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# Exploring promoter silencing and re-expression of SH3GL2/endophilin A1 in urothelial cancer

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BOSTON UNIVERSITY  
SCHOOL OF MEDICINE

Thesis

**EXPLORING PROMOTER SILENCING AND RE-EXPRESSION OF  
SH3GL2/ENDOPHILIN A1 IN UROTHELIAL CANCER**

by

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B.Sc., Queen's University, 2016

Submitted in partial fulfillment of the  
requirements for the degree of  
Master of Science

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**EXPLORING PROMOTER SILENCING AND RE-EXPRESSION OF  
SH3GL2/ENDOPHILIN A1 IN UROTHELIAL CANCER**

**ISAAC JAKE ZUCKER**

ABSTRACT

**INTRODUCTION:**

Bladder cancer (BC) is highly prevalent. It presents as either non-muscle invasive or muscle-invasive disease. The prognosis of muscle invasive disease is poor, with a 5-year survival rate of less than 50%. Treatment approaches for both types of BC have not advanced much in the last few years and new therapies are needed to overcome the large burden of BC. Recently, a large effort has been undertaken to classify BC into molecular subtypes. These analyses have revealed significant alterations in epigenetic modifiers in BC. A previous study from our group revealed that SH3GL2, a negative regulator of receptor tyrosine kinase (RTK) signaling, was lost with high frequency in BC, leading to increased growth of tumor cells *in-vitro* and *in-vivo*. Conversely, forced expression of SH3GL2 in BC cell lines attenuated oncogenic behaviors including growth and migration. In addition to genomic deletion, SH3GL2 is subject to methylation-induced silencing, a key epigenetic mechanism.

**OBJECTIVE:**

Epigenetic mechanisms of gene regulation are known to be perturbed in BC. The objectives of this study were to investigate methylation of the SH3GL2 promoter and to test whether agents that promote Deoxyribonucleic acid (DNA) demethylation could be used to re-express SH3GL2 thereby restoring regulation of RTK signaling.

## **METHODS:**

Methylation of a specific CpG island in the SH3GL2 promoter was analyzed using methylation-specific Polymerase Chain Reaction (PCR) in a panel of BC cell lines with known SH3GL2 messenger Ribonucleic Acid (mRNA) status. Selected BC cell lines were treated with a variety of demethylating agents at different doses and for different times to evoke the re-expression of silenced SH3GL2. Demethylation inhibitors were combined with the histone deacetylase inhibitor, trichostatin A (TSA), to determine whether further re-expression could be achieved.

## **RESULTS:**

The SH3GL2 promoter displayed differing extents of promoter methylation among cell lines examined. In RT4 cells, the only cell line with detectable expression of SH3GL2 mRNA and protein, the promoter was completely unmethylated. In contrast, T24 and 253J cells displayed significant promoter methylation with little to no SH3GL2 mRNA expressed, consistent with methylation-induced silencing. Treatment of T24 and 253J with 5-Aza-2'-deoxycytidine (5-Aza-dC, 20  $\mu$ M), a DNA methyltransferase (DNMT) inhibitor increased gene expression but this was not dose- or time-dependent. Two additional DNMT inhibitors, Zebularine and RG-108 were also tested. A much higher dosage of Zebularine was required to trigger activation (500  $\mu$ M) while RG-108 was unable to trigger gene reactivation at all. Combination treatment with 5-Aza-dC and TSA further increased SH3GL2 expression compared to either agent alone. These results suggest that DNA methyltransferase inhibition is an effective treatment to re-express SH3GL2 in cells with SH3GL2 promoter silencing.

**CONCLUSION:**

The present study shows silencing of SH3GL2 in a variety of BC cell lines as a consequence of DNA promoter hypermethylation. Treatment with demethylating agents was able to increase gene expression. Based on prior findings showing attenuation of tumor cell growth and migration with forced expression of SH3GL2, DNA methyltransferase inhibition represents an effective strategy to re-express SH3GL2 in BC and normalize tumor cell behavior.

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## LIST OF ABBREVIATIONS

5-Aza-dC.....	5-Aza-2'-deoxycytidine
BC.....	Bladder Cancer
C.....	Celsius
CCLE.....	Cancer Cell Line Encyclopedia
c-Met.....	Mesenchymal Epithelial Transition Factor
dH <sub>2</sub> O.....	Distilled Water
DMSO.....	Dimethyl Sulfoxide
DNA.....	Deoxyribonucleic Acid
DNMT.....	DNA Methyltransferase
dsRNA.....	Double Stranded Ribonucleic Acid
EGF.....	Epidermal Growth Factor
EGFR.....	Epidermal Growth Factor Receptor
FBS.....	Fetal Bovine Serum
FGFR3.....	Fibroblast Growth Factor Receptor 3
HAT.....	Histone Acetyltransferase
HDAC.....	Histone Deacetylase
HRP.....	Horseradish Peroxidase
L-Glut.....	L-Glutamine
LOH.....	Loss of Heterozygosity
MEM.....	Minimal Essential Media
MIBC.....	Muscle-Invasive Bladder Cancer

mM.....	Millimolar
mRNA.....	Messenger Ribonucleic Acid
MT.....	Methyltransferase
$\eta$ M.....	Nanomolar
NMIBC .....	Non-Muscle Invasive Bladder Cancer
PBS .....	Phosphate Buffered Saline
PBS/T .....	Phosphate Buffered Saline/Tween
PCR.....	Polymerase Chain Reaction
P/S.....	Penicillin-Streptomycin Solution
qRT-PCR .....	Quantitative Reverse Transcription-Polymerase Chain Reaction
RB .....	Retinoblastoma
RNA .....	Ribonucleic Acid
RTK.....	Receptor Tyrosine Kinase
RT .....	Reverse Transcription
SH3GL2 .....	Endophilin-A1
TCGA.....	The Cancer Genome Atlas
TKI.....	Tyrosine Kinase Inhibitors
TSA.....	Trichostatin A
$\mu$ L.....	Microliter
$\mu$ M.....	Micromolar

## INTRODUCTION

Bladder cancer (BC) is a highly prevalent cancer with over 70,000 new cases diagnosed last year (Siegel et al., 2017). While the majority of cases diagnosed are non-muscle invasive bladder cancer (NMIBC) and are treated with a transurethral bladder resection, they commonly recur (Knowles, 2006). Muscle-invasive BC (MIBC) has a much poorer prognosis with a five-year survival rate of less than 50% (Knowles & Hurst, 2015). For MIBC, the current standard of care involves radical cystectomy as well as chemotherapy. Treatment approaches for both types of BC have not advanced much in the last number of years and new therapies are needed to overcome the large burden of BC. Recently, there has been a significant effort to identify particular markers that might help determine which treatment would be most appropriate for a given patient, as well as those unlikely to be beneficial, to ensure optimal treatment. New biomarkers are needed to assess whether patients will respond to specific treatments.

### **Molecular mechanisms of BC**

BC originates from the urothelium of the bladder along two distinct pathways, which gives rise to either papillary NMIBC or non-papillary MIBC (Sanli et al., 2017). NMIBC has two common alterations: (i) a loss of heterozygosity (LOH) of chromosome 9 (Chow et al., 2000; Obermann et al., 2003) and (ii) activating mutations in the Fibroblast Growth Factor Receptor 3 gene (FGFR3) (Cappellen et al., 1999). With respect to the first common alteration, the LOH of chromosome 9 is found in greater than 50% of bladder tumors (Cairns et al., 1993). Chromosome 9, especially region 9p21, is a known location of many tumor suppressor genes, including CDKN2A/ARF which encodes two proteins,

p16 and p14<sup>ARF</sup> (Knowles, 2006). Both p16 and p14<sup>ARF</sup> are key cell cycle regulators. P16 is a negative regulator of Retinoblastoma (RB) activation, which allows continuous activation of Rb, enabling cells to continuously move through the cell cycle unencumbered (Benedict et al., 1999). P14<sup>ARF</sup> is also a negative regulator of another key cell cycle protein, p53 (Knowles, 2006). With respect to the second alteration, activating mutations in the FGFR3 gene are found in 74% of early low-grade bladder tumors (Billerey et al., 2001). When FGFR3 is mutated there is an increase in activation of the RAS-MAPK pathway. There is also an increase in activation of phospholipase C $\gamma$  which results in increased cell proliferation and survival ability (Di Martino et al., 2009).

In MIBC there are many changes and rearrangements of both oncogenes and tumor suppressor genes. Similar to NMIBC, there is LOH on chromosome 9p as well as gain-of-function mutations in FGFR3 in MIBC (Knowles, 2006). Additional mutations include changes in ERBB2, a tyrosine kinase of the Epidermal Growth Factor Receptor (EGFR) family (Gardiner et al., 1992). As with NMIBC, tumor suppressor genes are also altered in MIBC, including changes in p53, RB and CDKN2A/ARF (Knowles, 2006). However, the key alteration in the muscle invasive phenotype is the change in the PTEN pathway. Several studies have reported frequent LOH of chromosome 10, the location of PTEN, in MIBC (Cappellen et al., 1997; Kagan et al., 1998). PTEN is a known negative regulator of the key signaling pathway PI3K/AKT/mTOR and its loss or inactivation can result in increased AKT phosphorylation and activation (Houédé & Pourquier, 2015). The increase in AKT phosphorylation can activate many downstream effectors resulting in increased cell metabolism, growth, protein synthesis and increased cell survival ability (Hay, 2005).

Several studies have found that decreased PTEN expression is strongly associated with metastasis and poor survival across several cancer subtypes (Saal et al., 2007). Specifically, in MIBC the combination of PTEN and p53 loss often leads to aggressive disease (Puzio-Kuter et al., 2009).

### **EGFR and c-Met expression in BC**

In normal tissue, the binding of ligand to the EGFR results in dimerization with another EGFR molecule resulting in receptor activation. This will trigger intracellular signaling through several pathways such as the MAP kinase or AKT pathways which regulate many cellular functions including survival, proliferation or invasion (Black & Dinney, 2008). In tumor cells, EGFR activation will promote growth by increasing cell proliferation, motility, adhesion, invasive capacity and inhibiting apoptosis (Bellmunt et al., 2003). The level of expression of EGFR is altered in both NMIBC and MIBC with 50% of human tumors having an increase in EGFR (Bellmunt et al., 2003). EGFR expression levels have a significant correlation with recurrence and prognosis. In NMIBC, an increase in EGFR level was correlated with increased disease recurrence (Chow et al., 1997). In addition, the overexpression of EGFR is associated with adverse prognosis compared to BC with normal EGFR status (Mellon et al., 1995). Patients with advanced MIBC that expressed increased levels of EGFR had a much worse prognosis compared to patients with tumors with no EGFR expression (Nguyen et al., 1994). In addition to the effect of EGFR on cell growth, it also plays a key role in angiogenesis. Two key ligands of the EGFR, epidermal growth factor (EGF) and transforming growth factor-alpha, can increase the

secretion of vascular endothelial growth factor, a key pro-angiogenic protein (Bellmunt et al., 2003).

Another protein which is also overexpressed in MIBC is mesenchymal epithelial transition factor (c-Met). c-Met is also a receptor tyrosine kinase (RTK) and is expressed in a variety of epithelial tissues in the body. Like EGFR, upon binding of its ligand, hepatocyte growth factor, c-Met will dimerize to activate intracellular pathways including ERK, AKT and PI3K (Hass et al., 2017). In addition, the overexpression of c-Met has been correlated with metastatic spread of many cancers including breast, liver and kidney cancers (Cheng et al., 2002). In BC, c-Met expression was found to be positively correlated with tumor grade and stage, with no expression in normal urothelial cells (Miyata et al., 2009). Furthermore, the overexpression of c-Met is a strong indicator of a decrease in patient survival (Cheng et al., 2002).

The EGFR and c-Met pathways are closely linked together and often activate similar pathways. Many tumors which initially respond to EGFR inhibitors will acquire resistance and demonstrate an increase in activation of the c-Met receptor (Wang et al., 2012). This resistance has been observed in many cancers including colon, lung and breast (Liska et al., 2011). Following EGFR inhibition, the tumor cell will bypass this pathway and activate PI3K/AKT and MAPK pathways through c-Met activation (Zhang et al., 2018). The crosstalk between these two receptors explains why a monotherapy does not completely stop tumor growth and a combination is needed to activate both pathways (Zhang et al., 2018). Blockade of both pathways results in a decrease in tumor volume and

proliferating cells compared to a single agent in a variety of cancer types (Castoldi et al., 2013; Xu et al., 2011).

### **Epigenetic modifications in BC**

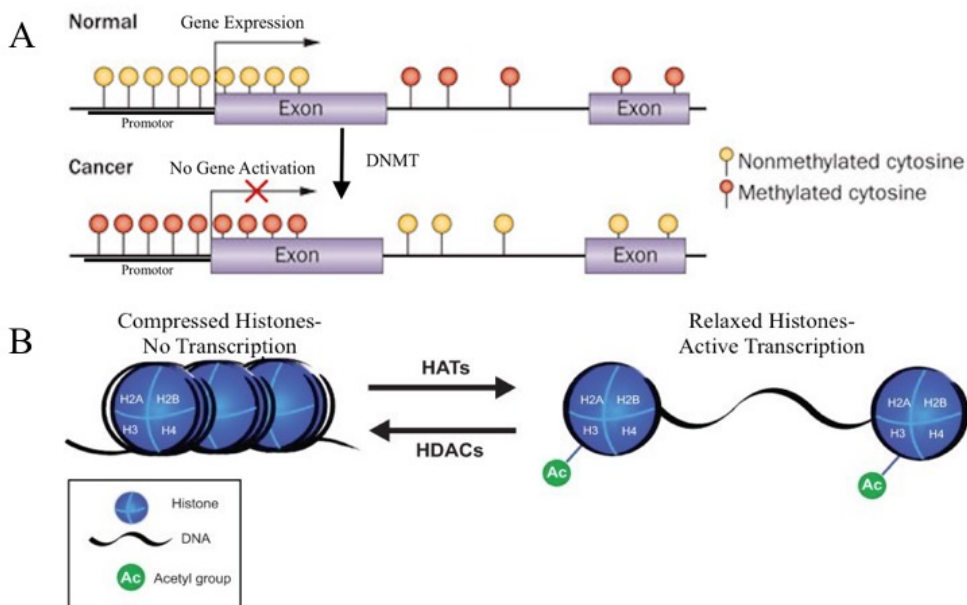
In 2014, the Cancer Genome Atlas (TCGA) initiative evaluated 131 high-grade MIBC in order to find genetic and genomic alterations (Cancer Genome Atlas Research Network, 2014). In BC, many genes have been found to have genomic alterations including deletion, amplification and mutations of several genes (Cancer Genome Atlas Research Network, 2014). In addition, the results showed a significant subgroup of tumors (76%) presented with epigenetic modifications (Cancer Genome Atlas Research Network, 2014). Epigenetic modifications are changes to gene expression without alteration in DNA sequences, in contrast to genomic alterations in which regions of DNA are amplified, deleted or mutated (Kanwal et al. 2015).

Epigenetic modifications have been found throughout all cells in the body and are seen in several disease states, including cancer (Chen et al., 2014). There are three main types of epigenetic modifications: DNA methylation, histone modification, and non-coding Ribonucleic Acid (RNA). DNA methylation has been shown to be a major contributor to all types of cancer throughout the body (Akhavan-Niaki & Samadani, 2013). Specifically, DNA methylation of promoter regions of several cancer-related genes are found to be hypermethylated (Hoque et al., 2006; Maruyama et al., 2001). DNA methylation is a stable modification which is produced during DNA replication. In eukaryotes, DNA methylation occurs on cytidine residues in sequences 5'CG3' or CpG dinucleotide (Akhavan-Niaki & Samadani, 2013). These CpG regions are not found throughout DNA but rather located in

specific sections called CpG islands (Ioshikhes & Zhang, 2000). Interestingly, CpG islands are found in 50% of the promoter regions of human genes, including both housekeeping and specific genes (Ioshikhes & Zhang, 2000). Normally, CpG islands located in the gene promoter are unmethylated, which allows for the gene to be active and DNA transcribed. Methylation of a gene can interfere with the transcriptional machinery interacting with the DNA and thus inhibit transcription (Figure 1A). This mechanism is used by tumor cells to silence many genes including tumor suppressor genes such as p16. DNA methylation is believed to occur early in tumor cell transformation and could facilitate tumorigenesis, as DNA hypermethylation has been seen in pre-neoplastic lesions in breast, colon and many other cancers (Gokul & Khosla, 2013). Wolff et al. examined a variety of genes in both NMIBC, MIBC and normal tissue from similarly age patients and found that in MIBC 38% of loci were hypermethylated compared to only 10% in NMIBC (Wolff et al., 2010).

The second major group of epigenetic modifications occur on histone proteins, and include acetylation, phosphorylation or methylation. Histones are a part of the DNA packaging unit called a nucleosome which involves a sequence of DNA that is wrapped around 2 subunits each consisting of histone proteins H2A, H2B, H3 and H4 (Hoffman & Cairns, 2011). The histones have a tail region which is accessible to covalent modifications. Two key proteins, histone acetyltransferases (HAT) and histone deacetylases (HDAC), are the enzymes which add or remove acetyl groups respectively. The addition of an acetyl group can neutralize the positive charge on the histone allowing a relaxed conformation and promoting gene transcription (Barneda-Zahonero & Parra, 2012) (Figure 1B). Histone methylation is mediated by histone methyltransferases (MT). In BC, there has been found

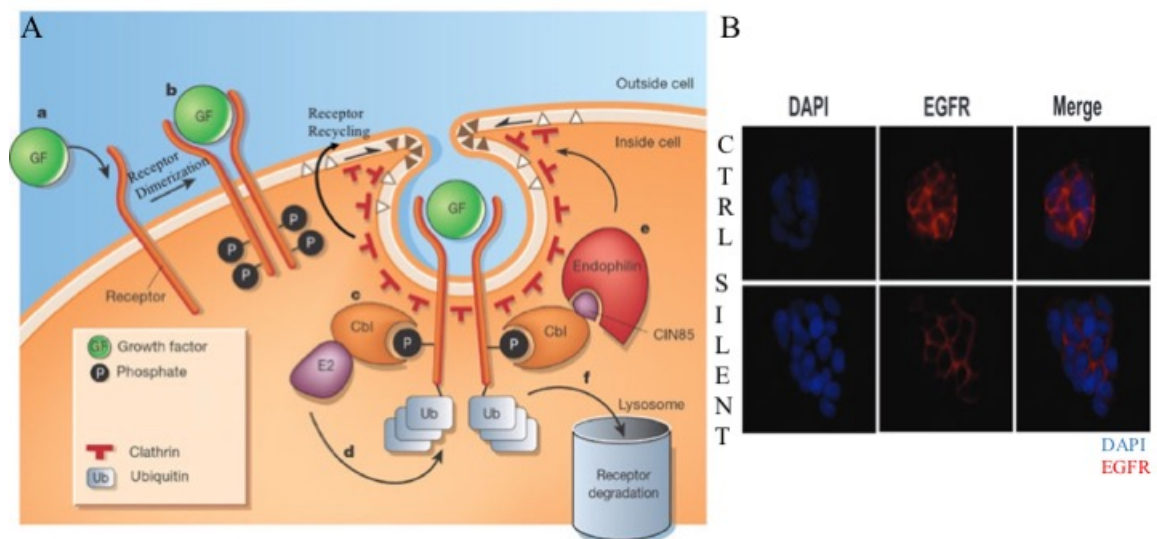
to be altered histone modification in 89% of cases (Cancer Genome Atlas Research Network, 2014). For example, Enhancer of Zest Homolog 2, an MT and a common oncogene, is overexpressed in BC and is associated with more aggressive tumors (Wang et al., 2012). In BC there is also an altered expression of HDAC, which is overexpressed compared to normal tissue (Tanji et al., 2011).



**Figure 1. Schematic depicting epigenetic modifications.** (A) The same gene is shown in two different conditions. During the normal state, the gene promoter is hypomethylated resulting in increased transcription of the exon. However, during cancer, the gene promoter becomes hypermethylated resulting in no gene transcription. The switch from hypo- to hyper-methylation status is completed by DNMTs. (Figure adapted from Kandimalla et al., 2013) (B) Histone modifications where the addition of acetyl groups by HAT cause histone relaxation and increased gene transcription or the removal of acetyl groups by HDACs cause histone compression and decreased gene transcription. (Figure adapted from Heerboth et al., 2014)

## SH3GL2/Endophilin-A1 and BC

SH3GL2/endophilin A1 was originally discovered in relation to its function in the brain, aiding in synaptic vesicle recycling (Ringstad et al., 1997). SH3GL2 is a major component of the endophilin-CIN85-Cbl complex which regulates recycling and therefore signaling of EGFR and c-MET (Petrelli et al., 2002; Soubeyran et al., 2002). Following EGFR/c-MET binding to their respective ligands and phosphorylation of tyrosine residues on the RTK, the phosphorylated receptor recruits the protein Cbl (Oved & Yarden, 2002), a ubiquitin ligase. Cbl ubiquitinates the receptor which triggers recruitment of the adaptor protein CIN85 which in turn will recruit SH3GL2 (Soubeyran et al., 2002). Once the complex is attached to the RTK, it changes the curvature of the plasma membrane in which the receptor is located leading to endocytosis and inactivation of the receptor (Oved & Yarden, 2002) (Figure 2A).



**Figure 2. SH3GL2/Endophilin is involved in receptor recycling and degradation.** (A) A cartoon schematic of the function of SH3GL2 function after growth factor binding to receptor. After binding, SH3GL2 will associate with Cbl and CIN85 to trigger receptor

endocytosis of the active growth receptor which either results in receptor recycling or degradation. (Figure 2A adapted from Oved & Yarden, 2002). (B) Immunofluorescence staining of cells after EGF treatment. In the control cells, after EGF binding, the EGFR undergoes internalization shown by merge of coloring of the red and blue. In cells with SH3GL2 knocked down, the EGFR remains on the surface after EGF treatment (published data from Majumdar et al., 2013.).

Recently our laboratory found that SH3GL2 was enriched in the bladder and the loss of function of SH3GL2 in BC was discovered to be an important regulator of the oncogenic activity of BC and had a role in tumor suppressor (Majumdar et al., 2013). The gene encoding SH3GL2 is located on chromosome 9p (9p22) (Majumdar et al., 2013), a known hotspot of genetic alterations in BC. Interestingly, p16, a gene lost frequently in BC, is located at 9p21. Several studies have found distal deletions of chromosome 9p relative to the p16 gene locus (Keen & Knowles, 1994; Pollock et al., 2001). Analysis of human tumors, found that with BC progression from superficial to invasive BC there is a significant decrease in mRNA and protein level of SH3GL2 and its loss appears to be a frequent phenomenon in BC pathogenesis (Majumdar et al., 2013). Additionally, when SH3GL2 was knocked down in BC cell lines there was an increase in EGFR on the surface following EGF treatment which resulted in increased growth and proliferation in both *in-vitro* and *in-vivo* experiments (Majumdar et al., 2013) (Figure 2B). These results indicate that the loss of SH3GL2 constitute a mechanism for EGFR, c-Met kinase activation in BC.

Apart from BC, SH3GL2 changes are also found in several other cancers. In breast cancer, a decrease in SH3GL2 is found as an early event in tumorigenesis (Sinha et al., 2008). Lung and head and neck cancers also have reduced SH3GL2 which leads to increased EGFR expression and increased cell growth (Dasgupta et al., 2013; Maiti et al.,

2013). The mechanism of silencing of SH3GL2 in breast cancer and several other cancers is through DNA methylation (Li et al., 2015; Sinha et al., 2008). Whether the loss of SH3GL2 in BC also occurs as a result of DNA methylation is unknown.

The overall goal of this study is to investigate the mechanisms of SH3GL2 silencing in BC cell lines. More specifically, the hypothesis is that pharmacological demethylation of the promoter together with inhibition of HDAC activity, will lead to re-expression of SH3GL2. To test this hypothesis two aims were explored.

**Aim I:**

To examine the methylation status of SH3GL2 in a variety of different BC cell lines.

**Aim II:**

- A. To explore the required dose of DNA demethylating agents and HDAC inhibitor needed to minimize apoptosis.
- B. To determine if demethylation along with an HDAC inhibitor is sufficient to get re-expression of SH3GL2 protein.

## METHODS

### Cell culture, chemicals and antibodies

253J and J82 BC cell lines were grown in Minimal Essential Media (MEM) cell culture media (Life Technologies, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Corning, Tewksbury, Massachusetts), 1% L-Glutamine Solution (L-Glut), 1% 10,000 U/mL Penicillin-Streptomycin Solution (P/S) (both from Life Technologies, Carlsbad, CA) and 1% MEM Non-Essential Amino Acids Solution (100x) (Life Technologies, cat# 11140050, Carlsbad, CA). RT4 and T24 cells were cultured in McCoy's 5A (modified) Medium cell culture media (Life Technologies, Carlsbad, CA) containing 10% FBS, 1% L-Glut and 1% P/S. All cells were incubated at 37° Celsius (C) and 5% Carbon Dioxide (CO<sub>2</sub>). 5-Aza-2'-deoxycytidine (Decitabine) (5-Aza-dC) was purchased from Sigma-Aldrich (cat # A3656, St. Louis, MO) and stocks of 50 millimolar (mM) were prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, cat# TS-20688, St. Louis, MO). Trichostatin A (TSA) was purchased from Sigma-Aldrich (cat# T1952, St. Louis, MO). Zebularine (cat# 10975, Ann Arbor, MI) and RG-108 (cat# 13302, Ann Arbor, MI) were purchased from Cayman Chemical. 50mM stocks of each drug were prepared in DMSO and stored at -80°C.

The following antibodies were used: primary antibody for SH3GL2 was a mouse monoclonal antibody (clone 4D12, Santa Cruz Biotechnology, cat# sc-134329) at a dilution of 1: 10,000 in Phosphate Buffered Saline/Tween (PBS/T).  $\beta$ -Actin antibody was a monoclonal mouse antibody (Thermofisher, cat# MA5-15739) at a dilution of 1: 10,000 in

PBS/T/5% non-fat dried milk. Horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibodies were used (Thermofisher, cat# 32430, Carlsbad, CA) at a dilution of 1: 20,000 in 10% milk/ PBS/T solution.

#### **5-Aza-dC and TSA treatment of cells**

Initially, 253J or T24 cells were cultured and 50,000 (253J) or 25,000 (T24) cells were counted and seeded into a 6-well plate (Corning, cat# 353046, Tewksbury, Massachusetts). After overnight incubation at 37°C, stock 5-Aza-dC was diluted in culture media and added at different concentrations to the medium and incubated at 37°C for varying times depending on the experiment. At the end of the incubation, 500µL of TRIzol Reagent (Life Technology cat# 15596026, Carlsbad, CA) was added to lyse the cells. Samples were frozen at -80°C.

After determining the appropriate dose to minimize toxicity, 50,000 253J cells were seeded and the repeat experiment was conducted with the addition of the proper concentrations of 5-Aza-dC and incubated for 48 hours. TSA was then added and incubated for an additional 24 hours. DMSO was added to the cells in the appropriate volume as a control. After treatment with TSA for 24 hours the medium was removed and 500µL of TRIzol Reagent was added to lyse the cells. Samples were frozen at -80°C.

#### **Zebularine and RG-108 treatment of cells**

Initially, 253J cells were cultured and 75,000 cells were counted, seeded into a 6-well plate and incubated overnight at 37°C. Using the protocol outlined in previous experiments using Zebularine (Cheng et al., 2004) 500µM was added to either 253J or T24 cells for 72 hours. Cells were then observed under a microscope to observe toxicity. Using

a similar procedure, cells were initially plated overnight at which point RG108 was then added at 500  $\mu$ M to the medium and incubated at 37°C for varying times depending on experimental conditions. Cells were then harvested in 500 $\mu$ L of TRIzol reagent. Samples were frozen at -80°C.

### **RNA isolation and cDNA synthesis**

The frozen samples were thawed and 100 $\mu$ L of chloroform was added. The solutions were vortexed and then centrifuged at 12,000 rpm at 4°C for 15 minutes, after which the top clear aqueous layer was removed and added to 200 $\mu$ L of 70% ethanol. This mixture was added to RNeasy spin columns (RNeasy kit, Qiagen, cat #74104, Germantown, MD) and centrifuged for 15 seconds at 10,000 rpm. Columns were washed with 700 $\mu$ L of wash buffer from the kit and centrifuged for 15 seconds at 10,000 rpm. Two additions of 500 $\mu$ L of RPE buffer from the RNeasy kit were added with 15 seconds and 2 minutes of centrifuging respectively after each addition. The spin column was air-dried for 10 minutes and 30 $\mu$ L of nuclease-free water (Qiagen, cat# 129114, Germantown, MD) was added to the column to elute the RNA. The column was added to a new centrifuge tubes and centrifuged for 2 minutes at 10,000 rpm at 4°C to collect eluted RNA. Purified RNA was quantified using a Nano-drop, and concentration and purity were obtained using absorbance of 260/280 as readouts. If impure RNA was obtained, an additional 30 $\mu$ L of nuclease-free water was added and re-centrifuged to improve RNA purity.

Depending on the yield of RNA from the isolation, a range of input RNA (175-1000ng) was added to the Reverse Transcription (RT) reaction to maximize cDNA yield. The Bio-Rad iScript cDNA synthesis Kit (cat# 1708891, Hercules, CA) was used in which

each 20 $\mu$ L reaction comprised 4 $\mu$ L of 5x iScript Reaction Mix, 1 $\mu$ L of iScript Reverse Transcriptase, RNA and nuclease-free water, depending on RNA concentrations in individual samples. Samples were incubated in a thermocycler for 5 minutes at 25°C for priming, 20 minutes at 46°C for RT and 1 minute at 95°C for RT inactivation. 20 $\mu$ L of the cDNA was diluted into 480 $\mu$ L of nuclease-free water and stored at -20°C.

### **Quantitative RT-PCR**

A MicroAmp 96-well reaction plate (Thermofisher Scientific, cat# 4346907) was used for the quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). Each cDNA sample was run in triplicate. A reaction mixture in the ratio of 10:1 of TaqMan Universal PCR Master Mix (Thermofisher Scientific, cat# 4304437) to TaqMan probe was used. The probes included SH3GL2 (Thermofisher Scientific cat# 4331182 Assay ID: Hs00182352\_m1), and  $\beta$ -Actin (Thermofisher Scientific, cat# 4331182, Assay ID: Hs01060665\_g1). For each sample 11 $\mu$ L of the reaction mixture was added to each well, followed by 9  $\mu$ L of the cDNA dilution.  $\beta$ -Actin was used as the house-keeping gene. The reaction plate was loaded into the StepOnePlus Real-Time PCR System. The PCR protocol was: 10 minutes at 95°C to activate the DNA polymerase followed by a two-step cycle of 95°C for 15 seconds, which allows the DNA to separate and 60°C for 1 minute which allows probe annealing. These two steps were repeated for 40 cycles to help amplify the gene of interest. The final read-out was a  $C_T$  which was used in further calculations.

### **Methylation analysis**

Previously isolated genomic DNA from a variety of BC cell lines were generated using the Qiagen DNeasy kit protocol (cat# 74104, Germantown, MD). DNA was

quantified using Nano-drop where concentration and purity were assessed. Dilutions of genomic DNA were made in nuclease free water to yield 25ng of genomic DNA. A reaction cocktail of 5μL of genomic DNA (125ng), 13μL of 5x Restriction Digestion Buffer from the Epiect Methyl II DNA Restriction Kit (Qiagen, cat# 335452, Germantown, MD) along with 42μL of nuclease-free water was generated. Four separate restriction enzyme digestions were set up. 14 μL of the reaction cocktail was added to each reaction as follows:

1. Mock digest (Mo) with no restriction enzyme
2. A methylation-sensitive restriction digest (Ms)
3. A methylation-dependent restriction digest (Md)
4. A double digest (Msd) where both the methylation-sensitive and dependent enzymes were added

All reactions were incubated at 37°C for 6 hours in a thermocycler in order to activate the enzymes. After incubation the reactions were heated to 65°C for 20 minutes in order to heat inactivate enzymes.

To determine the degree of methylation by qPCR, 5μL of each digest was added to a well in a MicroAmp 96-well reaction plate along with 12.5μL of RT<sup>2</sup> SYBR Green Mastermix (Qiagen, cat# 330500, Germantown, MD), 1μL of a SH3GL2 Primer (Qiagen, cat# EPHS114245-1A, Germantown, MD) for the CpG island, and 6.5μL of nuclease-free water.

Each sample was run in duplicate on the plate. The PCR was performed to obtain raw  $C_T$  values and cycled per the Epiect Methyl II DNA protocol:

Temperature	Time	Number of Cycles
95°C	10 minutes	1 cycle
99°C	30 seconds	3 cycles
72°C	1 minute	
97°C	15 seconds	40 cycles
72°C	1 minute	

A melting curve was also performed after the cycling program to ensure specificity of primers. The results were then exported into the Qiagen Methylation analysis software.

### **Immunoblot analysis**

40,000 J82 cells were seeded into four 6 cm dishes and allowed to adhere overnight at 37°C. The next day vehicle control (DMSO) and drugs (Zebularine, RG108 or 5-Aza-dC) were added to each dish and incubated for various times at 37°C. Following drug exposure, the media was then removed and washed twice with 3mL of ice cold PBS (Life Technology, cat# 10010023, Carlsbad, CA). Depending on cell confluence at the time of harvest either 100 or 150  $\mu$ L of cell lysis buffer (Cell Signalling, cat# 9803, Danvers, MA) with protease inhibitor cocktail (Thermofisher, cat# 78429, Carlsbad, CA) was added on ice. After 15 minutes, cells were scraped and lysates collected. Protein concentrations were determined using the MicroBCA protein assay (Thermofisher Scientific, Cat# 23235, Carlsbad, CA) to determine the appropriate amount of protein to add to the separating gel.

Following the protein assay, separating gels (distilled water (dH<sub>2</sub>O), 30% Acrylamide mix, 1.5M Tris (pH 8.8), 10% SDS, 10% APS, TEMED) and stacking gel (dH<sub>2</sub>O, 30% Acrylamide mix, 1M Tris (pH 6.8), 10% SDS, 10% APS, TEMED) were prepared. For each sample 4x sample buffer (100% glycerol, 1M Tris-HCl, 20% SDS, 1% Bromophenol blue and dH<sub>2</sub>O) and β-Mercaptoethanol was added and the samples were boiled for 10 minutes. After the gels polymerized, they were added to the electrode apparatus and running buffer (dH<sub>2</sub>O, Tris base, glycine and 0.1% SDS) was added. Appropriate amounts of protein were then added to specific wells. The gel was run at 100V to allow samples to pass through the stacking gel, and then at 160V for 75 minutes. When finished, the stacking gel was removed. The gel was placed in a tray with transfer buffer (dH<sub>2</sub>O, Tris base, glycine and 20% methanol) for 15 minutes to shrink gels prior to transfer. Assembly of the transfer cassette was performed and the transfer apparatus was filled with transfer buffer and an ice pack. The transfer was run overnight at 50V in the cold room. The next morning, the voltage was increased to 100V for an additional hour. Following the transfer, the membranes were washed in dH<sub>2</sub>O and then stained with Ponceau S dye to verify equal transfer and protein loading. The membranes were rinsed in dH<sub>2</sub>O and imaged on a flat-bed scanner. After rinsing in PBS/T (10x PBS, 10% Tween-20 Solution and dH<sub>2</sub>O), membranes were incubated in a solution of PBS/T and 10% milk for 1-hour at room temperature to block non-specific binding sites. Primary antibodies to proteins of interest were added and incubated overnight at 4°C. The membranes were then washed 3 times with PBS/T. Species-specific secondary antibodies, were added and allowed to incubate for 1-hour at room temperature. Membranes were washed again 3 times in PBS/T. To

detect proteins, membranes were incubated with SuperSignal West Pico PLUS Chemiluminescent Substrate (ThermoFisher Scientific, cat# 34580, Carlsbad, CA). Equal volumes of Luminol and Peroxide solutions from SuperSignal kit were mixed and added to membranes for 5 minutes at room temperature. Membranes were then exposed to X-ray film for variable times in the dark room to visualize signals.

### **Statistical analysis**

The output of all qRT-PCR reactions were Ct values. These Ct values were then normalized to the housekeeping gene,  $\beta$ -actin, to find the difference between the Ct values of each condition to its respective  $\beta$ -actin level yielding  $\Delta$ Ct. The average  $\Delta$ Ct of the vehicle control was then calculated and subtracted from each  $\Delta$ Ct yielding  $\Delta\Delta$ Ct.  $\Delta\Delta$ Ct was then analyzed using  $2^{(-\Delta\Delta Ct)}$  in order to find the relative gene expression value. All graphs showing qRT-PCR are shown with error bars indicating one standard deviation.

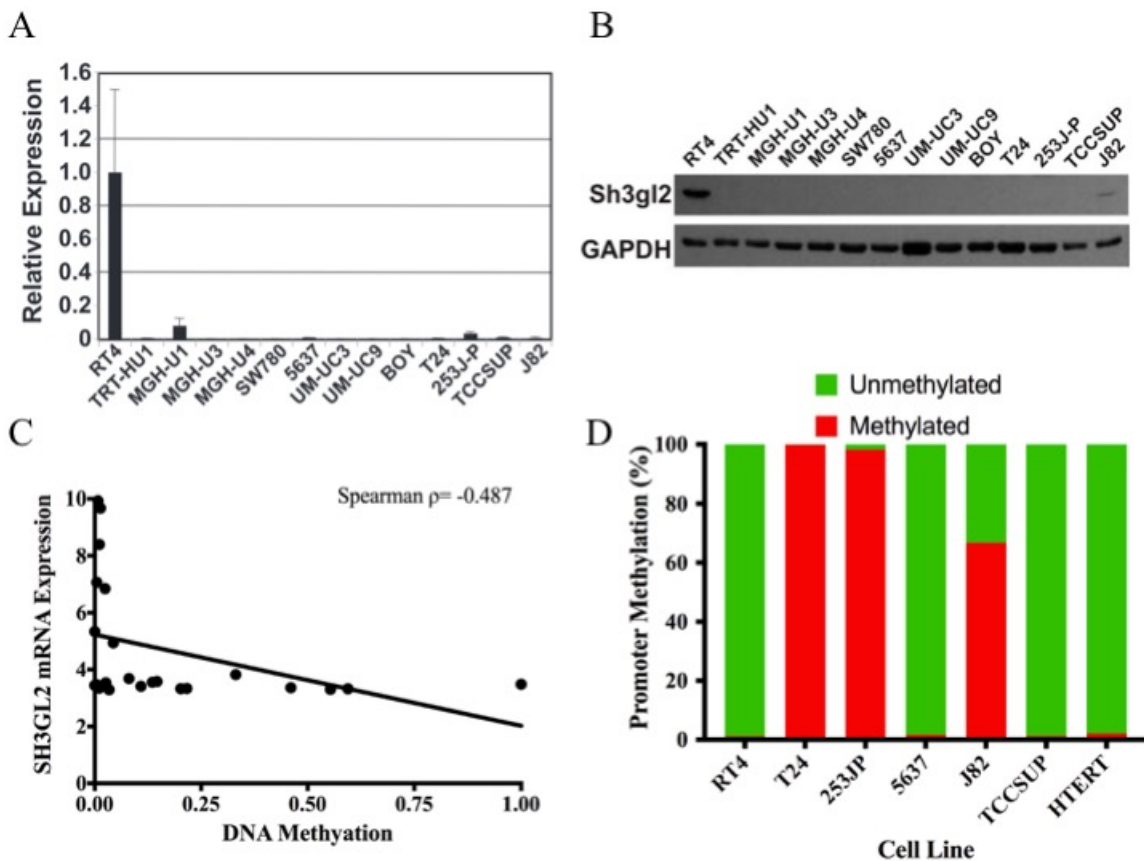
## RESULTS

SH3GL2 is normally expressed in the urothelium of the bladder and has previously been shown to have a major role in BC and its oncogenic properties (Majumdar et al., 2013). SH3GL2 is key in the regulation of EGFR and c-Met signaling and when it is lost, it leads to increased activation of EGFR in BC (Majumdar et al., 2013). Given the initial data suggesting little or no SH3GL2 mRNA or protein expression in the majority of BC cell lines and the inverse relationship between SH3GL2 DNA promoter methylation and mRNA expression (Figure 3), the hypothesis that promoter methylation is a likely mechanism to explain the lack of expression was explored. In addition, the potential for re-expression of SH3GL2 mRNA and protein using pharmacological approaches was also investigated.

### **Methylation status of SH3GL2 in BC cell lines**

Our laboratory found that in many BC tumor cell lines, there was little or no SH3GL2 expression at the mRNA or protein level (Figure 3A and B) (Data from Majumdar et al., 2013). Reports in the literature describe promoter methylation as a mechanism for downregulation of SH3GL2 expression (Li et al., 2015; Sinha et al., 2008). To explore this hypothesis a bioinformatics approach was used analyzing data from the Cancer Cell Line Encyclopedia (CCLE) (Figure 3C). This data showed a strong inverse correlation between DNA methylation and mRNA expression of SH3GL2 (Figure 3C). To determine the extent of SH3GL2 promoter methylation in BC, genomic DNA was isolated from a panel of BC cell lines and methylation-specific digestion and qPCR were performed. This demonstrated that RT4, 5637, TCCSUP and HTERT cell lines had close to a 100% unmethylated

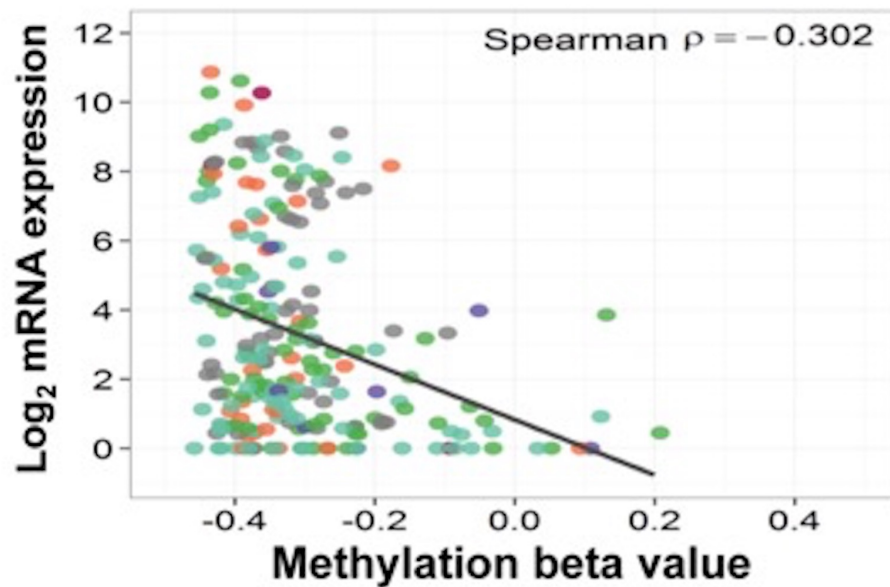
promoter while the T24 and 253J cell lines have almost 100% promoter methylation (Figure 3D). Interestingly, the SH3GL2 promoter region was approximately 30% unmethylated in the J82 cell line (Figure 3D). In T24 and 253J cells the high level of methylation is consistent with gene silencing shown with no mRNA and protein expression of SH3GL2 (Data from Majumdar et al., 2013) (Figure 3). However, several cell lines 5637, TCCSUP and hTERT, which do not express SH3GL2 (Figure 3A and B) but do have an unmethylated promoter region suggests that methylation is not the only mechanism of silencing.



**Figure 3. Promoter methylation affects mRNA and protein expression of SH3GL2.** (A) qRT-PCR analysis of SH3GL2 mRNA in a variety of BC tumor cell lines. Only RT4 had high gene expression (published data, contributed by Majumdar et al., 2013). (B) A

immunoblot analyzing SH3GL2 protein levels in a variety of BC tumor cell lines (published data, contributed by Majumdar et al., 2013). (C) A scatter plot of SH3GL2 mRNA data compared to SH3GL2 promoter DNA methylation. A strong negative correlation exists between mRNA levels and DNA methylation (Spearman  $\rho=-0.487$ ) (Data taken from <https://portals.broadinstitute.org/ccle>). (D). Methylation analysis of genomic DNA isolated from a variety of BC tumor cell lines. Genomic DNA was digested based upon protocol from the Epiect Methyl II DNA Restriction Kit and analyzed by qPCR.  $C_T$  values were then analyzed using Qiagen Methylation analysis software.

To explore if this method of silencing was seen beyond BC cell lines, the TCGA database was used to conduct an informatics-based analysis of expression and methylation of SH3GL2 from human BC tissue (Figure 4). This analysis was from 4 different DNA methylation sites on the SH3GL2 gene and a significant negative correlation between DNA methylation and SH3GL2 mRNA expression levels in BC was found. While a few cases showed genomic gains (orange circles) or losses (purple) the majority of cases showed the SH3GL2 gene unaltered by genomic rearrangements but rather showed silencing through DNA methylation (Figure 4). The genomic losses (purple circles) could explain the mechanism of silencing in 5637, TCCSUP and hTERT cell lines. This data illustrates a possible mechanism by which the intact chromosome is silenced in BC cells.



**Figure 4. DNA methylation correlation with SH3GL2 mRNA expression.** TCGA data of 228 human BC tumors analyzing the association between DNA methylation and mRNA expression of SH3GL2. Analysis is from 4 promoter sites. A strong negative correlation (Spearman  $\rho=-0.302$ ) exists in human BCs with only a few numbers of tumors having genomic changes (orange: genomic gains and purple/turquoise: genomic losses). (Data generated by the TCGA Research Network: <http://cancergenome.nih.gov>)

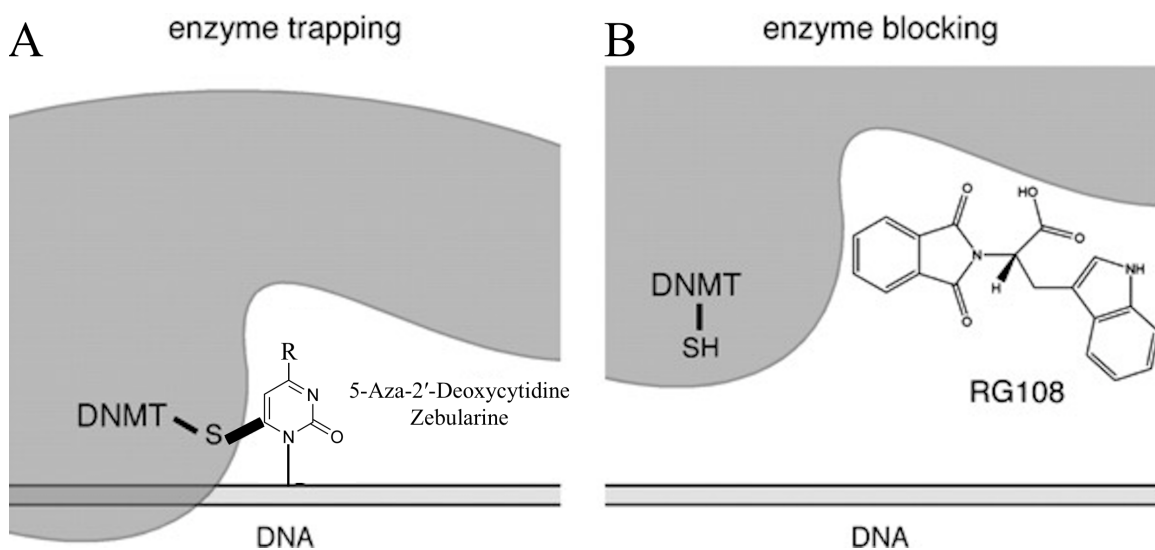
#### **Demethylating agents promote gene reactivation**

Given the previous observation that SH3GL2 was silenced in several BC cell lines and having observed a negative correlation in human BC tissues from TCGA, the ability to reactivate the gene in 253J and T24 BC tumor cells was explored. Several demethylating agents: 5-Aza-dC, Zebularine and RG108 were explored as to their ability to reactivate SH3GL2. Initially, 5-Aza-dC was used as this was a known cytidine analog that can be incorporated into DNA of cells leading to loss of DNMT activity due to the enzyme becoming permanently bound to the cytidine analog (Christman, 2002) (Figure 5A). However, like most DNA modifying agents, the drug can be toxic to cells and cause them

to undergo apoptosis. As a result, a dose-response was performed to determine the optimal dose that did not lead to cell toxicity (Figure 6A). Cell toxicity was observed by looking at cells under a microscope and assessing phenotype and adherence. From this result and previous experiments (unpublished data, Majumdar et al., 2013) a starting dose of 20 $\mu$ M was selected. After adding 5-Aza-dC at the appropriate dose, T24 cells exhibited a relative fold increase in SH3GL2 mRNA levels of 11.68 compared to vehicle control (1.48) analyzed through qRT-PCR (Figure 6B). We expanded this experiment to 253J cells using the same dose of 5-Aza-dC and were also able to see an increase of SH3GL2 expression (11.66) compared to vehicle control (1.38) (Figure 6B).

The largest issue encountered in these experiments was the problem of 5-Aza-dC stability in aqueous solution. Previous literature has shown that 5-Aza-dC is toxic and unstable in aqueous solutions which could complicate its use clinically (Cheng et al., 2004). As a result, another drug, Zebularine was tested. Zebularine is also an inhibitor of DNA methylation but is much more stable in aqueous solution and was proven effective in an *in-vivo* model (Cheng et al., 2003). Similarly to 5-Aza-dC, Zebularine is a cytidine analog which forms a complex with DNMT, thereby inhibiting their function (Cheng et al., 2003) (Figure 5A). As a result, a dose-response was performed using 253J to assess maximum gene reactivation with minimum cell toxicity. Taking into combination the toxicity observed under a microscope (floating cells) and the relative fold increase (7.19 times) in gene expression (Figure 7A), a dose of 500 $\mu$ M was selected. When compared to control (1), this was able to increase gene expression (9.21) (Figure 7B). However, when T24 cells were treated with Zebularine at the same dosage, we were only able to get a slight increase

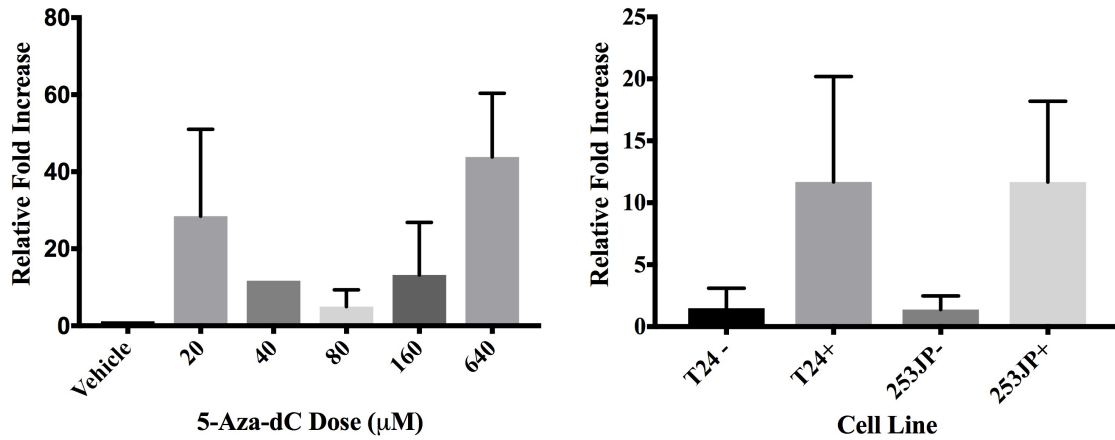
in gene activation compared to vehicle control (7B). Thus, the findings suggest that Zebularine is a valid alternative in selected cell lines and has much higher stability in solution compared to 5-Aza-dC.



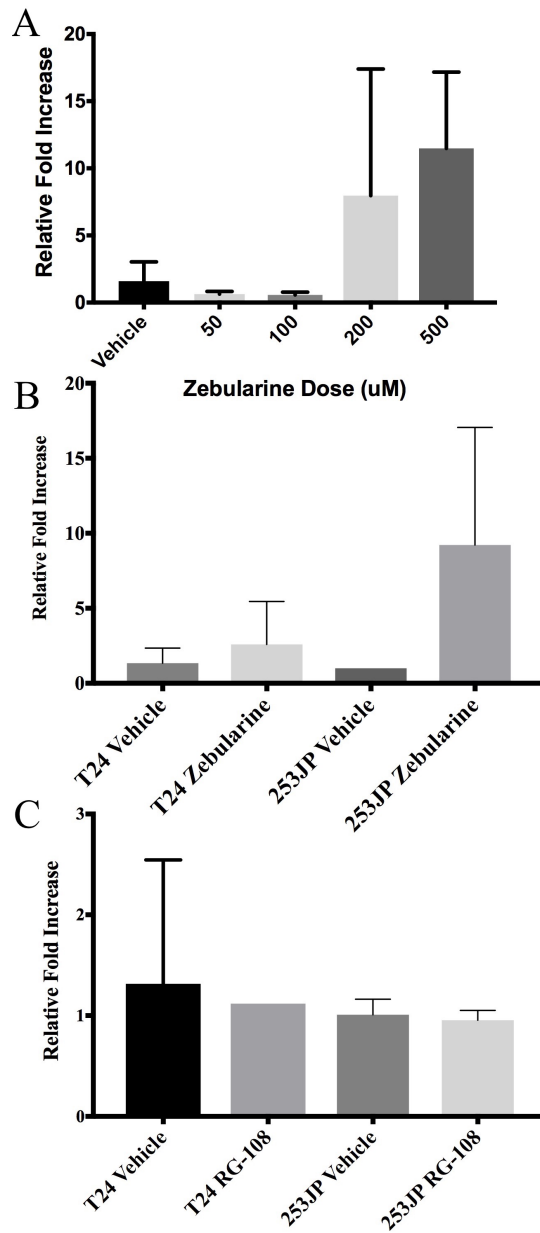
**Figure 5. Mechanism of action of the three drugs used in this study. (A)** Both 5-Aza-dC and Zebularine are cytosine analogs which will intercalate into DNA and covalently bind to DNMTs inhibiting their function. The R group represents the different functional groups between 5-Aza-dC and Zebularine. **(B)** RG-108 acts as an inhibitor of DNMT and will interact with the active site of the DNMT. (Figure adapted from Lyko & Brown, 2005)

Finally, to explore another method of gene reactivation, the drug RG-108 was tested. RG-108 is a direct methyltransferase inhibitor which does not covalently attach to the methyltransferase but rather will block the active site of DNA Methyltransferase 1 (Figure 5B). This modification was made in order to limit toxicity to cells, as covalent modifications, as seen with 5-Aza-dC and Zebularine, were found to be toxic to the cells. Similarly to Zebularine, 500  $\mu$ M was selected as the optimal dose to have gene reactivation while minimizing cell toxicity. However, unlike either 5-Aza-dC and Zebularine, RG-108

at this dose was unable to increase gene expression in either 253J or T24 cells (Figure 7C). Overall, these results indicate a stronger agent that directly binds as a cytidine analog rather than a competitive inhibitor is required to increase the expression of SH3GL2.



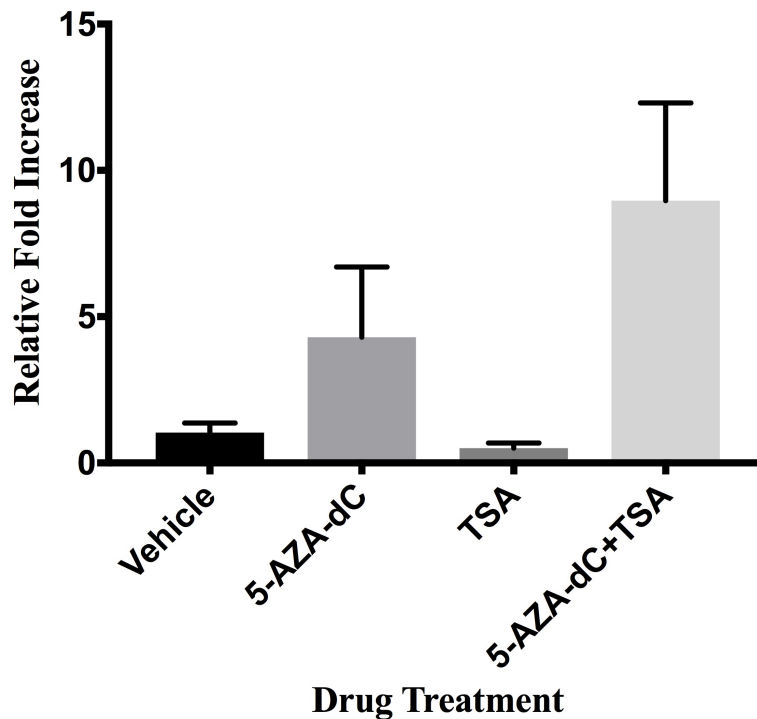
**Figure 6. Effect of 5-Aza-dC on SH3GL2 expression (A)** A dose-response of T24 cells exposed to a variety of 5-Aza-dC concentrations. T24 cells were grown for 24hrs and then treated with different 5-Aza-dC concentrations for 120 hrs. Cells were harvested and gene expression was detected by qRT-PCR. Error bars indicate standard deviation between technical triplicates. **(B)** The effect of 5-Aza-dC at a dose of 20μM on T24 and 253J cells. Both cell lines were grown for 24hrs after which 5-Aza-dC was added to the cells for 120hrs. Cells were harvested and gene expression was detected by qRT-PCR. Error bars indicate standard deviation between technical triplicates.



**Figure 7. Effect of Zebularine and RG-108 on SH3GL2 expression.** (A) A dose-response of 253J cells exposed to a variety of Zebularine concentrations. 253J cells were grown for 24hrs and then treated with different Zebularine concentrations for 72 hrs. (B) The effect of Zebularine at a dose of 500μM to T24 and 253J cells. Both cell lines were grown for 24hrs after which Zebularine was given to the cells for 72hrs. (C) The effect of RG-108 at a dose of 500 μM to T24 and 253J cells. Both cell lines were grown for 24hrs after which RG-108 was given to the cells for 96hrs. In all three figures cells were harvested and gene expression was detected by qRT-PCR. Error bars indicate standard deviation between technical triplicates.

## **Combining demethylation and histone deacetylation inhibitor further increases gene activation**

After observing that 5-Aza-dC was able to reverse silencing and increase SH3GL2 mRNA levels, the extent to which SH3GL2 protein could be re-expressed was also explored. Published literature suggests that demethylating agents alone are often not sufficient to achieve protein re-expression and that additional manipulation of the epigenetic machinery, namely histone modifications are required. On top of DNA methylation, a common silencing method in many cancer cells is histone deacetylation. In BC specifically, HDAC is overexpressed compared to normal tissue (Tanji et al., 2011). In these experiments, the HDAC inhibitor TSA, was used alone and in combination with 5-Aza-dC to try and induce protein expression. Using TSA along with 5-Aza-dC is a known mechanism as the two drugs have been shown to work synergistically to reactivate genes (Cameron et al., 1999). However, initial experiments showed the combination of drugs was very toxic to cells and as a result a lower dose (10 $\mu$ M) of 5-Aza-dC was needed along with a low dose of TSA (75 $\eta$ M). This combination increased gene expression by an average 8.62-fold compared to vehicle control (Figure 8). However, TSA alone was not sufficient to reactivate expression of SH3GL2. In fact the expression of SH3GL2 decreased after addition of TSA compared to the control (Figure 8). These results indicate that the combination of 5-Aza-dC along with TSA was sufficient to further increase gene activation compared to 5-Aza-dC alone (Figure 8) and could be used as a potential strategy to reactivate expression of SH3GL2.

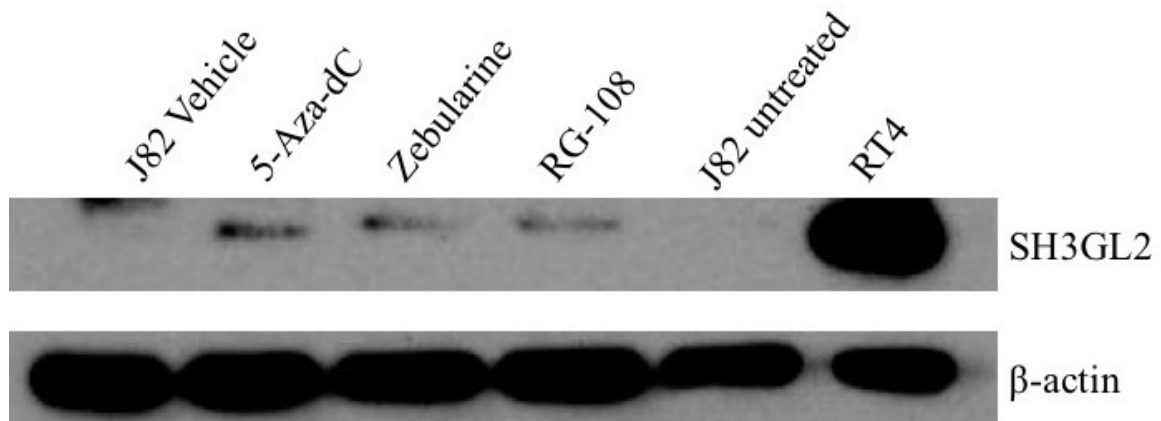


**Figure 8. Effect of 5-Aza-dC and TSA on SH3GL2 mRNA levels.** 253J cells were grown for 24hrs and then treated with 10 $\mu$ M of 5-Aza-dC for 48hrs. After which 75 $\eta$ M of TSA was added and cells were allowed to grow for an additional 24hrs. After which cells were harvested and gene expression was found using qRT-PCR. Error bars indicate standard deviation between technical triplicates.

### Demethylating agents effect on protein expression

In order to determine whether the three demethylating agents could increase protein levels, immunoblot analysis for SH3GL2 protein was performed using the J82 cell line. In J82 cells the extent of SH3GL2 promoter methylation is approximately 70% (Figure 3D). Interestingly, a small amount of SH3GL2 protein was detectable in J82 cells (Figure 3B, contributed by Majumdar et al., 2013), suggesting that this level could be increased by promoter demethylation. Consequently, J82 cells were exposed to the three demethylating agents, 5-Aza-dC (20 $\mu$ M), Zebularine (500 $\mu$ M) and RG-108 (500 $\mu$ M). SH3GL2 protein

expression was analyzed through immunoblot with RT4 used as a positive control. From the immunoblot, all three drugs were able to increase SH3GL2 protein levels above that observed in the untreated and vehicle control (DMSO) (Figure 9). Overall, these results indicate that demethylating agents can increase both mRNA and protein levels in J82 cells.

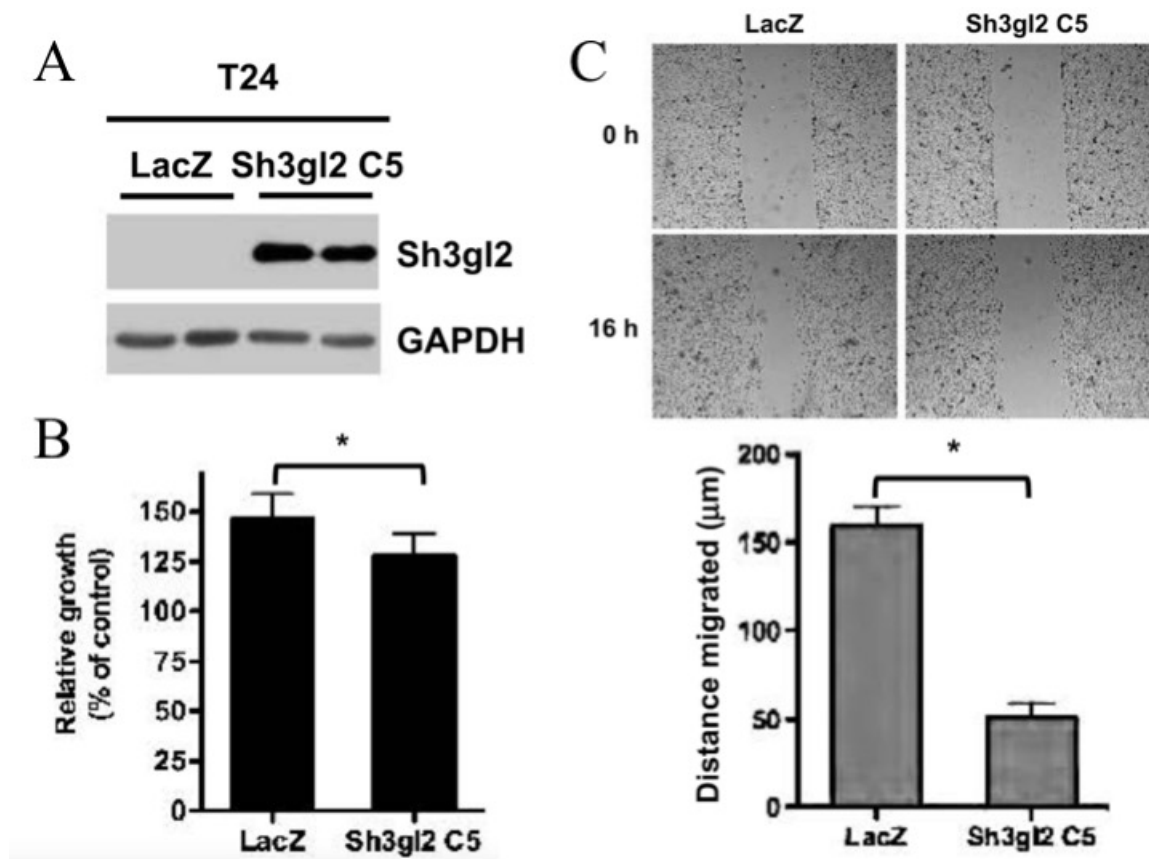


**Figure 9. Demethylating agents effect on SH3GL2 protein expression.** J82 cells were treated with 5-Aza-dC (20 $\mu$ M) for 48hrs with fresh drug added every 24hrs, after which the media was changed and cells were incubated for an additional 72 hrs. Zebularine (500 $\mu$ M) was given for 48hrs after which fresh media was added and cells were incubated for an additional 96hrs. RG-108 (500 $\mu$ M) was given for 96 hrs. Following addition of the chemiluminescent reagent, film was exposed to membranes for 1 hr. RT4 cells were used as a positive control.  $\beta$ -actin was used as a loading control.

#### **Effect of reactivation of SH3GL2 on BC cells**

While increasing SH3GL2 at the mRNA level is important to re-express the protein, it is important to determine how this re-expression affects tumor cell behaviour. To observe the impact of re-expression of SH3GL2 on tumor cell behaviour two key properties were explored: cell migration and growth. The re-expression of SH3GL2 was performed using viral re-expression in T24 cells (Figure 10A) (Data from Majumdar et al., 2013). This re-expression of SH3GL2 was able to decrease proliferation to a statistically significant extent

compared to cells transduced with virus encoding an irrelevant gene (LacZ) (Figure 10B). To assess the effect of SH3GL2 re-expression on cell migration, a migration assay was performed in the presence of EGF. When SH3GL2 was re-expressed, EGF-stimulated migration of T24 cells decreased by greater than 50% (Figure 10C). Overall, the re-expression of SH3GL2 had a major effect on two key tumor cell properties and can be seen as a possible mechanism to slow tumor cell growth.



**Figure 10. Re-expression of SH3GL2 slows tumor growth and migration.** (A) A immunoblot confirming the re-expression through viral transduction of SH3GL2 in T24 BC tumor cells. (B) Relative growth of T24 cells following transduction with SH3GL2 or an irrelevant gene (*LacZ*). T24 cells re-expressing SH3GL2 showed significantly less proliferation after 48hrs compared to *LacZ* expressing cells. (\*,  $P < 0.05$ ). (C) A migration assay showing the slowing of EGF-stimulated migration in T24 cells re-expressing SH3GL2 compared to those expressing *LacZ*. The graph represents distance migrated with SH3GL2-expressing cells migrating significantly less than *LacZ*-expressing cells ( $P < 0.05$ ). (All data contributed by Majumdar et al., 2013).

## DISCUSSION

This study describes the mechanism of SH3GL2 silencing in BC and how it could be re-expressed in BC cells. Using a panel of BC cell lines, it was demonstrated that: 1) the SH3GL2 promoter was hypermethylated to differing extents in different cell lines; (2) in several cell lines the extent of promoter methylation correlated inversely with mRNA and protein expression; (3) evaluation of data from the CCLE and TCGA initiatives verified the inverse relationship between SH3GL2 promoter methylation and expression; (4) treatment with demethylation agents induced re-expression of SH3GL2 mRNA and protein. Together, these findings provide a mechanism to increase SH3GL2 levels and a way to attenuate tumor cell growth beyond common treatments.

The mechanism of silencing was confirmed by looking at the methylation status of SH3GL2 promoter in several BC tumor cell lines. These findings were further supported when three different demethylating agents were added to methylation silenced BC tumor cell lines. This resulted in an increase in SH3GL2 mRNA levels, indicating that this was the mechanism of silencing. SH3GL2 silencing is found beyond BC. SH3GL2 was also found to be silenced through DNA methylation in breast and vulvar carcinoma (Sinha et al., 2008; Li et al., 2015). Interestingly, this relationship between methylation status and the gene expression does not hold in two BC tumor cell lines, TCCSUP and 5637 tumor cell lines. In addition, hTERT cells also exhibit no methylation of SH3GL2 with no protein expression. Previous studies on hTERT cells have analyzed their 9p status and found it to be homozygously deleted (Kim et al., 2011). In addition to hTERT cells, this homozygous deletion could also be found in TCCSUP and 5637 and explain the lack of SH3GL2

expression with low DNA methylation. Other changes could also include post-transcriptional changes such as mRNA and protein degradation. However, more research is needed to confirm this hypothesis. In human BC samples, while the majority of samples showed methylation changes to SH3GL2 there were several samples which exhibited a genomic change to the SH3GL2 gene, which would explain the lack of methylation with no gene expression in the cell lines. Therefore, while DNA hypermethylation may be the dominant mechanism for SH3GL2 silencing, genomic alteration could be an alternative mechanism in specific BC tumors.

In this work, three different demethylating drugs with three distinct mechanisms of action were used to assess the ability to reactivate silenced SH3GL2. Interestingly, each of the three drugs displayed differences in dose required for gene activation while minimizing cell toxicity. The instability of 5-Aza-dC in aqueous solution has been well documented and was a major limitation in this work as the half-life of 5-Aza-dC at 37°C was 20 hours (Stresemann & Lyko, 2008). Hence, for certain experiments, 5-Aza-dC was not able to reactivate the gene of interest. A possible explanation would be the lack of stability in aqueous solution. This lack of stability has led many researchers to utilize continuous addition of 5-Aza-dC in order to trigger its demethylating effects (Samlowski et al., 2005). However, this method was proven to be toxic in this work and therefore was not utilized to trigger gene reactivation. By virtue of this instability, a different drug, Zebularine was used. Like 5-Aza-dC, Zebularine is a cytidine analog that also binds to and inhibits DNMT's function. The benefit of using Zebularine is its high stability in aqueous solution, with a half-life of 508 hours at 37°C (Champion et al., 2010). Unlike 5-Aza-dC, a much higher

dosage of Zebularine was required to trigger gene reactivation. A dose of 5-Aza-dC at 20  $\mu\text{M}$  was equal to 500 $\mu\text{M}$  reactivation ability. This result is consistent with previous published data which explored the reactivation of silenced p16 in T24 cells where 500 $\mu\text{M}$  of Zebularine compared to a dose of 30 $\mu\text{M}$  of 5-Aza-dC was required to reactivate silenced p16 gene in BC cells (Cheng et al., 2003). In contrast to 5-Aza-dC, the cells were able to tolerate the increased dose of Zebularine, such that this drug was a viable alternative to the instability of 5-Aza-dC. However, unlike experiments exploring p16, Zebularine was only able to slightly increase gene expression in T24 cells compared to 253J cells, which displayed a large increase in expression. The difference in gene activation between these two cell lines could be the result of differing degrees of DNA methylation. At baseline, 253J cells were found to have slight SH3GL2 mRNA expression, while T24 had none. Thus, Zebularine was unable to decrease methylation to a significant degree in order to trigger the increase in mRNA level in T24 cells. 5-Aza-dC, which is a much stronger demethylating agent than Zebularine, as evidenced by the lower dose required to trigger gene activation, was able to overcome this barrier. The final drug explored was RG-108. Unlike Zebularine and 5-Aza-dC, RG-108 is a DNMT inhibitor that binds directly to the active site of the enzyme (Heerboth et al., 2014). It therefore appears that RG-108 is a much less toxic compound to cells compared to the cytidine analogs, 5-Aza-dC and Zebularine. Also, a much higher concentration (500 $\mu\text{M}$ ) was used with little observed cytotoxicity to cells. However, unlike Zebularine and 5-Aza-dC, RG-108 was unable to cause gene reactivation. RG-108 has been used in previous studies to explore its ability to reactivate a tumor suppressor gene silenced through DNA methylation and was unable to cause

significant demethylation and reactivation compared to 5-Aza-dC (Stresemann et al., 2006). Previous studies have used RG-108 at higher doses than those used during this experiment (Sun et al., 2016), and this may be an interesting avenue to explore in future studies. Interestingly, RG-108 at 500 $\mu$ M was able to increase SH3GL2 protein levels in J82 cells (Figure 9). J82 cells contain approximately 70% DNA hypermethylation of the promoter compared to approximately 100% in both T24 and 253J cells. The decrease in baseline DNA methylation of J82 cells compared to T24 and 253J could explain how RG-108 was able to increase protein expression compared to no increase in RNA expression in 253J and T24 BC cells. Overall, each drug utilizes different mechanisms with different stability and toxicity and a possible combination approach between RG-108 and the cytidine analogs could be used to achieve promoter demethylation.

SH3GL2 promotes EGFR internalization after activation and its loss is associated with increased cell proliferation both *in-vitro* and *in-vivo* (Majumdar et al., 2013). Conversely, SH3GL2 re-expression resulted in reduced tumor cell proliferation. Similar to the data presented above, which forced re-expression of SH3GL2 in a BC model, re-expression of SH3GL2 in a lung cancer model resulted in a significant decrease in cell growth, invasion and colony formation (Dasgupta et al., 2013). As a result, the targeting of SH3GL2 by the demethylating agents described above could be used as a cancer therapy to slow cancer proliferation. Previously, demethylating agents have only been approved to treat non-solid tumors such as leukemia and myelodysplastic syndrome (Li et al., 2016). These agents need to be given intravenously and can often lead to many off-target effects. However, because the bladder is easily accessible relative to many other organs in the body,

the use of these agents injected directly into the bladder could possibly help slow BC progression. Previous studies have used Zebularine to reactivate p16 expression in human bladder tumor cells *in-vivo* and have found it to be stable over long periods of time (Cheng et al., 2003). *In-vivo* models of reactivation of SH3GL2 are needed to assess the benefits of demethylating agents on tumor growth and behaviour.

Further research is also needed to assess the effect of demethylation and subsequent reactivation of SH3GL2 on regulation of RTK activation in BC cell lines. Normal SH3GL2 function would allow proper receptor internalization and inactivation after EGF binding. However, the impact of SH3GL2 re-expression on restoration of this function remains unknown. In addition, the loss of SH3GL2 may identify tumors in which the EGFR and/or c-MET remain active due to the lack of internalization, and therefore may be more sensitive to treatment with EGFR or c-MET tyrosine kinase inhibitors (TKI). The loss of SH3GL2 is an early event in BC pathogenesis (Majumdar et al., 2013). As a result, the level of methylation of SH3GL2 and by inference the extent of SH3GL2 gene and protein expression could serve as a novel biomarker to identify patients who need more aggressive treatment early in the disease. Further study is needed to correlate the level of methylation of the SH3GL2 promoter in BC specimens from patients and clinical outcomes, as well as the ability of SH3GL2 expression status to predict sensitivity to TKIs (EGFR, c-met inhibitors).

Beyond the possible impact of SH3GL2 status on response to TKI therapy, its loss has been found to impact patients' response to chemotherapy in ovarian cancer (Osterberg et al., 2009). This study found that the loss of SH3GL2 or CIN85 was associated with

chemotherapy-resistance in ovarian cancer. In chemotherapy-resistant tumors, 70% had losses in either SH3GL2 or CIN85 (Osterberg et al., 2009). This function of SH3GL2 suggests it can be involved in resistance to drugs in tumors. This drug resistance could possibly be extended beyond ovarian cancer and could be used to explain why certain patients do not respond to a given therapeutic intervention. Another possible hypothesis is that the reactivation of SH3GL2 could be used as an initial therapy to allow cancer cells to be more responsive to specific therapies. Further study is needed to assess the impact of SH3GL2 loss in drug resistance.

### **Study limitations**

There were several limitations in this study. While the demethylating agents are effective at demethylating promoter regions, the specificity of these agents was lacking. The addition of these drugs will likely cause systemic demethylation in many promoter regions and could result in reactivation of many silenced genes. A variety of other mechanisms have been found that could be used to directly activate genes silenced by methylation. Small non-coding double stranded RNA (dsRNA) have recently been found that can localize to specific promoter regions and produce an increase in gene expression (Li et al., 2006). These dsRNA are sequence-specific and will bind complementary to specific promoter regions and thus could be used as an alternative to upregulate SH3GL2 rather than the global demethylation effect caused by the demethylating drugs (Li et al., 2006).

Another limitation of this study was the variability in results achieved when adding these demethylating agents. Certain experiments did not lead to detectable gene

reactivation possibly due to the effect of stability of these drugs in solution or insufficient time needed in order to reactivate SH3GL2. More research is needed in order to identify a specific protocol that allows sufficient time for the agents to work while also maintaining stability in solution. In addition, all of the drugs were dissolved in DMSO. DMSO as a vehicle has been shown to affect the epigenetic profile and cause hypermethylation in several gene loci (Iwatani et al., 2006). As a result, the vehicle could be acting to counteract the activity of the demethylating agent which might explain the variable results as well as the changes in baseline SH3GL2 level.

In conclusion, the data presented here outlines three different demethylating agents that could be used to reactivate SH3GL2 in BC tumor cells that could be used to change tumor cell behavior. Interestingly, the SH3GL2 promoter was not methylated in all BC cell lines, suggesting alternative mechanisms beyond epigenetic modifications might lead to the lack of SH3GL2 expression. Overall, these findings suggest a mechanism for SH3GL2 reactivation in an *in-vitro* model. However, further exploration is needed to assess if the results could translate to an *in-vivo* model. In addition, further research is needed to explore what effect the reactivation of SH3GL2 by demethylation agents has on BC tumor cell behavior, response to TKI treatment and whether this method could be utilized as a new treatment for BC.

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## CURRICULUM VITAE

