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Promoter Methylation Analysis Reveals that KCNA5 Ion Channel Silencing Supports Ewing Sarcoma Cell Proliferation

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Abstract

Polycomb proteins are essential regulators of gene expression in stem cells and development. They function to reversibly repress gene transcription via post-translational modification of histones and chromatin compaction. In many human cancers, genes that are repressed by polycomb in stem cells are subject to more stable silencing via DNA methylation of promoter CpG islands. Ewing sarcoma is an aggressive bone and soft tissue tumor that is characterized by over-expression of polycomb proteins. This study investigates the DNA methylation status of polycomb target gene promoters in Ewing sarcoma tumors and cell lines and observes that the promoters of differentiation genes are frequent targets of CpG-island DNA methylation. In addition, the promoters of ion channel genes are highly differentially methylated in Ewing sarcoma compared to non-malignant adult tissues. Ion channels regulate a variety of biological processes, including proliferation, and dysfunction of these channels contributes to tumor pathogenesis. In particular, reduced expression of the voltage-gated Kv1.5 channel has been implicated in tumor progression. These data show that DNA methylation of the KCNA5 promoter contributes to stable epigenetic silencing of Kv1.5 channel. This epigenetic repression is reversed by exposure to the DNA methylation inhibitor decitabine, which inhibits Ewing sarcoma cell proliferation through mechanisms that include restoration of Kv1.5 channel function.

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Implications—This study demonstrates that promoters of ion channels are aberrantly methylated in Ewing sarcoma and that epigenetic silencing of KCNA5 contributes to tumor cell proliferation, thus providing further evidence of the importance of ion channel dyregulation to tumorigenesis.

Keywords

DNA methylation; Voltage-gated potassium channel; *KCNA5*; Ewing sarcoma

Introduction

A fundamental trait of cancer cells is their ability to sustain proliferation (1). Cancer cells accomplish this by hijacking physiologic pathways and silencing or mutating tumor suppressor genes that control cell cycle progression (1). Moreover, the proliferative phenotype of cancer cells is known to contribute to tumor relapse (2-4), a major impediment to cancer cures. Therefore, continued elucidation of the mechanisms that promote cancer cell proliferation is key for developing novel anti-cancer therapies.

Disruptions to gene expression in cancer can be achieved by both genetic and epigenetic mechanisms that lead to either aberrant induction or repression of target transcripts (reviewed in (5, 6)). While genetic loss of function is usually achieved by gene deletion or inactivating mutations, epigenetic loss of function is most often associated with aberrant repression of gene transcription mediated by post-translational histone modifications and DNA methylation. In normal stem cells, polycomb group proteins reversibly repress target genes and, in so doing, prevent differentiation, maintain stemness and control development until appropriate developmental cues are received (7, 8). Importantly, the promoters of these stem cell targets of polycomb complexes are frequently targeted for DNA hypermethylation in cancer (9-11). In this way, more stable, and potentially irreversible, gene silencing is effected and cancer cells become locked in a stem cell-like state.

Ewing sarcoma is an aggressive bone and soft tissue tumor that presents most often in adolescents and young adults. It is a tumor of presumed stem cell origin and is characterized by an undifferentiated cellular phenotype and the presence of a tumor-initiating oncogenic fusion gene, most commonly EWS- FLI1 (12). Ewing sarcoma is genetically quiet, with a low rate of genetic mutation (13-15) and tumor pathogenesis is largely mediated by EWS-FLI1 and its impact on the epigenome (16, 17). Over-expression of polycomb proteins, in particular EZH2 and BMI-1, is evident in nearly all Ewing sarcoma tumor cells and both proteins contribute to the tumor phenotype (18-21). In addition, altered DNA methylation of cancer-associated gene promoters has been described and preclinical studies demonstrate that exposure of Ewing sarcoma cells to hypomethylating agents inhibits tumor growth (22, 23).

In the current study we used a custom designed DNA methylation array to investigate whether the promoters of polycomb target genes are targeted for abnormal DNA methylation in Ewing sarcoma. Our results reveal that, like other cancer, CpG islands in the promoters of differentiation-associated genes are targets of DNA methylation. In addition, they uncover the unanticipated finding that promoters of ion channel genes are frequent targets of aberrant DNA methylation. In particular, these studies demonstrate that DNA

hypermethylation contributes to epigenetic repression of the *KCNA5* locus and that the resulting suppression of of the Kv1.5 ion channel supports cancer cell proliferation.

Materials and Methods

Cell lines and Tumor tissues

Ewing sarcoma cell lines were provided by Dr. Timothy Triche Children's Hospital Los Angeles (CHLA, Los Angeles, CA) and the Children's Oncology Group (COG) cell bank (www.cogcell.org). HuVECs were obtained from Lonza (191027). MRC-5 cells were obtained from ATCC (CCL-171). Human bone marrow-derived mesenchymal stem cell (MSC) lines and hESC-derived neural crest stem cells were obtained as previously described (24). Identities were confirmed by short tandem repeat profiling. All cell lines were cultured in standard cell culture media, supplemented with 10% FBS (Atlas Biologicals, Inc., F-500-A) at 37°C in 5% CO_2. Ewing sarcoma tumor specimens were acquired from the Vanderbilt University pathology archives. Approval from the Vanderbilt University Institutional Review Board was granted prior to tissue acquisition.

Proliferation Analysis

Cells were plated at a density of 200,000 cells per 6-well plate and left for 24 hours prior to a 72-hour drug treatment where the cells were treated every 24 hours. Brightfield images of the cells were captured on the Olympus CKX41 microscope on the 10× objective by the Lumenera Infinity 3-1 1.4 Megapixel camera. Proliferation was determined by trypan-blue exclusion and EdU incorporation. EdU was added to fresh medium of the cells after the 72-hour incubation for 2 hours before harvest at a concentration of 10 μM. EdU incorporation was determined with the Click-iT® Plus EdU Alexa Fluor 488 flow cytometry assay kit (Life Technologies, C10632) following the manufacturer's instructions, performed on the Accuri C6 flow cytometer and analyzed using FlowJo V10.

Pharmacologic Studies

Diphenyl phosphine oxide-1 (DPO-1) (310 nM, Tocris Bioscience, 2533) was prepared in ethanol, 4′Aminopyridine (4′AP) (50 μM, Sigma-Aldrich, 275875) was prepared in an aqueous solution and 5-aza-2′-deoxycytidine (5′AZA-CdR/decitabine) (100 nM, Sigma-Aldrich, A3656) was diluted in dimethyl sulfoxide (DMSO) (Fisher, D128-500). Cells were pre-treated at these concentrations for 72 or 96 hours.

Quantitative Real-Time PCR

Total RNA extraction was performed using the RNeasy® Plus Mini kit (Qiagen, 74136) or Quick-RNA™ Miniprep (Zymo, R1055) 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. Primers for *KCNA5* were: Forward: 5′-GTA ACG TCA AGG CCA AGA GC-3′; Reverse: 5′-TCC CAT TCC CTA CTC CAC TG-3′.

Statistical Analysis

Statistics were performed using GraphPad Prism 6. P-values of less than 0.05 across at least 3 independent experiments were considered significant.

DNA Methylation

DNA 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 >1,400 genes that are established targets of polycomb regulation in human embryonic stem cells (8). Two independent batches of samples were analyzed using this array, the first included 4 Ewing sarcoma tumors and 5 Ewing sarcoma cell lines and the second comprised 52 samples from 11 different non-transformed adult tissues that were obtained at rapid autopsy (25). Analysis of DNA methylation (as determined by beta values) was restricted to 1,297 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. Probes that contained repetitive sequence of more than ten base pairs or that contained single nucleotide polymorphisms within CpG islands were also excluded, resulting in evaluable data for 1,204 loci. Differential methylation was defined as a median difference in beta-value of at least 0.2 and a Bonferroni multiple test-corrected p-value of less than 0.05 between Ewing sarcoma and non-malignant adult tissues. Gene ontology was determined using DAVID and enriched categories determined relative to all genes, as well as relative to the 1,204 evaluable loci (26).

MethyLight Studies

MethyLight analyses were carried out as previously described (27). Briefly, genomic DNA was isolated 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 (27, 28), with the use of the EpiTect MethyLight PCR kit (Qiagen, 59496) and EpiTect PCR control DNA set (Qiagen, 59695). The assay for *KCNA5* consisted of: Forward primer: 5′- ATCGTAATCGGTTTAGTTTCGACG-3′; Reverse primer: 5′-AATAAAAACGTATCTCGTCCGCG-3′; Probe: 6FAM-CGTTAACCGACCTCCGCAAACGACC-BHQ1 (IDT, Applied Biosystems and Biosearch Technologies). Control probes and conditions have been published previously (27). Analysis was performed in triplicate using the Lightcycler® 480 System (Roche Applied Science) and the average percent of methylated reference (PMR) for each sample was determined as detailed previously (27).

Results

DNA Methylation of polycomb target gene promoters in Ewing sarcoma differs from normal adult tissues

Ewing sarcoma is characterized by an undifferentiated histology and transcriptional profiling and tumor biology studies suggest that this primitive state is a consequence of both

cellular origin and a block in cellular differentiation (18, 20, 21, 24). DNA hypermethylation at the promoters of developmentally critical polycomb target genes has been observed in many adult tumors and is associated with transcriptional silencing of genes that direct cell differentiation ((10, 11) and reviewed in (6)). Given the high level of polycomb protein expression that is evident in Ewing sarcoma, and the critical roles of these proteins in tumor pathogenesis, we hypothesized that abnormal DNA methylation of polycomb target genes might be a feature of Ewing sarcoma. To begin to address this we investigated the methylation status of gene promoters using a custom designed Illumina GoldenGate® bead array (see methods). Five Ewing sarcoma cell lines and four primary tumors were analyzed and their DNA methylation data compared to a panel of 52 normal adult tissues. As shown, despite their diverse origins, promoter DNA methylation was remarkably similar in all adult tissues but was distinct from Ewing sarcoma (Figure 1A). Notably, Ewing sarcoma tumors and cell lines clustered together and showed a similar overall pattern of DNA methylation, demonstrating that cell culture was not the primary driver of the abnormal DNA methylation profiles. Comparison of Ewing sarcoma to normal tissues identified 547 probes with differences in median beta value of ≥ 0.2 and 498 of these probes reached statistical significance, as described in methods (Supplemental Table S1). 265 loci showed increased methylation in Ewing sarcoma and 222 loci had lower levels of methylation than in adult tissues. As expected, an increase in DNA methylation was significantly more likely at CpG islands than non-islands. Specifically, 378 differentially methylated probes were located within CpG islands and nearly two-thirds of them (N=243, 64%) showed increased methylation in Ewing sarcoma. Conversely, 120 differentially methylated probes were not located within annotated CpG islands and the vast majority (N=98, 82%) showed reduced levels of methylation in Ewing sarcoma (Figure 1B).

Promoters of ion channel encoding genes are differentially methylated in Ewing sarcoma

In order to better characterize the biology of the genes with differentially methylated promoters, we performed gene ontology analyses to identify enriched biologic processes and molecular functions. To begin, we assessed enrichment of the gene ontology categories relative to the whole genome. As expected, given that the array was developed to directly interrogate polycomb targets, genes with differentially methylated promoters were highly enriched for embryonic transcription factors involved in morphogenesis, differentiation and development (Supplemental Table S2). In particular, numerous *HOX* genes were differentially methylated in Ewing sarcoma (Supplemental Table S1). This is consistent with our recent studies, which showed that expression of *HOX* genes is widely abnormal in Ewing sarcoma compared to adult tissues (29). In addition to the expected enrichment of developmental transcription factors, this analysis also identified an unanticipated enrichment of genes with molecular functions related to potassium ion binding, transport and channel activity (Supplemental Table S2B). To determine if enrichment of these functional categories was merely a reflection of the array design rather than a true enrichment, gene ontology analysis was repeated and enrichment determined relative to the set of 1,204 evaluable probes rather than the whole genome. This analysis confirmed that biologic and molecular processes involved in ion homeostasis were the most significantly enriched categories among differentially methylated genes (Figure 1C & D). In particular, genes involved in calcium homeostasis were most prominently associated with increased promoter

methylation (Figure 1C), while potassium-associated genes were over-represented among loci with reduced levels of DNA methylation (Figure 1D). Thus, in addition to the expected pattern of altered DNA methylation at developmental transcription factors, this analysis also identified ion regulatory genes as prime targets of differential methylation in Ewing sarcoma.

The promoter of the voltage-gated potassium ion channel gene *KCNA5* is DNA methylated in cancer

The flux of potassium, sodium, chloride and calcium ions across the cytoplasmic membrane is regulated by a complex array of voltage-gated channels and control of intracellular ion levels by these channels is essential for cell proliferation and survival. In particular, voltage-gated ion channels play critical roles in the maintenance of cellular homeostasis (30, 31) and specific deregulation of potassium ion channels has recently been broadly implicated in cancer pathogenesis (32). Twenty-two potassium ion channel genes have been reported to be differentially expressed in a wide variety of human cancers and 20 of these were found to be over-expressed in the context of malignant disease (reviewed in Ref. (32)). In contrast, expression of KCNQ1 and of Kv1.5 has been reported to be inversely correlated with clinical aggression in gastrointestinal tumors (33) and in lymphoma and gliomas (34-36), respectively. Thus, deregulated expression of potassium ion channels is common in cancer but the mechanisms underlying their deregulation remain largely ill-defined. Significantly, we recently reported that expression of the *KCNA5* gene, which encodes Kv1.5, is reduced in Ewing sarcoma and that the locus is reversibly epigenetically repressed by the polycomb proteins BMI-1 and EZH2 (37). Therefore, we hypothesized that more permanent silencing of the *KCNA5* locus by DNA methylation may be an additional mechanism of more stable channel suppression in Ewing sarcoma. To address this we first identified potassium ion channel genes among the list of 498 differentially methylated loci in Ewing sarcoma. Twenty potassium ion channel gene promoters were included in the list and 14 showed a relative reduction in DNA methylation in tumors compared to non-malignant adult tissues. Conversely, six showed an increase in DNA methylation (Figure 2A). Among relatively hypomethylated loci, increased expression of channels encoded by *KCNA1* (Kv1.1), *KCNC4* (Kv3.4), and *KCNK2* (K2P2.1) has been reported in cancer (32). Whether a relative reduction in DNA methylation at these gene promoters contributes to channel over-expression remains to be elucidated. With respect to the six loci with increased methylation in Ewing sarcoma, only down regulation of *KCNA5* (Kv1.5) has been implicated in cancer pathogenesis (34-37), thus leading us to continue to focus our studies on this channel. Significantly, *KCNA5* promoter methylation was detected in both Ewing sarcoma tumors and cell lines confirming that, in at least some Ewing sarcoma cells, polycomb-dependent repression of the locus is complemented by CpG-island DNA methylation (Figure 2B).

To validate the data from the smaller array-based study, we analyzed the *KCNA5* locus in a larger and completely independent set of primary tumors and cells using MethyLight analysis. A methylation specific probe was designed that allowed quantification of DNA methylation at the *KCNA5* locus, at a site that was several hundred bases downstream of the GoldenGate® array probe site, but still within the promoter-associated CpG-island (Figure 2C). This analysis confirmed that the *KCNA5* locus is methylated in Ewing sarcoma cell

lines and tumors compared to both non-malignant fibroblasts and endothelial cells as well as bone marrow derived mesenchymal stem cells (MSC), a putative cell of Ewing sarcoma origin (Figure 2D). In contrast, the *KCNA5* locus was highly methylated in neural crest stem cells (NCSC), another putative cell of tumor origin (Figure 2D). Thus, these data suggest that differences in DNA methylation exist between stem cells of different developmental origins. It is noteworthy that, compared to the GoldenGate® array probe, the MethyLight probe detected more abundant DNA methylation in the cell lines than in the primary tumors. This suggests that broader methylation of locus might be an adaptive response of cells to prolonged *in vitro* culture, or that Ewing sarcoma cells with more extensive DNA methylation of the *KCNA5* gene are more amenable to generating a cell line (Figure 2D). However, given that MSCs, fibroblasts, and endothelial cell lines showed no evidence of significant DNA methylation, it is clear that cell culture alone cannot explain increased methylation of the *KCNA5* locus in Ewing sarcoma cells. In support of this, data from the MethHC database (38) shows significant tumor-specific *KCNA5* promoter DNA methylation in all of the 18 different tumor types analyzed (Supplemental Figure S1). Thus, DNA methylation at the *KCNA5* promoter is a common attribute of multiple tumor types *in vivo*, thereby providing a potential mechanism to explain prior observations that Kv1.5 is down regulated in cancer.

Decitabine induces loss of DNA methylation at the *KCNA5* locus and an increase in gene expression

Having established that *KCNA5* is methylated in Ewing sarcoma cells, we next sought to determine if exposing cells to the hypomethylating agent, decitabine, would reverse DNA methylation and lead to increased gene expression. At high doses decitabine is cytotoxic, but at low doses it prevents DNA methylation without inducing cell death and the efficacy of decitabine as an epigenetic therapy is best achieved with subcytotoxic doses, which *in vitro* usually range from 20 to 300 nM (39). Exposure of Ewing sarcoma cells to 100nM decitabine did not induce cell death and led to reduced methylation of *KCNA5* in all three Ewing sarcoma cell lines (Figure 3A). Consistent with DNA methylation being a mechanism of gene repression, we also observed that expression of the *KCNA5* transcript increased concomitantly with loss of DNA methylation (Figure 3C). However, the correlation between loss of methylation and de-repression of gene expression was not linear over time, indicating that other mechanisms of gene regulation also contribute to modulation of *KCNA5* expression in these cells.

Kv1.5 channel function inhibits Ewing sarcoma cell proliferation

We next evaluated whether DNA methylation of *KCNA5* impacts on the proliferative phenotype of Ewing sarcoma cells. Exposure of Ewing sarcoma cells to low-dose decitabine for 72 hours had a profound impact on cell expansion (Figure 4A). Trypan blue exclusion displaying either total live cell count (Figure 4B) or total dead cell count (Figure 4C) and EdU incorporation (Figure 4D) were performed to determine if this effect was due to an increase in cell death or a decrease in proliferation. As shown, no significant loss of viability was observed (Figure 4C). In contrast, total live cell count was significantly reduced (Figure 4B) and incorporation of EdU was reduced (loss of 2nd peak in histogram, Figure 4D), revealing that decitabine inhibits cell cycle progression and proliferation of Ewing sarcoma

cells. To determine if reduced proliferation in decitabine-treated cells was a result of loss of *KCNA5* repression, we used the pharmacological compounds 4-aminopyridine (4′AP) and diphenyl phosphine oxide-1 (DPO-1) (Figure 5A). These two compounds are highly specific inhibitors of the Kv1.5 channel when used at pharmacologically validated doses (40-43). Notably, they function to block Kv1.5 channel function by inhibiting the flux of potassium ions across the channel pore and altering transmembrane current, without altering the levels of channel expression. Significantly, blocking the Kv1.5 channel with either agent partially restored the proliferative phenotype of decitabine-treated Ewing sarcoma cells (Figures 5B, C). Together these data reveal that epigenetic silencing of the Kv1.5 channel, by DNA methylation of *KCNA5*, contributes to Ewing sarcoma cell proliferation and that this effect is mediated, at least in part, by the function of Kv1.5 as a negative regulator of cell-cycle progression (Figure 6).

Discussion

Cancer cells hijack and dysregulate epigenetic mechanisms to dynamically alter gene expression and drive tumor pathogenesis. In the current studies, we assessed the DNA methylation profile of polycomb target gene promoters in the context of Ewing sarcoma. From these studies we discovered that, like other human cancers, CpG islands in the promoters of polycomb target genes are selectively methylated in Ewing sarcoma. However, we also discovered the unanticipated finding that genes involved in calcium and potassium homeostasis are also differentially methylated relative to non-malignant adult tissues. More specifically, our studies identified potassium ion channels as frequent targets of aberrant DNA methylation in Ewing sarcoma and revealed that the promoter of *KCNA5* is relatively hypermethylated in tumor cells. In addition, interrogation of public databases showed that the *KCNA5* locus is reproducibly hypermethylated, relative to adjacent normal tissues, in a wide variety of human cancers (38). We recently showed that polycomb-dependent repression of *KCNA5* contributes to Ewing sarcoma cell survival under conditions of hypoxia (37). Thus, the current study validates our prior observation that Kv1.5 channel suppression contributes to the cancer phenotype and provides new evidence that cancer cell proliferation in ambient conditions is promoted, in part, by stable DNA methylation-dependent repression of the *KCNA5* locus.

Potassium channels are transmembrane proteins that control cellular ion concentrations and homeostasis. Given that cell proliferation and survival are intimately linked to intracellular potassium ion levels (44-46), dysregulation of potassium channels can impact on the cancer phenotype. Consistent with this, aberrant expression of ion channels is prevalent in cancer (47, 48). Potassium-conducting channels also regulate the biophysical properties of a cell, controlling intracellular potassium concentrations and maintaining resting membrane potential (31). Thus, hijacking of potassium ion channel function by cancer cells has the potential to impact on cell proliferation, cell survival, migration, and differentiation (32). Interestingly, however, although most potassium channels have been found to be over expressed in cancer, Kv1.5 is one of only two potassium channels that is down regulated (32).

Knowledge regarding the physiologic functions of Kv1.5 in normal cell biology provides insights into why this channel in particular would be selectively silenced in cancer. 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 (49), suggesting the channel is a cell cycle checkpoint regulator and could function as a tumor suppressor gene. Our studies begin to illuminate the potential importance of this function in the context of Ewing sarcoma. Specifically, we have found that DNA methylation and repression of *KCNA5* contributes to cell cycle progression and that reversion of promoter methylation and *KCNA5* de-repression is associated with growth inhibition and cell cycle arrest. Moreover, specific pharmacologic inhibition of Kv1.5 channel function partially restores proliferation in Ewing sarcoma cells that have been exposed to decitabine. Thus, these studies together provide evidence that reactivation of silenced Kv1.5 channels can inhibit cancer proliferation, likely by permitting efflux of K+ ions and inhibiting G1 to S transition. Alternatively, emerging data suggest that there are other functions for potassium channels that do not depend on their roles as ion conductors (32, 50). The known function of both 4′AP and DPO-1 is to block K+ efflux and transmembrane current. Whether non-canonical roles exist for Kv1.5 outside of regulating K+ flux and membrane depolarization exist and whether they contribute to its function as a tumor suppressor remain to be elucidated.

The contribution of ion channel deregulation to tumor pathogenesis remains a relatively unexplored area of investigation in cancer biology. We have now shown that the Kv1.5 channel is reproducibly targeted for epigenetic repression, by both polycomb proteins (37) and by DNA methylation (this study), and that repression contributes to cancer cell survival and proliferation. These findings provide compelling evidence to support the designation of *KCNA5* as a tumor suppressor gene in human cancers, and suggest that further studies into its mechanism of action as a cell cycle regulator will reveal how loss of Kv1.5 function supports the proliferative cancer phenotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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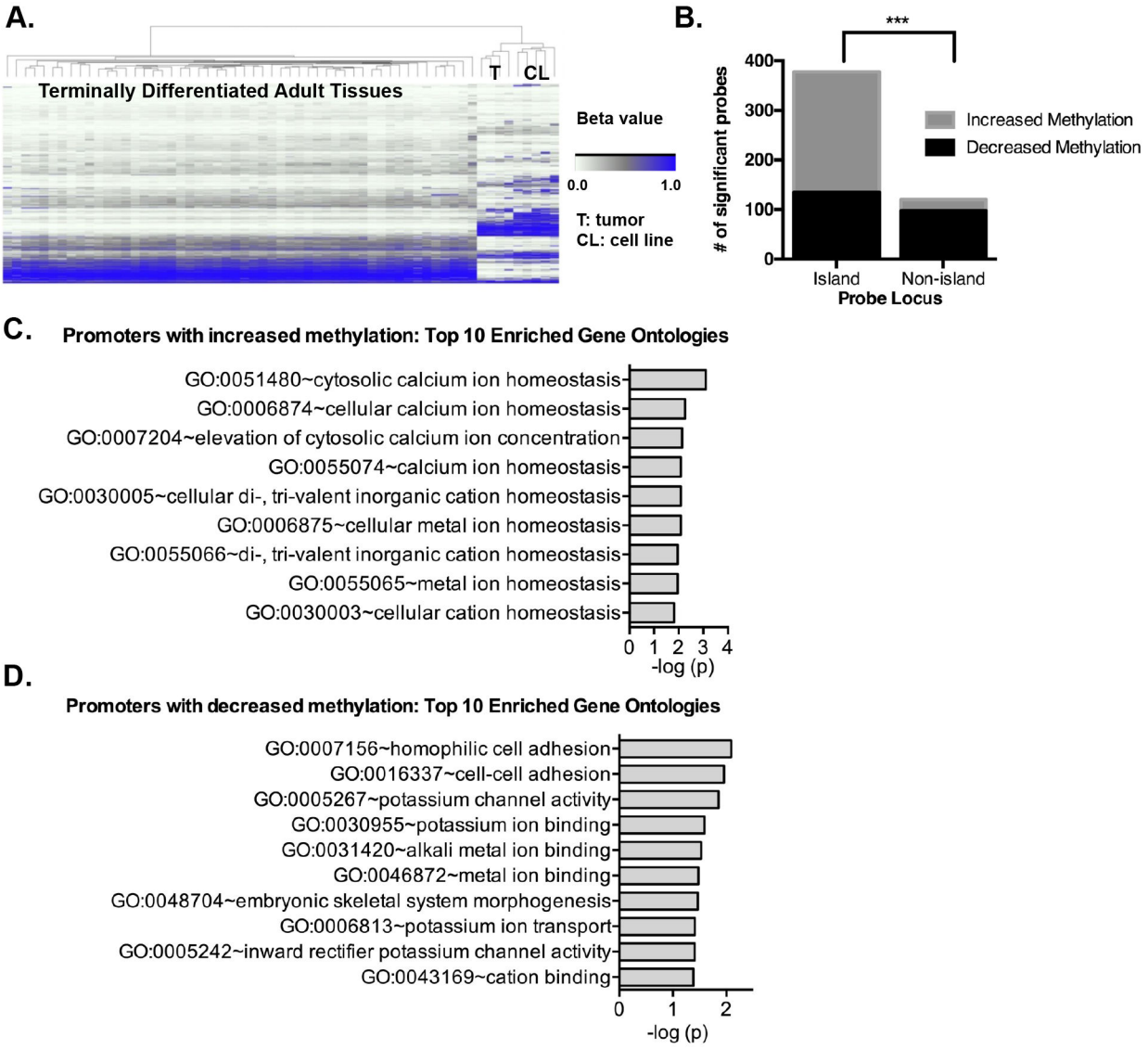

Figure 1. The abnormal DNA methylation profile of polycomb target genes in Ewing sarcoma

(A) Analysis of 52 normal adult tissues, 5 Ewing sarcoma cell lines (CL) and 4 primary Ewing sarcoma tumors (T) using a custom GoldenGate® bead array, demonstrates differences in the methylation profile of polycomb target gene promoters in Ewing sarcoma samples compared to normal adult tissue. (B) In Ewing sarcoma, increased methylation is more prevalent in promoters with CpG islands than in promoters that do not contain CpG islands. ***$p < 0.0001$ by Fisher's exact test. (C & D) Gene ontology analysis of differentially methylated promoters identifies calcium homeostasis genes as selective targets of increased promoter methylation (C) and potassium ion channel genes as being enriched among genes with decreased promoter methylation (D). Enrichment was determined relative to the set of 1,204 probes on the array and is expressed as the negative log of the enrichment score (p-value).

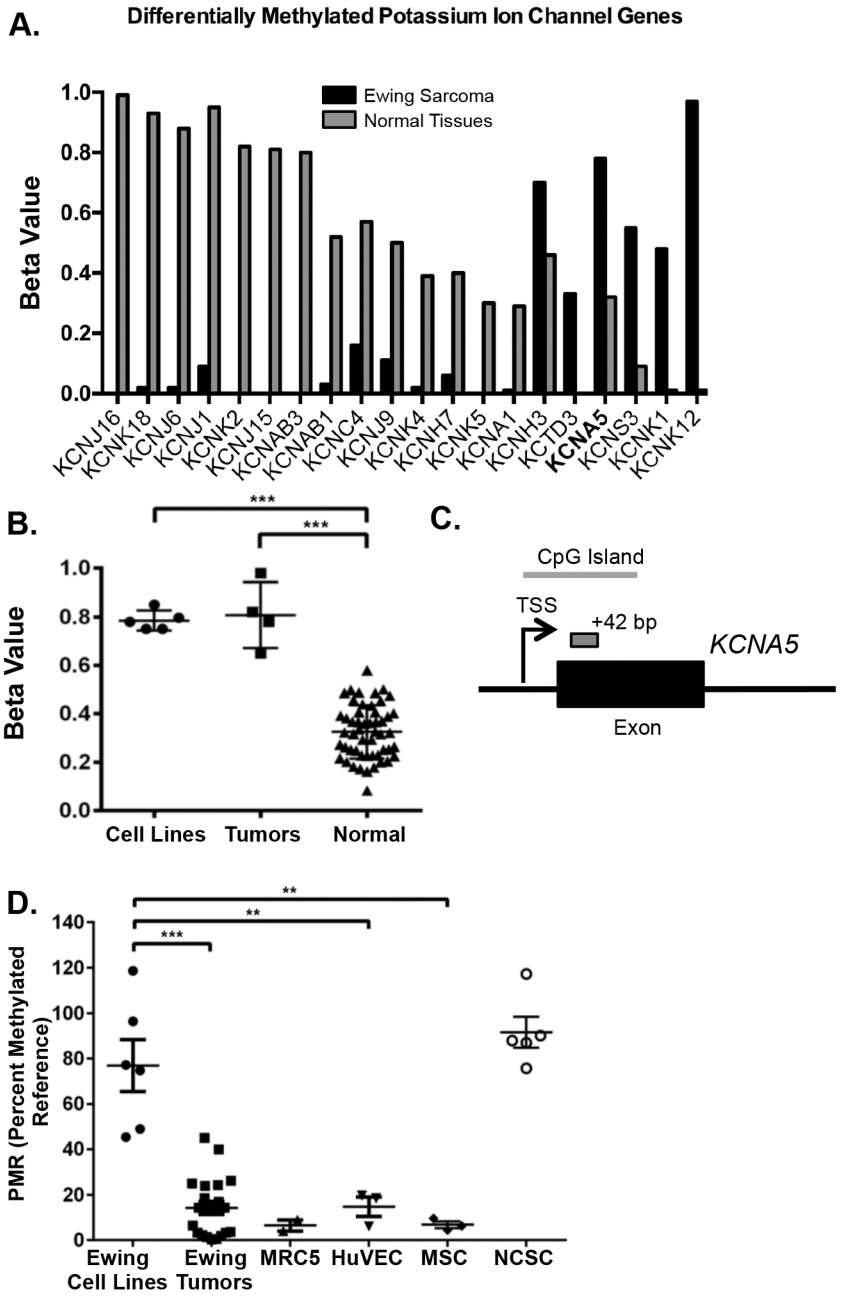

Figure 2. DNA methylation of *KCNA5* in Ewing sarcoma

(A) DNA methylation state (expressed as beta values) of differentially methylated potassium ion channel genes, as determined by a custom Illumina GoldenGate DNA methylation array. All twenty genes were significantly differentially methylated in Ewing sarcoma compared to normal adult tissues (median difference in beta-values ≥ 0.2 and corrected p-value <0.05). (B) *KCNA5* promoter DNA methylation. *** $p < 0.001$. (C) Site of MethyLight primers and probe for evaluation the *KCNA5* locus. (D) MethyLight analysis of 6 Ewing sarcoma cell lines, 21 Ewing sarcoma primary tumors, 2 non-transformed cell lines (human vascular endothelial cells: HuVEC, lung fibroblasts: MRC5), mesenchymal stem cells (MSC) and

neural crest stem cells (NCSC). Data points are presented as mean values of replicate experiments. MethyLight data is presented as percent of methylated reference (PMR). ** $p < 0.005$ and *** $p < 0.001$ by Student's *t*-test (mean ± SEM).

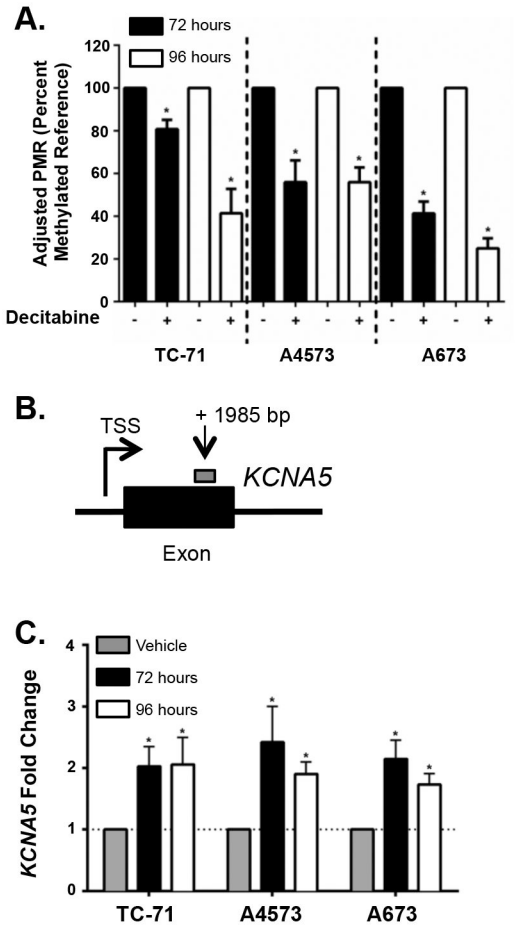

Figure 3. Demethylation of the *KCNA5* locus is associated with increased transcript expression

(A) MethyLight analysis of the *KCNA5* locus in Ewing sarcoma cells after treatment with 100 nM decitabine. Expression is presented as PMR relative to untreated cells. (B) Site of PCR primers for evaluation of *KCNA5* transcript expression. (C) qRT-PCR of *KCNA5* transcript expression in Ewing sarcoma cell lines after a 72 or 96 hour treatment with 100 nM decitabine. Expression is normalized to the geometric mean of *HPRT* and *GAPDH* within each sample and fold change in decitabine relative to the vehicle treated cells. * $p<0.05$ by Student's *t*-test (mean ± SEM, n=3).

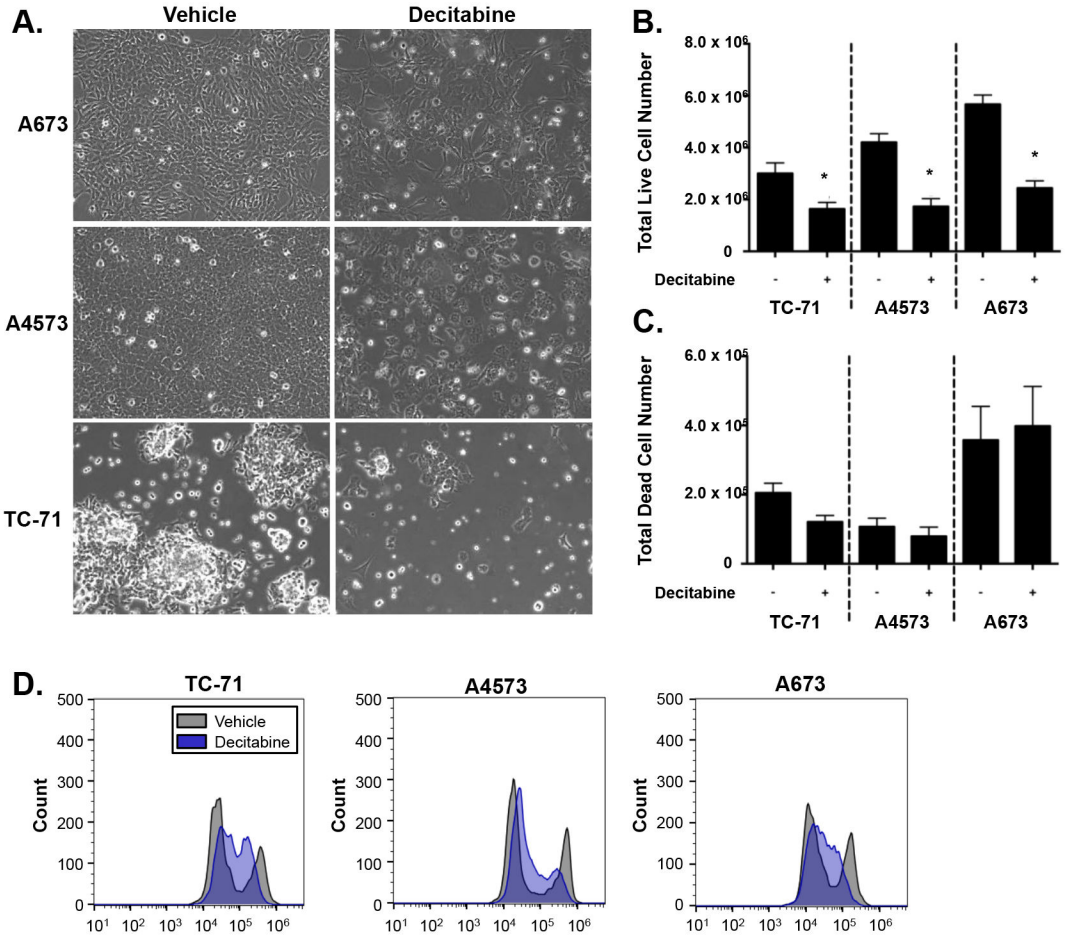

Figure 4. Decitabine inhibits Ewing sarcoma cell proliferation

(A) Brightfield images of three Ewing sarcoma cell lines with vehicle or 100nM decitabine. (B) Trypan-blue based proliferation assays determined TC-71, A4573 and A673 cells have a significant reduction in cell expansion after a 72 hour treatment with 100 nM decitabine. * $p < 0.05$ by Student's *t*-test (mean ± SEM, n=3-5). (C) Decitabine treatment has no effect on cell death as compared to vehicle control, as determined by direct counting of trypan blue stained cells. (D) Fluorescence signal from three Ewing sarcoma cells exposed to 10 μM EdU for 2 hours following 72 hour treatment with vehicle or 100 nM decitabine. The graphs show a clear separation of non-proliferating and proliferating cells in vehicle treated cells, while the decitabine treated cells lack a clear separation due to a reduction in or complete loss of proliferating cells.

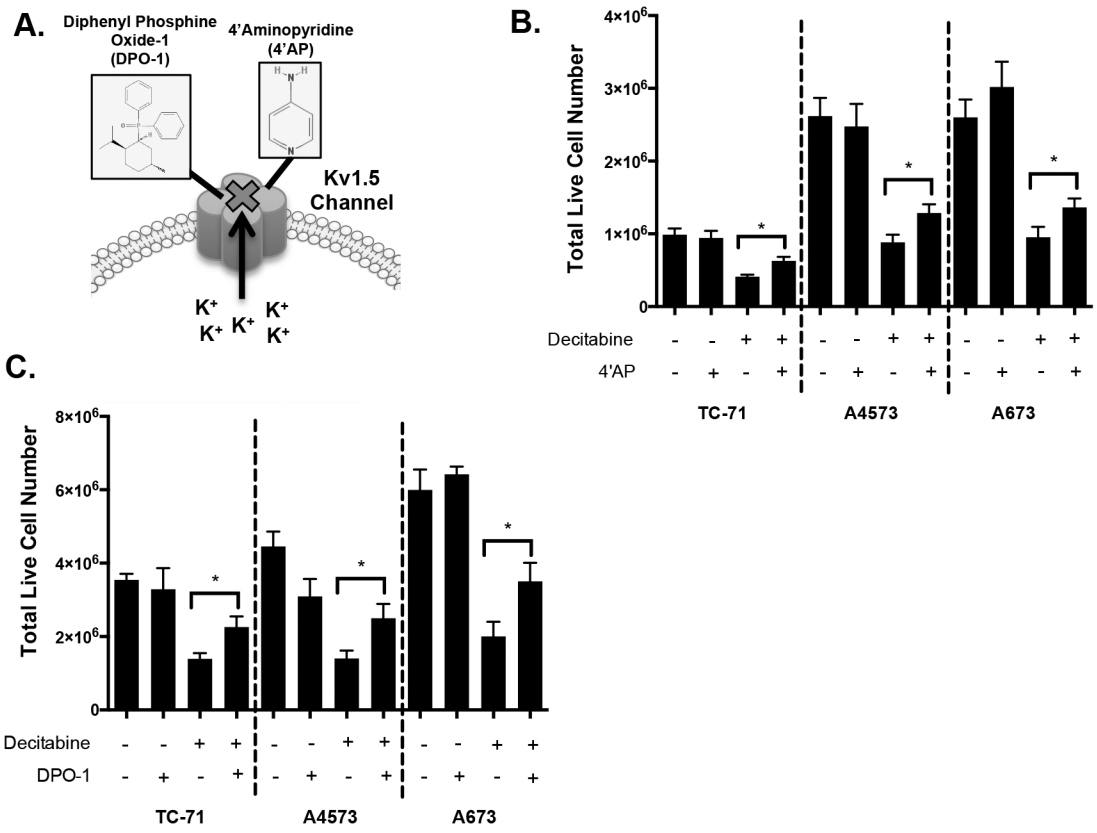

Figure 5. Blocking Kv1.5 channel function partially restores proliferative phenotype to decitabine-treated Ewing sarcoma cells

(A) Diagram demonstrating the open channel blockers, DPO-1 and 4′AP. Both compounds prevent K^+ flux out of the Kv1.5 channel. Blocking Kv1.5 channel function with 50 μM 4′AP (B) or 310 nM DPO-1 (C) in decitabine treated cells partially restores Ewing sarcoma cell proliferation. * $p < 0.05$ by Student's *t*-test (mean ± SEM, n=3-5).

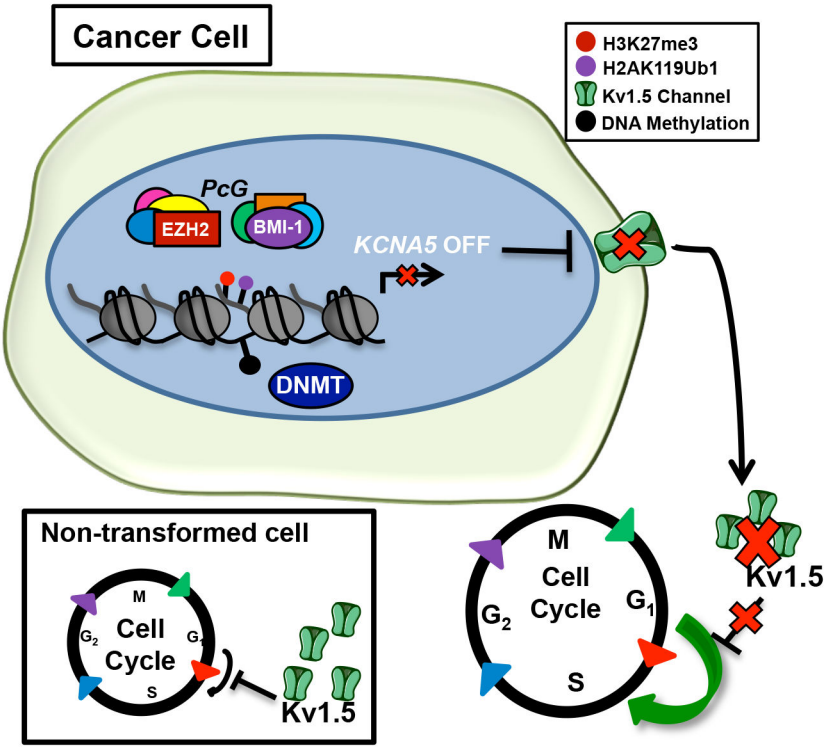

Figure 6. Overview of the epigenetic repression of *KCNA5* in Ewing sarcoma cancer cells

Survival of Ewing sarcoma cells in hypoxia, and maintenance of a proliferative state, are supported by epigenetic suppression of the *KCNA5* locus. The PcG proteins, BMI-1 and EZH2, and their respective histone modifications (H2AK119Ub1 and H3K27me3), along with DNA methylation, serve to reversibly and more stably silence the locus, respectively. Stable silencing of the *KCNA5* locus by promoter DNA methylation, contributes to Ewing sarcoma cell proliferation. The Kv1.5 channel has been shown to function as a G_1/S cell cycle checkpoint regulator. Therefore, silencing of this locus contributes to the ability of Ewing sarcoma cells to bypass cell cycle checkpoints and maintain a proliferative phenotype.