Evaluating the role of glutamine-dependent metabolic pathways in MYC-induced mammary gland tumourigenesis

Benedict Macintyre

University College London
and
The Francis Crick Institute
PhD Supervisor: Mariia Yuneva

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Declaration

I Benedict Macintyre confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.
Abstract

One of the major oncogenes dysregulated in breast cancer is MYC. MYC is a master regulator transcription factor that controls the expression of myriad genes involved in cell growth and metabolism. MYC transformation can render cells dependent on certain metabolic pathways, an important example of which is glutamine metabolism. Among MYC transcriptional targets are kidney-type glutaminase (GLS1), an enzyme regulating the first step of glutamine catabolism, and phosphoserine aminotransferase (PSAT1), an enzyme that utilises the amino group of glutamine for serine biosynthesis. Overexpression of MYC sensitises cells to glutamine deprivation and GLS1 inhibition. Hyperactivation of MYC is associated with increased GLS1 activity in basal-like and ER-negative breast tumours, and basal-like breast cancer cell lines are more sensitive to GLS1 inhibition. However, no direct relationship has been drawn between inhibiting GLS1, specific aminotransferase reactions downstream of GLS1, or both, and therapeutic effect in MYC-driven breast tumours. Herein we demonstrate that Gls1 deletion in the murine mammary gland delays MYC-induced tumour initiation, with tumours appearing histologically and metabolically distinct from controls. Consistently, Gls1KO tumours have decreased catabolism of glutamine carbon into the tricarboxylic acid cycle (TCA) cycle in comparison with control tumours. Nonetheless, Gls1KO tumours remain able to hydrolyse glutamine suggesting that compensatory mechanisms exist that permit MYC-induced tumour initiation and progression. We also find that deletion of Psat1 delays MYC-induced tumour initiation and progression. Psat1 deletion completely ablated serine biosynthesis in tumours and was sufficient to reduce total serine levels. Importantly, the effect of Psat1 deletion is enhanced by a serine- and glycine-deficient (-SG) diet. Psat1 deletion when combined with the -SG diet reduced glucose-derived carbon incorporation into nucleotides, while also decreasing total pools of nucleotides and one-carbon metabolites. The effects of either Gls1 deletion or perturbation of serine metabolism are markedly less pronounced in tumours induced by ERBB2, the murine ortholog of HER2, another oncogene commonly amplified in human breast cancers. These results reveal a clear functional connection between MYC overexpression and glutamine metabolism in mammary gland tumours in mice, providing further clinically relevant rationale for therapeutic
targeting of components of glutamine metabolism in human MYC-high breast tumours.
Impact Statement

The work presented herein demonstrates the importance of evaluating the context-specific roles of deregulated metabolic pathways to the design and implementation of novel therapeutic strategies in the treatment of cancer. *In vivo* models of MYC-driven breast cancer were used to assess the relative value of targeting different glutamine-dependent metabolic pathways to restrict the initiation and progression of tumourigenesis. Results demonstrated that targeting serine metabolism via PSAT1 inhibition and dietary serine and glycine restriction is an effective means of treating MYC-enriched tumours of the mammary gland in mice. GLS1 inhibition also demonstrated efficacy *in vivo*, while also being shown to synergise with inhibition of glutamine transport in MYC-driven tumour-derived cells *in vitro*. Taken together, these results, in combination with other data from the cancer metabolism field, support the potential of glutamine and serine metabolism as promising therapeutic targets for cancer therapy in humans.

How metabolic pathways are altered in specific cancers is a key question driving research in this field. Much work has focussed on the role that MYC plays in the aggressive basal-like subtype of breast cancer, while MYC’s role in regulating metabolic pathways is also becoming increasingly well-described. Our work is the first to draw direct links between MYC-induced mammary gland tumourigenesis, and the therapeutic efficacy that might be expected by specific targeting of glutamine-dependent metabolic pathways. Herein we describe in detail the metabolic effects of perturbing glutaminolysis or serine metabolism in MYC-driven breast tumours, and the phenotypic consequences that can be expected, from delayed tumour latency, to reduced metastatic potential.

Using genetically modified mice, we show that deletion of *Gls1* in the mammary gland is sufficient to significantly reduce the utility of glutamine as an anaplerotic metabolite in MYC-driven tumours, while delaying tumour initiation and growth. Importantly though, our research also exemplifies the importance of carefully considering environmental influences and experimental design upon experimental outcomes. We show how seemingly minor changes in cell culture medium can markedly influence
the efficacy of small molecule inhibitors targeting metabolic pathways, both in single and combined treatments. Furthermore, we show how the metabolic consequences of in vivo genetic ablation of Gls1 can be substantially reduced by changes in the housing environment of laboratory mice. This research also serves as a warning in this regard.

Previous work has demonstrated the potential therapeutic efficacy of targeting the serine synthesis pathway (SSP) in cancer, while a serine and glycine deficient diet has also shown efficacy in mouse cancer models. Our work demonstrates that inhibiting the second enzyme of the SSP, PSAT1, can reduce MYC-driven tumour initiation, latency, and metastasis in vivo. Furthermore, when combined with dietary serine and glycine deficiency, Psat1 deletion increased tumour latency by over 60%. The observations were accompanied by marked perturbations of one carbon metabolism and nucleotide biosynthesis, thereby revealing a possible mechanism by which targeting serine metabolism achieves its efficacy in these tumours.

While further research is required to translate our findings into clinical applications, our results contribute to the work that aims to identify suitable therapeutic targets for the treatment of MYC-enriched breast tumours in humans.
Acknowledgement

I would like to thank my PhD supervisor, Dr. Mariia Yuneva for giving me the opportunity to work in her lab at the Francis Crick Institute, and for all her help and guidance. It has been a truly incredible experience, and one that has provided memories and lessons that will stay with me for a lifetime. I would also like to thank my co-supervisor Dr. Gyorgy Szabadkai, and my thesis committee, Dr. Julian Downward, Dr. Markus Ralser, and Dr. Michael Duchen, for their expert knowledge and counsel throughout this process.

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Finally, to my family. My parents Jacqui and David, and sister Tia. Words cannot express the gratitude I feel for your continued love and support, and the joy you have brought me during my time as a PhD student and, indeed, my whole life. I couldn’t have done it without you.
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Abbreviations

-Q diet    Glutamine-deficient diet
-SG diet   Serine- and glycine-restricted diet
αKB        α-ketobutyrate
αKG        α-ketoglutarate
1C         One-carbon
3-PG       3-phosphoglycerate
AD         Anno Domini
ADP        Adenosine diphosphate
AMP        Adenosine monophosphate
AMPK       AMP-activated protein kinase
AOA        Aminooxyacetate
APS        Ammonium persulfate
ASCT2      Alanine, serine, cysteine transporter 2
ASNS       Asparagine synthetase
ATB0,+     Amino Acid Transporter B0,+ 
ATF4       Activating transcription factor 4
ATP        Adenosine triphosphate
B0AT1      System B0 Neutral Amino Acid Transporter AT1
BAT1       B0,+ -type amino acid transporter 1
BCA        Bicinchoninic acid
bHLHZIP    Basic helix-loop-helix leucine zipper
BLBC       Basal-like breast cancer
BPTES      Bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulphide
BRAF       V-Raf murine sarcoma viral oncogene homolog B1
CBS        Cystathionine β-synthase
CC3        Cleaved caspase-3
cDNA       Complementary DNA
CoA        Coenzyme A
Crick       The Francis Crick Institute
CRUK       Cancer Research UK
CTH        Cystathionine γ-lyase
DAPI       4′,6-diamidino-2-phenylindole
<table>
<thead>
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<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>E-box</td>
<td>Enhancer box</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced Chemiluminescence</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>EMT</td>
<td>Epithelial–mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
<td>Oestrogen receptor</td>
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<td>ERBB2</td>
<td>Erythroblastic leukaemia viral oncogene homolog 2</td>
</tr>
<tr>
<td>ET cells</td>
<td>MMTV-ERBB2 tumour-derived cells</td>
</tr>
<tr>
<td>F6P</td>
<td>Fructose-6-phosphate</td>
</tr>
<tr>
<td>FASN</td>
<td>Fatty acid synthase</td>
</tr>
<tr>
<td>GDH</td>
<td>Glutamate dehydrogenase</td>
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<td>GDP</td>
<td>Guanosine diphosphate</td>
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<td>GFAT</td>
<td>Glutamine fructose-6-phosphate amidotransferase</td>
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<td>GLDC</td>
<td>Glycine decarboxylase</td>
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<tr>
<td>GLS</td>
<td>Glutaminase</td>
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<tr>
<td>GLs1KO</td>
<td>Glsl knockout</td>
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<tr>
<td>Glu</td>
<td>Glutamate</td>
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<tr>
<td>GMP</td>
<td>Guanosine monophosphate</td>
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<td>GOT</td>
<td>Glutamic-oxaloacetic transaminase</td>
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<tr>
<td>GPNA</td>
<td>L-γ-Glutamyl-p-nitroanilide</td>
</tr>
<tr>
<td>GPT</td>
<td>Glutamic-pyruvic transaminase</td>
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<tr>
<td>GS</td>
<td>Glutamine synthetase</td>
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<tr>
<td>GSEA</td>
<td>Gene set enrichment analysis</td>
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<tr>
<td>GTP</td>
<td>Guanosine Triphosphate</td>
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<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
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<tr>
<td>HBP</td>
<td>Hexosamine biosynthesis pathway</td>
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<tr>
<td>Hcys</td>
<td>Homocysteine</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HRE</td>
<td>Hormone response element</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia-inducible factor</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IDP</td>
<td>Intrinsically disordered protein</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LAT</td>
<td>System L amino acid transporter</td>
</tr>
<tr>
<td>LCMS</td>
<td>Liquid chromatography–mass spectrometry</td>
</tr>
<tr>
<td>L-MYC</td>
<td>MYC lung carcinoma derived homologue</td>
</tr>
<tr>
<td>MAD</td>
<td>Max dimerisation protein</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAX</td>
<td>MYC-associated factor X</td>
</tr>
<tr>
<td>miR</td>
<td>miRNA</td>
</tr>
<tr>
<td>miRNA</td>
<td>MicroRNA</td>
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<tr>
<td>MMEC</td>
<td>Mouse mammary epithelial cell</td>
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<td>MMTV</td>
<td>Mouse mammary tumour virus</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<td>Methyltransferase</td>
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<td>MT cells</td>
<td>MMTV-MYC tumour-derived cells</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mechanistic target of rapamycin</td>
</tr>
<tr>
<td>mTORC</td>
<td>mTOR complex</td>
</tr>
<tr>
<td>MYC</td>
<td>Myelocytomatosis oncogene</td>
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<tr>
<td>N-MYC</td>
<td>MYC neuroblastoma derived homologue</td>
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<tr>
<td>NA</td>
<td>Natural abundance</td>
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<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
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<td>NADH</td>
<td>Reduced NAD</td>
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<tr>
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<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADPH</td>
<td>Reduced NADP</td>
</tr>
<tr>
<td>NEAA</td>
<td>Non-essential amino acid</td>
</tr>
<tr>
<td>NIMR</td>
<td>National Institute for Medical Research</td>
</tr>
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<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>P-Ser</td>
<td>3-phosphoserine</td>
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<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly-ADP ribose polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate-buffered saline, 0.05% Tween 20</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal component analysis</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDX</td>
<td>Patient-derived xenograft</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<td>PHGDH</td>
<td>Phosphoglycerate dehydrogenase</td>
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<td>PHP</td>
<td>Phosphohydroxypyruvate</td>
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<td>PPP</td>
<td>Pentose phosphate pathway</td>
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<tr>
<td>PRPP</td>
<td>Phosphoribosyl pyrophosphate</td>
</tr>
<tr>
<td>Psat1(^{\text{KO}})</td>
<td>Psat1 knockout</td>
</tr>
<tr>
<td>PSAT</td>
<td>Phosphoserine aminotransferase</td>
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<tr>
<td>PSPH</td>
<td>Phosphoserine phosphatase</td>
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<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
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<tr>
<td>R5P</td>
<td>Ribose 5-phosphate</td>
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<td>RAS</td>
<td>Rat sarcoma viral oncogene</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>RNA-seq</td>
<td>RNA sequencing</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>RPM</td>
<td>Revolutions per minute</td>
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<td>SAM</td>
<td>S-adenosylmethionine</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<td>SHMT</td>
<td>Serine hydroxymethyltransferase</td>
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<tr>
<td>Term</td>
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<tr>
<td>SIAM</td>
<td>Stable isotope-assisted metabolomics</td>
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<td>SLC</td>
<td>Solute carrier family</td>
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<td>Sodium-coupled neutral amino acid transporter</td>
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<td>Serine synthesis pathway</td>
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<td>TCA</td>
<td>Tricarboxylic acid</td>
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<tr>
<td>TEMED</td>
<td>$N,N',N'$-Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TET</td>
<td>Ten-eleven translocation</td>
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<tr>
<td>TNBC</td>
<td>Triple-negative breast cancer</td>
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<tr>
<td>UDP</td>
<td>Uridine diphosphate</td>
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<td>United Kingdom</td>
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<td>Uridine monophosphate</td>
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<td>Uridine Triphosphate</td>
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<td>World Health Organization</td>
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<tr>
<td>YME1L1</td>
<td>YME1-like 1</td>
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Chapter 1. Introduction

1.1 Cancer

‘Cancer’ refers to a group of heterogeneous diseases characterised by uncontrolled cell proliferation that can begin in almost any tissue and eventually spread to other tissues in the body. The first record of cancer in history dates back to ancient Egypt where a surgical textbook described cases of breast tumours, finishing with the remark ‘there is no treatment’ (Breasted, 1930). Hippocrates coined the term ‘cancer’ after suggesting that cross-sections of tumours had “veins stretched on all sides as the animal the crab (cancer) has its feet, whence it derives its name” (Paul of Aegina, 7th century AD). Over time, cancers have been recognised to be substantially different from one another depending on the tissue of origin. Indeed, even cancers that arise in the same tissue can vary substantially in terms of mutation profile and associated aspects of phenotype (Dagogo-Jack and Shaw, 2017). As such, the cancer research field is vast, and split into many different areas of expertise that aim to use different therapeutic approaches. In the present day, cancer continues to challenge the vast number of researchers who aspire to the eradication of the disease famously referred to as ‘the emperor of all maladies” (Mukherjee, 2010).

In 2018 cancer was ranked as the second leading cause of death worldwide, behind only cardiovascular diseases (World Health Organisation [WHO]; 2019). It is estimated that one-in-two people alive in the UK today will be diagnosed with cancer, an increase from the one-in-three estimate from Cancer Research UK (CRUK) in 2011, in large part due to the longer life expectancy of those born after 1960 (CRUK, 2019). While an increased capacity to detect cancers also contributes to this rise in incidence, carcinogenesis is driven by sequential genetic mutation, and thus can be referred to as age-dependent; the longer a person lives, the more time there is for carcinogenic mutations to occur. Of those that receive a cancer diagnosis, it is expected that approximately half will succumb to the disease (CRUK, 2019). There is no greater illustration as to the importance of research into the initiation and progression of cancers.
1.2 Breast cancer

Breast cancer is not only the most common cancer in women in the UK, but also the most common cancer overall, accounting for 15% of new cases yearly (CRUK, 2021). Despite this, survival statistics for breast cancer are among the best of all cancers, due to the relative ease of detection. Women diagnosed with stage one breast cancer are almost just as likely as those without the condition to survive five years after diagnosis, with survival of those diagnosed with stage two dropping to 90%, and stage three to 72% (Office for National Statistics, 2019). However, at stage four, five-year survival drops precipitously to just 26% (Office for National Statistics, 2019). Therefore, primary breast cancer is largely considered curable, while metastatic breast cancer is largely considered terminal; current treatment options for metastatic breast cancer focus on prolonging life rather than curing.

Following the establishment and appreciation of the extent of tumour heterogeneity, treatment options shifted toward approaches based on subtype classification. Five major subtypes of breast cancer have been identified: luminal A; luminal B; normal-like; basal cell-like; and HER2/neu (Perou et al., 2000). These molecular classifications have since been utilised for the clinical management of breast cancer patients, although patient outcomes vary widely depending on the subtype (Dai et al., 2015). The luminal-like subtypes are receptor positive, with luminal A tumours expressing both the oestrogen and progesterone receptors (ER and PR, respectively), and luminal B tumours largely additionally expressing the human epithelial growth factor receptor (HER2). HER2-enriched tumours, as the name suggests, are characterised by HER2 expression, while the basal-like subtype is classified as a ‘triple-negative breast cancer’ (TNBC). This designation comes from the fact that none of the aforementioned hormone receptors are expressed in this subtype, while they are further characterised by the presence of the histological markers cytokeratins 5, 6, and 17 (Alluri and Newman, 2015). The receptor-positive subtypes are amenable to hormonal therapy and antibody-based therapies such as trastuzumab, a monoclonal antibody specific to HER2, and concordantly exhibit significantly greater survival than basal-like subtype (Garnock-jones, Keating, and Scott, 2010; Vorobiof, 2016). Indeed, for the basal-like subtype there are currently no effective therapeutic options, with treatment consisting of either chemotherapy or
radiotherapy, and the survival rate reflects this; the basal-like subtype is particularly noteworthy for its especially poor clinical prognosis (Alluri and Newman, 2015).

Basal-like breast cancer (BLBC) is poorly defined, comprising breast cancers that do not express hormone receptors. BLBC accounts for approximately 15% of all breast cancers, and there is much concern regarding its aggression, and poor prognosis due to the current paucity of therapeutic options (Nielsen et al., 2004). While the triple-negative and basal-like classifications are often used interchangeably, BLBC represents a distinct class of tumours from others that are hormone receptor negative. After their original histological classification, gene-expression microarray analysis identified BLBC as its own subtype (Perou et al., 2000). Further subgroups have been proposed for this class of tumours, each with its own distinct clinical outcome (Milioli et al., 2017). Categorising tumours in this way is essential to stratify patients so that the appropriate therapeutic regimen may be applied, and research is focussed on identifying common characteristics shared between tumours in each category that could be therapeutically exploited.

1.3 MYC

c-MYC (cellular-MYC, hereafter referred to as ‘MYC’) was first discovered in humans as the cellular homologue of the v-myc oncogene carried by avian myelocytomatosis virus, and it is known to be one of the most frequently deregulated oncogenes in human cancers (Stehelin et al., 1976; Duesberg, Bister, and Vogt, 1977; Bister and Jansen, 1986; Dang, 2012; Gabay, Li, and Felsher, 2014). The MYC family includes two other members, N-MYC and L-MYC, named so for their observed deregulation in neuroblastoma and small cell lung cancer, respectively. Members of the MYC family are referred to as master transcriptional regulators, controlling the expression of approximately 15% of the genome (Pelengaris, Khan, and Evan, 2002; Adhikary and Eilers, 2005; Dang et al., 2006; Dang, 2012). The downstream effects of MYC signalling include significant metabolic changes and increased ribosome biogenesis to promote the accumulation of biomass, the promotion of cell proliferation and differentiation, and downregulation of genes involved in cell adhesion (Dang et al., 2006). MYC regulates the expression of myriad metabolic genes, including enzymes
involved in glycolysis and glutaminolysis, and glucose and amino acid transporters (Osthus et al., 2000; Kim et al., 2004; Wise et al., 2008; Wang et al., 2011; Yuneva et al., 2012; Yue et al., 2017; Méndez-Lucas et al., 2020). MYC can also enhance the translation of genes involved in metabolism, for example glutaminase 1 (GLS1) via inhibition of miR-23a/b (Gao et al., 2009). These activities of MYC are necessary in certain normal biological processes, such as the integral role it plays in the activated T-cell program as a potent activator of cell proliferation (Wang et al., 2011). However, inappropriate MYC hyperactivation is estimated to be involved in approximately 70% of cancers (Dang, 2012). Concordantly, MYC expression in cells is tightly regulated from transcription to post-translation under normal biological circumstances. Both MYC mRNA and protein have very short half-lives, while differential phosphorylation of MYC protein can stabilise or target it for proteasomal degradation (Sears et al., 1999; Gregory and Hann, 2000). MYC can only exert its transcriptional function by dimerising with MYC-associated factor X (MAX). However, MAX is provided with competition from the MAX dimerisation protein (MAD) family, which exert regulatory effects opposite to MYC/MAX dimers (Grinberg, Hi, and Kerppola, 2004). These proteins are basic helix-loop-helix leucine zipper (bHLHZIP) transcription factors and are collectively referred to as the MYC/MAX/MAD network. The relative levels of MYC and MAD proteins determine the proliferative state of the cell (Fig. 1-1).
Figure 1-1 The MYC/MAX/MAD network

a, MYC, MAX, and MAD protein domain organisation. MAX can associate with MYC by mutual interaction of the basic helix-loop-helix leucine zipper motifs, allowing dimer association with E-box sequences to promote transcription via the MYC transactivation domain. MAX can alternatively associate with MAD to repress transcription via the MAD SID domain. MYC must dimerise with MAX to bind E-box consensus sequences and activate target gene expression to promote cell growth, proliferation, and survival. MAD/MAX dimers directly oppose the function of MYC/MAX dimers by via transcriptional repression. The relative concentrations of MYC and MAD proteins determines the rate of cell growth. b, basic domain. HLH, helix-loop-helix domain. LZ, leucine zipper domain. MAD, Max dimerisation protein. MAX, MYC-associated factor X. MYC, Myelocytomatosis oncogene. SID, Sin3 interaction domain.
The importance of MYC overexpression in the initiation and progression of all types of breast cancer has been extensively studied (Efstratiadis, Szabolcs, and Klinakis, 2007; Chen and Olopade, 2008; Hynes and Stoelzle, 2009). First reported in 1986, MYC gene amplification and overexpression, rather than translocation, can contribute to the transformation of mammary epithelial cells (Escot et al., 1986). In 2000, a meta-analysis of studies investigating the clinical significance of MYC in breast cancer was performed and found that it was associated with poor prognosis (Deming et al., 2000). MYC can be found to be overexpressed in each of the aforementioned subtypes of breast cancer, although its significance in BLBC is due to its expression having been shown to be in strong concordance with the basal-like subtype classification (Chandriani et al., 2009; Alles et al., 2009; Horiuchi et al., 2012). Increased MYC activity in BLBC is thought to be key in its ability to achieve independence of hormone receptors, imitating growth-stimulatory input (Alles et al., 2009). The current relative lack of treatment options for BLBCs has led to MYC overexpression being highlighted as a potential therapeutic avenue to exploit.

Increased understanding of the requirement of metabolic alterations for tumour survival and progression has led to these alterations becoming novel therapeutic targets. If a tumour is metabolically reprogrammed to rely too much on a particular nutrient or pathway, a treatment that aims to restrict the availability of that nutrient (or a small molecule inhibitor targeting the pathway in question) may be sufficient to treat the tumour. This approach is referred to as ‘synthetic lethality’, whereby a particular mutation (in this case an activating MYC mutation) renders a cancer cell vulnerable to the targeting of a second gene product, leaving normal cells unaffected (O’Neil, Bailey, and Hieter, 2017).

MYC has a pronounced effect on glutamine metabolism, leading to glutamine availability and catabolism becoming essential to the survival of cells with increased MYC expression (Yuneva et al., 2012). However, the 3’ UTR of MYC mRNA has been found to contain a regulatory element that is responsive to intracellular levels of glutamine (Dejure et al., 2017). The authors found that, in colorectal cancer cells, glutamine deprivation inhibits MYC translation via the 3’ UTR due to decreased adenosine nucleotide concentrations, leading to cell cycle arrest rather than apoptosis (Dejure et al., 2017). Therefore, designing cytotoxic rather than cytostatic
therapeutic approaches to target MYC-driven tumours will require a more thorough understanding of the interplay between glutamine metabolism and MYC expression and activity. This provides rationale for investigating whether inhibiting glutamine metabolism, in a single or combinatorial therapy, represents a viable therapeutic strategy for MYC-driven tumours in vivo.

1.4 ERBB2

The human epidermal growth factor receptor (HER) family comprises four members: HER1, HER2, HER3, and HER4. HER2 is unique among its family members as it does not have a ligand, relying upon hetero- or homodimerisation for its activation (Gutierrez and Schiff, 2011). HER2 activation leads to signalling cascades mediated primarily by protein kinase C (PKC), phosphatidylinositol-4,5-biphosphate 3-kinase (PI3K), and mitogen-activated protein kinase (MAPK) that promote the transcription of genes involved in cell proliferation, differentiation, and survival (Fig. 1-2). HER2 is overexpressed in approximately 20% of human breast cancers and defines the ‘HER2-enriched’ subtype (Slamon et al., 1987; Perou et al., 2000). HER2 overexpression can both occur as an early driver of tumourigenesis or appear later during tumour progression, for example in response to the targeting of tumours expressing the oestrogen receptor (ER) with tamoxifen (Allred et al., 1992; Gutierrez et al., 2005). However, HER2-enriched breast cancer has a relatively good prognosis as the receptor can be targeted with the monoclonal antibody trastuzumab. The exact mechanism of action of trastuzumab is unclear but is likely to be either depletion of downstream signalling or antibody-dependent cellular cytotoxicity, or both (Maximiano et al., 2016). Clinical trials have demonstrated that trastuzumab can increase overall survival in patients with HER2-enriched tumours by 33%, while able to increase disease-free survival by 50% (Iqbal and Iqbal, 2014).

Notwithstanding the relatively favourable prognosis, HER2-enriched breast tumours represent some of the most aggressive, along with BLBCs (Carey et al., 2006). Distinctions in the lipid profiles between HER2-positive and HER2-negative breast cancer cells have been identified; HER2-positive cells have been shown to
upregulate fatty acid biosynthesis, with pro-lipogenic enzymes including fatty acid synthase (FASN) being upregulated (Yoon et al., 2007; Kourtidis et al., 2009; Kourtidis et al., 2010). It was subsequently demonstrated that this causes sensitivity to palmitate-induced cytotoxicity in HER2-enriched breast cancer cells (Baumann et al., 2018). Therefore, even though HER2-positive tumours do not separate significantly separate from HER2-negative tumours by metabolite signature, metabolic differences are present (Terunuma et al., 2014). In contrast, deregulated MYC expression in cancers and ectopic expression of MYC in cells are associated with marked metabolic rewiring (Yuneva et al., 2012). The ErbB2 gene is the murine ortholog of the human HER2 gene. To assess the oncogene-specificity of metabolic changes observed in MYC-driven breast tumours, in this project we compared some of the metabolic vulnerabilities of tumours from the MMTV-MYC and MMTV-ERBB2 mouse models. This should allow for the prediction of specific, metabolism-based therapeutic approaches for MYC-enriched human breast tumours.
HER2 is activated by homodimerisation, or heterodimerisation with HER1, 3, or 4, leading to kinase cascades that transmit signals to the nucleus for the transcription of genes involved in tumour cell growth, proliferation, and survival. Akt/PKB, protein kinase B. HER, human epidermal growth factor. PI3K, phosphatidylinositol-4,5-biphosphate 3-kinase. PLCε, phospholipase Cε. MAPK, mitogen-activated protein kinase. PKC, protein kinase C.
1.5 Therapeutic targeting of MYC in BLBC

Due to the plethora of effects that MYC has on cells, which are also context-dependent, there are a wide range of possibilities for targeting it therapeutically. The primary goal is to selectively target MYC-amplified cells without affecting normal tissues, but unfortunately the MYC protein has earned a reputation of being ‘undruggable’ as no clinically relevant inhibitors have been identified since it was discovered over 40 years ago. The importance of MYC in normal cells is in large part responsible for this problem, while targeted drug delivery to the nucleus is also extremely challenging due to the presence of drug-efflux pumps, detoxification enzymes, and sequestration of the drug molecules (Duvvuri et al., 2002; Deepthi et al., 2013). Furthermore, the structure of the MYC protein itself represents a challenge for therapeutic targeting as it exists as an intrinsically disordered protein (IDP), also referred to as a ‘protein cloud’, capable of existing in one of many conformational structures with a lack of effective binding pockets for drugs (Jin et al., 2013). However, as mentioned previously, MYC is a well characterised regulator of cellular metabolism, promoting dynamic upregulation of anabolic and catabolic pathways as part of its growth-promoting transcriptional program (Osthus et al., 2000; Kim et al., 2004; Wise et al., 2008; Gao et al., 2009; Wang et al., 2011; Yue et al., 2017). As such, targeted inhibition of specific metabolic reactions and nutrient deprivation represent viable therapeutic opportunities for MYC-amplified tumour cells.

1.6 Metabolism in cancer

It is now recognised that altered metabolic states contribute to the pathology of not only cancer, but other diseases such as neurodegeneration, obesity, and diabetes. Metabolic homeostasis is essential for the normal functioning of cells in many ways, including appropriate activity of signalling pathways, cell growth, and proliferation. Regulation of metabolism in healthy cells is governed by nutrient availability, which in turn activates pathways such as mTOR during nutrient excess to promote anabolism, or AMP-activated protein kinase (AMPK) signalling during deprivation to promote catabolism. Cancer cells undergo extensive metabolic rewiring to provide themselves with a competitive advantage over healthy cells in terms of energy and
biomass production. The first indication of this was in the 1920s when Otto Warburg observed that tumours, relative to normal tissues, consume large amounts of glucose and enhance lactate production, even in the presence of normal oxygen levels, a phenomenon now widely known as the ‘Warburg effect’ or ‘aerobic glycolysis’ (Warburg and Minami, 1923; Warburg, 1927). These findings led Warburg to propose that cancer was caused by impaired oxidative phosphorylation. It was later found that cancer cells retain their ability to utilise oxidative phosphorylation and that oxygen consumption in tumours can even exceed that of normal tissues (Weinhouse, 1976; Fantin, St-Pierre, and Leder, 2006).

The Warburg effect is observed in normal cells during rapid proliferation, as enhanced glycolysis is important to support branching biosynthetic pathways (Sun et al., 2019). The pathways in question are: the pentose phosphate pathway (PPP), responsible for the production of ribose for nucleotide synthesis and NADPH for reductive biosynthesis; the serine synthesis pathway (SSP), which produces serine and feeds carbon units into one-carbon (1C) metabolism; the hexosamine biosynthesis pathway (HBP), which is required for the glycosylation of proteins; and glycerol production for the synthesis of lipids. Furthermore, while also the primary mechanism for anaplerosis, the increased rate of glycolysis means roughly equivalent amounts of ATP can be produced by glycolysis alone per unit time versus the full oxidation of a glucose molecule, as glycolysis occurs at 10-100 times the rate of oxidative phosphorylation (Shestov et al., 2014).

Otto Warburg was the first to elucidate the increased requirement for glucose in tumour tissue versus normal tissue, but Harry Eagle concordantly demonstrated a necessity for a plentiful supply of amino acids, especially glutamine; HeLa cells were found to require glutamine at a 10 to 100-fold greater concentration than other amino acids (Eagle, 1955; Eagle et al., 1956). It has since been demonstrated that this increased requirement for glutamine in malignant rather than healthy tissue is widespread, while the metabolic profile of tumours with respect to glutamine is dependent on both the tissue of origin and initiating oncogenic lesion (Rivera et al., 1988; Márquez et al., 1989; Yuneva et al., 2012). The metabolic adjustments that take place in tumours have been proposed to bestow various selective advantages upon cancer cells, in terms of ATP production, competition for nutrients, and
providing proliferating cancer cells with metabolic intermediates that can be used for biosynthesis (Liberti and Locasale, 2016). A cell lacking growth inhibition must accumulate sufficient energetic and biosynthetic intermediates or face inevitable death, and two fundamental routes to produce these intermediates are glycolysis and glutaminolysis.

1.7 Glutamine metabolism in breast cancer

1.7.1 Glutamine

Glutamine is an α-amino acid with five carbon atoms and is the most abundant amino acid in human serum (Bergström et al., 1974). Glutamine also represents the most versatile amino acid in terms of amino acid metabolism. Glutamine is fundamental for nitrogen exchange between tissues and is utilised as a nitrogen donor to produce nucleotides and non-essential amino acids (NEAAs), while also serving as a precursor to produce glutathione and providing carbon to produce TCA cycle intermediates (Altman, Stine, and Dang, 2016). While designated as non-essential, it is also known as a ‘conditionally essential’ amino acid, referring to the fact that in certain pathophysiological circumstances, such as during infection or wound healing, glutamine becomes essential (Labow, Soubra, and Abcouwer, 2001; Rogero et al., 2007; Rodas et al., 2012).

1.7.2 Glutamine transport

The energetic and biosynthetic requirements of cancer cells mandate a plentiful supply of nutrients. However, it is necessary for glutamine to be transported across the cell membrane due to its hydrophilic nature, which prevents it from being able to freely cross the phospholipid bilayer. The mammalian glutamine transporters comprise four distinct families of transporters organised by amino acid sequence similarity (SLC1, SLC6, SLC7, and SCL38), with a total of fourteen transporters that either act as obligate exchangers or active transporters in a particular direction across the cell membrane. None of these transporters are exclusive to glutamine.
In the SLC6 family, A14 and A19 (also known as ATB$^{0,+}$ and B$^0$AT1 respectively) can transport a broad range of amino acids, including glutamine. ATB$^{0,+}$ has the broadest specificity, being able to transport eighteen of the twenty proteinogenic amino acids except aspartate and glutamate. It is a concentrative transporter, transporting amino acids against their concentration gradient into the cell, utilising the Na$^+$ and Cl$^-$ concentration gradients as well as membrane potential (Bhutia et al., 2014). B$^0$AT1 is specific for all neutral amino acids.

The SLC7 family comprises A5 and A8 (also known as system L amino acid transporters 1&2, LAT1 and LAT2 respectively), A6 and A7 ($\gamma$LAT1 and $\gamma$LAT2), and A9 (BAT1). Each transports all neutral amino acids and are all obligate exchangers, with $\gamma$LAT1, $\gamma$LAT2, and BAT1 exchanging for cationic amino acids. $\gamma$LAT1, $\gamma$LAT2 mediate influx of neutral amino acids coupled to efflux of cationic amino acids, while BAT1 mediates the reverse. LAT1 and LAT2 only mediate the exchange of neutral amino acids across the membrane, with a preference for large amino acids and in particular leucine. An important function of LAT1 is thought to be the coupling of glutamine export to leucine import across the cell membrane, which in turn activates mTORC1 and thus cell growth and proliferation (Lynch, 2001; Bothwell et al., 2018; Fig. 1-3). LAT1, along with most other transporters in the SLC7 family, functions as a heterodimer with a catalytic transport unit that is associated with a chaperone protein that takes it to the cell membrane (Fotiadis et al., 2013). LAT1 specifically interacts with SLC3A2, a member of the solute carrier family SLC3.
While glutamine transport into the cancer cell is necessary to sustain glutamine catabolism and downstream glutamine-dependent metabolic pathways, it also provides LAT1 with the substrate necessary to import leucine.

**Figure 1-3 Coordinated glutamine transport in cancer cells**

While glutamine transport into the cancer cell is necessary to sustain glutamine catabolism and downstream glutamine-dependent metabolic pathways, it also provides LAT1 with the substrate necessary to import leucine.
The SLC38 family has six members, A1, A2, A3, A5, A7, and A8. They are all also referred to as ‘SNAT’ (each with the same number suffix), which stands for ‘sodium-coupled neutral amino acid transporters’. SNAT1 and SNAT2 represent significant ports of entry of glutamine into cells, due to their ubiquitous expression and narrow substrate selectivity. SNAT1 and SNAT2 also play an important role in the central nervous system (CNS); they are expressed in cortical neurons where they mediate the ‘glutamine-glutamate’ cycle in synapses, along with glutamine synthetase (GS) and GLS (Conti and Melone, 2006). SNAT7 and SNAT8 are also expressed in the CNS and are purported to engage in this cycle (Hagglund et al., 2011; Hagglund et al., 2015). SNAT3 and SNAT5 expression is localised to specific organs, where they can mediate either influx or efflux of glutamine.

The final family is SLC1, and SLC1A5 is the only member that can transport glutamine. Alanine, serine, cysteine transporter 2 (ASCT2), the protein product of SLC1A5, is a sodium-dependent antiporter, and is selective for alanine, cysteine, glutamine, serine, and threonine. ASCT2 functions as an obligate exchanger i.e., it can transport glutamine in either direction depending on the concentration gradient across the membrane.

1.7.3 Glutamine catabolism

Glutamine contains two nitrogen atoms at the \( \alpha \) and \( \gamma \) positions along its carbon backbone, forming part of an amino group and an amido group, respectively (Fig. 1-4). The first step in glutamine metabolism is the removal of the amido group, which can be achieved via several different metabolic reactions. GLS is the first enzyme involved in glutamine catabolism, which generates glutamate and an ammonium ion. GLS1, a K-type (kidney-type) isozyme of GLS, is present in most adult tissues, whereas L-type (liver-type) GLS2 expression is largely confined to the liver (Pérez-Gómez et al., 2005).
Glutamine catabolism

*In vivo* glutamine demand is met by a combination of dietary intake and *de novo* glutamine synthesis by glutamine synthetase (GS). Glutamine is converted to glutamate primarily by the action of glutaminase (GLS), by removal of the γ-nitrogen amide group to produce ammonia. This amide group can also be utilised by amidotransferase enzymes for *de novo* synthesis of nucleotides, asparagine, and glucosamine. The α-nitrogen amine group can then be removed by glutamate dehydrogenase (GDH) to produce α-ketoglutarate. This amine group can also be utilised by aminotransferase enzymes for *de novo* non-essential amino acid (NEAA) synthesis. AMP, adenosine monophosphate. ASNS, asparagine synthetase. CTP, cytidine triphosphate. CTPS, cytidine triphosphate synthetase. GDH, glutamate dehydrogenase. GFAT, glutamine:fructose-6-phosphate aminotransferase. GLS1, glutaminase 1. GMP, guanosine monophosphate. GMPS, guanosine monophosphate synthetase. GS, glutamine synthetase. HBP, hexosamine biosynthesis pathways. PFAS, phosphoribosylformylglycinamidine synthase. PPAT, Phosphoribosylpyrophosphate amidotransferase.
The γ-nitrogen can also be utilised by amidotransferase enzymes during nucleotide synthesis (Fig. 1-4). Purine synthesis requires further nitrogen donation from aspartate and glycine, although this can be derived from glutamine’s α-nitrogen. Glutamine’s γ-nitrogen is also utilised for hexosamine synthesis, which is essential for the glycosylation of proteins; glutamine:fructose-6-phosphate amidotransferase (GFAT) transfers the nitrogen to fructose-6-phosphophate (F6P) to produce glucosamine-6-phosphate. Finally, glutamine can be used to produce asparagine by γ-nitrogen transfer to aspartate by asparagine synthetase (ASNS).

Following the removal of the γ-nitrogen, glutamate is produced. Glutamate can have many biochemical fates aside from protein synthesis, starting with the removal or transfer of the amine α-nitrogen. This can be achieved either by glutamate dehydrogenase (GDH), producing a second ammonium ion and αKG, or by a transaminase enzyme that also produces αKG but instead transfers the amino group to an α-keto acid, producing de novo NEAAs (Fig. 1-4). Glutamic-pyruvic transaminase (GPT) uses glutamate to produce alanine. GPT2 is the mitochondrial isoform and is present higher cellular concentrations than its cytosolic counterpart GPT1, and plays an important role in regulating gluconeogenesis, and glucose, lipid, and amino acid metabolism more generally (Yang et al., 2002; Qian et al., 2015). Phosphoserine aminotransferase 1 (PSAT1) produces 3-phosphoserine (P-Ser) as part of the SSP, which ultimately produces serine for use in a variety of cellular reactions (Fig. 1-5). Serine donates carbon units to 1C metabolism while simultaneously producing glycine and is also required for glutathione synthesis, as well as the production of nucleotides, sphingolipids, and phospholipids such as phosphatidylserine (Fig. 1-5). 1C units donated by serine can be used to replenish S-adenosyl methionine (SAM), which subsequently can be used in a variety of methyltransferase reactions. Furthermore, phospholipid methylation has been shown to play an important role in regulating the SAM/SAH ratio to balance cellular methylation potential (Ye et al., 2017). Glutamate is used directly and indirectly via the SSP in the production of glutathione, the most abundant endogenous antioxidant that is essential for host defence against free radical injury (Denek and Fanburg et al., 1989). Coenzyme A (CoA) produced via the transsulfuration pathway has also been implicated in antioxidant defence, having been shown to covalently bind a
variety of cellular proteins in response to oxidising agents, in a reversible manner (Tsuchiya et al., 2017). αKG produced following the catabolism of glutamate is a key TCA cycle metabolite. Therefore, glutamine also represents an important source of energy for the cell via the decarboxylation of αKG during oxidative phosphorylation.
Figure 1-5 The serine synthesis pathway (SSP) and one-carbon (1C) metabolism

Serine provides carbon units to the folate cycle via the action of serine hydroxymethyltransferase (SHMT), simultaneously producing glycine. Glycine also contributes carbon units via glycine decarboxylase (GLDC). 1C units supply the methionine cycle for methyltransferase reactions. The methionine cycle also provides homocysteine (HCys) for de novo cysteine biosynthesis via the transsulfuration pathway, contributing to the maintenance of the intracellular glutathione pool. αKB, α-ketobutyrate. αKG, α-ketoglutarate. 3-PG, 3-phosphoglycerate. CBS, Cystathionine β-synthase. CoA, coenzyme A. CTH, cystathionine γ-lyase. GLDC, glycine decarboxylase. Glu, glutamate. HCys, homocysteine. MT, methyltransferase. NAD, nicotinamide dinucleotide. NADH, reduced NAD. PHGDH, phosphoglycerate dehydrogenase. PHP, phosphohydroxypyruvate. PSAT1, phosphoserine aminotransferase 1. P-Ser, 3-
phosphoserine. ROS, reactive oxygen species. SHMT, serine hydroxymethyltransferase. SAM, S-adenosylmethionine.
1.7.4 Dysregulation of glutamine transport in breast cancer

While both ATB\(^{0,+}\) and B\(^0\)AT1 have theoretical potential for upregulation in cancer, this has only been shown to occur for ATB\(^{0,+}\), upregulated in ER+ breast cancer, colon cancer, pancreatic cancer, and cervical cancer (Gupta et al., 2005; Gupta et al., 2006; Karunakaran et al., 2008; Gutierrez et al., 2015). From the SLC7 family, only LAT1 has been linked to cancer (colon cancer, lung cancer, and melanoma in particular; Pochini et al., 2014; Wang and Holst, 2015). There is currently not much evidence for an increase in the expression of any of the SLC38 family transporters in cancer, although some are the targets of notorious oncogenes, and all have the potential to provide a significant route of entry of glutamine into cancer cells. For example, SNAT1 & SNAT2 have been shown to compensate for SLC1A5 deletion in osteosarcoma cells (Broer et al., 2019).

ASCT2 is a prominent example of a glutamine transporter that is upregulated in cancer. ASCT2 upregulation has been demonstrated in prostate cancer, breast cancer, gastric cancer, endometrial carcinoma, and colorectal cancer (Wang et al., 2014; van Geldermalsen et al., 2016; Lu et al., 2017; Marshall et al., 2017; Toda et al., 2017; van Geldermalsen et al., 2018;). Importantly, ASCT2 is highly expressed in human TNBC along with other glutaminolysis genes such as GLS and GS (van Geldermalsen et al., 2016). It is thought that upregulation of ASCT2 and LAT1 simultaneously could provide a cancer cell with the advantage of upregulated mTORC1 signalling, while also providing the cell with ample glutamine for anaplerosis into the TCA cycle, and nitrogen donation to produce other non-essential amino acids (NEAAs) and nucleic acids. Indeed, both ASCT2 and LAT1 are targets of MYC, and therefore represent potential therapeutic targets for cancers with high MYC expression (Hayashi et al., 2012; Gao et al., 2009).

1.7.5 Dysregulation of glutamine catabolism in breast cancer

GLS1 expression is higher in TNBC cell lines relative to their hormone receptor-positive counterparts (Wise et al., 2008). Furthermore, high GLS2 expression is associated with poor clinical outcome in breast cancer patients (Dias et al., 2019). However, while the prooncogenic activity of GLS1 has been widely studied, GLS2
has also been found to exhibit tumour suppressor activity in several types of cancer including glioblastoma and hepatocellular carcinoma (Suzuki et al., 2010; Liu et al., 2014; Szeliga et al., 2015). Recent work from our lab demonstrated that MYC-induced liver tumours in mice switch to Gls1 expression, with Gls2 re-expression emerging to compensate upon Gls1 deletion (Méndez-Lucas et al., 2020). Therefore, the effect of GLS1 inhibition specifically in MYC-high breast tumours, as well as the potential involvement of GLS2, remains to be determined.

Research has suggested that the amide nitrogen of glutamine is an important resource in cancer cells. The amidotransferases integrate glucose and glutamine metabolism and have been receiving increasing attention in recent years for their potential role in cancer. Phosphoribosylpyrophosphate amidotransferase (PPAT) expression is observed in lung adenocarcinoma, while GFAT expression has been correlated with poor clinical outcome for patients with TNBC (Goswami et al., 2015; Dong et al., 2016). Antisense GLS1 mRNA in MCF7 breast cancer cells led to a 50% reduction in proliferation and an 80% reduction in GFAT activity, suggesting that glutaminase can act as a cellular glutamine sensor (Donadio et al., 2008). MYC is known to upregulate the expression of enzymes involved in nucleotide biosynthesis, and recently work from our lab demonstrated that amidotransferase expression is upregulated in MYC-driven liver tumours in mice, and that they can compensate for the combined inhibition of GLS1 and GLS2 to fuel the TCA cycle (Liu et al., 2008; Méndez-Lucas et al., 2020).

ASNS has been shown to be a MYC transcriptional target (Demma et al., 2019). However, mammalian cells are unable to catabolise asparagine, which instead acts to induce the expression of GS for de novo synthesis of glutamine (Pavlova et al., 2018). Asparagine is essential to maintain protein translation in glutamine-deprived cells, and the addition of asparaginase can prevent cells from adapting to glutamine deprivation (Pavlova et al., 2018). Moreover, cancer cells have been shown to be completely rescued from glutamine deprivation by asparagine, but only minimally by aspartate, glutamate, and α-ketoglutarate (αKG) (Zhu et al., 2017). The role of the amidotransferases, including ASNS and those involved in nucleotide metabolism, in MYC-driven breast tumours is yet to be determined.
Chapter 1 Introduction

Transaminases have variously been shown to represent important nodes in the metabolic network of cancer cells and, as such, represent additional therapeutic targets. For example, Glutamic-oxaloacetic transaminase 1 & 2 (GOT1 & 2, cytosolic and mitochondrial, respectively) form part of the malate-aspartate shuttle, which has been shown to account for approximately 20% of the total respiratory rate in several types of tumours (Greenhouse and Lehninger, 1976). Furthermore, aspartate has been specifically shown to rescue breast cancer cells from cell death due to glutamine deprivation (Thornburg et al., 2009; Korangath et al., 2015).

GPT produces alanine and has been shown to have a role in mediating glutamine anaplerosis in a range of cancer cell lines (Vacanti et al., 2014; Yang et al., 2014; Smith et al., 2016). Furthermore, αKG production by GPT2 is thought to be a driving factor in extracellular matrix (ECM) production in metastatic breast cancer cells (Elia et al., 2019). PSAT1 has been shown to supply approximately 50% of glutamine derived αKG in breast cancer cells (Possemato et al., 2011), while PSAT1 overexpression is observed in tumours from breast cancer patients where it promotes cell proliferation, metastasis, and is associated with a poor clinical outcome (Gao et al., 2017). In addition to de novo synthesis, tumours are also able to import serine and glycine from the environment and withdrawal of both from the diets of mice has been shown to restrict tumour progression in mouse models of colorectal cancer and lymphoma (Maddocks et al., 2017). The requirement of dietary serine and glycine in a mouse model of breast cancer has not yet been evaluated.
1.8 Targeting glutamine transport and metabolism as an anti-cancer strategy

1.8.1 ASCT2 as a therapeutic target in cancer

Due to its upregulation in many types of cancer, ASCT2 is now regarded as an attractive therapeutic target (Liu et al., 2018). However, while the preferred substrate of ASCT2 is glutamine, it can transport other neutral amino acids. Therefore, targeting ASCT2 could have broader effects on tumour metabolism than those mediated by solely by glutamine deprivation. Serine, for example, which can be transported by ASCT2, is a crucial precursor of 1C metabolism, while cysteine is necessary to produce glutathione, contributing to normal cellular redox balance. ASCT2 is required for BLBC cell growth in vitro, but not luminal breast cancer cells, suggesting that its expression is subtype-dependent (van Geldermalsen et al., 2016).

There are many studies demonstrating that the knockdown or knockout of ASCT2 reduces glutamine flux into cancer cells both in vitro and in vivo, leading to growth inhibition of both cell lines and tumours (Hassanein et al., 2015; van Geldermalsen et al., 2016; Cormerais et al., 2018). However, some breast cancer cell lines with high ASCT2 expression do not respond to ASCT2 knockdown or knockout (van Geldermalsen et al., 2016; Bothwell et al., 2018; Comerais et al., 2018). One potential explanation for this is that some cancer cells express GS, reducing their dependency on exogenous glutamine uptake (Kung, Marks, and Chi, 2011).

Examples of ASCT2 inhibitors that have been used in cancer studies to date are benzylserine, L-$\gamma$-glutamyl-p-nitroanilide (GPNA) and V-9302. Benzylserine inhibits ASCT2 via a competitive mechanism and has been demonstrated to limit breast cancer cell growth and cell cycle progression (Grewer and Grabsch, 2004; van Geldermalsen et al., 2018). However, the actions of this inhibitor are not limited to ASCT2. Benzylserine also targets several of the other glutamine transporters concurrently, including ASCT1, LAT1, LAT2, SNAT1, and SNAT2 (Wang et al., 2014; van Geldermalsen et al., 2018). While pre-clinical studies have demonstrated that GPNA is effective at preventing glutamine uptake and has therapeutic effects in several cancer cell lines, like benzylserine it is also not a specific ASCT2 inhibitor as
it also targets LAT1, LAT2, SNAT1, and SNAT2 (Hassanein et al., 2015; van Geldermalsen et al., 2016; Marshall et al., 2017; Wang et al., 2015; Chiu et al., 2017; Broer et al., 2016). Finally, V-9302 was initially reported to be an ASCT2-specific inhibitor and was shown to significantly reduce cell viability in a variety of human cancer cell lines and tumours in vivo (Schulte et al., 2018). However, it was subsequently found that V-9302 does not target ASCT2, but in fact targets LAT1 and SNAT2, which underlies its biological effects (Broer et al., 2018). A specific ASCT2 inhibitor is yet to be identified.

1.8.2 GLS1 inhibition as an anti-cancer strategy

Exploiting high GLS1 expression as a method of treating MYC-driven tumours can be seen in several studies using inhibitors of GLS1. Bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulphide (BPTES) is an allosteric inhibitor of GLS1 and has no structural similarity to glutamine or glutamate, making off-target effects on glutamine metabolism unlikely (Robinson et al., 2007). CB-839 is like BPTES in its mechanism of GLS1 allosteric inactivation, but more potent with an IC$_{50}$ value at least thirteen times lower than BPTES (Gross et al., 2014).

CB-839 has been shown to be efficacious in multiple models of cancer both in vitro and in vivo, including breast, lung, liver, and lymphoma (Xiang et al., 2015; Boysen et al., 2019; Galan-Cobo et al., 2019; Jin et al., 2020). In breast cancer, CB-839 has demonstrated strong anti-proliferative activity against TNBC cell lines and was shown to inhibit tumour growth by 54% in mice bearing TNBC cell line tumour xenografts, with inhibition rising to 100% in combination with paclitaxel (Gross et al., 2014). It has recently been demonstrated that TNBC cell lines, especially those that are basal-like, are dependent on glutathione for survival by suppressing ROS (Beatty et al., 2018). The sensitivity of TNBC to GLS1 inhibition is concordant with these results and the works demonstrating the role of GLS1 and glutamine catabolism in protecting cells from oxidative stress (Son et al., 2013). CB-839 is currently being tested in multiple clinical trials for TNBC, both alone and in combination with the chemotherapeutic agent paclitaxel (clinicaltrials.gov, ongoing).
BPTES has been shown to increase survival in transgenic mice with MYC-driven liver tumours, while also being effective in preventing the MYC-dependent P493 human B-cell lymphoma cell line from proliferating both in vitro and in mouse tumour xenografts (Xiang et al., 2015). The in vivo effectiveness of GLS1 inhibition in MYC-driven breast tumours remains to be determined.

1.9 Targeting serine availability and the serine synthesis pathway as an anti-cancer strategy

The SSP comprises three enzymes that ultimately convert the glycolytic intermediate 3-phosphoglycerate (3-PG) to serine. The SSP is a ‘phosphorylated’ pathway and is widely considered to be the physiological route of serine biosynthesis (Ichihara and Greenberg, 1955). Expression of each SSP gene is upregulated by MYC and in response to nutrient deprivation (Sun et al., 2015). The pathway was first proposed in 1955, with the first research investigating its role in cancer in 1970 when the first enzyme of the pathway, phosphoglycerate dehydrogenase (PHGDH), was found to be higher in liver cancer cells relative to normal liver cells and that its expression in the cancer cells correlated with growth rate (Ichihara and Greenberg, 1955; Davis et al., 1970).

The less popularised ‘non-phosphorylated’ pathway is widely believed to synthesise serine exclusively in photosynthetic organisms, with the pathway only operating in reverse to catabolise serine in non-photosynthetic organisms (Suda, 1967; Cheung et al., 1969; Snell, 1984; Benstein et al., 2013; Ros, Munoz-Bertomeu, and Krueger, 2014; Fig. 1-6). However, it was shown in the 1950s that this non-phosphorylated route can produce serine in mammalian tissues (Sallach, 1956). The final enzyme in this non-phosphorylated pathway is serine-pyruvate aminotransferase (SPAT), analogous to PSAT1 of the phosphorylated pathway. Where PSAT1 utilises glutamate as the amino group donor, SPAT utilised alanine.
Serine biosynthesis is widely considered to be achieved only via the phosphorylated pathway, with the non-phosphorylated pathway operating exclusively toward serine catabolism. However, serine biosynthesis via this non-phosphorylated pathway is possible in mammals and has previously been demonstrated (Sallach, 1955).
In the last decade, there has been renewed interest in the SSP and its relevance to cancer following the discovery that there are PHGDH copy number gains in triple negative breast cancers, with cells remaining able to proliferate in the absence of extracellular serine (Locasale et al., 2011; Possemato et al., 2011). Furthermore, PHGDH knockdown has been shown to perturb tumour growth (Possemato et al., 2011; DeNicola et al., 2015). Small molecule inhibitors of PHGDH have demonstrated efficacy in breast cancer cells and tumours, while PSAT1 has also been revealed as a potential therapeutic target (Possemato et al., 2011; Mullarky et al., 2016; Pacold et al., 2016).

Surprisingly, it is also suggested that cancer cell proliferation relies on the SSP beyond its ability to produce serine, as intracellular serine concentration remains stable even upon PHGDH knockdown (Possemato et al., 2011; Chen et al., 2013). It has been suggested that this is due to a decrease in αKG availability, although this is disputed by other studies that observed no difference in αKG levels (Possemato et al., 2011; Locasale et al., 2011; Fan et al., 2015). Another suggestion is that intracellular ammonia levels are less likely to reach toxic levels because of transamination, although this is unlikely the case as there are other transaminases available that can produce the same outcome. Therefore, the requirement for an intact SSP in cancer cells can be due to one or more of many factors.

PSAT1 expression has been shown to be upregulated in human breast tumours and aminooxyacetate (AOA), a pan-transaminase inhibitor, has a pronounced anti-tumour effect on MYC-induced mammary gland tumours in mice (Korangath et al., 2015; Coloff et al., 2016). Therefore, evaluating the importance of specific transaminase enzymes such as PSAT1 in MYC-high breast tumours, and their potential to be effectively therapeutically targeted, could form the basis of new treatments in BLBC. This could be as a standalone treatment, or as part of a combination targeting one or more transaminases and GLS1 simultaneously. Similarly, if a particular NEAA is found to be critical to the metabolism of MYC-high tumours, alternative strategies such as nutrient deficient diets may synergise with small molecule inhibitors.
While serine can be synthesised *de novo*, cells can also take it up from the environment. It is important to note that studies investigating the requirement for extracellular serine in cancer must also consider glycine availability; serine can be synthesised from glycine using carbon units from the 1C pool (Fig. 1-5). Studies have shown removing serine and glycine reduces cancer cell proliferation and tumour xenograft growth, while also suggesting that the requirement for glycine increases upon serine depletion (Maddocks *et al.*, 2013; Labuschagne *et al.*, 2014). Cancer cells lacking exogenous serine but supplied with extra glycine fare worse than when both serine and glycine are depleted, while removal of serine can be rescued by adding formate to the culture media (Labuschagne *et al.*, 2014). This suggests that, upon serine depletion, the 1C pool preferentially diverts carbon units towards the synthesis of serine using glycine, to the detriment of other processes it governs such as nucleotide biosynthesis, glutathione production, and maintenance of the epigenome.

A major factor governing the requirement for serine in cancer cells is thought to be the maintenance of SAM levels, which provide carbon units for nucleotide synthesis and methylation reactions (Maddocks *et al.*, 2016). Furthermore, serine is required for the synthesis of phosphatidylserine and sphingolipids. Recent work has shown that lipid supplementation can partially rescue mitochondrial defects and cell proliferation observed in cancer cells following serine deprivation *in vitro* (Gao *et al.*, 2018). Dietary deficiency of serine and glycine has also been shown to synergise with the biguanide phenformin to inhibit tumour growth *in vivo* (Gravel *et al.*, 2014). This suggests that the role of serine in cancer extends beyond its ability to provide carbon units for nucleotide biosynthesis to supporting mitochondrial function via ceramide synthesis.

### 1.10 Stable isotope-assisted metabolomics for investigating tumour metabolism

Mass spectrometry has proven to be an exquisitely sensitive method for detecting relative metabolite levels between tissues and within tissues in different conditions, although on its own is insufficient to provide information regarding metabolic fluxes.
Chapter 1 Introduction

The rate at which a particular nutrient is transported across cell membranes, enzymatically synthesised, or consumed, cannot be inferred simply by observing total metabolite levels. Rather, stable isotopes of the constituent atoms of a particular nutrient are used to chemically generate a ‘labelled’ compound. For example, a glucose molecule in which the $^{12}$C atoms are replaced by $^{13}$C will alter the mass of the compound but, crucially, not its biochemical behaviour. By providing the cell or tissue of interest with the labelled compound, mass spectrometric measurement of the labelling patterns of metabolites provides information as to the activities of different metabolic pathways. By estimating metabolic fluxes from labelled precursors to their downstream products, a broader picture of the metabolic network of a particular specimen can be drawn. This method is known as stable isotope-assisted metabolomics (SIAM), and in the last decade has become widespread for studying metabolic phenotypes in cancer.

The application of SIAM has allowed researchers to interrogate multitudinous biochemical pathways, revealing metabolic network information at an unprecedented resolution. SIAM has also allowed known metabolic phenomena underlying the pathogenesis of cancer to be investigated in greater detail, such as the Warburg effect and glutamine addiction (Rodrigues et al., 2014; Wise et al., 2008). Furthermore, the metabolic consequences of perturbing certain pathways in cancer cells and tumours can be studied using this approach, which in turn informs the discovery of novel therapeutic targets (Sellers et al., 2015; Méndez-Lucas et al., 2020). However, there are important factors to consider when interpreting data from SIAM. Firstly, currently existing technologies only allow for total cellular levels of metabolites to be measured, meaning that higher resolution information regarding the specific levels within subcellular compartments cannot be distinguished. It is also important to carefully select which version of a labelled compound to use. If total carbon flux to the TCA cycle from glucose is to be measured, fully labelled glucose ([$U^{13}$C]-glucose) should be used, but not a positionally labelled variant as this would bias the results towards flux through a specific pathway that preferentially utilises that section of the molecule. For example, in cancer cells the pentose phosphate pathway is typically upregulated to grant increased nucleotide production, but intermediates in the pathway can also be shunted back into glycolysis. Therefore, if one were to use [$^{13}$C]-glucose as a label, a significant portion of the $^{13}$C atoms will
be lost as CO\textsubscript{2} before reaching the TCA. Nevertheless, using multiple, differentially labelled compounds can provide useful information, such as the relative contributions of disparate pathways toward the production of a certain metabolite. Naturally, this is only possible for metabolites that can be produced via multiple pathways.

An important factor to consider in SIAM is the natural abundance (NA) of stable isotopes. For example, \textsuperscript{13}C and \textsuperscript{15}N are present in the environment at 1.07\% and 0.364\%, respectively (Haynes, 2017). Not only can the NAs of isotopes being used in the experiment confound results, but the NAs of others can also. A glutamine molecule that is detected as M+1 may also be the result of \textsuperscript{17}O, which has a NA of 0.038\% (Haynes, 2017). Therefore, the proportion of metabolites detected at >M+0 that are naturally occurring must be stripped from the labelling data. While this would ideally be performed by correcting each individual metabolite using labelled versus unlabelled standards, this is too expensive and time consuming to be performed at the regularity required by such an approach. Therefore, we have chosen to apply correction factors to our measured metabolites computationally, based on the theoretical abundance of naturally occurring isotopes (see Materials and methods).

1.11 Thesis aim

The primary aim of this thesis is to evaluate the requirement of \textit{Gls1} and supported glutamine metabolism for the initiation and progression of MYC-driven mammary gland tumours in mice, using ERBB2-driven mammary gland tumours as a comparison. By comparing the metabolic profiles of cancer cells and tumours upon inhibition of GLS1, PSAT1, and ASCT2, and following the removal of specific nutrients from the diet, novel therapeutic strategies may be identified specifically for the treatment of MYC-high breast tumours in humans.

1.12 Thesis objectives

- Evaluate the requirement of \textit{Gls1} in MYC-driven and ERBB2-driven mammary gland tumours
- Evaluate the therapeutic potential of inhibiting the glutamine transporter ASCT2 in MYC-driven mammary gland tumours
• Evaluate the role of PSAT1 and serine/glycine metabolism in MYC-driven and ERBB2-driven mammary gland tumours
Chapter 2. Materials and methods

2.1 Reagents and chemicals

2.1.1 Animals

All procedures in mice were performed according to the Use of Animals in Research Policy (The Francis Crick Institute).

Figure 2-1 Mechanism of Cre-LoxP-mediated gene deletion in mouse models

To test the biological consequences of Gls1 deletion in MYC-driven and ERBB2-driven mammary gland tumours, transgenic mouse models were used. Control groups ectopically expressed either MYC or ERBB2, and Cre recombinase (Cre) specifically in the mammary gland via the murine mammary tumour virus (MMTV) promoter. eYFP was additionally inserted into the Rosa26 locus with a stop codon preceding it, which is flanked by LoxP sites (floxed). The mice developed spontaneous, ERBB2- or MYC-driven mammary gland tumours. Expression of eYFP was used as a marker of Cre activity (Srinivas et al., 2001). The experimental groups additionally carried either floxed Gls1 or floxed Psat1 alleles, resulting in mammary gland-specific Gls1 or Psat1 deletion.
All mice used are on the FVB/NJ background (internal stock, The Francis Crick Institute), and produced by crossing the following lines:

Tg(MMTV-Myc)141-3Led (The Jackson Laboratory, Bay Harbor, Maine, United States. MGI ID: 2447500)

Tg(MMTV-cre)4Mam (The Jackson Laboratory, Bay Harbor, Maine, United States. MGI ID: 2176166)

Tg(MMTV-Erbb2*,-cre)1Mul (imported from McGill University, Toronto, Canada. MGI ID: 4361017)

Gt(ROSA)26Sor\textsuperscript{tm1(EYFP)Cos} (The Jackson Laboratory, Bay Harbor, Maine, United States. MGI ID: 2176166)

\textit{Gls}\textsuperscript{tm2.1Sray/J} (imported from The University of California San Francisco, United States. MGI ID: 5430235)

\textit{Psat1}\textsuperscript{tm1a(KOMP)Wtsi} (imported from The University of California San Francisco, United States. MGI ID: 4363603)

From the age of approximately four months, mice were monitored for tumour growth with the help of the Biological Research Facility at the Francis Crick Institute. Upon detection of a tumour by palpation, the growth of the tumour was monitored until the experimental endpoint, which was when tumours reached dimensions of 10x10mm. After this point the mice were culled; this marks the humane endpoint for all tumour latency data. All mice used for this project were humanely killed by dislocation of the neck followed by confirmation of permanent cessation of the circulation.
2.1.2 Antibodies

The following antibodies were used:

Table 2-1 Antibodies

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<th>Western blot</th>
<th>Immunohistochemistry</th>
<th>Immunofluorescence</th>
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<tr>
<td>Actin</td>
<td>Sigma (A5228)</td>
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<td>ASCT2</td>
<td>Santa Cruz (sc-99002)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC2</td>
<td>Cell Signalling Technologies (9661)</td>
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<td>ERRB2</td>
<td>Abcam (ab26901)</td>
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2.1.3 Buffers and media

Buffers were prepared as follows:

**Table 2-2 DMEM MMEC media**

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<th>DMEM MMEC Media</th>
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<td>(+) 5g/L D-Glucose, [-] L-Glutamine, [-] Sodium Pyruvate</td>
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<tr>
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<tr>
<td>Penicillin/Streptomycin (10mg/ml)</td>
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</tr>
<tr>
<td>HEPES (1M)</td>
<td>Gibco (15630-056)</td>
<td>5ml</td>
<td>10mM</td>
</tr>
<tr>
<td>L-Glutamine (200mM)</td>
<td>Gibco (25030-024)</td>
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<td>2mM</td>
</tr>
<tr>
<td>Insulin (5mg/ml)</td>
<td>Sigma (I1882)</td>
<td>500ul</td>
<td>5ug/ml</td>
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<tr>
<td>Hydrocortisone (1mg/ml)</td>
<td>Sigma (H8888)</td>
<td>500ul</td>
<td>1ug/ml</td>
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<tr>
<td>Mouse epidermal growth factor (100µg/ml)</td>
<td>Gibco (PMG8043)</td>
<td>50ul</td>
<td>10ng/ml</td>
</tr>
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**Table 2-3 DMEM/F-12 MMEC media**

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**Table 2-4 Collagenase buffer**

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</tr>
</thead>
<tbody>
<tr>
<td>Gibco RPMI Medium 1640 (1X): [+]- L-Glutamine</td>
<td>Gibco (11875093)</td>
<td>500ml</td>
<td>-</td>
</tr>
<tr>
<td>Fetal bovine serum</td>
<td>Gibco (10270)</td>
<td>11.25ml</td>
<td>2.5%</td>
</tr>
<tr>
<td>HEPES (1M)</td>
<td>Gibco (15630-056)</td>
<td>5ml</td>
<td>10mM</td>
</tr>
</tbody>
</table>

**Table 2-5 10X Protein running buffer**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>10X Protein running buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trizma Base</td>
<td>25mM</td>
</tr>
<tr>
<td>Glycine</td>
<td>192mM</td>
</tr>
<tr>
<td>SDS</td>
<td>0.10%</td>
</tr>
</tbody>
</table>

10X protein running buffer was prepared by the media preparation team (Francis Crick Institute)

2.1.4 Cell lines

MMTV-MYC tumour-derived (MT) cell lines were produced as described in 2.2.4.1.

2.1.5 Cloning

The Gateway cloning system (Invitrogen) was used to produce pCW57.1-Cre-puro according to Gateway Cloning Protocols (Thermo scientific, 2019).
2.1.5.1 Plasmids

The following plasmids were used:

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Distributor</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAG-GFP-IRES-CRE</td>
<td>addgene (#48201)</td>
</tr>
<tr>
<td>pDONR221</td>
<td>Invitrogen (12536017)</td>
</tr>
<tr>
<td>pCW57.1</td>
<td>addgene (#41393)</td>
</tr>
</tbody>
</table>

2.1.5.2 Primers

The following primers were designed to amplify CRE with added attB sites from CAG-GFP-IRES-CRE:

Primer 1: GGGGACAAGTTTGATACCAAAAAAAGCAAGCTTAATGTCCAATTACTGACCGTACACCA
Primer 2: GGGGACCACCTTTGTACAAAGAAAGCTGGGTTTTAATCCCCATCTTCCAGCAGGC

2.1.6 Drugs

The following drugs were used:

<table>
<thead>
<tr>
<th>Drug</th>
<th>Distributor</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPNA (L-Glutamic acid γ(p-nitroanilide) hydrochloride)</td>
<td>Sigma (G6133)</td>
</tr>
<tr>
<td>CB-839</td>
<td>Cambridge Bioscience (CAY22038)</td>
</tr>
</tbody>
</table>

2.1.7 Enzymes

The following enzymes were used:

<table>
<thead>
<tr>
<th>Table 2-6 Enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme</td>
</tr>
<tr>
<td>Collagenase from Clostridium histolyticum, Type L</td>
</tr>
<tr>
<td>Gateway BP Clonase II Enzyme mix</td>
</tr>
<tr>
<td>Gateway LR Clonase II Enzyme mix</td>
</tr>
</tbody>
</table>
2.1.8 Miscellaneous

The following reagents were used:

**Table 2-7 Reagents**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Distributor</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Mercaptoethanol</td>
<td>Bio-Rad (1610710)</td>
</tr>
<tr>
<td>40% Acrylamide/Bis Solution 37.5:1</td>
<td>Bio-Rad (1610148)</td>
</tr>
<tr>
<td>4X Laemmli Sample Buffer</td>
<td>Bio-Rad (1610747)</td>
</tr>
<tr>
<td>Acetonitrile, 99.9%, for HPLC</td>
<td>Acros Organics (32573)</td>
</tr>
<tr>
<td>Ammonium Persulfate</td>
<td>Sigma (A3676)</td>
</tr>
<tr>
<td>Bovine Serum Albumin</td>
<td>Sigma (A2058)</td>
</tr>
<tr>
<td>Ethanol Absolute 99.8-99.9%</td>
<td>Thermo (10437341)</td>
</tr>
<tr>
<td>High-Capacity cDNA Reverse Transcriptase Kit</td>
<td>Thermo (4368814)</td>
</tr>
<tr>
<td>L-Valine-14C,15N</td>
<td>Sigma (600148)</td>
</tr>
<tr>
<td>Methanol Chromasolv LC-MS</td>
<td>Honeywell (34966)</td>
</tr>
<tr>
<td>Matrigel Growth Factor Reduced (GFR) Basement Membrane Matrix</td>
<td>Corning (356230)</td>
</tr>
<tr>
<td>Pierce BCA Protein Assay Kit</td>
<td>Thermo (23227)</td>
</tr>
<tr>
<td>Ponceau S Solution</td>
<td>Sigma (P7170)</td>
</tr>
<tr>
<td>Precision Plus Protein Dual Color Standards</td>
<td>Bio-Rad (1610374)</td>
</tr>
<tr>
<td>RNasey Mini Kit</td>
<td>QIAGEN (74104)</td>
</tr>
<tr>
<td>SDS (Sodium lauryl sulfate)</td>
<td>Sigma (1614363)</td>
</tr>
<tr>
<td>Skim Milk Powder</td>
<td>Sigma (70166)</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>Sigma (S2002)</td>
</tr>
<tr>
<td>TaqMan Gene Expression Master Mix</td>
<td>Thermo (4369016)</td>
</tr>
<tr>
<td>TEMED (N,N',N''-Tetramethylethlenediamine)</td>
<td>Sigma (T9281)</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>Sigma (T8787)</td>
</tr>
<tr>
<td>Tween 20 (Polysorbate)</td>
<td>VWR Chemicals (663684B)</td>
</tr>
<tr>
<td>VECTASHIELD Antifade Mounting Medium with DAPI</td>
<td>Vector Laboratories (H-1200)</td>
</tr>
</tbody>
</table>

2.1.9 SDS-PAGE gels

Gels were prepared as follows:

**Table 2-8 12% running gel**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>12% separating gel</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O</td>
<td>17.58 ml</td>
<td></td>
</tr>
<tr>
<td>Acrylamide/Bis (40%)</td>
<td>12 ml</td>
<td></td>
</tr>
<tr>
<td>1.5M Tris pH 8.8, SDS (0.4%)</td>
<td>10 ml</td>
<td></td>
</tr>
<tr>
<td>TEMED</td>
<td>20 ul</td>
<td></td>
</tr>
<tr>
<td>APS (10%)</td>
<td>400 ul</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>40 ml</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2-9 8% running gel**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>8% separating gel</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O</td>
<td>21.58 ml</td>
<td></td>
</tr>
<tr>
<td>Acrylamide/Bis (40%)</td>
<td>8 ml</td>
<td></td>
</tr>
<tr>
<td>1.5M Tris pH 8.8, SDS (0.4%)</td>
<td>10 ml</td>
<td></td>
</tr>
<tr>
<td>TEMED</td>
<td>20 ul</td>
<td></td>
</tr>
<tr>
<td>APS (10%)</td>
<td>400 ul</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>40 ml</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 2 Materials and Methods

Table 2-10 4% stacking gel

<table>
<thead>
<tr>
<th>Reagent</th>
<th>4% stacking gel</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td></td>
<td>9.774 ml</td>
</tr>
<tr>
<td>Acrylamide/Bis (40%)</td>
<td></td>
<td>2 ml</td>
</tr>
<tr>
<td>1.5M Tris pH 8.8, SDS (0.4%)</td>
<td></td>
<td>4 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td></td>
<td>16 ul</td>
</tr>
<tr>
<td>APS (10%)</td>
<td></td>
<td>160 ul</td>
</tr>
<tr>
<td>Bromophenol Blue (1%)</td>
<td></td>
<td>50 ul</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>16 ml</td>
</tr>
</tbody>
</table>

2.1.10 Stable isotopes

The following stable isotopes were used:

Table 2-11 Stable isotopes

<table>
<thead>
<tr>
<th>Labelled compound</th>
<th>Distributor</th>
</tr>
</thead>
<tbody>
<tr>
<td>[U¹³C₆]-glutamine</td>
<td>Cambridge Isotope Laboratories (184161-19-1)</td>
</tr>
<tr>
<td>[U¹³C₆]-glucose</td>
<td>Cambridge Isotope Laboratories (110187-42-3)</td>
</tr>
<tr>
<td>[α²⁰N]-glutamine</td>
<td>Cambridge Isotope Laboratories (80143-53-3)</td>
</tr>
</tbody>
</table>

2.1.11 Synthetic diets

Diets used for dietary deprivation studies were purchased from TestDiet (St. Louis, Missouri, US).

For dietary glutamine restriction (-Q) the following diets were used:
Control: Control Amino Acid Diet (1.6% GLN) – Yellow (5BYA; #1817157-203)
-Q: TestDiet GLN Deficient AA Diet – Orange (5BYB; #1817158-203)

For dietary serine and glycine restriction (-SG) the following diets were used:
Control: Baker Amino Acid Diet (5CC7; #1812426)
-SG: Baker Amino Acid Diet w/ No Added Gly or Ser (5W53; #1815774-203)

Mice were weaned onto diets over eight days as follows: (1) 50% normal chow plus 50% synthetic diet for four days; (2) 75% synthetic diet plus 25% percent normal chow for four days; (3) 100% synthetic diet.
2.1.12 TaqMan probes

The following TaqMan probes were used:

**Table 2-12 TaqMan probes**

<table>
<thead>
<tr>
<th>Probe</th>
<th>Distributor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gls1</td>
<td>Thermo (4351372)</td>
</tr>
<tr>
<td>Gls2</td>
<td>Thermo (4351372)</td>
</tr>
<tr>
<td>Rn18s</td>
<td>Thermo (4331182)</td>
</tr>
</tbody>
</table>

2.2 Experimental methods

2.2.1 Graph and figure generation

Unless otherwise indicated, all graphs were generated using Prism 7 for Mac OS X (GraphPad Software, Inc., La Jolla California, USA) and all figures were prepared using Adobe Illustrator CS5 (Adobe Systems Incorporated, 2011).

2.2.2 Histology

Tumours were fixed in 4% PFA for 24 hours then transferred to 70% ethanol for storage before histological preparation. Samples were paraffin-embedded and cut at 5μM by the Experimental Histopathology (EHP) team at the Francis Crick Institute. All haematoxylin and eosin staining was performed using a Tissue-Tek Prisma Plus & Film Automated Slide Stainer & Coverslipper (Sakura Finetek Europe B.V., Flemingweg, Alphen aan den Rijn, the Netherlands), with analysis and arbitrary grading by Professor Gordon Stamp (g.stamp@ahlab.co.uk). All immunohistochemistry was performed using a BenchMark ULTRA Automated IHC/ISH slide staining system (Roche, Grenzacherstrasse, Basel, Switzerland); Slides were scanned using a Zeiss Axio ScanZ1. Image analysis was performed using FIJI Is Just ImageJ (FIJI; Schindelin et al., 2012).

2.2.3 Immunofluorescence

Immunofluorescence staining was performed by Shravan Venkateswaran. Cells were plated in standard 12-well culture plates with inserted 19mm coverslips (VWR chemicals) at 5000 cells/well. Cells were fixed using 4% PFA at 37°C for 7 minutes then washed with PBS. Cells were permeabilised with 0.5% Triton X-100 for 10
minutes at 37°C then washed with 0.15% Triton X-100 (in PBS) at RT. Cells were blocked with 10% normal goat serum (SouthernBiotech) in 0.15% Triton X-100 for one hour. Primary antibodies were diluted in blocking buffer and cells were incubated overnight at 4°C. After 3x 5-minute washes with 0.15% Triton X-100 secondary antibodies were diluted in blocking buffer and cells were incubated for one hour at RT. Coverslips were washed 3 times with 0.15% Triton X-100 and mounted on SuperFrost slides (Thermo) using VECTASHIELD.

2.2.4 In vitro drug studies

2.2.4.1 Tumour-derived cell line production

Dissected tumours were transported in PBS followed by chopping with a razor blade for 2 minutes to disrupt the tissue. The chopped tumour was then placed in 10ml of 1mg/ml collagenase from Clostridium Histolyticum in collagenase buffer and then shaken for 30 minutes (37°C, 200RPM). Cells were spun by centrifugation at 530xG for 5 minutes and supernatant discarded. 5ml red blood cell lysis buffer was added to the pellet and vortexed for 6 seconds, followed by 1 minute incubation at room temperature (RT). Cells were spun again by centrifugation at 530xG for 5 minutes. The supernatant was discarded and 10ml PBS added vortexed for 6 seconds. Cells were spun again by centrifugation at 530xG for 5 minutes. Cells were passed through a 40μm cell strainer followed by centrifugation at 530xG for 5 minutes. Cells were plated and propagated in DMEM/F-12 MMEC media. For long term storage, cells were frozen in FBS (10% DMSO).

2.2.4.2 Drug treatments

10,000 cells per well were seeded in standard 96-well culture plates and left overnight to attach.

GPNA was prepared as follows: 5M GPNA was prepared in 20ul DMSO with 1 hour incubation at 65°C, then added to 20ml 65°C MMEC media for a 5mM solution. A serial dilution was then performed for final concentrations of 2mM, 1mM, 0.5mM, 0.2mM, 0.1mM, 0.05mM.
CB-839 was prepared as follows: 10uM CB-839 was prepared in MMEC media. A serial dilution was then performed for final concentrations of 1uM, 100nM, 10nM, 1nM 100pM, 10pM.

Media was aspirated from wells and MMEC media containing drugs added. Cell proliferation over time was measured using an Incucyte Live-Cell Analysis System.

Drug synergy calculations were performed using SynergyFinder.

2.2.5 Metabolomics

Stable isotopes were dissolved in saline. Following the completion of bolus and infusion experiments, blood samples were collected by cardiac puncture and allowed to coagulate at 4°C for 15 minutes, after which they were centrifuged at 2000xg for 10 minutes. To rapidly quench metabolism, dissected tissues and sera were snap-frozen in liquid nitrogen.

2.2.5.1 Boluses

The [U\textsuperscript{13}C\textsubscript{6}]-glucose boluses was given at 0.57mg per body weight in grams in 100ul of saline. Mice were terminally anaesthetised with isoflurane (Zeotis) and culled following a 15-minute incubation period and tissues collected.

2.2.5.2 Infusions

Mice were terminally anaesthetised with isoflurane (Zeotis) and given intravenous boluses of stable isotope labelled substrates via the tail vein before infusion.

[U\textsuperscript{13}C\textsubscript{6}]-glucose initial boluses were 0.4mg per body weight in grams, followed by infusion for 3 hours at 0.15ml per hour (0.012mg/g/min) using an Aladdin SyringeONE Programmable Syringe Pump (AL-100; World Precision Instruments).

[U\textsuperscript{13}C\textsubscript{5}]-glutamine and [\alpha\textsuperscript{15}N]-glutamine initial boluses were 0.187mg per body weight in grams, followed by infusion for 3 hours at 0.15ml per hour (0.005mg/g/min) using an AL-100 pump.
2.2.5.3 Tissue preparation and pulverisation

Tissues were dissected from animals and immediately flash-frozen using tissue clamps cooled in liquid nitrogen. Frozen tissues were then pulverised using a mortar and pestle submerged in liquid nitrogen.

2.2.5.4 Metabolite extraction

Metabolite extraction buffer (MEB) was prepared according to Kerr et al. (2016). 2ml tubes were placed in a cooling rack on ice and, on a sample-by-sample basis, approximately 4mg pulverised tissue powder was added, followed by 500μl MEB containing 10μM [U^{13}C,^{15}N]-valine as an internal standard. Samples were immediately vortexed for 30 seconds, then sonicated for 24 minutes in a sonicating water bath at 4°C. Samples were then centrifuged at 13,000RPM for 20 minutes, after which supernatant was transferred to a new 2ml tube and kept on ice. The tissue pellets were then re-extracted using 500ul MEB without internal standard. Supernatants were pooled with those from the first extraction step and stored at -80°C until analysis.

2.2.5.5 Protein quantification

Lysates were prepared from the protein pellets remaining from metabolite extraction as follows: pellets were sonicated for 24 minutes in a sonicating water bath after the addition of lysis buffer, vortexed for 10 seconds, and then placed in a 100°C heating block for 10 minutes before centrifugation at 20,000xG. Protein content of lysates were quantified according to Pierce BCA Protein Assay Kit – Microplate Procedure (Thermo scientific, 2015). Protein concentrations were measured using an Infinite M1000 PRO Multimode Microplate Reader (Tecan Group Ltd., Seestrasse, Männedorf, Switzerland).

2.2.5.6 Liquid chromatography/mass spectrometry

Samples were run with the help of the Metabolomics STP at the Francis Crick Institute using a Q Exactive Hybrid Quadrupole- Orbitrap Mass Spectrometer (Thermo Scientific). Data analysis was performed using Xcalibur version 4.1.31.9 and TraceFinder 4.1 – EFS (Thermo Scientific).
2.2.6 Natural abundance correction

Natural abundance correction was performed using MSIso, developed for use in our laboratory by Avinash Ghanate.

2.2.7 PCR genotyping

PCR genotyping for MMTV-ERBB2-\textit{Gls1}^{KO} tumours was performed by Wei Lin as previously described in Mingote \textit{et al.} (2015). The following primers were used:

Primer A: GG CCT GCTTA ATGG TTT CCTG

Primer C: GGCATATCCCTGAGTT CGAG

Primer X: GCCCCAAGCATC CCTCATC TCGAATA

\textbf{Figure 2-2 PCR genotyping strategy for MMTV-ERBB2-\textit{Gls1}^{KO} tumours}

\textbf{a}, Wild-type (WT) mice should only exhibit a 341bp amplicon using primers A and C, corresponding to a section of intron 1 of \textit{Gls1}. \textbf{b}, MMTV-ERBB2-\textit{Gls1}^{KO} mice should exhibit both a 430bp amplicon using primers A and C, corresponding to the same sequence of intron 1 including the 3’ LoxP site (present in extratumoural tissue extracted alongside the tumour), and a 385bp amplicon corresponding to part of the 5’ untranslated region (UTR), the recombined 5’ and 3’ LoxP sites, and part of intron 1 (demonstrating \textit{Gls1} deletion in tumoural tissue).
2.2.8 Quantitative polymerase chain reaction (qPCR)

qPCR was performed according to TaqMan Gene Expression Assays Protocol (Applied Biosystems, 2010) using an Applied Biosystems ViiA 7 Real-Time PCR system.

2.2.8.1 RNA isolation


2.2.8.2 cDNA synthesis


2.2.9 RNA sequencing (RNA-seq)

RNA was isolated as in 1.2.8.2. RNA-seq was performed by the Advanced Sequencing Facility at the Francis Crick Institute. Data analysis was performed by Probir Chakravarty.

2.2.10 SDS-PAGE/Western blot

2.2.10.1 Protein quantification

Lysates were produced by boiling pulverised tissue powder for 10 minutes in lysis buffer (62.5mM Tris, pH 6.8, 2% SDS), homogenised by pulling through a 30G syringe, centrifuged at 20,000xG for 30 minutes, and the supernatant collected. Protein content of lysates were quantified according to Pierce BCA Protein Assay Kit – Microplate Procedure (Thermo scientific, 2015). Protein concentrations were measured using an Infinite M1000 PRO Multimode Microplate Reader (Tecan Group Ltd., Seestrasse, Männedorf, Switzerland).
2.2.10.2 SDS-PAGE

20μg sample protein and 5μl of protein standard were loaded into wells of SDS-PAGE gels. Gels were run in 1X Protein running buffer at 100V until the protein standard reached the bottom of the gel.

2.2.10.3 Protein transfer and staining

According to General western blot protocol (abcam, 2018). The following modifications were made: after protein was transferred to the membrane, Ponceau solution was used to check the transfer quality; 5% milk (in phosphate-buffered saline 0.05% Tween 20 – PBST) was used to block the membrane; primary antibodies were diluted in blocking buffer (1% BSA, 0.05% sodium azide in PBST); secondary antibodies were diluted in 5% milk; a luminol-based detection system was used (Amersham ECL Prime Western Blotting Detection Reagent) according to Amersham ECL Prime Western Blotting Detection Reagent Product booklet – 7.5 Detection (GE Healthcare, Little Chalfont, Buckinghamshire, UK); exposure was performed using X-ray film (mol7016; SLS, Ruddington Lane, Wilford, Nottingham, UK) and a Curix 60 Processor (Agfa-Gevaert, Septestraat, Mortsel, Belgium); processed films were scanned and edited in Adobe Photoshop CS5 Extended and Adobe Illustrator CS5 (Adobe Systems Incorporated, 2011).

2.2.11 Sequencing

Sequencing was performed by Source BioScience.
Chapter 3.  *Gls1* deletion in the mammary gland restricts MYC-driven tumour growth

3.1 Introduction

It is well established that increased MYC activity upregulates glutamine metabolism (Wise *et al.*, 2008; Gao *et al.*, 2009; Yuneva *et al.*, 2012; Méndez-Lucas *et al.*, 2020). Furthermore, GLS1 inhibitors have demonstrated that glutamine addiction can be exploited therapeutically (Yuneva *et al.*, 2012; Gross *et al.*, 2014; Xiang *et al.*, 2015; Méndez-Lucas *et al.*, 2020). The GLS1 inhibitor CB-839 produced a significant anti-tumour response in both TNBC and BLBC patient-derived xenograft (PDX) mouse models (Gross *et al.*, 2014). Although BLBCs are characterised by high MYC expression (Alles *et al.*, 2009), the direct relationship between GLS1 and high MYC activity in breast tumours remains to be determined.

In this project, we aimed to evaluate whether *Gls1* is required for the initiation and development of MYC-driven mammary gland tumours in mice. To delete *Gls1* during MYC-induced tumour initiation in the mammary gland we used Cre-LoxP recombination. We crossed *Gls1*fl/fl mice (described in Mingote *et al.*, 2015) with the MMTV-MYC-Cre mouse line, which ectopically expresses murine MYC and Cre recombinase under the control of the mouse mammary tumour virus (MMTV) promoter. The MMTV promoter is stronger and more frequently used than other mammary gland-specific promoters described in the literature, due to the presence of hormone response elements (HREs) that increase its activity from puberty onwards in mice (Stewart, Pattenhale, and Leder, 1984; Ham *et al.*, 1988; Wagner *et al.*, 1997; Hennighausen, 2000). The excision of exon 1 of *Gls1* specifically in the mammary gland resulted in the formation of *Gls1* knockout (*Gls1KO*) MYC-driven mammary gland tumours (MMTV-MYC *Gls1KO*). MMTV-MYC-Cre mice with wild-type *Gls1* alleles were used as controls (MMTV-MYC).

To investigate the metabolic consequences of removing GLS1 activity in MMTV-MYC tumours, we employed SIAM. SIAM is a powerful technique that is crucial in determining changes in the activity of metabolic pathways, as it allows one to trace...
the fate of individual atoms from metabolic precursors to their products (Fan et al., 2012). By combining stable isotope labelling with LCMS, we probed the metabolic changes that occurred in tumours following Gls1 deletion. Glucose and glutamine metabolism are inextricably linked, not only regarding energy generation but also the production of biosynthetic precursors that are critical for the development of a proliferating cell. Therefore, we performed three separate infusion experiments using [U\textsuperscript{13}C]-glutamine, [α\textsuperscript{15}N]-glutamine, and [U\textsuperscript{13}C]-glucose to investigate the effect of Gls1 deletion on central carbon metabolism, glutamine-dependent, and glucose-dependent metabolic pathways.
3.2 *Gls1* deletion increases MYC-induced mammary gland tumour latency

Previous work from our lab has compared the metabolic profiles of MMTV-MYC tumours and ERBB2-driven mammary gland tumours (MMTV-ERBB2), induced by ectopic expression of the respective oncogenes under the control of the MMTV promoter. It demonstrated that glutamine catabolism is upregulated in MMTV-MYC tumours when compared to the normal mammary gland and MMTV-ERBB2 tumours (Still, 2018). Consistently, GLS1 is expressed at higher levels in MMTV-MYC tumours compared to MMTV-ERBB2 tumours and the normal mammary gland, while several of the transaminase enzymes, including PSAT1, are upregulated in both types of tumour (Fig. 3-1a). The expression of GLS2 was also significantly increased in MMTV-MYC tumours, indicating that this glutaminase isoform may also play a significant role in the initiation and progression of MYC-driven breast tumours (Fig. 3-1a).

Given the previously established role of GLS1 in MYC-induced tumourigenesis (Yuneva et al., 2012; Gross et al., 2014; Xiang et al., 2015; Méndez-Lucas et al., 2020), we first evaluated its role in the initiation of MYC-induced mammary gland tumours using Cre-LoxP recombination. Our results demonstrated that *Gls1* was efficiently deleted in MMTV-MYC *Gls1*KO tumours (Fig. 3-1b&c). The published work from our group evaluating the role of GLS1 in MYC-induced liver tumours demonstrated that GLS2 expression can compensate for *Gls1* deletion (Méndez-Lucas et al., 2020). My results presented herein show that although GLS2 expression is higher in MMTV-MYC tumours than in normal mammary gland, it is quite heterogeneous and significantly lower compared to normal mouse liver (Fig. 3-1a&b). Importantly, in contrast to liver tumours, *Gls1* deletion in MMTV-MYC tumours further decreases GLS2 protein expression, although mRNA levels are unaffected (Fig. 3-1b&c). These results build on previous observations that metabolic phenotypes in tumours are specific to tissue of origin (Yuneva et al., 2012) and suggest that, even though GLS2 expression is increased in MMTV-MYC tumours in comparison to normal mammary gland, it may not be essential for their formation and progression. Furthermore, GLS2 has been suggested to have tumour suppressor activity, with its upregulation having been associated with an antiproliferative response in
glioblastoma cells (López de la Oliva et al., 2020). Therefore, decreased GLS2 expression in MMTV-MYC $Gls1^{KO}$ tumours may be providing a selective survival advantage. We were also able to delete $Gls1$ in the same manner in MMTV-ERBB2 mice (MMTV-ERBB2 tumours $Gls1^{KO}$), although Western blot detected truncated protein product, so $Gls1$ deletion was confirmed by PCR genotyping (Fig. 3-1d; see Materials and methods Fig. 2-2).

Both control and $Gls1^{KO}$ MMTV-MYC and MMTV-ERBB2 tumours were collected when they reached 10mm in diameter. Our results demonstrated that $Gls1$ deletion in MMTV-ERBB2 tumours did not affect tumour latency (Fig. 3-1e). However, a statistically significant increase in tumour latency was observed in MMTV-MYC tumours, with median survival increasing from 237 days to 284 days (Fig. 3-1h). Our analysis also demonstrated that this was a result of decreased growth rate, rather than delayed tumour initiation (Fig. 3-1f, g, i & j).
Figure 3-1 MMTV-MYC tumour latency is increased following Glis1 deletion

a. Western blot comparing GLS1, GLS2, PSAT1, GOT1, GOT2, GPT2, ERBB2, and MYC expression between normal mammary glands, MMTV-ERBB2 (Cre-) tumours, and MMTV-MYC (Cre-) tumours from NIMR mice. The PSAT1 Western blot was
performed by Emma Still (Emma Still, 2018). b, Western blot comparing GLS1 and GLS2 expression between MMTV-MYC and MMTV-MYC Gls1KO tumours from NIMR mice. GLS1 positive (+) control is mouse MYC-induced liver tumour, negative (-) control is normal mouse liver. GLS2 positive (+) control is normal mouse liver, negative (-) control is mouse MYC-induced liver tumour (Gls2 knockdown). c, qPCR comparing Gls1 and Gls1 mRNA fold induction relative to 18S ribosomal RNA between MMTV-MYC and MMTV-MYC Gls1KO tumours. Data are presented as mean ± s.d. Statistical analysis was performed using a two-tailed Student’s t-test. d, MMTV-ERBB2 Gls1KO PCR genotyping as described in Materials and methods. Genotyping was performed by Wei Lin. fl/fl control is an ear biopsy from a Gls1fl/fl control mouse. WT control is an ear biopsy from a control mouse. Only samples displaying both the Gls1fl/fl band and the Gls1KO band were used for experiments e–g, Gls1 deletion in MMTV-ERBB2 tumours has no effect on tumour latency, tumour onset, or growth rate. e, Kaplan-Meier curve (MMTV-ERBB2 tumours n = 21; MMTV-ERBB2 Gls1KO tumours n = 8). Each data point represents a mouse that has reached the experimental endpoint. f, Scatter plot showing time taken for MMTV-ERBB2 tumour initiation. Data are presented as mean ± s.d. Statistical analysis was performed using a two-tailed Student’s t-test. g, Line graph showing MMTV-ERBB2 tumour growth rate. h–j, Gls1 deletion in MMTV-MYC tumours increases tumour latency with no difference in tumour onset or growth rate. h, Kaplan-Meier curve (MMTV-MYC tumours n = 49; MMTV-MYC Gls1KO tumours n = 29). Each data point represents a mouse that has reached the experimental endpoint. P value was calculated using the Log-rank (Mantel-Cox) test. i, Scatter plot showing time taken for MMTV-MYC tumour initiation. (MMTV-MYC tumours n = 28; MMTV-MYC Gls1KO tumours n = 16). Data are presented as mean ± s.d. Statistical analysis was performed using a two-tailed Student’s t-test. j, Line graph showing MMTV-MYC tumour growth rate.
It is important to note that this experiment was started in the National Institute for Medical Research (NIMR) and continued in the Francis Crick Institute (Crick). Comparing survival curves between mice that were bred in the NIMR versus those that were bred at the Crick revealed a difference in MMTV-MYC tumour latency between control and \( Gls1^{KO} \) mice. To investigate this further we separated mice into ‘NIMR’ and ‘Crick’ cohorts and replotted the Kaplan-Meier curves. These results demonstrated that indeed the difference in tumour latency was far less pronounced in the Crick mice compared to the NIMR mice, with the \( P \) value rising from 0.0003 to 0.1955 and thus rendering the difference statistically insignificant (Fig. 3-2a&b). We investigated whether \( Gls1 \) deletion was successful in MMTV-MYC \( Gls1^{ko} \) tumours from Crick mice via Western blot, which showed that \( Gls1 \) was deleted (Fig. 3-2c). However, it was not a decrease in \( Gls1^{ko} \) tumour latency that we observed between the NIMR and Crick cohorts, but an increase in control tumour latency (Fig. 3-2a&b). There are several potential environmental reasons for this occurrence, discussed further in 6.1.1. For example, differences in microbiome composition and housing conditions can affect experimental outcomes (Macrides, Bartke, and Dalterio, 1975; Nichols and Chevins, 1981; Franklin and Ericsson, 2017; Lloyd et al., 2018) The following sections describe the analysis of these tumours. Which mice were used for each experiment is clearly indicated.
Figure 3-2 MMTV-MYC tumour latency is unchanged following Gls1 deletion in Crick mice

a, Kaplan-Meier curve showing tumour latency in ‘Crick’ mice (MMTV-MYC tumours n = 31; MMTV-MYC Gls1\(^{KO}\) tumours n = 16). P value was calculated using the Log-rank (Mantel-Cox) test. b, Kaplan-Meier curve showing tumour latency in ‘NIMR’ mice (MMTV-MYC tumours n = 18; MMTV-MYC Gls1\(^{KO}\) tumours n = 13). P value was calculated using the Log-rank (Mantel-Cox) test. c, Western blot showing GLS1 expression in MMTV-MYC Gls1\(^{KO}\) tumours from Crick (1-5) and NIMR (6&7) mice. Positive (+) control is NIMR MMTV-MYC tumour. Negative (-) control is NIMR MMTV-MYC Gls1\(^{KO}\) tumour.
3.3 \textit{Gls1}^{KO} MYC-driven mammary gland tumours are more differentiated than controls

To investigate the effect of \textit{Gls1} deletion on tumour histology, haematoxylin and eosin (H&E) staining was performed on MMTV-MYC control and \textit{Gls1}^{KO} tumours. Tumours were analysed were from both the NIMR and Crick mouse cohorts. Histological analysis of H&E-stained tumour sections was performed by Professor Gordon Stamp and revealed significant differences in gross morphology between control and \textit{Gls1}^{KO} MMTV-MYC tumours from the NIMR cohort. Control tumours were largely found to have solid, undifferentiated growth patterns, displaying epithelial-to-mesenchymal transition (Fig. 3-3a-e). While there were instances of focal differentiation in a minority of control tumours, differentiated growth patterns of both tubuloglandular and microacinar histology were observed to a significantly greater extent in \textit{Gls1}^{KO} tumours (Fig. 3-3f-k).

On the other hand, control and \textit{Gls1}^{KO} tumour grades from the Crick cohort were not significantly different (Fig. 3.3k). Importantly, control Crick tumours tended towards a more differentiated state relative to control NIMR tumours, becoming more like the \textit{Gls1}^{KO} tumours. This observation pairs well with the increase in tumour latency observed in control Crick tumours compared to control NIMR tumours. There was still a difference, however, between control and \textit{Gls1}^{KO} tumours from the Crick cohort, with no \textit{Gls1}^{KO} tumours exhibiting poor/moderate or undifferentiated growth patterns (Fig. 3-3k). These results illustrate the importance of considering environmental factors in experimental design. Nonetheless, the observed differences in tumour grade from the both the NIMR and Crick cohorts suggests that \textit{Gls1} deletion results in more highly differentiated tumours compared to controls.
Chapter 3 Results

Figure 3-3 MMTV-MYC \(Gls^{1\text{KO}}\) tumours are morphologically distinct from controls

a-e, Control MMTV-MYC tumours from NIMR mice example photos. Average arbitrary grading score: 3.61 (\(n = 19\)).

a, Poorly differentiated (4).
b, Solid, poorly differentiated (4).
c, Solid, undifferentiated (5).
d, Solid/EMT, undifferentiated (5).
e, Solid/focal tubuloglandular (4).

f-j, MMTV-MYC \(Gls^{1\text{KO}}\) tumours from NIMR mice example photos. Average score: 2.94 (\(n = 17\)).

f, Moderate, tubuloglandular (2).
g, Moderate, tubuloglandular (2).
h, Moderate, microacinar (3).
i, Moderate/poor, mixed microacinar/tubuloglandular (3).
j, Moderate/poor, microacinar (3).
k, Stacked bar graph showing arbitrary grading scores as the percentage of tumours with that score.

Statistical analysis was performed on arbitrary grading scores using a two-tailed Student’s \(t\)-test.
Both GLS1 inhibition by BPTES and GLS1 knockdown have previously been shown to induce apoptosis in MYC-high cells (Yuneva et al., 2012). Therefore, we next evaluated whether Gls1 deletion affects either proliferation or survival in addition to differentiation status by comparing control and Gls1KO MMTV-MYC tumours from the NIMR cohort. To accomplish this, we performed immunohistochemical analysis and Western blotting to examine the relative expression levels of: Ki-67, a protein expressed in proliferating cells; proliferating cell nuclear antigen (PCNA), a DNA sliding clamp required for DNA replication; phosphohistone H3 (PHH3), a specific marker of mitosis; cleaved caspase-3 (CC3) and poly-ADP ribose polymerase (PARP), both markers of apoptosis.

The percentage of both Ki67- and CC3-positive cells did not significantly differ between control and Gls1KO MMTV-MYC tumour sections (Fig. 3-4a-f). The CC3 staining results were confirmed by Western blot (Fig. 3-4j). However, the trend towards increased Ki67 staining in Gls1KO tumours was very close to being significant ($P = 0.0512$; Fig. 3-4c). To confirm this result, we performed PHH3 staining. Our results demonstrated that the percentage of PHH3-positive cells was significantly higher in Gls1KO tumour sections compared to controls (Fig. 3-4g-i). This result was also confirmed by Western blot (Fig. 3-4j). Considering that the Gls1KO tumours exhibited increased tumour latency (Fig. 3-1f), the increased percentage of PHH3-positive cells may suggest that cells in these tumours are experiencing growth arrest during S-phase due to replicative stress, possibly caused by a depleted nucleotide pool and DNA damage, which has previously been reported (Chopra et al., 2016).

We are currently estimating relative cell cycle lengths between the two sets of tumours using pulse-labelling of halogenated pyrimidine derivatives (5-chloro-2'-deoxyuridine and 5-iodo-2'-deoxyuridine) according to the protocol from Martynoga et al. (2005).
Figure 3-4 Immunohistochemistry and Western blot analysis suggest enhanced proliferation in MMTV-MYC \textit{Gls}^\textit{fKO} tumours \textit{versus} controls, with no difference in apoptosis

Immunohistochemical staining was performed by the Experimental Histopathology Laboratory using the BenchMark ULTRA IHC/ISH System. \textbf{a-c}, Ki67 staining and quantification in control \textit{versus} \textit{Gls}^\textit{fKO} MMTV-MYC tumours. \textbf{a}, Control MMTV-MYC tumour. \textbf{b}, MMTV-MYC \textit{Gls}^\textit{fKO} tumour. \textbf{c}, Quantification of Ki67 staining between control and \textit{Gls}^\textit{fKO} MMTV-MYC tumours. Data are presented as mean ± s.d. Statistical analysis was performed using a two-tailed Student’s \textit{t}-test. \textbf{d-f}, Cleaved caspase-3 (CC3) staining in control and \textit{Gls}^\textit{fKO} MMTV-MYC tumours. \textbf{d}, Control MMTV-MYC tumour. \textbf{e}, MMTV-MYC \textit{Gls}^\textit{fKO} tumour. \textbf{f}, Quantification of CC3 staining between control and \textit{Gls}^\textit{fKO} MMTV-MYC tumours. Data are presented as mean ± s.d. Statistical analysis was performed using a two-tailed Student’s \textit{t}-test. \textbf{g-i}, Phosphohistone H3 (PHH3) staining and quantification in control and \textit{Gls}^\textit{fKO} MMTV-MYC tumours. \textbf{g}, Control MMTV-MYC tumour. \textbf{h}, MMTV-MYC \textit{Gls}^\textit{fKO} tumour. \textbf{i}, Quantification of PHH3 staining between control and \textit{Gls}^\textit{fKO} MMTV-MYC tumours. Data are presented as mean ± s.d. Statistical analysis was performed using a two-tailed Student’s \textit{t}-test. \textbf{j}, Western blot comparing CC3, poly-ADP ribose polymerase (PARP), PHH3, and proliferating cell nuclear antigen (PCNA) expression between control and \textit{Gls}^\textit{fKO} MMTV-MYC tumours.
3.4 Glutamine metabolism is perturbed in MYC-induced mammary gland tumours upon Gls1 deletion

To evaluate the effect of Gls1 deletion on metabolism in MMTV-MYC tumours we infused tumour-bearing mice with stable isotope-labelled tracers and analysed metabolites in tumours and non-tumoural mammary gland tissue by LCMS. The mice infused were part of the NIMR cohort. To first investigate the effect of Gls1 deletion on glutaminolysis and downstream glutamine metabolism, we infused mice bearing either control or Gls1KO MMTV-MYC tumours with [U\(^{13}\)C]-glutamine. The results of LCMS analysis demonstrated that the enrichment of [U\(^{13}\)C]-glutamine in serum was equal between control and Gls1KO groups (Fig. 3-5). \(^{13}\)C enrichment into TCA metabolites in control tumours was increased compared to mammary glands indicating upregulated glutamine catabolism, consistent with previous results from our lab (Fig. 3-5; Still, 2018). Deleting Gls1 in MMTV-MYC tumours resulted in decreased enrichment of glutamine-derived carbons in glutamate and other TCA cycle intermediates, including succinate, fumarate, malate, and aspartate (Fig. 3-5). However, no changes in \(^{13}\)C enrichment were observed in Gls1KO mammary glands compared to controls. The significant decrease in Gls1KO tumours of the +5 isotopologue of \(\alpha\)-ketoglutarate (\(\alpha\)KG), and the accompanying significant decreases in the +4 isotopologues of succinate, fumarate, and malate demonstrate that glutamine catabolism is impaired in MMTV-MYC Gls1KO tumours (Fig. 3-6). Concordantly, Gls1KO tumours also had decreased total levels of these metabolites (Fig. 3-7). Gls1 deletion also resulted in decreased incorporation of glutamine-derived carbon into ribose 5-phosphate (R5P) and a reduction in overall levels of nucleotides including AMP, ADP, and GDP (Figs. 3-5 & 3-7). Glutamine carbon is not directly incorporated into nucleotides but is contributed via the pentose phosphate pathway (PPP) and one carbon (1C) metabolism, using glycolytic intermediates produced by gluconeogenesis. This is demonstrated by the detection each of the +1 to +5 isotopologues in R5P (Fig. 3-6).

Gls1 deletion also resulted in decreased total levels of non-essential amino acids (NEAAs) including alanine, aspartate, serine, and glycine (Fig. 3-7). \(^{13}\)C enrichment into aspartate was significantly decreased, but not alanine or serine (Fig. 3-5). This
is because aspartate is produced directly from glutamine carbon via the TCA cycle, while glycolytic metabolites provide carbon backbones for the synthesis of alanine and serine. $^{13}$C enrichment was observed at each of the +1, +2, and +3 positions in alanine and lactate, which suggests pyruvate/malate cycling via malic enzyme activity (Fig. 3-6). These results demonstrate that glutamine catabolism represents a significant source of anaplerotic carbon in MYC-high mammary gland tumours, and thus Gls1 expression is important for energy generation and biomass production.

Total pools of the 1C metabolites methionine and dimethylglycine were significantly reduced in MMTV-MYC Gls1KO tumours (Fig. 3-7). Dimethylglycine is produced from betaine following carbon unit donation for folate-independent synthesis of methionine and can support 1C metabolism by carbon unit donation to the folate cycle. 1C metabolism is primarily supported by serine and glycine, which were also decreased in these tumours. 1C metabolism integrates cellular nutrient status via these inputs, and outputs such as nucleotide biosynthesis and methylation reactions. Therefore, compromised glutamine metabolism leading to decreased input into 1C metabolism may be causing decreased nucleotide biosynthesis, total levels of which were decreased (Fig. 3-7). Furthermore, total levels of glutathione disulphide levels were significantly reduced. Glutathione production is dependent on 1C metabolism, as well as glutamate availability, decreases in both of which are likely resulting in decreased glutathione synthesis. These results indicate that MMTV-MYC Gls1KO tumours may have impaired ability to sufficiently regulate cellular ROS levels. It has recently been demonstrated that TNBC cell lines, especially those that are basal-like, are dependent on glutathione for survival by suppressing ROS (Beatty et al., 2018). Furthermore, hydrogen peroxide treatment has been shown to synergise with GLS1 inhibition, indicating that perturbation of glutamine metabolism sensitises cancer cells to ROS (Son et al., 2013). We also found that total levels of nicotinamide, NAD, and NADP were reduced in Gls1KO tumours. Nicotinamide, a form of vitamin B3, is obtained via dietary intake, so further investigation as to whether Gls1 deletion affects the expression of transporters such as SLC12A8 is required (Grozio et al., 2019).

The decreases in total alanine and serine levels in Gls1KO tumours, and the significant decrease in the +1 isotopologue of alanine, may be indicative of
decreased transaminase activity. The transaminase enzymes transfer the amino group from glutamate to an α-keto acid to produce an NEAA and αKG. Serine is produced via the SSP, in which PSAT1 catalyses the conversion of PHP to P-Ser (Fig. 1-5). Alanine is produced by GPT1 and GPT2, which utilise pyruvate. Finally, aspartate is produced by GOT1 and GOT2 using oxaloacetate. To investigate this, we performed [α15N]-glutamine infusions. This experiment was performed using Crick mice and, consistent with the lack of difference in tumour latency between control and Gls1KO tumours, the total levels of metabolites were not different (Fig. 3-9). There was a tendency towards decreased 15N enrichment in alanine and aspartate, although this did not reach statistical significance (Fig. 3-8). Unexpectedly, αKG levels were significantly increased in in Gls1KO tumours compared to controls. However, while increased Gls2 expression can compensate for Gls1 deletion in MYC-driven liver tumours, we did not find evidence for this occurring in NIMR MMTV-MYC Gls1KO tumours (Fig. 3-1b&c). These results show that glutamine catabolism and TCA cycle activity is not inhibited in MMTV-MYC Gls1KO tumours from Crick mice but is inhibited to a significant extent in MMTV-MYC Gls1KO tumours from NIMR mice. Nonetheless, in both cases Gls1 deletion is insufficient to prevent tumour initiation and progression, indicating that metabolic compensation is occurring.

The disparity in the data between the NIMR and Crick cohorts of mice illustrates the problem of reproducibility faced by many researchers in the field. Simply changing the environment in which the mice were bred and housed was sufficient to eliminate the significant differences observed in the previous data. Collectively, these results demonstrate that deleting Gls1 in the mammary gland results in decreased glutamine catabolism, with an accompanying decrease in glutamine-dependent anaplerosis. MMTV-MYC Gls1KO tumours were nonetheless still able to compensate for Gls1 deletion and grew with increased latency. There are multiple mechanisms by which this can be achieved, such as increased Gls2 expression or amidotransferase activity, as previously demonstrated by our lab (Méndez-Lucas et al., 2020). However, MMTV-MYC Gls1KO tumours had no change in Gls2 mRNA levels and potentially a tendency toward decreased protein expression, as revealed by qPCR and Western blot (Fig. 3-1b&c). Therefore, for MMTV-MYC tumours it is more likely that amidotransferase activity, rather than GLS2, would be providing compensatory glutaminolytic activity.
Our results indicate that glutaminolysis via GLS1 represents an important route for glutamine anaplerosis in MMTV-MYC tumours, but also that it is not essential for tumour initiation or progression. The versatility of glutamine as a cellular resource of both carbon and nitrogen means that Gls1 deletion was sufficient to deplete the pools of many other metabolites due to reduced glutaminolytic capacity. Decreases in nucleotide levels and 1C metabolites indicate that a nutrient-deficient signal may be being relayed in MMTV-MYC Gls1\(^{KO}\) tumours, resulting in decreased nucleotide biosynthesis. Depleted total metabolite pools may also be secondary to the decreased rate of tumour growth. There are several possibilities, and we decided to investigate this further using RNA sequencing (RNA-seq, see 3.6).
Figure 3-5 Glutamine metabolism is decreased, but not blocked, in MMTV-MYC \(Gls^{1KO}\) tumours from NIMR mice

\(^{13}\)C incorporation into metabolites of the TCA cycle, glycolysis, and the pentose phosphate pathway following 3h infusions of \([U^{13}\text{C}]\)-glutamine. Control MMTV-MYC mammary glands \((n = 7)\) and tumours \((n = 7)\), and MMTV-MYC \(Gls^{1KO}\) mammary glands \((n = 5)\) and tumours \((n = 8)\) from NIMR mice are compared. \(^{13}\)C incorporation is shown as percentage enrichment of total metabolite pools on the y-axis. \(^{13}\)C enrichment (%) represents the sum of the enrichment of all detected isotopologues. Data are presented as mean ± s.d. Statistical analysis was performed using multiple two-tailed Student’s \(t\)-tests.
Figure 3-6 Isotopologue enrichment distributions from [U^{13}C]-glutamine experiment

$^{13}$C incorporation into metabolites of the TCA cycle, glycolysis, and the pentose phosphate pathway following 3h infusions of [U$^{13}$C]-glutamine. Control MMTV-MYC mammary glands ($n = 7$) and tumours ($n = 7$), and MMTV-MYC $Gls^{fKO}$ mammary glands ($n = 5$) and tumours ($n = 8$) from NIMR mice are compared. $^{13}$C incorporation is shown as relative percentage enrichment in metabolite isotopologues on the $y$-axis. Data are presented as mean ± s.d. Statistical analysis was performed using multiple two-tailed Student’s $t$-tests.
Figure 3-7 Total pools of metabolites are decreased following $Gls1$ deletion in MMTV-MYC tumours from NIMR mice

Key intermediates of glycolysis, the TCA cycle, and 1C metabolism, as well as amino acids and nucleotides are compared between Control MMTV-MYC mammary glands ($n=7$) and tumours ($n=7$), and MMTV-MYC $Gls1^{KO}$ mammary glands ($n=5$) and
tumours \((n = 8)\) from NIMR mice. Data are presented as mean \(\pm\) s.d. Statistical analysis was performed using multiple two-tailed Student’s \(t\)-tests.
There is a tendency towards a decrease in transaminase activity in MMTV-MYC Gls\(^{\text{fCO}}\) tumours. \(^{15}\text{N}\) incorporation into non-essential amino acids via transamination following 3h infusions of \([\alpha^{15}\text{N}]\)-glutamine. Control MMTV-MYC mammary glands \((n = 7)\) and tumours \((n = 7)\), and MMTV-MYC Gls\(^{\text{fCO}}\) mammary glands \((n = 6)\) and tumours \((n = 6)\) from Crick mice are compared. \(^{15}\text{N}\) incorporation is shown as percentage enrichment of total metabolite pools on the \(y\)-axis. \(^{15}\text{N}\) enrichment (%) represents the sum of the enrichment of all detected isotopologues. Data are presented as mean ± s.d. Statistical analysis was performed using multiple two-tailed Student’s \(t\)-tests.
Figure 3-9 Total pools of metabolites are unaffected following Gls1 deletion in MMTV-MYC tumours from Crick mice

Key intermediates of glycolysis, the TCA cycle, and 1C metabolism, as well as amino acids and nucleotides are compared between control MMTV-MYC mammary glands ($n = 7$) and tumours ($n = 7$), and MMTV-MYC Gls1$^{KO}$ mammary glands ($n = 6$) and
tumours (n = 6) from Crick mice. Data are presented as mean ± s.d. Statistical analysis was performed using multiple two-tailed Student’s t-tests.
3.5 Glucose metabolism is unaffected in MYC-induced mammary gland tumours following Glis1 deletion

The results of the [U\(^{13}\)C]-glutamine infusion experiment demonstrated a decrease in R5P synthesis and depleted nucleotide pools in MMTV-MYC Glis1\(^{KO}\) tumours compared to controls (Figs. 3-5 & 3-7). Although the carbon contribution for R5P comes from glucose, glutamine carbons can be incorporated into R5P, for example following gluconeogenesis in the liver and subsequent recirculation of the resultant glucose. The carbon contribution for purine nucleobases comes predominantly from glucose via the SSP, while for pyrimidines it comes mostly from glutamine-derived aspartate. To investigate whether the glucose contribution to purine production is truly decreased in these tumours, as well as whether there is any increased contribution from glucose to the TCA cycle to compensate for perturbed glutamine anaplerosis, [U\(^{13}\)C]-glucose infusions were performed in Crick mice bearing control and MMTV-MYC Glis1\(^{KO}\) tumours. Consistent with our previous results, MMTV-MYC Glis1\(^{KO}\) tumours had increased levels of glutamine and decreased levels of glutamate (Fig. 3-11). However, the levels of all other metabolites were unchanged. These results are consistent with the unchanged total metabolite levels in the MMTV-MYC Glis1\(^{KO}\) tumours from Crick mice following \([\alpha^{15}\text{N}]\)-glutamine infusions (Fig. 3-9), again highlighting the problem of irreproducibility between experiments performed in NIMR and Crick mice.

The only metabolite for which \(^{13}\text{C}\) enrichment was reduced in Glis1\(^{KO}\) MMTV-MYC tumours following [U\(^{13}\)C]-glucose infusions was aspartate, with no evidence for any difference in carbon contribution to any other metabolite analysed (Fig. 3-10). Unfortunately, the previous [U\(^{13}\)C]-glutamine experiment cannot be compared to this experiment, as the phenotype and the effect of Glis1 deletion on total MMTV-MYC metabolite levels is not comparable between Crick and NIMR mice. Therefore, whether the significant decrease in \(^{13}\text{C}\) enrichment from [U\(^{13}\)C]-glutamine in R5P, with the decrease in AMP also very close to reaching significance (\(P = 0.0897\)), is solely due to the diminished capacity to produce glycolytic intermediates from glutamine remains to be determined.
The decrease in aspartate synthesis observed in both $^{13}$C labelling experiments, and the tendency toward decrease in the $[\alpha^{15}\text{N}]$-glutamine labelling experiment, points to an effect of $Gls1$ deletion on GOT2 activity. The reduction we observed in aspartate synthesis is most likely due to the reduced glutamate pool that results from $Gls1$ deletion, as glutamate is required as the nitrogen donor to oxaloacetate in the transamination reaction, producing aspartate and $\alpha$KG. At time of writing, the specific \textit{in vivo} requirement for GOT1/2 in breast cancer has not been well described in the literature, but previous studies looking at its \textit{in vitro} requirement, along with our observations of reduced aspartate synthesis following $Gls1$ deletion make this an interesting point to pursue, both as a potential solitary therapeutic target, but also potentially in combination with $Gls1$ inhibition.
Figure 3-10 Glucose metabolism is largely unaffected by Gls1 deletion in MMTV-MYC tumours

$^{13}$C incorporation into metabolites of the TCA cycle, glycolysis, and the pentose phosphate pathway following 3h infusions of [U$^{13}$C]-glucose. Control MMTV-MYC tumours ($n = 7$), and Gls1$^{KO}$ MMTV-MYC tumours ($n = 7$) are compared. $^{13}$C incorporation is shown as percentage enrichment of total metabolite pools on the y-axis. $^{13}$C enrichment (%) represents the sum of the enrichment of all detected isotopologues. Data are presented as mean ± s.d. Statistical analysis was performed using multiple two-tailed Student’s $t$-tests.
Figure 3-11 Total pools of metabolites are unaffected following Gls1 deletion in MYC-driven mammary gland tumours from Crick mice
Key intermediates of glycolysis, the TCA cycle, and 1C metabolism, as well as amino acids and nucleotides are compared between control MMTV-MYC tumours (CT; \( n = 7 \)), and \( Gls^{1\text{KO}} \) MMTV-MYC tumours (\( n = 7 \)) following 3h infusions of \([^{13}\text{C}]-\text{glucose}\). Data are presented as mean ± s.d. Statistical analysis was performed using multiple two-tailed Student’s t-tests.
3.6 RNA-seq reveals transcriptional similarity between $Gls1^{KO}$ and control MYC-driven mammary gland tumours

Cellular metabolism and signalling pathways are inextricably linked, involving myriad species of biomolecules and vast, extremely complex interaction profiles. To identify potential mechanisms that support or delay tumour progression in the absence of $Gls1$, we performed RNA-seq of RNA extracts from control and $Gls1^{KO}$ MMTV-MYC tumours from NIMR mice. In collaboration with the bioinformatics STP at the Crick, we first performed principal component analysis (PCA), which revealed that there is very little difference between gene expression between the groups (Fig. 3-12a). Large intragroup variation reduces the number of statistically significantly different genes to those that have higher fold change differences. Indeed, there were only five differentially expressed genes between the groups, out of a possible 27,378 genes (Fig3-12b&c). These results demonstrate that there is more variation within the groups than between them. Therefore, in this case the sample size was too small to detect smaller differences in gene expression between the two groups. However, these results do not preclude the possibility of differences at the post-translational level.

Consistent with our previous results, the most differentially expressed gene between the groups was $Gls1$. In second place was Potegl, the human orthologue of which is POTEB (POTE ankyrin domain family, member B). The cellular role of POTE gene family proteins is unclear, although its expression in normal tissues is known to be restricted to the prostate, ovary, testis, and placenta. POTE proteins are thought to be involved in the regulation of apoptosis as their expression is associated with high levels of CC3 in apoptotic spermatids (Bera et al., 2012). POTEB has been reported to be a potential oncogene in many types of cancer, including ovarian cancer, and its expression is highest in metastatic malignancies (Bera et al., 2006; Redfield et al., 2013; Barger et al., 2018). Potegl is the second most differentially expressed, and most upregulated gene in MMTV-MYC $Gls1^{KO}$ tumours versus controls. Notwithstanding our IHC results for CC3 (Fig. 3-4f&j), this result suggests that the delay in tumour latency observed in $Gls1^{KO}$ tumours may be due to increased apoptosis signalling.
In third place is \textit{Yme1l1} (Yme1-like 1), \textit{YME1L1} in humans. This is the second most downregulated gene after \textit{Gls1} and is an ATP-dependent metalloprotease involved in apoptotic resistance that has recently suggested to exhibit tumour suppressor activity in neuroblastoma (Ando \textit{et al.}, 2019). Downregulation of a tumour suppressor upon \textit{Gls1} deletion suggests that the stress exerted upon tumour cells \textit{in vivo} might necessitate such a downregulation to promote survival. Considering that the tumours still initiate and progress, albeit with extended latency, this may be a facilitating factor.

The third most downregulated gene in \textit{Slc27a2}, the human orthologue of which codes for very long-chain acyl-CoA synthetase, a membrane protein that is involved in the trafficking of lipids. The expressed protein has a key role in lipid biosynthesis and fatty acid degradation, while its downregulation has recently been correlated with chemo-resistance in ovarian cancer (Chen \textit{et al.}, 2018). This suggests that downregulation of \textit{Slc27a2} expression in MMTV-MYC \textit{Gls1KO} tumours could promote resistance to the deleterious effects \textit{Gls1} deletion. The final gene upregulated was \textit{Gm13422}, a predicted long non-coding RNA gene that has had no investigation at time of writing.

We also carried out gene set enrichment analysis (GSEA), which uses the expression data of all genes rather than only those that are differentially expressed, allowing for the detection of differentially regulated pathways without requiring high numbers of differentially expressed genes. 22 pathways were downregulated, and 150 pathways were upregulated in MMTV-MYC \textit{Gls1KO} tumours compared to controls. Of the downregulated pathways, the most statistically significant were pathways involved in the production of proteins that contribute to the structure and function of extracellular matrices, known as the ‘matrisome’ (Table 3-1).

The increased production of ECM proteins promotes tumour progression by increasing stiffness and promoting the assembly and stabilisation of focal adhesions, enhancing tumour growth and perturbing tissue organisation (Paszek \textit{et al.}, 2005). Therefore, these results are consistent with the role of GLS1 in supporting \textit{\alpha}KG and thus \textit{\alpha}KG-dependent hydroxylation of collagen, downstream of HIF-1\textit{\alpha} (Stegen \textit{et al.}, 2019). Furthermore, glutamine-dependent proline synthesis plays an important
role in collagen production (Phang, Hancock, and Fischer, 2015). Our results have demonstrated that MMTV-MYC Gls1KO tumours have a significantly depleted proline pool (Fig. 3-7). The extent to which Gls1 deletion mediates an increase in tumour latency by affecting ECM production remains to be determined.

Of the upregulated pathways, most were generic pathways including those involved in the cell cycle and the metabolism of RNA and amino acids. However, several pathways involved in nucleotide biosynthesis were upregulated (Table 3-2). This could suggest that increased amidotransferase activity contributes to compensation of Gls1 deletion, providing the tumours with an alternative method of catabolising glutamine to glutamate. The RNA-seq results are from NIMR mice, and we have thus far demonstrated that the metabolic response to Gls1 deletion differs significantly between NIMR and Crick mice. However, increased amidotransferase activity could still represent a potential secondary therapeutic target in MMTV-MYC tumours, in addition to GLS1. To investigate this, the relative expression levels of the aminotransferases in tumour samples from NIMR mice can be compared by Western blot. This work is ongoing.

To investigate the differences between control and Gls1KO MMTV-MYC tumours further, analysing larger numbers of tumours would be necessary to first confirm that the transcriptional profiles are indeed as similar as is indicated by this data. Larger sample numbers could yield a larger list of differentially expressed genes, perhaps identifying further potential investigative targets. This could also reveal a more precise picture of the mechanism by which Gls1 deletion increases MMTV-MYC tumour latency.
Figure 3-12 The transcriptional profiles of control and Gls1KO MMTV-MYC tumours are highly similar

a, Principal component analysis (PCA) of 5 control MMTV-MYC tumours (MYC CT) and 5 MMTV-MYC Gls1KO tumours (MYC Gls1KO) using RNA-seq data. b, Volcano plot showing upregulated, downregulated, and not statistically significant genes in MMTV-MYC Gls1KO tumours versus controls. c, Heatmap of differentially expressed genes between control and Gls1KO MMTV-MYC tumours.

Table 3-1 Selected downregulated pathways in MMTV-MYC Gls1KO tumours compared to controls

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Size</th>
<th>ES</th>
<th>NES</th>
<th>P adj</th>
</tr>
</thead>
<tbody>
<tr>
<td>NABA_MATRISOME</td>
<td>797</td>
<td>-0.1580682</td>
<td>-5.112538</td>
<td>1E-08</td>
</tr>
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<td>NABA_CORE_MATRISOME</td>
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<td>-4.7155747</td>
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<tr>
<td>NABA_ECM_GLYCOPROTEINS</td>
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<td>-0.2605228</td>
<td>-3.9236138</td>
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<tr>
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<td>558</td>
<td>-0.11307757</td>
<td>-3.0945497</td>
<td>1E-08</td>
</tr>
</tbody>
</table>

Table 3-2 Selected upregulated pathways in MMTV-MYC Gls1KO tumours compared to controls

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Size</th>
<th>ES</th>
<th>NES</th>
<th>P adj</th>
</tr>
</thead>
<tbody>
<tr>
<td>KEGG_PYRIMIDINE_METABOLISM</td>
<td>94</td>
<td>0.2890062</td>
<td>3.2770815</td>
<td>1E-08</td>
</tr>
<tr>
<td>KEGG_PURINE_METABOLISM</td>
<td>145</td>
<td>0.18407144</td>
<td>2.5701156</td>
<td>7.2E-04</td>
</tr>
<tr>
<td>REACTOME_METABOLISM_OF_NUCLEOTIDES</td>
<td>68</td>
<td>0.2549906</td>
<td>2.4397833</td>
<td>0.001771484</td>
</tr>
<tr>
<td>REACTOME_PYRIMIDINE_METABOLISM</td>
<td>23</td>
<td>0.3266152</td>
<td>1.9063646</td>
<td>0.044498954</td>
</tr>
</tbody>
</table>
3.7 Chapter 3 summary

The aim of this project section was to evaluate whether GLS1 and supported glutamine metabolism are essential for MYC-induced tumourigenesis in the mammary gland. We achieved this by generating a mouse model that develops spontaneous MMTV-MYC \textit{Gls1}\textsuperscript{KO} tumours. These tumours develop following activation of \textit{Myc} overexpression by the MMTV promoter during puberty, while also expressing Cre recombinase to delete \textit{Gls1} specifically in the mammary gland. Unfortunately, the environmental shift between NIMR and Crick mice led to the observed phenotype disappearing, so much of the follow up work that was performed in Crick mice was unable to reveal the more precise mechanisms at play following \textit{Gls1} deletion in MMTV-MYC tumours from NIMR mice.

\textit{MMTV-MYC Gls1}\textsuperscript{KO} tumours from NIMR mice, compared to controls, exhibited increased latency. This was confirmed by Western blot to be caused by successful \textit{Gls1} deletion. Our comparative MMTV-ERBB2 model did not exhibit and change in tumour latency following \textit{Gls1} deletion. These results demonstrate that MMTV-MYC tumours are susceptible to perturbation of glutamine metabolism by inhibition of \textit{Gls1} expression, but that this is insufficient to prevent the initiation and progression of these tumours.

We found that MMTV-MYC \textit{Gls1}\textsuperscript{KO} tumours were more differentiated than controls, while also staining more for PHH3, a marker of mitosis. Increased PHH3 staining can be indicative of mitotic arrest; it has previously been shown that glutamine deprivation and GLS1 inhibition can prevent cancer cells from transitioning from S-phase to G2/M (Gaglio \textit{et al.}, 2009; Columbo \textit{et al.}, 2011; Gwangwa, Joubert, and Visagie, 2019). This would provide an explanation for the observed increase in tumour latency. Work is ongoing to confirm this.

We next utilised SIAM to evaluate the effectiveness of targeting GLS1 as a therapeutic strategy in MMTV-MYC tumours from NIMR mice, along with allowing potential secondary targets to be identified based on the specific metabolic changes that take place following \textit{Gls1} deletion. We found that \textit{Gls1} deletion in MMTV-MYC tumours significantly reduced glutaminolysis and glutamine-dependent TCA cycle
anaplerosis, with $^{13}$C enrichment decreasing in metabolites including glutamate, succinate, fumarate, and malate. Total levels of these metabolites were also decreased, as were NEAAs including alanine, aspartate, serine, and glycine, suggesting that transaminase activity might also have been affected. Furthermore, $^{13}$C enrichment into R5P was reduced, along with total levels of AMP, ADP, and GDP. This suggests that inhibition of $Gls1$ expression reduces nucleotide biosynthetic capacity in these tumours. The $^{13}$C isotopologue enrichment profile of R5P, and decreases in 1C metabolite levels, further suggest this may be caused by perturbation of the PPP and/or 1C metabolism.

Finally, to further investigate the mechanistic basis of increased tumour latency in MMTV-MYC $Gls1^{KO}$ tumours from NIMR mice, we performed RNA-seq. We found that control and $Gls1^{KO}$ MMTV-MYC tumours were very similar in terms of transcriptional profile, with only five differentially expressed genes. The upregulation of $Potegl$ suggests upregulation of apoptosis signalling, and thus was consistent with increased latency in $Gls1^{KO}$ tumours. Downregulation of $Slc27a2$, a plasma membrane lipid-trafficking protein, has previously been shown to promote resistance to chemotherapy in cancer, and was found to be downregulated in MMTV-MYC $Gls1^{KO}$ tumours. Downregulation of $Yme1l1$, previously reported to be a tumour suppressor, might suggest that removal of YME1l1 activity is required for tumour initiation in the mammary gland following $Gls1$ deletion. This suggests that YME1l1 may have a role in ameliorating the consequences of $Gls1$ deletion to promote tumourigenesis in the mammary gland.

GSEA demonstrated that pathways involved in ECM production were the most significantly downregulated in MMTV-MYC $Gls1^{KO}$ tumours. GLS1 activity is a major source of glutamate, the precursor to $\alpha$KG. Therefore, these results suggest that the decreased synthesis of $\alpha$KG we observed in these tumours might impact $\alpha$KG-depended collagen hydroxylation. This is further supported by our observation that total proline levels in MMTV-MYC $Gls1^{KO}$ tumours are decreased, with proline playing an important role in collagen biosynthesis. Considering the significant differences in gross tumour morphology we also observed in these tumours, the effect of $Gls1$ deletion on ECM production, and subsequent consequences to tumour initiation and progression, is worthy of further investigation.
Our finding that pathways involved in nucleotide biosynthesis were upregulated agrees with previous results from our lab that have shown that Gls1 deletion in MYC-induced liver tumours can be compensated for by amidotransferase enzymes. In MMTV-MYC tumours, Gls2 expression is not upregulated in response to Gls1 deletion, therefore the amidotransferases may represent secondary therapeutic targets alongside GLS1.

One of the major drawbacks of our mouse model is that Gls1 is deleted in the mammary gland before tumour initiation. This means that our model is not evaluating the effect of acute inhibition of GLS1, but rather the effect of the absence of GLS1 on tumour initiation and progression. An alternative method would be to genetically ablate Gls1 following tumour initiation, to simulate therapeutic intervention with a small molecule. This would have the additional advantage of allowing the effect of Gls1 targeting to be evaluated in the absence of any side effects caused by the small molecule inhibitor in question. I have modified the pCW57.1 tetracycline-inducible vector to express Cre recombinase in tumour cells for this purpose (Fig. 3-13). This will allow us to evaluate the effect of acute Gls1 deletion on MMTV-MYC tumour growth in the mammary gland following cell transplantation. This vector can also be used to evaluate the requirement other enzymes whose activity can support glutamine catabolism and tumour progression in the absence of GLS1.

In the next section, we investigated the potential of targeting the glutamine transporter ASCT2 as a therapeutic approach to MYC-enriched tumours, building on previous work from our lab. Given that deleting Gls1 was insufficient to prevent MMTV-MYC tumour initiation and progression, targeting the ability of tumours to import glutamine may be a more efficacious alternative. We also investigated the effectiveness of targeting ASCT2 alongside GLS1 in a combinatorial approach.
Figure 3-13 Inducible Cre vector cloning strategy
Using the Gateway Cloning technique, a tetracycline-inducible Cre expression vector (pCW57.1-Cre-puro) was produced. Figure generated using SnapGene.
Chapter 4. Targeting glutamine transport inhibits proliferation in MYC-driven mammary gland tumour cells, and synergises with GLS1 inhibition

4.1 Introduction

We established in the previous section that targeting GLS1 is unlikely to be sufficient to inhibit the formation of MYC-driven mammary gland tumours. Previous work from our group has shown that there are multiple mechanisms contributing to metabolic flexibility in MYC-driven liver tumours in mice, which allow tumours to initiate and progress following Gls1 deletion (Méndez-Lucas et al., 2020). The results presented in the previous section suggest that these compensatory mechanisms may also play a role in MMTV-MYC tumours.

In addition to glutamine-dependent metabolic enzymes, targeting glutamine transporters is an attractive potential therapeutic route. Unpublished results from our lab demonstrate that ASCT2 expression, and its glycosylation-mediated localisation to the plasma membrane, are significantly increased in MMTV-MYC tumours compared to MMTV-ERBB2 tumours and normal mammary glands (Still, 2018). Furthermore, siRNA-mediated knockdown of Asct2 decreased cell MMTV-MYC tumour cell proliferation in vitro with an associated decrease in glutamine catabolism (Still, 2018). Importantly, ASCT2 localisation to the plasma membrane in tumour-derived cells increased following Gls1 knockdown or GLS1 inhibition by BPTES (Still, 2018).

In this project section we investigated whether inhibiting ASCT2 using GPNA, a small molecule inhibitor of glutamine transport, affected the proliferation of MMTV-MYC tumour cells. We also evaluated whether dual inhibition of ASCT2 and GLS1 would have a synergistic effect. Finally, we investigated whether dietary glutamine restriction in mice affects MMTV-MYC tumour growth in the mammary gland, which has previously been shown to be effective in a mouse model of medulloblastoma (Niklison-Chirou et al., 2017).
4.2 Aim

To evaluate the efficacy restricting glutamine availability to MMTV-MYC tumour cells
in vitro by pharmacological blockade, or in vivo by dietary restriction.
4.3 Glutamine deprivation \textit{in vitro} decreases MYC-driven mammary gland tumour cell proliferation

To evaluate the \textit{in vitro} effectiveness of pharmacological ASCT2 and GLS1 inhibition in MMTV-MYC tumour-derived cells (MT cells) and MMTV-ERBB2 tumour-derived cells (ET cells), I first established primary cell cultures from MMTV-MYC and MMTV-ERBB2 tumours. Although the initial aim was to compare the effectiveness between MT and ET cells, we found that ET cells lose \textit{ErbB2} expression after as few as two passages in culture, which is accompanied by a significant upregulation of \textit{Myc} expression (data not shown). Most commonly used cell culture media were formulated with the aim of keeping cells alive, not to recapitulate \textit{in vivo} conditions. Therefore, the artificially high concentrations of nutrients can cause profound changes in terms of gene expression and metabolism. For example, hepatocytes have been shown to develop drastic changes in metabolite levels and respiratory capacity when isolated and cultured \textit{in vitro}, while gene expression in smooth muscle cells has been shown to be significantly altered (Zaitseva \textit{et al.}, 2006; Cassim \textit{et al.}, 2017). Therefore, only MT cells were used for the \textit{in vitro} experiments described in this chapter. The MT cells used for each experiment were kept within ten passages of one another to minimise the biological differences that appear over time because of \textit{in vitro} culture conditions. Three MT cell lines, derived from three different MMTV-MYC tumours, were used as biological replicates in each of the experiments.

MT cells are isolated and propagated in DMEM/F-12 MMEC media (see \textit{Materials and methods}). In contrast to DMEM, DMEM/F-12 contains all 20 proteinogenic amino acids and pyruvate. Pyruvate, and NEAAs including glutamate have been shown to support tumour cell proliferation following inhibition of glutamine catabolism (Yuneva \textit{et al.}, 2007; Cheng \textit{et al.}, 2011; Zhang \textit{et al.}, 2014). Therefore, I performed each experiment using either DMEM or DMEM/F-12 MMEC media (hereafter referred to as DMEM and DMEM/F-12, respectively).

I first investigated whether targeting the glutamine transporter ASCT2 with the small molecule inhibitor GPNA would affect growth in MT cells. GPNA is a competitive antagonist of glutamine transport that is widely used to inhibit ASCT2 activity (Esslinger \textit{et al.}, 2005; Ren \textit{et al.}, 2015; Wang \textit{et al.}, 2015; van Geldermalsen \textit{et al.},
2016; Marshall et al., 2017). However, it has recently been demonstrated to inhibit several other transporters from the SLC7 and SLC38 transporter families (Broer et al., 2016). Currently, there are no more specific ASCT2 inhibitors. My results demonstrated that GPNA inhibited proliferation of MT cells grown in DMEM in a dose-dependent manner, with an IC50 value of 25.34 μM at 24 hours (Fig. 4-1a). Consistent with previously published works, both glutamine deprivation and GPNA treatment were less efficient in inhibiting MT cell proliferation when grown in DMEM/F-12 (Fig. 4-1b). The IC50 of GPNA was calculated to be 49.43 μM at 24 hours for MT cells grown in DMEM/F-12.

The concentrations of amino acids in DMEM/F-12 more closely resemble those observed in serum compared to DMEM. However, these results demonstrate that glutamine-deprivation and GPNA treatment are still able to reduce proliferation in MT cells growing in DMEM/F-12. This suggests that targeting glutamine transport could be effective against MMTV-MYC tumours *in vivo*.
The ASCT2 inhibitor GPNA reduces the proliferative capacity of MT cells, with F-12 in the culture medium providing a protective effect.

**Figure 4-1** The ASCT2 inhibitor GPNA reduces the proliferative capacity of MT cells, with F-12 in the culture medium providing a protective effect.

**a**, GPNA treatment of MT cells (passage numbers = 2; n = 3) at the concentrations indicated, cultured in DMEM MMEC media. **b**, GPNA treatment of MT cells (passage numbers = 2; n = 3) at the concentrations indicated, cultured in DMEM/F-12 MMEC media. **c**, The structural formula of GPNA, drawn using MarvinSketch. n indicates the number of biological replicates (different cell lines) used. Three repeats (technical replicates) were performed for each cell line. Graphs represent averages of three technical replicates for each cell line used.
4.4 A combination of GLS1 inhibition by CB-839 and glutamine transport inhibition by GPNA produced a synergistic therapeutic effect in MYC-driven mammary gland tumour cells

Unpublished results from our lab demonstrated that inhibiting GLS1 activity, using either siRNA-mediated knockdown or BPTES, increases ASCT2 expression and localisation at the plasma membrane (Still, 2018). This suggests that GLS1 inhibition might synergise with ASCT2 inhibition if this makes cells more dependent on glutamine transport. To test whether combined inhibition of GLS1 and glutamine transport produces a synergistic antiproliferative effect in MT cells, I performed a drug combination experiment using GPNA and CB-839. CB-839 is a potent, non-competitive inhibitor of GLS1 that has been shown to restrict growth in a range of TNBC cell lines, as well as tumour xenografts in vivo (Gross et al., 2014). Furthermore, published work from our lab has demonstrated that CB-839 inhibits MYC-driven liver tumour cell proliferation in vitro (Méndez-Lucas et al., 2020). Before combining GPNA and CB-839 in vitro, I first evaluated the effect of CB-839 alone on MT cell proliferation.

I found that CB-839 inhibited the proliferation of MT cells in a dose-dependent manner, with IC\textsubscript{50} values at 24 hours of 68.75pM in DMEM and 154.3nM in DMEM/F-12 (Fig. 4-2). Consistent with the previous results, MT cells were more sensitive to CB-839 treatment in DMEM than in DMEM/F-12. To pinpoint the rescue factor exactly, further experiments are required that selectively remove each amino acid from the F-12 mix. Finding the source of rescue may reveal further therapeutic targets that can be utilised in a combinatorial approach for the treatment of MYC-high tumours.
The GLS1 inhibitor CB-839 reduces the proliferative capacity of MT cells, with F-12 in the culture medium providing a protective effect.

**Figure 4-2**

- **a**, CB-839 treatment of MT cells (passage numbers = 7, 7, and 9; *n* = 3) at the concentrations indicated, cultured in DMEM MMEC media.
- **b**, CB-839 treatment of MT cells (passage numbers = 7, 7, and 9; *n* = 3) at the concentrations indicated, cultured in DMEM/F-12 MMEC media.
- **c**, The structural formula of CB-839, drawn using MarvinSketch. *n* indicates the number of biological replicates (different cell lines) used. Three repeats (technical replicates) were performed for each cell line. Graphs represent averages of three technical replicates for each cell line used.
I next tested whether a combination of GPNA and CB-839 would have a stronger therapeutic effect than each of the treatments individually. We first confirmed our previous results demonstrating increased ASCT2 localisation at the plasma membrane upon GLS1 inhibition. We performed immunofluorescence staining to visualise ASCT2 protein expression in MT cells at increasing concentrations of CB-839 in either DMEM or DMEM/F-12. Our results demonstrated that increasing concentrations of CB-839 promoted increased levels of membrane-localised ASCT2 in MT cells grown in DMEM (Fig. 4-3a). The dose-response curve for GPNA showed that at later passages (compared to the initial GPNA treatment experiment performed in earlier passages; Fig. 4-1) the response to lower concentrations of GPNA is lost, with inhibition of proliferation only occurring at concentrations above 2mM (Fig. 4-3b). However, the dose-response curve for CB-839 remained consistent with our previous observations (Fig. 4-3b). This illustrates the importance of considering passage number when conducting drug studies using primary cell lines; after only a few passages the response of MT cells to GPNA treatment was reduced, possibly due to differences in baseline ASCT2 expression and localisation.

Notwithstanding the difference in drug response observed at later passages, the increase in ASCT2 expression observed with increasing concentrations of CB-839 was consistent with our previous results using BPTES and siRNA-mediated Gls1 knockdown (Still, 2018). This provided further rationale to perform a combined GPNA and CB-839 drug study. The dose-response matrix for this experiment showed that different combinations of the two drugs could be synergistic, antagonistic, or have no interaction with one another, as visualised by a drug synergy heatmap showing zero-interaction point (ZIP) delta (δ) -score (Fig. 4-3c&d). The greatest synergy score was observed when MT cells cultured in DMEM were treated with 5mM GPNA plus 10nM CB-839, while increasing concentrations of CB-839 beyond this point resulted in increasing inter-drug antagonism (Fig. 4-3d). These results suggest that when MT cells are cultured in DMEM, increasing concentrations of CB-839 increases ASCT2 expression and localisation at the plasma membrane, which reduces the effectiveness of ASCT2 inhibition by GPNA.

In contrast, when MT cells were cultured in DMEM/F-12, increasing concentrations of CB-839 did not affect ASCT2 expression or membrane localisation (Fig. 4-3a).
When these cells were treated with the drug combination, synergy was observed at most concentrations tested (Fig. 4-3d). These results suggest that when MT cells are cultured in DMEM/F-12 and treated with CB-839, the additional amino acids and pyruvate content of the media prevents upregulation of ASCT2 expression and membrane localisation, resulting in an increased response to the additional treatment of GPNA. Because DMEM/F-12 better recapitulates the nutrient profile of serum compared to DMEM, this suggests that combined CB-839 and GPNA treatment could be efficacious against MMTV-MYC tumours in vivo.
Figure 4-3 CB-839 synergy with GPNA is dependent on ASCT2 expression
a, Immunofluorescence staining for ASCT2 of MT cells treated with CB-839 alone at the indicated concentrations in DMEM or DMEM/F-12 MMEC media (passage number = 7; n = 1). Staining was performed by Shravan Venkateswaran. b, Dose-response curves for GPNA and CB-839 in MT cells (passage numbers = 7, 7, and 9; n = 3) in DMEM or DMEM/F-12 MMEC media. c, Dose-response matrices showing percentage inhibition of MT cell proliferation and the indicated concentrations of combined GPNA and CB-839 treatment in DMEM or DMEM/F-12 MMEC media (passage numbers = 7, 7, and 9; n = 3). Percentage inhibition was calculated as follows: 100 – ((percent confluency untreated cells/percent confluency treated cells) x 100). d, Drug synergy heatmap for combined GPNA and CB-839 treatment in MT cells (passage numbers = 7, 7, and 9; n = 3) in DMEM or DMEM/F-12 MMEC media showing delta (δ) -score. δ-scores >0, <0, and 0 represent drug synergy, antagonism, and zero interaction, respectively. The zero-interaction potency (ZIP) model assumes no changes to dose-response curves for drugs that do not interact. ZIP synergy score represents the overall δ-score for the drug combination. Drug synergy calculations were performed using SynergyFinder. Graphs were generated using SynergyFinder. n indicates the number of biological replicates (different cell lines) used. Three repeats (technical replicates) were performed for each cell line. Graphs represent averages of three technical replicates for each cell line used.
4.5 Short-term dietary glutamine deprivation is not sufficient to decrease serum glutamine concentration and thus affect tumour growth

The results presented herein demonstrate that deleting Glis1 in the mammary gland delays MYC-driven tumour formation, while glutamine deprivation and small molecule inhibition of glutamine transport reduces the proliferative capacity of MT cells in vitro, even in the presence of NEAAs and pyruvate. I next investigated whether dietary glutamine deprivation would affect either MMTV-MYC or MMTV-ERBB2 tumour growth. A glutamine-deficient diet has been shown to decrease tumour growth in medulloblastoma xenografts (Niklison-Chirou et al., 2017). To overcome the time limitations of our spontaneous mammary gland tumour mouse models, tumours were induced by injection of one million freshly isolated MMTV-MYC or MMTV-ERBB2 tumour cells into the mammary fat pads of wild-type mice (Fig. 4-4a). Mice were weaned onto either a control (CT) or glutamine-deficient (-Q) diet between four and six weeks of age, and cells were injected between the ages of six and eight weeks. The median appearance of tumours following injection was three weeks, although some were palpable as early as nine days, while others appeared as late as 112 days.

While we previously observed that MT cells responded to glutamine deprivation in vitro, we did not observe a significant difference in ET cell or MT cell tumour latency or initiation upon dietary glutamine restriction (Fig. 4-4b-d). Measuring serum glutamine levels of the mice at the experimental end point demonstrated that, while there was a trend towards a decrease in serum glutamine levels in the glutamine deprivation condition, this was not significant (Fig. 4-4e). Serum glutamate levels were also decreased in mice bearing MYC tumours on the glutamine-deprived diet, although this did not reach statistical significance either ($P = 0.0587$). While Niklison-Chirou et al. (2017) demonstrated that the glutamine-deficient diet reduced tumour growth in a mouse model of medulloblastoma, the tumour xenografts in their study were produced by injecting tumour cells into the cerebellum and the authors found that the diet was able to reduce glutamine concentration in the cerebrospinal fluid (CSF; Niklison-Chirou et al., 2017). In our case, the diet was not sufficient to reduce
serum glutamine levels. Niklison-Chirou et al. (2017) showed that a mouse cohort provided with the -Q diet for four months exhibited significantly decreased CSF serine levels, whereas our mice were on the -Q diet for an average of 3.2 months. Therefore, mice may have to be kept on a -Q diet for a longer period to achieve a significant decrease in serum glutamine levels. However, Niklison-Chirou et al., (2017) observed tumour growth inhibition after approximately eight weeks, which was not observed in our experiment. This suggests that the CSF may be more susceptible to dietary elimination of glutamine compared to serum.

It has been demonstrated that glioblastoma cells cultured in human serum-like media are less sensitive to glutamine restriction than cells cultured in DMEM, with compensation being provided via \textit{de novo} glutamine synthesis by GS (Tardito et al., 2015). Our results indicate that the lack of glutamine in the diet can be compensated for by endogenous glutamine production to sustain serum concentrations. This may be provided by GS activity, which is post-translationally stabilised in response to glutamine deprivation (Van Nguyen et al., 2016). GS is ubiquitously expressed in mammalian tissues, with particularly high expression in the liver, brain, and muscle (Haussinger and Sies, 1984). Overall, these results suggest that longer-term dietary glutamine deprivation may be required to affect MYC-driven mammary gland tumour growth.
Figure 4-4 A glutamine-restricted diet does not alter ERBB2- or MYC-driven tumour latency or appearance

**a**, The experiment comprised four cohorts of mice injected with tumour-derived cells: 12x MMTV-MYC and 12x MMTV-ERBB2 fed a control (CT) diet, and 12x MMTV-MYC and 12x MMTV-ERBB2 fed a glutamine-deficient (-Q) diet. 4 mice did not develop tumours (1x MMTV-MYC CT diet, 2x MMTV-MYC -Q diet, and 1x MMTV-ERBB2 CT diet). **b**, Kaplan-Meier curve (ERBB2 tumours CT diet \( n = 12 \); ERBB2 tumours -Q diet \( n = 11 \)). **c**, Kaplan-Meier curve (MYC tumours CT diet \( n = 12 \); MYC tumours -Q diet \( n = 12 \)). \( P \) value was calculated using the Log-rank (Mantel-Cox) test. **d**, Scatter plot showing time taken for observable tumours to appear following tumour
cell injection into the mammary gland. Data are presented as mean ± s.d. Statistical analysis was performed using multiple two-tailed Student’s t-tests. Total pools of glutamine and glutamate in serum from cardiac puncture at experimental end point (ERBB2 tumours CT diet mice n = 8; ERBB2 tumours -Q diet mice n = 11; MYC tumours CT diet mice n = 11; MYC tumours -Q diet mice n = 8). Data are presented as mean ± s.d. Statistical analysis was performed using multiple two-tailed Student’s t-tests.
4.6 Chapter 4 summary

GLS1 inhibition has been investigated in multiple *in vitro* and *in vivo* studies to date (reviewed in Wang *et al.*, 2020), while the GLS1 inhibitor CB-839 is currently being utilised in multiple clinical trials (summarised in Meng *et al.*, 2019). However, no studies to date have investigated the specific link between MYC overexpression in breast tumours and GLS1 requirement. We aimed to address that in this section of the project. Furthermore, we evaluated whether inhibiting glutamine transport via ASCT2 by using the glutamine transport inhibitor GPNA would be more effective, either alone or in combination with GLS1 inhibition.

Our results demonstrated that both GPNA and CB-839 have therapeutic efficacy against MMTV-MYC tumour cells *in vitro*. However, this effect was diminished when the cells were cultured in DMEM/F-12 rather than DMEM. This was likely due to the additional presence of amino acids and pyruvate provided by the F-12. DMEM/F-12 better represents serum nutrient concentrations compared to DMEM alone, so we next evaluated the effectiveness of combined CB-839 and GPNA treatment in MT cells cultured in either DMEM/F-12 or DMEM. Importantly, our results showed that the drugs synergised at most of the concentrations tested when cells were cultured in DMEM/F-12, whereas CB-839 treatment primed MT cells cultured in DMEM to upregulate ASCT2 expression and plasma membrane localisation, diminishing the efficacy of GPNA for an overall antagonistic drug combination. These observations were in direct opposition to our original hypothesis that increased ASCT2 expression would sensitise cells to GPNA treatment. This highlights the necessity of carefully designing *in vitro* culture conditions when exploring the potential of drug combinations for *in vivo* studies. Nonetheless, these results suggest that combined GPNA and CB-839 treatment may represent a viable therapeutic treatment for MYC-enriched mammary gland tumours *in vivo*.

A previous study utilising the LAT1/SNAT2 inhibitor V-9302 in combination with CB-839 demonstrated that single treatment with CB-839 significantly reduced the concentrations of several glutamine-derived metabolites in liver cancer cells, including glutathione (Jin *et al.*, 2020). The authors found that additional treatment
with V-9302 further decreased glutathione levels, leading to sufficiently increased ROS levels to cause severe DNA damage and subsequent cell death (Jin et al., 2020). This was consistent with another study demonstrating reduced glutathione levels upon V-9302 treatment (Shulte et al., 2018). This suggests that the synergy we observed between GPNA and CB-839 in MT cells may be caused by a ROS-mediated decrease in proliferative capacity. These results are also concordant with our previous observation that glutathione disulphide levels are decreased in MMTV-MYC Gls1KO tumours.

We observed that ASCT2 expression and localisation at the plasma membrane was upregulated in MT cells upon increasing GLS1 inhibition by CB-839 when cultured in DMEM, but not DMEM/F-12. This is consistent with previous observations from our lab following Gls1 knockdown or GLS1 inhibition with BPTES in the cells, which was also found to increase ASCT2 N-glycosylation (Still et al., 2018). ASCT2 expression is upregulated in response to endoplasmic reticulum stress, which can be induced by glutamine deprivation (Chen et al., 2014). Furthermore, asparagine has been shown to completely rescue tumour cell proliferation in glutamine-deprived cells by restoring protein synthesis via asparagine-dependent induction of GS expression (Pavlova et al., 2018). When considered alongside our observations, this indicates that when MT cells are cultured in DMEM/F-12, the effect of GLS1 inhibition by CB-839 may be attenuated by de novo glutamine synthesis using asparagine. Indeed, MT cells were also far less responsive to glutamine deprivation when cultured in DMEM/F-12 compared to DMEM. The following model is proposed: ASCT2 expression and membrane-localisation is not induced upon GLS1 inhibition when MT cells are cultured in DMEM/F12 due to a lack of the necessary stress stimulus; this lack of increased ASCT2 expression makes cells more susceptible to pharmacological blockage by GPNA, resulting in a synergistic effect. This requires further confirmation by repeating the experiment using DMEM/F-12 lacking asparagine. This work is ongoing.

Finally, we explored the potential of dietary glutamine deprivation as a therapeutic intervention for MYC-enriched mammary gland tumours in vivo, using ERBB2-enriched tumours as a comparison. Our results showed that a glutamine-restricted diet was not able to significantly reduce serum glutamine levels. As such, no
differences in time taken for tumours to appear or tumour latency were observed. These results suggest that metabolic compensation was occurring to replenish serum glutamine levels in response to the restricted diet. This compensation could come from several sources, for example \textit{de novo} glutamine synthesis via GS.
Chapter 5. 

Psat1 deletion in the mammary gland must be combined with dietary serine and glycine deprivation to restrict MYC- and ERBB2-driven tumour growth

5.1 Introduction

Increased diversion of glucose carbon into serine synthesis has been observed in many types of cancer, including breast (Davis et al., 1970; Snell et al., 1984; Locasale et al., 2011). The SSP provides cancer cells with one carbon units for nucleotide biosynthesis, methylation reactions, alteration of cellular redox status, and the production of αKG from glutamate via PSAT1 (Fig. 1-5). Antifolate treatment as a cancer therapy dates to 1948, demonstrating that further interrogation of the SSP may provide novel, more sophisticated treatments (Farber and Diamond, 1948). For instance, antifolate therapy targets rapidly dividing cells in a non-specific manner, producing adverse effects in normal tissues such as the skin and bone marrow, while presenting significant risk of teratogenesis to a developing foetus if administered during pregnancy (Vogelzang et al., 2003; Lopes, Vincek, and Raez, 2005; Matok et al., 2009). On the other hand, a significantly more targeted therapeutic strategy that specifically disrupts proliferative capacity in specific cancer cells would ameliorate these issues.

Genes encoding SSP enzymes, including PSAT1, are transcriptional targets of MYC (Vazquez et al., 2011; Sun et al., 2015). Furthermore, these genes have been found to be elevated in human breast cancer (Possemato et al., 2011; Locasale et al., 2011). More recently, the expression of SSP enzymes has been specifically linked to MYCN in neuroblastoma cell lines (Xia et al., 2019). The recently published work from our lab has demonstrated that simultaneously inhibiting Psat1 expression and removing dietary serine and glycine is required to restrict tumour progression in MYC-driven liver tumours (Méndez-Lucas et al., 2020). These results suggested that targeting serine metabolism can be a plausible therapeutic strategy for MYC-driven tumours in general, and in MYC-driven breast tumours specifically.
PSAT1 catalyses the second reaction of the three that comprise the SSP, converting PHP to P-Ser, simultaneously converting glutamate to αKG. In PHGDH-amplified breast cancer cells, PSAT1 has been estimated to provide approximately 50% of glutamine-derived carbon to the TCA cycle, while it has also been demonstrated to promote tumour progression in vivo (Possemato et al., 2011; Gao et al., 2017). PSAT1 requires glutamate as a nitrogen donor to produce serine and has recently been shown to be upregulated in response to glutamine deprivation and GLS1 inhibition by CB-839 in non-small cell lung cancer cells (Jin et al., 2019). Concordantly, results from our lab have demonstrated that total serine levels are increased in MMTV-ERBB2 and MMTV-MYC tumours relative to the normal mammary gland (Still, 2018). Furthermore, serine synthesis from glucose and glutamine is greater in MMTV-MYC tumours than MMTV-ERBB2 tumours (Still, 2018). However, PSAT1 expression was increased in both types of tumours to same extent in comparison with normal mammary glands (Fig. 3-1a; Still, 2018). We therefore evaluated the requirement for PSAT1 in both MMTV-MYC and MMTV-ERBB2 tumours by deleting Psat1 via Cre-loxP-mediated recombination as previously described for the MMTV-MYC Gls1KO model (see Materials and methods).

5.2 Aim

In this section of the project, we aimed to investigate the requirement of PSAT1 in MYC-driven mammary gland tumours in vivo and to evaluate whether a serine- and glycine-deficient (-SG) diet can synergise with PSAT1 inhibition to therapeutic benefit.
5.3 Dietary serine and glycine deprivation is required to inhibit MYC- and ERBB2-driven mammary gland tumour growth in the absence of PSAT1

To investigate whether inhibiting serine biosynthesis has any effect on either MYC- or ERBB2- induced tumourigenesis in the mammary gland, we used Cre-loxP recombination (as previously described) to delete Psat1 (Psat1\(^{\text{KO}}\)) at the onset of either MMTV-ERBB2 or MMTV-MYC tumourigenesis. These experiments were performed in Crick mice. Both MMTV-MYC and MMTV-ERBB2 tumours formed in the absence of PSAT1 (Fig. 5-1a). Surprisingly, neither tumour latency nor initiation was affected (Fig. 5-2). To evaluate the effect of Psat1 deletion on serine biosynthesis and central carbon metabolism, tumour-bearing mice were given [U\(^{13}\)C]-glucose boluses, and tumour metabolites were analysed by LCMS. Psat1 deletion completely abolished serine biosynthesis from glucose and resulted in significantly decreased levels of serine in both types of tumours (Fig. 5-1b). Interestingly, while glycine synthesis from glucose was also inhibited, total levels of glycine in both types of tumours were much less affected (Fig. 5-1b). These results suggested that to maintain tumour progression in the absence of de novo serine and glycine biosynthesis, MMTV-MYC and MMTV-ERBB2 tumours replenish serine and glycine levels via import from circulation. Indeed, it has been shown that tumour growth is significantly reduced in mouse models of colorectal cancer and lymphoma upon dietary serine and glycine restriction (Maddocks et al., 2013; Maddocks et al., 2017). Serum levels of serine in MMTV-ERBB2 control mice were found to be approximately twice that of MMTV-MYC control mice, although tumour serine levels are approximately equivalent. However, MMTV-MYC tumours exhibit greater \(^{13}\)C incorporation into serine and glycine compared to MMTV-ERBB2 tumours, while total glycine levels are also higher in MMTV-MYC tumours by approximately two-fold. Together these data suggest greater uptake of serine from serum by MMTV-MYC tumours, and an increased rate of carbon flux through the SSP.

We next evaluated whether dietary serine and glycine restriction (-SG diet) would be sufficient to inhibit tumour progression in the absence of de novo serine and glycine biosynthesis. Mice were placed either on a synthetic -SG diet or a control (CT) diet
containing serine and glycine (see Materials and methods). Mice were transitioned gradually onto the diet between four and six weeks of age. In mice bearing either MMTV-MYC or MMTV-ERBB2 tumours, the -SG diet significantly depleted serum serine levels (Fig.5-1b). However, while tumour serine levels were significantly reduced in MMTV-ERBB2 tumours compared to controls, and less so in MMTV-MYC tumours, they were significantly increased relative to Psat1KO tumours, which was associated with significantly increased biosynthesis (Fig.5-1b).

Tumour latency and initiation of MMTV-ERBB2 tumours were unaffected by the -SG diet (Fig.5-2a&b). In contrast, placing mice bearing MMTV-MYC tumours on the -SG resulted in a modest but significant increase in tumour latency, but not initiation or growth rate (Fig.5-2e&f). These results demonstrate that both MMTV-ERBB2 and MMTV-MYC tumours can compensate for lack of dietary serine and glycine via de novo serine biosynthesis, although the -SG diet was sufficient to increase the latency of MMTV-MYC tumours.

Finally, we evaluated the effect of combining Psat1 deletion in the mammary gland with the -SG diet on MMTV-ERBB2 and MMTV-MYC tumour initiation and progression. The Psat1KO/-SG combination led to further decreases in serum and tumour serine levels compared to controls, and total abolition of de novo synthesis in tumours (Fig.5-1b). MMTV-ERBB2 tumours exhibited significantly increased tumour latency, with an increase in median tumour latency from 150 to 177.5 days, which was associated with delayed tumour initiation but not growth rate (Fig.5-2b&c). However, the Psat1KO/-SG combination produced a far more striking delay in tumour latency in MMTV-MYC tumours, increasing from a median of 234.5 days in controls to 373 days in Psat1KO/-SG tumours, which was associated with significantly delayed initiation, but no clear difference in growth rate (Fig.5-2e&f).

These data demonstrate that MMTV-MYC tumours are more susceptible to perturbations of serine metabolism than MMTV-ERBB2 tumours and provides good rationale for targeting serine metabolism in MYC-high breast tumours in humans.
Figure 5-1 Following Psat1 deletion in the mammary gland, serine and glycine synthesis in both MMTV-ERBB2 and MMTV-MYC tumours is completely abolished.

**a**, Western blot comparing PSAT1 expression between control and Psat1 KO MMTV-ERBB2 and MMTV-MYC tumours. **b**, Relative serum levels of serine, and tumour levels plus 13C incorporation into serine and glycine in tumours following boluses of [U13C]-glucose, detected by LCMS. Glycine in serum could not be detected. MMTV-ERBB2 tumours CT (n = 7), MMTV-ERBB2 Psat1KO tumours CT (n = 7), MMTV-ERBB2 tumours -SG (n = 6), MMTV-ERBB2 Psat1KO tumours -SG (n = 7), MMTV-MYC tumours CT (n = 9), MMTV-MYC Psat1KO tumours CT (n = 7), and MMTV-MYC tumours -SG (n = 6), MMTV-MYC Psat1KO tumours -SG (n = 7) are compared. Data are presented as mean ± s.d. Statistical analysis was performed using multiple two-tailed Student’s t-tests.
Figure 5-2 MMTV-ERBB2 tumour latency is increased following Psat1 deletion, and MMTV-MYC tumour latency is increased both by a serine- and glycine-deficient alone and when combined with Psat1 deletion

a-c, Psat1 deletion in MMTV-ERBB2 tumours only increases tumour latency and tumour initiation when combined with a serine- and glycine-deficient (-SG) diet, with no observable difference in growth rate. a, Kaplan-Meier curve. MMTV-ERBB2 tumours CT (n = 14); MMTV-ERBB2 Psat1KO tumours CT (n = 15); MMTV-ERBB2 tumours -SG (n = 12); MMTV-ERBB2 Psat1KO tumours -SG (n = 16). P value was calculated using the Log-rank (Mantel-Cox) test.

b, Scatter plot showing time taken for MMTV-ERBB2 tumour initiation. Data are presented as mean ± s.d. Statistical analysis was performed using multiple two-tailed Student’s t-tests.

c, Line graph showing MMTV-ERBB2 tumour growth rate. d-f, Psat1 deletion in MMTV-MYC tumours increases both tumour latency and tumour initiation when combined with a -SG diet, with no observable difference in growth rate. Latency alone also increases with the -SG diet. d, Kaplan-Meier curve. MMTV-MYC tumours CT (n = 18); MMTV-MYC Psat1KO tumours CT (n = 19); MMTV-MYC tumours -SG (n = 14); MMTV-MYC Psat1KO tumours -SG (n = 11). P values were calculated using the Log-rank (Mantel-Cox) test.

e, Scatter plot showing time taken for MMTV-MYC tumour initiation. Data are presented as mean ± s.d. Statistical analysis was performed
using multiple two-tailed Student’s $t$-tests. 

\[ f \]

Line graph showing MMTV-MYC tumour growth rate.
5.4 *Psat1* deletion, in combination with a serine- and glycine-deficient diet, alters ERBB2- and MYC-driven mammary gland tumour morphology

To investigate whether either *Psat1* deletion or the -SG diet, or their combination affected MMTV-ERBB2 and MMTV-MYC tumour histology, H&E staining was performed on control and *Psat1*KO tumours from mice either on the control diet or the -SG diet. Histological analysis of H&E-stained tumour sections was performed by Professor Gordon Stamp. MMTV-ERBB2 tumours in each group had a consistent, solid morphology, with every tumour receiving an arbitrary grading score of 2, signifying a moderate degree of differentiation (Fig. 5-3a-g).

MMTV-MYC tumour groups displayed far greater morphological differences than MMTV-ERBB2 tumours, consistent with our previous results (Fig. 3-3). However, while *Gls1*KO tumours exhibited a significantly larger degree of differentiation than controls, this was not the case following *Psat1* deletion, in mice either provided with the control or -SG diet (Fig. 5-4g). Notably though, the undifferentiated category was only found in control tumours. The greatest tendency towards significance was between the *Psat1*KO group and the *Psat1*KO/-SG diet combination group, with the latter being the most differentiated (*P* = 0.097 Fig. 5-4g). A therapeutic regimen that reduces the capacity of tumours to dedifferentiate is desirable, as this is known to be a significant factor in a tumour’s ability to acquire drug resistance and metastasise (Gabbert *et al.*, 1985). While further work is required to confirm whether this is the case, we investigated whether tumours from each group had differing abilities to metastasise (see 5.6).

In MMTV-ERBB2 tumours, Ki67 staining revealed tumours from both the *Psat1*KO and *Psat1*KO/-SG combination groups were significantly more positive for Ki67 (Fig. 5-3h). However, this is unlikely to be the result of increased proliferative capacity, given that tumour growth measurements between each group are not clearly distinct, and tumours from the combination group have increased latency and initiate later than controls (Fig. 5-2a-c). As discussed previously, increased positivity for markers such as Ki67 may be caused by DNA replication stress causing cell cycle arrest; the
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The aforementioned experiment involving pulse labelling of halogenated thymidine analogues would be required to confirm this. In MMTV-MYC tumours, consistent with significantly increased tumour latency, the percentage of Ki67-positive tumour cells was significantly decreased in the $Psat1^{\text{KO}}$-SG diet group in comparison with $Psat1^{\text{KO}}$ and -SG groups (Fig. 5-4h).

MMTV-ERBB2 $Psat1^{\text{KO}}$ tumours from mice on the -SG diet exhibited ischaemic features with increased dyscohesion of cells and more prominent infarction, leading to a characteristic “shotgun-like” pattern in tissue sections (Fig. 5-3d-f). These differences may be indicative of increased vascular fragility, but characterisation by immunohistochemical staining for a vascular marker such as CD31 is required to confirm this. Furthermore, staining for E-cadherin and/or $\beta$-catenin would confirm whether loss of cell-cell adhesion is responsible for the disruption observed in this group. This work is ongoing. These results suggest that $Psat1$ deletion in the mammary gland and removal of serine/glycine from the diet promotes differences in vascularisation that increases MMTV-ERBB2 tumour latency. This is further supported by work that has demonstrated that perturbation of the SSP in endothelial cells causes lethal vascular abnormalities in mice (Vandekeere et al., 2018).

The most striking feature to emerge in the MMTV-MYC $Psat1^{\text{KO}}$-SG combination group is the presence of dilated, branching vascular channels that are noticeably devoid of erythrocytes (Fig. 5-4a-c), compared to the other three groups (Fig. 5-4d-f). This was also observed, but to a far subtler degree, in MMTV-MYC tumours from mice on the -SG diet. Staining for vascular markers followed by comparative assessment of vascular diameter/density measurements would highlight these observed differences. This work is ongoing. However, these results suggest that the increase in tumour latency observed in the -SG and $Psat1^{\text{KO}}$-SG groups could be, in a similar manner to MMTV-ERBB2 tumours, the result of differences in vascular architecture between the tumours.
Figure 5.3 MMTV-ERBB2 \( Psat1^{\text{KO}} \) tumours from mice on a serine- and glycine-deficient diet are morphologically distinct from other groups

a-c, MMTV-ERBB2 tumours control diet example photos. All were solid/moderately differentiated and given an arbitrary grading score of 2 \( (n = 7) \). d-f, MMTV-ERBB2 \( Psat1^{\text{KO}} \) tumours -SG diet example photos. All were solid/moderately differentiated with a “shotgun-like” pattern and given an arbitrary grading score of 2 \( (n = 7) \). g, Stacked bar graph showing arbitrary grading scores as the percentage of tumours with that score. h, Quantification of Ki67 staining between MMTV-ERBB2 and MMTV-ERBB2 \( Psat1^{\text{KO}} \) tumours from mice either on the CT or -SG diets. Data are presented as mean ± s.d. Statistical analysis was performed using multiple two-tailed Student’s t-tests.
Figure 5-4 Psat1KO MYC-induced mammary gland tumours from mice on a serine- and glycine-deficient diet are morphologically distinct from other groups

a-c, MMTV-MYC Psat1KO tumours -SG diet example photos showing empty core/staghorn-like vasculature, compared to randomly selected tumours from the other groups (d-f). g, Stacked bar graph showing arbitrary grading scores as the percentage of tumours with that score. Average scores: MMTV-MYC tumours CT = 3.46; MMTV-MYC Psat1KO tumours CT = 3.25; MMTV-MYC tumours -SG = 3.59; MMTV-MYC Psat1KO tumours -SG = 3. h, Quantification of Ki67 staining between MMTV-MYC and MMTV-MYC Psat1KO tumours from mice either on the CT or -SG diets. Data are presented as mean ± s.d. Statistical analysis was performed using multiple two-tailed Student’s t-tests.
5.5 Serine metabolism is perturbed in ERBB2- and MYC-driven mammary gland tumours upon Psat1 deletion and dietary deficiency of serine and glycine

To evaluate the effect of Psat1 deletion and the -SG diet on the metabolism of MMTV-ERBB2 and MMTV-MYC tumours I performed [U\(^{13}\)C]-glucose boluses on tumour-bearing mice. As previously described, LCMS analysis revealed that in both MMTV-ERBB2 and MMTV-MYC tumours Psat1 deletion resulted in complete abolition of serine and glycine synthesis, with \(^{13}\)C enrichment decreasing to zero. This was accompanied by significant decreases in total serine pools. However, the -SG diet alone produced compensation via de novo serine synthesis in both MMTV-ERBB2 and MMTV-MYC tumours.

In MMTV-ERBB2 tumours, \(^{13}\)C enrichment into TCA cycle metabolites in each experimental group was unchanged (Fig. 5-5). However, increased \(^{13}\)C enrichment was observed into glycolytic metabolites in each experimental group compared to controls, suggesting an upregulation of glycolytic flux upon perturbation of serine metabolism. There was a significant decrease in \(^{13}\)C enrichment into AMP in the Psat1\(^{KO}\)/-SG combination group compared to controls and the -SG diet group, but a significant increase in the -SG group compared to every other group. Furthermore, a decrease in the total pools of GDP was observed in the combination group compared to controls (Fig. 5-6). Increases in 1C metabolites including homocysteine and SAH were also observed in the combination group compared to controls. When considered alongside the increase in serine and glycine enrichment observed in the -SG group, these results suggest that glycolysis is upregulated in response serine deprivation with the purpose of diverting carbon flux through the SSP, and that a significant portion of 1C units are utilised for nucleotide biosynthesis. However, either in the absence of Psat1 or when Psat1\(^{KO}\) is combined with the -SG diet, this is not possible. This leads to a decrease in the ability of the tumours to produce nucleotides de novo. Therefore, the increase in tumour latency observed in the combination group may be cause by DNA replication stress caused by inadequate nucleotide biosynthesis.
In MMTV-MYC tumours, $^{13}$C enrichment into TCA cycle metabolites was also unaffected in each experimental group (Fig. 5-7). However, in contrast to MMTV-ERBB2 tumours, total pools of TCA cycle metabolites in MMTV-MYC tumours were decreased in the combination group compared to controls (Fig. 5-8). Decreases in the total pools of amino acids including arginine and proline was observed. These decreases are likely caused by downregulation of amino acid synthesis, which can be associated with the increase in tumour latency observed in this group.

Consistent with the more pronounced effect on tumour latency in the -SG diet and Psat1$^{KO/-}$-SG diet combination groups, changes in $^{13}$C enrichment and total metabolite levels were more pronounced in MMTV-MYC tumours compared to MMTV-ERBB2 tumours. While MMTV-ERBB2 tumours exhibited decreased $^{13}$C enrichment into only AMP in the Psat1$^{KO/-}$-SG combination group compared to control and -SG diet groups, MMTV-MYC tumours additionally showed decreased enrichment into GMP and UMP in the Psat1$^{KO/-}$-SG group compared to all other groups. Furthermore, the decreases in enrichment were associated with decreased total pools of AMP, ADP, GMP, and GDP. Decreases in the total pools of metabolites involved with 1C metabolism, including methionine and dimethylglycine, were also observed in the Psat1$^{KO/-}$-SG group. These results suggest that 1C metabolism and nucleotide biosynthesis are also perturbed in MMTV-MYC tumours in the combination group, but to a greater extent. These observations are concordant with the larger effect size in terms of increased tumour latency observed for MMTV-MYC tumours compared to MMTV-ERBB2 tumours in the Psat1$^{KO/-}$-SG combination condition.

It has been recently demonstrated that exogenous serine is required for maximal proliferation in breast cancer cells with PHGDH copy number gain (Diehl et al., 2019). The SSP requires NAD+ to function, and the authors demonstrated that intracellular demand for NAD+ exceeded supply in these cells for adequate de novo serine synthesis, and that the provision of exogenous purine nucleobases can rescue cells deprived of serine (Diehl et al., 2019). It must be confirmed whether nucleotide deficiency caused by SSP ablation is the cause of the observations in MMTV-MYC and MMTV-ERBB2 tumours. This can be achieved via an in vitro rescue experiment, for example by providing Psat1$^{KO}$ cells deprived of serine and glycine with exogenous
purine nucleobases. If this can rescue cell growth it would indicate that replication stress caused by nucleotide insufficiency is the proximate cause for the observed increase in tumour latency.

Altogether this suggests that perturbation of serine metabolism by PSAT1 inhibition and removal of dietary serine and glycine may represent valuable therapeutic opportunities for both HER2- and MYC- enriched human breast tumours, but that MYC-enriched tumours would exhibit a relatively greater therapeutic response.
Figure 5-5 The serine synthesis pathway is upregulated upon dietary serine- and glycine deprivation, and blocked in MMTV-ERBB2 Psat1KO tumours

$^{13}$C incorporation into metabolites of the TCA cycle, glycolysis, and the pentose phosphate pathway following boluses of [U$^{13}$C]-glucose. MMTV-ERBB2 tumours CT ($n = 7$), MMTV-ERBB2 Psat1KO tumours CT ($n = 7$), MMTV-ERBB2 tumours -SG ($n = 6$), and MMTV-ERBB2 Psat1KO tumours -SG ($n = 7$) are compared. $^{13}$C incorporation is shown as percentage enrichment of total metabolite pools on the y-axis. $^{13}$C enrichment (%) represents the sum of the enrichment of all detected isotopologues. Data are presented as mean ± s.d. Statistical analysis was performed using multiple two-tailed Student’s t-tests.
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MMTV-ERBB2 tumours CT
MMTV-ERBB2 Psat1<sup>HC</sup> tumours CT
MMTV-ERBB2 tumours -SG
MMTV-ERBB2 Psat1<sup>HC</sup> tumours -SG

Glutamine
Glutamate
αKG
Succinate
Fumarate
Malate
Citrate
Aspartate
PEP
Lactate
Alanine
Glycine
Arginine
Asparagine
Proline
AMP
ATP
GMP
GDP
GTP
GDP
F6P
DHAP
Nicotinamide
NAD
NADP
Homocysteine
SAH
Methionine
Dimethylglycine
Cystathionine
Cysteine/sulfenic acid
GSH
GSSG
UMP
UDP
UTP
Urate
Figure 5-6 Total pools of metabolites are largely unaffected following *Psat1* deletion and serine/glycine removal from the diet in MMTV-ERBB2 mammary gland tumours

Key intermediates of glycolysis, the TCA cycle, and 1C metabolism, as well as amino acids and nucleotides are compared between MMTV-ERBB2 tumours CT (*n* = 7), MMTV-ERBB2 *Psat1*KO tumours CT (*n* = 7), MMTV-ERBB2 tumours -SG (*n* = 6), and MMTV-ERBB2 *Psat1*KO tumours -SG (*n* = 7) following boluses of [U^13C]-glucose. Data are presented as mean ± s.d. Statistical analysis was performed using multiple two-tailed Student’s *t*-tests.
The serine synthesis pathway is upregulated upon dietary serine- and glycine deprivation, and blocked in MMTV-MYC Psat1KO tumours. 13C incorporation into metabolites of the TCA cycle, glycolysis, and the pentose phosphate pathway following boluses of [U13C]-glucose. MMTV-MYC tumours CT (n = 9), MMTV-MYC Psat1KO tumours CT (n = 7), MMTV-MYC tumours -SG (n = 6), and MMTV-MYC Psat1KO tumours -SG (n = 7) are compared. 13C incorporation is shown as percentage enrichment of total metabolite pools on the y-axis. 13C enrichment (%) represents the sum of the enrichment of all detected isotopologues. Data are presented as mean ± s.d. Statistical analysis was performed using multiple two-tailed Student’s t-tests.
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Figure 5-8 Decreases in total metabolite pools are observed following *Psat1* deletion and serine/glycine removal from the diet in MMTV-MYC tumours

Key intermediates of glycolysis, the TCA cycle, and 1C metabolism, as well as amino acids and nucleotides are compared between MMTV-MYC tumours CT (n = 9), MMTV-MYC *Psat1*KO tumours CT (n = 7), MMTV-MYC tumours -SG (n = 6), and MMTV-MYC *Psat1*KO tumours -SG (n = 7) following boluses of [U\(^{13}\)C]-glucose. Data are presented as mean ± s.d. Statistical analysis was performed using multiple two-tailed Student’s *t*-tests.
5.6 Perturbation of serine metabolism decreases the metastatic potential of MYC- and ERBB2-driven mammary gland tumours

Thus far my results have demonstrated that deletion of *Psat1* in the mammary gland, combined with a -SG diet, significantly increases MMTV-ERBB2 and MMTV-MYC tumour latency, with a much larger effect observed in the latter. Both tumour types also exhibited changes in vascular architecture in the *Psat1*^KO/-SG groups. To evaluate whether this affected the abilities of these tumours to metastasise, we compared H&E-stained lung sections from each of the experimental groups. Characterisation of the observed lung tumours was performed by Professor Gordon Stamp.

MMTV-ERBB2 tumours did not produce any secondary tumours in the lung parenchyma but rather pulmonary intravascular tumours (Fig. 5-9a-c). Significantly fewer metastases were observed in the -SG group compared to controls, whereas the decreases observed in the *Psat1*^KO group (*P* = 0.1479) and the *Psat1*^KO/-SG group (*P* = 0.0568) narrowly avoided statistical significance (Fig. 5-9d). This likely reflected the low n numbers utilised. Most sets of lungs from MMTV-ERBB2 mice contained only a single tumour (Fig. 5-9e). In contrast, MMTV-MYC tumours produced metastatic lesions within the lung parenchyma (Fig. 5-10a-c). Lungs from the control group contained multiple tumours, with one set of lungs containing as many as 43 separate lesions (Fig. 5-10d&e). However, in each of the experimental groups, MMTV-MYC tumour metastasis was significantly decreased. Furthermore, the only set of lungs from each of the *Psat1*^KO and *Psat1*^KO/-SG groups that were positive for metastasis had only a single tumour each (Fig. 5-10e). It is clear from these results that *Psat1* deletion in the mammary gland and the -SG diet, both alone and in combination, significantly decreased metastatic potential of MMTV-MYC tumours.

It was recently demonstrated that TNBC cells are dependent on *PHGDH* expression to metastasise to the brain, as the brain CSF contains significantly lower concentrations of both serine and glycine (Ngo *et al.*, 2020). The authors also showed that *PHGDH* expression is enriched in human TNBC compared to other
subtypes, with the highest expression in metastatic tumours (Ngo et al., 2020). Pharmacological inhibition of PHGDH combined with serine- and glycine-deficient media resulted in DNA damage in TNBC cells, preventing cell cycle progression, which was rescued by nucleoside supplementation (Ngo et al., 2020). Furthermore, HER2-positive breast cancer cells implanted into the mouse brain significantly upregulated PHGDH expression, while increasing de novo serine biosynthesis (Ngo et al., 2020).

Our previous results demonstrated that 1C metabolism and nucleotide production is perturbed by targeting serine metabolism. Altogether this suggests that the inability of primary MMTV-MYC and MMTV-ERBB2 tumours to generate and/or acquire serine may be constraining their ability to metastasise to the lung, because of a diminished capacity to synthesise nucleotides.
Figure 5-9 More intra-arterial tumours were observed in lungs from mice bearing MMTV-ERBB2 tumours on the control diet

**a-c**, Example photos of lungs with intra-arterial tumours from mice bearing MMTV-ERBB2 tumours on the control (CT) diet. No tumours were observed in lung parenchyma. **d**, Bar graph showing percentage of lungs with metastatic lesions. MMTV-ERBB2 tumours CT ($n$ = 6), MMTV-ERBB2 $Psat^{fKo}$ tumours CT ($n$ = 5), MMTV-ERBB2 tumours -SG ($n$ = 6), MMTV-ERBB2 $Psat^{fKo}$ tumours -SG ($n$ = 7). Data are presented as mean $\pm$ s.d. Statistical analysis was performed using multiple two-tailed Student’s $t$-tests. **e**, Scatter plot showing number of malignant lung nodules per set of lungs with metastases.
Figure 5-10 More metastases were observed in lungs from mice bearing MMTV-MYC tumours on the control diet

a-c, Example photos of lung metastatic deposits from mice bearing MYC-driven mammary gland tumours. d, bar graph showing percentage of lungs with metastatic lesions. MMTV-MYC tumours CT (n = 12), MMTV-MYC Psat1KO tumours CT (n = 10), MMTV-MYC tumours -SG (n = 11), MMTV-MYC Psat1KO tumours -SG (n = 9). Data are presented as mean ± s.d. Statistical analysis was performed using multiple two-tailed Student’s t-tests. e, Scatter plot showing number of malignant lung nodules per set of lungs with metastases.
5.7 Chapter 5 summary

A direct link between MYC overexpression in breast cancer and the requirement for PSAT1 and/or dietary serine and glycine has not been established. We aimed to address this by first generating a mammary gland-specific \( Psat1^{KO} \) MMTV-MYC mouse tumour model, followed by evaluating how this affects tumour latency and tumour metabolism in comparison to the MMTV-ERBB2 mouse model. We then provided control mice with a -SG diet to investigate the effects of dietary serine and glycine deprivation on tumour initiation and progression. Finally, we combined the -SG diet and \( Psat1 \) deletion in the mammary gland.

Our results demonstrated that \( Psat1 \) deletion in the mammary gland significantly reduces tumoural serine levels and both MMTV-MYC and MMTV-ERBB2 tumours, and \textit{de novo} serine synthesis was abolished. There was no effect on tumour latency, as dietary serine and glycine was sufficient to compensate. We next evaluated the effectiveness of feeding mice a -SG diet upon tumour initiation and growth. Similarly, tumours were able to compensate by upregulating \textit{de novo} serine biosynthesis. However, only MMTV-MYC tumours exhibited increased latency. Finally, we combined \( Psat1 \) deletion in the mammary gland with the -SG diet and found that total tumour serine levels were significantly reduced in both MMTV-ERBB2 and MMTV-MYC tumours, and \textit{de novo} synthesis was abolished. Tumour latency increased in both tumour models, which was determined to be a result of delayed tumour initiation. The largest effect was observed in MMTV-MYC tumours, for which median tumour latency was increased by over 60%.

Further investigation revealed significant histological differences in both MMTV-ERBB2 and MMTV-MYC tumours from the \( Psat1^{KO}\) -SG combination groups. MMTV-ERBB2 tumours from each group were homogeneous with respect to their differentiation status, with each being moderately- to well-differentiated. However, MMTV-ERBB2 tumours from the combination group exhibited a characteristic “shotgun-like” pattern, suggesting blood vessels in these tumours may be more fragile and more prone to ischaemia and infarction. MMTV-MYC tumours were relatively heterogeneous compared to MMTV-ERBB2 tumours and tended towards more differentiation following perturbation of serine metabolism in each of the three
experimental groups. However, MMTV-MYC tumours from the combination group had a markedly different vasculature, with a characteristic “empty core/staghorn” pattern. Blood vessels in these tumours were relatively dilated with more branches, while also strikingly devoid of erythrocytes. The differences in vasculature for both MMTV-ERBB2 and MMTV-MYC combination groups require further confirmation. However, at this stage these results suggest that perturbation of serine metabolism by \textit{Psat1} deletion in the mammary gland and removal of serine and glycine from the diet increases tumour latency potentially due to changes in tumour vasculature.

Metabolomics revealed reduced glucose-derived carbon incorporation into, and decreased total levels of, nucleotides in the combination groups. However, MMTV-MYC tumours additionally had decreased total levels of 1C metabolites. This suggests that both MMTV-ERBB2 and MMTV-MYC tumours experience dysregulated nucleotide biosynthesis due to disrupted serine metabolism, although to a more significant extent in MMTV-MYC tumours. Further work is required to confirm whether disrupted nucleotide biosynthesis contributes to the increased latency of these tumours.

Finally, while lungs from mice bearing MMTV-ERBB2 tumours exhibited metastatic lesions presenting as intra-arterial tumours, lungs from mice bearing MMTV-MYC tumours contained metastases within the lung parenchyma. Perturbation of serine metabolism lowered the metastatic potential of both types of tumours. However, consistent with our other observations, the greatest effect size was observed in mice bearing MMTV-MYC $\text{Psat1}^{\text{KO}}$ tumours on the -SG diet. The metastatic potential of MMTV-MYC tumours was reduced by $\text{Psat1}$ deletion alone, the -SG diet alone, and the $\text{Psat1}^{\text{KO}}$/-SG combination. Considered alongside our metabolomics results, this suggests that targeting serine metabolism may reduce the metastatic potential of MMTV-MYC and MMTV-ERBB2 tumours due to insufficient nucleotide biosynthesis, caused by perturbed 1C metabolism.

Altogether, these results demonstrate that targeting PSAT1 alone may be therapeutically beneficial for human patients with MYC-enriched breast tumours, but that dietary serine and glycine restriction would likely also be required to achieve the maximum therapeutic response.
Chapter 6. Discussion

Metabolic reprogramming is now widely recognised as a hallmark of cancer (Hanahan and Weinberg, 2011). The activities of a variety of metabolic pathways have been shown to be upregulated in tumours compared to normal tissue, allowing for the increased production of macromolecules, ATP, and reducing equivalents to meet the increased bioenergetic and growth demands of cancer cells. These pathways include glycolysis, glutaminolysis, the PPP, the SSP, lipogenesis and lipolysis, and mitochondrial biogenesis (Warburg, 1924; Warburg, 1927; Jonas et al., 1992; Wise et al., 2008; Locasale et al., 2011; Kuhajda et al., 1994; Li et al., 2005; Samudio et al., 2010; Pike et al., 2011). Importantly, the precise metabolic profile of a tumour is dependent on both tissue of origin and initiating oncogenic lesion (Yuneva et al., 2012).

In addition to MYC, other oncogenes including RAS and BRAF have been noted for their ability to promote metabolic network rearrangement. RAS, for example has been shown to upregulate glycolysis to allow for increased shunting of intermediates through the PPP and HBP, while also encumbering pancreatic ductal adenocarcinoma cells with GOT1 and GOT2 dependence for glutamine metabolism (Ying et al., 2012; Son et al., 2013). BRAF on the other hand decreases the capacity of melanoma cells to conduct oxidative phosphorylation, resulting in dependence on glycolysis for ATP production (Ferretta et al., 2016). These specific metabolic dependencies, caused by specific oncogenic lesions, have allowed researchers to explore the possibility of targeting them therapeutically as a novel approach to cancer treatment. MYC is known to regulate metabolic genes involved in nucleotide biosynthesis, glycolysis, and glutaminolysis, and increasing attention has been paid to the role of metabolic reprogramming by MYC in cancer over the past few decades (Pusch et al., 1997; Osthus et al., 2000; Kim et al., 2004; Wise et al., 2008; Morrish et al., 2009; Wang et al., 2011; Yue et al., 2017).

Much research has focussed on exploiting metabolic differences between tumours and healthy tissues, with the aim of perturbing specific metabolic pathways that will leave healthy tissues unaffected while being cytostatic or cytotoxic to cancer cells. Genetic deletion of enzymes involved in each of the aforementioned pathways have
demonstrated therapeutic effect in mouse models of cancer (Kuemmerle et al., 2011; Possemato et al., 2011; Xiang et al., 2015; Davidson et al., 2016; Li et al., 2016; Zou et al., 2016; Benito et al., 2017). However, it is becoming increasingly appreciated that metabolic flexibility must be considered when targeting tumours using a metabolism-based approach, as exemplified by recent published results from our lab (Méndez-Lucas et al., 2020). Therapeutic targeting of a particular metabolic pathway can be compensated for by expression of an alternative isoform of the target enzyme, or upregulation of an alternative metabolic pathway. It is therefore essential to identify these metabolic compensations and investigate whether a combinatorial approach would be more efficacious.

The results presented herein demonstrate that cancer cells can resist therapeutically exerted metabolic pressures in vivo and highlights the requirement to identify further metabolic targets for the purpose of developing an effective combination therapy. The MMTV-MYC model was more susceptible to perturbations of serine metabolism than the MMTV-ERBB2 model, consistent with the role of MYC in coordinating a distinct growth-promoting metabolic programme and previous results from our lab (Still, 2018). However, we also found that the effects of Glis1 deletion could be masked by control tumours following a change in experimental environment. Be that as it may, by deleting specific metabolic genes in the mammary glands of mice, we evaluated their potential as therapeutic targets and were able to generate tumours with the aim of identifying further metabolic vulnerabilities that, when targeted, might synergise to increased therapeutic benefit.

6.1 The susceptibility of MYC-driven breast tumours to perturbation of glutamine metabolism

6.1.1 GLS1 inhibition as a therapeutic strategy

We first deleted Glis1 in the mammary glands of mice and compared tumour latency between MMTV-MYC and MMTV-ERBB2 models. We performed this experiment while relocating to a new institution and found that, while a significant increase in tumour latency was observed for the Glis1KO ‘NIMR’ cohort, this effect was reduced in the ‘Crick’ cohort. This was a striking observation and highlights the widespread
problem of reproducibility in biomedical research. Simply changing the environment in which the mice were bred and housed was sufficient to eliminate the biological effect we observed from Gls1 deletion.

The environmental impact upon in vivo mouse model research is a crucial determining factor in the reproducibility of experiments. As such, laboratory conditions are strictly controlled. However, these conditions inevitably vary between institutions, resulting in reproducibility issues. For example, variations in gut microbiota in mice can impact the severity of a range of pathological conditions, including metabolic syndromes (Su et al., 2016; Zachariassen et al., 2017; Franklin and Ericsson, 2017). The two cohorts of mice used herein to investigate the effect of Gls1 deletion in MMTV-MYC tumours were from either open-top cages (NIMR) or individually ventilated cages (Crick), which can affect the endocrinological profiles and levels of anxiety experienced of both male and female mice (Macrides, Bartke, and Dalterio, 1975; Nichols and Chevins, 1981; Polissidis et al., 2017). Even whether mice are housed in mixed-sex or single-sex rooms has been shown to affect experimental outcomes (Lloyd et al., 2018). These observations highlight the necessity to report the laboratory conditions in which in vivo experiments are conducted.

In the present report, SIAM using [U$^{13}$C]-glutamine revealed that glutamine catabolism is significantly decreased in MMTV-MYC Gls1$^{KO}$ tumours from NIMR mice, with an associated decrease in glutamine-dependent anaplerosis. However, glutaminolytic flux was still observed, albeit to a significantly lower extent. This indicates that metabolic compensation is occurring. Our lab has shown that, in MYC-driven liver tumours, this compensation can come from upregulation of Gls2 and amidotransferase expression (Méndez-Lucas et al., 2020). Western blot and qPCR revealed that GLS2 is unlikely to be the source of this compensation in MMTV-MYC tumours, but the RNA-seq results suggest that the amidotransferases involved in nucleotide biosynthesis may be involved. In Crick mice, control tumours were more resemblant of Gls1$^{KO}$ tumours and SIAM suggested that their metabolism had changed independent of GLS1 activity. Nonetheless, our results demonstrate the efficacy of Gls1 deletion in the mammary glands of NIMR mice.
Interestingly, $Gls1^{KO}$ MMTV-MYC tumours exhibited identifiable differentiated growth patterns, in contrast to control tumours that were largely undifferentiated, solid masses. It has been shown that increased glutamine metabolism in tumours can lead to a significant decrease in glutamine concentration relative to adjacent normal tissue (Kamphorst et al., 2016). Furthermore, low regional intratumoural glutamine levels in solid tumours have been shown to promote dedifferentiation due to histone hypermethylation, caused by depleted $\alpha$KG levels and thus $\alpha$KG-dependent histone demethylase activity (Pan et al., 2016). Epigenetic modification is highly dynamic and dependent on a variety of input signals, a significant portion of which are relayed by cellular metabolic pathways. Another example are the ten-eleven translocation (TET) proteins, which are $\alpha$KG-dependent dioxygenase enzymes that contribute to epigenetic regulation via the oxidation of 5-methylcytosine residues to promote DNA demethylation. TET2 signalling has been found to regulate cellular differentiation state in luminal breast epithelial cells (Wu et al., 2017). Glutamine is a significant source of $\alpha$KG, thereby linking epigenetic regulation with glutamine metabolism.

Dedifferentiation is crucial for a tumour to acquire drug-resistance, as well gaining the ability to seed metastatic lesions in other tissues (Gabbert et al., 1985). It therefore follows that a reduced capacity to dedifferentiate is desirable as a therapeutic outcome if complete destruction of cancer cells is not possible. Although MMTV-MYC $Gls1^{KO}$ tumours did not differ significantly from controls in terms of total tumoural glutamine concentration, this does not necessarily imply that glutamine concentrations were equal in each tumoural region. While further work is required to assess the relative intratumoural levels of glutamine following $Gls1$ deletion in MMTV-MYC tumours, our results may suggest that control MMTV-MYC tumours experience depleted regional glutamine levels, whereas $Gls1^{KO}$ tumours do not due to their impaired ability to catabolise glutamine. This may affect the ability of MMTV-MYC $Gls1^{KO}$ tumours to dedifferentiate by perturbing metabolic regulation of epigenetic modification, possibly mediated by $\alpha$KG levels.

Our RNA-seq data demonstrated that pathways involved in ECM production were downregulated in MMTV-MYC $Gls1^{KO}$ tumours. The ECM can promote cancer cells to dedifferentiate via the induction of the epithelial-mesenchymal transition (EMT), for example through collagen-mediated activation of NF$\kappa$B signalling (Mani et al.,
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2008; Medici and Nawshad, 2010; Liu et al., 2018). Furthermore, Gls1KO tumours had depleted proline pools, while decreased αKG synthesis in these tumours may have affected αKG-dependent hydroxylation of collagen (Stegen et al., 2019). This may suggest that Gls1 deletion in MMTV-MYC tumours resulted in downregulated ECM production due to reduced αKG synthesis and total proline levels, impairing their capacity to dedifferentiate.

Associated with the increased tumour latency and differentiated morphology of Gls1KO tumours was an increase in Ki67- and PHH3-positive cells. While these markers are typically associated with proliferative capacity, Gls1KO tumours had increased latency, indicating that cells were arresting during mitosis rather than proliferating at an increased rate. Breast cancer cells have been shown to occupy S-phase at a significantly increased incidence upon glutamine deprivation, while provision of exogenous nucleotides can rescue abortive S-phase in KRAS-transformed fibroblasts (Gaglio et al., 2009; Gwangwa, Joubert, and Visagie, 2019). Furthermore, GLS1 activity has been shown to be higher in S-phase, while cells in which GLS1 has been silenced fail to progress from S-phase to G2/M (Columbo et al., 2011). In this context, our results suggest that Gls1 deletion increases the incidence of mitotic arrest in MMTV-MYC tumours, due to diminished glutamine catabolism. To confirm this, we are estimating relative cell cycle lengths between the two sets of tumours using pulse-labelling of halogenated pyrimidine derivatives (Martynoga et al., 2005). At time of writing, tumour bearing mice have been injected with 5-chloro-2′-deoxyuridine (ClDU) and 5-iodo-2′-deoxyuridine (IDU) and tumour samples have been collected. This work is ongoing.

The [U^{13}C]-glutamine labelling experiment must be repeated in control and Gls1KO MMTV-MYC tumours from Crick mice, while also investigating the relative expression of GLS2 and transamidase enzymes between control and Gls1KO tumours. While we demonstrated that GLS2 expression is not upregulated following Gls1 deletion in MMTV-MYC tumours from the original NIMR cohort, the disparity in the data we observed between those mice and Crick mice warrants further investigation.
6.1.2 Dual targeting of GLS1 and ASCT2 as a therapeutic strategy

ASCT2, the product of the SLC1A5 gene, is one of fourteen glutamine transporters found in mammals. Numerous studies utilising different models of cancer have demonstrated the efficacy of shRNA-mediated knockdown of SLC1A5 in preventing cancer cell proliferation both in vitro and in vivo (Hassanein et al., 2015; van Geldermalsen et al., 2016; Cormerais et al., 2018). Further to this work, previous results from our laboratory demonstrated a relative increase in glutamine catabolism in MMTV-MYC versus MMTV-ERBB2 tumours, and that this was associated with significantly increased presence of ASCT2 at the plasma membrane in MMTV-MYC tumour cells (Still, 2018). Concordantly, decreasing the expression of ASCT2 inhibited MMTV-MYC-derived tumour cell proliferation in vitro (Still, 2018). In a non-pathological setting, ASCT2 has been shown to mediate rapid influx of glutamine into naïve T-cells upon activation, upon which signal transduction from the T-cell receptor to mTORC1 was dependent (Nakaya et al., 2014). However, following full-body deletion of Slc1a5 in mice no defects in immune function were observed, with normal B-cell and T-cell development and a normal B-cell germinal centre response (Masle-Farquhar et al., 2017). Therefore, targeted inhibition of ASCT2 using a small molecule represented a viable potential therapeutic strategy for treating MMTV-MYC tumours in vivo.

At time of writing, there are no available specific ASCT2 inhibitors. Much of the work demonstrating the efficacy of glutamine transport inhibition in cancer has utilised glutamine antagonists including benzylserine and GPNA (Grewer and Grabsch, 2004; Hassanein et al., 2015; Wang et al., 2015; van Geldermalsen et al., 2016; Broer et al., 2016; Marshall et al., 2017; Chiu et al., 2017; van Geldermalsen et al., 2018). However, V-9302 was recently published as a specific ASCT2 inhibitor and demonstrated both in vitro and in vivo efficacy against colorectal cancer cells (Schulte et al., 2018). Unfortunately, V-9302 was subsequently shown to inhibit LAT1 and SNAT2, not ASCT2 (Broer et al., 2018). Nevertheless, the importance of developing small molecule inhibitors that target different glutamine transporters is highlighted by studies demonstrating compensation from other transporters when ASCT2 is inhibited. For example, increased SNAT1 and SNAT2 expression has been
shown to rescue osteosarcoma cells following an amino acid starvation response elicited by \textit{SLC1A5} deletion (Broer \textit{et al.}, 2019).

Using MMTV-MYC-derived tumour cells, the work presented herein demonstrates that inhibiting glutamine transport using GPNA adversely affects proliferative capacity. However, inhibition of proliferation is greater when cells are cultured in DMEM rather than DMEM/F-12 MMEC media. It has previously been shown that \textit{SLC1A5} deletion only modestly inhibited the proliferation of colon and lung adenocarcinoma cells \textit{in vitro}, whereas when the same cells were transplanted into mice tumour growth was strongly inhibited (Cormerais \textit{et al.}, 2018). This exemplifies that the relative nutrient profiles of both cell culture media and serum can significantly affect the outcome of drug testing experiments.

Building on the observation that inhibiting GLS1 activity increases ASCT2 expression and membrane localisation, I tested whether GLS1 inhibition by CB-839 would have an antiproliferative effect in MT cells. Like GPNA treatment, CB-839 reduced MT cell proliferation cultured in either medium, but to a greater extent in DMEM MMEC media. To evaluate whether the associated increase in ASCT2 expression would sensitise MT cells further to inhibition of glutamine transport by GPNA, I next used both CB-839 and GPNA in a combinatorial approach.

We identified regions of synergy, antagonism, and no interaction between the drugs. When MT cells were cultured in DMEM MMEC media, CB-839 induced ASCT2 upregulation and localisation at the plasma membrane, consistent with previous observations from our lab (Still, 2018). This resulted in overall drug antagonism between CB-839 and GPNA, with the antiproliferative effect of GPNA reducing with increasing ASCT2 expression. Synergy was observed however between the lowest concentration of CB-839 tested and each concentration of GPNA, indicating that in this context ASCT2 upregulation was not sufficient to counteract glutamine transport inhibition by GPNA. Antagonism driven by GLS1 inhibition was observed with increasing CB-839 concentration past this point.

However, when the experiment was repeated using cells cultured in DMEM/F-12 MMEC media, synergy between the drugs occurred at most concentrations tested.
Concordantly, CB-839 was unable to increase ASCT2 expression when MT cells were cultured in DMEM/F-12 MMEC media. F-12 contains all 20 proteinogenic amino acids, including glutamine, although the DMEM/F-12 we use lacks glutamine, which we add in later during media preparation. It is likely that when cells were cultured in media containing F-12, the additional presence of amino acids and pyruvate made the cells less sensitive to inhibition of GLS1 by CB-839. Therefore, GLS1 inhibition was unable to produce ASCT2 upregulation, thereby sensitising the cells to GPNA treatment. Previous work indicates that the rescue factor could be asparagine, which was found to become essential upon glutamine deprivation in breast cancer cells (Pavlova et al., 2018). This was found to be via asparagine-dependent upregulation of GS, which allowed the cells to become self-sufficient regarding glutamine. The authors found that ectopic expression of asparaginase prevented the cells from accumulating asparagine, re-sensitising the cells to glutamine deprivation (Pavlova et al., 2018).

Our results suggest that targeting glutamine transport and GLS1 as a combination strategy could be therapeutically viable. However, rescue factors must be identified if dual inhibition of GLS1 and glutamine transport fail to completely prevent the initiation and progression of MYC-driven breast tumours in vivo. For example, to identify if asparagine is preventing GLS1 inhibition-mediated upregulation of ASCT2, removing asparagine as a constituent of the cell culture medium would be necessary. Furthermore, asparaginase could be used alongside the CB-839/GPNA drug combination. Using asparaginase is a well characterised method of depleting serum asparagine levels for the treatment of acute lymphoblastic leukaemia (Egler, Ahuja, and Matloub, 2016). Asparaginase has also been shown to synergise with GLS1 inhibition in colon cancer cells (Li et al., 2017). Moreover, asparaginase has shown efficacy in breast cancer cell xenografts (Shiromizu et al., 2018). Whether asparaginase has any potential utility in combination with GLS1 inhibition and glutamine transport inhibition in MYC-high mammary gland tumour cells remains to be determined.

Drug combination therapy has proven to be an invaluable tool for cancer treatment, as it allows for the bypassing of adaptive mechanisms used by cancer cells to develop drug resistance. Breast tumours, like all tumours, exist as heterogeneous
populations of cells and thus subclonal populations of cells within the same tumour respond differently to the same therapeutic (Stephens et al., 2012; Duncan et al., 2012). Furthermore, therapeutics act as a selection pressure upon cancer cells, leading to drug-resistant subclonal outgrowth (Turke et al., 2010; Sequist et al., 2011; Choi et al., 2010). It also usually takes 10-15 years for a new drug to be developed following novel therapeutic target identification (DiMasi et al., 2003; Paul et al., 2010). Therefore, finding new, effective combinations would be more time efficient.

Our results suggest that a combination therapy comprising GLS1 plus glutamine transport inhibition could be utilised for the treatment of MYC-high breast tumours. However, these results must be validated in vivo. This combination could also be useful for other cancers that overexpress GLS1 and ASCT2 simultaneously. However, as previously discussed, the search for a specific ASCT2 inhibitor is still ongoing. Recently, a splice variant of ASCT2 specific for mitochondrial glutamine transport was discovered (Yoo et al., 2020). Therefore, further investigation is required, not only into specific targeting of ASCT2, but even more specifically targeting of cytoplasmic versus mitochondrial ASCT2.

6.1.3 Dietary glutamine deprivation as a therapeutic strategy

In vitro glutamine deprivation has proven to be efficacious in killing cancer cells, with this effect being mediated by what is referred to as ‘glutamine addiction’ in the literature (Qing et al., 2012; Chen et al., 2015). However, the in vivo efficacy of dietary glutamine restriction as treatment strategy for breast tumours remains to be determined. While Niklison-Chirou et al. (2017) were able to demonstrate that a -Q diet could reduce growth of medulloblastoma xenografts by mediating a decrease in glutamine levels in the CSF, we were unable to reproduce this effect in our MYC-driven breast cancer model as dietary glutamine deprivation was insufficient to lower serum glutamine levels. As the lengths of time that mice were kept on the -Q diet were approximately the same between Niklison-Chirou et al. (2017) and our own study, this indicates that the CSF is more susceptible to dietary glutamine deprivation than serum. This highlights the importance of considering the ability of certain nutrient-deficient diets to modulate metabolite levels in different compartments (e.g., serum versus CSF).
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The most likely source of compensation for lack of dietary glutamine is glutamine synthetase (GS). GS is subject to feedback regulation by glutamine, which promotes its degradation via ubiquitylation (Arad, Freikopt, and Kulka, 1976; Crook and Tomkins, 1978; Van Nguyen et al., 2016). Furthermore, glioblastoma cells cultured in human serum-like media exhibited resistance to glutamine restriction due to de novo glutamine synthesis by GS (Tardito et al., 2015). When considered alongside our results, this suggests that dietary glutamine deprivation alone will likely be insufficient as a therapeutic strategy to treat human breast tumours. However, it has also been shown that GS expression is increased in sarcoma cells upon glutamine deprivation, which rescues proliferation (Issaq et al., 2019). Moreover, knockdown of GS abolishes proliferation in sarcoma cells both in vitro and in vivo (Issaq et al., 2019). Therefore, assessing GS activity in MMTV-MYC tumours following glutamine deprivation in vivo could reveal GS as a secondary, synergistic therapeutic target.

6.2 The susceptibility of MYC-driven breast tumours to perturbation of serine metabolism

Serine deprivation has been shown to inhibit cancer cell proliferation both in vitro and in vivo in numerous different models of cancer, while serine has also been shown to be the second-highest consumed amino acid across 60 cancer cell lines, after glutamine (Jain et al., 2012; Maddocks et al., 2013; Labuschagne et al., 2014; Maddocks et al., 2017). In breast cancer cells the serine biosynthesis genes PHGDH, PSAT1, and PSPH, and the serine transporter SLC1A4 are each upregulated, while survival of these cells in serine-free conditions was shown to be dependent on PSAT1 expression (Pollari et al., 2010). In addition to de novo synthesis, serine can also be obtained via dietary intake. Breast cancer cells have been shown to be able to survive serine deprivation due to the increased expression of the SSP enzymes, while siRNA-mediated knockdown or small molecule-mediated inhibition of PHGDH inhibits breast cancer cell line proliferation in vitro and xenografts in vivo (Possemato et al., 2011; Pacold et al., 2016; Mullarky et al., 2016). Upon serine starvation, PKM2-mediated conversion of phosphoenolpyruvate (PEP) to pyruvate is downregulated, promoting diversion of glucose carbon toward serine synthesis via the serine synthesis pathway (SSP). The SSP enzymes are transcriptionally upregulated by
MYC, and SSP inhibition is selectively toxic to MYC-amplified cells (Sun et al., 2015; Xia et al., 2019). These studies suggest that targeting serine metabolism could be a valuable therapeutic option for breast cancer patients with MYC-enriched tumours, although dietary serine must be considered.

6.2.1 PSAT1 inhibition and dietary serine and glycine deprivation as a therapeutic strategy

The work presented herein aimed to evaluate the requirement of PSAT1 for the initiation and progression of MMTV-MYC tumours, using the MMTV-ERBB2 model for comparison. Furthermore, we aimed to investigate whether a SG diet could provide a therapeutic benefit both alone and in combination with Psat1 deletion in the mammary gland. We found that a -SG diet could effectively reduce serum serine levels and increased tumour latency both alone and in combination with Psat1 deletion in the MMTV-MYC model. This contrasted with the MMTV-ERBB2 model, which only showed a modest effect when the two were combined. Our results showed that this increase in tumour latency can be attributed to an increase in the amount of time it takes tumours to initiate for both models but did not result in different tumour growth rate once established.

6.2.2 Psat1 deletion and the -SG diet perturbs breast tumour metabolism

Following Psat1 deletion in MMTV-ERBB2 and MMTV-MYC tumours, de novo biosynthesis of serine, and consequently glycine, was completely abolished. When provided with a diet including serine and glycine, tumours were able to compensate for this shortfall with enhanced exogenous uptake. Likewise, enhanced de novo synthesis could compensate when mice were fed the -SG diet. These results highlight the necessity of taking both endogenous and exogenous sources of serine into account for the development of a therapeutic strategy to target human breast tumours.

In each of the experimental groups, MMTV-ERBB2 tumours exhibited increased $^{13}$C labelling in glycolytic metabolites compared to controls, indicating increased
glycolytic flux. The same was also true for MMTV-MYC tumours, except for the combination group. The most likely reason for these observations is an attempt by the tumours to increase carbon flux into the SSP to compensate for the decreases to total serine availability in each of the experimental conditions, which is supported by the observation that carbon labelling into serine and glycine is significantly increased in MMTV-ERBB2 tumours from mice on the -SG diet, and glycine in MMTV-MYC tumours. However, while total pools of 1C metabolites in MMTV-ERBB2 tumours were increased in the Psat1KO/-SG diet group, decreases were observed in the same group for MMTV-MYC tumours. This demonstrates that 1C metabolism was perturbed in both types of tumours, but in different ways.

The importance of serine as a primary contributor of carbon units to 1C metabolism is well established, which in turn is essential for nucleotide biosynthesis and the methylation of DNA and histones (Herbig et al., 2002; Davis et al., 2004). As such, altering cellular metabolism to support these processes drives tumour initiation and progression. Tumours have been shown to display significant alterations in terms of DNA methylation relative to their tissues of origin, and methylation patterns are inherited by daughter cells (Feinberg and Vogelstein, 1983; Goelz et al., 1985; Lorincz et al., 2002). These studies provide evidence for epigenetic abnormalities preceding cancer cell initiation. Considering this in the context of our results, the disruption of 1C metabolism caused by insufficient intracellular serine levels could delay tumour initiation via an epigenetic mechanism. To reveal the exact mechanism of tumour initiation delay, experiments designed to eliminate successive possibilities should be performed. For example, performing rescue experiments with either formate or hypoxanthine and comparing the extent of the rescue. This would reveal the extent to which the contribution of serine to nucleotide biosynthesis versus methyltransferase reactions is responsible for the observed increase in tumour latency, while a methyltransferase assay and genome-wide methylation profiling would further detail any differences produced by Psat1 deletion and the -SG diet.

In MMTV-ERBB2 tumours we found that $^{13}$C labelling into AMP was increased compared to controls in the -SG condition and decreased in the combination, with total nucleotide pools following this trend. Similarly, there was a trend toward an increase in labelling in the -SG condition for MMTV-MYC tumours and a significant
decrease in the combination, both of carbon labelling and total pools. These results suggest that in the \textit{Psat1}^{\text{KO}} condition, exogenous serine and glycine can fully compensate for impaired nucleotide biosynthesis due to lack of endogenous serine production, which is also supported by the fact that latency in these tumours is unchanged \textit{versus} controls. Furthermore, total pools of urate, the end product of purine metabolism, are significantly decreased in these tumours. These results suggest that impaired nucleotide biosynthesis could also be a causal factor of increased tumour latency, but, as previously mentioned, a rescue experiment utilising exogenous nucleotides is required to strengthen this claim.

The overexpression of SSP and 1C metabolism genes has been specifically linked to \textit{MYCN} amplification in neuroblastoma cell lines. MYCN directly binds the promoter regions of SSP and 1C metabolism genes, including \textit{PSAT1}, with \textit{MYCN} knockdown reducing their expression (Xia \textit{et al.}, 2019). MYCN was shown to inflict dependence upon glucose-derived carbon for serine and glycine production in these cells, as demonstrated by sensitivity to PHGDH inhibition (Xia \textit{et al.}, 2019). This reduced glucose flux through the SSP, leading to metabolic stress and G1 arrest (Xia \textit{et al.}, 2019). The SSP has also been found to be essential for nucleotide biosynthesis, even in the presence of abundant exogenous serine in breast cancer cells. The SSP was shown to be required for central carbon metabolism mass balance by coordinating anabolic pathways, with glucose flux into the PPP and TCA cycle significantly reduced following PHGDH inhibition (Reid \textit{et al.}, 2018). Although in the present work glucose flux into the TCA cycle was unaffected by \textit{Psat1} deletion and/or the -SG diet, we did observe decreased enrichment into nucleotides as well as altered 1C metabolite profiles. Altogether this suggests that perturbation of serine metabolism in MMTV-MYC and MMTV-ERBB2 tumours may increase \textit{in vivo} latency by perturbing nucleotide biosynthesis.

To test whether this is the case, rescue experiments can be performed using exogenous nucleotides. Diehl \textit{et al.} (2019) showed that the requirement of the SSP upon sufficient cellular NAD+ levels meant that even when the SSP was intact, maximal proliferation of breast cancer cells could not be achieved without the addition of exogenous serine. These cells were rescued using exogenous purine nucleobases (Diehl \textit{et al.}, 2019). Therefore, the perturbation of central carbon
metabolism that has been reported upon SSP inactivation might be alleviated by supporting nucleotide metabolism. Determining whether MMTV-MYC and MMTV-ERBB2 cells can be rescued in the same manner is required.

In MMTV-ERBB2 tumours, $^{13}$C enrichment into alanine is increased in the $Psat1^{KO}$ alone and combination conditions, which is associated with a trend towards an increased total alanine pool for the combination. The same is not true for MMTV-MYC tumours, which in fact display the opposite trend. Furthermore, MMTV-MYC tumours show decreased enrichment into, and total pools of, lactate in the combination condition compared to $Psat1^{KO}$ alone. Pyruvate can be converted into lactate by lactate dehydrogenase (LDH), or to alanine by the action of the transaminase enzymes GPT1 and GPT2, simultaneously consuming glutamine and producing $\alpha$KG. In MMTV-ERBB2 tumours, $^{13}$C enrichment into TCA cycle metabolites is largely unchanged in each group, while lactate production is unchanged in each group. This suggests that tumours preferentially push glucose carbon towards the synthesis of alanine.

The diversion of glucose carbon toward alanine biosynthesis in $Psat1^{KO}$ tumours could suggest an alternative pathway for serine biosynthesis has been activated (Fig. 1-6). It therefore stands to reason that an increase in alanine synthesis in these tumours could be driving serine synthesis via the non-phosphorylated pathway. Considering that we do not see $^{13}$C enrichment into serine in $Psat1^{KO}$ tumours, it is unlikely that the carbon contribution comes from glucose. However, glycerol derived from lipolysis can also provide carbon for this pathway.

While previous work from our lab demonstrated that alanine levels increase in MYC-driven liver tumours following $Psat1$ deletion and dietary glutamine restriction, in MMTV-MYC tumours this did not occur (Méndez-Lucas et al., 2020). We observed an increase in MMTV-ERBB2 tumours, which could also be indicative of increased utilisation of alanine in the absence of serine to produce sphingolipids. It has been shown that an increased alanine to serine ratio promotes the production of cytotoxic deoxysphingolipids, as serine levels are inadequate to maintain normal sphingolipid metabolism (Esaki et al., 2015; Muthusamy et al., 2020). Developing an LCMS
method to measure sphingolipid species could reveal if this contributes to the increased latency observed in these tumours.

While glycolytic flux is upregulated in each of the experimental groups from MMTV-ERBB2 tumours, with increased alanine and unchanged lactate, in MMTV-MYC tumours glycolytic flux is only upregulated in the Psat1KO and -SG groups, with the combination group returning to control levels. This is also reflected in alanine enrichment. Furthermore, overall lactate pools decrease in tumours from the combination group. These results may indicate a metabolic switch towards increased glycolysis for MMTV-MYC tumours in the Psat1KO and -SG groups, and a reversion to oxidative phosphorylation in the combination group. It is now known that cancer cells utilise both glycolysis and oxidative phosphorylation, and it has been demonstrated that targeting only one of these processes in TNBC cells is therapeutically insufficient, as cancer cells can enhance the other to compensate (Jia et al., 2019). This highlights the requirement to target both glycolysis and oxidative phosphorylation if central carbon metabolism is the therapeutic target of interest, and this has been shown to be the case in preclinical models (Cheong et al., 2011).

6.2.3 Therapeutic efficacy via vascular disruption following intratumoural serine deprivation

We investigated whether Psat1 deletion and the -SG diet affected the pathology of either MMTV-MYC or MMTV-ERBB2 tumours. Our results showed striking differences between MMTV-MYC and MMTV-ERBB2 control tumours and Psat1KO tumours from the -SG diet cohort. MMTV-ERBB2 tumours were consistently morphologically homogenous, each receiving the same arbitrary grading score of ‘moderately differentiated’, while Psat1KO -SG tumours exhibited a ‘shotgun-like’ pattern, indicative of relatively high levels of ischaemia and infarction. More pronounced differences were observed in the MMTV-MYC tumours, with controls exhibiting greater morphological heterogeneity and Psat1KO tumours from mice on the -SG diet exhibiting an empty core/staghorn-like vasculature.

A role for serine metabolism in the proper functioning of blood vessels has previously been described. Specifically, human endothelial cells are sensitive to perturbations
of the SSP \textit{in vitro}, which is likely due to the highly proliferative nature of the cells and the impairment of pyrimidine synthesis and mitochondrial respiration observed upon PHGDH knockdown (Vandekeere \textit{et al.}, 2018). Furthermore, PHGDH deletion results in vascular defects in mice (Vandekeere \textit{et al.}, 2018). It has also been shown that PKM2, which is allosterically activated by serine, promoted blood vessel formation in tumours (Azoitei \textit{et al.}, 2016). Our findings that only the combination of Psat1 deletion and the -SG diet produced the observed apparent vascular differences are concordant with these results and previous results that demonstrated that supplementation with exogenous serine and glycine can rescue mitochondrial respiration in retinal cells (Zhang \textit{et al.}, 2018). However, further confirmation is required to confirm whether the observed vascular differences in Psat1\textsuperscript{K0}/-SG MMTV-ERBB2 and MMTV-MYC tumours are caused by disruption of vascular integrity.

\subsection*{6.2.4 Serine metabolism as a driver of primary breast tumour metastasis}

While MMTV-ERBB2 tumours produced intra-arterial secondary tumours in the lungs, MMTV-MYC tumours produced what might be considered more 'conventional' lung metastases embedded within the parenchyma. In both cases Psat1 deletion, the -SG diet, and the combination of the two were able to reduce the metastatic potential of the primary tumours. Serine metabolism has been previously linked to metastatic potential in the MDA-MB-231 breast cancer cell line, a particular bone metastatic variant of which had serine metabolism as its most significantly enriched pathway, with a 648-fold increase in PHGDH expression (Pollari \textit{et al.}, 2010). Ngo \textit{et al.} (2020) recently demonstrated that PHGDH was the most highly upregulated protein in aggressive metastatic TNBC cells that exhibit tropism for brain metastasis. This was associated with increased glucose flux through the SSP (Ngo \textit{et al.}, 2020). The authors also found that PHGDH expression was enriched in 90\% of brain metastases arising from primary breast tumours (Ngo \textit{et al.}, 2020). Furthermore, while primary TNBC tumours had four-fold higher PHGDH expression compared to other subtypes, the highest expression was found in primary tumours that turned out to be the most metastatic (Ngo \textit{et al.}, 2020). Importantly, the authors demonstrated that serine and glycine levels in the CSF are significantly lower than plasma
concentrations, and that PHGDH activity was required to prevent DNA damage and consequent cell cycle arrest in TNBC cells cultured in serine- and glycine-deficient media (Ngo et al., 2020).

Our results demonstrate that SSP ablation by Psat1 deletion or a -SG diet, both alone and in combination, reduced lung metastatic burden from MMTV-MYC and MMTV-ERBB2 tumours. However, while metastasis to the lung was decreased in each of the in MMTV-MYC tumour experimental groups, the most metastases were observed in the -SG group, which also contained multiple tumours. Considered alongside our results that serine and glycine synthesis is upregulated in primary tumours from this group, this suggests that PSAT1 activity allows MMTV-MYC tumours to retain more metastatic potential when environmental serine and glycine levels are limiting.

Ngo et al. (2020) found that provision of exogenous nucleotides could rescue proliferation in aggressive TNBC cells in vitro when cultured in media resembling the relatively low concentrations of serine and glycine observed in mouse CSF. Our data suggest that nucleotide biosynthesis is affected in MMTV-ERBB2 and MMTV-MYC tumours upon perturbation of serine metabolism. Thus, it is possible that perturbed de novo nucleotide biosynthesis in MMTV-ERBB2 and MMTV-MYC tumours due to insufficient levels of serine and glycine is the cause of decreased metastatic potential. Further work is required to confirm this.

While Ngo et al. (2020) found that ectopic expression of PHGDH only promoted brain metastasis in breast cancer cells and not lung metastasis, our data suggest that the capacity to both synthesise and acquire serine are required for MMTV-MYC and MMTV-ERBB2 tumours to metastasise to the lung. This is a promising outcome regarding a potential new avenue for breast cancer treatment in humans, as serum serine levels can be lowered by dietary modification in mice, which synergises with SSP perturbation. Further work evaluating the ability of SSP perturbation and the -SG diet to prevent MYC-enriched human breast cancer cells from metastasising is required, for example using patient-derived xenograft (PDX) models.
Chapter 7. Conclusion

The work presented herein demonstrates that the perturbation of metabolic pathways represents a viable therapeutic opportunity for human breast cancer. Using both *in vitro* and *in vivo* models, we demonstrated that specifically targeting glutamine metabolism and serine metabolism significantly impairs MYC-induced mammary gland tumour cell proliferation. We observed that MMTV-ERBB2 tumours respond differently, and to a lesser extent, than MMTV-MYC tumours to the targeting of these pathways. Thus, these results suggest that these methods may be useful in targeting HER2-enriched breast tumours, but greater benefits would likely be seen in MYC-enriched tumours.

*Gls1* deletion in the mammary gland was sufficient to increase MMTV-MYC tumour latency, but not MMTV-ERBB2 tumour latency. However, environmental differences were shown to have a significant impact on experimental outcomes, as the effect size observed in tumour from NIMR mice was reduced in Crick mice. Nonetheless, reduced glutaminolysis in MMTV-MYC *Gls1* KO tumours demonstrate the importance of GLS1 in these tumours. However, the fact that glutamine catabolism continued, albeit to a significantly lower extent, demonstrates that metabolic compensation for *Gls1* deletion is occurring. Our RNA-seq results, and previous work from our lab on the metabolism of MYC-driven liver tumours, suggest that this compensation can be from GLS2 and/or transamidase enzymes (Méndez-Lucas *et al.*, 2020). Therefore, further work is required for confirmation of the source of compensation in MMTV-MYC tumours. MMTV-MYC *Gls1* KO tumours from the NIMR cohort exhibited significantly less morphological dedifferentiation, as well as increased PHH3 staining. Considered alongside RNA-seq results that showed downregulation of ECM-related pathways, this suggests perturbation of glutamine catabolism decreases dedifferentiation and thus tumour progression.

MMTV-MYC-derived tumour cells exhibited sensitivity to small molecule inhibition of GLS1 by CB-839, and glutamine transport by GPNA. This sensitivity was reduced for individual drug treatments when cells were cultured in DMEM/F-12 MMEC media but increased for combination drug treatment. This exemplifies that differences in the nutrient profiles of cell culture can have a significant impact on experimental results,
and thus the design of subsequent \textit{in vivo} experiments. Whether combined GLS1 and glutamine transport inhibition is efficacious against MMTV-MYC tumours \textit{in vivo} remains to be determined.

We also demonstrated that targeting serine metabolism in MMTV-MYC tumours, and to a lesser extent MMTV-ERBB2 tumours, was effective \textit{in vivo}. In MMTV-ERBB2 tumours, only combined \textit{Psat1} deletion and dietary serine and glycine deficiency (\textit{-SG} diet) was sufficient to increase tumour latency. However, the \textit{-SG} diet alone had therapeutic effect against MMTV-MYC tumours, while the \textit{Psat1}^{\textit{KO}}/-SG tumours exhibited the greatest delay in tumourigenesis. Importantly, we observed that in each experimental group (\textit{Psat1}^{\textit{KO}}, \textit{-SG} diet, and combined \textit{Psat1}^{\textit{KO}}/-SG) the metastatic potential of tumours to the lungs was decreased, again with the greatest effect for MMTV-MYC tumours. While primary breast tumours are relatively rarely lethal, metastatic breast cancer is associated with significant mortality. Therefore, our results demonstrate that targeting serine metabolism could result in significant survival improvements for patients with MYC-enriched breast cancers such as TNBC.

Altogether, our results suggest that targeting serine metabolism may be more efficacious than targeting glutamine metabolism, as the metabolic perturbations that we observed due to genetic ablation of \textit{Psat1} were greater than \textit{Gls1}. However, dietary serine and glycine deprivation was necessary for the maximal effect to be achieved. Our results, and others, suggest that the primary mechanism for the anti-cancer effect is perturbation of 1C metabolism, and subsequent deregulation of \textit{de novo} nucleotide biosynthesis.


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