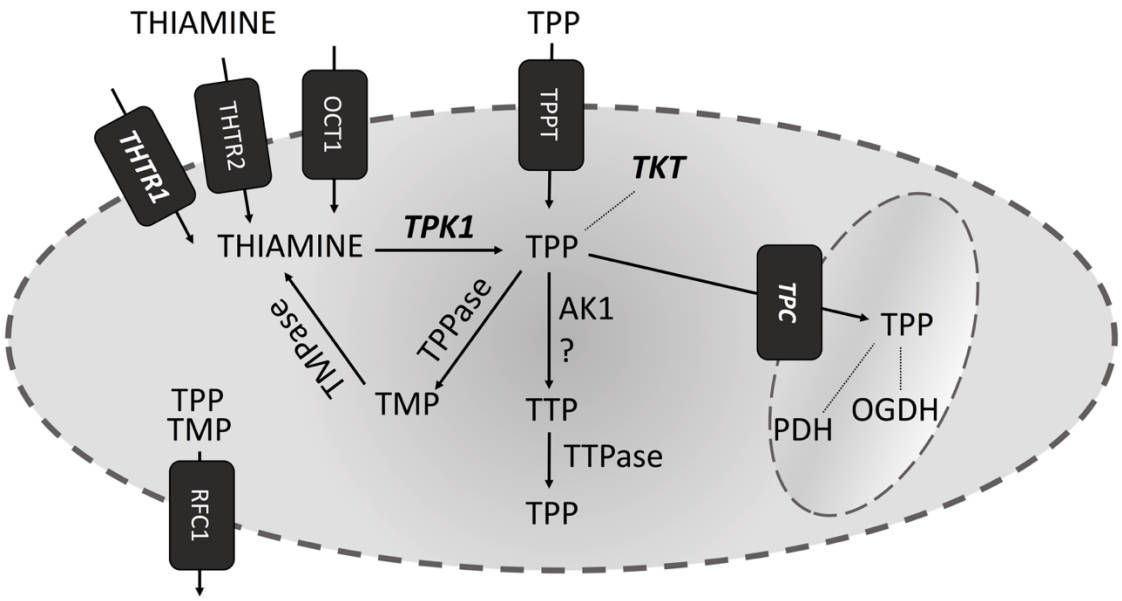


Fig 1.4 Schematic diagram of intracellular thiamine homeostasis Intracellular thiamine accumulation is dependent on the activity of the dedicated thiamine transporters *THTR1* and THTR2. The organic cation transporter OCT1 also impacts intracellular thiamine accumulation in tissue-specific cases. *TPK1* catalyzes the subsequent intracellular phosphorylation of thiamine into thiamine pyrophosphate (TPP). TPP can also enter the cell via the membrane bound thiamine pyrophosphate transporter (TPPT). Cytosolic TPP can be transported into the mitochondria by the *TPC*, where it serves as a cofactor for mitochondrial enzymes including pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (OGDH). Alternatively, TPP can serve as a cofactor for *TKT* in the cytoplasm. If not used as a metabolic cofactor, TPP can be converted to TMP by the activity of thiamine pyrophosphatase (TPPase). TMP can be further dephosphorylated to thiamine by the activity of thiamine monophosphatase (TMPase). Both TPP and TMP can be effluxed out of the cell by the activity of the reduced folate carrier 1 (RFC1). TPP may also be further phosphorylated by the activity of an undefined enzyme, potentially adenylate kinase 1 (AK1), to form thiamine triphosphate (TTP). TTP can be back-converted to TPP by the activity of thiamine triphosphatase (TTPase). Thiamine-related homeostasis genes and enzymes that are up-regulated during cancer are italicized.

1.4.3.4 Oncogenic miR-155 regulates thiamine levels in malignant cells

miR-155 is a well-characterized oncogenic miR involved in the development and progression of multiple tumor types from different tissue origins including breast, blood, colon, lung, and pancreatic (Mattiske *et al.* 2012; Sandhu *et al.* 2012; Wan *et al.* 2016; Yanaihara *et al.* 2006; Habbe *et al.* 2009). Using a combined bioinformatic and metabolomic analysis in breast cancer, six metabolites were found to be significantly correlated with miR-155 expression including: glycodeoxycholate; inositol mono-phosphate; fructose 1,6 diphosphate; N-carbamoyl-beta-alanine; uracil; and thiamine. (Kim *et al.* 2015). Of these, thiamine was the only metabolite significantly up-regulated in the high miR-155 expressers demonstrating a positive correlation. Lentiviral knockdown of miR-155 expression and activity resulted in significant decreases to the expression of *SLC19A2* (THTR1), *SLC25A19* (TPC), and TPK1 at both the mRNA and protein level (Kim *et al.* 2015). Furthermore, the average amount of thiamine present in human Triple Negative Breast Cancer (TNBC) tissues can be stratified based on high and low expression of miR-155, with high expressers of miR-155 containing increased levels of thiamine measured by LC-MS/MS (Kim *et al.* 2015). Based on their analysis, Kim *et al*. propose that the induction of miR-155 during oncogenesis results in the induction of thiamine homeostasis genes and increases the overall thiamine status of tumor cells. This relationship relies on miR-155 silencing the expression of an unknown "Mediator X" instead of having a direct binding relationship with mRNA sequences of the thiamine homeostasis genes (Kim *et al.* 2015). These findings provide support for the hypothesis that tumor cells actively acquire thiamine through a defined mechanism to adaptively up-regulate thiamine homeostasis gene expression.

1.5 Physiological function of thiamine in tumor cells: cofactor vs. non-cofactor roles

The next major hurdle at the forefront of thiamine research in regard to cancer becomes elucidating the intracellular function of the compound and mechanistically defining how thiamine's supplemental presence impacts tumor growth. The most obvious explanation for why tumor cells may up-regulate thiamine homeostasis depends on the premise that thiamine provides a critical cofactor for multiple metabolic enzymes. Thus, tumor cells may enhance the presence of the cofactor in order to support their highly metabolic nature by increasing thiamine homeostasis (Zastre *et al.* 2013b). Alternatively, recent research suggests that thiamine and its derivatives may play other non-cofactor roles within tumor cells that are directly related to tumor growth and thus require consideration when defining thiamine's mechanistic action.

1.5.1 General overview of thiamine dependent enzymes (TDEs)

Thiamine dependent enzymes (TDEs) are essential for carbohydrate metabolism. Functioning in multiple metabolic networks, TDEs support ATP production as well as the biosynthesis of neurotransmitters, nucleic acids, and reducing equivalents (Singleton & Martin 2001). Loss of TDE activity is associated with oxidative stress, excitotoxicity, and inflammation (Hazell & Butterworth 2009).

1.5.1.1 Transketolase

As described in section 1.2.2, the cytosolic TDE transketolase (TKT) reversibly links glycolysis to the pentose phosphate pathway (PPP). TKT maintains flux through the non-oxidative branch of the PPP by catalyzing two-carbon unit transfers between

carbohydrates, specifically the conversion of xylulose-5-phosphate (X5P) and ribose 5-phosphate (R5P) to glyceraldehyde-3-phosphate (G3P) and sedoheptulose-7-phosphate (S7P) (Schenk *et al.* 1998). R5P is subsequently converted into phosphoribosyl pyrophosphate and used for nucleotide synthesis (Patra & Hay 2014). Depending on the metabolic requirements of the cell, TKT can also convert xylulose-5-phosphate (X5P) and erythrose 4-phosphate (E4P) into G3P and F6P (Schenk *et al.* 1998). Both G3P and F6P can reenter glycolysis and fuel energy production or maintain redox status through NADPH production in the oxidative branch of the PPP (Patra & Hay 2014).

1.5.1.2 Pyruvate Dehydrogenase

In the mitochondria, the pyruvate dehydrogenase complex (PDC) catalyzes the oxidative decarboxylation of pyruvate irreversibly linking glycolysis with the TCA cycle. The PDC consists of three subunit catalytic enzymes including pyruvate dehydrogenase (E1), dihydrolipoamide acetyltransferase (E2), and dihydrolipoamide dehydrogenase (E3) (Patel *et al.* 2014). Of these, the E1 subunit requires TPP as a cofactor for functional activity and carries out the initial step in converting pyruvate to acetyl-CoA (Patel *et al.* 2014).

1.5.1.3 α-Ketoglutarate Dehydrogenase

Glutaminolysis results in the production of α-Ketoglutarate (α-KG), which can be used as an additional carbon source to carbohydrates in fueling metabolism (DeBerardinis *et al.* 2007). Within the mitochondria, α-KG can be oxidized by the α-Ketoglutarate Dehydrogenase Complex (OGDC) to form succinyl CoA (Vatrinet *et al.*

2017). Like the PDC, the OGDC is also a multienzyme complex composed of three subunits. The dehydrogenase E1 subunit depends on TPP as a cofactor and facilitates the decarboxylation of α-KG, which is the first step in its conversion to succinyl CoA (Vatrinet *et al.* 2017).

1.5.2 Cofactor function: altered TDE activity in cancer

Thiamine dependent enzyme activity may be decreased or enhanced during malignancy depending on the specific enzyme's function within the complex metabolic network required to sustain tumor cell proliferation. As discussed in Section 1.2.1 and 1.2.2, oncogenic transcription factors confer a glycolytic phenotype in tumor cells, which may be partly dependent on inhibiting aspects of mitochondrial-dependent metabolism. Considering both PDH and OGDH function within the mitochondria, the deregulation of their activity during cancer may serve to promote the glycolytic nature of tumor cells. An increase in the cytosolic conversion of α-KG to isocitrate by isocitrate dehydrogenase-1 in cancer cells reduces the amount of α-KG used to fuel OGDC activity within the mitochondria (Vatrinet *et al.* 2017). HIF-1α has also been demonstrated to negatively regulate OGDC, suggesting that the enzyme's activity may be downregulated by oncogenic signaling during malignancy (Vatrinet *et al.* 2017). As discussed in Section 1.2.2, cancer cells have been demonstrated to down regulate PDH activity *via* phosphorylation through pyruvate dehydrogenase kinases (PDKs) to promote a more glycolytic metabolic phenotype (Saunier *et al.* 2016).

TKT's function at the junction of glycolysis and the PPP in the cytosol supports the anabolic capacity of tumor cells, and therefore TKT activity appears to be exploited

for a growth advantage by tumor cells. Boros *et al.* found that ~85% of ribose in malignant cells is generated through the TKT dependent non-oxidative branch of the PPP (Boros *et al.* 1997). TKT up-regulation occurs in cancerous tissue compared to non-cancerous control and associates with more advanced disease staging (Xu *et al.* 2016; Ricciardelli *et al.* 2015). shRNA inhibition of TKT directly impedes the growth of hepatocellular carcinoma cells both *in vitro* and i*n vivo* (Xu *et al.* 2016). It has been hypothesized that the up-regulation of thiamine homeostasis during malignancy may serve to promote TKT activity through TPP's cofactor function (Zastre *et al.* 2013b). This hypothesis may be supported by a recent report demonstrating that thiamine homeostasis serves as a critical factor in the radiosensitivity of tumor cells. In this study, the effects of thiamine homeostasis coincided with the maintenance of nucleotide pools through TKT activity (Tiwana *et al.* 2015). Knockdown of TPK1, as well as THTR1, but not TPC, resulted in increased sensitivity to ionizing radiation treatment for a wide variety of tumor cell lineages (Tiwana *et al.* 2015). Pyrithiamine (PT), a chemical inhibitor of TPK1, also increased tumor cell sensitivity to radiation treatment (Tiwana *et al.* 2015). Tiwana *et al.* associated these findings with data demonstrating that PT treatment resulted in reduced nucleotide pools and, therefore, the working model that TPK1 inhibition results in decreased cofactor to support TKT activity for nucleotide synthesis was proposed. The authors suggest that this limits substrate flow through the non-oxidative PPP, theoretically limiting a tumor cell's ability to respond to radiation treatment by decreasing its capacity to synthesize nucleotides and repair radiation-induced DNA damage (Tiwana *et al.* 2015).

The function of TKT in malignant growth may be tied to factors other than enhancing anabolic capacity and supporting nucleotide synthesis. In hepatocellular carcinoma, the activity of TKT supports tumor cell proliferation by maintaining cellular redox status through the maintenance of NADPH pools necessary for glutathione production (Xu *et al.* 2016). Furthermore, TKT was recently shown to regulate breast cancer oncogenesis by mediating the dynamic switch between glycolysis and oxidative phosphorylation through an α-ketoglutarate driven signaling pathway (Tseng *et al.* 2018). Additionally, TKT possesses two structural isomers, known as transketolase-like 1 (TKTL1) and transketolase-like 2 (TKTL2) (Zhao & Zhong 2009). Both TKTL1 and TKTL2 share high sequence homology with TKT, 61% and 66% respectively (Mitschke *et al.* 2010). Expression of TKTL1 in clinical samples correlates with disease progression and poor disease prognosis in patients suffering from ovarian, colorectal, and urothelial cancer as well as glioblastomas (Langbein *et al.* 2006; Krockenberger *et al.* 2007; Volker *et al.* 2008). Supporting its classification as an oncogene, the overexpression of TKTL1 in non-malignant cells induces proliferation, while genetic silencing of TKTL1 through RNAi directly inhibits tumor cell proliferation (Hartmannsberger *et al.* 2011; Zhang *et al.* 2007). Knockdown of TKTL1 reduces total cellular transketolase activity suggesting the effects of TKTL1 on tumor cell proliferation are related to the activity of the non-oxidative branch of the PPP (Zhang *et al.* 2007). Despite this demonstrated transketolase-like activity *in vitro*, it remains unresolved if TKTL1 requires TPP as a cofactor for functional activity (Mitschke *et al.* 2010). The genetic mutation differentiating TKTL1 from TKT consists of a 38-amino acid deletion within the cofactor and catalytic domains of TKTL1. This deletion affects a residue (His^{77}) that has been shown to be critical in

TPP association with TKT (Mitschke *et al.* 2010). The deletion also alters protein folding making it theoretically unlikely that the aminopyrimidine ring and thiazolium ring of TPP associate with TKTL1 in the same fashion as TKT (Mitschke *et al.* 2010).

1.5.3 Non-cofactor function: antioxidant potential of thiamine and TPP

A further evaluation of the data proposed by *Tiwana et al*. reveals other potential mechanistic roles for thiamine and its metabolites in tumor growth. As suggested by the authors, considering TPK1 is responsible for producing TPP, knockdown of TPK1 should reduce the production of new TPP molecules (Tiwana *et al.* 2015). TPK1 activity has also been shown to be a driving force in intracellular thiamine accumulation in a neuroblastoma cell line as well as in isolated rat hepatocytes (Bettendorff 1995; Yoshioka 1984). This evidence suggests TPK1 knockdown will also halt accumulation of thiamine further limiting TPP production. This sequence of events becomes important when considering the predominant pathway for cellular toxicity associated with ionizing radiation (IR). Following IR exposure, ROS are produced from the radiolysis of water, which results in damage to DNA, proteins, and lipids and subsequent cell death (Azzam *et al.* 2012). It has been proposed that antioxidant supplementation during treatment with IR may be beneficial in protecting normal cells from impending death due to the induction of ROS that coincides with radiation exposure (Borek 2004). However, if antioxidant supplementation can protect normal cells, tumor cells may also exploit antioxidant pathways for protection against oxidative stress induced by ionizing radiation.

In vivo evidence demonstrates the antioxidant potential of thiamine and, more so, TPP. Multiple studies have analyzed the effects of the administration of thiamine and/or TPP on oxidative stress in multiple target organs. Thiamine was shown to protect against ethanol induced liver toxicity by limiting the extent of lipid peroxidation (Portari *et al.* 2016). In a second hepatotoxicity model, intraperitoneal injections of TPP demonstrated protection against alcohol-induced liver damage marked by reduced malonialdehyde (MDA) levels, increased concentrations of endogenous antioxidants, and reduced DNA damage (Yilmaz *et al.* 2015). TPP administration also protects against hyperglycemia-induced retinopathy in rats by balancing the oxidant/antioxidant status of rats, thus preventing oxidative stress (Cinici *et al.* 2016a). Similar protective effects were reported for TPP in ethambutol-induced ocular toxicity, cisplatin induced liver and cardiotoxicity, as well as ischemia-reperfusion induced kidney and ovarian toxicity (Cinici *et al.* 2016b; Coskun *et al.* 2014; Altuner *et al.* 2013; Turan *et al.* 2013; Demiryilmaz *et al.* 2013).

Both thiamine and TPP are suggested to have antioxidant potential *ex vitro*, but of the two TPP appears more powerful (Okai *et al.* 2007). In its proposed antioxidant mechanism, thiamine undergoes an oxidation reaction, where $2H^+ + 2e^-$ are transferred from its aminopyrimidine ring to radical groups resulting in the formation of a thiochrome (Lukienko *et al.* 2000). One interesting dataset in support of the antioxidant hypothesis suggests that liquidators exposed to ionizing radiation during the catastrophic Chernobyl nuclear accident (April 26, 1986) demonstrated dramatic decreases in blood TPP concentration (Donchenko *et al.* 1997). In this case, TPP may have been consumed as an antioxidant to counteract ROS induced by IR. Identifying how supplementation with thiamine can alter basal ROS level in tumor cells will require a dedicated effort in

future work. In addition, it remains of interest how the presence of the thiamine and its derivatives may protect tumor cells against therapeutics that mechanistically rely on oxidative stress (i.e. ionizing radiation, doxorubicin) for their effectiveness. Data in support of the antioxidant hypothesis will provide a logical reasoning for why tumor cells up-regulate genes controlling thiamine homeostasis.

1.5.4 Thiamine impacts p53 activity in tumor cells

To fully elucidate the impact of supplemental thiamine on malignant growth, a dedicated effort must also be focused on understanding thiamine's capability to impact p53 signaling. For the ~50% of clinical cases where p53 signaling isn't silenced by genetic mutation, the ability to mute p53 by other means may serve as an important factor in malignant progression. McLure *et al*. identified that thiamine's molecular structure confers the ability to bind p53 and prevent its transactivation of target gene expression (McLure *et al.* 2004). Compared with thiamine, TPP demonstrates an enhanced affinity for p53 with capability to silence its downstream signaling (McLure *et al.* 2004). Thiamine may also directly inhibit p53 expression *in vitro* evidenced by the finding that retinal neurons of diabetic rats treated with thiamine demonstrate less p53 compared with control neurons (Yang *et al.* 2004). Limited effort has been dedicated to evaluating how supplemental thiamine may inhibit p53 signaling to promote tumor growth, and in so, this remains a critical void for thiamine research.

1.6 Evidence for thiamine's anti-proliferative property against tumor growth

Although moderate thiamine supplementation enhances malignant growth, super-physiological pharmacologic concentrations of the vitamin directly impede tumor cell proliferation (Hanberry *et al.* 2014). This finding coincides with observations for high-dose supplementation of other vitamins (i.e. vitamin A, vitamin C), which have been considered as potential nutraceutical approaches to chemotherapy (Mamede *et al.* 2011). Vitamin A and its retinoid derivatives (retinoic acid) have been demonstrated to have anti-carcinogenic effects both *in vitro* and *in vivo* (Mamede *et al.* 2011). Treatment with retinoids directly inhibits cell growth and induces differentiation or apoptosis in tumor cell lines from multiple malignant subtypes including: non-small cell lung cancer; hematopoietic malignancies; neuroblastoma; cervical carcinoma; and head and neck carcinomas (Lotan 1995). Pharmacologic concentrations of vitamin C (ascorbic acid, dehydroascorbic acid) have also been demonstrated to have chemotherapeutic potential (Mamede *et al.* 2011). Administration of ascorbic acid has been demonstrated to be selectively toxic to cancer cells *in vitro* at IC50 doses ranging from 1-10 mM (Verrax & Calderon 2009; Chen *et al.* 2008). These findings translate *in vivo* where pharmacologic ascorbate treatment (4g/kg, once or twice daily) significantly reduces growth rates of ovarian, pancreatic, and glioblastoma tumors (Chen *et al.* 2008). The chemotherapeutic effects of high-dose vitamin C therapy are considered to be linked to its pro-oxidant properties, which result in hydrogen peroxide-dependent cytotoxicity to cancer cells (Chen *et al.* 2008).

1.6.1 Thiamine and TPP inhibit tumor growth in vivo

As detailed in Section 1.3.1, Comin-Anduix *et al.* demonstrated that supplementing mice with low doses of thiamine, ranging from 12.5-37.5 times the RDI, significantly stimulated tumor cell proliferation (Comin-Anduix *et al.* 2001). Strikingly, at higher doses of thiamine, 75-2500 times the RDI, there was no proliferative advantage to tumor growth (Comin-Anduix *et al.* 2001). At 2500 times RDI, a slight decrease in tumor cell proliferation was noted (Comin-Anduix *et al.* 2001). The effects of direct supplementation with TPP on tumor growth have also been assessed. Mice implanted with subcutaneous Erlichs tumor cells and dosed with hydroxyethylthiamine diphosphate through intraperitoneal injections demonstrated that the TPP derivative significantly reduced tumor volume by approximately 75% relative to control tumor-bearing mice (Gevorkian & Gambashidze 2013).

1.6.2 Mechanistic action of thiamine as chemotherapeutic strategy

Thiamine's mechanism of action for inhibiting tumor growth is considered to be directly related to tumor cell metabolism. As discussed in Section 1.2, tumor cells display an enhanced reliance on glycolytic metabolism compared to non-cancerous cells (Warburg *et al.* 1927; Cairns *et al.* 2011). Preferentially exploiting glycolysis provides tumor cells with a proliferative advantage by promoting the rapid generation of ATP and the necessary synthesis of macromolecules required to produce daughter cells (Cairns *et al.* 2011). Targeting the metabolic phenotype of tumor cells has seen a recent resurgence in scientific interest for developing novel chemotherapeutic strategies (Hirschey *et al.* 2015).

1.6.2.1 Current therapeutic strategies targeting tumor cell metabolism through glycolysis

Inhibiting glucose transport at the level of GLUT1 through the small molecule inhibitor WZB117 as well as through RNA interference (RNAi) has demonstrated anticancer activity *in vitro* and *in vivo* (Ooi & Gomperts 2015). In addition to glucose transport, the effectiveness of targeting the activity of key glycolytic enzymes for anticancer therapy has been explored. Hexokinase (HK), phosphofructokinase 2 (PFKFB3), and pyruvate kinase isoform M2 (PKM2) have all been validated as effective targets *in vitro* and *in vivo* (Martinez-Outschoorn *et al.* 2017). **Table 1.5** provides examples of current compounds and strategies under consideration to target tumor cell metabolism.

1.6.2.2 Dichloroacetate (DCA): targeting tumor cell metabolism through mitochondrial metabolism

In addition to directly inhibiting glycolysis, strategies have been developed to re-establish mitochondrial dependent metabolism in cancer cells. As discussed in section 1.2.1, pyruvate dehydrogenase kinases (PDKs) serve to phosphorylate pyruvate dehydrogenase (PDH) and blockade mitochondrial metabolism (Saunier *et al.* 2016). **Table 1.6** highlights the four known isoforms of PDK and details regarding each isoform's unique tissue specificity and PDH regulatory sites (Gudi *et al.* 1995; Kolobova *et al.* 2001). PDKs are overexpressed in multiple cancer types including breast, colon, gastric, glioblastoma, head and neck, hepatocellular

Table 1.5 Chemotherapeutic strategies targeting tumor cell metabolism

Compound	Target	Stage of Investigation	Observations	Reference
WZB117	GLUT1	Preclinical	Anticancer activity *in vitro* and *in vivo*	(Ooi & Gomperts 2015)
2-deoxyglucose	HK	Clinical	Anticancer activity *in vitro* and *in vivo*, but discontinued for clinical development	(Maschek *et al.* 2004; Dwarakanath *et al.* 2009; Martinez-Outschoorn *et al.* 2017)
Lonidamine	HK	Clinical	Anticancer activity *in vitro*, *in vivo,* and in Phase III clinical trial	(Cervantes-Madrid *et al.* 2015)
3-bromopyruvic acid	HK	Clinical	Anticancer activity *in vitro* and *in vivo*, but discontinued for clinical development	(Gong *et al.* 2014; Martinez-Outschoorn *et al.* 2017)
Methyl jasmonate	HK	Preclinical	Anticancer activity *in vitro* and *in vivo*	(Li *et al.* 2017)
PFK15/8	PFKFB3	Clinical	Anticancer activity *in vitro* and *in vivo,* ongoing clinical trial of PFK158	(Clem *et al.* 2013; Lu *et al.* 2017)
TLN-232	PKM2	Clinical	Anticancer activity *in vitro*, *in vivo,* and in Phase II clinical trial	(Hersey *et al.* 2009; Martinez-Outschoorn *et al.* 2017)
DCA	PDK1	Clinical	Anticancer activity *in vitro* and *in vivo*, ongoing Phase II clinical evaluation	(Michelakis *et al.* 2008)

Table adapted from (Martinez-Outschoorn *et al.* 2017)

Table 1.6 Isoform specific properties of pyruvate dehydrogenase kinases

Isoform	Tissue Expression	PDH Phosphorylation Residues	Cancer-Specific Expression
PDK1	Heart, pancreas	S293, S232, S300	Colon Gastric Glioblastoma Head and neck Hepatocellular carcinoma Lung Melanoma Myeloma Renal cell carcinoma
PDK2	Universal	S300, S232	Breast Colon Glioblastoma Head and neck Hepatocellular carcinoma Lung Melanoma
PDK3	Testis	S300, S232	Colon Glioblastoma Hepatocellular carcinoma Lung Melanoma
PDK4	Skeletal muscle, heart, and liver	S300, S232	Colon Glioblastoma Hepatocellular carcinoma Lung Melanoma

carcinoma, lung, melanoma, myeloma, and renal cell carcinoma (Zhang *et al.* 2015). Therefore, targeting PDKs has been the interest of recent pre-clinical and clinical investigation for developing anticancer strategies focused on restoring mitochondrial-dependent metabolism (Stacpoole 2017). DCA, a small-molecule inhibitor of PDK1, has demonstrated considerable pre-clinical efficacy. Treatment with DCA *in vitro* activates pyruvate dehydrogenase and increases mitochondrial conversion of pyruvate into acetyl-CoA (Michelakis *et al.* 2008). In cancerous cells, this leads to depolarization of the mitochondrial membrane, enhanced generation of ROS, and ultimately apoptotic cell death (Bonnet *et al.* 2007; Wong *et al.* 2008; Cao *et al.* 2008). In addition to reducing tumor cell proliferation *in vitro*, DCA significantly reduces *in vivo* tumor growth (Bonnet *et al.* 2007). Despite its promising pre-clinical results, a recent phase II clinical trial for DCA in treating recurring metastatic breast and non-small-cell lung cancer was terminated early due to higher than expected safety concerns (Martinez-Outschoorn *et al.* 2017).

1.6.2.3 Thiamine inhibits tumor cell proliferation through a DCA-like mechanism

Like DCA, high-dose thiamine supplementation has recently been demonstrated to promote mitochondrial-dependent metabolism in tumor cells (Hanberry *et al.* 2014; Liu *et al.* 2018). Interestingly, in a direct comparison of two compounds, thiamine exhibited a lower IC50 value than DCA (Hanberry *et al.* 2014). Treatment with thiamine in low millimolar concentrations (IC50 ~5mM) results in a reduction of tumor cell proliferation, which corresponds with a reduction in total PDH-phosphorylation (Hanberry *et al.* 2014). Following thiamine treatment, tumor cells consume less glucose

and produce reduced levels of lactate (Hanberry *et al.* 2014). These findings are corroborated by a second study demonstrating that thiamine supplementation promotes PDH activity and increases oxygen consumption in malignant cells (Liu *et al.* 2018). Although thiamine treatment results in mitochondrial membrane depolarization and activation of pro-apoptotic pathways similar to DCA, no increase in ROS production following thiamine treatment has been observed (Hanberry *et al.* 2014). This suggests there may be subtle differences between the mechanistic actions for DCA and thiamine requiring further elucidation.

More evidence is also required to elucidate the mechanism governing thiamine's effect on PDH phosphorylation. It currently remains unclear if thiamine or one of its phosphate ester metabolites serves as the active species mediating reduced PDH phosphorylation. Some evidence points to TPP as the mediator, which may function through direct PDK inhibition. Pyruvate, the primary substrate of PDH, directly binds to inhibit PDK activity, therefore serving as a physiological inhibitor to aid in the regulation of PDH function (Hucho *et al.* 1972) In addition to pyruvate, PDK activity is inhibited by other physiological molecules including ADP, NAD^+, and CoA-SH (Saunier *et al.* 2016). TPP possesses structural similarity to ADP and has been demonstrated to mimic ADP binding *ex vitro* (McLure *et al.* 2004). Furthermore, a presumable change in confirmation caused by the cofactor association of TPP to the E1 subunit of PDH has been shown to mediate the rate at which PDK isoforms can phosphorylate PDH (Kolobova *et al.* 2001). Therefore, TPP bound to form holoenzyme dictates the total amount of phosphate incorporated into the regulatory sites of PDH (Kolobova *et al.* 2001).

1.6.2.4 Limitations of thiamine as an anticancer strategy

Clinical research in healthy adults and patients suffering diseases other than cancer demonstrates that supplementation with high-dose thiamine is generally well-tolerated. Other than nausea and indigestion, no overt symptoms of toxicity were noted following administration of pharmacological thiamine at doses of 3-8 g/day over a 1 year period of time (Meador *et al.* 1993). Oral administration of pharmacologic thiamine three times per day over the course of 2-12 months has been reported in multiple studies with no reported adverse effects (Meador *et al.* 1993; Blass *et al.* 1988; Nolan *et al.* 1991). Instead, the overall bioavailability of thiamine is expected to be the primary limitation associated with its use as a chemotherapeutic strategy. Primary absorption of thiamine occurs *via* a saturable active transport mechanism driven by THTR1 and THTR2, although passive transport of the molecule has been demonstrated at high concentrations (Davis & Icke 1983; Smithline *et al.* 2012). Based on this mechanism and a slow rate of absorption, thiamine (thiamine hydrochloride) has an extremely low bioavailability estimated between 3.7 and 5.3% (Weber & Kewitz 1985; Tallaksen *et al.* 1993).

1.6.2.5 Synthetic thiamine analogs as alternative chemotherapeutic treatments

Commercially available synthetic analogs of thiamine including benfotiamine and sulbutiamine demonstrate higher bioavailability than thiamine (Volvert *et al.* 2008b). S-benzoylthiamine O-monophosphate, or benfotiamine, is an S-acyl derivative of thiamine. Following dephosphorylation by alkaline phosphatases present at the brush border membrane, S-benzoylthiamine diffuses through membranes of intestinal and epithelial cells and accumulates within the bloodstream (Volvert *et al.* 2008b). Within erythrocytes,

S-benzoylthiamine is converted to free thiamine through non-enzymatic transfer of its S-benzoyl group to SH groups of glutathione (Wada *et al.* 1961; Volvert *et al.* 2008b). After opening of their respective thiazole rings, two molecules of thiamine are joined by a disulfide bridge to form O-isobutyrylthiamine disulfide, or sulbutiamine (Bettendorff *et al.* 1990b). To further decrease polarity, an isobutyryl functional group is attached to the alcohol moiety of each thiamine ring (Bettendorff *et al.* 1990b). The lipophilic nature of sulbutiamine yields the ability of the molecule to pass all biological membranes where subsequent intracellular conversion to independent thiamine molecules occurs (Bettendorff 1994b). Compared to an equivalent dose of thiamine, independent oral administration of both benfotiamine and sulbutiamine leads to a greater accumulation of thiamine, TPP, and TTP (Volvert *et al.* 2008b; Bettendorff *et al.* 1990b). Therefore, both benfotiamine and sulbutiamine may be effective means to elevate intracellular levels of thiamine and its phosphate ester metabolites for PDK-targeted cancer therapy. Benfotiamine has already been shown to possess anti-tumor activity *in vitro*, but through an unrelated mechanism of action corresponding with parapoptotic cell death mediated through JNK1/2 activation in leukemia cells (Sugimori *et al.* 2015).

1.7 Research rationale and goal

As described above, many lines of evidence suggest supplemental thiamine demonstrates a dose-dependent, biphasic impact on malignant growth. Thiamine's effects in promoting and inhibiting tumor cell proliferation appear to occur through independent mechanisms and suggest non-canonical intracellular actions of the vitamin outside its canonical function as a cofactor. However, the complete mechanism of action for thiamine in each context remains unknown. Therefore, the objective of this work is two-fold:

1. We aim to understand how moderate thiamine supplementation provides tumor cells a proliferative advantage in relation to the exploitation of critical thiamine homeostasis genes during malignancy.
2. We aim to determine the impact of exploiting synthetic thiamine analogs to maximize thiamine bioavailability on thiamine's chemotherapeutic effect both *in vitro* and *in vivo*. Furthermore, we aim to identify the active thiamine species mediating its anticancer property through the inhibition of PDH-phosphorylation.

CHAPTER 2

THE ADAPTIVE REGULATION OF THIAMINE PYROPHOSPHOKINASE-1 FACILITATES MALIGNANT GROWTH DURING SUPPLEMENTAL THIAMINE CONDTIONS[1]

[1]Jonus, H.C., Hanberry, B.S., Khatu, S., Kim, J., Luesch, H., Dang, L.H., Bartlett, M.G., and Zastre, J.A. Accepted by *Oncotarget*. Reprinted here with permission of publisher.

2.1 Overview

The beginning of this chapter comprises a manuscript that has been accepted for publication in the journal *Oncotarget*. The manuscript is provided in the publisher's formatting, and therefore, the discussion for this chapter preludes the provided Materials and Methods section. Supplemental figures and tables from the publication are provided within the main text for the reader's ease of access.

Previous work has demonstrated that thiamine supplementation promotes tumor growth and that thiamine homeostasis may be altered during malignancy. Therefore, the overarching goal for this manuscript was to elucidate a mechanism for how increasing intracellular thiamine may be advantageous to tumor cell proliferation. In so, we identified the adaptive regulation of TPK1 in response to hypoxic and oxidative stress in tumor cells. Our results suggest that the adaptive response of TPK1 during malignant stress may maintain TPP production and balance redox homeostasis. It appears that TPK1 expression serves as a key component in facilitating tumor growth during supplemental thiamine conditions. In addition to the manuscript, unpublished supplemental data provided at the end of the chapter further details a HIF-dependent mechanism for the translational regulation of TPK1 in tumor cells. We also provide evidence for the adaptive regulation of TPK1 in response to hypoxia using a non-cancerous *in vitro* cell culture model. To our knowledge, the data presented within this chapter stands as the first report detailing the adaptive regulation of TPK1 in response to hypoxic stress under both malignant and non-malignant conditions. This supports that increasing TPK1 expression may be a universal response to cellular stress.

2.2 Abstract

Supplemental levels of vitamin B1 (thiamine) have been implicated in tumor progression. Tumor cells adaptively up-regulate thiamine transport during hypoxic stress. Upon uptake, thiamine pyrophosphokinase-1 (TPK1) facilitates the rapid phosphorylation of thiamine into thiamine pyrophosphate (TPP). However, the regulation of TPK1 during hypoxic stress is undefined. Understanding how thiamine homeostasis changes during hypoxia will provide critical insight into the malignant advantage supplemental thiamine may provide cancer cells. Using Western blot analysis and RT-PCR, we have demonstrated the post-transcriptional up-regulation of TPK1 in cancer cells following hypoxic exposure. TPK1 expression was also adaptively up-regulated following alterations of redox status by chemotherapeutic and antioxidant treatments. Although TPK1 was functionally up-regulated by hypoxia, HPLC analysis revealed a reduction in intracellular TPP levels. This loss was reversed by treatment with cell-permeable antioxidants and corresponded with reduced ROS production and enhanced cellular proliferation during supplemental thiamine conditions. siRNA-mediated knockdown of TPK1 directly enhanced basal ROS levels and reduced tumor cell proliferation. These findings suggest that the adaptive regulation of TPK1 may be an essential component in the cellular response to oxidative stress, and that during supplemental thiamine conditions its expression may be exploited by tumor cells for a redox advantage contributing to tumor progression.

2.3 Introduction

Malignant cells experience elevated levels of reactive oxygen species (ROS) during tumor progression. This multifactorial byproduct arises from events including enhanced metabolic activity, increased activity of ROS-producing enzymes, dysfunctional mitochondrial metabolism, activated immune responses, intratumoral hypoxia, and therapeutic intervention (i.e. chemotherapeutics, ionizing radiation) (Liou & Storz 2010; Gorrini *et al.* 2013). Moderate increases in ROS are tumorigenic as oxygen radicals provide critical secondary messengers in oncogenic signaling cascades and promote genomic instability. However, a delicate balance of ROS must be maintained as excessive levels cause damage to DNA, proteins, and lipids resulting in cellular senescence and apoptosis (Liou & Storz 2010). During tumor initiation and progression, antioxidant pathways are up-regulated aiding to limit the damaging effects of ROS (Gorrini *et al.* 2013). For example, constitutive activation of the transcription factor Nuclear factor erythroid-2–related factor 2 (NRF2) in tumor cells up-regulates levels of the endogenous glutathione machinery, promoting tumorigenicity (Singh *et al.* 2008). In hypoxic tumor microenvironments, the stabilization of the oncogenic transcription factor hypoxia-inducible factor-1α (HIF-1α) regulates the expression of pyruvate dehydrogenase kinase-1 (PDK1), which subsequently limits ROS production by phosphorylating pyruvate dehydrogenase (PDH) and restricting mitochondrial metabolism (Kim *et al.* 2006). siRNA-mediated silencing of HIF-1α increases intracellular ROS levels both *in vitro* and *in vivo*, highlighting HIF-1α's critical role in ROS maintenance (Gu *et al.* 2017).

Tumor cells may also exploit dietary antioxidants as an alternative means to balance intracellular redox status (Gorrini *et al.* 2013). Dietary antioxidants, which consist of a broad range of molecular classes including polyphenols, carotenoids, and tocopherols, commonly exist in the form of vitamins (Borek 2017). Vitamin E and its cell-permeable mimetic Trolox have been demonstrated to accelerate tumor progression *in vivo* and enhance the migrative and invasive properties of tumor cells *in vitro* (Sayin *et al.* 2014; Le Gal *et al.* 2015). Supplemental vitamin E also protects against protein oxidation during hypoxia and hypoglycemia induced oxidative stress (Shahrzad *et al.* 2005). Vitamin B1 (thiamine) and its activated cofactor form, thiamine pyrophosphate (diphosphate; TPP) have also exhibited antioxidant activity and can suppress the generation of superoxide, hydroperoxide, and hydroxyl radicals (Okai *et al.* 2007). Supplemental doses of thiamine can promote the *in vivo* growth of malignant tumors (Comin-Anduix *et al.* 2001; Daily *et al.* 2012). The uptake of vitamin B1, or thiamine, was recently demonstrated to be adaptively up-regulated in tumor cells during hypoxic stress, but it remains unclear how increasing intracellular thiamine could be advantageous to hypoxic tumor cells (Sweet *et al.* 2010).

As an essential micronutrient, thiamine must be obtained from the diet to maintain metabolism in all cells. The Solute Carrier (SLC) transporters THTR1 (*SLC19A2*) and THTR2 (*SLC19A3*) facilitate the absorption and cellular uptake of thiamine from the plasma (Ganapathy *et al.* 2004). Thiamine pyrophosphokinase-1 (TPK1) subsequently phosphorylates thiamine into TPP (Nosaka *et al.* 2001). The resulting TPP moiety canonically functions as a required cofactor for multiple enzymes in the metabolic network including PDH, α-ketoglutarate dehydrogenase (OGDH), and transketolase

(TKT). Of these, up-regulation of TKT occurs in cancerous tissue and promotes tumor progression (Xu *et al.* 2016; Ricciardelli *et al.* 2015). TKT links glycolysis with the pentose phosphate pathway (PPP) by catalyzing a reversible reaction that produces ribose-5-phosphate for use in nucleotide synthesis essential to tumor cell proliferation. Boros *et al.* found that malignant cells generate 85% of their necessary ribose through the non-oxidative portion of the PPP (Boros *et al.* 1997). The activity of TKT within the PPP also facilitates the maintenance of NADPH pools and balance of the cellular redox status (Xu *et al.* 2016). Though the functionality remains unresolved, TKT expression has been shown to increase ~15-fold in hypoxia (Haseloff *et al.* 2006). Therefore, increasing thiamine supply during hypoxia may support TKT activity in a canonical cofactor fashion. Alternatively, thiamine as well as TPP may serve other non-canonical functions during hypoxic stress potentially as antioxidants.

We have previously established an increase in the expression of *SLC19A2* and *TPK1* in breast cancer tissue when compared to normal breast tissue (Zastre *et al.* 2013a). Furthermore, HIF-1α directly transactivates the adaptive expression of *SLC19A3* and enhances thiamine uptake during hypoxic stress (Sweet *et al.* 2010; Zera *et al.* 2016). Despite thiamine's implicit requirement for cellular metabolism within hypoxic tumor microenvironments, how changes in thiamine homeostasis impact malignant progression remain unclear. Tiwana *et al.* recently demonstrated TPK1, the enzyme responsible for the production of TPP, as a critical component of tumor cell survival following exposure to ionizing radiation (Tiwana *et al.* 2015). Unfortunately, there exists limited knowledge regarding the regulation of TPK1 in cancer cells and how thiamine supplementation functions to enhance malignant progression.

2.4 Results

2.4.1 Induction of TPK1 protein during hypoxia correlates with HIF-1α

TPK1 expression increased following 24, 48, and 72 h exposure to 1% O_2 in an array of cancer cell lines from multiple tissue origins including breast (MCF7, MDA-MB-231), brain (LN 18, U-87 MG), and intestine (Caco-2, HCT 116, HuTu 80) **(Fig 2.1A)**. To establish the role of HIF-1α in the regulation of TPK1, we utilized HCT 116 cells since an isogenic HIF-1$\alpha^{-/-}$ knockout was previously developed in this cell line. Wild type and HIF-1$\alpha^{-/-}$ HCT 116 cells were exposed to either 1% O_2 or the prolyl hydroxylase inhibitor DMOG for 24 h. In wild type cells, DMOG and 1% O_2 resulted in the stabilization of HIF-1α and the ~2 and 3-fold induction of TPK1, respectively **(Fig 2.1B and 2.1C)**. DMOG and 1% O_2 treatment also resulted in the induction of LDHA protein expression in wild type cells, confirming the transcriptional functionality of HIF-1α **(Fig 2.1B)**. In contrast to wild type, HIF-1$\alpha^{-/-}$ cells demonstrated no induction of TPK1 or LDHA protein following treatment with DMOG or 1% O_2 **(Fig 2.1B and 2.1D)**.

To further confirm a role for HIF-1α in mediating TPK1 expression, we utilized a constitutively active form of HIF-1α (HIF-1α CA) kindly provided by Dr. Hayakawa (Fujino *et al.* 2009). Wild type cells transfected with HIF-1α CA demonstrated an ~2-fold increase in TPK1 expression **(Fig 2.1E and 2.1F)**. Transfection of HIF-1$\alpha^{-/-}$ cells with the HIF-1α CA resulted in an ~4-fold enhancement in TPK1 protein expression **(Fig 2.1E and 2.1G)**.

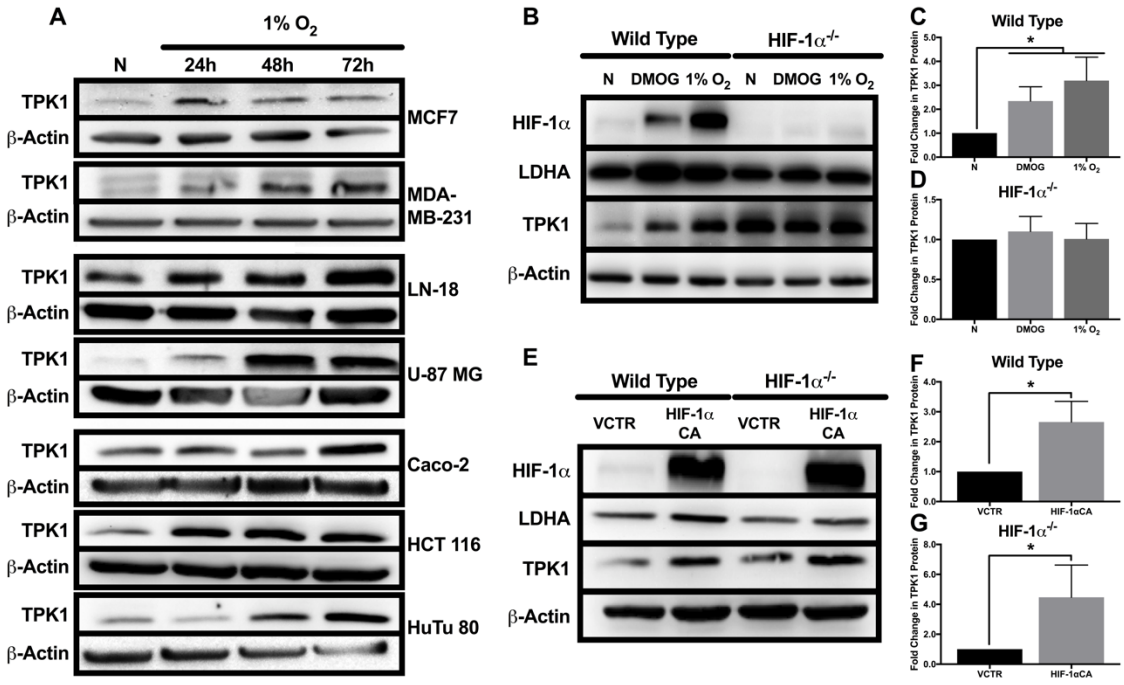


Fig 2.1. Effect of hypoxic stress and HIF-1α on TPK1 expression (A) Representative Western blots demonstrating TPK1 protein expression in WCLs isolated from seven tumor cell lines with tissue origins including breast (MCF7, MDA-MB-231), brain (LN-18, U-87 MG), and intestine (Caco-2, HCT 116, HuTu 80) following treatment with 1% O_2 for 24, 48, and 72 h relative to normoxic control (N). β-Actin expression serves as the loading control. **(B)** Representative Western blots demonstrating HIF-1α, LDHA, and TPK1 protein expression in WCLs isolated from wild type and HIF-1$\alpha^{-/-}$ HCT 116 cells seeded at 1250 cells/cm^2 and treated with 150 μM DMOG or 1% O_2 for 24 h relative to normoxic control (N). **(C,D)** Densitometry analysis of the fold change in TPK1 expression +/- standard deviation (SD) following DMOG and 1% O_2 treatment in wildtype and HIF-1$\alpha^{-/-}$ HCT 116 cells compared to normoxic control (N) including *n = 4* independent experiments for wild type and *n = 3* independent experiments in HIF-1$\alpha^{-/-}$ cells. **(E)** Representative Western blots demonstrating HIF-1α, LDHA, and TPK1 protein expression in WCLs isolated from wild type and HIF-1$\alpha^{-/-}$ HCT 116 cells seeded at 2500 cells/cm^2 and transfected with 2.5 μg of HIF-1α CA plasmid DNA relative to vector control (VCTR) for 72 h. **(F,G)** Densitometry analysis of the fold change in TPK1 expression +/- SD following HIF-1α CA overexpression in wildtype and HIF-1$\alpha^{-/-}$ HCT 116 cells compared to vector control including *n = 3* independent experiments. (★) Represents statistically significant difference ($p<0.05$) based on results of (C,D) one-way ANOVA with Tukey's post-hoc test or (F,G) unpaired student's t-test.

2.4.2 Pharmacological inhibition of HIF-1α and reoxygenation attenuates TPK1 up-regulation during hypoxia

To establish the effects of HIF-1α inhibition on TPK1 expression in hypoxia, we employed YC-1 as a pharmacological means to reduce both HIF-1α protein stabilization and its functional activity (Li *et al.* 2008). Treatment of HCT 116 cells with YC-1 resulted in the reduced expression of HIF-1α and its target gene LDHA in hypoxia **(Fig 2.2A)**. YC-1 treatment also decreased basal expression of HIF-1α during normal oxygen conditions **(Fig 2.2A)**. Inhibition of HIF-1α activity by YC-1 significantly attenuated the up-regulation of TPK1 during hypoxic stress **(Fig 2.2A and 2.2B)**.

To determine the dynamics of TPK1 regulation following hypoxic stress, HCT 116 cells were re-oxygenated (21% O_2) after 48 h of 1% O_2 exposure. We observed a loss of HIF-1α and a trending reduction of TPK1 protein expression within 4 h of re-oxygenation **(Fig 2.2C and 2.2D)**. After 24 h of re-oxygenation, restoration of TPK1 expression back to the basal normoxic level was achieved **(Fig 2.2C and 2.2D)**.

2.4.3 Increased TPK1 expression during hypoxic stress lacks transcriptional induction

In contrast to the increase in TPK1 protein during hypoxia, no increase in mRNA expression of *TPK1* (combined variant 1 and 2) was observed after 24, 48, or 72 h of 1% O_2 treatment in HCT 116 cells **(Fig 2.3A)**. Instead, these treatments resulted in a slight, but significant decrease in *TPK1* transcript expression **(Fig 2.3A)**. Consistent with the hypoxic driven transcriptional activity of HIF-1α, a significant increase in the mRNA of three well-defined gene targets, including *SLC2A1*, *LDHA*, and *VEGF* was observed **(Fig 2.3A)**. Treatment of HCT 116 cells with the hypoxia mimetic DMOG for 24 h

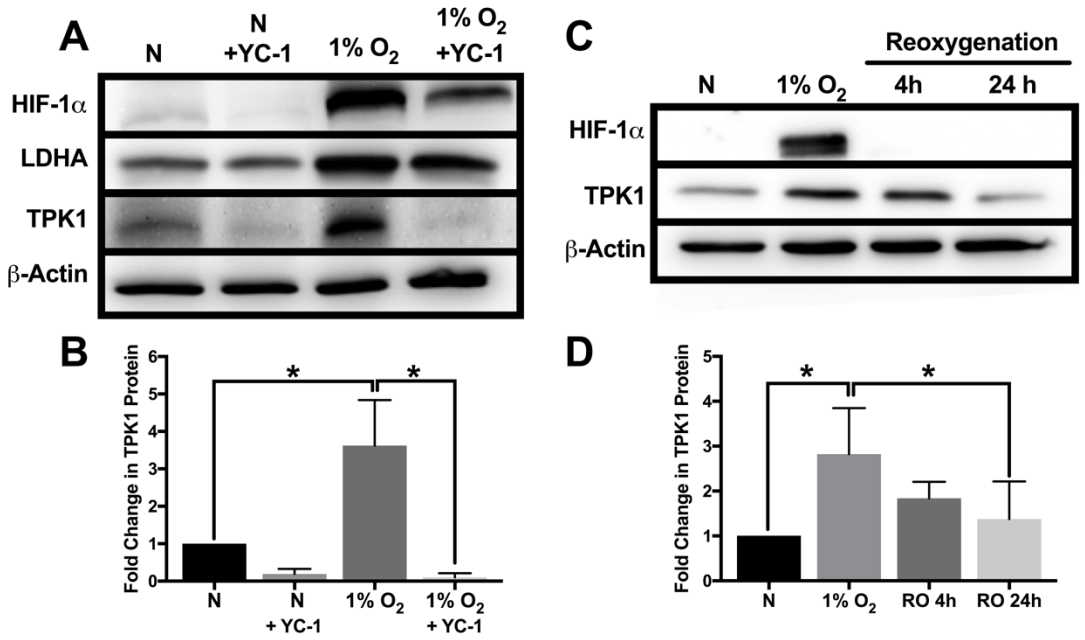


Fig 2.2. Attenuation of TPK1 expression using pharmacological inhibition of HIF-1α and reoxygenation (A) Representative Western blots demonstrating HIF-1α, LDHA, and TPK1 protein expression under normoxic (N) and hypoxic conditions +/- YC-1 in WCLs isolated from wild type HCT 116 cells seeded at 2500 cells/cm^2 and pre-treated with 5 μM YC-1 for 24 h prior to 48 h hypoxic exposure in the presence of 5 μM YC-1. β-Actin expression serves as the loading control. **(B)** Densitometry analysis of the fold change in TPK1 expression +/- SD following exposure to 1% O_2 in the presence or absence of YC-1 for wildtype HCT 116 cells compared to untreated normoxic control (N) including *n = 3* independent experiments. **(C)** Representative Western blots demonstrating HIF-1α and TPK1 in WCLs isolated from wild type HCT 116 cells seeded at 1250 cells/cm^2 and treated in 1% O_2 for 48 h with subsequent reoxygenation at 21% O_2 for 4 and 24 h. **(D)** Densitometry analysis of the fold change in TPK1 expression +/- SD following exposure to 1% O_2 and subsequent reoxygenation in wildtype HCT 116 cells compared to untreated normoxic control (N) including *n = 4* independent experiments. (★) Represents statistically significant difference ($p<0.05$) based on results of one-way ANOVA with Tukey's post-hoc test.

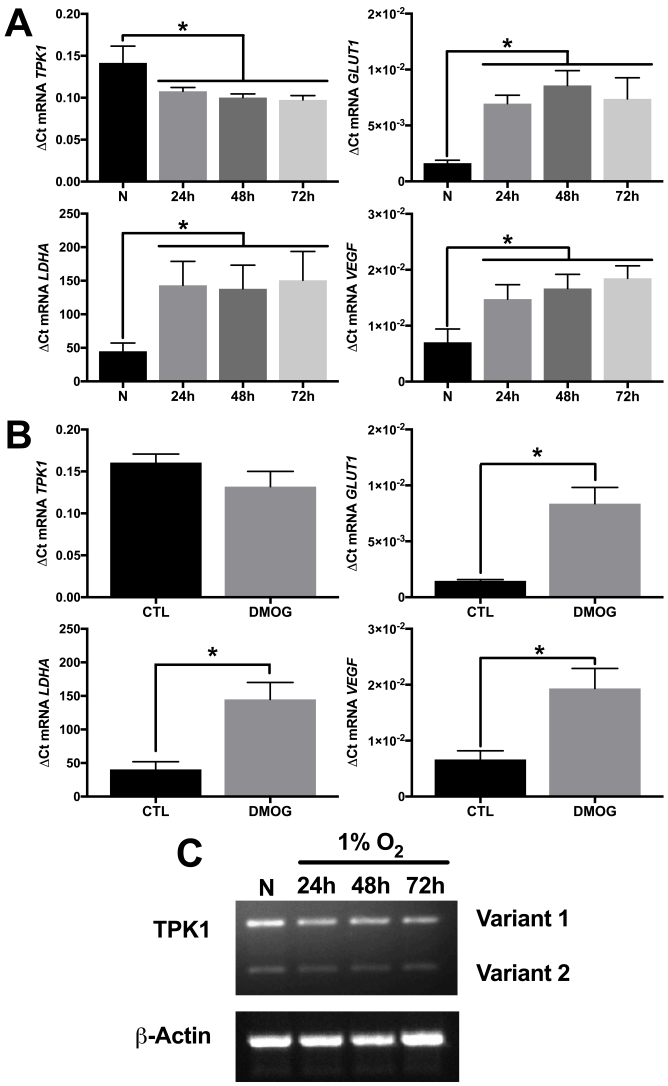


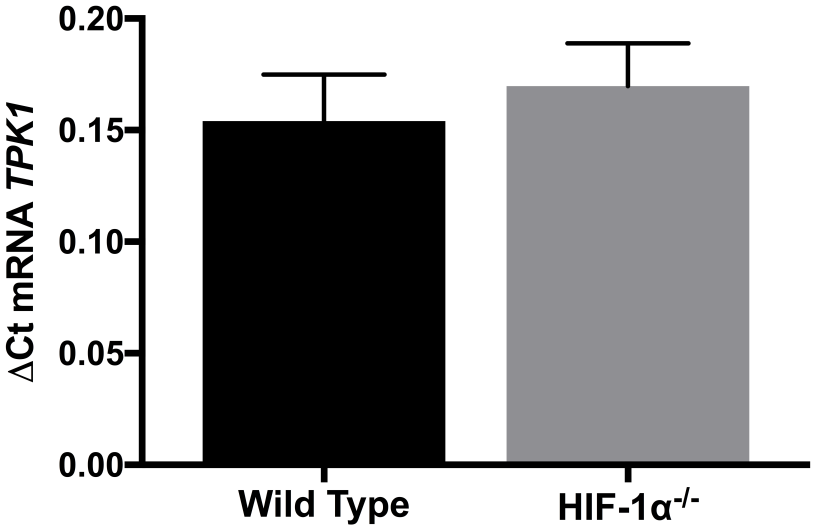
Fig 2.3. Increase in TPK1 protein expression lacks transcriptional involvement (A) Relative mRNA expression levels of *TPK1*, *SLC2A1*, *LDHA*, and *VEGF* determined by qRT-PCR analysis in wild type HCT 116 seeded at 1250 cells/cm^2 and treated in 1% O_2 for 24, 48, and 72 h relative to normoxic control (N) representative of *n = 4* independent experiments normalized by the $2^{-\Delta Ct}$ method. **(B)** Relative mRNA expression levels of *TPK1*, *SLC2A1*, *LDHA*, and *VEGF* determined by qRT-PCR analysis in wild type HCT 116 cells seeded at 1250 cells/cm^2 and treated with 150 μM DMOG for 24 h relative to untreated control (CTL) representative of *n = 3* independent experiments normalized by the $2^{-\Delta Ct}$ method. **(C)** Qualitative *TPK1* splice variant expression (1 and 2) in wild type HCT 116 cells during normoxic (N) and 24, 48, and 72 h 1% O_2 exposure. *β-Actin* expression serves as the loading control. (✶) Represents statistically significant difference ($p<0.05$) based on results of (A) one-way ANOVA with Tukey's post-hoc test or (B) unpaired student's t-test.

demonstrated a similar lack of mRNA induction for *TPK1* and a significant increase in the transcription of the HIF-1α target genes *SLC2A1*, *LDHA*, and *VEGF* **(Fig 2.3B)**. Since our primers designed for the quantitative detection of TPK1 gene expression simultaneously detect both splice variant 1 and 2, we developed alternative primers to independently analyze the expression of both variants. Qualitative analysis of the individual expression of both *TPK1* genetic splice variants in HCT 116 cells further confirmed that neither variant 1 nor variant 2 was up-regulated during hypoxic exposure up to 72 h **(Fig 2.3C)**.

2.4.4 HIF-1α independent induction of TPK1 involves ROS

While establishing the differential regulation of TPK1 in wild type and HIF-1$\alpha^{-/-}$ HCT 116 cells, we noted a significant difference in the basal level of TPK1 protein between these two isogenic cell lines **(Fig 2.1B and 2.4A)**. HIF-1$\alpha^{-/-}$ HCT 116 cells exhibited an ~4-fold increase in TPK1 protein compared to wild type **(Fig 2.4B)**. Despite significant up-regulation of TPK1 protein following HIF-1α knockout, we found no change in TPK1 mRNA expression between wild type and HIF-1$\alpha^{-/-}$ HCT 116 cells **(Supplemental Fig 2.1)**. It was previously reported that the genetic knockdown of HIF-1α results in increased levels of ROS (Gu *et al.* 2017). Consistent with these findings, we demonstrate that the knockout of HIF-1α in HCT 116 cells resulted in an ~1.5-fold increase in ROS **(Fig 2.4C)**.

To determine the potential influence of ROS on TPK1 expression, we cultured HIF-1$\alpha^{-/-}$ HCT 116 cells with the antioxidants N-acetylcysteine (NAC) or ascorbate (ASC) for 24 and 48 h. NAC and ASC supplementation have both been previously



Supplemental Fig 2.1. mRNA expression of TPK1 in wild type and HIF-1α$^{-/-}$ HCT 116 cells Relative mRNA expression level of *TPK1* determined by qRT-PCR analysis in wild type and HIF-1α$^{-/-}$ HCT 116 cells seeded at 1250 cells/cm^2 and cultured for 96 h normalized by the $2^{-\Delta Ct}$ method.

demonstrated to reduce tumor cell ROS levels *in vitro* (Kim *et al.* 2008; Yadav *et al.* 2015). Following 48 h of treatment, both NAC and ASC supplementation significantly reduced basal TPK1 expression in HIF-1$\alpha^{-/-}$ cells to comparable levels observed in wild type cells **(Fig 2.4D and 2.4E)**. Since antioxidant treatment of HIF-1$\alpha^{-/-}$ HCT 116 reduced the level of TPK1 protein, we tested whether the induction of ROS in wild type cells would induce TPK1 expression. Wild type HCT 116 cells were treated with two chemotherapeutics, doxorubicin (DOX) and cisplatin (CDDP), previously demonstrated to generate ROS (Lupertz *et al.* 2010; Marullo *et al.* 2013). Cells were also treated with Antimycin A (AA), which inhibits complex III of the electron transport chain to elevate ROS and the oxidant *tert*-butyl hydroperoxide (TBHP) for 24 h. Each treatment significantly induced the expression of TPK1 compared to control **(Fig 2.4F and 2.4G)**.

2.4.5 Thiamine pyrophosphate production and consumption supports antioxidant function

To test the functionality of TPK1 in HCT 116 cells after hypoxic treatment and in HIF-1$\alpha^{-/-}$ cells, we used an *ex vitro* functionality assay. As a positive control that the assay system was capable of producing TPP, we overexpressed TPK1 in HCT 116 cells. TPK1 overexpression resulted in an ~200-fold increase in the amount of TPP produced compared to control cells **(Supplemental Fig 2.2)**. A significant increase in TPP production in HIF-1$\alpha^{-/-}$ cells relative to wild type as well as in hypoxia treated HCT 116 wild type cells relative to normoxic control was observed confirming the functional increase in TPK1 expression **(Fig 2.5A)**. Although HIF-1$\alpha^{-/-}$ HCT 116 cells demonstrated

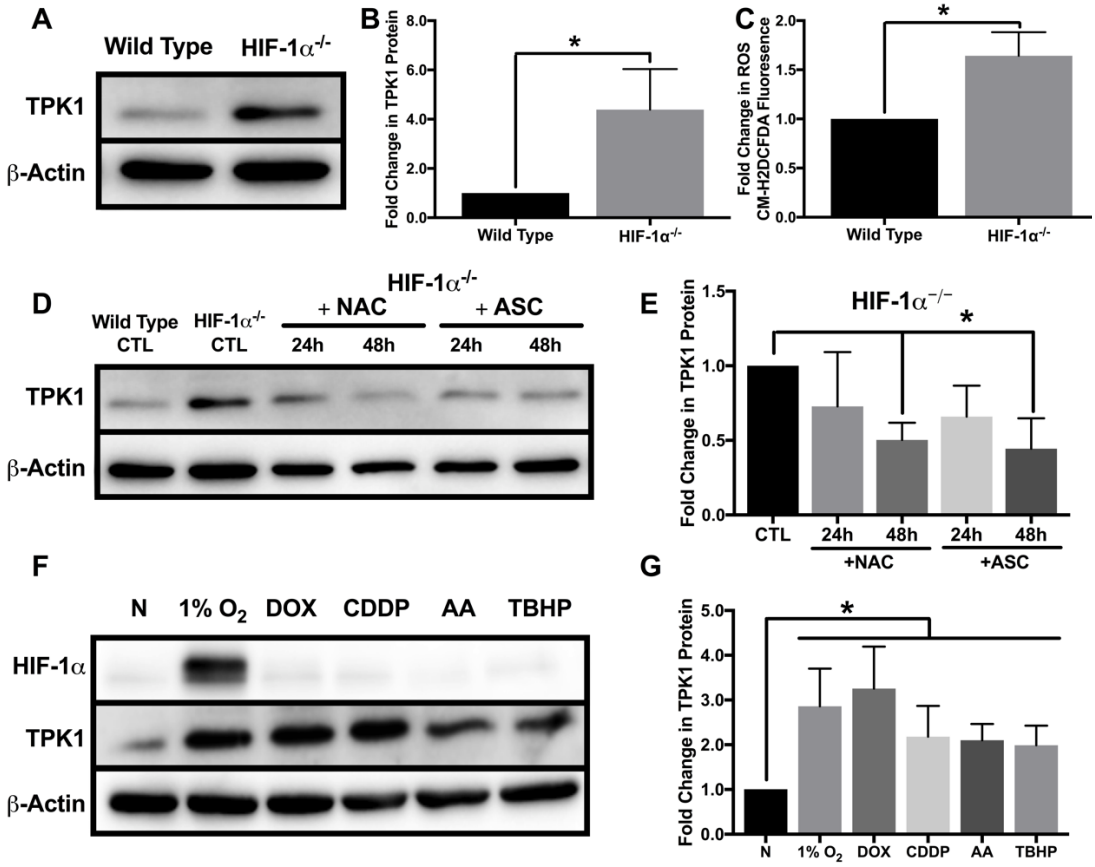


Fig 2.4. Oxidative stress mediated regulation of TPK1 (A) Representative Western blot demonstrating TPK1 expression in WCLs isolated from isogenic wild type and HIF-1$\alpha^{-/-}$ HCT 116 cells. β-Actin expression serves as the loading control. **(B)** Densitometry analysis of the fold change in TPK1 expression +/- SD for HIF-1$\alpha^{-/-}$ HCT 116 compared to wild type HCT 116 cells including *n = 5* independent experiments. **(C)** Fold change in CM-H2DCFDA median fluorescence intensity +/- SD demonstrating fold change in ROS levels between HIF-1$\alpha^{-/-}$ relative to wild type HCT 116 cells including *n = 4* independent experiments. **(D)** Representative Western blot demonstrating TPK1 expression in WCLs isolated from HIF-1$\alpha^{-/-}$ HCT 116 cells seeded at 1250 cells/cm^2 and treated with 1 mM NAC or 100 μM ASC for 24 and 48 h relative to untreated HIF-1$\alpha^{-/-}$ HCT 116 CTL (expression of TPK1 in wild type cells provided for comparison). **(E)** Densitometry analysis of the fold change in TPK1 expression +/- SD for HIF-1$\alpha^{-/-}$ HCT 116 cells treated with NAC and ASC compared to untreated HIF-1$\alpha^{-/-}$ HCT 116 control including *n = 4* independent experiments. **(F)** Representative Western blots demonstrating HIF-1α and TPK1 protein expression in WCLs isolated from wild type HCT 116 cells seeded at 1250 cells/cm^2 and treated with 1% O_2, 0.1 μM DOX, 10 μM CDDP, 5 μM AA or 10 μM TBHP for 24 h relative to normoxic control (N). **(G)** Densitometry analysis of the fold change in TPK1 expression +/- SD for wild type HCT 116 cells treated with 1% O_2,

DOX, CDDP, AA or TBHP compared to untreated normoxic control (N) including *n = 6* independent experiments. (★) Represents statistically significant difference ($p<0.05$) using (B, C) an unpaired student's t-test or (E,G) one-way ANOVA with Tukey's post-hoc test.

a greater ability to produce TPP *ex vitro* relative to wild type cells, no significant differences in the intracellular levels of thiamine or TPP between the two cell lines were detected **(Fig 2.5B, Supplemental Fig 2.3)**. However, significant decreases in thiamine and TPP levels were observed in HCT 116 cells treated with 1% O_2 for 24 h compared to normoxic conditions **(Fig 2.5B, Supplemental Fig 2.4A)**.

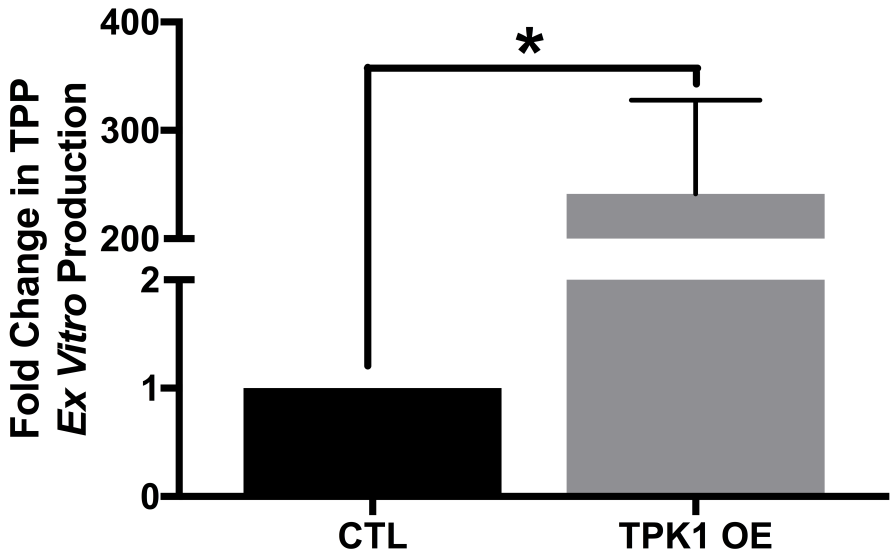
To assess changes in TPP during hypoxic stress, HCT 116 cells were treated with sodium fluoride (NaF) and methotrexate (MTX). As a cell permeable phosphatase inhibitor, NaF was used to limit dephosporylation of TPP to either thiamine monophosphate (TMP) or thiamine. MTX was used as a recognized inhibitor of the Reduced Folate Carrier (*SLC19A1*) transporter, which was previously demonstrated to function in the extracellular transport of TPP (Zhao *et al.* 2001b). Neither of these treatments significantly preserved or accumulated thiamine, TMP, or TPP levels in hypoxic HCT 116 cells **(Fig 2.5C, Supplemental Fig 2.4A+B)**. Alternatively, we considered that TPP may be consumed as an intracellular antioxidant. **Figure 2.5D** demonstrates that treatment with two different cell permeable antioxidants Trolox (TRLX) and MitoQuinone (MitoQ), resulted in a significant preservation of TPP during hypoxia in HCT 116 cells. TRLX treatment also significantly increased intracellular thiamine concentration under the same conditions **(Supplemental Fig 2.4A)**.

Considering intracellular TPP concentrations may be highly dynamic, HCT 116 cells were next cultured in a more physiologically relevant concentration of thiamine (10

nM) to assess if the dose of thiamine has any effect on TPP homeostasis. Culturing cells in the supplemental 3 μM dose of thiamine lead to significantly higher intracellular thiamine and TPP levels under normoxic conditions compared to cells cultured with 10 nM thiamine **(Fig 2.5E, Supplemental Fig 2.5)**. The exposure of cells cultured in 10 nM thiamine to 1% O_2 demonstrated no significant difference in intracellular TPP levels compared to those cultured in normoxia **(Fig 2.5E)**. However, a significant loss in TPP was observed for cells cultured with 3 μM thiamine in 1% O_2 compared to normoxic conditions **(Fig 2.5E)**. We next questioned whether altering the intracellular level of TPP through supplemental thiamine influences hypoxia induced-ROS levels. When HCT 116 cells were placed in 1% O_2 for 48 h, there was a significant reduction in ROS for cells grown in the presence of 3 μM thiamine compared to 10 nM thiamine **(Fig 2.5F)**. A significant increase in HCT 116 proliferation during hypoxic stress was also observed when supplemented with 3 μM thiamine compared to 10 nM thiamine **(Fig 2.5G)**.

2.4.6 TPK1 facilitates cellular proliferation in the presence of supplemental thiamine

HCT 116 cells were also used to define the role of TPK1 expression in tumor cell proliferation with supplemental thiamine levels. Cells were cultured in either the physiological thiamine level of 10 nM or the supplemental dose of 3 μM, and under these conditions basal ROS levels decreased with 3 μM compared to 10 nM thiamine **(Fig 2.6A)**. In addition, HCT 116 cells cultured in 3 μM thiamine proliferated more rapidly than those grown in 10 nM thiamine **(Fig 2.6B)**. To understand how these effects may relate to TPK1 expression, a validated siRNA construct was utilized to mediate the



Supplemental Fig 2.2. *Ex Vitro* functionality of TPK1 overexpression in HCT 116 cells HPLC analysis demonstrating *ex vitro* TPP production as fold change in TPP +/- SD established in lysates isolated from wild type HCT 116 transfected with pcDNA-*TPK1* (TPK1 OE) vector for 72 h relative to control (CTL) cells. (*) Represents statistically significant difference ($p<0.05$) based on results of an unpaired student's t-test.

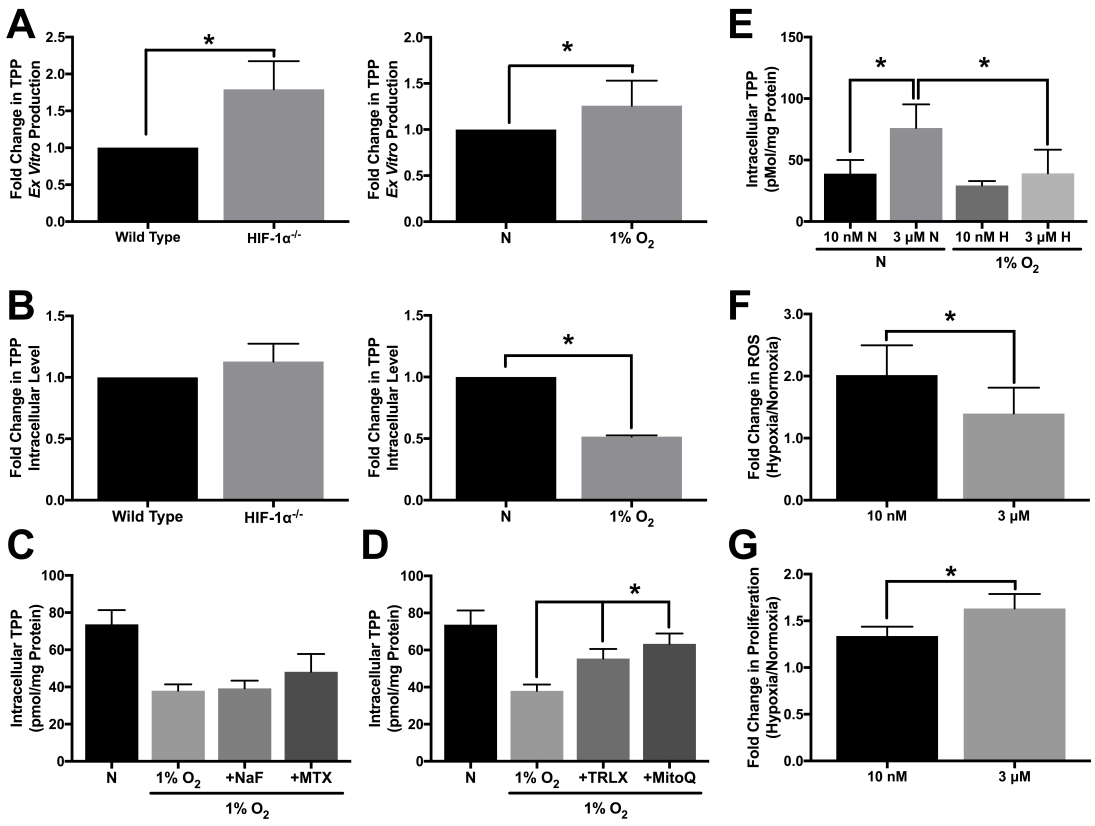
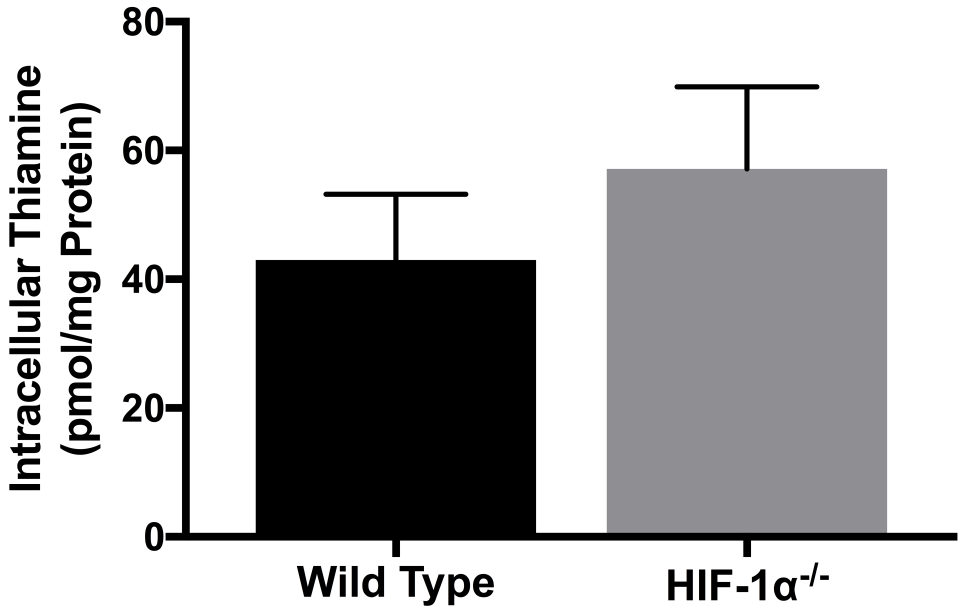
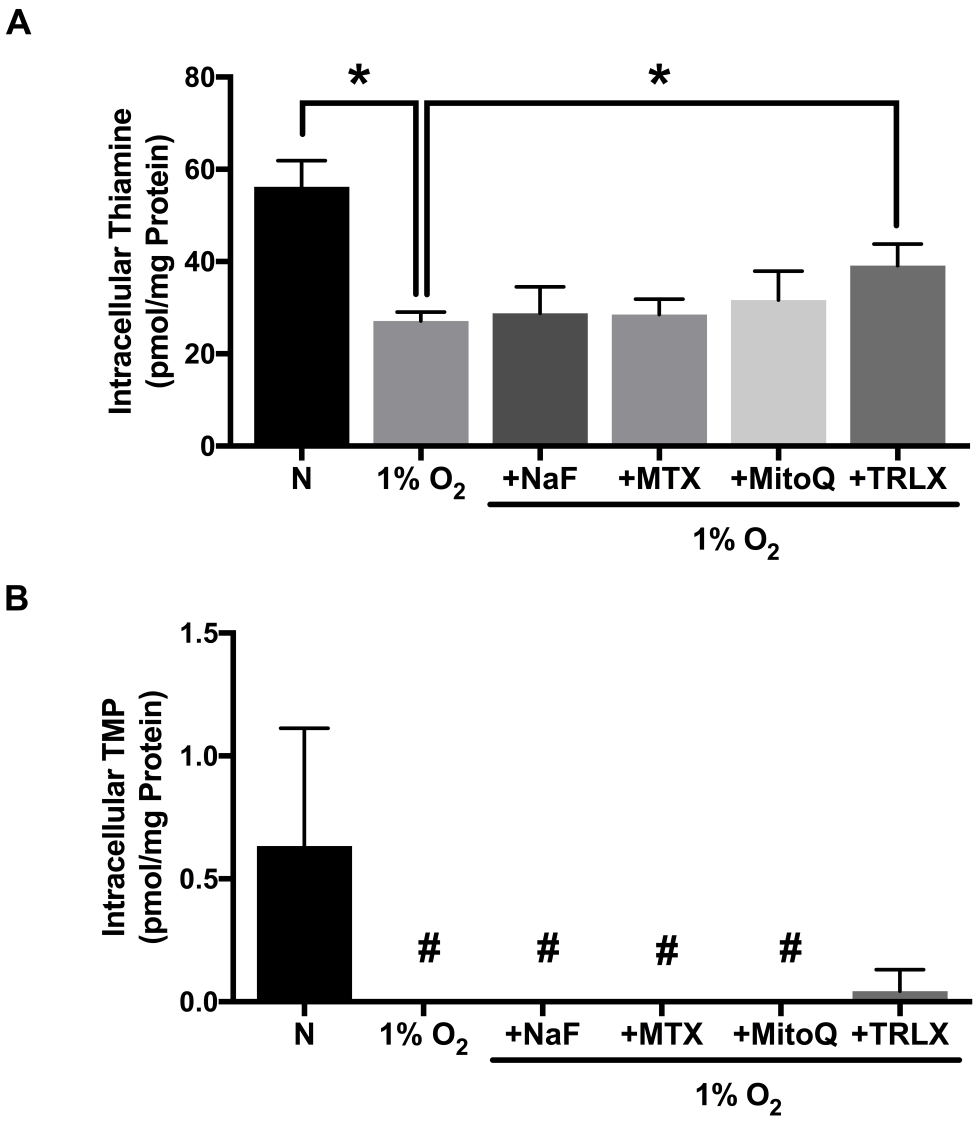


Fig 2.5. Altered TPP homeostasis during hypoxic stress (A) HPLC analysis demonstrating *ex vitro* TPP production as fold change in TPP +/- SD comparing wild type and HIF-1$\alpha^{-/-}$ HCT 116 cells seeded at 1250 cells/cm^2 and cultured for 96 h or wild type cells seeded at 1250 cells/cm^2 and treated with 1% O_2 for 24 h relative to normoxic control (N) including $n = 5$ independent experiments. **(B)** HPLC analysis demonstrating intracellular TPP levels as fold change in TPP +/- SD comparing wild type and HIF-1$\alpha^{-/-}$ HCT 116 cells seeded at 1250 cells/cm^2 and cultured for 96 h or wild type cells seeded at 1250 cells/cm^2 and treated with 1% O_2 for 24 h relative to normoxic control (N) including $n = 3$ independent experiments. **(C, D)** HPLC analysis demonstrating mean intracellular TPP levels +/- SD established in wild type HCT 116 cells seeded at 25,000 cells/cm^2 and pretreated with 500 μM NaF, 100 μM MTX, 500 μM TRLX or 10 μM MitoQ for 12 h prior to hypoxic exposure for 24 h with sustained exposure to each compound including $n = 5$ independent experiments. **(E)** HPLC analysis demonstrating mean intracellular TPP levels +/- SD established in wild type HCT 116 cells exposed to 10 nM or 3μM thiamine for 5 d prior to seeding at 50,000 cells/cm^2 and exposure to 1% O_2 for 24 h including $n = 3$ independent experiments for 10 nM and $n = 7$ independent experiments for 3 μM. **(F)** Effect of thiamine dose on hypoxia-induced ROS levels demonstrated by hypoxic to normoxic fold change in MitoSOX median fluorescence intensity +/- SD in wild type HCT 116 cultured in 10 nM or 3μM thiamine for 5 d prior to seeding at 50,000 cells/cm^2 and exposure to 1% O_2 for 48 h including $n = 3$ independent experiments. **(G)** Effect of

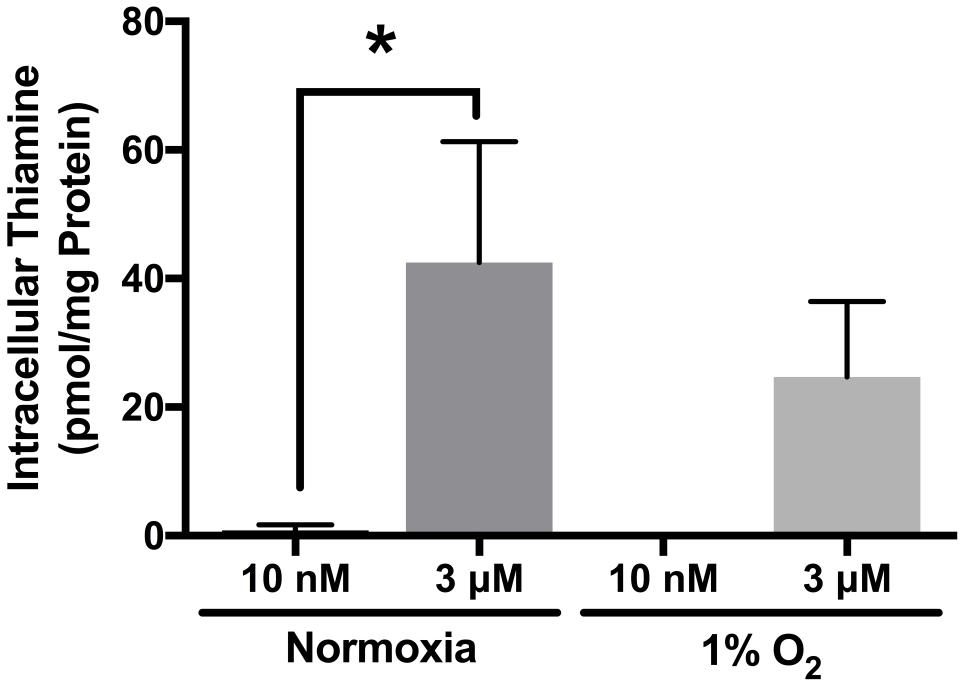
thiamine dose on hypoxic tumor cell proliferation demonstrated by hypoxic to normoxic fold change in live cell count +/- SD determined by trypan blue exclusion. Wild type HCT 116 cells were seeded at 500 cells/cm^2 were grown for 5 d in either 1% O_2 or normoxia including *n = 5* independent experiments. (★) Represents statistically significant difference ($p<0.05$) based on results of (A,B,F,G) an unpaired student's t-test or (C-E) one-way ANOVA with Tukey's post-hoc test.



Supplemental Fig 2.3. Thiamine levels in wild type and HIF-1α$^{-/-}$ HCT 116 Cells HPLC analysis demonstrating intracellular thiamine level +/- SD established in wild type and HIF-1α$^{-/-}$ HCT 116 cells seeded at 1250 cells/cm^2 and cultured for 96 h.



Supplemental Fig 2.4. Thiamine and TMP levels in HCT 116 cells treated with NaF, MTX, TRLX and MitoQ HPLC analysis demonstrating intracellular **(A)** thiamine and **(B)** TMP levels +/- SD established in wild type HCT 116 cells seeded at 25,000 cells/cm^2 and pretreated with 500 μM NaF, 100 μM MTX, 500 μM TRLX or 10 μM MitoQ for 12 h prior to hypoxic exposure for 24 h with sustained exposure to each compound. (✶) Represents statistically significant difference ($p<0.05$) based on results of an unpaired student's t-test. (#) Represents concentration result below the limits of quantification for assay design.



Supplemental Fig 2.5. Thiamine levels in wild type HCT 116 cells grown under physiological and supplemental thiamine conditions HPLC analysis demonstrating intracellular thiamine levels +/- SD established in wild type HCT 116 cells exposed to 10 nM or 3µM thiamine for 5 d prior to seeding at 50,000 cells/cm^2 and exposure to 1% O_2 for 24 h. (*) Represents statistically significant difference ($p<0.05$) based on results of one-way ANOVA with Tukey's post-hoc test for multiple comparisons.

knockdown of TPK1 in HCT 116 cells **(Fig 2.6C and 2.6D)**. In the presence of supplemental thiamine (3 μM), TPK1 knockdown resulted in a significant reduction in the intracellular TPP levels **(Fig 2.6E)**, while no significant change in the intracellular thiamine pool was observed **(Supplemental Fig 2.6A)**. **Figure 2.6F** demonstrates a significant increase in basal ROS in TPK1 knockdown cells compared to CTL. TPK1 knockdown also significantly reduced HCT 116 proliferation **(Fig 2.6G and 2.6H)**. The effects of TPK1 knockdown on HCT 116 proliferation were confirmed using a second siRNA construct with an alternative target sequence to mediate knockdown **(Supplemental Fig 2.7)**.

A pcDNA3.1+ vector containing *TPK1* was used to achieve TPK1 overexpression **(Fig 2.6I and 2.6J)**. In the presence of supplemental thiamine (3 μM), exogenous TPK1 overexpression resulted in a significantly greater intracellular TPP level **(Fig 2.6K)** and a significant reduction in the level of intracellular thiamine **(Supplemental Fig 2.6B)**. The overexpression of TPK1 had no significant effect on ROS levels **(Fig 2.6L)**. TPK1 overexpression induced a modest but significant increase in tumor cell proliferation **(Fig 2.6M and 2.6N)**. Altering intracellular TPP levels, either due to TPK1 knockdown or overexpression, had no impact on the functional activity of the thiamine dependent enzyme TKT **(Fig 2.6O)**. Furthermore, no intracellular deficiency of TPP cofactor was detected due to TPK1 knockdown or overexpression as demonstrated by a lack of TKT activity enhancement with exogenous addition of TPP to the TKT activity assay **(Fig 2.6O)**.

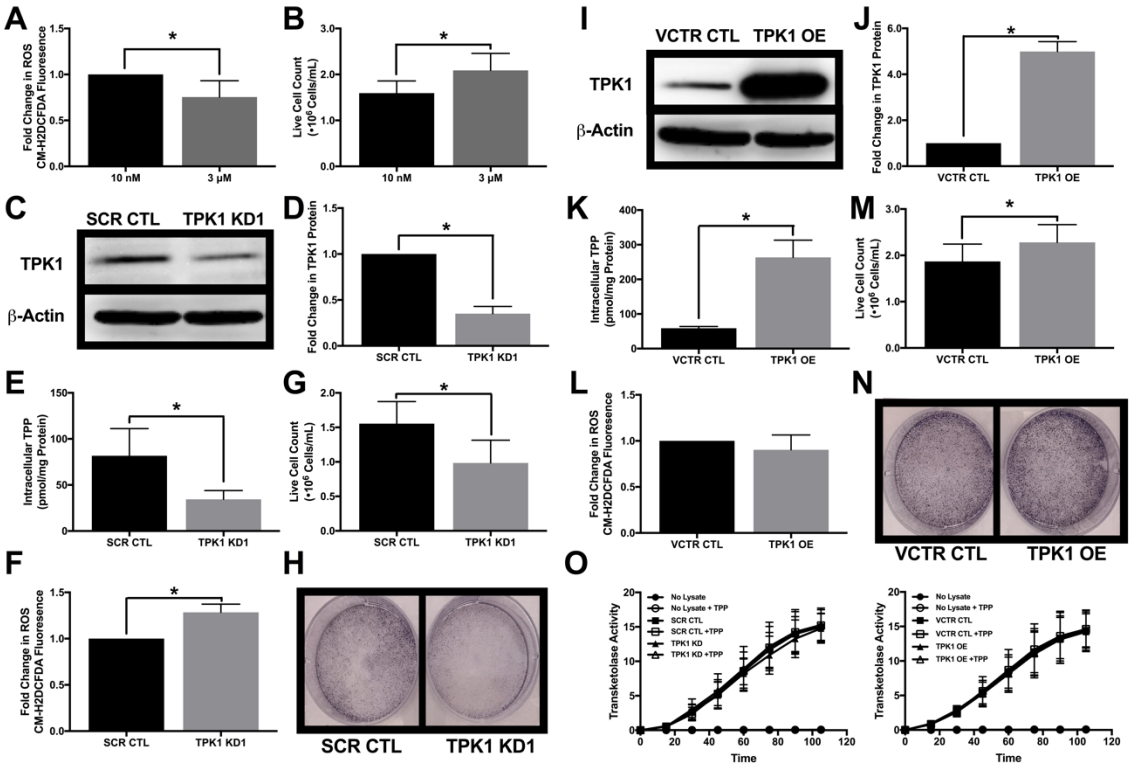
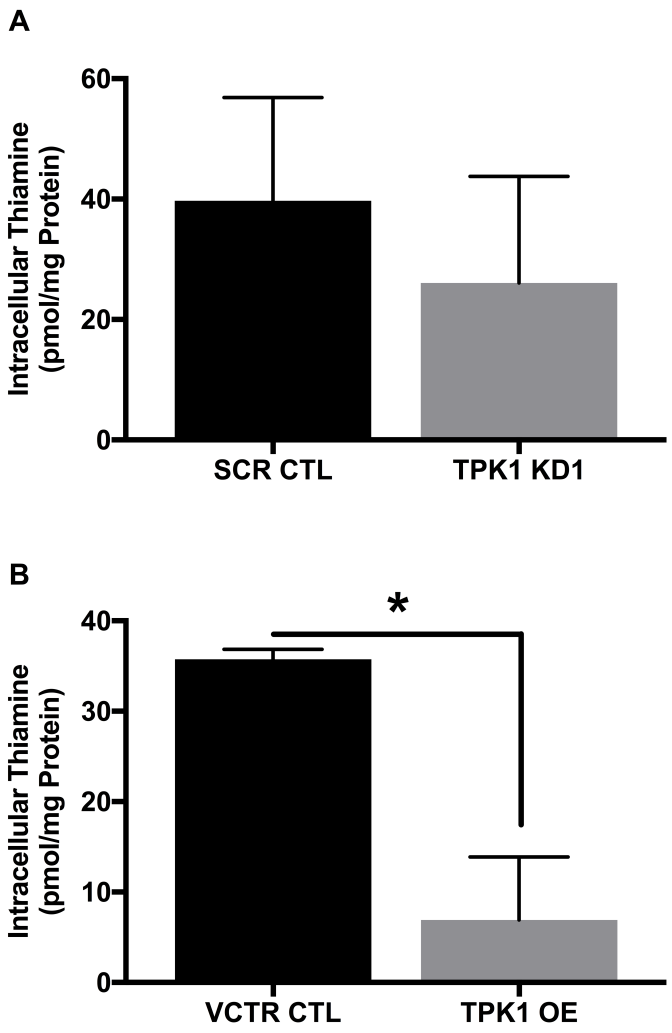
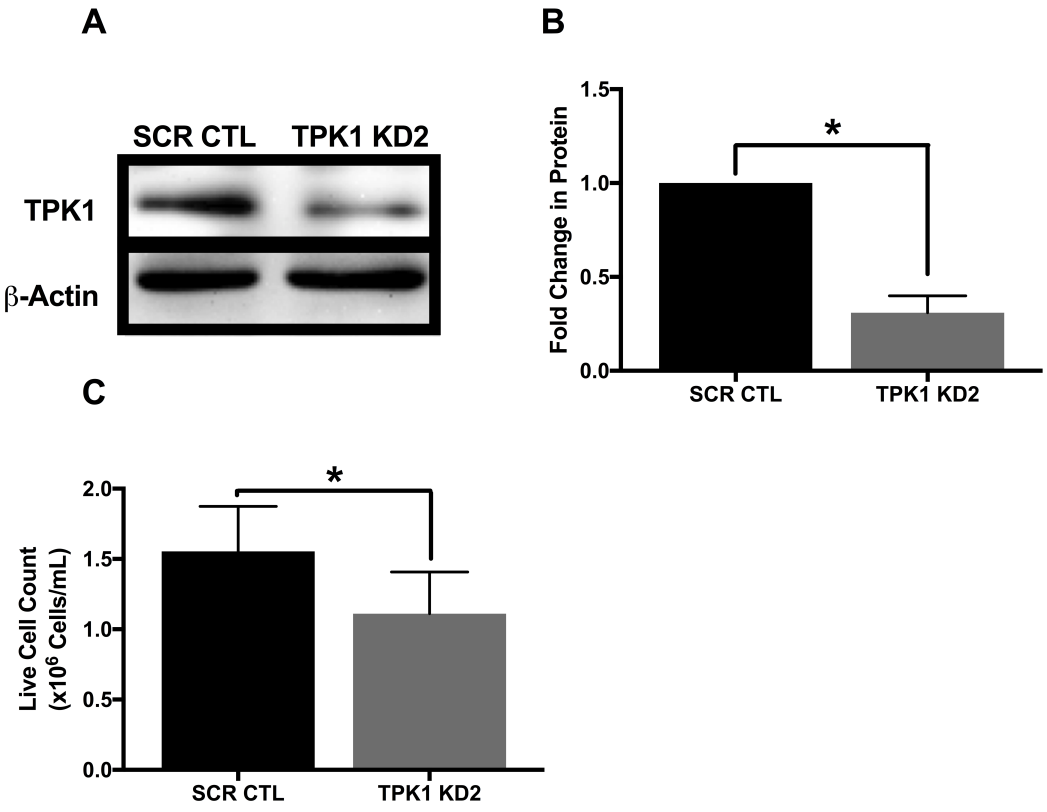


Fig 2.6. Impact of TPK1 on tumor cell proliferation during supplemental thiamine conditions (A) Fold change in CM-H2DCFDA median fluorescence intensity +/- SD comparing fold change in ROS levels of wild type HCT 116 cells seeded at 10,000 cells/cm^2 and supplemented with 3 μM thiamine compared to 10 nM for 5 d including *n = 4* independent experiments. **(B)** Effect of thiamine dose on cell proliferation demonstrated by mean live cell count +/- SD determined by trypan blue exclusion of wild type HCT 116 seeded at 1250 cells/cm^2 supplemented with 3 μM thiamine or 10 nM for 5 d including *n = 5* independent experiments. **(C)** Representative Western blot demonstrating TPK1 expression in WCLs isolated from wild type HCT 116 cells transfected with non-silencing scramble control (SCR CTL) or TPK1 targeted (TPK1 KD1) siRNA for 72 h. β-Actin expression serves as the loading control. **(D)** Densitometry analysis of the fold change in TPK1 expression +/- SD for wild type HCT 116 cells transfected with TPK1 KD1 siRNA compared to SCR CTL including *n = 3* independent experiments. **(E)** HPLC analysis demonstrating mean intracellular TPP levels +/- SD established in wild type HCT 116 cells transfected with SCR CTL or TPK1 KD1 siRNA for 72 h including *n = 5* independent experiments. **(F)** Fold change in CM-H2DCFDA median fluorescence intensity +/- SD comparing fold change in ROS levels of wild type HCT 116 cells transfected with TPK1 KD1 compared to SCR CTL siRNA for 96 h including *n = 6* independent experiments. **(G)** Effect of TPK1 knockdown on tumor cell proliferation demonstrated by mean live cell count +/- SD determined by trypan blue exclusion of wild type HCT 116 cells transfected with SCR CTL or TPK1 KD1 siRNA for 96 h including *n = 5* independent experiments. **(H)** Images of formalin

fixed cells stained with crystal violet following transfection with SCR CTL or TPK1 KD1 siRNA for 96 h. **(I)** Representative Western blot demonstrating TPK1 expression in WCLs isolated from wild type HCT 116 cells transfected with pcDNA3.1+ vector control (VCTR CTL) or pcDNA-*TPK1* (TPK1 OE) vectors for 72 h. **(J)** Densitometry analysis of the fold change in TPK1 expression +/- SD for wild type HCT 116 cells transfected with TPK1 OE compared to VCTR CTL including $n = 3$ independent experiments. **(K)** HPLC analysis demonstrating mean intracellular TPP levels +/- SD established in wild type HCT 116 cells transfected with VCTR CTL or TPK1 OE vectors for 72 h including $n = 3$ independent experiments. **(L)** Fold change in CM-H2DCFDA median fluorescence intensity +/- SD comparing fold change in ROS levels of wild type HCT 116 cells transfected with TPK1 OE compared to VCTR CTL for 96 h including $n = 3$ independent experiments. **(M)** Effect of TPK1 overexpression on tumor cell proliferation demonstrated by mean live cell count +/- SD determined by trypan blue exclusion of wild type HCT 116 cells transfected with VCTR CTL or TPK1 OE vectors for 96 h including $n = 5$ independent experiments. **(N)** Images of formalin fixed cells stained with crystal violet following transfection with VCTR CTL or TPK1 OE vectors for 96 h. **(O)** The average rate of TKT activity quantified from the reduction of NADH over time +/- the addition of exogenous TPP in lysates isolated from wild type HCT 116 cells transfected with SCR CTL and TPK1 KD1 siRNA or VCTR CTL and TPK1 OE vectors for 72 h including $n = 3$ independent experiments for both conditions. (★) Represents statistically significant difference ($p<0.05$) based on results of an unpaired student's t-test.



Supplemental Fig 2.6. Thiamine levels following TPK1 knockdown and overexpression in HCT 116 cells HPLC analysis demonstrating mean intracellular thiamine levels +/- SD established in wild type HCT 116 cells transfected with **(A)** SCR CTL and TPK1 KD1 siRNA or **(B)** VCTR CTL and TPK1 OE vectors for 72 h. (*) Represents statistically significant difference ($p<0.05$) based on results of an unpaired student's t-test.



Supplemental Fig 2.7. Validation of TPK1 knockdown and growth effects using alternative siRNA construct (A) Representative Western blot demonstrating TPK1 expression in WCLs isolated from wild type HCT 116 cells transfected with non-silencing scramble control (SCR CTL) or TPK1 targeted (TPK1 KD2) siRNA for 72 h. **(B)** Densitometry analysis of fold change in TPK1 expression +/- SD in wildtype HCT 116 cells transfected with TPK1 KD2 compared to SCR CTL siRNA for 72 h. **(C)** Effect of TPK1 knockdown on tumor cell proliferation demonstrated by mean live cell count +/- SD determined by trypan blue exclusion for wild type HCT 116 cells transfected with SCR CTL or TPK1 KD2 siRNA for 96 h. (*) Represents statistically significant difference ($p<0.05$) based on results of an unpaired student's t-test.

2.5 Discussion

Recent evidence has demonstrated that thiamine supplementation supports malignant progression by increasing tumor proliferation. Using the spontaneous tumor mouse model FVB/N-Tg(MMTV-neu), Daily *et al.* found that thiamine supplementation reduced tumor latency (Daily *et al.* 2012). An increase in Ehrlich ascites tumor proliferation was also observed in mice administered thiamine at 12.5 times the recommended daily allowance (RDA) (Comin-Anduix *et al.* 2001). Our lab has previously established that the effects of thiamine supplementation on tumor proliferation may be supported by the up-regulation of thiamine homeostasis genes. An increase in the expression of the thiamine transporter, *SLC19A2* was found in breast cancer tissue when compared to normal breast tissue (Zastre *et al.* 2013a). Correspondingly, the level of thiamine was higher in three out of four breast cancer cell lines compared to human mammary epithelial cells. An increase in the gene expression of *TPK1* was also observed in breast tumor tissue when compared to normal breast tissue (Zastre *et al.* 2013a). In hypoxia, an adaptive increase in the expression of the thiamine transporter *SLC19A3* and increase in thiamine transport was found in malignant cells (Sweet *et al.* 2010). Here, we demonstrate a similar adaptive up-regulation for the thiamine activating enzyme TPK1 during hypoxic and oxidative stress.

Supported by both gain-of-function (hypoxia, DMOG, HIF-1α CA) and loss-of-function (YC-1, HIF-1$\alpha^{-/-}$) studies, the activity of the oncogenic transcription factor HIF-1α appeared to be responsible for enhancing TPK1 expression during hypoxic conditions. HIF-1α also mediates *SLC19A3*'s adaptive up-regulation during hypoxia (Sweet *et al.* 2010). However, the regulatory pathways for the two thiamine homeostasis genes diverge

in that TPK1 expression was found to be translationally enhanced, while HIF-1α directly transactivates *SLC19A3* gene expression (Zera *et al.* 2016). Our findings for the inhibitory effects of YC-1 on TPK1 expression may inadvertently provide further support for the translational regulation of TPK1 expression. In this study, the intended use for YC-1 was as a HIF-1α inhibitor (Li *et al.* 2008). However, it has also been demonstrated that YC-1 treatment may downregulate cap-dependent mRNA translation through inhibiting the phosphorylation of eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) (Sun *et al.* 2007; Qin *et al.* 2016). Therefore, reduced TPK1 expression under hypoxic conditions following YC-1 treatment may have been due to direct inhibition of TPK1 translation instead of through HIF-1α inhibition. The observed decrease in TPK1 expression during normoxic conditions supports inhibition of TPK1 translation by YC-1 treatment.

Rapid cellular adaptations to stress are often facilitated by translationally regulated protein responses rather than transcriptionally mediated gene expression alone (Spriggs *et al.* 2010). Translational up-regulation of TPK1 in hypoxia may suggest an immediate requirement for TPP production in response to oxidative stress. This coincides with our finding that TPK1 expression was also regulated in a HIF-independent manner related to oxidative stress. Although we did not observe HIF-1α stabilization in our ROS-inducing treatments (i.e. DOX, CDDP, AA, TBHP), convergence of the two pathways cannot be ruled out as ROS has previously been defined as a factor in both the normoxic and hypoxic stabilization of HIF-1α (Movafagh *et al.* 2015). Identifying secondary mediators related to both HIF-1α and ROS will be critical in further elucidating TPK1's adaptive regulation in response to malignant stress. The oncogenic microRNA miR-155

may be a candidate as it was recently demonstrated to regulate thiamine homeostasis during malignancy by mediating the transcript and protein expression of THTR1 (*SLC19A2*) and TPK1 (Kim *et al.* 2015). In addition, miR-155 has been demonstrated to be functionally up-regulated by hypoxia and has also been shown to deregulate redox homeostasis highlighting a potential connection to ROS (Babar *et al.* 2011; Onodera *et al.* 2017). Future work should validate the role of miR-155 in the translational regulation of TPK1 expression.

Both thiamine and TPP have been demonstrated to act as antioxidants, scavenging superoxide and hydroxyl radicals as well as peroxide molecules (Okai *et al.* 2007). The transfer of 2 H^+ + 2 e^- from the aminopyrimidine ring of thiamine to free radicals has been shown to drive its direct antioxidant property (Lukienko *et al.* 2000). Our findings demonstrate that TPP may act as an intracellular antioxidant consumed during oxidative stress in malignant cells. Like TPP, thiamine levels also decreased during the ROS-associated stress of hypoxia suggesting it may also have been consumed as an antioxidant. However, TPP has previously been demonstrated to provide greater protective effect against oxidative stress-induced damage (i.e. DNA hydroxylation) compared with thiamine (Coskun *et al.* 2014). Therefore, the observed loss of thiamine may have been due to an increased conversion to TPP following antioxidant consumption of TPP. Further investigation regarding the kinetics for thiamine conversion into TPP during hypoxia and oxidative stress will be required to provide these mechanistic details. *In vivo* evidence supports TPP's ability to act as an antioxidant by establishing that TPP administration protects against CDDP-induced neuro, liver, and cardiotoxicity (Turan *et al.* 2014; Turan *et al.* 2013; Coskun *et al.* 2014). Likewise, the administration of TPP

prevents against oxidative damage in ischemia reperfusion induced kidney toxicity, methotrexate induced liver toxicity, and alcohol induced hepatotoxicity (Demiryilmaz *et al.* 2012; Yilmaz *et al.* 2015; Altuner *et al.* 2013). In the brain, thiamine deficiency (lack of TPP) and oxidative stress appear to coincide and potentially contribute to neurodegenerative disorders such as Alzheimer's disease (Liu *et al.* 2017). These findings suggest that the antioxidant potential of TPP may be far reaching amongst different disease pathologies and not simply limited to the malignant state.

A dichotomy presents for the effects of dietary antioxidants on tumor progression. Epidemiological studies suggest an inverse correlation between diets rich in dietary antioxidants and cancer risk, while clinical data reveals that β-carotene, vitamin A, and vitamin E may increase the risk of cancer-associated death (Bjelakovic *et al.* 2007; Borek 2017). Supplementation with the antioxidants NAC and vitamin E have recently been shown to promote tumor progression and enhance metastatic potential *in vivo* (Sayin *et al.* 2014; Le Gal *et al.* 2015). Here, the adaptive up-regulation of TPK1 during ROS-inducing conditions appeared to be a cellular response to maximize TPP production. With supplemental thiamine, the level of ROS was reduced and proliferation increased. Loss of TPK1 expression sensitized tumor cells to enhanced ROS-levels, while the exogenous overexpression of TPK1 produced no further reduction in basal ROS, despite significant TPP accumulation. The lack of effect for TPK1 overexpression may be contingent on supplemental thiamine, considering that with 3 μM thiamine basal ROS was already minimal compared to the more physiological relevant level of 10 nM thiamine. The minimal impact for the exogenous overexpression of TPK1 to further reduce ROS in the presence of supplemental thiamine suggests that TPP has limited capacity to reduce

cellular redox status compared to other endogenous systems (i.e. glutathione, NADPH). Our findings support that the endogenous adaptive regulation of TPK1 in response to malignant stress facilitates the necessary TPP production for the molecule to serve as both a cofactor and intracellular antioxidant.

Tiwana et al. found TPK1 expression to be a significant factor in the susceptibility of cancer cells to ionizing radiation (Tiwana *et al.* 2015). siRNA-mediated knockdown of TPK1 decreased tumor cell survival following radiation treatment. This finding was attributed to the necessity of thiamine homeostasis to maintain TKT activity for the production of nucleotides and DNA repair following oxidative stress (Tiwana *et al.* 2015). However, radiation sensitization due to the silencing of TPK1 could also be linked to TPP's apparent role as an intracellular antioxidant. Using TKT activity as a probe for thiamine-dependent enzyme activity, we found the cofactor and non-cofactor roles for TPP to be mutually exclusive. No change was found for TKT activity due to an apo-holo enzyme effect despite significant losses in TPP with siRNA directed against TPK1. There was also no demonstrated "TPP effect" through the addition of TPP to the assay system or enhancement of TKT activity following TPP accumulation with the exogenous overexpression of TPK1 suggesting no intracellular TPP deficiency impacting thiamine dependent enzyme activity. One factor for further consideration will be the effects of supplemental thiamine. There was no change for intracellular TPP levels detected in hypoxia compared to normoxia when cells were grown in the physiological relevant concentration of 10 nM. This may be indicative of the basal level of TPP required to maintain function of thiamine dependent enzymes. However, in the presence of supplemental thiamine there was a loss of accumulated TPP in hypoxia compared to

normoxia. We suggest this corresponds with the antioxidant function of TPP. Despite the observed loss, a similar baseline level of TPP was maintained comparable to that of 10 nM conditions highlighting the minimum required level for functional enzymatic activity. These findings are indicative of disadvantageous effects for the consumption of supplemental dietary thiamine during malignancy. Furthermore, vitamin supplements often contain 1000-6000% of the RDA for thiamine and may pose significant health hazards for cancer patients (Zastre *et al.* 2013b).

In conclusion, we propose that the adaptive up-regulation of TPK1 occurs during malignant stress to facilitate TPP production and offset its consumption as an intracellular antioxidant independent of its cofactor function. This coincides with the model proposed by *Bettendorf et al*. that two independent intracellular pools of TPP exist with separate regulatory and functional fates (Bettendorff 1994b). The "cofactor" pool, which undergoes little turnover, provides the necessary amount of cofactor required to maintain the activity of thiamine dependent enzymes. The second "free" pool undergoes rapid intracellular turnover, consistent with our antioxidant consumption hypothesis **(Fig 2.7)**. In response to elevated TPP turnover during stressed conditions, it appears that tumor cells may exploit convergent mechanisms to rapidly maximize TPP production. First, up-regulation of thiamine transport should ensure the availability of necessary substrate to rapidly produce new TPP molecules. Supporting this proposal, thiamine transport enhances via *SLC19A3* expression during hypoxic conditions in addition to the up-regulation of thiamine homeostasis that occurs during malignancy related to *SLC19A2* expression (Zastre *et al.* 2013a; Sweet *et al.* 2010). Second, our findings for the adaptive up-regulation of TPK1 during oxidative stress suggest an attempt to maximize

intracellular conversion of thiamine to TPP. Together, the dual up-regulation of thiamine transport and TPK1 expression should serve to maximize TPP production and counterbalance TPP loss during oxidative stress. In doing so, the adaptive regulation of thiamine transport and TPK1 may prevent an intracellular TPP deficit despite rapid turnover and simultaneously maintain required TPP levels for thiamine dependent enzyme activity. Consumption of TPP may provide some insight into the numerous clinical reports of thiamine deficiency in advanced stage cancer patients (Isenberg-Grzeda *et al.* 2016a). It remains undefined as to why these patients become thiamine deficient, however one of the associated risk factors is the occurrence of a rapidly developing tumor (Kuo *et al.* 2009). Thiamine and its phosphate ester metabolites are also elevated in tumor tissue, while uninvaded control tissues show perpetual declines of the moieties throughout tumor growth (Baker *et al.* 1981). Together, this may suggest that the body's thiamine stores can be redistributed through up-regulation of thiamine homeostasis genes and exploited by tumor cells for growth and survival purposes.

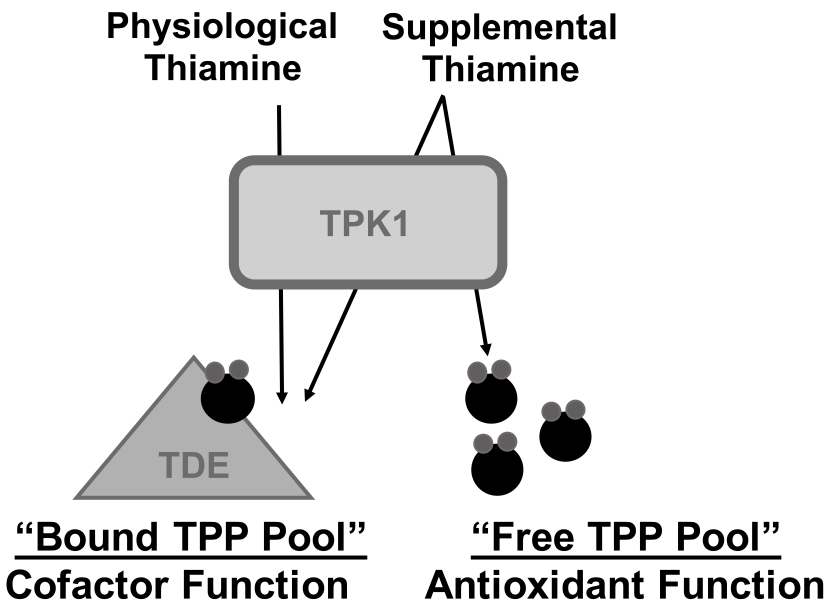


Fig 2.7. Schematic representation for the hypothesized role of TPK1 in mediating the effects of supplemental thiamine on malignant progression We hypothesize that in the presence of physiological thiamine levels, TPP produced by TPK1 maintains thiamine dependent enzyme activity (TDE) representing the bound TPP pool. However, during supplemental thiamine conditions, TPK1 up-regulation facilitates both the production of bound TPP, as well as a "free TPP pool," which may be consumed by tumor cells through antioxidant reactions to maintain redox status and promote growth.

2.6 Materials and Methods

Standard cell culture reagents including RPMI 1640 media, penicillin/streptomycin, and trypsin/EDTA were purchased from Corning (Manassas, VA). Fetal bovine serum (FBS) was obtained from Seradigm (Radnor, PA). Cell culture treated flasks and dishes were purchased from Greiner Bio-One (Monroe, NC). Chemicals including DMOG, DOX, CDDP, MTX, NAC, ASC, NaF, AA, TBHP and TRLX were purchased from Sigma Aldrich (St. Louis, MO). MitoQ was purchased from GlycoSyn Technologies (New Zealand) and YC-1 from Tocris (Minneapolis, MN).

2.6.1 Cell culture

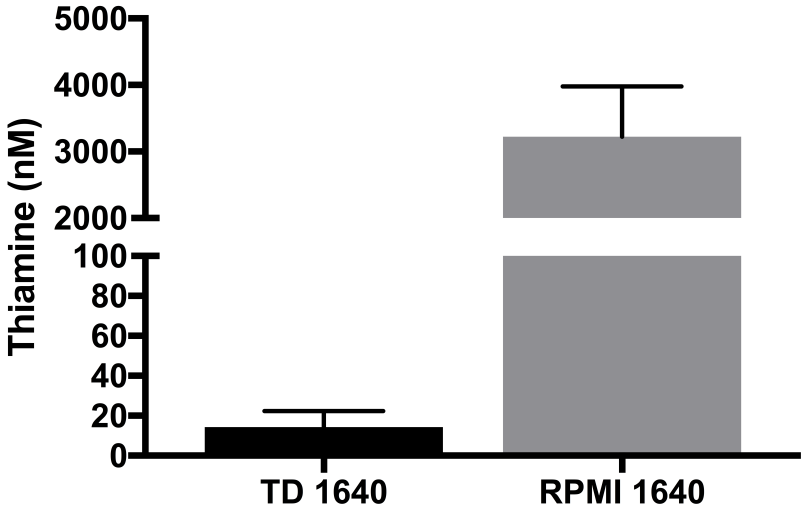
Tumor cell lines including MCF7, MDA-MB-231, LN-18, U-87 MG, Caco-2, HCT 116, and HuTu 80 were obtained from ATCC (Manassas, VA). Research Registry IDs are provided in Supplemental Table 2.1. HCT 116 HIF-1$\alpha^{-/-}$ cells were developed previously (Dang *et al.* 2006). All cells were routinely cultured in complete RPMI 1640, which contained 10% FBS and 1% penicillin/streptomycin, at 37°C with 5% CO_2 designated as normoxic conditions. Routine culture media also contained 0.1% Mycozap (Lonza, Verviers, Belgium) to prevent mycoplasma contamination. Wild type HCT 116 cells were confirmed to be mycoplasma free in June 2018 by IDEXX Bioresearch (Columbia, MO) Impact 1 PCR profile. Maintenance flasks of cells were grown to 60-70% confluency prior to splitting into dishes for experimental treatments. All cells used for experimentation ranged from passage 4-14.

Supplemental Table 2.1. Research Registry Identification (RRID) for cell lines

Cell Line	ATCC Catalog Number	RRID
MCF7	HTB-22	CVCL_0031
MDA-MB-231	CRM-HTB-26	CVCL_0062
LN-18	CRL-2610	CVCL_0392
U-87 MG	HTB-14	CVCL_0022
Caco-2	HTB-37	CVCL_0025
HCT 116	CCL-247	CVCL_0291
Hutu 80	HTB-40	CVCL_1301

An incubator equipped with a ProOX oxygen sensor and regulator (Biospherix, Lacona, NY) was used for hypoxia treatments. The regulator supplies nitrogen gas to maintain a designated level of 1% O_2 within the incubator. The sensor was calibrated on a weekly basis to ensure consistent hypoxic conditions. For hypoxic treatments, culture media was replaced with complete RPMI 1640 that had been pre-equilibrated in hypoxia for a minimum of 24 h. Dishes were transferred to hypoxic incubator for the remainder of experiment and hypoxia pre-equilibrated media was changed every 24 h as necessary during extended hypoxia treatment times.

RPMI 1640 contains 3 μM thiamine, an amount approximately 300 times greater than the ~10 nM concentration found in human serum (Gangolf *et al.* 2010). When necessary to adjust the thiamine concentration to be consistent with physiological levels, custom formulated thiamine deficient RPMI 1640 (TD 1640) (United States Biological, Salem, MA) was utilized. TD 1640 was supplemented with 10% FBS, 1% penicillin/streptomycin, and 0.1% Mycozap to complete the medium. Supplementation of the TD 1640 medium with 10% FBS resulted in the physiologically relevant concentration of ~ 10 nM **(Supplemental Fig 2.8)**.



Supplemental Fig 2.8. Thiamine concentration of cell culture medium HPLC analysis demonstrating thiamine concentration +/- SD established in TD 1640 and RPMI 1640 following supplementation with 10% fetal bovine serum (FBS) to prepare complete growth medium.

2.6.2 Cloning and over-expression of TPK1

Although two isoforms of the *TPK1* gene exist, only variant 1 (NM_022445.3) has been shown to catalyze the activation of thiamine (Nosaka *et al.* 2001). *TPK1* splice variant 2 (NM_001042482.1) lacks exon 7, which results in a 49-amino acid deletion in a conserved portion of the full-length protein. It is unclear if this deletion alters the functional activity for thiamine diphosphorylation (Mayr *et al.* 2011). *TPK1* variant 1 was cloned from human testis cDNA (Clontech Laboratories Inc., Mountain View, CA) by PCR amplification. Cloning primers for *TPK1* were constructed (F: 5'-TCC***GCTAGC***ATGGAGCATGCC-3' and R: 5'-TCC**GGTACC**TTAGCTTTTGACGGCC-3') to be flanked with ***NheI*** (Forward) and **KpnI** (Reverse) restriction sites. PCR was performed with an annealing temperature of 57°C for 32 cycles and the resulting 734 bp product was excised and purified using a Gel Extraction Kit (Omega Bio-tek, Norcross, GA). The resulting fragment was then digested and ligated into pcDNA3.1(+) (Life Technologies, Grand Island, NY). The sequence was verified using the Georgia Genomics and Bioinformatics Core (Athens, GA).

The effects of TPK1 overexpression were studied by transfecting the pcDNA3.1(+)-*TPK1* vector into HCT 116 cells. A pcDNA3.1(+) vector containing *EGFP* was used as a vector transfection control for each experiment. For ROS measurements relying on fluorescence signal, an empty pcDNA3.1(+) vector was used. Cells were seeded at 5000 cells/cm^2 and allowed to attach for 12 h. Cells were then transfected with 17.5 ng of DNA/cm^2 at a final concentration of 0.01% lipofectamine (Promega, Madison, WI).

2.6.3 siRNA-mediated TPK1 knockdown

siRNA knockdown of TPK1 was achieved using functionally validated constructs purchased from Qiagen (Supplemental Table 2.2). HCT 116 cells were seeded at 5000 cells/cm^2 and constructs targeting TPK1 or scrambled control sequences were reverse transfected using a final siRNA concentration of 20 nM with Qiagen HiPerfect transfection reagent at a ratio of 1:400 (final volume).

Supplemental Table 2.2. siRNA constructs used to mediate TPK1 knockdown

Construct	Sense and Antisense Sequences	Target Sequence
Hs_TPK1_5 TPK1 KD1	5'-ccuggugcaucgaaauguatt-3'(sense) 5'-uacauuucgaugcaccaggtt-3' (antisense)	5'-aacctggtgcatcgaaatg-3'
Hs_TPK1_6 TPK1 KD2	5'-gguugucuguagagaaugatt-3' (sense) 5'-ucauucucuacagacaacctg-3'(antisense)	5'-caggttgtctgtagagaatga-3'

2.6.4 Assessment of gene expression

Differential gene expression of *TPK1* splice variants was qualitatively assessed using PCR analysis. RNA was extracted using the E.Z.N.A Total RNA Kit I (Omega Bio-Tek, Norcross, GA) following the manufacturer's provided protocol. RNA concentration was determined using a Nanodrop 2000c Spectrophotometer (Thermo Scientific, Rockford, IL). cDNA was reverse transcribed from 1μg of isolated RNA using the qScript cDNA Synthesis Kit (Quanta BioSciences, Gaithersburg, MD). The expression of *TPK1* splice variants was analyzed by designing primers to simultaneous amplify *TPK1*

variant 1 (438 bp) and variant 2 (291 bp). Primer sequences were: F 5'-CCTGAATTCATCAATGGAGACTTTG-3' and R 5'-AGCAAGCACATCATTTGTGAGG-3'. PCR reaction was carried out using EconoTaq Plus Green 2X Master Mix (Lucigen, Middleton, WI) in a DNA Thermal Cycler (Thermo Scientific, Rockford, IL) with annealing at 57°C for 32 cycles. The resulting product was electrophoresed on a 1.5% agarose ethidium bromide gel. Fragments were visualized with ultraviolet light using a Biorad Gel Doc EZ Imager (BioRad, Hercules, CA).

The combined total expression of *TPK1* variants 1 and 2 and the HIF-1α target genes lactate dehydrogenase (*LDHA*), vascular endothelial growth factor (*VEGF*), and *SLC2A1* (GLUT1) was determined by quantitative real-time PCR using a Light-Cycler 480 II (Roche Applied Science, Indianapolis, IN). For detection, gene specific primers were designed using the Roche Universal Probe Library assay design center in correspondence with a specific Roche hydrolysis probe labeled with fluorescein (FAM). The primer/probe pairs used for this study are listed in Supplemental Table 2.3. Human TATA-binding protein (*TBP*) was used as a reference gene to calculate relative gene expression based on the $2^{-\Delta Ct}$ method with an assumed efficiency of 2.

Supplemental Table 2.3. Primer sequences and Roche UniversalProbe Library probe pairs for Real Time-PCR analysis

Gene	Forward and Reverse Primer Sequences	Probe
TBP	F: 5'-cggctgtttaacttcgcttc-3' R: 5'-cacacgccaagaaacagtga-3'	3
TPK1	F: 5'-gcctttaccccgttggag-3' R: 5'-ccaaaggctgattaagaattacaag-3'	48
LDHA	F: 5'-gtccttggggaacatggag-3' R: 5'-ttcagagagacaccagcaaca-3'	47
VEGF	F: 5'-cagactcgcgttgcaaga-3' R: 5'-gagagatctggttcccgaaa-3'	12
SLC2A1	F: 5'-gcccatgtatgtgggtgaa-3' R: 5'-agtccaggccgaacacct-3'	81

2.6.5 Evaluation of protein expression

To assess changes in TPK1 protein expression, cells were harvested as whole cell lysates (WCL) for Western blot analysis. WCLs were prepared by washing treated cells in ice-cold phosphate buffered saline (PBS) followed by immediate lysis using lysis buffer (1% Nonidet P-40 (NP40), 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 0.01% sodium azide, 50 mM tris, 250 mM NaCl, and 1 mM ethylenediaminetetraacetic acid (EDTA) at pH=8.5) supplemented with phenylmethanesulfonylfluoride (Calbiochem, La Jolla, CA) and protease/phosphatase inhibitors (G-Biosciences, St. Louis, MO). Lysates were collected and centrifuged at 17,000xg using a Microfuge 22R Centrifuge (Beckman Coulter, Brea, CA) for 20 min at 4°C. The supernatant was collected and total protein content was determined using a BCA protein assay (Thermo Scientific, Rockford, IL). WCLs (50 μg) from each

treatment were resolved by electrophoresis using a 12% SDS-PAGE gel. Separated proteins were then transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% non-fat milk in tris buffered saline-tween 20 (TBS-T) for 1 h at room temperature. Membranes were then immunoblotted with primary antibody for 12 h at 4°C. TPK1 and HIF-1α expression were assayed with β-Actin (ACTB) expression serving as loading control. The expression of LDHA was also assayed as a marker of functionally active HIF-1α (Semenza *et al.* 1996). Supplemental Table 2.4 provides all information regarding manufacturer and dilution (in TBS-T) for individual antibodies. After the primary antibody incubation, blots were washed three times with TBS-T (10 min each) and then exposed to horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit secondary antibody (Bethyl Laboratories, Montgomery TX) at a 1:10,000 dilution in TBS-T for 1 h at room temperature. Blots were again washed three times with TBS-T. Protein expression was visualized using Supersignal-PLUS West Pico Solution (Thermo Scientific, Rockford, IL) according to manufacturer's instruction. Signal was imaged using a Fluorchem SP digital imager (Alpha Innotech, San Leandro, CA). Densitometry analysis comparing each protein of interest relative to β-Actin was performed using Fluorchem SP Software (Alpha Innotech, San Leandro, CA).

Supplemental Table 2.4. Primary antibodies used for Western blot analysis

Protein of Interest	Manufacturer (Catalog Number)	Dilution	Secondary Antibody	Registry ID
TPK1 (27kDa)	Genetex (GTX103943)	1:500	Goat Anti-Rabbit	AB_1952355
LDHA (37kDa)	Genetex (GTX101416)	1:1000	Goat Anti-Rabbit	AB_10726413
β-Actin (42kDa)	Sigma-Aldrich (A2228)	1:1000	Goat Anti-Mouse	AB_476697
HIF-1α (93kDa)	Genetex (GTX127309)	1:1000	Goat Anti-Rabbit	AB_2616089

2.6.6 Quantitation of thiamine and thiamine phosphorylates

The effect of treatment on intracellular thiamine, TMP, and TPP was established using ion-paired reversed phase high-performance liquid chromatography (HPLC) as previously described (Basiri *et al.* 2016). Media was aspirated from treatment dishes and cells were trypsinized at 37°C. Cells were collected, rinsed with an equal volume of ice-cold TD 1640 medium and pelleted by centrifugation at 500xg for 5 min at 4°C in an Allegra X-22R centrifuge (Beckman Coulter, Brea, CA). The supernatant was aspirated and cells were subsequently washed an additional two times with ice-cold PBS. If not immediately used for extraction and analysis via HPLC, pellets were stored at -80°C. Precipitated protein pellets isolated from extraction process were solubilized with 100 mM NaOH. Protein concentration was determined using a BCA protein quantification kit (Thermo Scientific, Rockford, IL) according to manufacturer's protocol. The total

thiamine or thiamine phosphate metabolite level (pmol) determined by HPLC was then normalized to total protein (mg).

2.6.7 Determination of TPK1 enzymatic activity

The functionality of TPK1 was determined by an *ex vitro* enzymatic assay (Nishimura *et al.* 1991). TPK1 catalyzes the transfer of two phosphate groups onto thiamine in the presence of adenosine triphosphate (ATP) and Mg^{2+}. Cell lysates were prepared by washing treated cells with ice-cold PBS followed by lysis using mammalian protein extraction reagent (M-PER, Thermo Scientific, Rockford, IL) containing 10 μL/mL of EDTA free-100X Mammalian Protease Arrest (G-Biosciences, St. Louis, MO). Lysates were collected and centrifuged at 17,000xg using a Microfuge 22R Centrifuge (Beckman Coulter, Brea, CA) for 10 min at 4°C to pellet cellular debris. Protein concentration of the resulting supernatant were quantitated by BCA protein assay (Thermo Scientific, Rockford, IL). Lysates (0.5 mg total protein) were then combined with ATP (5 mM, disodium salt, Cayman Chemical, Ann Arbor, MI) and $MgSO_4$ (10 mM) in 0.02 M Tris-HCl reaction buffer (pH8.6). Thiamine (10 μM) was added to initiate the TPK1 reaction, which was allowed to proceed at 37°C for 30 min. To stop the reaction, proteins were precipitated by the addition of 10% trichloroacetic acid and the mixture was immediately placed on ice. The extent of TPP produced was determined via HPLC as described above.

2.6.8 Determination of TKT enzymatic activity

Methodology to determine TKT enzymatic activity was adapted from Chamberlain *et al*. (Chamberlain *et al.* 1996). This assay determines the functionality of TKT protein present in lysate samples based on the proportional reduction of NADH over time. In the clinic, thiamine deficiency can be diagnosed based on the changes of TKT activity with the addition of exogenous TPP using this assay (Chamberlain *et al.* 1996). Cell lysates were prepared by washing treated cells with ice-cold PBS followed by lysis using mammalian protein extraction reagent (M-PER, Thermo Scientific, Rockford, IL) containing 10 μL/mL of EDTA free-100X Mammalian Protease Arrest (G-Biosciences, St. Louis, MO). Lysates were collected and centrifuged at 17,000xg using a Microfuge 22R Centrifuge (Beckman Coulter, Brea, CA) for 10 min at 4°C. Protein concentration of the resulting supernatant were quantitated by BCA protein assay (Thermo Scientific, Rockford, IL). 50 μg of total protein was used in a 250 μL reaction mixture with 100 mM Tris-HCl (pH 8.0), 15 mM ribose-5-phosphate, 200 μU/μL α-glycerophosphate dehydrogenase, 2.5 mU/μL triosephosphate-isomerase, and 250 μM β-nicotinamideadeninedinucleotide reduced sodium salt (NADH). The reaction mixture was incubated at 37°C and the change in absorbance (340 nm) was recorded for 120 min at 15 min increments and normalized to total protein content.

2.6.9 Determination of intracellular ROS levels

Intracellular levels of ROS were determined by the general oxidative stress indicator CM-H2DCFDA. For hypoxic quantification, the mitochondrial targeted probe MitoSOX was also used (Invitogen, Eugene, OR). Following treatments, plated cells

were washed with 37°C PBS and then loaded with either cell permeable fluorescent probe dissolved in Hank's Buffered Salt Solution (HBSS) at a concentration of 5 μM for 30 min at 37°C. Cells were subsequently washed with PBS to remove any remaining probe and then trypsinized for 3 min at 37°C. Cells were collected, washed with ice-cold PBS, then suspended in HBSS with 1 μg/mL propidium iodide (PI). Sample fluorescence was determined by flow cytometry using the FL1 and FL3 channels of a CyAn ADP analyzer (Bechman Coulter, Brea CA). Data were analyzed using FlowJo v.10 software (FlowJO, LLC, Ashland, OR).

2.6.10 Quantitation of cellular proliferation

Cellular proliferation was determined by cell counting with trypan blue exclusion. Media from treatment dishes was aspirated and cells were trypsinized for 3 min at 37°C. An equal volume of cold RPMI 1640 was added to neutralize trypsin. Live cell count was determined using a 1:1 dilution with trypan blue using a TC-20 automated cell counter (BioRad, Hercules, CA). Crystal violet staining was utilized as a visual representation for the changes in proliferation. Adherent cells were fixed in buffered formalin at room temperature for 30 min. Formalin was removed and replaced with 0.5% crystal violet for 10 min at room temperature. Crystal violet was then removed and the culture dishes were washed three times with deionized water and allowed to dry prior to imaging.

2.6.11 Statistical analysis

All experiments were performed with a minimum of three independent replicates. Depending on the data set, statistical significance ($p<0.05$) was established using either

an unpaired student's T-test or a one-way analysis of variance (ANOVA) with Tukey's post hoc test using GraphPad Prism 6® (GraphPad Software, La Jolla, CA).

2.7 Further evidence for HIF-mediated regulation of TPK1 expression

2.7.1 Introduction

Our lab has previously demonstrated that HIF-1α serves as a transcriptional activator for thiamine transport during hypoxic conditions (Zera *et al.* 2016). The work detailed above suggests that HIF-1α also facilitates the adaptive up-regulation of TPK1 expression during hypoxic stress. However, TPK1 may also be regulated independently of HIF-1α, specifically under conditions of oxidative stress. This demonstrates that other factors are most likely important for TPK1 expression. Hypoxia-inducible factor-2α (HIF-2α) is a paralog of HIF-1α sharing ~48% sequence homology to its isoform. Expression of both HIF-1α and HIF-2α are tightly regulated by prolyl hydroxylation within an oxygen dependent degradation domain (Kaelin & Ratcliffe 2008). Therefore like HIF-1α, HIF-2α expression increases during hypoxic conditions (Gordan & Simon 2007). Though both transcription factors target hypoxia response elements (HREs) at target gene loci, the two isoforms demonstrate non-redundant target gene profiles (Gordan & Simon 2007). For example, HIF-1α, but not HIF-2α, has been identified to regulate the expression of genes necessary to induce a glycolytic phenotype (Hu *et al.* 2003). Interestingly, unlike HIF-1α, HIF-2α demonstrates direct target genes associated with cellular redox homeostasis (Majmundar *et al.* 2010). Loss of HIF-2α results in increased cellular ROS associated with enhanced p53-mediated tumor cell death following ionizing radiation (Bertout *et al.* 2009). Considering our previous findings for

TPK1's relationship to oxidative stress, the importance of HIF-2α in the adaptive regulation of TPK1 expression following hypoxic treatment was investigated.

2.7.2 Materials and Methods

Standard cell culture reagents including RPMI 1640 media, penicillin/streptomycin, and trypsin/EDTA were purchased from Corning (Manassas, VA). Fetal bovine serum (FBS) was obtained from Seradigm (Radnor, PA). Cell culture treated flasks and dishes were purchased from Greiner Bio-One (Monroe, NC). The prolyl-hydroxylase inhibitor dimethyloxalylglycine (DMOG) was purchased from Sigma Aldrich (St. Louis, MO).

2.7.2.1 Cell culture

Wild type HCT 116 cells were obtained from ATCC (Manassas, VA). HCT 116 HIF-1$\alpha^{-/-}$, HIF-2$\alpha^{-/-}$**,** and HIF-1α2$\alpha^{-/-}$cells were developed previously (Dang *et al.* 2006; Burkitt *et al.* 2009). The isogenic cell lines were routinely cultured in complete RPMI 1640, which contained 10% FBS, 1% penicillin/streptomycin, and 0.1% Mycozap at 37°C with 5% CO_2 designated as normoxic conditions. Maintenance flasks of cells were grown to 60-70% confluency prior to splitting into dishes for experimental treatments. All cells used for experimentation ranged from passage 4-16.

An incubator equipped with a ProOX oxygen sensor and regulator (Biospherix, Lacona, NY) was used for hypoxic conditions. To maintain a designated level of 1% O_2 within the incubator, the regulator supplies nitrogen gas to the chamber. The sensor was calibrated weekly to ensure hypoxic conditions. For hypoxic treatments, culture media

was replaced with complete RPMI 1640 that had been pre-equilibrated in hypoxia for 24 h.

2.7.2.2 Evaluation of protein expression

To assess changes in protein expression, cells were harvested as WCLs for Western blot analysis. WCLs were prepared as described in Section 2.6.5. WCLs (50 μg) from each treatment were resolved by electrophoresis using a 12% SDS-PAGE gel. Separated proteins were then transferred to polyvinylidene difluoride membranes. Membranes were blocked and immunoblotted for TPK1, HIF-1α, and HIF-2α with β-Actin expression serving as loading control as described in Section 2.6.5. **Table 2.1** provides all information regarding manufacturer and dilution (in TBS-T) for individual antibodies used for this experimentation. Protein expression was visualized as described in Section 2.6.5. and densitometry analysis comparing TPK1 expression relative to β-Actin was performed using Fluorchem SP Software (Alpha Innotech, San Leandro, CA).

Table 2.1. Primary antibodies used for Western blot analysis

Protein of Interest	Manufacturer (Catalog Number)	Dilution	Secondary Antibody	Antibody Registry ID
TPK1 (27kDa)	Genetex (GTX103943)	1:500	Goat Anti-Rabbit	AB_1952355
LDHA (37kDa)	Genetex (GTX101416)	1:1000	Goat Anti-Rabbit	AB_10726413
β-Actin (42kDa)	Sigma-Aldrich (A2228)	1:1000	Goat Anti-Mouse	AB_476697
HIF-1α (93kDa)	Genetex (GTX127309)	1:1000	Goat Anti-Rabbit	AB_2616089
HIF-2α (96kDa)	Genetex (GTX30123)	1:1000	Goat Anti-Mouse	AB_836038

2.7.3 Results

2.7.3.1 Induction of TPK1 protein during hypoxia correlates with HIF-2α stabilization

Wild type and HIF-1$\alpha^{-/-}$ HCT 116 cells were exposed to either 1% O_2 or the prolyl hydroxylase inhibitor DMOG for 24 h. In wild type cells, DMOG and 1% O_2 treatment resulted in the stabilization of HIF-2α **(Fig 2.8A)**. A similar induction of HIF-2α was observed in HIF-1$\alpha^{-/-}$ cells **(Fig 2.8B)**. Following isogenic elimination of HIF-2α, HCT 116 cells were treated with DMOG and 1% O_2 for 24 h. Under these conditions, HIF-1α stabilization was observed but there was no detection of HIF-2α induction **(Fig 2.8C)**. Treatment with DMOG resulted in a modest but significant ~1.5-fold enhancement of TPK1 expression **(Fig 2.8C and 2.8D)**. However, no significant change for TPK1 expression during 1% O_2 treatment was observed in HIF-2$\alpha^{-/-}$ cells **(Fig 2.8C and 2.8D)**.

LDHA expression increased following DMOG and 1% O_2 treatment, confirming the intact transcriptional functionality of HIF-1α in HIF-2α$^{-/-}$ cells **(Fig 2.8C)**. The regulation of TPK1 expression was also observed in HIF-1α2α$^{-/-}$ HCT 116 cells. Following the simultaneous genetic elimination of both HIF-1α and HIF-2α, no induction of HIF-1α or HIF-2α protein was observed following 24 h treatment with DMOG or 1% O_2 **(Fig 2.8E)**. There was no enhancement of TPK1 protein in response to either treatment **(Fig 2.8E and 2.8F)**. The lack of HIF-1α transcriptional activity was confirmed by no demonstrated induction for LDHA **(Fig 2.8E)**.

2.7.3.2 Enhanced basal expression of TPK1 following HIF knockout

The basal expression of TPK1 was compared between wild type, HIF-1α$^{-/-}$, HIF-2α$^{-/-}$ and HIF-1α2α$^{-/-}$ HCT 116 cells grown to either sub-confluent or confluent conditions. As demonstrated in **Figures 2.1B, 2.4A, 2.4B, and 2.9A** TPK1 expression was enhanced ~4-fold in HIF-1α$^{-/-}$ cells compared to wild type when cultured at sub-confluent conditions. In both HIF-2α$^{-/-}$ and HIF-1α2α$^{-/-}$ cells, a slightly higher ~5-fold induction of TPK1 protein was observed when comparing knockout cells to their wildtype counterparts **(Fig 2.9A-2.9C)**. Culturing cells at confluent conditions resulted in enhanced TPK1 expression relative to sub-confluent culture conditions in all four isogenic cell lines **(Fig 2.9A)**. When grown at confluency, no upregulation of TPK1 was observed following genetic elimination of HIF-1α$^{-/-}$ and/or HIF-2α$^{-/-}$ compared to wild type cells **(Fig 2.9A)**.

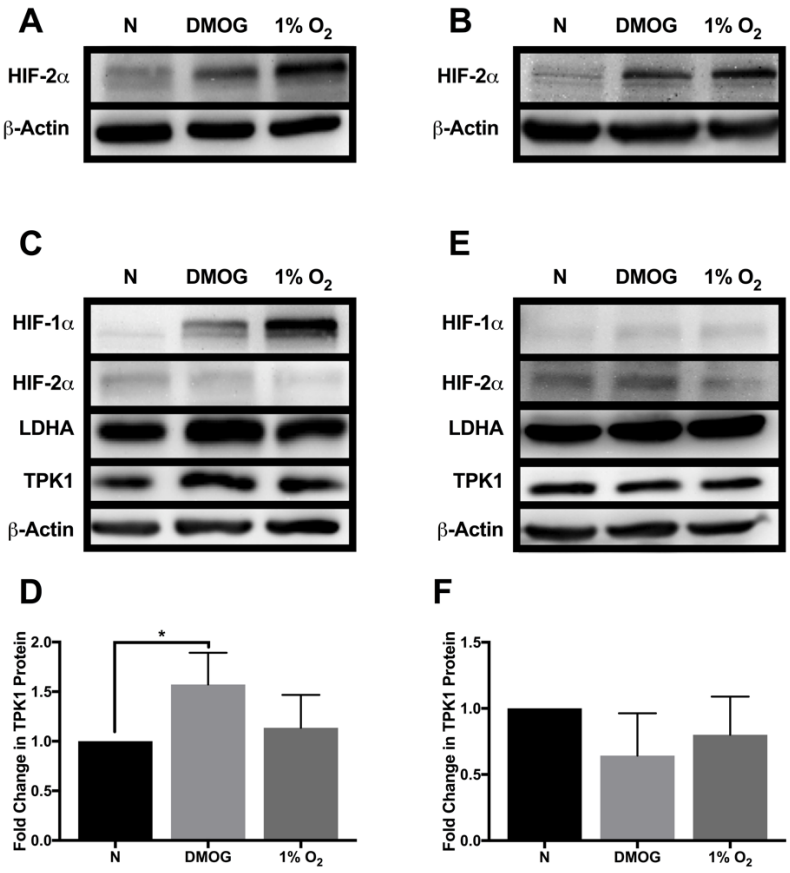


Fig 2.8. Regulation of TPK1 expression in HIF-2α$^{-/-}$ and HIF-1α2α$^{-/-}$ HCT 116 cells Representative Western blots demonstrating HIF-2α expression in WCLs isolated from isogenic **(A)** wild type and **(B)** HIF-1α$^{-/-}$ HCT 116 cells seeded at 1250 cells/cm^2 and treated with 150 μM DMOG or 1% O_2 for 24 h relative to normoxic control (N). β-Actin expression serves as the loading control. **(C)** Representative Western blots demonstrating HIF-1α, HIF-2α, LDHA, and TPK1 protein expression in WCLs isolated from HIF-2α$^{-/-}$ HCT 116 cells seeded at 1250 cells/cm^2 and treated with 150 μM DMOG or 1% O_2 for 24 h relative to normoxic control (N). **(D)** Densitometry analysis of the fold change in TPK1 expression +/- SD following DMOG and 1% O_2 treatment in HIF-2α$^{-/-}$ HCT 116 cells compared to normoxic control (N) including *n = 3* independent experiments. **(E)** Representative Western blots demonstrating HIF-1α, HIF-2α, LDHA, and TPK1 protein expression in WCLs isolated from HIF-1α2α$^{-/-}$ HCT 116 cells seeded at 1250 cells/cm^2 and treated with 150 μM DMOG or 1% O_2 for 24 h relative to normoxic control (N). **(F)** Densitometry analysis of the fold change in TPK1 expression +/- SD following DMOG and 1% O_2 treatment in HIF-1α2α$^{-/-}$ HCT 116 cells compared to normoxic control (N) including *n = 3* independent experiments. (★) Represents statistically significant difference ($p<0.05$) based on results of one-way ANOVA with Tukey's post-hoc test.

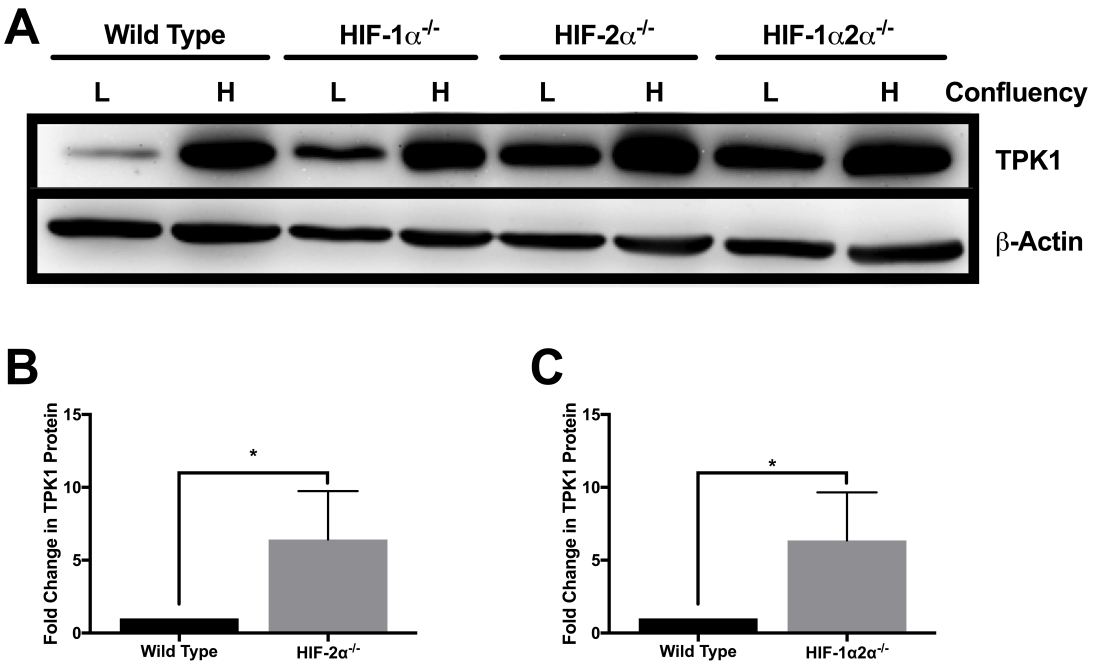


Fig 2.9. TPK1 up-regulation following HIF knockout in HCT 116 cells (A) Representative Western blot demonstrating TPK1 expression in WCLs isolated from isogenic wild type, HIF-1$\alpha^{-/-}$, HIF-2$\alpha^{-/-}$, and HIF-1α2$\alpha^{-/-}$ HCT 116 cells at both sub-confluent (L) and confluent (H) conditions. HCT 116 cells were seeded at 1250 cells/cm^2 (L) or 50,000 cells/cm^2 (H) and cultured for 96 h under normal growth conditions. β-Actin expression serves as the loading control. **(B)** Densitometry analysis of the fold change in TPK1 expression +/- SD comparing HIF-2$\alpha^{-/-}$ to wildtype HCT 116 including *n = 3* independent experiments. **(C)** Densitometry analysis of the fold change in TPK1 expression +/- SD comparing HIF-1α2$\alpha^{-/-}$ to wildtype HCT 116 including *n = 3* independent experiments. (★) Represents statistically significant difference ($p<0.05$) based on results of an unpaired student's t-test.

2.7.4 Discussion

The lack of induction for TPK1 protein expression in hypoxia following HIF-2α elimination supports that TPK1 expression may be regulated by both HIF-1α (as described above) and HIF-2α during hypoxic conditions. Considering previous findings demonstrate HIF-2α's involvement in redox homeostasis, this may further support that TPK1 up-regulation functions to maintain oxidative status. Since the HIF-1α gain-of-function experiments detailed in Section 2.4.1 were performed in wild type cells with functional HIF-2α activity, it cannot be concluded that HIF-1α regulates TPK1 expression independently of HIF-2α. Therefore, TPK1 up-regulation may be a coordinated response requiring both HIF-1α and HIF-2α signaling activity.

The effect of confluency on TPK1 expression was an unexpected but critical variable of this experimentation. We hypothesized that TPK1 induction at confluency was related to HIF-1α as previous reports suggest density-mediated HIF-1α accumulation under normoxic conditions (Sheta *et al.* 2001; Dayan *et al.* 2009). Pericellular hypoxia induced by high cell density has been shown to result in enhanced nuclear accumulation of HIF-1α as well as increased HRE activity (Sheta *et al.* 2001). We also considered that confluent conditions may promote nutrient deprivation and lactate accumulation conferring a pseudohypoxic event that leads to HIF-1α stabilization and TPK1 up-regulation (Zera & Zastre 2018). A preliminary experiment where cells were cultured in media containing galactose as the primary carbon source to prevent lactate accumulation demonstrated no change in TPK1 expression at confluency. After obtaining HIF-1α$^{-/-}$ cells, the relationship of HIF-1α and confluency in TPK1 expression was directly tested leading to the conclusion that confluency-dependent TPK1 up-

regulation occurs independent of HIF-1α. It remains unclear why cells cultured at confluent conditions demonstrate enhanced TPK1 expression compared to those grown at sub-confluent conditions.

2.8 Further evidence for translational regulation of TPK1 expression

2.8.1 Introduction

The evidence detailed within the manuscript portion of this chapter strongly suggests the post-transcriptional, rather than transcriptional activation of TPK1 expression in response to malignant stress. However, it remains unclear if TPK1 up-regulation occurs as a translational response or at an alternative level of gene regulation. Cycloheximide (CHX) inhibits eukaryotic translation by binding the ribosome and inhibiting translocation, therefore blocking the elongation phase (Donohoe *et al.* 2012). CHX treatment serves as the most common laboratory technique to inhibit new protein synthesis (Schneider-Poetsch *et al.* 2010). Therefore, CHX was used to determine if TPK1 up-regulation following treatment with hypoxia or doxorubicin may be a translationally regulated response.

2.8.2 Materials and Methods

Standard cell culture reagents including RPMI 1640 media, penicillin/streptomycin, and trypsin/EDTA were purchased from Corning (Manassas, VA). Fetal bovine serum (FBS) was obtained from Seradigm (Radnor, PA). Cycloheximide (CHX) and doxorubicin (DOX) were purchased from Sigma (St. Louis,

MO). Cell culture treated flasks and dishes were purchased from Greiner Bio-One (Monroe, NC).

2.8.2.1 Cell culture

Wild type HCT 116 cells were obtained from ATCC (Manassas, VA) and maintained in complete RPMI 1640, which contained 10% FBS, 1% penicillin/streptomycin, and 0.1% Mycozap at 37°C with 5% CO_2 designated as normoxic conditions. Maintenance flasks of cells were grown to 60-70% confluency prior to splitting into dishes for experimental treatments. All cells used for experimentation ranged from passage 4-14.

An incubator equipped with a ProOX oxygen sensor and regulator (Biospherix, Lacona, NY), which supplies nitrogen gas to maintain a level of 1% O_2 was used for hypoxia treatments. The sensor was calibrated weekly to ensure a consistent hypoxic environment. For hypoxic treatments, culture media was replaced with complete RPMI 1640 that had been pre-equilibrated in hypoxia for a minimum of 24 h and dishes were transferred to hypoxic conditions for the remainder of experiment.

2.8.2.2 Evaluation of protein expression

To assess changes in protein expression, cells were harvested as WCLs for Western blot analysis. WCLs were prepared as described in Section 2.6.5. WCLs (50 μg) from each treatment were resolved by electrophoresis using a 12% SDS-PAGE gel. Separated proteins were then transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% non-fat milk in TBS-T for 1 h at room temperature.

Following, membranes were immunoblotted as described in Section 2.6.5 for TPK1 with β-Actin expression serving as loading control. **Table 2.2** provides all information regarding manufacturer and dilution (in TBS-T) for individual antibodies used in this experimentation. Protein expression was visualized as described in Section 2.6.5.

Table 2.2. Primary antibodies used for Western blot analysis

Protein of Interest (Predicted MW)	Manufacturer (Catalog Number)	Dilution	Secondary Antibody	Antibody Registry ID
TPK1 (27kDa)	Genetex (GTX103943)	1:500	Goat Anti-Rabbit	AB_1952355
β-Actin (42kDa)	Sigma-Aldrich (A2228)	1:1000	Goat Anti-Mouse	AB_476697

2.8.3 Results

The effect of inhibiting mRNA translation on TPK1 expression was determined in wild type HCT 116 cells exposed to 1% O_2 or doxorubicin (DOX) in the presence and absence of cycloheximide (CHX). Densitometry analysis of previous experiments demonstrated significant accumulation of TPK1 following 24 h exposure to 1% O_2 or DOX **(Fig 2.4G)**. Similarly, TPK1 accumulation was observed following 1% O_2 and DOX in this experiment **(Fig 2.10)**. Treatment with CHX lead to a slight decrease observed for TPK1 expression under normoxic conditions **(Fig 2.10)**. Furthermore, CHX treatment attenuated the induction of TPK1 following exposure to 1% O_2 and DOX **(Fig 2.10)**.

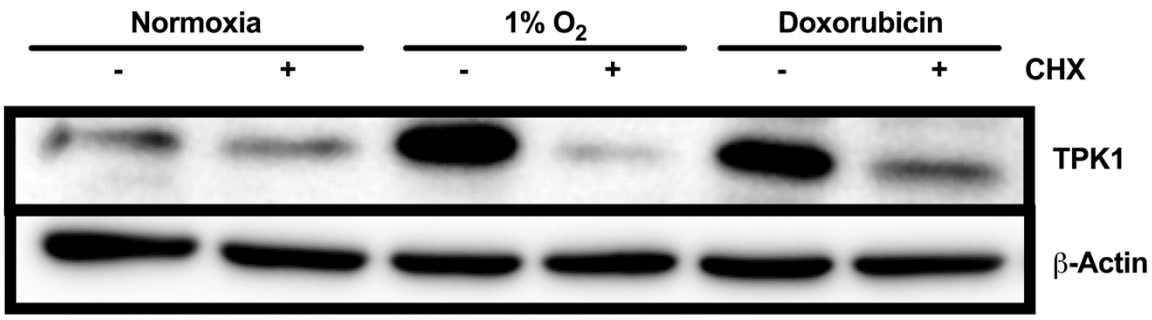


Fig 2.10. Translation inhibition with cycloheximide blocks TPK1 up-regulation during treatment with 1% O_2 and doxorubicin Representative Western blot demonstrating TPK1 expression in WCLs isolated from wild type HCT 116 cells seeded at 1250 cells/cm2 and treated with 1% O_2 or 0.1 μM DOX for 24 h in the presence (+) and absence (-) of 5 μg/mL cycloheximide (CHX). β-Actin expression serves as the loading control. Results are representative of *n = 3* independent experiments.

2.8.4 Discussion

The absence of transcriptional regulation for both TPK1 splice variants demonstrated within earlier sections of this chapter suggests that the adaptive regulation of TPK1 may be mediated downstream of transcription. This leaves many alternative levels of regulation left for investigation including mRNA localization and sequestration, micro-RNA regulation, translation, post-translational modification, and protein degradation (Spriggs *et al.* 2010). Here, the lack of induction for TPK1 during hypoxia or DOX exposure in the presence of CHX supports that TPK1 up-regulation may be mediated through mRNA translation. YC-1 has previously been shown to inhibit protein translation by mediating 4EBP1 phosphorylation (Sun *et al.* 2007). Therefore, the inhibition of TPK1 expression by both CHX and YC-1 **(Fig 2.2A and 2.2B)** supports translational regulation of the protein.

The translational up-regulation of TPK1 following malignant stress appears counterintuitive considering the high-energy burden associated with synthesizing new protein results in general repression of mRNA translation during conditions of cellular stress (Liu & Qian 2014). In so, multiple mechanisms have been described for translation inhibition during hypoxia. Phosphorylation of the eukaryotic initiation factor 2 α (eIF2 α) results in reduced numbers of competent initiation complexes available for protein translation during hypoxia (Spriggs *et al.* 2010). In addition, hypoxic generated ROS have also been shown to activate the integrated stress response (ISR) and decrease global protein synthesis (Liu *et al.* 2008). Our results suggest that TPK1 may evade the global suppression of translation that has been described for hypoxic conditions. Similar

findings have been documented for other proteins critical to cell survival during hypoxic stress, highlighting TPK1's potential role as a stress-response protein (Lai *et al.* 2016).

Of the most prominent examples of stress-response proteins, HIF-1α demonstrates increased protein expression during hypoxia in association with its pro-survival functions (Semenza 2000b). Post-translational modifications to stabilize HIF-1α and prevent its degradation partly account for the up-regulation of HIF protein during hypoxic conditions (Semenza 2007). However, HIF-1α mRNA also evades global translation shutdown demonstrated by enhanced HIF-1α translation during hypoxic conditions (Spriggs *et al.* 2010). One proposal for the enhanced translation of HIF-1α during hypoxic conditions suggests that its mRNA sequence contains an internal ribosome entry site (IRES) (Lang *et al.* 2002). In addition to HIF-1α, many other proteins that mediate cellular responses to stress are believed to contain IRESs including: c-Myc; p53; X-linked inhibitor of apoptosis (XIAP); B-cell CLL/lymphoma 2 (BCL2); HIAP2; and cold inducible RNA binding protein (CIRP) (Liu & Qian 2014) IRESs promote the recruitment of translation machinery within the 5'UTR of the mRNA sequence (downstream from typical cap-dependent translation sites), allowing continued translation despite overall repression of cap-mediated protein synthesis during stressed conditions (Spriggs *et al.* 2010). In addition to cap-independent translation through IRES, other factors can promote protein translation during stressed conditions including the presence of upstream open reading frames (uORF) with alternative start codons (CUG) (Liu & Qian 2014). Considering these mechanisms for translational activation despite general repression of translation during cellular stress, future effort should aim to further define TPK1 regulation and identify how this may relate to its importance for cell survival.

2.9 Evidence for hypoxic adaptive regulation of TPK1 in non-cancerous model

2.9.1 Introduction

One of the primary conclusions drawn within this chapter regards the HIF-mediated adaptive up-regulation of TPK1 expression following hypoxic stress in malignant cells. Like malignant cells, non-transformed cells also demonstrate functional HIF-signaling (Ratcliffe 2007). HIF-1α activation has been demonstrated in multiple non-malignant pathophysiological responses including those to hypoxic pulmonary hypertension associated with chronic lung disease, ischemic stroke, and inflammation (Ke & Costa 2006; Semenza 2000a). Furthermore, normal embryonic development requires HIF-1α signaling demonstrating the importance of this protein under a non-pathological condition (Semenza 2000a). Despite the demonstration of functional HIF-signaling in normal tissues, no knowledge exists regarding HIF-1α's impact on TPK1 expression in non-cancerous tissue.

2.9.2 Materials and Methods

Standard cell culture reagents including DMEM:F12 media, penicillin/streptomycin, and trypsin/EDTA were purchased from Corning (Manassas, VA). Fetal bovine serum (FBS) was obtained from Seradigm (Radnor, PA). HEPES, cholera toxin, insulin, transferrin, hydrocortisone, and human recombinant EGF were purchased from Sigma (St. Louis, MO). Cell culture treated flasks and dishes were purchased from Greiner Bio-One (Monroe, NC).

2.9.2.1 Cell culture

Fetal human colon (FHC) cells were obtained from ATCC. This normal colon epithelium cell line derived from fetal tissue at 13 weeks gestation has previously been used as a non-cancerous comparison for colorectal cancer cells *in vitro* (Wang *et al.* 2011; Donohoe *et al.* 2012). Per ATCC protocol, FHC cells were maintained in complete DMEM:F12, containing an additional 10 mM HEPES, 10 ng/mL cholera toxin, 0.005 mg/mL insulin, 0.005 mg/mL transferrin, 100 ng/mL hydrocortisone, 20 ng/mL human recombinant EGF, 10% FBS, and 1% penicillin/streptomycin. Mycozap (0.1%) was also added as a preventative measure for mycoplasma contamination. All cells used for experimentation ranged from passage 2-6.

For hypoxia treatments, an incubator equipped with a ProOX oxygen sensor and regulator (Biospherix, Lacona, NY) was used. This system supplies nitrogen gas to the incubation chamber, maintaining a designated level of 1% O_2 within the incubator. The sensor was calibrated weekly to ensure consistent hypoxic treatments. At the onset of hypoxic exposure, culture media was replaced with complete medium that had been pre-equilibrated in hypoxia for a minimum of 24 h.

2.9.2.2 Evaluation of protein expression

To assess changes in protein expression, cells were harvested as WCLs for Western blot analysis. WCLs were prepared as described in Section 2.6.5. WCLs (50 μg) from each treatment were resolved by electrophoresis using a 12% SDS-PAGE gel. Separated proteins were transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% non-fat milk in tris buffered saline-tween 20 (TBS-T) for 1 h at

room temperature. Membranes were then immunoblotted with primary antibody for 12 h at 4°C as previously described in Section 2.6.5. TPK1 and HIF-1α expression were assayed with β-Actin (ACTB) expression serving as loading control. **Table 2.3** provides all information regarding manufacturer and dilution (in TBS-T) for individual antibodies. Protein expression was visualized as described in Section 2.6.5

Table 2.3. Primary antibodies used for Western blot analysis

Protein of Interest (Predicted MW)	Manufacturer (Catalog Number)	Dilution	Secondary Antibody	Antibody Registry ID
TPK1 (27kDa)	Genetex (GTX103943)	1:500	Goat Anti-Rabbit	AB_1952355
β-Actin (42kDa)	Sigma-Aldrich (A2228)	1:1000	Goat Anti-Mouse	AB_476697
HIF-1α (93kDa)	Genetex (GTX127309)	1:1000	Goat Anti-Rabbit	AB_2616089

2.9.3 Results

FHC cells were used to determine the regulation of TPK1 following hypoxic conditions in a non-malignant model system. As demonstrated in **Figure 2.11A**, exposure to 1% O_2 for 24 h resulted in HIF-1α accumulation in the FHC cell line. There was a corresponding increase in TPK1 expression, which demonstrated ~15-fold enhancement compared to expression in cells grown under normal oxygen conditions **(Fig 2.11A and 2.11B)**.

2.9.4 Discussion

The observed increase of TPK1 expression in the non-malignant FHC cell line demonstrates that the adaptive regulation of TPK1 may not be limited to malignant cells. Furthermore, in HCT 116 tumor cells, TPK1 up-regulation following hypoxic conditions was quantified to be ~3-fold. In FHC cells, TPK1 expression was enhanced nearly 15-fold following hypoxia. The reasoning behind the difference in magnitude of TPK1 induction between the cancerous and non-cancerous colorectal cell lines remains unclear. However, this finding may suggest that TPK1 serves an even greater importance to the survival of normal cells during hypoxic stress. It must be considered that FHC cells represent a fetal cell line derived at 13 weeks gestation, which may be a complicating factor limiting the validity of this conclusion. Evidenced by the demonstration that mice homozygous for a HIF-1α loss-of-function mutation die at midgestation due to defects in vascularization, cardiac morphogenesis, and neural tube closure, HIF-1α signaling serves as a critical component in embryogenesis and fetal maturation (Semenza 2000a; Kotch *et al.* 1999; Iyer *et al.* 1998). Therefore, TPK1 up-regulation in response to hypoxia may be over exaggerated in the fetal FHC model due to a high-dependence of the cell line on HIF-signaling. Regardless, the adaptive regulation of TPK1 in a non-cancerous cell line provides promising evidence for the importance of TPK1 as a universal stress response during physiological and pathophysiological conditions.

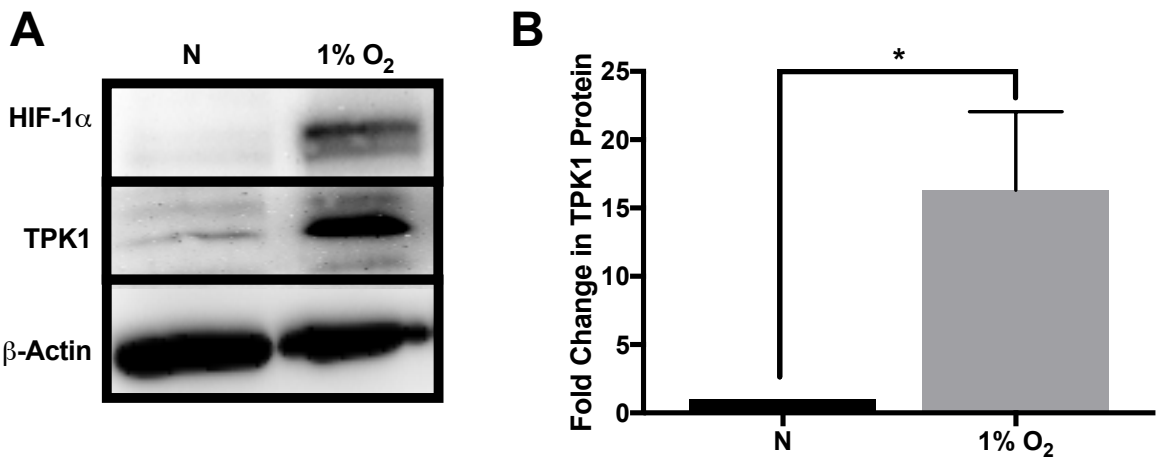


Fig 2.11. TPK1 up-regulation following hypoxia exposure in non-malignant FHC cell line (A) Representative Western blot demonstrating TPK1 expression in WCLs isolated from FHC cells exposed to 1% O_2 for 24 h compared to normoxic control (N). Cells were collected from confluent T75 maintenance flask, seeded 1:14 in 60 mm treatment dishes, and allowed to gain confluency for 96 h prior to starting hypoxia treatments. β-Actin expression serves as the loading control. **(B)** Densitometry analysis of the fold change in TPK1 expression +/- standard deviation (SD) comparing FHC cells treated in 1% O_2 compared to normoxic control (N) including $n = 3$ independent experiments. (★) Represents statistically significant difference ($p<0.05$) based on results of an unpaired student's t-test.

CHAPTER 3

THE ADAPTIVE REGULATION OF THIAMINE PYROPHOSPHOKINASE-1 IN BREAST CANCER AND IMPACTS OF SUPPLEMNTAL THIAMINE ON CELLULAR REDOX STATUS[1]

[1] Jonus, H.C. and Zastre, J.A. To be submitted to *Breast Cancer Research.*

3.1 Overview

This chapter contains a series of unpublished experiments that support the findings detailed in Chapter 2. Although primarily supplemental data, this chapter expands the scope for future work regarding thiamine homeostasis and oxidative stress. Alterations in thiamine homeostasis have predominantly been described in breast cancer compared with all other cancer types. Therefore, this chapter focuses on the regulation of TPK1 expression and the proliferative effects of supplemental thiamine in a model of breast cancer. The immortalized tumor cell line MCF7, which was used for all experimentation described within this chapter, was derived from the mammary gland of a 69-year old Caucasian female suffering from breast adenocarcinoma. The experiments detailed using MCF7 cells provide further evidence for the adaptive regulation of TPK1 in response to oxidative stress. Using MCF7 cells as supporting model to our previous work in colorectal cancer cells, we confirmed the importance of TPK1 expression for tumor cell proliferation. In addition, the findings here demonstrate that supplemental thiamine may directly reduce oxidative stress in breast tumor cells similar to what was identified for colorectal cancer cells. Furthermore, preliminary evidence suggests that exposure of MCF7 cells to supplemental thiamine may directly alter the nuclear localization of NRF2, which serves as the master transcriptional regulator orchestrating the intracellular antioxidant response. Therefore, the findings within this chapter further support an antioxidant function for thiamine and/or its derivatives in cancer cells that may contribute to malignant proliferation.

3.2 Abstract

Alterations in the expression of critical thiamine homeostasis genes have been extensively described in pre-clinical models of breast cancer as well as clinical breast tumor tissue. Despite the effort that has been placed on delineating alterations to thiamine homeostasis that occur during breast cancer, there remains little evidence demonstrating a molecular advantage for supplemental thiamine in breast cancer cells. This finding would provide logical reasoning as to why thiamine homeostasis may be exploited in cancer. Our previous work in colorectal cancer cells suggests that the proliferative benefits of thiamine may be related to the maintenance of oxidative stress. This appears to be facilitated through the adaptive regulation of TPK1 expression for thiamine pyrophosphate production. Using MCF7 cells as a model of breast cancer, Western blot analysis revealed that TPK1 expression was up-regulated in response to enhanced levels of ROS induced by hypoxia and chemotherapeutic treatment. TPK1 knockdown mediated by shRNA reduced the proliferation of MCF7 cells, while the presence of supplemental thiamine promoted their proliferation as determined by cell counting. The effect of supplemental thiamine on MCF7 proliferation corresponded with a direct impact to the antioxidant status of tumor cells demonstrated by reduced nuclear accumulation of the transcription factor nuclear factor erythroid 2-related factor. Molecular probes detecting intracellular ROS revealed that supplemental thiamine did not reduce the basal level of ROS in MCF7 cells. However, enhanced thiamine reduced intracellular superoxide levels following stimulation with Antimycin A. These findings support that during supplemental thiamine conditions thiamine homeostasis may be exploited in breast cancer for a redox advantage contributing to tumor progression.

3.3 Introduction

The up-regulation of thiamine homeostasis has been defined in breast cancer more so than any other cancer subtype. Detailed in both pre-clinical and clinical settings, evidence supports that thiamine exploitation may be a factor in the progression of breast tumors. Compared with non-cancerous primary human mammary epithelial cells (HMECs), immortalized tumorigenic cells derived from breast cancers (MDA-MB-231, MCF7, BT474) demonstrate enhanced thiamine accumulation (Zastre *et al.* 2013a). This finding may be accounted for through alterations in the expression of several thiamine homeostasis genes. Up-regulation of the thiamine transporter THTR1 (*SLC19A2*) and mitochondrial thiamine pyrophosphate carrier (TPC, *SLC25A19*) as well as the thiamine activating enzyme thiamine pyrophosphokinase-1 (TPK1) have been demonstrated for breast cancer cells compared to non-malignant control *in vitro* (Zastre *et al.* 2013a). Oncogenic signaling may account for this up-regulation as the oncogenic microRNA-155 (miR-155) has been demonstrated to regulate the gene and protein expression of THTR1, TPC, and TPK1 (Kim *et al.* 2015). Furthermore, the heterogeneous nature of breast cancer may also impact thiamine homeostasis between subtypes. *SLC19A2* and *SLC25A19* up-regulation are stronger in estrogen receptor alpha positive (ERα+) tumor cell lines (BT474, MCF7), compared with those lacking estrogen receptor alpha (ERα-) expression (BT20, MDA-MB-231) (Zastre *et al.* 2013a). Gene expression of a second thiamine transporter (THTR2, *SLC19A3*) down-regulates in breast cancer cells, but in most cases, this down-regulation does not translate into reduced protein expression (Zastre *et al.* 2013a). Despite general repression of *SLC19A3* in breast cancer cells, oncogenic signaling mediated by hypoxia-inducible factor-1α (HIF-1α) transactivates the

gene and increases thiamine transport in BT474 cells during tumor hypoxia (Sweet *et al.* 2010; Zera *et al.* 2016). Likewise, following hypoxic conditions, TPK1 protein expression enhances in MCF7 and MDA-MB-231 cells (Jonus *et al.* Accepted 2018).

Pre-clinical findings demonstrating the up-regulation of thiamine homeostasis during breast cancer are corroborated by clinical findings suggesting a similar effect. Patients suffering from breast malignancies often present to the clinic as thiamine deficient, signifying that alterations of thiamine homeostasis within tumor cells may deplete the body's peripheral thiamine store (Basu & Dickerson 1976). Supporting this hypothesis, Zastre *et al.* found enhanced expression of *SLC19A2*, *SLC25A19*, and *TPK1* in breast tumor tissue relative to non-cancerous control (Zastre *et al.* 2013a). Similar to pre-clinical findings, ERα+ tissue demonstrated a stronger expression of *SLC19A2* compared to ERα- samples. However, no differences were identified for *SLC25A19* or *TPK1* expression based on estrogen receptor status of tissue samples (Zastre *et al.* 2013a). Interestingly, supporting the deregulation of transcript levels found using *in vitro* models, Ng *et al.* identified that promoter hypermethylation represses the expression of *SLC19A3* in 80% of breast tumor tissues (Ng *et al.* 2011).

Considering the alterations that occur to thiamine homeostasis gene expression at the cellular level during malignancy, increasing thiamine supply may promote the development and growth of breast tumors. Daily *et al.* exposed the genetically predisposed MMTV^{neu} mouse model of spontaneous breast tumor development to diets with varying thiamine content (Daily *et al.* 2012). In this model, lower thiamine consumption resulted in a significant increase for tumor latency (Daily *et al.* 2012). In addition to speeding the development of tumors, higher thiamine consumption decreased

the overall percent survival time associated with breast tumor burden (Daily *et al.* 2012). In a second model, subcutaneous growth of MDA-MB-231 tumors was restricted when mice were fed thiamine-free chow compared with normal chow (Liu *et al.* 2010). Although significant advances have been made in detailing alterations in thiamine homeostasis during breast cancer, there remains very little evidence for the molecular advantage that increasing intracellular thiamine content may provide tumor cells. Our most recent report identified that a supplemental supply of thiamine may promote the proliferation of colorectal cancer cells through the TPK1's production of thiamine pyrophosphate (TPP) and maintenance of cellular redox status (Jonus *et al.* Accepted 2018). Therefore, the objective of this work was to identify the regulation of TPK1 in response to oxidative stress in the ERα+ MCF7 breast cancer cell line, and to relate this regulation to the overall proliferation of breast cancer cells during supplemental thiamine conditions.

3.4 Materials and Methods

Standard cell culture reagents including RPMI 1640 media, penicillin/streptomycin, and trypsin/EDTA were purchased from Corning (Manassas, VA). Fetal bovine serum (FBS) was obtained from Seradigm (Radnor, PA). Cell culture treated flasks and dishes were purchased from Greiner Bio-One (Monroe, NC). Doxorubicin (DOX), cisplatin (CDDP), and MG-132 were purchased from Sigma Aldrich (St. Louis, MO).

3.4.1 Cell culture

The estrogen receptor positive MCF7 (HTB-22, CVCL_0031) breast cancer cell line was obtained from ATCC (Manassas, VA). MCF7 cells were routinely cultured in complete RPMI 1640, which contained 10% FBS, 1% penicillin/streptomycin and 0.1% Mycozap (Lonza) at 37°C with 5% CO_2 designated as normoxic conditions. RPMI 1640 contains 3 μM thiamine, which is an amount approximately 300 times greater than the ~10 nM concentration found in human serum (Gangolf *et al.* 2010). Custom formulated thiamine deficient RPMI 1640 (TD 1640) (United States Biological, Salem, MA) was utilized to study the effect of supplemental thiamine compared to physiological conditions. TD 1640 was supplemented with 10% FBS, 1% penicillin/streptomycin, and 0.1% Mycozap to complete the medium. Supplementation of the TD 1640 medium with 10% FBS resulted in the physiologically relevant concentration of ~ 10 nM thiamine **(Supplemental Fig 2.8)**. Maintenance flasks of cells were grown to 80-90% confluency prior to splitting into dishes for experimental treatments. All cells used for experimentation ranged from passage 4-30.

An incubator equipped with a ProOX oxygen sensor and regulator (Biospherix, Lacona, NY) was used for hypoxia treatments. Nitrogen gas is supplied through the regulator to maintain a designated level of 1% O_2 within the incubation chamber. To ensure consistency between hypoxia treatments, the sensor was calibrated to 1% O_2 on a weekly basis. Culture media was replaced with complete RPMI 1640 that had been pre-equilibrated in hypoxia for a minimum of 24 h to begin hypoxia treatments. Dishes were subsequently transferred to hypoxic incubator for the remainder of experiment.

3.4.2 Determination of intracellular ROS level

Intracellular levels of ROS were determined by the general oxidative stress indicator CM-H2DCFDA. Following DOX and CDDP treatments, plated cells were washed with 37°C PBS and then loaded with CM-H2DCFDA dissolved in Hank's Buffered Salt Solution (HBSS) at a concentration of 5 μM for 30 min at 37°C. Cells were subsequently washed with PBS to remove any remaining probe and then trypsinized for 3 min at 37°C. Cells were collected, washed with ice-cold PBS, then suspended in HBSS for analysis. Sample fluorescence was determined by flow cytometry using the FL1 channel of a CyAn ADP analyzer (Bechman Coulter, Brea CA). Data were analyzed using FlowJo v.10 software (FlowJO, LLC, Ashland, OR).

Alternatively, basal and AA-induced ROS were detected in lysates of MCF7 cells using CM-H2DCFDA or the superoxide specific indicator dihydroethidium (DHE). Treated cells were washed then loaded with 5 μM CM-H2DCFDA or 0.5 μM DHE in HBSS for 20 min at 37°C. Plated cells were subsequently washed with PBS to remove any remaining probe and lysed using lysis buffer (1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 0.01% sodium azide, 50mM tris, 250mM NaCl, and 1mM EDTA at pH=8.5). Lysates were collected and centrifuged at 17,000xg using a Microfuge 22R Centrifuge (Beckman Coulter, Brea, CA) for 5 min at 4°C to pellet debris. Protein concentration of the resulting supernatants were quantitated by BCA protein assay (Thermo Scientific, Rockford, IL). Sample fluorescence was measured in duplicate using a Spectra Max M2e (Molecular Devices, Sunnyvale, CA) 96-well microplate reader at excitation/emission bandwidth of 535-635 with automatic cutoff at 570 nm. Sample fluorescence was subsequently normalized to total protein content.

3.4.3 Evaluation of protein expression

To assess changes in protein expression, cells were harvested as whole cell lysates (WCL) for Western blot analysis. WCLs were prepared as described in Section 2.6.5. WCLs (50 μg) from each treatment were resolved by electrophoresis using a 12% SDS-PAGE gel. Separated proteins were then transferred to polyvinylidene difluoride membranes. Membranes were blocked and immunoblotted for TPK1 and HIF-1α with β-Actin expression serving as loading control as described in Section 2.6.5. **Table 3.1** provides all information regarding manufacturer and dilution (in TBS-T) for individual antibodies used for this experimentation. Protein expression was visualized as described in Section 2.6.5 and densitometry analysis comparing TPK1 expression relative to β-Actin was performed using Fluorchem SP Software (Alpha Innotech, San Leandro, CA).

Alternatively, to assess the compartmental localization of NRF2 independent nuclear and cytosolic extracts were prepared. Treated cells were washed with ice-cold 1X phosphate buffered saline (PBS). Cells were then lysed in cytoplasmic extraction buffer (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM ethylene glycol tetraacetic acid (EGTA)). Lysates were collected, vortexed and incubated on ice for 10 min. Following incubation, 10% NP-40 was added to each lysate at the ratio of 62.5 μL/1mL of lysate. Samples were vortexed and incubated on ice for 1 min. Nuclei were then pelleted by centrifugation at 17,000xg for 10 min at 4°C using a Microfuge 22R Centrifuge (Beckman Coulter, Brea, CA). The resulting supernatant was collected as the cytosolic extract. Nuclei were then lysed by resuspension in nuclei extraction buffer (20 mM HEPES, 0.4 M NaCl, 1mM EDTA, 1 mM EGTA). Nuclear lysates were incubated on ice for 40 min and vortexed for 30 secs every 10 min. Lysates were then centrifuged at

17,000xg for 10 min at 4°C using a Microfuge 22R Centrifuge (Beckman Coulter, Brea, CA). The resulting supernatant was collected and used as nuclear extract. Total protein of WCLs as was as nuclear and cytosolic extracts was quantified using the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL).

To determine protein expression and localization, 50 μg of total protein from each treatment was resolved by electrophoresis using a 12% SDS-PAGE gel. Separated proteins were then transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% non-fat milk in TBST for 1 h at room temperature. Membranes were subsequently immunoblotted with primary antibody for 12 h at 4°C. NRF2 expression was assayed with β-Actin expression provided as loading control for cytosolic extracts and p84 as loading control for nuclear extracts. **Table 3.1** provides all information regarding manufacturer and dilution (in TBS-T) for individual antibodies. After the primary antibody incubation, blots were washed three times with TBS-T (10 min each) and then exposed to horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit secondary antibody (Bethyl Laboratories, Montgomery TX) at a 1:10,000 dilution in TBS-T for 1 h at room temperature. Blots were again washed three times with TBS-T. Protein expression was visualized using Supersignal-PLUS West Pico Solution (Thermo Scientific, Rockford, IL) according to manufacturer's instruction. Signal was imaged using a Fluorchem SP digital imager (Alpha Innotech, San Leandro, CA). Densitometry analysis comparing each protein of interest relative to its loading control was performed using Fluorchem SP Software (Alpha Innotech, San Leandro, CA).

Table 3.1. Primary antibodies used for Western blot analysis

Protein of Interest (Predicted MW)	Manufacturer (Catalog Number)	Dilution	Secondary Antibody	Antibody Registry ID
TPK1 (27kDa)	Genetex (GTX103943)	1:500	Goat Anti-Rabbit	AB_1952355
HIF-1α (93kDa)	Genetex (GTX127309)	1:1000	Goat Anti-Rabbit	AB_2616089
NRF2 (68kDA)	Genetex (GTX103322)	1:1000	Goat Anti-Rabbit	AB_1950993
β-Actin (42kDa)	Sigma-Aldrich (A2228)	1:1000	Goat Anti-Mouse	AB_476697
p84 (84kDa)	Genetex (GTX70220)	1:1000	Goat Anti-Mouse	AB_372637

3.4.4 shRNA mediated TPK1 knockdown

Stable knockdown of TPK1 in MCF7 cells was generated using a commercially available and validated shRNA construct introduced by MISSION™ Lentiviral Transduction Particles (Sigma, St. Louis, MO). Briefly, MCF7 cells were seeded at 47,500 cells/cm^2 and allowed to attach ~12 h prior to transduction. Cells were subsequently transduced using a 5X multiplicity of infection (MOI). Transduction particles were combined with hexadimethrine bromide (polybrene, 8 μg/mL) in complete RPMI 1640 and applied to plated cells for 24 h. After 24 h, transducing media was removed and cells were supplied fresh RPMI 1640 for a 24 h recovery period prior to selection with 1.5 μg/mL puromycin in RPMI 1640. TPK1 knockdown was validated in two passages following transduction by RT-PCR **(Fig 3.1)**.

3.4.5 Validation of TPK1 knockdown by RT-PCR

Quantitative real-time PCR was used to confirm the genetic knockdown of *TPK1* in MCF7 cells. Total RNA was extracted using the E.Z.N.A Total RNA Kit I (Omega Bio-Tek, Norcross, GA) following the manufacturer's protocol. The RNA concentration was determined using a Nanodrop 2000c Spectrophotometer (Thermo Scientific, Rockford, IL), and 1 μg of isolated RNA was reverse transcribed using the qScript cDNA Synthesis Kit (Quanta BioSciences, Gaithersburg, MD). Gene detection was performed using a Light-Cycler 480 II (Roche Applied Science, Indianapolis, IN). Primers for TPK1 (F: 5'-gcctttaccccgttggag-3' and R: 5'-ccaaaggctgattaagaattacaag-3') were designed using the Roche Universal Probe Library assay design center. The primers corresponded with Roche hydrolysis probe #48 labeled with fluorescein (FAM). The expression of *ACTIN (ACTB)* was determined using the Universal ProbeLibrary Human ACTB Gene Assay and used as a reference gene to calculate relative gene expression based on the $2^{-\Delta\Delta Ct}$ method with an assumed efficiency of 2.

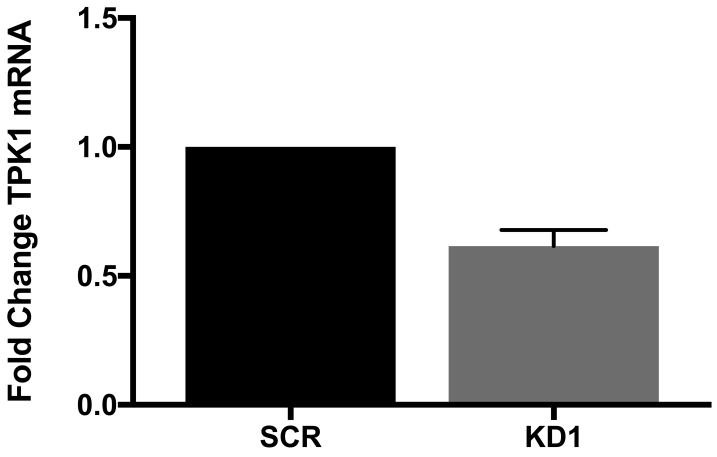


Fig 3.1. Validation of shRNA-mediated TPK1 knockdown in MCF7 cells Fold change +/- SD in *TPK1* expression determined by qRT-PCR analysis comparing MCF7 cells expressing TPK1 targeted shRNA construct (KD1) to non-targeted control (SCR) vector representative of two passages following original transduction and selection.

3.4.6 Determination of cellular proliferation

A clonogenic assay was used to determine the impact of TPK1 knockdown on MCF7 proliferation. Cells stably expressing shRNA vectors were seeded at 500 cells/well in 6-well plates containing RPMI 1640 with either 10 nM or 3 μM thiamine and 1.5 μg/mL puromycin. Cells were cultured 7 d and then provided fresh RPMI 1640 with either 10 nM or 3 μM thiamine and 1.5 μg/mL puromycin. Following media change, cells were cultured an additional 7 d before determining extent of proliferation by crystal violet staining. Adherent cells were fixed in buffered formalin at room temperature for 30 min. Formalin was removed and plates were washed two times with diH_2O. Fixed cells were immediately stained with 0.1% crystal violet solution for 30 min at room temperature. Crystal violet stain was then removed and plates were washed with diH_2O. Plates were allowed to dry in a biosafety cabinet under laminar air flow. When completely dry, 1% Trition X-100 was used to lyse and destain fixed cells. Absorbance of resulting solution was determined using a Spectra Max M2e (Molecular Devices, Sunnyvale, CA) 96-well microplate reader at 550 nm.

Cellular proliferation was also determined by cell counting with trypan blue exclusion. Spent media was aspirated and cells were trypsinized for 3 min at 37°C. An equal volume of RPMI 1640 was added to neutralize trypsin. Live cell count was determined using a 1:1 dilution with trypan blue using a TC-20 automated cell counter (BioRad, Hercules, CA). Additionally, crystal violet staining was utilized as a visual representation for the changes in proliferation. Adherent cells were fixed in buffered formalin at room temperature for 30 min. Formalin was removed and replaced with 0.5% crystal violet for 10 min at room temperature. Crystal violet was then removed and the

culture dishes were washed three times with deionized water and allowed to dry prior to imaging.

3.5 Results

3.5.1 Induction of ROS in MCF7 cells corresponds with enhanced TPK1 expression

Previously, inducing hypoxic and oxidative stress in colorectal cancer cells by treatment with 1% O_2 and the chemotherapeutics doxorubicin (DOX) and cisplatin (CDDP) resulted in enhanced expression of the thiamine activating enzyme TPK1. Therefore, the impact of all three treatments on TPK1 expression was assessed in MCF7 breast cancer cells. First, each treatment condition was confirmed to induce ROS levels. Following treatment with 1% O_2, DOX, or CDDP for 24 h, there was a significant increase in the level of basal ROS in MCF7 cells determined by CM-H2DCFDA fluorescence intensity **(Fig 3.2A)**. The induction of ROS corresponded with significant up-regulation of TPK1 expression following treatment with 1% O_2, DOX, and CDDP compared to control **(Fig 3.2B and 3.2C)**. Lysates treated with 1% O_2 also demonstrated accumulation of HIF-1α, confirming the induction of hypoxic conditions, while no notable HIF-1α was detected following treatment with DOX and CDDP **(Fig 3.2B)**.

3.5.2 TPK1 knockdown restricts MCF7 proliferation under basal and supplemental conditions

A validated shRNA construct targeting TPK1 was used to stably knockdown TPK1 expression in MCF7 cells **(Fig 3.1)**. MCF7 cells expressing shRNA targeting TPK1 or a scrambled non-targeting sequence were selected with puromycin prior to

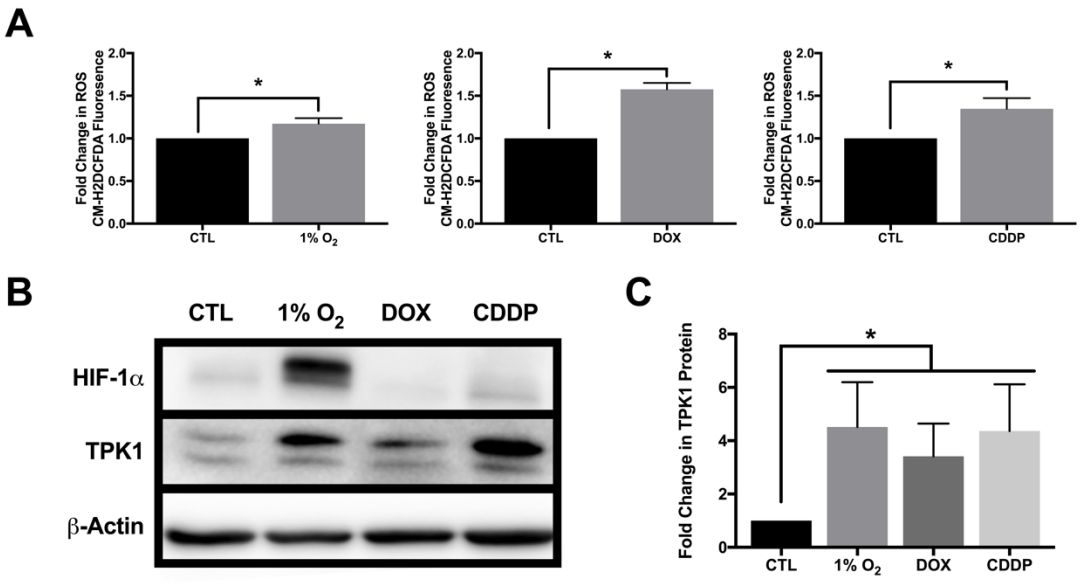


Fig 3.2. TPK1 up-regulation corresponds with ROS induction in MCF7 cells (A) Fold change in CM-H2DCFDA median fluorescence intensity +/- SD demonstrating fold change in ROS levels between MCF7 cells seeded at 3000 cells/cm^2 and treated with 1%O_2, 0.1 μM DOX, or 10 μM CDDP for 24 h relative to untreated normoxic control (CTL). **(B)** Representative Western blot demonstrating HIF-1α and TPK1 expression in WCLs isolated from MCF7 cells seeded at 3000 cells/cm^2 and treated with 1%O_2, 0.1 μM DOX, or 10 μM CDDP for 24 h relative to untreated normoxic control (CTL). β-Actin expression serves as the loading control. **(C)** Densitometry analysis of the fold change in TPK1 expression +/- SD following 1% O_2, DOX or CDDP treatment in MCF7 cells compared to untreated normoxic control (CTL) including *n = 4* independent experiments. (✶) Represents statistically significant difference ($p<0.05$) based on results of (A) unpaired student's t-test or (C) one-way ANOVA with Tukey's post-hoc test.

assessing proliferation rates under both basal and supplemental thiamine conditions. **Figures 3.3A and 3.3B** demonstrate that MCF7 cells expressing the TPK1 targeted shRNA construct (KD1) proliferated at nearly half the rate of vector control (SCR) cells when cultured in TD 1640 (10 nM thiamine) media. Supplementing the cells with excess thiamine by culturing in RPMI 1640 (3 μM thiamine) demonstrated no protection to proliferation as TPK1 KD1 cells also proliferated at nearly half the rate of SCR control MCF7 cells **(Fig 3.3C and 3.3D)**.

3.5.3 Thiamine supplementation promotes MCF7 proliferation

While demonstrating the effects of TPK1 knockdown on tumor cell proliferation under basal and supplemental thiamine conditions, it was noticed that control cells cultured with supplemental thiamine appeared to proliferate more rapidly than those cultured with basal thiamine **(Fig 3.3B and 3.3D)**. To confirm this effect, wild type MCF7 cells were routinely cultured in 10 nM thiamine or the supplemental dose of 3 μM thiamine and growth rates were compared. MCF7 cells cultured in supplemental thiamine demonstrated an ~2-fold increase in proliferation compared to cells grown in 10 nM thiamine **(Figure 3.4)**.

3.5.4 Alterations in NRF2 localization following exposure to supplemental thiamine

The transcription factor nuclear factor erythroid 2-related factor (NRF2) serves as the master transcriptional regulator coordinating the cellular response to ROS (Kansanen *et al.* 2013). During conditions of oxidative stress, NRF2 ubiquitination is blocked resulting in reduced proteolytic degradation (Li, 2013). This results in NRF2

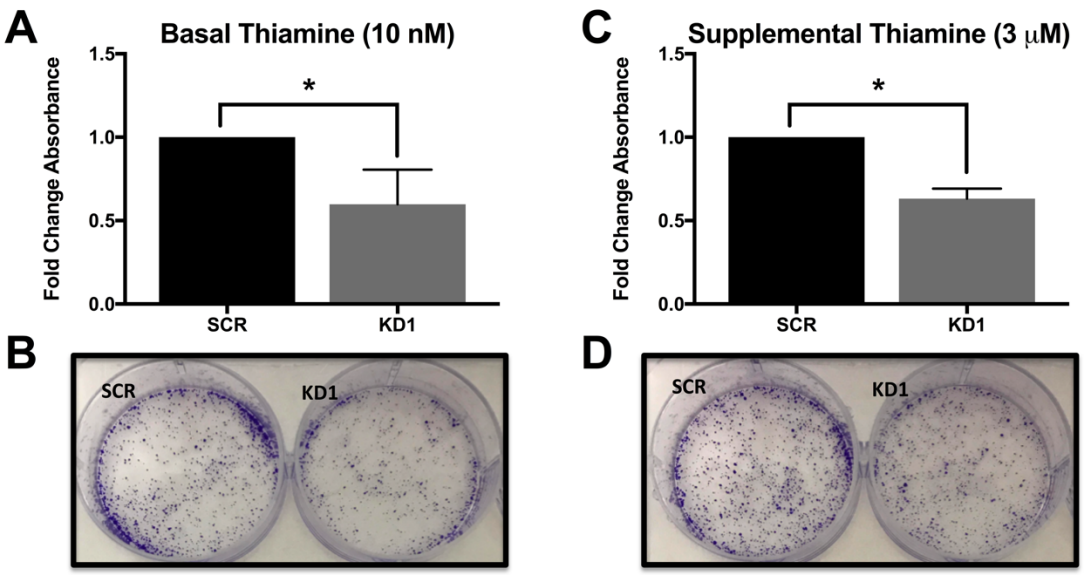


Fig 3.3. TPK1 knockdown reduces proliferation of MCF7 cells regardless of thiamine concentration (A) Effect of TPK1 knockdown on MCF7 proliferation in 10 nM thiamine determined by clonogenic assay and quantitated as fold change +/- SD in crystal violet absorbance (550 nm) of cells transduced with scramble control (SCR) or TPK1 knockdown (KD1) shRNA and selected with puromycin. Results are representative of $n = 3$ independent experiments. **(B)** Images of formalin fixed cells stained with crystal violet following clonogenic assay of SCR and KD1 MCF7 cells in 10 nM thiamine. **(C)** Effect of TPK1 knockdown on MCF7 proliferation in the presence of 3 μM thiamine determined by clonogenic assay and quantitated as fold change +/- SD in crystal violet absorbance (550 nm) of cells transduced with scramble control (SCR) or TPK1 knockdown (KD1) shRNA and selected with puromycin. Results are representative of $n = 3$ independent experiments. **(D)** Images of formalin fixed cells stained with crystal violet following clonogenic assay of SCR and KD1 MCF7 cells in 3 μM thiamine. (★) Represents statistically significant difference ($p<0.05$) based on results of unpaired student's t-test.

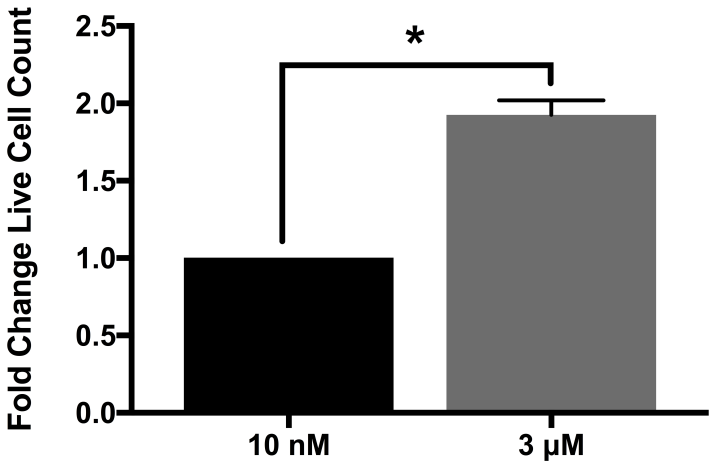


Fig 3.4. Supplemental thiamine promotes MCF7 proliferation Effect of thiamine supplementation on tumor cell proliferation demonstrated by fold change in live cell count +/- SD determined by trypan blue exclusion of MCF7 cells seeded at 10,000 cells/cm^2 and grown 6 d in either 10 nM or 3 µM thiamine representative of *n = 3* independent experiments. (*) Represents statistically significant difference ($p<0.05$) based on results of unpaired student's t-test.

accumulation and subsequent translocation to the nucleus where it transactivates the expression of genes harboring antioxidant response elements (AREs) in their regulatory domains (Kansanen *et al.* 2013). Therefore, the cytosolic and nuclear localization of NRF2 were used as a probe to describe the oxidative status of tumor cells under supplemental thiamine conditions compared to the basal level of 10 nM thiamine. As a positive control for the detection of NRF2, MCF7 cells were treated with MG-132, a proteasome inhibitor, to induce its accumulation by blocking proteolytic degradation (Li *et al.* 2012). MG-132 treatment resulted in enhanced NRF2 expression in both the cytosolic and nuclear cell lysates **(Fig 3.5A)**. Following a dose-dependent fashion, there was a trending reduction in NRF2 nuclear localization when supplemental thiamine was supplied to MCF7 cells **(Fig 3.5A and 3.5B)**. **Figure 3.5A** demonstrates that compared with the nucleus, only faint detection of NRF2 was found in the cytosolic fraction of the cell lysate. Based on densitometry analysis, NRF2's presence in the cytosol was not impacted by thiamine dose **(Fig 3.5B)**.

3.5.5 Effects of supplemental thiamine on basal and AA-induced ROS levels

Considering supplemental thiamine reduced nuclear accumulation of NRF2, it was hypothesized this may have been due to a reduction in the basal level of ROS present in MCF7 cells during supplemental conditions. Therefore, CM-H2DCFDA fluorescence was used to detect general ROS levels in the cytoplasm. MCF7 cells cultured in the presence of 300 nM and 3μM thiamine demonstrated no difference in ROS compared with cells cultured in the basal level of 10 nM thiamine **(Fig 3.6A)**. Although thiamine supplementation did not directly alter basal ROS levels, it was considered that the

presence of supplemental thiamine may offer protection during ROS generating conditions. To test thiamine's ability to protect against oxidative stress during ROS stimulation, Antimycin A (AA) was used as a chemical means to induce ROS production. AA inhibits complex III of the electron transport chain, resulting in e^- leakage and the generation of superoxide radicals. During AA stimulation, there was a trending reduction in general oxidative stress with increasing thiamine dose **(Fig 3.6B)**. In addition to CM-H2DCFDA, AA-induced ROS was also detected with DHE, which directly detects the presence of superoxide radicals within the cytoplasm. Using DHE as a probe, there was a significant decrease in the intracellular level of superoxide following AA treatment in the presence of 3 μM thiamine compared to the basal level of 10 nM **(Fig 3.6C)**.

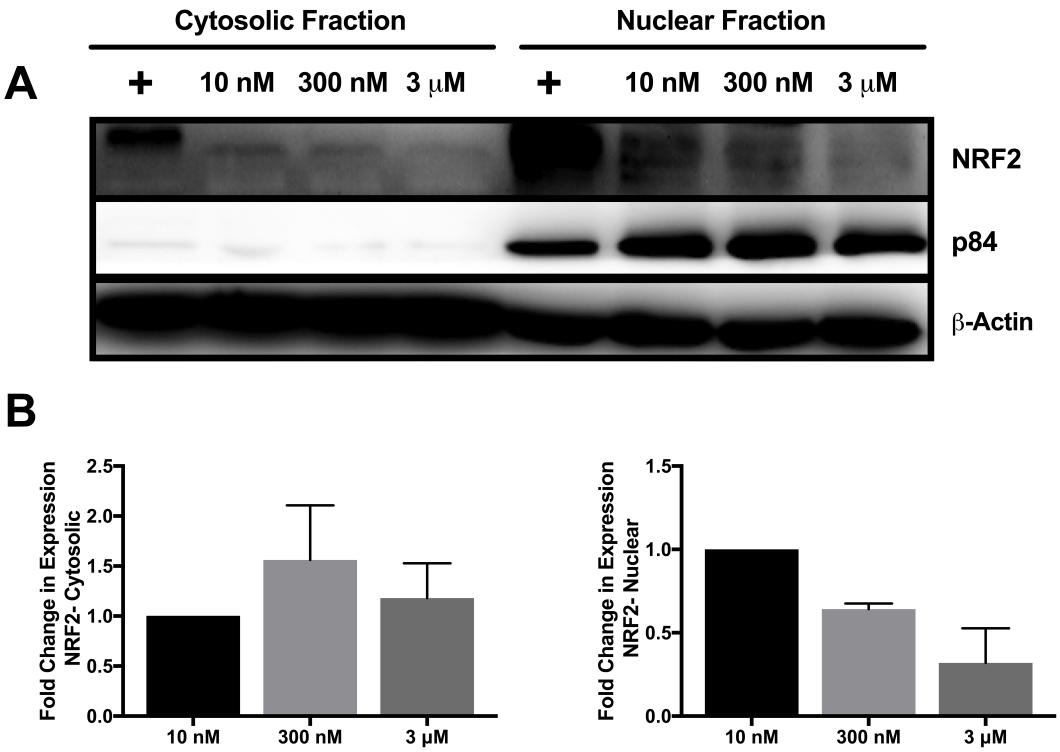


Fig 3.5. Supplemental thiamine reduces NRF2 localization (A) Representative Western blots demonstrating NRF2 protein expression in cytosolic and nuclear cell lysates isolated from MCF7 cells seeded at 40,000 cells/cm^2 and cultured 6 d in the presence of 10 nM, 300 nM, or 3 μM thiamine. For NRF2 positive control (+), cells were treated with 10 μM MG-132 for 12 h prior to harvest. β-Actin expression serves as the cytosolic loading control and p84 serves as the nuclear loading control. **(B)** Densitometry analysis of the fold change in NRF2 expression +/- SD in cytosolic and nuclear cell fractions following supplementation with 300 nM or 3 μM thiamine compared to basal 10 nM control including *n = 2* independent experiments.

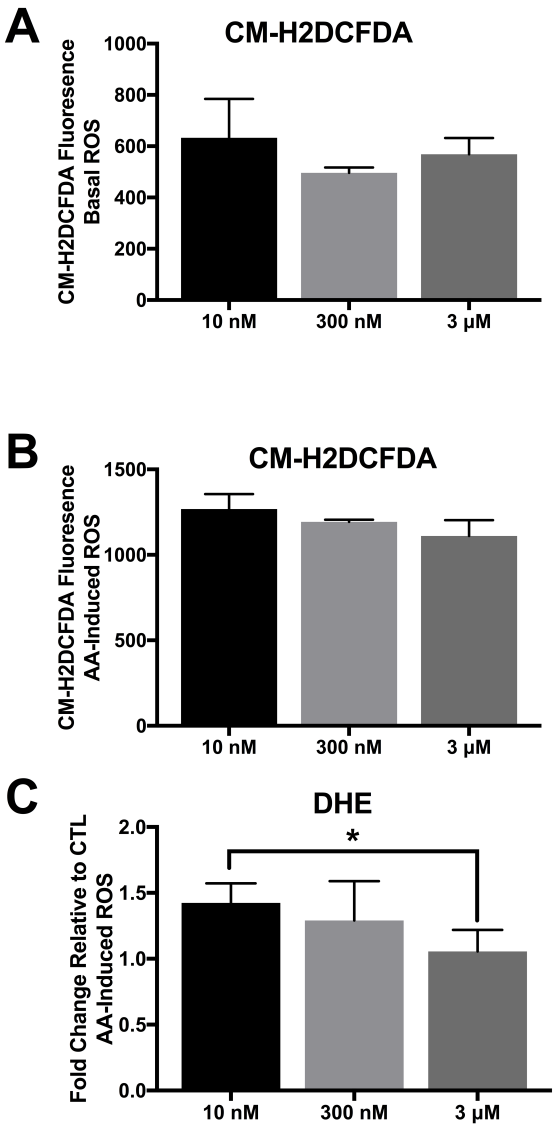


Fig 3.6. Thiamine protects against AA-induced oxidative stress (A) CM-H2DCFDA fluorescence intensity +/- SD comparing ROS levels of MCF7 cells seeded at 40,000 cells/cm^2 and supplemented with 300 nM or 3 µM thiamine compared to 10 nM for 6 d including *n = 3* independent experiments. **(B)** CM-H2DCFDA fluorescence intensity +/- SD comparing ROS levels of MCF7 cells seeded at 40,000 cells/cm^2 and supplemented with 300 nM or 3 µM thiamine compared to 10 nM for 6 d prior to treating with 5 µM AA for 6 h including *n = 3* independent experiments. **(C)** Fold change in DHE fluorescence intensity +/- SD comparing fold change in ROS levels of MCF7 cells seeded at 40,000 cells/cm^2 and supplemented with 300 nM or 3 µM thiamine compared to 10 nM for 6 d prior to treating with 5 µM AA for 6 h including *n = 5* independent experiments.

3.6 Discussion

The global burden of breast cancer exceeds that of all other cancers for women, with rates of new incidences continuing to rise (Giordano & Gradishar 2017). Many advances have been made in developing novel therapies for the treatment of breast cancer with some of the most promising including inhibitors targeting poly(ADP-ribose) polymerase, cyclin-dependent kinases 4 and 6, the PI3K/Akt/mTOR pathway, histone deacetylation, multi-targeting tyrosine kinases, and immune checkpoints (Tong *et al.* 2018). However, despite these recent advances the disease remains the second leading cause of cancer-related death among women (Tong *et al.* 2018). Thiamine has been considered as a common dietary factor that may contribute to the progression of breast cancer (Zastre *et al.* 2013b). Although thiamine homeostasis up-regulates at the cellular level in breast cancer, the molecular advantage that supplemental thiamine provides tumor cells remains unclear (Zastre *et al.* 2013a).

Here, it was demonstrated that expression of the thiamine activating enzyme TPK1 up-regulates following oxidative stress in MCF7 breast cancer cells. Furthermore, TPK1 expression was required for maximal tumor cell proliferation. This finding supports our previous work, which demonstrates the adaptive regulation of TPK1 in response to hypoxic and oxidative stress in colorectal cancer cells (CRC) (Jonus *et al.*, Accepted 2018). Considering the similar response for TPK1 regulation across tumor types, this mechanism of thiamine homeostasis may also transcend the heterogeneity associated with breast cancer, which directly contributes to complication in treating the disease (Turashvili & Brogi 2017). Supporting this hypothesis, TPK1 up-regulates in the ERα+ MCF7 cell line as well as the triple negative breast cancer (TNBC) cell line MDA-

MB-231 following hypoxic stress (Jonus *et al.* Accepted 2018). Therefore, if certain aspects of the thiamine homeostasis pathway are uniformly exploited across all breast cancer sub-types, elucidating their intracellular functions should contribute to a further understanding of the disease progression and enable more effective treatment strategies.

Supplemental vitamin E, which demonstrates antioxidant potential, reduces levels of ROS in MCF7 cells and promotes *in vitro* and *in vivo* proliferation (Diao *et al.* 2016). Like vitamin E, thiamine and, more so, TPP, demonstrate antioxidant activity (Okai *et al.* 2007). In CRC, TPK1 up-regulation following oxidative stress appears to facilitate the production of TPP (Jonus *et al*. Accepted 2018). In addition to serving in its canonical role as a cofactor, the TPP produced during supplemental thiamine conditions may also serve as an intracellular antioxidant counteracting oxidative stress and promoting tumor cell proliferation (Jonus *et al*. Accepted 2018). In support, supplemental thiamine increases the proliferation rate of the colorectal cancer cell line HCT 116 and decreases basal cellular ROS (Jonus *et al*. Accepted 2018). Unlike HCT 116 cells, exposure of MCF7 breast cancer cells to supplemental thiamine resulted in no reduction in basal ROS levels. However, as was seen with HCT 116 cells, an increase in cellular proliferation was observed with 3 μM thiamine compared to 10 nM thiamine. It remains unclear why supplemental thiamine directly reduced basal ROS in HCT 116 cells but not MCF7 cells. However, the antioxidant effect of TPP may still be supported in the breast cancer model considering the observed reduction in NRF2 nuclear localization. During supplemental thiamine conditions, TPP production and antioxidant consumption in MCF7 cells may result in a lower cellular perception of oxidative stress resulting in decreased NRF2 nuclear localization. The reduction in superoxide observed with supplemental thiamine

during AA-stimulation further supports the antioxidant activity of thiamine and/or TPP. Interestingly, this reduction was detected with the superoxide-specific DHE probe but not the general oxidative stress indicator CM-H2DCFDA. This necessitates further investigation to confirm which ROS thiamine derivatives preferentially neutralize. Furthermore, it should be considered that TPP localization within the cytosol or its accumulation in the mitochondria following transport *via* the TPC (*SLC25A19*) may impact interaction with ROS.

Based on our model, it was expected that supplemental thiamine may, at least in part, rescue proliferation following TPK1 knockdown in MCF7 cells by salvaging TPP production. However, there was no demonstrated difference in proliferation of MCF7 cells following TPK1 knockdown with 10 nM thiamine compared to 3 μM thiamine. This may suggest a complete inhibition of TPP production due to TPK1 knockdown regardless of thiamine concentration. Alternatively, TPK1 may confer other TPP-independent effects on tumor cell proliferation. Future efforts should further investigate TPP levels following TPK1 knockdown under basal and supplemental thiamine conditions. This will allow a more detailed conclusion for the role of TPK1 expression in the maintenance of tumor cell proliferation

CHAPTER 4

THE CHEMOTHERAPEUTIC POTENTIAL OF THIAMINE ANALOGS *IN VITRO* AND *IN VIVO*[1]

[1] Jonus, H.C., Darkhal, P., Byrnes, C., Kim, J., Barlett, M.G., Said, H.M., and Zastre, J.A. To be submitted to *Biomedicine & Pharmacotherapy*.

4.1 Overview

This chapter opens with a manuscript currently in preparation for submission to *Biomedicine & Pharmacotherapy* later this year. Supplemental figures and tables from the publication are provided within the main text for the reader's ease of access. The work detailed within the manuscript describes the chemotherapeutic potential of the commercially available thiamine analogs sulbutiamine and benfotiamine both *in vitro* and *in vivo*. In addition, the findings presented suggest that the anticancer properties of sulbutiamine, benfotiamine, and thiamine are all dependent on the intracellular accumulation of TPP. It is concluded that TPP serves as the active species in reducing tumor cell proliferation through its ability to inhibit pyruvate dehydrogenase kinase activity. Additional data provided at the end of the chapter demonstrate the chemotherapeutic potential of thiamine analogs in regard to chemoresistance. It was found that cancer cells demonstrate less relative resistance to both sulbutiamine and benfotiamine compared with other standard chemotherapies. This work provides the basis for future studies in identifying any additive and/or synergistic effects of sulbutiamine or benfotiamine that may support their use as adjuvant therapies with standard chemotherapies.

4.2 Abstract

Malignant cells demonstrate an oncogenic-driven reliance on glycolytic metabolism to support their highly proliferative nature. The up-regulation of pyruvate dehydrogenase kinase (PDK) expression promotes glycolysis through the restriction of mitochondrial metabolism. PDK silencing restores mitochondrial function and leads to reduced tumor cell proliferation. Vitamin B1, or thiamine, possesses antitumor properties related to its capacity to inhibit PDH phosphorylation and promote PDH activity, presumably through PDK inhibition. Though a promising nutraceutical approach for cancer therapy, thiamine's low bioavailability may limit clinical effectiveness. Here, we have demonstrated that increasing thiamine bioavailability through exploiting the commercially available lipophilic thiamine analogs sulbutiamine and benfotiamine increases thiamine's anticancer effect. Determined by crystal violet and MTS proliferation assays, both sulbutiamine and benfotiamine reduced thiamine's millimolar IC50 value to micromolar equivalents. Furthermore, pharmacologic administration of benfotiamine significantly reduced tumor growth in a subcutaneous mouse model. HPLC analysis revealed that sulbutiamine and benfotiamine increased intracellular thiamine and thiamine pyrophosphate (TPP) concentrations *in vitro* corresponding with reduced levels of PDH phosphorylation. Through an *ex vitro* kinase screen, thiamine's activated cofactor form TPP was found to inhibit the function of multiple PDK isoforms. Attempts to maximize intracellular TPP through thiamine homeostasis gene expression resulted in enhanced apoptosis in tumor cells. Based on these results, we conclude that TPP most likely serves as the active species mediating thiamine's inhibitory effect on tumor cell proliferation.

4.3 Introduction

Reengineering metabolism provides tumor cells with a proliferative advantage by allowing the rapid generation of ATP and accumulation of biomass, while simultaneously maintaining redox homeostasis (Cairns *et al.* 2011). Several oncogenic driven processes confer a glycolytic phenotype in tumor cells including the up-regulation of glucose transporters, overexpression of glycolytic and pentose phosphate pathway (PPP) enzymes, and restriction of mitochondrial metabolism (Cairns *et al.* 2011). The latter is facilitated by pyruvate dehydrogenase kinases (PDK), which inactivate pyruvate dehydrogenase (PDH) *via* phosphorylation. This phosphorylation event limits pyruvate from entering the tricarboxylic acid (TCA, Kreb's) cycle and subsequently reduces mitochondrial ATP production (Saunier *et al.* 2016). Four isoenzymes of PDK (1-4) exist, each demonstrating unique tissue specificity and PDH phosphorylation sites (Gudi *et al.* 1995; Kolobova *et al.* 2001). PDKs are overexpressed in multiple cancer types by the influence of oncogenic transcription factors including hypoxia-inducible factor-1α (HIF-1α) and MYC (Zhang *et al.* 2015). Inhibiting PDK activity restores mitochondrial-dependent metabolism resulting in mitochondrial membrane depolarization and the accumulation of mitochondrial derived reactive oxygen species (ROS) (Shen *et al.* 2013; Bonnet *et al.* 2007). PDK silencing ultimately results in enhanced apoptosis and an overall reduction in tumor cell proliferation (Woolbright *et al.* 2018; McFate *et al.* 2008; Bonnet *et al.* 2007). Targeting PDKs has been the focus of recent pre-clinical and clinical investigation as a chemotherapeutic strategy (Stacpoole 2017). Dichloroacetate (DCA), a PDK inhibitor approved for the treatment of lactic acidosis, demonstrated promising pre-clinical chemotherapeutic efficacy (Michelakis *et al.* 2008). However, a recent phase II

clinical trial of DCA for treating metastatic breast and non-small-cell lung cancer was terminated early due to higher than expected safety concerns (Papandreou *et al.* 2011; Martinez-Outschoorn *et al.* 2017). Therefore, overcoming this limitation for DCA with other compounds targeting tumor cell metabolism at the PDK-PDH axis may prove promising as a chemotherapeutic strategy.

Vitamin B1, or thiamine, may be one effective alternative to DCA for targeting cancer cell metabolism. Thiamine is an essential micronutrient, whose activated co-factor form thiamine pyrophosphate (TPP) canonically functions as a required cofactor for the maintenance of metabolism in all cells. Comin-Anduix *et al.* found that high-dose thiamine supplementation (~2500 times the regular daily allowance) inhibits tumor proliferation *in vivo* (Comin-Anduix *et al.* 2001). Thiamine supplementation has also been demonstrated to reduce glycolysis in tumor cells (Liu *et al.* 2018). We previously identified that high-dose thiamine decreased tumor cell proliferation corresponding with a reduction in PDH phosphorylation (Hanberry *et al.* 2014). These results suggest that thiamine may function to inhibit tumor growth by stimulating PDH activity through a mechanism similar to DCA (Hanberry *et al.* 2014). Once inside the cell, thiamine is rapidly phosphorylated into thiamine pyrophosphate (TPP) by the activity of thiamine pyrophosphokinase-1 (TPK1) (Nosaka *et al.* 2001). TPP serves as a cofactor for enzymes involved in carbohydrate metabolism including PDH as well as α-ketoglutarate dehydrogenase (ODGH) and transketolase (TKT). In addition to its role as a cofactor, the presence of TPP promotes PDH activity by regulating its phosphorylation (Kolobova *et al.* 2001). *In vivo* administration of TPP in the form of hydroxyethylthiamine diphosphate reduced tumor burden by ~75% (Gevorkyan & Gambashidze 2014). These findings

suggest that TPP may mediate the growth-inhibiting effects of high-dose thiamine during malignancy.

Thiamine administration in doses greater than 1g/day have been clinically reported to be well-tolerated with only minor side-effects including nausea and indigestion (Smithline *et al.* 2012; Meador *et al.* 1993). Compared to xenobiotic chemotherapeutic treatments, a nutraceutical approach using thiamine would not be expected to be limited by systemic toxicity. However, a major limitation for thiamine's clinical effectiveness will be its poor bioavailability estimated to be as low as 3.7-5.3% (Weber & Kewitz 1985; Tallaksen *et al.* 1993). Similar to DCA, thiamine demonstrated an IC50 value in the mM range required to reduce tumor cell proliferation (Hanberry *et al.* 2014). This was most likely due to limited thiamine accumulation within tumor cells. The quaternary nitrogen located within thiamine's thiazole ring and overall hydrophilicity of the molecule necessitates carrier mediated transport across the plasma membrane for absorption and cellular accumulation **(Fig 4.1)**. To circumvent this, lipophilic thiamine analogs that do not require facilitative transport may be exploited. Sulbutiamine and benfotiamine are two synthetic analogs of thiamine that are commercially available as vitamin supplements. Sulbutiamine (isobutyrylthiamine disulfide) is a lipophilic precursor of thiamine that possesses the ability to cross biological membranes (Bettendorff 1994b). Following thiazole ring opening, two thiamine molecules are bound together through a disulfide bridge to form the sulbutiamine molecule **(Fig 4.1)** (Bettendorff *et al.* 1990a). Sulbutiamine is rapidly converted into individual thiamine molecules by thioesterase activity inside the cell (Yagi 2012). Benfotiamine (S-benzoylthiamine-O-Monophosphate) is dephosphorylated in the intestine and absorbed

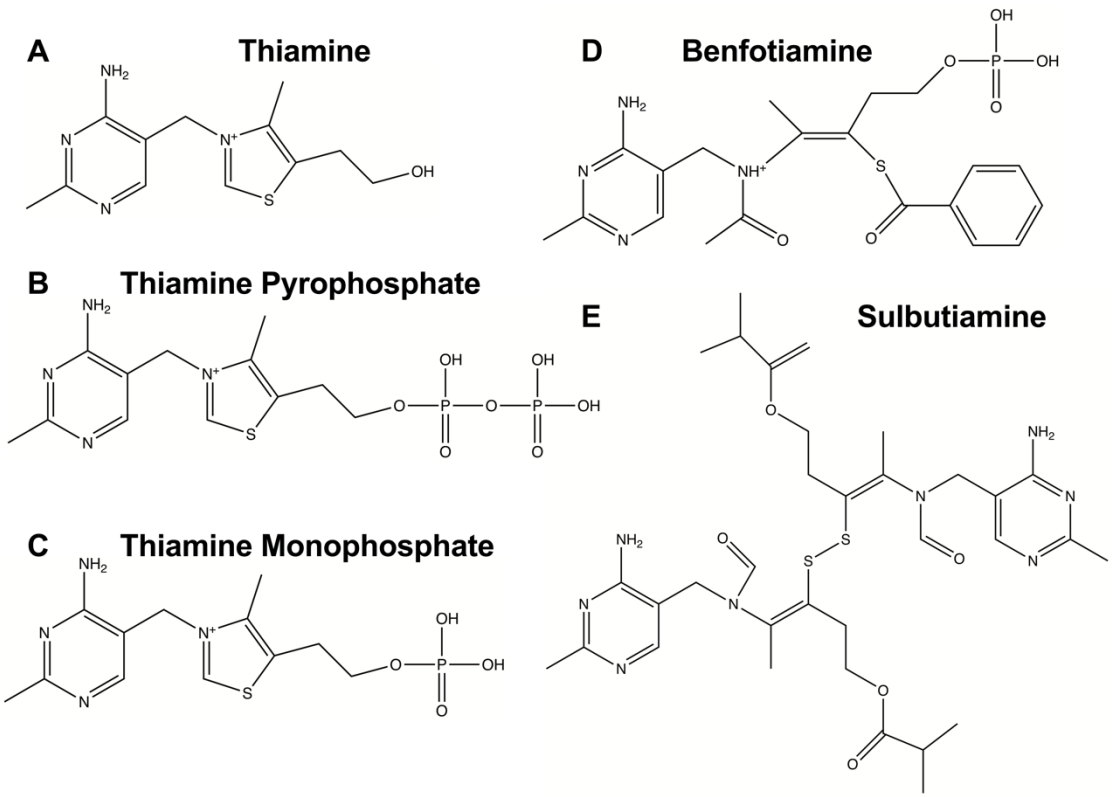


Fig 4.1. Schematic diagram representing chemical structures of thiamine, thiamine derivatives, and thiamine analogues. The rapid intracellular phosphorylation of **(A)** thiamine produces its active cofactor form **(B)** thiamine pyrophosphate (TPP). Dephosphorylation of TPP forms a single phosphate thiamine derivative **(C)** thiamine monophosphate (TMP). Lipophilic thiamine analogues **(D)** benfotiamine and **(E)** sulbutiamine can be utilized to increase cellular thiamine status by bypassing thiamine's requirement for carrier-mediated transport.

by diffusion **(Fig 4.1)**. Due to its lipophilicity benfotiamine passes the cell membrane and undergoes subsequent conversion into thiamine after thiazole ring closure (Volvert *et al.* 2008a). Both of these compounds build up higher concentrations of intracellular TPP compared with thiamine, suggesting they may enhance thiamine-related toxicity in cancer cells (Volvert *et al.* 2008a; Bettendorff 1994a). Therefore, the present study was undertaken to determine the sensitivity of cancer cells to lipophilic analogs of thiamine and identify the active species mediating a reduction in PDH phosphorylation.

4.4 Materials and Methods

Cell culture reagents including RPMI 1640 media, penicillin/streptomycin, and trypsin/EDTA were purchased from Corning (Manassas, VA). Fetal bovine serum (FBS) was purchased from Seradigm (Radnor, PA). All flasks and dishes used for routine maintenance of cultures were purchased from Greiner Bio-One (Monroe, NC). Thiamine was purchased from Sigma (St. Louis, MO). Both sulbutiamine and benfotiamine were purchased from Toronto Research Chemicals (North York, ON).

4.4.1 Cell culture

Human cancer cell lines including HCT 116 (CVCL_0291), U-87 MG (CVCL_0022), and MDA-MB-231 (CVCL_0062) cells were obtained from ATCC. Spontaneously immortalized Fetal Human Colon (FHC, CVCL_3688) cells were also purchased from ATCC and used as a non-cancerous comparison. Cancer cell lines were routinely cultured at 37°C with 5% CO_2 in complete RPMI 1640 medium, which contained 10% FBS and 1% penicillin/streptomycin. Following ATCC protocol, FHC

cells were cultured in DMEM:F12 supplemented with 10 mM HEPES, 10 ng/mL cholera toxin, 0.005 mg/mL insulin, 0.005 mg/mL transferrin, 100 ng/mL hydrocortisone, 20 ng/mL human recombinant EGF, 10% FBS, and 1% penicillin/streptomycin. All culture media was also supplemented with 0.1% Mycozap (Lonza, Verviers, Belgium) as a prophylactic treatment for prevention of mycoplasma contamination. Cells used for experimentation ranged from passage 4-25.

4.4.2 Quantitation of cellular proliferation

Cellular proliferation was determined using a crystal violet assay and the commercially available CellTiter 96® AQueous One Solution Proliferation Assay (MTS) (Promega, Madison, WI). HCT 116 (1000 cells/well), U-87 MG (1700 cells/well), and MDA-MB-231 (1000 cells/well) were seeded into 96-well plates at the stated densities. Cells were allowed to attach ~12h in complete growth medium. Media was then aspirated and replaced with complete growth medium containing serial dilutions of either thiamine, sulbutiamine, benfotiamine, or mannitol. Plates were incubated under normal growth conditions for 5 d in the presence of each compound. Crystal violet staining of the cells was done as previously described (Hanberry *et al.* 2014). Briefly, the treatment media was aspirated from each well and cells were washed with ice-cold phosphate buffered saline (PBS). PBS was replaced by buffered formalin (EMD Millipore, Darmstadt, Germany) and plates were incubated at 4°C for 30 min to fix cells. Formalin was removed and plates were washed two times with diH_2O. Fixed cells were immediately stained with 0.1% crystal violet solution for 30 min at room temperature. Crystal violet stain was then removed and plates were washed with diH_2O. Plates were allowed to dry

in a biosafety cabinet under laminar air flow. When completely dry, 1% Trition X-100 was used to lyse and destain fixed cells. Absorbance was determined using a Spectra Max M2e (Molecular Devices, Sunnyvale, CA) 96-well microplate reader at 550 nm.

Additionally, the sensitivity of HCT 116, U-87 MG, and MDA-MB-231 cells to thiamine, sulbutiamine, or benfotiamine was determined using the commercially available CellTiter 96® AQueous One Solution Proliferation Assay (MTS) (Promega, Madison, WI). Following 5 d of treatment, CellTiter 96® AQueous One Solution Reagent was added to each well following manufacturer's protocol. The plate was incubated in the presence of MTS solution for 3 h under normal growth conditions. The resulting absorbance of each well was immediately determined at 490 nm using a Spectra Max M2e (Molecular Devices, Sunnyvale, CA) 96-well microplate reader. For both the crystal violet and MTS assay, proliferation was normalized by comparing the absorbance of treated wells to untreated control wells. A non-linear regression ([inhibitor] vs. normalized response) was fitted using GraphPad Prism 6® (GraphPad Software, La Jolla, CA) and used to determine IC50 values.

4.4.3 Detection of apoptotic cell death

The Cell Death Detection ELISA^{PLUS} (Roche LifeScience, Indianapolis, IN) was used to quantitate cell death induced by various treatments through the analysis of cytoplasmic histone-associated DNA fragments. The manufacturer's provided protocol was followed for quantification. Briefly, the provided lysis buffer was applied to treated cells and incubated at room temperature for 30 min with gentle shaking. The resulting lysate was centrifuged at 200xg for 10 min at 15°C. Supernatant was combined with

immunoreagent in the supplied microplate for 2 h at room temperature with gentle shaking at 300 rpm. After 2 h, individual wells were aspirated and washed 3 times with incubation buffer. ABTS developing solution was immediately added to each well. The plate was incubated for 10 min at room temperature with gentle shaking at 250 rpm. Following development, stop solution was added and the plate's absorbance was read at 405 nm using a SpectraMax M2 spectrophotometer (Molecular Devices, Sunnyvale, CA). Cell death was calculated as enrichment factor representing the fold change comparing treated sample to untreated control.

4.4.4 Quantitation of intracellular thiamine, TPP, sulbutiamine, and benfotiamine

The effect of thiamine, sulbutiamine, and benfotiamine treatment on intracellular thiamine and TPP concentration was established using ion-paired reversed phase high-performance liquid chromatography (HPLC) as previously described (Basiri *et al.* 2016). Cells were collected by scraping and immediately placed on ice. Cells were washed with ice-cold PBS, then pelleted by centrifugation at 600xg for 5 min at 4°C in an Allegra X-22R centrifuge (Beckman Coulter, Brea, CA). Cells were subsequently washed two additional times with ice-cold PBS. Prior to pelleting following the final wash, suspended cells were counted using a TC-20 automated cell counter (BioRad, Hercules, CA). If not immediately used for extraction and analysis via HPLC, pellets were stored at -80°C. The thiamine/TPP metabolite level (pmol) determined by HPLC was normalized to total cell count. Cell pellets were also used to detect intracellular sulbutiamine and benfotiamine using reversed-phase HPLC as previously described (Kim *et al.* 2016). Prior to analysis,

cells were collected and prepared as described above for intracellular thiamine and TPP quantitation.

4.4.5 Evaluation of protein expression and phosphorylation

To assess the extent of PDH phosphorylation and PDK protein expression, Western blot analysis was performed on whole cell lysates (WCL). To prepare WCLs, treated cells were washed with ice-cold PBS followed by immediate lysis using 1% Nonidet P-40 (NP40), 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 0.01% sodium azide, 50mM tris, 250mM NaCl, and 1mM ethylenediaminetetraacetic acid (EDTA) at pH=8.5 supplemented with phenylmethanesulfonylfluoride (Calbiochem, La Jolla, CA) and protease/phosphatase inhibitors (G-Biosciences, St. Louis, MO). Lysates were collected and cellular debris was pelleted by centrifugation at 17,000xg using a Microfuge 22R Centrifuge (Beckman Coulter, Brea, CA) for 20 min at 4°C. The supernatant was collected and total protein content was determined using a BCA protein assay (Thermo Scientific, Rockford, IL). WCLs (50 μg) from each treatment were resolved by electrophoresis using a 10% SDS-PAGE gel. Separated proteins were then transferred to polyvinylidene difluoride membranes. Membranes for detection of total PDH and PDK1-4 expression were blocked with 5% non-fat milk in tris buffered saline-tween 20 (TBS-T) for 1 h at room temperature. Alternatively, membranes used to detect PDH phosphorylation were blocked in 3% bovine serum albumin (Sigma, St. Louis, MO) for 1 h at room temperature. Membranes were then immunoblotted with primary antibody for 12 h at 4°C. **Table 4.1** provides all information regarding manufacturer and dilution (in TBS-T) for individual antibodies. After the primary antibody incubation, blots were

washed three times with TBS-T (10 min each) and then exposed to horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit secondary antibody (Bethyl Laboratories, Montgomery TX) at a 1:10,000 dilution in TBS-T for 1 h at room temperature. Blots were washed three additional times with TBS-T. Protein expression was visualized using Supersignal-PLUS West Pico Solution (Thermo Scientific, Rockford, IL) according to manufacturer's instruction. Signal was imaged using a Fluorchem SP digital imager (Alpha Innotech, San Leandro, CA). Densitometry analysis was performed using Fluorchem SP Software (Alpha Innotech, San Leandro, CA).

4.4.6 Quantitation of PDH activity

The activity of PDH following treatment with thiamine, sulbutiamine, benfotiamine, or DCA was determined using the commercially available PDH Enzyme Activity Microplate Assay Kit (Abcam) following manufacturer's provided protocol. Adherent cells were collected following treatment by scraping, suspended in ice-cold PBS, and pelleted at 1000xg for 5 min at 4°C in an Allegra X-22R centrifuge (Beckman Coulter, Brea, CA). Cells were washed two additional times using ice-cold PBS. Following final wash, cells were suspended in ice-cold PBS containing phosphatase inhibitor and protease inhibitor (G-Biosciences, St. Louis, MO). To determine sample protein concentration, an aliquot of the cell suspension was combined with

Table 4.1 Primary antibodies used for Western blot analysis

Protein of Interest	Manufacturer (Catalog Number)	Dilution	Secondary Antibody	Antibody Registry ID
PDK1	Genetex (GTX107405)	1:1000	Rabbit	AB_11168810
PDK2	ProteinTech (15647-1-AP)	1:1000	Rabbit	AB_2268006
PDK3	Genetex (GTX104286)	1:1000	Rabbit	AB_1951161
PDK4	ProteinTech (12949-1-AP)	1:1000	Rabbit	AB_2161499
PDH	Genetex (GTX104015)	1:1000	Rabbit	AB_1951155
PDH p232	CalBiochem (AP1063)	1:1000	Rabbit	AB_10616070
PDH p293	CalBiochem (AP1062)	1:1000	Rabbit	AB_10616069
PDH p300	CalBiochem (AP1064)	1:1000	Rabbit	AB_10618090
β-Actin	Sigma-Aldrich (A2228)	1:1000	Mouse	AB_476697

an equal volume of lysis buffer (1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 0.01% sodium azide, 50mM tris, 250mM NaCl, and 1mM EDTA at pH=8.5). The sample was incubated on ice for 10 min to allow for lysis. Cellular debris was pelleted by centrifugation at 17,000xg using a Microfuge 22R Centrifuge (Beckman Coulter, Brea, CA) for 20 min at 4°C. The protein concentration of supernatant was determined using the BCA protein assay (Thermo Scientific, Rockford, IL) following manufacturer's protocol. After determination of protein concentration, detergent solution provided in the

kit was added to each sample at a final dilution of 1/10. Samples were incubated on ice for 10 min to allow solubilization and then centrifuged at 1000xg for 10 min at 4°C. Protein concentration was adjusted to account for the addition of detergent. Protein from supernatant (1mg) was loaded into each individual well provided in the kit's microplate. Plate was covered with foil and placed on rotating shaker (200 rpm) for 3 h at room temperature. After 3 h, wells were aspirated and washed three times with 1X Stabilizer. Following washes, developing solution was added to each well and the plate's absorbance was measured kinetically at 450 nm over 15 min using a SpectraMax M2 spectrophotometer. Based on the kinetic read, the maximal velocity (V_{max}) of PDH activity was determined for each sample through SoftMax Pro Software (Molecular Devices, Sunnyvale, CA).

4.4.7 Assessment of PDK gene expression

Differential gene expression of *PDK* isoforms 1-4 was determined by quantitative real-time PCR. For analysis, RNA was extracted using the E.Z.N.A Total RNA Kit I (Omega Bio-Tek, Norcross, GA) following the manufacturer's protocol. The RNA concentration was determined using a Nanodrop 2000c Spectrophotometer (Thermo Scientific, Rockford, IL). Using the qScript cDNA Synthesis Kit (Quanta BioSciences, Gaithersburg, MD), 1 μg of isolated RNA was reverse transcribed. Gene detection was performed using a Light-Cycler 480 II (Roche Applied Science, Indianapolis, IN) For each *PDK,* specific primers were designed using the Roche Universal Probe Library assay design center. Each primer set corresponded with a specific Roche hydrolysis probe labeled with fluorescein (FAM). Primer/probe pairs used for this study are listed in **Table**

4.2. The expression of *ACTIN (ACTB)* was determined using the Universal ProbeLibrary Human ACTB Gene Assay and used as a reference gene to calculate relative gene expression based on the $2^{-\Delta Ct}$ method with an assumed efficiency of 2.

Table 4.2. Primer sequences and Roche UniversalProbe Library probe pairs for Real Time-PCR analysis

Gene	Forward and Reverse Primer Sequences	Probe
PDK1	F: 5'-GAGTCTTCAGGAGCT-3' R: 5'-TGCAACCATGTTCTTCTACCG-3'	5
PDK2	F: 5'-TGAAGCAGTTTCTGGACTTCG-3' R: 5'-AGGTTGATCTCTTTCATGATGTTG-3'	5
PDK3	F: 5'-TGTGTGAACAGTATTACCTGGTAGC-3' R: 5'-GTTTGTCTGCTTTGG-3'	5
PDK4	F: 5'-CAGTGCAATTGGTTAAAAGCTG -3' R: 5'-GGTCATCTGGGCTTTTCTCA -3'	5

4.4.8 Determination of PDK Inhibition

To determine the species of thiamine capable of inhibiting PDK activity, the commercially available PDH Enzyme Activity Microplate Assay Kit (Abcam) was adapted to screen kinase activity. Recombinant human protein for PDK1-4 was obtained from Abcam. To carry out PDK inhibition screening assay, 25 μg of bovine heart mitochondria (Abcam) as a source of PDH protein was added to each well of the provided antibody-coated microplate. The plate was covered with foil and placed on rotating shaker (200 rpm) for 2.5 h at room temperature to allow protein binding. Following incubation, wells were washed four times using 1X stabilizer. PDK's (1

μg/mL) were pre-incubated with serial dilutions of inhibitor (thiamine, TMP, TPP, sulbutiamine, or benfotiamine) at 37°C for 15 min. In a second reaction tube, 0.2X ATP (Cayman Chemical, Ann Arbor, MI) was also pre-incubated with serial dilutions of inhibitor. PDK/inhibitor solution and ATP/inhibitor solution was added 1:1 into each well of microplate. Plate was covered with foil and placed on rotating shaker (200 rpm) for 20 min at room temperature. After 20 min, each well was aspirated and washed four times with 1X stabilizer. Developing solution was immediately added to each well and the plate's absorbance was measured kinetically at 450 nm over 15 min. Based on the kinetic read, the maximal velocity (V_{max}) of PDH activity was determined for each PDK with/without inhibitor through SoftMax Pro Software (Molecular Devices, Sunnyvale, CA). Uninhibited PDH activity from isolated bovine heart mitochondrial extract (Abcam, City, State) was used as a negative control. PDK4 protein was unable to provide a positive control for the inhibition of PDH activity and was excluded from analysis.

4.4.9 Overexpression of thiamine homeostasis genes

HCT 116 cells were used to determine the sensitivity of tumor cells to thiamine and TPP following the transient overexpression of critical thiamine homeostasis genes. THTR1 (thiamine transporter) overexpression was mediated through the introduction of *SLC19A2* using the expression plasmid pEFGP-N3 developed and kindly provided by Dr. Hamid Said (Subramanian *et al.* 2003). TPPT (TPP transporter) overexpression was mediated through the introduction of *SLC44A4* using the expression plasmid pFLAG-CMV-2 also developed and provided by Dr. Said (Nabokina *et al.* 2014). TPK1 (TPP producing enzyme) overexpression was mediated through the introduction of *TPK1* using

the expression plasmid pcDNA3.1(+) that our lab previously developed (Jonus *et al*. Accepted 2018). For all transfections, cells were seeded at 6000 cells per well into 96-well plates and allowed to attach ~12 h. Normal culture media was then replaced with transfection media (100 μL) containing 500 ng plasmid DNA and 2.5% lipofectamine. Cells were transfected for 24 h, following which transfection media was removed and replaced with complete growth medium for a 24 h recovery period. After recovery, cells were dosed with thiamine or TPP for 24 h and cell death was determined by Cell Death Detection ELISA^{PLUS} as described above.

4.4.10 Xenograft studies

Six-week old female athymic nude (Nu/Nu) mice were purchased from Taconic Biosciences (Rensselaer, NY). Following arrival, mice were given a 1-week acclimatization period prior to introducing a liquid diet (Lieber-DeCarli '82 Shake and Pour control liquid diet, BioServ, Flemington, NJ). Mice were allowed to adjust to liquid consumption for 7-days before tumor implantation. To establish xenografts, 100 μL containing $1x10^6$ HCT 116 cells in a 4X dilution of BD Matirgel Matrix (Corning, Manassas, VA) with PBS was injected subcutaneously into the flank of each mouse. Approximately 7-days following tumor implant all mice demonstrated palpable tumors.

To test the efficacy of thiamine, sulbutiamine, and benfotiamine in inhibiting *in vivo* tumor growth, each compound was administered daily through the diet with a bolus intraperitoneal (IP) injection every other day. Dietary dosing commenced on the seventh day following tumor implant. Thiamine (2 mg/mL), benfotiamine (2 mg/mL), and sulbutiamine (1 mg/ml) were introduced into the diets of mice along with 5% (w/v)

chocolate flavoring (BioServ, Flemington, NJ) and 1% (w/v) Splenda sweetener to mask any aversive taste properties associated with each compound. Sulbutiamine dosing had to be reduced from 2 mg/mL to 1 mg/mL because of strong food aversion demonstrated at the higher concentration. Based on their daily consumption, animals received ~20 mg of thiamine or benfotiamine and ~10 mg of sulbutiamine through their diet each day. In addition to dietary consumption, mice also received bolus IP injections of each compound every second day starting on day 14 post-tumor implant. Due to the low solubility of sulbutiamine and benfotiamine, 25 mg/ml suspensions of the test compounds were prepared using 15% Arabic gum in normal saline as previously described (Trovero *et al.* 2000). As a vehicle control, 15% Arabic gum solutions were administered to control animals. All injections were adjusted to pH = 6 to avoid any irritation to the abdominal cavity. For benfotiamine and sulbutiamine, animals were dosed at 400 mg/kg. Animals receiving thiamine were dosed at 150 mg/kg through IP injection and supplemented the remaining 250 mg/kg through their diet. Other than lethargy, which is consistent with previous findings for these compounds, no other adverse symptoms were noted. Tumor measurements, animal weights, and food consumption were tracked over the course of the study.

4.4.11 Statistical Analysis

For each *in vitro* experiment described, a minimum of three independent replicates were performed ($n = 3$). For animal studies, exact *n* numbers for each treatment arm are presented in the figure legend. GraphPad Prism 6® (GraphPad Software, La Jolla, CA) was used for statistical analysis. Statistical significance ($p<0.05$) was determined

through either an unpaired student's T-test or a one-way analysis of variance (ANOVA) with Tukey's post hoc test for multiple comparisons.

4.5 Results

4.5.1 Tumor cells demonstrate increased susceptibility to lipophilic thiamine analogs

The sensitivity of tumor cell lines including HCT 116 (colorectal carcinoma), U-87 MG (glioblastoma), and MDA-MB-231 (metastatic breast adenocarcinoma) to thiamine, sulbutiamine, and benfotiamine was determined by crystal violet proliferation **(Fig 4.2A)** and MTS assay **(Fig 4.2B)**. All three cell lines demonstrated a decrease in proliferation with increasing concentration of thiamine, sulbutiamine, or benfotiamine. IC50 values for each compound were calculated in each individual cell line and provided in **Table 4.3** (crystal violet) and **Table 4.4** (MTS). Sulbutiamine and benfotiamine demonstrated IC50s ranging from ~75 to ~250 μM, compared with thiamine, which required doses ranging from ~4 to ~15 mM to reduce tumor cell proliferation by ~50% over the course of 5 d. To rule out osmotic effects in the toxicity profiles, HCT 116, U-87 MG, and MDA-MB-231 were treated with similar concentrations of the cell impermeable compound mannitol. No toxicity was found for mannitol treatment up to 10 mM **(Fig 4.2C)**. Due to the extremely slow proliferation rate of the non-cancerous FHC cell line, the Cell Death Detection ELISA^{PLUS} was used to quantitate differences in the sensitivity of non-cancerous and cancerous cells to thiamine, sulbutiamine, or benfotiamine treatment. HCT 116 cells treated with sulbutiamine or benfotiamine demonstrated an ~5- and 4-fold increase in cell death, respectively **(Fig 4.2D)**. Though there was no significant effect for thiamine treatment, the fold change in cell death for HCT 116 cells

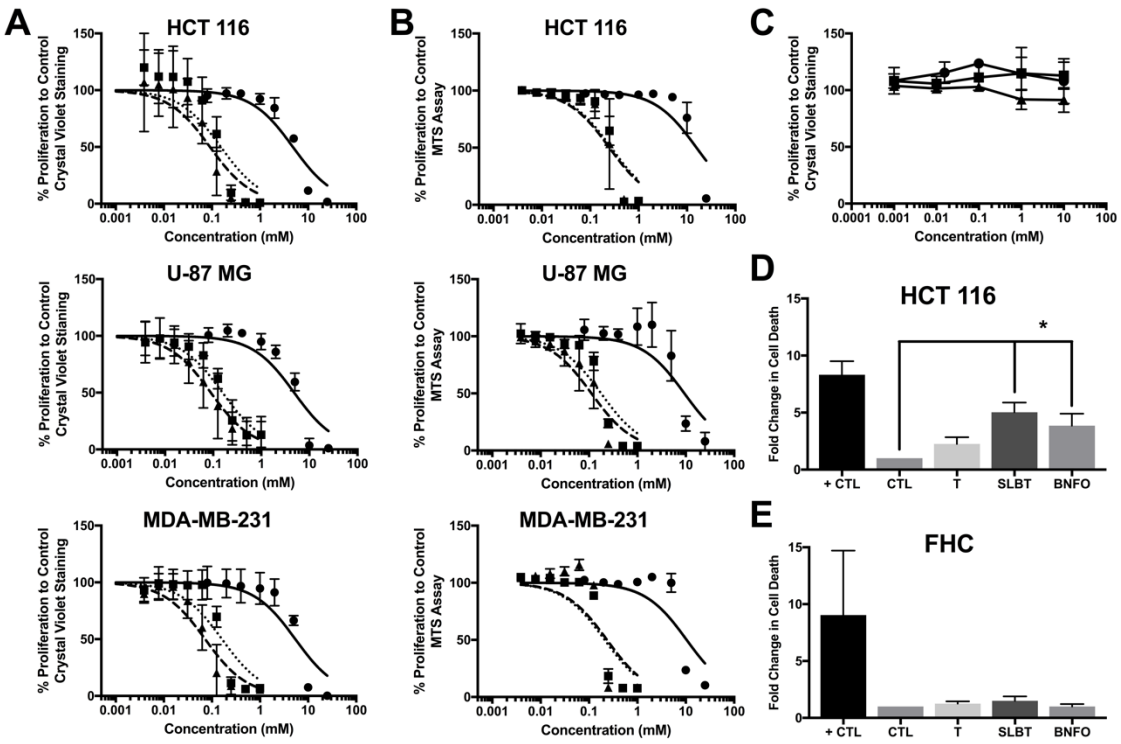


Fig 4.2. Lipophilic thiamine analogues inhibit tumor cell proliferation (A) Proliferation of HCT 116, U-87 MG, and MDA-MB-231 cells determined by crystal violet assay demonstrating the effect of thiamine (●), sulbutiamine (■), and benfotiamine (▲) treatment following 5 days of exposure. Results are normalized as mean percent proliferation +/- standard deviation (SD) comparing treated cells to untreated control. **(B)** Proliferation of HCT 116, U-87 MG, and MDA-MB-231 cells determined by MTS assay demonstrating the effect of thiamine (●), sulbutiamine (■), and benfotiamine (▲) treatment after 5 days of exposure. Results are normalized as mean percent proliferation +/- SD comparing treated cells to untreated control. **(C)** Proliferation of HCT 116 (▲), U-87 MG (■), and MDA-MB-231 (●) cells determined by crystal violet assay demonstrating the effect of mannitol treatment after 5 days of exposure. Results are normalized as mean percent proliferation +/- SD comparing treated cells to untreated control. **(D)** Apoptotic cell death demonstrated as fold change +/- SD in HCT 116 cells seeded at 6000 cells/cm^2 and treated with thiamine (T, 25 mM), sulbutiamine (SLBT, 0.5 mM), or benfotiamine (BNFO, 1mM) for 24 h relative to untreated control (CTL). Treatment of HCT 116 cells with 15 μM comptothecin serves as positive control (+ CTL) for detection of apoptosis. **(E)** Apoptotic cell death demonstrated as fold change +/- SD in FHC cells treated with thiamine (T, 25 mM), sulbutiamine (SLBT, 0.5 mM), or benfotiamine (BNFO, 1mM) for 24 h relative to untreated control (CTL). Due to slow growth rate of FHC cell line, cells were seeded at 30,000 cells/cm^2 and cultured 1 week prior to initiating treatments. Treatment of FHC cells with 15 μM comptothecin serves as positive control (+ CTL) for apoptotic. (✶) Represents statistically significant difference ($p<0.05$) based on results of one-way ANOVA with Tukey's post-hoc test.

Table 4.3. IC50 values with 95% confidence intervals for thiamine, sulbutiamine, and benfotiamine determined by crystal violet proliferation assay

Cell Line	Thiamine (mM)	Sulbutiamine (μM)	Benfotiamine (μM)
HCT 116	**4.83** (3.44, 6.81)	**153** (117, 202)	**91** (71, 118)
U-87 MG	**4.85** (3.45, 6.83)	**157** (135, 182)	**90** (75, 107)
MDA-MB-231	**5.43** (3.71, 7.98)	**162** (135, 194)	**72** (60, 87)

Table 4.4. IC50 values with 95% confidence intervals for thiamine, sulbutiamine, and benfotiamine determined by MTS assay

Cell Line	Thiamine (mM)	Sulbutiamine (μM)	Benfotiamine(μM)
HCT 116	**15.8** (10.8, 23.4)	**273**(194, 386)	**250** (171, 367)
U-87 MG	**9.37** (5.95, 15.1)	**178** (132, 240)	**112** (81, 156)
MDA-MB-231	**10.4** (6.85, 16.3)	**211** (147, 306)	**234** (146, 386)

trended higher **(Fig 4.2D)**. Unlike their cancerous counterparts, FHC cells demonstrated no significant increase in cell death markers following treatment with 25 mM thiamine, 0.5 mM sulbutiamine, or 1 mM benfotiamine for 24 h **(Fig 4.2E)**.

4.5.3 Thiamine and its lipophilic analogs increase intracellular thiamine and TPP concentration

To determine how dosing with thiamine, sulbutiamine, or benfotiamine impacts intracellular thiamine and TPP levels, HCT 116 cells were treated with each compound for 24 and 48 h. Based on MTS toxicity profiles, concentrations of 25 mM thiamine and 250 μM sulbutiamine and benfotiamine were chosen for short-term studies at 24 h and 48 h to minimize the impact of cellular toxicity. At these doses, there was no significant change in cell count compared to control cells after 24 h and only a moderate reduction after 48 h **(Fig 4.3A)**. Treatment with each compound significantly increased intracellular thiamine levels after 24 h and 48 h of treatment, with thiamine treatment causing exaggerated increases compared to both sulbutiamine and benfotiamine **(Fig 4.3B)**. No intracellular sulbutiamine or benfotiamine was detected following treatment with either analog **(Supplemental Fig 4.1)**. Treatment with thiamine, sulbutiamine, or benfotiamine also significantly increased intracellular TPP levels after 24 h and 48 h by nearly 2-fold **(Fig 4.3C)**.

4.5.4 Activation of PDH by thiamine, sulbutiamine, and benfotiamine

Next, it was assessed whether treatment with thiamine, sulbutiamine, or benfotiamine can reduce PDH phosphorylation and increase its activity in HCT 116 cells.

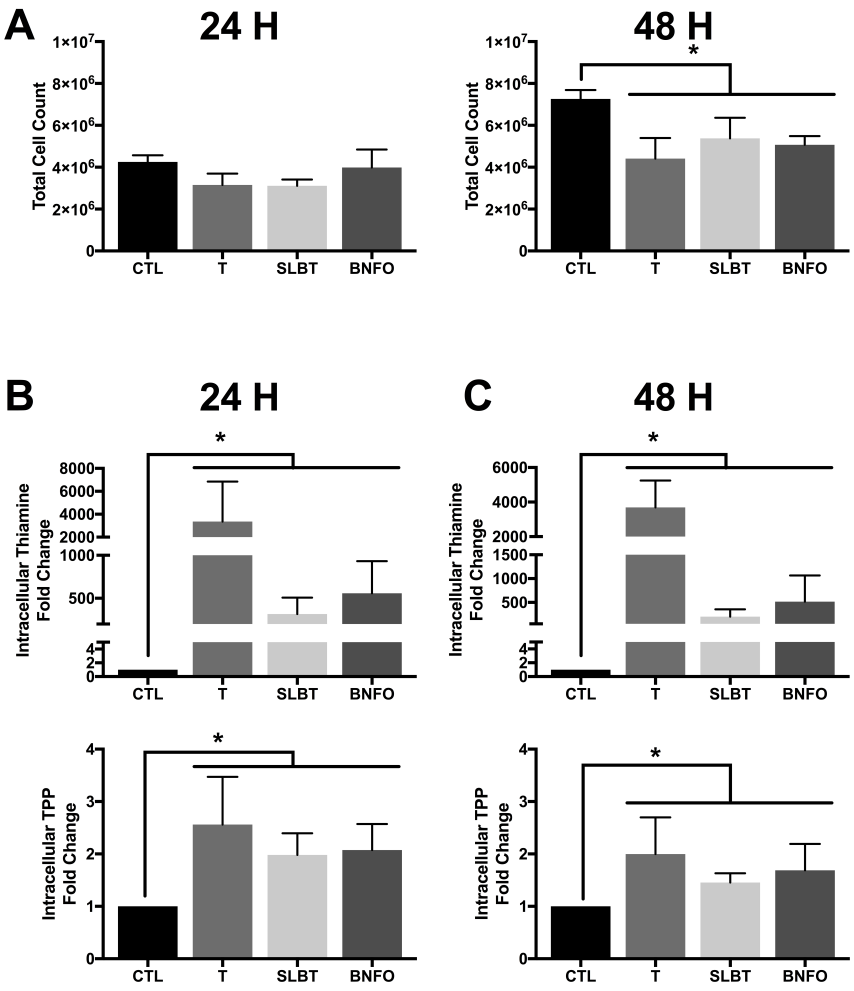
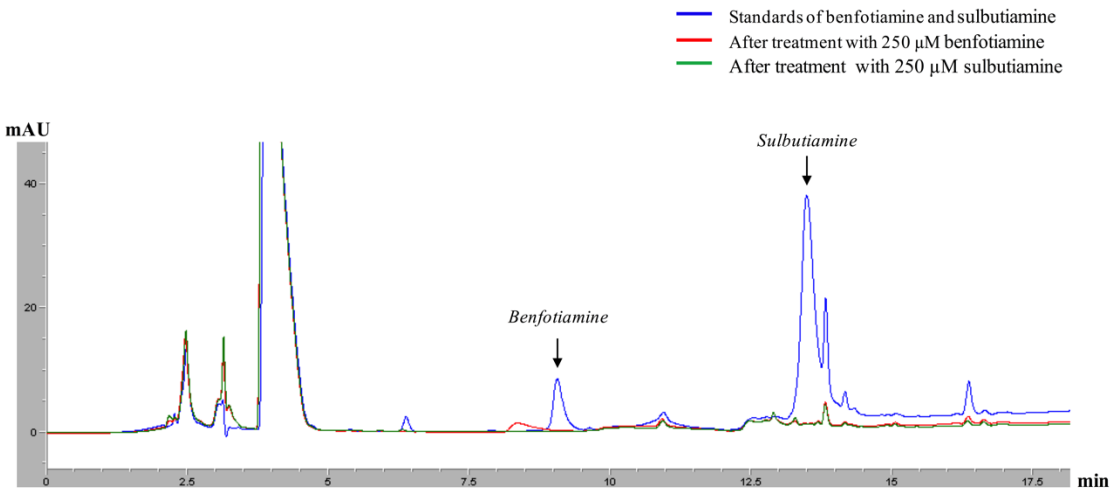
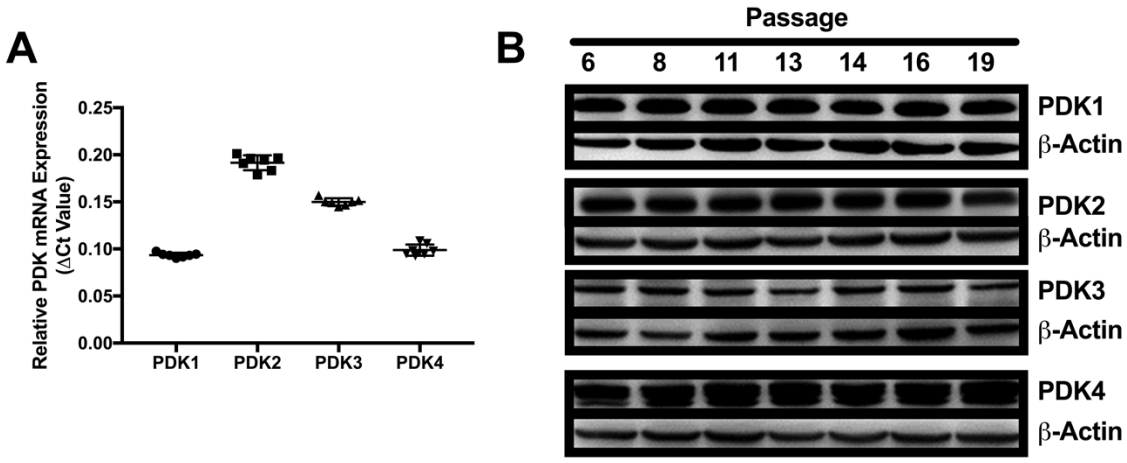


Fig 4.3. Enhanced intracellular thiamine status following pharmacologic treatment with thiamine and its lipophilic analogues (A) Effect of thiamine (T, 25 mM), sulbutiamine (SLBT, 250 μM**)**, or benfotiamine (BNFO, 250 μM**)** on tumor cell proliferation demonstrated by mean cell count +/- SD of HCT 116 cells seeded at 20,000 cells/cm^2 and treated for 24 or 48 h compared to untreated control (CTL). **(B)** HPLC analysis demonstrating fold change in intracellular thiamine and TPP levels +/- SD established in HCT 116 cells seeded at 20,000 cells/cm^2 and treated with 25 mM thiamine (T) , 250 μM sulbutiamine (SLBT), or 250 μM benfotiamine (BNFO) compared to untreated control (CTL) for 24 h. **(C)** HPLC analysis demonstrating fold change in intracellular thiamine and TPP levels +/- SD established in HCT 116 cells seeded at 20,000 cells/cm^2 and treated with 25 mM thiamine (T), 250 μM sulbutiamine (SLBT), or 250 μM benfotiamine (BNFO) compared to untreated control (CTL) for 48 h (*) Represents statistically significant difference ($p<0.05$) based on results of (A) one-way ANOVA with Tukey's post-hoc test or (B,C) an unpaired student's t-test for each individual treatment with CTL.



Supplemental Fig 4.1. Lack of detection for intracellular sulbutiamine and benfotiamine Representative chromatograms demonstrating presence of sulbutiamine and benfotiamine in standard used for detection/quantification (blue) and lack of compound detection in cell extracts following treatment with 250 µM sulbutiamine (green) and 250 µM benfotiamine (red).

First, the expression of all four PDK isoenzymes in HCT 116 cells was confirmed at the gene and protein levels. In 7 different passages, HCT 116 cells demonstrated consistent gene expression of *PDK1*, *PDK2*, *PDK3*, and *PDK4* **(Supplemental Fig 4.2A)**. Of the four isomeric forms, *PDK2* demonstrated the highest relative gene expression followed by *PDK3* > *PDK4* = *PDK1* **(Supplemental Fig 4.2A)**. Protein expression for each PDK isoform was also detectable and stable over the course of several passages **(Supplemental Fig 4.2B)**. To detect changes in PDH phosphorylation and activity due to treatment with thiamine, sulbutiamine, or benfotiamine, DCA (25 mM) was used as a positive control. DCA has previously been shown to enhance PDH activity by decreasing phosphorylation through inhibiting PDK activity (Knoechel *et al.* 2006). Three phosphorylation residues (Ser232, Ser293, Ser300) are involved in PDK-mediated inhibition of PDH (Korotchkina & Patel 2001). **Figures 4.4A and 4.4B** demonstrate that DCA, as well thiamine, sulbutiamine, and benfotiamine significantly reduced PDH phosphorylation by approximately half at all three residue sites. There was no significant change on total PDH protein with DCA, thiamine, sulbutiamine or benfotiamine treatment **(Fig 4.4A and 4.4B)**. Decreased phosphorylation was associated with a significant increase in PDH activity with DCA, thiamine, sulbutiamine, or benfotiamine treatment **(Fig 4.4C)**.



Supplemental Fig 4.2. Individual expression of PDK isoforms in HCT 116 cells (A) Relative mRNA expression levels of *PDK1*, *PDK2*, *PDK3* and *PDK4* determined by qRT-PCR and normalized by the $2^{-\Delta Ct}$ method in HCT 116 cells isolated from 7 independent cell passages. **(B)** Representative Western blot demonstrating PDK1-PDK4 expression in WCLs prepared from HCT 116 cells isolated from 7 independent cell passages. β-Actin expression serves as loading control.

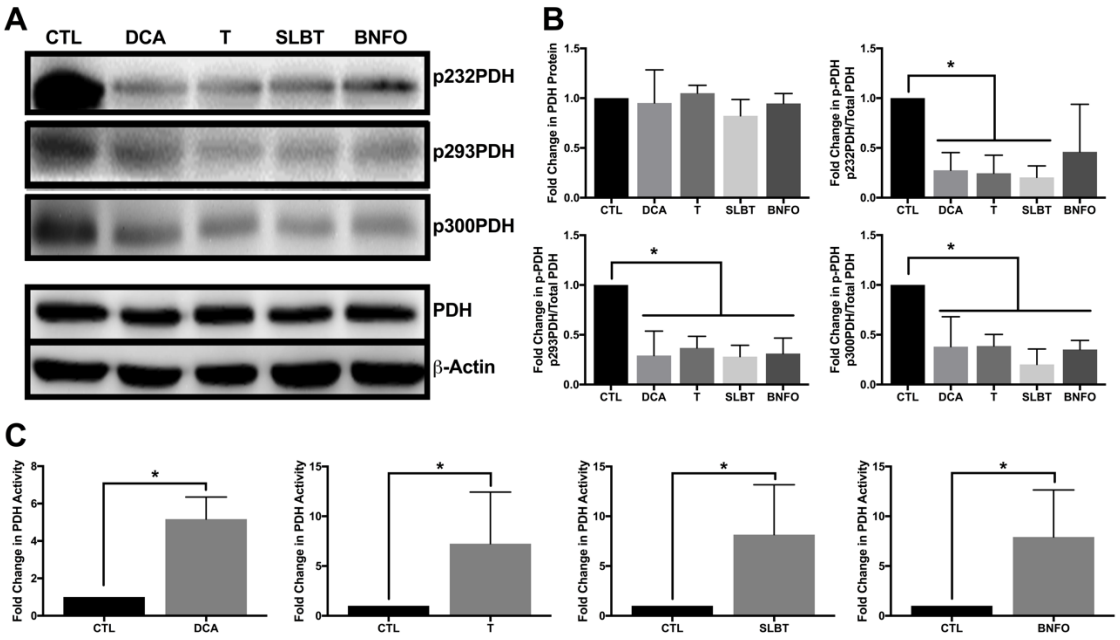


Fig 4.4. Activation of PDH activity through treatment with thiamine, sulbutiamine, and benfotiamine (A) Representative Western blots demonstrating PDH expression and the extent of phosphorylation at its three regulatory sites in WCLs isolated from HCT 116 cells seeded at 10,000 cells/cm^2 and treated with dichloroacetate (DCA, 25 mM), thiamine (T, 25 mM), sulbutiamine (SLBT, 250 μM**)**, or benfotiamine (BNFO, 250 μM**)** for 48 h compared to untreated control (CTL). **(B)** Densitometry analysis of the fold change in PDH expression and its phosphorylation at serine residues 232, 293, and 300 +/- SD in HCT 116 cells seeded at 10,000 cells/cm^2 treated with dichloroacetate (DCA, 25 mM), thiamine (T, 25 mM), sulbutiamine (SLBT, 250 μM**)**, or benfotiamine (BNFO, 250 μM**)** for 48 h compared to untreated control (CTL). **(C)** Fold change +/- SD of PDH activity in lysates isolated from HCT 116 cells following treatment with dichloroacetate (DCA, 25 mM), thiamine (T, 25 mM), sulbutiamine (SLBT, 250 μM**)**, or benfotiamine (BNFO, 250 μM**)** for 48 h compared to untreated control (CTL). (✶) Represents statistically significant difference ($p<0.05$) based on results of (B) one-way ANOVA with Tukey's post-hoc test or (C) of an unpaired student's t-test.

4.5.5 TPP inhibits ex vitro PDK activity

To determine if thiamine, TPP, thiamine monophosphate (TMP), sulbutiamine, or benfotiamine inhibit PDK activity an *ex vitro* kinase assay was used. When screening each kinase, uninhibited PDH activity from isolated bovine mitochondria provided a negative control **(Fig 4.5A-5E)**. The addition of PDK1, PDK2, and PDK3 significantly reduced detectable PDH activity **(Fig 4.5A-5E)**. DCA increased PDH activity in the presence of PDK1, PDK2, and PDK3 demonstrating the assay was able to detect kinase inhibition **(Supplemental Fig 4.3)**. Addition of TPP inhibited the activity of PDK1 in a dose-dependent manner with moderate inhibition at 1 μM and significant inhibition at higher doses of 10 μM, 100 μM, and 1 mM **(Fig 4.5A)**. The greatest inhibitory effect for TPP on PDK1 was found between 10 and 100 μM **(Fig 4.5A)**. TPP inhibited PDK2 in a similar profile found for PDK1. Moderate inhibition was found at 1 μM and increased in a dose-dependent manner from 10 to 100 μM **(Fig 4.5A)**. For PDK2, maximal inhibition occurred at 100 μM TPP **(Fig 4.5A)**. TPP demonstrated PDK3 inhibition, but only at higher concentrations of 100 μM and 1 mM **(Fig 4.5A)**. No effect for thiamine, TMP, sulbutiamine or benfotiamine was observed at any of the doses considered for inhibition of PDK1, PDK2, or PDK3 **(Fig 4.5B-4.5E)**.

4.5.6 TPP demonstrates direct anticancer potential in vitro

The anticancer effect of TPP was analyzed based on results above suggesting an active role for TPP in inhibiting PDK. To do so, various thiamine homeostasis genes were overexpressed in HCT 116 cells to encourage intracellular TPP accumulation.

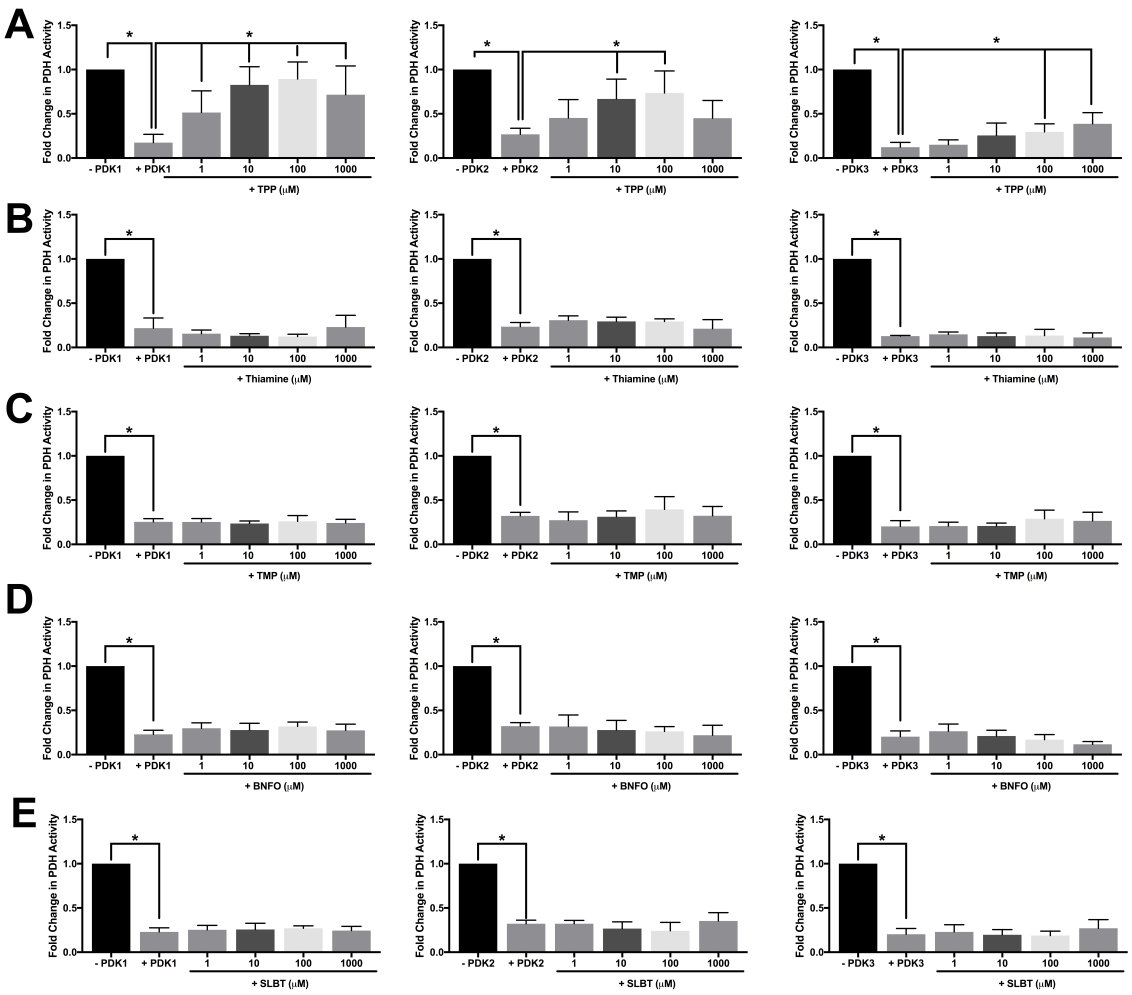
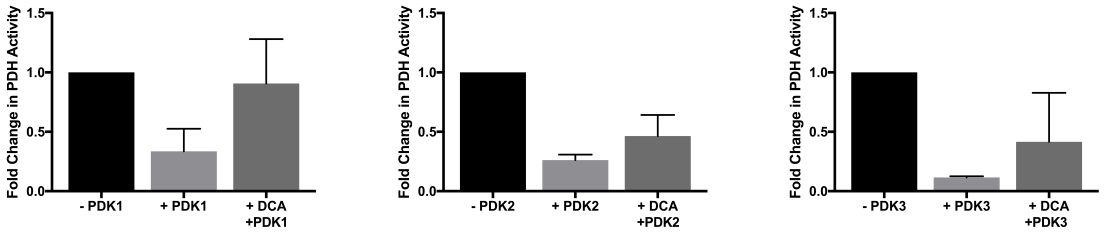


Fig 4.5. TPP inhibits *ex vitro* PDK activity *Ex vitro* demonstration of PDK1, PDK2, and PDK3 activity in the presence and absence of **(A)** TPP, **(B)** thiamine (T), **(C)** TMP, **(D)** benfotiamine (BNFO), or **(E)** sulbutiamine (SLBT) demonstrated by fold change in PDH activity +/- SD. Uninhibited PDH activity (-PDK) from isolated bovine mitochondria lysate determined as V_{max} of reaction serves as the negative control for normalization in each assay. The fold change +/- SD in PDH activity in the presence of each PDK (+PDK) compared to uninhibited control (-PDK) serves as the positive control for functional PDK activity. The ability of each test compound to inhibit PDK activity and restore PDH function demonstrated as fold change +/- SD in PDH activity was tested by introducing TPP, thiamine (T), TMP, sulbutiamine (SLBT) and benfotiamine (BNFO) in logarithmic dilutions from 1000 μM (+ compound, μM). (★) Represents statistically significant difference ($p<0.05$) based on results of one-way ANOVA with Tukey's post-hoc test.



Supplemental Fig 4.3. DCA inhibits *ex vitro* PDK activity Demonstration of PDK1, PDK2, and PDK3 activity in the presence and absence of DCA demonstrated by fold change in PDH activity +/- SD. Uninhibited PDH activity (-PDK) from isolated bovine mitochondria lysate determined as V_{max} of reaction serves as the negative control for normalization in each assay. The fold change +/- SD in PDH activity in the presence of each PDK (+PDK) compared to uninhibited control (-PDK) serves as the positive control for functional PDK activity. The capability of DCA (+DCA,+PDK) to inhibit PDK activity and restore PDH function is demonstrated as fold change +/- SD in PDH activity.

SLC44A4 encodes the thiamine pyrophosphate transporter (TPPT), responsible for transporting TPP across the plasma membrane and intracellular accumulation of TPP within the colon (Nabokina *et al.* 2016). HCT 116 cells transfected with *SCL44A4* demonstrated an ~3.5-fold increase in cell death in the presence of TPP compared to vector control cells **(Fig 4.6A)**. There was no effect on cell death due to treatment with thiamine, which is not known to be transported by TPPT **(Fig 4.6A)**. Overexpression of *SLC19A2*, the gene encoding for the high-capacity thiamine transporter THTR1, demonstrated enhanced cell death (~2-fold) following thiamine treatment in HCT 116 cells **(Fig 4.6B)**. TPP treatment resulted in no increase in apoptotic index following overexpression of *SLC19A2* **(Fig 4.6B)**. **Figure 4.6C** demonstrates that overexpression of TPK1, the enzyme responsible for the intracellular conversion of thiamine into TPP resulted in an ~4-fold enhancement in HCT 116 sensitivity to thiamine, but had no effect following TPP treatment.

Tumor cells have previously been demonstrated to upregulate the expression of critical thiamine homeostasis genes (Zastre *et al.* 2013a). Expression of *SLC44A4* was compared between the androgen-sensitive human prostate adenocarcinoma cell line LNCaP and the normal prostate epithelial cell line RWPE-1 by Dr. Hamid Said and colleagues. Their analysis revealed LNCaP cells demonstrate greater than 200-fold enhancement in *SLC44A4* expression compared to RWPE-1 cells **(Fig 4.6D)**. Therefore, *SLC44A4* expression may be a factor in the susceptibility of tumor cells to high-dose TPP administration. **Figure 4.6E** demonstrates no significant change in cell death when RWPE-1 cells were exposed to 1mM TPP for 24 h. However, TPP treatment resulted in a nearly 3-fold enhancement of cell death for the cancerous LNCaP cell line. No increase

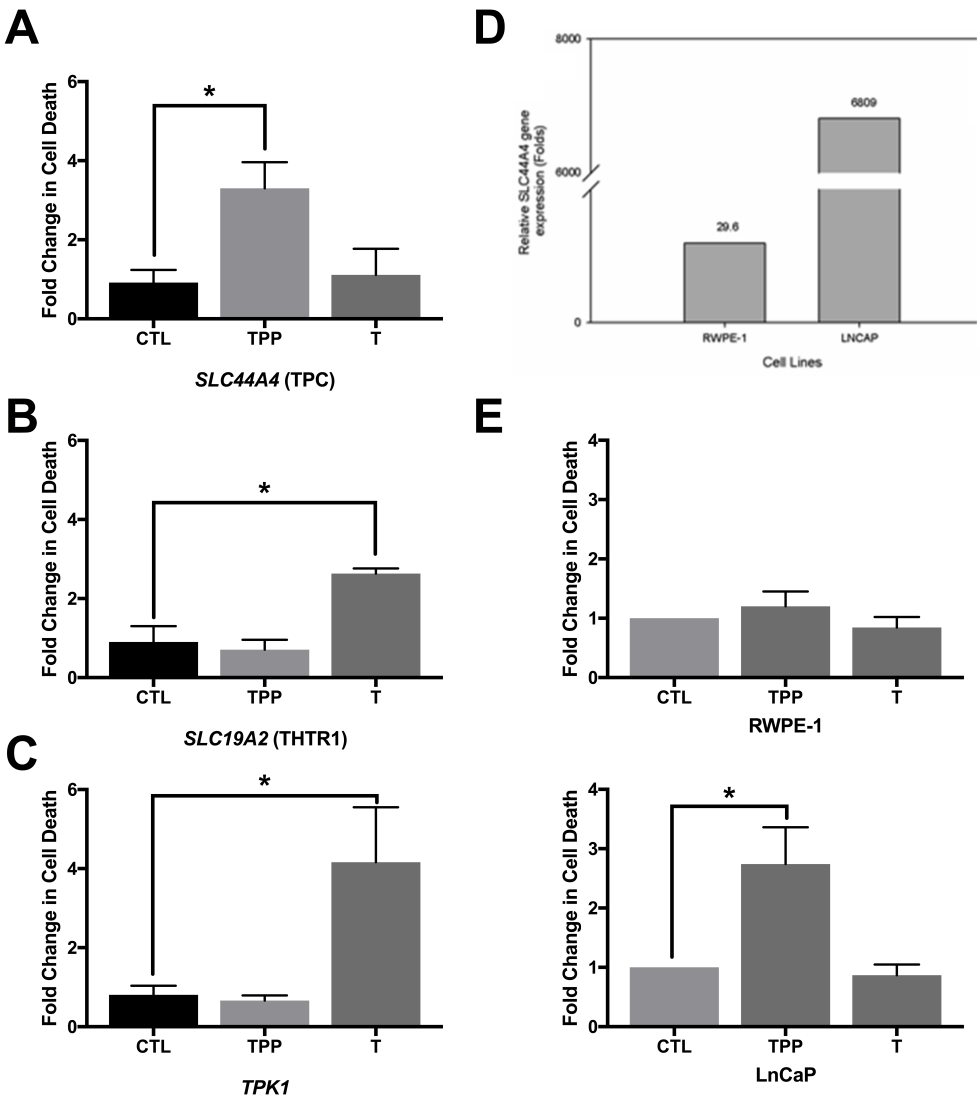


Fig 4.6. Targeted TPP accumulation induces apoptotic tumor cell death Analysis of apoptotic cell death in HCT 116 cells following overexpression **(A)** *SLC44A4,* **(B)** *SLC19A2*, or **(C)** *TPK1* and treatment with 1 mM thiamine (T) or TPP for 24 h relative to untreated control (CTL). Fold change in cell death +/- SD compares treated or untreated HCT 116 cells overexpressing each gene of interest (described in materials and methods) to its treated or untreated vector control. **(D)** Data provided by Dr. Hamid Said demonstrating relative mRNA expression levels of *SLC44A4* determined by qRT-PCR in RWPE-1 (non-cancerous) and LNCAP (cancerous) prostate derived cell lines. **(E)** Apoptotic cell death demonstrated as fold change +/- SD in RWPE-1 and LNCAP cells following treatment with 1mM thiamine (T) or TPP for 24 h compared to untreated control (CTL). (★) Represents statistically significant difference ($p<0.05$) based on results of one-way ANOVA with Tukey's post-hoc test.

in apoptotic index was observed in either cell line following treatment with 1 mM thiamine for 24 h **(Fig 4.6E)**.

4.5.7 Benfotiamine reduces in vivo tumor growth

To determine the impact of pharmacologic treatment with thiamine, sulbutiamine, and benfotiamine on *in vivo* tumor growth mice were administered each compound through their diet on a daily basis. In addition, mice received a bolus dose through IP injection every second day. Over the course of the study, tumor volume tracked lower for mice administered benfotiamine compared with control animals, with a significant reduction by nearly half at the conclusion of the study **(Fig 4.7A and 4.7B)**. There was also a trending reduction ($p=0.08$) in the total mass of tumors isolated (~2-fold) from benfotiamine treated groups compared with control **(Fig. 4.7C)**. No effect on tumor growth rate or final tumor mass was observed for thiamine or sulbutiamine treatment **(Fig 4.7A and 4.7C).** Animals supplemented with dietary thiamine trended to consume more food on a daily basis compared to control and those supplemented with benfotiamine and sulbutiamine **(Supplemental Fig 4.4A)**. However, no significant effect on animal weight was observed with increased consumption **(Supplemental Fig 4.4B)**. Mice fed supplemental sulbutiamine demonstrated a modest, but significant reduction in mass at the conclusion of the study corresponding with their noted food aversion **(Supplemental Fig 4.4A and 4.4B)**.

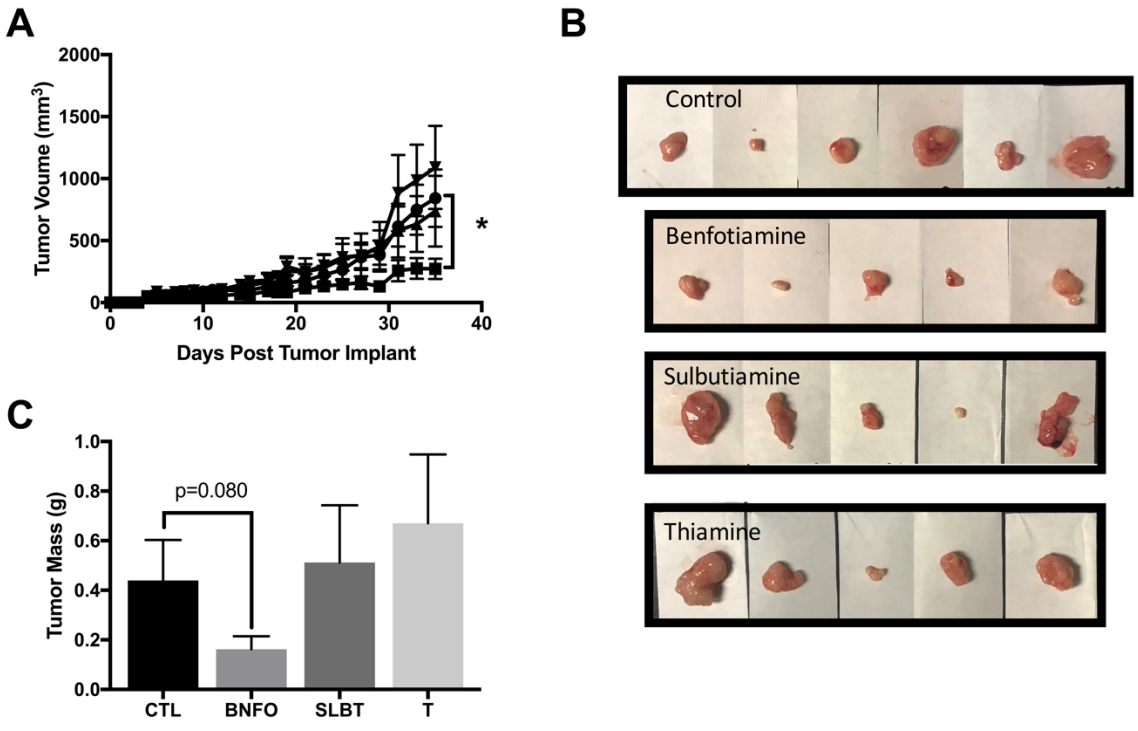
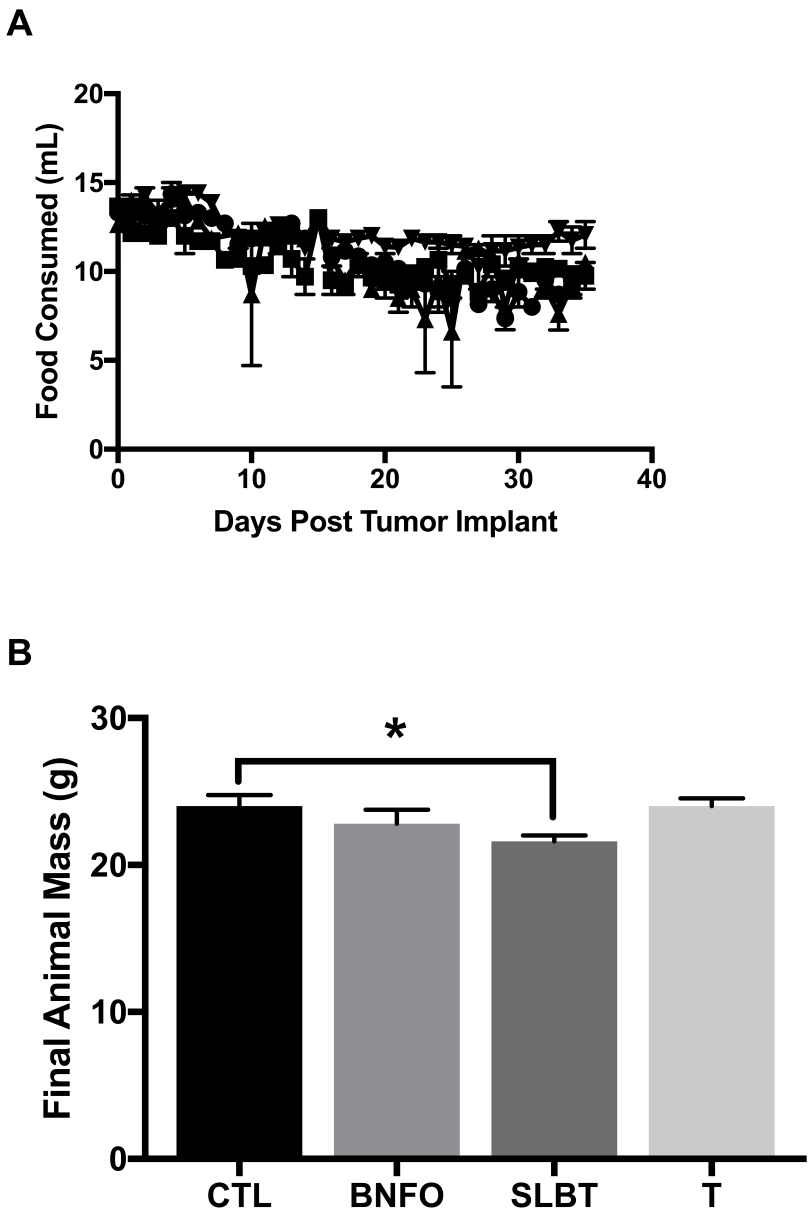


Fig 4.7. Benfotiamine reduces *in vivo* tumor growth (A) Quantitative analysis demonstrating the effect of pharmacologic treatment with benfotiamine (■), sulbutiamine (▲), and thiamine (▼) compared to vehicle control (●) on HCT 116 tumor volume over the course of 35 days following tumor implant (Day 0). Average tumor volume is presented as the mean +/- SEM of $n = 6$ control animals and $n = 5$ animals for each treatment arm. **(B)** Representative images demonstrating the effect of pharmacologic treatment with benfotiamine, sulbutiamine, or thiamine compared to vehicle control on HCT 116 tumor volume at the conclusion of the 35-day tumor growth period following subcutaneous implant. **(C)** Average tumor mass +/- SEM of $n = 6$ control animals and $n = 5$ animals for each treatment arm depicting effect of pharmacologic treatment with benfotiamine (BNFO), sulbutiamine (SLBT), or thiamine (T) compared to vehicle control (CTL) at the conclusion of 35-day growth period. (★) Represents statistically significant difference ($p<0.05$) based on results of an unpaired student's t-test.



Supplemental Fig 4.4. Food consumption and animal mass at study conclusion (A) Average food intake (per animal) +/- SEM for vehicle control (●), sulbutiamine (▲), benfotiamine (■) and thiamine (▼) treated animals over the course of tumor growth. **(B)** Average animal mass +/- SEM at study conclusion for vehicle control (CTL), sulbutiamine, benfotiamine, and thiamine treated animals. (✶) Represents statistically significant difference ($p<0.05$) based on results of an unpaired student's t-test.

4.6 Discussion

Considering their presumable safety and notable pre-clinical anticancer effects, exploiting pharmacologic doses of nutraceutical compounds (i.e. vitamins) provides a promising alternative approach to chemotherapy for cancer treatment (Watson 2003; Mamede *et al.* 2011). The early work of Linus Pauling and colleagues, demonstrated high-dose vitamin C (ascorbic acid, ascorbate, dehydroascorbic acid) as a safe and effective therapy to improve symptoms and prolong the lifespan of terminal cancer patients (Cameron & Pauling 1974; Cameron & Campbell 1974). Pharmacologic treatment of vitamin C with dosages in low mM concentrations has since been demonstrated to selectively kill cancer cells *in vitro* (Chen *et al.* 2008; Verrax & Calderon 2009). These findings translate *in vivo* where pharmacologic ascorbate (4g/kg, once or twice daily) significantly reduces tumor growth rate (Chen *et al.* 2008). The anticancer effects of vitamin C are attributed to its pro-oxidant properties and generation of an ascorbate radical resulting in H2O2-dependent cytotoxicity (Chen *et al.* 2008; Chen *et al.* 2007). Like vitamin C, vitamin B1, or thiamine, has also been shown to slow *in vivo* tumor growth at pharmacologic concentrations (Comin-Anduix *et al.* 2001). Here, we provide evidence that exploiting lipophilic thiamine analogs devoid of a requirement for carrier-mediated transport, may enhance thiamine's anticancer effect.

The sensitivity of three independent tumor cell lines from different tissue origins was increased following treatment with sulbutiamine and benfotiamine compared with thiamine. Micromolar IC50 values for thiamine analogs compared with millimolar concentrations for thiamine reduced tumor cell proliferation by ~50%. Reduction in tumor cell proliferation corresponded with increased apoptotic cell death in the colorectal

cancer cell line HCT 116 following treatment with sulbutiamine and benfotiamine. This is congruent with DCA treatment, which has previously been shown to enhance apoptosis and G2 phase cell-cycle arrest in colorectal cancer cells (Madhok *et al.* 2010). DCA-mediated apoptosis is associated with the depolarization of the mitochondrial membrane potential (MMP) resulting in the release of pro-apoptotic factors (Madhok *et al.* 2010; Michelakis *et al.* 2008). We previously demonstrated that like DCA, high-dose thiamine supplementation also results in reduction of MMP and subsequent cell death through an apoptotic associated mechanism (Hanberry *et al.* 2014). Following treatment with sulbutiamine and benfotiamine, total intracellular levels of thiamine and TPP were increased but the accumulation of the lipophilic derivatives was not detected. Therefore, the apoptotic response observed following sulbutiamine and benfotiamine treatment may be associated with a reduction of MMP and subsequent release of pro-apoptotic factors related to intracellular thiamine/TPP accumulation. Of note, the non-cancerous cell lines HB2 and HK-293 demonstrate no reduction in MMP following DCA treatment and a lack of sensitivity to DCA at comparable concentrations that inhibit tumor cell proliferation (Madhok *et al.* 2010). A similar effect was observed for sulbutiamine and benfotiamine, which induced significant apoptotic cell death in HCT 116 cancer cells but not their non-cancerous FHC counterparts.

The *in vitro* chemotherapeutic effect for benfotiamine translated to an *in vivo* model where its administration was able to significantly reduce tumor growth in a subcutaneous mouse model. To our knowledge, this is the first *in vivo* evidence for the anticancer effect of a commercially available thiamine analog. Highlighting the therapeutic safety of benfotiamine, no systemic toxicity was observed following daily

benfotiamine consumption (~20 mg/day) combined with bolus pharmacologic doses (400 mg/kg) administered *via* IP injection every second day. This supports the good tolerability of daily benfotiamine administration (600-900 mg/day) that has previously been demonstrated in clinical trials of diabetic nephropathy (Stracke *et al.* 2008; Alkhalaf *et al.* 2010). Interestingly, no significant effect was observed for sulbutiamine and thiamine administration on tumor growth. It remains unclear if this was due to a factor of bioavailability or if benfotiamine reduces tumor growth through an alternative mechanistic action *in vivo*. Benfotiamine has previously been shown to induce cell cycle arrest and parapoptotic cell death by inhibiting the activity of constitutively active ERK1/2 while concomitantly increasing phosphorylation of JNK1/2 in leukemia cells (Sugimori *et al.* 2015). The antitumor effects of benfotiamine in this capacity were attributed to similar concentrations (50-100 μM) found to be IC50 values in this study. However, the parapoptotic effects of benfotiamine were attributed directly to the benfotiamine molecule itself related to the compounds benzoyl group or its S-acyl moiety (Sugimori *et al.* 2015). In our *in vitro* model system, no intracellular traces of sulbutiamine or benfotiamine were found suggesting that the anticancer effects were mediated by thiamine or one of its phosphate-ester metabolites.

Mechanistically it remains undefined how thiamine inhibits PDH phosphorylation to reduce tumor cell proliferation. PDKs phosphorylate PDH to tightly regulate its activity (Saunier *et al.* 2016). Pyruvate, the primary substrate of PDH, serves as a physiological inhibitor of PDK, functioning through direct binding (Hucho *et al.* 1972). DCA, a pyruvate mimetic, also binds to inhibit PDK activity (Knoechel *et al.* 2006). In addition to pyruvate, PDK activity is inhibited by other physiological molecules

including ADP, NAD^+, and CoA-SH (Saunier *et al.* 2016). TPP possesses structural similarity to ADP and has been demonstrated to mimic ADP binding *ex vitro* (McLure *et al.* 2004). We demonstrate that TPP inhibits PDK activity in an *ex vitro* reaction following a dose-dependent manner for PDK1 and PDK2. TPP also inhibited PDK3 activity, but only at the highest concentrations assayed. Neither thiamine, sulbutiamine, nor benfotiamine demonstrated any ability to inhibit PDK activity in our assay system. These results suggest that TPP may be the active species mediating a reduction in PDH phosphorylation by directly inhibiting PDK activity. HPLC analysis of intracellular TPP levels demonstrated an ~2-fold enhancement of TPP following treatment with thiamine, sulbutiamine, or benfotiamine. Considering the delicate intracellular TPP balance, this level of TPP induction may be of enough significance to produce the observed effect on PDH phosphorylation. Supporting a role for a TPP-mediated anticancer effect, exogenous overexpression of *SLC44A4,* which encodes the thiamine pyrophosphate transporter (TPPT), resulted in increased apoptosis of HCT 116 cells following TPP treatment but not thiamine treatment. Furthermore, attempts to maximize TPP production through increasing thiamine transport (*SLC19A2*, THTR1) and conversion to TPP (TPK1) resulted in toxicity following only thiamine treatment. TPP administration had no effect in the latter cases most likely due to its requirement for carrier-mediated transport to cross the plasma membrane.

Significant increases for the intracellular concentration of thiamine were detected following treatment with thiamine, sulbutiamine, or benfotiamine. This increase was exaggerated compared to the modest induction identified for TPP. Therefore, other effects of intracellular thiamine cannot be ruled out. High-dose supplementation with

vitamins (i.e. vitamin C) has been demonstrated to reduce malignant growth in an association with the ability to modulate redox status (Verrax *et al.* 2011). Thiamine has been previously demonstrated to have redox potential (Lukienko *et al.* 2000). Therefore, alternative properties of thiamine (i.e. redox potential) may reduce tumor cell proliferation in combination with TPP's effects on PDK activity (Lukienko *et al.* 2000).

In conclusion, we have demonstrated that lipophilic thiamine analogs decrease *in vitro* tumor cell proliferation in a mechanism similar to that of DCA and high-dose thiamine supplementation. Like thiamine and DCA, sulbutiamine and benfotiamine reduce PDH phosphorylation and activate its activity. However, sulbutiamine and benfotiamine demonstrate enhanced potency requiring only micromolar concentrations to exert their effects. Benfotiamine demonstrates promising *in vivo* evidence to reduce tumor growth with minimal systemic toxicity. Furthermore, our analysis has revealed that TPP may be the active species mediating the effects of thiamine, sulbutiamine, and benfotiamine on PDH phosphorylation. Importantly, concentrations of thiamine, sulbutiamine, and benfotiamine that demonstrate anticancer effects in tumor cells demonstrate no toxicity to normal cells. Future work should aim to maximize intracellular TPP concentrations within cancer cells to determine if enhanced chemotherapeutic potential can be achieved.

4.7 The chemotherapeutic potential of thiamine analogs during chemoresistance

4.7.1 Introduction

Chemoresistance stands as a forefront obstacle challenging cancer therapy (Zheng 2017). Many cancer patients will not respond to applied therapies due to intrinsic resistance or will develop acquired resistance over the course of treatment (Wilson *et al.* 2009). Factors contributing to therapy resistance in tumor cells include alterations to drug transport and metabolism, mutation of drug targets (i.e. oncogenes), and genetic rewiring to circumvent drug-induced apoptosis (Zahreddine & Borden 2013). Strategies to overcome and target chemoresistance are desperately needed to improve disease progression and promote patient survival. Of the available strategies, nutraceuticals offer a promising approach because of their presumed safety, cost-effectiveness, demonstrated anticancer activity, and capability to target pathways of chemoresistance (Bharti & Aggarwal 2018). Multiple natural compounds are under ongoing pre-clinical and clinical evaluation for use as adjuvant therapy in combination with existing chemotherapeutics to determine their impact as anticancer chemosensitizers (Bharti & Aggarwal 2018). As demonstrated by Sugimori *et al.* and our findings described above, both sulbutiamine and benfotiamine demonstrate direct anticancer effects *in vitro* (Sugimori *et al.* 2015). Furthermore, combination treatment with benfotiamine has been shown to synergistically sensitize acute myeloid leukemia (AML) cells to the cytotoxic drug cytarabine commonly used to treat AML (Sugimori *et al.* 2015). Despite this promising finding there remains little evidence for the effectiveness of sulbutiamine and benfotiamine as anticancer and adjuvant therapy strategies during chemoresistance. Therefore, the present study was

undertaken to determine the sensitivity of the ovarian cancer cell line A2780 and its drug resistant counterpart 2780^{ADR} to both sulbutiamine and benfotiamine.

4.7.2 Materials and Methods

Cell culture reagents including RPMI 1640 media, penicillin/streptomycin, and trypsin/EDTA were purchased from Corning (Manassas, VA). Fetal bovine serum (FBS) was purchased from Seradigm (Radnor, PA). All flasks used for routine maintenance of cultures as well as 96-well plates used for toxicity profiles were purchased from Greiner Bio-One (Monroe, NC). Common chemotherapeutics compounds including cisplatin, doxorubicin, paclitaxel, and 7-Ethyl-10-hydroxycamptothecin (SN-38) were purchased from Sigma (St. Louis, MO). Both sulbutiamine and benfotiamine were purchased from Toronto Research Chemicals (North York, ON).

4.7.2.1 Cell Culture

The human ovarian cancer cell line A2780 derived from an ovarian endometrioid adenocarcinoma of an untreated patient and its adriamycin (doxorubicin) resistant subclone (2780^{ADR}) were kindly provided by Dr. Shelley Hooks. 2780^{ADR} were generated by exposure of A2780 to doxorubicin. Prior to experimentation, lineage was challenged with 100 nM doxorubicin to ensure resistance. Both cell lines were routinely cultured at 37°C with 5% CO_2 in complete RPMI 1640 medium, which contained 10% FBS and 1% penicillin/streptomycin. Mycozap (0.1%, Lonza, Verviers, Belgium) was also added to medium as a prophylactic treatment for prevention of mycoplasma contamination.

4.7.2.2 Quantitation of cellular proliferation

Cellular proliferation was determined using a MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay. In viable cells, mitochondrial activity results in the reduction of the tetrazolium MTT dye to a formazan crystal, which can be solubilized and detected through absorbance. Based on its ability to detect viable cells, the MTT assay has been deemed suitable for the measurement of drug sensitivity in cancer cell lines. A2780 and 2780^{ADR} cells were seeded into 96-well plates at 1000 cells/well. Cells were allowed to attach ~12h in complete growth medium. Media was then aspirated and replaced with complete growth medium containing serial dilutions of either cisplatin, doxorubicin, paclitaxel, SN-38, sulbutiamine, or benfotiamine. Plates were incubated under normal growth conditions for 72 h in the presence of each compound. Following 72 h of treatment, MTT solution was added to each well at a final concentration of 0.5mg/mL and incubated ~2h. Following formation of formazan, media from each well was aspirated. DMSO was added to solubilize formazan crystal and plates were placed on orbital shaker (200 RPM) for ~15 min at room temperature. The resulting absorbance of each well was immediately determined at 590 nm using a Spectra Max M2e (Molecular Devices, Sunnyvale, CA) 96-well microplate reader. Proliferation was normalized by comparing the absorbance of treated wells to untreated control wells. A non-linear regression ([inhibitor] vs. normalized response) was fitted using GraphPad Prism 6® (GraphPad Software, La Jolla, CA) and used to determine IC50 values.

4.7.3 Results

The sensitivities of the ovarian cancer cell line A2780 and its doxorubicin-resistant counterpart 2780^{ADR} to treatment with cisplatin, doxorubicin, paclitaxel, SN-38, sulbutiamine, or benfotiamine were determined by MTT assay **(Fig 4.8)**. Both cell lines demonstrated a decrease in proliferation with increasing concentration of each compound **(Fig 4.8)**. IC50 values along with the relative resistance (fold change) in sensitivity comparing 2780^{ADR} to A2780 for each compound were calculated and provided in **Table 4.5**. For each compound, the drug resistant 2780^{ADR} demonstrated decreased sensitivity to treatment in comparison to A2780. When compared to standard chemotherapeutics with IC50 values ranging from 0.002 μM to 0.68 μM, both sulbutiamine and benfotiamine required much higher concentrations, 77.9 μM and 234 μM respectively, to reduce A2780 proliferation by 50%. Likewise, much higher concentrations of sulbutiamine and benfotiamine, 433 μM and 1.76 mM respectively, were required to inhibit 2780^{ADR} proliferation by half compared to standard chemotherapeutic treatments ranging from 0.03 μM to 3.9 μM. When comparing the relative resistance, 2780^{ADR} demonstrated strongest resistance to paclitaxel (406), followed by doxorubicin (62.3) > SN-38 (15.0) > benfotiamine (7.52) > cisplatin (5.74) = sulbutiamine (5.56).

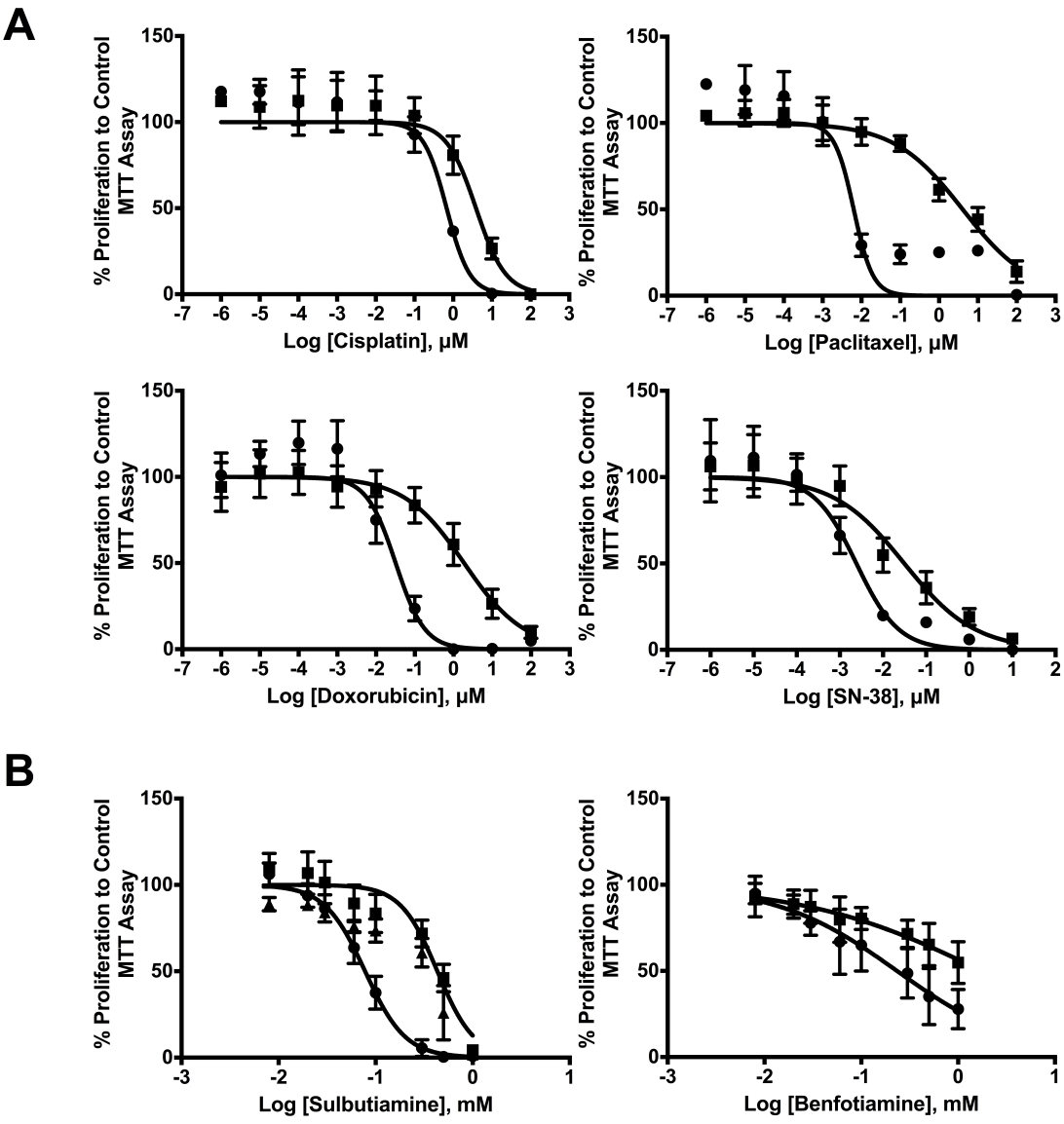


Fig 4.8. Sensitivity A2780 and 2780^{ADR} cells to chemotherapeutic treatment (A) Proliferation of A2780 (●) and 2780^{ADR} (■) cells determined by MTT assay demonstrating the effect cisplatin, doxorubicin, paclitaxel, and SN-38 treatment following 72 h exposure. Results are normalized as mean percent proliferation +/- SD comparing treated cells to untreated control. **(B)** Proliferation of A2780 (●) and 2780^{ADR} (■) cells determined by MTT assay demonstrating the effect sulbutiamine and benfotiamine treatment following 72 h exposure. Results are normalized as mean percent proliferation +/- SD comparing treated cells to untreated control.

Table 4.5. Comparison of IC50 values (with 95% confidence intervals) for A2780 and 2780^{ADR} following treatment with various chemotherapeutics

Compound	A2780	2780^{ADR}	Relative Resistance
Cisplatin	0.68 μM (0.43, 0.97)	3.90 μM (2.64, 5.69)	5.74
Doxorubicin	0.03 μM (0.02, 0.05)	1.87 μM (1.21, 2.92)	62.3
Paclitaxel	0.01 μM (0.003, 0.02)	4.06 μM (2.73, 6.08)	406
SN-38	0.002 μM (0.001, 0.004)	0.03 μM (0.02, 0.05)	15.0
Sulbutiamine	77.9 μM (71.8, 84.7)	433 μM (366, 497)	5.56
Benfotiamine	234 μM (174, 323)	1.76 mM (1.00, 4.53)	7.52

4.7.3 Discussion

Similar to the findings detailed in previous sections of this chapter for HCT 116 (colorectal), U-87 MG (glioblastoma), and MDA-MB-231 (breast) cells, both sulbutiamine and benfotiamine inhibited the proliferation of ovarian cancer cells *in vitro*. This finding expands the chemotherapeutic usefulness of the two analogs to a novel cancer type and highlights the potential for a universal response of the compounds across cancer subtypes. Compared with parental A2780 cells, the doxorubicin-resistant strain 2780^{ADR} demonstrated less sensitivity to both sulbutiamine and benfotiamine. This may suggest that drug resistant cell lines can also demonstrate resistance to sulbutiamine and benfotiamine. However, differences in relative resistance in sensitivity between 2780^{ADR} and A2780 cells when comparing the chemotherapeutics tested may suggest otherwise. In

our study, 2780^{ADR} demonstrated ~5-fold more resistance to cisplatin than A2780 compared with ~62-fold more resistance to doxorubicin. 2780^{ADR} cells were specifically developed to be resistant to doxorubicin, while a second clone was developed to be resistant to cisplatin (2780^{CIS}, A2780CIS) (Beaufort *et al.* 2014). Interestingly, 2780^{ADR} relative resistance to sulbutiamine and benfotiamine parallels that of cisplatin instead of the chemotherapeutics with much higher resistance profiles (doxorubicin, paclitaxel). This may demonstrate less resistance associated with thiamine analogs compared with other standard therapies. If so, this supports the potential usefulness of thiamine analogs as treatments and/or adjuvant therapies for chemoresistant cancers.

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

5.1 Summary

Malignancy associates with cellular adaptations to confer unrestricted proliferation (Cairns *et al.* 2011). Together, oncogenic activation and tumor suppressor inhibition contribute to the redirection of metabolic flux so that glycolysis and its subsidiary pathways are preferentially exploited in tumor cells (Tarrado-Castellarnau *et al.* 2016). This metabolic shift supports energy maintenance by allowing rapid ATP generation, while simultaneously enhancing biomass production necessary for replication (Cairns *et al.* 2011). In addition to metabolic adaptations, tumor cells tightly regulate their intracellular antioxidant concentration to maintain a delicate balance of ROS and promote a proliferative oxidative state over oxidative stress (Liou & Storz 2010; Gorrini *et al.* 2013). One factor that may impact both the metabolism and redox status of tumor cells is the supply of dietary nutrients. In addition to providing the necessary substrates and cofactors required for metabolic activity, nutrient molecules often possess antioxidant properties (Borek 2017). Therefore, the extent of their accumulation in tumor cells may directly impact proliferation. Furthermore, dietary nutrients and their derivatives can also effect other intracellular pathways (i.e. cell signaling, protein activity) to alter the proliferation rate of tumor cells (Lu'o'ng & Nguyen 2013).

Vitamin B1, or thiamine, and its activated cofactor form thiamine pyrophosphate (TPP) provide an example of an essential micronutrient implicated to influence tumor

growth in pre-clinical models (Comin-Anduix *et al.* 2001; Daily *et al.* 2012; Liu *et al.* 2010). Thiamine presents a unique paradigm in that the extent of its presence dictates the observed impact to proliferation (Comin-Anduix *et al.* 2001). Low-to-moderate supplemental doses of thiamine increase the growth rate of solid tumors, while high-dose pharmacologic supplementation with the vitamin restricts tumor growth (Comin-Anduix *et al.* 2001). The molecular mechanisms dictating the duality of thiamine's effect on tumor growth are unresolved, and therefore further inquiry into its bioactivity during malignancy is warranted. Prior investigation supports that tumor cells actively enhance thiamine homeostasis by up-regulating thiamine transporter expression (Zastre *et al.* 2013a). During stressed conditions, such as hypoxia, thiamine transport may be further increased through oncogenic signaling (Sweet *et al.* 2010). This up-regulation may demonstrate an attempt by tumor cells to acquire excess amounts of the vitamin compared to non-proliferative, non-cancerous cells. The high-prevalence of thiamine deficiency among cancer patients supports that tumor cell acquisition of thiamine may result in a redistribution of thiamine homeostasis during malignancy (Isenberg-Grzeda *et al.* 2016b). Based on this premise, the research goal of this investigation was two-fold. First, we aimed to elucidate a molecular action for thiamine or one of its derivatives that may confer a proliferative advantage during low-to-moderate supplemental thiamine conditions. Second, we aimed to further delineate the anticancer property of pharmacologic thiamine by identifying the active thiamine moiety functioning to reduce tumor cell proliferation.

5.1.1 Insight into the malignant advantage of supplemental thiamine

Due to its cofactor nature, there is an implicit requirement for TPP in tumor cell metabolism (Zastre *et al.* 2013b). Up-regulation of the thiamine dependent enzyme transketolase (TKT) has also been demonstrated in malignancy, and its activity directly contributes to malignant growth (Xu *et al.* 2016; Ricciardelli *et al.* 2015). Therefore, it remains reasonable that supplemental thiamine may support thiamine dependent enzyme activity to drive tumor cell proliferation. However, our work reveals an alternative non-canonical intracellular function for TPP (and potentially thiamine) in tumor cells that may directly contribute to the growth benefit observed during supplemental thiamine conditions.

During hypoxic and oxidative stress, the expression of thiamine pyrophosphokinase-1 (TPK1) was post-transcriptionally enhanced in malignant cells through what appeared to be a stress response mechanism. Despite TPK1 up-regulation, an intracellular consumption of TPP was defined during hypoxia, which coincides with its previously identified antioxidant properties (Okai *et al.* 2007). Alongside TPK1 expression, thiamine transport also enhances during malignant stress *via* oncogenically-driven *SLC19A3* expression (Sweet *et al.* 2010). We propose that these convergent mechanisms maximize TPP production in tumor cells and offset its antioxidant consumption during oxidative stress, which is independent of its canonical cofactor function. This agrees with the previous *Bettendorf et al*. proposal describing two intracellular pools of TPP, each with independent cellular functions, and one of which, undergoes rapid intracellular turnover, (Bettendorff 1994b). The adaptive regulation of thiamine transport and TPK1 expression may prevent an intracellular TPP deficit during

supplemental thiamine conditions despite this proposed rapid turnover. Our finding that supplemental thiamine protected against hypoxia and AA-induced oxidative stress may support this hypothesis. Supplemental thiamine also promoted tumor cell proliferation, corresponding with a reduction in basal ROS or reduced nuclear NRF2 accumulation. Loss of intracellular TPP mediated by the knockdown of TPK1 expression increased intracellular ROS and reduced tumor cell proliferation independent of TPP cofactor activity. Together, these results signify a non-canonical intracellular antioxidant activity for TPP that would benefit from the availability of supplemental thiamine and may be required for optimal tumor cell proliferation.

5.1.2 Insight into the anticancer property of pharmacologic thiamine

The adaptive regulation of thiamine homeostasis during malignancy may be detrimental to tumor cells during pharmacologic supplementation with high-dose thiamine. High-dose thiamine supplementation inhibits tumor cell proliferation through a mechanism similar to the chemotherapeutic dichloroacetate (Hanberry *et al.* 2014). Our previous work, and that of others, demonstrates that the effects of pharmacologic thiamine are mediated by activating pyruvate dehydrogenase (PDH), which forces mitochondrial metabolism in tumor cells and overrides their primary reliance on glycolysis (Hanberry *et al.* 2014; Liu *et al.* 2018). Despite understanding that the downstream effect of pharmacologic thiamine relies on obstructing the signature metabolic phenotype of tumor cells, it stands unclear how this effect is mediated at the molecular level. Our work reveals TPP as the probable active species facilitating the inhibitory effects of thiamine on tumor cell proliferation. TPP inhibited the *ex vitro*

activity of pyruvate dehydrogenase kinases 1-3 (PDK1-3), which are responsible for inactivating PDH activity through phosphorylation (Gudi *et al.* 1995; Kolobova *et al.* 2001). This supports that the intracellular accumulation of TPP observed following high-dose thiamine therapy may have inhibited PDK activity, resulting in decreased PDH phosphorylation and the overall increase in PDH activity that was observed. Maximizing intracellular TPP by exploiting thiamine homeostasis gene expression resulted in enhanced apoptosis. This supports that intracellular accumulation of TPP may confer apoptotic cell death and corresponds with previous reports demonstrating that activation of PDH by PDK inhibition induces apoptosis in cancer cells (Bonnet *et al.* 2007; Woolbright *et al.* 2018; McFate *et al.* 2008).

Our work also revealed that circumventing the requirement for carrier-mediated transport by exploiting lipophilic thiamine analogs may increase the therapeutic effectiveness of thiamine for treating malignancy. Therefore, the commercially available nutraceuticals sulbutiamine and benfotiamine may be more effective alternatives to high-dose thiamine supplementation. The lipophilic nature of both sulbutiamine and benfotiamine diminish the requirement for carrier-mediated transport associated with thiamine. Sulbutiamine and benfotiamine demonstrated micromolar IC50 concentrations, whereas thiamine was required in millimolar doses to reduce tumor cell proliferation by 50%. Concentrations of sulbutiamine and benfotiamine (250 μM) approximately 20-times lower than thiamine (5 mM) produced a similar increase in TPP, reduction in PDH phosphorylation, and activation of PDH activity. Furthermore, the *in vitro* effects of benfotiamine translated into an *in vivo* model where it reduced the growth of subcutaneous tumor implants. It is not clear why sulbutiamine did not show a similar

effect to benfotiamine *in vivo*, but the compared bioavailability of the two molecules as well as their alternative intracellular effects should be considered. Also adding to the promise of sulbutiamine and benfotiamine as cancer therapies, chemoresistant cancer cells demonstrated less relative resistance to sulbutiamine and benfotiamine treatment compared with the standard chemotherapies doxorubicin and paclitaxel. Going forward, developing compounds to maximize intracellular TPP content should be considered when developing novel therapies to target cancer cells.

5.1.3 Graphical Conclusion

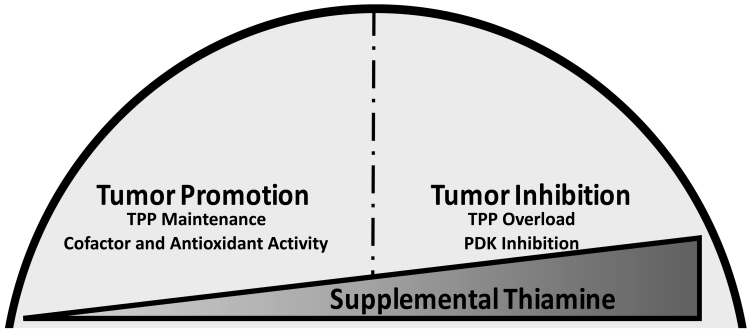


Fig 5.1 Schematic diagram demonstrating TPP's hypothesized role in tumor cell proliferation We propose that depending on the context of its concentration, TPP demonstrates both pro-proliferative and anti-proliferative properties during malignancy. It appears tumor cells may exploit moderate thiamine supplementation to maintain the optimal TPP concentration required to meet both its canonical cofactor and non-canonical antioxidant functions. However, following pharmacologic treatment with thiamine and its commercially available analogs, the concentration of TPP produced may become a burden to tumor cells resulting in decreased proliferation through PDK inhibition.

5.2 Experimental Limitations

5.2.1 Confluency and in vitro culture systems

In vitro culture of immortalized tumor cells has long been used as a model to determine mechanistic insights into tumor cell proliferation. In many cases, *in vitro* analysis can reveal significant findings that translate into the clinical setting. For example, our group previously identified up-regulation of the thiamine homeostasis genes *SCL19A2*, *SLC25A19*, and *TPK1* using breast tumor cells cultured *in vitro* (Zastre *et al.* 2013a). The clinical validity of these findings was confirmed by a similar pattern of up-regulation of these genes in breast tumor tissue compared with corresponding non-tumor tissue (Zastre *et al.* 2013a). However, many limitations exist within the standard model of *in vitro* culture. One issue that presented within this study was confluency of *in vitro* cultures when applying experimental variables. Basal TPK1 expression was much higher at confluent conditions compared with cultures harvested at sub-confluent conditions. Therefore, in order to observe the inductive nature of TPK1, some experiments had to be performed prior to cells reaching confluency. However, the high expression of TPK1 at confluency was intriguing as it may more accurately represent TPK1 expression in the tumor microenvironment where cells are in constant close contact. Alternatively, the effect of confluency on TPK1 expression may also be an artifact induced by events including pericellular hypoxia or nutrient deprivation during *in vitro* culture.

Depicting another limitation of *in vitro* culture systems, we found that sulbutiamine and benfotiamine produced similar toxicity profiles *in vitro,* but only benfotiamine exerted tumor inhibition *in vivo.* This demonstrates the complexity of applying a treatment to a system as a whole (mouse with tumor) compared with just the

partial system (tumor cells *in vitro*). Culture of tumor cells *in vitro* does not account for the bioavailability or metabolism of potential treatment strategies that may significantly impact their effectiveness *in vivo* and in clinical settings.

5.2.2 Thiamine dosing

The structure of our report requires the analysis of low-dose and high-dose thiamine supplementation using both *in vitro* and *in vivo* models. However, the translation of our dosing parameters into a clinical setting, and vice-versa, is not clear cut. The concentration of 10 nM thiamine was chosen to represent the "basal" thiamine condition. This was established based on previous research demonstrating that healthy human serum contains a concentration of ~10 nM thiamine (Gangolf *et al.* 2010). It has been extensively detailed that cancer patients often present as thiamine deficient, so 10 nM thiamine may over represent the actual basal plasma thiamine level of cancer patients. However, the addition of 10% FBS to formulate TD 1640 produced a thiamine level of ~10 nM, making it the lowest achievable concentration without the introduction of extraneous variables by using dialyzed FBS. Based on this limitation and the congruence with healthy human serum, we chose 10 nM thiamine as the basal condition. For our low dose supplementation *in vitro* models, the supplemental dose most commonly used was 3 μM thiamine consistent with the normal thiamine concentration of RPMI 1640 medium. This dose represents a concentration ~300 times greater than the thiamine content of TD 1640 medium (basal thiamine). Previous *in vivo* studies demonstrate that the greatest proliferative benefits of thiamine were observed at 25 times

the RDI for thiamine intake in mice. Although the *in vitro* and *in vivo* dosing schemes are not directly comparable, there appears to be incongruence between the two models.

Another limitation regarding thiamine dose within our research arose during high-dose *in vivo* thiamine supplementation. The lowest reported tolerated dose for thiamine varies drastically among different species. The first toxic symptoms caused by intravenous thiamine injection occur in mice at 125 mg/kg, in rats at 250 mg /kg, rabbits at 300 mg/kg, dogs at 350 mg/kg, and in monkeys 600 mg/kg causes the first signs of toxicity (Perla 1937). We used a dose of 150 mg/kg for high-dose thiamine administered *via* intraperitoneal injection based on similar reports (Moallem *et al.* 2008). This bolus dose, combined with excess thiamine supplied through the diet, demonstrated no inhibitory effect on tumor growth suggesting its lack of effectiveness for chemotherapy. However, previous reports demonstrate that larger species can tolerate much higher bolus doses of thiamine. Therefore, these higher doses may prove chemotherapeutic usefulness in humans. Unfortunately, because of potential lethality, a higher bolus dose cannot be tested using subcutaneous implants in mice as a model system. Further research is necessary to determine the most effective model system for testing the anticancer effects of thiamine and its analogs *in vivo*.

5.2.3 Limitations of detection when describing TPP as an antioxidant

Our analysis demonstrating the potential antioxidant consumption of TPP during hypoxia characterizes a single 24 h time point. Therefore, our study excludes factors regarding the kinetics of thiamine conversion to TPP when determining the *in vitro* functionality of TPK1. Although we determined that TPK1 up-regulation was functional

ex vitro, more detail in an *in vitro* setting is needed to confirm that TPK1 up-regulation actually results in enhanced TPP production in tumor cells. Furthermore, there is currently a limited capacity to track the intracellular fate of individual thiamine molecules making it impossible to conclusively define the intracellular fate of supplemental thiamine/TPP. Therefore, TPP's function as an intracellular antioxidant has not been molecularly confirmed. Although our study lays the necessary groundwork for us and others to answer this important question, more sophisticated techniques are needed to track thiamine and its derivatives inside tumor cells. This work remains actively ongoing as we are currently developing an isotopically-labeled model coupled with LC-MS/MS detection to track the intracellular fates of thiamine and TPP.

5.3 Future Directions

5.3.1 Mechanistic detail for post-transcriptional regulation of TPK1

A further understanding of the mechanism supporting the post-transcriptional up-regulation of TPK1 will aid in defining TPK1 as a stress-response protein. Considering TPK1 up-regulation does not occur due to enhanced transcript production, many alternative levels of regulation must be investigated. Potential factors include mRNA localization and sequestration, micro-RNA regulation, translation, post-translational modification, and protein degradation (Spriggs *et al.* 2010). Regulation of TPK1 through microRNAs (miRs) may be a promising research avenue as miR-155 has previously been demonstrated to impact thiamine homeostasis through inhibition of an undefined mediator (Kim *et al.* 2015). Alternatively, the lack of induction for TPK1 during hypoxia in the presence of the translational inhibitors CHX and YC-1 supports that TPK1 up-

regulation may be a factor of its increased mRNA translation. The translational up-regulation of TPK1 following malignant stress seems counterintuitive since repression of mRNA translation generally occurs during conditions of cellular stress (hypoxia, oxidative stress). Our results suggest that TPK1 may evade the global suppression of translation that has been described for hypoxic conditions. Similar findings have been documented for other proteins critical to cell survival during hypoxic conditions (Lai *et al.* 2016). One proposal for the enhanced translation of stress response proteins during hypoxic conditions suggests the presence of internal ribosome entry site (IRES) in their mRNA sequence (Lang *et al.* 2002). If an IRES is present in TPK1's mRNA sequence, it would allow the recruitment of translation machinery within the 5'UTR of the mRNA sequence and support continued translation during stressed conditions (Spriggs *et al.* 2010). Other factors in TPK1's mRNA sequence could also promote its enhanced expression during global translation inhibition including the presence of upstream open reading frames (uORF) with alternative start codons (CUG) (Liu & Qian 2014). These mechanisms, among others, should be considered when defining the post-transcriptional regulation of TPK1 expression in the future.

5.3.2 Dynamics of thiamine homeostasis in TPP's antioxidant function

As was described for a limitation of this work, a further understanding of the dynamics of intracellular thiamine homeostasis is required to fully delineate the intracellular fate and function of TPP in malignant cells. There are many factors that might affect the TPP pool during hypoxic and oxidative stress including the rate of thiamine conversion to TPP, dephosphorylation of TPP to TMP or thiamine, generation

of thiamine triphosphate (TTP) or adenylated thiamine derivatives, consumption of TPP as an antioxidant, and cellular efflux of thiamine and/or its derivatives. Although we touched on many of these potentials, more effort must be focused on defining thiamine homeostasis at the molecular level in tumor cells during conditions of malignant stress. Tracking isotopically labeled thiamine molecules using LC-MS/MS will allow a more complete understanding of whether TPP acts as an intracellular antioxidant. Furthermore, this methodology will help to define if thiamine also acts as an intracellular antioxidant, or if thiamine up-regulation during malignancy simply facilitates the production of TPP. These findings are important to further defining the active thiamine species mediating tumor growth.

5.3.4 Thiamine's impact on basal ROS levels and NRF2 activation

Among the most intriguing findings within our work was the preliminary demonstration that supplemental thiamine may reduce NRF2 nuclear accumulation. As a master transcriptional regulator with aberrant activation in cancer, interest in NRF2's role in malignant progression has rapidly grown in the recent decade (Taguchi, 2017). Therefore, the ability of thiamine to impact NRF2 expression in relation to tumor proliferation provides a promising new focus for thiamine research in regard to malignancy. In the immediate future, thiamine's impact on the expression of NRF2 target genes should be considered. This will provide further evidence that a relationship between supplemental thiamine and NRF2 activity exists in malignant cells.

5.3.5 Maximizing TPP to inhibit tumor growth

Sulbutiamine and, more so, benfotiamine provide promising evidence for their potential effectiveness in treating malignancy. The consideration that both compounds are relatively inexpensive and generally well-tolerated further enhances their appeal as chemotherapeutic therapies. Developing more potent thiamine analogs through structure based design should further increase the anticancer effectiveness observed for sulbutiamine and benfotiamine. It is also of great interest to generate a lipophilic analog of TPP with the ability to penetrate the cellular membrane. This molecule would allow an increase in intracellular TPP concentration independent of TPK1 activity, which based on our evidence could produce even more potent anticancer effects. In the immediate future, it appears that the most logical strategy for the anticancer use of thiamine analogs will be to consider their impacts as adjuvant therapies to increase the effectiveness of current standards of care.

5.3.6 Importance of TPP as an antioxidant outside the malignant state

Our work using the non-cancerous model of FHC cells demonstrates that the adaptive regulation of TPK1 during hypoxia is not restricted to tumor cells. Therefore, our conclusion that TPP production may be an important factor in limiting oxidative stress during supplemental thiamine conditions should also not be limited to malignancy. Interestingly, in other disease pathologies, specifically those involving neurodegeneration, oxidative stress coincides with thiamine deficiency (defined by reduction of TPP) (Liu *et al.* 2017). There are many possible causes for increased generation of ROS during thiamine deficiency including mitochondrial dysfunction and

neuroinflammation due to loss of thiamine dependent enzyme activity (Liu *et al.* 2017). However, in light of our results it should also be considered that a lack of TPP's antioxidant function during thiamine deficiency may also directly impact oxidative stress. If so, supplemental thiamine may protect against ROS-associated toxicity and be advantageous for patients suffering neurodegenerative diseases including Alzheimer's Disease, Parkinson's Disease, and Huntington's Disease.

REFERENCES

Ahn, C. S. and Metallo, C. M. (2015) Mitochondria as biosynthetic factories for cancer proliferation. *Cancer Metab* **3,** 1.

Aksoy, M., Basu, T. K., Brient, J. and Dickerson, J. W. (1980) Thiamin status of patients treated with drug combinations containing 5-fluorouracil. *Eur J Cancer* **16,** 1041-1045.

Alkhalaf, A., Klooster, A., van Oeveren, W. et al. (2010) A double-blind, randomized, placebo-controlled clinical trial on benfotiamine treatment in patients with diabetic nephropathy. *Diabetes Care* **33,** 1598-1601.

Altuner, D., Cetin, N., Suleyman, B., Aslan, Z., Hacimuftuoglu, A., Gulaboglu, M., Isaoglu, N., Demiryilmaz, I. and Suleyman, H. (2013) Effect of thiamine pyrophosphate on ischemia-reperfusion induced oxidative damage in rat kidney. *Indian J Pharmacol* **45,** 339-343.

Azzam, E. I., Jay-Gerin, J. P. and Pain, D. (2012) Ionizing radiation-induced metabolic oxidative stress and prolonged cell injury. *Cancer Lett* **327,** 48-60.

Babar, I. A., Czochor, J., Steinmetz, A., Weidhaas, J. B., Glazer, P. M. and Slack, F. J. (2011) Inhibition of hypoxia-induced miR-155 radiosensitizes hypoxic lung cancer cells. *Cancer Biol Ther* **12,** 908-914.

Bailey, R. L., Gahche, J. J., Miller, P. E., Thomas, P. R. and Dwyer, J. T. (2013) Why US adults use dietary supplements. *JAMA Intern Med* **173,** 355-361.

Baker, H., Frank, O., Chen, T., Feingold, S., DeAngelis, B. and Baker, E. R. (1981) Elevated vitamin levels in colon adenocarcinoma as compared with metastatic liver adenocarcinoma from colon primary and normal adjacent tissue. *Cancer* **47,** 2883-2886.

Barthel, A., Okino, S. T., Liao, J., Nakatani, K., Li, J., Whitlock, J. P., Jr. and Roth, R. A. (1999) Regulation of GLUT1 gene transcription by the serine/threonine kinase Akt1. *J Biol Chem* **274,** 20281-20286.

Basiri, B., Sutton, J. M., Hanberry, B. S., Zastre, J. A. and Bartlett, M. G. (2016) Ion pair liquid chromatography method for the determination of thiamine (vitamin B1) homeostasis. *Biomed Chromatogr* **30,** 35-41.

Basu, T. K. and Dickerson, J. W. (1976) The thiamin status of early cancer patients with particular reference to those with breast and bronchial carcinomas. *Oncology* **33,** 250-252.

Beaufort, C. M., Helmijr, J. C., Piskorz, A. M. et al. (2014) Ovarian cancer cell line panel (OCCP): clinical importance of in vitro morphological subtypes. *PLoS One* **9,** e103988.

Bensaad, K., Tsuruta, A., Selak, M. A., Vidal, M. N., Nakano, K., Bartrons, R., Gottlieb, E. and Vousden, K. H. (2006) TIGAR, a p53-inducible regulator of glycolysis and apoptosis. *Cell* **126,** 107-120.

Bertout, J. A., Majmundar, A. J., Gordan, J. D., Lam, J. C., Ditsworth, D., Keith, B., Brown, E. J., Nathanson, K. L. and Simon, M. C. (2009) HIF2alpha inhibition promotes p53 pathway activity, tumor cell death, and radiation responses. *Proc Natl Acad Sci U S A* **106,** 14391-14396.

Bettendorff, L. (1994a) The compartmentation of phosphorylated thiamine derivatives in cultured neuroblastoma cells. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research* **1222,** 7-14.

Bettendorff, L. (1994b) The compartmentation of phosphorylated thiamine derivatives in cultured neuroblastoma cells. *Biochim Biophys Acta* **1222,** 7-14.

Bettendorff, L. (1995) Thiamine homeostasis in neuroblastoma cells. *Neurochem Int* **26,** 295-302.

Bettendorff, L., Lakaye, B., Kohn, G. and Wins, P. (2014) Thiamine triphosphate: a ubiquitous molecule in search of a physiological role. *Metab Brain Dis* **29,** 1069-1082.

Bettendorff, L., Weekers, L., Wins, P. and Schoffeniels, E. (1990a) Injection of sulbutiamine induces an increase in thiamine triphosphate in rat tissues. *Biochemical pharmacology* **40,** 2557-2560.

Bettendorff, L., Weekers, L., Wins, P. and Schoffeniels, E. (1990b) Injection of sulbutiamine induces an increase in thiamine triphosphate in rat tissues. *Biochem Pharmacol* **40,** 2557-2560.

Bharti, A. C. and Aggarwal, B. B. (2018) *Role of nutraceuticals in cancer chemosensitization,* Vol. volume 2: Cancer sensitizing agents for chemotherapy,. Elsevier/Academic Press, London, United Kingdom.

Bjelakovic, G., Nikolova, D., Gluud, L. L., Simonetti, R. G. and Gluud, C. (2007) Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: systematic review and meta-analysis. *JAMA* **297,** 842-857.

Blass, J. P., Gleason, P., Brush, D., DiPonte, P. and Thaler, H. (1988) Thiamine and Alzheimer's disease. A pilot study. *Arch Neurol* **45,** 833-835.

Bonnet, S., Archer, S. L., Allalunis-Turner, J. et al. (2007) A mitochondria-K+ channel axis is suppressed in cancer and its normalization promotes apoptosis and inhibits cancer growth. *Cancer Cell* **11,** 37-51.

Borek, C. (2004) Antioxidants and radiation therapy. *J Nutr* **134,** 3207S-3209S.

Borek, C. (2017) Dietary Antioxidants and Human Cancer. *Journal of Restorative Medicine* **6,** 53-61.

Boros, L. G. (2000) Population thiamine status and varying cancer rates between western, Asian and African countries. *Anticancer research* **20,** 2245-2248.

Boros, L. G., Puigjaner, J., Cascante, M. et al. (1997) Oxythiamine and dehydroepiandrosterone inhibit the nonoxidative synthesis of ribose and tumor cell proliferation. *Cancer Res* **57,** 4242-4248.

Boroughs, L. K. and DeBerardinis, R. J. (2015) Metabolic pathways promoting cancer cell survival and growth. *Nat Cell Biol* **17,** 351-359.

Boulware, M. J., Subramanian, V. S., Said, H. M. and Marchant, J. S. (2003) Polarized expression of members of the solute carrier SLC19A gene family of water-soluble multivitamin transporters: implications for physiological function. *Biochem J* **376,** 43-48.

Bourdon, A., Minai, L., Serre, V. et al. (2007) Mutation of RRM2B, encoding p53-controlled ribonucleotide reductase (p53R2), causes severe mitochondrial DNA depletion. *Nat Genet* **39,** 776-780.

Buesa, J. M., Garcia-Teijido, P., Losa, R. and Fra, J. (2003) Treatment of ifosfamide encephalopathy with intravenous thiamin. *Clin Cancer Res* **9,** 4636-4637.

Burkitt, K., Chun, S. Y., Dang, D. T. and Dang, L. H. (2009) Targeting both HIF-1 and HIF-2 in human colon cancer cells improves tumor response to sunitinib treatment. *Mol Cancer Ther* **8,** 1148-1156.

Cairns, R. A., Harris, I. S. and Mak, T. W. (2011) Regulation of cancer cell metabolism. *Nat Rev Cancer* **11,** 85-95.

Cameron, E. and Campbell, A. (1974) The orthomolecular treatment of cancer. II. Clinical trial of high-dose ascorbic acid supplements in advanced human cancer. *Chem Biol Interact* **9,** 285-315.

Cameron, E. and Pauling, L. (1974) The orthomolecular treatment of cancer. I. The role of ascorbic acid in host resistance. *Chem Biol Interact* **9,** 273-283.

Cancarini, I., Krogh, V., Agnoli, C. et al. (2015) Micronutrients Involved in One-Carbon Metabolism and Risk of Breast Cancer Subtypes. *PLoS One* **10,** e0138318.

Cao, W., Yacoub, S., Shiverick, K. T., Namiki, K., Sakai, Y., Porvasnik, S., Urbanek, C. and Rosser, C. J. (2008) Dichloroacetate (DCA) sensitizes both wild-type and over expressing Bcl-2 prostate cancer cells in vitro to radiation. *Prostate* **68,** 1223-1231.

Cefalo, M. G., De Ioris, M. A., Cacchione, A., Longo, D., Staccioli, S., Arcioni, F., Bernardi, B. and Mastronuzzi, A. (2014) Wernicke encephalopathy in pediatric neuro-oncology: presentation of 2 cases and review of literature. *J Child Neurol* **29,** NP181-185.

Centeno, C., Sanz, A. and Bruera, E. (2004) Delirium in advanced cancer patients. *Palliat Med* **18,** 184-194.

Cervantes-Madrid, D., Romero, Y. and Duenas-Gonzalez, A. (2015) Reviving Lonidamine and 6-Diazo-5-oxo-L-norleucine to Be Used in Combination for Metabolic Cancer Therapy. *Biomed Res Int* **2015,** 690492.

Chamberlain, B. R., Buttery, J. E. and Pannall, P. R. (1996) A stable reagent mixture for the whole blood transketolase assay. *Ann Clin Biochem* **33 (Pt 4),** 352-354.

Chen, L., Shu, Y., Liang, X. et al. (2014) OCT1 is a high-capacity thiamine transporter that regulates hepatic steatosis and is a target of metformin. *Proc Natl Acad Sci U S A* **111,** 9983-9988.

Chen, Q., Espey, M. G., Sun, A. Y. et al. (2007) Ascorbate in pharmacologic concentrations selectively generates ascorbate radical and hydrogen peroxide in extracellular fluid in vivo. *Proc Natl Acad Sci U S A* **104,** 8749-8754.

Chen, Q., Espey, M. G., Sun, A. Y., Pooput, C., Kirk, K. L., Krishna, M. C., Khosh, D. B., Drisko, J. and Levine, M. (2008) Pharmacologic doses of ascorbate act as a prooxidant and decrease growth of aggressive tumor xenografts in mice. *Proc Natl Acad Sci U S A* **105,** 11105-11109.

Cho, I. J., Chang, H. J., Lee, K. E., Won, H. S., Choi, M. Y., Nam, E. M., Mun, Y. C., Lee, S. N. and Seong, C. M. (2009) A case of Wernicke's encephalopathy following fluorouracil-based chemotherapy. *J Korean Med Sci* **24,** 747-750.

Cinici, E., Ahiskali, I., Cetin, N. et al. (2016a) Effect of thiamine pyrophosphate on retinopathy induced by hyperglycemia in rats: A biochemical and pathological evaluation. *Indian J Ophthalmol* **64,** 434-439.

Cinici, E., Cetin, N., Ahiskali, I., Suleyman, B., Altuner, D., Alp, H. H., Sener, E., Calik, I. and Suleyman, H. (2016b) The effect of thiamine pyrophosphate on ethambutol-induced ocular toxicity. *Cutan Ocul Toxicol* **35,** 222-227.

Clem, B. F., O'Neal, J., Tapolsky, G. et al. (2013) Targeting 6-phosphofructo-2-kinase (PFKFB3) as a therapeutic strategy against cancer. *Mol Cancer Ther* **12,** 1461-1470.

Comerford, K. B. (2013) Recent developments in multivitamin/mineral research. *Adv Nutr* **4,** 644-656.

Comin-Anduix, B., Boren, J., Martinez, S. et al. (2001) The effect of thiamine supplementation on tumour proliferation A metabolic control analysis study. *Eur J Biochem* **268,** 4177-4182.

Coskun, R., Turan, M. I., Turan, I. S. and Gulapoglu, M. (2014) The protective effect of thiamine pyrophosphate, but not thiamine, against cardiotoxicity induced with cisplatin in rats. *Drug Chem Toxicol* **37,** 290-294.

Daily, A., Liu, S., Bhatnagar, S., Karabakhtsian, R. G. and Moscow, J. A. (2012) Low-thiamine diet increases mammary tumor latency in FVB/N-Tg(MMTVneu) mice. *Int J Vitam Nutr Res* **82,** 298-302.

Dang, D. T., Chen, F., Gardner, L. B., Cummins, J. M., Rago, C., Bunz, F., Kantsevoy, S. V. and Dang, L. H. (2006) Hypoxia-inducible factor-1alpha promotes nonhypoxia-mediated proliferation in colon cancer cells and xenografts. *Cancer Res* **66,** 1684-1936.

Davis, R. E. and Icke, G. C. (1983) Clinical chemistry of thiamin. *Adv Clin Chem* **23,** 93-140.

Dayan, F., Bilton, R. L., Laferriere, J., Trottier, E., Roux, D., Pouyssegur, J. and Mazure, N. M. (2009) Activation of HIF-1alpha in exponentially growing cells via hypoxic stimulation is independent of the Akt/mTOR pathway. *J Cell Physiol* **218,** 167-174.

DeBerardinis, R. J., Lum, J. J., Hatzivassiliou, G. and Thompson, C. B. (2008) The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. *Cell Metab* **7,** 11-20.

DeBerardinis, R. J., Mancuso, A., Daikhin, E., Nissim, I., Yudkoff, M., Wehrli, S. and Thompson, C. B. (2007) Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. *Proc Natl Acad Sci U S A* **104,** 19345-19350.

Demiryilmaz, I., Sener, E., Cetin, N., Altuner, D., Akcay, F. and Suleyman, H. (2013) A comparative investigation of biochemical and histopathological effects of thiamine and thiamine pyrophosphate on ischemia-reperfusion induced oxidative damage in rat ovarian tissue. *Arch Pharm Res* **36,** 1133-1139.

Demiryilmaz, I., Sener, E., Cetin, N., Altuner, D., Suleyman, B., Albayrak, F., Akcay, F. and Suleyman, H. (2012) Biochemically and histopathologically comparative review of thiamine's and thiamine pyrophosphate's oxidative stress effects generated with methotrexate in rat liver. *Med Sci Monit* **18,** BR475-481.

DeNicola, G. M., Karreth, F. A., Humpton, T. J. et al. (2011) Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis. *Nature* **475,** 106-109.

Diao, Q. X., Zhang, J. Z., Zhao, T., Xue, F., Gao, F., Ma, S. M. and Wang, Y. (2016) Vitamin E promotes breast cancer cell proliferation by reducing ROS production and p53 expression. *Eur Rev Med Pharmacol Sci* **20,** 2710-2717.

Donchenko, H. V., Chernukhina, L. O., Kuz'menko, I. V. and Parkhomenko Iu, M. (1997) [Blood vitamin levels in various population groups in the Ukraine suffering from sequelae to the accident at the Chernobyl power plant]. *Ukr Biokhim Zh (1978)* **69,** 48-53.

Donohoe, D. R., Collins, L. B., Wali, A., Bigler, R., Sun, W. and Bultman, S. J. (2012) The Warburg effect dictates the mechanism of butyrate-mediated histone acetylation and cell proliferation. *Mol Cell* **48,** 612-626.

Dutta, B., Huang, W., Molero, M., Kekuda, R., Leibach, F. H., Devoe, L. D., Ganapathy, V. and Prasad, P. D. (1999) Cloning of the human thiamine transporter, a member of the folate transporter family. *J Biol Chem* **274,** 31925-31929.

Dwarakanath, B. S., Singh, D., Banerji, A. K. et al. (2009) Clinical studies for improving radiotherapy with 2-deoxy-D-glucose: present status and future prospects. *J Cancer Res Ther* **5 Suppl 1,** S21-26.

Eudy, J. D., Spiegelstein, O., Barber, R. C., Wlodarczyk, B. J., Talbot, J. and Finnell, R. H. (2000) Identification and characterization of the human and mouse SLC19A3 gene: a novel member of the reduced folate family of micronutrient transporter genes. *Mol Genet Metab* **71,** 581-590.

Fikhman, G., Berger, J. R. and Gal, T. J. (2011) Wernicke's encephalopathy in the course of chemoradiotherapy for head and neck cancer. *Am J Otolaryngol* **32,** 250-252.

Fleming, R. A. (1997) An overview of cyclophosphamide and ifosfamide pharmacology. *Pharmacotherapy* **17,** 146S-154S.

Fujino, T., Murakami, K., Ozawa, I. et al. (2009) Hypoxia downregulates farnesoid X receptor via a hypoxia-inducible factor-independent but p38 mitogen-activated protein kinase-dependent pathway. *FEBS J* **276,** 1319-1332.

Galadari, S., Rahman, A., Pallichankandy, S. and Thayyullathil, F. (2017) Reactive oxygen species and cancer paradox: To promote or to suppress? *Free Radic Biol Med* **104,** 144-164.

Ganapathy, V., Smith, S. B. and Prasad, P. D. (2004) SLC19: the folate/thiamine transporter family. *Pflugers Arch* **447,** 641-646.

Gangolf, M., Czerniecki, J., Radermecker, M. et al. (2010) Thiamine status in humans and content of phosphorylated thiamine derivatives in biopsies and cultured cells. *PLoS One* **5,** e13616.

Gevorkian, L. and Gambashidze, K. (2013) The novel hypothesis of carcinogenesis and anti-cancer treatment perspectives - Hydroxyethylthiamine diphosphate. *Georgian medical news***,** 57-68.

Gevorkyan, L. and Gambashidze, K. (2014) Anticancer efficacy of hydroxyethylthiamine diphosphate in vivo. *Experimental oncology* **36,** 48-49.

Giordano, S. B. and Gradishar, W. (2017) Breast cancer: updates and advances in 2016. *Curr Opin Obstet Gynecol* **29,** 12-17.

Gong, L., Wei, Y., Yu, X., Peng, J. and Leng, X. (2014) 3-Bromopyruvic acid, a hexokinase II inhibitor, is an effective antitumor agent on the hepatoma cells : in vitro and in vivo findings. *Anticancer Agents Med Chem* **14,** 771-776.

Gordan, J. D. and Simon, M. C. (2007) Hypoxia-inducible factors: central regulators of the tumor phenotype. *Curr Opin Genet Dev* **17,** 71-77.

Gorrini, C., Harris, I. S. and Mak, T. W. (2013) Modulation of oxidative stress as an anticancer strategy. *Nat Rev Drug Discov* **12,** 931-947.

Gu, J., Li, Y., Zeng, J., Wang, B., Ji, K., Tang, Y. and Sun, Q. (2017) Knockdown of HIF-1alpha by siRNA-expressing plasmid delivered by attenuated Salmonella enhances the antitumor effects of cisplatin on prostate cancer. *Sci Rep* **7,** 7546.

Gudi, R., Bowker-Kinley, M. M., Kedishvili, N. Y., Zhao, Y. and Popov, K. M. (1995) Diversity of the pyruvate dehydrogenase kinase gene family in humans. *J Biol Chem* **270,** 28989-28994.

Habbe, N., Koorstra, J. B., Mendell, J. T. et al. (2009) MicroRNA miR-155 is a biomarker of early pancreatic neoplasia. *Cancer Biol Ther* **8,** 340-346.

Hanberry, B. S., Berger, R. and Zastre, J. A. (2014) High-dose vitamin B1 reduces proliferation in cancer cell lines analogous to dichloroacetate. *Cancer Chemother Pharmacol* **73,** 585-594.

Hartmannsberger, D., Mack, B., Eggert, C., Denzel, S., Stepp, H., Betz, C. S. and Gires, O. (2011) Transketolase-like protein 1 confers resistance to serum withdrawal in vitro. *Cancer Lett* **300,** 20-29.

Haseloff, R. F., Krause, E., Bigl, M., Mikoteit, K., Stanimirovic, D. and Blasig, I. E. (2006) Differential protein expression in brain capillary endothelial cells induced by hypoxia and posthypoxic reoxygenation. *Proteomics* **6,** 1803-1809.

Hatzivassiliou, G., Zhao, F., Bauer, D. E., Andreadis, C., Shaw, A. N., Dhanak, D., Hingorani, S. R., Tuveson, D. A. and Thompson, C. B. (2005) ATP citrate lyase inhibition can suppress tumor cell growth. *Cancer Cell* **8,** 311-321.

Hazell, A. S. and Butterworth, R. F. (2009) Update of cell damage mechanisms in thiamine deficiency: focus on oxidative stress, excitotoxicity and inflammation. *Alcohol Alcohol* **44,** 141-147.

Heier, M. S. and Dornish, J. M. (1989) Effect of the fluoropyrimidines 5-fluorouracil and doxifluridine on cellular uptake of thiamin. *Anticancer Res* **9,** 1073-1077.

Hersey, P., Watts, R. N., Zhang, X. D. and Hackett, J. (2009) Metabolic approaches to treatment of melanoma. *Clin Cancer Res* **15,** 6490-6494.

Hirschey, M. D., DeBerardinis, R. J., Diehl, A. M. E. et al. (2015) Dysregulated metabolism contributes to oncogenesis. *Semin Cancer Biol* **35 Suppl,** S129-S150.

Hockel, M. and Vaupel, P. (2001) Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. *J Natl Cancer Inst* **93,** 266-276.

Homma, S., Ishii, Y., Morishima, Y. et al. (2009) Nrf2 enhances cell proliferation and resistance to anticancer drugs in human lung cancer. *Clin Cancer Res* **15,** 3423-3432.

Hu, C. J., Wang, L. Y., Chodosh, L. A., Keith, B. and Simon, M. C. (2003) Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. *Mol Cell Biol* **23,** 9361-9374.

Hucho, F., Randall, D. D., Roche, T. E., Burgett, M. W., Pelley, J. W. and Reed, L. J. (1972) -Keto acid dehydrogenase complexes. XVII. Kinetic and regulatory properties of pyruvate dehydrogenase kinase and pyruvate dehydrogenase phosphatase from bovine kidney and heart. *Arch Biochem Biophys* **151,** 328-340.

Institute of Medicine (U.S.). Standing Committee on the Scientific Evaluation of Dietary Reference Intakes., Institute of Medicine (U.S.). Panel on Folate Other B Vitamins and Choline. and Institute of Medicine (U.S.). Subcommittee on Upper Reference Levels of Nutrients. (1998) *Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B_6, folate, vitamin B_{12}, pantothenic acid, biotin, and choline*. National Academy Press, Washington, D.C.

Isenberg-Grzeda, E., Alici, Y., Hatzoglou, V., Nelson, C. and Breitbart, W. (2016a) Nonalcoholic Thiamine-Related Encephalopathy (Wernicke-Korsakoff Syndrome) Among Inpatients With Cancer: A Series of 18 Cases. *Psychosomatics* **57,** 71-81.

Isenberg-Grzeda, E., Hsu, A. J., Hatzoglou, V., Nelso, C. and Breitbart, W. (2015) Palliative treatment of thiamine-related encephalopathy (Wernicke's encephalopathy) in cancer: A case series and review of the literature. *Palliat Support Care* **13,** 1241-1249.

Isenberg-Grzeda, E., Shen, M. J., Alici, Y., Wills, J., Nelson, C. and Breitbart, W. (2016b) High rate of thiamine deficiency among inpatients with cancer referred for psychiatric consultation: results of a single site prevalence study. *Psychooncology*.

Iyer, N. V., Kotch, L. E., Agani, F. et al. (1998) Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. *Genes Dev* **12,** 149-162.

Jung, E. S., Kwon, O., Lee, S. H., Lee, K. B., Kim, J. H., Yoon, S. H., Kim, G. M., Jeung, H. C. and Rha, S. Y. (2010) Wernicke's encephalopathy in advanced gastric cancer. *Cancer Res Treat* **42,** 77-81.

Kabat, G. C., Miller, A. B., Jain, M. and Rohan, T. E. (2008) Dietary intake of selected B vitamins in relation to risk of major cancers in women. *Br J Cancer* **99,** 816-821.

Kaelin, W. G., Jr. and Ratcliffe, P. J. (2008) Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. *Mol Cell* **30,** 393-402.

Kandoth, C., McLellan, M. D., Vandin, F. et al. (2013) Mutational landscape and significance across 12 major cancer types. *Nature* **502,** 333-339.

Kansanen, E., Kuosmanen, S. M., Leinonen, H. and Levonen, A. L. (2013) The Keap1-Nrf2 pathway: Mechanisms of activation and dysregulation in cancer. *Redox Biol* **1,** 45-49.

Kantor, E. D., Rehm, C. D., Du, M., White, E. and Giovannucci, E. L. (2016) Trends in Dietary Supplement Use Among US Adults From 1999-2012. *JAMA* **316,** 1464-1474.

Kastenhuber, E. R. and Lowe, S. W. (2017) Putting p53 in Context. *Cell* **170,** 1062-1078.

Kasznicki, J., Sliwinska, A. and Drzewoski, J. (2014) Metformin in cancer prevention and therapy. *Ann Transl Med* **2,** 57.

Ke, Q. and Costa, M. (2006) Hypoxia-inducible factor-1 (HIF-1). *Mol Pharmacol* **70,** 1469-1480.

Kim, J., Hopper, C. P., Connell, K. H., Darkhal, P., Zastre, J. A. and Bartlett, M. G. (2016) Development of a novel method for the bioanalysis of benfotiamine and sulbutiamine in cancer cells. *Anal. Methods* **8,** 5596-5603.

Kim, J. E., Jin, D. H., Lee, S. D., Hong, S. W., Shin, J. S., Lee, S. K., Jung, D. J., Kang, J. S. and Lee, W. J. (2008) Vitamin C inhibits p53-induced replicative senescence through suppression of ROS production and p38 MAPK activity. *Int J Mol Med* **22,** 651-655.

Kim, J. W., Tchernyshyov, I., Semenza, G. L. and Dang, C. V. (2006) HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. *Cell Metab* **3,** 177-185.

Kim, K. H. (2013) Wernicke-korsakoff syndrome in primary peritoneal cancer. *Case Rep Oncol* **6,** 593-597.

Kim, S., Rhee, J. K., Yoo, H. J. et al. (2015) Bioinformatic and metabolomic analysis reveals miR-155 regulates thiamine level in breast cancer. *Cancer Lett* **357,** 488-497.

Kim, S. H., Kim, K. H., Yoo, B. C. and Ku, J. L. (2012) Induction of LGR5 by H2O2 treatment is associated with cell proliferation via the JNK signaling pathway in colon cancer cells. *Int J Oncol* **41,** 1744-1750.

Kim, Y. R., Oh, J. E., Kim, M. S., Kang, M. R., Park, S. W., Han, J. Y., Eom, H. S., Yoo, N. J. and Lee, S. H. (2010) Oncogenic NRF2 mutations in squamous cell carcinomas of oesophagus and skin. *J Pathol* **220,** 446-451.

Kizilocak, H., Ozdemir, G. N., Dikme, G., Hasiloglu, Z. I. and Celkan, T. (2017) Wernicke's Encephalopathy in a Child with Acute Lymphoblastic Leukemia. *Turk J Haematol* **34,** 99-100.

Knoechel, T. R., Tucker, A. D., Robinson, C. M., Phillips, C., Taylor, W., Bungay, P. J., Kasten, S. A., Roche, T. E. and Brown, D. G. (2006) Regulatory roles of the N-terminal domain based on crystal structures of human pyruvate dehydrogenase kinase 2 containing physiological and synthetic ligands. *Biochemistry* **45,** 402-415.

Kolobova, E., Tuganova, A., Boulatnikov, I. and Popov, K. M. (2001) Regulation of pyruvate dehydrogenase activity through phosphorylation at multiple sites. *Biochem J* **358,** 69-77.

Kondo, K., Fujiwara, M., Murase, M., Kodera, Y., Akiyama, S., Ito, K. and Takagi, H. (1996) Severe acute metabolic acidosis and Wernicke's encephalopathy following chemotherapy with 5-fluorouracil and cisplatin: case report and review of the literature. *Jpn J Clin Oncol* **26,** 234-236.

Koppenol, W. H., Bounds, P. L. and Dang, C. V. (2011) Otto Warburg's contributions to current concepts of cancer metabolism. *Nat Rev Cancer* **11,** 325-337.

Korotchkina, L. G. and Patel, M. S. (2001) Probing the mechanism of inactivation of human pyruvate dehydrogenase by phosphorylation of three sites. *J Biol Chem* **276,** 5731-5738.

Kotch, L. E., Iyer, N. V., Laughner, E. and Semenza, G. L. (1999) Defective vascularization of HIF-1alpha-null embryos is not associated with VEGF deficiency but with mesenchymal cell death. *Dev Biol* **209,** 254-267.

Krockenberger, M., Honig, A., Rieger, L., Coy, J. F., Sutterlin, M., Kapp, M., Horn, E., Dietl, J. and Kammerer, U. (2007) Transketolase-like 1 expression correlates with subtypes of ovarian cancer and the presence of distant metastases. *Int J Gynecol Cancer* **17,** 101-106.

Kuo, S. H., Debnam, J. M., Fuller, G. N. and de Groot, J. (2009) Wernicke's encephalopathy: an underrecognized and reversible cause of confusional state in cancer patients. *Oncology* **76,** 10-18.

Kwon, J., Lee, S. R., Yang, K. S., Ahn, Y., Kim, Y. J., Stadtman, E. R. and Rhee, S. G. (2004) Reversible oxidation and inactivation of the tumor suppressor PTEN in cells stimulated with peptide growth factors. *Proc Natl Acad Sci U S A* **101,** 16419-16424.

Kwon, K. A., Kwon, H. C., Kim, M. C., Kim, S. H., Oh, S. Y., Lee, S. and Kim, H. J. (2010) A case of 5-fluorouracil induced encephalopathy. *Cancer Res Treat* **42,** 118-120.

Lai, M. C., Chang, C. M. and Sun, H. S. (2016) Hypoxia Induces Autophagy through Translational Up-Regulation of Lysosomal Proteins in Human Colon Cancer Cells. *PLoS One* **11,** e0153627.

Lang, K. J., Kappel, A. and Goodall, G. J. (2002) Hypoxia-inducible factor-1alpha mRNA contains an internal ribosome entry site that allows efficient translation during normoxia and hypoxia. *Mol Biol Cell* **13,** 1792-1801.

Langbein, S., Zerilli, M., Zur Hausen, A. et al. (2006) Expression of transketolase TKTL1 predicts colon and urothelial cancer patient survival: Warburg effect reinterpreted. *Br J Cancer* **94,** 578-585.

Laptenko, O. and Prives, C. (2006) Transcriptional regulation by p53: one protein, many possibilities. *Cell Death Differ* **13,** 951-961.

Lawlor, P. G., Gagnon, B., Mancini, I. L., Pereira, J. L., Hanson, J., Suarez-Almazor, M. E. and Bruera, E. D. (2000) Occurrence, causes, and outcome of delirium in patients with advanced cancer: a prospective study. *Arch Intern Med* **160,** 786-794.

Le Gal, K., Ibrahim, M. X., Wiel, C. et al. (2015) Antioxidants can increase melanoma metastasis in mice. *Sci Transl Med* **7,** 308re308.

Lemos, C., Faria, A., Meireles, M., Martel, F., Monteiro, R. and Calhau, C. (2012) Thiamine is a substrate of organic cation transporters in Caco-2 cells. *Eur J Pharmacol* **682,** 37-42.

Li, J., Chen, K., Wang, F. et al. (2017) Methyl jasmonate leads to necrosis and apoptosis in hepatocellular carcinoma cells via inhibition of glycolysis and represses tumor growth in mice. *Oncotarget* **8,** 45965-45980.

Li, S. H., Shin, D. H., Chun, Y. S., Lee, M. K., Kim, M. S. and Park, J. W. (2008) A novel mode of action of YC-1 in HIF inhibition: stimulation of FIH-dependent p300 dissociation from HIF-1{alpha}. *Molecular cancer therapeutics* **7,** 3729-3738.

Li, Y., Paonessa, J. D. and Zhang, Y. (2012) Mechanism of chemical activation of Nrf2. *PLoS One* **7,** e35122.

Lindhurst, M. J., Fiermonte, G., Song, S. et al. (2006) Knockout of Slc25a19 causes mitochondrial thiamine pyrophosphate depletion, embryonic lethality, CNS malformations, and anemia. *Proc Natl Acad Sci U S A* **103,** 15927-15932.

Liou, G. Y. and Storz, P. (2010) Reactive oxygen species in cancer. *Free Radic Res* **44,** 479-496.

Liu, B. and Qian, S. B. (2014) Translational reprogramming in cellular stress response. *Wiley Interdiscip Rev RNA* **5,** 301-315.

Liu, D., Ke, Z. and Luo, J. (2017) Thiamine Deficiency and Neurodegeneration: the Interplay Among Oxidative Stress, Endoplasmic Reticulum Stress, and Autophagy. *Mol Neurobiol* **54,** 5440-5448.

Liu, L., Wise, D. R., Diehl, J. A. and Simon, M. C. (2008) Hypoxic reactive oxygen species regulate the integrated stress response and cell survival. *J Biol Chem* **283,** 31153-31162.

Liu, S., Monks, N. R., Hanes, J. W., Begley, T. P., Yu, H. and Moscow, J. A. (2010) Sensitivity of breast cancer cell lines to recombinant thiaminase I. *Cancer Chemother Pharmacol* **66,** 171-179.

Liu, X., Montissol, S., Uber, A., Ganley, S., Grossestreuer, A. V., Berg, K., Heydrick, S. and Donnino, M. W. (2018) The Effects of Thiamine on Breast Cancer Cells. *Molecules* **23**.

Longley, D. B., Harkin, D. P. and Johnston, P. G. (2003) 5-fluorouracil: mechanisms of action and clinical strategies. *Nat Rev Cancer* **3,** 330-338.

Lotan, R. (1995) Retinoids and apoptosis: implications for cancer chemoprevention and therapy. *J Natl Cancer Inst* **87,** 1655-1657.

Lu, H., Forbes, R. A. and Verma, A. (2002) Hypoxia-inducible factor 1 activation by aerobic glycolysis implicates the Warburg effect in carcinogenesis. *J Biol Chem* **277,** 23111-23115.

Lu, L., Chen, Y. and Zhu, Y. (2017) The molecular basis of targeting PFKFB3 as a therapeutic strategy against cancer. *Oncotarget* **8,** 62793-62802.

Lu'o'ng, K. V. and Nguyen, L. T. (2013) The role of thiamine in cancer: possible genetic and cellular signaling mechanisms. *Cancer Genomics Proteomics* **10,** 169-185.

Lukienko, P. I., Mel'nichenko, N. G., Zverinskii, I. V. and Zabrodskaya, S. V. (2000) Antioxidant properties of thiamine. *Bull Exp Biol Med* **130,** 874-876.

Lupertz, R., Watjen, W., Kahl, R. and Chovolou, Y. (2010) Dose- and time-dependent effects of doxorubicin on cytotoxicity, cell cycle and apoptotic cell death in human colon cancer cells. *Toxicology* **271,** 115-121.

Macheda, M. L., Rogers, S. and Best, J. D. (2005) Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer. *J Cell Physiol* **202,** 654-662.

Maden, M., Pamuk, G., Celik, Y. and Unlu, E. (2016) Wernicke's Encephalopathy in an Acute Myeloid Leukemia Patient: A Case Study. *Turk J Haematol* **33,** 78-79.

Madhok, B. M., Yeluri, S., Perry, S. L., Hughes, T. A. and Jayne, D. G. (2010) Dichloroacetate induces apoptosis and cell-cycle arrest in colorectal cancer cells. *Br J Cancer* **102,** 1746-1752.

Magda, D., Lecane, P., Prescott, J. et al. (2008) mtDNA depletion confers specific gene expression profiles in human cells grown in culture and in xenograft. *BMC Genomics* **9,** 521.

Majmundar, A. J., Wong, W. J. and Simon, M. C. (2010) Hypoxia-inducible factors and the response to hypoxic stress. *Mol Cell* **40,** 294-309.

Mamede, A. C., Tavares, S. D., Abrantes, A. M., Trindade, J., Maia, J. M. and Botelho, M. F. (2011) The role of vitamins in cancer: a review. *Nutr Cancer* **63,** 479-494.

Manson, J. E. and Bassuk, S. S. (2018) Vitamin and Mineral Supplements: What Clinicians Need to Know. *JAMA* **319,** 859-860.

Martinez-Outschoorn, U. E., Peiris-Pages, M., Pestell, R. G., Sotgia, F. and Lisanti, M. P. (2017) Cancer metabolism: a therapeutic perspective. *Nat Rev Clin Oncol* **14,** 113.

Marullo, R., Werner, E., Degtyareva, N., Moore, B., Altavilla, G., Ramalingam, S. S. and Doetsch, P. W. (2013) Cisplatin induces a mitochondrial-ROS response that contributes to cytotoxicity depending on mitochondrial redox status and bioenergetic functions. *PLoS One* **8,** e81162.

Maschek, G., Savaraj, N., Priebe, W., Braunschweiger, P., Hamilton, K., Tidmarsh, G. F., De Young, L. R. and Lampidis, T. J. (2004) 2-deoxy-D-glucose increases the efficacy of adriamycin and paclitaxel in human osteosarcoma and non-small cell lung cancers in vivo. *Cancer Res* **64,** 31-34.

Matoba, S., Kang, J. G., Patino, W. D., Wragg, A., Boehm, M., Gavrilova, O., Hurley, P. J., Bunz, F. and Hwang, P. M. (2006) p53 regulates mitochondrial respiration. *Science* **312,** 1650-1653.

Mattiske, S., Suetani, R. J., Neilsen, P. M. and Callen, D. F. (2012) The oncogenic role of miR-155 in breast cancer. *Cancer Epidemiol Biomarkers Prev* **21,** 1236-1243.

Mayr, J. A., Freisinger, P., Schlachter, K. et al. (2011) Thiamine pyrophosphokinase deficiency in encephalopathic children with defects in the pyruvate oxidation pathway. *Am J Hum Genet* **89,** 806-812.

McFate, T., Mohyeldin, A., Lu, H. et al. (2008) Pyruvate dehydrogenase complex activity controls metabolic and malignant phenotype in cancer cells. *J Biol Chem* **283,** 22700-22708.

McLure, K. G., Takagi, M. and Kastan, M. B. (2004) NAD+ modulates p53 DNA binding specificity and function. *Mol Cell Biol* **24,** 9958-9967.

Meador, K., Loring, D., Nichols, M., Zamrini, E., Rivner, M., Posas, H., Thompson, E. and Moore, E. (1993) Preliminary findings of high-dose thiamine in dementia of Alzheimer's type. *J Geriatr Psychiatry Neurol* **6,** 222-229.

Michelakis, E. D., Webster, L. and Mackey, J. R. (2008) Dichloroacetate (DCA) as a potential metabolic-targeting therapy for cancer. *Br J Cancer* **99,** 989-994.

Mitschke, L., Parthier, C., Schroder-Tittmann, K., Coy, J., Ludtke, S. and Tittmann, K. (2010) The crystal structure of human transketolase and new insights into its mode of action. *J Biol Chem* **285,** 31559-31570.

Moallem, S. A., Hosseinzadeh, H. and Farahi, S. (2008) A study of acute and chronic anti-nociceptive and anti-inflammatory effects of thiamine in mice. *Iran Biomed J* **12,** 173-178.

Movafagh, S., Crook, S. and Vo, K. (2015) Regulation of hypoxia-inducible factor-1a by reactive oxygen species: new developments in an old debate. *J Cell Biochem* **116,** 696-703.

Muwakkit, S., Al-Aridi, C., Saab, R., Hourani, R., Yazbeck, N. and Abboud, M. (2009) Wernicke's encephalopathy during total parenteral nutrition in a child with acute lymphoblastic leukemia and acute pancreatitis. *Neuropediatrics* **40,** 249-251.

Muz, B., de la Puente, P., Azab, F. and Azab, A. K. (2015) The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy. *Hypoxia (Auckl)* **3,** 83-92.

Nabokina, S. M., Inoue, K., Subramanian, V. S., Valle, J. E., Yuasa, H. and Said, H. M. (2014) Molecular identification and functional characterization of the human colonic thiamine pyrophosphate transporter. *J Biol Chem* **289,** 4405-4416.

Nabokina, S. M., Subramanian, V. S. and Said, H. M. (2016) The human colonic thiamine pyrophosphate transporter (hTPPT) is a glycoprotein and N-linked glycosylation is important for its function. *Biochim Biophys Acta* **1858,** 866-871.

Ng, E. K., Leung, C. P., Shin, V. Y., Wong, C. L., Ma, E. S., Jin, H. C., Chu, K. M. and Kwong, A. (2011) Quantitative analysis and diagnostic significance of methylated SLC19A3 DNA in the plasma of breast and gastric cancer patients. *PLoS One* **6,** e22233.

Nishimura, H., Kawasaki, Y., Nosaka, K., Kaneko, Y. and Iwashima, A. (1991) A constitutive thiamine metabolism mutation, thi80, causing reduced thiamine pyrophosphokinase activity in Saccharomyces cerevisiae. *J Bacteriol* **173,** 2716-2719.

Nolan, K. A., Black, R. S., Sheu, K. F., Langberg, J. and Blass, J. P. (1991) A trial of thiamine in Alzheimer's disease. *Arch Neurol* **48,** 81-83.

Nosaka, K., Onozuka, M., Kakazu, N., Hibi, S., Nishimura, H., Nishino, H. and Abe, T. (2001) Isolation and characterization of a human thiamine pyrophosphokinase cDNA. *Biochim Biophys Acta* **1517,** 293-297.

Okai, Y., Higashi-Okai, K., E, F. S., Konaka, R. and Inoue, M. (2007) Potent radical-scavenging activities of thiamin and thiamin diphosphate. *J Clin Biochem Nutr* **40,** 42-48.

Okamura, S., Ng, C. C., Koyama, K., Takei, Y., Arakawa, H., Monden, M. and Nakamura, Y. (1999) Identification of seven genes regulated by wild-type p53 in a colon cancer cell line carrying a well-controlled wild-type p53 expression system. *Oncol Res* **11,** 281-285.

Olivier, M., Hollstein, M. and Hainaut, P. (2010) TP53 mutations in human cancers: origins, consequences, and clinical use. *Cold Spring Harb Perspect Biol* **2,** a001008.

Omenn, G. S., Goodman, G. E., Thornquist, M. D. et al. (1996) Risk factors for lung cancer and for intervention effects in CARET, the Beta-Carotene and Retinol Efficacy Trial. *J Natl Cancer Inst* **88,** 1550-1559.

Onishi, H., Ishida, M., Kagamu, H., Murayama, Y., Kobayashi, K., Sato, I., Uchida, N. and Akechi, T. (2018a) Wernicke encephalopathy in a lung cancer patient during treatment with nivolumab. *Palliat Support Care***,** 1-3.

Onishi, H., Ishida, M., Tanahashi, I., Takahashi, T., Taji, Y., Ikebuchi, K., Furuya, D. and Akechi, T. (2018b) Wernicke encephalopathy without delirium in patients with cancer. *Palliat Support Care* **16,** 118-121.

Onodera, Y., Teramura, T., Takehara, T., Obora, K., Mori, T. and Fukuda, K. (2017) miR-155 induces ROS generation through downregulation of antioxidation-related genes in mesenchymal stem cells. *Aging Cell* **16,** 1369-1380.

Ooi, A. T. and Gomperts, B. N. (2015) Molecular Pathways: Targeting Cellular Energy Metabolism in Cancer via Inhibition of SLC2A1 and LDHA. *Clin Cancer Res* **21,** 2440-2444.

Osiezagha, K., Ali, S., Freeman, C., Barker, N. C., Jabeen, S., Maitra, S., Olagbemiro, Y., Richie, W. and Bailey, R. K. (2013) Thiamine deficiency and delirium. *Innov Clin Neurosci* **10,** 26-32.

Papandreou, I., Goliasova, T. and Denko, N. C. (2011) Anticancer drugs that target metabolism: Is dichloroacetate the new paradigm? *Int J Cancer* **128,** 1001-1008.

Papila, B., Yildiz, O., Tural, D., Delil, S., Hasiloglu, Z. I., Ayan, F. and Papila, C. (2010) Wernicke's Encephalopathy in Colon Cancer. *Case Rep Oncol* **3,** 362-367.

Pardanani, A., Harrison, C., Cortes, J. E. et al. (2015) Safety and Efficacy of Fedratinib in Patients With Primary or Secondary Myelofibrosis: A Randomized Clinical Trial. *JAMA Oncol* **1,** 643-651.

Patel, M. S., Nemeria, N. S., Furey, W. and Jordan, F. (2014) The pyruvate dehydrogenase complexes: structure-based function and regulation. *J Biol Chem* **289,** 16615-16623.

Patra, K. C. and Hay, N. (2014) The pentose phosphate pathway and cancer. *Trends Biochem Sci* **39,** 347-354.

Perla, D. (1937) *Proc. Soc. Exp. Bio. Med.* **37**.

Portari, G. V., Ovidio, P. P., Deminice, R. and Jordao, A. A., Jr. (2016) Protective effect of treatment with thiamine or benfotiamine on liver oxidative damage in rat model of acute ethanol intoxication. *Life Sci* **162,** 21-24.

Qin, X., Jiang, B. and Zhang, Y. (2016) 4E-BP1, a multifactor regulated multifunctional protein. *Cell Cycle* **15,** 781-786.

Rajgopal, A., Edmondnson, A., Goldman, I. D. and Zhao, R. (2001) SLC19A3 encodes a second thiamine transporter ThTr2. *Biochim Biophys Acta* **1537,** 175-178.

Ratcliffe, P. J. (2007) HIF-1 and HIF-2: working alone or together in hypoxia? *J Clin Invest* **117,** 862-865.

Rathmell, J. C., Fox, C. J., Plas, D. R., Hammerman, P. S., Cinalli, R. M. and Thompson, C. B. (2003) Akt-directed glucose metabolism can prevent Bax conformation change and promote growth factor-independent survival. *Mol Cell Biol* **23,** 7315-7328.

Rhee, S. G. (2006) Cell signaling. H2O2, a necessary evil for cell signaling. *Science* **312,** 1882-1883.

Ricciardelli, C., Lokman, N. A., Cheruvu, S., Tan, I. A., Ween, M. P., Pyragius, C. E., Ruszkiewicz, A., Hoffmann, P. and Oehler, M. K. (2015) Transketolase is upregulated in metastatic peritoneal implants and promotes ovarian cancer cell proliferation. *Clin Exp Metastasis* **32,** 441-455.

Riganti, C., Gazzano, E., Polimeni, M., Aldieri, E. and Ghigo, D. (2012) The pentose phosphate pathway: an antioxidant defense and a crossroad in tumor cell fate. *Free Radic Biol Med* **53,** 421-436.

Rufa, A., Rosini, F., Cerase, A., Giannini, F., Pretegiani, E., Buccoliero, R., Dotti, M. T. and Federico, A. (2011) Wernicke encephalopathy after gastrointestinal surgery for cancer: causes of diagnostic failure or delay. *Int J Neurosci* **121,** 201-208.

Salmeen, A., Andersen, J. N., Myers, M. P., Meng, T. C., Hinks, J. A., Tonks, N. K. and Barford, D. (2003) Redox regulation of protein tyrosine phosphatase 1B involves a sulphenyl-amide intermediate. *Nature* **423,** 769-773.

Sandhu, S. K., Volinia, S., Costinean, S. et al. (2012) miR-155 targets histone deacetylase 4 (HDAC4) and impairs transcriptional activity of B-cell lymphoma 6 (BCL6) in the Emu-miR-155 transgenic mouse model. *Proc Natl Acad Sci U S A* **109,** 20047-20052.

Saunier, E., Benelli, C. and Bortoli, S. (2016) The pyruvate dehydrogenase complex in cancer: An old metabolic gatekeeper regulated by new pathways and pharmacological agents. *Int J Cancer* **138,** 809-817.

Sayin, V. I., Ibrahim, M. X., Larsson, E., Nilsson, J. A., Lindahl, P. and Bergo, M. O. (2014) Antioxidants accelerate lung cancer progression in mice. *Sci Transl Med* **6,** 221ra215.

Schenk, G., Duggleby, R. G. and Nixon, P. F. (1998) Properties and functions of the thiamin diphosphate dependent enzyme transketolase. *Int J Biochem Cell Biol* **30,** 1297-1318.

Schneider-Poetsch, T., Ju, J., Eyler, D. E., Dang, Y., Bhat, S., Merrick, W. C., Green, R., Shen, B. and Liu, J. O. (2010) Inhibition of eukaryotic translation elongation by cycloheximide and lactimidomycin. *Nat Chem Biol* **6,** 209-217.

Schwartzenberg-Bar-Yoseph, F., Armoni, M. and Karnieli, E. (2004) The tumor suppressor p53 down-regulates glucose transporters GLUT1 and GLUT4 gene expression. *Cancer Res* **64,** 2627-2633.

Schwingshackl, L., Boeing, H., Stelmach-Mardas, M., Gottschald, M., Dietrich, S., Hoffmann, G. and Chaimani, A. (2017) Dietary Supplements and Risk of Cause-Specific Death, Cardiovascular Disease, and Cancer: A Systematic Review and Meta-Analysis of Primary Prevention Trials. *Adv Nutr* **8,** 27-39.

Sechi, G. and Serra, A. (2007) Wernicke's encephalopathy: new clinical settings and recent advances in diagnosis and management. *Lancet Neurol* **6,** 442-455.

Seligmann, H., Levi, R., Konijn, A. M. and Prokocimer, M. (2001) Thiamine deficiency in patients with B-chronic lymphocytic leukaemia: a pilot study. *Postgrad Med J* **77,** 582-585.

Semenza, G. L. (2000a) HIF-1: mediator of physiological and pathophysiological responses to hypoxia. *J Appl Physiol (1985)* **88,** 1474-1480.

Semenza, G. L. (2000b) Surviving ischemia: adaptive responses mediated by hypoxia-inducible factor 1. *J Clin Invest* **106,** 809-812.

Semenza, G. L. (2002) HIF-1 and tumor progression: pathophysiology and therapeutics. *Trends Mol Med* **8,** S62-67.

Semenza, G. L. (2007) Hypoxia-inducible factor 1 (HIF-1) pathway. *Sci STKE* **2007,** cm8.

Semenza, G. L. (2010) HIF-1: upstream and downstream of cancer metabolism. *Curr Opin Genet Dev* **20,** 51-56.

Semenza, G. L. (2013) HIF-1 mediates metabolic responses to intratumoral hypoxia and oncogenic mutations. *J Clin Invest* **123,** 3664-3671.

Semenza, G. L., Jiang, B. H., Leung, S. W., Passantino, R., Concordet, J. P., Maire, P. and Giallongo, A. (1996) Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. *J Biol Chem* **271,** 32529-32537.

Seo, J. H., Kim, J. H., Sun, S., Won, H. S., Park, J. W. and Ko, Y. H. (2017) Wernicke encephalopathy as initial presentation of lymphoma. *Korean J Intern Med* **32,** 1112-1114.

Seth, D. and Rudolph, J. (2006) Redox regulation of MAP kinase phosphatase 3. *Biochemistry* **45,** 8476-8487.

Shahrzad, S., Quayle, L., Stone, C., Plumb, C., Shirasawa, S., Rak, J. W. and Coomber, B. L. (2005) Ischemia-induced K-ras mutations in human colorectal cancer cells: role of microenvironmental regulation of MSH2 expression. *Cancer Res* **65,** 8134-8141.

Shen, Y. C., Ou, D. L., Hsu, C., Lin, K. L., Chang, C. Y., Lin, C. Y., Liu, S. H. and Cheng, A. L. (2013) Activating oxidative phosphorylation by a pyruvate dehydrogenase kinase inhibitor overcomes sorafenib resistance of hepatocellular carcinoma. *Br J Cancer* **108,** 72-81.

Sheta, E. A., Trout, H., Gildea, J. J., Harding, M. A. and Theodorescu, D. (2001) Cell density mediated pericellular hypoxia leads to induction of HIF-1alpha via nitric oxide and Ras/MAP kinase mediated signaling pathways. *Oncogene* **20,** 7624-7634.

Shibata, T., Ohta, T., Tong, K. I., Kokubu, A., Odogawa, R., Tsuta, K., Asamura, H., Yamamoto, M. and Hirohashi, S. (2008) Cancer related mutations in NRF2 impair its recognition by Keap1-Cul3 E3 ligase and promote malignancy. *Proc Natl Acad Sci U S A* **105,** 13568-13573.

Singh, A., Boldin-Adamsky, S., Thimmulappa, R. K. et al. (2008) RNAi-mediated silencing of nuclear factor erythroid-2-related factor 2 gene expression in non-small cell lung cancer inhibits tumor growth and increases efficacy of chemotherapy. *Cancer Res* **68,** 7975-7984.

Singleton, C. K. and Martin, P. R. (2001) Molecular mechanisms of thiamine utilization. *Curr Mol Med* **1,** 197-207.

Smithline, H. A., Donnino, M. and Greenblatt, D. J. (2012) Pharmacokinetics of high-dose oral thiamine hydrochloride in healthy subjects. *BMC Clin Pharmacol* **12,** 4.

Spriggs, K. A., Bushell, M. and Willis, A. E. (2010) Translational regulation of gene expression during conditions of cell stress. *Mol Cell* **40,** 228-237.

Stacpoole, P. W. (2017) Therapeutic Targeting of the Pyruvate Dehydrogenase Complex/Pyruvate Dehydrogenase Kinase (PDC/PDK) Axis in Cancer. *J Natl Cancer Inst* **109**.

Stiefel, F., Fainsinger, R. and Bruera, E. (1992) Acute confusional states in patients with advanced cancer. *J Pain Symptom Manage* **7,** 94-98.

Stracke, H., Gaus, W., Achenbach, U., Federlin, K. and Bretzel, R. G. (2008) Benfotiamine in diabetic polyneuropathy (BENDIP): results of a randomised, double blind, placebo-controlled clinical study. *Exp Clin Endocrinol Diabetes* **116,** 600-605.

Subarsky, P. and Hill, R. P. (2003) The hypoxic tumour microenvironment and metastatic progression. *Clin Exp Metastasis* **20,** 237-250.

Subramanian, V. S., Marchant, J. S., Parker, I. and Said, H. M. (2003) Cell biology of the human thiamine transporter-1 (hTHTR1). Intracellular trafficking and membrane targeting mechanisms. *J Biol Chem* **278,** 3976-3984.

Sugimori, N., Espinoza, J. L., Trung, L. Q., Takami, A., Kondo, Y., An, D. T., Sasaki, M., Wakayama, T. and Nakao, S. (2015) Paraptosis cell death induction by the thiamine analog benfotiamine in leukemia cells. *PLoS One* **10,** e0120709.

Sun, H. L., Liu, Y. N., Huang, Y. T., Pan, S. L., Huang, D. Y., Guh, J. H., Lee, F. Y., Kuo, S. C. and Teng, C. M. (2007) YC-1 inhibits HIF-1 expression in prostate cancer cells: contribution of Akt/NF-kappaB signaling to HIF-1alpha accumulation during hypoxia. *Oncogene* **26,** 3941-3951.

Sweet, R., Paul, A. and Zastre, J. (2010) Hypoxia induced upregulation and function of the thiamine transporter, SLC19A3 in a breast cancer cell line. *Cancer Biol Ther* **10,** 1101-1111.

Sweet, R. L. and Zastre, J. A. (2013) HIF1-alpha-mediated gene expression induced by vitamin B1 deficiency. *Int J Vitam Nutr Res* **83,** 188-197.

Tallaksen, C. M., Sande, A., Bohmer, T., Bell, H. and Karlsen, J. (1993) Kinetics of thiamin and thiamin phosphate esters in human blood, plasma and urine after 50 mg intravenously or orally. *Eur J Clin Pharmacol* **44,** 73-78.

Tarrado-Castellarnau, M., de Atauri, P. and Cascante, M. (2016) Oncogenic regulation of tumor metabolic reprogramming. *Oncotarget* **7,** 62726-62753.

Thompson, C. B. (2011) Rethinking the regulation of cellular metabolism. *Cold Spring Harb Symp Quant Biol* **76,** 23-29.

Tiwana, G. S., Prevo, R., Buffa, F. M. et al. (2015) Identification of vitamin B1 metabolism as a tumor-specific radiosensitizing pathway using a high-throughput colony formation screen. *Oncotarget* **6,** 5978-5989.

Tong, C. W. S., Wu, M., Cho, W. C. S. and To, K. K. W. (2018) Recent Advances in the Treatment of Breast Cancer. *Front Oncol* **8,** 227.

Trachootham, D., Zhou, Y., Zhang, H. et al. (2006) Selective killing of oncogenically transformed cells through a ROS-mediated mechanism by beta-phenylethyl isothiocyanate. *Cancer Cell* **10,** 241-252.

Trebukhina, R. V., Ostrovsky, Y. M., Shapot, V. S., Mikhaltsevich, G. N. and Tumanov, V. N. (1984) Turnover of [14C]thiamin and activities of thiamin pyrophosphate-dependent enzymes in tissues of mice with Ehrlich ascites carcinoma. *Nutr Cancer* **6,** 260-273.

Trebukhina, R. V., Ostrovsky Yu, M., Shapot, V. S., Petushok, V. G., Velichko, M. G., Tumanov, V. N. and Mikhaltsevich, G. N. (1982) Thiamine metabolism in the liver of mice with Ehrlich ascites carcinoma. *Neoplasma* **29,** 257-268.

Trovero, F., Gobbi, M., Weil-Fuggaza, J., Besson, M. J., Brochet, D. and Pirot, S. (2000) Evidence for a modulatory effect of sulbutiamine on glutamatergic and dopaminergic cortical transmissions in the rat brain. *Neurosci Lett* **292,** 49-53.

Tsao, S. M., Yin, M. C. and Liu, W. H. (2007) Oxidant stress and B vitamins status in patients with non-small cell lung cancer. *Nutr Cancer* **59,** 8-13.

Tseng, C. W., Kuo, W. H., Chan, S. H., Chan, H. L., Chang, K. J. and Wang, L. H. (2018) Transketolase Regulates the Metabolic Switch to Control Breast Cancer Cell Metastasis via the alpha-Ketoglutarate Signaling Pathway. *Cancer Res* **78,** 2799-2812.

Turan, M. I., Cayir, A., Cetin, N., Suleyman, H., Siltelioglu Turan, I. and Tan, H. (2014) An investigation of the effect of thiamine pyrophosphate on cisplatin-induced oxidative stress and DNA damage in rat brain tissue compared with thiamine: thiamine and thiamine pyrophosphate effects on cisplatin neurotoxicity. *Hum Exp Toxicol* **33,** 14-21.

Turan, M. I., Siltelioglu Turan, I., Mammadov, R., Altinkaynak, K. and Kisaoglu, A. (2013) The effect of thiamine and thiamine pyrophosphate on oxidative liver damage induced in rats with cisplatin. *Biomed Res Int* **2013,** 783809.

Turashvili, G. and Brogi, E. (2017) Tumor Heterogeneity in Breast Cancer. *Front Med (Lausanne)* **4,** 227.

Ulusakarya, A., Vantelon, J. M., Munck, J. N., Fenaux, P. and Rerat, K. (1999) Thiamine deficiency in a patient receiving chemotherapy for acute myeloblastic leukemia. *Am J Hematol* **61,** 155-156.

van Zaanen, H. C. and van der Lelie, J. (1992) Thiamine deficiency in hematologic malignant tumors. *Cancer* **69,** 1710-1713.

Vander Heiden, M. G., Cantley, L. C. and Thompson, C. B. (2009) Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* **324,** 1029-1033.

Vatrinet, R., Leone, G., De Luise, M., Girolimetti, G., Vidone, M., Gasparre, G. and Porcelli, A. M. (2017) The alpha-ketoglutarate dehydrogenase complex in cancer metabolic plasticity. *Cancer Metab* **5,** 3.

Verrax, J., Beck, R., Dejeans, N. et al. (2011) Redox-active quinones and ascorbate: an innovative cancer therapy that exploits the vulnerability of cancer cells to oxidative stress. *Anticancer Agents Med Chem* **11,** 213-221.

Verrax, J. and Calderon, P. B. (2009) Pharmacologic concentrations of ascorbate are achieved by parenteral administration and exhibit antitumoral effects. *Free Radic Biol Med* **47,** 32-40.

Volker, H. U., Hagemann, C., Coy, J. et al. (2008) Expression of transketolase-like 1 and activation of Akt in grade IV glioblastomas compared with grades II and III astrocytic gliomas. *Am J Clin Pathol* **130,** 50-57.

Volvert, M.-L., Seyen, S., Piette, M., Evrard, B., Gangolf, M., Plumier, J.-C. and Bettendorff, L. (2008a) Benfotiamine, a synthetic S-acyl thiamine derivative, has different mechanisms of action and a different pharmacological profile than lipid-soluble thiamine disulfide derivatives. *BMC pharmacology* **8,** 10.

Volvert, M. L., Seyen, S., Piette, M., Evrard, B., Gangolf, M., Plumier, J. C. and Bettendorff, L. (2008b) Benfotiamine, a synthetic S-acyl thiamine derivative, has different mechanisms of action and a different pharmacological profile than lipid-soluble thiamine disulfide derivatives. *BMC Pharmacol* **8,** 10.

Vousden, K. H. and Ryan, K. M. (2009) p53 and metabolism. *Nat Rev Cancer* **9,** 691-700.

Wada, T., Takagi, H., Minakami, H., Hamanaka, W., Okamoto, K., Ito, A. and Sahashi, Y. (1961) A new thiamine derivative, S-benzoylthiamine O-monophosphate. *Science* **134,** 195-196.

Wallace, D. C. (2012) Mitochondria and cancer. *Nat Rev Cancer* **12,** 685-698.

Wan, J., Xia, L., Xu, W. and Lu, N. (2016) Expression and Function of miR-155 in Diseases of the Gastrointestinal Tract. *Int J Mol Sci* **17**.

Wang, H., Wu, J., Meng, X., Ying, X., Zuo, Y., Liu, R., Pan, Z., Kang, T. and Huang, W. (2011) MicroRNA-342 inhibits colorectal cancer cell proliferation and invasion by directly targeting DNA methyltransferase 1. *Carcinogenesis* **32,** 1033-1042.

Wang, J., Lin, D., Peng, H., Huang, Y., Huang, J. and Gu, J. (2013) Cancer-derived immunoglobulin G promotes tumor cell growth and proliferation through inducing production of reactive oxygen species. *Cell Death Dis* **4,** e945.

Warburg, O., Wind, F. and Negelein, E. (1927) The Metabolism of Tumors in the Body. *J Gen Physiol* **8,** 519-530.

Ward, P. S. and Thompson, C. B. (2012) Signaling in control of cell growth and metabolism. *Cold Spring Harb Perspect Biol* **4,** a006783.

Watson, R. R. (2003) *Functional foods & nutraceuticals in cancer prevention*. Iowa State Press, Ames, Iowa.

Weber, W. and Kewitz, H. (1985) Determination of thiamine in human plasma and its pharmacokinetics. *Eur J Clin Pharmacol* **28,** 213-219.

Whitfield, K. C., Bourassa, M. W., Adamolekun, B. et al. (2018) Thiamine deficiency disorders: diagnosis, prevalence, and a roadmap for global control programs. *Ann N Y Acad Sci*.

Wilson, T. R., Johnston, P. G. and Longley, D. B. (2009) Anti-apoptotic mechanisms of drug resistance in cancer. *Curr Cancer Drug Targets* **9,** 307-319.

Wong, J. Y., Huggins, G. S., Debidda, M., Munshi, N. C. and De Vivo, I. (2008) Dichloroacetate induces apoptosis in endometrial cancer cells. *Gynecol Oncol* **109,** 394-402.

Woolbright, B. L., Choudhary, D., Mikhalyuk, A., Trammel, C., Shanmugam, S., Abbott, E., Pilbeam, C. C. and Taylor, J. A., 3rd (2018) The Role of Pyruvate Dehydrogenase Kinase-4 (PDK4) in Bladder Cancer and Chemoresistance. *Molecular cancer therapeutics*.

Xu, I. M., Lai, R. K., Lin, S. H. et al. (2016) Transketolase counteracts oxidative stress to drive cancer development. *Proc Natl Acad Sci U S A* **113,** E725-734.

Yadav, N., Kumar, S., Marlowe, T. et al. (2015) Oxidative phosphorylation-dependent regulation of cancer cell apoptosis in response to anticancer agents. *Cell Death Dis* **6,** e1969.

Yagi, T. (2012) *B vitamins and folate: chemistry, analysis, function and effects,* Vol. 4. Royal Society of Chemistry.

Yanaihara, N., Caplen, N., Bowman, E. et al. (2006) Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. *Cancer Cell* **9,** 189-198.

Yang, Z., Ge, J., Yin, W., Shen, H., Liu, H. and Guo, Y. (2004) [The expression of p53, MDM2 and Ref1 gene in cultured retina neurons of SD rats treated with vitamin B1 and/or elevated pressure]. *Yan Ke Xue Bao* **20,** 259-263.

Yates, A. A., Schlicker, S. A. and Suitor, C. W. (1998) Dietary Reference Intakes: the new basis for recommendations for calcium and related nutrients, B vitamins, and choline. *J Am Diet Assoc* **98,** 699-706.

Yilmaz, I., Demiryilmaz, I., Turan, M. I., Cetin, N., Gul, M. A. and Suleyman, H. (2015) The effects of thiamine and thiamine pyrophosphate on alcohol-induced hepatic damage biomarkers in rats. *Eur Rev Med Pharmacol Sci* **19,** 664-670.

Yoshioka, K. (1984) Some properties of the thiamine uptake system in isolated rat hepatocytes. *Biochim Biophys Acta* **778,** 201-209.

Zahreddine, H. and Borden, K. L. (2013) Mechanisms and insights into drug resistance in cancer. *Front Pharmacol* **4,** 28.

Zastre, J. A., Hanberry, B. S., Sweet, R. L., McGinnis, A. C., Venuti, K. R., Bartlett, M. G. and Govindarajan, R. (2013a) Up-regulation of vitamin B1 homeostasis genes in breast cancer. *J Nutr Biochem* **24,** 1616-1624.

Zastre, J. A., Sweet, R. L., Hanberry, B. S. and Ye, S. (2013b) Linking vitamin B1 with cancer cell metabolism. *Cancer Metab* **1,** 16.

Zera, K., Sweet, R. and Zastre, J. (2016) Role of HIF-1alpha in the hypoxia inducible expression of the thiamine transporter, SLC19A3. *Gene* **595,** 212-220.

Zera, K. and Zastre, J. (2018) Stabilization of the hypoxia-inducible transcription Factor-1 alpha (HIF-1alpha) in thiamine deficiency is mediated by pyruvate accumulation. *Toxicol Appl Pharmacol* **355,** 180-188.

Zhang, Q., Zhang, Y., Diamond, S. et al. (2014) The Janus kinase 2 inhibitor fedratinib inhibits thiamine uptake: a putative mechanism for the onset of Wernicke's encephalopathy. *Drug Metab Dispos* **42,** 1656-1662.

Zhang, S., Yang, J. H., Guo, C. K. and Cai, P. C. (2007) Gene silencing of TKTL1 by RNAi inhibits cell proliferation in human hepatoma cells. *Cancer Lett* **253,** 108-114.

Zhang, W., Zhang, S. L., Hu, X. and Tam, K. Y. (2015) Targeting Tumor Metabolism for Cancer Treatment: Is Pyruvate Dehydrogenase Kinases (PDKs) a Viable Anticancer Target? *Int J Biol Sci* **11,** 1390-1400.

Zhao, J. and Zhong, C. J. (2009) A review on research progress of transketolase. *Neurosci Bull* **25,** 94-99.

Zhao, R., Gao, F. and Goldman, I. D. (2001a) Molecular cloning of human thiamin pyrophosphokinase. *Biochim Biophys Acta* **1517,** 320-322.

Zhao, R., Gao, F. and Goldman, I. D. (2002) Reduced folate carrier transports thiamine monophosphate: an alternative route for thiamine delivery into mammalian cells. *Am J Physiol Cell Physiol* **282,** C1512-1517.

Zhao, R., Gao, F., Wang, Y., Diaz, G. A., Gelb, B. D. and Goldman, I. D. (2001b) Impact of the reduced folate carrier on the accumulation of active thiamin metabolites in murine leukemia cells. *J Biol Chem* **276,** 1114-1118.

Zheng, H. C. (2017) The molecular mechanisms of chemoresistance in cancers. *Oncotarget* **8,** 59950-59964.

Zhong, H., Chiles, K., Feldser, D., Laughner, E., Hanrahan, C., Georgescu, M. M., Simons, J. W. and Semenza, G. L. (2000) Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. *Cancer Res* **60,** 1541-1545.