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TRPM2 INHIBITION INCREASES OXIDATIVE STRESS AND REDUCES TUMOR GROWTH IN NEUROBLASTOMA THROUGH MODULATION OF GSH

A Thesis in

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by

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Abstract

The transient receptor potential melastatin 2 (TRPM2) ion channel modulates cell signaling pathways in response to oxidative stress and is highly expressed in neuroblastoma and other cancers. Previous *in vitro* experiments generated from neuroblastoma cells in which TRPM2 was depleted with CRISPR/Cas9 technology have demonstrated increased levels of reactive oxygen species (ROS) and an increase in sensitivity to doxorubicin. Xenograft experiments with these cell lines have exhibited an increased sensitivity to doxorubicin and inhibition of tumor growth. Here, the NF-E2 related factor 2 (Nrf2) signaling cascade, which regulates expression of proteins involved in the antioxidant response, was suppressed by TRPM2 depletion. Upstream inhibitors of Nrf2 activity showed increased expression in TRPM2 depleted cells. TRPM2 depleted SH-SH5Y neuroblastoma cells also demonstrated reduced glutathione (GSH) concentrations after doxorubicin treatment. These findings demonstrate the importance of TRPM2 in modulating cell signaling pathways involved in antioxidant production in response to increased ROS. Thus, TRPM2 is a potential target for inhibition as a therapy to reduce tumor growth and increase susceptibility to chemotherapeutic agents through modulation of ROS.

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List of Abbreviations

ADPR	Adenosine diphosphate ribose
ALDH1L2	Aldehyde dehydrogenase 1 family, member L2
AP-1	Activator protein 1
ARE	Antioxidant response element
BNIP3	BCL2/adenovirus E1B 19 kDa protein interacting protein 3
BTB	BR-C, ttk and bab
bZIP	Basic leucine zipper
cADPR	Cyclic ADP ribose
CaM	Calmodulin
CCR	CC chemokine receptors
C_P	Peroxidatic cysteine
C_R	Resolving cysteine
CREB	cAMP response element-binding protein
CRL	Cullin-RING ligase
CTR	C-terminal region
Cul3	Cullin-3
DGR	Double glycine repeat
ECH	Erythroid cell-derived protein with CNC homology
ERK	Extracellular signal-regulated kinase
FOXO3a	Forkhead box O3a
GCL	Glutamate cysteine ligase
GCLC	Glutamate cysteine ligase catalytic subunit

GCLM	Glutamate cysteine ligase modifier subunit
GR	Glutathione reductase
GSH	Glutathione
GSK-3	Glycogen synthase kinase 3
GSS	Glutathione synthetase
GSSG	Glutathione disulfide
GST	Glutathione *S*-transferase
GSTP	Glutathione *S*-transferase pi
HIF	Hypoxia-inducible factor
IVR	Intervening region
JNK	c-Jun N-terminal kinase
Keap1	Kelch-like ECH-associated protein 1
LDHA	Lactate dehydrogenase A
MAPK	Mitogen-activated protein kinase
MTHFD1	Methylenetetrahydrofolate dehydrogenase 1
NADP	Nicotinamide adenine dinucleotide phosphate
NDUFA4L2	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4-like 2
Neh	Nrf2-ECH homology
NF-κB	Nuclear factor kappa-light-chain enhancer of activated B cells
Nrf2	NF-E2 related factor 2
NTR	N-terminal region
PARG	Poly (ADP-ribose) glycohydrolase
PARP-1	Poly [ADP-ribose] polymerase 1

PRX	Peroxiredoxin
RARα	Retinoic acid receptor alpha
RBX1	RING-box protein 1
RING	Really interesting new gene finger domain
ROS	Reactive oxygen species
RXRα	Retinoid X receptor alpha
SCF	Skp1, Cul3, RBX1, and F-box E3 ubiquitin ligase complex
TNFα	Tumor necrosis factor alpha
TRP	Transient receptor potential channel
TRPM	Transient receptor potential channel melastatin
TRPM2	Transient receptor potential channel melastatin 2
TXN	Thioredoxin
TxrR	Thioredoxin reductase
VEGF	Vascular endothelial growth factor
β-TrCP	F-box/WD repeat-containing protein 1A
γ-GC	*Gamma*-L-glutamyl-L-cysteine

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Chapter 1: Introduction

1.1 Neuroblastoma

1.1.1 Neuroblastoma Causes

Neuroblastoma is the most common extracranial solid tumor found in pediatric patients. Approximately 7% of pediatric malignancies are neuroblastomas. Neuroblastoma originates from the sympaticoadrenal neural crest lineage and can form tumors anywhere in the sympathetic nervous system with common sites including abdomen, neck, chest, and pelvis. Nearly 65% of primary tumors arise in the abdomen with the majority of those tumors being found in the adrenal medulla (12).

1.1.2 Neuroblastoma Presentation

There are a variety of signs and symptoms associated with neuroblastoma with primary tumor site and metastatic disease affecting the presentation. These various presentations are generally organized into three main clinical scenarios: localized tumors, metastatic disease, and 4S disease (12).

Approximately 40% of neuroblastoma patients present with localized tumors. These tumors are highly variable from intra-adrenal masses discovered via prenatal ultrasound to extensive tumor networks along the sympathetic chain ganglia. Paraspinal tumors, which occur in between 5-15% of patients, can arise in the thoracic, abdominal, and pelvic regions. These tumors have the potential to extend into the neural foramina, which can lead to symptoms of nerve root and spinal cord compression. Such tumors are often associated with neurological

signs such as motor weakness, pain, and sensory loss which are all related to spinal cord impingement (12).

Patients with localized tumors may present with vasoactive intestinal peptide secretion and opsoclonus-myoclonus syndrome. Secretion of vasoactive intestinal peptide results in watery diarrhea and failure to thrive. Opsoclonus-myoclonus syndrome is seen in between 2-4% of neuroblastoma patients and presents with rapid eye movement, ataxia, and irregular muscle movement (12).

Approximately 50% of neuroblastoma patients present with hematogeneous metastasis. Distant metastasis to the cortical bone, bone marrow, liver, and non-contiguous lymph nodes is distinct from local tumor spread adjacent to the primary tumor. Patients with metastatic neuroblastoma often present with high tumor burdens and usually are very ill. Metastasis often occurs about the bony orbit which can lead to both periorbital ecchymoses and proptosis, making these signs classic to the presentation of metastatic neuroblastoma. Metastasis to the bone and bone marrow can present as bone pain, limping, or general irritability. Compromise of the renal vasculature can result in renin-mediated hypertension (12).

4S disease occurs in approximately 10% of neuroblastoma patients and is defined as occurring in children less than 12 months of age. These affected infants have localized primary tumors with associated metastases in three specific sites, liver, skin, or bone marrow that usually regress spontaneously. Infants who are younger than two months may present with intrahepatic expansion of neuroblastoma that can result in respiratory compromise (12).

Neuroblastoma is diagnosed based upon the histolopathological characteristics of primary tumor tissue, or the presence of tumor cells, usually in bone marrow, along with increased catecholamines. Increased concentrations of catecholamines in the urine are often used to reinforce a neuroblastoma diagnosis (12).

1.1.3 Neuroblastoma Treatments

Neuroblastoma treatments include surgery, chemotherapy, radiation, and even observation in select circumstances. The appropriate treatment is selected based upon multiple risk factors including age, extent of disease at diagnosis and biological variables including prognastic N-myc expression and ploidy (12).

Localized tumors sometimes only require surgical treatment. Recurrences are generally manageable with localized tumors sometimes only requiring surgery (12).

Neuroblastoma presenting with more advanced stages and metastatic tumors requires more intensive treatment than localized tumors. While the majority of patients fall into a low-risk category with a high probability of survival, high-risk cases remain a substantial challenge to treat. Generally high-risk patients undergo intensive induction therapy with common chemotherapy agents including cisplatin, etoposide, doxorubicin, cyclophosphamide, and vincristine. In metastatic disease (Stage IV), outcome has been poor but with improved chemotherapy regimens and biologically-based therapies, survival has recently increased to 50% of patients or greater (12).

1.2 TRPM2

1.2.1 TRP and TRPM Channels

The transient receptor potential (TRP) superfamily consists of a group of calcium-permeable cation channels expressed in mammalian cells. Members of the TRP superfamily demonstrate a wide variety of cation selectivities and activation mechanisms. This superfamily is divided into six protein subfamilies: C (canonical), V (vanilloid receptor), M (melastatin), A (ANKTM), P (polycystin), and ML (mucolupin) (17).

TRP proteins function as either homotetramers or heterotetramers and are composed of six transmembrane domains with a pore domain between the fifth and the sixth transmembrane domains. The pore itself has a tetrameric structure through which various ionic species may pass with differing degrees of selectivity. TRP channels include additional interaction motifs and regulatory domains that assist in the regulation of the entire protein. Some of these factors which influence channel opening include extracellular signaling, secondary messengers, channel subunit assembly, and macromolecular complex formation (17, 18).

The TRPM subfamily of TRP channels is named after its first described member TRPM1 or melastatin. The members of this subfamily share a region of high coiled coil character (CCR) in the C-terminus. However, these channels demonstrate an extensive variability in other aspects of the C-terminus which serve to distinguish the individual members from one another (17, 18).

1.2.2 TRPM2

TRPM2 was the second member of the TRPM subfamily to be cloned. TRPM2 channels are permeable to Na^+, K^+, and Ca^{2+}. Activation of the TRPM2 channel occurs when ADP-ribose (ADPR) binds to the TRPM2 COOH-terminal NUDT9-H domain, which is an ADPR hydrolase (7). ADPR arises from mitochondria or activation of poly-ADPR polymerase (PARP). PARP-1 catalyzes the poly (ADP-ribosylation) of nuclear proteins (28). These are eventually hydrolyzed into free ADPR by poly ADP-ribose glycohydrolase (PARG) (17, 18).

Intracellular Ca^{2+} also serves as a positive regulator of TRPM2 and in the TRPM2-L, TRPM2-ΔN, and TRPM2-ΔC isoforms, intracellular Ca^{2+} is sufficient for TRPM2 activation. Calmodulin (CaM) serves as a sensor of intracellular Ca^{2+} where influx of Ca^{2+} into the cell enhances the interaction of CaM with the IQ-like motif in the N-terminus of TRPM2 thus creating a positive feedback loop of channel activation for both intracellular Ca^{2+} and ADPR/Ca^{2+} mediated pathways (7, 17, 18, 25).

Extracellular signals such as oxidative stress, TNFα, amyloid β-peptide, and concavalin A are known to activate TRPM2 through stimulation of ADPR production (25, 28). TRPM2 can also be activated by cyclic ADPR (cADPR) alone at high concentrations, which also amplifies ADPR effects at lower concentrations (7, 17, 18).

1.2.3 TRPM2 Isoforms

The TRPM2-L (full-length or wild-type), TRPM2-S (short), TRPM2-ΔN, TRPM2-ΔC, and TRPM2-TE (tumor-enriched) isoforms comprise the five physiological variants of the TRPM2 that have been identified. The specific mechanisms which control the splicing of the different TRPM2 isoforms are unknown. TRPM2-S is missing the last four transmembrane domains and the C-terminus, resulting in the loss of its calcium pore. These features cause TRPM2-S to behave as a dominant negative inhibitor of TRPM2-L via suppression of Ca^{2+} influx (7, 25). Although TRPM2-L and TRPM2-S are known to directly interact, the specific mechanism through which TRPM2-S suppresses TRPM2-L is unknown (27). TRPM2-ΔN is characterized by a deletion of amino acids 538-557 at its N-terminus. Its failure to respond to H_2O_2 and ADPR indicate that TRPM2-ΔN may disrupt channel gating. TRPM2-ΔC is characterized by a deletion of amino acids 1292-1325 at its C-terminus). It has a decreased affinity for ADPR but remains responsive to H_2O_2. This phenomenon suggests that oxidative stress may activate TRPM2 via pathways independent for ADPR (17). TRPM2-TE is a C-terminal fragment which is highly expressed in tumor cells such as melanoma and in the lung. When expressed with TRPM2-L, TRPM2-TE may protect cells from apoptosis (20).

1.3 Oxidative Stress

1.3.1 ROS

Oxidative stress is a physiological process that results from an imbalance between oxidants and antioxidants. Severe and prolonged oxidative stress can result in tissue damage and plays a significant role in the physiological processes associated with aging, cancer, neurodegenerative disorders, diabetes mellitus, atherosclerosis, ischemia-reperfusion injury (15), and autoimmune diseases (13).

Reactive oxygen species (ROS) are highly reactive molecules and include both free radicals, such as superoxide (O_2^{-}) and hydroxyl ($HO^{\cdot}$), and non-radical molecules, such as hydrogen peroxide (H_2O_2) (13). Superoxide, hydroxyl, and hydrogen peroxides are all derived from oxygen as it has been consumed in metabolic processes in the mitochondria, the peroxisomes, and the endoplasmic reticulum (9).

ROS are produced in enzymatic and non-enzymatic reactions under normal physiological conditions. At lower and even moderate concentrations, ROS behave as signaling molecules for cellular proliferation and differentiation processes. Additionally, ROS serve to activate pathways in response to oxidative stress. However, regulation of the intracellular ROS levels is crucial in maintaining homeostasis. ROS at higher concentrations results in lipid, protein, and nucleic acid damage that is characteristic of oxidative stress (13).

1.3.2 Cancer and Oxidative Stress

The role of ROS in cancer is not entirely elucidated. Low to moderate levels of ROS may contribute to tumor formation through signaling mechanisms and result in genomic DNA mutations which promote cell proliferation (10). Under certain conditions, ROS is shown to stimulate the phosphorylation of mitogen-activated protein kinase (MAPK), the phosphorylation of extracellular signal-regulated kinase (ERK), expression of cyclin D1, and activation of JUN N-terminal kinase (JNK) which are all linked to tumor cell proliferation and survival (13). In contrast, high levels of ROS increase susceptibility to cell death and reduce tumor growth (10).

Oxidative stress has also been shown to inhibit distant metastasis in human melanoma cells. Oncogenic signals can promote metastatic survival by increasing glucose uptake via the pentose phosphate pathway. This pathway generates NADPH and regenerates glutathione (GSH), which protect against oxidative stress thus increasing cell survival and increasing metastasis (21). NADPH is necessary to convert glutathione disulfide (GSSG) to GSH with increased production of NADPH promoting increased regeneration of GSH (5). NADPH and NADP levels have been observed to be increased in metastatic cells compared to subcutaneous tumors (21). The increased level of NADPH in metastasizing cells may increase their capacity to regenerate GSH. Conversely, the GSH/GSSG ratio has been observed to be decreased in metastatic cells compared to subcutaneous tumors due to increased GSSG. These metastatic

cells have increased levels of ROS compared with subcutaneous tumors and may they consume greater quantities of GSH to reduce oxidative stress (5).

ALDH1L2 knockdown and MTHFD1 knockdown have been demonstrated to inhibit metastasis. ALDH1L2 is a NADPH-regenerating enzyme and when depleted, is linked to decreased GSH/GSSG levels. ALDH1L2 knockdown has been shown to significantly reduce the number of melanoma cells circulating in blood. MTHFD1 is another NADPH-regenerating enzyme that when knocked down is associated with reduced frequency of melanoma cells in the blood (21). The effect of the enzymes' depletion on metastatic burden suggests that inhibition of NADPH pathways may serve to increase oxidative stress and reduce cell survival (6).

1.3.3 Oxidative Stress and TRPM2

TRPM2-L expression and Ca^{2+} influx (9) have been shown to increase in response to oxidative stress (4). TRPM2-L function has been inhibited through several approaches including downregulation of the protein with RNAi , and utilizing calcium chelation. The TRPM2-S isoform acts as a dominant negative inhibitor when co-expressed with TRPM2-L and reduces Ca^{2+} influx (27). Under conditions which generate oxidative stress, TRPM2-S expression is associated with increased ROS and reduced cell viability (3, 14). As stated previously, the specific mechanisms by which TRPM2-S inhibits TRPM2-L function are currently unknown (27).

TRPM2 is highly expressed in neuroblastoma and many other cancers. TRPM2 enhances viability of cells under low to moderate oxidative stress (14). Cells expressing the TRPM2-S isoform demonstrate increased ROS, reduced levels of forkhead box transcription factor 3a (FOXO3a), a transcription factor associated with apoptosis, and decreased expression of superoxide dismutase 2, an enzyme that converts superoxide into oxygen and hydrogen peroxide. TRPM2-S expression reduces cell viability and tumor growth after exposure of cells to hydrogen peroxide (4) or doxorubicin (3).

The hypoxia-inducible factor 1, alpha subunit (HIF-1α) and 2 (HIF-2α) are up-regulated in many cancers and are associated with poor outcomes. These and other hypoxia-inducible transcription factors regulate genes involved in glycolysis (LDHA), oxidative stress (FOXO3a), and angiogenesis (VEGF). With regards to TRPM2, HIF-1α and HIF-2α and genes which it regulates downstream have greater expression in TRPM2-L as opposed to TRPM2-S expressing cells (3).

BNIP3 and NDUFA4L2 are proteins expressed in mitochondria and regulated by HIF-1α and HIF-2α. BNIP3 is a pro-apoptotic factor and its inhibition results in decreased mitochondrial autophagy and enhanced cell death. NDUFA4L2 is component of NADH dehydrogenase of the mitochondria (15). It has been observed that both BNIP3 and NDUFA4L2 have lower levels of expression in TRPM2-S expressing cells when compared with TRPM2-L (3). This decreased expression is associated with reduced mitophagy which allows for the accumulation of dysfunctional mitochondria. Dr. Miller's laboratory has recently

demonstrated that TRPM2 depletion with CRISPR technology (1) or inhibition with TRPM2-S expression results in reduced tumor growth in mice through the mechanisms described above (3).

1.4 Nrf2

1.4.1 Nrf2

NF-E2 related factor 2 (Nrf2) is a transcription factor that regulates the expression of approximately 250 genes related to antioxidant defense systems in both homeostatic and stress-induced conditions (23). These systems involve cellular functions including cytoprotection against endogenous and exogenous stressors and lipid and carbohydrate metabolism (13). Most specifically, downstream of Nrf2 activity is related to the expression of the antioxidants glutathione (GSH) and NADPH (10, 26).

In humans, Nrf2 is a modular protein that consists of 605 amino acids. The sequence identity shared between human Nrf2 and the orthologous chicken protein, known as erythroid cell-derived protein with CNC homology (ECH), can be divided into distinct regions referred to as Nrf2-ECH homology (Neh) domains. Seven Neh domains have been described with each individual Neh domain having different functions (24).

The Neh1 domain of Nrf2 is a conserved CNC-bZIP region, first described as a DNA-binding peptide sequence in *Drosophila melanogaster*. It acts as a transcription factor and heterodimerizes with other bZIP proteins (24).

The Neh2 domain lies at the N-terminus of Nrf2. It negatively controls Nrf2 activity through Kelch-like ECH-associated protein 1 (Keap1)-mediated repression. There are two highly conserved peptide sequences, the high-affinity ETGE motif and lower-affinity DLG motif, to which Keap1 binds (24).

At the C-terminus, the Neh3 domain is involved in the transcriptional activation of Nrf2. Removal of 16 amino acids from the Neh3 domain results in the inactivation of CNC-bZIP factor, suggests there may be some role in the transactivation of target genes. The Neh3 domain also interacts with the chromodomain helicase DNA-binding protein 6 offering further support that the Neh3 domain is involved in transcriptional activation (24).

Both the Neh4 and the Neh5 domains serve as Nrf2 transactivation domains. Together, they cooperatively bind the coactivator CREB binding protein which increases the rate of gene transcription (24).

The Neh6 domain negatively controls Nrf2 through Keap1-independent regulation. The two conserved peptide motifs, DSGIS and DSAPGS, are recognized by β-transducin repeat-containing protein (β-TrCP). The DSGIS motif includes a glycogen synthase kinase-3 (GSK-3) phosphorylation site called the DSGIS-containing phosphodegron, which controls the degradation rate of Nrf2. When phosphorylated by GSK-3, β-TrCP recruits Cul3 to degrade Nrf2 (24).

The most recently described domain is the Neh7 domain. There is a region that engages in a direct protein-protein interaction between Nrf2 and the DNA-binding domain of retinoid X receptor α (RXRα) which suppresses Nrf2 activity by preventing recruitment of coactivators to the Neh4 and Neh5 domains.

The RXRα ligand bexarotene inhibits Nrf2-target gene expression by promoting interaction between RXRα and the Neh7 domain. Additionally, it is possible that the retinoic acid receptor α (RARα) ligand all-*trans*-retinoic acid acts as an inhibitor of Nrf2 (24).

1.4.2 Keap1

Keap1 is a dimeric protein that is mainly located in the cytoplasm. Nrf2 is inhibited by Keap1 through proteasomal degradation. Keap1 enables the Cul3 RING-box 1 (RBX1) E3 ubiquitin ligase complex (CRL) to ubiquitinate Nrf2 under homeostatic conditions (24).

Keap1 is comprised of five domains: an N-terminal region (NTR); a conserved N-terminal broad complex, tram-track, bric-a-brac (BTB) protein-protein interaction domain; an intervening region (IVR); a C-terminal double-glycine repeat (DGR) domain (which consists of six subdomains with distinct Gly-Gly motif and is called the Kelch-repeat domain); and a C-terminal region (CTR). Cul3 is recruited to the protein-protein interaction domain of BTB. The Neh2 domain of Nrf2 interacts with a six-bladed β-propeller structure formed by the DGR and the CTR (24).

There are two conserved Keap1 binding sites in the Neh2 domain of Nrf2, the high-affinity ETGE motif and the low-affinity DLG motif. Mutation of either the ETGE or the DLG motif prevents Keap1-mediated Nrf2 degradation. These conserved high and low-affinity binding sites are theorized to participate in a “two-site tethering” model of binding between Keap1 and Nrf2. According to this

model, Keap1 initially binds Nrf2 via the high-affinity ETGE motif followed by the binding of the low-affinity DLG motif onto the β-propeller of Keap1. Oxidative stress modifies cysteine residues in Keap1 which prevents the ubiquitination of Nrf2. This is in turn results in Keap1 failing to target the CNC-bZIP factor for proteasomal degradation which leads to an accumulation of Nrf2 (24).

1.4.4 GSK-3

GSK-3 acts as regulator of glycogen metabolism by inhibiting glycogen synthase. Additionally, GSK-3 is a regulator for many pathways including insulin signaling, cell fate specification in embryo development, the cell cycle, and apoptosis. There is a GSK-3 phosphorylation site within the atypical DSGIS β-TrCP-binding site in the Neh6 domain of Nrf2. This DSGIS motif increases the activity of phosphodegron inhibiting Nrf2 activity through ubiquitination via the E3 ligase complex ($SCF^{\beta\text{-}TrCP}$) (24).

The active form of GSK-3 requires its eventual substrate to be first phosphorylated by a “priming” kinase at an amino acid that resides four residues to the C-terminal side of the GSK-3 site. However, no specific kinase that primes Nrf2 for GSK-3 phosphorylation has been identified. There are two GSK-3 isoforms, GSK-3α and GSK-3β, which are active in unstressed cell and regulated by growth factors via Akt phosphorylation of their N-terminal Ser-21 and Ser-9 residues, respectively. This inhibitory phosphorylation occurs because the N-terminal regions act as a pseudo-substrate which competes for the binding site of the primed substrate (24).

1.5 Glutathione

1.5.1 Glutathione

GSH is antioxidant that forms the glutathione system of redox reactions used to maintain homeostasis and counter oxidative stress in most aerobic organisms. In its reduced form, GSH is a nucleophile that serves to convert various electrophilic substances under physiological conditions (5).

The cysteine moiety of GSH leads to the glutathione system being centered about common sulfur biochemistry and requires an electron relay linking the reducing agent NADPH to thiol/disulfide-metabolism as well as a thiol-containing adapter molecule to transfer electrons to a set of different acceptors (21). Reducing equivalents from NADPH enter the glutathione system with the help of the FAD-dependent enzyme glutathione reductase (GR) or the thioredoxin reductase/thioredoxin couple (TrxR/TRX). These electrons are then transferred to glutathione disulfide (GSSG) which yields two molecules of GSH. GSH serves as a reducing agent for disulfides and hydroperoxides or may be conjugated with 2-OA and other electrophilic substances. Additionally, GSSG can oxidize thiols depending on thermodynamic and kinetic conditions (5).

1.5.2 Glutamate cysteine ligase

Glutamate cysteine ligase (GCL) is an enzyme that serves as a catalyst in the production of GSH. Catalyzing the first and rate-limiting step, GCL utilized ATP-dependent condensation of cysteine and glutamate to form g*amma*-L-

glutamyl-L-cysteine (γ-GC). In turn, γ-GC is condensed with glycine via the enzyme, glutathione synthestase (GSS), to form GSH. Due to its position as the rate-limiting step of GSH synthesis, GCL activity directly affects GSH levels and biosynthetic capacity (8).

The structure of GCL is a heterodimeric holoenzyme with the two protein subunits being coded on independent genes on separate chromosomes. The glutamate cysteine ligase catalytic subunit (GCLC) possesses the substrate and cofactor binding sites for catalysis of GSH. The smaller glutamate cysteine ligase modifier subunit (GCLM) has no independent enzymatic activity. GCLM increases the catalytic potential of GCLC when both subunits form a complex (8).

GCL expression is regulated at multiple levels including transcriptional, posttranscriptional, translational, and posttranslational levels. Baseline expression has been demonstrated to be affected by oxidative stress, GSH depletion, and toxic exposure. The transcriptional factors Nrf2, AP-1, and NF-κB have been demonstrated to regulate expression of both the GCLC and the GCLM subunits (9). Additionally, GSH itself acts to inhibit GCL activity (8).

1.5.3 Glutathione *S*-transferase

Glutathione *S*-transferases (GSTs) are a family of phase II detoxification enzymes with a dimeric structure. Their primary role is catalyzing the conjugation of electrophilic substrates to GSH. However, GSTs also have peroxidase and isomerase activities, they can inhibit Jun N-terminal kinase protecting cells

against H_2O_2-induced cell death, and they bind non-catalytically to a variety of endogenous and exogenous ligands (5, 22).

Mammalian GSTs have been well-characterized and fall into one of seven classes: Alpha, Mu, Pi, Theta, Kappa, Zeta, and Omega. These genes associated with these GST classes differ from each other in size, intron/exon structure, and in their chromosomal localizations. Within these classes are clearly defined subfamilies that represent unique subunit types. Each subfamily may include as many as five separate but highly homogenous polypeptides (22).

Among the GSTs with activity in the cytosol, GSTP1 is reported to have the greatest number of interactions with different proteins and affect the greatest number of cellular pathways. In particular, GSTP1 utilizes GSH in redox interactions that reduce ROS and other electrophilic compounds (5, 22). Like other proteins involved in the antioxidant response, GSTP1 expression seems to be highly regulated by Nrf2 activity (2).

1.5.4 Peroxiredoxin

The peroxiredoxin (PRX) family of enzymes serves as antioxidants and regulators of cytokine-induced peroxide levels. There are six members of the PRX family in humans that are then grouped as either 1-Cys enzymes or 2-Cys enzymes based on the presence or absence of a semi-conserved cysteine residue (C_R). The absolutely conserved cysteine residue or peroxidatic cysteine (C_P) resides in the PRX active site where it is oxidized to a sulfenic acid by the

peroxide substrate (5). Nrf2 has been demonstrated to have a regulatory effect on the six members of the PRX family, including PRX3 (19).

PRX has the ability to reduce ROS substrates, namely peroxide, leaving PRX in its inactive, oxidized form. There are several reducing agents that can return PRX to reduced state with regulation via thioredoxin (TXN) being the most well documented. PRX is also reduced by glutathione reductase (GR) and GSTP1 in the presence of GSH (5).

Chapter 2: Hypothesis and Specific Aims

2.1 Hypothesis

In response to oxidative stress, cells with different TRPM2 isoforms express differing levels of specific transcription factors and mitochondrial proteins. Understanding the specific mechanisms through which TRPM2 inhibition modulates mitochondrial proteins, mitochondrial function, and cell survival could lead to the design of novel therapies which target TRPM2 (14). For example, TRPM2 inhibition may modulate HIF-1α or HIF-2α thus affecting mitochondrial function, ROS production, bioenergetics (16), and mitophagy and reducing tumor growth (Figure 1) (1, 3).

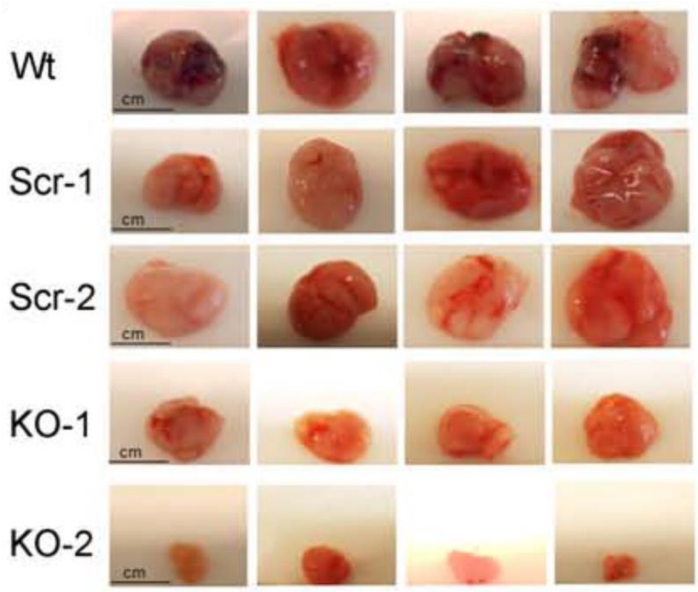

Figure 1: SH-SY5Y neuroblastoma xenografts show reduced growth with TRPM2 depletion
Athymic female mice were injected with 1.5×10^7 SH-SY5Y cells in which TRPM2 was depleted with CRISPR (two clones, KO-1 and KO-2), scrambled control cells (two clones, Scr-1 and Scr-2), or wild type parental cells (Wt). Photographs of representative tumors are shown. (1)

TRPM2-S expression is associated with increased ROS and reduced cell viability when compared with TRPM2-L (4, 27). Additionally, TRPM2-S expressing cells demonstrated reduced HIF-1α, HIF-2α, BNIP3, and NDUFA4L2 expression (3). These patterns have also been demonstrated through TRPM2 depleted via CRISPR/Cas9 technology (Figure 2) (1). Recently oxidative stress has been shown to be a major factor in metastasis formation (1). Further elucidating the relationship between these transcription factors, ROS production, and mitochondrial function is vital toward designing novel therapies to kill cancer cells through TRPM2 inhibition.

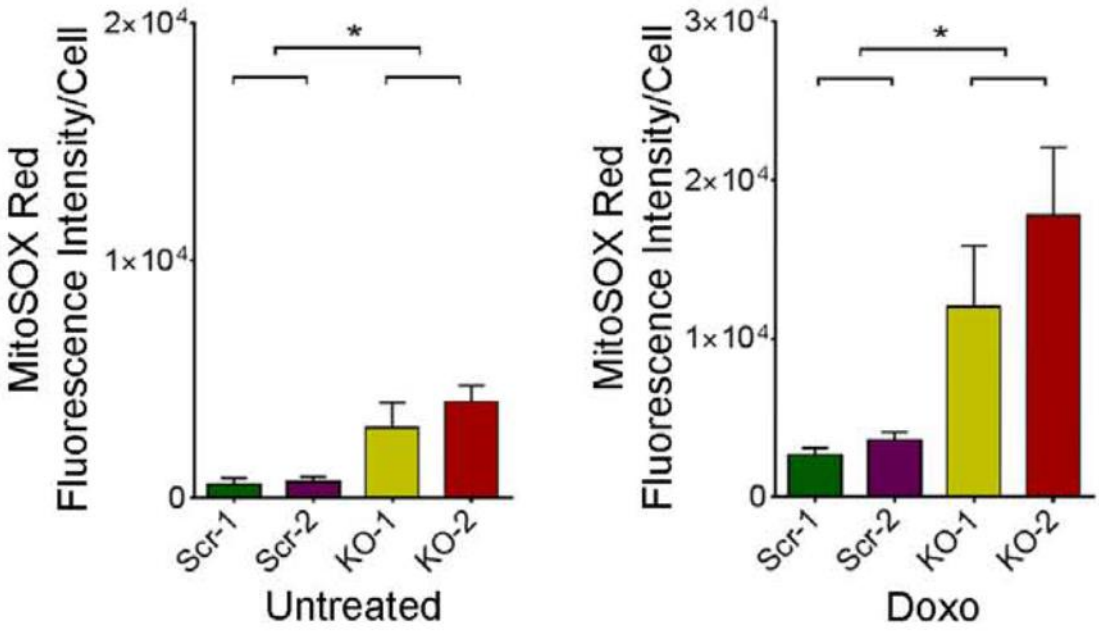

Figure 2: TRPM2 depletion results in increased mitochondrial ROS with and without doxorubicin treatment

Mitochondrial superoxide was measured with MitoSOX Red via confocal microscopy in SH-SY5Y cells depleted of TRPM2 (two clones, KO-1 and KO-2) and scrambled control (two clones, Scr-1 and Scr-2) cells treated with or without 0.3 μM doxorubicin for 24 hours. Four experiments were performed with similar results and mean ± S.E. of a minimum of 100 cells in at least 10 fields were quantified. The results of a representative experiment are shown. *,$p<0.05$. (1)

It has previously been shown that successfully metastasizing cells may increase their ability to withstand oxidative stress through upregulation of GSH generating pathways. Because oxidative stress may limit distant metastasis, we will determine if cells in which TRPM2 is inhibited have decreased levels of GSH, and reduced metastasis. The hypothesis of this research is that inhibition of TRPM2 increases oxidative stress and ROS; increased ROS occurs through increased mitochondrial dysfunction (1, 11) and decreased antioxidant responses including those modulated through Nrf2 and antioxidant enzymes (13, 24). The increase in ROS is sufficient to reduce cell survival (16), decrease tumor growth in the TRPM2 knockout, and may also be an approach to reduce metastasis.

2.2 Specific Aims

2.2.1

The first aim is to investigate whether TRPM2 inhibition by CRISPR depletion influences levels of glutathione (GSH/GSSG) in neuroblastoma.

2.2.2

The second aim is to investigate if TRPM2 inhibition by CRISPR depletion influences levels of Nrf2 expression in neuroblastoma, which may a mechanism through which GSH levels are reduced.

Chapter 3: Methods and Materials

3.1 Glutathione Assay

We measured the concentrations of GSH from whole cell lysates via fluorescence microplate reader. SH-SY5Y wild-type (Wt) cells, three TRPM2 scrambled control (Scr) clones, and two TRPM2 knockout (KO) clones, which were generated and characterized previously, were studied. The cells were plated at 13×10^6 cells in tissue culture dishes and treated without doxorubicin or 0.3 μM doxorubicin for 24 hours. The cells were harvested and GSH levels measured using Abcam's ab138881 GSH/GSSG Ratio Detection Assay Kit (Fluorometric-Green) and Abcam's ab204708 Deproteinizing Sample Kit – TCA. The GSH samples were diluted in the kit's Mammalian Lysis Buffer at 1:500 and pH was adjusted to 6.0 for all samples. The GSH standard curve was made in the kit's Mammalian Lysis Buffer with concentrations of 0.0 μM, 0.1563 μM, 0.3125 μM, 0.625 μM, 1.25 μM, 2.5 μM, 5.0 μM, and 10.0 μM. The prepared samples were plated on a 96-well plate and measured with the Clariostar microplate reader. The settings for the microplate reader were 482-15 for excitation, 502 for dichroic filter, and 520-10 for emission.

The GSH concentrations were calculated from a standard curve and the respective means and standard errors of samples were determined. The Student's *t*-test was performed to determine the statistical significance of GSH concentrations measured between TRPM2 knockout cells and scrambled control clones and treated with different doxorubicin concentrations.

3.2 Immunoblot Analysis

We prepared whole cell lysates and tumor lysates with Triton lysis buffer (50mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10 mM NaF, protease inhibitor and phosphatase inhibitor) and a 10-minute centrifugation at 10,000 rpm at 4 °C. The supernatants were collected and subjected to 10-15% SDS-PAGE. These gels were trans-blotted onto nitrocellulose membranes. Blots were probed with antibodies to GCLC (1:2000; Abcam), GSK-3α/β (1:1000; Cell Signaling), GSTP1 (1:1000; Cell Signaling), Keap1 (1:1000; Cell Signaling), Nrf2(1:1000; Cell Signaling), Phospho GSK-3α/β (1:1000; Cell Signaling), PRX3 (1:7500; Cell Signaling), and Txnrd1 (1:7500; Cell Signaling). Enhanced chemiluminescence (ECL) was used for detection of signal. Intensity of bands was quantitated with densitometry.

Chapter 4: Results

4.1 Glutathione Assay

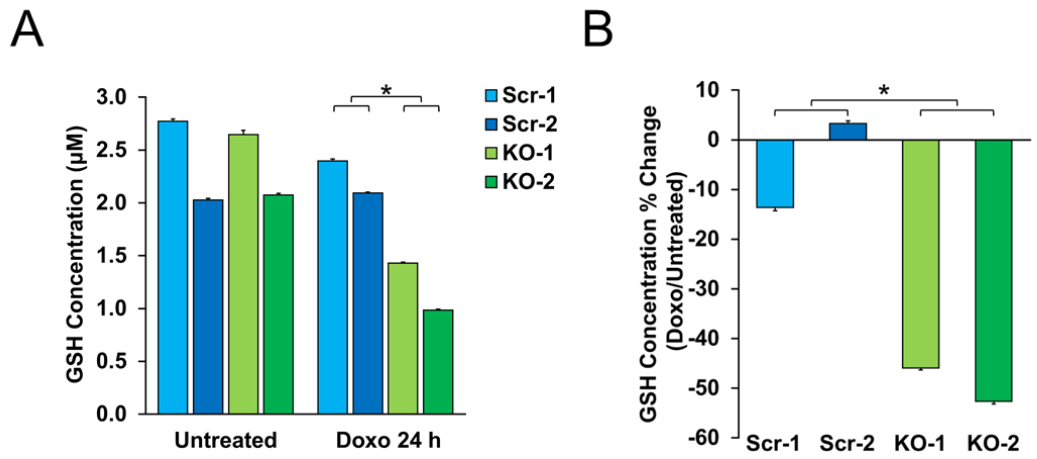

Figure 3: Glutathione (GSH) concentrations are reduced following doxorubicin treatment in TRPM2-depleted neuroblastoma cells.
A, GSH concentration was measured in SH-SY5Y cells depleted of TRPM2 (two clones, KO-1 and KO-2) and scrambled control (two clones, Scr-1 and Scr-2) cells treated with or without 0.3 μM doxorubicin for 24 hours. Four experiments were performed with similar results and one representative experiment is shown. Results expressed as mean ± S.E. (n=6 each) are shown. *,$p<0.05$. *B*, Percentage change in GSH concentration was calculated by dividing the mean doxorubicin-treated GSH concentrations by the mean untreated GSH concentration for each group. Four experiments were performed with similar results and results expressed as mean ± S.E. of one representative experiment (n=6 each) are shown. *,$p<0.05$.

TRPM2 depleted SH-SY5Y cells have been demonstrated to show increased ROS. Because the antioxidant glutathione (GSH) is a major regulator of cellular ROS, the concentrations of glutathione in scrambled and TRPM2 KO cells were measured in untreated cells and cells at 24 hours after doxorubicin (0.3 μM) treatment. The GSH concentrations in doxorubicin treated KO cells were statistically less than that in scrambled control cells (Figure 3A). Untreated knockout and scrambled control cells were not statistically different from each other. Both of the knockout clones demonstrated much larger decreases in GSH concentration, greater than 40 percent, compared to scrambled cells (Figure 3B).

These data indicate that TRPM2 modulates important antioxidant pathways in response to doxorubicin treatment.

4.2 Immunoblot Analysis

Western blotting was performed on lysates of xenograft tumors derived from TRPM2 depleted (KO) and scrambled control cells that were harvested six weeks after SH-SY5Y cell injection. A statistically significant decrease in expression of the transcription factor Nrf2 was observed in xenograft tumors expressing the TRPM2 knockout (Figure 4). Western blotting demonstrated modulation of expression of proteins that regulate the activity of Nrf2 including Keap1, GSK-3α/β, and phosphorylated GSK-3α/β, with TRPM2 knockout cells demonstrating greater expression of these inhibiting factors than the scrambled control (Figure 4). Enzymes downstream of Nrf2, namely GCLC (a rate-limiting enzyme in glutathione production), GSTP1, and PRX3, demonstrated decreased expression in the TRPM2 knockout relative to the scrambled control (Figure 4). These data support the conclusion that TRPM2 activity regulates expression of Nrf2 through upstream inhibitors. Additionally, some of the proteins downstream of Nrf2 important in the antioxidant response including in GSH production are downregulated.

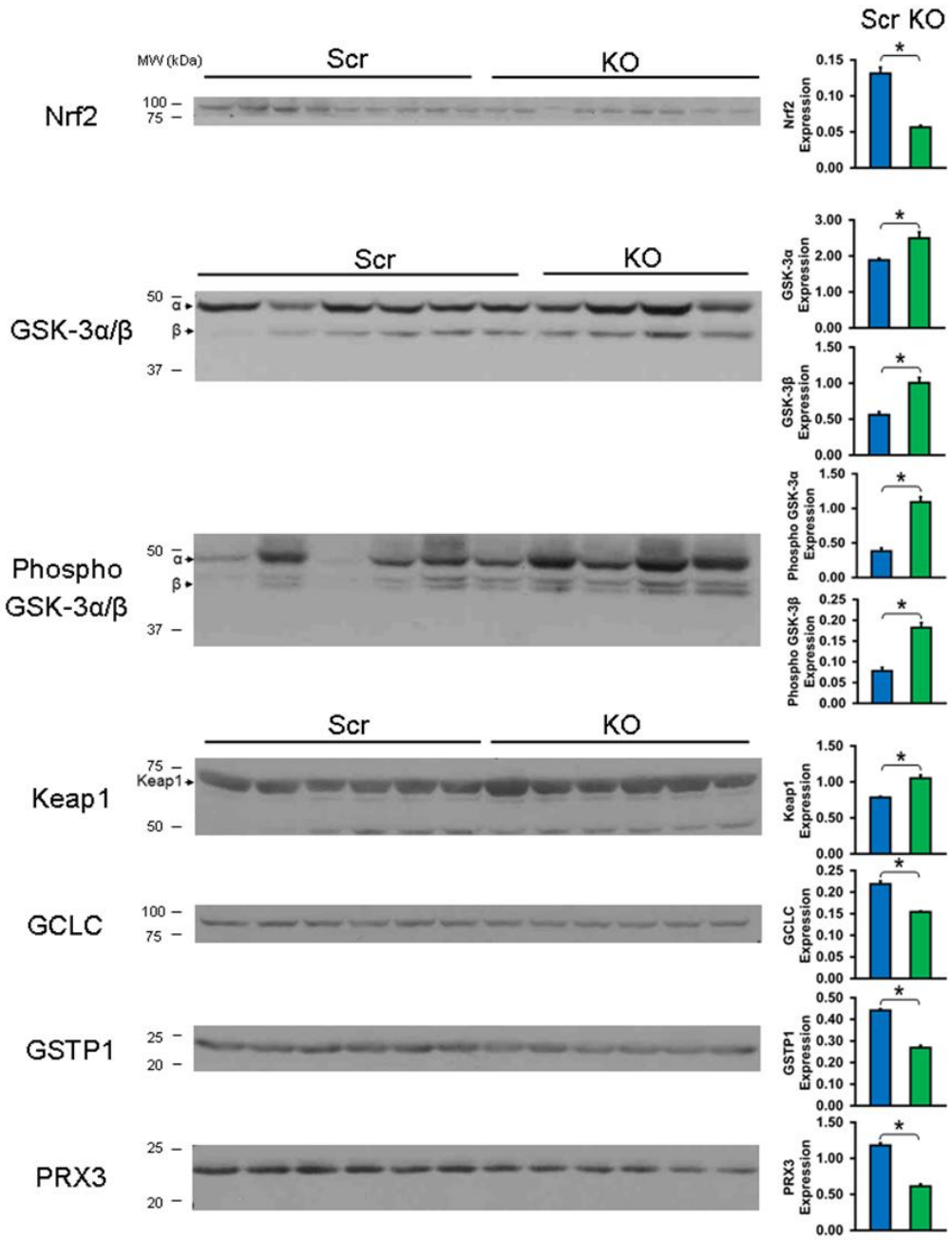

Figure 4: Tumors depleted of TRPM2 demonstrate reduced expression of Nrf2 and downstream antioxidants response proteins and increased expression of upstream Nrf2 regulators.

Lysates were prepared from tumor xenografts harvested 6 weeks after injection with SH-SY5Y cells depleted of TRPM2 (KO) or scrambled control (Scr) cells. Western blotting analysis was performed and blots were probed with anti-Nrf2 and antibodies to the downstream target genes GCLC, GSTP1, and PRX3 and antibodies to the upstream Nrf2 regulators Keap1, GSK-3α/β, and phospho GSK-3α/β. Equivalent protein amounts were added to each lane. Western blotting was performed on at least four tumors for each cell type. Protein expression was quantified by densitometry and the mean ± S.E. of protein was calculated. *,$p<0.05$.

Chapter 5: Discussion

TRPM2 plays an important role in the cellular response to oxidative stress. Several studies demonstrated that TRPM2 is highly expressed in many malignancies including melanoma, neuroblastoma, breast cancer, and lung cancer, suggesting that TRPM2 plays an important role in tumor growth. Previous work demonstrated that TRPM2 is involved in regulation of mitochondrial function, cellular bioenergetics, and ROS production (Figure 5) (1, 3, 11), suggested that inhibition of TRPM2 may serve as a novel therapy for a number of malignancies (14). The studies performed here utilized neuroblastoma cells in which TRPM2 was depleted with CRISPR/Cas9 technology as a model to study the importance of TRPM2 in ROS production (16), tumor growth and sensitivity to doxorubicin treatment (1). Our findings indicate that TRPM2 depletion plays a significant role in the inhibition of the transcription factor Nrf2 (Figure 4), altering activity of downstream targets of Nrf2, and reducing the concentration of the antioxidant GSH (Figure 3). This work identifies an additional pathway through which TRPM2 inhibition results in increased levels of ROS (Figure 2).

The first important finding of this work is that for cells treated with doxorubicin, TRPM2 depletion significantly decreases the levels of the antioxidant GSH. In its reduced form, GSH reduces ROS and other electrophilic substances in many redox reactions (5). Decreased concentration of reduced GSH has been previously shown to be correlated with oxidative stress.

The second key finding is the decreased expression of Nrf2 associated with TRPM2 depletion. In its role as a transcription factor, Nrf2 regulates the expression of many genes through binding to the antioxidant response element (ARE) (13, 23). Through the decreased expression of these downstream targets, decreased expression of Nrf2 has been demonstrated to mediate reduced levels of the antioxidant GSH.

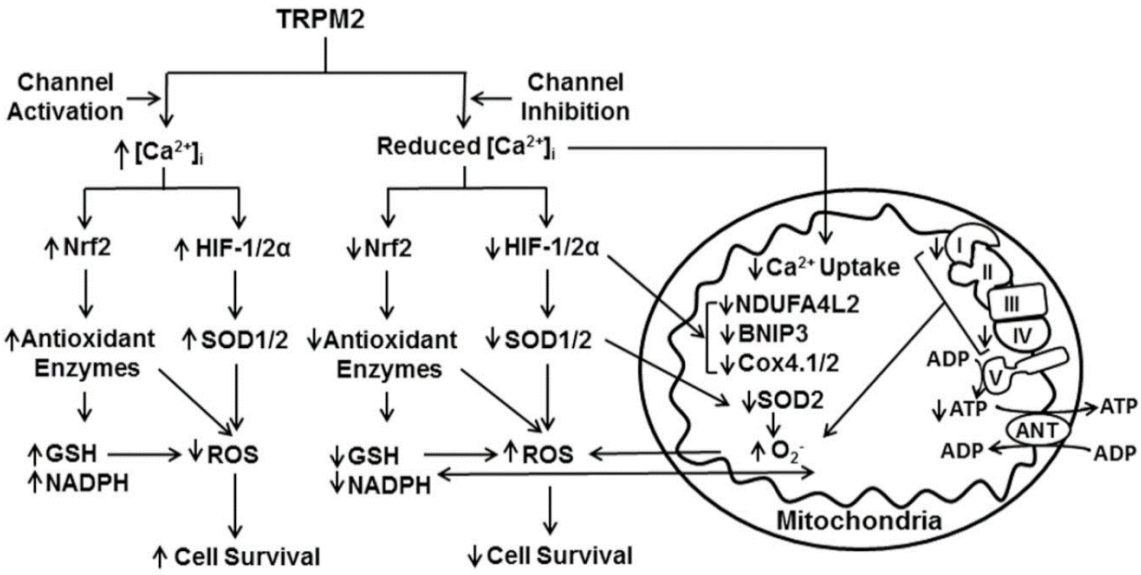

Figure 5: Scheme of the influence of TRPM2 inhibition on Nrf2 expression, antioxidant levels, and cell survival.
Inhibition of TRPM2 results in reduced intracellular calcium entry. Decreased intracellular calcium results in reduced Nrf2 expression as well as genes transcriptionally regulated by Nrf2 including GCLC, which catalyzes GSH production, and GSTP1 and PRX3, which reduce GSSG to active form GSH. This results in decreased GSH levels which further contribute to increased ROS, and reduced cell survival and tumor growth. (Miller Lab unpublished)

Immunoblot analysis has demonstrated that Nrf2 downstream targets, GCLC (10), GSTP1 (2), and PRX3 (19) are reduced in the TRPM2 knockout compared to the scrambled control. GCL acts as a catalyst in the first and the rate-limiting step in the production of GSH. Generally, GCL activity determines GSH levels and antioxidant capabilities. More specifically, GCLC is the subunit of

GCL that possesses all of the substrate and cofactor binding sites that are responsible in catalyzing GSH production. Like other GSTs, GSTP1 has been found to act as a catalyst in reactions where glutathione reduces ROS and other electrophilic compounds.The peroxiredoxin PRX3 acts as an antioxidant which is reduced to its active form via enzymatic reaction with GSH and GSTP1 (5, 19). The decreased expression of these proteins associated with the antioxidant response element (ARE) serves to partially explain the relatively low levels of the antioxidant GSH in the knockout when compared to the scrambled control. Previous publications have demonstrated the regulation of GCLC, GSTP1, and PRX3 expression via Nrf2 (13) and this current work supports that established connection.

Additionally, immunoblot analysis demonstrated that the proteins Keap1 and GSK-3α/β have increased expression in the TRPM2 knockout. Keap1 inhibits the binding of Nrf2 to the ARE through the formation of the Keap1/Nrf2 complex in the cytosol. Keap1 binds to Nrf2 via the Neh2 domain and in this bound state, Nrf2 is ubiquitinated by CRL and summarily degraded via proteasomes. Conversely the failure of Keap1 binding to Nrf2 results in the en masse bypass of Nrf2 from the cytosol into the nucleus where Nrf2 binds to ARE sequences driving gene expression. GSK-3α/β inhibits Nrf2 activity through its influence on β-TrCP mediated degradation of Nrf2. When GSK-3α/β phosphorylates the DSGIS motif of the Neh6 domain of Nrf2, β-TrCP more readily represses Nrf2 through ubiquitination via $SCF^{\beta\text{-}TrCP}$ (24). While the decreased expression of Nrf2 correlates with the increased expression of these

upstream inhibitors Keap1 and GSK-3α/β, it is unclear which TRPM2 depletion related mechanism makes the key contribution.

TRPM2 depletion and the resulting decrease in intracellular Ca^{2+} have been previously demonstrated to increase ROS within neuroblastoma cells through a reduction in HIF-1/2, mitochondrial proteins involved in electron transport, and reduced cellular bioenergetics (1). The decreased expression of the transcription factor Nrf2, the decreased expression of its downstream targets associated with the ARE, and the reduced levels of the antioxidant GSH were all found to be associated with TRPM2 depletion. These observations suggest a mechanism that may play an important role in the increased ROS levels that are observed in TRPM2 depleted cells, in addition to the mitochondrial dysfunction previously demonstrated (1).

The data shown here demonstrating the increased expression of the upstream Nrf2 inhibitors, Keap1 and GSK-3α/β, suggest a pathway by which Nrf2 activity is reduced. Further study into the pathways associated with Keap1 and GSK-3α/β expression may serve to elucidate the connection between TRPM2 depletion and reduced expression in Nrf2 (24). Additionally, there are several other pathways associated with Nrf2 binding to the ARE that involve the production and reduction of GSH which should be explored. There are also NADPH production pathways (21), TXN production pathways, and other various enzymatic pathways which have been previously demonstrated to be regulated by Nrf2 expression in oxidative stress (10).

Understanding the mechanisms by which TRPM2 depletion increases intracellular ROS levels is invaluable in developing future therapies for treating neuroblastoma and other cancers (10), as well as other diseases involving increased oxidative stress and ischemic injury (14). Cells that experience high levels of ROS often demonstrate reduced cell viability and increased cell death (16). Therapies could modulate TRPM2 activity to either increase or decrease ROS levels, which would subsequently affect cell viability (1, 3, 14, 15). Specifically, in the case of cancers, the goal of depletion of TRPM2 would be to increase ROS levels above a cytotoxic threshold leading to reduced cell survival and increased tumor cell death. A nanoparticle based therapy may have the potential to form the basis for such TRPM2 modulation in target cancer cells without affecting TRPM2 function or survival of adjacent cells. In contrast, in ischemic injury, the objective would be to maintain TRPM2 levels which would optimize protection of cell viability (10, 15). A goal for further research on TRPM2 in cancer is to elucidate the cascade of signaling pathways that are regulated by TRPM2 while at the same time identifying the most appropriate approach to modulate TRPM2 to influence cell survival (14).

References

1. Bao L, Chen SJ, Conrad K, Keefer K, Abraham T, Lee JP, Wang J, Zhang XQ, Hirschler-Laszkiewicz I, Wang HG, Dovat S, Gans B, Madesh M, Cheung JY, Miller BA. Depletion of the human ion channel TRPM2 in neuroblastoma demonstrates its key role in cell survival through modulation of mitochondrial reactive oxygen species and bioenergetics. *The Journal of Biological Chemistry* 291(47): 24449-24464, 2016.

2. Bartolini D, Galli F. The functional interactome of GSTP: A regulatory biomolecular network at the interface with the Nrf2 adaptation response to oxidative stress. *Journal of Chromatography B* 1019: 29-44, 2016.

3. Chen SJ, Hoffman NE, Shanmughapriya S, Bao L, Keefer K, Conrad K, Merali S, Takahashi Y, Abraham T, Hirschler-Laszkiewicz I, Wang J, Zhang XQ, Song J, Barrero C, Shi Y, Kawasawa YI, Bayerl M, Sun T, Barbour M, Wang HG, Madesh M, Cheung JY, Miller BA. A splice variant of the human ion channel TRPM2 modulates neuroblastoma tumor growth through hypoxia-inducible factor (HIF)-1/2α. *Journal of Biological Chemistry* 289(52): 36284-36302, 2014.

4. Chen SJ, Zhang W, Tong Q, Conrad K, Hirschler-Laszkiewicz I, Bayerl M, Kim JK, Cheung JY, Miller BA. Role of TRPM2 in cell proliferation and susceptibility to oxidative stress. *American Journal of Physiology – Cell Physiology* 304: C548-C560, 2013.

5. Deponte M. Glutathione catalysis and the reaction mechanisms of glutathione-dependent enzymes. *Biochimica et Biophysica Acta* 1830: 3217-3266, 2013.

6. Di A, Gao XP, Qian F, Kawamura T, Han J, Hecquet C, Ye RD, Vogel SM, Malik AB. The redox-sensitive cation channel TRPM2 modulates phagocyte ROS production and inflammation. *Nature Immunology* 13(1): 29-34, 2011.

7. Du J, Xie J, Yue L. Intracellular calcium activates TRPM2 and its alternative spliced isoforms. *Proceedings of the National Academy of Sciences of the United States of America* 106(17): 7239-7244, 2009.

8. Franklin CC, Backos DS, Mohar I, White CC, Forman HJ, Kavanagh TJ. Structure, function, and post-translational regulation of the catalytic and modifier subunits of glutamate cysteine ligase. *Molecular Aspects of Medicine* 30(1-2): 86-98, 2009.

9. Görlach A, Bertram K, Hudecova S, Krizanova O. Calcium and ROS: A mutual interplay. *Redox Biology* 6: 260-271, 2015.

10. Gorrini C, Harris IS, Mak TW. Modulation of oxidative stress as an anticancer strategy. *Natural Reviews Drug Discovery* 12: 931-947, 2013.

11. Hoffman NE, Miller BA, Wang J, Elrod JW, Rajan S, Gao E, Song J, Zhang XQ, Hirschler-Laszjhkiewicz I, Shanmughapriya S, Koch WJ, Feldman AM, Madesh M, Cheung JY. Ca^{2+} entry via TRPM2 is essential for cardiac myocyte bioenergetics maintenance. *American Journal of Physiology: Heart and Circulatory Physiology* 308(6): H637-H650, 2015.

12. Maris JM, Hogarty MD, Bagatell R, Cohn SL. Neuroblastoma. *Lancet* 369(9579): 2106-2120, 2007.

13. Matés JM. Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology. *Toxicology* 153: 83-104, 2000.

14. Miller BA, Cheung JY. TRPM2 protects against tissue damage following oxidative stress and ischemia-reperfusion. *Journal of Physiology* 000(00): 1-11, 2015.

15. Miller BA, Hoffman NE, Merali S, Zhang XQ, Wang J, Rajan S, Shanmughapriya S, Gao E, Barrero CA, Mallilankaraman K, Song J, Gu T, Hirschler-Laszkiewicz I, Koch WJ, Feldman AM, Madesh M, Cheung JY. TRPM2 channels protect against cardiac ischemia-reperfusion injury: role of mitochondria. *Journal of Biological Chemistry* 289(11): 7615-7629, 2014.

16. Miller BA, Wang J, Hirschler-Laszkiewicz I, Gao E, Song J, Zhang XQ, Koch WJ, Madesh M, Mallilankaraman K, Gu T, Chen SJ, Keefer K, Conrad K, Feldman AM, Cheung JY. The second member of transient receptor potential-melastatin channel family protects hearts from ischemia-reperfusion injury. *American Journal of Physiology: Heart and Circulatory Physiology* 304: H1010-H1022, 2013.

17. Miller BA, Zhang W. TRP channels as mediators of oxidative stress. *Advances in Experimental Medicine and Biology* 704: 531-544, 2011.

18. Miller BA. The role of TRP channels in oxidative stress-induced cell death. *Journal of Membrane Biology* 209: 31-41, 2006.

19. Miyamoto N, Izumi H, Miyamoto R, Kondo H, Tawara A, Sasaguri Y, Kohno K. Quercetin induces the expression of peroxiredoxins 3 and 5 via the Nrf2/Nrf1 transcription pathway. *Investigative Ophthalmology & Visual Science* 52: 1055-1063, 2011.

20. Orfanelli U, Wenke AK, Doglioni C, Russo V, Bosserhoff AK, Lavorgna G. Identification of novel sense and antisense transcription at the TRPM2 locus in cancer. *Cell Research* 18: 1128-1140, 2008.

21. Piskounova E, Agathocleous M, Murphy MM, Zeping H, Huddlestun SE, Zhao Z, Leitch AM, Johnson TM, DeBerardinis RJ, Morrison SJ. Oxidative stress inhibits distant metastasis by human melanoma cells. *Nature* 527: 186-191, 2015.

22. Sheehan D, Meade G, Foley VM, Dowd CA. Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. *Biochemical Journal* 360: 1-16, 2001.

23. Strom J, Xu B, Tian X, Chen QM. Nrf2 protects mitochondrial decay by oxidative stress. *The GASEB Journal* 30(1): 66-80, 2017.

24. Tebay ET, Robertson H, Durant ST, Vitale SR, Penning TM, Dinkova-Kostova AT, Hayes JD. Mechanisms of activation of the transcription factor Nrf2 by redox stressors, nutrient cues, and energy status and the pathways through which it attenuates degenerative disease. *Free Radical Biology and Medicine* 88: 108-146, 2015.

25. Tong Q, Zhang W, Conrad K, Mostoller K, Cheung JY, Peterson BZ, Miller BA. Regulation of the transient receptor potential channel TRPM2 by the Ca^{2+} sensor calmodulin. *Journal of Biological Chemistry* 281(14): 9076-9085, 2006.

26. Wu KC, Cui JY, Klaasen CD. Beneficial role of Nrf2 in regulating NADPH generation and consumption. *Toxicological Sciences* 123(2): 590-600, 2011.

27. Zhang W, Chu X, Tong Q, Cheung JY, Conrad K, Masker K, Miller BA. A novel TRPM2 isoform inhibits calcium influx and susceptibility to cell death. *Journal of Biological Chemistry* 278(8): 16222-16229, 2003.

28. Zhang W, Hirschler-Laszkiewicz I, Tong Q, Conrad K, Sun SC, Penn L, Barber DL, Stahl R, Carey DJ, Cheung JY, Miller BA. TRPM2 is an ion channel that modulates hematopoietic cell death through activation of caspases and PARP cleavage. *American Journal of Physiology: Cell Physiology* 290(4): C1146-C1159, 2006.