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Role and regulation of Sphingomyelin synthase 1 in Leukemia

A Dissertation Presented

by

Sitapriya Moorthi

to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Doctor of Philosophy

in

Genetics

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Abstract of the Dissertation

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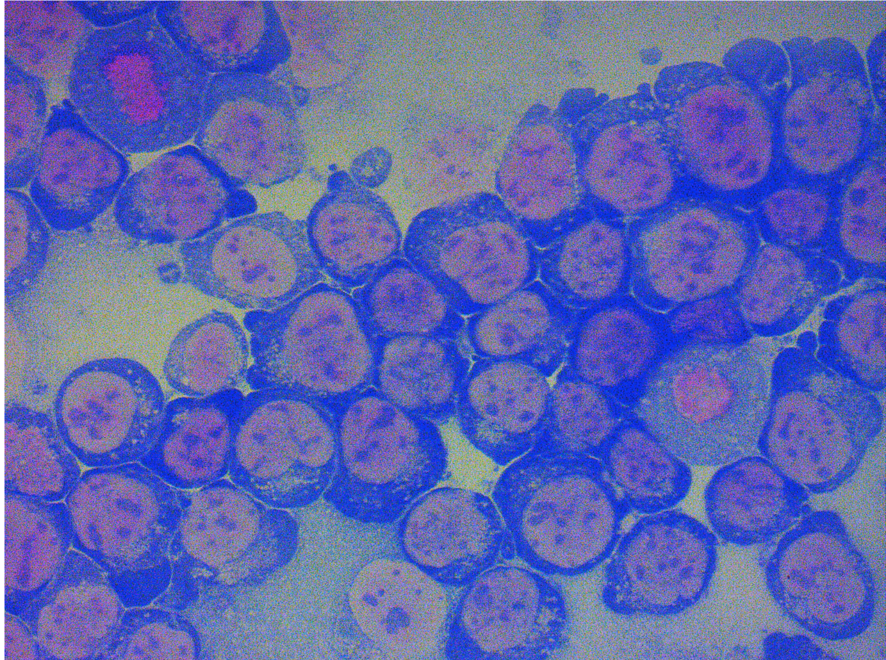
The Sphingomyelin synthase (SMS1 and SMS2) enzymes constitute a class of transferases, belonging to the sphingolipid pathway and they catalyze the synthesis of sphingomyelin (SM). The role of sphingolipids has transformed, from being vital components of cellular membranes to mediators of various biological processes, such as proliferation, regulation of the cell cycle and differentiation. SMS catalyzes the transfer of the phosphorylcholine moiety from phosphatidylcholine to ceramide (Cer), generating SM and diacylglycerol (DAG). The role of SMS in cancer stems from its ability to regulate the levels of two bioactive lipids, Cer (mediator of anti-proliferative effects) and DAG (a mitogenic lipid), which have opposing effects on cell proliferation. Previous work from our laboratory has shown that *SMS1* expression and activity is elevated in Chronic Myelogenous Leukemia (CML) and is mediated by the oncogene Bcr-Abl, a fusion tyrosine kinase. High *SMS1* expression in CML has a pro-proliferative effect in these leukemic cells. My dissertation establishes the precise molecular mechanism via which Bcr-Abl regulates *SMS1* expression. In the first part of my

dissertation, I show that Bcr-Abl up-regulated the mRNA expression of *SMS1* through a transcription-translation mechanism. Herein Bcr-Abl increased *SMS1* transcription from a novel alternative transcriptional start site. This resulted in the generation of an mRNA with a short 5'UTR, which was translated 20-fold more efficiently, resulting in an increased SMS1 protein expression. Thus for the first time these results show that an oncogene (Bcr-Abl) increased protein abundance of its downstream target (SMS1) via mediating a shift in transcription initiation. In the second part of my dissertation, I elucidated the molecular mechanism resulting in the shift in transcription initiation of *SMS1* in CML cells. I identified that the transcription factor GATA-1 bound to the promoter region and regulated transcription from it. This is the first description of an up-stream transcriptional regulator of *SMS1.* Interestingly, I showed that in a subset of AML cells with high *GATA-1* expression, *SMS1* transcription was also regulated in a similar manner and affected progression of cells through the cell cycle. Herein I identified a novel role of SMS in affecting cellular proliferation in AML cells.

To my parents, my “second parents” and Ramanan, for all your love, support and guidance



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LIST OF ABBREVIATIONS

ALK	Anaplastic lymphoma kinase
AML	Acute myeloid leukemia
APL	Acute promyelocytic leukemia
AP	Accelerated Phase
AP-1	Activator protein-1
ATI	Alternative transcription initiation
ATP	Adenosine triphosphate
ATRA	All-trans retinoic acid
BCR-ABL	Break-point Cluster Region- Abelson
Bax	Bcl-2 associate X protein
BCL2	B-Cell Lymphoma 2
BCL-xL	B-Cell Lymphoma extra large
BP	Blast Phase
BRCA1	Breast Cancer gene 1
BTK	Brutons Tyrosine Kinase
CBF-AML	Core-binding factor AML
cDNA	complementary DNA
CDase	Ceramidase
Cer	Ceramide
CerS	Ceramide synthase
C1P	Ceramide-1 Phosphate
CERK	Ceramide kinase
CERT	Cermaide transfer protein
ChIP	Chromatin Immuno-Precipitation
CPE	Ceramide PhosphoEthanolamine
CML	Chronic Myelogenous Leukemia
CP	Chronic Phase
DAG	Diacylglycerol
DES	Dihydroceramide destaurase
DLC	Deleted in liver cancer
DhCer	Dihydroceramide
DMS	Dimethylsphingosine
DMSO	Dimethyl sulphoxide
ER	Endoplasmic Reticulum
ERK	Extracellular signal regulated kinase
FISH	Fluorescense in-situ hybridization
GCS	Glucosylceramide synthase
GlcCer	Glucosyl Ceramide
GalCer	Galactosyl ceramide
GM-CSF	Granulocyte-macrophage colony stimulating factor
GSK3	Glycogen synthase kinase 3 alpha
HSC	Hematopoietic Stem Cells
IFNγ	Interferon gamma
IR	Ionizing radiation
IRES	Internal Ribosome Entry Site

IRF8	Interferon regulatory factor 8
IRIS	International Randomized Study of Interferon and STI571
LC-MS	Liquid chromatography-tandem mass spectrometry
MAPK	Mitogen-activated protein kinases
Mcl-1	Induced myeloid leukemia cell differentiation protein
MMP2	Matrix metalloprotease 2
mRNA	messenger Ribo-Nucleic Acid
miRNA	micro RNA
MRP1	Multi-drug resistance-associated protein 1
Myc	Myelocytomatosis viral oncogene
NFkB	Nuclear Factor Kappa Beta
ORF	Open Reading Frame
PC	Phosphatidylcoline
PFS	Progression Free Survival
P-gp	P-glycoprotein
PI3K	Phosphoinositide 3-kinase
PKC	Protein Kinase C
PKD	Protein Kinase D
PM	Plasma membrane
PMA	Phorbol myristate acetate
PML	Promyelocytic leukemia
PP2A	Protein phosphatase 2
qRT-PCR	quantitative Real Time Polymerase Chain Reaction
RA	Retinoic acid
RACE	Rapid Amplification of cDNA ends
RUNX	Runt-related transcription factor-1
SDM	Site Directed Mutagenesis
S1P	Sphingosine-1-Phosphate
siRNA	small interfering RNA
SL	Sphingolipids
SM	Sphingomyelin
SMase	Sphingomyelinase
SMS	Sphingomyelin Synthase
SPT	Serine palmitoyl transferase
Sph	Sphingosine
SphK	Sphingosine Kinase
STAT5	Signal Transducer and Activator of Transcription 5
SV-40	Simian vacuolating virus 40
TG	Triglycerides
TF	Transcription Factor
TFBS	Transcription Factor Binding Sites
TKI	Tyrosine Kinase Inhibitor
TNFα	Tumor necrosis factor alpha
TP	Prostanoid Thromboxane Receptor
TSS	Transcriptional Start Site
UGT2B7	UDP-glucuronosyltransferase 2B7

UTR	Untranslated region
uORF	upstream Open Reading Frame
Vit D3	Vitamin D3
WHO	World Health Organization

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PUBLICATIONS

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2. Moorthi S., Yu G., Luberto C. GATA1 regulates the expression of Sphingomyelin Synthase 1; Implications in CML and AML. *In preparation.*

3. Moorthi S., Luberto C. (2015) Role of Sphingolipids in Hematological Malignancies: Myeloproliferative Disorders. In: Hannun Y., Luberto C., Mao C., Obeid L. (eds) Bioactive Sphingolipids in Cancer Biology and Therapy. Springer, Cham.

CHAPTER 1

INTRODUCTION AND SCOPE OF DISSERTATION

1.1 The Sphingolipid pathway, a brief introduction.

Sphingolipids (SL) (phospholipids and glycolipids built on a sphingoid base backbone) are a diverse and important class of lipids associated with all eukaryotic cellular membranes. Since their discovery by J.L.W Thudichum in 1884 (1), sphingolipids were thought to primarily have a structural role in cell membranes. But, over the last few decades, several members of the sphingolipid family have been shown to play central roles in several cellular processes such as proliferation, cell death, signal transduction and inflammation (2,3).

The SL pathway **(Figure 1.1)** commences with the condensation of L-serine and palmitoyl-CoA to generate 3-keto-dihydrosphingosine, which is catalyzed by the enzyme serine palmitoyl transferase (SPT) (4,5). Following this the enzyme 3-keto-dihydrosphingosine reductase (KDSR) catalyzes the reduction of 3-keto-dihydrosphingosine to dihydrosphingosine or sphinganine (6). Dihydrosphingosine is then N-acylated by ceramide synthase (7) (also known as Lass) to dihydroceramide (DhCer) (8,9). Finally DhCer is desaturated to from ceramide (Cer) by the action of the enzyme dihydroceramide destaurase (DES) (10).

Once synthesized, Cer is then transported either via the ceramide transfer protein (CERT) or vesicular transport to the luminal side of the Golgi apparatus where it is the substrate for more complex SLs (11). In the Golgi, sphingomyelin synthase (SMS) catalyzes the synthesis of sphingomyelin (SM) using ceramide as a substrate **(discussed further in Chapter 1.2)**. There are two SM synthesizing enzymes, SMS1 found in the Golgi and SMS2 found in the Golgi and plasma membrane (12,13). Also in the Golgi, glucosylceramide synthase transfers glucose from UDP-glucose onto

ceramide to generate glucosyl ceramide (GlcCer) (14) while, with a similar reaction, galactosylceramide synthase produces galactosylceramide in the ER (15) (16). Additionally ceramide can be phosphorylated by ceramide kinase (CERK) to generate ceramide-1-phosphate (C1P) (17).

Cer once produced can be converted to sphingosine (Sph) through fatty acid cleavage by ceramidases (CDases), which are characterized based on the pH at which they catalyze the reaction, acidic, neutral or alkaline (18). Sphingosine kinase (SK) can then phosphorylate Sph to generate sphingosine-1-phosphate (S1P) (19). S1P can then either be broken down into hexadecenal and ethanolamine phosphate by a lyase (the only exit from the SL pathway) (20) or converted back to Cer by the reverse reaction of Cers.

Once generated SLs are transported to the cells membrane and other organelles for further metabolism, to elicit their functions (21,22). Most of the SL breakdown however occurs in the lysosome in response to specific stimuli by sphingomyelinases (SMases, characterized by acidic, neutral or alkaline pHs), which hydrolyse SM into Cer at cellular membranes (23). GluCer and GalCer are also catabolized by their own glycosidases and C1P is dephosphorylated by ceramide phosphatase.

Thus Cer occupies a central position in the pathway for two main reasons: a) ceramide formation occurs through several different routes along the sphingolipid pathway, namely the *de novo* pathway, the salvage pathway and the SM cycle via SMase; and b) ceramide is the substrate in the synthesis of other complex sphingolipids.

Thus the sphingolipid pathway is extremely complex; i) it is constituted of several interconnected biochemical reactions; ii) the pathway is distributed into several sub-cellular compartments adding further complexity associated with substrate availability and re-localization; iii) substrates and enzymes of the SL pathway have diverse functions; and iv) the SL pathway cross talks with several other metabolic and signaling pathways.

1.2 Sphingomyelin synthase

SMS catalyzes the synthesis of SM from Cer and phosphatidylcholine (PC) to generate SM and DAG (24-34) **(Figure 1.2)**. Additionally SMS also catalyzes the reverse reaction (35,36). It was recently discovered that all SMS family members in mouse have the ability to catalyze the reaction for the synthesis of ceramide phosphoethanolamine synthesis as well (37).

There are two human SM synthesizing enzymes, SMS1 and SMS2 , encoded by two different genes, *SMS1 (SGMS1*, Chr.10 q11.2) and *SMS2* (*SGMS2*, Chr.4 q25) (12,13,38,39) respectively. SM synthase activity was predominantly found in the Golgi (luminal orientation) and the remaining activity at the plasma membrane (40-43). It is now known that SMS1 is located in the Golgi while SMS2 is localized to both the Golgi as well as the PM due to S-palmitoylation of its COOH terminus (29,41,44-47). SMS activity was also shown in the nucleus however the nuclear SMS identity and role are yet to be elucidated (48).

Both SMS1 and SMS2 proteins have six membrane spanning domains with their N and C termini facing the cytosolic side (41). Moreover both proteins form homo-dimers in the ER, which is important for their transport to the Golgi. For SMS2, the C-terminal tails interact with each other to cause dimerization however for SMS1 the C-terminus interacts with another region of its partner protein (49).

The *SGMS1* gene, which spans 320 kbp, is constituted of 11 exons and 10 introns, which encodes for the *SMS1* mRNA that is 3734 nucleotides in length **(Figure 1.3).** The *SMS1* transcript is characterized by its exceptionally long 5’ and 3’-UTR , which are 954 bp and1538 bp in length respectively. The annotated translational start

site for *SMS1* is a “weak” ATG sequence within exon 7. It is pertinent to note that the 5’ UTR of *SMS1* has three upstream open reading frames (uORF), which may play a role in regulating the translation of the mRNA. Several reports have described that the *SMS1* mRNA undergoes massive regulation at the post-transcriptional level and 16 distinct mRNA variants have been described, whose analysis potentially suggests the existence of alternative promoters and transcriptional start sites (50,51). Recently it was found that the human *SMS1* mRNA is present as circular RNA predominantly in the brain (52). Its current biological relevance is being explored but it is hypothesized that the circular RNA acts as molecular sponges for miRNA and thereby affect gene expression.

The biological functions of SMS can be attributed to its ability to regulate the levels of its products (DAG and SM) and substrates (Cer and PC). As mentioned earlier, while Cer, the central sphingolipid molecule has an anti-proliferative effect on the cells (8,53,54), DAG primarily has mitogenic effect on cells through recruitment and/or activation of PKCs, PKDs and Ras (indirectly) (45,55-62). Additionally SMS regulates the relative ratio of SM to PC, which is intrinsically connected to the DAG/Cer ratio and is vital to maintaining the cell membranes lipid fluidity and osmotic fragility (63-70). Deevska *et al.,* showed that increased expression of SMS1 in HepG2 (liver cell line) signals a “low PC-state” (PC is required for membrane biogenesis and cell survival) and thereby affects the partitioning of DAG away from the TG and glycero-phospholipid pathways (71), thus showing that SMS can also affect other biochemical pathways.

Increased SMS activity has been observed in conditions of enhanced cell proliferation (72,73) and cell transformation, such as hepatocellular carcinoma versus

normal liver and SV-40 transformed fibroblasts versus their normal counterpart (57,74,75). Moreover inhibition of SMS in U937 cells by D609 (an inhibitor of SMS activity) resulted in cell death mediated by increase in Cer and decrease in DAG (76).

SMS has also been shown to play a role in opposing apoptosis through its ability to shunt ceramide to complex SLs and therefore preventing cell death (77). In Jurkat cells it was seen that upon photodynamic therapy (PDT) by Pc 4, there was an accumulation of Cer and induction of apoptosis due, in part, to the inhibition of GCS and SMS (78). Indeed, over expression of SMS1 could overcome this accumulation of ceramides and prevent apoptosis (77,79). In contrast, another study showed that, in the brain cancer cell line U118, activation of SMS by treatment with 2-hydroxy oleic acid (2OHOA) promoted cell death possibly through autophagy (80,81). However in this case cell death was possibly due to an accumulation of Cer caused by the SL pathway in an "over-drive" mode, to sustain the increased demand for SMS substrate.

SM biosynthesis in itself is important for maintaining membrane integrity and formation of rafts, which in turn is critical for cellular signaling and cell survival. Interestingly this role of SMS derived SM is important in mediating cell migration via the CXCL12-CXCR4 pathways. Binding of CXCL12 to CXCR4 promotes its homo-dimerization and inclusion in lipid rafts (82-84), a step necessary for its function in promoting migration (85-88). SMS-derived SM (and not ceramide) inhibits CXCL12-CXCR4 mediated migration of mouse fibroblast cells by preventing its inclusion in lipid rafts (89). On the other hand, down-regulation of *SMS1* in Neuro 2a cells inhibits migration and is correlated with a decrease in the expression of MMP2 (matrix

metalloprotease 2), a marker of migration (90). These studies thus show that the role of SMS is cell-type dependent.

SMS plays a role in regulating progression through the cell cycle, it does this is by regulating Cer levels in the cell (91). It was shown that SMS inhibition with D609 augments ceramide-induced cell cycle arrest after stroke (92). Cer can affect progression through the cell cycle by inhibiting c-Myc through the activation of the protein phosphatase 2A (73,93). Activation of PP2A lifts c-Myc-induced inhibition of p21 and p27 leading to inhibition of CDK and arrest of the cell cycle (94,95). Indeed in Neuro-2a cells, it was shown that inhibition of SMS1 reduced proliferation and progression through the cell cycle with cells accumulating in the G0/G1 (90). The cell cycle arrest was accompanied by increase in the CDK inhibitor p27 and decrease in the expression of Cyclin D1.

One of the least studied aspects of SMS is its regulation. There is little evidence in the literature that identifies mediators able to regulate the expression and activity of SMS. During induction of macrophages into foam cells upon treatment with the PPAR delta agonist GW501516, an increase of SMS2 expression and activity was observed (96). Although this suggests regulation of SMS2 by GW501516, the molecular mechanism remains unknown. In Jurkat cells upon Fas-L treatment, SMS activity is inhibited as SMS1 undergoes caspase mediated degradation resulting in ceramide increase and cell death (97). And inhibition of SMS activity by D609 reverses this cell death phenotype (98). Another interesting aspect of SMS regulation and its dependence from other components of the SPL pathway was shown with Gentamycin. In lymphoma cell lines, Gentamycin activated cellular SMase while inhibiting nuclear SMase and this

led to the activation of SMS (99). One of the clearest mechanisms of regulation of SMS is by the oncogene Bcr-Abl. Burns *et al.*, showed that Bcr-Abl is capable of inducing the expression and activity of SMS in CML cells (100). Recently it was also found that ABCA8, an ATP binding cassette subfamily-A, was capable of inducing the expression and activity of SMS1 (101).

Thus SMS, with its impact on diverse cellular functions, both in normal and in disease conditions, is an interesting enzyme for investigation. However one of the key aspects of its biology is its regulation that is vastly understudied and is the primary focus of this dissertation.

1.3 Myeloid Leukemias

Leukemia refers to the malignances of the white blood cells originating in the bone marrow. Myeloid neoplasms broadly encompass clonal disorder of hemopoiesis, characterized by over-production of dysfunctional cells within the myeloid lineage such as granulocytic, erythroid and megakaryocytic cells. Myeloid leukemia can be broadly classified into Chronic and Acute type based on the duration of the disease.

1.3.1 Chronic myelogenous leukemia (CML)

Chronic Myelogenous (or Myelocytic/Myeloid/Granulocytic) Leukemia (CML) is a myeloproliferative disorder of the hematopoietic stem cells (HSC) and accounts for 11% of all leukemia's affecting adults and was first described in 1951 (7,102). It is characterized by a consistent chromosomal abnormality, which involves the reciprocal translocation between chromosome 9 and 22 [t(9;22)(q34;q11)] resulting in a shortened chromosome 22, known as the Philadelphia Chromosome (103,104). This translocation results in the fusion gene, *Bcr-Abl* that is translated into an abnormal protein kinase, which is constitutively active and is responsible for CML pathology (105,106).

CML affects 1.8 individuals per 100,000, with a median age of diagnosis at 64 (107). CML progresses through three clinical stages, each characterized by specific hematological and molecular features.

The diagnosis of CML is confirmed by karyotypic demonstration of the Philadelphia chromosome by visualization of the Bcr-Abl fusion genes by

fluorescence in-situ hybridization (FISH). Most commonly today RT-PCR of Bcr-Abl transcripts is utilized to monitor disease prevalence and efficacy of therapy. Response to treatment is categorized into haematological, cytogenetic and molecular, based on increasing quality of response (108-111).

The three clinical stages of CML are: (i) the Chronic Phase (CP) characterized by the expansion of the granulocytic cell lineage with a median duration of 3-4 years, if left untreated; (ii) the Accelerated Phase (AP) which involves the acquisition of additional cytogenetic aberrations and is characterized by the presence of 10-20% blast cells (abnormal and immature cells) in blood and bone marrow; and the Blast Phase (BP) characterized by a block in differentiation of HSC and increased proliferation of immature myeloid or lymphoid (blast) cells in the bone marrow and peripheral blood which account for more than 30% of cells.

Apart from distinguished clinical characteristics, CML presents certain molecular features unique to this Bcr-Abl-driven disorder. The Bcr-Abl onco-protein prolongs cell survival and inhibits cell death rather than solely promoting cellular proliferation (112-115). In fact it has been shown that CML cells, precursors and progenitors display proliferation characteristics (mitotic indices, rate and maturation) similar to their normal counterparts, yet there is an accumulation of cells at disease initiation (CP)(107,116,117). Additionally, the prolonged cell survival of CML cells fosters the acquisition of secondary genetic aberrations that promote the progression from the CP to the BP of the disease (118,119). This mitotic “immortality” is characteristic of cancer cells and it is elicited by alterations in molecular pathways that govern normal cellular turnover. These include (not limited to) expression of growth factor ligands and/or of

their receptors, hyper-responsiveness to limiting proliferative signals and stimulation of stromal cells to supplement available growth factor repertoire, in other words cancer cells become ‘growth factor-independent’. In CML, Bcr-Abl constitutively activates signaling pathways that normally mediate growth factor-dependent hematopoietic signaling and these pathways are key drivers of neoplastic transformation in CML (120). Studies have shown that CML cells from CP are still partially dependent on extracellular supplemented factors whereas cells from BP acquire total independence from such factors through the acquisition of secondary genetic changes (121).

The therapeutic landscape of CML has changed dramatically with the introduction of Imatinib/Glivec/Gleevec/STI571, a tyrosine kinase inhibitor (122-124) in 2001. Imatinib competitively occupies the ATP binding site of Bcr-Abl thus inhibiting its ability to phosphorylate its substrates. Subsequently cells dependent of Bcr-Abl signaling undergo cell death (122,125). The response of patients to Imatinib treatment has been remarkable, a multi-center study following patients for 11 years treated with Imatinib showed that almost 82.8% of the patients showed a complete cytological response with minimal cumulative or late cytotoxic events (126).

While Imatinib has received an overall successful outcome 10-30% of patients treated with Imatinib develop resistance. Resistance to imatinib treatment is most commonly either due to point mutation in Bcr-Abl in the kinase domain affecting the binding of Imatinib (127) or due to amplification of Bcr-Abl increasing its expression (128,129). To overcome this significant rate of resistance a new generation TKIs with increased efficacy has been developed, these are Dasatinib, Nilotinib, Bosutinib and Ponatinib (130). However treatment with these is also subject to development of

resistance and severe toxicities. The key reason of eventual inefficacies associated with TKIs is the lack of elimination of the disease causing HSC (131,132). Thus a critical task of current research is the identification of the mechanisms by which resistance is developed and ways these can be prevented or overcome.

1.3.2 Acute Myeloid Leukemia (AML)

AML is the most common acute leukemia, affecting adults; nevertheless, it is a rare disease and for instance in the United States it causes an overall 1.2% deaths among all cancer related deaths (133,134). AML arises from the combination of a block of differentiation and additional genetic alterations that promote unchecked proliferation of immature cells. Differently from CML, AML is characterized by great heterogeneity because genetic alterations can occur in myeloid cells at different stages of differentiation (135). These alterations often consist of recurrent chromosomal translocations leading to the generation of fusion proteins with oncogenic functions, and specific genetic alterations are often associated with different prognosis (136).

The best characterized chromosomal translocations are the t(8:21) in core-binding factor AML (CBF-AML) and the t(15:17) in acute promyelocytic leukemia (APL) resulting in the formation of chimeric proteins (RUNX1-RUNX1T1 and PML-RARA, respectively), which affect the normal maturation of myeloid precursor cells. Progression of the disease occurs rapidly and, depending on the AML subtype, the 5 years survival rates may vary from 15 to 70%.

The most current classification of AML has been revised by the WHO and takes into consideration differences in morphology, cyto-chemistry, immune-phenotype, genetic and clinical features to group disease entities. On the other hand, most older manuscripts refer to the French-American-British (137,138) classification of AML which instead consists of 8 subtypes (M0 to M7), organized by the type of cell from which the leukemia developed and its level of maturity.

Chemotherapy is the treatment of choice for AML, and most commonly it consists in the combination of cytarabine and anthracycline. This first intervention (induction phase of therapy) aims to bring the patients in complete remission (no detectable leukemic cells) and it is followed by the consolidation therapy that aims at the eradication of any residual leukemic cell. The consolidation therapy varies depending on the specific prognostic factors of the patient, and can range from high dose of cytarabine to allogeneic hematopoietic stem cell transplantation for patients at high risk of relapse, with a 5 years overall survival rate of 40-45% in younger patients (139). Generally, the relapse rate varies from 33 to 78% depending on the subtype of AML, and if AML relapses or it does not respond to induction phase therapy from the beginning, treatment options are almost not existent. Thus alternative therapies targeting resilient AML or relapses are warranted (140).

1.4 Sphingolipids and Leukemia

Hematological malignancies have been a powerful model to discover and dissect sphingolipid-related functions in the context of cell proliferation, differentiation, autophagy, immunological responses and neoplastic transformation. From a historical point of view, hematological malignancies have been instrumental in the discovery of the first link between sphingolipids and their bioactive role in apoptosis and differentiation. Molt-4 and HL-60 cell lines were in fact the experimental models used in those first seminal reports (141,142). More recent studies have also translated the discoveries carried out *in vitro* to clinically relevant models of patient samples and/or *in vivo* animal models. In the following sections,, I will discuss the role of sphingolipids in hematological malignancies of myeloid origins, specifically in Chronic and Acute myeloid leukemias following the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemias revised in 2008 (143).

1.4.1 Role of Sphingolipids in CML

Sustenance of cell proliferation

The role of the sphingolipid pathway in sustaining proliferation of CML cells has only been recently explored, and a discovered link is *via* the Sphingomyelin synthase enzyme (SMS) (144). Of relevance, Burns *et* al., have shown that Bcr-Abl specifically up-regulated the *SMS1* isoform in K562 cells (a human cell line derived from a patient in BC). Pharmacological inhibition or silencing of *SMS1* caused a clear block in cell growth, with minimal effect on cell death, highlighting SMS1's specific role in sustaining cellular proliferation of Bcr-Abl positive CML cells. Importantly, inhibition of SMS1 in these cells caused an accumulation of ceramide (its substrate) and a drastic reduction

of DAG (its product), suggesting that the effect of SMS1 on cell proliferation may be elicited through the regulation of either or both these bioactive lipids (144). A possible mechanism by which ceramide may exert an anti-proliferative effect is *via* stimulation of the tumor suppressor protein phosphatase PP2A, an established ceramide target with a well documented role in regulating cell cycle and apoptosis in CML cells (145,146). In fact, it has been reported that Bcr-Abl up-regulates SET, a physiological inhibitor of PP2A, resulting in the repression of PP2A, and that reactivation of PP2A has detrimental effects on CML cells (147,148). All together these observations suggest that Bcr-Abl may employ at least two different mechanisms to keep the activity of PP2A under-check: up-regulation of SET and up-regulation of *SMS1* that quenches the "PP2A-activating pool of ceramides". Additionally, given the profound effects that inhibition of SMS1 exerts on DAG levels in K562 cells and the recent work on the role of DAG-responsive classical and novel PKCs and PKDs in cell proliferation (including work in Bcr-Abl positive cells), a DAG-mediated mechanism by which *SMS1* may contribute to sustaining proliferation of CML cells can also potentially take place (55,76,149-159). All together, these results and their potential implications in the regulation of important signaling pathways warrant further investigations.

Resistance to cell death

The sphingolipid pathway is known to regulate cell death in different cell types (160). In the context of CML the major breadth of research focuses on understanding the role of ceramides and its metabolizing enzymes in: 1) mediating apoptosis and 2) mechanisms involved to keep ceramide levels in check and promote survival. Ceramides are known to mediate apoptosis (161-164) but we are just beginning to

understand their role and regulation in the context of CML. Initial studies tested the effects of exogenous synthetic ceramide treatments. However, reports on the sensitivity of CML cells to treatment with such exogenous analogues (C2-, C6- and C8-ceramide) have been inconsistent. For instance, Maguer-Satta *et al.* showed that primary CML progenitor cells and Bcr-Abl transformed cell lines (BAF3-Bcr-Abl+) were more sensitive than their 'normal' or non-transformed counterparts to apoptosis by exogenous treatment with C2-ceramide. In fact such a treatment resulted in a transient increase of Bcr-Abl phosphorylation, which appeared to surprisingly accelerate the rate of apoptosis in these cells (165,166). However in contrast to this study, Amarante-Mendes *et al.,* showed that exogenous C2-ceramide treatment of CML cell lines (K562 and Bcr-Abl transformed HL60 cells) did not result in apoptosis (167). These cell lines failed to induce release of cytochrome C from the mitochondria (possibly due to the high levels of anti-apoptotic Bcl-xl in these cells), and to activate caspase 3, which instead occurred in control cells (HL60) when treated similarly. Moreover, Nica and colleagues showed that, in Bcr-Abl positive K562 and KBM5 cells, C6-ceramide induced apoptosis in a caspase 8-dependent manner. This was shown to occur via activation of the JNK/SAPK cascade, which in turn led to phosphorylation and inhibition of the anti-apoptotic protein, Mcl-1 (a member of the Bcl-2 family of proteins) (168,169). Additionally, the authors describe that ceramide treatment did not affect Bcl-xl levels in K562 cells nor did it activate PP2A, thus eliminating two other possible mechanisms of induction of apoptosis (170). In contrast to these investigations, McGahon and co-workers showed that K562 cells were protected from apoptosis induced by C6-ceramide (171).

Thus studies based on treatment with exogenous analogues do not provide clear insights into the role of ceramides in cell death of CML cells. However studies looking at endogenous ceramides have shed light upon the possible pro-apoptotic roles of specific ceramide species and re-directed our understanding on how endogenous ceramide levels are regulated in CML cells in order to resist cell death. Baran *et al.,* for instance, have shown that, in K562 cells, upon induction of apoptosis by pharmacological inhibition of the kinase activity of Bcr-Abl with Imatinib [tyrosine kinase inhibitor (TKI)] (122), there was an increase in endogenous C18-ceramide generated *via* the activation of the ceramide synthase 1 enzyme (CerS1). This increase in C18-ceramide preceded apoptosis and it was not observed in imatinib-resistant cells (172). Accumulation of ceramide following Imatinib treatment was also observed in the other CML-derived cell line, LAMA84 (173).

While there seems to be a consensus on the accumulation of ceramide in response to inhibition of the kinase activity of Bcr-Abl (144,172,173) the role of S1P in Imatinib-induced cell death is still not clear. In fact, while in LAMA84 cells Imatinib treatment caused a decrease of S1P possibly due to inhibition of SK1,(173) in K562 cells the drug caused an increase of S1P; thus the contribution of Imatinib-induced S1P changes to the overall functional effects of Imatinib needs further investigation.

Changes in sphingolipid-metabolizing enzymes consistent with elevation of ceramide levels were also reported upon treatment of K562 cells with dasatinib and nilotinib, second generation TKIs (174,175). In fact both TKIs caused an up-regulation of the expression of ceramide synthase genes, mainly *CerS1* and an inhibition of *SK1*.

Therefore, all together these results suggest that ceramide generation precedes and signals TKI-mediated programmed cell death in CML cells.

In addition to these observations, Beverly *et al.* showed that, in K562 cells, pharmacological inhibition of anti-apoptotic Bcl-2 by ABT-263 activated pro-apoptotic BAK and BAX, causing an increase in the activity of CerS and resulted in the accumulation of C16-ceramide, which possibly drove forward a pro-apoptotic response (176). This study implicates the activation of pro-apoptotic molecules along with ceramide generation in the induction of apoptosis thus perhaps providing an explanation for why exogenous ceramide alone could not consistently induce apoptosis in CML cells. These observations point to an important role for ceramides as effectors of apoptotic cell death of CML cells, and the studies that employed TKIs suggest that Bcr-Abl activity contributes to keep ceramide levels in check in these cells.

These conclusions are further corroborated by studies investigating the role of other sphingolipid metabolic pathways in CML. For instance, Hu *et* al. have shown that Acid Ceramidase (A-CDase), the enzyme that cleaves ceramide and produces sphingosine, is overexpressed in CML cells through a mechanism involving regulation of its transcription (177). In particular, while A-CDase is normally transcriptionally repressed by IFN regulatory factor 8 (IRF8), activation of STAT5 by Bcr-Abl leads to IRF-8 down-regulation and consequent de-repression of the A-CDase transcription (178). In line with the functional significance of these observations, IRF8 is frequently lost in myeloid leukemias (179). Accordingly, restoration of IRF8 expression in CML cells caused repression of A-CDase expression, accumulation of C16-ceramide and restored susceptibility of myelogenous leukemia cells to Fas L-induced apoptosis (177).

Thus all together these results suggest that CML cells have in place a fine tuned mechanism to keep ceramide levels in check *via* elevation of A-CDase expression.

Another enzyme whose regulation is also affected by Bcr-Abl is sphingosine kinase (SK), which catalyzes the phosphorylation of sphingosine to sphingosine-1-phosphate (S1P) and it is known to promote cell proliferation and inhibit apoptosis (180). Li *et* al. have shown that Bcr-Abl is capable of up-regulating SK1 expression and its cellular activity (181). Moreover, it was also shown that SK1 and S1P up-regulated the anti-apoptotic protein and pro-survival factor of early hematopoietic progenitors, Mcl-1 (182,183) implicating an anti-apoptotic function of SK1/S1P in CML. This conclusion is also supported by the fact that pharmacological inhibition of SK with dimethylsphingosine (DMS) in CML cell lines and blast cells derived from patients was shown to be cytotoxic (184).

An additional mechanism employed by CML cells to regulate ceramide levels may be represented by inactivation of the first step of sphingolipid *de novo* synthesis regulated by the serine-palmitoyl transferase (SPT). In Taouji *et al.*, data are provided to show that Bcr-Abl inactivates SPT through phosphorylation of the SPTLC-1 subunit at Tyr164 in K562 and LAMA84 cells (185). Additionally the authors showed that the inhibition of Bcr-Abl by Imatinib reversed the phosphorylation on SPTLC-1, activated the enzyme, and resulted in an increase in ceramide levels preceding apoptosis. All these events were also accompanied by the unexpected re-localization of SPTLC-1 from the ER to the Golgi.

All together these studies illustrate how expression of Bcr-Abl causes the re-programming of the sphingolipid pathway with the net effect of keeping ceramide levels in check.

TKI resistance and therapeutic strategies

The completion of phase III clinical trial of Imatinib, has established this drug as the first line of therapy for CP, with the best cytological response of 82% (as determined by IRIS - International Randomized Study of Interferon and STI571) (186-188). While the patient outcome for CP patients on Imatinib is very successful, its inability to kill Bcr-Abl positive stem cells necessitates lifelong treatment (189,190). A problem associated with this long-term usage of Imatinib has been the insurgence of resistance to the drug, which, if not bypassed with alternative therapies, can lead to the clinical progression from CP to the resilient BP (187,191-195). The development of second and third generation TKIs has tackled in part this problem, however resistance to TKIs remains an obstacle in CML therapy (196). Thus the understanding of the molecular mechanisms that lead to TKI resistance has become one of the most studied aspects of CML research (197,198).

The sphingolipid pathway is significantly altered in CML cells and evidence has suggested that regulation of ceramide is central to its pathogenesis **(Figure 1.4)**. Thus targeting ceramide metabolism poses as an attractive clinical strategy and several approaches for inducing ceramide accumulation in CML cells have been explored as potential novel therapeutic means, mainly to overcome TKI resistance. For instance, it has been reported that, in Imatinib-resistant K562 CML cells, there is an increased expression of glucosylceramide synthase (GCS) and the over-expression of GCS in

Imatinib-sensitive K562 cells conferred resistance to Imatinib (199). While the levels of ceramide don't dramatically differ between the resistant and sensitive sub-lines, inhibition of GCS with PDMP (a GCS inhibitor) in the resistant cells dramatically elevated ceramide accumulation and enhanced apoptosis. All together these results suggest that GCS may have a role to play in mediating imatinib resistance by preventing the accumulation of C18-ceramide and that GCS could represent a viable target to re-sensitize TKI resistant cells.

Although the precise mechanism by which ceramide exerts its cytotoxic effects in CML cells is not entirely clear, one possible mechanism may be through reactivation of the glycogen synthase kinase 3 (GSK3) (200). GSK3, is a well-described tumor suppressor with a wide array of targets (201-203) and in CML cells GSK-3B is inactivated by Bcr-Abl (204). On the other hand, GSK3 is potently activated by ceramide (205). Interestingly, Huang *et al.* have shown that Imatinib resistant patient samples harboring the Bcr-Abl mutation T315I or cells transformed with mutant Bcr-Abl could be re-sensitized to TKIs by forced accumulation of ceramide following inhibition of GCS, and they demonstrated that this combined cytotoxic effect occurred through reactivation of GSK3.(200)

In the context of re-sensitization to TKI, the natural phenol resveratrol has also been shown to cause an increase in the intracellular ceramide level in K562 cells and to promote apoptosis, and along with inhibitors of GCS has been proposed as a potential combinatorial option for CML (206). Thus all together these observations support the idea that increasing intracellular levels of ceramide represents an intervention that could sensitize TKI resistant CML cells.

SK1 up-regulation has also been implicated in TKI resistance in CML. In imatinib resistant-K562 cells, Baran *et al.* have observed that there is an increased expression of SK1 and a concomitant shift of the ceramide to S1P ratio, in favor of S1P (172). Additionally, upon SK1 silencing in resistant K562 cells, sensitivity to Imatinib was significantly increased. This was also the case in Imatinib-resistant LAMA84 cells (173). These data point to a role of SK1 in contributing to TKI resistance in CML cells. Moreover, overexpression of SK1 and production of S1P in parent K562 or LAMA84 cells was sufficient to induce resistance suggesting an active role of SK1 in this process, independently from the regulation of ceramide levels (172,173). In line with these observations, Salas *et al*. have shown that engagement of the S1P2 receptor *via* S1P promoted the stabilization of Bcr-Abl through inhibition of PP2A. In fact, PP2A causes the dephosphorylation and subsequent proteosomal degradation of Bcr-Abl, thus SK1 signaling, by blocking PP2A, promotes TKI-resistance (199). All together these observations strongly support a link between resistance to TKI and expression of SK1.

Recently, resistance to nilotinib, a second generation TKI, has been associated with increased GCS and SK1, and decreased CerS1. Down-regulation of GCS and SK-1 restored nilotinib sensitivity (207) reinforcing the concept that targeting the sphingolipid pathway might represent a viable strategy to overcome TKI resistance.

In terms of viable drugs, FTY720 can reactivate the tumor suppressor PP2A (147,208-210) and, once converted into its phosphorylated form, FTY720-P, it can also down-regulate S1P receptors (except S1PR2), inhibiting S1P-receptor mediated signaling. Unexpectedly, FTY720 has also been reported to induce ceramide

accumulation in cells (211). Thus these multifactorial functions of FTY720, all converging towards a pro-apoptotic phenotype, make it an attractive candidate for an alternative CML therapeutic strategy.

In a recent study it was shown that CD34 cells derived from patients in blast phase of CML had low ceramide compared to CD34 from normal individuals. The authors attribute the low ceramide levels to reduced formation of ceramide in response to lower SMS1 and SMS2 expression. On the other hand, the authors have not confirmed decreased SMS activity in these samples, making it difficult to truly assess the significance of the link between expression of SMSs and ceramide levels (212).

1.4.2 Role of Sphingolipids in AML

The sphingolipid metabolic pathway has been implicated in the regulation of differentiation, cell cycle progression, apoptosis, and autophagy of AML cells and SPLs have been linked to the response and/or resistance to treatment of AML. Hence regulation of SL metabolism may represent a way to trigger therapeutic response of AML.

Regulation of differentiation

Most of the studies addressing the roles of SPLs in differentiation of AML cells were performed in cell culture models mainly employing HL-60 or U937 cells. HL-60 cells are a valuable model to study the molecular mechanisms leading to maturation since they retain the ability to differentiate either towards the monocytic or the granulocytic lineages. Retinoic acid (RA) induces differentiation of HL-60 cells towards the granulocytic lineage and during the differentiation process accumulation of ceramide has been reported. Indeed treatment with all trans RA (ATRA) in HL-60 cells induced accumulation of ceramide in the nucleus whereas treatment of promyelocytic NB4 cells induced elevation of ceramide levels potentially via increased expression of acid sphingomyelinase (214,215). Since, at the time of these reports, very limited molecular tools were available to dissect and study the SPL metabolism, the causative link between these observations and the differentiation pathway was not established.

Moreover it has been also shown that the addition of sphinganine enhanced the differentiating activity of RA, possibly through inhibition of Protein kinase C (PKC) and it blocked proliferation (139,216). Similarly, sphinganine enhanced the granulocytic differentiation of HL-60 cells also induced by Adriamycin, Daunomycin and DMSO

(217,218). Thus treatments that would increase sphinganine levels in combination with inducers of granulocytic differentiation might have clinical benefits. On the other hand, C2-ceramide was shown to inhibit the granulocytic differentiation induced by ATRA (215), highlighting the potential counteracting effects of the inter-conversion of sphinganine into ceramide and raising the question whether the accumulation of ceramide in response to ATRA is the actual effector in induction of differentiation or it is a step for the formation of other active intermediate(s), like sphinganine.

Contrary to granulocytic differentiation, low concentrations of sphinganine and sphingosine or treatment with exogenously added sphingomyelinase (which also caused an increase of sphingoid bases) were found to inhibit the monocytic differentiation of HL-60 cells induced by the phorbol ester, PMA and thus activation of PKC (219,220). On the other hand, glucosylceramide and GM3 were shown to specifically favor adherence of HL-60 cells undergoing monocytic differentiation in response to PMA as the GCS inhibitor, PDMP hindered attachment of these cells to the plates without affecting other morphological aspects characteristic of monocytic differentiation or growth inhibition (221,222). Interestingly, the effect of sphinganine on monocytic differentiation depends specifically on the inducer. In fact sphinganine was unable to block monocytic differentiation of HL-60 cells induced by 1 alpha-25-dihydroxyvitamin D3 (Vit D3). These results suggest that, in the case of PMA treatment, it is the inhibitory effect of sphinganine on PKC responsible for the effects and that PKC may not play a role in differentiation to Vit D3 (223).

Furthermore, sphingolipids have been implicated in the regulation of the differentiation process itself. Indeed a role for ceramide generated from hydrolysis of

sphingomyelin (SM) was proposed to be critical for monocytic differentiation of HL-60 cells induced by Vit D3, TNFα and IFNγ (142,224). These were among the first reports on the existence of a SM cycle that, in the interval of few hours (2-4 hours), caused the generation of ceramide from SM and SM re-synthesis thereafter. Interestingly, the activation of the SM cycle seemed to be inducer-specific since it did not occur during granulocytic differentiation or upon monocytic differentiation promoted by PMA. The activation of a neutral sphingomyelinase (NSMase) enzyme, responsible for SM hydrolysis, was described and the involvement of its activation in the differentiation process was strengthened by the addition of exogenous bacterial sphingomyelinase, which enhanced the effects of sub-threshold concentrations of Vit D3. Following studies supported a signaling role for ceramide generated through the SM cycle in Vit D3 and IFNγ-induced differentiation, as the kinetics of ceramide generation matched those of SM hydrolysis, treatment with C2-ceramide enhanced the effect of sub-threshold concentrations of Vit D3 and higher concentrations of C2-ceramide were sufficient to mimic Vit D3 effects (225,226). In the case of IFNγ, production of arachidonic acid through PLA2 seemed to be responsible for the activation of the SM cycle whereas the PI3-K/PKC/JNK/ERK pathways were later indicated as responsible for the differentiation-enhancing activity of ceramide on Vit D3 (227). Of interest, is that mutations in the neutral sphingomyelinase 2 gene (*SMPD3)* were found in 5% of acute myeloid leukemias and 6% of acute lymphoid leukemias but not in other tumor types, suggesting, in a small fraction of these leukemias, the existence of a block of ceramide generation (228).

Involvement of sphingosine phosphorylation has been instead implicated in PMA-induced differentiation of human erythroleukemia (HEL) and megakaryoblastic cells (229,230). In fact, in both cases, prolonged treatment with PMA induced an increase of SK activity, which was inhibited by staurosporine and calphostin C, implicating the upstream activation of PKC. The PKC-mediated activation of SK1 was not through direct phosphorylation but rather through regulation of SK1 expression. Interestingly though, other differentiating agents did not exert the same activating effects on SK, implicating the existence of a specific PKC-SK pathway.

All together these observations suggest that the SPL metabolism has different outcomes depending on the specific molecular pathway invoked during differentiation and that increasing sphinganine/ceramide levels might represent a beneficial strategy against AML when used in combination with differentiating agents.

Regulation of apoptosis

Ceramide treatment and apoptosis

Several studies have shown that treatment of AML cell lines with exogenous ceramide causes cell death. Indeed treatment with low micro-molar concentrations of short chain ceramide (C2-and C8-ceramide) (or addition of bacterial sphingomyelinase) was reported to induce DNA fragmentation in both U937 and HL-60 cells (141,231-233). Interestingly, it was also noted that activation of PKC, either via treatment with DAG analogues or phorbol ester, counteracted the cytotoxic effects of ceramide. Ceramide-induced DNA fragmentation was shown to occur through stimulation of p46-JNK1/p54-JNK2 activity, increased expression of c-jun and ultimately the activation of the transcription factor AP-1; indeed AP-1 inhibition blocked ceramide-induced DNA

damage (234-236). Importantly, while activation of AP-1 by ceramide was shown to regulate apoptosis, this pathway was not involved in ceramide-induced differentiation (237). Together with the activation of AP-1, down-regulation of the anti-apoptotic protein Bcl2 was also observed upon treatment with C2-ceramide in both HL-60 and U937 cells (238). Involvement of Bcl2 in ceramide-induced cell death was strengthened by the observation that stable overexpression of this protein in HL-60 cells protected from C2-ceramide-induced caspase 3 activation and cell death (239). It has also been reported that treatment with C2-ceramide in HL-60 cells exerts a G1 cell cycle arrest via the induction of the Cdk inhibitor p27 (Kip1), and that this growth arrest may be necessary for the onset of apoptosis (232,240,241). Furthermore, ceramide-induced expression of p27 (Kip1) was also significantly decreased by overexpression of Bcl2 (240).

Another proposed indirect downstream target of ceramide in HL-60 cells is Bax. In fact ceramide has been shown to increase Bax expression and to induce its translocation to the mitochondria, while antisense Bax inhibited ceramide-induced caspase activation and apoptosis (242). Following studies have proposed a regulatory role for p38 MAPK in the induction of ceramide-dependent translocation of Bax to mitochondria, as both pharmacological inhibition and dominant negative p38 attenuated this process, as well as DNA fragmentation and caspase 3 activation (243).

An additional downstream target for C2-ceramide in HL-60 cells is the oncogene c-Myc (244). In fact low concentrations of ceramide down-regulated c-Myc mRNA and this process seemed to be mediated by activation of an okadaic acid sensitive protein phosphatase, most likely PP2A.

Treatment with short chain ceramide analogs has been linked also to oxidative damage in HL-60 cells, and it has been proposed that this may be due to the inhibitory effect of ceramide-induced caspase-3 activation on catalase (245,246). On the other hand, accumulation of ceramide (and dihydrosphingosine) is also downstream of H_2O_2 treatment suggesting that potentially the cytotoxic effect of H_2O_2 is amplified by the inhibitory effect of ceramide on catalase (247).

Complementary observations have indicated that conditions in which ceramide metabolism is stimulated (i.e. conversion into glucosylceramide promoted by the expression of p-glycoproteins) confer resistance to ceramide in AML cells (248).

(Chemo)therapeutics and ceramide-mediated apoptosis

Ceramide accumulation has been observed in response to different chemotherapeutic agents. In particular, daunorubicin treatment of HL-60 and U937 cells caused an early increase in ceramide (10 minutes post treatment) through activation of a neutral SMase (249). Daunorubicin-induced activation of neutral SMase, generation of ceramide and induction of apoptosis were all blocked by inhibition of serine proteases or by activation of PKC (250,251). Interestingly though, a report suggested that ceramide accumulation per se is not the apoptotic signal but rather its metabolic conversion to produce the ganglioside GD3 (252).

In addition to daunorubicin, ceramide accumulation was observed following adriamycin treatment of HL-60 cells in both microsomal fraction and nucleus, although the role of these pools of ceramide in the cytotoxic effect of the drug was not established (253). Moreover, it has been reported that ceramide increased also in response to 1-beta-D-Arabinofuranosylcytosine (AraC) in HL-60 cells (254) but, in this

case, its contribution to drug-induced apoptosis seems marginal since it was later shown that inhibition of neutral SMase activity and ceramide accumulation did not affect AraC-mediated cell death (255). On the other hand, co-treatment with AraC and sphingoid bases enhanced AraC cytotoxicity possibly because of inhibition of PKC (255).

Neutral SMase, ceramide synthase activation and ceramide accumulation were all reported following treatment of HL-60 cells with irradiated riboflavin. These events were followed by inhibition of MAPK and up-regulation of Bax and they seemed to be specific to leukemic cells since normal cells were not sensitive to irradiated riboflavin, highlighting the potential beneficial therapeutic effect of this treatment (256).

More recently, involvement of acid and neutral SMase activation and consequent ceramide generation in mitochondrial-mediated cell death induced by Stichoposide C was reported, and the antitumor activity of Stichoposide C, accompanied by ceramide accumulation, was also confirmed in an *in vivo* HL-60 xenograph model (257). Other studies pointed to a role for ceramide in mediating in part the cytotoxic effect of arsenic trioxide in acute promyelocitic leukemia (128) cells, NB4 (258). In this model, ceramide accumulation was shown to derive from both *de novo* biosynthesis and reduced conversion to glucosylceramide, and the study suggested that ceramide accumulation could represent an important step in the therapeutic benefit exerted by arsenic trioxide whether in combination with ATRA as standard treatment for APL or for treatment of relapsed APL. Ceramide accumulation through ceramide synthase activity was involved also in fenretinide-mediated apoptosis of HL-60 cells since the ceramide synthase inhibitor, fumonisin B1 blocked fenretinide-mediated ceramide accumulation and its

cytotoxic effects;(259) in this model, ceramide generation seemed to be triggered by production of reactive oxygen species.

Another clinically relevant chemotherapeutic agent found to induce ceramide accumulation is DT(388)-GM-CSF, a fusion toxin which conjugates the human granulocyte-macrophage colony-stimulating factor (GM-CSF) with the catalytic and translocation domains of diphteria toxin. In cell culture using either parental or vincristine resistant HL-60 cells, DT(388)-GM-CSF caused a substantial accumulation of ceramide (potentially generated through SM hydrolysis) and subsequent caspase activation and inhibition of protein synthesis, resulting in cell death (260). A phase I clinical trial with DT(388)-GM-CSF for patients with highly chemotherapy refractory AML produced some clinical remissions but it also showed dose-limiting toxicity for liver injury (261).

A potentially additional beneficial drug treatment for AML, found to induce ceramide production, is the combination of histone deacetylase inhibitors (HDACIs) and the alkyl-lysophospholipid, perifosine (262). This drug combination in fact synergistically induced apoptosis in HL-60 and U937 cells by triggering mitochondrial dysfunction and caspase activation. Several events concurred to this highly cytotoxic phenotype, such as inhibition of anti-apototic proteins ERK and Akt and induction of pro-apoptotic factors such as ceramide accumulation, oxidative damage, translocation of Bax to mitochondria and elevated Bak. Beside the fact that inhibition of ERK and Akt was shown to play an active role in the regulation of ceramide accumulation, the apoptotic chain of events were not ordered.

Finally, FTY720, a sphingosine analogue approved for treatment of multiple sclerosis, has been shown to induce apoptosis of M2 AML cells by increasing intracellular ceramide levels (263). Indeed FTY720 has shown anti-tumorigenic activity against Kasumi-1 cells, xenograft mouse models and blast cells isolated from AML-M2 patients with the t(8;21) translocation. In Kasumi-1 cells, FTY720 induced ceramide accumulation possibly through activation of both neutral SMase2 and ceramide synthase, and it was proposed that the accumulated ceramide exerts its cytotoxic effects through activation of the tumor suppressor protein phosphatase 2A by binding and displacing its inhibitor, IPP2A.

Early on, lack of ceramide accumulation has been also proposed to play a role in conferring resistance of human erythromyeloblastic cells to apoptosis induced by ionizing radiations (IR) (264). In fact, two sub-clones of the human TF-1 cells (one CD34 positive and one CD34 negative) were characterized by different sensitivity to IR which was not due to different DNA repair mechanisms; on the other hand, sensitivity to IR of the CD34 negative cells corresponded to activation of a neutral SMase and consequent increase of ceramide which did not occur in CD34 positive cells.

Sphingoid bases and apoptosis

Sphingoid bases have also been shown to be an integral part of the apoptosis pathway in AML cells. Treatment with exogenously added sphingosine or sphinganine induced apoptosis in HL-60 and U937 cells, causing DNA fragmentation, nuclear condensation, production of apoptotic bodies and reduced clonogenic capability, and similarly to ceramide, exogenously added sphingosine also down-regulated c-Myc mRNA (265-268). Also, addition of sub-lethal concentrations of sphingoid bases (below

750nM) synergized with the ability of short chain ceramides or bacterial sphingomyelinase to induce apoptosis. This synergistic effect was also mimicked by selective pharmacological inhibitors of PKC, suggesting that the cytotoxic actions of sphingoid bases might be due to their inhibitory effect on PKC (266). Interestingly, a study of the effect of different caspase inhibitors on cell death induced by sphingoid bases versus ceramide revealed that sphingoid bases and ceramide operate independently on the apoptotic pathway with sphingoid bases acting upstream of caspase 8 and ceramides activating late caspases (269).

Endogenous sphingosine accumulation was observed upon monocytic differentiation of HL-60 cells with PMA and the elevation of sphingosine paralleled the amount of apoptotic cells during differentiation. Interestingly, during this process, the conversion of ceramide into sphingosine was greatly stimulated suggesting the activation of a ceramidase (265).

Finally, the optimization of the use of sphingoid bases as anti-leukemic agents was investigated by studying a liposomal encapsulation of safingol (L-threo-sphinganine) with minimal hemolytic activity in a panel of AML cell lines and patients samples. It was found that this preparation exhibited effective cytotoxicity in cells and it also prolonged the median survival time of SCID mice inoculated with U937 cells (270). Moreover, a synergistic effect of a 1:1 mixture of safingol and C2-ceramide was also reported in AML cell culture and in *in vivo* xenografts of U937 cells (271).

S1P and apoptosis

A cytoprotective role for SK and S1P has been observed in AML cell lines in response to different stimuli. For instance, long-term treatment with Vit D3 in HL-60 cells

was shown to induce a late activation of SK, and activation of SK achieved through prolonged Vit D3 treatments provided protection from ceramide-induced apoptosis (272). Exogenous treatment with S1P also protected from ceramide-induced apoptosis and dimethylsphingosine (DMS), a competitive inhibitor of SK, eliminated the protective effect of long-term Vit D3 treatment. Furthermore, addition of S1P bypassed the cytotoxic effects of inhibition of SK by DMS, suggesting that S1P mediates the cyto-protective effects of long-term treatment with Vit D3.

S1P was found to prevent apoptosis of HL-60 and U937 cells induced by Fas ligation, TNF, ceramide and serum deprivation by preventing the translocation of cytochrome c and Smac/DIABLO from mitochondria to the cytosol (273). In line with these observations, inhibition of SK with DMS enhanced the cytotoxic effects of these treatments and accelerated cytochrome c and Smac/DIABLO release from mitochondria, while addition of S1P reverted these effects. More recent studies using an SK1 specific inhibitor (SK1-I), revealed that the activity of this SK isoform is critical for survival of U937 cells and blasts isolated from AML patients, and implicated SK1/S1P in the positive regulation of phosphorylation of ERK1/2 and Akt. Importantly SK1-I also significantly reduced growth of AML xenografts (274). The cyto-protective effect of SK1 in AML cells was confirmed by the use of SKI-178 (275). Importantly, multidrug resistant AML cells were similarly sensitive to the inhibitor highlighting the potential broad-spectrum therapeutic application of this drug.

In line with an anti-apoptotic and pro-proliferative function of SK1 in AML, an oncogenic role for this protein was reported in erythroleukemic progression (276). Indeed, taking advantage of a transgenic multistage erythroleukemic mouse model in

which Sp-1/PU.1 is overexpressed in hematopoietic cells, it was first shown that expression of *SK1* was significantly higher in tumorigenic proerythroblasts (HS2) as compared to that of non tumorigenic proerythroblasts (HS1). Furthermore, overexpression of SK1 in HS1 cells conferred increased proliferation, clonogenicity, resistance to apoptosis and tumorigenicity when engrafted *in vivo*. Complementary to these observations, expression of dominant negative SK1 in HS2 cells reduced cell growth and resistance to apoptosis, suggesting that SK1 might represent a viable drug target for AML.

Sphingolipid metabolism and chemoresistance

As discussed, several chemotherapeutic agents exert their cytotoxic effects by elevating intracellular levels of ceramide. On the other hand, it has been observed that some chemo-resistant AML cell lines (i.e. HL-60/ADR) are unable to accumulate ceramide in response to chemotherapeutic drugs (i.e. doxorubicine) even though the uptake of the drug is comparable to chemo-sensitive cells (277). In these cells, a lower level of endogenous ceramide was reported and this was accompanied by an augmented basal activity of enzymes that convert ceramide into more complex sphingolipids, like glucosylceramide and SM synthases. Importantly, these observations were also confirmed in chemo-resistant leukemic patients samples. Since the levels of Bcl2 were also increased in the chemo-resistant samples, it is possible that the lower level of ceramide might have consequences on Bcl2 expression, thus bolstering the anti-apoptotic capacity of these cells. Interestingly, ceramide resistant HL-60 cells were also found to be resistant to anticancer drugs (daunorubicin, etoposide, ara-C) and differentiation-inducing agents (Vit D3, RA, PMA) (278,279). In these cells, the levels of

Bax and PKCδ were significantly decreased while the levels of anti-apoptotic PKCs, such as PKCα and βI, were increased.

All together these results indicate that ceramide action is able to modulate both pro-apoptotic (PKCδ delta, Bax) and anti-apoptotic effectors (Bcl2, PKCα and βI) and they suggest that the block of the metabolic conversion of ceramide into complex sphingolipids may be a valuable approach to sensitize resistant AML cells.

Finally, sphingolipid metabolism has been found to influence p-glycoprotein function, and, in particular, inhibition of glucosylceramide synthase resulted in increased drug retention and cytotoxicity (280). In immature AML KG1a cells, it was observed that inhibition of glucosylceramide synthase activity with PDMP induced accumulation of ceramide and concomitant reduction of the levels of glucosylceramide and other glycosilated derivatives, including gangliosides GD3 and GM3 (281). On the other hand, only the addition of gangliosides and not of ceramide or other glycosilated sphingolipids stimulated P-gp function, indicating a specific role for gangliosides in the regulation of P-gp.

In addition to the glycosylation pathway, SK activity was also linked to multi drug resistance. In fact, it was observed that while chemo-sensitive HL-60 cells responded to doxorubicine and etoposide treatment by accumulating ceramide and inhibiting SK1 activity, chemorestistant HL-60 cells overexpressing MRP1 and MDR1 retained SK1 activity and failed to increase intracellular ceramide (282). Incubation of chemo-resistant cells with short chain ceramide caused inhibition of SK1 and apoptosis, which was also triggered by pharmacological inhibition of SK1 with F-12509a. Thus all together, these

observations suggest that inhibition of SK1 may be an effective way to overcome multi-drug resistance.

It is clear from the above discussion that investigations of the basic molecular mechanisms contributing to myelocytic transformation are essential to the development of targeted interventions. As for the role of sphingolipids in the homeostasis of the myelocytic arm of hematopoiesis, they intervene in critical aspect of physiological processes, such as differentiation and their deregulation contributes to the development of myelogenous neoplasms. As such, one could envision the targeting of sphingolipid metabolism to prevent progression of these disorders.

It is also increasingly evident that the alteration of the sphingolipid metabolism plays a role in drug resistance, thus combination therapy is something that is being actively investigated in order to maximize the effectiveness of each intervention and decrease the toxic side effects of chemotherapy.

1.5 Alternative transcription initiation in cancer

In their pioneering paper Hanahan and Weinberg describe ten biological competencies or "Hallmarks of Cancer" that drive the malignant transformation of cells (283). One common mechanism that results in the acquisition of these biological competencies is mediated by the alteration in gene expression (284). Gene expression includes all those mechanisms involved in the synthesis of a functional gene product, which is either RNA or protein **(Figure 1.5)**. The first step in gene expression is the transcription of a gene into RNA. Following which, the RNA may be translated into a protein or may function as a non-coding RNA. Traditionally each gene was thought to encode a single RNA form, but with advent of improved technologies capable of sequencing long-stretches of RNA (285-287), we now know that several different RNA isoforms can be generated from the same DNA sequence. It is estimated that roughly each multi-exon gene can encode 6.3 different RNA variants (288) with potentially diverse functional consequences in the cell.

Apart from altered levels of TFs there are four broad mechanisms that regulate the transcription of a gene and could alter the quantity and quality of the RNA expressed: 1) epigenetic modifications of the chromatin (such as histone methylation, or acetylation) which affect accessibility of the transcription machinery to the gene (289-291); 2) genetic changes such as chromosomal translocations, mutations, insertions and deletions that alter the DNA sequence and consequently the encoded RNA sequence (292,293); 3) alternative transcription initiation and termination, which influence both qualitative and quantitative output of RNA (294); 4) post-transcriptional regulatory mechanisms such as splicing, RNA editing, 5' capping, 3' poly-adenylation,

mRNA stability and transport, which determine the final functional RNA formed (295-298).

The last decade has seen a massive influx of information implicating the regulation of these processes in normal tissues and its deregulation in diseases such as cancer (284,294,299-302). In the context of cancer, all these mechanisms have been shown to affect RNA both qualitatively as well as quantitatively and we are only beginning to understand the functional relevance of this altered “RNA state” in cancer.

Transcriptional and translational mechanisms of gene regulation are inter-connected, one influencing the other therefore making the entire process of gene expression extremely complex and diverse. As highlighted earlier, one mechanism by which cells acquire a malignant phenotype is by an altered state of gene expression. In cancer this is due to the de-regulation of one or more of these processes. This chapter focuses on highlighting alterations in the process of transcription initiation that results in “altered RNAs” of protein encoding genes, focusing on those examples, which have a defined consequence on important cellular functions.

Alternative transcription initiation occurs frequently in humans

Alternative transcription initiation (ATI) is the mechanism in which a gene is transcribed from a start site different/alternate to its canonically annotated transcriptional start site. ATI is commonly used to generate a repertoire of diverse mRNA isoforms in the human genome (303). It is estimated that on an average each gene is transcribed from at least four different transcriptional start sites (304). More recently ATI has been studies in the context of cancer with a myriad of functional consequences (305,306). In the context of cancer, Vitting-Seerup and Sandelin

developed a method to identify “isoform switching” across 12 different cancer types (307). Herein they identified genes that express different mRNA isoforms in normal individuals versus cancer patients, which they term as “isoform switching”. In addition they focused on identifying functional consequences of switching that resulted in a modified protein and excluded those mRNA isoforms that resulted in the same protein in both normal and cancer cells. The authors identified that alternative transcription initiation accounts for 40.4% of mRNA isoform diversity observed and alternative transcription termination only accounts for 8.1% of transcriptome diversity **(Figure 1.6).** In this part of the chapter I highlight recent findings that describe ATI events that up-regulate expression of both canonical and non-canonical protein isoforms.

Epigenetic alterations in cancer mediate transcription from alternative TSSs

ATI occurs at a genome wide scale, especially in the context of cancer (304,308,309). The most well studied mechanism that regulates transcription initiation from alternative TSS’s in the context of cancer is through altered epigenetic regulation. The gene deleted in liver cancer-1 (DLC-1), a tumor suppressor that inhibits cell growth, migration and invasion (310), is transcribed from two alternative TSSs. This results in the synthesis of three different mRNA isoforms which are translated into three distinct protein isoforms, DLC-1 α, β, and γ. DLC-1 α is down-regulated in hepatocellular carcinomas and meningiomas and has been shown to occur due to the hyper methylation of its promoter (311,312). However the mechanism that triggers the hyper-methylation of its promoter remains to be elucidated.

In contrast hypo-methylation is implicated in the regulation of p53 delta, a truncated p53 isoform, generated from an alternative promoter P2 within intron 4 (313). A recent study by Blackburn *et al.*, has suggested that intra-genic de-methylation of CpG within intron4/exon 5 causes the transcription initiation from P2 (314). This truncated isoform is expressed in several different cancer types and has an inhibitory effect on wild-type tumor suppressor p53. Another example implicating decreased methylation is described for the Anaplastic lymphoma kinase (ALK) gene. ALK is transcribed from an alternative TSS within intron 19, resulting in a truncated protein isoform that can induce the transformation of IL-3 dependent BaF3 cells (315). The alternative mRNA isoform is present in melanoma and thyroid cancers and absent from normal tissues. Bisulphite sequencing revealed a low methylation of region flanking the ATI site and a positive H3K27ac mark, which associated with actively transcribed regions. Importantly these epigenetic signatures were absent from non-melanoma cells.

Thus together, these examples show that in cancer epigenetic alterations at alternative promoters can result in the transcription initiation from these sites and as a consequence increase the expression of aberrant proteins that support malignant transformation.

ATI results in aberrant proteins

One of the most well described repercussions of alternative transcription initiation in cancer is the production of RNA that encode for alternative protein isoforms that are not expressed in normal tissues. These aberrant proteins have altered functions and promote disease progression. One cause of the aberrant nature of these proteins is that certain domain-coding exons are excluded or included. Moreover it has been described

by a few studies that ATI sites influences down-stream AS and therefore the final mRNA produced (316,317). One gene exemplifying this process is *TBXA2R*, which encodes for the Prostanoid Thromboxane Receptor (TP). *TBXA2R* has three alternative promoters, Prm1/P1, Prm2/P2 and P3. P1 and P2 initiated transcription encodes for the TPα form of the receptor, while P3 is responsible for the transcription of TPβ (318). TPβ expression is highly elevated in prostate cancer and is correlated with increased Gleason grade and pathological stage (319). This high expression of TPβ in cancer is due to hypo-methylation of the P3 promoter in prostate cancer compared to benign tissue. The mRNA isoforms generated from all three TSSs are mostly similar, however the TPβ protein has a different cytoplasmic tail, due to an alternative 3' end (mediated by splicing), which is suggested to be largely responsible for the down-stream signaling differences. Coyle *et al.*, suggest that the alternative initiation of transcription from P3 dictates the alternative down-stream splicing pattern thus resulting in a different C-terminal of TPβ.

Menard *et al,* describe another example of such a down-stream consequence of ATI for the gene UDP-glucuronosyltransferase 2B7 (UGT2B7), which catalyzes the detoxification of small molecules from both endogenous and exogenous sources. UGT2B7 is transcribed from two promoters, the canonical "promoter 1" and a promoter 44kb up-stream known as "promoter 1b" (320). In kidney tumors, transcription is primarily up regulated from Promoter 1b. The resulting mRNA undergoes alternative splicing to either include or exclude the down-stream exon1. Exon1 encodes for the part of the protein responsible for its enzymatic activity and its absence results in a non-functional protein. The authors show that exon 1-excluded isoform predominates in

tumor sample and is translated (shown by its association with ribosomal fraction) and accounts for the low glucuronidation activity in these samples.

ATI influences translation

One of the most prominent consequences of ATI is the generation of alternative 5' mRNA regions. This region of the transcript contain features such as upstream open reading frame (uORF) that regulate translation. In general, cancer-stem cells or cells that initiate tumorigenesis have a low rate of translation. However cancer cells have developed mechanisms to overcome this and up-regulate the expression of tumor promoting genes. In a recent paper it was shown that SOX2 increases the rate of translation of a subset of mRNAs from a uORFs using an unconventional translational machinery involving eIF2A (321). It is known that eIF2A competes with the canonical mediator of translation eIF2-alpha. However, in tumor initiating cells eIF2-alpha is suppressed by phosphorylation and therefore eIF2A activity drive the translation of a specific subset of mRNAs. However the authors fail to describe the consequence of translation initiated from these uORFs as they could not detect peptides for the predicted proteins.

mRNA isoforms generated by ATI can also alter the efficiency of translation mediated by alternative 5' sequences, that different in length and structure. The best-studied example of this is BRCA1, whose expression is frequently down regulated in breast cancer. BRCA1 has two TSS encoding for mRNA with either long or short 5' UTR. In normal breast tissue, the mRNA with the shorter 5' UTR is generated and it is efficiently translated. On the other hand, in breast cancer, an mRNA with a longer 5'

UTR is generated from an ATI site and this reduces translational efficiency owing to inhibitory stable mRNA secondary structures forming at the long 5'UTR (322).

Differential translation efficiency due to ATI is implicated in the expression of Dicer, a ribonuclease mediating RNA interference (323,324). In the context of cancer, it has been shown that the expression of Dicer is correlated with increased invasiveness of breast, endometrial cancer (325-327). In a study by Irvin-Wilson and Chaudhuri it was shown that, in different breast cancer cell lines, the transcription of Dicer was initiated at about 16 kb up stream of its canonical exon 1 and resulted in an alternative first exon, 80 bp in length. The resultant mRNA had ta longer 5' UTR and presented a number of upstream AUG sequences thought to slow down the efficiency of protein translation. Through S1 nuclease protection assay, the authors show that this mRNA isoform is present in breast cancer cell lines but absent from the immortalized cell line MCF-10A, which is often considered a control cell line in breast cancer studies.

Another interesting example is the p65 isoform of Brutons Tyrosine Kinase (p65BTK) that is transcribed from an alternative TSS 15kb up stream of its canonical TSS (328). p65BTK is a novel oncoprotein over expressed in colon cancer cells, affecting cell growth and survival and activated by the Ras/Erk pathway. Three dimensional modeling of the p65BTK mRNA suggested that the canonical translation start site is hidden in a hairpin loop thus promoting translation from a downstream ATG resulting in the synthesis of the oncogenic protein isoform.

Thus in conclusion, alternative transcription initiation is a common occurrence in the context of cancer. The literature is predominated by studies describing functional consequences of ATI however very few studies explore its mechanism. Moreover the

mechanisms that are described are predominantly incomplete, failing to attribute a clear up-stream molecular regulator of ATI. An understanding of such a molecular regulator would be key in developing novel therapeutic strategies to combat the malignant phenotypes elicited by their resultant proteins.

1.6 Scope of dissertation

Previous work from the lab has shown that Bcr-Abl, the oncogenic trigger of Chronic Myelogenous Leukemia (CML), up regulates the expression and activity of Sphingomyelin synthase 1 (SMS1) which contributes to the proliferative capacity of CML cells. However, the mechanism by which this increased expression of SMS1 is mediated remains unknown. This dissertation identifies the mechanism of transcriptional regulation of *Sphingomyelin synthase 1* in the context of Leukemia. Chapter 1 provides a review of the literature relevant to this dissertation and the subsequent chapters are structured as follows:

Chapter 2: In this chapter, I show that Bcr-Abl initiates the transcription of *SMS1* from four alternative TSSs. I developed a method to measure abundance of mRNA generated from each TSS, named First Exon Profiling (FEP). Through FEP, I identified that Bcr-Abl enhances transcription of *SMS1* predominantly from its novel transcription start site- TSS 7, just upstream of its open reading frame. The Bcr-Abl mediated utilization of TSS 7 in CML cells results in the accumulation of an mRNA with a shorter 5' UTR which imparts a 20-fold greater translational efficiency compared to the longer SMS1 5'UTR found in Bcr-Abl negative cells. Therefore, Bcr-abl increases SMS1 abundance exponentially through a concerted mechanism that involves up-regulation of both transcription and translation. This is the first time an oncogene is shown to be drive such a mechanism of gene regulation.

Chapter 3: This chapter characterizes the precise molecular mechanism that regulates the transcription of *SMS1* from its alternative transcriptional start site, TSS 7. Firstly, I characterized the promoter of TSS 7 and showed that it has two regulatory

regions, namely Region I and Region II, which are important for maintaining its activity. Region II was found to be the core region, paramount in maintaining the full function of the TSS 7 promoter. Investigation of transcription factor (TF) binding sites at this region identified GATA-1 as a strong candidate TF. I show that GATA-1 in fact binds to Region II and regulates the transcriptional activity via TSS 7. Furthermore Bcr-Abl causes the up-regulation of GATA-1, which in turn causes the increase in *SGMS1* expression.

Interestingly, while comparing *GATA-1* and *SGMS1* mRNA expression across all leukemia cell lines I found a strong correlation in their expression in a subset of AML cell lines. Herein I showed that *SGMS1* transcription is increased via TSS 7, similar to the mechanism identified in CML cells. Finally, I show that in AML cells, SMS1 expression and activity is important for progression through the cell cycle and thus plays a role in sustaining proliferation of these cells.

1.7 Figures and Legends

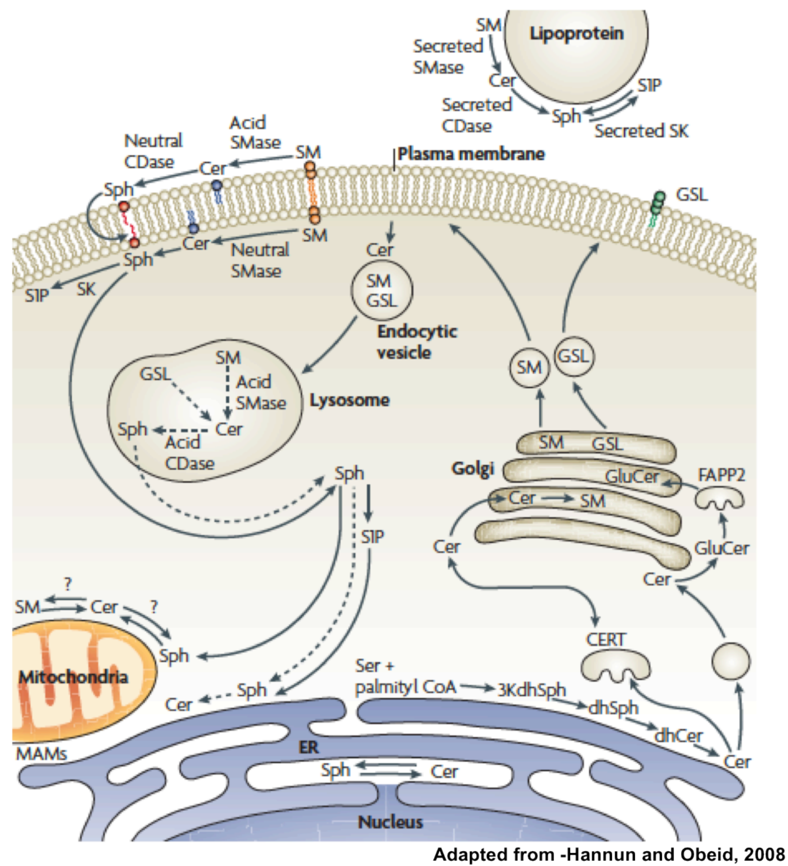


Figure 1.1: The SL pathway. The figure shows the compartmentalization of the pathway in different organelles in the cell. Additionally it shows the routes of SL transport to their final location. Also shown in the figure is the inter-connection of the different SLs reactions either via their substrates or products.

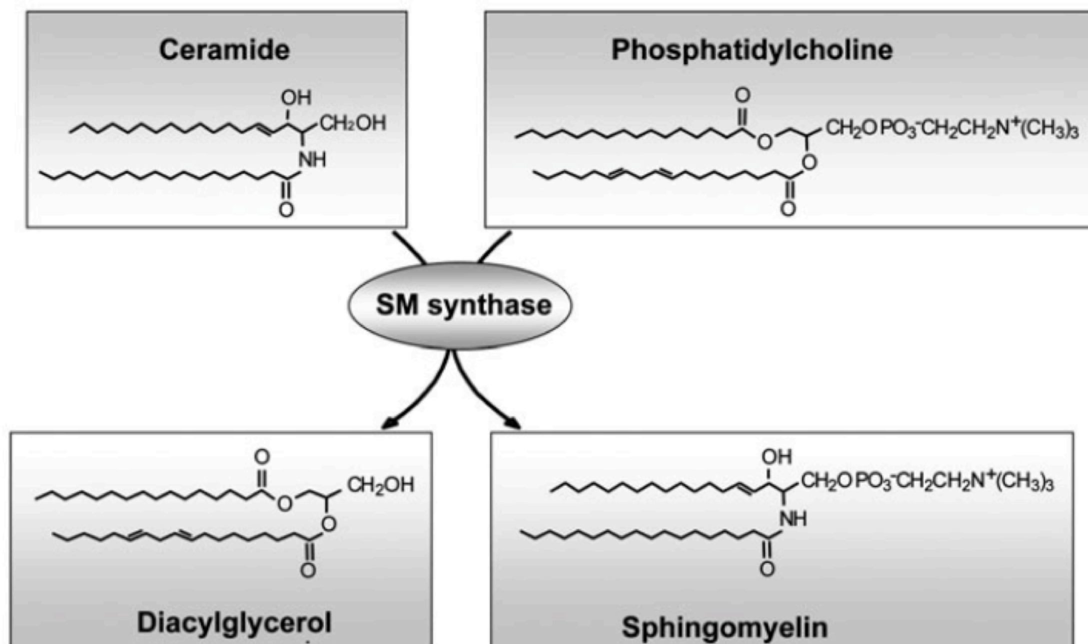


Figure 1.2: Sphingomyelin synthase reaction. SMS catalyzes the transfer of the phosphorylcholine moiety from phosphatidylcholine on to ceramide, producing both SM and diacylglycerol (DAG).

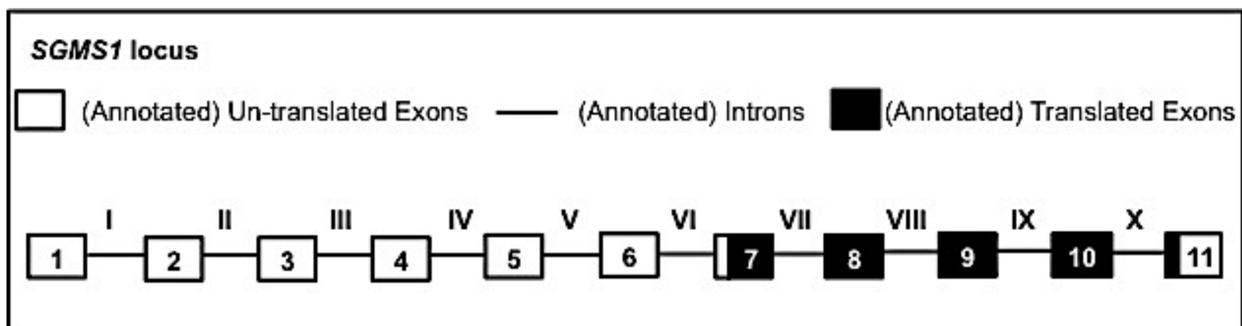


Figure 1.3: Schematic representation of the SGMS1 locus. The SGMS1 gene is made of 11 exons and 10 introns. Exons are annotated as boxes and exon numbers are indicated in alpha-numeric. Introns are represented as solid black lines and are identified in Roman numeral. White exon boxes indicate exons that are un-translated and black colored exon boxes indicate exons that are translated. Note: the locus is not drawn to scale.

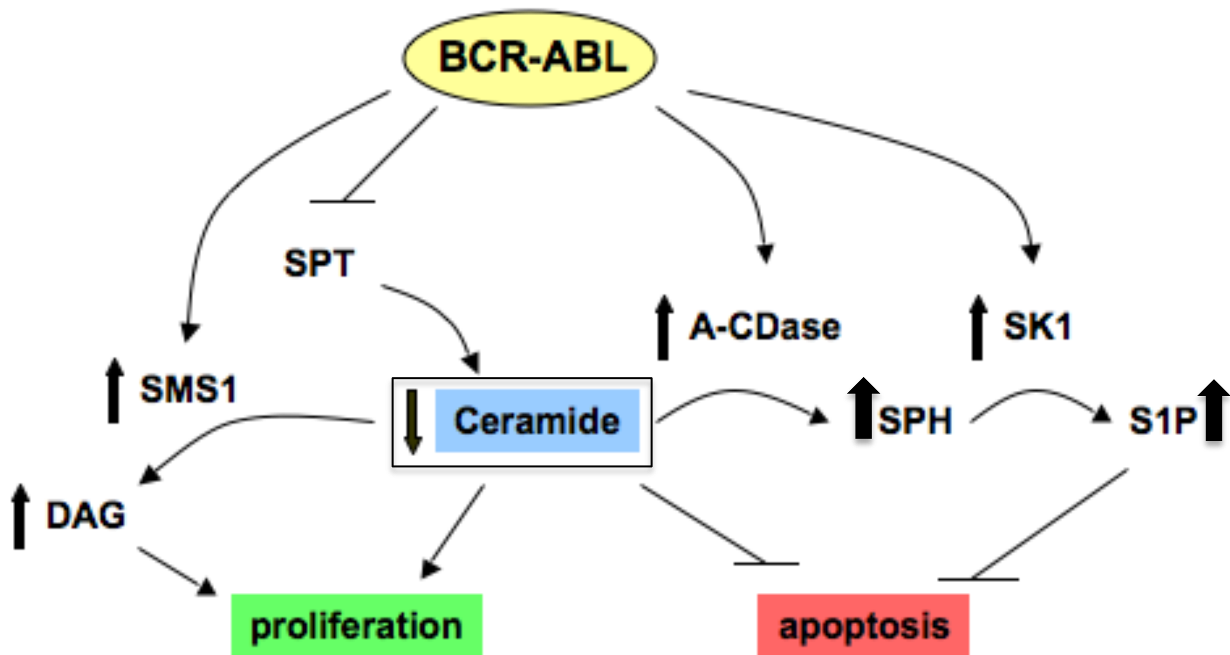
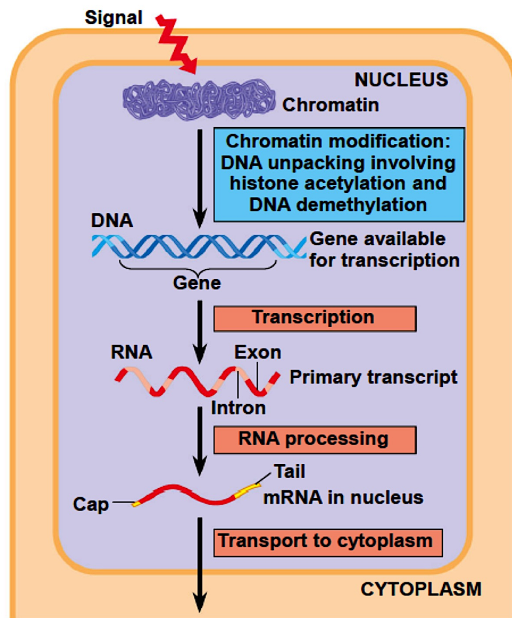


Figure 1.4: Reprogramming of sphingolipid-metabolizing enzymes in CML cells. Expression of Bcr-Abl induces the reprogramming of some sphingolipid metabolizing enzymes ultimately favoring a proliferative and antiapoptotic phenotype in CML cells. The reprogramming results in the overall control of ceramide production through reduced SPT activity and increased SMS1 and A-CDase expression, and it feeds into the formation of S1P through the combination of the increased A-CDase and SK1 activities.

A



B

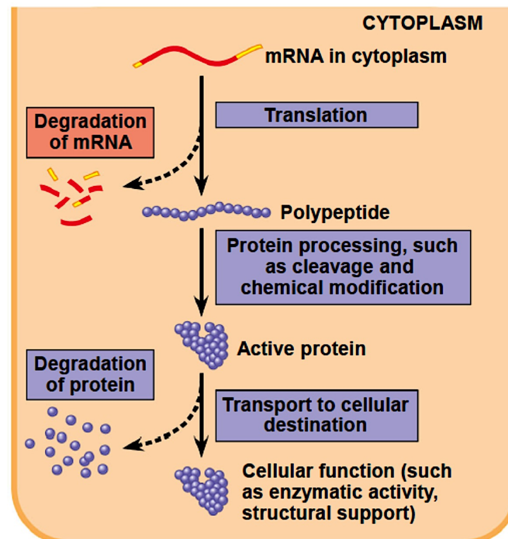


Figure 1.5: Mechanisms regulating gene expression. (A) Gene expression regulation at the level of transcription. (B) Gene-expression regulation at the level of translation. Figure adapted from Campbell Biology, 9th Edition.

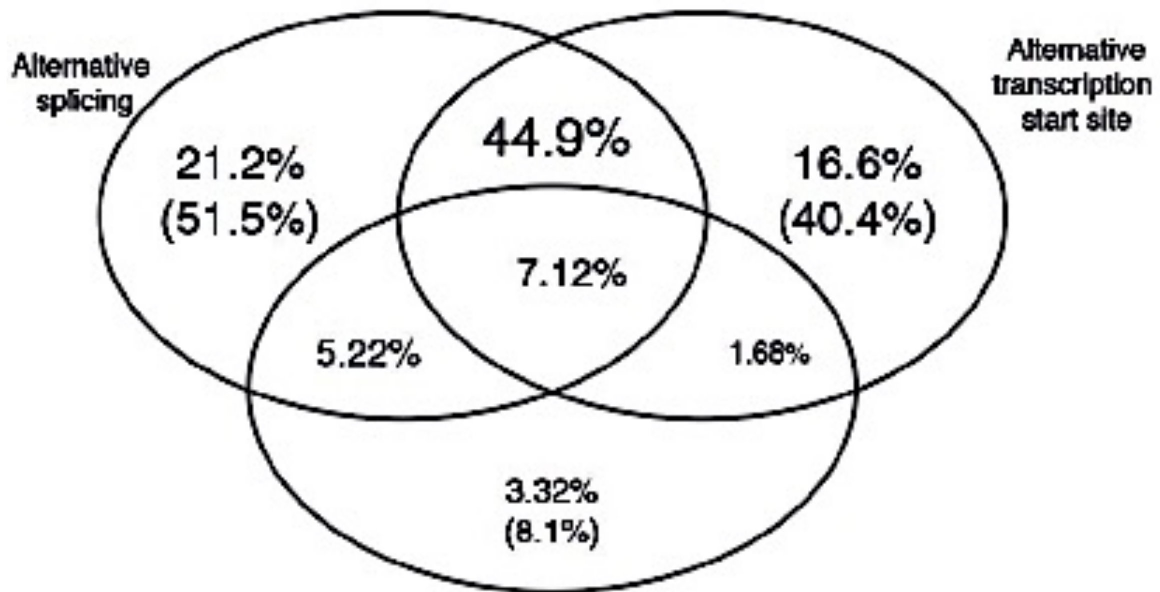


Figure 1.6: Mechanisms of isoform switching. Venn diagram shows the percentage genes that show isoform switching caused by alternative splicing, alternative transcription start sites, alternative transcription termination sites or combinations thereof. Percentages in parentheses are calculated from isoform switches that only utilize a single mechanism. (Figure adapted from Vitting-Seerup and Sandelin, 2017)

CHAPTER 2

BCR-ABL UP-REGULATES THE TRANSCRIPTION OF *SMS1* VIA ALTERNATIVE TRANSCRIPTION INITIATION

2.1 Introduction

Sphingomyelin synthase (SMS/*SGMS*) is a class of mammalian transferases that synthesize the phospholipid sphingomyelin (SM), and are encoded by two distinct genes, *SGMS1* and *SGMS2* (12,13). SMS catalyzes the transfer of the phosphorylcholine moiety from phosphatidylcholine to ceramide, producing both SM and diacylglycerol (DAG) (24-31). SMS can affect important cell functions by directly regulating the abundance of SM, a critical structural component of plasma membranes and lipid microdomains (67) and the levels of ceramide and DAG, two important bioactive lipids (53,163,329-335). The ability of SMS to affect the integrity of lipid microdomains at the plasma membrane has been shown to have biological implications in cell survival and inflammation due to regulation of receptor mediated-signaling by Fas ligand, Tumor Necrosis Factor alpha, engagement of T cell receptor and Toll Like Receptor 4 (48,65,70,97,336,337). Moreover, regulation of plasma membrane microdomains by SMSs contributes to the cytotoxic activity of alkyl lysophospholipids (68).

SMS activity is associated with cell survival and cell proliferation (56,73,338), independently from changes in SM levels and rather due to regulation of ceramide, generally associated with negative effects on cell proliferation and/or DAG, a well-established mitogenic lipid (335). For example, in HeLa cells, down-regulation of either *SGMS1* or *SGMS2* caused accumulation of ceramide, reduced levels of SM and impaired cell growth; since cell growth was not restored upon addition of exogenous SM, this suggested that the effects on cell growth were triggered by changes in the bioactive lipids, ceramide and/or DAG (58). In Neuro-2a cells, inhibition of *SGMS1*

caused a G0/G1 cell cycle arrest, which resulted in decreased proliferation (90), while up-regulation of SMS1 in response to hydrogen peroxide (H_2O_2) counteracted the cytotoxic effect of H_2O_2, possibly by keeping the levels of ceramide in check (339). In line with a pro-survival function, silencing of SMS1 enhanced the accumulation of ceramide in response to photodamage and exacerbated cell death (79).

In cancer, increased SMS activity has been associated with both pro and anti tumorigenic effects. In fact, while activation of SMS and enhanced production of SM has been shown to mediate in part the anti-cancer activity of 2-hydroxyoleic acid in glioma cells (75), increased SMS activity correlated with drug-resistance in samples derived from patients with Acute Myelogenous Leukemia (340) and Chronic Myelogenous Leukemia (CML) (277). Furthermore, in a study from our laboratory, it was shown that increased SMS activity contributed to proliferation of the CML cell line, K562 (144).

CML is a myeloproliferative disorder of the hematopoietic stem cells (HSC) and accounts for 20% of all leukemias affecting adults (132). CML is caused by the reciprocal translocation between the long arms of chromosome 22 and 9 resulting in the formation of the chimeric Bcr-Abl oncogene. The resulting Bcr-Abl oncogene generates a constitutively active abl tyrosine kinase (TK), responsible for initiating CML (103,341,342). CML progresses through three clinical stages – chronic phase (hyper expansion of mature myeloid cells), accelerated phase (acquisition of additional cytogenetic aberrations) and blast phase (proliferation of immature cells), each identified by specific hematological and molecular features. Treatment of patients in the chronic phase with small molecule tyrosine kinase inhibitors such as Imatinib (Gleevec/STI571) (123) has greatly increased progression free survival of patients

(343,344). On the other hand, since treatment with TKIs does not eradicate the disease, as it does not kill the leukemic stem cells (189), CML patients are on TKI therapy for life. The need for long-term therapy favors the insurgence of TKI resistance in 20-30% of all CML patients, constituting the foremost challenge for the treatment of CML.

Previous work from our laboratory discovered Bcr-Abl as the first known up-stream regulator of *SGMS1* (345). Bcr-Abl increased the expression of *SGMS1* mRNA and protein*,* which I showed contributed to the proliferative capacity of the CML cell line, K562 through modulation of ceramide and DAG. However, the precise molecular mechanism involved in the regulation of *SGMS1* expression by Bcr-Abl remained to be elucidated.

In the current study, first, I demonstrate that Bcr-Abl increases transcription of *SGMS1*. Second, I show that Bcr-Abl shifts the *SGMS1* transcription start site (304) just upstream of the translation start site. Third, I demonstrate that this Bcr-Abl-induced shift in TSS generates an mRNA which has a very short 5' untranslated region (UTR) compared to Bcr-Abl negative cells. Finally, I demonstrate that this "short" *SGMS1* mRNA isoform displays a greatly enhanced efficiency of translation.

2.2 Results

2.2.1 Bcr-Abl up-regulates the transcription of *SGMS1.*

Previous work from our lab showed that Bcr-Abl increased SMS1 protein and activity by significantly elevating *SGMS1* mRNA (346). To investigate the mechanism responsible for the elevation of *SGMS1* mRNA, I first determined the effect of Bcr-Abl on *SGMS1* transcription.

Transcription was measured by quantification of hetero-nuclear RNA (the newly transcribed and un-spliced mRNA; hnRNA) (347-349) by qRT-PCR in Bcr-Abl positive (K562) and Bcr-Abl negative (HL-60) cells **(Figure 2.1(A))**. Specific quantification of hnRNA (without contamination from mRNA) was obtained by using primers spanning the junction of Intron VII and Exon 8, thus downstream of the translation start site **(primer sequences reported in Table 2.1)**. As shown in the figure, *SGMS1* hnRNA abundance in K562 cells was ~200 fold higher compared to HL-60 cells. Importantly, when the reverse transcriptase (Superscript III) was not added to the cDNA preparation, no qRT-PCR signal was detected demonstrating that the samples were devoid of contamination from genomic DNA. These results indicated enhanced accumulation of hnRNA of *SGMS1* in K562 cells.

Next, contribution of mRNA stability to the elevated *SGMS1* mRNA expression in the Bcr-Abl positive cells was examined. Herein, K562 and HL-60 cells were treated with either vehicle (H_2O) or Actinomycin D (5 μg/mL) to block transcription, and mRNA degradation was measured over a 2-hour time course, **(Figure 2.1B)**. Primers used to measure *SGMS1* mRNA levels were designed within Exon 7 (down-stream of the translation start-site) and expression was quantified by qRT-PCR **(primer sequences**

reported in Table 2.1). As shown in the figure, there was no significant difference in the rate of mRNA degradation between the two cell-lines. All together these results indicated that, in K562 cells, *SGMS1* mRNA expression was up regulated by increased transcription and not through altered mRNA stability.

To determine if Bcr-Abl mediated the observed increase of *SGMS1* transcription in K562 cells, hnRNA levels were measured in HL-60 cells stably over-expressing Bcr-Abl, using primers at the junction of Intron VII and Exon 8, as in the previous experiment. Similar to K562 cells, HL-60-Bcr-Abl cells showed a 70-fold higher *SGMS1* hnRNA level compared to HL-60 cells **(Figure 2.2, black bars)**. Importantly, increased *SGMS1* hnRNA abundance was also observed in other CML cell lines, LAMA-84 and JURL-MK-1.

Next I evaluated the role of Bcr-Abl kinase activity in regulating the transcription of *SGMS1*. To this end K562 cells were treated with 1 uM of the Bcr-Abl kinase inhibitor, Imatinib/STI571 for 6 hours. As shown in **Figure 2.3(A)**, after treatment for 6 hours STAT5 phosphorylation is completely inhibited. STAT5 phosphorylation is a direct measure of Bcr-Abl kinase activity. At this time point cells were collected and hnRNA abundance was measured using the same primers as above. **Figure 2.3(B)** shows that inhibition of Bcr-Abl kinase activity with Imatinib results in a down-regulation of *SGMS1* transcription.

Together these results indicated that, in CML cells, elevated *SGMS1* mRNA expression can be caused by a Bcr-Abl-mediated increase in transcription.

2.2.2 Bcr-Abl initiates *SGMS1* transcription from alternative start sites.

Interestingly, when *SGMS1* hnRNA was measured in K562 cells using a second set of primers further up-stream in the locus, precisely at the junction of Intron V-Exon 6 **(Figure 2.2, white arrows and bars) (primer sequences reported in Table 2.1)**, the hnRNA levels was found to be ~50 fold lower compared to hnRNA levels measured at the junction of Intron VII-Exon 8 **(Figure 2.2, black bars versus white bars)**. This difference in *SGMS1* hnRNA levels between the two regions was also observed in other CML cell lines and in HL-60/Bcr-Abl cells **(Figure 2.2, black bars versus white bars)**. However, in HL-60 cells, the hnRNA levels up-stream and down-stream were comparable. Consequently, whereas there was an increase of *SGMS1* hnRNA of about 200 fold in K562 versus HL-60 cells when using primers downstream of the open reading frame (Intron VII-Exon 8) (black bars), the increase was 4 fold when the hnRNA was probed using primers at the junction of Intron V-Exon 6 (white bars). These differences in hnRNA were also observed in the other CML cells.

Thus, our results indicate first that, in Bcr-Abl positive cells, transcription of *SGMS1* is initiated from at least two different start-sites, one upstream of Intron V and one downstream of Exon 6, and second, that Bcr-Abl increases transcription primarily from an initiation site downstream of Exon 6.

Additionally, it was determined whether the differences observed in hnRNA levels were reflected in the mRNA expression pattern as well. Primer sets were designed to target mRNA by probing exon-exon junctions, up-stream (Exon 5-Exon 6) and downstream (within Exon 7) of exon 6 **(primer sequences reported in Table 2.1)**. As shown in **Supplementary Figure 2.S1**, the mRNA data followed a pattern similar to

hnRNA indicating that the specific transcriptional pattern induced by Bcr-Abl is also maintained at the mRNA level.

2.2.3 Identification of *SGMS1* transcription start sites in K562 by 5' RLM-RACE.

To identify the distinct TSSs, a 5' RLM-RACE was performed on total RNA from the Bcr-Abl positive cell line, K562. The 5' end of *SGMS1* transcripts was targeted using two different reverse primers, one within Exon 6 (Exon 6-reverse) and the second within Exon 7 (Exon 7-reverse) **(primer sequences reported in Table 2.1)**. The 5' RLM-RACE identified four different TSSs resulting in five different mRNA transcript variants **(Figure 2.4(A))**. Three of these transcription initiation sites resided within Introns (I, II and VI, respectively), and the fourth TSS within Exon 7, up-stream of the translation start site **(Figure 2.4(B)) (sequences of the different 5'RLM-RACE products are reported in Table 2.2)**. Each TSS was annotated by the intron or exon in which it resided: TSS I, TSS II, TSS VI and TSS 7, respectively, and they each generated transcripts with alternative first-exons. Thus, consistent with the results in **Figure 2.2**, transcription of *SGMS1* in K562 is initiated from two TSSs upstream of Intron V (TSS I and TSS II) and two TSSs down-stream of Exon 6 (TSS VI and TSS 7).

2.2.4 Bcr-Abl transforms the transcriptional landscape of *SGMS1*.

To identify which of these TSSs accounted for increased *SGMS1* transcription in Bcr-Abl positive cells, I developed the First Exon Profiling (FEP) method by Real Time PCR. The FEP method takes advantage of the fact that the first exon of a transcript is never spliced out, representing therefore an accurate measure of the total mRNA generated from individual TSSs, regardless of downstream splicing. Thus, I designed primers targeting each *SGMS1* alternative first exon and measured their abundance by RT-PCR **(for primer sequences refer to Table 2.1**) (**Figure 2.5(A)**). FEP results in **Figure 2.5(B)** clearly showed that, in all CML cell lines tested, TSS 7 bore the greatest transcriptional burden for the *SGMS1*. In K562 cells (and other CML cell lines), the mRNA levels generated from TSS 7 was approximately 80-fold higher compared to that generated from TSS II, the second most abundant *SGMS1* mRNA isoform. To evaluate if mRNA stability contributed to the differences observed in the mRNA levels of the different transcripts, mRNA degradation was assessed in K562 cells and no significant differences were observed among the half-life of the different transcripts **(Figure 2.5(C))**. Together these results indicate that, in Bcr-Abl positive cells, transcription of *SGMS1* primarily occurred through TSS 7. It is to be noted here that FEP expression measured for TSS 7 is a sum of expression of transcriptional burden of TSS 7 and up-streams TSSs as well, since exon 7 is present in all mature transcripts generated from the different TSSs.

To determine whether the preferential utilization of TSS 7 is dependent on Bcr-Abl, I generated a map of the transcriptional landscape of *SGMS1* comparing HL-60 to K562 and HL-60/Bcr-Abl cells **(Figure 2.6(A))**. The hnRNA abundance was plotted in

the context of the *SGMS1* locus. First, FEP primers targeting TSS I and TSS II were used to quantify transcriptional abundance in HL-60 cells (white bars). As shown in the figure, both primer sets picked up signals whose intensity was in the range of hnRNA levels of *SGMS1* in HL-60 cells (MNE values in HL-60 cells are 0.03-0.16 x10^{-4} for hnRNA versus 8.8-38.63 x10^{-4} for mRNA) thus indicating that, in these cells, these portions of the transcript were not alternative first exons retained in the mRNA as in CML cells, but part of hnRNAs. Furthermore, in HL-60 cells, the *SGMS1* hnRNA levels at TSS I and TSS II were comparable to those at the junctions of Intron V-Exon 6 and Intron VII-Exon 8 **(Figure 2.6(A))**, indicating the existence of a single transcript starting upstream of TSS I. On the other hand, in K562 and HL-60/Bcr-Abl cells, transcripts originating from TSS II and TSS 7 are both elevated compared to HL-60 cells. However, while transcripts originating from TSS II in K562 cells are ~4-5 fold higher than the single, long transcript in HL-60 cells **(Figure 2.6(B))**, transcripts originating from TSS 7 are 200 fold higher, indeed indicating that Bcr-Abl is shifting transcription initiation to TSS 7.

2.2.5 Identification of the TSS 7 promoter and its regulation by Bcr-Abl.

To further validate TSS 7 as an authentic transcriptional start site, I set out to identify, isolate and assess the activity of its putative promoter. Based on the promoter prediction software, Genomatix a stretch of 832 bp up-stream of TSS 7 was isolated from genomic DNA of K562 cells and cloned into the pGL3-basic luciferase reporter vector and named Promoter 7 **(sequence of promoter 7 reported in Table 2.3)**. Promoter 7-pGL3 and pCMV-β-galactosidase (transfection control) constructs were co-transfected into Bcr-Abl positive (HL60-Bcr-Abl and K562) and negative (HL-60) cells and luciferase activity measured and normalized to β-galactosidase activity **(Figure 2.7(A))**. As shown in the figure, the isolated genomic region corresponding to the putative promoter 7 induced transcription as indicated by the high luciferase activity obtained in K562 cells. Importantly, the promoter activity in both K562 and HL-60/Bcr-Abl cells was ~20 fold higher compared to Bcr-Abl negative HL-60 cells. These results indicate that Promoter 7 is a *bona fide* promoter and is activated by Bcr-Abl, thus validating the increased utilization of TSS 7 in CML cells shown in **Figure 2.7(A)**.

Next I evaluated the effect of inhibition of Bcr-Abl kinase activity by Imatinib on TSS 7 promoter. To this end K562 cells were transfected with the TSS 7 promoter construct and after 24 hours transfected cells were treated with 1 uM Imatinib. After 8 hours of treatment of treatment with Imatinib cells were collected to assess relative luciferase activity of TSS 7 promoter. **Figure 2.7(B)** shows inhibition of Bcr-Abl kinase activity resulted in a 40% decrease in promoter activity.

2.2.6 Increased translational efficiency of *SGMS1* Transcript-7.

Based on the results from FEP and mRNA, transcript IIb and Transcript 7 are the two most abundant *SGMS1* mRNA transcripts in CML cells, with overwhelming preference for transcript 7. Interestingly, transcript 7 is characterized by a short 5'UTR (~135 bp) while transcript IIb has the longest 5'UTR (574 bp) among all the mRNA variants present in K562 cells. Thus, I wondered whether the preferential utilization of TSS 7 had a functional consequence on its transcript due to the very short 5'UTR. It has been reported that the length and structure of the 5'UTR can interfere with the translation efficiency of an mRNA (350,351), therefore I set out to test whether there was a difference in the efficiency of translation between Transcript 7 and Transcript IIb. Mammalian expression plasmids were generated which contained the 5'UTR either from Transcript 7 or Transcript IIb and the full *SGMS1* coding sequence with a flag-tag at the carboxy-terminus **(sequences of vectors are reported in Table 2.4)**. To determine whether the intrinsic features of the 5'UTRs directly affected the efficiency of translation (independently of any effect mediated by Bcr-Abl), I first transfected the constructs in HeLa cells. After transfection, samples were collected at 10h and 26h (expression was not saturated at these time-points) and assessed by western blotting to measure protein levels **(Figure 2.8(A) and Supplementary Figure 2.S2(A))** and by qRT-PCR to measure mRNA levels **(Supplementary Figure 2.S2(B)).** To obtain translational efficiency, western blot bands corresponding to SMS1 were quantified **(Supplementary Figure 2.S2(A))** and divided by *SGMS1* mRNA levels measured by qRT-PCR using FEP primers **(Supplementary Figure 2.S2(B)) (Figure 2.8(B)).** As

shown, the translational efficiency of transcript 7 was ~20 fold higher compared to that of transcript IIb **(Figure 2.8(B)).**

To assess if Bcr-Abl exerted an additional effect on regulation of translation of these transcripts, the constructs were transfected in K562 cells and, at 8 and 10 hours post transfection, proteins and mRNA levels were measured. Similar to HeLa cells, also in K562 cells, transcript 7 was translated approximately 20 fold more efficiently than transcript IIb **(Figure 2.8(C) and 2.8(D)) (Supplementary Figure 2.S3),** with no additional effect due to the presence of Bcr-Abl.

All together these results show that the intrinsic features in the longer 5’UTR of transcript IIb exert an inhibitory effect on translation of *SGMS1* compared to the short 5’UTR in transcript 7.

2.3 Discussion

Our study shows that Bcr-Abl increases SMS1 abundance via a concerted mechanism that involves regulation of both transcription and translation: first, Bcr-Abl stimulates transcription of *SGMS1* shifting the TSS close to the translation start and second, this shift in TSS generates an mRNA which is now translated with greater efficiency **(Figure 2.9).** The coordinated action of these mechanisms leads to an geometric increase of SMS1 abundance in CML cells.

Several observations in our study support the transcriptional regulation of *SGMS1* by Bcr-Abl. First, I show elevated *SGMS1* hnRNA levels in CML cells as compared to Bcr-Abl negative HL-60 cells. Second, I show that Bcr-Abl is sufficient to elevate *SGMS1* hnRNA as the increase in *SGMS1* hnRNA is also observed in HL-60 cells over-expressing Bcr-Abl. Additionally, I identified four *SGMS1* TSSs in the CML cell line K562, and through my newly developed method, FEP, I determined that in CML cells, *SGMS1* mRNA is primarily generated from TSS 7. The preferential usage of TSS 7 in CML cells was also confirmed by the up regulation of its promoter activity in Bcr-Abl positive cells. Taken together these results unveil, for the first time, Bcr-Abl as an upstream transcriptional regulator of *SGMS1* and characterize promoter 7 as the first *bona fide SGMS1* promoter.

Since its identification (12,13), the regulation of the *SGMS1* gene has been largely unexplored, primarily because of lack of study that could directly implicate regulation of its expression in physiological or pathological conditions. *SGMS1* is a large gene spanning 320 kb of human chromosome 10 and contains 11 annotated exons. The *SGMS1* transcript is characterized by its exceptionally long predicted 5’ and

3'UTRs, of 954 bp and 1538 bp, respectively (50). Dergunova *et al.*, reported that the lengthy 3'UTR of *SGMS1* contains alternative 3' polyadenylation sites which could possibly affect mRNA stability and therefore its abundance (352). However, our assessment of *SGMS1* mRNA stability showed no differences between Bcr-Abl positive and negative cells thus validating that increased abundance of *SGMS1* in CML cells was due to increased transcription.

Regulation of transcription initiation of *SGMS1* is complex. Indeed, preliminary experiments from our lab investigating the usage of the alternative TSSs identified in this study, suggest that, even within the hematopoietic lineage, *SGMS1* utilizes different transcription initiation sites (preliminary data not shown). Moreover, blast of our 5'RACE sequences against the EST databases for cells and tissues revealed that these sequences might be also expressed in few other cancer cell lines, like Ramos and Jurkat and potentially also in normal adult tissues such as the uterus, trachea and testis. Since entries in the EST databases can represent partial sequences, this type of analysis is only suggestive and not conclusive, though it supports heterogeneity in the *SGMS1* 5'UTRs **(Table 2.5).** This diversity is not unique to humans in fact *Mus musculus SGMS1* has four different TSSs resulting in three different protein isoforms with varied tissue distribution (353). In humans, the existence of a vast repertoire of *SGMS1* 5'UTRs is corroborated by studies conducted by Rozhkova *et al.* and Sudarkina *et al.* (51,354) on mRNA from different human tissues, although the corresponding TSSs could not be identified except for TSS VI.

Our study, on the other hand, offers an exhaustive investigation of transcriptional regulation from diverse TSSs previously unreported for human *SGMS1*. Indeed, hnRNA

data, results from 5'-RACE, FEP and promoter studies all supported the preferential utilization of TSS 7 in CML cells as compared to HL-60 cells. Thus, this is the first time that a mechanism of upstream regulation of *SGMS1* has been identified and it shows that the Bcr-Abl oncogene up-regulates *SGMS1* transcription from a newly identified and confirmed alternative start site (TSS 7).

Our study further establishes that the observed Bcr-Abl-mediated shift in transcription initiation of *SGMS1* has the functional consequence of enhancing *SGMS1* translation, independently and in addition to the observed increase in transcription. In fact, I clearly show, in two different cell types, that transcripts from TSS 7 display a 20 fold higher translation efficiency compared to transcript IIb, the second most abundant transcript type in CML cells.

It is well accepted that the structural characteristics of 5'UTRs such as their length, presence of upstream ORFs (uORFs), secondary structures and terminal oligopyrimidine tracts can have repressive effects on translation (351,355-358) and this seems to be the case for *SGMS1*. Most of the alternative transcripts that I identified in K562 cells have uORFs: transcript I has 2, transcript IIb has 1, transcript VI has 1, while transcript IIa and transcript 7 have no uORF. However the total expression of transcript IIa is extremely low compared to transcrip IIb, so it does not significantly contribute to SMS1 expression. Furthermore, our own analysis of the 5'UTR of annotated *SGMS1*, the longest transcript of *SGMS1* starting at canonical exon 1, revealed two uORFs on the mRNA derived from the minus strand of genomic DNA, where *SGMS1* resides. Furthermore, the 5'UTR of transcript 7 is short compared to the average length of 5'UTRs of human genes (200 bp), it has minimal secondary structures, low comparative

G+C content and low total free energy **(Table 2.6)**. The lack of all these translational inhibitory features in the 5'UTR of transcript 7 supports and provides a mechanism for the increased translational efficiency of TSS 7 compared to TSS IIb.

The preferential usage of TSS 7 in Bcr-Abl positive cells is driven by a shift in promoter activation. Classically, in normal human tissues, alternative promoters are utilized to allow spatio-temporal expression of specific proteins, via differential expression of specific transcription-factor regulatory programs (359-361). In cancer, the use of alternative TSSs has been primarily associated with production of different isoforms of relevant proteins, causing either loss or gain of function. This is the case for instance of LEF1, DLC1 and p73 (311,312,362-365). In our case, the shift in promoter utilization induced by Bcr-Abl increased translation of full-length SMS1 protein, as detected by western blotting and increased SMS activity ((346) and Figure 7). A very limited number of studies have described alteration in the levels of cancer-associated full-length proteins as consequence of a promoter shift. Similar to *SGMS1*, increased expression of *Axin2* and *mdm2* has been associated with a shift in promoter utilization resulting in increased translational efficiency (306,358,366-369), while, opposite to SMS1, lower BRCA1 levels in breast cancers are a consequence of inefficient translation due to a shift in promoter utilization towards a longer 5'UTR (322,370-372). However, in the reported cases, the trigger for the shift in promoter utilization is yet to be identified. Remarkably, our study not only demonstrated the association of the shift in promoter utilization with increased translation efficiency of SMS1, a protein important for proliferation of Bcr-Abl positive cells (346) but, for the first time, I have established a causative link between an oncogene (Bcr-Abl) and such mechanism of up-regulation.

2.4 Materials and methods

Cell-lines and media:

K562, HL-60 and HeLa cell-lines were purchased from American Type Culture Collection (ATCC) (Rockville, MD). Human acute myeloid leukemia, HL-60 cells stably expressing p185 Bcr-Abl (HL-60/Bcr-Abl) was a generous gift from Dr. K. Bhalla (167). LAMA-84 and JURL-MK-1 cell-lines were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Brunswick, Germany). K562, LAMA-84 and JURL-MK-1 were grown in RPMI-1640 (Life Technologies Corporation, Carlsbad, CA), supplemented with 10% FBS (Fetal Bovine Serum, heat inactivated) (Life Technologies Corportion, Carlsbad, CA) or 20% FBS for HL-60 and HL-60-Bcr-Abl, and 100 U/mL Penicillin and 100 µg/mL Streptomycin (Life Technologies Corporation, Carlsbad, CA). HeLa cells were grown in DMEM supplemented with 10% FBS, 100 U/mL Penicillin and 100 ug/mL Streptomycin (Life Technologies Corporation, Carlsbad, CA). Cells were maintained at 37°C and 5% CO_2 in a humidified incubator at supplier suggested cell concentrations.

RNA isolation and cDNA synthesis:

To analyze RNA expression (hnRNA and mRNA) total RNA was isolated from 1-$4x10^6$ cells, collected by centrifugation at 3000 RPM, 5 minutes at 4°C. Cells were immediately re-suspended in lysis buffer with β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) and total RNA was isolated using RNeasy mini kit (Qiagen, Hilden, Germany) according to manufacturer's protocol, including the on-column DNase digestion. Additional round of DNase digestion was performed using TURBO DNA free kit (Life Technologies Corporation, Carlsbad, CA) according to manufacturer's protocol

to ensure complete elimination of genomic DNA. cDNA was prepared by a two step method; first an RNA mix was prepared with 1 µg of total RNA, 1 µL of Oligo $(dT)_{20}$ primer (50 µM) (Life Technologies Corporation, Carlsbad, CA) and 1 µL of 10 mM dNTP (Life Technologies Corporation, Carlsbad, CA) mixed to a final volume of 12 µL and incubated at 65°C for 5 minutes. After this step, a second master mix of 4 µL of 5x 1st Strand Buffer, 2 µL of 0.1 M DTT, 1 µL each of RNase Out and Superscript III (Life Technologies Corporation, Carlsbad, CA) was added to the RNA mix. The 20 µL reaction was then incubated at 42°C for 52 minutes and then 70°C for 15 minutes.

Quantitative Real-Time PCR (qRT-PCR):

To evaluate mRNA and hnRNA expression, qRT-PCR was performed using the SYBR green mixture (Bio-Rad, Hercules, CA) with primers within exons or at intron-exon junctions respectively **(primer sequences are reported in Table 2.1**). All experiments were performed on Applied Biosystem, 7500 Real time PCR system. The following cycling conditions were used, 1 cycle of 3min at 94°C, 40 cycles of 20s at 94°C and 30s at 60°C. The melt curve stage was as follows, one cycle of 1min at 95°C, one cycle of 1 min at 60°C followed by each cycle increasing by 1°C for 30s starting at 60°C followed by a final step at 60°C for 15s. Amplification efficiency for each primer pair was assessed being between 90-100% across a range of cDNA concentrations. Results were normalized to internal control gene, β-actin and amplification efficiency. qRT-PCR results were analyzed using Q-Gene software, as mean of normalized expression (MNE) (373). To assess genomic DNA contamination, cDNA was prepared without reverse transcriptase. These controls did not amplify during qRT-PCR thus demonstrating the absence of genomic DNA.

mRNA stability:

Cells were re-suspended in complete medium (without antibiotics) at a concentration of $0.3x10^6$ cells /ml and allowed to rest for 2 hours. Cells were treated with 5 µg/ ml of Actinomycin D or H_2O (control). Cells were treated for 30 minutes, 1, 2, and 4 hours and collected by centrifugation at 900 RPM for 5 minutes and re-suspended in RLT buffer containing β-mercaptoethanol. RNA and cDNA was prepared as described above. The mRNA expression was plotted against time to calculate the half-life using an exponential one-phase decay model.

Imatinib treatment

K562 cells were treated with Imatinib 1 uM (Santa Cruz, CA) prepared in DMSO. Cells were seeded at $0.1x10^6$ cells/mL for all treatment experiments. Cells were treated for 6 hours at which point they showed complete inhibition of kinase activity as shown by measurement of pSTAT5 (Cell Signaling Technology, MA) versus total STAT5 (Santa Cruz, CA) by western-blotting (method described below).

5' RLM-RACE:

RNA Ligase Mediated Rapid Amplification of cDNA ends (RLM-RACE) was used to identify the transcriptional start site of SGMS1 in the Bcr-Abl positive K562 cells according to the manufacturer's specified protocol for Ambion FirstChoice RLM-RACE (Invitrogen, Carlsbad, CA). For all the following enzymatic reactions, enzymes activities were terminated by the addition of phenol/chloroform and RNA was subsequently extracted as suggested by manufacturer. In summary, 1µg of RNA from K562 cells was either de-phosphorylated with calf intestine phosphatase (CIP) or left untreated at 37°C for 1hr. To remove the mRNA Cap structure, CIP treated and non-treated RNA were

either incubated with tobacco acid pyrophosphatase (128) or left untreated, at 37°C for 1 hour. All control reactions were treated similarly to the experimental samples in the subsequent RLM-RACE. Dephosphorylated and un-capped mRNA was added to the GeneRacer RNA oligo and incubated with T4 RNA ligase at 37°C for 1hr. The un-capped, full-length mRNA ligated to the GeneRacer RNA oligo was used for reverse transcription of mRNA into cDNA using Superscript III reverse transcriptase and random primers following the standard protocol (Invitrogen, Carlsbad, CA). This cDNA was used as a template for an initial outer PCR to amplify cDNA ends. The outer PCR was performed using 0.25U of platinum high fidelity Taq polymerase and all primers were used at 0.4uM for a standard 50µL PCR reaction as suggested by the manufacturer (Invitrogen, Carlsbad, CA). The primers used for the outer PCR included in the GeneRacer 5' primer (kit component) and the following gene specific primer (GSP2R), 5'-CAA GAA CGG CCA TGC CAA TGG-3'. The following negative controls were added: PCR reaction without template cDNA, PCR reaction without GSP2, and a PCR reaction without the GeneRacer 5' primer. Additionally, to ensure fidelity of the kit and all its enzymes, 1µL of HeLa cDNA was used as template with the GeneRacer 5'primer and control primer B.1 provided with the kit. Two additional PCR reactions were used as positive controls for verification of the presence of the target RNA and for testing RLM-RACE procedure. These PCR reactions utilized primers synthesized upstream to GSP2 and far enough downstream to produce a resolvable PCR product as visualized by agarose gel electrophoresis. The combination of primers used for these positive control reactions are as follows, GSP/outer1F (5'-TGC CAA ACA AGT CTC TGC TC-3') with GSPR, or GSP/outer1F with GSP3R (5'-AAG TCC TGG CCT GTG AAA TG-3').

PCR amplification conditions followed for the outer PCR were as follows: 94ºC for 3min and 35 cycles of 94ºC for 30s, 60 ºC for 30s, 72ºC for 30s, followed by an extension at 72ºC for 7min. The product from the outer PCR was used as template for the inner 5' RLM-RACE PCR with the following primers, GSP3R and 5'RACE inner primer provided with kit. Negative controls were added as above for inner PCR and PCR application was performed using the same PCR amplification conditions as above. Following both outer and inner PCR reactions, 10µL of the reactions and all above-mentioned controls were added to a 2% agarose gel containing 100 ug/mL ethidium bromide for visualization of PCR products. PCR products were excised from gel, and purified under standard conditions (Qiagen, Valencia CA). Gel-purified products were then ligated into the pCR-2.1-TOPO cloning vector, transformed into DH5α cells according to standard protocol (Invitrogen, Carlsbad CA) and grown overnight on LB plates containing 100 ug/ml ampicillin for selection. Bacterial colonies were selected and plasmid DNA was purified from these colonies according to manufacturer recommended protocol (Qiagen, Valencia CA) and was used for sequencing with both M13F and M13R primers. The entire 5' RLM RACE protocol was repeated twice.

First-Exon Profiling (FEP):

First-exon profiling is a method developed in our laboratory to measure the abundance of mRNA generated from alternative transcriptional start sites by qRT-PCR. Primers are designed within the first exon of each identified mRNA isoform generated from the different TSSs as identified by 5'RLM-RACE. Since the first exon is never spliced out this method allows for the measurement of mRNA abundance of specific transcripts. For SGMS1, 4 TSSs were identified by 5' RLM-RACE in K562 cells. Each

TSS had a unique alternative first exon residing within Intron I, Intron II, Intron VI and Exon 7, respectively. Primers sequences used for each mRNA isoform are reported in **Table 2.1**. Amplification efficiency was determined to be between 90-100% for each primer pair prior to abundance measurement in Bcr-Abl positive and negative cell lines.

Generation of SGMS1 promoter-7 luciferase construct:

Genomic DNA was isolated from exponentially growing K562 cells. $15x10^6$ cells were centrifuged at 3000 RPM for 5 minutes at 4ºC and re-suspended in 500 µl of freshly prepared lysis buffer containing 50 mM EDTA (pH-8), 1% SDS, 50 mM Tris pH 7.4, 100 mM $NaCl_2$ and 750 µg/ml Proteinase K (Roche Molecular Biochemical, Indianapolis, Indiana). K562 cell lysate was then incubated for 1 hour at 60ºC, vortexed briefly, and then incubated at 60ºC overnight. Following overnight incubation, 200 µl of ice-cold 5M KOAc was added, the lysate was vortexed for 30s and incubated on ice for 1.5 hours, centrifuged at 12,000 RPM for 15 minutes and 500 µl of the supernatant was removed. To precipitate DNA, 1 ml of ice cold Ethanol was added to the top of supernatant and the mixture was gently rocked a few times at 90ºC. DNA was spooled from the interface of the supernatant, washed first with 70% Ethanol and then with 100% Ethanol. DNA precipitate was air-dried for 10 minutes and re-suspended in 100 µl of sterile double-distilled H_2O. Promoter 7 was amplified from K562 genomic DNA by PCR using primers as indicated in **Table 2.1**, in a 100 µl volume reaction containing approximately 40 ng of DNA and the following PCR conditions: 0.2 mM dNTP, 1.5 mM MgCl2, 0.75 uM of primers, and 2.5U Taq DNA polymerase (Invitrogen Life Technologies, Carlsbad CA). PCR amplification conditions for Promoter 7 isolation were as follows: 95ºC for 5 min and 35 cycles of 95ºC for 50 s, 52ºC for 50 s, and 72ºC for 2

min, followed by an extension at 72ºC for 10 min. PCR amplicons were cloned into the pCR 2.1 TOPO-TA sub-cloning vector and transformed into DHA5α bacterial cells using standard conditions (Invitrogen, Carlsbad, CA). The plasmids were extracted from ampicillin resistant bacterial colonies, and inserts were excised from the pCR 2.1 TOPO-TA vector using KpnI and ZhoI (Invitrogen Life Technologies, Carlsbad, CA) and cloned into the final pGL3-basic vector (Promega, Madison, WI). All sequences were verified (Genewiz, Southplainfield, NJ). Sequence analysis and verification was carried out using the Jellyfish software **(sequence in Table 2.3**). DNA was prepared for transfections using the Endofree Plasmid Maxi Kit (Qiagen, Valencia, CA).

Transient transfection and promoter 7 luciferase activity assay:

Two million cells were transfected with 5 µg each of pGL3 basic vector or pGL3 basic-promoter 7 construct and pCMV-β-galactosidase reporter construct. Plasmids were transfected into cells using the Neon Electroporation system (Life Technologies Corporation, Carlsbad, CA). K562 cells were electroporated at 1000V, 50 ms, 1 pulse while HL-60 and HL-60-Bcr-Abl cells at 1000 V, 35 ms, 2 pulses. Cells were harvested after an overnight incubation and then lysed with 1X reporter lysis buffer (RLB), and luciferase activity was measured using a Promega kit as per manufacturer recommended protocol (Promega, Madison, WI). Luminescence was measured using a Sirius Luminometer (Bethold Technologies, GMBh & Co. KG, Germany) programmed with a 2-second measurement delay followed by a 10-second measurement read. β-Galactosidase activity was used to normalize cell transfection efficiency using a colorimetic method as suggested by the manufacturer (Promega, Madison, WI). Promoter activity from K562 or HL-60 lysates was calculated after normalization with the

amount of cell lysate used for the luciferase activity assay, to the β-Galactosidase activity of each sample and by subtraction of background values (as determined from pGL3 vector only controls).

Translational efficiency of TSS II and TSS 7 mRNA:

Transcript II and Transcript 7 expression constructs (including SGMS1 open reading frame) were synthesized by GenScript USA (Piscataway, NJ, USA) and cloned into pcDNA3.1 expression vector (for HeLa) and pEF1 (for K562) (sequence in Table 4). DNA was prepared for transfections using the Endofree Plasmid Maxi Kit (Qiagen, Valencia, CA). HeLa cells : $1.5x10^5$ HeLa cells were plated in 6 wells plate 24h before transfection. Two hundred µL of Opti-MEM (Life Technologies Corporation, Chicago, IL) were mixed with 6 µL of Xtreme gene (Roche, Atlanta, GA) and incubated with 2 µg of Vector, Transcript IIb or Transcript 7 for 20 minutes at room temperature. Cell medium in the wells was replaced with 2.8 ml of fresh growth medium without antibiotics and the transfection mix was then added drop by drop. Cells were collected after 18h and 26h for mRNA and western-blotting. Isolation of mRNA, cDNA synthesis and qRT-PCR were performed as described above. FEP primers were used for real-time experiments. For western-blotting, cell pellets were re-suspended in 100 µL of 0.5% SDS (Sodium Dodecyl Sulphate) (on ice). Samples were sonicated for 20s. Cell lysates were then re-suspended in loading buffer and boiled for 8 minutes. Ten µg of proteins were run and blotted with primary antibodies against SMS1 (Exalpha, Shirley, MA) and β-Actin (Santa Cruz Biotechnology, Santa Cruz, CA). Western-blot band intensity was measured using Image J (374) and values were recorded after subtracting background. K562 cells: Transcript II and 7 were sub-cloned into pEF6/V5-His-TOPO Vector (Life Technologies

Corporation, Carlsbad, CA). Five μg of plasmids were then transfected into K562 cells via electroporation as described above. Cells were collected at 8h and 10h to process for mRNA expression and western-blotting as described for HeLa cells. Translation efficiency was calculated by dividing the protein band intensity (amount of protein) by the MNE of mRNA expression.

2.5 Figures and legends

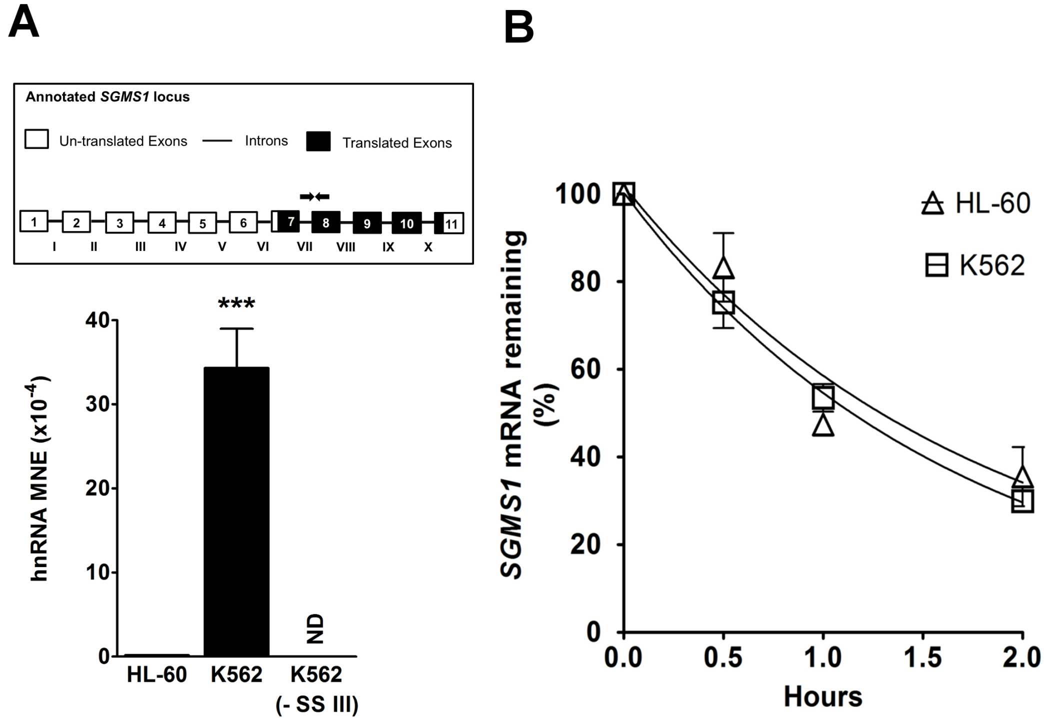


Figure 2.1: SGMS1 is transcriptionally up regulated in K562 cells. (A) SGMS1 transcription was assessed by quantifying hnRNA by qRT-PCR. Primers used target Intron VII-Exon 8 (primer sequences provided in table 2.1) to quantify hnRNA expression, normalized to β-actin and expressed as MNE. Primer locations are indicated on the SGMS1 locus. Negative control for cDNA synthesis (without Superscript III) in K562, shows no amplification, thus verifying the absence of genomic DNA. Results from three independent experiments are shown here. (B) K562 and HL-60 cells were treated with Vehicle (H_2O) or Actinomycin D (5 µg/mL) over a 2 hour time-course. SGMS1 mRNA abundance was measured at different time intervals by qRT-PCR using primers within Exon 7 (primer sequences provided in table 2.1) to quantify percent of remaining mRNA. Calculated half-life values are shown in figure. Asterisks indicate significance; *** $p<0.0005$. ND- Not detectable; SGMS1- Sphingomyelin synthase 1; hnRNA- hetero-nuclear RNA; qRT-PCR: quantitative Real-Time Polymerase Chain Reaction; MNE: Mean of Normalized Expression; mRNA: messenger RNA; SSIII: superscript III.

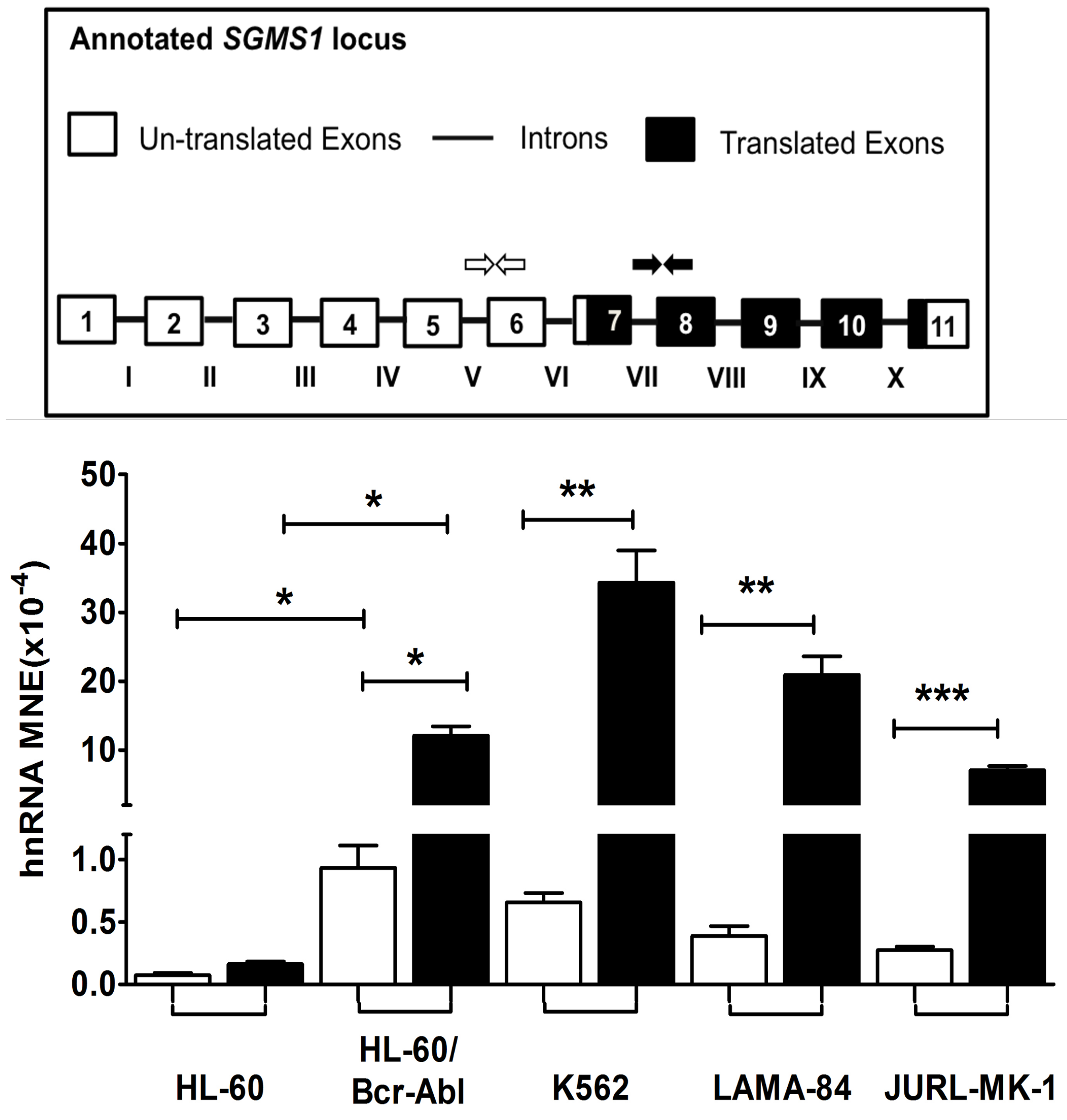


Figure 2.2: Bcr-Abl up-regulates SGMS1 transcription and shifts transcription initiation. Transcriptional up-regulation of SGMS1 was verified by qRT-PCR of hnRNA (normalized to β-actin) using two primer pairs, one designed to span Intron V-Exon 6 (white arrows and white bars) and a second primer pair across the junction of Intron VII-Exon 8 (black arrows and black bars) (primer sequences provided in table 2.1) in HL-60, HL-60/Bcr-Abl, K562, LAMA-84 and JURL-MK-1. Approximate primer locations are indicated on the SGMS1 locus. All results represent three independent experiments. Asterisks indicate significance; *** p<0.0005; ** p<0.005; *p<0.05

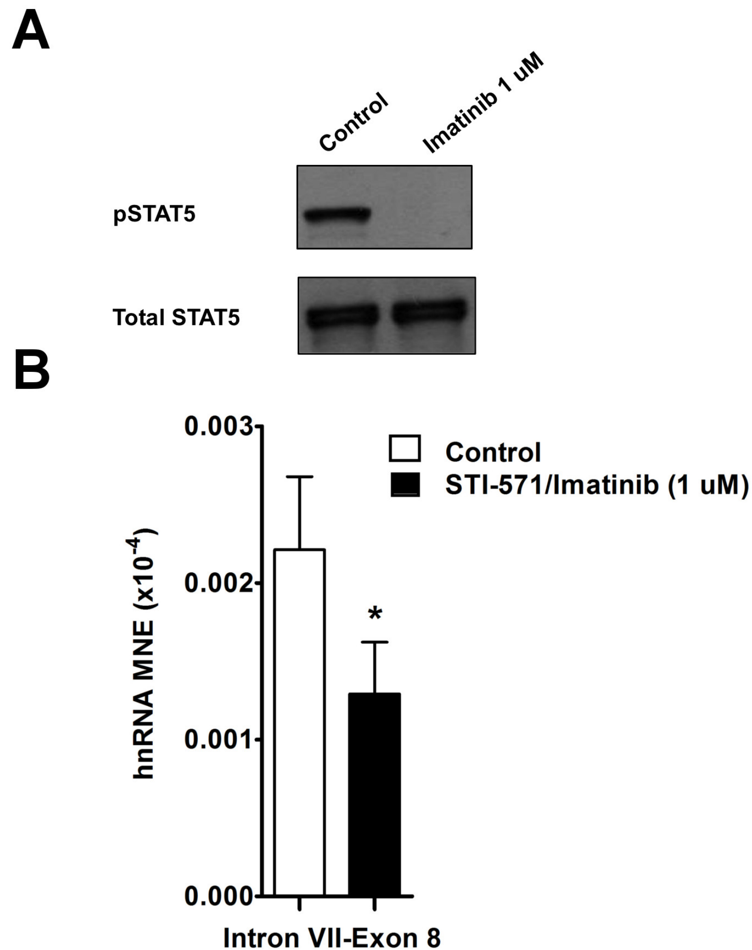
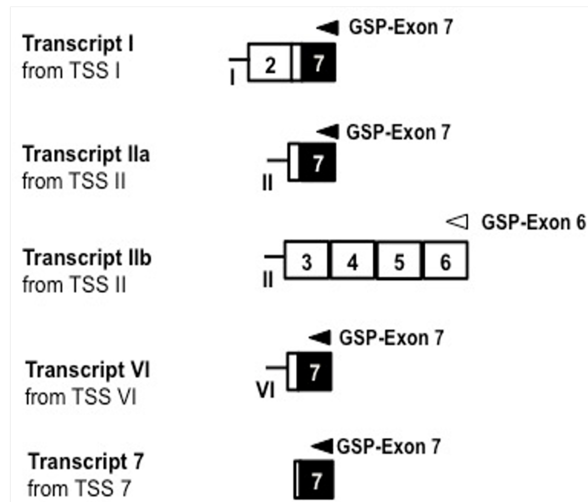


Figure 2.3: Bcr-Abl kinase activity regulates the transcription of *SGMS1.*(A) K562 cells were treated with Imatinib 1uM for 6 hours. Cells were harvested and lystaes prepared for Western-blot. Total STAT-5 and pSTAT5 levels were measured in control and treated cells. (B) In control and Imatinib treated cells RNA was extracted to measure hnRNA levels of *SGMS1.* Intron VII-Exon 8 specific RT-PCR primers were used. These results represent three independent experiments. Asterix indicate significance; *p<0.05.

A



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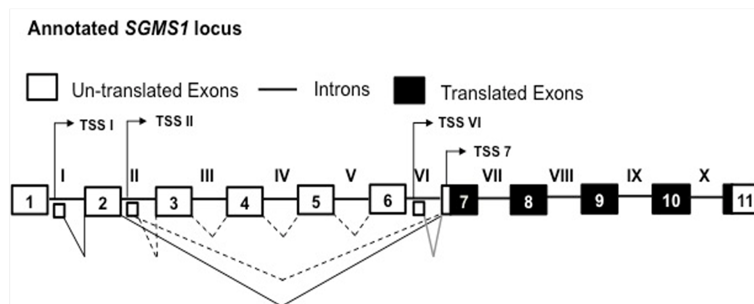


Figure 2.4: Identification of SGMS1 transcriptional start sites in K562. (A) Representation of the 5'RLM-RACE products of SGMS1. Five different transcript variants were identified and were annotated based on location of transcription initiation site, i.e. TSS I, TSS II, TSS VI and TSS 7. Gene-specific primers within Exon 7 (black arrow) and Exon 6 (white arrow) used to perform the 5'RLM-RACE in K562 cells are indicated above the respective exons. These results are representative of two independent 5'RLM-RACE experiments (sequences of clones are given in table 2.2). (B) Graphical representation of the four transcriptional start sites identified by 5'RLM-RACE positioned on the SGMS1 locus. Large white boxes with alpha-numerals represent annotated exons; straight connecting solid lines with Roman numerals represent annotated introns; small white boxes represent new first-exons identified by 5'RLM-RACE. Solid/dashed connector lines correspond to potential splicing events that result in mature transcripts as seen in figure above. RLM-RACE: RNA Ligase Mediated Rapid Amplification of cDNA Ends; TSS: Transcriptional Start Site; GSP: gene specific primer.

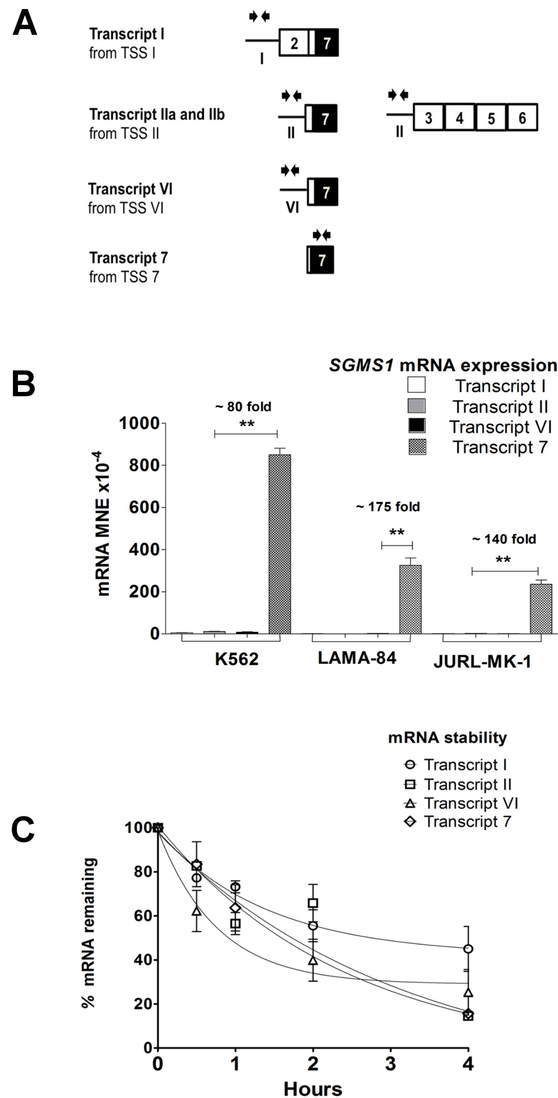
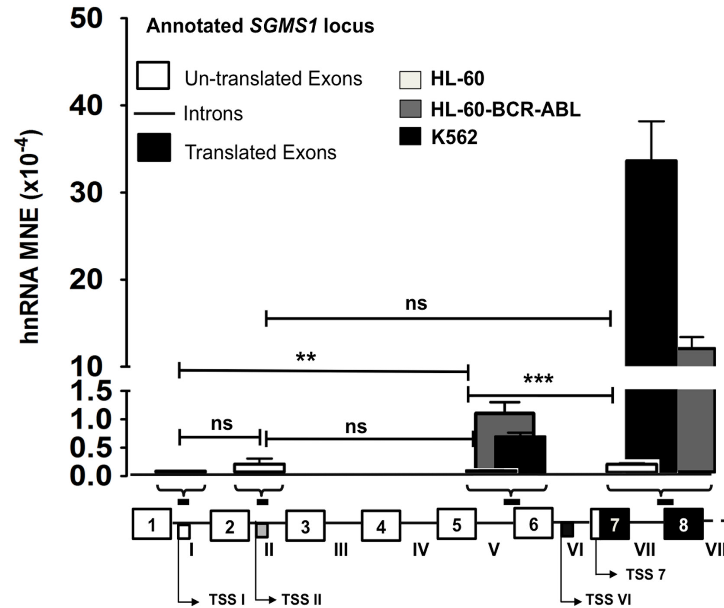


Figure 2.5: SGMS1 is transcriptionally up-regulated via TSS 7 in CML cells. (A) Representation of primers used for FEP analysis to quantify the transcriptional abundance from each TSS. Black arrows (facing each other, above each new first-exon) represent the location of primers at each new first-exon (primer sequences in Table 1). (B) Abundance of mRNA from each TSS measured using the FEP method in Bcr-Abl positive cells (K562, LAMA-84 and JURL-MK-1). Expression of mRNA was normalized to β-actin and expressed as MNE. (C) Stability of mRNA of the different transcripts in K562. Cells were treated with Vehicle (H_2O) or Actinomycin D (5 µg/mL) for different intervals over a 4 hour time-course (described in Materials and Methods). Abundance of mRNA was measured by FEP to quantify percent of remaining mRNA. Results represent three independent qRT-PCR experiments. There is not significant difference in the stability of different SGMS1 transcripts. Asterisks indicate significance; ** p<0.005; *p<0.05. FEP: First-Exon-Profiling by qPCR.



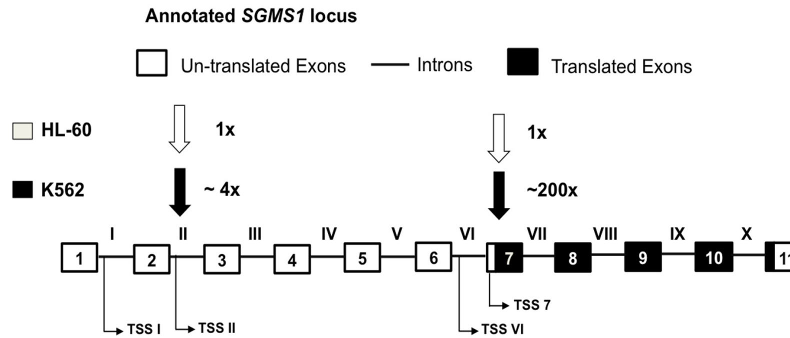
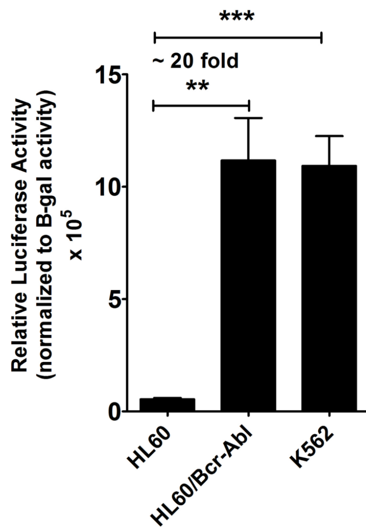


Figure 2.6: Bcr-Abl transforms the transcriptional landscape of SGMS1. (A) Bar-graph shows qRT-PCR data of hnRNA abundance with primers at Intron I, II, Intron V-Exon 6, Intron VII-Exon 8, in Bcr-Abl positive (K562 and HL-60-Bcr-Abl) and negative (HL-60) cells. Below the bar graph are shown the (approximate) regions on the SGMS1 locus targeted by the primers used (primer sequences in table 2.1) and the position of the different TSSs identified in K562. Results represent three independent qRT-PCR experiments. Asterisks indicate significance; *** $p<0.0005$; ** $p<0.005$. (B) Graphical representation of the transcriptional landscape of *SGMS1* in Bcr-Abl positive versus negative cells. Arrows indicate the utilization of different TSSs in Bcr-Abl positive and negative cells with fold-abundance compared to TSS II in HL-60 cells, as quantified from qRT-PCR data.



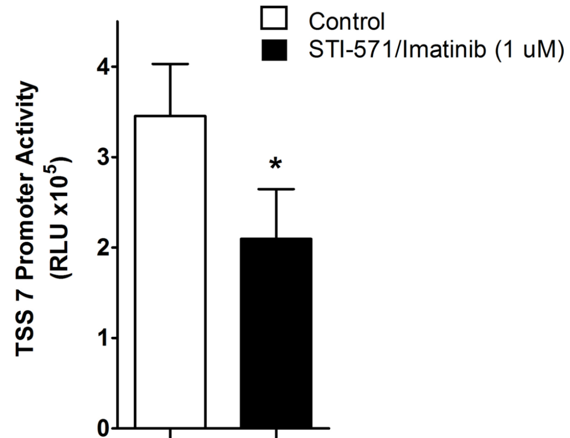
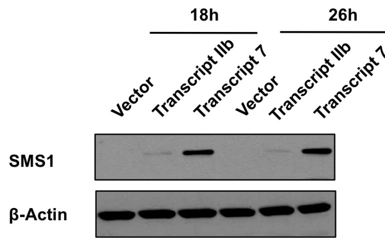
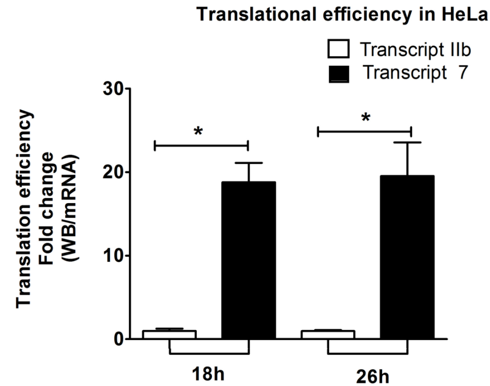


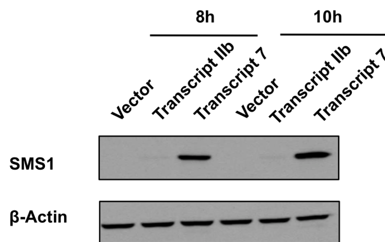
Figure 2.7: Identification of promoter up-stream of TSS 7 and its regulation by Bcr-Abl. (A) A stretch of 832 bp up-stream of TSS 7 was isolated from K562 genomic DNA and cloned into the pGL3-basic vector to assess promoter activity (construct sequence in table 3). HL-60, HL-60 Bcr-Abl and K562 cells were transfected with 5 µg of promoter-7 pGL3 constructs and pCMV-β-galactosidase plasmid vectors (transfection control). Promoter activity was measured by luciferase activity normalized to β-galactosidase (β-gal) activity, vector subtracted, expressed as RLU. Results represent three independent experiments. (B) TSS 7 promoter construct was transfected in K562 and treated with Imatinib for 8 hours. Cells were then analyzed for promoter activity. Asterisks indicate significance; ***p<0.0005; **p<0.005; *p<0.05. RLU- Relative Luciferase Units.

A **B**





C **D**



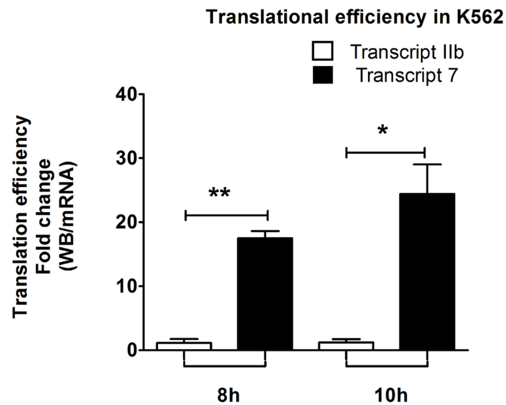


Figure 2.8: *SGMS1* mRNA from TSS 7 is translated more efficiently. (A) Representative western-blot of *SGMS1* protein in HeLa cells over-expressing either Transcript IIb or Transcript 7 (upper panel) (sequences in Table 2.4) and β-actin (lower panel). (B) Translational efficiency of the two transcripts was calculated by dividing the intensity of the western-blot band over mRNA, 10h and 26h post-transfection. The mRNA was quantified using transcript-specific primers as in FEP. (C) Representative western-blot of *SGMS1* protein in K562 cells over-expressing either Transcript IIb or Transcript 7 (upper panel) and β-actin (lower panel). (D) Translational efficiency of the two transcripts was calculated by dividing the intensity of western-blot band over mRNA 8h and 10h post-transfection. The mRNA was quantified using transcript-specific primers. All results represent three independent experiments. Asterisks indicate significance; *** $p<0.0005$; **$p<0.005$; *$p<0.05$.

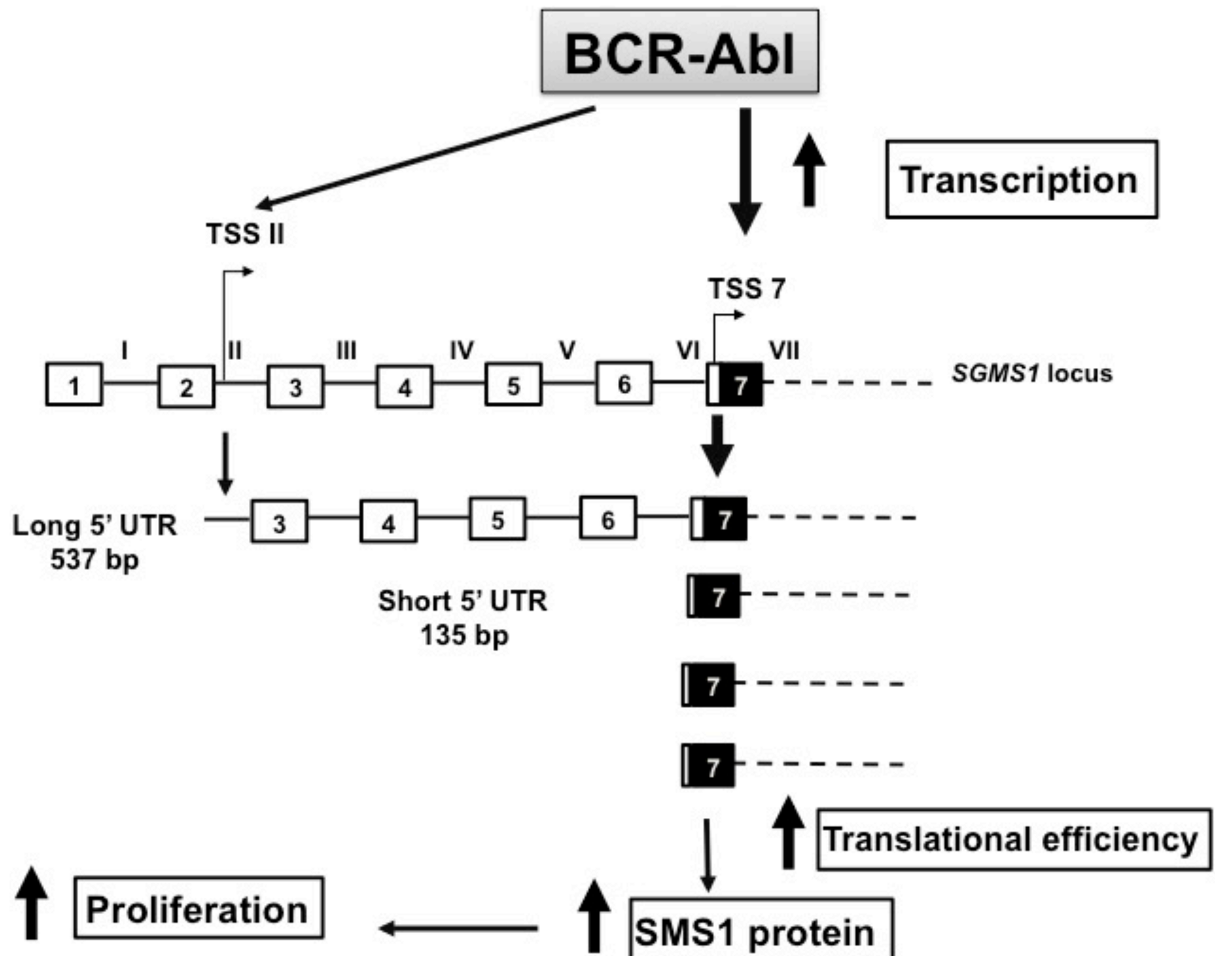


Figure 2.9: Model for the regulation of SMS1 expression by Bcr-Abl. Bcr-Abl up-regulates *SGMS1* mRNA by promoting transcription from 2 alternative transcriptional start sites, TSS II and TSS 7. However, in Bcr-Abl positive cells, maximum transcription of *SGMS1* occurs from TSS 7. The mRNA from TSS 7 is characterized by a very short 5'UTR (135 bp) which is devoid from translational inhibitory features present in the longer *SGMS1* 5'UTR found in Bcr-Abl negative HL-60 cells. The Bcr-Abl induced TSS 7 transcript is therefore translated more efficiently resulting in further increase of the SMS1 protein. Overall, Bcr-Abl exponentially enhances the expression of *SGSM1* by up-regulating the transcription of a specific *SGMS1* mRNA isoform that is translated more efficiently. This increased *SGMS1* expression supports Bcr-Abl mediated proliferation .

2.6 Supplementary figures

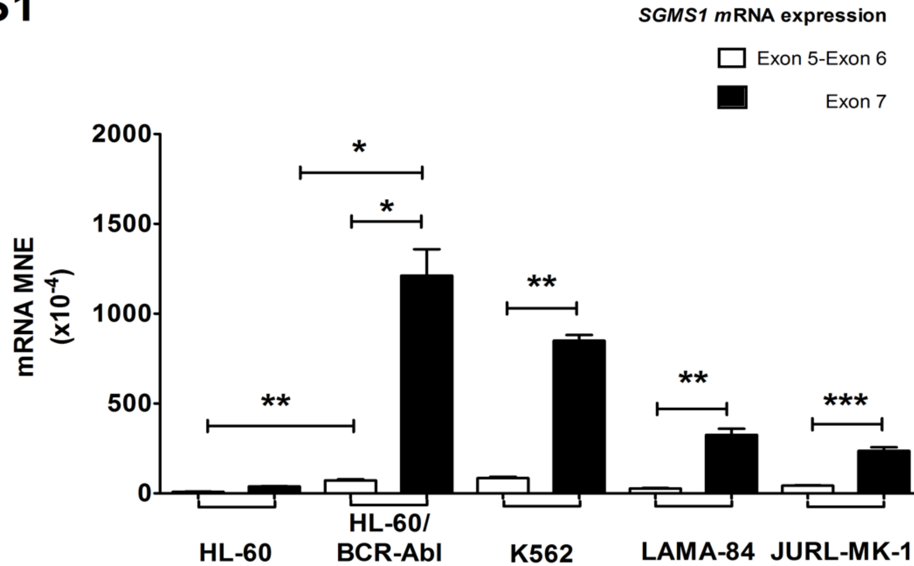
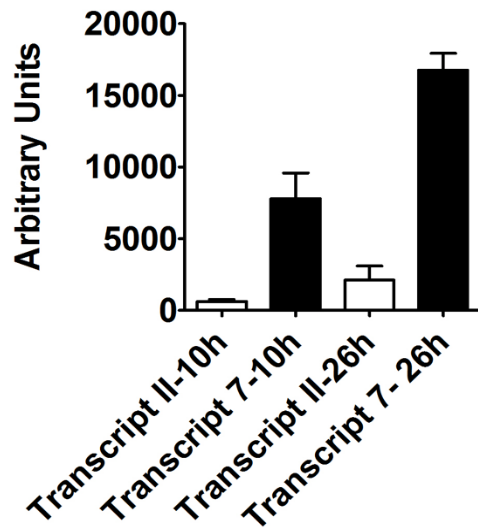


Figure 2.S1: mRNA expression of *SGMS1*. Abundance of mRNA was evaluated by qRT-PCR with two sets of primers spanning Exon 5-Exon 6 or within Exon 7(primer sequences in table 2.1). All results represent three independent experiments. Asterisks indicate significance; *** p<0.0005; ** p<0.005; *p<0.05.



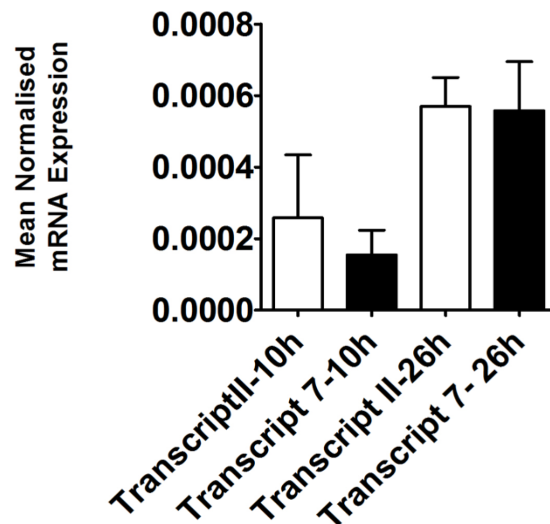


Figure 2.S2: Efficiency of translation of SMS1 from Transcript IIb or Transcript 7 in HeLa cells. HeLa cells were transfected with pCDNA3.1 to over-express either transcripts from TSS IIb or TSS 7. Cells were collected after 10h and 26h and processed for western blotting and mRNA measurements. (A) Quantification of SMS1 bands in western blots (vector subtracted) for three different experiments. (B) Measurements of mRNA abundance; for cells over-expressing TSS IIb transcript, primers from FEP Intron II were used while FEP primers for Exon 7 were utilized for cells over-expressing TSS 7 transcript.

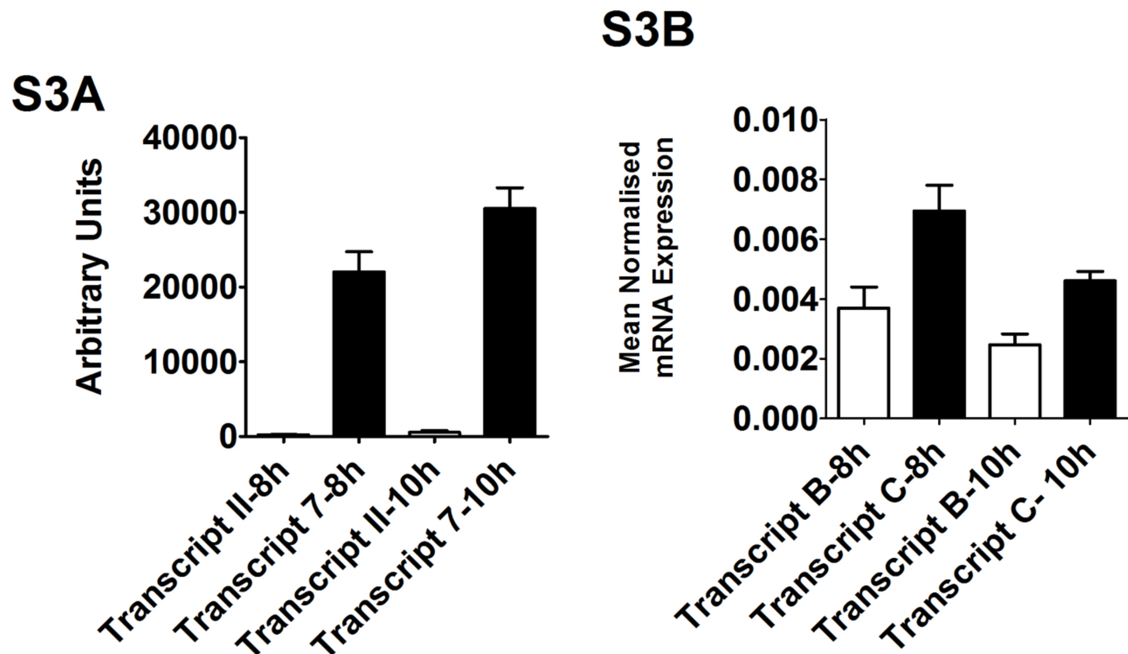


Figure 2.S3: Efficiency of translation of SMS1 from Transcript IIb or Transcript 7 in K562 cells. K562 cells were transfected with pEF1 to over-express either transcript IIb or transcript 7. Cells were collected after 8h and 10h and processed for western blotting and mRNA measurements. (A) Quantification of SMS1 bands in western blots (vector subtracted) for three different experiments. (B) Measurements of mRNA abundance; for cells over-expressing TSS IIb transcript, primers from FEP Intron II were used while FEP primers for Exon 7 were utilized for cells over-expressing TSS 7 transcript.

2.7 Tables

Table 2.1: Primers

Primer Set	Primer	Sequence (5' -3')
Intron VII-Exon 8	Intron VII-forward	ATTGAGTCATTGAAAAATTACAG
	Exon 8-reverse	GCCAACTATGCAGAAAAATC
Exon 7	Exon 7-forward	GCCAGGACTTGATCAACCTAACC
	Exon 7-reverse	CCATTGGCATGGCCGTTCTTG
Intron V-Exon 6	Intron V-forward	TGACTGGACTTTTCTATGTCTTGTG
	Exon 6-reverse	GCTACCAGCTTCTCCCATTG
Exon 5-Exon 6	Exon 5-forward	AAGACGAGAACCTGCCTCAGCA
	Exon 6-reverse	AGTTCCATCACCTGCAACAGCC
Intron I	Intron I-forward	CACCGTCTTAACCCGAACTC
	Intron I-reverse	TGACAACCCTTATCCCGATT
Intron II	Intron II-forward	AAGCAGGAAGATGTGAACTTT
	Intron II-reverse	TCCAAAATACATCTGGATTGGAG
Intron VI	Intron VI-forward	AAACTTGGTGCCCCAGGAAC
	Intron VI-reverse	GAGGCAGTGTCTCTGGAAAG
β- Actin	β- Actin-forward	ATTGGCAATGAGCGGTTCC
	β- Actin-reverse	GGTAGTTTCGTGGATGCCACA
Promoter - 7	Promoter 7-forward	TTCTGTGAGTTGCTGTATGTTATG
	Promoter 7-reverse	ATGGTCAGGGCAGTTTTTAAA

Table 2.2: 5'RLM-RACE products from K562

Adaptor sequence **Transcriptional start site**

TSS I (Clone: MP1-720 #10)

NNNNNNNNNNNNNNNGGGCGNNTTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGCCCTTGCCGGATCCGA
ACACTGCGTTTGCTGGCTTTGATGAAA**AAT**CGGCGCAGCCCTGAACTGGACAGTCTCTCCAGGACGCCGCTTTTCCGTGACAGAGGGGGTCTGCGCT
ATCCCCCAGCGACGTGTCAAGGGCAAAACAGCAGCAGCAGCGGCTGCCTCTCCCACCGTCTTAACCCGAACTCGGGCTTGTGGCGTCCTCCCGTGGG
CCGGATAGAGTGCCCTAGTCCAGCTAACGGATTCAGCCTGGAGCGTTCTGGTGAATGCTGCTTTCCTCTTTGGTGCCGCGAAGTAGAAATCGGGATA
AGGGTTGTCATGGCCGGAGGGAGCGAAGAGAATCCAGCGTGACGACAGCATGTACGATCCCTGAATGGGCCCGTGGGCATATTTAGTCATTCATTTT
TGATCAGGAGAAAGAAGACAGCCATCATTTGAAAGAAGGAAGAATCCTGCTCAAAAATGAGGTGAATTAATACTTGGGCGCTCAGGAACCCTGGACA
GCTACATGAGGTGTTTAAAAACTGCCCTGACCATCTTGCCAAACAAGTCTCTGCTCATGAGGCCCCACAGGATGAACGAGCCGAGGCAGGACAGTGT
CCTGCCCTGTCGGAACAGTGACTGCTGACCTGCCAAGAGAGAGCTGGGGACTGCCTGCTGTCTGCCAGTACAATGAAGGAAGTGGTTTATTGGTCAC
CCAAGAAGGTGGCAGACTGGCTGCTGGAGAATGCTATGCCAGAATACTGTGAGCCTCTGGAGCATTTCACAGGCCAGGACTTANGGNGAATTCCAGC
ACACTGGCGGGCCGTTACTAGTGGNTCCNAGCTCGGTACCAAGCTTGGCGTAATCATGGNCATAGCTGTTTCCTGNGTGAAATTGTNATCCGCTCNC
NATTCCNCNCAANNNTACGANCCNGAAGCNTNAAGNNNNAAANNCCTGGNNNNGCCTAATGN

TSS II (374) (Clone: MP8-R1 # 4a)

NNNNNNNNNNNTAGGGCGANTTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGCCCTTCGCGGATCCGAA
CACTGCGTTTGCTGGCTTTGATGAAA**AAA**GCAGGAAGATGGTGAACTTTATGCAATTCTGAGTTGACTCCAATCCAGATGTATTTTGGAACTAGGTA
GAATATGACTGGTAAAGCTTCAGCGACTGAAGGAGTGTGGACTTTTTGAATTTCAAGAACAGTAAAGTTGGAACACAGCAGAGGGTGTGTAATAAAA
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TGGAACTAAGGGCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCCGAGCTCGGTACCAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTG
TGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATT
AATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGT
ATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAANGGNGGTAATACGGT
TATCCACAGANCNGGGGANACGCNNNAAGANNATGTGANCAAAAGCAGCAANNCAGANNTAAAAGCNCGTNGCTGNGTTTTCNTNGNTCNNCCCCTG
ANNANCNTNNNAAATCNNNNCTCAGTNNANGNNGGNNAANNCCGANNGGACTNNNN

TSS IIb (Clone: MP450031210 #2)

TTNCCTGGGGTTATCCCCGGATTNTGGGGNATAACCGGTTTTCCCGCCTTTGAAGTAAGTGAAACCGCTTCGCCGCAGCCGAACGACCAAGGGCAAC
GAGTCANTAAGCGAGGAAGCGGAANAGCGCCCCANTACGCAAACCGCCTTTTCCCCGNGGGGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGT
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CGTATGTTGTGGGAATTGTGAGCGGATAACANTTTCACACAGGAAACAGCTATGACCATGATTACGCCAAGCTTGGTACCGAGCTCGGATCCACTAG
TAACGGCCGCCAGTGTGCTGGAATTCGCCCTTCGCGGATCCGAACACTGCGTTTGCTGGCTTTGATGAAA**AAA**AGCAGGAAGATGGTGAACTTTATG
CAGTTCTGAGTTGACTCCAATCCAGATGTATTTTGGAACTAGGTAGAATATGACTGAAGGAAGAATCCTGCTCAAAAATGAGGTGAATTAATACTTG
GGCGCTCAGGAACCCTGGACAGCTACATGAGGTGTTTAAAAACTGCCCTGACCATCTTGCCAAACAAGTCTCTGCTCATGAGGCCCCACAGGATGAA
CGAGCCGAGGCAGGACAGTGTCCTGCCCTGTCGGAACAGTGACTGCTGACCTGCCAAGAGAGAGCTGGGGACTGCCTGCTGTCTGCCAGTACAATGA
AGGAAGTGGTTTATTGGTCACCCAAGAAGGTGGCAGACTGGCTGCTGGAGAATGCTATGCCAGAATACTGTGAGCCTCTGGAGCATTTCACAGGCCA
GGACTTAAGGGCGAATTCTGCAGATATCCATCACACTGGCGGCCGCTCGAGCATGCATCTAGAGGGCCCAATTCGCCCTATAGTGAGTCGTATTCAN
TNNNNNNNNNNNNNNCNNNNNNNNNNT

TSS VI (Clone: 450-3-3-9 # 3)

ANNCCAGCAANNNGGCCTTTTNCGNTNCNGNCCTTTGCTGNNTTTGCTNNNCATGTTCTTNNNGNGTNTNCCCNGNTCTGNNGATAACCNTATTACC
NNCTTTGAGTGAGCTGATNCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCC
TCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAG
CTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGC
TATGACCATGATTACGCCAAGCTTGGTACCGAGCTCGGATCCACTAGTAACGGCCGCCAGTGTGCTGGAATTCGCCCTTCGCGGATCCGAACACTGC
GTTTGCTGGCTTTGATGAAA**TTC**CGCCCGCGCCAGGGCTGGCTGCGCGCACCCTGAGCGCGAGGAGCCGCGCGCAAACTTGGTGCCCCAGGAACTTT
CCAGGAGACACTGCCTCCGGGGACGAGAGACCAAGGAAGAATCCTGCTCAAAAATGAGGTGAATTAATACTTGGGCGCTCAGGAACCCTGGACAGCT
ACATGAGGTGTTTAAAAACTGCCCTGACCATCTTGCCAAACAAGTCTCTGCTCATGAGGCCCCACAGGATGAACGAGCCGAGGCAGGACAGTGTCCT
GCCCTGTCGGAACAGTGACTGCTGACCTGCCAAGAGAGAGCTGGGGACTGCCTGCTGTCTGCCAGTACAATGAAGGAAGTGGTTTATTGGTCACCCA
AGAAGGTGGCAGACTGGCTGCTGGAGAATGCTATGCCAGAATACTGTGAGCCTCTGGAGCATTTCACAGGCCAGGACTTAAGGGCGAATTCTGCAGA
TATCCATCACACTGGCGGCCGCTCGAGCATGCATCTAGAGGGCCCAATCNNNNNNNNNNNNNNNNNNN

TSS 7 (Clone: MP8-300 #5)

NNNNNNNNNNNNANNNGGCGATTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGCCCTTCGCGGATCCGAA
CACTGCGTTTGCTGGCTTTGATGAA**CTT**GCCAAACAAGTCTCTGCTCATGAGGCCCCACAGGATGAACGAGCCGAGGCAGGACAGTGTCCTGCCCTG
TCGGAACAGTGACTGCTGACCTGCCAAGAGAGAGCTGGGGACTGCCTGCTGTCTGCCAGTACAATGAAGGAAGTGGTTTATTGGTCACCCAAGAAGG
TGGCTGACTGGCTGCTGGAGAATGCTATGCCAGAATACTGTGAGCCTCTGGAGCATTTCACAGGCCAGGACTTAAGGGCGAATTCCAGCACACTGGC
GGCCGTTACTAGTGGATCCGAGCTCGGTACCAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACA
CAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAG
TCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTG
ACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCNGGGATNACGCNNNAGAN
ATGTGAGCAAANNCAGCAAAGGCCNGNAACCGTAAAAAGCCGCGTTGCTGGCGTTTTNCATAGGNTCCGCCCCCNGACNANCATCACAAAATCGACG
CTCAGTCNNAGNNGGNGAAANCCGNCNNNCTNNNANATNCNGNGTTCCCCNGGNNCTCCNNNNNGNGNNNNNCNNTNCGACCNNN

Table 2.3: TSS 7 promoter

Promoter up-stream of TSS 7 (832 bp)

TTCTGTGAGTTGCTGTATGTTATGTACATTTTTAAACATTCTTCACGAGGTAGAGTAAGCAAATATATTTTATTCTTTTAAATTATCTCAGGAAGCA
TGTAAGGACAAAGAGAATACCTGGTCTCTTGACCAGGTTGAAAAGTGTCCAAAGCACTGTCAAGTTGCTTCTTAGAAAATAGACCATTGGAAATTAT
TATTATTTTTTTTTTTTGAGAAGGAATCTCGCTCTGTCGCCCAGGCTGGAGTACAGTGGCTGATCTCGGCTCACTGCAAGCTCCGCCTCCCGGGTTC
ACACCATTCTCCTGCCTCAGCCTGGGCAGGAGTAGCTGGGACTACAGGCGCCCGCCACCACACCCGGCTAATTTTTTGTATTTTTAGTAGAGACAGG
GTTTCACCGTGTTAGCCAGGATGCTCTCTATCTCCTGACCTCGTGATCCGCCCGCCTCGTTCTCCCAAAGCGCTGGGATTACAGGCGTGAGCCACCA
CGCCCGGCTGACCGTTGGAAATTCTATAAAATATGTCTCCTTTGACAACATCCATTTTCAGATCCAATTCAATGTGTGTCGCAAAAGACTAATAACC
AGGAAATCAGTAGTCCCTGAAACGAATACATGTTTGAAAACTATTAAGTTTACCAATAACTGGTTTTCTACTTTCCTTATGTATATAAGGCTT*TGCG*
*TTTTGAGAGGGGTAAT*TTGGAAGTACAGCATCTTGATATTTTCTCTTGCTTTCACAGAAGGAAGAATCCTGCTCAAAAATGAGGTGAATTAATACTT
GGGCGCTCAGGAACCCTGGACAGCTACATGAGGTGTTTAAAAACTGCCCTGACCAT

Table 2.4: *SGMS1* Transcript IIb and 7 synthesized

mRNA Transcript - TSS IIb

AAAGCAGGAAGATGGTGAACTTTATGCAATTCTGAGTTGACTCCAATCCAGATGTATTTTGGAACTAGGTAGAATATGACTGGTAAAGCTTCAGCGA
CTGAAGGAGTGTGGACTTTTTGAATTTCAAGAACAGTAAAGTTGGAACACAGCAGAGGGTGTGTAATAAAATACAGATTGGAAAGAAGACTGATGAA
GAAAAATAACAGCATGATTCAGGCACACCATTGAGTTCTTTGGGATGCCCGAAAGTGTCTGGTTGGGAAACATGAAATAGAAGACGAGAACCTGCCT
CAGCAAGCGTACCTGAGAATACTTTTAAAGGAACCTGTTGATAAATGCAAAACAGTGGCTGTTGCAGGTGATGGAACTGTACTGGAACAATGGGAGA
AGCTGGTAGCTTATAGGAAGCCAAGATGACCAGAATGTTTTTAAATAATCCAAGGAAGAATCCTGCTCAAAAATGAGGTGAATTAATACTTGGGCGC
TCAGGAACCCTGGACAGCTACATGAGGTGTTTAAAAACTGCCCTGACCATCTTGCCAAACAAGTCTCTGCTCATGAGGCCCCACAGGATGAACGAGC
CGAGGCAGGACAGTGTCCTGCCCTGTCGGAACAGTGACTGCTGACCTGCCAAGAGAGAGCTGGGGACTGCCTGCTGTCTGCCAGTACAATGAAGGAA
GTGGTTTATTGGTCACCCAAGAAGGTGGCAGACTGGCTGCTGGAGAATGCTATGCCAGAATACTGTGAGCCTCTGGAGCATTTCACAGGCCAGGACT
TGATCAACCTAACCCAAGAGGATTTCAAAAAACCCCCCTTGTGCCGAGTCTCCTCTGACAATGGGCAGCGGCTCCTGGACATGATAGAAACCCTGAA
AATGGAGCACCATTTGGAAGCACACAAGAACGGCCATGCCAATGGGCACCTCAACATTGGCGTAGACATCCCCACCCCCGACGGCAGCTTCAGCATC
AAGATTAAACCCAACGGGATGCCAAATGGGTATAGGAAAGAGATGATAAAGATCCCCATGCCAGAACTGGAGCGCTCTCAGTACCCCATGGAGTGGG
GCAAGACTTTTCTGGCCTTTCTTTATGCACTTTCCTGTTTCGTTCTCACCACAGTGATGATCTCGGTCGTCCACGAACGAGTACCTCCTAAGGAGGT
GCAGCCTCCACTACCGGACACATTTTTTGACCATTTTAACCGGGTGCAGTGGGCCTTTTCTATTTGTGAAATTAATGGCATGATCCTTGTAGGACTC
TGGTTAATTCAGTGGCTGCTCTTAAAATACAAGTCTATTATTAGCAGAAGATTTTTCTGCATAGTTGGCACGCTGTACCTGTATCGGTGTATTACAA
TGTATGTAACTACACTCCCAGTACCTGGTATGCATTTCAACTGTTCTCCGAAGCTTTTCGGAGACTGGGAAGCCCAACTGCGAAGAATAATGAAGCT
CATTGCTGGAGGTGGCTTGTCTATCACTGGCTCTCACAACATGTGTGGGGACTATCTGTACAGCGGCCACACGGTCATGCTAACACTTACCTACTTA
TTTATCAAAGAGTATTCCCCTCGGCGACTCTGGTGGTATCACTGGATTTGCTGGCTTCTCAGCGTAGTTGGAATCTTCTGTATTCTCTTAGCGCATG
ACCACTACACTGTGGACGTGGTGGTGGCATATTACATCACCACGAGACTCTTCTGGTGGTATCACACTATGGCCAATCAGCAAGTGCTAAAGGAAGC
TTCCCAGATGAACCTCCTGGCCAGGGTGTGGTGGTACAGGCCATTTCAGTACTTTGAAAAGAATGTCCAAGGAATTGTACCTCGATCTTACCATTGG
CCTTTCCCCTGGCCAGTAGTCCACCTCAGTAGGCAAGTTAAATACAGCCGGCTGGTGAATGACACAGACTACAAAGACGATGACGACAAGTAA

mRNA Transcript - TSS 7

CTTGCCAAACAAGTCTCTGCTCATGAGGCCCCACAGGATGAACGAGCCGAGGCAGGACAGTGTCCTGCCCTGTCGGAACAGTGACTGCTGACCTGCC
AAGAGAGAGCTGGGGACTGCCTGCTGTCTGCCAGTACAATGAAGGAAGTGGTTTATTGGTCACCCAAGAAGGTGGCTGACTGGCTGCTGGAGAATGC
TATGCCAGAATACTGTGAGCCTCTGGAGCATTTCACAGGCCAGGACTTGATCAACCTAACCCAAGAGGATTTCAAAAAACCCCCCTTGTGCCGAGTC
TCCTCTGACAATGGGCAGCGGCTCCTGGACATGATAGAAACCCTGAAAATGGAGCACCATTTGGAAGCACACAAGAACGGCCATGCCAATGGGCACC
TCAACATTGGCGTAGACATCCCCACCCCCGACGGCAGCTTCAGCATCAAGATTAAACCCAACGGGATGCCAAATGGGTATAGGAAAGAGATGATAAA
GATCCCCATGCCAGAACTGGAGCGCTCTCAGTACCCCATGGAGTGGGGCAAGACTTTTCTGGCCTTTCTTTATGCACTTTCCTGTTTCGTTCTCACC
ACAGTGATGATCTCGGTCGTCCACGAACGAGTACCTCCTAAGGAGGTGCAGCCTCCACTACCGGACACATTTTTTGACCATTTTAACCGGGTGCAGT
GGGCCTTTTCTATTTGTGAAATTAATGGCATGATCCTTGTAGGACTCTGGTTAATTCAGTGGCTGCTCTTAAAATACAAGTCTATTATTAGCAGAAG
ATTTTTCTGCATAGTTGGCACGCTGTACCTGTATCGGTGTATTACAATGTATGTAACTACACTCCCAGTACCTGGTATGCATTTCAACTGTTCTCCG
AAGCTTTTCGGAGACTGGGAAGCCCAACTGCGAAGAATAATGAAGCTCATTGCTGGAGGTGGCTTGTCTATCACTGGCTCTCACAACATGTGTGGGG
ACTATCTGTACAGCGGCCACACGGTCATGCTAACACTTACCTACTTATTTATCAAAGAGTATTCCCCTCGGCGACTCTGGTGGTATCACTGGATTTG
CTGGCTTCTCAGCGTAGTTGGAATCTTCTGTATTCTCTTAGCGCATGACCACTACACTGTGGACGTGGTGGTGGCATATTACATCACCACGAGACTC
TTCTGGTGGTATCACACTATGGCCAATCAGCAAGTGCTAAAGGAAGCTTCCCAGATGAACCTCCTGGCCAGGGTGTGGTGGTACAGGCCATTTCAGT
ACTTTGAAAAGAATGTCCAAGGAATTGTACCTCGATCTTACCATTGGCCTTTCCCCTGGCCAGTAGTCCACCTCAGTAGGCAAGTTAAATACAGCCG
GCTGGTGAATGACACAGACTACAAAGACGATGACGACAAGTAA

Table 2.5: EST analysis of *SGMS1* transcripts

***SGMS1* transcripts**	**GenBank Accession (Organ/Cell-line)**
Canonical exon-1 containing mRNA	DA211727 (Brain), DB163207 (Thymus), DB240815 (Trachea)
TSS I	DC423654 (Uterus), DC415603 (Trachea)
TSS IIb	BX405356 (Jurkat cells), DB081510 (Testis)
TSS VI	DA113748 (Cerebellum), AL560778 (Ramos cells)
TSS 7	*Unique ESTs not identifiable*

ESTs that map to the transcripts identified by 5’RLM-RACE in K562 cells. These ESTs have all the unique splice junctions as found in the K562 transcripts.

Table 2.6: Analysis of *SGMS1* transcripts 5'UTRs.

Transcript type	Length (bp)	uORFs	5' TOP	Free energy estimates	GC Content (%)
TSS I	627	2	No	-213.42	55.4
TSS IIb	670	1	No	-158.70	44.9
TSS VI	340	1	No	-103.90	58.8
TSS 7	135	0	No	-46.20	59.2

Analysis of the different 5'UTRs of *SGMS1* transcripts found in K562 cells for features known to affect translation. The 5'UTRs of annotated canonical *SGMS1* mRNA transcript and the four others identified here were analyzed for various features. uORF's were identified using uORFdb. 5' TOP were manually assessed. Free energy estimates and percent GC content were evaluated using mFOLD(375).

Appendix A: Identification of other genes that show a Bcr-Abl mediated shift in transcription initiation with translational consequences

In Chapter 2, I show that Bcr-Abl causes a reprogramming of the *SGMS1* transcriptional landscape. Bcr-Abl promotes the transcription initiation of *SGMS1* from four alternative start-sites, however it up-regulates the transcription of *SGMS1* primarily from TSS 7. As a consequence of this, the mRNA produced has a short 5' UTR with a 20-fold greater translational efficiency. I attribute this advantage to the lack of up-stream ORFs that decrease the rate of translation of a given transcript.

Next I wanted to identify if this unique oncogene-mediated mechanism to increase expression of SMS1 was relevant for the expression of other genes, using a genome-wide approach. The following results are the preliminary efforts for the identification of other genes, which show a regulation similar to SMS1. These results are a continuation of Chapter 2, however they require further investigation (described below).

A.1 Results

A.1.1 Identification of proteins up regulated by Bcr-Abl

The mechanism described in Chapter 2 involves a Bcr-Abl mediated shift in transcription initiation resulting in increased protein expression. In order to identify other genes that may be regulated in a similar manner, I first identified, through a proteomics approach, all those proteins that are elevated in a Bcr-Abl-mediated fashion. Herein I extracted proteins from K562, HL-60 and HL-60/Bcr-Abl cells and performed trypsin mediated digestion followed by a fractionation of the peptides generated **(method described in Chapter A.3)**. Fractionated peptides were then identified by mass spectrometry (MS), performed at the Stony Brook Proteomics facility by Justin Snider. Protein abundance at a 90% confidence was then evaluated using normalized spectral abundance factor (NSAF) **(method described in Chapter A.3)**. As shown in **Figure A.1(A),** the two experiments resulted in the identification of about 3000 proteins. **Figure A.1(B),** shows the proteins identified in each cell line and the number of proteins that are common between cell-lines. It can be seen that in all three cell lines, 1615 proteins are commonly detected. Two hundred and thirty two proteins are detectable only in the Bcr-Abl positive cell lines, K562 and HL-60/Bcr-Abl. **Figure A.1(C)** is an analysis of all the gene ontology (GO) terms associated with the proteins identified in all the three cell lines.

A.1.2 Identification of protein over-expressed in Bcr-Abl positive cells

Next, I identified those proteins that are exclusively up regulated in Bcr-Abl positive cells. For this, proteins were normalized to the NSAF value of Actin. Proteins that showed a 5 fold greater expression in Bcr-Abl positive cells (both K562 and HL-60/Bcr-Abl) or undetectable in HL-60 cells, were demarcated as having an increased expression **(method described in Chapter A.3)**. After such a NSAF based sorting 451 proteins identified were undetectable in HL-60 cells but were detectable in Bcr-Abl positive cells **(Table A.1)** and 215 proteins, had a 5-fold greater expression in Bcr-Abl positive cells compared to HL-60 **(Table A.2)**.

A.1.3 RNA-sequence analysis of HL-60 and K562

Next, I evaluated the distribution of RNA-seq reads for those genes identified above across their respective gene loci. K562 and HL-60 RNA-seq reads **(datasets used provided in Table A.3)** were mapped to the human genome **(method described in Chapter A.3)** (mapping done by Dr. Kaustav Mukherjee). Visualization of RNA-read abundance was done using the IGV visualization software. Each protein that was over-expressed in Bcr-Abl cells was individually evaluated using this software. Genes were classified as having a potential shift in transcription initiation if an accumulation of reads was observed down-stream of its annotated exon-1 in Bcr-Abl positive K562 cells but not in HL-60 cells.

This is exemplified for the gene Rock-1 in **Figure A.2**. As it can be seen, the RNAseq distribution of the Rock-1 gene in K562 cells shows an accumulation of reads down-stream of exon-1 closer to the translational start site **(Figure A.2 top panel)**. This is similar to the situation in *SGMS1* wherein TSS 7 lies close to its translational start site. In contrast, in HL-60 **(Figure A.2 bottom panel)** it is seen that the read distribution is more or less equal across the entire exon-1. Thus I analyzed the identified targets and identified the following potential candidate genes: TLE3, Rock-1, Rab10, ZNF800, PLD3, S100A13 and CDKN2C.

A.2 Conclusions

Preliminary analysis shows that Bcr-Abl is capable of mediating a shift in transcription initiation not only for *SGMS1* but potentially for other genes as well. Moreover this shift in transcription initiation results in an increased abundance of proteins in the Bcr-Abl positive cells. Of the 211 genes analyzed using this method, 3.5% of the genes show a shift in transcription initiation with a translational consequence. In relation to the shift in transcription, I hypothesize three possible oncogenic-driven mechanisms that could alter protein expression and these are:

1. Mechanism 1 (SMS1-like, increased transcription and translation): herein Bcr-Abl mediates a shift in transcription initiation from a down-stream TSS and up-regulates transcription from it. The resultant mRNA has a shorter 5' UTR compared to its canonical mRNA and therefore is translated more efficiently.

2. Mechanism 2 (increased translation): Bcr-Abl may shift transcription initiation to a down-stream alternative TSS without increasing transcription from it. However the shift in transcription initiation is sufficient to increase translational efficiency of the mRNA and therefore protein abundance in Bcr-Abl positive cells.

3. Mechanism 3 (decreased translation): Bcr-Abl may shift transcription initiation to a more up-stream start site. This could potentially generate an mRNA with a longer 5' UTR that is translated much slower and therefore there is a down-regulation in protein expression. This subset of genes includes possibly tumor suppressors and in normal condition their transcription is initiated from a TSS that is down-stream.

A.3 Methods

Proteomics Analysis

Cell preparation

Cells were harvested in 5% SDS containing 50mM Ammonium Bicarbonate. Cell lysate was sonicated with nano-tip sonicator for 15 second then centrifuged at 4°C and 10,000g for 10 minutes. Protein was quantified and 200 ug was aliquoted for trypsin digestion. Protifi S-trap MS sample Prep Columns (376) were utilized follow established procedures that included: MTT reduction, followed by TCEP alkylation, Trypsin digest at a substrate ratio of 10:1, and multi-step elution and dried to 1ul in a Thermo CentriVac. Samples were then brought back to volume in 100ul HipH (20mM ammonium formate pH 9 in water) Mobile phase A and sonicated into solution. 100ug of protein digest is then loaded onto a Phenomenex Gemini-C18 (3 µm, 110 Å, 1 × 250 mm) and separated utilizing gradient fractionation. Fractions were collected at 1 minute intervals and fractions 15-55min were later recombined in a concatenated fashion, to generate 8 samples. Samples were dried on a CetriVac and brought to volume in 0.1% formic acid in water. 1ug of peptides were then analyzed by nLC-MS/MS on a Eksigent NanoLC 400 couple directly to an ABsciex 5600+ TripleTOF mass spectrometer.

nLC-MS/MS analysis

Peptides were separated by reverse phase chromatography utilizing a 10cm Phenomenex peptide Aeris XBC-18 1.7uM column at a flow rate of 300nl/min using a 90 min discontinuous gradient of ACN as follows: 1% for 5 min, 1% to 20% B over 45 min, 20% to 40% B over 40 min (Mobile phase A: 0.1% formic acid in water, Mobile phase B: 0.1% formic acid in 97% ACN). The mass spectrometer operated in Data-dependent

(IDA) acquisition mode. A single acquisition cycle comprised a single full-scan mass spectrum (m/z = 400 – 1800) in the TOF, followed by CID fragmentation on the top 20 most intense precursor ions.The following methods parameters were used: Charge state 2-4 exceeding 200cps, exclusion of former target ion for 15sec,rolling collision energy. The following instrument parameters were used: Ion source gas 1 was set at 2, Curtain gas set to 30, ionSpray voltage set at 2200.

Data processing and analysis

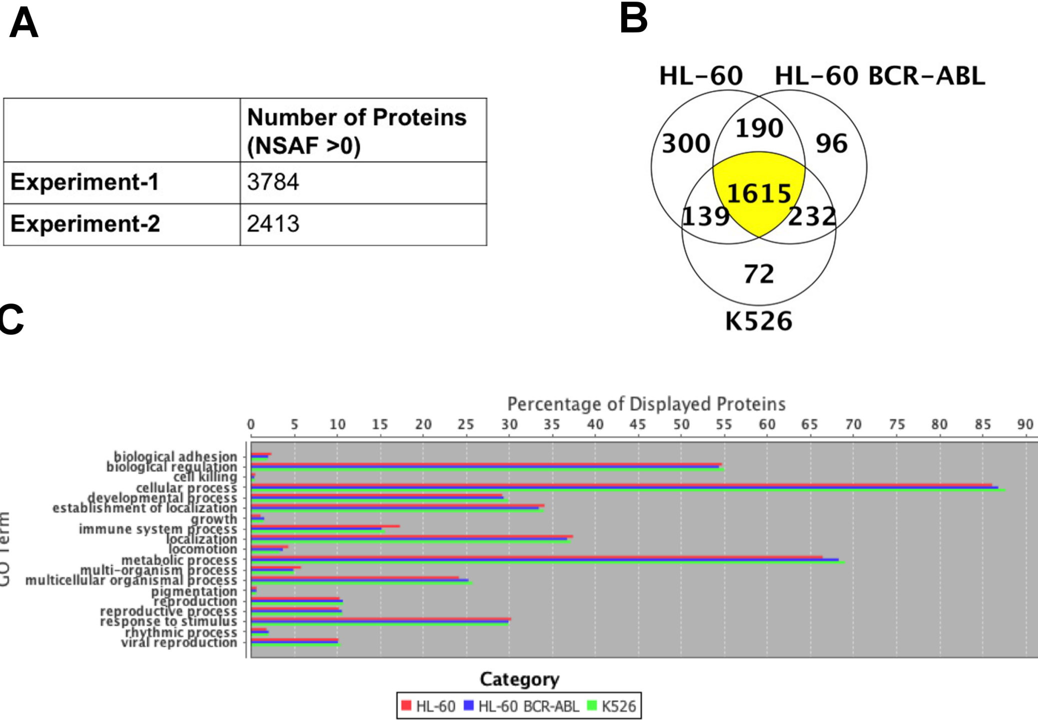
MS/MS spectra from raw files corresponding to single biological samples (N=2, 8 HipH fractions each) were extracted/submitted to Protein Pilot (AbSciex) for database searching against the UniProt SwissProt sequence human proteome database (11/2016) containing the subset of human, herpesvirus, and common contaminant sequences (21,570 entries). Spectra were searched against indexed peptide databases, generated from the forward and reverse protein sequence entries, using the following settings: Biological modifications for ID Focus, thorough ID as search effort, a FDR of 90%, a False discovery rate analysis. Maximum of 3 missed cleavages, parent and fragment ion mass tolerances of 50 ppm and 0.5 Da, respectively, static modification of methylthio (+46 Da), variable modification of methionine oxidation (+16 Da). Peptide spectrum matches (PSMs) were then loaded into Scaffold (ver. 4; Proteome Software, Inc.) and post-search validation performed using using X!Tandem (GPM 2010.12.1.1). The high mass accuracy search option was enabled. PSMs from all three cell lines were loaded into a single Scaffold session and confidence filters were selected at an FDR of < 1%: 99% protein confidence, 90% peptide confidence, minimum of 2 unique peptides per protein in at least one biological samples. Proteins passing these filters were

analyzed with respective “normalized spectral abundance factor (NSAF)”. NSAF was chosen as it takes into account protein variability and size between runs to normalize relative protein abundance between samples (377).

RNA-seq Analysis

Reads were extracted from NCBI GEO database using the sratoolkit. The extracted fastq files were aligned to human genome build hg38 using STAR with default parameters for a human genome(378). The aligned reads were visualized using Integrative Genomics Viewer (379). Read counts were generated using kallisto, which uses an alignment-free algorithm to generate read counts directly from fastq files (286). Counts were normalized using the R package DESeq2, which uses a negative binomial model for variance estimation, and subsequent normalization (380). DESeq2 was also used to determine differentially expressed genes between K562 and HL60 cell lines.

A.4 Figures and Legends



	Number of Proteins (NSAF >0)
Experiment-1	3784
Experiment-2	2413

Figure A.1 Proteomic analysis. (A) Table showing the total number of proteins identified in two experiments in all three cell lines, K562, HL-60 and HL-60/Bcr-Abl. The number of proteins identified are those that have an NSAF value greater than 0. (B) Venn diagram showing the proteins identified which are common and unique to each cell line. (C) GO term analysis of all the proteins identified.

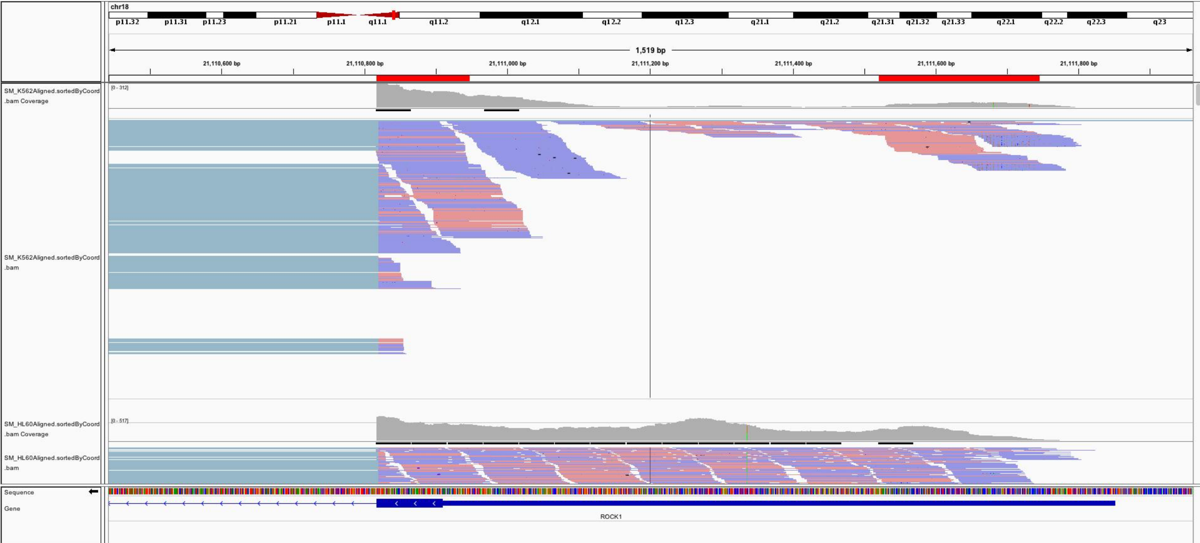


Figure A.2 Rock-1 RNA-seq reads. RNA read distribution for Rock-1 in K562 (top panel) and HL-60 (bottom panel).

A.5 Tables

Table A.1: Proteins detected in Bcr-Abl positive cells but undetectable in HL-60 cells.

HBE1,HBZ,ANXA2,CISD1,HSPB1,KRT18,MAPK1,PSMD5,FSCN1,OAT,GAGE1,LSM3,HBA1,ATP5I,PYCR1,GLOD4,NQO1,FTH1,CKB,POR,STAT5A,PDLIM1,SERPINH1,PAGE5,MYL4,FAM136A,PKLR,MAGEB2,CCNB2,HMBS,ISOC1,UAP1,PRDX2,AK1,TUBB3,AUP1,ALDH1A2,SAMSN1,NUP35,SBDS,LYRM7,SCAMP3,UBAC1,ADD1,NGDN,TBCE,ECI2,STAU1,FLNC,ZYX,TRMT112,RFC2,FLOT1,AKR1C1,PTRF,BLVRB,CBSL,CDC5L,PIP4K2A,ISYNA1,RBM34,TPX2,SMIM1,CRYZ,PTP4A2,DECR1,CDC26,COX6B1,PPIL2,ARMC6,DYNLL2,BANF1,LYRM4,RNH1,HIGD1A,SERPINB9,FUNDC2,S100A13,THOC7,TPD52L1,PCBD1,GOSR2,ATPIF1,PYCR2,RBX1,TUBB2A,ANK1,MYH10,GABARAPL2,KIF2A,COX20,GRSF1,UROD,CMBL,BAG3,RCL1,GSPT1,DNAJC8,CLIC4,SNCG,NDUFB6,USE1,PEA15,PIN4,PAFAH1B1,ZW10,HAT1,FECH,RBBP4,FLOT2,PSMD1,KIFC1,RDX,CDK5,PAGE1,DDX20,KHDRBS1,DUSP23,CPPED1,PKP3,POLR1E,CCDC47,PBK,MRPL1,CORO1B,FDXR,SPTA1,GLS,NDUFAF2,DCAF7,SNX12,CETN2,SAE1,DCTD,CPNE3,ACSL3,CCDC115,NUDT4,SURF6,SH3GLB1,SH3GL1,PPP4R1,RAB3IL1,HIBCH,HGH1,MARCKSL1,EXOSC1,SPC24,APOO,PLK1,RRAS2,MRPS26,RAB1A,PTPN2,MKRN2,GMNN,AGK,SMAP2,EPB41,ERAL1,CHMP4A,DCAF13,EIF2B3,FAM207A,GOPC,CCDC6,RCOR1,PLD3,RNF138,ROCK2,NCAPH,CHTOP,PRAME,NUP160,CHD1,SKIV2L2,SPR,EIF2B4,COPS7B,AHCYL1,UQCRFS1,GCLM,CTTN,CLCC1,YRDC,RPP38,CDC45,COIL,STX3,MLEC,SGF29,BLVRA,TATDN1,ENDOG,SLC25A4,PPIE,PPT1,APOE,AIMP2,PICALM,MLST8,CBFA2T3,GRHPR,GFI1B,RABGGTB,CDC123,NT5C3A,DCPS,SLC25A13,UBIAD1,GNB2,TGM2,AURKB,HMG20A,EIF2B2,CLPB,CAST,MCUR1,IST1,SMARCC1,LARP4B,PRPSAP2,GLUL,GPN1,MRPL38,CERS2,WDR74,IDH3B,SMARCB1,BMP2K,MRPS9,XPO5,SCCPDH,WDR18,COPG2,GCDH,EXOSC10,CPOX,NARF,NFS1,PPHLN1,PWP2,NUCB1,VPS11,HDAC7,KIF23,CTNND1,HEMGN,SRSF4,PKMYT1,MOV10,ZC3HC1,CTU2,PREX2,STAM,BAIAP2,RIOK2,AGFG1,ORC2,NUP133,CDKAL1,TRAFD1,FIP1L1,UTP6,IGF2BP2,ANAPC7,PPP2R1B,UBQLN4,WDR46,PPP1R18,KIAA0907,KIF4A,VPS33B,ETFDH,TOX4,KEAP1,CCDC93,DARS2,PRKCB,NDC1,NCAPD2,ADD2,PLOD1,PLOD3,LIMA1,YME1L1,STAT5B,GTF3C4,TFIP11,EPS15L1,DNM1,ACO1,CTNNA1,MLLT4,WDR44,MAP1A,WDR3,UNC45A,GOLGA2,EPB41L2,KIF11,SUGP2,BUB1,FHOD1,CTR9,PC,PUM1,TJP2,MTR,BCR,SYMPK,BMS1,AGL,SCRIB,TJP1,LTN1,APOB,NBAS,MACF1,RAP1GDS1,CPSF2,LGALS1,GINS3,SPTB,PYCRL,UBE2M,PHF19,TBCC,DHRS4,RER1,ABL1,MRPS33,DRG2,SCO1,YIF1B,WDHD1,THUMPD1,RPAP3,HEXB,CDCA5,ANKMY2,QPRT,QDPR,TPMT,NUF2,SETD2,TIMM23,UTP3,CNP,LAS1L,CWC15,MLH1,DHX16,IQGAP2,RAB13,NEFH,CDK9,HSPA14,NOL9,ADO,NDUFB3,ATOX1,WDR75,ARL2,GEMIN6,WDR82,KNOP1,TOR1AIP2,MFN2,TEX10,PCF11,FAM129B,CSNK1A1,ELAC2,GTF2E2,ASPH,SMS,ETNK1,NCDN,AIFM2,YLPM1,REEP6,PXN,DSG2,SPAG5,TRNT1,CRLF3,MAGED2,TAF6,FASTKD2,MPZL1,RANBP9,GGPS1,CASP3,DBN1,TCEB3,CTSB,RPF1,CMAS,HSDL2,WBSCR22,DPP9,FGFR1OP,PGM3,HMGN4,TMEM106B,GTF2H3,AMFR,MAZ,PTPN12,ALG13,ARFGAP3,CTH,CLASRP,DYNLT1,ELF2,EML2,HMGN2,ISY1,GUK1,KTI12,NCOR1,NDUFB7,NDUFB9,NES,SF3B5,SHTN1,STX4,TRPV2,UNC119B,UBA5,TRAPPC1,USP3,GABARAP,SMAD2,RNF14,DNAJC1,CDC42BPA,ATL2,FLYWCH2,MRRF,ENPP3,ASMTL,CWC22,PRKAG1,MRPL16,ATP2B1,SRA1,GALNT2,RAD23A,XRCC1.

Table A.2: Protein with an expression 5-fold or more in Bcr-Abl positive cells compared to HL-60 cells.

PABPC4,CRKL,ATP5D,SERPINB6,VIM,TBL3,MRPS36,NQO2,MAP4,PDLIM5,GFPT1,CHERP,ACACA,NUP214,CLUH,NUDT5,DHCR7,CYCS,DHX30,LAMTOR1,CD2AP,AK6,FLNB,MRPS34,HSPA1A,EIF3F,SFXN1,NOP16,SRSF10,DAP3,MPC2,SDF4,NRBP1,POLR1C,TUBB6,RRS1,NUP93,TFB2M,TXLNG,SDHA,LRRC47,HK1,ALDOC,TNIK,RNF40,PDCD10,FKBP8,EIF2S3,MRPS7,NUP153,MDN1,HDDC2,NUP107,XAB2,DIMT1,THOP1,CSTB,HNRNPDL,ATP6V1A,KLC1,USP15,AAMP,NOC4L,UQCRQ,TMUB1,PPP2R1A,SCAF11,PGAM5,ACAT2,EMC1,SPAG9,PELP1,RPA1,UBR4,ATL3,PTPN1,DPM1,AK4,RALA,OXSR1,ACY1,PPME1,HBG1,SARS,RRP1,ACTL6A,BRIX1,PSMA5,PPP2R2A,DIABLO,COPS4,RPL21,FH,EZR,NUP62,PSIP1,COPS3,VAPB,DYNLRB1,RAB3GAP1,EMD,NDUFA6,UBLCP1,NRDC,QKI,PPP2CA,RPS6KA1,FAM98B,EIF1AD,ERMP1,TUBG1,ZNF24,ABHD14B,DCTN1,LTV1,VDAC3,SRPK1,DDX56,HACD3,WDR36,SLC2A1,RACGAP1,EIF2AK2,CCDC124,TRIP13,PRPS1,CPT2,ACADM,ADI1,HDDC3,P3H1,AP3B1,PIN1,FTSJ3,PPP1R12A,HSPA4,LMNA,KPNA2,PMPCB,GSTK1,EIF5B,PAFAH1B3,SNAP29,EIF1AX,PRPF19,SCFD1,SAR1A,NAMPT,RTF1,HEATR1,OGDH,FAM50A,PPP1CC,RANBP2,PFKP,EIF2B1,DNAJB1,U2SURP,UBE2L3,PTBP3,KIF2C,SRRM2,APRT,GEMIN5,DNAJC7,MAP2K2,YTHDF2,MPP1,HIST1H1D,AHCTF1,UBE2T,RBM22,RTN4,DDX19A,TXNDC17,ATAD3B,TNPO1,RELA,EXOSC9,GLTSCR2,RRP12,PFDN1,CMPK1,TROVE2,RBM28,RAE1,IGF2R,UBA6,CEP170,CPSF7,CASP2,ZC3HAV1,RBM14,TELO2,SAFB,LAMTOR4,ADH5,NUP37,NUDCD2,CLSPN,UBR5,PPAN,SEC31A,HK2,ETF1,MFAP1,TXNDC12,CTNNBL1,SMARCE1,EIF3G,MAT2A,ZNF787,AP2M1,PFDN4,NDUFB10.

Table A.3: Dataset used for RNA-seq analysis

Cell line	Datasets used
K562	ERR1855448, ERR1855449, ERR1855450, K562 (Sitapriya Moorthi's dataset)
HL-60	SRR3302979, SRR3302980

CHAPTER 3

GATA-1 REGULATES THE TRANSCRIPTION OF *SMS1* FROM TSS 7

3.1 Introduction

The previous chapter, identifies three important aspects about the regulation of *SMS1* expression, firstly Bcr-Abl initiates the transcription of *SGMS1* from four start sites in CML cell lines. Second Bcr-Abl up-regulates the mRNA expression of *SGMS1* predominantly from TSS 7. Third, the shift in transcription initiation and up-regulation of *SGMS1* from TSS 7 generates an mRNA containing an extremely short 5' UTR, which enhances translation. As discussed before, the regulation of *SGMS1* at the transcription level is highly complex. Previous work from our lab and others has described the diversity in expression and distribution of *SGMS1* mRNA in normal and disease cells and tissues. However no study thus far has clearly elucidated the molecular mechanisms involved in the regulation of *SGMS1* gene expression. This chapter elucidates the mechanism involved in regulation of transcription of *SGMS1* from TSS 7, the start site responsible for the majority of *SGMS1* transcription in the context of CML.

It is estimated that there are about 20,000 coding genes, transcribing for almost ten times as many different RNA isoforms which in turn account for about 250,000 – 1 million proteins (Ensembl). This vast repertoire of RNA isoforms is generated through various epigenetic, genetic, transcriptional and post-transcriptional regulatory mechanisms. At the transcriptional level, alternative transcription initiation is one of the key mechanisms for generating RNAs with different 5' leader sequences (306).

The consequences of generation of different varieties of mRNA are diverse, however in the context of *SGMS1*, I have shown that the impact of this alternative mRNA isoform results in an increased protein abundance. However the major gap that remains to be addressed is the understanding of the precise mechanism that mediates

the utilization of this alternative *SGMS1* TSS. In this chapter, I identify the transcription factor (TF) that binds to the promoter of *SGMS1* TSS 7 and mediates its transcription. As described in **Chapter 1.2,** SMS1 expression and activity is associated with several different cancers and plays a significant role in maintenance of their oncogenic phenotype. However until this day, there has been no clear identification of an up-stream molecular regulator, which controls the expression of *SGMS1*.

The role of SMS in cancer has been addressed in the context of different cancer types or in the context of cell functions whose deregulation could promote cancer. It has been shown that SMS activity is associated with proliferation and apoptosis (73,79). For example in HeLa cells it was shown that both SMS1 and SMS2 expression are required to sustain cellular proliferation of the cell line (58). The primary effector sphingolipid eliciting the observed phenotype is ceramide. Ceramide has been shown to increase the levels of the CDK inhibitor p21 and has been shown to affect progression through the cell cycle (92).

One study has shown that the utilization of D609, the SMS activity inhibitor, affects progression through the cell cycle (69,76,92,381). Gusain *et al.*, show that in BV-2 (immortalized murine microglia) cells treatment with D609 (dose and time regulated) results in the inhibition of cells through an arrest in the G_0/G1 phase of the cell cycle (91). The authors show that external addition of ceramide in these cells caused inhibition of proliferation and the downstream p21 response. Thus suggesting that accumulation of ceramide and its downstream effects cause cell cycle inhibition. In contrast, another study shows that activation of SMS activity by 2-hydroxyoleic acid in A549 (adenocarcinomic human alveolar basal epithelial cells) in fact causes a G0/G1

cell cycle arrest (75), initiated by the re-organization of the lipid domains on the cell surface affecting receptor-mediated signaling cascades. Additionally, increased activity of SMS explained the increase in DAG and therefore activation of PKCs, which are known to increase the expression of CDK inhibitors p27 and p21 (90).

A more recent study by Weseley *et al.*, performed a more specific evaluation of the role of SMS1 in Neuro-2a cells (murine neuroblast) (90). Herein inhibition of SMS1 resulted in an arrest in cell cycle progression at the G0/G1 phase of the cell cycle. They also showed a concomitant increase in p27 and inhibition of cyclin-D1 and phospho-Akt (known to down-regulated the expression and localization of p27).

It can be concluded from these studies that the role of SMS in regulating progression through the cell cycle is highly cell-context dependent and cannot be universally defined. Most importantly the response of a cell to modulation of SMS expression and activity depends on the status of other sphingolipid enzymes and oncogenic pathways active in the given cell type and their ability to respond to changes in ceramide, SM and DAG levels.

In this chapter, I provide uncharted analysis of the molecular mechanism regulating Bcr-abl-mediated increase of *SGMS1* transcription through TSS 7; I uncover a novel link between the transcription factor, GATA1 and SGMS1; I extend the molecular significance of this novel regulatory link to GATA1-positive AML cells; and I establish the importance of regulation of SMS1 in cell cycle progression of this subclass of AML cells.

3.2 Results

3.2.1 Identification of the proximal-regulatory region(s) of *SGMS1* TSS 7 promoter

As shown in the previous chapter Bcr-Abl up regulates the transcription of *SGMS1* via TSS 7. This increase in transcription is due to a Bcr-Abl mediated by the increase in activity of its upstream promoter. In order to understand the molecular mechanism involved it was important to identify the cis-regulatory region(s) important for maintaining high promoter activity.

Progressive 5' deletions were generated to the full-length TSS 7 promoter and were cloned into the luciferase based reporter pGL3-basic vector **(method described in Chapter 3.4)**. These deletion constructs were co-transfected with pCMV-β-galactosidase plasmid into K562 cells and promoter activity was assessed as a function of fold-change in relative luciferase activity compared to the activity of the full-length TSS 7 promoter **(method described in Chapter 3.4).** From **Figure 3.1(A)**, it can be clearly seen that there are two regions, which I demarcate as Region I (-160 to -127) and Region II (-118 to -115), which when deleted from the promoter significantly inhibits its activity. **Figure 3.1(B)** shows a graphical representation of the cis-regulatory regions of the TSS 7 promoter in the context of the full-length TSS 7 promoter.

However one draw back of this experiment is that it is difficult to decipher the relative contributions of these regions to the overall activity of the promoter because; a) In the Δ-127 construct, Region II is present, which may contribute to half the promoter activity observed (Asssumption 1: both Region I and Region II contribute equally to the promoter activity) b) The Δ-115 construct shows a complete loss of promoter activity,

which might be the results of either the loss of its core promoter (Region II) or due to loss of both Region I and II (Assumption 2: Region II controls the full activity of the promoter).

To resolve this a second set of promoter constructs were generated in the full-length-TSS 7-pGL3-basic promoter backbone. These constructs had deletions for either Region I or Region II or were deleted for both Region I and II **(method described in Chapter 3.4)**. All three constructs, the full-length TSS 7 promoter and the empty vector were co-transfected with the pCMV-β-galactosidase plasmid into K562 cells and relative luciferase activity was calculated as before **(method described in Chapter 3.4)**. In **Figure 3.1(C)** it is seen that Δ Region I resulted in a loss of half of the promoter activity while Δ Region II completely abrogated the activity of the TSS 7 promoter.

3.2.2 GATA-1 binds to Region II and regulates the expression and activity of *SGMS1*

Since deletion of Region II is sufficient to completely abolish the activity of the TSS 7 promoter, it can be concluded that Region II comprises of the minimal sequence required for maintaining the full activity of the TSS 7 promoter.

To identify the potential transcription factor(s) that bind to Region II and regulate transcription from it, an *in silico* analysis was performed using TFBind (382). The analysis revealed two TFs with consensus sequences within Region II, GATA-1 and GATA-2, as shown in **Figure 3.2(A).**

Next the mRNA expression pattern of *GATA-1*, *GATA-2* was compared with that of *SGMS1* across an array of CML cell-lines. To this end mRNA expression data extracted from the Cancer Cell Line Encyclopedia (CCLE) (383) and plotted as a heat-map **(method described in Chapter 3.4)**. The correlation in expression between all three genes was calculated across CML cell-lines. As seen in **Figure 3.2(B)**, there is a strong correlation in expression of *GATA-1*, *GATA-2* and *SGMS1*. This data shows that in cell-lines with high *GATA-1* and *GATA-2*, *SGMS1* is also has a high expression, thus making these TFs suitable candidates involved in regulating the transcription of *SGMS1*.

Next the role of GATA-1 and GATA-2 was evaluated in regulating endogenous *SGMS1* expression. Herein the effect of down-regulation of GATA-1 and GATA-2 was evaluated on the endogenous mRNA expression of *SGMS1*. GATA-1 and GATA-2 were down regulated in K562 cells by siRNA **(method described in Chapter 3.4).** Knock down of GATA-1 was confirmed by western blotting **(method described in Chapter**

3.4) of GATA-1 protein as shown **in Figure 3.2(C)(top)**. Next, in these cells, knocked down for GATA-1 the endogenous mRNA expression of *SGMS1* was measured by qRT-PCR **(method described in Chapter 3.4)**. Mean normalized expression was calculated **(method described in Chapter 3.4)** after normalization with the mRNA expression of β-Actin. As shown in **Figure 3.2(C)(bottom)** the expression of *SGMS1* mRNA was down regulated to 50% compared to cells transfected with control siRNA.

Similar to GATA-1, GATA-2 was also knocked-down as shown in **Figure 3.S1 (method described in Chapter 3.3)**. Knock-down of GATA-2 was confirmed by western-blotting and endogenous *SGMS1* mRNA expression was measured. **Figure 3.S1** shows that inhibition of expression of GATA-2 had no effect on *SGMS1* mRNA expression compared to cells transfected with control siRNA. Thus preliminarily showing that GATA-1 and not GATA-2 regulated the expression of *SGMS1* in the CML cell line K562.

Previous work from the lab has shown that inhibition of Bcr-Abl with the tyrosine kinase inhibitor STI-571/Imatinib resulted in a decrease in the total activity of SMS in cells.

To understand if GATA-1 knock-down had a similar effect I assessed endogenous SMS activity in these cells. K562 cells where GATA-1 had been knocked-down, protein lysates were prepared and endogenous SMS activity was assayed **(method described in Chapter 3.4)**.

As seen in the **Figure 3.2(D)**, inhibition of GATA-1 expression decreased the endogenous SMS activity by about forty percent. Together these results show that

GATA-1 regulates at least fifty percent of the mRNA expression and activity in CML cells.

It should however be noted that the GATA-1 siRNA experiment might not have abolished all the GATA-1 present in the cells and therefore GATA-1 could potentially be responsible for regulation of a greater percentage of *SGMS1* expression.

Next it was evaluated if GATA-1 down-regulation affected the expression of *SGMS1* by specifically inhibiting the activity of TSS 7 promoter and therefore transcription through it. To this end GATA-1 expression was down-regulated in K562 cells and then these cells were co-transfected with the pGL3-basic TSS 7 full-length promoter construct and pCMV-β-galactosidase plasmid **(method described in Chapter 3.4)**. The relative luciferase activity was calculated after normalization to β-galactosidase activity followed by subtraction of empty vector activity. As shown in **Figure 3.2(E)**, knock down of GATA-1 expression significantly inhibits the activity of the full-length TSS 7-promoter. This data suggests that GATA-1 might be responsible for the majority of regulation of expression of the TSS 7 promoter and transcription from it.

Finally to establish that GATA-1 directly binds to Region II, Chromatin Immuno-precipitation (176) was performed of GATA-1 in K562 cells **(method described in Chapter 3.4)**. To assess GATA-1 binding to Region II of the TSS 7 promoter, qRT-PCR primers were designed targeting Region II.

Additionally as positive controls primers were designed targeting the promoter regions of the beta-globin gene, specifically the HS2 region and Addmodulin (ADD), which are bound by GATA-1 (383). Additionally anti-IgG antibodies that do not target

any specific protein were used to control for the GATA-1 IP. As show in **Figure 3.2(F)** GATA-1 is bound to Region II of the TSS 7 promoter.

3.2.3 Bcr-Abl up-regulates the expression of GATA-1

As I demonstrated in chapter 2, up-regulation of promoter 7 depends on Bcr-abl, thus it became important to investigate whether the regulation of promoter 7 by GATA 1 was dependent on Bcr-abl. To this aim, I measured GATA-1 protein expression by western blotting across different CML cell-lines **(method described in Chapter 3.4)** as compared to Bcr-abl negative cells**,** HL-60**.** Cell lysates from HL-60, HL-60 over-expressing Bcr-Abl, K562, LAMA-84, JURKL-1 and JK-1 cell lines were probed with anti-GATA-1 antibodies and β-actin as a loading control. **Figure 3.3(A)** shows that GATA-1 is expressed in all CML cell lines. In fact over expression of Bcr-Abl in the HL-60 control up regulates the protein expression of GATA-1.

As a complementary approach, I evaluated if inhibition of Bcr-abl would decrease GATA-1. To this aim, I treated HL-60/Bcr-Abl cells with the Bcr-abl kinase inhibitor, Imatinib and evaluated its effect on GATA-1 protein levels **(method described in Chapter 3.4)**. K562 cells were not used in this experiment as treatment with Imatinib in these cells induces cell death/differentiation. **Figure 3.3B** shows that treatment with 1 μM Imatinib causes a significant reduction of GATA 1 levels at 48 hours and a complete loss by 72 hours. These results show that GATA 1 expression is regulated by the kinase activity of Bcr-Abl.

3.2.4 *GATA-1* regulates *SGMS1* expression in a subset of AML cell lines

In the analysis of mRNA expression of *GATA 1* and *SGMS1* across cell lines from CCLE, the expression analysis was extended to incorporate all leukemia cell lines (method described in Chapter 3.4). Interestingly it was observed that in a subset of Acute Myeloid leukemia cell lines there was a strong positive correlation between the expression of *SGMS1* and *GATA-1*. However this correlation was not as significant between *SGMS1* and *GATA-2*, as shown in Figure 3.4A. The AML cell-lines that showed a strong correlation in expression are TF-1, MO7E, UT7, MOLM-16, HEL, HEL 9217, CMK, CMK115, CMK 86, F36P and OCM 1, which belong either to the M6 or to the M7 AML subgroups

The expression level of GATA-1 and SMS1 protein was measured by western blotting in MOLM-16 and HEL cells, as representatives of the two AML types **(method described in Chapter 3.4). Figure 3.4(B)** shows that in MOLM-16 and HEL cells the level of GATA 1 and SMS1 protein are high compared to the AML control cell line HL-60. Thus the correlation at the mRNA level is recapitulated at the protein level.

Additionally the total SMS activity was also compared between these three cell lines **(method described in Chapter 3.4)** and as shown in the **3.4(C)** SMS activity in MOLM-16 and HEL cells was 5-10 fold higher compared to that of HL-60 cells.

Based on the observations in CML cells, GATA 1 upregulates SGMS1 expression by binding to promoter 7. To evaluate if GATA-1 drives the expression of *SGMS1* with a similar mechanism also in this subset of AML cells, hnRNA expression was evaluated upstream and downstream of exon 7. Since *SGMS1* TSS is not known in

AML cells (nor 5’ RACE data), FEP primers from CML transcriptional analysis could not be used.

Primers for qRT-PCR probing the junction of Intron VI –Exon 7 or the junction of Intron VII-Exon 8 were employed **(primer sequences provided in Table 3.4)**. HnRNA expression levels were measured in HL-60 control cells, MOLM-16 and HEL cells and mean of normalized expression was calculated **(method described in Chapter 3.4)**. As shown in **Figure 3.4(D)**, hnRNA levels measured using primers down-stream of TSS 7 show a 17-23 fold increase compared to levels measured using primers up-stream of TSS 7 in MOM-16 and HEL cells. The high expression of *SGMS1* down-stream of the putative TSS 7 suggests that, like in CML, in M6 and M7 AML cell lines, SGMS1 is transcribed from an alternative TSS. Thus as in CML cells, in a subset of AML cell lines *SGMS1* expression is up-regulated and this is possibly from TSS 7. Moreover the expression of TSS 7 is potentially regulated by GATA-1 as seen by the strong correlation in expression

To evaluate if GATA-1 mediated the regulation of *SGMS1* expression was conserved in AML cells, GATA-1 expression was down-regulated by siRNA **(method described in Chapter 3.4)** in HEL cells. Knock-down of GATA-1 was validated by western-blotting as shown in **Figure 3.4(E), (top panel), (method described in Chapter 3.4)** and from these cells, mRNA was extracted and endogenous *SGMS1* mRNA expression was measured by qRT-PCR using primers within exon 7 **(method described in Chapter 3.3)**. As seen in **Figure 3.4(E) (bottom panel),** inhibition of GATA-1 expression resulted in 50% down-regulation of *SGMS1* expression.

3.2.5 Inhibition of SMS1 causes a delay in G2/M transition in HEL cells

Inhibition of SMS1 affects the proliferation of the CML cell line K562 (346). This suggests that SMS1 activity contributes to proliferation of these cells. In this chapter it has been clearly demonstrated that a subset of AML cells also show a high expression of SMS1. Thus I evaluated whether SMS1 had a role to play in regulating the proliferation of AML cells.

Briefly HEL cells were treated with different doses of the SMS inhibitor D609 **(method described in Chapter 3.4)** and their growth was tracked over 72 hours by cell counting **(method described in Chapter 3.4).** As can be seen in **Figure 3.5(A)**, inhibition of SMS1 activity with D609 severely impairs growth of HEL cell at both doses starting at 48 hours. These results suggest that SMS activity has a role in supporting proliferation of these cells.

Next it was evaluated if the inhibition of proliferation was due to an effect on progression through the cell cycle. To this end, stable knock-down of SMS1 cells was generated in HEL cells **(method described in Chapter 3.4).** As shown in **Figure 3.S2**, these cells showed a down-regulation of *SMS1* mRNA of about 50%. To further reduce the expression of *SGMS1*, these stable cells were transfected with siRNA targeting SMS1 **(method described in Chapter 3.4).** ShSMS1 + siRNA SMS1 HEL cells were then synchronized in the G2/M stage of the cell cycle using nocodazole treatment **(method described in Chapter 3.4)**. Time and dose of nocodazole for synchronization were previously standardized (data not shown). After 12 hours of synchronization, cells were released from the arrest and collected at different time points for cell cycle

analysis **(method described in Chapter 3.4)**. As seen in **Figure 3.5(B)**, cells with *SGMS1* knock-down show a delay of about 7-10 percent in re-entering the cell cycle from the G2/M arrest.

3.3 Discussion

In this chapter I identify that, in CML cells, GATA 1 mediates transcription of *SGMS1* by binding to Region II of promoter 7. Furthermore Bcr-Abl up regulates the expression of GATA-1 in CML cells. Interestingly, while evaluating the expression pattern of *GATA-1* and *SGMS1* across different types of leukemia cell lines, I found this mechanism of regulation is conserved in a subset of AML cells. Moreover *SGMS1* expression and activity is important in maintaining the progression through the cell cycle and therefore sustaining proliferation in AML cells.

In the previous chapter I show that *SGMS1* transcription is up regulated by Bcr-Abl from an alternative transcriptional start site. In fact studies estimate that almost 90% of all coding genes have alternative transcription initiation sites (308). However no studies thus far have established a clear molecular mechanism that regulates transcription initiation from these alternative start sites, especially in the context of cancer. In an attempt to elucidate a common mechanism that regulates transcription initiation from these alternative TSSs, high-throughput sequence analyses have been employed to identify common features in their promoters (305). It has been proposed that the promoter regions of these alternative TSSs are evolutionarily conserved (384,385) and several databases have been developed to assess this (386,387). Furthermore these promoters are said to be associated with increased DNAse I hypersensitivity, histone methylation and acetylation marks (388). These “characteristic” features have been shown to be associated with actively transcribed regions of the genome, however there is a lack of experimental evidence to deem these features as universal. Analysis of TSS 7 promoter region did not reveal any of these distinguishing

characteristics associated with active transcription. Nonetheless experimentally, I have shown that promoter 7 is capable of mediating transcription.

Several recent reports suggest that alternative transcription initiation could be one feature recurrent in different cancers and may have an important role in its pathogenesis (as discussed in **Chapter 1.5**). Ours is the first study wherein a well-defined oncogene, Bcr-Abl, mediates a shift in transcription initiation, as shown in the previous chapter. The first step in elucidating the precise molecular players participating in the regulation of *SGMS1* transcription from TSS 7 involved the identification of its proximal regulatory regions or core promoter. Classically, core promoters are defined by the presence of key elements such as the TATA-box, BRE elements, Inr sequences etc (308,389,390). However, with the advent of modern molecular tools, it is widely accepted that these regions are more often than not absent from core promoters (388,390,391). The TSS 7 promoter is unique, as it does not contain these classical promoter elements, however here I identify two important proximal-promoter regions and possibly an insulator element (between -235 and -160 bp). Of the two proximal promoter regions, Region I activity is consistent with that of an enhancer while Region II seems to represent the Core promoter 7 and found to be regulated by the TF GATA-1.

GATA family of transcription factors includes six different TFs, which are expressed in both hematopoietic as well as non-hematopoietic cells (392,393). GATA-1 and GATA-2 (and GATA-3) are primarily expressed in the hematopoietic cell lineages (392,393). However in certain specific stages of hematopoietic differentiation, they are temporally separated (394,395). For example, GATA-2 expression exclusively precedes that of GATA-1 in Hematopoietic Stem and Progenitor cells (HSPCs) and erythroid

precursor cells (396). The consensus sequence for GATA TFs is WGATAR, however millions of this sequence motif are scattered over the genome and it is estimated that a GATA TF occupies less than 1% of these (397). Recent studies have revealed through ChIP sequencing experiments that GATA 1 and GATA 2 occupancy can be very similar if not identical making it impossible to determine their binding based on sequence alone (398). Several studies have described a unique mechanism of GATA factor occupancy in the context of hematopoietic differentiation, known as the GATA switch. Herein at specific stages of differentiation, the various GATA factors may replace each other at the same site and elicit a differential regulation in gene expression (399-402). In fact GATA 1 is shown to be associated with a co-regulator, Friend of GATA 1 or FOG 1 that plays a role in mediating this switch and regulating down-stream transcription in a cell-type dependent manner (403). Additionally, there is evidence that GATA 2 might prime a specific chromatin site for subsequent GATA 1 binding In the Region II of *SGMS1* TSS 7 promoter the GATA-1 binding site identified exhibits a non-canonical sequence motif, however through genetic manipulation of GATA-1 expression and ChIP analysis I clearly show that GATA-1 binds and regulates transcription from this start site. Additionally, I have shown that GATA-1 and not GATA-2 regulates transcription from TSS 7. Moreover Bcr-Abl up-regulates the expression of GATA-1 which has not been described before.

In the context of hematopoietic malignancies one mechanism involved in maintaining the cancer phenotype is an inhibition of the differentiation pathways. This block in differentiation is crucial to maintain increased proliferation in cancer cells (404). The role of GATA 1 has been classically explored in the context of regulating

differentiation and genetic mutations in GATA 1 have been explored in the context of AML (405). The literature is sparse in evaluating the expression levels of GATA 1 and therefore it is difficult to decipher if the absolute expression of GATA 1 is altered in these leukemias (406-409). In the context of CML it is known that Bcr-Abl mediates a lineage shift and studies have shown the circulating CD34+ cells resemble that of a common-myeloid progenitor with a multipotent lineage (410-414). Interestingly, it has been shown that GATA 1 is important in commitment to the erythroid-megakaryocytic lineage of the common myeloid progenitor (415,416). These studies therefore suggest that in CML cells, GATA 1 expression must be elevated. Some very early studies also showed that GATA 1 expression is increased in specific subtypes of AML patient samples (M6 and M7) and is usually associated with a worse prognosis (417). In this chapter, I have shown that GATA 1 expression is up regulated by Bcr-Abl and in a subtype of AML cell lines belonging to the M6 and M7 class as well.

Xu *et al.* and Morceau *et al.* have shown that the kinase activity of Bcr-Abl inhibits the expression and activity of GATA 1. However, data shown in this chapter and preliminary results not shown provide evidence that regulation of GATA 1 by Bcr-Abl is highly complex, responding to both the kinase and non-kinase domains of Bcr-abl (418,419). In this chapter I show that in Bcr-Abl positive cells there is an increased GATA 1 activity. In fact over expression of Bcr-Abl in HL-60 cells (low GATA 1 basal expression) results in an increased GATA-1 expression and activity, as assessed by increased transcription from TSS 7. However it is to be noted that the regulation of *SMS1* via GATA-1 maybe only partially dependent of the kinase activity of Bcr-Abl and it is possible that kinase independent effects may also regulate the expression of *SMS1*.

The role of SMS1 in regulating proliferation via modulating progression through the cell cycle has recently gained momentum (as discussed in introduction). However most studies have shown that SMS inhibition results in a G0/G1 cell cycle arrest. A recent study has shown that GATA 1 is involved in mitotic bookmarking and is required for effective re-entry into G1 phase of the cell cycle (420). Another study shows that erythroid specific genes are highly up regulated during transition from the G2-M phase of the cell cycle and the persistent association of GATA 1 to the chromatin throughout the mitotic phase provides further support to this observation (421). In this chapter I show that in the AML cell line HEL, knock-down of *SGMS1* results in a slightly delayed progression from the G2/M phase of the cell cycle or inhibition of re-entry into the G1 phase of the cell cycle. It can be postulated that one way by which GATA 1 elicits these effects is via SMS1. However the mechanism involved remains to be elucidated.

3.4 Materials and Methods

Cell-lines

K562, HL-60 cell-lines were purchased from American Type Culture Collection (ATCC) (Rockville, MD). Human acute myeloid leukemia, HL-60 cells stably expressing p185 Bcr-Abl (HL-60/Bcr-Abl) was a generous gift from Dr. K. Bhalla (167). LAMA-84 JURL-MK-1,JK-1, HEL and MOLM-16 cell-lines were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Brunswick, Germany). K562, LAMA-84, JURL-MK-1, HEL and MOLM-16 were grown in RPMI-1640 (Life Technologies Corporation, Carlsbad, CA), supplemented with 10% FBS (Fetal Bovine Serum, heat inactivated) (Life Technologies Corportion, Carlsbad, CA) or 20% FBS for HL-60 and HL-60-Bcr-Abl, and 100 U/mL Penicillin and 100 µg/mL Streptomycin (Life Technologies Corporation, Carlsbad, CA). HeLa cells were grown in DMEM supplemented with 10% FBS, 100 U/mL Penicillin and 100 ug/mL Streptomycin (Life Technologies Corporation, Carlsbad, CA). Cells were maintained at 37°C and 5% CO_2 in a humidified incubator at supplier suggested cell concentrations.

Deletion constructs

1. 5' Progressive deletion constructs

Deletion constructs were generated using the forward primers and Promoter-7-reverse primer as indicated in **Table 3.1**. Each forward primer name indicates the total bp-distance from TSS 7. Constructs were generated using the full-length TSS 7-pGL3-basic plasmid as the template for PCR **(as described in Chapter 2.4).** TSS 7 deletion PCR products were cloned into pCR 2.1 TOPO-TA sub-cloning vector and transformed into DH5α bacterial cells using standard transformation conditions (Invitrogen, Carlsbad,

CA). The plasmids were extracted from ampicillin resistant bacterial colonies, and inserts were excised from the pCR 2.1 TOPO-TA vector using KpnI and ZhoI (Invitrogen Life Technologies, Carlsbad, CA) and cloned into the final pGL3-basic vector (Promega, Madison, WI). All sequences were verified (Genewiz, Southplainfield, NJ). Sequence analysis and verification was carried out using the 4Peaks software. Plasmid DNA was prepared for transfections using the Endofree Plasmid Maxi Kit (Qiagen, Valencia, CA).

2. Region I, Region II and Region I+II promoter deletion constructs

Promoter constructs deleted for Region I, Region II and both Region I and II were generated by Life Technologies Corporation (Carlsbad, CA). Promoter sequences provided in **Table 3.2**. These constructs were sub-cloned into the pGL3-basic luciferase reporter vector as described above.

Site-directed mutagenesis

The Region II of the TSS 7 promoter was predicted to contain putative consensus sequence for GATA-1 and GATA-2. Thus, in order to determine if Evi-1 is responsible for activation of TSS 7 promoter in Bcr-Abl positive cells, a mutated TSS 7 promoter was generated and tested for luciferase reporter activity in K562 cells. Nucleotides substitutions were made to mutate the GATA-1 and GATA-2 consensus sequence (SDM construct) using the Agilent software. The oligonucleotides generated for site directed mutagenesis, are provided in **Table 3.3**. Site directed mutagenesis was performed according to the standard protocol, as suggested by the manufacturer (Agilent, CA) and using the full length TSS 7 promoter construct cloned in the pGL3-basic vector as a template. All sequences were verified by sequencing (Genewiz, Southplainfield, NJ). Sequence analysis and verification was carried out using the

4Peaks software. Plasmid DNA was prepared for transfections using the Endofree Plasmid Maxi Kit (Qiagen, Valencia, CA).

Transient transfection of TSS 7 full-length promoter, deletion (both 5' progressive deletions and cis-regulatory regions) constructs and SGM constructs for promoter activity

K562 cells ($2x10^6$) were transfected with 5 µg each of pGL3 basic vector or pGL3 basic-TSS 7 constructs and pCMV-β-galactosidase reporter construct (Promega, Madison, WI). Plasmids were transfected into cells by the Neon Electroporation system (Life Technologies Corporation, Carlsbad, CA). K562 cells were electroporated at 1000V, 50 ms and 1 pulse.

Luciferase activity

After transfection cells were harvested after an overnight incubation and then lysed with 1X reporter lysis buffer (RLB), and luciferase activity was measured using a Promega kit as per manufacturer recommended protocol (Promega, Madison, WI). Luminescence was measured using a Sirius Luminometer (Bethold Technologies, GMBh & Co. KG, Germany) programmed with a 2-second measurement delay followed by a 10-second measurement read. β-Galactosidase activity was used to normalize for cell transfection efficiency determined by using a colorimetic method as suggested by the manufacturer (Promega, Madison, WI). Promoter activity was calculated as Relative Luciferase Unites after normalization with the amount of cell lysate used for the luciferase activity assay, to the β-Galactosidase activity of each sample and by subtraction of background values (as determined from pGL3 vector only controls). Results were expressed as fold change in relative luciferase activity of constructs over relative luciferase activity of full-

length TSS 7 promoter, or as RLU. Statistical significance was determined by paired t-test.

Cancer Cell Line Encyclopedia analysis and expression correlation

mRNA expression for the different cell lines was derived from CCLE and were analyzed using R packages gplots and RColorBrewer to generate the heat map and correlation matrix.

siRNA knock-down of GATA-1 and GATA-2

The siRNA used to knockdown human GATA-1 was from a pool of 3 target-specific 19-25 nucleotides siRNA (Santa Cruz Biotechnologies, Santa Cruz, CA). The Allstar Negative Control (Qiagen, Valencia, CA) was used in experiments as control. The siRNA used to knochdown human GATA-2 was a single specific 21-bp nucleotide siRNA, s5598 (Ambion Life Technologie, CA).

K562 and HEL cells were transfected with siRNA using the same electroporation protocol (as described before). The protocol was optimized regarding the concentration of *GATA-1* and *GATA-2* siRNA, transfection and post-transfection conditions in preliminary dose-response experiments. Consequently, the following optimal conditions were employed. For GATA-1- cells ($2x10^6$ K562 cells) were transfected with 20nM of siRNA for *GATA-1.* After 1 hours of incubation at 37°C, 5% CO_2 in 2 mL of complete growth medium, cells were seeded at $0.2x10^6$ cells/ml. Cells were collected for RNA isolation or western blotting at 24 hours and the remaining cells were re-transfected for subsequent time points. For GATA-1 siRNA in HEL cells the same transfection conditions were used (cell number and electroporation parameters) as K562 however only a single transfection was sufficient to down regulate GATA-1 for up to 48 hours.

To evaluate the effect of GATA-1 siRNA on TSS 7 promoter activity, K562 cells were transfected with siRNA as above after 24 hours cells were transfected with the respective luciferase constructs. Cells were then collected for luciferase activity after 16 hours of transfection with the plasmid constructs.

For GATA-2, cells ($2x10^6$ K562 cells) were transfected with and 40 nM of siRNA for *GATA-2.* After 1 hour of incubation at 37°C, 5% CO_2 in 2 mL of complete growth medium, cells were seeded at $0.2x10^6$ cells/ml. Cells were collected for RNA isolation or western blotting at 24 and 48 hours.

Lentiviral knock-down of SMS1

For lentiviral experiments cells were transduced with either non-targeting control particles (shRNA-NTCP) or Mission lentiviral transduction particles (both from Sigma-Aldrich) that contained the pLKO.1-puro vector including the SMS1 targeting sequence, 5'-CCG GCC AAC TGC GAA GAA TAA TGA ACT CGA GTT CAT TAT TCT TCG CAG TTG GTT TTT TG-3' (sh-RNA-SMS1). During transduction, $3x10^4$ cells/ml re-suspended in RPMI-1640 containing 10% FBS and 10 μg/ml of Hexabromide (Sigma) were infected at a Multiplicity of Infection (MOI) of 10 with the appropriate lenti-viral particles. Cells were incubated at 37^oC, 5% CO_2 and 3 days post-transduction, 10 μg/ml of puromycin (Sigma) was added to the media to select for pLK0.1-puro positive clones and re-added every 3-4 days to maintain selection. The concentration of puromycin needed to select for vector expressing clones was based on a kill-curve analysis showing 70-80% cell death after 3 days in non-transduced cells (data not shown). SMS1 down-regulation was confirmed by quantitative real time PCR.

siRNA Knock-down of SMS1

The siRNA for human SMS1 targeted the sequence CAC ACT ATG GCC AAT CAG CAA (45). In preliminary experiments, the effects on SMSs RNA expression, SMS activity, and cell viability of the AllStars Negative Control siRNA (Qiagen) was compared to that of the transfection reagent by itself and found no difference, therefore the Allstars Negative Control was used in subsequent experimentation as baseline. K562 and HEL cells were transfected with siRNAs by electroporation. The protocol was optimized regarding the concentration of SMS1 siRNA, transfection and post-transfection conditions in preliminary dose-response experiments. Consequently, the following optimal conditions were employed. shRNA cells were transfected with 60 nM siRNA. After 1 hour of incubation at 37°C, 5% CO_2 in 500 μl of complete growth medium, cells were s diluted to 0.2×10^6 cells/ml concentration. At the indicated time points after transfection, cells were processed according to the required analysis.

Western blotting SMS1

Twenty million cells were harvested on ice, washed in PBS and resuspended in 800 μl of homogenization buffer containing 250 mM Tris, 50 mM EDTA pH 7.4, Pierce Halt phosphatase inhibitor, Pierce Halt protease inhibitor (Pierce, Rockville, Il) and 5 mM PMSF. Cells were then lysed as indicated for SMS activity and centrifuged at 1000g for 10 minutes at 4°C. Supernatants were collected and centrifuged at 120,000g for 1 hour at 4°C. Membrane pellets were re-suspended in 200 μl of homogenization buffer and passed through a 21 gauge needle until uniform. Membrane protein concentration was determined using the Bio-rad protein determination assay reagent (Bio-rad, Hercules, CA). Membranes were diluted with 4X sample buffer and incubated at 37°C for 30 minutes. Cell membrane proteins (80 μg) were separated by 10% SDS-

PAGE under reducing conditions and transferred to nitrocellulose membranes. Membranes were blocked with 5% milk PBS-Tween 0.1% for 1 hour at room temperature. Membranes were then incubated with Sms1 antibodies raised against the full-length protein (1:1000) (Ex-alpha Biologics, Maynard, MA) for 4 hours at room temperature in 5% milk PBS-Tween 0.1%. After extensive washings, membranes were next incubated with peroxidase-conjugated goat-anti-rabbit (1:3500, Santa Cruz) in 5% milk PBS-Tween 0.1% for 1 hour at room temperature. Signals were visualized using Super Signal (Pierce Chemical Co) and exposure to Kodak BioMax MR Film (Eastman Kodak Co., Rochester, NY).

Western blotting β-Actin, GATA-1 and GATA-2

For western-blotting, cell pellets were re-suspended in 100 μL of 0.5% SDS (Sodium Dodecyl Sulphate) (on ice). Samples were sonicated for 20s. Cell lysates were then re-suspended in loading buffer and boiled for 8 minutes. Following this 20 μg of proteins were run and blotted with primary antibodies against GATA-1 and GATA-2 (Santa Cruz Biotechnology, Santa Cruz, CA) and β-Actin (Santa Cruz Biotechnology, Santa Cruz, CA).

mRNA extraction and cDNA synthesis

To analyze mRNA expression, total RNA was isolated from 1-4x10^6 cells, collected by centrifugation at 3000 RPM, 5 minutes at 4°C. Cells were immediately re-suspended in lysis buffer with β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) and total RNA was isolated using RNeasy mini kit (Qiagen, Hilden, Germany) according to manufacturer's protocol, including the on-column DNase digestion. Additional round of DNase digestion was performed using TURBO DNA free kit (Life Technologies

Corporation, Carlsbad, CA) according to manufacturer's protocol to ensure complete elimination of genomic DNA. cDNA was prepared by a two step method; first an RNA mix was prepared with 1 µg of total RNA, 1 µL of Oligo $(dT)_{20}$ primer (50 µM) (Life Technologies Corporation, Carlsbad, CA) and 1 µL of 10 mM dNTP (Life Technologies Corporation, Carlsbad, CA) mixed to a final volume of 12 µL and incubated at 65°C for 5 minutes. After this step, a second master mix of 4 µL of 5x 1st Strand Buffer, 2 µL of 0.1 M DTT, 1 µL each of RNase Out and Superscript III (Life Technologies Corporation, Carlsbad, CA) was added to the RNA mix. The 20 µL reaction was then incubated at 42°C for 52 minutes and then 70°C for 15 minutes.

Quantitative Real-Time PCR (qRT-PCR)

To evaluate mRNA expression, qRT-PCR was performed using the SYBR green mixture (Bio-Rad, Hercules, CA) with primers sequences are reported in **Table 3.4** All experiments were performed on Applied Biosystem, 7500 Real time PCR system. The following cycling conditions were used, 1 cycle of 3min at 94°C, 40 cycles of 20s at 94°C and 30s at 60°C. The melt curve stage was as follows, one cycle of 1min at 95°C, one cycle of 1 min at 60°C followed by each cycle increasing by 1°C for 30s starting at 60°C followed by a final step at 60°C for 15s. Amplification efficiency for each primer pair was assessed being between 90-100% across a range of cDNA concentrations (annealing temperatures were also standardized for each pair of primers). Results were normalized to internal control gene, β-actin and amplification efficiency. qRT-PCR results were analyzed using Q-Gene software, as mean of normalized expression (MNE) (373). To assess genomic DNA contamination, cDNA was prepared without

reverse transcriptase. These controls did not amplify during qRT-PCR thus demonstrating the absence of genomic DNA.

SMS activity

SMS activity was performed as previously described with few modifications (45). Briefly, three to four million cells were harvested on ice, washed in PBS and resuspended in 150 μl of homogenization buffer containing 250 mM Tris, 50 mM EDTA pH 7.4, Pierce Halt phosphatase inhibitor, Pierce Halt protease inhibitor (Pierce, Rockville, Il) and 5 mM PMSF. Cells were then lysed by sonication at 11% power for 20 seconds (preliminary optimization experiments showed 95% cell lysis with these conditions). Unbroken cells were pelleted by centrifugation at 400g for 5 minutes at 4°C and supernatant was collected and assayed for protein concentration using the Bio-rad protein determination assay reagent (Bio-Rad, Hercules, CA). Fifty micrograms of proteins were used for SMS activity which was carried out and analyzed as previously described (45).

Chromatin Immuno-precipitation (ChIP)

ChIP was done using the Magna ChIP –G-immunoprecipitation kit protocol (EMD Millipore, MA). K562 cells ($20x10^6$) were cross-linked with formaldehyde (final concentration 1%) for 10 minutes at room temperature. Cross-linking was stopped by the addition of 10x Glycine (final concentration 1x). Cells were then pelleted down and washed with ice cold 1x PBS, twice. Cells were then re-suspended in 500 uL of cell lysis buffer with protease inhibitor added and allowed to lyse on ice for 15 minutes. Nuclei were spin down at 800g for 5 minutes, and re-suspended in 500 uL of nuclear lysis buffer with protease inhibitor. Nuclear lysate was sonicated (10 % output, 20sx6 cycles

with 50 second rest). Standardization was carried out for sonication and shearing of cross-linked chromatin as well. Sonicated chromatin was spun down at 10,000g for 10 minutes. Cross-linked DNA, 250 ug was diluted to a final volume of 500 uL with the kit provided dilution buffer. Input chromatin of 5 uL or 1% was alquoted. Protein G beads, 20 uL, was added to the tube and allowed to rotate for 1 hour at 4°C to pre-clear the nuclear lysate. Beads were separated from the supernatant and 6 ug of IgG (Santa Cruz, CA) and GATA-1 antibody (Abcam, MA) were added to two aliquots of cross-linked chromatin. The immuno-precipitation with GATA-1 antibodywas standardized to allow for complete pull-down and enrichment of DNA bound-GATA-1. The beads and antibody were incubated overnight with constant rotation at 4°C. Following this all steps were followed as in manufacturer suggested protocol. Eluted DNA was detected by qRT-PCR using primers for ChIP provided in **Table 3.4**

STI-571/Imatinib and D609 treatment

Pharmacological inhibition of Bcr-abl was achieved by incubation of cells with Imatinib mesylate (Santa Cruz, CA) and inhibition of SMS activity was achieved by treatment with D609 (Tocris, UK) as indicated in the individual experiments in complete growth medium (in absence of antibiotics) using $0.1x10^6$ cells/ml cultures.

Cell counting

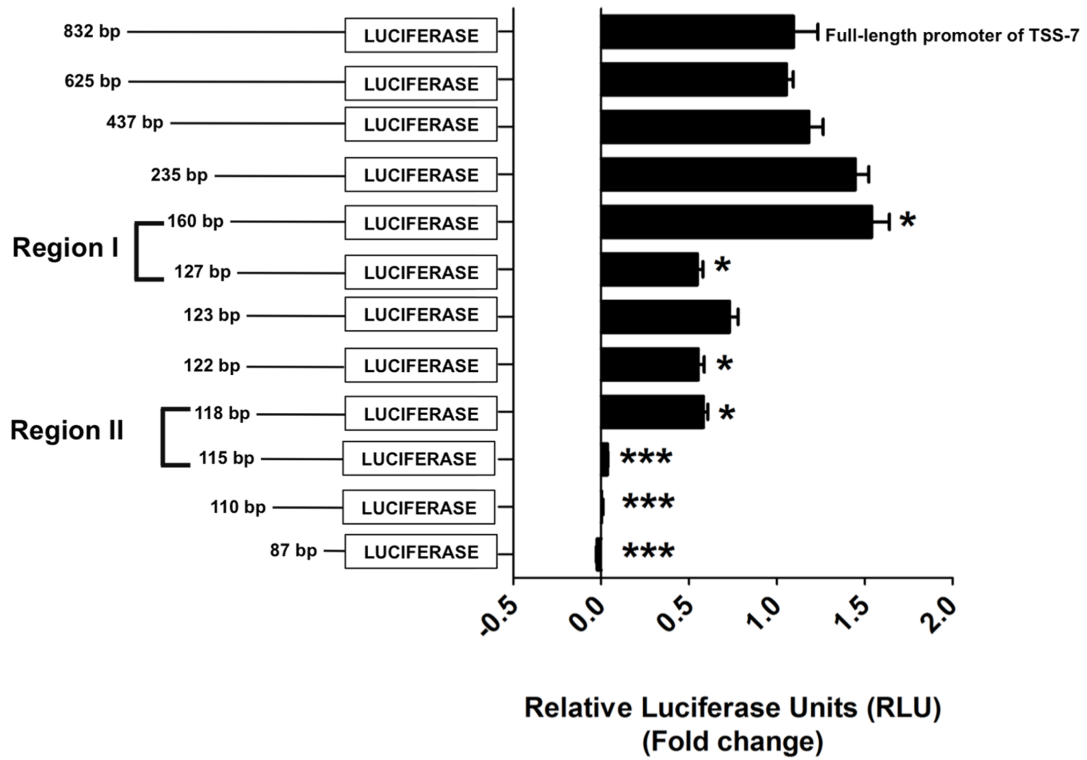
To count the cell survival after treatment with D609, an aliquot of cells was mixed with trypan blue (Life Technologies, CA) and counted manually in a hemocytometer. Live cells were counted separately from dead, which were trypan blue positive cells.

Cell cycle analysis

HEL cells that had been knocked down for *SMS1* (lenti-viral +siRNA (24 hours), described above) were treated with 100 ng/mL Nocodozole (Sigma, MO) for 12 hours at a cell concentration of $0.2x10^6$ cells/mL. Cells were released from the G2/M arrest by Nocodozole, by spinning cells down and washing with 1x PBS, two times. Cells were re-suspended in full media at the same cell concentration. Cells were collected immediately after re-suspension for the 0h time-point and after 3h for cell cycle analysis. Cells were collected by spinning down and aspiration of media. Cells were then washed twice with ice-cold 1x PBS and re-suspended in chilled 70% ethanol. Cells were stored at 4°C for further processing. Cells we pelleted by centrifugation and resuspended in PI/RNAse (Cell Signalling, MA) solution with 0.2% Triton X. Cells were incubated for one hour, protected from light. After the incubation cells were spun down and washed twice with 1x PBS and re suspended in 500 uL 1x PBS for analysis by flow-cytometry. Flow cytometry was analyzed using the FACSCaliber (Becton Dickinson) and results were provided by the flow cytometry core facility at Stony Brook University.

3.5 Figures and legends

A



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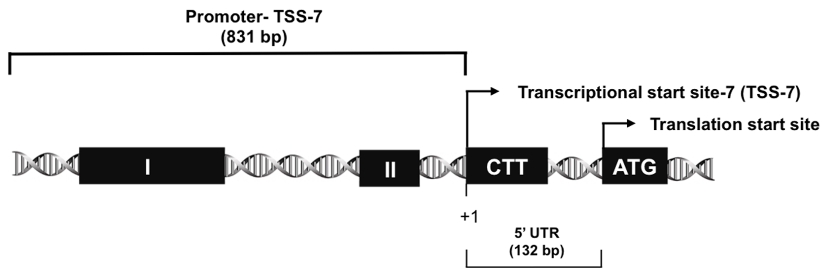

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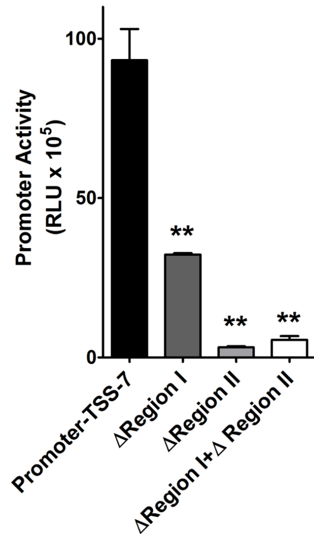


Figure 3.1: Identification of cis-regulatory regions of the TSS 7 promoter. (A) Promoter activity of the different 5' deletion constructs was measured as a function of the fold change in relative luciferase activity compared to full length TSS 7 promoter. The y-axis shows the length of each promoter construct identified by its bp-distance from TSS 7. Region I and II are the two regions when deleted, results in a significant loss in TSS 7 promoter activity. This result represents three independent experiments. (B) Graphical representation of the two cis-regulatory regions identified by promoter deletion constructs. Region I and II are depicted in the context of their full-length promoter. (C) The contribution of the two proximal regions identified in (A) was evaluated for their role in maintaining the activity of the TSS 7 promoter. TSS 7 constructs deleted for either Region I, Region II or both regions were transfected into K562 cells. Relative luciferase activity was calculated for these constructs and compared to that of the full-length TSS 7 promoter. This result represents two independent experiments performed in duplicates. Asterisks indicate significance; *** $p<0.0005$; **$p<0.005$; *$p<0.05$.

A

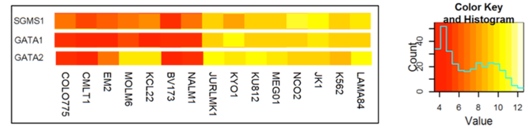
GATA-1/GATA-2

cttGATattt

REGION I REGION II

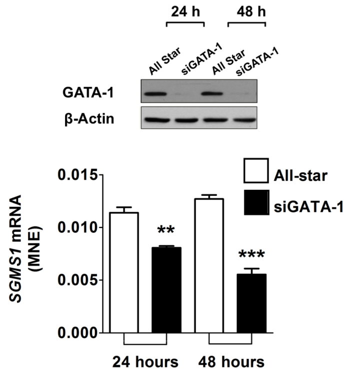
GGGGTAATTTGGAAGTACAGCATCTTGATATTTTCTCTTGCTTTCAC
CCCCATTAAACCTTCATGTCGTAGAACTATAAAAGAGAACGAAAGTG

B



Variable vs. Variable	*Correlation (R)*
GATA1 vs. SGMS1	0.84314
GATA2 vs. GATA1	0.73049
GATA2 vs. SGMS1	0.72682

C



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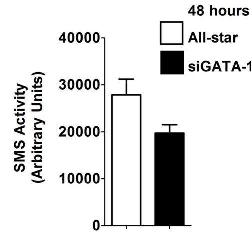

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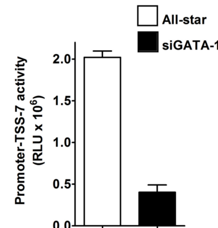

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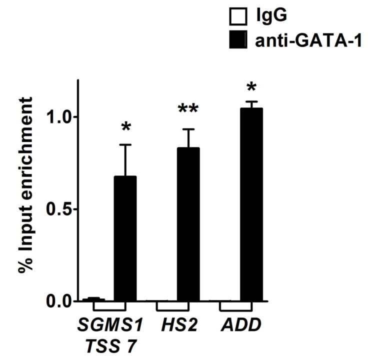
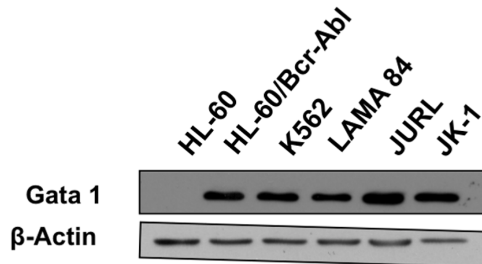


Figure 3.2: GATA-1 binds to Region II of the TSS 7 promoter. (A) Figure shows the base-sequence map of Region I and Region II (and some flanking sequences). GATA-1 and 2 consensus sequences are indicated above the base-sequence map as identified by TFBind. The capitalized sequence indicate the core region recognized by the TFs. (B) Heat-map showing the relative mRNA expression of *SGMS1*, *GATA-1* and *GATA-2* as derived from CCLE. The expression is shown across an array of CML cell lines indicated below the heat-map. The colors in the heat-map are lava-color coded and their relation to expression is shown in the color-key. (Bottom) Correlation in mRNA expression was calculated across CML cell lines and is shown in the table. (C) siRNA down regulation of GATA-1 in K562 was assessed by western blotting. GATA-1 anti-bodies were used to measure abundance in GATA-1 expression 24h and 48h post-siRNA transfection of Control (AllStar) or siGATA-1 (siRNA for GATA-1). β-Actin protein blotted as loading control. This figure is representative of three independent experiments. (Bottom) Endogenous *SGMS1* expression was measured by qRT-PCR in K562 cells in which GATA-1 expression had been down-regulated. The figure shows mean-normalized expression of *SGMS1* calculated after normalization to mRNA expression of β-Actin. This figure is representative of three independent experiments. (D) Endogenous SMS activity was estimated in K562 cells where GATA-1 knock-down was performed. Total SMS activity is shown in the figure as quantification of SM bands on TLC. This figure is representative of two independent experiments. (E) Effect of GATA-1 down-regulation on TSS 7 promoter activity was assessed after down-regulation of *GATA-1* in K562 cells. In the figure luciferase activity is shown in cells transfected with Control (AllStar) and siGATA-1 (siRNA for GATA-1) siRNA. This figure is representative of two independent experiments performed in duplicates. (F) Chromatin-immuno precipitation was performed in K562 cells using Control IgG and GATA-1 antibodies. Primers targeting the Region II of *SGMS1*-TSS 7, promoter of HS2 and ADD were used to quantify abundance of GATA-1 bound DNA by qRT-PCR. Primers used for RT-PCR are provided in Table 3.4. This figure is representative of two independent experiments and qRT-PCR performed in triplicates. Asterisks indicate significance; *** p<0.0005; **p<0.005; *p<0.05.



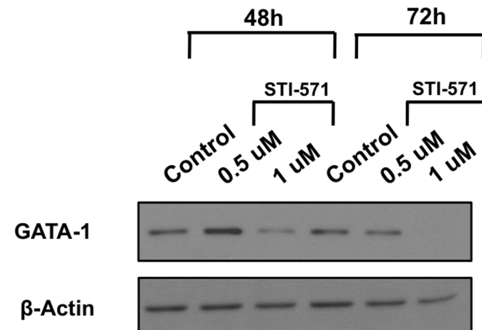
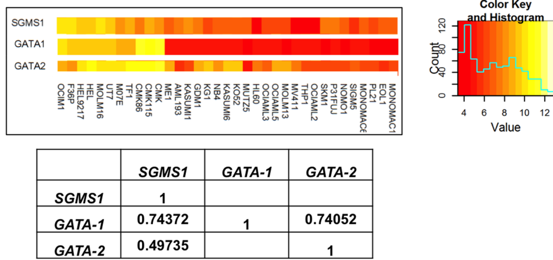


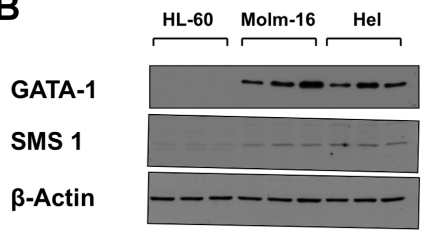
Figure 3.3: Regulation of expression of GATA-1 by Bcr-Abl (A) Cell lysates from CML cell lines K562, LAMA-84, JURL-MK-1 and JK-1, control Bcr-Abl negative HL-60 cells and HL-60 cells over-expressing Bcr-Abl were prepared for western blotting. Lysates were probed for their expression of GATA-1 and β-Actin as a loading control. (B) HL-60/Bcr-Abl cells were treated with Imatinib at 0.5 uM and 1 uM for 24h and 48h. Cell-lysates were prepared at the two time-points for western blotting. GATA-1 and β-Actin preotein expression was evaluated. This figure is representative of two independent experiments.

A

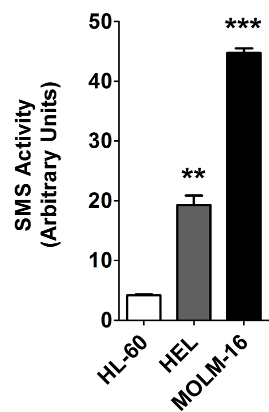


	SGMS1	*GATA-1*	*GATA-2*
SGMS1	1		
GATA-1	0.74372	1	0.74052
GATA-2	0.49735		1

B



C



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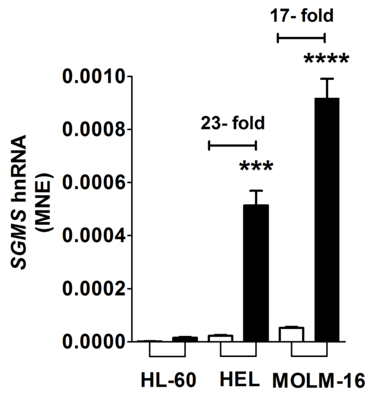


E

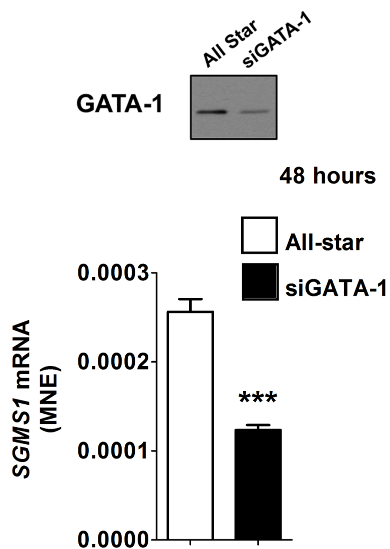
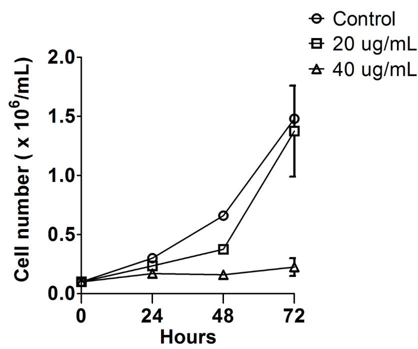


Figure 3.4: GATA-1 regulates the expression of *SMS1* in AML cells. (A) (Top) Heat-map showing the relative mRNA expression of *SGMS1*, *GATA-1* and *GATA-2* as derived from CCLE. The expression is shown across an array of AML cell lines indicated below the heat-map. The colors in the heat-map are lava-color coded and their relation to expression is shown in the color-key. (Bottom) Correlation in mRNA expression was calculated across AML cell lines and is shown in the table. (B) Protein expression of GATA-1 and SMS1 was evaluated in AML cell lines HEL, MOLM-16 and HL-60. Cell lysates were extracted from these cell lines and blotted using gene specific antibodies. β-Actin protein blotted as loading control. This figure is representative of three independent experiments. (C) Total SMS activity was measured in HL-60, MOLM-16 and HEL cells. Cell lysates were prepared from healthy cultures for SMS activity. The SMS activity shown in figure is derived from band-quantification from a TLC plate. This result is representative of three independent experiments. (D) *SGMS1* hnRNA expression was measured in HL-60, HEL and MOLM-16 cell lines. hnRNA abundance was measured using two pairs of primers, one at the junction of Intron VI-Exon-7 (up-stream of TSS 7) and the second at the junction of Intron VII-Exon 8 (down-stream of TSS 7). Mean normalized expression was calculated after normalization with β-Actin. This figure is representative of three independent experiments. (E) (Top) siRNA down regulation of GATA-1 in HEL was assessed by western blotting. GATA-1 anti-bodies were used to measure extent of down-regulation in GATA-1 expression 48h post-siRNA transfection of Control (AllStar) or siGATA-1 (siRNA for GATA-1). β-Actin protein blotted as loading control. This figure is representative of two independent experiments. (Bottom) Endogenous *SGMS1* expression was measured by qRT-PCR in HEL cells in which GATA-1 expression had been down-regulated. The figure shows mean-normalized expression of *SGMS1* calculated after normalization to mRNA expression of β-Actin. This figure is representative of three independent experiments. (Asterisks indicate significance; **** p<0.00005; *** p<0.0005; ** p<0.005.



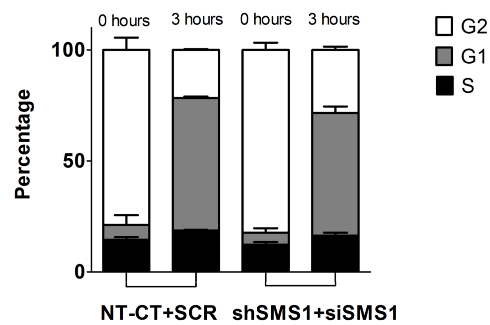


Figure 3.5: SMS1 inhibition affect progression through the cell cycle (A) HEL cells were treated with the SMS inhibitor D609 at 20 ug/mL and 40 ug/mL. Cell growth was monitored by cell counting and cell death evaluated by trypan blue exclusion over 72 hours. Cell numbers were plotted against time points of measurement. This figure is a representative of two independent experiments. (B) shSMS1 HEL cells were transfected either control siRNA (AllStar) or siSMS1 siRNA. Cells were arrested in the G2/M phase by Nocodazole treatment. After 12h cells were released from the arrest and cell-cycle progression was evaluated. This results shows % of cells in different phases of the cell cycle at 3h compared to 0h (after release). This figure is representative of two independent experiments.

3.6 Supplementary figures

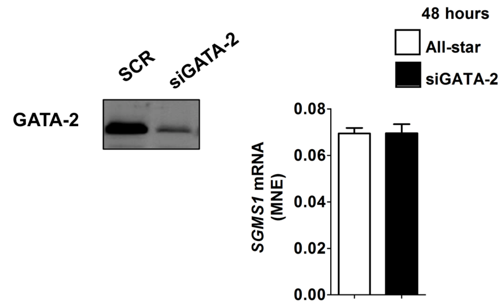


Figure 3.S1: GATA-2 siRNA in K562 cells. K562 cells transfected with either control siRNA (SCR) or GATA-2 siRNA (siGATA-2). Western blot showing the extent of GATA-2 down-regulation. In K562 cells knocked down with siRNA Endogenous *SGMS1* mRNA expression was measured by qRT-PCR in cells in which GATA-2 expression had been down-regulated. The figure shows mean-normalized expression of *SGMS1* calculated after normalization to mRNA expression of β-Actin. This figure is representative of one experiment and qRT-PCR performed in triplicates.

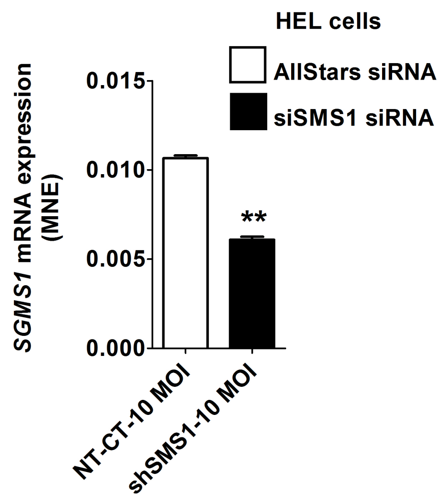


Figure 3.S2: Down-regulation of SMS1 in HEL. Stable knocked down stably using shRNA targeting SMS1 were generated in HEL cells. After developing such stable knock-down cells *SGMS1* expression was further down-regulated using siRNA. To measure the reduction in expression qRT-PCR performed, shown in the figure. Asterix indicate significane of ** p<0.005

3.7 Tables

Table 3.1: Primer sequences for 5' progressive deletion constructs

Primer	Sequence (5' to 3')
Promoter 7-reverse	ATGGTCAGGGCAGTTTTTAAA
Δ625	TTGAGAAGGAATCTCGCTCTGTCG
Δ437	CGTGTTAGCCAGGATGCTCT
Δ235	CCTGAAACGAATACATGTTTGAA
Δ160	TGCGTTTTGAGAGGGGTAAT
Δ127	AGCATCTTGATATTTTCTCTTGCTTTC
Δ123	TCTTGATATTTTCTCTTGCTTTCA
Δ122	CTTGATATTTTCTCTTGCTTTCACAGA
Δ118	ATATTTTCTCTTGCTTTCACAG
Δ115	TTTTCTCTTGCTTTCACAGAAGG
Δ110	TCTTGCTTTCACAGAAGGAAGAATC
Δ87	TCCTGCTCAAAAATGAGGTG

Table 3.2: Region I and Region II promoter deletion construct sequences

Δ Region I: 825 bp

TAAGCAGGTACCTCTGTGAGTTGCTGTATGTTATGTACATTTTTAAACATTCTTCACGAGGTAGAGTAAGCAAATATATTTTATTCTTTTAAATTAT
CTCAGGAAGCATGTAAGGACAAAGAGAATACCTGGTCTCTTGACCAGGTTGAAAAGTGTCCAAAGCACTGTCAAGTTGCTTCTTAGAAAATAGACCA
TTGGAAATTATTATTATTTTTTTTTTTTGAGAAGGAATCTCGCTCTGTCGCCCAGGCTGGAGTACAGTGGCTGATCTCGGCTCACTGCAAGCTCCGC
CTCCCGGGTTCACACCATTCTCCTGCCTCAGCCTGGGCAGGAGTAGCTGGGACTACAGGCGCCCGCCACCACACCCGGCTAATTTTTTGTATTTTTA
GTAGAGACAGGGTTTCACCGTGTTAGCCAGGATGCTCTCTATCTCCTGACCTCGTGATCCGCCCGCCTCGTTCTCCCAAAGCGCTGGGATTACAGGC
GTGAGCCACCACGCCCGGCTGACCGTTGGAAATTCTATAAAATATGTCTCCTTTGACAACATCCATTTTCAGATCCAATTCAATGTGTGTCGCAAAA
GACTAATAACCAGGAAATCAGTAGTCCCTGAAACGAATACATGTTTGAAAACTATTAAGTTTACCAATAACTGGTTTTCTACTTTCCTTATGTATAT
AAGGCTTAGCATCTTGATATTTTCTCTTGCTTTCACAGAAGGAAGAATCCTGCTCAAAAATGAGGTGAATTAATACTTGGGCGCTCAGGAACCCTGG
ACAGCTACATGAGGTGTTTAAAAACTGCCCTGACCATCTCGAGTAAGCA

Δ Region II: 853 bp

TAAGCAGGTACCTCTGTGAGTTGCTGTATGTTATGTACATTTTTAAACATTCTTCACGAGGTAGAGTAAGCAAATATATTTTATTCTTTTAAATTAT
CTCAGGAAGCATGTAAGGACAAAGAGAATACCTGGTCTCTTGACCAGGTTGAAAAGTGTCCAAAGCACTGTCAAGTTGCTTCTTAGAAAATAGACCA
TTGGAAATTATTATTATTTTTTTTTTTTGAGAAGGAATCTCGCTCTGTCGCCCAGGCTGGAGTACAGTGGCTGATCTCGGCTCACTGCAAGCTCCGC
CTCCCGGGTTCACACCATTCTCCTGCCTCAGCCTGGGCAGGAGTAGCTGGGACTACAGGCGCCCGCCACCACACCCGGCTAATTTTTTGTATTTTTA
GTAGAGACAGGGTTTCACCGTGTTAGCCAGGATGCTCTCTATCTCCTGACCTCGTGATCCGCCCGCCTCGTTCTCCCAAAGCGCTGGGATTACAGGC
GTGAGCCACCACGCCCGGCTGACCGTTGGAAATTCTATAAAATATGTCTCCTTTGACAACATCCATTTTCAGATCCAATTCAATGTGTGTCGCAAAA
GACTAATAACCAGGAAATCAGTAGTCCCTGAAACGAATACATGTTTGAAAACTATTAAGTTTACCAATAACTGGTTTTCTACTTTCCTTATGTATAT
AAGGCTTTGCGTTTTGAGAGGGGTAATTTGGAAGTACAGCATCTTGATTTTCTCTTGCTTTCACAGAAGGAAGAATCCTGCTCAAAAATGAGGTGAA
TTAATACTTGGGCGCTCAGGAACCCTGGACAGCTACATGAGGTGTTTAAAAACTGCCCTGACCATCTCGAGTAAGCA

Δ Region I+II: 823 bp

TAAGCAGGTACCTCTGTGAGTTGCTGTATGTTATGTACATTTTTAAACATTCTTCACGAGGTAGAGTAAGCAAATATATTTTATTCTTTTAAATTAT
CTCAGGAAGCATGTAAGGACAAAGAGAATACCTGGTCTCTTGACCAGGTTGAAAAGTGTCCAAAGCACTGTCAAGTTGCTTCTTAGAAAATAGACCA
TTGGAAATTATTATTATTTTTTTTTTTTGAGAAGGAATCTCGCTCTGTCGCCCAGGCTGGAGTACAGTGGCTGATCTCGGCTCACTGCAAGCTCCGC
CTCCCGGGTTCACACCATTCTCCTGCCTCAGCCTGGGCAGGAGTAGCTGGGACTACAGGCGCCCGCCACCACACCCGGCTAATTTTTTGTATTTTTA
GTAGAGACAGGGTTTCACCGTGTTAGCCAGGATGCTCTCTATCTCCTGACCTCGTGATCCGCCCGCCTCGTTCTCCCAAAGCGCTGGGATTACAGGC
GTGAGCCACCACGCCCGGCTGACCGTTGGAAATTCTATAAAATATGTCTCCTTTGACAACATCCATTTTCAGATCCAATTCAATGTGTGTCGCAAAA
GACTAATAACCAGGAAATCAGTAGTCCCTGAAACGAATACATGTTTGAAAACTATTAAGTTTACCAATAACTGGTTTTCTACTTTCCTTATGTATAT
AAGGCTTAGCATCTTGATTTTCTCTTGCTTTCACAGAAGGAAGAATCCTGCTCAAAAATGAGGTGAATTAATACTTGGGCGCTCAGGAACCCTGGAC
AGCTACATGAGGTGTTTAAAAACTGCCCTGACCATCTCGAGTAAGCA

Table 3.3: Primers used for Site-directed mutagenesis of GATA-1 and GATA-2 binding site

Primer Name	Primer Sequence (5' to 3')
SDM-GATA-Forward	gctttgcgttttgagaggggtaatttggaagtacatttttttttttttttctcttgctttcacagaaggaagaatcctgct
SDM-GATA-Reverse	agcaggattcttccttctgtgaaagcaagagaaaaaaaaaaaaaaaatgtacttccaaattacccctctcaaaacgcaaagc

Table 3.4: Primers used for qRT-PCR

Primer set	Primer	Primer sequence (5' to 3')
Exon 7	Exon 7-forward	GCCAGGACTTGATCAACCTAACC
	Exon 7-reverse	CCATTGGCATGGCCGTTCTTG
β- Actin	β- Actin-forward	ATTGGCAATGAGCGGTTCC
	β- Actin-reverse	GGTAGTTTCGTGGATGCCACA
ChIP HS2	HS2-forward	TGCCCAGATGTTCTCAGCCT
	HS2-reverse	TGATGCCGTTTGAGGTGGAGT
ChIP ADD	ADD-forward	AGAGAGAGGCTGTGCTTTGTG
	ADD-reverse	CAGAGGCAGTTATTCAGTTTGC
ChIP-SGMS1-TSS 7	TSS 7-forward	AAGGCTTTGCGTTTTGAGAG
	TSS 7-reverse	GGGCAGTTTTTAAACACCTCAT
Intron VII-Exon 8	Intron VII-forward	ATTGAGTCATTGAAAAATTACAG
	Exon 8-reverse	GCCAACTATGCAGAAAAATC
Intron VI-Exon 7	Intron VI-forward	AAGGCTTTGCGTTTTGAGAGG
	Exon 7-reverse	AGCTGTCCAGGGTTCCTGAG

Appendix B: Effect of SMS inhibition with D609 on Imatinib resistance.

Imatinib, the Bcr-Abl inhibitor is the first line of therapy in the treatment of patients diagnosed with CML. However a vast majority of patients show a differential response to Imatinib either responding sub-optimally (not reaching treatment milestones at correct times) or not responding at all (422).

Additionally about 10-30% patients that do respond to primary Imatinib treatment develop resistance. There are a diverse set of mechanisms by which resistance is developed the most common of which is increased expression of Bcr-Abl or the T315I mutation that prevents Imatinib binding (423).

While the precise molecular mechanism involved in the development of resistance has been vastly studied the question that remains to be addressed is whether drug resistant-stem cells (attributed to the cause of resistance) exist prior to therapy or are developed as a consequence of therapy.

Several studies have shown the leukemic stem cells with mutations that make them resistant to TKI therapy exist prior to the onset of the treatment, suggesting that resistance is an eventuality (424). In this appendix I explore some initial findings suggesting that inhibition of SMS with D609 increases Imatinib sensitivity and prevents development of resistance to Imatinib in the K562 model cell line.

B.1 Results

B.1.1 SMS inhibition with D609 specifically inhibits proliferation of Bcr-Abl positive cells.

SMS has been known to affect proliferation of cells however work from Chapter 2 and 3 shows that Bcr-Abl specifically up-regulates the expression and activity of SMS. Furthermore SMS inhibition in these cells results in an inhibition of proliferation. First I evaluated if SMS inhibition affected cell growth specifically in Bcr-Abl positive cells. To this end K562, HL-60 and HL-60/Bcr-Abl cells were treated with different doses of the D609 **(method described in B.3)**. **Figure B.1(A)** shows that in K562 cells with increasing dose of D609 there is an inhibition of cell proliferation **(method described in B.3)** as shown before as well (345). Next I measured cell proliferation in HL-60 cells over-expressing Bcr-Abl and **Figure B.1(B)** shows that treatment with D609 in these cells results in a decrease in proliferation however this is not comparable to the extent of inhibition seen in K562 cells. Interestingly the rate of proliferation of HL-60/Bcr-Abl cells after treatment matches that of HL-60 cells, **Figure B.1(C)**. This data suggests that the proliferative advantage acquired through the expression of Bcr-Abl can be affected in part by inhibition of SMS. Interestingly HL-60 cells are moderately affected by D609 treatment; here SMS expression is lower compared to Bcr-Abl positive cells.

B.1.2 Co-treatment of SMS inhibitor D609 sensitizes K562 cells to the Imatinib.

SMS has an important role to play in regulating proliferation and signaling. There are several complimentary reasons by which SMS can affect cell proliferation, either by affecting the relative levels of Cer and DAG that directly effect cell growth or by modulating the level of SM on the cell surface which affects down-stream pro-proliferative signaling. It has been previously shown that Imatinib inhibits SMS1 but only by 50% (144). Given the proproliferative role of SMS1 in K562 cells and being SMS1 a target of Imatinib but only partially inhibited by the drug, I evaluated the effect of co-treatment of the SMS inhibitor D609 with the Bcr-Abl kinase inhibitor Imatinib in K562 cells on cell growth **(method described in B.3)**. Interestingly I observed that using sub-inhibitory concentrations of both inhibitors resulted in a cumulative decrease in cell proliferation, **Figure B.2**. This data shows that inhibition of SMS results in a sensitization of K562 cells to a lower dose of Imatinib.

B.1.3 SMS inhibition with D609 impairs development of TKI resistance.

Development of resistance to TKI can occur through several different mechanisms and is increasingly prevalent in the context of CML. However one over-arching mechanism that governs the development of resistance is the ability of cancer stem-cells to survive and proliferate (425-427). Thus strategies involving inhibition of proliferation of is a lucrative therapeutic option in combating the development of resistance. The above data reveals that inhibition of SMS with D609 impedes the proliferation of K562 cells and it sensitizes the cells to Imatinib, suggesting that a combination therapy could allow the use of lower concentrations of Imatinib with potential beneficial effects on the onset of resistance. Therefore, I next evaluated the effect of SMS inhibition in the context of development of resistance to Imatinib using an in-vitro resistance model in K562 cells **(method described in B.3)**. I treated K562 cells with increasing doses of Imatinib and cultured the cells that survived, generating a pool of cells that were resistant to otherwise lethal doses of Imatinib (1 uM). Additionally another set of cells was co-treated with a low dose of D609 and Imatinib. **Method shown in Figure B.3**. After ~30 days of culturing, several Imatinib-resistant clones were obtained. Cells that were treated with only low doses of D609 were not affected and the lines were closed. Importantly, only very few Imatinib-resistant clones in the co-treatment condition were obtained, **Figure B.4**.

B.2 Conclusion

In this appendix I show that inhibition of SMS activity with D609 impedes the proliferative capacity of the Bcr-Abl positive cell line K562. This effect is more pronounced in Bcr-Abl positive cells suggesting that SMS contributes partly to the pro-proliferative capacity of these cells. Additionally co-treatment of D609 with Imatinib results in a cumulative effect on cellular proliferation. These results can be attributed to several causes: first, an increased sensitivity to Imatinib due to the effect of inhibition of SMS on plasma membrane structure, second, increased ceramide content and third, decreased DAG. However the precise molecular mechanism involved is yet to be elucidated.

Increased proliferative capability of cancer stem cells is associated to transformation and development of resistance. Since D609 affects proliferation of Bcr-Abl positive cells, its role in development of resistance to TKI was evaluated. Herein it was seen that co-treatment with D609 significantly reduced the ability of Bcr-Abl positive cells to develop resistance to Imatinib. The mechanism involved in this effect warrants further investigation.

B.3 Methods

Inhibitor treatments

For all inhibitor treatments cells were seeded at 0.1x 10^6 cells/ mL in antibiotic free RPMI-1640 (Life Technologies, CA) supplemented with 10% FBS (Life Technologies, CA). D609 (Tocris Biosciences, MA) was re-suspended in water and Imatinib (Santa Cruz, CA) in DMSO.

Cell counting

To count the cell survival after treatment, an aliquot of cells was mixed with trypan blue (Life Technologies, CA) and counted manually in a hemocytometer. Live cells were counted separately from dead, which were trypan blue positive cells.

Imatinib resistance

Cells were seeded at concentrations mentioned above and treated at a starting dose of 0.05 uM of Imatinib with and without D609 (10 and 20 ug/mL). Cells were re-seeded at starting cell concentrations after they reached confluence (1x10^6 cells/mL). Single-clones were isolated by diluting resistant culture and seeding in 96-well plates.

B.4 Figures and legends

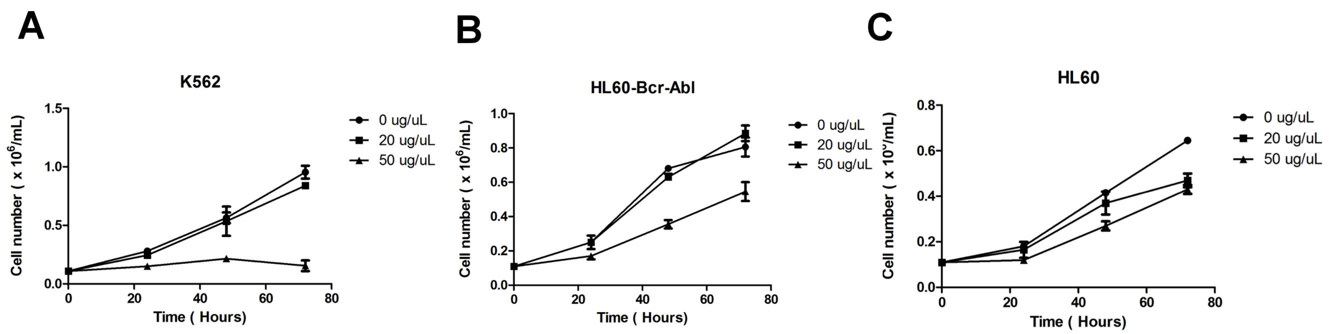


Figure B.1: Effect of SMS inhibition on cell proliferation in (A) K562 (B) HL-60/Bcr-Abl (C) HL-60 cells. This figure represents two independent experiments.

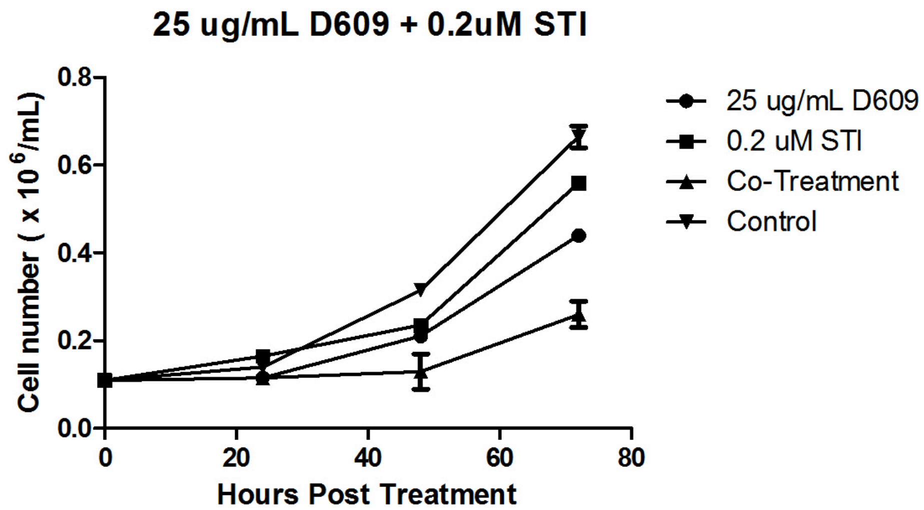


Figure B.2: Effect of co-treatment of Bcr-Abl and SMS inhibitors. K562 cells were treated with 0.2 uM of Imatinib and 25 ug/mL D609. These treatments only moderately affected cell proliferation of K562 cells. However when added together resulted in a cumulative inhibition of proliferation. This figure is representative of two independent experiments.

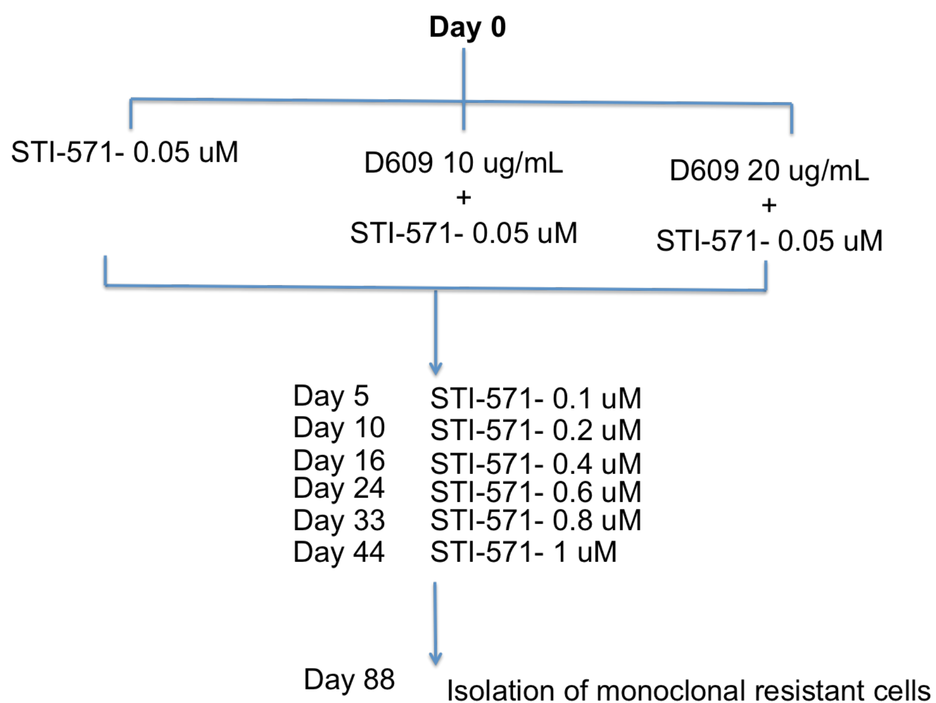


Figure B.3: Outline of procedure used to develop monoclonal lines resistant to Imatinib (with and without D609).

Treatment	Number of resistant clones
Imatinib (1uM)	37
Imatinib (1 uM) + D609 (10 ug/mL)	10
Imatinib (1 uM) + D609 (20 ug/mL)	3

Figure B.4: Number of resistant clones developed from treatment of K562 cells with Imatinib with or without D609 treatment.

CHAPTER 4

CONCLUDING REMARKS AND FUTURE DIRECTIONS

SMS regulates key cellular functions such as proliferation, migration, inhibition of apoptosis and cell survival, thus making it a unique target in the context of cancer. The primary way in which SMS elicits these functions is by regulating the levels of its substrates (Cer and PC) and products (SM and DAG), which have been shown to affect these biological processes. Thus understanding the regulation of SMS would potentially offer novel therapeutic strategies for cancer and other diseases. Unfortunately, till date, very limited information is available in this area.

Previous work from our lab was the first study to establish Bcr-Abl, the oncogene that causes CML, as an up-stream regulator of SMS1 expression and activity. Bcr-Abl increases the expression of SMS1 mRNA and as a consequence its protein level in CML cells. Moreover the kinase activity of Bcr-Abl in part is responsible for the elevated expression of SMS1. This study thus established a defined molecular framework to understand the regulation of SMS1.

This chapter is subdivided by key findings described in this dissertation and discusses the overall relevance and the future research it entails.

Bcr-Abl transforms the transcriptional landscape of *SMS1* to up-regulate its expression.

In the second chapter, I show that Bcr-Abl increases SMS1 expression via a transcriptional mechanism, which also affects its translation. Firstly, I show that Bcr-Abl initiates the transcription of *SGMS1* from four alternative TSSs. However *SMS1* expression is primarily up regulated from TSS 7. The mRNA generated from this TSS is translated more efficiently owing to its shorter 5’ UTR and thus increasing SMS1 protein abundance in CML cells. This part of the dissertation establishes three novel findings; a)

Bcr-Abl is an up-stream transcriptional regulator of *SMS1*; b) SMS1 transcription occurs primarily from the novel TSS 7; c) TSS selection by Bcr-Abl has a direct consequence on the protein abundance owing to the enhanced translation efficiency of the mRNA generated.

Important questions arise from these observations, some specific to the transforming ability of Bcr-Abl and some of more general nature. For instance, is this mechanism a general way through which Bcr-Abl up-regulates or down-regulates important molecular targets? In other words, is SMS1 the only target of such regulatory mechanism by Bcr-abl? A preliminary analysis identified that more genes may be regulated in a similar manner by Bcr-Abl **(Appendix A)**. As the analysis is only partial, the discovery of other genes regulated like SMS1 may suggest the existence of a novel Bcr-Abl-mediated regulatory mechanism to collectively affect the expression of several downstream targets. If so, does such regulation take place through coordinated modulation of specific transcription factors and/or epigenetic events? Is this mechanism an underappreciated way of coordinating protein expression patterns (translatome) in other types of cancers by other oncogenes as well? Furthermore, in a non-cancer context, such regulation may also explain the disconnect between mRNA and protein levels, often observed but vastly unexplained.

In the context of SMS1, I identified that SMS expression is up regulated by differential start site selection. As mentioned before this over-expression of SMS has been associated with cancer and may, in part, be explained by the usage of alternative TSSs. I developed a novel technique, FEP to monitor the abundance of mRNA generated from different TSSs. This method can be potentially applied to monitor SMS1

TSS utilization in leukemia patient samples and thereby identify the subset of patients where targeting SMS may be therapeutic. Additionally this method can be applied for other genes with known alternative TSSs to monitor their abundance in different tissues. This is an important development, as there is a growing body of evidence suggesting that TSS selection is cell type dependent and maybe altered in the context of cancer.

Finally, the mechanism of translational regulation of mRNAs generated in Bcr-Abl positive cells remains to be elucidated. As discussed in Chapter 2, the primary difference in the different SGMS1 mRNA transcripts is the number of uORFs. Longer transcripts having more uORFs than the shorter transcript generated from TSS 7. However a clear evaluation is warranted of whether these uORFs have an inhibitory effect on translational efficiency.

GATA-1 mediates transcription initiation from TSS 7

Chapter 3 establishes several novel findings: a) I identify GATA-1 as the first molecular regulator of *SMS1* transcription: b) Bcr-Abl up regulates the expression of GATA-1 and this results in an increased expression of *SMS1*; c) in a subset of AML cells, GATA-1 up-regulates transcription of *SMS1* from TSS 7 similar to that shown in CML; and d) inhibition of SMS1 affects the proliferation of AML cells

These observations give rise to several important questions. Firstly, thus far no study has shown that Bcr-Abl increases the expression of GATA-1. Some studies have shown that Bcr-Abl affects the activity of GATA-1. However, at a basal level I show that mere over-expression of Bcr-Abl increases the level of GATA-1. It remains to be elucidated how Bcr-Abl causes an increase in GATA-1 expression. This finding offers a new therapeutic strategy in the context of CML as GATA-1 has not been characterized

a target in the context of CML. Additionally targeting down-stream targets of Bcr-Abl offers a new approach in conditions where Bcr-Abl targeting is not possible, such as in the case of CML resistance due to T315I mutations.

Next using the correlation in expression of GATA-1 and SMS1, I identified a subset of AML cells where *SGMS1* transcription is regulated from TSS 7. Moreover inhibition of SMS1 resulted in a decrease in cellular proliferation. However it remains to be elucidated how SMS1 inhibition affects progression through the cell cycle. One possible mechanism is through the increase in Cer level that induces p27 expression and inhibition of CDKs. Thus this study establishes for the first time SMS1 as a potential therapeutic target in AML.

The role of SMS1 in regulating cell proliferation and its implication in TKI resistance.

Burns *et al.,* have previously shown that SMS1 inhibition affects the proliferation of CML cells, and in chapter 3 I have described a similar effect in AML cells. Interestingly, as described in **Appendix B**, inhibition of SMS activity has a cumulative impact on proliferation of CML cells when co-treated with low dose of the TKI, Imatinib. From the perspective of CML, this is a very interesting finding with therapeutic implication. In patients being treated with TKIs, it has been shown that there is a wide-range of responses to therapy, resulting in a diverse remission time-line. The co-treatment experiment suggests that inhibition of SMS could potentially increase sensitivity of cells to even low dose of Imatinib. Burns *et al.*, have shown that an inhibition of SMS with D609 results in a decrease in total SM levels. Therefore it can be hypothesized that altered SM levels upon SMS inhibition could affect membrane fluidity

and could possibly affect Imatinib influx. Further studies are required to establish this mechanism as a cause for increased sensitivity to Imatinib on D609 co-treatment.

Another perspective on the role of SMS in regulation of proliferation was established in a model for TKI resistance. Increased proliferative capacity of stem cells has been attributed to disease progression and development of resistance. As described in **Appendix B,** by inhibiting the rate of proliferation of cells the development of resistance can be substantially delayed if not prevented. These observations are significant in light of the increasing number of patients developing resistance to TKI therapy. It would be important to study this effect in the context of patient bone marrow derived stem cells to establish SMS inhibition in affecting disease progression. Additionally the role of SMS in affecting proliferation could be used as strategy in preventing or delaying the development of resistance to drugs in other cancers as well.

Thus in summary I show that Bcr-Abl regulates the transcription of *SMS1* from a novel TSS with translational consequences which causes an increased expression of SMS1 in CML cells. This effect of Bcr-Abl is mediated by the binding of GATA-1 to the promoter up-stream of the alternative TSS. Moreover Bcr-Abl up-regulates the expression of GATA-1, which resulted in the identification of a subset of AML cells wherein SMS is transcriptionally up regulated in a similar manner. Additionally SMS regulates the proliferation of both CML and AML cells and affects the development of resistance to tyrosine kinase inhibitors.

CHAPTER 5

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