EXAMINING THE ROLE OF METABOLISM IN MYC-DRIVEN TUMORIGENESIS

Tacha Zi Plym Forshell
This work is protected by the Swedish Copyright Legislation (Act 1960:729)
ISSN: 0346-6612
Omslagsbild/Cover Image: Melissa M. Fraser (www.melissamfraserphotography.com)
Elektronisk version tillgänglig på http://umu.diva-portal.org/
Tryck/Printed by: Print and Media, Umeå, Sweden 2011
For my Family – My parents, siblings and husband

Men love to wonder, and that is the seed of science.
~Ralph Waldo Emerson

Sheldon: I made tea.
Leonard: I don't want tea.
Sheldon: I didn't make tea for you. This is my tea.
Leonard: Then why are you telling me?
Sheldon: It's a conversation starter.
Leonard: That's a lousy conversation starter.
Sheldon: Oh, is it? We're conversing. Checkmate.

(The Big Bang Theory - The Maternal Congruence Episode)
# TABLE OF CONTENTS

ABSTRACT ..........................................................................................................................6

LIST OF ARTICLES ............................................................................................................7

DISSERTATION SURVEY ....................................................................................................8

ABBREVIATIONS ................................................................................................................9

INTRODUCTION ..................................................................................................................10
  GENERAL BACKGROUND TO CANCER DEVELOPMENT .................................................10
  MODELS OF IMMORTALIZATION AND CANCER ............................................................10
  ONCOGENES AND TUMOR SUPPRESSORS .......................................................................11
  THE CELL CYCLE ............................................................................................................13
  CELL DEATH .....................................................................................................................14
  MYC: INSTIGATOR OF CELL PROLIFERATION AND CELL DEATH .............................16
    Myc: Instigator of cell proliferation ..............................................................................17
    Myc: Instigator of cellular senescence and death .........................................................17
  MYC OVER-EXPRESSION AND CANCER ........................................................................19
    Myc deregulation .........................................................................................................19
  MYC AND THE HALLMARKS AND ENABLING CHARACTERISTICS OF CANCER ......20
    Deregulation of cellular energetics ..............................................................................21
  GLUCOSE METABOLISM .................................................................................................21
    Glycolysis .....................................................................................................................22
    Lactate dehydrogenase A .............................................................................................22
    Glycolysis and Ldha in cancer .......................................................................................23
    Targeting Ldha in cancer ..............................................................................................24
  SERINE METABOLISM ......................................................................................................25
    Serine biosynthesis: the phosphorylated pathway and Phgdh ......................................25
    Serine biosynthesis: glycine degradation and Shmt .......................................................26
    Serine biosynthesis in cancer .......................................................................................27
    Targeting Serine biosynthesis in cancer ......................................................................27
  POLYAMINE METABOLISM ............................................................................................28
    Polyamine biosynthesis: Spermidine synthase .............................................................29
    Polyamine biosynthesis in cancer ...............................................................................29
    Targeting polyamine biosynthesis in cancer ................................................................30

AIMS OF THIS DISSERTATION ............................................................................................32

SUMMARY OF PUBLICATIONS ........................................................................................33

GENERAL CONCLUSIONS AND DISCUSSION ................................................................40

ACKNOWLEDGMENTS .........................................................................................................41

REFERENCES ......................................................................................................................43

APPENDIX ...........................................................................................................................57
Myc transcriptionally regulates genes involved in processes such as cell proliferation, metabolism, differentiation, and angiogenesis. MYC expression is deregulated in many types of human cancer; therefore discovering the mechanisms behind MYCs role in tumorigenesis is essential. In this dissertation, I have focused on several Myc target genes, Spermidine synthase (Srm); Lactate dehydrogenase (Ldh); 3-phosphoglycerate dehydrogenase (Phgdh); Serine hydroxymethyltransferase (SHMT) 1 and 2; and Pim-3 (a member of the Pim family of serine/threonine kinases). These enzymes play a role in various functions: Spermidine synthase (polyamine synthesis); Lactate dehydrogenase (glycolysis); Phgdh and Shmt (serine metabolism); and Pim-3 (cell signaling). In order to elucidate the impact Myc over-expression has on metabolism in tumorigenesis, we use human cell lines, and transgenic mice as well as cell lines and tissues derived from these mice. The impact of inhibition of these target genes on Myc-driven tumorigenesis was done by genetically inhibiting the target gene (using RNAi or mouse models) or inhibiting the protein with a chemical inhibitor. Investigating these Myc target genes will help determine if inhibition of Myc target genes is a viable approach for chemotherapeutics, and under what conditions this inhibition may be the most valuable.

In paper I, we examine SRM; a highly expressed enzyme in the polyamine synthesis pathway that converts putrescine to spermidine, and is important for actively growing cells. Genetic inhibition via RNAi against Srm, or chemical inhibition of Srm, resulted in decreased proliferation of B-cell tumor lines from transgenic mice in vitro. In vivo treatment of λ-Myc transgenic mice with a chemical SRM inhibitor exhibited a significant chemopreventative effect on tumor formation. These results support previous findings that inhibition of polyamine synthesis pathway enzymes has a place in cancer therapy. Many Myc target genes have been suggested as attractive targets in battling Myc-driven tumorigenesis. Surprisingly in paper II, when we analyzed the inhibition of other Myc target genes, such as Ldh, Shmt, and Phgdh, we found that inhibition of these genes did not inhibit Myc-driven tumorigenesis to any significant degree. However, inhibition of Ldh, Phgdh and Shmt2 had a notable effect on in vitro Ras-driven transformation. These findings suggest that chemotherapeutic inhibition of metabolic genes such as Ldh, Phgdh and Shmt2 may be effective in genetically defined settings, keeping in mind the oncogenic lesion behind the tumor. The Pim kinase family consists of three serine/threonine kinases, Pim1-3. In paper III, we found that Pim-3 is a direct Myc target gene and that Pim-3 expression is high in Burkitt Lymphoma samples taken from human patients, as well as spontaneously arising lymphomas from Myc transgenic mice. We also found that inhibition of Pim-3 using a pan-Pim kinase inhibitor, Pimi, in these spontaneously arising Myc lymphomas resulted in caspase independent cell death. These results indicate that Pim kinase inhibition may be a potential chemotherapeutic strategy in human lymphomas that rely on Pim-3 kinase expression.
List of Articles

This dissertation is based on the following papers and manuscripts referred to in the text by their roman numerals.

**Paper I.**


**Paper II.**


**Paper III.**

# DISSERTATION SURVEY

## Inhibition of Myc metabolic target genes *in vitro* and *in vivo*

<table>
<thead>
<tr>
<th>Paper</th>
<th>Aims in short</th>
<th>Results in Short</th>
</tr>
</thead>
</table>
| I     | Chemoprevention of B-cell lymphomas by inhibition of the Myc target spermidine synthase. | • *Srm* is a direct Myc target gene.  
• Inhibition of *Srm* has limited effect in transformation *in vitro* but a chemopreventative effect *in vivo*. |
| II    | Mouse genetics suggest that metabolic enzymes dispensable for Myc-induced lymphomagenesis can play critical roles in Ras-induced fibrosarcoma growth. | • *Ldh*, *Phgdh* and *Shmt* are dispensable for Myc-driven tumorigenesis *in vivo*.  
• However, *Ldh*, *Shmt2* and *Phgdh* are needed for rapid subcutaneous growth of Ras transformed fibroblasts. |
| III   | The direct target Pim-3 cooperates with other Pim kinases in supporting viability of Myc-induced B-cell lymphomas. | • *Pim-3* is a direct Myc target gene.  
• Inhibition of Pim kinases in Myc induced lymphomas results in caspase independent cell death. |
**ABBREVIATIONS**

In order to separate genes from proteins, throughout this dissertation genes and proteins are written differently. Genes are written in italics: non-human genes are written as such *Myc*, with human genes written in all capitals (MYC). Proteins are written the same way but are not italicized, for example Myc (non-human) and MYC (human).

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4HT</td>
<td>4-hydroxytamoxifen</td>
</tr>
<tr>
<td>4-MCHA</td>
<td>trans-4-methylcyclohexamine</td>
</tr>
<tr>
<td>Amd1</td>
<td>S-adenosylmethionine decarboxylase</td>
</tr>
<tr>
<td>APC</td>
<td>adenomatous polyposis coli</td>
</tr>
<tr>
<td>Arf</td>
<td>alternate reading frame of INK4a/ARF locus</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin dependent kinase</td>
</tr>
<tr>
<td>CDKI</td>
<td>cyclin dependent kinase inhibitor</td>
</tr>
<tr>
<td>CHX</td>
<td>cyclohexamide</td>
</tr>
<tr>
<td>dcSAM</td>
<td>decarboxylated S-adenosylmethionine</td>
</tr>
<tr>
<td>DFMO</td>
<td>α-difluoromethylornithine</td>
</tr>
<tr>
<td>DFR</td>
<td>dihydrofolate reductase</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>GSK3</td>
<td>glycogen synthase kinase</td>
</tr>
<tr>
<td>Glut</td>
<td>glucose transporter</td>
</tr>
<tr>
<td>HIF1α</td>
<td>hypoxia inducible factor alpha</td>
</tr>
<tr>
<td>Ldh</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>Max</td>
<td>Myc-associated protein X</td>
</tr>
<tr>
<td>miRNA</td>
<td>micro RNA</td>
</tr>
<tr>
<td>Mnt</td>
<td>Max binding protein</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MSCV</td>
<td>murine stem cell virus</td>
</tr>
<tr>
<td>Myc</td>
<td>c-Myc</td>
</tr>
<tr>
<td>Odc</td>
<td>ornithine decarboxylase</td>
</tr>
<tr>
<td>Pdh</td>
<td>pyruvate dehydrogenase</td>
</tr>
<tr>
<td>Pdk1</td>
<td>pyruvate dehydrogenase kinase 1</td>
</tr>
<tr>
<td>Phgdh</td>
<td>3-phosphoglycerate dehydrogenase</td>
</tr>
<tr>
<td>PKA</td>
<td>cAMP-dependent kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>Pkm</td>
<td>pyruvate kinase M</td>
</tr>
<tr>
<td>PPP</td>
<td>pentose phosphate pathway</td>
</tr>
<tr>
<td>Puro</td>
<td>puromycin</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative Reverse Transcriptase PCR</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosylmethionine</td>
</tr>
<tr>
<td>Serine</td>
<td>L-serine</td>
</tr>
<tr>
<td>Shmt1</td>
<td>serine hydromethyltransferase (cytoplasmic form)</td>
</tr>
<tr>
<td>Shmt2</td>
<td>serine hydromethyltransferase (mitochondrial form)</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
</tr>
<tr>
<td>Sme</td>
<td>spermine synthase</td>
</tr>
<tr>
<td>Smr</td>
<td>spermidine synthase</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofolate</td>
</tr>
<tr>
<td>Ub</td>
<td>Ubiquitin</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular-endothelial growth factor</td>
</tr>
</tbody>
</table>
INTRODUCTION

General Introduction to Cancer

Recent advances in modern medicine have led us to believe that we can conquer almost any illness. However, one steadfast challenger to this belief is cancer. Although we are coming closer to understanding the molecular and cellular mechanisms leading up to cancer, we still have not managed to fully design treatment protocols that guarantee survival. The main obstacle in the treatment of cancer is that cancer cells are essentially normal cells that have escaped normal cellular regulations and are no longer playing by the rules. This means that in treating tumors we have to find a way to kill cancer cells while not damaging the rest of the normal cells in the body at the same time. Since cancer cells are simply normal cells “uncensored”, identifying specific markers or characteristics of cancer cells allowing us to target and eliminate them is essential.

The reigning model of transformation is that cancer is a multistep process, where a normal cell acquires more and more mutations until it is completely transformed. Fortunately, this model of transformation offers opportunities for treatment and prevention. Strategies that prevent the acquisition of additional mutations and block the “next step” on the road to transformation are valuable because they can prevent a tumor from becoming more malignant or spreading to new sites in the body. Some of these new acquired characteristics make tumor cells more vulnerable to specific compounds than normal cells. This helps us design specific treatments that target tumor cells, while leaving normal cells relatively undamaged. These characteristics of cancer cells, acquired over time in a multistep process, are collectively known as “hallmarks of cancer” (Hanahan and Weinberg, 2011).

Models of immortalization and cancer

In order to study the characteristics of cancer cells, researchers often use mammalian cell lines they can grow and manipulate in the laboratory. Cell lines allow researchers to study the process of transformation (from a normal cell to a cancer cell) in a genetically and environmentally defined setting. These cell lines, can also be used as tools to test inhibition of potential target genes (using RNAi or chemical inhibitors) on transformation rates. These non-transformed cells are not exactly normal however, because in order to become an established cell line, they have had to adapt to life in cell culture. The selection pressure of culture conditions such as specific O2, CO2, pH, and nutrient level in cell culture media is such that not all cells will be able to adjust.

Some cell types, such as fibroblasts, are easier to establish in culture than others. Fibroblasts, immature cells from connective tissue, attach to the plastic of the culturing vessel and exhibit growth inhibition when too confluent. Fibroblasts are density sensitive and must be “passaged”, or split regularly in order to grow consistently. In fact, simply passaging mouse embryonic fibroblasts at a specific density over time can select for fibroblasts of a specific genetic background, such as loss of Arf (Quelle et al., 1995; Todaro and Green, 1963). By following this “3T3 Protocol” the resulting cells are immortalized but not transformed, meaning that they have escaped cellular senescence but are not actually cancer cells. In order to become cancer cells, primary cells must over-express two collaborating oncogenes (Land et al., 1983) or one oncogene together with functional loss of a tumor suppressor (Boehm et al., 2005). These 3T3 cells can be infected with retro or lenti-viruses for stable expression of oncogenes of interest, in order to study transformation. The effect of inhibition of specific target genes can also be analyzed in these cells with the use of RNAi constructs due to the ease of infection exhibited by these cells.

When studying the characteristics of transformed cells, sometimes it is the most effective to study already transformed cells. These cell lines are often established from spontaneously arising tumors in mice or humans, and once established can be extremely useful tools in the hunt for chemotherapeutics. Potential chemotherapeutic agents can be tested using cell lines from various cancer types in order to establish dose and effectiveness in specific cancer types. Targeting specific genes, such as Myc target genes, can be done in cancer cells lines to establish the effect of genetic inhibition on proliferation or cell death.
However, the amount of knowledge you can obtain from cell culture is limited because it is an artificial setting that is not like what is seen in the body. Therefore, mouse models of cancer are valuable tools in understanding how tumor cells behave in the body, and also indentifying successful ways of targeting tumor cells while limiting damage to normal cells. Typically tumorigenesis in mice is studied one of two ways: xenograft models where tumor cells are transplanted into mice and then studied, and genetically engineered mouse models (GEMMs) in which genes are deleted, mutated or over-expressed, in order to model various human diseases (Sharpless and Depinho, 2006). We have used mice lacking p53, a key tumor suppressor; as well as various mouse models directly or indirectly over-expressing Myc. The p53 knock-out (KO) mouse lacks functional p53 due to targeted insertion of a neomycin cassette resulting in mice with elevated rates of tumorigenesis, most commonly thymoma (Jacks et al., 1994). In this dissertation, data from experiments using GEMM's modeling direct as well as indirect, Myc-driven tumorigenesis will be discussed and the specific mouse models are described in the Myc deregulation section.

Oncogenes and Tumor Suppressors

Genetic screens looking at the mechanisms of transformation have identified specific genes that are often mutated or deleted in various types of malignancies. These genes often encode proteins important for maintaining the “status quo" in cells by either regulating cell proliferation and growth, or policing the cells genome for damage or mistakes. Cancer is considered a genetic disease, in the sense that it is often activating or inactivating mutations of genes that result in the transformation of a cell from a normal cell into a tumor cell. In this sense, genes can be categorized into two categories: oncogene or tumor suppressor.

Oncogenes are characterized by the fact that activating mutations in these genes promote tumorigenesis by driving cellular proliferation, unlimited replication, angiogenesis or other characteristic hallmarks of cancer. Before they are mutated genes such as Myc or Ras are known as proto-oncogenes, but after activating mutation effecting either protein expression or function, they are known as oncogenes. Some oncogenes can drive transformation alone (e.g. Myc), while other “weak oncogenes" (e.g. Pim kinases) require help from cooperating oncogenes.

<table>
<thead>
<tr>
<th>Mechanism of activation</th>
<th>Gene</th>
<th>Function</th>
<th>Malignancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retroviral oncogene</td>
<td>v-src</td>
<td></td>
<td></td>
</tr>
<tr>
<td>v-myc</td>
<td>Non-receptor Tyrosine Kinase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>v-raf</td>
<td>Transcription factor</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Serine/Threonine Kinase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>avian sarcoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>avian myeloid leukemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>mouse sarcoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proviral Insertion</td>
<td>Mos</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Serine/Threonine Kinase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>mouse plasmacytoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Plm2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Serine/Threonine Kinase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>mouse B-cell lymphoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bmi1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Transcriptional repressor</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>mouse T-cell lymphoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amplification</td>
<td>ERB1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Receptor Tyrosine Kinase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glioblastoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>c-MYC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Transcription factor</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Carcinomas</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AKT1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Serine/Threonine Kinase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>gastric cancers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overexpression due to translocation</td>
<td>c-MYC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B &amp; T cell malignancies</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BCL-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B-cell lymphoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BCL-6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B-cell lymphoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fusion protein due to translocation</td>
<td>BCR/ABL</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Non-receptor Tyrosine Kinase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CML &amp; AML</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>E2A/PBX1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Transcription factor</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pre-B-cell leukemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NPM/ALK</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Receptor Tyrosine kinase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Large cell lymphoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Point mutation</td>
<td>H-K-N-Ras</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>G protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Many</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CDK4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cell cycle protein (Cdk)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Melanoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>JAK2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Receptor Tyrosine Kinase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>diffuse large cell B-cell lymphoma</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Examples of oncogenes as well as the genetic mechanism of activation

Several of the most well known oncogenes are listed in Table 1, along with the mechanism activating these oncogenes and the tumor types they are associated with. Fortunately, oncogenic signaling typically triggers response from signaling pathways monitoring cell cycle, DNA quality, etc which results in cell cycle arrest or apoptosis.
In the case of tumor suppressors, their function is apparent in their name; they function to inhibit tumor formation by responding to uncontrolled proliferation signals or DNA damage by causing cell cycle arrest or inducing programmed cell death. Due to the central role they play in preventing tumorigenesis, tumor suppressors are often inactivated in many malignancies (See Table 2).

<table>
<thead>
<tr>
<th>Mechanism of inactivation</th>
<th>Gene</th>
<th>Function</th>
<th>Malignancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promoter Methylation</td>
<td>CDKN2A</td>
<td>Encodes for p14/p19 ARF</td>
<td>colon cancer; lymphoma</td>
</tr>
<tr>
<td></td>
<td>APC</td>
<td>Induces degradation of β-Catenin</td>
<td>colon carcinomas</td>
</tr>
<tr>
<td></td>
<td>RB</td>
<td>Cell cycle regulator</td>
<td>retinoblastoma</td>
</tr>
<tr>
<td></td>
<td>CDKN1C</td>
<td>Cell cycle CDK inhibitor</td>
<td>gastric; pancreatic; hepatic; AML</td>
</tr>
<tr>
<td></td>
<td>APAF1</td>
<td>Pro-apoptotic cascade</td>
<td>melanoma</td>
</tr>
<tr>
<td>Mutation or Deletion</td>
<td>TP53</td>
<td>Transcription factor</td>
<td>many</td>
</tr>
<tr>
<td></td>
<td>CDKN2A</td>
<td>Encodes for p14/p19 ARF</td>
<td>colon cancer; lymphoma</td>
</tr>
<tr>
<td></td>
<td>RB</td>
<td>Cell cycle regulator</td>
<td>retinoblastoma</td>
</tr>
<tr>
<td></td>
<td>VHL</td>
<td>Induces degradation of HIF1α</td>
<td>sporadic kidney carcinomas; VHLS</td>
</tr>
<tr>
<td></td>
<td>NF1</td>
<td>Negative regulator of Ras</td>
<td>neurofibromatosis</td>
</tr>
<tr>
<td></td>
<td>PTEN</td>
<td>Inhibits Akt/PKB signaling</td>
<td>many</td>
</tr>
</tbody>
</table>

Table 2. Examples of tumor suppressors and the genetic mechanism of inactivation

p53 “the guardian of the genome”, is a prime example of a tumor suppressor and found to be inactivated, by deletion or mutation, in roughly 50% of human malignancies (Hollstein et al., 1994). DNA damage or oncogenic signaling normally activates p53 which sets in motion pathways resulting in cell cycle arrest and repair of DNA damage, or if this is not possible, programmed cell death (Levine, 1997). p53 functions as a transcription factor inducing downstream genes responsible for cell cycle arrest (e.g. p21<sup>Cip1</sup>), DNA repair (e.g. p53R2) or apoptosis (e.g Bad, Bax, Puma, Noxa) (Ozaki and Nakagawara, 2011; Zuckerman et al., 2009). p53 is regulated by a variety of factors, which are summarized and simplified in Figure 1.

Figure 1. Regulation of p53, the “guardian of the genome”
A: A simplified view of physiological factors stimulating p53, and the downstream effects of p53 activation. The tumor suppressive role of p53 is so potent that it is inactivated in many tumors. (modified from Robert A. Weinberg “The Biology of Cancer” textbook Garland Science 2007)
B: The Arf-Mdm2-p53 loop of tumor suppression. This regulation of p53 control is so important that if the p53 protein itself is not inactivated, proteins regulating p53 such as Arf, are often inactivated instead.
p53 is targeted for degradation by Mdm2 (Hdm2 in humans), which also inhibits activation of p53 target genes (Momand et al., 1992). DNA damage or induction of Mdm2 inhibitor p19Arf (p14Arf in humans) results in phosphorylation of p53 and/or Mdm2, disassociation of p53-Mdm2, and activation of p53 (Moll and Petrenko, 2003). p53 also regulates itself through a negative-feedback loop by initiating transcription of Mdm2 (Wu et al., 1993) which results in rapid degradation of p53 in cell. p53, and the downstream pathways it activates, play a central role in responding to uncontrolled proliferation or DNA damage, frequently the result of oncogenic signaling in transformed cells, so it is not surprising that p53 signaling is frequently inactivated in tumors. p53 itself can be inactivated by gene deletion, or by dominant negative mutation which results in a nonfunctional protein (Sherr, 1998). Perturbation of p53 regulation such as over-expression of Mdm2 or p19Arf deletion is also seen in many cancers and results in inactivation of p53 dependent pathways (Sherr, 1998). p53 regulation is complex and previous studies have found that Arf may have p53 independent functions (Holley and St Clair, 2009), and that p53 can respond to DNA damage even in the absence of Arf (Suzuki et al., 2003).

The Cell Cycle

Oncogenes and tumor suppressors are fundamentally different, as illustrated by how they impact normal cellular cycling. The cell cycle is regulated by a complex web of signaling pathways that tell the cell when to rest, when to proliferate and therefore synthesize new DNA and proteins, or when to die. These signaling pathways receive input from the outside environment of the cell as well as communicate with each other within the cell to ensure that cells resulting from cellular division have no mutations or flaws that will impact normal function. Oncogenes are typically involved in signaling for growth and proliferation, whereas tumor suppressors typically function in quality assurance and inducing repair or cell death signaling.

Figure 2. Cell Cycle

Upon mitogen stimulation cells begin a process of replication and division called the cell cycle. This is a carefully orchestrated process controlled by the expression and interaction of a variety of proteins namely CDK’s (cyclin dependent kinases) and their binding partner proteins, cyclins. The restriction point, or R point labeled here in red, is the point of no return for entering the cell cycle and the interactions required to reach this point are tightly controlled by proteins such as pRb (in black), an important tumor suppressor gene as well as CDK inhibitors such as the INK4 and CIP/KIP family of proteins (in dark gray).
When cells receive a signal to divide from the environment, a signaling program called the cell cycle is put in motion (Figure 2) (Vermeulen et al., 2003). Most cells in the body are not dividing and in the G0 phase of the cell cycle. However once cells are stimulated to divide, they enter into different phases of the cell cycle: G1, S and G2 phases are collectively called Interphase; and Mitosis or M phase. G1 is a resting phase where the cell prepares for the DNA synthesis that will take place in S phase. G2 phase is another resting phase where DNA integrity is checked before M phase when the cells divide into two individual daughter cells. These phases are distinct and tightly controlled by specific protein complexes called Cyclin dependent kinase (CDK)-cyclin complexes. These CDK-cyclin complexes are in turn activated by CDK activating kinase (CAK) that causes CDK conformational changes leading to elevated cyclin-CDK binding. The specific CDK-cyclin complexes responsible for various phases of the cell cycle are shown in figure 2. CDK proteasomal degradation by anaphase promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase, keeps CDK levels low and even throughout the cell cycle. Although CDK levels are regulated, the amount of specific cyclin levels present has the most impact on which CDK-cyclin complex is formed. An important CDK activity is the phosphorylation of pRb, thereby causing the release E2F, resulting in E2F transcription of proteins necessary for S and M phases. CDK inhibitors (CKIs) consisting of the INK4 and Cip/Kip family of proteins, regulate CDK activity by binding CDKs directly (INK4 family) or binding the CDK-cyclin complex (Cip/Kip family) (Blain, 2008). These CKIs provide regulation of cell cycle checkpoints, and respond to stress or anti-proliferative signals in order to halt cell cycling. Tumor suppressors impact cell cycling by sequestering important regulators (e.g. pRb sequestering E2Fs), or initiating transcription of CKIs (e.g. p53 transcription of p21\(^{cip1}\)). This complicated regulation of proliferation and cell cycling exists solely to ensure that there are no mutations or mistakes in the resulting daughter cells, however in tumors, many of these same players are either activated (e.g. oncogenic Myc or Ras signaling) or inactivated (p53) resulting in deregulation of this network.

**Cell Death**

During the lifetime of a cell, if there is damage to cellular components such as DNA, or if the cell has simply reached the end of its replicative potential, cell death programs are initiated. Several cell death programs can be induced depending on the conditions signaling for cell death: autophagy, necrosis and apoptosis (Kroemer et al., 2009). These programs are distinct and are triggered by various factors, however there are some similar features as well as cross talk between them. Autophagy is characterized by the breakdown, and lysosomal digestion of cytoplasmic organelles resulting in the building blocks (proteins, amino acids, etc) from these organelles to be reused by other cellular machinery (Mizushima, 2007). Interestingly, this type of cell death actually functions as a survival mechanism for cells undergoing metabolic starvation, and disruption of autophagy has been shown to make cells more susceptible to apoptosis (Rosenfeldt and Ryan, 2009). Autophagy has been shown to serve important biological functions in a variety of normal cellular processes as well as pathological diseases (Levine and Kroemer, 2008). Necrosis is morphologically defined by a drastic increase in cell volume, expansion of organelles and subsequent membrane rupture triggering an immune response (Han et al., 2008). Necrosis used to be associated with physical injury or damage to cells that resulted in uncontrolled cell death, however recent studies indicate that defined signaling pathways might actually be responsible for necrotic death regulation (Golstein and Kroemer, 2007). Although autophagy and necrosis signaling pathways are important and involved in many biological settings, the bulk of the discussion on cell death will be concerning apoptosis.

Apoptotic cell death is morphologically characterized by: rounding up of the cells and withdrawal of pseudopodes, reduction of cellular volume, limited structural modification of organelles, nuclear volume reduction followed by fragmentation, plasma membrane “blebbing” and finally (*in vivo*) phagocytic engulfment without triggering the immune response (Han et al., 2008; Kroemer et al., 2009). Apoptosis is a tightly controlled, programmed response resulting in cell death that can be induced by external or internal signaling pathways (Figure 3) (Han et al., 2008; Kerr et al., 1972). Apoptosis can be induced through extrinsic signaling, such as activation of cell membrane receptors (e.g. Fas or TNFR1) resulting in death inducing signaling
complex (DISC) formation, or intrinsic signaling mediated through the mitochondria. Extrinsic and intrinsic signals typically trigger proteolytic cascades (e.g. caspase cascades) that cleave various target proteins, such as other caspases, finally resulting in cell death. Caspases can be separated into two groups, the initiator caspases such as Caspase-8 and 10 (extrinsic) or Caspase-9 (intrinsic) and the executioner caspases such as Caspase-3, and 5-7. Initiator caspases are activated by homodimerization, whereas executioner caspases are activated by cleavage. The end result of this caspase cascade is the proteolysis of proteins involved in all areas of cellular life, resulting in the morphological characteristics that define apoptosis.

Mitochondrial signaling plays an important role in apoptosis, regardless if the initiating signal for apoptosis originated from the mitochondria (intrinsic) or if the signaling is simply passing through the mitochondria (extrinsic) (Elmore, 2007). Mitochondria release pro-apoptotic proteins such as AIF, Endonuclease G and CAD that aid in nuclear DNA disintegration. Importantly, cytochrome-c release from the mitochondria results in the activation of apoptosis activating factor 1 (Apaf-1), formation of the apoptosome, and activation of Caspase-9 thereby activating executioner caspases. Cytochrome-c release is tightly controlled by the interaction of membrane permeability transition (MPT) pore proteins and pro-apoptotic and anti-apoptotic members proteins of the Bcl family (Adams and Cory, 2007). Anti-apoptotic family members Bcl-2 and Bcl-XL prevent pro-apoptotic Bak and Bax from initiating conformational changes in MPT pore proteins causing release of cytochrome-c. Pro-apoptotic BH3-only proteins Bim, Bid, Bad, Noxa and Puma also influence this delicate balance by inhibiting Bcl-2 anti-apoptotic proteins. Tumor suppressor p53 can initiate apoptosis by transcriptionally activating pro-apoptotic proteins Bax, Noxa and Puma in response to DNA damage or oncogenic hyperproliferative signaling (Ozaki and Nakagawara, 2011). Although p53 is deregulated in 50% of human cancers (Hollstein et al., 1994), many tumors express functional p53 therefore anti-apoptotic proteins such as Bcl-2 are often deregulated instead (Adams and Cory, 2007; Yip and Reed, 2008).

Figure 3: Extrinsic and Intrinsic Apoptotic signaling
A simplified view of apoptotic signaling is depicted here. Apoptotic signaling can begin at the cell surface and be transmitted into to cell, where it converges on the intrinsic, mitochondrial mediated, pathway. Alternatively, it can begin inside the cell and be transmitted via the intrinsic pathway. There is cross-talk, as well as common mediators, between the pathways.
MYC: instigator of cell proliferation and cell death

The Myc family, c-Myc, N-Myc and L-Myc, is a group of helix-loop-helix leucine zipper transcription factors mainly targeting genes involved in metabolism and cell proliferation (http://www.myccancergene.org). Although these proteins share similar functions N-Myc is expressed in brain tissue whereas L-Myc is expressed in lung tissues, and over-expression of these Myc genes also results in transformation in these tissues (Malynn et al., 2000). However, in this dissertation I will focus on c-Myc therefore all mentions of Myc will refer to c-Myc unless clearly stated otherwise.

Myc transcription is induced by signals telling the cell to grow or proliferate such as mitogens, cytokines, Wnt and Notch signaling (Figure 4) (Vervoorts et al., 2006; Wierstra and Alves, 2008). Myc is temporarily expressed in the G1 phase to induce cell cycle entry, and then is quickly turned off again via negative feedback regulation (Hooker and Hurlin, 2006). It is also important to note that in addition to proliferation signals, tumor suppressors Arf and p53 also transcriptionally regulate Myc. Arf binds the Myc Box II domain and relocates Myc from the nucleus to the nucleolus interfering with Myc transcriptional activity resulting in growth arrest, independent of p53 (Amente et al., 2006). p53 transcriptionally represses Myc by directly binding to the c-Myc promoter, as well as reduced acetylation of H4 at the c-Myc promoter and recruitment of the co-repressor mSin3a (Ho et al., 2005). Myc protein has a short half-life of around 15-20 minutes resulting in low Myc protein levels in S phase (Hooker and Hurlin, 2006). Mitogen activation of receptor tyrosine kinases results in Ras driven activation of the Raf/ERK and PI-3K kinase pathways, stabilization of Myc protein and diminished Myc degradation (Sears et al., 2000). The Raf/ERK kinase pathway stabilizes Myc due to phosphorylation of Serine 62 (S62), whereas PI-3K kinase impacts Myc degradation by inhibiting glycogen synthase kinase (GSK-3) phosphorylation of threonine 58 (T58) thereby blocking Myc proteasomal degradation.

Figure 4: Factors regulating Myc transcription or protein function
Upon mitogen stimulation, transcription of Myc, as well as stabilization of Myc protein, results in transcription of many genes important for cellular proliferation and growth.
Myc: Instigator of cellular proliferation

Myc forms dimers with Max, binds to E-box sequences in DNA (usually CACGTG) to initiate transcription of target genes (Blackwood et al., 1991). Myc initiates transcription of target genes in several different ways: binding Max and initiating transcription directly (Eisenman, 2000), relieving transrepression by Max; Mnt/Mxd dimers (Nilsson and Cleveland, 2004), or associating with TRRAP and recruiting histone acetyltransferases (McMahon et al., 2000). Myc can also negatively regulate transcription by binding and interfering with other transcriptional proteins like Miz-1, or bind Sin3A and Sin3B to recruit histone deacetylases to repress Inr element driven transcription (Peukert et al., 1997).

Myc transcriptional activities are carefully balanced and depend on levels of the various players such as Max, Mad, Mnt and Miz-1, which themselves are strictly regulated (Hooker and Hurlin, 2006). In order for Myc to dimerize with Max and initiate transcription, it must compete with the other Max dimerization partners, such as the Mxd family (Mxd1, Mxi1 Mxd3 and Mxd4) but particularly constitutively expressed Mnt. The Mxd family is transiently expressed at different stages of the cell cycle: Mxd3 during S phase whereas Mxd1, Mxi1 and Mxd4 are expressed during differentiation, senescence and G0. Previous studies have shown that Myc over-expression cells and cells lacking Mnt have similar transformation phenotypes (Hooker and Hurlin, 2006; Hurlin et al., 2003; Nilsson et al., 2004).

In addition to direct transcriptional activation of genes, Myc has been shown to impact general transcription by modifying chromatin and allowing access to gene promoters (Knoepfler, 2007). Myc transcriptional impact also includes recruitment of RNA polymerase (pol) II to target genes, as well as transcriptional elongation due to RNA pol II binding (Kanazawa et al., 2003). Myc also upregulates translational machinery by effecting rRNA expression through RNA pol I binding (Grandori et al., 2005), as well as binding TFIIIB resulting in activation of RNA pol III and transcription of 5S rRNA and tRNA (Gomez-Roman et al., 2003). Myc again exhibits translational effects by promoting RNMT 5'cap methylation of specific mRNA’s thereby inducing translation of these specific mRNA’s (Cole and Cowling, 2008). These studies all support the conclusion that Myc transcriptional activation of target genes, as well as general transcriptional and translational effects, is important in actively proliferating cells and therefore highly controlled.

Myc plays an important role in cell cycle regulation due to the Myc induced transcription of cyclin D2 and CDK4 (which promote cell cycle entry by impacting pRb phosphorylation) as well as various E2Fs and proteins responsible for degrading CDK inhibitor p27\(^{Kip1}\) (Dang et al., 2006). Myc and Miz1 dimers also directly repress transcription of CDK inhibitors p15\(^{INK4B}\), p21\(^{Cip1}\) and p27\(^{Kip1}\), resulting in the promotion of cyclinD-CDK4/6 activity and entry into cell cycle (Eilers et al., 1991). The effect of Myc over-expression is so potent that it has been shown to drive cell cycle entry in the absence of mitogens and growth signals (Soucek and Evan, 2010). The importance of Myc in cellular proliferation is well established, and deletion of c-Myc is embryonic lethal in mice (Davis et al., 1993). Cells lacking Myc can sometimes survive, although they are smaller in size and even manage cell cycling depending on cell type and tissue (Mateyak et al., 1997; Sansom et al., 2007; Soucek et al., 2008). However it is possible to get too much of a good thing, and in normal cells too much Myc also has a detrimental effect on cellular proliferation and growth (Murphy et al., 2008).

Myc: Instigator of cellular senescence and death

Myc mediated transcriptional targets account for 15% of the human genome and Myc is deregulated in 70% of human cancers indicating that Myc target genes are clearly important for cellular proliferation and transformation (Dang et al., 2006). However, because Myc plays such a central role in cellular proliferation and the consequences of elevated Myc signaling are so severe, Myc deregulation triggers senescence or apoptosis in normal cells (Hoffman and Liebermann, 2008; Nilsson and Cleveland, 2003; Sherr, 1998). Importantly, the choice between senescence and apoptosis depends on the level of Myc expression (Murphy et al., 2008). Myc over-expression can trigger apoptosis in several different ways, notably activation of the Arf/p53 tumor suppression pathway. Myc over-expression triggers FoxO-mediated transcription of Arf leading to elevated p53 protein levels, resulting in senescence or apoptosis (Bouchard et al., 2007).
2007; Zindy et al., 1998). In the presence of wild-type p53, Myc deregulation typically results in apoptosis due to Myc down-regulation of p21 and Gadd45 (cell cycle regulators and p53 transcriptional targets), therefore tipping the balance in favor of apoptosis over growth arrest (Amundson et al., 1998; Seoane et al., 2002; Vousden and Lu, 2002). Myc over-expression in vivo results in genomic instability and DNA damage activating the ATM signaling pathway, a major pathway responsible for cellular response to double stranded DNA breaks, culminating in the activation of p53 (Pusapati et al., 2006; Sargent et al., 1996; Vafa et al., 2002). This p53 mediated response is so potent, that in order for Myc driven transformation to occur in vivo, Arf or p53 is mutated or deleted in tumors occurring in Eµ-Myc transgenic mice (Eischen et al., 1999).

However, p53 activation is not always necessary in order to initiate apoptosis. Myc also triggers intrinsic apoptotic pathways via the mitochondria by up-regulating pro-apoptotic factors such as Bax and Bim, or down-regulating anti-apoptotic factors such as Bcl-2 and Bcl-XL, resulting in p53 independent apoptosis (Egle et al., 2004; Hoffman and Liebermann, 2008; Maclean et al., 2003; Mitchell et al., 2000). Myc directly activates Bax transcription and may transcriptionally activate Bim, although if this is direct or indirect is still unknown (Egle et al., 2004; Hemann et al., 2005; Mitchell et al., 2000). Myc indirectly down-regulates Bcl-XL and Bcl-2, and down-regulation of Bcl-2 is dependent on Myc binding to, and inactivating the transcriptional activator Miz-1 (Eischen et al., 2001a; Patel and McMahon, 2007). Myc has been shown to activate intrinsic apoptotic pathways through the mitochondria, but it has also been shown to modulate death receptor mediated extrinsic apoptotic signaling, as well as survival pathways activated by death receptors. Myc can alter expression of death receptors themselves as well as components of DISC (death inducing signaling complex). Myc transcriptionally activates death receptors FasL as well as the TRAIL receptor DR5 (Kasibhatla et al., 2000; Wang et al., 2005). Myc transcriptionally down-regulates FLIP (Fas-associated death domain-like II-1β-converting protein), a caspase-8 inhibitor, resulting in increased caspase-8 activation and apoptosis (Rici et al., 2004). Rat1a cells over-expressing Myc were more sensitive to CD95/Fas death signaling activation, and this activation was downstream of the CD95/Fas death receptor, suggesting that Myc plays a role in amplifying the death signal at the level of the mitochondria (Hueber et al., 1997). Myc repression of survival pathways, such as those activated by c-Jun or NF-κB, in response to death receptor and disc activation also favors apoptosis (Kleifstrom et al., 1997).

So if the cellular response to Myc deregulation is apoptosis, how does Myc over-expression result in tumors? Tumors over-expressing Myc have been shown to exhibit inactivating mutations or deletions in many pro-apoptotic pathways, such as the Arf/p53 pathway, that would normally initiate apoptosis in response to Myc deregulation (Eischen et al., 1999). Pre-cancerous cells and lymphomas from Eµ-Myc transgenic mice exhibited marked upregulation of anti-apoptotic Bcl-2 or Bcl-XL, highlighting the importance of these proteins in tumorigenesis (Eischen et al., 2001b). Analysis of human Burkitt Lymphoma tumors, as well as tumors arising in mouse models of Burkitt Lymphoma, revealed methylation and silencing of genes encoding pro-apoptotic proteins such as the BH3-only protein Puma (Garrison et al., 2008). Co-expression of another oncogene, such as Ras, has been shown to augment Myc driven transformation possibly due to Ras suppression of Myc apoptotic inducing functions as well as Myc suppression of Ras-induced senescence (Land et al., 1983).

Unlike Myc, the expression level of Ras is not what results in transformation; it is a mutation resulting in a conformational change in the protein, rendering it perpetually activated. Ras is a membrane bound GTPase that is only active in the GTP-bound conformation. Mitogen activation of receptor tyrosine kinases is relayed through Grb2-SOS, resulting in a Ras conformational change causing the release of GDP and the binding of GTP (Margolis and Skolnik, 1994). However in many tumors, one of the three Ras proteins (H-, K- and N-Ras) are mutated rendering it incapable of hydrolyzing GTP, thus locking Ras in an activated conformation (Bos, 1989). Mitogen activation of receptor tyrosine kinases results in Ras driven activation of the Raf/ERK and PI-3K kinase pathways resulting in transcriptional activation of AP-1, Ets-1, CREB, NF-κB, fos, jun and Myc (Chang et al., 2003). As previously mentioned, the Raf/ERK kinase pathway stabilizes Myc due to phosphorylation of Serine 62 (S62), and PI-3K kinase impacts Myc degradation by inhibiting glycogen synthase kinase (GSK-3) phosphorylation of threonine 58 (T58) thereby blocking Myc proteosomal degradation (Sears et
al., 2000). Ras can enable Myc transformation by increasing survival signals via the Raf/ERK and PI-3K kinase pathways that balance out the pro-apoptotic signals Myc deregulation normally activates (Figure 5). In return, Myc inhibition of CKI's such as p21^Cip1 and p27^Kip1, can block the growth arrest and senescence normally triggered by oncogenic Ras signaling activation of the ARF/p53 pathway (Eilers et al., 1991; Groth et al., 2000). In vitro transformation experiments as well as in vivo tumor analysis have shown that Myc and Ras collaborate in tumorigenesis (D'Cruz et al., 2001; Land et al., 1983).

**Myc over-expression and cancer**

**Myc deregulation**

As discussed in previous sections, Myc is a potent transcription factor that is tightly regulated by a variety of mechanisms. However, it is also theorized that Myc over-expression contributes to anywhere between 40-70% of human cancers due to the massive Myc target gene network (Dang et al., 2006; Nilsson et al., 2005). Since Myc regulation is so tightly controlled, and the consequences of Myc over-expression are so catastrophic, how does Myc become deregulated? The deregulation of upstream signaling pathways, such as the receptor tyrosine kinase and Ras pathways, could result in over-expression of Myc (Sears, 2004). Stabilizing point mutation, gene amplification or chromosomal translocations of the Myc gene also contribute to deregulation of Myc (Boxer and Dang, 2001). Some malignancies, such as Burkitt Lymphoma, are characterized by Myc deregulation. Burkitt Lymphoma (BL) is a B-cell lineage malignancy characterized by Myc over-expression due to a translocation from its normal position on chromosome 8 to the promoter region of the immunoglobulin (Ig) heavy chain enhancer \( \mu \) (chromosome14), or light chain enhancer \( \kappa \) or \( \lambda \), on chromosome 2 and 22 respectively. This translocation results in B-cell specific Myc over-expression, and lymphomagenesis. Not only is Myc transcriptionally over-expressed in these cells, it is also resistant to repression while the normal Myc allele remains sensitive to transcriptional repression and is therefore down-regulated (Kelly and Siebenlist, 1985). As discussed previously, genetically engineered mouse models (GEMMs) of cancer are valuable tools in the search for effective treatment strategies for many malignancies, one of which is Myc-driven lymphoma. The E\( _{\mu} \)-Myc, \( \lambda -\)Myc, and iMyc\(^{5+}\) mice model the human disease Burkitt Lymphoma.
by over-expressing Myc in the B-cell compartment therefore developing B-cell lymphomas of diverse maturity. In the Eµ-Myc transgenic mouse, c-Myc remains under the control of its own promoter but is also regulated by the enhancer element of the immunoglobulin heavy chain, which results in the development of pre-B and immature B-cell lymphomas (Adams et al., 1985). In the λ-Myc mouse, c-MYC is placed under the control of the λ light chain enhancer resulting in the formation of more matures B-cell lymphomas (Kovalchuk et al., 2000). The iMycE mouse is a knock-in mouse, where c-Myc has been knocked-in to the IgH locus and develops immature as well as mature B-cell lymphomas therefore more closely modeling what happens in Burkitt Lymphoma (Zhu et al., 2005). In this dissertation we will be presenting data from experiments preformed in Eµ-Myc, λ-Myc, and iMycE mice which model Myc-driven lymphomagenesis, as well as an indirect Myc tumorigenesis model, the ApcMin mouse. In the ApcMin mouse model, mice develop adenomatous polyps due to an inactivating point mutation in Apc, resulting in loss of Wnt signaling and upregulation of Myc transcription (See Figure 4) (McCart et al., 2008; Moser et al., 1995).

**Myc and the Hallmarks of Cancer**

In normal cells, proliferation and cell cycling is under strict control ensuring that cells divide only when needed, and in a controlled manner. Damage to the cell, such as mutation or breakage of DNA, is sensed by pathways that respond by either repairing the damage or initiating cell death depending on the severity of the damage. These pathways act as a kind of security force in cell signaling and are necessary to make sure that cells adhere to the rules of behavior according to tissue specific environments. Since cell cycling is regulated so tightly, and aberrations in DNA are dealt with so harshly, the characteristics needed to evade these control are fairly rare and specific. Hanahan and Weinberg initially summarized these six characteristics in a publication ‘Hallmarks of Cancer’ in 2000 and earlier this year they expanded this list, which now includes eight characteristics as well as two enabling characteristics (Hanahan and Weinberg, 2011). The revised list of hallmarks of cancer include: sustaining proliferation signaling, evading growth suppressors, resisting cell death, avoiding immune destruction, enabling replicative immortality, activating invasion and metastasis, inducing angiogenesis, deregulating cellular energetics. Two enabling characteristics or qualities that enable the various hallmarks of
cancer in tumorigenesis, genome instability and mutation as well as tumor promoting inflammation, are also described. For the purposes of this discussion, I will depict the hallmarks of cancer highlighting the role of Myc in each of these processes (Figure 6). I refer the reader to the original article by Hanahan and Weinberg for a more detailed and thorough evaluation of these topics.

**Deregulation of cellular energetics: Emerging Hallmark of Cancer**

As outlined in “Hallmarks of Cancer: The next generation”, in order for tumor formation to occur, cells need to acquire many characteristics enabling the cells to escape normal control mechanisms that would normally prevent the high rate of proliferation commonly exhibited by aggressive tumor cells (Hanahan and Weinberg, 2011). Characteristics such as sustained proliferation signaling and evasion of growth suppressors results in increased proliferation of transformed cells. Therefore, cellular energy metabolism must also be rewired to compensate for the increase in proliferation. One of the metabolic pathways deregulated in many tumors is the glucose metabolism pathway. Not only do many tumors increase glucose transport into the cell, they also exhibit significant changes in how the glucose is metabolized once it enters the cell (Hsu and Sabatini, 2008). In many tumors, the rapid proliferation of tumor cells results in increased DNA and protein synthesis, requiring increases in serine biosynthesis (de Koning et al., 2003). This rapid proliferation also results in alterations in the metabolism of polyamines, small polycationic molecules required for eukaryotic cell growth. Polyamines have been shown to be required for rapidly proliferating cells, possibly due to the binding and stabilization of RNA, DNA and proteins (Larque et al., 2007). The pathways mentioned above are known to be altered in many different cancers, and are also known to be upregulated by Myc (Casero and Marton, 2007; Dang et al., 2009). These pathways, and the role that Myc target genes play in them, will be discussed in the following sections. The publications that are included in this dissertation have focused on the these pathways, as well as the Myc target genes within them, for the purpose of ascertaining if targeting of these genes and these pathways provide novel strategies in combating Myc driven tumorigenesis.

**Glucose metabolism**

Figure 7. Glycolysis
A simplified version of anaerobic glycolysis is depicted here, as well some notable pathways that are linked to glycolysis via intermediate substrates. Abbreviations of enzymes shown here are: PKM (pyruvate kinase M1/M2); LDH (lactate dehydrogenase); PDH (pyruvate dehydrogenase).
**Glycolysis**

The metabolism of carbohydrates, lipids, amino acids, vitamins, hormones and nucleotides occurs throughout the cell in complicated, often overlapping or interconnected, metabolic pathways. Glucose metabolism, glycolysis coupled with the citric acid cycle, is a central pathway in cellular metabolism providing the majority of ATP required the fuel many cellular processes. Through an intermediate step in glycolysis, glucose metabolism is linked to the pentose phosphate pathway (PPP) resulting in the formation of ribose-5-phosphate required for nucleotide biosynthesis, as well as NADPH required for fatty acid synthesis (Figure 7). In the presence of oxygen, glucose metabolism begins with glycolysis in the cytoplasm, and finishes with the citric acid cycle and oxidative phosphorylation in the mitochondrial membrane providing energy in the form of ATP production. In the cytosol, glycolysis breaks down glucose into pyruvate, which is then shuttled in the citric acid cycle and oxidative phosphorylation in the mitochondria resulting in the formation of ATP and carbon dioxide (see Figure 7). In hypoxic conditions, pyruvate cannot be converted to Acetyl-CoA due to HIF1α induction of pyruvate dehydrogenase kinase 1 (Pdk1), which then inhibits pyruvate dehydrogenase (Pdh) essentially blocking entry into the citric acid cycle (Kim et al., 2006). Therefore, under anaerobic conditions glycolysis alone is used and pyruvate is not shuttled into the citric acid cycle, but instead converted into lactate by the cytosolic enzyme lactate dehydrogenase (Ldh). Glycolysis coupled with the citric acid cycle is much more efficient than glycolysis alone, producing 36 instead of 2 ATP molecules per glucose molecule, so in normal cells reliance on glycolysis is usually used as a short-term solution to cope with anaerobic conditions.

**Lactate dehydrogenase A**

Mice and humans have two genes that encode the two Ldh subunits, Ldha and Ldhb, residing on different chromosomes (Seawright et al., 1988). Ldha is transcriptionally regulated by signals activating the Protein Kinase A (PKA) and C (PKC) pathways (Huang et al., 1995). PKA and PKC signaling also results in post-transcriptional modification, increased stabilization, of Ldha mRNA transcripts (Jungmann et al., 1998). Expression of lactate dehydrogenase A (Ldha) is also transcriptionally activated by Myc as well as HIF1α, resulting in increased expression of Ldha in hypoxic conditions or in response to cell proliferation signaling (Semenza et al., 1994; Shim et al., 1997).

![Ldh enzyme](image)

**Figure 8. Ldh enzyme**

The Ldh enzyme is a tetramer made up of Ldha or Ldhb subunits, with the number of subunits of each type affecting the substrate specificity. The higher the number of Ldhb subunits, the higher the preference for lactate to pyruvate conversion, and vice versa. Ldha is also known as “muscle-type” as it is highly expressed in muscle, whereas Ldhb is also known as “heart-type”.

The two Ldh subunits, Ldha and Ldhb, and various combinations of these two subunits make five different Ldh isozymes. The Ldh subunits have also been named according to which tissues they tend to be expressed: Ldha is also known as Ldh-M for muscle-type, and Ldhb is also known as Ldh-H, for heart type (Jungmann et al., 1998). There is also a third subunit, Ldhc that is exclusively expressed in the testis (Goldberg et al., 2010). LDH5 consists entirely of Ldha subunits, and LDH1 consists entirely of Ldhb subunits, whereas LDH2-4 is made up of a mix of Ldha and Ldhb subunits (Figure 8). The Ldh enzyme is a tetramer made up of Ldh subunits and the type of subunits making up the tetramer determines the direction the Ldh catalyzed reaction, more Ldha subunits favor conversion of pyruvate to lactate, whereas more
Ldhb subunits favor the conversion of lactate to pyruvate (Fritz et al., 1969). The cytosolic enzyme Ldh plays a significant role in glycolysis converting pyruvate to lactate, and reoxidizing NADH to NAD⁺. The Ldh mediated production of NAD⁺ is then used by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that reduces NAD⁺ to NADH, enabling further ATP production (See Figure 7). Ldh can also convert lactate to pyruvate, using NADH as a cofactor. In addition to its well-established role in glycolysis, Ldha has been found to bind single-stranded DNA and interact with DNA-polymerase α-primase as well as acting as a DNA-helix destabilizing protein suggesting it may also play a role in transcription (Cattaneo et al., 1985; Grosse et al., 1986; Williams et al., 1985). Recently, Ldha was also found to be a component of OCA-S, a complex that activates Oct-1, and is responsible for the S-phase activation of Histone H2B (Zheng et al., 2003). Although this evidence is supportive of an Ldha role in transcription, its contribution in pathways outside of glycolysis have not been firmly established.

**Glycolysis and Lactate dehydrogenase A in Cancer**

Rapidly proliferating cells require higher amounts of glucose than do slower proliferating cells. Tumor cells have especially high rates of glucose uptake, in fact the increased uptake of radioactively labeled glucose is utilized to visualize tumor cells by positron emission tomography (PET) scanning (Hsu and Sabatini, 2008). Tumor cells increase uptake of glucose by increasing the expression of genes encoding membrane glucose transporters, particularly glucose transporter (GLUT) 1 and 3 (Airley and Mobasher, 2007). Not only is uptake of glucose altered in tumor cells, glucose metabolism in tumor cells is markedly different than that of normal cells in that tumor cells preferentially utilize anaerobic glycolysis even in the presence of oxygen (Warburg, 1956). Tumor cells up-regulate glycolytic genes, such as Ldha, while inhibiting entry of pyruvate into the mitochondria, decreasing the conversion of pyruvate into Acetyl-CoA and slowing citric acid cycling by regulating genes such as pyruvate kinase (PK) (Christofk et al., 2008). Although this has been confirmed in many different tumors, for many years the cancer field struggled with this idea because it made little sense, why would tumor cells preferentially use an energy pathway that was so inefficient? One explanation is that by utilizing glycolysis alone, the metabolites produced are then available for use in other cellular processes such as nucleotide, amino acid, and fatty acid biosynthesis instead of shuttled into the citric acid cycle (See Figure 7) (Vander Heiden et al., 2009). This is accomplished, in part, by increased expression of Ldha resulting in increased conversion of pyruvate into lactate, which is then secreted into the extracellular matrix (Feron, 2009). As previously stated, Ldha is transcriptionally activated by Myc as well as HIF1α, resulting in increased expression of Ldha in hypoxic conditions or in response to cell proliferation signaling (Semenza et al., 1994; Shim et al., 1997). Therefore the increased production, and excretion, of lactate functions to favor glycolysis but also as a mechanism for hypoxic cells to survive anaerobic conditions (Fantin et al., 2006). The excreted lactate lowers the pH of the extracellular matrix around the tumor cell and favors blood vessel formation in response to angiogenic growth factors, such as vascular endothelial growth factor (VEGF), produced by the tumor cells (Hunt et al., 2007). The increased lactate concentrations have been shown to be beneficial in tumor cell invasion of surrounding tissues, due to extracellular matrix degradation, as well as evasion of the immune system (Swietach et al., 2007; Wigfield et al., 2008). Interestingly, in some tumors there appears to be two types of tumor cells: cells that rely on “aerobic glycolysis” metabolism and cells that utilize normal glycolysis and citric acid cycle metabolism of glucose (Feron, 2009). In these tumors there appears to be a reciprocal relationship between the normoxic and hypoxic cells, the lactate “waste” produced by the hypoxic cells is utilized by the more oxygenated tumor cells (Feron, 2009).

Glycolytic genes are regulated by oncogenic signaling pathways such as Myc, NFκB, AKT, and tyrosine kinase receptor pathways resulting in activation of Ras, MAPK and PI3K and mammalian target of rapamycin (mTOR) pathways (Levine and Puzio-Kuter, 2010). Oncogenic Myc signaling results in increased glucose transport, breakdown of glucose to trioses and pyruvate due to transcriptional activation of glycolytic genes such as GLUT1, hexokinase 2 (HK2), phosphofructokinase (PFKM) and enolase 1 (ENO1) and Ldha (Dang et al., 2009). AKT and mTOR signaling regulate HIF1α, with elevated AKT and mTOR signaling resulting in increased HIF1α signaling (Wouters and Koritzinsky, 2008). Hypoxic conditions result in the
stabilization of HIF1α transcripts, and increases in HIF1α protein levels resulting in transcription of HIF1α target genes, many of which are glycolytic genes such as GLUT1, Ldha and pyruvate dehydrogenase kinase (Pdk1) (Airley and Mobasheri, 2007). Increased expression of Ldha and Pdk1 result in decreased Acetyl-CoA production and mitochondrial citric acid processing due to increased conversion of pyruvate into lactate, as well as decreased pyruvate dehydrogenase (Pdh) mediated conversion of pyruvate into Acetyl-CoA (Wouters and Koritzinsky, 2008). Interestingly, many of the glycolytic genes transcriptionally stimulated by Myc such as GLUT1, Ldha, and Pdk1 are also activated by HIF1α (Dang et al., 2008) suggesting that within tumors, hypoxia stimulated HIF1α activation cooperates with the oncogenic Myc stimulation of these genes to drive glycolysis (Dang et al., 2008).

As mentioned previously, increased lactate production and excretion can benefit tumor cells in several ways, by stimulating of angiogenesis, increasing metastatic potential and aiding evasion of the immune system (Hunt et al., 2007; Swietach et al., 2007; Wigfield et al., 2008). Increases in Ldha expression result in increased lactate production and increases in Ldha levels are reported in many tumors often due to transcriptional activation by Myc or HIF1α. However, a phosphorylating form of Ldha (Tyr238) with increased single-stranded DNA binding capabilities has been identified in the nucleus of tumor cells (Cooper et al., 1984; Zhong and Howard, 1990). Later, this Tyr238 form of Ldh was found to be complexed with Ras in several human ovarian cancer samples (Anderson and Kovack, 1981; Li et al., 1988). This Tyr238 form of Ldha was also found to correlate with higher malignancy grade in ovarian cancer samples (Chow et al., 1997).

Targeting Lactate dehydrogenase in Cancer

Ldha is an important enzyme in glycolysis, and has been shown to be upregulated in many tumors therefore it is interesting to determine if Ldha inhibition is a viable method in battling tumorigenesis. Results from experiments examining Ldha inhibition on Myc driven tumorigenesis on tumor cells in vitro, as well as in vivo xenograft transplants, have found that inhibition of Ldha results in significant effects on Myc transformed cells (Fantin et al., 2006; Le et al., 2010; Shim et al., 1997). Antisense mediated suppression of Ldha in Myc transformed Rat1a fibroblasts and several human lymphoid tumor lines resulted in significantly decreased soft agar colony growth, indicating that Ldha activity is important in Myc driven tumorigenesis (Shim et al., 1997). Mouse mammary tumor cells and human lung carcinoma cells expressing RNAi against Ldha transplanted into recipient mice exhibited significantly reduced tumor formation (Fantin et al., 2006; Le et al., 2010). These results indicate that Ldha inhibition has a significant effect on tumorigenesis, but what are the consequences of complete removal of Ldha? Interestingly, there are reports of families exhibiting LDHA or LDHB deficiencies due to inheritance of a recessive allele (Kanno et al., 1983). This Ldha deficiency doesn’t effect general health or viability but is only apparent itself after strenuous exercise when the patient exhibits muscle stiffness and pain (Kanno et al., 1988). Mice containing an inactivating point mutation (D223H) in Ldha, effecting Ldha transcript stability and enzymatic function, are also viable and outwardly normal (Charles and Pretsch, 1981). There is no phenotype exhibited by mice expressing a single mutated Ldha allele but mice expressing two mutated Ldha alleles possess a significantly increased spleen size due to increased hemolysis that stimulates splenic hematopoiesis (Kremer et al., 1986). The severe hemolysis of the Ldha mutant mouse is caused by reliance on Ldha expression stemming from the complete absence of Ldhb expression in erythrocytes due to a polymorphism expressed by the B6 mouse (the background strain used in many laboratory mouse models) (Engel et al., 1972). The slight hemolytic anemia exhibited by the Ldha mutant mice, does not significantly impact their lifespan and they are fertile and generally healthy (Kremer et al., 1986). As we have discussed before tumor cells are sensitive to perturbations in Ldha, so the fact that humans and mice are viable and generally healthy without Ldha activity suggests that Ldha could be a potential safe target in cancer therapy.
Serine metabolism

L-Serine (hereafter referred to as Serine unless specifically noted) plays a central role in cellular proliferation, and is considered a conditionally essential amino acid, meaning that although it does not play a role in maintaining nitrogen balance in the cell under certain circumstances cellular biosynthesis of serine cannot meet cellular demands so serine must be taken up from the environment (de Koning et al., 2003). Serine metabolic pathways provide the precursors for synthesis of amino acids such as cysteine and taurine, lipid messenger molecules (ceramide and phosphatidylserine) as well as glycine and D-serine that are important in neurological function (de Koning et al., 2003). Importantly, in mammalian cells serine is the main source of single carbon groups necessary for the synthesis of purine nucleotides and deoxythymidine monophosphate, thereby effecting nucleotide synthesis (Snell et al., 1987). Serine can be derived from four possible sources: dietary intake; glycine degradation; protein and phospholipid degradation; or synthesis from 3-phosphoglycerate, a glycolytic intermediate (see Figure 9). The importance of any one of these sources varies from tissue to tissue, and from one developmental stage to the next (de Koning et al., 2003).

![Figure 9. Sources of L-Serine](image)

The major sources of L-serine are shown here, with key enzymes involved in the phosphorylated synthase pathway as well as glycine degradation highlighted in bold. Abbreviations of enzymes shown is as follows: LDH (lactate dehydrogenase); PHGDH (3-phosphoglycerate dehydrogenase); PSAT (phosphoserine aminotransferase); PSPH (phosphoserine phosphotase); SHMT (serine hydroxymethyltransferase 1 and 2).

Although the various catabolic pathways and extracellular uptake mechanisms contributing to serine are important, this dissertation will focus on the enzymes involved in the biosynthesis of serine from 3-phosphoglycerate as well as glycine degradation, and the consequences of disruption of these enzymes on tumorigenesis.

**Serine biosynthesis: The phosphorylated pathway and PHGDH**

Serine is synthesized via two pathways: the “phosphorylated” pathway and the “non-phosphorylated” pathway (See figure 10). The phosphorylated pathway is considered to be the main source of serine biosynthesis, and the enzymes making up the phosphorylated pathway are found in most tissues whereas the enzymes in the non-phosphorylated pathway are only found in the liver and kidney (Snell, 1984). In fact, the non-phosphorylated pathway is not strictly a serine biosynthetic pathway at all because when it is reversed, it is involved in
Gluconeogenesis (de Koning et al., 2003). The phosphorylated pathway is not involved in gluconeogenesis because the last step of the pathway, the dephosphorylation of 3-phosphoserine yielding serine, is irreversible (Snell and Weber, 1986). The first step of the phosphorylated pathway is the 3-phosphoglycerate dehydrogenase (PHGDH) mediated conversion of 3-phosphoglycerate (a glycolytic intermediate) to phosphohydroxypyruvate. The next step, the conversion of phosphohydroxypyruvate to phosphoserine is performed by phosphohydroxypyruvate aminotransferase (PSAT). The last, and irreversible step, the conversion of phosphoserine to serine is catalyzed by phosphoserine phosphotase (PSP).

Phosphorylated serine synthesis pathway enzymes are highly active in the brain, spleen, kidney and testis and less active in heart and skeletal muscle (Snell, 1984). In the brain, the phosphorylated serine synthesis pathway is particularly important and perturbation of this pathway results in severe neurological defects in mice and in humans (Furuya et al., 2008; Jaeken et al., 1996). In general, serine biosynthesis via the phosphorylated pathway is regulated by expression of enzymes, such as Phgdh and Psp, involved in the pathway (Possemato et al., 2011; Strunck et al., 2001). In vitro studies examining breast cancer cells over expressing PHGDH found that over-expression of PHGDH alone was enough to increase serine synthesis via the phosphorylated pathway (Possemato et al., 2011). In most tissues dietary intake of serine does not regulate serine synthesis via the phosphorylated pathway, but this is not true of serine synthesis of the liver (Snell, 1984). Studies examining liver serine synthesis in rats have found that Phgdh and Psp activity is effected by the protein content in the dietary intake, on a low protein/high carbohydrate diet, Phgdh and Psp activity was high whereas on a high protein/low carbohydrate diet Phgdh and Psp activity was low (Snell, 1984).

**Serine biosynthesis: Glycine degradation and SHMT**

Serine hydroxymethyltransferase (SHMT) catalyzes the reversible conversion of glycine into serine, providing metabolites such as formyl groups important for purine biosynthesis, as well as methylation reactions like methyl groups for pyrimidine synthesis, remethylation of homocysteine and other reactions important for cellular homeostasis (de Koning et al., 2003) (See Figure 11). SHMT is actually two isozymes: a cytoplasmic form (Shmt1) and a mitochondrial form (Shmt2). In the cytosol, Shmt1 is an important mediator of folate dependent deoxyribonucleotide synthesis and synthesis of S-adenosylmethionine (SAM) by providing the one-carbon units for thymidylate synthesis, as well as the methyltetrahydrofolate necessary for SAM production (Herbig et al., 2002). In the mitochondria, Shmt2 is an important supplier of glycine degradation and SHMT.

---

**Figure 10. Phosphorylated and non-phosphorylated pathways of serine synthesis**

Abbreviations of enzymes involved in these pathways is as follows: LDH (lactate dehydrogenase); PHGDH (3-phosphoglycerate dehydrogenase); PSAT (phosphoserine aminotransferase); PSPH (phosphoserine phosphotase); SPT/AGT (serine:pyruvate/alanine:glyoxylate aminotransferase); SRR (serine racemase).
one-carbon units, such as 5,10-methylenetetrahydrofolate, from the preferential conversion of serine to glycine (Fu et al., 2001). Shmt1 and Shmt2 have both been shown to undergo post-translational modification by the small ubiquitin-like modifier (SUMO) and be translocated to the nucleus during S and G2/M phases where they contribute to nuclear thymidylate synthesis (Anderson and Stover, 2009; Woeller et al., 2007).

Figure 11. SHMT mediated Serine and folate metabolism
SHMT mediates important metabolic reactions that help produce the L-Serine and the folate necessary for DNA synthesis as well as substrates for methylation reactions. Abbreviations of enzymes shown is as follows: SHMT (serine hydroxymethyltransferase 1 and 2); DHFR (Dihydrofolate reductase); TYMS (Thymidylate synthase).

Serine biosynthesis in Cancer

Enzymes involved in the phosphorylated serine synthesis pathway such as Phgdh, Psat, and Psp, as well as SHMT involved in serine synthesis from glycine degradation, are over-expressed in many types of tumor tissue (Locasale et al., 2011; Snell et al., 1988; Ulrich, 2005). Interestingly, while many genes responsible for serine synthesis is up-regulated in these tumors, genes involved in the non-phosphorylated or gluconeogenic pathway are down-regulated (Snell et al., 1988). This suggests that it is not simply serine synthesis that is important for tumor cells but the coupling of serine synthesis to nucleotide biosynthesis. The folate metabolites provided by SHMT mediated reactions are also important in maintaining normal methylation patterns and DNA stability, and perturbation of folate levels has been shown to contribute to abnormal methylation patterns as well as contributing to DNA instability observed in many tumors as well as in lung cancer patients (Duthie, 2011; Piskac-Collier et al., 2011).

Targeting Serine biosynthesis in Cancer

As described in the previous section, the synthesis of adequate serine is important for tumor cells, but does this mean that serine synthesis enzymes would make good chemotherapeutic targets? Studies looking at the effect of serine synthesis inhibition on tumor cell growth have found that inhibition of serine biosynthesis enzyme Phgdh using RNAi in a subset of breast cancer cells results in significantly decreased rates of tumor cell growth (Possemato et al., 2011). Unfortunately, absolute removal of Phgdh is complicated by the fact that humans with low levels of Phgdh activity, caused by missense mutations in the C-terminal region of the Phgdh gene, suffer from severe neurological symptoms such as congenital microcephaly, seizures, and extreme psychomotor retardation (Jaeken et al., 1996). In some patients, the
neurological symptoms of Phgdh deficiency, such as seizures, were treatable by high doses of serine or a combination of serine and glycine (Jaeken et al., 1996). The mutations in Phgdh cause reduced enzymatic activity but do not completely abolish Phgdh function suggesting that the neurological defects stemming from complete Phgdh removal would be embryonic lethal (Jaeken et al., 1996; Klomp et al., 2000). This was shown to be the case when disruption of Phgdh in mice, creating a Phgdh “knock-out” mouse, was embryonic lethal on day E13.5 due to neurological defects (Yoshida et al., 2004). However, in this dissertation we present data illustrating that removal of Shmt1 is compatible with life and mice lacking Shmt1 due to a germline mutation of Shmt1, essentially resulting in a Shmt1 “knock-out” mouse, are viable, outwardly normal, and fertile. This is consistent with findings from other reports examining Shmt1 loss in mice (MacFarlane et al., 2008).

**Polyamine metabolism**

There are many biogenic multiple-amine molecules, such as histamine and cadaverine but the term “polyamine” is reserved for spermidine and spermine, and the diamine precursor to spermidine, putrescine. Polyamines are essential for eukaryotic cell growth and proliferation, making them the focus of many studies both in general eukaryotic cell signaling as well as proliferative diseases like cancer (Casero and Marton, 2007). Polyamines are water soluble, and are fully protonated at physiological pH, making them positively charged (Gugliucci, 2004). They are present at fairly high (millimolar) concentrations within the cell, but most polyamines are not free but are bound to negatively charged macromolecules in the cell such as DNA, RNA, proteins and phospholipids. The proper balance of polyamines (putrescine, spermidine and spermine) is essential for cell growth therefore cellular polyamine concentrations are tightly regulated through synthesis, catabolism and transport (Figure 12) (Casero and Marton, 2007).

Agmatine (AGM), a polycationic molecule synthesized from the decarboxylation of L-arginine, is a new addition to the polyamine family. Agmatine was originally identified as a neurotransmitter, and inhibits cellular proliferation by depleting intracellular polyamine levels (Agostinelli et al., 2010). Metabolism of agmatine, and its role in cellular proliferation, is currently being explored but will not be covered in this dissertation.

![Figure 12. Polyamine metabolism](image)

Polyamines can be taken up from the extracellular environment, or produced within the cell by biosynthetic enzymes (labeled in blue) or catabolic pathways (labeled in gray). Polyamine biosynthesis is regulated by the availability and activity of Odc and Amd, as well as dc-AdoMet, an important co-factor required to synthesize spermidine and spermine. Abbreviations of enzymes depicted is as follows: ODC (ornithine decarboxylase); SRM (spermidine synthase); SMS (spermine synthase); AMD (Ado-Met decarboxylase); SMOX (spermine oxidase); SAT1 (spermine/spermidine acetyltransferase 1); PAOX (polyamine oxidase).
**Polyamine biosynthesis: Spermidine Synthase**

The main precursors of polyamine synthesis are L-arginine and L-methionine, which are often taken up from the environment (See Figure 12) (Larque et al., 2007). L-arginine is converted into L-ornithine by arginase, and methionine adenosyltransferase (MAT) mediates the conversion of L-methionine to S-adenosylmethionine (SAM) (Gugliucci, 2004). Growth stimuli in the environment stimulate ornithine decarboxylase (ODC), which converts L-ornithine into putrescine; spermidine synthase (SRM) then converts putrescine into spermidine and lastly, spermine synthase (SMS) converts spermidine into spermine. Spermidine and spermine synthesis require aminopropyl group donation from decarboxylated S-adenosylmethionine (dcSAM), yielded from the decarboxylation of SAM mediated by SAM decarboxylase (SAM-DC). Since they represent the first enzyme in polyamine synthesis or regulate the availability of aminopropyl groups, the rate-limiting enzymes in polyamine biosynthesis are ODC and SAM-DC (Casero and Marton, 2007). Myc stimulates polyamine biosynthesis by transcriptionally activating several genes encoding polyamine biosynthetic enzymes; Odc was first identified as a Myc transcriptional target gene in 1993, and we have recently shown that Srm and AdoMetDC (Adm1) (the gene encoding SAM-DC) are also regulated by Myc (Bello-Fernandez et al., 1993; Forshell et al., 2010). Polyamine binding to DNA, RNA, and protein has been shown to play an important role in transcription and translation (Balasundaram and Tyagi, 1991; Liu et al., 2009). The number of charges on the polyamine molecule has been suggested to have a strong effect on the binding to DNA, RNA, and protein, and therefore on the role in cellular metabolism. The finding that spermine, with a greater number of positive charges, is more active in biological processes than putrescine seems to support this hypothesis (Yuan et al., 2001). However, of all the polyamines, only spermidine has a unique function. It is essential in the hypusination modification of eukaryotic initiation factor 5A (eIF5A) (Wolff et al., 1990). The unique modification of this enzyme, called hypusination, by attaching spermidine onto Lys50 (human protein) has been identified long before and is the only unique function of a polyamine (Joe and Park, 1994). eIF5A is involved in the ribosomal translation initiation of RNA, and in order to function correctly, eIF5A must be modified at Lys50 (human protein) (Joe and Park, 1994). Many of the other polyamines are interchangeable when it comes to general effects on protein folding, DNA packaging etc.

**Polyamine biosynthesis in Cancer**

Polyamines are essential for eukaryotic cell proliferation and the central role they have in tumor formation is being rigorously studied (Casero and Marton, 2007; Gerner and Meyskens, 2004). Polyamine concentrations are especially high in rapidly proliferating tissues and tumors, conditions in which Myc is also active, and the polyamine biosynthetic enzyme genes Odc, Srm and AdoMetDC are Myc transcriptional target genes (Bello-Fernandez et al., 1993; Forshell et al., 2010). It has been shown in many tumors that genes involved in polyamine metabolism are over-expressed, suggesting that polyamine synthesis is upregulated in response to oncogenic signaling, such as Myc and Ras (Forshell et al., 2010; Nilsson et al., 2005; Shantz and Levin, 2007). Interestingly, it has also been shown in vitro and in vivo that deregulated ODC activity can cause transformation (Auvinen et al., 1992; Shantz et al., 1996b; Shantz and Pegg, 1994). When NIH-3T3 cells over-expressing Odc were injected into recipient mice they formed aggressive, invasive tumors (Auvinen et al., 1997). Importantly, the transformation mediated by deregulated ODC activity can be reversed by ODC inhibition (Auvinen et al., 1992; Shantz et al., 1996a). Studies using transgenic mice that over-express Odc, where Odc is put under the transcriptional control of tissue specific promoters in the outer root sheath of the hair follicle, have shown that Odc over-expression alone is sufficient to promote skin tumorigenesis (O’Brien et al., 1997). In these same Odc transgenic mice, Odc over-expression has also been shown to collaborate with oncogenic Ras to drive skin tumorigenesis (Hayes et al., 2006). Interestingly, over-expression of SRM, or increasing intracellular spermidine concentration does not have the same transformation favoring effect in cells or in mice (Kauppinen et al., 1993; Poulin et al., 1993). Odc inhibition coupled with addition of exogenous spermidine in B-cells induces apoptosis instead of transformation due to the cytotoxic amounts of N1-acetyl-spermidine.
Polyamines across the intracellular membrane has also been proposed are sequestered into first transported in to the cell by an unidentified carrier/transporter complex, where they are then perturbation of polyamine pools are low despite the fact that polyamines are essential for cellular proliferation, and tumor cells are especially sensitive to perturbation of polyamine biosynthesis, why does polyamine synthesis inhibition have a chemopreventative effect? To answer this, we look to one of the main mechanisms of regulating polyamine levels, polyamine transport (Figure 12). The human diet is rich with polyamines as well as polyamine precursors, and the normal flora of the intestinal tract also excrete large amount of polyamines, so import from the extracellular environment has the potential to compensate for disturbances in polyamine biosynthesis (Larque et al., 2007). Interestingly, polyamine synthesis inhibition in cells with defective polyamine transport results in significantly reduced cellular proliferation when compared to control cells suggesting that coupling polyamine synthesis inhibition with polyamine transport inhibition is a promising methodology in cancer therapy (Ask et al., 1992; Belting et al., 1999; Nilsson et al., 2005; Soulet et al., 2004; Welch et al., 2008). Although the selective, energy-dependent, polyamine transport system present in mammalian cells has not been conclusively identified, there are studies that provide insight on some mechanisms of polyamine transport (Belting et al., 2002; Belting et al., 1999; Mani et al., 2007; Soulet et al., 2004). One proposed mechanism is that polyamines are first transported in to the cell by an unidentified carrier/transporter complex, where they are then sequestered into vesicles via a vacuolar-ATPase pH gradient and proton exchange mechanism (Soulet et al., 2004). These vesicles can be stimulated to release the sequestered polyamines by depletion of intracellular polyamine pools, creating a system able to rapidly respond to perturbation of polyamine pools (Soulet et al., 2004). This sequestering event could explain why free polyamine pools are low despite the fact that intracellular polyamine concentrations are in the millimolar range. Heparan sulfate proteoglycans (HSPG) mediated transport of polyamines across the intracellular membrane has also been proposed (Belting et al., 1999).
HSPGs have been shown to facilitate spermine uptake, and depletion of intracellular polyamine pools results in increased HSPG synthesis as well as polyamine uptake (Belting et al., 1999). Combining polyamine synthesis inhibition with interference with HSPG synthesis, or availability of polyamine-HSPG binding due to competition, results in dramatic effects on tumor cells growth (Belting et al., 1999; Mani et al., 2007; Welch et al., 2008). Currently the search is underway for efficient polyamine transport inhibitors, but unfortunately the design of polyamine transport inhibitors is complicated by the fact that the specific mechanism of polyamine transport is unknown and perturbation of polyamine transport or synthesis results in activation of other proteins and enzymes in the polyamine metabolic pathway (Mitchell et al., 2007; Seiler et al., 1996).
Aims of this dissertation

The overall aim of this dissertation is to examine the efficacy of inhibition of Myc target genes, particularly metabolic genes, as a viable way of treating Myc-driven tumorigenesis. Specifically, we attempted to answer some key questions:

I. Myc over-expression results in upregulation of many polyamine biosynthetic genes, therefore will *in vitro* and *in vivo* chemical and genetic inhibition of spermidine synthase (Srm) result in inhibition of Myc-driven tumorigenesis?

II. Myc over-expression results in upregulation of genes involved in glycolysis and serine biosynthesis therefore will genetic inhibition of Lactate dehydrogenase (*Ldh*), Serine hydromethyltransferase (*Shmt*), and 3-phosphohydroxyglycerate dehydrogenase (*Phgdh*) have an effect on Myc-driven tumorigenesis?

III. Pim Kinases have been shown to collaborate with Myc in tumorigenesis therefore will chemical inhibition of Pim kinases have an effect on Myc-driven lymphomagenesis?
Summary of Publications

Paper I: Chemoprevention of B-cell lymphomas by inhibition of the Myc target spermidine synthase.

On paper, cellular polyamine metabolism is straightforward, but in reality is a complicated pathway consisting of synthesis, catabolism and uptake from the environment. Polyamine synthesis is increased in actively proliferation cells, as well as many types of cancer (Casero and Marton, 2007). Studies inhibiting ornithine decarboxylase (Odc), an initial and bottleneck enzyme in the polyamine synthesis pathway, have shown promise in chemoprevention both in studies using transgenic mouse models of cancer (Nilsson et al., 2005) and clinical trials (Gerner and Meyskens, 2009). The ubiquitous presence of polyamines in the environment (e.g. food and cell culturing media) could explain why Odc inhibition results in a chemopreventative rather than a chemotherapeutic effect in these studies. Polyamines are necessary for growing cells, both normal and cancer cells, so the complete removal of polyamines from the body would result in collateral damage to normal tissue. Therefore the aim in this study, as well as previous studies, has been to bring the level of polyamines back down to normal cellular levels and study the effect of Myc driven tumorigenesis. Myc transgenic mouse models such as Eµ-Myc, λ-Myc, and iMyc5−, where Myc is over-expressed in the B-cell compartment, are invaluable tools in analyzing the value of specific targets in Myc driven lymphomagenesis. The “gold standard” of Myc target genes has been Odc, as one of the first Myc target genes identified, as well as over-expressed in many malignancies (Shantz and Levin, 2007). Previous studies have shown that inhibition of Odc, gene, chemically or genetically, has shown promise in chemoprevention (Gerner and Meyskens, 2009; Nilsson et al., 2005). Due to the success with Odc inhibition, we examined the chemotherapeutic potential of other enzymes in the polyamine pathway, notably spermidine synthase (Srm).

Srm is a direct Myc transcriptional target and is over-expressed in Myc driven B-cell lymphoma

Previous studies (Nilsson et al., 2005), as well as the Myc target gene database (http://myccancergene.org), have identified Srm as a potential Myc target gene. To examine Srm expression in spontaneously arising lymphomas resulting from Myc over-expression, we analyzed mRNA from spontaneously arising lymphomas in λ-Myc transgenic mice. λ-Myc precancerous material, as well as all tumors screened show higher mRNA expression of Srm, than WT mice. Interestingly, Srm was expressed at levels higher than that of Odc. This result suggests that Srm may be more important in Myc driven lymphomas than Odc. Western blot analysis revealed that P493-6, a human B-cell line with inducible Myc expression (Pajic et al., 2000), also exhibited increased Srm expression when under Myc driven proliferation.

Although previous studies investigating Myc target genes had shown that Srm is over expressed in settings of Myc driven proliferation (Nilsson et al., 2005), the precise relationship between Myc and Srm was not known. The presence of five E-boxes in the Srm promoter region led to the assumption that Srm was a direct Myc target gene, although this was not formally proven. Direct transcriptional activation of Srm, due to Myc binding, was determined using NIH 3T3 cells expressing a Myc-ER fusion protein. The Myc-ER fusion protein allows for tamoxifen inducible Myc transcriptional activation (Littlewood et al., 1995). In this system, Myc-ER is held in the cytoplasm bound by heat shock proteins and addition of 4-HT (4-hydroxytamoxifen) causes a conformational change in the Myc-ER protein, resulting in the release of Myc-ER, which is then free to translocate to the nucleus and activate transcription of target genes. Activation of Myc driven transcription results in a marked increase in Srm, as well as Amd1, mRNA expression even in the absence of protein translation (CHX treatment). These results classify Srm and a direct Myc target gene.
Inhibition of *Srm* in *Myc* driven lymphoma results in a decrease in cellular proliferation, and a reduced rate of transformation, but has no effect on established tumors

Genetic inhibition of Odc, and upstream enzyme in the polyamine biosynthesis pathway, has shown a delay in tumorigenesis in previous studies (Nilsson et al., 2005). Like Odc, we show that Srm is a direct Myc target gene that is over-expressed in Myc driven lymphomas and Myc over expressing cells. Therefore, the effect of genetic Srm inhibition was explored using RNAi in B-cell lymphoma cell lines established from \( \text{E}_\mu \)-*Myc*, \( \lambda \)-*Myc*, and \( i\text{Myc}^E \) transgenic mice as well as NIH 3T3 fibroblasts. In NIH 3T3 fibroblasts, western blot analysis reveals that RNAi inhibition of Srm results in reduced levels of Srm protein. HPLC analysis of these cells show that the reduction in Srm protein results in a severe reduction in spermidine, as well as a back up of the spermidine precursor putrescine. However, the expected reduction in spermine levels, given the decreased in precursor spermidine, was not observed and remained roughly similar all samples. One possible explanation for this finding is that dCDCAdoMet is needed by both Srm and Sms so when Srm levels are depleted, Sms has no competition for the available dCDCAdoMet and the little spermidine remaining this therefore rapidly converted to spermine. These Srm depleted cells also exhibited a marked decreased in proliferation rate that over-expression of Myc in these cells could not rescue. Myc driven transformation in these fibroblasts were also analyzed using soft agar assay, and although the size of the colonies were smaller in the Srm RNAi expressing cells, the over all number of colonies did not vary significantly. This finding suggests that although inhibition of Srm results in a decrease in proliferation rate, it cannot inhibit Myc driven transformation in fibroblasts *in vitro*.

To examine the effect of Srm inhibition in another type of cell, primary tumor cell lines established from \( \text{E}_\mu \)-*Myc* mice, were infected with Srm RNAi constructs. The expression of Srm in these cells was reduced by 75%, and they also exhibited a marked decrease in proliferation rate. However, when these vector control or Srm RNAi expressing fibroblasts were injected into recipient mice, no difference in tumor reoccurrence was observed. Chemical inhibition of Srm using 4-MCHA, an efficient chemical inhibitor of Srm used in previous studies (Beppu et al., 1995; Shirahata et al., 1993), was also examined. Treatment of uninfected \( \text{E}_\mu \)-*Myc* tumor cells with 4-MCHA resulted in a similar decrease in proliferation rate. We considered the fact that these tumor cell lines have been established in culture, which can result in changes in the cell line due adaptation to culture conditions. To evaluate the effect of Srm inhibition on uncultured tumor cells, two never before cultured \( i\text{Myc}^E \) tumor cell lines (one p53 mutant, one p53 WT) were injected into recipient mice and given 4-MCHA in their drinking water. However, Srm inhibition in this setting also did not alter tumor reoccurrence. These data also suggest that p53 status does not play an obvious role in the effectiveness of Srm inhibition tumor reoccurrence. These data taken together suggest that Srm inhibition alone is not an efficient chemotherapeutic method.

The chemopreventative value of Srm inhibition in Myc driven lymphomagenesis was evaluated by treating \( \lambda \)-*Myc* transgenic mice with 0.025% 4-MCHA drinking water starting at weaning age. \( \lambda \)-Myc transgenic mice develop B-cell lymphomas with a median survival time of 100 days, but 4-MCHA treatment significantly delayed tumor onset (\( p=0.0001 \)) by 41 days. Analysis of tumor material of untreated and treated mice for p53/Arf mutation rate revealed a similar rate of mutation, so this was not the reason for the delayed tumorigenesis. Western blot analysis and qRT-PCR analysis show a similar rate of mutation or deletion of p53, and deletion of p19\(^{Arf}\), as was previously reported in other studies suggesting that tumors in 4-MCHA treated mice did not arise via an alterative route of transformation (Eischen et al., 1999).

Previous studies have hypothesized that tumors arising in DFMO treated \( \text{E}\mu\text{Myc} \) mice, and \( \text{E}\mu\text{Myc};\text{Odc}^{\text{WT}} \) mice show an altered route of transformation due to a change in Arf deletion rate when compared to \( \text{Odc} \) wt or untreated \( \text{E}\mu\text{Myc} \) mice (Nilsson et al., 2005). This suggests that polyamine inhibition causes a slower proliferation rate of tumor cells, and that although the Myc over-expressing cells still transform, they must use altered route of transformation. Interestingly, we show that inhibition of Srm also has a chemopreventative effect on Myc-driven lymphomagenesis, although we see no alteration in the mutation or deletion rates of Arf or p53. This could mean that tumor cells are not forced into an alternate route of transformation, or it could simply indicate that the altered route of transformation does not require Arf or p53 inactivation. This suggests that although Odc and Srm inhibition result in polyamine depletion...
and a chemopreventative effect on tumorigenesis, the mechanism behind the effect may be different. Recent clinical trials have shown that targeting polyamine biosynthesis via DFMO has a chemopreventative effect colon cancer, and combining DFMO with NSAIDs yields even better chemopreventative results (Meyskens et al., 2008; Umar et al., 2001). If the mechanism behind the chemopreventative effect of Odc and Srm inhibition on tumorigenesis is different, Srm inhibition could be used in cases where Odc inhibition is no longer effective, due to Odc gene duplication for example.

**Paper II: Mouse genetics suggest that metabolic enzymes dispensable for Myc-induced lymphomagenesis can play critical roles in Ras-induced fibrosarcoma growth.**

Myc transcriptionally activates many genes and many of these genes are involved in cellular metabolism. In fact, several classical Myc target genes are metabolic enzymes such as ornithine decarboxylase (Odc) and lactate dehydrogenase A (Ldha). The “Warburg effect” or anaerobic glycolysis is observed in many tumor cells, and the glucose from this increased glucose uptake is used to provide metabolites for processes such as nucleotide synthesis (Dang et al., 2009). Ldha is a key enzyme in glycolysis, and studies have shown that although Ldha over-expression alone is not sufficient to cause transformation (Lewis et al., 2000), is does give tumor cells growth advantages highlighting the importance of targeting glycolysis in tumorigenesis (Fantin et al., 2006; Shim et al., 1997). Glycolysis and serine biosynthesis are linked by the pentose phosphate shunt as well as the phosphorylated pathway of serine synthesis. Enzymes involved in the phosphorylated serine synthesis pathway such as 3-phosphoglycerate dehydrogenase (Phgdh), as well as serine hydroxymethyltransferases (SHMT) involved in serine synthesis from glycine degradation, are over expressed in many types of tumor tissue (Locasale et al., 2011; Snell et al., 1988; Ulrich, 2005). Phgdh catalyzes the first step in the phosphorylated pathway of serine synthesis (de Koning et al., 2003), whereas Shmt catalyzes the reversible transfer of the hydroxymethyl group of serine to tetrahydrofolate (THF) to form methyleneTHF and glycine (Anderson and Stover, 2009; Stover, 2009), thereby producing the methyleneTHF that is then used in methylation and thymidylate synthesis. Ldha, Phgdh, and SHMT are over expressed in many tumors, and the pathways they participate in are commonly upregulated in Myc driven tumorigenesis (Dang et al., 2009; Locasale et al., 2011). These results suggest that targeting these metabolic pathways regulated by Myc could be a viable treatment strategy. To explore this possibility we have performed in vitro and in vivo experiments, examining the effect of genetic inhibition of Ldha, Phgdh, as well as Shmt, on tumorigenesis.

**Myc target genes Ldha, Shmt1, and Phgdh are dispensable in Myc driven lymphomagenesis**

The in vivo expression of 153 metabolic genes in λ-Myc transgenic mice (described previously) was analyzed using Illumina bead chip arrays on RNA taken from 4-6 week old wild type and pre-cancerous mice. Unsupervised hierarchical clustering revealed that the precancerous Myc transgenic B-cells grouped together, simply by looking at expression of genes involved in metabolic pathways like glycolysis, TCA cycle, and serine synthesis alone. These data support findings from other genetic studies that Myc over-expression results in significant metabolic changes (Dang et al., 2009), and that targeting metabolism in Myc over-expression settings is an option worth exploring. qRT-PCR analysis of pre-cancerous B-cells harvested from 4-6 week old wild type or λ-Myc transgenic mice showed increased expression of Ldha, Shmt1 and 2, as well as Phgdh confirming the Illumina array results that metabolic genes are upregulated in Myc over-expressing cells.

Serine hydroxymethyltransferase (Shmt) is an isozyme present in both the cytoplasm (Shmt1) and mitochondria (Shmt2), and previous in vitro data suggest that Shmt1 cannot rescue loss of Shmt2 (Stover et al., 1997). To examine the effect of Shmt1 inhibition on tumorigenesis in vivo, we created an Shmt1 gene-trap mouse where the Shmt1 transcript is
“trapped” and therefore not expressed, resulting in an \textit{Shmt1} “knock-out” mouse. In order to examine how \textit{Shmt1} loss would effect tumorigenesis, we crossed the \textit{Shmt1} KO mouse to the \textit{p53} knockout, \textit{APC\textsuperscript{Min}}, or \textit{\lambdacyan}-Myc mouse models of cancer. The \textit{p53} knockout mouse lacks the tumor suppressor \textit{p53} and typically presents with T-cell malignancies (Jacks et al., 1994). The \textit{APC\textsuperscript{Min}} mouse is an intestinal tumorigenesis mouse model in which mice develop adenomatous polyps due to an inactivating point mutation in \textit{Apc}, resulting in loss of Wnt signaling and upregulation of Myc transcription (McCott et al., 2008; Moser et al., 1995). \textit{Shmt1} loss in these mouse models did not significantly alter tumor formation. However, \textit{Shmt1} loss in the \textit{\lambdacyan}-Myc mouse model resulted in accelerated in lymphomagenesis and significant impact on survival (\textit{p}=0.0002) suggesting a B-cell specific effect. \textit{Shmt1} loss did not impact tumorigenesis in the \textit{p53} KO mouse or in the \textit{APC\textsuperscript{Min}} mouse, and significantly accelerated lymphomagenesis in the \textit{\lambdacyan}-Myc mouse, which suggests that \textit{Shmt1} is a poor chemotherapeutic target. \textit{Shmt2} knockout or gene-trap mice were not available so the effect of \textit{Shmt2} inhibition in tumorigenesis was evaluated using NIH 3T3 fibroblasts over-expressing Myc or HRasG12V and shRNA against \textit{Shmt1}, \textit{Shmt2} and \textit{Phgdh}. Previous reports have shown that subcutaneous growth of Myc over-expressing cells is unreliable due to adaptation problems in the transplant environment (Dews et al., 2006) and this may explain the similar phenotype we observed in our cells. However, when Ras over-expressing cells were injected into recipient mice \textit{Shmt2} and \textit{Phgdh}, but not \textit{Shmt1}, inhibition resulted in smaller fibrosarcoma growth. Myc expression is impacted by Ras (Sears et al., 2000), and in this context, Myc might contribute the serine metabolic requirements that Ras needs in order to drive transformation.

\textit{Phgdh} catalyzes the first step in the serine biosynthesis pathway, and shown to be essential in mammalian neuronal development resulting in embryonic lethality at E13.5 in mice completely lacking \textit{Phgdh} (Jaeken et al., 1996; Yoshida et al., 2004). Therefore, with traditional crossing of \textit{Phgdh} knockout mouse to the \textit{APC\textsuperscript{Min}} or \textit{\lambdacyan}-Myc mouse models we were limited to analysis of the effect of removal of one allele of \textit{Phgdh}. Previous studies have shown that \textit{Odc} heterozygosity is sufficient to impact tumorigenesis (Nilsson et al., 2005), however in \textit{APC\textsuperscript{Min}} or \textit{\lambdacyan}-Myc mouse models, \textit{Phgdh} heterozygosity is not sufficient to impact tumorigenesis. In order to determine the effect to complete \textit{Phgdh} removal on Myc driven lymphomagenesis, we created lymphoma prone mice expressing varying amounts of \textit{Phgdh} by crossing \textit{\lambdacyan}-Myc and \textit{Phgdh} knockout mouse, then transplanting hematopoietic stem cells from these mice into lethally irradiated recipient mice. Despite the complete removal of \textit{Phgdh} the mice had functional hematopoiesis, however the lymphomagenesis in these mice was not impacted indicating that \textit{Phgdh} is dispensable in normal hematopoiesis as well as Myc-driven lymphomagenesis.

Previous RNAi or antisense studies have indicated that \textit{Ldha} plays an important role in established tumors (Fantin et al., 2006; Le et al., 2010; Shim et al., 1997), but the role of \textit{Ldha} in tumorigenesis in \textit{vivo} was unclear. In order to examine the importance of \textit{Ldha} in Myc driven tumorigenesis, we used an \textit{Ldha} mutant mouse carrying a germ-line point mutation (D223H) in \textit{Ldha}, resulting in impaired enzymatic activity and reduced stability (Charles and Pretsch, 1981). The \textit{Ldha} mutant mouse was crossed to the \textit{\lambdacyan}-Myc mouse, and \textit{Ldha} activity was measured in precancerous B-cells from these mice. We found elevated \textit{Ldha} activity in \textit{\lambdacyan}-Myc;\textit{Ldha}\textsuperscript{wt} precancerous B-cells, whereas \textit{Ldha} mutant mice showed a marked decrease in \textit{Ldha} activity irrespective of \textit{\lambdacyan}-Myc status. Surprisingly, the absence of functional \textit{Ldha} did not significantly impact tumorigenesis in these mice. Multiple studies have shown \textit{Ldha} to be important in tumor cells, so the finding that \textit{Ldha} is dispensable in Myc driven lymphomagenesis was a surprise with several possible explanations: rescue by another form of \textit{Ldh}; lack of \textit{Ldha} leads to altered routes of transformation or simply that \textit{Ldha} activity really is dispensable for Myc driven tumorigenesis. Tumors from \textit{\lambdacyan}-Myc;\textit{Ldha}\textsuperscript{wt} and \textit{\lambdacyan}-Myc;\textit{Ldha}\textsuperscript{mut/mut} mice were analyzed for activity of \textit{Ldhb}, another form of \textit{Ldh}, and were found to be low or absent suggesting that compensation by \textit{Ldhb} activity was not the explanation. To examine if \textit{Ldha} deficiency results in alternate routes of transformation, we crossed the \textit{Ldha} mutant mouse, the \textit{\lambdacyan}-Myc mouse and the \textit{p53} knockout mouse to create a more genetically defined population. Regardless of \textit{Ldha} genotype, the \textit{\lambdacyan}-Myc;\textit{p53}\textsuperscript{wt} mice exhibited accelerated lymphomagenesis compared to the \textit{\lambdacyan}-Myc;\textit{p53}\textsuperscript{mut} mice, and PCR analysis revealed that these tumors had lost the remaining \textit{p53} allele. The rate of \textit{p53} mutation in tumors from mice arising in \textit{\lambdacyan}-Myc;\textit{Ldha}\textsuperscript{wt} and \textit{\lambdacyan}-Myc;\textit{Ldha}\textsuperscript{mut/mut} mice showed
no significant difference based on Ldha genotype. These data taken together suggest that Ldha is dispensable in Myc driven lymphomagenesis.

In the \(\lambda\)-Myc transgenic mouse model of lymphomagenesis, Myc is the primary oncogenic lesion driving transformation. To explore the effect of Ldha deficiency in settings where Myc is a downstream oncogenic lesion, we crossed the Ldha mutant mouse to the \(APC^{\text{Min}}\) or \(p53\) knockout mouse models. Contrary to expectations, tumorigenesis in these settings was also not significantly affected by Ldha deficiency. However, when \(p53^{\text{wt}};\text{Ldh}^{\text{wt}}\) or \(\text{Ldh}^{\text{mut/mut}}\) mouse embryonic fibroblasts (MEFs) infected with retrovirus encoding oncogenic H-Ras, were injected into recipient mice, \(\text{Ldh}^{\text{mut/mut}}\) cells generated significantly smaller fibrosarcoma (\(p<0.0001\)). We examined if Ldha was important to transformed cell growth in a hypoxic environment, such as that often found in vivo, using NIH 3T3 fibroblasts over-expressing Myc or H-Ras also expressing RNAi against Ldha. As expected, hypoxia induced downstream Hif1\(\alpha\) targets Ldha and Pdk1, and 3H-Thymidine analysis revealed that cells expressing Ldha shRNA exhibited a decreased proliferation rate when compared to control cells. This data indicates that Ldha function is required under certain settings, although dispensable in Myc-driven lymphomagenesis.

Myc over-expression studies have shown that many metabolic genes are also upregulated (Dang et al., 2009) and that many of these genes are direct Myc targets. However, this does not mean that targeting of these enzymes would be successful (e.g. Ldh, Phgdh) or even a good idea (e.g Shmt1) in Myc-driven tumorigenesis. The altered metabolic pattern of tumor cells, such as the Warburg effect, is known and has been discussed in many studies over the years. We also know that the transformation process is not the same in every cell, and that different oncogenic lesions may result in different metabolic requirements. In this study we have examined the importance of three genes involved in glycolysis and folate metabolism on Myc-driven tumorigenesis. Interestingly, our data indicate that although Phgdh and Ldha are dispensable in Myc over-expressing context they are important in an oncogenic Ras context. Phgdh function is necessary for neurological development, but we have shown that Phgdh knockout bone marrow transplanted into recipient mice does not impact hematopoiesis. This suggests that inhibition of Phgdh by use of a chemical inhibitor, one that would not cross the blood brain barrier and impact neurological Phgdh function, could be a useful chemotherapeutic strategy. Importantly, this study highlights the importance of genetic stratification of malignancies in developing successful chemotherapeutic treatments.

**Paper III:** The direct Myc target gene Pim3 cooperates with other Pim kinases in supporting viability of Myc-induced B-cell lymphomas.

The Pim kinases (Pim-1, 2 and 3) are serine/threonine kinases, identified as hotspots for proviral integration of Moloney murine leukemia virus (Pim) in retroviral induced lymphomas. Pim kinase proteins are highly homologous, and roughly the same size and it is unclear exactly how much redundant function exists between the different Pim kinases members. Previous studies have found that NfkB and Jak/STAT signaling regulate Pim1 and Pim2 (Allen et al., 1997; van der Lugt et al., 1995), although this has not been confirmed for Pim3. Pim kinase phosphorylation activities impact cell cycle proteins (e.g. CDC25A and CDC 25C (Bachmann et al., 2006; Mochizuki et al., 1999) and cytokine signaling via SOCS1 and SOCS3 (Chen et al., 2002; Peltola et al., 2004). Pim kinases have also been shown to impact transcription via phosphorylation of transcriptional activators such as Myc and Myb (Winn et al., 2003; Zhang et al., 2008); as well as translation via eIF4B and 4EBP1 (Fox et al., 2003; Peng et al., 2007). Apoptotic signaling is also impacted due to the phosphorylation of Bad, a pro-apoptotic signaling factor, which results in inhibition of apoptosis (Macdonald et al., 2006). Pim-1 has been shown to bind Myc-Max and be recruited to E-boxes, where it then phosphorylates serine 10 on histone H3, thereby impacting up to 20% of Myc transcriptional target genes (Zippo et al., 2007). Pim kinase over-expression has been linked with radiation resistance (Peltola et al., 2009), chemoresistance (Chen et al., 2009) as well as poor prognosis (Liu et al., 2010) in various tumor types. Transgenic mouse studies have shown Pim kinases to be weakly oncogenic alone (Matikainen et al., 1999; Miura et al., 1994; Zhu et al., 2002), but greatly accelerate Myc-driven lymphomagenesis when over-expressed with Myc (Allen et al., 1997). However, transgenic mice lacking all three Pim kinases are viable and fertile (Mikkers et al.,
indicating that Pim kinases may be good chemotherapeutic targets without resulting in excessive damage to normal cells. In this study, we investigate the role of Pim kinases, especially Pim-3, in Myc-driven lymphomagenesis.

**Pim3 is over-expressed in Myc driven lymphomas**

To investigate Pim expression in a Myc over-expression setting *in vivo*, we analyzed mRNA from spontaneously arising lymphomas in the λ-Myc transgenic mouse model using quantitative reverse transcriptase PCR (qRT-PCR). Pim1 and Pim2 expression was high in several tumors, but Pim3 expression was significantly increased in all tumors, and this pattern was also exhibited in pre-cancerous B-cells harvested from 4-6 week old λ-Myc mice. When we analyzed mRNA from cell lines established from E12-Myc and λ-Myc transgenic mice we found that Pim3 expression was also elevated in these lines suggesting that Pim3 is important for the transformation process as well as maintenance of Myc-driven lymphomas. To verify if this was also true in humans we examined Pim mRNA expression in human Burkitt lymphoma lines and found that Pim1 and Pim2 were consistently highly expressed, however threshold count (Ct) values from the PCR indicate that the Pim3 transcript was the predominant Pim transcript in these cells. This finding was supported by immunohistochemistry staining for Pim3 in mantle cell, follicular, diffuse large B-cell, and Burkitt Lymphoma that clearly showed that Burkitt lymphoma exhibited the highest Pim3 expression. These data indicate that Pim1 and Pim2 are intermittently over-expressed in Burkitt lymphoma tumors, and Pim3 is consistently over-expressed and the predominant Pim kinase in this tumor setting.

**Pim3 is a direct Myc transcriptional target**

Although Pim3 mRNA is over-expressed in the setting we have analyzed, we wanted to determine if this was due to direct transcriptional induction by Myc. The promoter region of Pim3, in both mice and man, contains two Eboxes suggesting that the over-expression of Pim3 in Myc driven lymphomagenesis could be due to direct Myc binding, but this had not been proven. To do this, we analyzed Myc binding to the Eboxes present in the Pim3 promoter region by chromatin immunoprecipitation (ChIP) on DNA from a tumor cell line established from the λ-Myc transgenic mouse. qRT-PCR analysis of ChIP material, using primers designed to flank Ebox-1 of Pim3, revealed that Myc binding to Ebox-1 of Pim3 was comparable to Odc or Srm, two confirmed direct Myc target genes.

We also looked at Pim3 mRNA expression and translation in P493-6 cells, a human B-cell line expressing inducible Myc expression (Pajic et al., 2000), comparing when Myc is off (72hrs) or on (8hrs). Consistent with results in our mouse experiments, qRT-PCR and western blotting analysis showed Pim3 mRNA expression and translation was elevated in “Myc on” samples. To determine if this was due to direct Myc binding to human Pim3 Eboxes, we performed ChIP analysis of DNA from P493-6 cells when Myc is either off (72hrs) or on (8hrs). ChIP material from “Myc on” cells showed Myc binding to Cyclin D2 (CCND2), a confirmed Myc target gene (Bouchard et al., 1999), as well as Pim3 Ebox-1. These findings indicate that Myc binds directly to Ebox-1 in Pim3 in human and mouse cells, resulting in elevated amount of Pim3 mRNA and translation in Myc-driven lymphomagenesis.

**Pim kinase inhibitor, Pimi, hampers cellular proliferation and causes a reduction in Myc regulated transcripts**

We have shown that Pim kinases, Pim3 in particular, are over-expressed in Myc driven lymphomas. To examine the effect that Pim kinase inhibition has on Myc driven lymphomagenesis we obtained a pan kinase inhibitor, Compound 14j (Tao et al., 2009) that we will call Pimi, from Abbott laboratories. Pimi exhibits a high degree of selectivity for Pim kinases, Pim3 in particular, and is effective at nanomolar concentrations. We treated cell lines E239 and 820, established from spontaneously arising B-cell lymphomas in E12-Myc or λ-Myc transgenic mice, with Pimi or Pim-cl (a significantly less effective Pim inhibitor) to verify Pim kinase inhibition and to investigate the effect Pim inhibition would have on proliferation. Pim kinases phosphorylate Ser112 on Bad, a member of the BH3-only Bcl-2 family (Macdonald et
al., 2006), therefore we determined Pim kinase inhibition efficiency by monitoring Bad Ser112 phosphorylation by western blot analysis. We found a slight effect of Bad Ser112 phosphorylation in cells treated with 10nM Pimi but a significant effect at 10µM Pimi, at this concentration no Bad Ser112 phosphorylation was observed. Pim-ci and DMSO had no effect on Bad Ser112 phosphorylation at these concentrations suggesting that Pimi inhibition of Pim kinases was responsible for this effect. To determine the effect of Pim kinase inhibition on cellular proliferation, we measured 3H-Thymidine incorporation in Eµ239 and λ820 cell lines treated with 10nM to 10µM Pimi for 48 hours. We found that Pimi had an EC₅₀ of 2.6µM in Eµ239 cells, and 6µM in λ820 cells, suggesting that Eµ239 cells are more sensitive to Pim kinase inhibition than λ820 cells. Growth curves on various mouse and human B-cell lymphoma lines treated with 10nM to 10µM Pimi showed some cell lines, notably Eµ239 and Akata, exhibited decreased cell proliferation and an induction of cell death.

A possible explanation behind the observed decrease in cell proliferation and induction of cell death in some cell lines could be the impact of Pim kinase inhibition on Myc transcriptional activities. As mentioned previously, Pim-1 has been shown to bind Myc-Max and be recruited to E-boxes, where it then phosphorylates serine 10 on histone H3, thereby impacting up to 20% of Myc transcriptional target genes (Zippo et al., 2007). To investigate this possibility, we analyzed mRNA expression of Myc regulated genes Amd1, Aurkb, Cad, Shmt2, and Srm in Eµ239 cells treated with 10nM to 10µM Pimi for 24 hours. qRT-PCR analysis revealed decreased mRNA expression of these genes in Pimi treated cells supporting previous findings that Pim kinase inhibition impacts Myc transcriptional activation, and suggesting that this impact may result in decreased cellular proliferation in Myc driven lymphomagenesis.

**Pimi induces Caspase-3 independent cell death**

Pim kinases phosphorylate Ser112 on Bad rendering it inactive, and because Bad is a member of the BH3-only Bcl-2 family, this results in an inhibition of apoptotic cell death. Thus, Myc driven over-expression of Pim kinases may aid in evasion of apoptotic cell death. FACS profiles of Propidium Iodide (PI) stained Pimi treated Eµ239 cells show an elevated amount of cells in sub-G1, indicating increased cell death in these cells. Immunofluorescence analysis on Pimi treated Eµ239 cells using antibodies against the cleaved form of Caspase-3, as well as complementary FACS analysis, indicated that Pimi treated Eµ239 cells exhibited increased amounts of cleaved Caspase-3. FACS analysis revealed that DNA histograms of Pimi treated Eµ239 cells indicated that the Caspase-3 positive cells were primarily hyperdiploid and in the G2/M phase of the cell cycle, which suggests mitotic problems and may be the cause of the observed cell death. In order to determine if this cell death was dependent on Caspase-3, we treated Eµ239 cells with Pimi in combination with QVD-OPH, a pan caspase inhibitor, and monitored Caspase-3 cleavage and cell death. Immunofluorescence and FACS analysis showed that although Caspase-3 cleavage was decreased in these cells cell death was not significantly decreased. These results indicate that the Pimi induced cell death is independent from Caspase-3 activation, and therefore other cell death programs outside classical apoptosis could be responsible for this phenotype.

Although Pim kinases have been shown to promote cell survival due to Bad phosphorylation and inhibition of apoptotic signaling, they have also been implicated in metabolic signaling. As discussed previously, loss of Pim kinases impact Myc-driven transcription and this was confirmed in this study by examination of Myc metabolic target genes in cells treated with a Pim kinase inhibitor. In addition a previous study has shown that Pim-3 expression modulates Myc and proliferator-activated receptor gamma coactivator 1α (PGC-1α) protein levels thereby modulating cell growth and metabolism (Beharry et al., 2011). In this paper, we evaluated the impact of Pim kinase inhibition on tumor cell survival, and found that Pim kinase inhibition triggers cell death. However if this effect is strictly due to Pim kinase role in blocking cell death, or of it is due to Pim kinase promotion of cell survival is unknown and is an interesting area for future study.
GENERAL CONCLUSIONS AND DISCUSSION

The main aim of this dissertation was to explore the role of metabolism in Myc-driven tumorigenesis, and the efficacy of inhibition of key metabolic genes in treating Myc-driven tumors. Papers presented in this dissertation have shown that many genes involved in polyamine, glycolysis, serine metabolism, and cell survival are direct Myc transcriptional targets. We show in paper II that Myc over-expressing pre-cancerous cells also over-express many metabolic genes, and that Myc over-expressing cells can be differentiated from normal cells simply by examining metabolic genes alone. Data presented in this dissertation illustrate that although Myc over-expression results in increased expression of many metabolic genes, targeting these pathways does not always result in inhibition of Myc-driven tumor formation, but can effect Ras-driven tumorigenesis. Importantly, this study highlights the importance of genetic stratification of malignancies in developing successful chemotherapeutic treatments.
ACKNOWLEDGMENTS

This dissertation is the result of many hours of benchwork and computer work that was made possible (as well as fun) by help from many people that I would like to acknowledge.

I would like to thank my supervisor Jonas “Captain Krk” Nilsson for all the help and enthusiasm for science that you have shown over the years...as well as your encyclopedic knowledge of papers and journals! It has been fun to learn lessons during my PhD via quotes from “Pulp Fiction”, “Fletch” or “Dumb and Dumber” – teaching through humor is a valuable skill and it has been much appreciated!

Thank you Lisa Nilsson for being my “partner in pain” on the metabolism project and for being such a good example of a dedicated scientist, we have all learned a lot from you and thanks to you will always remember to have the proper controls for our experiments! Your mantra of “fast is slow and slow is fast” has helped ensure that our experiments are through and well thought out...of course whether they worked or not is another issue!

Thank you to the “Nilsson Team”, Jonas and Lisa, for having us over to your house for football games, crayfish parties and Thanksgiving dinners – they were a lot of fun. Thank you also for inviting us to your family home in Croatia, that is a trip that great on many levels – beautiful scenery, exciting scientific discussions and lots of sunshine!

Sara – Thanks for being as silly as I am and for making those late nights counting cells fun and full of laughter - I'm telling you our “Caspase 9” song will be a hit someday...as long as we are not the ones singing it! We had a lot of fun over the years collaborating on projects, teaching courses and making spex movies, and I have really enjoyed it so thank you!

Andreas – Thank you for the good times and all the laughter, it has been fun and you have been a good sport! You have helped make some good spex movies, even though you have somehow managed to avoid the craziness and chaos that goes with it - although why you know so much about “Twilight” is still a mystery...

Sanna - Thanks for all the laughs and fun times, it was fun working with you and I’m glad we have stayed friends even after you moved to Stockholm! Your cheer and happiness are contagious, and made my first few months here fun and that still continues – although now we go shoe shopping and visit museums instead of miniprepping and cell culturing!

Birgitta – Thank you for helping me learn and run the HPLC machine, my paper would not have been possible without you! Your patience and thoroughness was a good example to us, and I try to apply your saying of “less work from the brain makes more work for the fingers”.

To the post-docs we have had in the lab: Thank you Yongmei for your cheer and happiness and I wish you luck! Thank you Lisandro for your sense of humor and good advice, it was fun to have you in the lab!

We have had a lot of students come through our lab and I have enjoyed working with all of them: Thank you Susanne for your sense of humor and your dedication, it was fun to take “Molecular Genetics” with you and to start our projects in Jonas’s lab together – good luck in your PhD! Thank you Per-Anders, Sofie, Nicolin, Hakim, Shruthi, Helene and Som for your cheerfulness and smiles, it was fun to work with you! Thank you Ina and Christiane, it was fun to collaborate with you and make spex movies with you – you guys are just as silly as the rest of us and fit in perfectly into the group so thank you! It was nice to work with you Kerstin and it was fun to have you in the lab, good luck in the future and if you decide to start a PhD you will rock at it!

Maria N, thank you for being a cheerful and happy collaborator and making all day cell harvesting and sorting experiments fun – I didn’t think when we planned those long days we were going to have fun but thanks to you we did! Of course now we get eye twitches whenever someone mentions B-cell sorting but such is science! Good luck on the rest of your PhD!

Thank you to Sonja, Kristina R and Gunilla for your help, you have helped me run (and more importantly – understand) machines and techniques, even though you were both busy with your own projects and I really appreciated it!

Thank you to all of the people who have helped me at various points throughout my PhD: Olle, Lennart, Tord, Micke W, Anders B, Martin, Thomas, Ethel, Berit, Maria W, Eva-Christine, Anitha, Johnny, Ulla-Britt, Carola, and Maria. You have helped me get started here, given me valuable feedback on my projects, made sure all the i’s were dotted and t’s were
crossed on my paperwork, and made sure I had all the reagents and tools I needed to perform my experiments so thank you!

Thank you to the people who have taught with me over the years, or just offered valuable teaching assistance and advice! Thank you to: Linus, Sara, Pramoud, Linda, Micke S, Sreenivas, Christina S, Tiago, Maria N, Barbara W, and Edmund.

The “Friday Fika Group” has made my Fridays fun since I first started here, and many of the people in have become my friends (and partners in crime as far as spex movies goes – see the “Coffee Stain Productions” films): David, Regina, Sa, Linn, Anna, Erik T, John, Sara, Andreas, Linus, Therese, Lina, Margarida, Caroline, Viktoria, and Hande. We have made movies, crafts, pottery, and lots of bad jokes…okay, mostly on my part! I have had a lot of fun and appreciated your sense of humor and fun more than I can say so thank you!

Thank you to my fellow crafty friends from work for all the good times, dinners, fikas, craft evenings, laughter and memories: Sara, Sa, Linn, Erik T, Lina, Therese, John, Marie and Erik N. You have given me good advice, encouragement, and “Eurovision song contest’ commentary!

Thank you to “The Girls” in Umeå, Sanna, Anna, Inger, Robyne, and Iliana, for keeping me sane and offering me support, friendship and lots of laughter as well as many good memories. Sanna, as I said before you are a sunny person who brings light wherever you go, and that is always welcome, especially in Umeå in winter! Thank you Anna for being such a warm and funny friend, you have given me a shoulder to cry on and someone to laugh with during some hard times and that means more than words can say. Inger, my hilarious friend - it is not often that I laugh until my stomach hurts but it happens a lot with you…even while working out (or watching seahorses) at IKSU! Robyne you are an adventurer that is not afraid to take chances, and face challenges with grace and calm – I hope that by being friends with you some of that rubs off on me! Iliana, you have a warm and generous heart and always have a kind word and smile for your friends – even when the going gets tough.

Thank you to my friends and coworkers from St. Jude Children’s Research Hospital in Memphis – Angie, Andrew, Tania, Diane, Dave, Missy and “The Oracle” Carol. I had so much fun working with you, and watching you work that it inspired me to get a Phd…so maybe I should blame you instead! Thank you to Dr. Gronemeyer and Clare for getting me started in basic research and for giving me an opportunity to work in your lab. I never thought when I first left Montana to go to Memphis to be a POE student it would lead me to Sweden, but it has been and fun and exciting ride!

Thank you Jen and Jean (as well as Shawn and Robert) for being there for me over the years and encouraging me to leave Missoula and chase my dreams. Thank you to Jen for being a (loving) voice of reason in the chaos and for always being supportive and warm – I could not have moved from Memphis to Sweden without you! Thank you to Jean for always being adventurous and for cheering me on, even when my ideas are wild and not that well planned - you make it easy to be brave when you are standing beside me…and laughing!

I have a large and loving family that has supported me from the beginning of my education made it possible for me to get to this point. To my mother, thank you for believing in me and for always being there for me – I am blessed to be your daughter. To my father for teaching me that science is fun and that the world is a wonderful place I should explore…I got my silly side from you. To Dan, for all the encouragement, love and laughter you shower us with everyday. Thank you to my aunts and uncles for being surrogate parents to me as I was growing up – your support and love helped me make it here. To my brothers and sisters, you have made my life fun and make me smile whenever I am with you or talk to you – thank you for reminding me there is life outside of school! Thank you to my Swedish family, Lasse, Linda, Frida, Lars, Maja, Emma and Karl for making me feel at home here and for always showing me support and love…and tolerating my love of craft fairs and Skansen!

To my husband Linus, you made all of this possible and I love you. Home is where you are…so my love, now where should we go?

Hi 5!

Chaz
REFERENCES


