The Epigenetic Regulation of *KCNA5* in Pediatric Solid Tumors and its Role in Cancer Biology

by

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To my family and fiancé

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LIST OF ABBREVIATIONS

ES	Ewing Sarcoma
NB	Neuroblastoma
K^+	Potassium Ion
Kv	Voltage-Gated Potassium Channel
Kir	Inward Rectifying Potassium Channels
K_{Ca}	Calcium-activated Potassium Channels
K2P	Two-Pore Domain Potassium Channels
TM	Transmembrane Domain
I_{Kur}	Ultra-rapid Delayed Rectifier Current (Kv1.5 Channel Current)
I_{to}	Transient Outward Current
KCNA5	Kv1.5 Channel Encoding Gene
AF	Atrial Fibrillation
PAH	Pulmonary Arterial Hypertension
PASMCs	Pulmonary Arterial Smooth Muscle Cells
VSMCs	Vascular Smooth Muscle Cells
HPV	Hypoxic Pulmonary Vasoconstriction
ROS	Reactive Oxygen Species
KRE	Kv1.5 Repressor Element
V_m	Membrane Potential
H_2O_2	Hydrogen Peroxide
MSC	Mesenchymal Stem Cell
NCSC	Neural Crest Stem Cell
HAT	Histone Acetyltransferases

HDAC	Histone Deacetylases
HMT	Histone Methyltransferases
PcG	Polycomb Group Protein
PRC1	Polycomb Repressive Complex 1
PRC2	Polycomb Repressive Complex 2
DNMT	DNA Methylatransferase Inhibitor
H2AubK119	Mono-ubiquitination of Histone 2A at Lysine residue 119
H3K27me3	Tri-methylation of Histone 3 at Lysine residue 27
ChIP	Chromatin Immunoprecipitation
DPO-1	Diphenyl Phosphine Oxide-1
4’AP	4’Aminopyridine
DMSO	Dimethyl Sulfoxide
WT	Wild-Type
PD	Pore-Dead
sGFP	soluble-GFP
PDK	Pyruvate Dehydrogenase Kinase
DCA	Dichloroacetate
PMR	Percent Methylated Reference
BrdU	Bromodeoxyuridine
PI	Propidium Iodine
GBM	Glioblastoma Multiforme
MDS	Myelodysplastic Syndrome
Kv11.1	HERG
Kv10.1	EAG1
PDX	Patient Derived Xenograft
CSC	Cancer Stem Cell

CHAPTER 1

INTRODUCTION

Rationale

It is well known that potassium channels are essential for maintaining cellular ionic homeostasis, a key requirement for numerous biological functions, including proliferation, migration and apoptosis (1-5). Abundant evidence demonstrates that cancer cells hijack the normal physiologic function of potassium channels to promote tumor pathogenesis (reviewed in (6, 7)). One such channel deregulated in cancer, is the voltage-gated potassium (Kv) channel, Kv1.5. Various studies demonstrate that the Kv1.5 channel is downregulated in cancer and propose its downregulation contributes to cancer progression by promoting cancer cell survival and growth (8-15). Despite these observations, the mechanisms governing Kv1.5 expression in cancer have yet to be elucidated. Therefore, in this thesis, we begin to investigate the mechanisms underlying aberrant regulation of Kv1.5 channel expression and determine the consequences this misregulation has on cancer cell survival and cell growth. Investigating the contribution of Kv1.5 channel misregulations in cancer will

provide new insights into molecular mechanisms of cancer pathogenesis and offer innovative therapeutic strategies to tackle resistant cancer cells and relapsed disease.

Great strides have been made in advances for cancer treatment, however cancer still remains the second leading cause of death in children (16). This thesis focuses on two aggressive pediatric solid tumors, Ewing Sarcoma (ES) and Neuroblastoma (NB). ES is the second most prevalent form of bone tumor in children and young adults and NB is the most common extra-cranial solid tumor of childhood. Though effective therapies have been developed, patients diagnosed with metastases have a low rate of survival and relapse is predominant as it occurs in 1/3rd of ES patients (16, 17) and 50-60% of high-risk NB patients (18). This indicates that at least some tumor cells survive despite exposure to cytotoxic drugs and an increasingly hostile microenvironment (e.g. hypoxia, necrosis, nutrient deprivation) (19, 20). Furthermore, relapsed ES and NB tumors are often highly drug resistant, thus limiting treatment options (16, 17). These predominant issues re-enforce the need for new therapeutic approaches, ones that specifically target resistant cells.

Potassium Channel Overview

Potassium channels are a large and diverse family of ion channels. They are transmembrane proteins, which selectively move potassium ions (K^+) into and out of intracellular and extracellular compartments, in response to various stimuli (voltage or ligand-gated) (5, 21, 22). Potassium channels are found in

nearly all cell types, including skeletal muscle cells, glial cells, cardiomyocytes, erythrocytes, pancreatic β cells, smooth muscle cells and lymphocytes (5, 22). They have diverse biological roles, including regulating membrane potential (23, 24), cell volume (25), calcium signaling (26) and determining the rate of repolarization, especially in excitable cells such as neuronal and cardiac cells (23).

Kv Channel Family

The potassium channel family is comprised of seventy-eight members and its largest subset consists of the voltage-gated potassium channels (Kv) (22). The three other classes of the potassium channel family include, inward rectifying potassium channels (Kir), calcium-activated potassium channels (K_{Ca}) and two-pore domain potassium channels (K2P). The Kv channels are a superfamily comprised of twelve subfamilies (Kv1-12) encoded by forty genes (22). The first four Kv channel subfamilies were originally cloned from *Drosophila* and are known as the *Shaker* (Kv1), *Shab* (Kv2), *Shaw* (Kv3) and *Shal* (Kv4) families (27). Further cloning studies led to the identification of additional Kv subfamilies, designated Kv5-Kv12 (22). Within each subfamily, multiple members exist (e.g. Kv1.1-Kv1.8); these are known as Kv α subunits.

To form a functional Kv channel protein complex, four α subunits, each with six transmembrane domains (TMs), assemble into a tetrameric structure (Illustrated in Figures 1.1A and 1.1B). The TM1-4 helices form the voltage-sensing region, as TM4 possesses conserved arginine residues that respond to

electrostatic forces (Illustrated in Figure 1.2) (28). TM5-6 and the intracellular loop between TM5 and TM6 form the ion-conducting pore (29-32). Within the pore helix is a highly conserved GYG amino acid sequence, which is crucial for the regulation of potassium selectivity (29-32). Kv channels can form functional homotetramers or heterotetramers (22). Heterotetramerization occurs with four Kv α subunits within the same subfamily (22). In addition to α subunits, there are three Kvβ subunits (Kv β1-3), which can alter the kinetics, stability and expression of the Kv α subunits (22).

Kv channels play key roles in a wide variety of cellular processes including regulating cell growth, cell volume, motility, apoptosis, establishing resting membrane potential and vessel tone (1, 5, 7, 21, 22, 24). In the cardiovascular system, Kv channels are important for generating myocardial action potentials and determining action potential duration. Myocardial action potentials are generated from ion channels that conduct inward, depolarizing (Na^+ and Ca^{2+}) currents and outward, repolarizing (K^+) currents (23). The Kv channels underlie the repolarization currents, which are important for returning membrane potential to its resting state. Kv channels play a role in both the early and late phases of repolarization in the action potential, as they encode two types of Kv currents: transient outward currents (I_{to}) and delayed rectifier currents (23). Similar to myocardial action potentials, Kv channels are also important in neuronal excitability, as they are important for the repolarization of the neuronal action potential (33). In the vasculature (e.g. pulmonary and cerebral), Kv channels play an integral role in the myogenic response. In particular, Kv channels establish

vessel tone, maintain resting membrane potential and regulate blood pressure as they promote vasodilation (21, 34).

It is well known that Kv channels play an essential role in maintaining a balance between cell survival and apoptosis and cell growth/proliferation, as they regulate intracellular potassium concentrations (1, 5). Seminal work in lymphocytes demonstrated that changing intracellular potassium concentrations altered a cell's ability to respond to cell death signals (35). In healthy cells, the intracellular potassium concentration ($[K^+]_i$) sits at ~145-150 mM (36-38). If a cell's $[K^+]_i$ is at normal to high levels (~150-200 mM), caspase activation is suppressed, however once $[K^+]_I$ falls to low levels (≤100 mM), caspases are activated (35, 36, 38, 39). Therefore, a threshold level of $[K^+]_i$ must be reached before caspase activation occurs (35, 40). Mechanistically, caspase activation is inhibited by normal to high levels of $[K^+]_i$ (150-200 mM) as a result of K+-dependent inhibition of Apaf-1 oligomerization, a key requirement for formation of the apoptosome (40, 41). In cell growth and proliferation, Kv channels contribute to the regulation of membrane potential (V_m) (1, 3, 4, 24, 42). The V_m of cells changes throughout the cell cycle. Specifically, a hyperpolarization occurs at the G1-S transition due to potassium efflux through Kv channels (1, 43) and depolarization occurs at the G2-M transition (43, 44). Therefore, changes in Kv channel expression can significantly alter potassium flux in the cell and affect cell cycle regulation.

Given the vast roles of Kv channels in cell physiology, it is not unexpected that dysregulation of Kv channels is linked to numerous disease states, including

atrial fibrillation (AF) (45), pulmonary arterial hypertension (PAH) (46), heart failure (47), and cancer (7, 48, 49).

Structure of the Kv1.5 Channel

Within the Kv channel family, a key channel of interest, and the focus of this thesis, is the Kv1.5 channel. The Kv1.5 channel consists of four α subunits, which generate homo- or hetero-tetramers. The Kv1.5 α subunits can associate with Kvβ ancillary subunits. Three Kvβ subunits have been shown to interact with the Kv1.5 channel, Kvβ1.2, Kvβ1.3 and Kvβ2.1 (50-52). Kvβ subunits are important as they participate in Kv1.5 α subunit assembly in the endoplasmic reticulum (53), aid in transport to the plasma membrane (51) and modulate activation and inactivation kinetics of the Kv1.5 channel current, I_{Kur} (54-56).

There are two types of inactivation mechanisms for Kv1 channels, N-type and C-type (57-59). Co-expression of Kv1.5 with a Kvβ subunit results in N-type inactivation. This occurs when the inactivation peptide, located on the N-terminus of the β subunit, enters the inner cavity of the channel pore and obstructs ion passage (60). Inactivation of the Kv1.5 channel alone is of the C-type. This form of inactivation is due to conformational changes around the exterior mouth of the pore, which inhibit access to the selectivity filter (56).

The Kv1.5 channel is part of the voltage-gated Kv channel family, whose role is to regulate potassium ion flux in response to alterations in the membrane potential, making Kv channels responsible for establishing the resting membrane potential in cells (23, 61, 62). Kv channels possess voltage-sensor domains,

which are positioned at the periphery of the channel and consist of the S1-S4 transmembrane segments (Figure 1.2) (31, 63). The voltage-sensor domains respond to changes in membrane potential, in particular the S4 segment, as it possesses positively charged arginines, causing structural re-arrangement (31, 63). The structural re-arrangement results in a conformational change in the conduction pore, which can either promote or occlude passage of potassium through the channel and down its concentration gradient (31, 63).

Another unique feature of the Kv1.5 channel is that it is an oxygen sensor, meaning it possesses an oxygen (O_2)-sensitive current. Specifically, potassium flux through the channel can be increased or decreased upon exposure to reactive oxygen species (ROS) (46, 64). Though controversial as to its effect, it is known that the Kv1.5 channel current is responsive to ROS (46, 64-66). Currently, the molecular physiology underlying O_2 sensitivity of the Kv1.5 channel remains unclear. However, work with other O_2-sensitive Kv channels (i.e. Kv1.2, Kv2.1, Kv3.1, Kv3.3, Kv4.2 and Kv9.3), has identified key channel components regulated by ROS (67). Specifically, ROS oxidize thiol-containing residues (cysteine and methionine) in the N-terminus of the Kv channel, which results in the formation of disulfide bridges with other thiol-residues in the channel (68). The disulfide bridges physically prevent channel inactivation and therefore promote Kv channel current (69-71). The Kvβ subunits also play a role in redox sensing, as each subunit possesses the oxygen-binding protein, NAPDH oxidase (72). In the presence of oxygen, NAPDH becomes oxidized (NADP+) and produces ROS, which can oxidize thiol-residues and contribute to Kv channel

inactivation (70-72). However, this can only occur if the β subunit is coupled to a Kv channel (70-72).

Kv1.5 Channel Function in Physiological and Pathological Conditions

Kv1.5 channel expression is detected in many cell types in the human body, including atrial myocytes (45), pulmonary arterial smooth muscle cells (PASMCs) (73, 74), GH_3 pituitary cells (75, 76), oligodendrocyte precursor cells (77, 78), macrophages (79), somato dendritic Purkinje cells of the cerebellum (80) and cancer cells (7, 81). Aside from its diverse expression, the Kv1.5 channel is of particular importance in O_2-sensitive tissues, as it possesses an O_2-sensitive channel and its expression, at both a protein and transcript level, is directly altered by oxidative factors, mainly HIF-1α (82) and hydrogen peroxide (H_2O_2) (83). Therefore, it is no surprise that due to its voltage and oxygen-sensitivity the Kv1.5 channel has a dynamic role in physiologic and pathophysiological states that are associated with ROS and oxidative stress. In fact, most of what is known about Kv1.5 channel function stems from its role in disease, particularly atrial fibrillation (AF), pulmonary arterial hypertension (PAH) and brain ischemia.

Atrium

In the cardiovascular system, specifically in the human atrium, Kv1.5 underlies the ultra-rapid delayed recitifier current (I_{Kur}), important for atrial repolarization and action potential duration (84, 85). Therefore, the Kv1.5 channel is important for returning the membrane potential of atrial myocytes from

a depolarized state (~ 52 mV) back towards its resting membrane potential (~-80 mV) (23). This is accomplished through the movement of potassium down its concentration gradient, which results in the efflux of potassium out of the cell, driving the membrane potential towards a negative membrane voltage. In atrial myocytes, the Kv1.5 channel current is activated at potentials in the range of -20 mV to + 50 mV (86, 87). Interestingly, the reported activation of the voltage-sensitive Kv1.5 channel current is slightly different in varying tissues. For example, heterologous expression of the Kv1.5 channel in mouse cells, Kv1.5 channel current activates at potentials between -10 to +60 mV and partially inactivates at +60 mV (88). Despite the slight differences, the purpose of the activation range of the Kv1.5 channel current is to establish the resting membrane potential in many cells, particularly in vascular smooth muscle.

Vascular smooth muscle cells, and likely cancer cells, are both examples of tissues with small-size cells and high input resistance. This makes the cells particularly sensitive to changes in membrane potential, with small changes in potential leading to large changes in the opening of channels and therefore potassium ion current. Because the flux of potassium is essential for maintaining cellular ionic homeostasis and critical for regulating cellular processes, this implies that small changes in membrane potential can have profound effects on a cells ability to function normally. Thus, the precise biophysical characterization of the Kv1.5 channel remains an area of extensive research in relation to both healthy and diseased states.

Kv1.5 is best known for its role in cardiovascular disease, in particular atrial fibrillation (AF) (45). AF is the most common cardiac arrhythmia present in the population. Shortening of the atrial effective refractory period of the atrium is an important factor in acute and persistent AF. As previously mentioned Kv1.5 underlies the I_{Kur} current, which is important for atrial repolarization and action potential duration (84, 85). An early study established that patients in chronic AF have electrophysiological and physiological remodeling and a consequence of this remodeling is the downregulation of Kv1.5 channel expression (45). One aspect associated with structural and electrical remodeling of the heart in AF, is the excessive production of ROS (89). The modulation of Kv1.5 by ROS still remains unclear, however it was discovered that H_2O_2 activates Kv1.5 channel current in acute AF (83). Furthermore, the activation of Kv1.5 reduces action potential duration and leads to the shortening of the atrial refractory period and therefore it is hypothesized to initiate and/or contribute to the duration of AF (83). In particular, it is thought that initial activation of the Kv1.5 channel in acute AF contributes to the continuation of AF, leading to chronic AF and the eventual downregulation of the Kv1.5 channel (83). Therefore, it is clear that the Kv1.5 channel and its I_{Kur} current function to maintain normal physiologic cardiac rhythm.

Pulmonary Vasculature

PASMCs rely on Kv channels, in particular the Kv1.5 channel, to determine resting membrane potential and the concentration of cytosolic free Ca^{2+} (90). In the pulmonary vasculature, exposure to acute hypoxia inhibits Kv

channels, increases cytosolic Ca^{2+}, depolarizes the PASMCs and causes pulmonary vasoconstriction (46). In the pathophysiological disease, pulmonary arterial hypertension (PAH), hypoxia initiates vasoconstriction as an adaptive mechanism to redistribute blood flow to better-oxygenated areas of the lungs (91). Hypoxic pulmonary vasoconstriction is mediated by the inhibition of Kv potassium channels in PASMCs, predominantly via acute downregulation of the O_2-sensitive Kv1.5 channel (82, 90, 92, 93). In PAH, conditions of acute and chronic hypoxia result in the significant downregulation of Kv1.5 channel expression and the Kv1.5-encoding gene transcript *KCNA5* (82, 94-97). Therefore, loss of Kv1.5 expression in is a contributing factor in the pathogenesis of PAH (95, 98, 99).

Recently, it was discovered that the Kv1.5 channel is part of a key pathway, the mitochondria-ROS-HIF-Kv pathway, whose disruption contributes to the development of PAH (100). It was elucidated that under hypoxic conditions, HIF-1α represses *KCNA5* and ROS withdrawal inhibits the opening of Kv1.5 channels (46). This results in pulmonary vasoconstriction, the eventual hyperpolarization of the mitochondria and ultimately disrupts the mitochondria-ROS-HIF-Kv pathway (46). Furthermore, re-introducing the Kv1.5 channel improves pulmonary hypertension, as it restores the O_2-sensitive current and reduces pulmonary vascular resistance (82). Interestingly, the Kv1.5 channel also underlies mitochondrial-mediated cell death in PAH, due to its involvement in the mitochondria-ROS-HIF-Kv pathway and the discovery that the pro-apoptotic activator cytochrome-c activates Kv channels, while the anti-apoptotic

bcl-2 inhibits Kv channels (101). Together, this demonstrates that the function of Kv1.5 in the pulmonary vasculature is to maintain vessel tone, regulate an adaptive response to hypoxic stress and mediate apoptotic cell death.

Brain

Impaired blood flow in the brain produces injury and brain ischemia. Exposure of the brain to short periods of noninjurious ischemia (pre-conditioning), results in ischemic tolerance, meaning the brain does not sustain injury when exposed to a subsequent ischemic insult (102, 103). It was discovered that in response to ischemia, ischemic-tolerant brains suppressed gene expression (103). Interestingly, genomic analysis revealed that a unique feature of ischemic-tolerant brains was the suppression of outward potassium currents, in particular the downregulation of *KCNA5* and its β-subunit *KCNAB2* (103). Furthermore, it was demonstrated that an epigenetic mechanism, involving polycomb group (PcG) proteins, suppressed *KCNA5*. The PcG protein BMI-1 was found to bind and alter *KCNA5* expression, ultimately affecting the functionality of potassium currents (103) (See Section Below: Epigenetic Mechanisms in Cancer). Thus, the function of Kv1.5 in the brain, aside from maintaining vessel tone in the vasculature (104), is to regulate ischemic tolerance, an adaptive response to oxygen limitation.

Regulation of Kv1.5 Channel Expression

Kv1.5 channel expression is regulated at the level of the protein and at the transcript through diverse mechanisms. This section focuses on protein regulation, post-translational modifications and transcription factors.

Protein Trafficking and Localization

Steady-state expression of the Kv1.5 channel at the cell-surface is a function of protein trafficking and localization. Scaffolding proteins and anterograde and retrograde trafficking pathways all function to regulate Kv1.5 channel surface expression. Scaffolding proteins function to stabilize Kv1.5 at the cell surface through regulating its localization within the plasma membrane (105, 106). In particular, membrane-associated guanylate kinase (MAGUK) proteins, such as synapse-associated protein 97 (SAP97), bind to Kv1.5 and target it to special domains at the cell surface (e.g. intercalated disk) (105). Anterograde trafficking of the channel occurs via the dynein motor along microtubules (107-109) and the myosin-V-A motor along actin filaments (110). Retrograde trafficking of the Kv1.5 channel is a Rab-GTPase specific process where individual Rab proteins have unique functions. Association with Rab4- or Rab11-endocytic vesicles recycles Kv1.5 to the surface, while association with the Rab7-endocytic vesicle results in Kv1.5 degradation (108, 111). Recent evidence also demonstrates that the myosin-V-B motor mediates post-endocytic recycling of Kv1.5 and this is dependent on coupling to Rab11 (110).

Post-Transcriptional Modifications

Post-translational modifications of Kv1.5 channels regulate surface expression, stability and biophysical properties of the channel. Numerous post-translational modifications of the channel have been identified, including phosphorylation, sumoylation, nitrosylation, palmitoylation, glycosylation, thioacylation, ubiquitination and sulfenic acid modification. Sumoylation, phosphorylation and nitrosylation all affect the biophysical properties of the Kv1.5 channel. Sumoylation regulates Kv1.5 channel inactivation (112), nitrosylation has been shown to decrease the Kv1.5 channel current I_{Kur} (113), and phosphorylation impacts the amplitude of the I_{Kur} current. Specifically, PKA increases (114-116) whereas PKC-mediated phosphorylation decreases I_{Kur} amplitude (117). Palmitoylation (118) and glycosylation (119) both impact Kv1.5 channel localization to the plasma membrane and therefore affect its surface expression. Thioacylation regulates steady-state expression of Kv1.5 as mutations of these residues increased Kv1.5 cell surface expression (120). Lastly, sulfenic acid modification (121) and ubiquitination (122) of Kv1.5 targets the channel for degradation.

Transcription Factors

As Kv1.5 channel expression is significantly altered in PAH and AF, the transcriptional machinery controlling expression of *KCNA5* has predominantly been determined in these pathophysiological diseases. Currently, three main transcription factors, Sp1, HIF-1α and NFAT, have been determined to directly affect *KCNA5* expression (92, 123, 124).

Sp1 is a triple zinc finger protein that binds G/C rich nucleotide sequences, including GC and CACCC boxes, and is a modulator of tissue specific transcription (124). The *KCNA5* promoter activity in vascular smooth muscle is dependent on Sp1 regulation through CACCC box motifs. Inhibition of Sp1-KCNA5 promoter interactions prevented promoter activity, while expression of exogenous Sp1 augmented promoter activity (124). While Sp1 promotes *KCNA5* transcription, HIF-1α and NFAT repress *KCNA5* transcription. Interestingly, a negative correlation between the transcription factors HIF-1α and NFAT, and Kv1.5 expression has been found in PASMCs (92, 123). Evidence demonstrates that blocking HIF-1α activation using a dominant-negative HIF-1α restores *KCNA5* transcript and Kv1.5 expression and function (100). Furthermore, the presence of the RCGTG binding motif, a core-binding motif of HIF-1α, in the *KCNA5* promoter suggests that HIF-1α directly binds the *KCNA5* locus (125, 126). Though HIF-1α has been demonstrated to repress *KCNA5* transcription, opposing literature suggests HIF-1α induces Kv1.5 channel expression (127), adding debate to the field. In PAH, loss of Kv channels, especially Kv1.5, causes membrane depolarization in PASMCs and results in an elevation of cytosolic Ca^{2+}. The increase in Ca^{2+} then activates the transcription factor, NFAT (128). Activation of NFAT reinforces the downregulation of the Kv1.5 channel through repression of *KCNA5 (128).* Multiple NFAT binding elements, GGAAA, are present in the *KCNA5* promoter, suggesting that NFAT binds the *KCNA5* locus. Of the four isoforms of NFAT, NFATc1-c4, NFATc2 is

the predominant isoform in PAH that has been demonstrated to repress *KCNA5* (123, 129).

Aberrant Expression of Kv1.5/*KCNA5* in Cancer

Aberrant expression of ion channels is prevalent in cancer and dysregulation of potassium channels has, in particular, been well documented (see recent reviews (7, 13, 49)). In general, expression of potassium channels is mostly upregulated in cancer (Illustrated in Table 1), and upregulation is a consequence of cancer cell transformation (7). Since potassium channels influence many cell behaviors, all of which are important for cancer pathogenesis, the hijacking of potassium ion channel function by malignant cells in not surprising (Illustrated in Table 2). Significantly, however, though most channels are upregulated in cancer, two channels are uniquely downregulated, and one of these is Kv1.5 (*KCNA5*) (9-12, 14, 15, 81) (Illustrated in Table 1). Overall, the net effect of potassium channel deregulation can have a tumorigenic or tumor suppressive impact, depending on the channel and the cellular context. Thus, elucidation of the role and mechanism of Kv1.5 downregulation in cancer has the potential to offer new insights into tumor pathogenesis.

Genetic Abnormalities

Recent studies observe a dysregulation in Kv1.5 expression in human cancer. Specifically, Kv1.5 expression is downregulated and its expression is inversely correlated with tumor aggression in gliomas and lymphomas (8, 10-12, 14, 15, 130). Further observations note that the mutation rate of *KCNA5* across

multiple cancer types is less that 10% and only a few of these somatic mutations are predicted to result in a gain or loss of *KCNA5* function (the results shown here are in part based on data generated by the TCGA Research Network: http://cancergenome.nih.gov/ and (131)). Furthermore, recurrent deletions have yet to be described for this gene. Together these observations suggest that the downregulation of *KCNA5* in cancer is unlikely to be caused by genetic mutations. Thus, the mechanism(s) underlying Kv1.5 channel and *KCNA5* transcript downregulation remain to be elucidated and we aim to fill this gap with the work presented in this thesis.

Cell Proliferation and Apoptosis

The Kv1.5 channel has been shown to regulate the G1/S transition in the cell cycle (132, 133). It was discovered that Kv1.5 inhibited skeletal muscle cell proliferation at the G1/S transition, through a mechanism that increased cyclin-dependent kinase inhibitors p21 and p27 and decreased expression of cyclins A and D1 (133). Therefore, it is hypothesized that the Kv1.5 channel is a G1/S checkpoint regulator and cancer cells downregulate its expression to promote cell cycle progression. However, in contrast to the previous study, pharmacological inhibition of the Kv1.5 channel was shown to block B-lymphocyte proliferation and arrest cells in the G1 phase (15). Together, this evidence suggests that the Kv1.5 channel has cell-type specific function.

As mentioned previously, the Kv1.5 channel is important for maintaining a balance between cell survival and apoptosis in non-transformed cells (1, 8, 45, 46, 134, 135). As Kv1.5 and *KCNA5* expression are downregulated in many

human cancers (e.g. glioblastoma and lymphoma), it is hypothesized that this downregulation contributes to cancer cell survival (8, 10-12, 14, 15). Two studies specifically demonstrate an effect of Kv1.5 expression on apoptosis in cancer cells. In lymphocytes, downregulation of Kv1.5 expression prevented apoptosis (10), while in several cancer cell lines, including M059K (glioblastoma), A549 (non-small-cell lung) and MCF-7 (breast) cancer cells (8), reactivation of the Kv1.5 channel induced apoptosis. Interestingly, in the cell line study it was discovered that the transcription factor, NFAT directly represses *KCNA5,* and release of this repression leads to apoptotic cell death (8). Combined, these studies demonstrate that the Kv1.5 channel has an inherent role in governing cancer cell fate. However, despite these studies, a gap remains in understanding the mechanism(s) underlying regulating Kv1.5 expression.

As discussed above, potassium channels have many roles in tumor biology. However, the involvement of potassium channels in cancer tumorigenesis is still an emerging field and it is clear that potassium channels have cell-type specific functions. Therefore, it is necessary to continue to define the roles of specific potassium channels such as Kv1.5, in cancer biology. We focus on the Kv1.5 channel, in particular, as it possesses oxygen and voltage-sensitivity, which we hypothesize, contributes to its role in cancer. As mentioned previously, the oxygen-sensitivity and voltage-sensitivity of the Kv1.5 channel is dependent on its environment and these aspects of the Kv1.5 channel have yet to be explored in cancer. This thesis begins to address those gaps in knowledge by investigating the function and role of the Kv1.5 channel in the two pediatric

solid tumors, ES and NB. In addition to understanding the roles of potassium channels in cancer biology, studies also need to address the mechanisms regulating channel expression, as it will be necessary for the development of targeted therapies that seek to exploit the tumor-specific nature of potassium channel deregulation. Finally, understanding the influence of the microenvironment and its stressors on potassium channel regulation will also be pertinent to understanding how to manipulate such mechanisms.

Pediatric Cancer: A Clinical Problem

As previously mentioned this thesis focuses on the two pediatric solid tumors, Ewing Sarcoma (ES) and Neuroblastoma (NB). Though effective treatment regimens have been developed, a predominant issue associated with these tumors is they are plagued by relapse. However, before proposing new therapeutic avenues, let me first describe the treatment backbone of these diseases at diagnosis.

Upon diagnosis and following tumor staging, standard chemotherapy induction agents used in ES patients include alternating cycles of vincristine, doxorubicin, cyclophosphamide, ifosfamide and etoposide, known as the VDC-IE regime (17). In NB patients, the standard chemotherapy induction agents used include, vincristine, doxorubicin, cyclophosphamide and topotecan (16, 18). Given the aggressive nature of ES and NB, treatment regimens consist of a combination of chemotherapy, surgery and radiation.

Relapsed ES and NB tumors are often highly resistant to cytotoxic drugs and micro-environmental stressors, limiting treatment options and re-enforcing the need for new therapeutic avenues. Recent discoveries have determined that ES and NB are epigenetically driven diseases, as disruptions in epigenetic machinery contribute to the initiation and progression of these tumors. Therefore, understanding how these tumors manipulate epigenetic mechanisms will provide novel therapeutic targets that can be incorporated into ES and NB treatment regimens.

Epigenetic Mechanisms in Cancer

Epigenetics

Traditionally, cancer was considered to be a disease solely driven by an accumulation of genetic mutations (19). Over the last decade, we have learned that epigenetic abnormalities are equally important drivers of cancer tumorigenesis, thus causing a paradigm shift (136). Epigenetics is defined as a heritable change in gene expression not caused by alterations in DNA sequence. Epigenetic regulation of gene expression is achieved through three over-arching mechanisms: DNA methylation, histone modifications and chromatin remodeling (Illustrated in Figure 1.3).

CpG rich sequences, known as CpG islands, are targets of DNA methyltransferases (DNMTs) and these enzymes methylate cytosines at the 5'-position (Figure 1.3A). There are two groups of DNMTs, de *novo* DNMTs (137), which newly methylate cytosines, and maintenance DNMTs (138), which

methylate a DNA strand if its partner strand is already methylated. In general, DNA methylation modifies gene expression by silencing genes. This occurs through hypermethylation of promoters, which then can directly (139) and sterically (140-142) prevent transcription factors from accessing the DNA and initiating transcription.

Chromatin is a condensed form of DNA that serves as a way to control gene expression. The basic structure of chromatin consists of the nucleosome, which is a complex of DNA wrapped around a histone octamer. Nucleosomes can be packed together to form condensed chromatin, known as heterochromatin, or they can be loosely spaced apart, a chromatin state known as euchromatin. Gene expression occurs in the euchromatin state, while heterochromatin lacks gene transcription. Epigenetic regulation influences gene expression by altering the chromatin state largely through the use of post-translational modifications and chromatin remodeling complexes.

Post-translational histone modifications occur either on histone tails or the core histones, and they contribute to gene regulation. There are a variety of histone modifications, including acetylation, methylation, phosphorylation, ubiquitination and sumoylation, and these modifications are associated with transcription activation or repression (Figure 1.3B). Proteins that regulate histone modifications are known as histone regulators and three classes exist: writers, which add the marks; erasers, which remove the marks; and readers, which recognize specific modifications and recruit translational machinery. The two main and well-studied histone modifications are acetylation and methylation.

Acetylation of lysine residues on the tails of histones H3 and H4 is associated with transcription activation. The writers for acetylation are histone acetyltransferases (HATs) (e.g. CBP) and these marks are in turn erased by histone deacetylases (HDACs) (i.e. HDAC1-3) (143). There are three levels of methylation, mono-, di- and tri-methylation that occur at lysine residues on the tails of histone 3. The writers for methylation are histone methyltransferases (HMTs) and the erasers are histone demethylases (e.g. LSD1, JMJD2C) (144, 145). There are two main classes of HMTs, the proteins that promote transcription and the proteins that silence transcription. The Trithorax group proteins methylate lysine 4 on histone 3 by the mixed lineage leukemia (*MLL*) genes, *MLL1-3*, and they promote transcription. PcG proteins are composed of two main complexes, polycomb repressive complex 1 (PRC1) that monoubiquinates lysine 119 on histone H2A and polycomb repressive complex 2 (PRC2), which functions to methylate lysine 27 on histone 3, both histone modifications silence transcription. In stem cells, PcG proteins are important for silencing genes involved in differentiation, and in so doing maintain self-renewal and pluripotency (146). Lastly, the readers of all histone modifications are chromatin-associated proteins (e.g. HP1, MeCP2), which maintain or alter the chromatin, respectively (147, 148).

The mechanisms that alter the structure of chromatin are ATP-dependent multiprotein complexes, known as chromatin remodelers. These complexes use the energy from the hydrolysis of ATP to reposition nucleosomes and remove or exchange histones, creating open or closed regions of DNA (149, 150) (Figure

1.3C). There are five families of chromatin remodelers: SWI/SNF, ISWI, NuRD/Mi-2/CHS, INO80 and SWR1 (Figure 1.3C). These complexes can restructure chromatin to either an active or inactive state, which influences accessibility of transcription factors to promoter regions; therefore chromatin remodelers can both activate and silence gene expression.

Epigenetic Dysregulation

Cancer cells hijack epigenetic mechanisms to alter gene expression. In particular, cancer cells use epigenetic mechanisms to aberrantly silence regulatory and tumor suppressor genes, to promote uncontrolled tumor growth and survival.

Early studies revealed cancer cells have different DNA methylation patterns compared to normal cells. It was discovered that cancer cells hypomethylate intergenic regions of genes and hypermethylate the promoter region of genes (141, 151-153). The regions that are targets of hypermethylation contain CpG islands and are frequently associated with tumor suppressor genes, (e.g. Retinoblastoma (pRb)) (154). While the role of promoter hypermethylation in cancer is generally understood, the role of intergenic hypomethylation remains unclear. It is currently proposed that loss of methylation results in genomic instability, which promotes overexpression of certain genes (e.g. CD30, CYCLIND2) (155, 156) and thus drives disease progression (157). Furthermore, though it is observed that DNMTs and DNA methylation binding proteins are slightly elevated in human tumors, it is currently unknown as to their role in the epigenome changes that occur in cancer.

In cancer cells, a global change is also seen in histone acetylation and methylation. Genome-wide profile analysis of histone modifications in cancer cells discovered a loss of monoacetylation of lysine 16 and trimethylation of lysine 20 on histone 4 (158). Furthermore, a gain in methylation of the specific lysine residue 27 on histone 3 is seen in cancer cells (143, 159). The alterations of histone modifications in cancer are due to a disruption in their regulators. The loss of acetylation is due to translocations and inactivating mutations of HATs, as seen with CBP in leukemia (158). HDACs can also be targeted for inactivation, as with HDAC2 (160); however they can also be overexpressed in other cancer types (143). The alteration of HMTs in cancer varies depending on its associated histone modification, as HMTs associated with lysine 27 on histone 3 can be overexpressed (e.g. EZH2), while HMTs associated with lysine 9 on histone 3 can be downregulated (e.g. RIZ1) (143). Therefore, the changes in expression of histone regulators are tumor specific and can have various effects on tumor biology.

Nucleosome remodeling is an important component of gene silencing in cancer. It is well known that mutations in the SWI/SNF complex and the nine genes encoding SW/SNF complex subunits, occur in 20% of all human cancers (161-163). Furthermore, recent studies have determined that these mutations result in the dysregulation of numerous pathways (e.g. retinoblastoma (164) and MYC (165) function), which directly contribute to tumorigenesis (166-168). Ultimately, the importance of chromatin remodelers is linked to DNA methylation

(169, 170) and histone modifications (171, 172), as it is the interaction amongst all three players that dynamically regulates gene expression (136).

Abnormalities in ES and NB

Recent studies have determined that ES and NB are epigenetically driven diseases, as disruptions in epigenetic machinery were found to contribute to the initiation and progression of these tumors (reviewed in ((173)). Key drivers of ES and NB tumorigenicity are the PcG proteins, BMI-1 and EZH2 (174-178). Overexpression of BMI-1 and EZH2 are important in ES and NB tumors, as they maintain cancer cells in a stem-like state and support oncogenesis by suppressing tumor suppressor genes and developmental regulators (146, 174-179). Furthermore, preliminary studies suggest that the overexpression of PcG proteins contribute to alterations in another epigenetic mechanism, DNA methylation. Initial studies in ES reveal that CpG islands of PcG target genes are hypermethylated (Lawlor lab, unpublished) and genome-wide microarray data in NB reveals a direct correlation between *EZH2* and *DNMT1 (173)*. Together, these data suggest that PcG proteins may recruit DNMTs to induce permanent gene silencing of key loci. Therefore, targeting specific epigenetic mechanisms, such as PcG proteins, is a promising approach to inhibiting ES and NB tumorigenicity and preventing relapse.

In this thesis, we test the hypothesis that downregulation of the Kv1.5 channel in ES and NB cells promotes tumor pathogenesis, and that this is achieved through the impact of channel dysregulation on cell death and proliferation. Further, in light of the lack of evidence for genetic mutation of

KCNA5, this thesis investigates the contribution of epigenetic mechanisms as mediators of Kv1.5 channel suppression.

Thesis Summary

The aim of this thesis is to better understand the mechanisms underlying Kv1.5 channel dysregulation in human cancer and to elucidate the contribution of Kv1.5 downregulation to cancer cell biology. In Chapter Two, we investigate epigenetic silencing of *KCNA5* by PcG proteins and how it promotes cancer cell survival under conditions of physiological stress. This was accomplished through genetic and pharmacologic modulation of Kv1.5 channel function and PcG proteins and *KCNA5* promoter analysis under ambient (21% O_2) and stress-inducing (hypoxia (1% O_2) and growth-factor deprivation) conditions. We discovered that ES and NB cells are resistant to stress-induced cell death and this resistance is mechanistically linked to downregulation of Kv1.5 channel activity. Further, we determined that suppression of the Kv1.5 channel is mediated by polycomb-dependent repression of the *KCNA5* locus. In Chapter Three, we investigate whether the *KCNA5* promoter is hypermethylated in Ewing sarcoma and its consequence on ES cell biology. This was determined using two methylation assays: an Illumina GoldenGate® methylation array and MethyLight, and pharmacologic modulation of DNA methyltransferases and Kv1.5 channel function. In these studies, we found that the *KCNA5* promoter is hypermethylated in ES and other cancer types. Furthermore, we determined that the promoter can be demethylated and transcript expressed upon exposure to the hypomethylating

agent, decitabine. Interestingly, decitabine treatment leads to a significant decrease in ES cell growth, which is partially reversed through pharmacologic inhibition of the Kv1.5 channel. Together these chapters implicate that epigenetic repression of *KCNA5* promotes two key components of tumor pathogenesis, cell survival (Chapter Two) and cell proliferation (Chapter Three).

The final chapter of this thesis discusses the therapeutic implications of understanding how the *KCNA5* locus is epigenetically regulated. Specifically, it focuses on epigenetic modifiers and ion channel targeted therapy in cancer. Furthermore, this chapter explores the future potential of Kv1.5 channel research in relation to cancer stem cells and the mitochondria. Together, the Kv1.5 channel makes an important target in cancer due to its unique properties of voltage and oxygen-sensitivity, which contribute to its involvement in mediating cell survival and cell proliferation. Therefore, fully understanding its regulation will provide another mechanism that can be therapeutically exploited, which is of key importance in cancer relapse.

I would like to acknowledge the work from our collaborators, as their help contributed to the formulation of this thesis. Dr. Daniel J. Weisenberger and Dr. Peter W. Laird at the University of Southern California carried out the MethyLight analysis shown in Figure 3.1B. The primary Ewing sarcoma tumor samples analyzed in the studies in Figure 3.1B were kindly provided by Dr. Scott Borinstein at Vanderbilt.

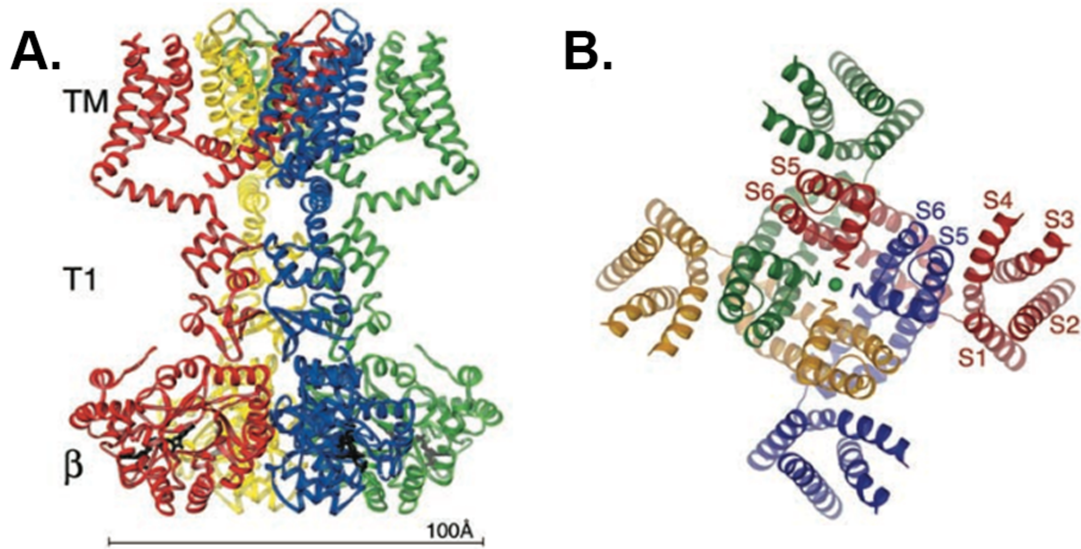


Figure 1.1. Representation of a Shaker Kv Channel structure.

(A) Stereoview of a Kv channel from the side, represented in ribbon diagram. The four α-subunits are a different color and include the voltage sensor and pore, the T1 domain and the β-subunit tetramer. TM represents the transmembrane spanning segments. (B) Tetramer structure of a Kv channel from the extracellular side of the pore. Provided by (32) in (A) and (31) in (B).

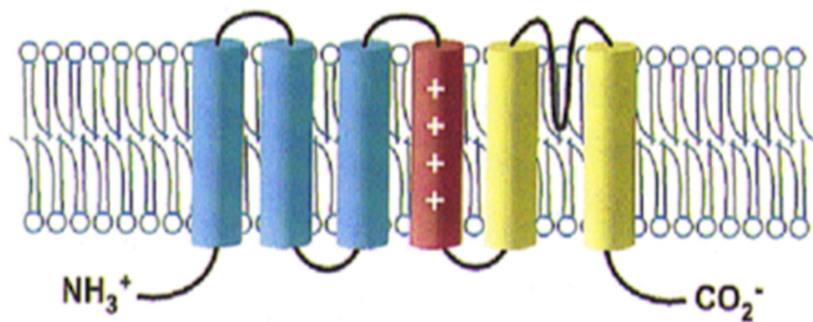


Figure 1.2. Topology of the α-Subunit of a Kv Channel.

The α-subunit consists of six transmembrane segments (S1-S6). S1-S3 segments are colored blue, the positively charged S4 region is red and S5-S6 segments are yellow. The N- and C-terminal domains of the α-subunit are located on the intracellular side. Provided by (30).

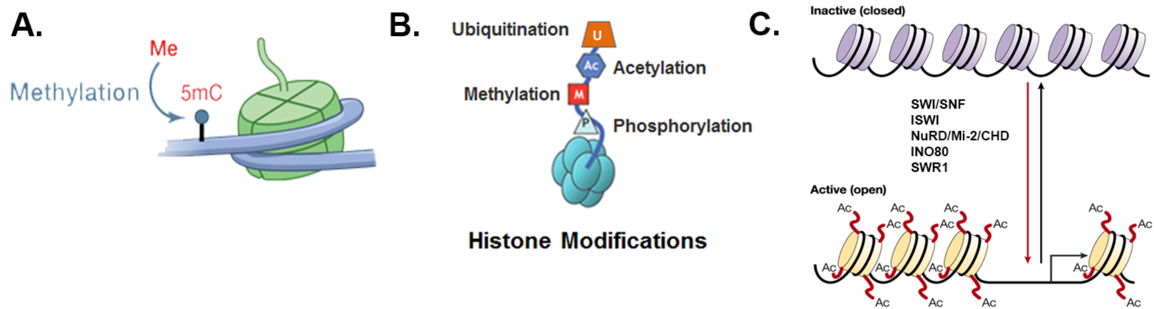


Figure 1.3. Gene Expression is Regulated by Three Epigenetic Mechanisms.

(A) DNA methylation is a biochemical process where a methyl group is placed on cytosine nucleotides. DNA methylation predominantly occurs at CpG islands in the genome. DNA methyltransferases (DNMTs) place a methyl group on the 5' position of cytosine nucleotides. (B) DNA wraps around histones, composed of two H2A-H3B dimers and a H3-H4 tetramer. Histones have a feature of long tails and this is the location of post-translational modifications, known as histone modifications. There are numerous modifications that can occur and they include methylation, acetylation, phosphorylation and ubiquitination, all of which can positively or negatively regulate gene expression. (C) Modification of the chromatin structure to allow access to regulatory transcriptional machinery occurs via chromatin remodeling complexes. There are currently five known chromatin remodelers, SWI/SNF, ISWI, NuRD/MI-2/CHD, INO80 and SWR1. Adapted from (180) in (A), Luong, P. Basic Principles of Genetics Lecture Series in (B) and provided by (181) in (C).

Table 1. Potassium Channel Expression in Cancer.

Information provided by (7).

Cancer type	Channel gene																					
	Kv1.1 KCNA1	Kv1.3 KCNA3	Kv1.5 KCNA5	Kv3.4 KCNC4	Kv4.1 KCND1	Kv7.1 KCNQ1	Kv10.1 KCNH1	Kv10.2 KCNH5	Kv11.1 KCNH2	K_{Ca}1.1 KCNMA1	K_{Ca}2.3 SK3	K_{Ca}3.1 IK1	Kir2.2 KCNJ12	Kir3.1 KCNJ3	Kir3.4 KCNJ5	Kir4.1 KCNJ10	Kir6.1 KCNJ8	Kir6.2 KCNJ11	K2P2.1 KCNK2	K2P3.1 KCNK3	K2P5.1 KCNK5	K2P9.1 KCNK9
Adrenal															Recurring Somatic mutation					Overexpressed		
Blood			Downregulated				Overexpressed		Overexpressed													
Bone							Overexpressed			Overexpressed												
Brain	Overexpressed		Downregulated					Overexpressed	Overexpressed	Overexpressed		Overexpressed				Overexpressed	Overexpressed	Overexpressed				
Breast		Overexpressed			Overexpressed		Overexpressed		Overexpressed	Overexpressed	Overexpressed	Overexpressed	Overexpressed	Overexpressed			Overexpressed	Overexpressed			Overexpressed	Overexpressed
Cervix							Overexpressed															
GI tract					Overexpressed	Downregulated	Overexpressed		Overexpressed		Overexpressed	Overexpressed	Overexpressed									Overexpressed
Head and neck				Overexpressed			Overexpressed		Overexpressed													
Kidney							Overexpressed		Overexpressed													
Lung		Overexpressed							Overexpressed					Overexpressed								Overexpressed
Lymphoma		Overexpressed																				
Melanoma									Overexpressed		Overexpressed	Overexpressed					Overexpressed	Overexpressed				Overexpressed
Ovary							Overexpressed		Overexpressed	Overexpressed												
Pancreas		Overexpressed					Overexpressed							Overexpressed								
Prostate		Overexpressed								Overexpressed		Overexpressed	Overexpressed						Overexpressed			
Sarcoma							Overexpressed															
Thyroid									Overexpressed													
Uterine																	Overexpressed	Overexpressed				

Overexpressed
Downregulated
Recurring Somatic mutation

Table 2. Cell Behaviors Potassium Channels Influence in Cancer.

Information provided by (49).

Protein	Gene	Migration	Proliferation	Apoptosis	Angiogenesis
Kv1.1	*KCNA1*			Macrophages	
Kv1.3	*KCNA3*			Blood Cells	
Kv1.5	*KCNA5*			Macrophages	
Kv10.1	*KCNH1*	AML and Breast	Cervix, Neuroblastoma and Breast		Cellular models
Kv10.2	*KCNH5*		Medulloblastoma		
Kv11.1	*KCNH2*	AML, Thyroid and Breast			Glioblastoma
Kir1.1	*KCNJ1*			Blood cells	
Kir3.4	*KCNJ5*		Adrenal		
K2P5.1	*KCNK5*		Breast		
K2P9.1	*KCNK9*	Breast	Breast	Breast, Neurons and Glioma	
KCa1.1	*KCNMA1*	Neurons and Glioma		Glioma	
KCa2.3	*KCNN3*	Breast			
KCa3.1	*KCNN4*	Glioma	Vascular smooth muscle	Glioma and Colon	

CHAPTER 2

POLYCOMB-DEPENDENT REPRESSION OF THE POTASSIUM CHANNEL-ENCODING GENE *KCNA5* PROMOTES CANCER CELL SURVIVAL UNDER CONDITIONS OF STRESS[1]

Abstract

Relapse after clinical remission remains a leading cause of cancer-associated death. Although the mechanisms of tumor relapse are complex, the ability of cancer cells to survive physiologic stress is a prerequisite for recurrence. Ewing sarcoma (ES) and neuroblastoma (NB) are aggressive cancers that frequently relapse after initial remission. In addition, both tumors over-express the polycomb group (PcG) proteins BMI-1 and EZH2, which contribute to tumorigenicity. We have discovered that ES and NB resist hypoxic stress-induced death and that survival depends on PcG function. Epigenetic repression of developmental programs is the most well established cancer-associated function of PcG proteins. However, we noted that voltage-gated potassium (Kv) channel genes are also targets of PcG regulation in stem cells. Given the role of potassium in regulating apoptosis, we reasoned that repression

[1] **Ryland KE**, Svoboda LK, Vesely ED, McIntyre JC, Zhang L, Martens JR, et al. Polycomb-dependent repression of the potassium channel-encoding gene KCNA5 promotes cancer cell survival under conditions of stress. Oncogene. 2014. doi: 10.1038/onc.2014.384.

of Kv channel genes might play a role in cancer cell survival. Here, we describe our novel finding that PcG-dependent repression of the Kv1.5 channel gene, *KCNA5,* contributes to cancer cell survival under conditions of stress. We show that survival of cancer cells in stress is dependent upon suppression of Kv1.5 channel function. The *KCNA5* promoter is marked in cancer cells with PcG-dependent chromatin repressive modifications that increase in hypoxia. Genetic and pharmacologic inhibition of BMI-1 and EZH2, respectively, restore *KCNA5* expression, which sensitizes cells to stress-induced death. In addition, ectopic expression of the Kv1.5 channel induces apoptotic cell death under conditions of hypoxia. These findings identify a novel role for PcG proteins in promoting cancer cell survival via repression of *KCNA5*.

Introduction

The ability to resist cell death is a hallmark of cancer (19). Cancer cells are able to survive despite exposure to cell intrinsic (e.g. metabolic and genotoxic) and extrinsic (e.g. hypoxia, nutrient deprivation) stress (19). This ability to survive conditions of stress allows cancer cells to escape physiologic death responses that are induced upon exposure to hostile microenvironments. In rapidly growing solid tumors the cancer outstrips its blood supply, thereby subjecting the cancer cells to a hostile microenvironment that is characterized by oxygen, growth factor and nutrient deprivation (19, 182). Furthermore, chemotherapy and radiation exacerbate the hostile local microenvironment by inducing tumor necrosis. Despite exposure to these tremendous stresses, however, solid tumors often

recur after clinical remission and relapse remains a leading cause of cancer-associated death. The mechanisms of tumor relapse are both diverse and complex but elucidating how cancer cells resist stress-induced death has the potential to uncover novel opportunities for cancer therapy.

Stem cells harbor the ability to self-renew indefinitely and are epigenetically programmed to resist differentiation and to survive in hypoxic niche environments (183). Tumor cells often hijack normal stem cell processes to support their propagation and this is particularly evident in cancer cell populations that display tumor-initiating properties (19, 184). Although the mechanisms that support maintenance of stem cell traits are complex (183), chromatin repressive complexes are essential mediators of stemness and also crucial contributors to cancer pathogenesis (185). Among the best characterized of the chromatin repressive complexes are the polycomb group protein complexes PRC1 and PRC2 (reviewed in (185) and (179)). Polycomb proteins function to silence target gene loci *via* direct post-translational modification of histones. In particular, the PRC1 complex proteins BMI-1 and RING1B cooperate to induce ubiquitination of histone 2A at lysine residue 119 (H2AubK119), while the PRC2 member EZH2 mediates methylation of histone 3 at lysine residue 27 (H3K27me3) (185). Together these chromatin marks support maintenance of a repressed chromatin state that inhibits transcriptional activation (179). Both BMI-1 and EZH2 are highly over-expressed by many human cancers and play central roles in tumor initiation and tumor progression (179). In particular, over-expression of polycomb proteins is evident in tumor-initiating cell populations (186) and in the aggressive

pediatric solid tumors Ewing sarcoma (ES) and neuroblastoma (NB) (174-178). The precise targets of polycomb-dependent regulation are cell type and context specific but, in general, polycomb repressive complexes support maintenance of stemness and oncogenesis by suppressing the expression of tumor suppressor genes and developmental regulators (146, 179)

Controlled regulation of intracellular levels of elemental ions is essential for normal cellular homeostasis. Transmembrane channels control ion flux across cellular membranes and there is abundant evidence that deregulation of calcium and sodium channel function can contribute to cancer pathogenesis in diverse fashions (187, 188). In addition, altered expression, regulation and function of potassium ion channels has been implicated in several cancer hallmarks including abnormal proliferation, resistance to cell death, and enhanced migration (7). In the current study we have identified the voltage-gated potassium channel Kv1.5-encoding gene, *KCNA5*, as a novel target of polycomb-dependent repression in aggressive cancer cells. Significantly, our studies show that epigenetic repression of *KCNA5* contributes to selective survival of cancer cells under conditions of hypoxic stress and implicate activation of the Kv1.5 channel as a central mediator of hypoxia-induced apoptotic cell death.

Experimental Procedures

Cell Culture and Viability

ES cells were cultured in RPMI-1640 media (Gibco, Grand Island, NY, USA) and NB cells in MEM 1x media (Gibco, Grand Island, NY, USA). Media was supplemented with 10% FBS (Atlas Biologicals, Inc., Fort Collins, CO, USA)

and 6 mM L-glutamine (Life Technologies, Grand Island, NY, USA). Identities were confirmed by short tandem repeat profiling. HuVEC cells were cultured in EBM-2 Basal Medium (Lonza, Pittsburgh, PA, USA) supplemented with EGM-2 SingleQuot Kit Suppl. & Growth Factors (Lonza, Pittsburgh, PA, USA). HL-1 cells were cultured in Claycomb media (Sigma-Aldrich, St. Louis, MO, USA-Aldrich) supplemented in 10% FBS (Sigma-Aldrich, St. Louis, MO, USA-Aldrich), 2 mM L-glutamine, 100 µg/mL Penicillin/Streptomycin and 0.1 mM Norepinephrine (Sigma-Aldrich, St. Louis, MO, USA-Aldrich). Cells were maintained in ambient conditions at 37°C in 5% CO_2. For growth factor deprivation and hypoxia studies FBS was removed and cells were placed in 1% O_2 in an xVivo system (Biospherix, Lacona, NY, USA). Viability was determined by cell counting and trypan blue. Data were normalized relative to 0 hours.

Pharmacologic inhibitor studies

4'Aminopyridine (4'AP) (50 µM, Sigma-Aldrich, St. Louis, MO, USA-Aldrich) was prepared in an aqueous solution and diphenyl phosphine oxide-1 (DPO-1) (310 nM, Tocris Bioscience, Bristol, UK) and GSK-126 (1 µM, 10 µM, Active Biochem, Maplewood, NJ, USA) were diluted in dimethyl sulfoxide (DMSO). Cells were pre-treated for 72 hours.

Generation of genetically modified cells

Cell lines were transduced with pLKO.1-puro vectors (Sigma-Aldrich, St. Louis, MO, USA-Aldrich) containing one of two short hairpin RNAs targeting BMI-1 (shBMI-1#156: 5'- CCTAATACTTTCCAGATTGAT-3' or shBMI-1 #157: 5'-CGGAAAGTAAACAAAGACAAA-3') or a non-silencing control sequence (shNS:

5'-CAACAAGATGAAGAGCACCAA-3'). Cells were selected in puromycin (2 μg/mL, Sigma-Aldrich, St. Louis, MO, USA-Aldrich) for 48 hours prior to experimentation.

Adenoviral constructs were generated using pAD/V5/-dest (Invitrogen, Grand Island, NY, USA). Virus was produced using ViraPower (Invitrogen, Grand Island, NY, USA) and purified using ViraPur Adenovirus mini purification (ViraPur, San Diego, CA, USA). Cells were infected with Kv1.5-GFP-WT, KV1.5-GFP-PD and soluble GFP 24 hour's prior to use in an experiment. Response of the infected cells to hypoxia was tested by transferring cells to a 1% O_2 chamber as above.

Quantitative Real-Time PCR

Total RNA extraction was performed using RNeasy® Plus Mini kit (Qiagen, Valencia, CA, USA) and cDNA generated using iScript (Bio-Rad, Hercules, CA, USA). qRT-PCR was performed using validated SYBR primers (sequences below). Analysis was performed in triplicate using the Lightcycler® 480 System (Roche Applied Science, Indianapolis, IN, USA). Data were analyzed by normalizing average Ct values of the gene of interest to the geometric mean of reference genes (*HPRT* and *GAPDH*) using ΔΔCt method.

qRT-PCR Primers

Primers	**Sequence (5' to 3')**
KCNA5 (Human)	Forward: GTA ACG TCA AGG CCA AGA GC
BMI-1 (Human)	Forward: CGT GTA TTG TTC GTT ACC TGG A

KCNA5 (Mouse)	Forward: CAG CGG GTC CTC ATA AAC AT

Western Blot and Immunocytochemistry

Levels of BMI-1 (Millipore, Mouse mAb #05-637, Billerica, MA), EZH2 (Cell Signaling Technology, Rabbit mAb #4905, Danvers, MA), or GAPDH (Cell Signaling Technology, Rabbit mAb #2118) were determined using standard western blot assays as previously described (189).

For apoptosis studies TC-71 cells were treated with 4 µg Etoposide or infected with Kv1.5-WT, Kv1.5-PD or sGFP. After 24 hours, cells were placed in ambient (21% O_2) or hypoxic (1% O_2) conditions. After 8 hours, cells were fixed with 4% paraformaldehyde (10 min.), permeabilized with 0.1% Triton X-100 (10 min.) and blocked with 2% goat serum (20 min.). Cells were incubated with polyclonal anti-cleaved capase-3 (1:200 dilution, Cell Signaling Technology, Rabbit mAb #9661) in 2% goat serum (45 min.), followed by incubation with goat anti-rabbit Alexa Fluor-594 antibody (1:200 dilution, Life Technologies, Grand Island, NY, USA, Grand Island, NY, USA) in 2% goat serum (40 min.). Nuclei were labeled with DAPI (Life Technologies, Grand Island, NY, USA, Grand Island, NY, USA) and mounted with ProLong Gold (Invitrogen, Grand Island, NY, USA, Grand Island, NY, USA).

Cell images were acquired on a Nikon TI81 A1R confocal microscope with a 60x by 1.40x N.A. oil objective. For every experiment 5-10 images were acquired for each condition and 50-100 cells were analyzed. Z-stack images were compiled with ImageJ software (NIH, Bethesda, MD). The resolution obtained in

these imaging experiments was 512 by 512 pixels with a z resolution of 0.5μm for each filter set.

Electrophysiology

The isolation and recording of the I_{Kur} current, specific current of the Kv1.5 channel, was recorded by a whole-cell patch clamping protocol as previously described (190). Total potassium current was recorded using 10mV step depolarizations, from a holding potential of -80mV, to +60mV. The intracellular pipette solution contained (in mM): KCl 148, $MgCl_2$ 1, EGTA 5, HEPES 5, K_2ATP 5; and was adjusted to pH 7.2 with KOH. The bath solution contained (in mM): NaCl 148, NaH_2PO_4 0.4, $MgCl_2$ 1, KCl 5.4, $CaCl_2$ 1, HEPES 15; and was adjusted to pH 7.4 with NaOH.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR) was performed using established protocols, with modifications. Cells were fixed with 1% formaldehyde, lysed, and sonicated (Qsonica cup horn sonicator, 100% amplitude). DNA fragments were in the 200-700 bp range. The following antibodies were then added to the pre-cleared sample and incubated overnight at 4°C: Anti-Trimethyl Histone H3Lys27 (Millipore 07-449, 2μg) and Anti-Ubiquityl-Histone H2ALys119 D27C4 XP (Cell Signaling Technology #8240, 10μl). The complexes were purified using protein-G dynabeads (Invitrogen, Grand Island, NY, USA) followed by elution and crosslink reversal. DNA was recovered using QIAquick PCR Purification Kit (Qiagen, Valenica, CA, USA). Target sequences were amplified by PCR using iTaq SYBR green (Bio-Rad, Hercules, CA, USA).

Primer sequences for the KCNA5 promoter were KCNA5 #1 (Forward: 5'-TCCAGCATCATCAGTTTCCA -3' and Reverse: 5'-TGGCTCTCATTATGCACCAG-3') and KCNA5 #2 (Forward: 5'-GCTGAAGGTTGCATCTGCT-3' and Reverse: 5'- GGCCCTGACGTCAAGAAG-3'). Data were analyzed using the percent input method where Percent input = 100*2^(Average Ct Input – Average Ct IP).

Statistical Analysis

Statistics were performed using GraphPad Prism 6 (San Diego, CA, USA) and values of $p<0.05$ between groups were considered significant.

Results

Polycomb proteins promote cancer cell survival under conditions of hypoxic stress. Most pediatric solid tumors, including NB and ES, respond to chemotherapy and tumors exhibit extensive necrosis at the time of surgery. However, a significant number of patients relapse following initial clinical remission demonstrating that at least some cells are capable of surviving the stress of a necrotic microenvironment. In order to explore the potential mechanisms that underlie resistance to stress-induced death we studied non-malignant and cancer cells in *in vitro* conditions that mimic the hostile microenvironment of a necrotic solid tumor. Specifically, cells were exposed to either ambient, unstressed conditions (21% oxygen, 10% FBS) or microenvironmental stress (1% oxygen, 0% FBS) and cell viability monitored over time. Exposure of non-malignant endothelial (HUVEC) and atrial (HL-1) cells to stress resulted in significant cell death that was evident within 24 hours and

increased over time (Figure 1.1A). In contrast, ES (Figure 1.1B) and NB (Figure 1.1C) cells exhibited no significant loss of viability after up to 72 hours. Thus, these studies confirmed that ES and NB cells are relatively resistant to microenvironmental stress.

NB and ES are highly undifferentiated tumors that are thought to arise from stem and progenitor cells of neural crest (NB, ES) and/or mesenchymal (ES) origin. Stem cells thrive in conditions of hypoxia, leading us to hypothesize that the ability of NB and ES to survive stress may be linked to their primitive stem-like biology. Both ES and NB cells express high levels of the polycomb complex proteins BMI-1 and EZH2 and over-expression of these proteins contributes to stemness, tumorigenicity and tumor progression (174-178, 191). To test whether polycomb proteins contribute to survival of cancer cells under conditions of stress we evaluated viability in NB and ES cells that had been modified to down-regulate polycomb function. First, we assessed survival of cells that had been engineered to down-regulate BMI-1 as a result of RNA interference (Figure S1.1A). Significantly, NB and ES cells with reduced levels of BMI-1 showed no change in viability in ambient conditions but died upon transfer to stress conditions (Figure 1.2A-B). Next, we used the pharmacologic inhibitor GSK-126 to inhibit the methyltransferase activity of EZH2 (192). Once again, inhibition of polycomb function had no impact on cell survival under ambient conditions but resulted in loss of cell viability under conditions of stress (Figure 1.2C-D). Thus, survival of both ES and NB cells under conditions of hypoxia and nutrient

deprivation is, at least in part, dependent on the continued activity of the polycomb proteins BMI-1 and EZH2.

***KCNA5* is repressed by polycomb in cancer cells.** Having established that polycomb proteins promote cancer cell survival under conditions of stress we next sought to define the mechanism underlying the death-resistant phenotype. Polycomb proteins repress thousands of gene loci in a highly context specific manner. In human embryonic stem cells they specifically bind the promoters of nearly 2,000 protein-encoding genes involved in orchestration of normal embryonic development (146). In addition, in stem cells, fibroblasts and cancer cells the most prevalent targets of polycomb regulation are transcription factors that instruct cell fate and organogenesis and genes that are known to function as tumor suppressors, such as the cell cycle inhibitor *CDKN2A* (31, 146, 179). However, we noted on further analysis of the published data that promoters of potassium ion channel genes were also discovered to be highly enriched ($p<3.8x10^{-11}$) among directly bound polycomb targets in stem cells (See Supplementary Table 10 in (146)) and also figured prominently among polycomb-regulated targets in human embryonic fibroblasts (See Supplementary Table 3 in (31)). Potassium flux out of a cell is a key requirement for apoptosis (8, 193) and, although altered expression of over 20 potassium ion channels has been described in human cancers, only two (Kv1.5 and Kv7.1) are down-regulated in tumors (7). Significantly, BMI-1 dependent silencing of the *KCNA5* gene, which encodes Kv1.5, was recently shown to promote ischemic tolerance in neurons (103). Thus, we hypothesized that tolerance of hypoxic stress in cancer cells

might be mediated by polycomb-dependent repression of the *KCNA5* locus. To address this we first interrogated publicly available microarray databases to determine if expression of the *KCNA5* transcript is altered in NB and ES. As shown in Figure 1.3A, basal levels of *KCNA5* are reduced in both tumor types compared to non-malignant embryonic and adult tissues. In addition, levels of *KCNA5* expression were found to be higher in a subset of ES tumors that did not express high levels of BMI-1 (194), thus providing indirect evidence that BMI-1 might repress *KCNA5* in ES (Figure 1.3B). Further support for this relationship was evidenced by an inverse relationship between *BMI-1* and *EZH2* expression and *KCNA5* expression in primary NB tumors (Figure S1.1B). To directly assess whether *KCNA5* is targeted for polycomb-mediated silencing in ES and NB, we performed chromatin immunoprecipitation (ChIP) experiments using antibodies to the polycomb-dependent histone modifications H2AubK119 and H3K27me3. H2A119ub is mediated by the BMI-1 partner protein RING1B and H3K27me3 by EZH2. In each of the cancer types we detected enrichment of both polycomb-dependent modifications at the *KCNA5* promoter, in particular H2A ubiquitination was highly enriched over background in both ES and NB cells (Figure 1.3C-D). Moreover, exposure of the cells to stress resulted in a near two-fold increase in the H2A119Ub mark demonstrating that the cells acutely respond to microenvironmental stress by further repressing the *KCNA5* promoter (Figure 1.3E). Consistent with polycomb-dependent repression of the locus in ES and NB cells, we detected little to no *KCNA5* transcript in ambient conditions and no measurable increase was detected following exposure to stress (Figure 1.3F and

Figure S1.1C). In contrast, *KCNA5* transcription was increased in BMI-1-knockdown following transfer to stress conditions (Figure 1.3F) and EZH2 inhibitor-treated cells (Figure S1.1D). Thus, the *KCNA5* promoter is subject to epigenetic regulation by polycomb group proteins in ES and NB and persistent polycomb-dependent repression of the locus prevents up-regulation of the transcript when cells are exposed to microenvironmental stress.

The Kv1.5 channel regulates hypoxic stress-induced cell death. After confirming that polycomb proteins epigenetically repress *KCNA5* in ES and NB cells we next sought to define the functional consequences of this repression. Specifically, we wished to determine whether loss of Kv1.5 channel function contributes to cell survival under conditions of hypoxic stress. To address this we took advantage of the pharmacologic compound diphenyl phosphine oxide-1 (DPO-1), a highly specific inhibitor of the Kv1.5 channel which effectively blocks potassium efflux through the channel with an IC_{50} = 310 nM (195) (Figure S1.2A). Non-malignant cells were exposed to DPO-1 and then transferred to stress conditions as above. Consistent with Kv1.5 being a key mediator of stress-induced death, channel blockade using DPO-1 rescued cell viability in both HUVEC and HL-1 cells (Figure 1.4A). Viability of cells under ambient conditions was unaffected by channel blockade (Figure 1.4A). To confirm these results we next exposed cells to 4'aminopyridine (4'AP), another potent inhibitor of the Kv1.5 channel (IC_{50} = 50 μM) (135) (Figure S1.2A). Pharmacologic blockade of Kv1.5 using 4'AP also largely prevented death of stress-exposed HUVEC and HL-1 cells (Figure S1.2B). Thus, these findings indicate that hypoxic stress-

induced death of non-malignant cells is, at least in part, dependent on a functional Kv1.5 channel.

Next, we tested the impact of Kv1.5 channel blockade on cancer cells that had been sensitized to stress-induced death as a consequence of polycomb modulation. Significantly, exposure of BMI-1 knockdown cancer cells to DPO-1 prevented stress-induced cell death (Figure 1.4B). Viability of ES cells was restored by DPO-1 while partial rescue was evident in the NB cells (Figure 1.4B). To test whether blockade of Kv1.5 would also restore the viability of EZH2-inhbited cells we exposed GSK-126 treated cells to DPO-1 prior to transfer to stress conditions. As shown (Figure 1.4C), DPO-1 restored cell viability in GSK-126 treated cells. Exposure of unmodified ES and NB cells to DPO-1 had no significant impact on cell death and cells remained viable in both ambient and stress conditions (Figure S1.2C). Together these data reveal the critical nature of polycomb proteins in promoting cancer cell viability under conditions of stress. In particular, they confirm that survival under conditions of hypoxic stress is, at least in part, dependent on polycomb-dependent suppression of the Kv1.5 channel. In addition, they further implicate both PRC1 and PRC2 in mediating epigenetic repression of the *KCNA5* locus.

Ectopic expression of Kv1.5 restores sensitivity of cancer cells to hypoxic stress-induced apoptosis. Potassium ion channels can affect cellular physiology through mechanisms that are both dependent or independent of their role as regulators of ion flux across the cell membrane (7) Having established that pharmacologic blockade of Kv1.5 prevents cell death under conditions of

stress, we next sought to more directly test if this effect on cell survival was mediated by blockade of potassium efflux. To address this question we transduced TC-71 ES cells with wild-type (WT) and non-conducting, pore-dead (PD) Kv1.5 mutant constructs or a control soluble GFP (sGFP) using adenoviral infection. Both Kv1.5 constructs carry full-length human *KCNA5* cDNA, tagged in the extracellular loop with GFP, and the trafficking and biophysical properties of both WT and PD ectopic proteins recapitulate those of the endogenous channel (108, 196). However, unlike Kv1.5-WT, which is fully functional, Kv1.5-PD generates a non-functional channel that cannot conduct potassium across the cell membrane as a result of an amino acid change (W472F) within the pore region (Figure 1.5A) (196). Confocal microscopy confirmed successful infection of ES cells with all constructs and cytoplasmic membrane localization of both Kv1.5-WT and Kv1.5-PD proteins (Figure 1.5B). Whole cell patch clamp experiments confirmed the presence of a robust and functional channel in Kv1.5-WT expressing cells and only background current in Kv1.5-PD that was equivalent to that of control sGFP infected cells (Figure 1.5C). To determine if the presence of ectopic channel altered cell viability we cultured infected cells in either room air (21% O2) or hypoxia (1% O2) for 24 hours. In these experiments, stress was limited to hypoxia alone without serum deprivation in order to more precisely assess the impact of channel re-expression on the hypoxic response. Expression of either Kv1.5-WT or Kv1.5-PD had no impact on cell viability in ambient conditions but transfer to hypoxic conditions resulted in death of the Kv1.5-WT cells (Figure 1.5D). In contrast, Kv1.5-PD cells experienced no loss of

viability in hypoxia (Figure 1.5D). To confirm that the hypoxia-associated death of Kv1.5-WT cells was due to efflux of potassium through the ectopic channel we exposed cells to DPO-1 and 4'AP. Significantly, survival of Kv1.5-WT cells in hypoxia was largely restored if cells were treated with either of the two compounds (Figure 1.5E). Finally, to test the specificity of Kv1.5 as a mediator of hypoxia-induced death we tested whether ectopic expression of two other voltage-gated potassium channels, Kv1.4 and Kv1.3, would mimic the effect of Kv1.5. In contrast to Kv1.5, ectopic expression of Kv1.4 and Kv1.3 induced no appreciable death in cells that were exposed to hypoxia (Figure S1.3A). After 24 hours the Kv1.3 infected cells began to die but death was less than that of Kv1.5-transduced cells and equivalent to that of cells that were infected with control vectors sGFP and Kv1.5-PD (Figure S1.3A, B). Kv1.4 infected remained viable at 24 hours, suggesting that the lower titer of the Kv1.4 virus afforded protection against the non-specific adenoviral death that we observed with Kv1.5-PD, Kv1.3 and sGFP infection (Figure S1.3A, B). Thus, these data demonstrate that the effects of Kv1.5 in mediating hypoxia-induced death are not generalizable to other Kv channels.

Finally, we investigated the mechanism of cell death in Kv1.5 expressing cells. It has been previously shown that potassium efflux promotes caspase activation and apoptosis (5, 35). To determine if TC-71 cells expressing Kv1.5-WT protein were dying by apoptosis we assessed cleavage of caspase-3 in ambient and hypoxic conditions. Whereas Kv1.5-WT cells showed little evidence of caspase-3 cleavage in ambient conditions, after 8 hours in hypoxia cells with cleaved

caspase-3 were readily detected (Figure 1.6A). By comparison, unmodified TC-71 cells showed no evidence of caspase cleavage in either ambient or hypoxic conditions but apoptosis could be dramatically induced in both conditions by etoposide, a cytotoxic topoisomerase II inhibitor that is routinely used in ES therapeutic regimens (Figure 1.6B). By contrast, cells with cleaved caspase-3 were only rarely detected in Kv1.5-PD and sGFP transduced populations in both ambient and hypoxic conditions (Figure 1.6C & D). Thus, these studies together demonstrate the specificity of Kv1.5 as a key mediator of hypoxia-associated cell death and confirm that, under conditions of cell stress, efflux of potassium through the channel promotes apoptotic cell death.

Discussion

Oxygen tension in normal tissues ranges from 2-9% but is often considerably less in stem cell niches and solid tumors (182). Normal sensing of oxygen tension is critical for cell proliferation and cell survival and cells that exist in hypoxic niches, such as cancer cells and stem cells, utilize sophisticated molecular tools to withstand the relatively ischemic microenvironment (183, 197-199). Cell proliferation and survival are also intimately linked to the concentration of intracellular ions including potassium, sodium and calcium (5, 42, 48). In particular, high levels of intracellular potassium inhibit caspase activation and promote cell survival, revealing a role for potassium efflux in execution of apoptotic cell death (35, 40, 41). Intracellular potassium concentrations are controlled by transmembrane ion channels, which actively regulate the flux of

potassium ions through central pores (200, 201). There are 78 different potassium ion channels (7, 202) and each is expressed in a context specific manner and alters potassium flux in response to different cellular signals (200, 201). Interestingly, recent studies of ion channel expression in cancer have suggested that deregulation of potassium channels is a characteristic of human tumors (reviewed in (7) and (49)). Moreover, recent high-throughput drug screening studies showed that the potassium ionophore salinomycin is selectively cytotoxic to cancer stem cell populations (203, 204). These findings together support further investigation of ion-channel targeted approaches as novel opportunities for cancer therapy.

In the current study we have focused on defining the mechanism of Kv1.5 channel suppression in aggressive pediatric solid tumors and the contribution of this suppression to tumor pathogenesis. Our results implicate polycomb-dependent epigenetic repression of *KCNA5* as a key mechanism of channel inhibition and reveal the critical role of this inhibition in promoting cancer cell survival under conditions of microenvironmental stress, in particular hypoxic stress. In addition, the inability of an ectopic pore-dead Kv1.5 channel to restore stress-induced death demonstrates that survival of Kv1.5 suppressed cancer cells is linked to its canonical function as a potassium conductor rather than to other potential non-canonical roles that might also impact on cell signaling (7)

The Kv1.5 channel is a voltage-gated potassium channel that conducts potassium in response to changes in cell membrane potential (202). In addition, Kv1.5 is a putative oxygen sensor and contributes to the regulation of cell

proliferation and survival under conditions of hypoxia by altering intracellular potassium levels in response to shifts in redox states (1, 46, 83, 135, 205). The role of Kv1.5 as an oxygen-sensing channel is well established in both cardiac and pulmonary physiology and aberrant expression and regulation of Kv1.5 channels contributes to pulmonary hypertension and cardiac arrhythmias (45, 46, 97, 134, 205). It was recently shown that exposure of neuronal cells to acute hypoxia and glucose deprivation leads to apoptosis and that this ischemia-induced cell death is associated with up-regulation of Kv1.5 expression (103). Significantly, tolerance to the ischemic insult could be elicited in this study if cells were first exposed to repetitive low-dose stress rather than an acute insult. At a molecular level this ischemic tolerance was found to be due to up-regulation of BMI-1 and recruitment of polycomb to the *KCNA5* promoter (103). Thus, BMI-1 dependent repression of the *KCNA5* gene can be an adaptive, physiologic response to chronic stress. Our current studies reveal that, in BMI-1 over-expressing cancer cells, this adaptive physiologic response has been co-opted to promote the survival of the cancer cells under conditions of acute stress. Whether this survival mechanism is an inherent feature of the cancer cell of origin (e.g. a stem cell) or an acquired characteristic that arises during the process of malignant transformation remains to be determined and will likely vary between different cancer types.

There have been several reports that Kv1.5 expression is reduced in human cancers (6, 9, 12, 14, 15) and in gliomas loss of expression is directly correlated with tumor aggression, suggesting that loss of the channel might contribute to

tumor progression (11). Moreover, recent studies demonstrated that restoration of normal mitochondrial function and redox tension by use of the pyruvate dehydrogenase kinase (PDK) inhibitor dichloroacetate (DCA), resulted in de-repression of *KCNA5* transcription and induction of Kv1.5-dependent apoptosis (8). Unfortunately, although these initial findings suggested that DCA might be useful as a relatively non-toxic anti-cancer agent, later studies failed to show significant impact of DCA in other preclinical models (206). In particular, pediatric tumors including NB and ES were found to be completely insensitive to the cytotoxic effects of DCA (206, 207). Our discovery that the *KCNA5* locus is targeted for polycomb-dependent repression in these tumors suggests that the disappointing results that have been encountered with DCA as an anti-cancer therapy may be due to the inaccessible chromatin state of the *KCNA5* promoter in cancer cells that over-express BMI-1 and other polycomb proteins, such as pediatric solid tumor and cancer stem cells,

In summary, we have identified that epigenetic repression of the *KCNA5* gene by polycomb proteins, in particular BMI-1, inhibits Kv1.5 expression and function in ES and NB. These findings provide new evidence that the Kv1.5 cha1nnel plays a seminal role in execution of hypoxia-induced apoptotic cell death in aggressive cancers. In addition, they suggest that cancer cells hijack physiologic regulation of the Kv1.5 channel to promote their survival in hostile microenvironments. Future studies are now needed to determine how this knowledge about altered epigenetic regulation of *KCNA5* can be therapeutically

exploited to maximize cell death in the context of aggressive solid tumors that are prone to relapse.

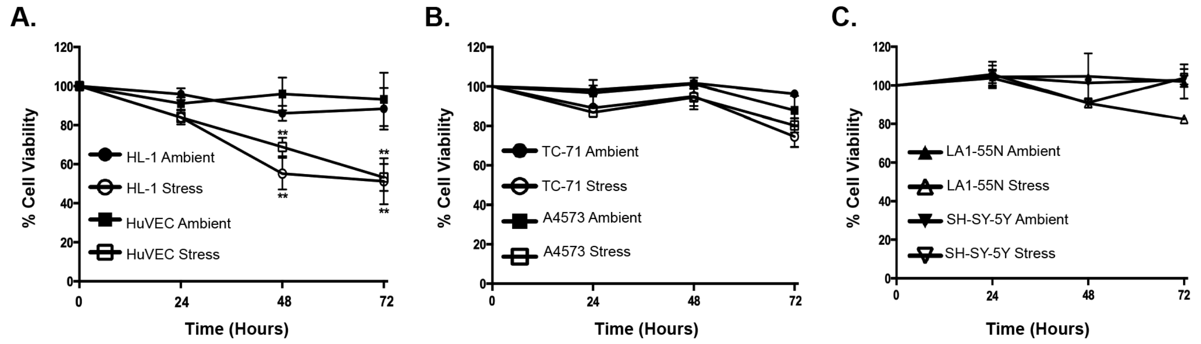


Figure 2.1. ES and NB cancer cells survive physiologic stress.

Under conditions of stress (serum starvation + hypoxia) non-malignant HuVEC and HL-1 cells experience a significant reduction in cell viability (A). In contrast ES cells, TC-71 and A4573, (B) and NB cells, LA1-55N and SH-SY-5Y, (C) cells survive. ** $p<0.005$ (mean ± SEM, n=3).

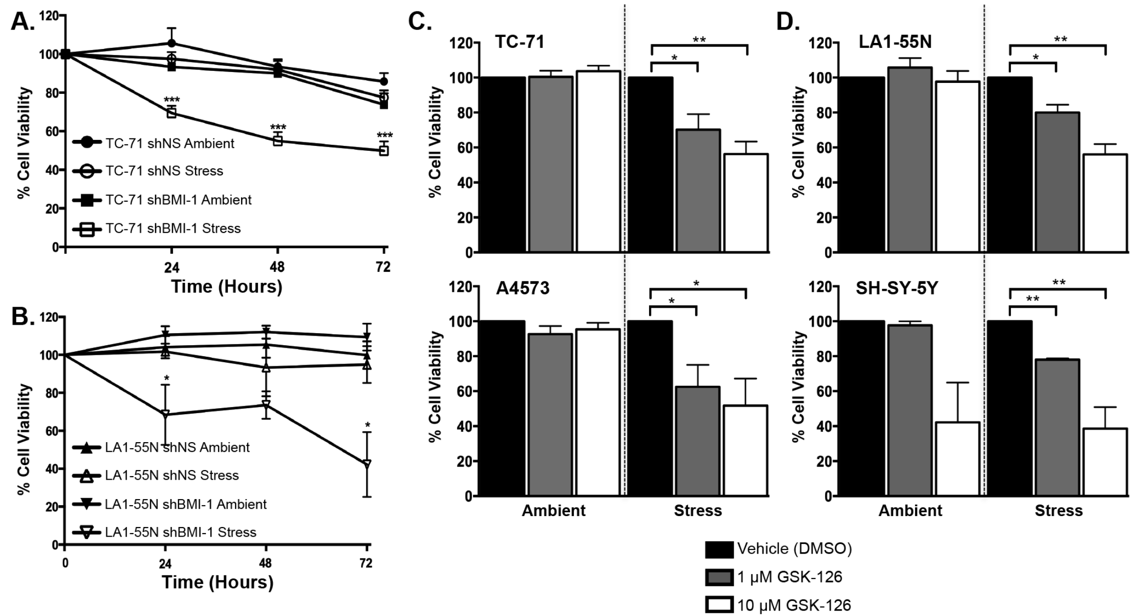


Figure 2.2. Polycomb proteins promote survival of cancer cells under conditions of stress.

Analysis of cell survival in control (shNS) and BMI-1 knockdown (shBMI-1) ES (A) and NB (B) cells after transfer to stress conditions shows loss of viability in shBMI-1 cells over a 72-hour time course. Exposure of ES cells, TC-71 and A4573, (C) and NB cells, LA1-55N and SH-SY-5Y, (D) to the EZH2 inhibitor GSK-126 at 1 μM and 10 μM for 72 hours results in loss of viability when cells are exposed to stress for 24 hours. * $p<0.05$, ** $p<0.005$ and *** $p<0.001$ (mean ± SEM, n=3) .

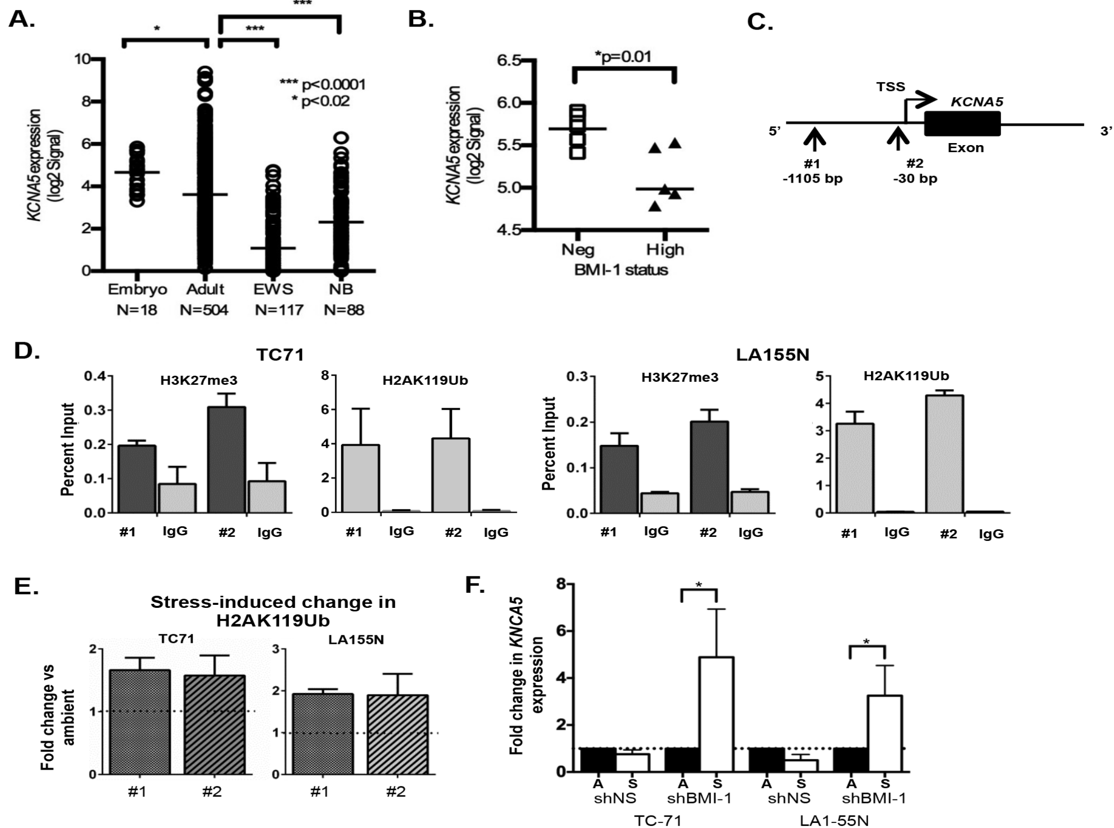


Figure 2.3. Polycomb group proteins repress KCNA5 in ES and NB cells.

(A) Publicly available microarray data were analyzed using the R2: microarray analysis and visualization platform (http://r2.amc.nl). Expression of *KCNA5* is significantly lower in ES (GSE34620) and NB (GSE16476) tumors compared to normal embryonic (GSE15744) and adult (GSE7307) tissues. (B) Expression of *KCNA5* is increased in ES tumors that do not express high levels of BMI-1 (GSE16016). * p<0.02 and *** p<0.001 (C) Site of PCR primers for evaluation of histone modifications at the KCNA5 promoter. (D) Chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR) of ES and NB cells in ambient conditions shows enrichment of both H3K27me3 and H2AK119Ub marks at the KCNA5 promoter (relative to IgG control ChIP). (E) ChIP of ES and NB cells in ambient and stress conditions shows a near 2-fold increase in enrichment of H2AK119Ub at the KCNA5 promoter after exposure to stress. Data are from 2 independent experiments and are expressed as mean +/- SEM. (F) qRT-PCR analysis demonstrates significant upregulation of *KCNA5* expression in BMI-1 knockdown ES and NB cells after 8 hours in stress conditions. Expression normalized to the geometric mean of *HPRT* and *GAPDH* in each sample and expressed as fold change in stress relative to ambient conditions. * p<0.05 by Mann-Whitney Test (mean ± SEM, n=3).

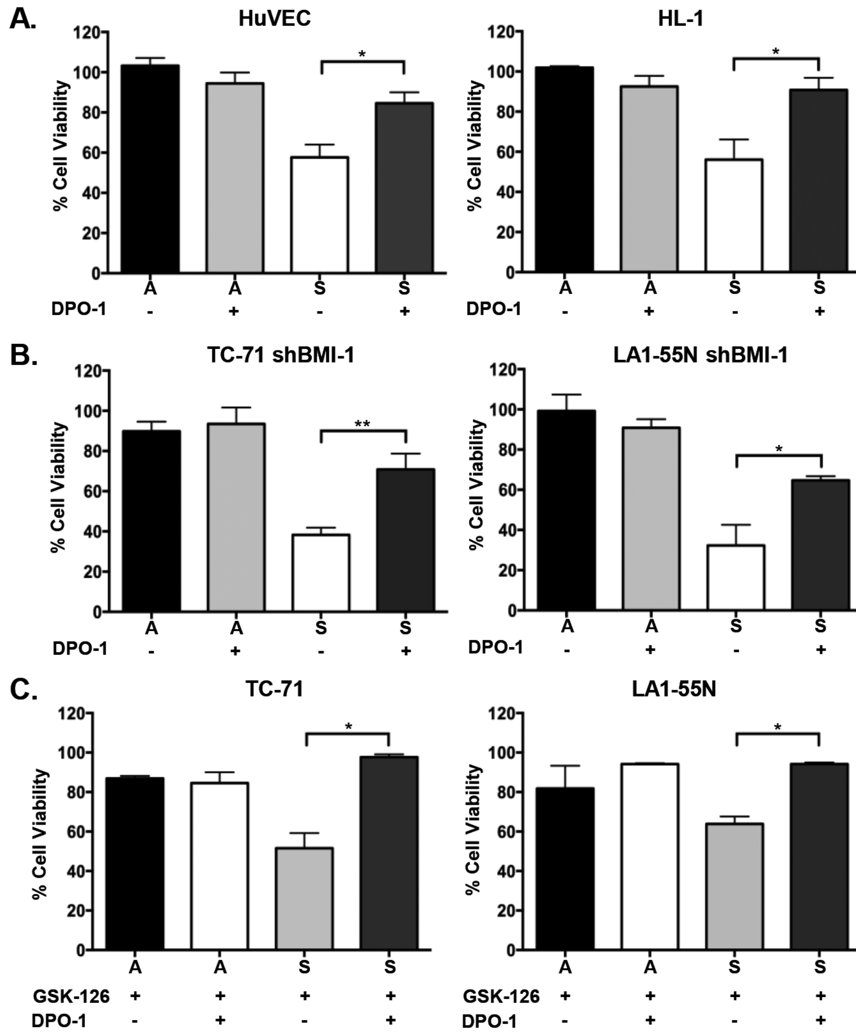


Figure 2.4. Stress-induced death is prevented by pharmacologic blockade of Kv1.5 channel function.

Cell viability analysis demonstrates that treating cells with 310 nM DPO-1 largely prevents stress-induced death in non-malignant cells (A). Death is also prevented in cancer cells engineered to express reduced levels of BMI-1 (shBMI-1) (B) and in cancer cells that have been exposed to 10 µM GSK-126 prior to transfer to stress conditions (C). Viability in ambient conditions is unchanged by exposure to 310 nM DPO-1. * $p<0.05$ and ** $p<0.005$ (mean ± SEM, n=3).

Figure 5

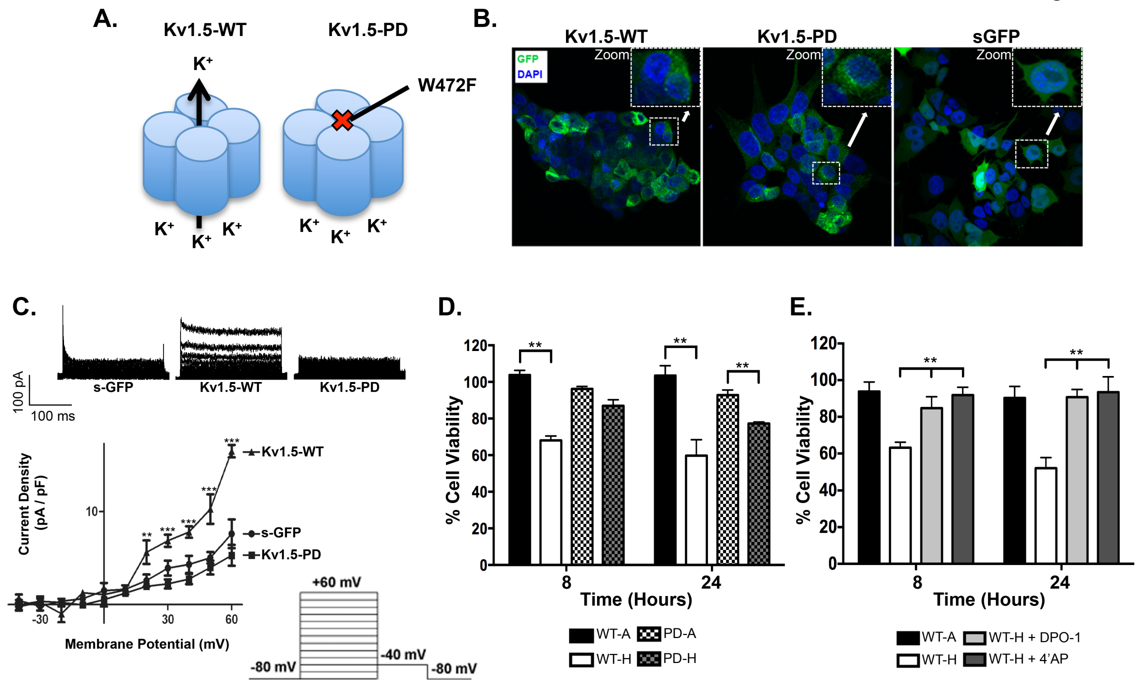


Figure 2.5. Ectopic expression of the Kv1.5 channel restores stress-induced death in ES cells.

(A) Cartoon of the wild-type (Kv1.5-WT) and pore-dead (Kv1.5-PD) channels, depicting the amino acid change W472F which prevents efflux of potassium out of the Kv1.5 channel. (B) GFP-fluorescent immunocytochemistry and confocal microscopy reveals robust expression of ectopic Kv1.5-WT, Kv1.5-PD and soluble GFP (sGFP) in TC-71 cells 24 hours post adenoviral infection. (C) Electrophysiology confirms induction of functional Kv1.5 current in TC-71 cells transduced with Kv1.5-WT but not with Kv1.5-PD or sGFP. (D) TC-71 cells expressing Kv1.5-WT channel have a significant reduction in cell viability at 8 and 24 hours in hypoxia while the TC-71 cells expressing the Kv1.5-PD channel are unaffected, in particular at 8 hours. (E) Pharmacological block of the Kv1.5 channel with either 50 μM 4'AP or 310 nM DPO-1 largely prevents hypoxia-induced death in Kv1.5-WT expressing ES cells. ** $p<0.005$ and *** $p<0.001$ (mean± SEM, n=3).

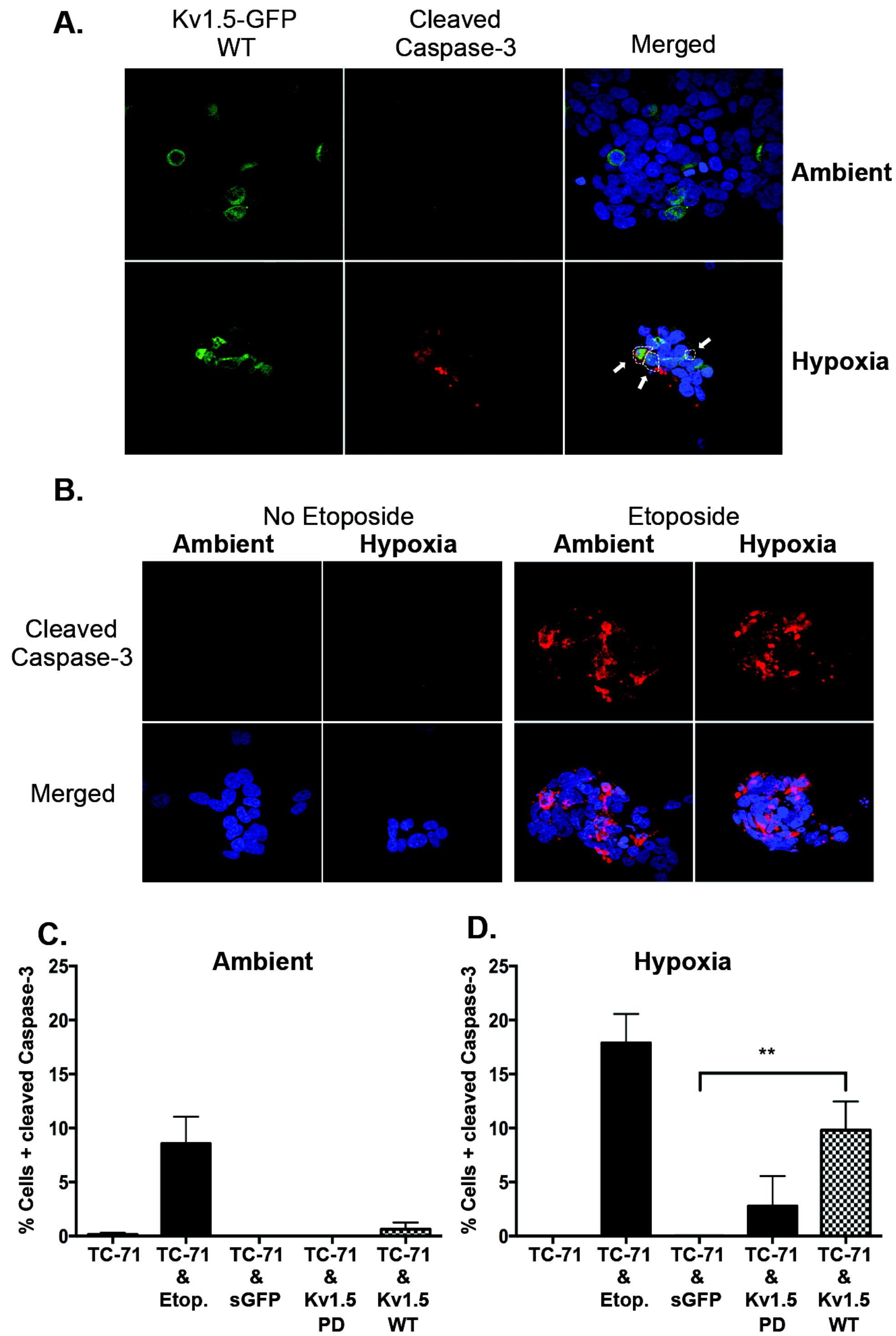
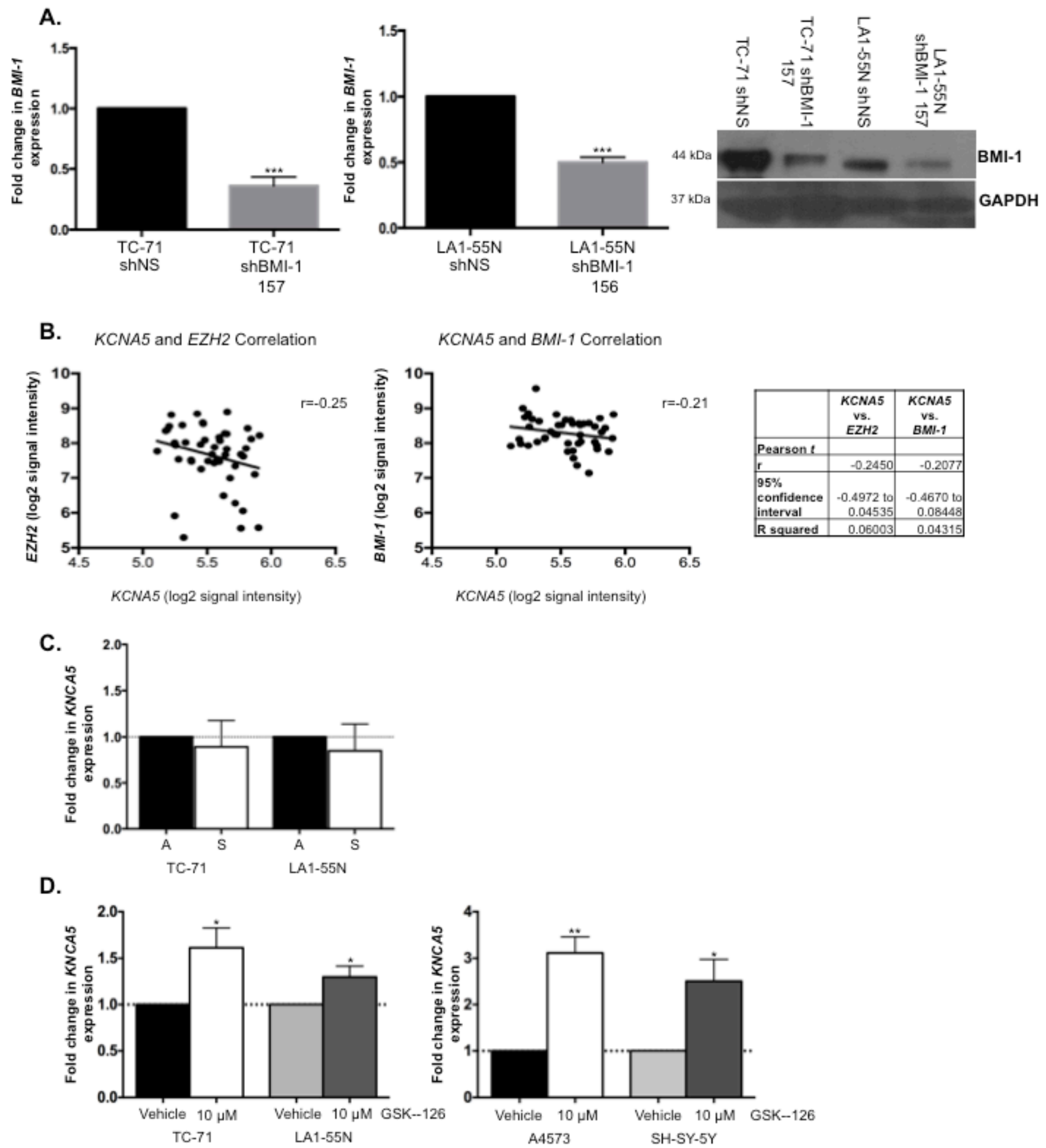


Figure 2.6. The Kv1.5-WT channel mediates cell death through caspase-3 activation.

(A) Immunocytochemistry and confocal microscopy of Kv1.5-WT expressing TC-71 cells shows induction of caspase-3 cleavage in Kv1.5+ (GFP+) cells (Indicated by white arrows) following exposure to hypoxia. No significant cleavage is detected in Kv1.5+ cells in ambient conditions. (B) Cleaved caspase-3 staining is not detected in parent TC-71 cells in either ambient or hypoxic conditions. To serve as a positive control for cleaved caspase-3 staining, cells were exposed to 4 µg Etoposide. This exposure resulted in robust induction of caspase-3 cleavage in both ambient and hypoxic conditions. Quantification of cells with cleaved caspase-3 is shown for cells in A & B, under ambient (C) (21% O_2) and hypoxic (1%O_2) (D) conditions. ** $p<0.005$ (mean ± SEM, n=2-3. 200-400 cell nuclei were counted for each condition.).



	KCNA5 vs. EZH2	KCNA5 vs. BMI-1
Pearson t		
r	-0.2450	-0.2077
95% confidence interval	-0.4972 to 0.04535	-0.4670 to 0.08448
R squared	0.06003	0.04315

Figure S2.1. Validation of BMI-1 knockdown cell lines and KCNA5 expression in GSK-126 treated ES and NB cells.

(A) Successful BMI-1 knockdown in TC-71 shBMI-1 and LA1-55N shBMI-1 was verified by qRT-PCR (top) and western blot analysis (bottom). (B) Publicly available microarray data were analyzed using the R2: microarray analysis and visualization platform (http://r2.amc.nl). Expression of *KCNA5* is inversely correlated with both *EZH2* and *BMI-1* in NB tumors (GSE27608). (C) qRT-PCR analysis demonstrates TC-71 and LA1-55N lack *KCNA5* expression in ambient and stressed conditions. (D) qRT-PCR analysis demonstrates an upregulation of *KCNA5* expression in stress after pre-treatment with 10 μM GSK-126 in TC-71, A4573, LA1-55N and SH-SY-5Y cells. Expression was normalized to the geometric mean of housekeeping genes *HPRT* and *GAPDH* in each sample and expressed as fold change relative to scrambled control for BMI-1 knockdown cells and vehicle for GSK-126 treated cells. * $p<0.05$, ** $p<0.005$ and *** $p<0.001$ (mean ± SEM, n=3).

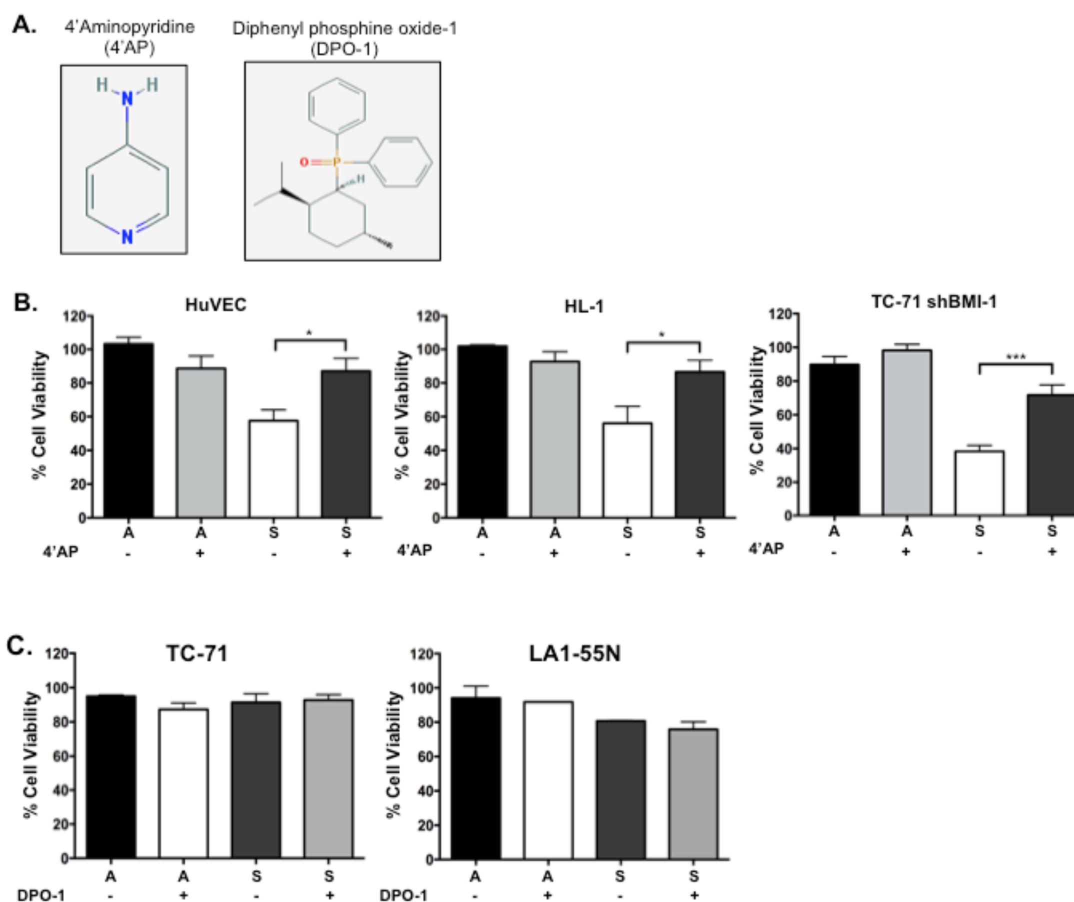


Figure S2.2. Blocking Kv1.5 channel function with 4'AP prevents stress-induced death.

(A) Chemical structures of 4'aminopyridine (4'AP) (left) and diphenyl phosphine oxide-1 (DPO-1) (right) obtained from the PubChem Compound database. (B) Cell viability graphs demonstrate that stress-induced death in HuVEC, HL-1 and TC-71 shBMI-1 cells are prevented by treating cells with 50 μM 4-AP. Treatment with 4'AP under ambient conditions has no toxic effect on any cell line. (C) Treatment with 310 nM DPO-1 under ambient and stressed conditions has no toxic effect on TC-71 or LA1-55N cell lines. * $p<0.05$ and *** $p<0.001$ (mean ± SEM, n=3).

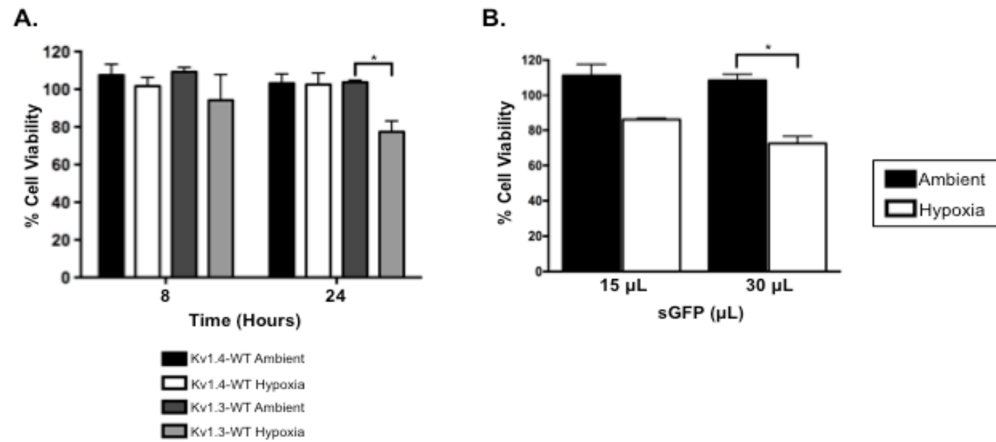


Figure S2.3. The effects of other Kv channels, Kv1.4 and Kv1.3, and adenoviral infection on TC-71 cell viability.

(A) TC-71 cells transduced with Kv1.4 or Kv1.3 adenoviral vectors remain viable in hypoxic conditions. (B) TC-71 cells transduced with 15 µL and 30 µL of sGFP have a marginal decrease in cell viability at 24 hours in hypoxia, demonstrating a non-specific effect of adenoviral infection on cell viability under conditions of stress. * $p<0.05$ (mean ± SEM, n=2-3).

CHAPTER 3

THE *KCNA5* PROMOTER IS HYPERMETHYLATED IN EWING SARCOMA AND SILENCING CONTRIBUTES TO CELL PROLIFERATION[2]

Abstract

Potassium channels regulate a variety of biological processes and dysfunction of these channels contributes to cancer initiation and progression. We recently showed that the *KCNA5*-encoded channel, Kv1.5, is a target of polycomb-group (PcG)–dependent repression in the aggressive pediatric solid tumor, Ewing Sarcoma (ES), and that this epigenetic silencing of the Kv1.5 channel contributes to cell survival under hypoxic stress. In human cancer, the promoters of PcG target genes are often subject to aberrant DNA hypermethylation. Given that *KCNA5* is a target of PcG mediated repression, we reasoned that this gene might also be a target of DNA hypermethylation. Here, we describe our finding that the *KCNA5* promoter is hypermethylated in ES and that inactivation of the Kv1.5 channel promotes cancer cell proliferation. We show that ES cancer cell proliferation is dependent on suppression of Kv1.5 channel function. The *KCNA5* promoter is marked by hypermethylation and

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[2] **Ryland KE**, Hawkins AG, Weisenberger DJ, Borinstein SC, Laird PW, Martens JR and Lawlor ER. The KCNA5 Promoter is Hypermethylated in Ewing Sarcoma and Silencing Contributes to Cell Proliferation. *Manuscript in Preparation*.

pharmacologic inhibition of DNA methyltransferases reduces the methylation present at the promoter, restores *KCNA5* transcript expression and slows ES cell proliferation. This work identifies DNA methylation as another epigenetic mechanism that regulates *KCNA5* expression in cancer and further implicates epigenetic repression of the Kv1.5 potassium ion channel in tumor pathogenesis.

Introduction

A fundamental trait of cancer cells is their ability to sustain proliferation (19). Cancer cells accomplish this by hijacking and altering many pathways and silencing or mutating tumor suppressor genes involved in the regulation of cell proliferation (e.g. TP53 and pRB) (19). Importantly, the ability to sustain proliferative signaling is tied to another hallmark, resisting cell death, as cancer cells disable cell death circuitry to promote excessive cell expansion (19). Significantly, the proliferative phenotype of cancer cells contributes to relapse in numerous tumor types (e.g. breast cancer, melanoma, prostate cancer) (208-210). Relapse is of particular importance in cancer treatment, especially in the pediatric solid tumor, Ewing Sarcoma (ES), as it remains a central issue and a leading cause of cancer-associated death (16, 17). Therefore, continuing to elucidate the mechanisms that promote cancer cell proliferation and cell expansion is key for developing novel anti-relapse therapies.

In cancer, epigenetic gene inactivation is as common, if not more frequent than genetic mutations as a means of gene silencing and it significantly contributes towards the development of cancer ((211-214) and reviewed in (136,

215)). One epigenetic mark, DNA methylation, has gained significant attention in the cancer field over the last decade, due to the clinical applications of hypomethylating agents. DNA methylation occurs when DNA methyltransferase (DNMT) enzymes add a methyl group to the 5' carbon position of a cytosine; this projects into the major groove of DNA and inhibits transcription (216). DNA methylation occurs at CpG islands, which typically reside in the promoter region of genes (217). The role of DNA methylation in mammalian cells is to maintain transcriptional silencing in regions of the genome. This mechanism is of importance, as DNA hypermethylation suppresses expression of harmful sequences that have over time integrated into the genome (e.g. viral sequences) (211, 216, 218). In cancer, DNA methylation is frequently used as a means to silence tumor suppressor genes that would otherwise function to inhibit tumorigenesis (e.g. VHL, MGMT, BRCA1, p16/CDKN2A) (214, 219, 220). In the aggressive pediatric cancer ES, a DNA methylation profile study identified that the genes subject to hypermethylation were those important for cell adhesion, cell cycle, cell regulation, development, transcription regulation and signal transduction (221). Therefore, continuing to elucidate the genes targeted by DNA methylation and understanding their biological significance, particularly in ES, can provide further support for clinical applicability of hypomethylating agents in ES cancer therapy.

Potassium channels are transmembrane proteins that control cellular ionic homeostasis and are important for all cellular processes. Two key biologic processes in particular, cell proliferation and survival, are intimately linked to

intracellular potassium ion concentrations (5, 42, 48). As the cellular concentration of potassium ions is controlled by potassium channel expression and function, dysregulation of potassium channels can alter many biologic processes, including proliferation, migration and response to cell death triggers (7). Dysregulation of potassium channel expression has been identified in numerous cancers and recent studies suggest that this deregulation is a characteristic of cancer (7, 49). However, the mechanisms causing loss of controlled expression of potassium channels in cancer are currently not well understood. In the current study we have identified the voltage-gated potassium (Kv) channel Kv1.5-encoding gene, *KCNA5*, as a target of DNA methylation and epigenetic repression in ES. Our studies show that the repression of *KCNA5* and inhibition of Kv1.5 channel activation, promotes ES cell proliferation.

Experimental Procedures

Cell Culture and Proliferation Assay

The ES cell lines, TC-71, A673 A4573, were cultured in RPMI-1640 media (Gibco, 11875) and supplemented with 10% FBS (Atlas Biologicals, Inc., F-500-A) and 6 mM L-glutamine (Life Technologies, 25030). Identities were confirmed by short tandem repeat profiling. Pellets of two non-transformed cell lines, HuVEC and MRC5, and five cancer cell lines, A549, LoVo, UM5, UM53 and U2OS, were collected for MethyLight studies. All cells were cultured in an incubator at 37°C in 5% CO_2. For the proliferation studies, cells were plated at a density of 200,000 cells per 6-well plate and left for 24 hours prior to a 72-hour

course drug treatment where the cells were treated every 24 hours. Proliferation was determined by trypan-blue exclusion.

Pharmacologic studies

Diphenyl phosphine oxide-1 (DPO-1) (310 nM, Tocris Bioscience, 2533) was prepared in ethanol and 5-aza-2'-deoxycytidine (5'AZA-CdR, 100 nM, Sigma-Aldrich, A3656) was diluted in dimethyl sulfoxide (DMSO) (Fisher, D128-500). Cells were pre-treated at these concentrations for 72-96 hours.

Quantitative Real-Time PCR

Total RNA extraction was performed using the RNeasy® Plus Mini kit (Qiagen) and cDNA was generated using iScript (Bio-Rad, BIO1708891). qRT-PCR was performed using validated SYBR primers (*KCNA5, GAPDH, HPRT;* IDT). Analysis was performed in triplicate using the Lightcycler® 480 System (Roche Applied Science). Data were analyzed by normalizing average Ct values of the gene of interest (*KCNA5*) to the geometric mean of reference genes (*HPRT* and *GAPDH*) within each sample using ΔΔCt method.

qRT-PCR Primers

Primers	**Sequence (5' to 3')**
KCNA5 (Human)	Forward: GTA ACG TCA AGG CCA AGA GC Reverse: TCC CAT TCC CTA CTC CAC TG

Illumina GoldenGate® Methylation Array

Initial assessment of *KCNA5* methylation was interrogated on a previously custom-designed Illumina Golden-Gate bead array. This array consists of 1,536 probes designed to detect DNA methylation at CpG islands within the promoters

of >1400 genes that are enriched for established targets of polycomb regulation in human embryonic stem cells (146). Two independent batches of samples were analyzed using this array, the first including 12 samples comprising 4 ES tumors, 5 ES cell lines and 3 stem cell populations and the second comprised of 52 samples from 11 different non-transformed adult tissues that were obtained at rapid autopsy (222). Analysis of methylation (as determined by beta values) was restricted to probes that were not targeted to promoters of X- or Y-linked genes and were successfully and reproducibly detected in over 75% of the samples in each experiment. This left a total of 1297 probes for analysis. A comparison of two groups was considered statistically significant if the difference in beta-values was ≥ 0.2 with an associated p-value <0.05 (2-tailed *t*-test).

MethyLight Studies

Initial MethyLight studies of Ewing Sarcoma tumor samples and cell lines were carried out by our collaborator Dr. Dan Weisenberger and Dr. Peter Laird using a previously described method (223). The Ewing sarcoma tumor samples analyzed in these studies were kindly provided by our collaborator Dr. Scott Borinstein. The later MethyLight studies involving decitabine were performed in the Lawlor laboratory. The process entailed, genomic DNA isolation using the Genomic DNA Clean & Concentrator Kit (Zymo Research, D4011). Sodium bisulfite conversion of genomic DNA was performed using EZ DNA Methylation Kit (Zymo Research, D5002). After sodium bisulfite conversion, the genomic DNA was amplified using MethyLight, a fluorescence-based real-time quantitative PCR, as previously described (223, 224). The primers and probes for *KCNA5*,

ALU-C4, and quality-control PCR reactions are listed below (IDT, Applied Biosystems and Biosearch Technologies). Control probes and conditions have been published previously (223). Analysis was performed in triplicate using the Lightcycler® 480 System (Roche Applied Science). Data was analyzed by calculating the average mean value from the Ct value using a standard curve generated by a dilution series of the positive control, fully methylated gDNA. Mean values were then used to find the average percentage of methylated reference (PMR) for each sample using the following formula: 100 x ((Average mean value of sample amplified with KCNA5 primers)/(Average mean value of sample amplified with ALUC4 primers))/((Average mean value of fully methylated gDNA amplified with KCNA5 primers)/(Average mean value of fully methylated gDNA amplified with ALUC4 primers)). This same method of analysis had been previously described (1).

MethyLight Primers

Primers	**Sequence (5' to 3')**
KCNA5	Forward: ATCGTAATCGGTTTAGTTTCGACG
	Reverse: AATAAAAACGTATCTCGTCCGCG
ALU-C4	Forward: GGTTAGGTATAGTGGTTTATATTTGTAATTTTAGTA
	Reverse: ATTAACTAAACTAATCTTAAACTCCTAACCTCA
CONV-C1	Forward: AAATTGGAGATGAGGGATGGGT
	Reverse: TTATCCTCCACTCATTCCCCAA

CONV-C2	Forward: AAATTGGAGATGAGGGATGGGT
	Reverse:TTATCCTCCACTCATTCCCCAA
CONV-C11	Forward: AAATTGGAGATGAGGGATGGGT
	Reverse: TTATCCTCCACTCATTCCCCAA
CONV-C16	Forward: AAATTGGAGATGAGGGATGGGT
	Reverse: TTATCCTCCACTCATTCCCCAA
CONV-C21	Forward: AAATTGGAGATGAGGGATGGGT
	Reverse: TTATCCTCCACTCATTCCCCAA

MethyLight Probes

Probe	**Sequence (5' to 3')**
KCNA5 Probe	6FAM-CGTTAACCGACCTCCGCAAACGACC-BHQ1
ALU-C4 Probe	6FAM-CCTACCTTAACCTCCC-MGBNGQ
CONV-C1 Probe	6FAM-TCTTACAAACTAATCCTTAACTTT-MGBNFQ
CONV-C2 Probe	6FAM-AACTGGTCCTTGGCTTT-MGBNFQ
CONV-C11 Probe	6FAM-TTACAAACTAGTCCTTGGCTTT-MGBNFQ
CONV-C16 Probe	6FAM-TTACAAACTAGTCCTTAGCTTT-MGBNFQ
CONV-C21 Probe	6FAM-TTACAAACTAATCCTTAGCTTT-MGBNFQ

Statistical Analysis

Statistics were performed using GraphPad Prism 6. Values of $p<0.05$ were considered significant.

Results

The *KCNA5* locus is hypermethylated in cancer. DNA methylation is a form of epigenetic regulation that is associated with gene silencing when methylation occurs in CpG islands of gene promoters. Cancer cells alter the epigenome and dysregulate DNA methylation patterns of genes, such that gene promoters become hypermethylated and intergenic regions become hypomethylated (136, 141, 211, 215). Interplay between DNA methylation and other epigenetic processes exist to heritably silence genes (147, 169, 170). Interestingly, in human cancer, the promoters of PcG target genes are often subject to aberrant DNA hypermethylation (225-227). Previous work from our group demonstrated that the *KCNA5*-encoded channel, Kv1.5, is a target of PcG–dependent repression in the aggressive pediatric solid tumors, Ewing Sarcoma (ES) and Neuroblastoma (NB). Furthermore, this epigenetic silencing of the Kv1.5 channel was shown to contribute to cancer cell survival under conditions of hypoxic stress (228). Therefore, we sought to determine whether the *KCNA5* locus is hypermethylated in ES. To address this we assessed the DNA methylation status of the *KCNA5* promoter by interrogating data from a custom Illumina GoldenGate® methylation array and by performing MethyLight studies. The GoldenGate® array analysis of ES cell lines and tumors and non-transformed tissues shows that the *KCNA5* promoter is hypermethylated in ES samples compared to non-transformed tissues, as the beta-values for the ES samples are significantly greater than the beta-values of the non-transformed tissues (Figure 3.1A). Collaborators, Dr. Peter Laird and Dr. Dan Weisenberger,

completed the initial MethyLight studies, while subsequent studies were completed in the Lawlor laboratory. By MethyLight analysis, we confirmed that the *KCNA5* promoter is hypermethylated in ES samples, using an independent set of primary tumors and cell lines (Figure 3.1B). Furthermore, these studies demonstrate that the methylation of *KCNA5* is relatively higher in ES cell lines than ES primary tumor samples (Figure 3.1B), potentially suggesting an adaptive mechanism for cancer cells to survive in culture. To compare the methylation status of *KCNA5* to non-transformed cells, MethyLight analysis was conducted in the two non-transformed cell lines, HuVEC and MRC5. Similar to the GoldenGate® array data, methylation of *KCNA5* is drastically lower in the two non-transformed cell lines compared to the ES cell lines and only slightly lower in comparison to the ES primary tumors (Figure 3.1B). Taking it a step further, we also analyzed the methylation profile of *KCNA5* in mesenchymal stem cells (MSC), one of the putative stem cells for ES. Interestingly, we found that the methylation profile of *KCNA5* in MSCs matched that found in the non-transformed cells, with it being lower in comparison to the ES cell lines and primary tumors (Figure 3.1B). Next, we interrogated the degree to which *KCNA5* is methylated in other cancer cell lines. We discovered that in the aggressive cancer cell lines osteosarcoma (U2OS), non-small-cell-lung (A549), pancreatic (UM53) and adenocarcinoma cancer cells (LoVo), five out of six cell lines have high percentages of *KCNA5* methylation (90-150% methylation) (Figure 3.1C). Interestingly, the pancreatic cancer cell line, UM5, a stem-like cancer cell line (personal communication from Simeone Laboratory), has a low percentage of

KCNA5 methylation (35-45%) (Figure 3.1C). Together, these studies demonstrate that DNA methylation is present at the *KCNA5* locus in human cancer cells.

Decitabine treatment results in loss of methylation at the *KCNA5* locus. Having established that DNA methylation epigenetically represses *KCNA5;* we next sought to determine if the *KCNA5* locus could be demethylated using a hypomethylating agent. The hypomethylating agent chosen was decitabine, a nucleoside analog that inhibits DNMTs (229). Current research demonstrates that chronic exposure to epigenetic modifiers at sub-cytotoxic levels alters the epigenetic landscape in cancer cells (215, 230). Interestingly, decitabine treatment has a biphasic effect, such that at sub-cytotoxic levels it exerts a hypomethylating effect, while at high levels it has cytotoxic effects promoting cell death (231). Thus, we treated three ES cell lines over a course of 72 or 96 hours with a sub-cytotoxic level of decitabine (100 nM). Exposure of ES cells to decitabine led to the demethylation of the *KCNA5* locus in all three ES cell lines, TC-71, A673 and A4573 (Figure 3.2A). These studies confirm that exposure to 100 nM of decitabine hypomethylates the *KCNA5* locus, partially restoring the epigenetic landscape of *KCNA5* to that seen in non-transformed cells. Next, we investigated whether demethylation of the *KCNA5* locus would lead to the upregulation of *KCNA5* transcript. To address this, we performed qRT-PCR analysis of ES cells exposed to 100nM decitabine for 72 or 96 hours. We discovered that demethylation of the *KCNA5* locus was associated with increased expression of the *KCNA5* transcript in all three ES cell lines, TC-71,

A673 and A4573 (Figure 3.2B). Together, these studies confirm that the *KCNA5* promoter is subject to epigenetic regulation by DNA methylation in ES cells.

Kv1.5 channel function inhibits ES cell proliferation. Understanding that decitabine treatment at sub-cytotoxic levels inhibits ES cell proliferation (173, 232), we sought to replicate this effect in our own hands in three ES cell lines (TC-71, A673 and A4573). To test this, we conducted proliferation assays using a set number of ES cancer cells. Cells were either treated with no drug, vehicle (DMSO) or a sub-cytotoxic treatment of decitabine (100 nM) for 72 hours in ambient conditions (21% O_2). Exposure of all three ES cells to decitabine resulted in significant decreases in cell proliferation compared to vehicle control (Figure 3.3A). Thus, ES cell proliferation *in vitro* is at least in part dependent on the activity of DNMTs and DNA methylation.

Having established that decitabine treatment at sub-cytotoxic levels slows the proliferative rate of ES cells, we next sought to determine if the reduction in proliferation was mediated by restoration of Kv1.5 channel expression. To address this question we used the pharmacological compound diphenyl phosphine oxide-1 (DPO-1), a specific inhibitor of the Kv1.5 channel which blocks potassium efflux through the channel with an IC_{50} = 310 nM (195). Proliferation assays were repeated and ES cells (TC-71, A673, A4573) were exposed to DPO-1 and decitabine over a course of 72 hours. Blocking the Kv1.5 channel with DPO-1 partially restores the proliferative phenotype of all three ES cell lines (Figure 3.3B). Importantly, the total number of dead cells across all conditions is largely consistent, demonstrating that a sub-cytotoxic level of

decitabine does not affect the viability of ES cells (Figure 3.3C, D). Lastly, exposure of ES cells to DPO-1 alone does not affect ES cell proliferation (Figure 3.3E). Thus, these findings indicate that the Kv1.5 channel influences the proliferation of ES cells and potentially functions as a regulator of the cell-cycle in cancer cells.

Discussion

Potassium-conducting channels are essential, as they predominantly regulate and maintain resting membrane potential, an essential biophysical component of a cell (24). The membrane potential (Vm) of a cell functions as a biophysical signal, important for controlling many biological properties, such as proliferation, migration and differentiation (7, 24). Interestingly, as cells proliferate, their Vm changes due to alterations in potassium flux, which is caused by differential expression of potassium channels within the cell cycle (1, 200, 233). The Vm of a cell is unique, because it regulates a cells progression through the cell cycle. Recent evidence has shown that cancer cells hijack the bioelectric regulator, the Vm, to be in a depolarized state by dysregulating potassium channel expression ((234) and see review (24)). These changes instruct the cancer cell to rapidly advance through cell cycle checkpoints, thereby sustaining proliferative signaling. Due to the diversity of potassium channels, identification of the key channels and the mechanisms regulating them have been slow, however it is clear that each channels contribution is cell-type and function specific (reviewed in (233, 235, 236)). Of the potassium channels, one of

prime interest is the Kv channel, Kv1.5, an important oxygen-sensitive channel, that has been implicated in mediating both apoptosis (8, 46, 83, 205) and cell cycle regulation (1, 133). In this study, we focused on an epigenetic mechanism regulating the Kv1.5 channel-encoding gene, *KCNA5*, and its impact on cell proliferation in ES.

Cancer cells hijack and dysregulate epigenetic machinery to dynamically alter gene expression and drive tumor pathogenesis (136, 141). In ES, the PcG proteins, BMI-1 and EZH2, are induced and function as oncogenes contributing to ES tumorigenesis (175, 176). Previous work demonstrated that in ES, the *KCNA5* promoter is targeted and repressed by BMI-1 and EZH2 (228). The consequence of PcG-dependent repression of *KCNA5* is to promote ES cell survival under conditions of physiological stress, hypoxia (1% O_2) and growth factor deprivation). In this study, we discovered that *KCNA5* is also epigenetically silenced by DNA methylation. In particular, we determined that this locus is hypermethylated in cancer cells compared to non-transformed cells. Unlike PcG proteins, the importance of *KCNA5* methylation is to promote cell proliferation under ambient conditions (21% O_2 and growth factors). Together these studies suggest that the effect of *KCNA5* expression on cancer biology is dependent on the epigenetic mechanism modulated. To specifically address this, future work will need to determine whether demethylation of the *KCNA5* locus sensitizes cancer cells to physiological stress (hypoxia 1% O_2 and growth-factor deprivation) and if this sensitization is dependent on Kv1.5 channel function. As recent advances have discovered the PcG protein, EZH2, recruits DNMTs to

establish a permanent repressive state in cancer cells (136, 237), future studies will also tease out the mechanistic crosstalk between DNMTs and PcG proteins at the *KCNA5* locus in both ambient and stressed conditions. To accomplish this, we will use pharmacologic or genetic means to remove one epigenetic mechanism and determine its impact on the regulation of the *KCNA5* locus by the other epigenetic mechanism using chromatin immunoprecipitation and MethyLight analyses. Lastly, our results reveal that demethylation of the *KCNA5* promoter leads to inhibition of ES cell proliferation. Therefore, this data suggests a role of the Kv1.5 channel in the modulation of cell-cycle progression in cancer cells.

Cancer cells dysregulate potassium channels to promote proliferation, migration and differentiation (reviewed in (7)). Seminal work, demonstrated that Kv channels are the predominant channels involved in cell proliferation (238). Accumulating evidence demonstrates that the Kv1.5 channel is implicated in cell growth and proliferation (77, 132, 133, 239, 240). In myoblasts, high Kv1.5 expression leads to accumulation of cyclin-dependent kinase inhibitors and impairs cell-cycle progression at the G1 to S transition, suggesting the channel to be a cell cycle checkpoint (133). As this channel is downregulated in numerous cancers (6, 9, 12, 14, 15) and inversely correlated to tumor aggression in gliomas, it suggests that downregulation of Kv1.5 contributes to tumor pathogenesis (11). Our studies begin to elucidate the role of the Kv1.5 channel in ES cell proliferation and the consequence of its downregulation. We discovered that the global hypomethylating agent, decitabine, directly promotes *KCNA5*

transcript and leads to a significant decrease in the proliferation of ES cells. Interestingly, we found that pharmacologically blocking the Kv1.5 channel partially restores the proliferative phenotype of ES cells. This study provides compelling evidence that potassium efflux through the Kv1.5 channel directly contributes to cancer cell proliferation. However, further studies are needed to investigate the role of the Kv1.5 channel in cancer cell proliferation and cell cycle regulation. To begin to address this, we will conduct another proliferation assay by trypan blue exclusion using another Kv1.5 channel blocker, 4'aminopyridine (4'AP). Next, an additional proliferation assay involving bromodeoxyuridine (BrdU) incorporation will be used to verify that the Kv1.5 channel activation inhibits ES cell proliferation. Furthermore, to specifically determine if Kv1.5 channel activation is affecting cell cycle progression, we will conduct cell cycle analysis using propidium iodine (PI) staining. Lastly, to directly determine if Kv1.5 channel function affects ES cell proliferation, we will re-introduce the Kv1.5 channel in ES cells using adenoviral constructs containing either the wild-type channel or the pore-dead channel, and conduct a proliferation assay by trypan blue exclusion. As the Kv1.5 channel contributes to membrane repolarization and resting membrane potential, it suggests that modulation of cell membrane polarization affects tumorigenesis. Thus, modulating membrane polarization through other ion channels (e.g. chloride) could re-capitulate the sensitization of cancer cells to physiological stress (24) and inhibit cell proliferation, as seen with Kv1.5. Ultimately, this could incorporate ion channel targeted therapies into cancer treatment.

In summary, we have identified another epigenetic mechanism, DNA methylation that contributes to the repression of the *KCNA5* locus in cancer. This data provides additional evidence, that cancer cells hijack the physiological regulation of the Kv1.5 channel to promote tumorigenicity. Future work needs to elucidate how we can therapeutically exploit the epigenetic regulation of *KCNA5,* to eliminate aggressive cancers, such as ES, and prevent patient relapse.

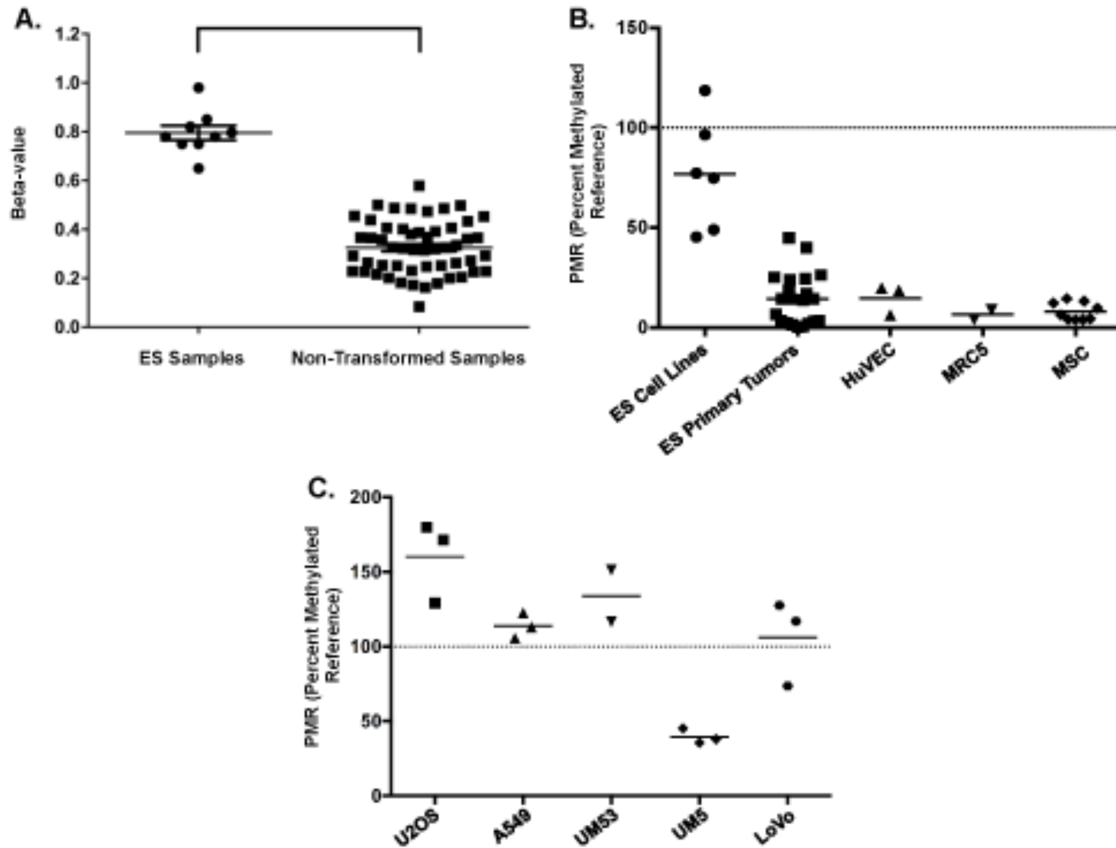

Figure 3.1. The methylation status of *KCNA5* in cancer cells.

(A) Analysis of ES samples, consisting of 4 ES primary tumors and 5 ES cell lines, and 52 non-transformed tissue samples by a GoldenGate® assay demonstrates that in ES samples the *KCNA5* promoter has a significantly higher level of methylation in comparison to non-transformed samples. *** p-value < 0.001 and the difference beta-value ≥ 0.2 (mean ± SEM) between the two groups. (B) MethyLight analysis of 6 ES cell lines, 21 ES primary tumor samples, 2 non-transformed cell lines (HuVEC, MRC5) and mesenchymal stem cells (MSC) reveals a broad range in the methylation of *KCNA5*. In particular, the ES cell lines have a diverse range in the methylation of *KCNA5* and the mean percentage of methylation is higher compared to the ES primary tumors. As in (A) the non-transformed samples, HuVEC and MRC5, along with MSCs, have a lower percentage of *KCNA5* methylation compared to ES samples. (C) MethyLight analysis of 6 other aggressive cancer cell lines, including osteosarcoma (U2OS), non-small-cell-lung (A549), pancreatic (UM53, UM5) and human colon adenocarcinoma (LoVo). Of the 6 cell lines, the UM5 cells have a low percentage of KCNA5 methylation (35-45%) in comparison to the 5 other cell lines that have a high amount of KCNA5 methylation (>100%). Data points are presented with mean values (n=2-3). Expression is presented as percent methylated reference (PMR).

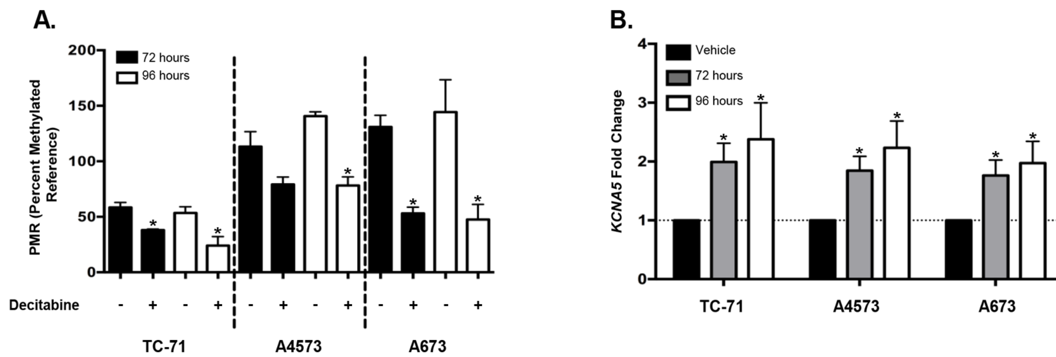

Figure 3.2. Demethylation of the *KCNA5* locus is associated with increased transcript expression.

(A) MethyLight analysis demonstrates a significant decrease in the percentage of methylation at the *KCNA5* promoter in TC-71, A4573 and A673 cells, after a 72-hour treatment with 100 nM decitabine. Expression is presented as percent methylated reference (PMR). (B) qRT-PCR analysis demonstrates a significant upregulation of *KCNA5* transcript expression in 3 ES cell lines (TC-71, A4573, A673) after a 72 hour treatment with 100 nM decitabine. Expression is normalized to the geometric mean of HPRT and GAPDH within each sample and the decitabine treated samples are represented as a fold change relative to the vehicle treated cells. * $p<0.05$ (mean ± SEM, n=3).

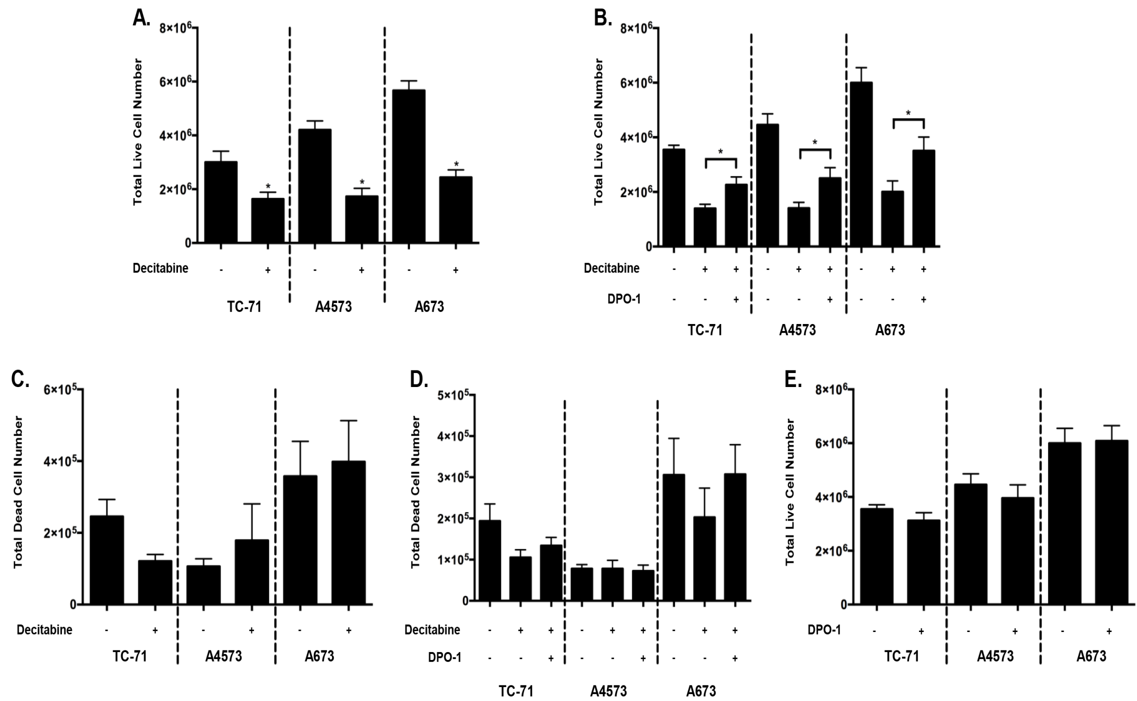

Figure 3.3. Blocking Kv1.5 channel function partially prevents the decitabine-induced reduction in ES cell proliferation.

(A) TC-71, A4573 and A673 cells have a significant reduction in cell proliferation, after a 72-hour treatment with 100 nM decitabine in ambient conditions. (B) Live cell count graphs demonstrate that blocking Kv1.5 channel function with 310 nM DPO-1 partially restores ES cell proliferation and therefore reverses the decitabine-induced reduction in cell proliferation. * $p<0.05$ (mean ± SEM, n=3). Dead cell count graphs demonstrate that treatment with 100 nM decitabine alone (C + D) and the combination of 100 nM decitabine and 310 nM DPO-1 (D) have no toxic effect on the ES cells. (E) Treatment with 310 nM DPO-1 under ambient conditions has no effect on TC-71, A4573 or A673 cell proliferation.

CHAPTER 4

CONCLUSION

Introduction

The dysregulation of ion channels is an emerging field in cancer research (7, 13, 49). In particular, growing evidence suggests dysregulation of potassium channels directly impacts tumor progression (7, 49). However, as potassium channel expression and function is relatively unexplored in cancer, many gaps exist in our understanding of the mechanisms underlying their aberrant expression. Through the work of this thesis, we have discovered a novel mechanism underlying expression of the unique O_2-sensitive channel, Kv1.5, in the pediatric solid tumors, ES and NB. Specifically, we have discovered that the epigenetic suppression of *KCNA5* by the PcG proteins, BMI-1 and EZH2, and by DNA hypermethylation contributes to Kv1.5 channel and *KCNA5* transcript downregulation (Illustrated in Figure 4.1). Furthermore, we determined PcG-dependent suppression of *KCNA5* is a mechanism by which cancer cells resist physiological stressors (i.e. hypoxia and growth factor deprivation) to promote cell survival, while the suppression of *KCNA5* by DNA methylation promotes proliferative signaling. Importantly, this work determined that potassium efflux out

of the Kv1.5 channel is the trigger that drives apoptotic-mediated cell death and prevents cell proliferation. Therefore, this work provides further evidence that cancer cells manipulate intracellular potassium concentrations to promote tumor progression. To our knowledge, this work is the first to describe an epigenetic mechanism underlying the regulation of the potassium channel, Kv1.5, in human cancer. Overall, this thesis is significant because it provides a mechanism that can be therapeutically exploited to eliminate aggressive solid tumors prone to relapse.

Implications for Cancer Pathogenesis and Future Biologic Study

The Mitochondria and Dichloroacetate (DCA)

Cancer cells dysregulate mitochondrial function to shift their energy metabolism to rely on glycolysis, as opposed to oxidative phosphorylation (Warburg effect) (241). This metabolic adaptation allows cancer cells to survive in a hypoxic microenvironment (242), increases their metastatic potential (242), offers a proliferative advantage (242) and induces resistance to apoptosis (8, 243). As mitochondrial remodeling promotes tumorigenesis, reversing it may promote apoptosis, inhibit cell proliferation and reduce metastatic potential.

Dichloroacetate (DCA) is a chemical modulator that selectively targets cells with abnormal mitochondrial function (8, 244). It was found to possess anti-cancer properties due to its inhibition of cancer cell growth in a wide range of tumors (8, 206). *In vitro* studies discovered DCA induces apoptosis and inhibits proliferation of cancer cells and *in vivo* studies found DCA decreases metastases

and reduces tumor growth (8, 244-246). In addition, DCA has no effect on non-cancerous cells, demonstrating its potential as a selective anti-cancer agent that possesses a low toxicity profile (8, 244). In a Phase I trial in patients with recurrent malignant brain tumors, such as Glioblastoma multiforme (GBM), (clinicaltrials.gov, NCT01111097), DCA was well tolerated at an individualized dose (247). The success of DCA in adult cancers, in the context of *in vitro* studies and a Phase I trial, led others to determine if DCA would have similar results in pediatric cancers. Regrettably, however, DCA was found to be completely ineffective at inducing apoptosis and inhibiting proliferation in six pediatric tumor cell lines, including ES and NB (207). Currently, the mechanism(s) preventing DCA from exerting its anti-cancer effect have not been addressed in these pediatric cancers.

It is known that the Kv1.5 channel is intimately linked to the mitochondria through the mitochondria-ROS-HIF-Kv pathway (8, 101). The channel functions as the downstream trigger that mediates potassium efflux from the cell, which is critical for regulating apoptotic mediated cell death and proliferation (8, 101). Recent research elucidated in glioblastoma, non-small-cell-lung and breast cancers that DCA induces apoptosis through a Kv1.5-dependent mechanism (8). DCA treatment forces cancer cells to increase reliance on oxidative phosphorylation, which in turn increases production of ROS. Kv1.5 channels are then activated by ROS, which through a positive feedback loop involving removal of NFAT and de-repression of *KCNA5*, increases Kv1.5 channel expression and together induces caspase activation (8). It is known that the PcG protein, BMI-1,

is upregulated in ES and NB and in Chapter Two we demonstrate that BMI-1 represses *KCNA5* in ES and NB (228). Furthermore, BMI-1 is demonstrated to regulate mitochondrial function and redox balance (248-250). Together, this suggests that BMI-1 is a key element regulating cellular metabolism in cells. Therefore, we hypothesize that the dysregulation of BMI-1 in ES and NB cells prevents DCA treatment from having a biological effect. Furthermore, due to the direct repression of *KCNA5* by BMI-1 in ES and NB, we also hypothesize that BMI-1 prevents DCA-mediated induction of *KCNA5* transcript and the upregulation of Kv1.5 expression. Thus, if these hypotheses stand correct, then targeting and inhibiting BMI-1 would sensitize ES and NB to DCA treatment.

To begin to address these hypotheses, we completed a few preliminary studies. First, to demonstrate that we could sensitize cells to DCA, we treated LoVo cells, a colon cancer cell line previously reported to be sensitive to DCA, with 20 and 40 mM of DCA for 48 hours (251, 252) (Figure 4.2A). After confirming that DCA treatment results in a decrease in LoVo cell proliferation, a phenotype previously described in the literature (251, 252), we moved on to determine whether BMI-1 prevents DCA from its anti-cancer effect in ES and NB. To address this, we utilized the BMI-1 knockdown ES cell line, TC-71 shBMI-1 156, and its scrambled control, TC-71 shNS, and exposed these cells to either vehicle control (DMSO) or 10 mM DCA for 48 hours. We assessed both cell viability and cell proliferation by trypan blue exclusion. Interestingly, we discovered that BMI-1 knockdown cells have a significant decrease in cell proliferation when treated with DCA (Figure 4.2B). This provides preliminary

evidence that BMI-1 dysregulation does prevent DCA from having an effect in ES and potentially in NB. Taking it a step further, we utilized the TC-71 shNS and TC-71 shBMI-1 cells and the Kv1.5 specific blocker DPO-1, to determine if the DCA-induced proliferation inhibition is mediated by restoration of Kv1.5 channel function. We found that inhibition of the Kv1.5 channel partially restores cell proliferation in the TC-71 shBMI-1 cells (Figure 4.2B). These data provide preliminary evidence that the Kv1.5 channel regulates the DCA-induced effect on cell proliferation and implying that Kv1.5 channel expression is restored in the BMI-1 knockdown cells. Interestingly, these data describe the Kv1.5 channel mediating a proliferative phenotype associated with DCA, which is a novel finding, as the literature previously reports Kv1.5 mediating DCA-induced apoptosis (8). All together, these data point to BMI-1 as the key player in preventing the anti-cancer effects of DCA and Kv1.5 as the trigger in promoting DCA's anti-cancer effect.

Based on the promising data gathered from the preliminary studies, we will continue to elucidate the mechanism behind DCA insensitivity in ES and NB by focusing on the PcG protein, BMI-1 and the potassium channel, Kv1.5. The first set of experiments will determine whether BMI-1 prevents DCA from having an anti-cancer effect in other ES cell lines (A673, A4573) and in NB cell lines (LA1-55N, SH-SY-5Y). In these cell lines, we will utilize lentiviral constructs to knockdown BMI-1 and then expose the cells either to vehicle control (DMSO) or 10 mM DCA. Cell proliferation will be assessed by trypan blue exclusion. As with the TC-71 shBMI-1 cell line (Figure 4.2B), we predict that the additional BMI-1

knockdown ES and NB cell lines will also have a significant decrease in proliferation upon DCA treatment. Taking it a step further, we will also determine if the DCA-induced reduction in proliferation is mediated by restoration of Kv1.5 channel function. To accomplish this, we will expose the BMI-1 knockdown cells to DCA and the Kv1.5 blocker, DPO-1. We predict that inhibition of the Kv1.5 channel will partially restore the proliferative phenotype in the other ES and NB cell lines, as seen in the TC-71 shBMI-1 cell line. Combined these experiments, if correct, will provide concrete evidence that BMI-1 is a key player in preventing the anti-cancer effects of DCA.

Previously, we hypothesized that direct repression of *KCNA5* by BMI-1 prevents DCA-induced upregulation of the *KCNA5* transcript. Therefore, we will determine if exposure of BMI-1 knockdown ES and NB cells to DCA upregulates *KCNA5* transcript by qRT-PCR. We predict that in the BMI-1 knockdown cells, *KCNA5* transcript will be detected, but only in the cells treated with DCA. If this holds true, then it suggests the effect of DCA works through a Kv1.5-dependent mechanism. To verify this, we will conduct an experiment that directly determines if the Kv1.5 channel and its potassium flux mediate the DCA-induced reduction in cell proliferation. In this study, we will ectopically express the Kv1.5 channel in ES and NB cells and then expose them to vehicle control or 10 mM DCA. Cell proliferation will be analyzed by trypan blue exclusion. In this experiment, two Kv1.5 channel constructs will be expressed, either Kv1.5 Wild-Type (functional channel) or Kv1.5 Pore-Dead (non-functional channel), to specifically determine if it is the flux of potassium out of the channel that affects cell proliferation. Based

on our preliminary data (Figure 4.2B), we hypothesize that the efflux of potassium out of the Kv1.5 channel mediates the DCA-induced reduction in proliferation. Therefore, we predict that only the cells expressing functional wild-type Kv1.5 channel and treated with DCA will have a reduction in proliferation. So far, if all of our combined hypotheses stand correct, it would provide rationale for a combination treatment in ES and NB involving either the inhibition of BMI-1 or the ectopic expression of Kv1.5 followed by DCA treatment.

DCA selectively targets the mitochondria (8, 244) and BMI-1 is demonstrated to have a role in mitochondrial regulation and redox homeostasis (248-250). Therefore, it can be suggested that BMI-1 exerts control over mitochondrial regulation and ROS generation and this contributes to the inability of DCA to exert an effect in ES and NB. To determine this, we will assess whether BMI-1 plays a role in regulating ROS generation and mitochondrial membrane polarization in ES and NB cells. Based on the literature, we hypothesize that BMI-1 overexpression in NB and ES contributes to the suppression of ROS and the hyperpolarization of the mitochondrial membrane. To address this, we will knockdown BMI-1 in ES and NB cell lines and measure ROS levels using an Amplex Red® assay and determine mitochondrial membrane polarization using the JC-1 MitoProbe™ Assay. We predict that in the BMI-1 knockdown cell lines there will be a significant increase in ROS generation and a depolarization of the mitochondrial membrane. Together, these studies would provide important mechanistic insight that could contribute to the rationale

for developing therapeutic strategies to target BMI-1 and ultimately incorporate DCA as a therapeutic treatment avenue in ES and NB.

The proposed studies discussed so far focus on BMI-1 and its role in DCA treatment, however we are also interested in EZH2, the other PcG protein that is upregulated in ES and NB and represses the *KCNA5* locus (see Chapter 2). As with BMI-1, we will determine if EZH2 contributes to the inability of DCA from exerting an effect in ES and NB cells and if this is due to repression of *KCNA5* by EZH2. Based on the role of EZH2 in regulating *KCNA5,* as demonstrated in Chapter 2, we hypothesize that loss of EZH2 will sensitize ES and NB cells to DCA and it will be partially mediated through Kv1.5 channel function. To address this hypothesis, we will inhibit EZH2 by pre-treating ES and NB cells with GSK-126 (an EZH2 inhibitor) and then exposure the cells to either 10 mM DCA or 10 mM DCA and the Kv1.5 blocker, DPO-1. We will then conduct a cell proliferation assay using trypan blue exclusion. We predict that the cells treated with GSK-126 and DCA will have a reduction in cell proliferation and this will be partially reversed by Kv1.5 channel inhibition. If this outcome occurs, it suggests that modulation of either EZH2 or BMI-1 is sufficient to promote Kv1.5 channel expression and sensitize ES and NB cells to DCA. Furthermore, this outcome could lead to the investigation of a potential cooperative mechanism between EZH2 and BMI-1 and its effect on DCA treatment. If however, we find that inhibition of EZH2 does not sensitize cells to DCA, then we will conclude that BMI-1 is the central PcG component regulating this effect.

KCNA5 is repressed by BMI-1, EZH2 and DNA hypermethylation in ES and NB and as we have discussed the contribution of BMI-1 and EZH2 towards the ineffective nature of DCA treatment, we will turn our attention to DNA methylation. To re-iterate, we hypothesize that it is the epigenetic repression of *KCNA5* that prevents the anti-cancer effect of DCA in ES and NB. Therefore, we would like to determine if a reduction in the hypermethylation of *KCNA5* would sensitize ES and NB cells to DCA and if this effect is mediated through the Kv1.5 channel. To address this we will utilize decitabine, which at sub-cytotoxic doses (100 nM) has a hypomethylating effect. We will pre-treat ES and NB cells with decitabine, expose them to 10 mM DCA or 10 mM DCA and 310 nM DPO-1 and then conduct a cell proliferation assay. Similar to the other sections, we predict that the cells pre-treated with decitabine will be sensitized to DCA and this sensitization will be reversed in the DPO-1 treated cells. If this occurs, there is also the potential that inhibition of BMI-1 or EZH2 and DNA methylation could further increase the sensitization of ES and NB cells to DCA. Together this would provide multiple combination treatments (GSK-126 and DCA or decitabine and DCA), to sensitize ES and NB cells to DCA. However, if pre-treatment with decitabine has no effect then as mentioned in the previous paragraph we will conclude that BMI-1 is the central component regulating the DCA effect.

In summary, our preliminary work and future studies suggest with modulation of BMI-1, EZH2, DNA hypermethylation and/or Kv1.5, DCA stands as a promising anti-cancer treatment in primary or recurrent ES and NB.

Ion Channel Therapy

Potassium channels are cell-surface proteins that are accessible drug targets and have significant potential in disease states that aberrantly regulate their expression. Currently, potassium channels (e.g. Kv11.1 (HERG)) are successful drug targets in treating numerous diseases, including diabetes, hypertension and cardiac arrhythmias (49, 253, 254). As potassium channel expression is dysregulated in cancer, they could serve as useful drug targets in the treatment of cancer.

The Kv1.5 channel is one of two potassium channels that are downregulated in cancer (Kv1.5 and Kv7.1). This thesis and other work (7, 8, 10, 228) demonstrate that cancer cells downregulate the Kv1.5 channel in order to modulated its physiologic effects, such as promoting cell death and inhibiting cell proliferation, that inherently suppress cancer. Therefore, a novel therapeutic approach could be to develop tools that result in increased expression of Kv1.5, in cancer. Such methods that could be utilized include epigenetic modifiers (see Therapeutic Implications for Epigenetic Modifiers) or a novel way of delivering the channel directly to tumors (e.g. gene therapy). The clinical success of immunotherapy, oncolytic virotherapy and gene transfer has brought back the possibility of gene therapy as a potential treatment strategy in cancer (255). Therefore, as we aim to promote overexpression of the Kv1.5 channel in cancer, this could be accomplished through the use of gene therapy. In 2013, neurosurgeons at UCSD were able to utilize MRI guidance for the delivery of gene therapy (TOCA 511) as treatment for GBM in combination with

chemotherapy (https://health.ucsd.edu). In this study, which has now become a clinical trial, they used the TOCA 511 retrovirus to make tumors more susceptible to cancer killing drugs (clinicaltrials.gov, NCT01156584). This study opens up the possibility of engineering a gene therapy treatment aimed to overexpress the Kv1.5 channel, which could then be specifically injected into the core of an ES or NB tumor. We hypothesize that overexpression of the Kv1.5 channel within a tumor would increase the sensitivity of cancer cells to standard treatment (and also to tumor hypoxia) and result in significant cell death and a decrease in tumor size. To test this hypothesis, we could utilize a patient derived xenograft (PDX) ES mouse model (256) or the MYC-N driven NB mouse model (257), and using MRI guidance inject a viral vector engineered to overexpress the Kv1.5 channel, into the core of these tumors. These mice would then be exposed to standard chemotherapy agents specific for ES or NB and during this time we would monitor tumor size and analyze the tumors for markers of cell death (e.g. necrosis and apoptosis). These measures would allow us to determine if Kv1.5 channel expression in combination with standard treatment had a greater tumor suppressive impact than in the control mice exposed to chemotherapy alone. In conclusion, regardless of the method, expression of the Kv1.5 channel in cancer cells could be of great therapeutic value, particularly if paired with therapeutic agents, such as DCA or with chemotherapeutic agents or radiotherapy, since re-establishment of potassium efflux would be expected to sensitize cancer cells to oxidative, chemical and/or radiation stress.

Other potassium channels that could serve as targets in cancer would be those that are overexpressed and contribute to tumor pathogenesis as illustrated in Table 1 (Chapter 1). Currently, Kv10.1 (EAG1) is an appealing candidate as it is overexpressed in 70% of human cancers and its overexpression confers tumorigenic potential (258, 259). Preliminary work has demonstrated that inhibition of EAG1 by the small molecule blockers imipramine and astemizole is effective in reducing EAG1-driven cancer cell growth *in vivo* (260, 261). Furthermore, targeting channels like Kv10.1 in cancer could allow for re-purposing of existing therapeutic agents. This would be beneficial for two reasons: first, it does not require development of new compounds and second, any undesired side effects could be accurately predicted based on their use in other diseases.

The importance of targeting potassium dysregulation in cancer is also demonstrated with the potassium ionophore salinomycin. In cancer research, salinomycin has been shown to selectively target a subpopulation of cancer cells, known as cancer stem cells (CSCs) in some types of cancers (e.g. breast (203), lung (262), colorectal (263) and leukemia (264)). Salinomycin exerts its anti-cancer effect by interfering with transmembrane potential and promoting significant potassium efflux from the CSCs, resulting in apoptosis and an inhibition of proliferation (203, 265, 266). Prior studies have demonstrated a direct link between potassium efflux and the induction of apoptosis and cytotoxicity in cancer cells (36, 267). Together, these data demonstrate the therapeutic impact of manipulating potassium in cancer cells and provides

rationale for the use of salinomycin in cancer treatment. However, as salinomycin treatment would affect cancerous and non-cancerous cells alike, it would have to be aimed at cancer cells directly. A potential method of delivery is nanocarrier, which have been shown to increase tumor accumulation of small molecular drugs as they can specifically target tumors through tumor specific recognition molecules (e.g. epidermal growth factor) (268-271).

The role of potassium channels in cancer biology is still an emerging field and drugs that target potassium channels are yet to be developed successfully. However, current studies can begin to design appropriate targets by elucidating the mechanistic function of potassium channels in cancer biology from *in vitro* and *in vivo* standpoints.

Cancer Stem Cells (CSCs)

CSCs are a specialized subpopulation of cells, possessing stem-like properties, which have emerged as a driving force behind tumor growth and recurrence (203, 272). According to the CSC hypothesis, CSCs possess the ability to reform a tumor (known as tumorigenicity) (273, 274). Furthermore, CSCs regenerate biologically heterogeneous tumors, consisting of CSCs and non-tumorigenic bulk tumor cells, therefore demonstrating the existence of cell hierarchy within a tumor (275). The CSC hypothesis has been demonstrated in numerous adult cancers (e.g. breast cancer, glioma, melanoma), as the CSC population in each tumor type is shown to reform tumors and re-establish hierarchical cell organization (273, 276-281). In contrast to adult tumors, CSCs have been isolated in relatively few pediatric tumors (282). Of those tumors, CSC

populations have been identified in both ES and NB (283, 284). Though, follow-up studies demonstrate that within both solid tumors, CSCs are isolated in only a small fraction of the tumors analyzed (285, 286).

A major concern with CSCs is their resistance to available therapy, consisting of chemotherapy and radiation (280, 287-291). Therefore, to prevent cancer relapse we need to develop strategies to increase the sensitivity of CSCs to current treatment. To accomplish this task, the mechanisms governing the maintenance of the CSC state need to be elucidated and exploited. One such mechanism could be the epigenetic regulation of the potassium channel, Kv1.5. As this was discovered and elucidated in pediatric tumors that innately possess stem-like properties, this mechanism could be applicable to adult cancers in the context of CSCs.

We are interested in the role and regulation of potassium channels, specifically the Kv1.5 channel, in CSCs. To date, only one channel, the Kv11.1 (HERG) channel, has been identified in CSCs, though its function is unknown (292). Furthermore, research with salinomycin, a potassium ionophore, demonstrates that potassium flux is important in CSCs, as significant potassium efflux from CSCs results in apoptosis and a decrease in proliferation (203, 265). In normal stem cells, it is known that potassium channels have a heterogeneous expression and their role is to regulate bioelectrical signals within the cell membrane (293). Of the potassium channels, the Kv channel family is important for controlling stem cell proliferation, specifically at the G_0 and G_1 checkpoints (293). Though the regulation of Kv channels in stem cells is unclear, it is known

that they are targets of PcG proteins (146, 228). Together, it can be suggested that potassium channels have an important function in stem cells and CSCs, as the salinomycin data suggests CSCs dysregulate potassium channel expression to promote tumorigenic potential. As our interest lies with the Kv1.5 channel, future work will focus on the regulation of the Kv1.5 channel encoding gene, *KCNA5*, and its biological impact on CSCs. In these studies we will utilize breast cancer cell lines (i.e. MCF-7, MDA-MB-231) to isolate CSCs, as they are well-established model cell lines for CSC isolation.

In this thesis, we discovered that *KCNA5* is epigenetically repressed in ES and NB, therefore we hypothesize that epigenetic repression of the *KCNA5* locus is an inherent feature in CSCs. To begin, we will assess the expression profile of *KCNA5* transcript in CSCs using qRT-PCR, to establish that *KCNA5* expression is silenced in these cells. If *KCNA5* transcript is undetectable, we will determine if the PcG proteins, BMI-1 and EZH2, and DNA hypermethylation epigenetically repress the *KCNA5* locus. To accomplish this we will perform ChIP experiments for the two PcG proteins, BMI-1 and EZH2, along with their histone modifications, H2AK119Ub1 and H3K27me3, and MethyLight studies to determine the methylation status of *KCNA5*. In these studies, the results for CSCs will be compared to bulk tumor cells, therefore we will also determine if there are any differences in the epigenetic regulation of *KCNA5* in bulk tumor cells compared CSCs. As we hypothesize that the CSC population has similar properties to ES and NB cells, we predict that the degree to which *KCNA5* is epigenetically repressed will be significantly higher in CSCs compared to bulk tumor cells. The

predicted difference in the epigenetic repression of *KCNA5* could be an underlying mechanism contributing to the tumorigenicity of CSCs, in regards to cell survival and proliferation.

In Chapters Two and Three we demonstrate that the epigenetic repression of *KCNA5* promotes cancer cell survival and cell proliferation. Therefore, we hypothesize that the epigenetic repression of *KCNA5* in CSCs promotes cell survival and proliferative signaling. To address this hypothesis, we will express the Kv1.5 channel in CSCs and assess its affect on cell viability and proliferation. Expression of the Kv1.5 channel in CSCs will be achieved through two methods. The first method will utilize genetic and pharmacologic reagents to inhibit the proteins contributing to the epigenetic repression of *KCNA5*, thereby allowing for expression of the *KCNA5* transcript and the Kv1.5 channel. The second method will utilize adenoviral constructs to ectopically express functional and pore-dead versions of the Kv1.5 channel. Use of the Kv1.5 pore-dead channel is of particular importance, as it will inform us of whether it is the efflux of potassium out of the Kv1.5 channel that mediates CSC survival and proliferation. After expressing the Kv1.5 channel, CSCs will either be placed under conditions of stress, consisting of hypoxia and growth factor deprivation, or left in ambient conditions. The effect of the Kv1.5 channel on cell viability and cell proliferation will be determined by trypan blue exclusion. Based on the data in Chapters Two and Three, we hypothesize that expression of functional Kv1.5 channel will trigger CSC death under conditions of stress and slow the proliferation of CSC under ambient conditions. Together, the studies addressing the epigenetic

regulation of *KCNA5* and its impact on CSC biology could provide concrete evidence that CSCs dysregulate Kv1.5 to promote tumorigenicity and a potential therapeutically exploitable mechanism.

As previously mentioned, developing ways of therapeutically targeting CSCs is key in preventing tumor recurrence. Therefore we will determine if silencing of the *KCNA5* locus contributes to the resilient nature of CSCs to current therapeutic agents. We hypothesize that expression of the Kv1.5 channel will sensitize CSCs to chemotherapeutic reagents. To address this hypothesis, the two methods, as previously described, will be employed to express the Kv1.5 channel in CSCs. After expressing Kv1.5 in CSCs, the cells will be exposed to various chemotherapeutic agents often used in breast cancer treatment (e.g. doxorubicin and paclitaxel) at increasing concentrations (0.1 μM – 60 μM) and cell viability will be assessed using trypan blue exclusion. If expression of functional Kv1.5 channel sensitizes CSCs to chemotherapeutic agents, it could mean the discovery of a new therapeutic avenue in targeting CSCs either through the use of epigenetic modifiers (discussed below) or ion channel therapy.

Suppression of KCNA5: A Consequence of Tumor Relapse or Cancer Transformation?

The proposed studies for investigating the role of *KCNA5* in CSCs brings up the question of whether the suppression of *KCNA5* is associated with tumor relapse or a consequence of cancer transformation in ES and NB. As either outcome is plausible, we will conduct experiments testing both possibilities.

First, we will determine if the repression of *KCNA5* is a consequence of tumor relapse by analyzing pre-existing clinical data from ES and NB patients. In this analysis, we will compare the expression profile of *KCNA5* in relapsed patients versus patients upon initial diagnosis. This correlative clinical data would provide indirect evidence as to whether *KCNA5* expression is further repressed in relapsed patients. On a side note, in this analysis, we could also determine if *KCNA5* would serve as a predictive biomarker for response to standard treatment and/or survival outcome. To experimentally determine whether *KCNA5* is repressed in relapsed ES and NB cells, we will take *in vitro* and *in vivo* models of relapse for both ES and NB and analyze the expression profile of *KCNA5*. In the *in vitro* model, we will select and expand an ES and a NB cell population that became resistant to a chemotherapy agent (e.g. doxorubicin) and analyze *KCNA5* transcript expression. With the *in vivo* model, we will take relapsed ES and NB tumors from mice and analyze them for *KCNA5* transcript by quantitative real-time PCR and in situ hybridization. Assuming the *in vitro* and *in vivo* relapse models exist, these experiments would provide sufficient evidence for us to determine whether *KCNA5* suppression is a consequence of tumor relapse.

Next, to assess whether repression of *KCNA5* is a consequence of ES and NB cancer transformation, we will analyze the expression profile of *KCNA5* in the ES and NB cells of origin before and after exposure to their oncogenic drivers. In ES, the two proposed stem cells of origin are mesenchymal (MSC) and neural crest stem cells (NCSC) and the driver is an EWS-ETS oncogenic fusion gene, which often is the EWS-FLI1 fusion. In NB, the stem cell of origin is

the neural crest stem cells and the oncogenic driver is usually MYC-N. Assuming that the MSC and NCSC cells express *KCNA5*, this experiment would determine if the repression of *KCNA5* is a result of the oncogenic drivers for both ES and NB.

Together, these preliminary studies would allow us to discern whether repression of *KCNA5* is an inherent feature of ES and NB cancer transformation or factor associated with tumor relapse.

Therapeutic implications of Epigenetic Modifiers

Epigenetic abnormalities play a large role in cancer development and progression (136, 179, 215, 294). This thesis and current research demonstrate that cancer cells are reliant on specific epigenetic pathways and are therefore considered to be epigenetically vulnerable (180, 295, 296). Due to the dynamic nature of epigenetic modifications and the reliance of cancer cells on epigenetic pathways, epigenetic modifiers have become appealing pharmacologic agents.

The current epigenetic agents approved by the FDA for anti-cancer therapy include DNMT inhibitors (5-azacytidine and Decitabine) for myelodysplastic syndrome (MDS) and leukemia and HDAC inhibitors (vorinostat, romidepsin and belinostat) for cutaneous and peripheral T-cell lymphoma (173, 215). Currently, no FDA approved epigenetic therapies exist for pediatric cancers. However, multiple clinical trials, both completed and ongoing, are assessing tolerance, combination therapies and optimal dosing of DNMT and HDAC inhibitors in pediatric patients (www.cancer.gov/clinicaltrials). Of the completed trials, Phase I trials with the HDAC inhibitors vorinostat and

bortezomib in children with refractory solid tumors and recurrent solid tumors showed promise as the drugs were well tolerated and had minimal toxicities (297). The data gathered from these clinical trials provide a starting point towards the incorporation of epigenetic modifiers in pediatric treatment regimens, for both initial and relapsed treatments.

The clinical success of DNMT and HDAC inhibitors propelled scientific investigations and led to the discovery of numerous other epigenetic regulators that can be therapeutically targeted (e.g. EZH2 (298) and LDS1 (299)). In the pediatric cancers ES and NB, targets that show promise include the aberrantly upregulated PcG proteins, BMI-1 (228, 300, 301) and EZH2 (178, 228, 302, 303), the histone demethylase LSD1 (304, 305) and DNA methylation (173, 306-308). Inhibition of these proteins results in anti-cancer effects ranging from decreases in tumor growth and cell proliferation to cancer cell death. Work from Chapters Two and Three of this thesis specifically demonstrate the therapeutic potential of inhibiting the PcG proteins, BMI-1 and EZH2, and DNA methylation in both ES and NB. In Chapter Two, the data clearly demonstrate that inhibiting BMI-1 or EZH2 sensitizes ES and NB cancer cells to physiological stress, consisting of hypoxia and growth factor deprivation. This data are significant because they suggest that targeting and inhibiting BMI-1 and/or EZH2 could sensitize cancer cells to their existing hypoxic microenvironment, prominent within rapidly expanding tumors, and its death signals. Chapter Three of this thesis, and work by others (221, 307), provide convincing data that low-dose exposure to DNMT inhibitors could have therapeutic gain in ES and NB. The data demonstrate that

exposure to low-dose DNMT inhibitors causes re-expression of tumor suppressors (e.g. *KCNA5* (Chapter 3), *CLU (309, 310), RASSF1A (311)*) and leads to a significant reduction in cell proliferation. Together, this work contributes to the strong rationale for the utilization of epigenetic modifiers as therapeutic agents in ES and NB.

A novel finding in this thesis is that the PcG proteins, BMI-1 and EZH2, and DNMT enzymes all target and repress the potassium channel gene *KCNA5*. Furthermore, inhibition of these epigenetic regulators led to the re-expression of *KCNA5* and its channel, Kv1.5, which sensitized cancer cells to physiological stress or had an anti-proliferative effect. Therefore, epigenetic modifiers could be used in the context of promoting Kv1.5 expression in ES and NB, which could then open up the possibility of pairing epigenetic modifiers with drugs that utilize a Kv1.5-dependent mechanism, such as DCA. Preliminary evidence in Figure 4.2B suggests that the combination of an epigenetic modifier with DCA has therapeutic benefit as the BMI-1 knockdown ES cells treated with DCA had a significant anti-proliferative effect. Pairing epigenetic modifiers with drugs that utilize tumor suppressor re-expression could provide novel combination therapies useful in combating primary and recurrent ES and NB.

A major challenge moving forward will be determining where and when to incorporate epigenetic-targeted therapies in current pediatric treatment regimens, as we are currently unaware of how to appropriately utilize these drugs. Ongoing clinical trials (www.cancer.gov/clinicaltrials) and research (173, 295, 312-314) are working towards determining how to incorporate epigenetic modifiers in therapy.

Data from Chapters Two through Four of this thesis, suggest utilizing epigenetic modifiers either as a pre-treatment strategy or in combination with chemotherapy agents. The rationale for use of epigenetic modifiers as a pre-treatment strategy stems from the data demonstrating that inhibition of EZH2, BMI-1 or DNA methylation alone sensitized cancer cells to their existing microenvironment. Therefore, utilizing epigenetic modifiers as pre-treatment could re-express numerous tumor suppressors pathways and increase the efficacy of existing treatments. A second option could be utilizing epigenetic modifiers in combination with chemotherapeutic agents. In solid tumors, many resistant cancer cells reside in the core of the tumor and are therefore important targets to eliminate (315). Due to their location within a tumor, these cancer cells are exposed to hypoxic conditions and growth-factor deprivation. In Chapter Two, I demonstrated through inhibition of BMI-1 or EZH2, I was able to sensitize cancer cells to these two stressors. Logistically these cancer cells are difficult to target as they have many layers of cancer cells surrounding them. Therefore, in order to target these cells we would have a treatment regimen that started with a combination of chemotherapeutic agents, then treated with an epigenetic modifier and then finished with a second combination of chemotherapeutic agents. The rationale for starting with chemotherapeutic agents would be to rid of the outer layer of cancer cells, so to increase the probability the cancer cells at the core of the tumor are targeted by the epigenetic modifiers and second round of chemotherapeutic agents. Future *in vitro* and *in vivo* studies will specifically determine if the second option of treatment is viable in ES and NB. These studies

will determine if this treatment regimen effectively targets and eliminates these cancer cells and whether this combination is tolerated *in vivo*.

Though my initial studies support the utilization of epigenetic modifiers in treatment strategies, continued work needs to determine whether epigenetic modifiers should be used prior to, in combination with or after chemotherapy and/or radiation. Furthermore, we need to determine if we can use epigenetic modifiers as a therapeutic option in relapsed disease. To address this, studies will need to conduct *in vitro* and *in vivo* studies exposing resistant cancer cells and relapsed tumors to epigenetic modifiers and determine if they can be re-sensitized to chemotherapeutic agents. Though current work demonstrates the therapeutic benefit of utilizing epigenetic modifiers in pediatric cancer, further research, an example of which is discussed above, is required before we can successfully incorporate epigenetic modifiers into standard practice for primary or recurrent disease.

Summary

In summary, the work in this thesis demonstrates a novel mechanism by which epigenetic suppression of the unique Kv channel, Kv1.5 promotes tumorigenesis. We show that the PcG proteins, BMI-1 and EZH2, and DNA methylation suppress the *KCNA5* locus in the pediatric cancers, ES and NB. Specifically, we demonstrate that physiological stress upregulates PcG-dependent suppression of *KCNA5* and inhibition of either BMI-1 or EZH2 promotes Kv1.5-mediated apoptotic cell death in response to hypoxia. In

addition, we elucidated that under ambient conditions hypermethylation of the *KCNA5* promoter promotes cell proliferation and that inhibition of DNMTs restores *KCNA5* expression and results in decreased cell proliferation. Significantly, both functions of Kv1.5 in mediating cancer cell proliferation and survival were found to be, at least in part, dependent on inhibition of potassium efflux. Together, these studies identify the Kv1.5 channel as a novel tumor suppressor that is epigenetically silenced in cancer cells to promote tumorigenesis. This thesis has provided a starting point for further studies elucidating the impact of the Kv1.5 channel on cancer biology and contributed to the growing literature demonstrating the importance of understanding ion channel regulation in cancer.

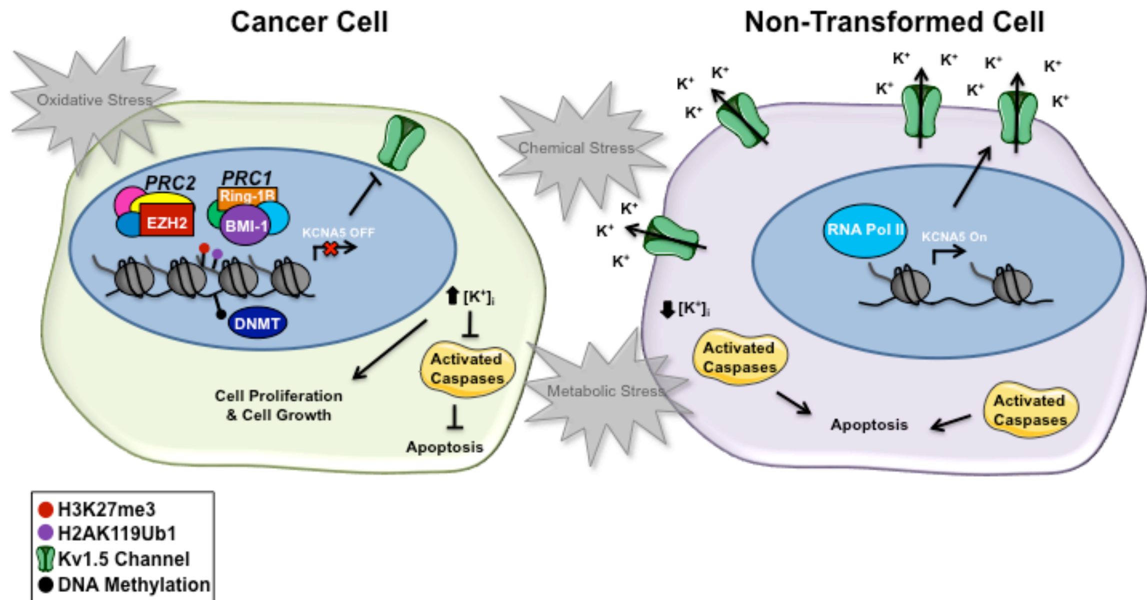

Figure 4.1. Model

To survive physiological stressors (i.e. oxidative, chemical and metabolic stress) and promote cell proliferation, cancer cells epigenetically suppress the *KCNA5* locus. The PcG proteins, BMI-1 and EZH2, and their respective histone modifications (H2AK119Ub1 and H3K27me3), along with DNA hypermethylation, silence the locus and prevent transcription. Silencing the *KCNA5* locus downregulates the redox sensitive Kv1.5 channel, which specifically prevents apoptotic-mediated cell death in cancer cells and promotes cell proliferation. Downregulation of Kv1.5 prevents potassium efflux out of the channel, which in turn increases the intracellular potassium concentration and prevents caspase activation. As the Kv1.5 channel is suspected to regulate the G_1/S transition in the cell cycle, downregulation of Kv1.5 would promote cell proliferation. In comparison to cancer cells, non-transformed cells express *KCNA5* transcript and Kv1.5 channels, as they do not dysregulate the epigenetic machinery to silence the *KCNA5* locus. Therefore, when non-transformed cells are exposed to physiological stressors they undergo apoptotic-mediated cell death.

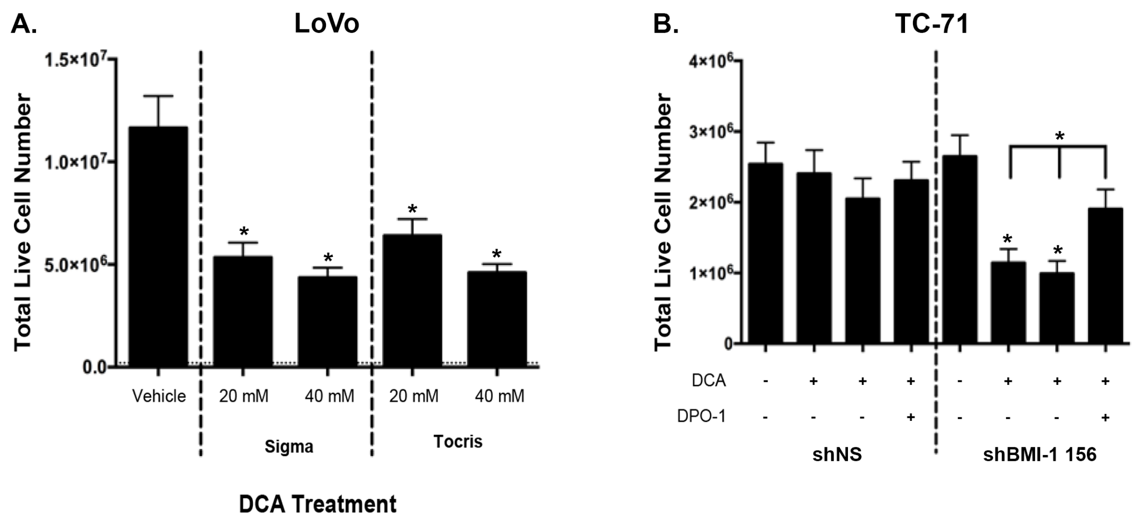

Figure 4.2. DCA Treatment in LoVo and BMI-1 Knockdown ES Cells.

(A) A cell assay in LoVo cells treated with vehicle control (DMSO) or DCA at 20 or 40 mM from either Sigma or Tocris. After 48 hours, DCA at both 20 and 40 mM significantly reduces cancer cell proliferation compared to vehicle control. (B) A 48-hour cell proliferation assay was conducted in the control ES cell line, TC-71 shNS, and the BMI-1 knockdown cell line, TC-71 shBMI-1 156. The cells were treated with vehicle control (DMSO), 10 mM DCA, 10 mM DCA and DMSO or 10 mM DCA and 310 μM DPO-1 (Kv1.5 channel blocker). The control cells (TC-71 shNS) are not affected by DCA or DPO-1, as the total numbers of live cells are similar across all conditions. While, the BMI-1 knockdown cells treated with 10 mM DCA or 10 mM DCA and DMSO have a significant decrease in cell proliferation compared to vehicle control. Upon treatment with 310 μM DPO-1, a specific blocker of Kv1.5, the proliferative phenotype is partially restored in the BMI-1 knockdown cells. * $p<0.05$ (mean ± SEM, n=3)

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