

**Investigating oncogenic FMS-like tyrosine kinase
3 (*FLT3*)-induced metabolic reprogramming in
acute myeloid leukaemia**

Stefan Bjelosevic

ORCID ID: 0000-0002-3012-9273

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Peter MacCallum Cancer Centre
and

The Sir Peter MacCallum Department of Oncology
Faculty of Medicine, Dentistry and Health Sciences
The University of Melbourne

We have not slain our enemy, the cancer cell, or figuratively torn the limbs from his body. In our adventures, we have only seen our monster more clearly, and described his scales and fangs in new ways – ways that reveal a cancer cell to be... a distorted version of our normal selves”.

Harold E. Varmus

Acceptance speech for the 1989 Nobel Prize in Physiology and Medicine

For the discovery of the cellular origin of retroviral oncogenes

Abstract

Mutations in the FMS-like tyrosine kinase 3 (*FLT3*) gene are among the most frequently occurring somatic mutations in acute myeloid leukaemia (AML), a disease that presents with devastating prognosis. FLT3 is primarily expressed on hematopoietic progenitor cells, and during early haematopoiesis coordinates a ligand-dependent signalling cascade that regulates the proliferation and maturation of the progenitor pool. FLT3 internal tandem duplication (FLT3-ITD), the most common type of FLT3 mutation, promotes constitutive FLT3 kinase activity and hyperactivation of downstream signalling pathways including STAT5, PI3K/mTOR and MAPK. Though our understanding of the molecular pathways downstream of mutant FLT3 have greatly improved in the modern genomic sequencing era, the repertoire of molecular signalling events induced by mutant-FLT3 to drive leukaemogenesis has not been fully characterised.

In this thesis, a murine model of *MLL*-rearranged AML harbouring inducible FLT3-ITD expression was developed and used, along with human AML cell lines, to demonstrate that FLT3-ITD promotes serine uptake and serine biosynthesis via transcriptional regulation of neutral amino acid transporters (*SLC1A4* and *SLC1A5*) and genes in the *de novo* serine biosynthesis pathway (*PHGDH* and *PSAT1*). Mechanistically, dysregulation of serine metabolism in FLT3-ITD-driven AML is dependent on the mTORC1-ATF4 axis, which drives RNA-Pol II occupancy at *PHGDH*, *PSAT1*, *SLC1A4* and *SLC1A5*. Genetic or pharmacological inhibition of the *de novo* serine biosynthesis pathway, *in vitro* and *in vivo*, selectively inhibited the proliferation of FLT3-ITD-driven AML cells. Pharmacological inhibition of the *de novo* serine biosynthesis pathway using WQ-2101, an inhibitor of PHGDH, the first rate-limiting enzyme of the *de novo* serine biosynthesis pathway, sensitises FLT3-ITD-driven AML cells, including primary patient samples, to the standard of care chemotherapy agent cytarabine via exacerbation of DNA damage.

Given that transcriptional activation of *de novo* serine biosynthesis and serine uptake was mediated by mTORC1 (a master regulator of biomass production and cellular metabolism), and that therapeutic responses *in vitro* and *in vivo* to mTORC1 inhibitors are poor, a small molecule compound screen utilising 181 epigenetic inhibitory compounds to determine novel synthetic lethal interactions between epigenetic regulators and mTORC1 inhibition was performed. This analysis revealed that inhibition of lysine-

specific methyltransferase SETD8, the only known enzyme that catalyses H4K20 monomethylation (H4K20me1), synergised with mTORC1 inhibition. Transcriptional profiling suggested dual SETD8/mTORC1 inhibition preferentially suppressed mTORC1 target genes mediating amino acid biosynthesis and transamination (including *de novo* serine biosynthesis) to a greater extent than either single agent SETD8 or mTORC1 inhibition alone. Importantly, these observations were independent of global transcriptional repression induced by impaired cell viability or suppression of global transcription. Thus, this preliminary work suggests mTORC1 and/or its target genes and pathways may be dependent on SETD8 and/or H4K20me1 or, alternatively, mTORC1 functionally regulates SETD8.

Collectively, the results presented herein provide novel insights into FLT3-ITD-induced metabolic reprogramming events in AML and identify a targetable metabolic dependency in this poor prognosis subtype of disease. In addition, these results provide the preliminary basis of a SETD8/mTORC1 synthetic dependency that can be exploited in FLT3-ITD-driven AML.

Declaration

This declaration certifies that:

- i. This thesis comprises only my original work towards the Ph.D. except where indicated in the Preface;
- ii. Due acknowledgement has been made in the text to all other material used;
- iii. The thesis is fewer than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

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Stefan Bjelosevic

Gene Regulation Laboratory, Translational Haematology Program
Peter MacCallum Cancer Centre
Melbourne, VIC, Australia.

The Sir Peter MacCallum Department of Oncology
Faculty of Medicine, Dentistry and Health Sciences
The University of Melbourne
Parkville, VIC, Australia

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Preface

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List of abbreviations

1-MNA	1-methylnicotinamide
1C	1 carbon unit
2-DG	2-deoxy-D-glucose
2-HG	2-hydroxyglutarate
2PG	2-phosphoglycerate
3PG	3-phosphoglycerate
3PHP	3-phosphohydroxypyruvate
3PS	3-phosphoserine
4E-BP1	Eukaryotic transcription initiation factor 4E-binding protein
5-hmC	5-hydroxymethylcytosine
5-mC	5-methylcytosine
a-KG	Alpha-ketoglutarate
AHCY	Adenosylhomocysteinase
AKT	Protein kinase B
ALDH1L	10-formyltetrahydrofolate dehydrogenases 1-like
ALL	Acute lymphoblastic leukaemia
alloHSCT	Allogenic haematopoietic stem cell transplantation
AML	Acute myeloid leukaemia
AMPK	AMP-activated protein kinase
AraC	Cytosine arabinoside (cytarabine)
ARG1	Arginase 1
ASL	Argininosuccinate lyase
ASNS	Asparagine synthetase
ASS1	Argininosuccinate synthase 1
ASXL1	Additional sex combs like 1
ATF4	Activating transcription factor 4
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
B-ALL	B-cell acute lymphoblastic leukaemia
BAX	BCL-2-associated X protein
BBB	Blood-brain-barrier
BCAT	Branched chain amino acid transaminase
BCL-2	B-cell lymphoma 2
BCL-xL	B-cell lymphoma-extra-large
BCOR	BCL6 corepressor
BET	Bromodomain and Extra-Terminal motif
BHMT	Betaine-homocysteine S-methyltransferase
BIM	BCL-2-like protein 11
bp	Base pairs
bZIP	Basic leucine-zipper
C-KIT	Tyrosine-protein kinase KIT

C/EBP	CCAAT/enhancer-binding protein
CARE	C/EBP-ATF4 response element
CBF	Core binding factor
CBL	Casitas B-lineage lymphoma
CBS	Cystathionine gamma-synthase
Cd135	Cluster of differentiation 135
CDK	Cyclin-dependent kinase
CEBPA	CCAAT/enhancer binding protein alpha
CHAC1	ChaC glutathione specific gamma-glutamylcyclotransferase 1
CK	Casein kinase
CLP	Common lymphoid progenitor
CML	Chronic myeloid leukaemia
CMML	Chronic myelomonocytic leukaemia
CMP	Common myeloid progenitor
CoA	Co-enzyme A
CpG	Cytosine guanine island
CR	Complete remission
CRE	cAMP responsive element
CRISPR-Cas9	Clusters of regularly interspaced short palindromic repeats, CRISPR-associated protein 9
CTG	Cell Titer Glo
CTH	Cystathionine gamma-lyase
CTV	Cell Trace Violet
CYP3A4	Cytochrome P450 3A4
DBZ	Dibenzazepine
DDIT4	DNA damage inducible transcript 4
DERL3	Derlin-3
DFG	Asp-Phe-Gly motif
DFS	Disease-free survival
DHF	Dihydrofolic acid
DHFR	Dihydrofolate reductase
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA (cytosine-5) methyltransferase
DOT1L	Disrupter of telomeric silencing 1-like
DSBs	Double-stranded DNA break
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
dTMP	2-deoxy-thymidine-5-monophosphate
dUMP	2-deoxy-uridine-5-monophosphate
ECL	Enhanced chemiluminescence
EHMT2	Euchromatic histone-lysine N-methyltransferase 2
EIF2AK4	Eukaryotic translation initiation factor 2-alpha kinase 4
ELN	European LeukaemiaNet

ER-positive	ERR alpha-positive
ERR-alpha	Estrogen-related receptor alpha
ESRP1	Epithelial splicing regulatory protein 1
EZH2	Enhancer of zeste 2 polycomb repressive complex 2 subunit
FBS	Foetal bovine serum
FBXW1A	F-box/WD repeat-containing protein 1A
FDA	Food and Drug Administration
FGF2	Fibroblast growth factor 2
FGFR	Fibroblast growth factor receptor
FH	Fumarate hydratase
FKBP12	FK-binding protein 12
Flk2	Foetal liver tyrosine kinase 2
FLT3	FMS-like tyrosine kinase 3
FLT3-ITD	FMS-like tyrosine kinase 3 - internal tandem duplication
FLT3-TKD	FMS-like tyrosine kinase 3 - tyrosine kinase domain mutation
FLT3L	FMS-like tyrosine kinase 3 ligand
FOXO3	Forkhead box O3
FTase	Farnesyltransferase
G6PD	Glucose-6-phosphate dehydrogenase
GAB2	GRB2-associated binding protein 2
GCN2	General control nonderepressible 2
GDP	Guanosine diphosphate
GGTase I	Geranylgeranyltransferase-1
GLDC	Glycine decarboxylase
GLS	Glutaminase
GMP	Granulocyte macrophage progenitors
GNMT	Glycine <i>N</i> -methyltransferase
GO	Gene ontology
GRB2	Growth factor receptor bound protein 2
GSEA	Gene set enrichment analysis
GSK3	Glycogen synthase kinase 3
HCT	Haematopoietic cell transplantation
HDAC	Histone deacetylase
HGB	Haemoglobin
HILIC	Hydrophilic interaction liquid chromatography
HK	Hexokinase
HMA	Hypomethylation agents
HMT	Histone methyltransferase
HOX	Homeobox
HSC	Haematopoietic stem cell
HSP	Heat shock protein
HSPC	Haematopoietic stem and progenitor cell
IDH	Isocitrate dehydrogenase

IGFBP1	Insulin-like growth factor binding protein 1
IK	Interkinase domain
IKZF1	Ikaros family zinc finger 1
IRS-1	Insulin receptor substrate-1
JAK	Janus kinase
JMD	Juxtamembrane domain
JMJD	Jumonji C-domain-containing domain
JOSD2	Josephin domain-containing 2
kb	Kilobase
kDa	kilo Daltons
KDM	Lysine-specific demethylase
KMT2A	Lysine methyltransferase 2A
LDHA	Lactate dehydrogenase A
LKB1	Liver kinase B1
MAPK	Mitogen activated protein kinase
MAT	S-adenosyl-methionine synthetase
MDM2	Mouse double minute 2
MDS	Myelodysplastic syndromes
MDS	Multidimensional scaling
Me	Methyl group
MEP	Megakaryocyte-erythroid progenitors
MLL	Mixed lineage leukaemia
MPN	Myeloproliferative neoplasms
MPP	Multipotent progenitor
MS	Methionine synthase
MTA	5'-S-methyl-5'-thioadenosine
MTHFD	Methylenetetrahydrofolate dehydrogenase-cyclohydrolase
MTHFR	Methylenetetrahydrofolate reductase
mTOR	Mechanistic target of rapamycin
NADH	Nicotinamide adenine dinucleotide hydrogen
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NEPC	Neuroendocrine prostate cancer
NGS	Next generation sequencing
NK	Natural Killer
NNMT	Nicotinamide N-methyltransferase
NO	Nitric oxide
NOTCH1	Notch homolog 1
NPM1	Nucleophosmin 1
NRF2	Nuclear factor erythroid 2-related factor 2
NSCLC	Non-small-cell lung carcinoma
NSG	NOD scid gamma mouse
OS	Overall survival

OTC	Ornithine transcarbamyl transferase
OXPHOS	Oxidative phosphorylation
p70S6K	p70 S6-kinase
PAX5	Factors paired box 5
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDH	Pyruvate dehydrogenase
PEI	Polyethyleneimine
PEP	Phosphoenolpyruvic acid
PGC-1a	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PHGDH	Phosphoglycerate dehydrogenase
PI3K	Phosphoinositide 3-kinase
PIM	Proviral Integration site for Moloney murine leukaemia virus
PKC	Protein kinase C
PKM2	M2 pyruvate kinase
PLT	Platelets
PP2A	Serine/threonine-protein phosphatase 2A
PPP	Pentose phosphate pathway
PRC	Polycomb repressive complex
PRMT	Protein arginine methyltransferase
PSAT1	Phosphoserine Aminotransferase 1
PSPH	Phosphoserine phosphatase
PTPRCA	Protein-tyrosine phosphatase, receptor type c
PVDF	Polyvinylidene difluoride
RAC1	RAS-related C3 botulinum toxin substrate 1
RAD21	Double-strand-break repair protein 21
RAS	Rat sarcoma family of oncogenes
RBC	Red blood cell
rDNA	Ribosomal DNA
RNA	Ribonucleic acid
RNA-Pol II	Ribonucleic acid polymerase II
RNF5	Ring finger protein 5
ROS	Reactive oxygen species
RPL	Ribosomal protein
RPMI	Roswell Park Memorial Institute
RPS	40S ribosomal protein
RT-qPCR	Quantitative real-time polymerase chain reaction
RTK	Receptor tyrosine kinase
S6	Ribosomal protein S6
SAH	S-adenosyl-L-homocysteine
SAM	S-adenosyl methionine
SCF	Stem cell factor
SCLC	Small cell lung carcinoma

SDH	Succinate dehydrogenase
SETD8	Su(var)3-9, Enhancer of Zeste, Trithorax domain 8
SF3B1	Splicing factor 3b subunit 1
SHC	Src homology 2 containing protein
SHIP	Src homology 2 domain containing inositol polyphosphate 5-phosphatase
SHMT	Serine hydroxymethyltransferase
SHP2	Src homology 2 domain containing tyrosine phosphatase 2
SLCXAY	Solute Carrier Family X Member Y
SOS	Guanine nucleotide exchange factor
SRSF2	Serine and arginine rich splicing factor 2
STAG2	Stromal antigen 2
STAT5	Signal transducer and activator of transcription 5
Stk-1	Stem cell tyrosine kinase 1
STR	Short-tandem repeat
SYK	Spleen tyrosine kinase
T-reg	T-regulatory cells
TCA	Tricarboxylic acid
TDG	Thymine DNA glycosylase
TET2	Ten-eleven translocation-2 methylcytosine dioxygenase 2
THF	Methylene-tetrahydrofolate
TKD	Tyrosine kinase domain
TKI	Tyrosine kinase inhibitor
TYK2	Tyrosine kinase 2
TYMS	Thymidylate synthase
U2AF1	U2 small nuclear RNA auxiliary factor 1
uORF	Upstream open reading frame
UTR	5'-untranslated region
VHL	Von Hippel-Lindau tumour suppressor
WHO	World Health Organisation
WT	Wild-type
ZIP	Zero Interaction Potency

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Chapter 1: Literature Review

1.1. Introduction to the haematopoietic system

Haematopoiesis refers to the sustained production, regulation and renewal of cells of the haematopoietic system, wherein a small number of multipotent stem cells undergo self-renewal, proliferation and differentiation into one of two haematopoietic lineages in a carefully choreographed process driven by defined sets of transcription factors (Blank et al., 2008; Wilson and Trumpp, 2006). The components of the haematopoietic system have traditionally been divided into the *myeloid compartment*, which include the bone marrow and its cellular derivatives, and the *lymphoid compartment*, consisting of the lymph nodes, spleen and thymus and cells that localise in these organs (Jagannathan-Bogdan and Zon, 2013). While the two lineages have defined anatomical and functional niches, this separation is largely artificial with reference to the normal physiology of the haematopoietic system. This section provides a brief overview of the development and maintenance of the haematopoietic system in both steady-state and malignant settings.

1.1.1 Development and maintenance of the haematopoietic system

Haematopoietic progenitors first appear in the third week of embryonic development, and defined haematopoietic stem cells (HSCs) arise in the intraembryonic aorta/gonad/mesonephros region several weeks later (Dzierzak and Speck, 2008). In approximately the third month of development, HSCs migrate to the liver which becomes the principal site of hematopoietic formation. The relevance of this developmental step in pre-clinical and clinical research has long been appreciated; murine foetal liver cells have formed the basis of many models of malignant haematopoiesis, wherein harvested cells are virally transduced with leukaemia-inducing oncogenes and subsequently transplanted into primary recipients which develop penetrant disease (Almosailekh and Schwaller, 2019; Milne, 2017; Ottersbach et al., 2018). The clinical use of foetal liver transplantations has been explored as an alternative to allogenic stem cell transplants in the setting of bone marrow failure in haematological malignancy, autoimmune disease and acute radiation exposure (Gale, 1992). In the fourth month of development, the HSCs migrate from the liver and constitute the bone marrow, where they reside for the remainder of life. After birth, the haematopoietic function of the liver is rapidly depleted, and until early adulthood the entire skeleton is haematopoietically active. At adulthood however, haematopoiesis is restricted to the axial skeleton.

The formation of mature cells of the haematopoietic system, including erythrocytes, monocytes, granulocytes, lymphocytes and megakaryocytes, arises from clonal proliferation and differentiation of HSCs in what has classically been thought of as being hierarchical in nature. HSCs, which retain the unique ability to self-renew (and thus maintain the HSC pool) are largely quiescent but can undergo differentiation into multipotent progenitors (MPPs) which maintain multipotent capability but can no longer self-renew (Wilson et al., 2008). MPPs can subsequently differentiate into two multipotent lineage progenitors, referred to as the common myeloid progenitor (CMP) and common lymphoid progenitor (CLP). CLPs give rise to T-cells, B-cells, and Natural Killer (NK) cell precursors. CMPs differentiate into one of several intermediary myeloid progenitors which are restricted to mature along particular lineages – namely granulocyte macrophage progenitors (GMPs) and megakaryocyte-erythroid progenitors (MEPs), along with dendritic cells which can also arise from the CLP compartment (Pinho and Frenette, 2019). GMPs can undergo further lineage commitment and give rise to dedicated precursors, such as myeloblasts, eosinophiloblast and basophiloblasts, which finally differentiate into functional neutrophils, eosinophils and basophils. MEPs can likewise undergo lineage commitment to form megakaryoblasts and proerythroblasts, with subsequent enucleation resulting in mature platelets and erythrocytes respectively (Doulatov et al., 2012).

Whilst the above descriptions of hierarchical haematopoiesis represent decades-old dogma in the field, the advent of next generation sequencing (NGS) has revealed that HSC differentiation is far more complex. An integrated approach employing examination of cell surface markers and transcriptomics at single-cell resolution revealed that HSCs acquire lineage biases without necessarily transitioning through the defined stages of maturation described above, in what could be thought of as a continuous development spectrum (Velten et al., 2017). Evidence has emerged that progenitors within the HSC compartment may become lineage-restricted, further challenging the classic model of haematopoiesis (Haas et al., 2018). Understanding of the fundamental biology of haematopoiesis is important, as it provides important insights into the development of haematological malignancies, which typically arise in and concern uncommitted progenitor cells.

1.2 Tumours of haematopoietic and lymphoid tissues

Cells of the haematopoietic compartment (blood, bone marrow) and lymphoid system (lymph and lymphatic system) can undergo neoplastic transformation (Küppers, 2009). This diverse group of cancers presents with a varied aetiology, prognosis and survival, and are classified primarily on the tissue compartment affected, the aggressiveness of the tumour, and relevant driver mutations. Indeed, since the early 2000s, the World Health Organisation (WHO) has produced guidelines to differentiate haematological neoplasms based primarily on the cell lineage in question and relevant genetic abnormalities/drivers. This is a distinct departure from historical management of these diseases, in which treatment was based on whether the disease was concentrated in the blood and bone marrow (leukaemia) or lymph structures (lymphomas) (Deschler and Lübbert, 2008).

Haematological malignancies are derived from the two major blood cell lineages and involve oncogenic transformation of lineage progenitors. Malignancies of the myeloid lineage, which are derived from a common myeloid progenitor, gives rise to leukaemias such as acute myeloid leukaemia (AML), chronic myeloid leukaemia (CML), myeloproliferative neoplasms (MPNs), and myelodysplastic syndromes (MDSs) (Estey and Döhner, 2006). Malignancies affecting the lymphoid lineage (arising from a common lymphoid progenitor), result in a variety of distinct diseases including mature T-cell and NK-cell neoplasms, B-cell acute lymphoblastic leukaemia (B-ALL) and lymphomas affecting B-cells, including Hodgkin and most non-Hodgkin lymphomas (Küppers, 2009). Malignancies of the plasma cell, a subtype of B-cell, are termed multiple myeloma (Raab et al., 2009). In contrast to many adult solid tumours, approximately 50% of acute leukaemias (encompassing both myeloid and lymphoid) are driven by chromosomal abnormalities (such as translocations), which activate inappropriate growth-inducing transcriptional and epigenetic programs whilst concomitantly downregulating pro-apoptotic pathways and growth suppression machinery (Medinger and Passweg, 2017). Of note however, some 50% of paediatric solid tumours harbour chromosomal translocations (Gröbner et al., 2018). The next section of this review shall focus on the epidemiology, genetics, and treatment of acute myeloid leukaemia (AML), a disease which typically presents with particularly poor prognosis, dismal overall survival and with limited therapeutic options currently available.

1.3 Acute myeloid leukaemia (AML)

Acute myeloid leukaemia (AML) is a heterogeneous clonal disorder arising from haematopoietic progenitors of the myeloid lineage or from multipotent stem cells (Slany, 2009). The disease is characterised by a block in myelogenous differentiation and subsequent accumulation of non-functional cells in various stages of incomplete maturity (typically myeloblastic cells) in the bone marrow compartment (Pollyea et al., 2014). Unlike solid neoplasms which typically feature dozens of driver mutations, AML typically manifests with a far smaller number of genetic drivers. These include genomic rearrangements and mutations in genes that regulate transcriptional and epigenetic control of cell growth, differentiation, and apoptotic signalling (Hou et al., 2014).

1.3.1 Epidemiology

AML is the most commonly occurring myeloid neoplasm, with an incidence of approximately 4.1 cases per 100,000 people and a mean age of incidence of 64.2 years in the United States (US) (Ferrara and Schiffer, 2013). In general, the disease presents in three men for every two women (Estey and Döhner, 2006). Whilst the precise initiating events of AML are not fully understood, several risk factors have been identified. Exposure to DNA-damaging agents such as ionizing radiation, benzene, and asbestos has been associated with higher risk for developing the disease (Kane et al., 1999; Travis et al., 1994). A well-defined subtype of secondary disease, known as chemotherapy-related AML, exists in patients who have previously undergone treatment with genotoxic agents that inhibit topoisomerase II function (often in the context of solid malignancies) (Head, 1996). Two primary subtypes of this disease typically manifest in patients. Treatment with drugs such as anthracyclines (e.g., doxorubicin) and epipodophyllotoxins (e.g., etoposide) are characterised by a secondary myeloid malignancy marked by cytogenetic abnormalities at the 11q23 (lysine methyltransferase 2A, *KMT2A/MLL*) locus, and a rapidly proliferating and primarily monocytic disease, within several months to 2 years post treatment (Bacher et al., 2010). More commonly however, a myelodysplastic-like syndrome harbouring deletions in chromosomes 5 and 7 and complex karyotype manifests 5-7 years after treatment (known as alkylator agent-induced disease) (Gilliland, 2002). Of note, as these phenomena are often observed in elderly patients who have undergone several rounds of chemotherapy, there is the probability that repeated exposure

to DNA damaging agents could contribute to the development of AML (Ferrara and Schiffer, 2013).

1.3.2 Current treatment of AML

The treatment regimen pursued by clinicians in the management of AML depends greatly on patient age, relevant comorbidities and the patient's overall health and ability to tolerate high dose chemotherapy. The standard treatment regimen for AML has not changed in over 30 years and consists of two key phases (i.) high dose induction chemotherapy and (ii.) subsequent consolidation, with the use of an allogenic stem cell (bone marrow) transplant if suitable donors are available and the patient is able to sustain immunosuppression (Lichtman, 2013). The aim of therapy is to achieve complete remission via induction therapy (defined as bone marrow blast counts of less than 5%, no extramedullary leukaemia, and physiologically relevant neutrophil and platelet counts), and eradication of residual disease with consolidation therapy with an intent to cure (Wiernik et al., 1992). The standard chemotherapeutic agents utilised are a combination of an anthracycline (such as daunorubicin, idarubicin or doxorubicin) for 3 days, along with continuous infusion of high dose cytosine arabinoside (cytarabine, a cytosine nucleoside analogue) for 7 days (the so-called "7+3" regimen) (Weick et al., 1996). If patients harbour FMS-like tyrosine kinase 3 internal tandem duplication (FLT3-ITD) mutations, the multi-kinase inhibitor midostaurin is used in combination with cytarabine induction chemotherapy as standard-of-care (Stone et al., 2017). Upon successful remission, and if the patient presents with favourable risk features (such as core binding factor (*CBF*) AML, or *NPM1*-mutant AML with no co-occurring FLT3-ITD mutation), they are subsequently considered for consolidation therapy, which typically consists of lower dose 7+3 therapy or cytarabine and mitoxantrone (a topoisomerase II inhibitor) (Stone et al., 2001). Patients who do not enter remission, or enter remission but harbour poor prognosis disease features (such as complex karyotype or mutations such as *FLT3-ITD*) are considered for allogeneic haematopoietic cell transplantation (HCT), or monotherapy with small molecule inhibitors (such as gilteritinib) in the context of FLT3-ITD mutant relapsed/refractory disease which carries the greatest likelihood of cure in this context (McClune et al., 2010; Perl et al., 2019). This stratification approach is curative in approximately 35-45% of patients under the age of 60 years. However, given the median age of AML patients at diagnosis is 68 years, therapy is curative in less than

15% of patients over the age of 60 years. This cohort of patients typically are unable to tolerate high dose chemotherapy or HCT, or possess a greater chance of poor risk genetics (Döhner et al., 2017) which contributes to the poor prognosis of this high-risk patient cohort (Short et al., 2018). The treatment options for these patients have traditionally been limited, and includes low-dose cytarabine or treatment with hypomethylation agents (HMAs) such as DNA methyltransferase inhibitors (azacitidine or decitabine) (Gardin and Dombret, 2017; Zeidan et al., 2020). Whilst these less-intensive therapies are associated with lower rates of treatment-related mortality compared to high-dose chemotherapy or HCT, median survival is modest, at only 7-10 months (Li et al., 2011). More recently however, the use of the BCL2 inhibitor venetoclax for elderly treatment-naïve patients in combination with hypomethylating agents such as decitabine or azacitidine (DiNardo et al., 2019), or low-dose cytarabine (Wei et al., 2019) has shown promise, producing remissions in elderly patients unable to tolerate intensive chemotherapy. Indeed, regulators in the US (FDA) and Europe (European Medicines Agency, EMA) have approved these combination regimens as standard-of-care in elderly patients. Of note, however, some controversy remains regarding the fitness criteria that dictates whether patients undergo intensive chemotherapy or venetoclax + hypomethylating agent/low-dose cytarabine treatment, and modest complete remission rates and durations in the trials underpinning these regulatory approvals has been noted (Estey et al., 2020). Thus, significant effort has been dedicated to developing novel, better-tolerated therapies to treat AML particularly in this high-risk cohort of patients. This has necessitated the need to better understand the cytogenetic and mutational landscape of AML, and how these events influence disease phenotype and prognosis.

1.3.3 Cytogenetic and mutational landscape of AML

The widespread use of next-generation sequencing (NGS) technologies has accelerated the discovery of novel molecular abnormalities as well as the identification of commonly recurring mutations in the AML genome (Ilyas et al., 2015). Comprehensive genetic analysis of 200 cases of *de novo* adult AML using whole genome, whole exome, RNA and micro-RNA sequencing revealed that the AML genomes typically have far fewer mutations than most solid tumours, with an average of 13 genetic mutations, 5 of which are recurrently mutated in AML (Ko et al., 2011; Li et al., 2011; Moran-Crusio et al., 2011; Quivoron et al., 2011; Shide et al., 2012). Cytogenetic mutations, which involve

chromosomal translocations, deletions or inversions, have historically been one of the primary determinants of prognosis in AML. The World Health Organisation's (WHO) 2016 revision to the classification of myeloid neoplasms categorised AMLs by the presence of recurrent genetic translocations, fusions or the presence of CCAAT/enhancer binding protein α (*CEBPA*) or Nucleophosmin 1 (*NPM1*) mutations (Arber et al., 2016). This global effort improved on the previously published 2008 version of the classification, by incorporating new NGS findings that improve diagnostic criteria and prognostic relevance for each of the disease subtypes (Vardiman et al., 2009).

Table 1.1. WHO classification of acute myeloid leukaemia (AML) and related neoplasms; AML with recurrent genetic abnormalities.

AML with t(8;21)(q22;q22.1); <i>RUNX1-RUNX1T1</i> (<i>AML1/ETO</i>)	
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); <i>CBFB-MYH11</i>	
APL with <i>PML-RARA</i>	
AML with t(9;11)(p21.3;q23.3); <i>MLLT3-KMT2A</i>	
AML with t(6;9)(p23;q34.1); <i>DEK-NUP214</i>	
AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); <i>GATA2, MECOM</i>	
AML (megakaryoblastic) with t(1;22)(p13.3;q13.3); <i>RBM15-MKL1</i>	
AML with biallelic mutations in <i>CEBPA</i>	
AML with mutated <i>NPM1</i>	
Provisional Entities	AML with <i>BCR-ABL1</i>
	AML with mutated <i>RUNX1</i>

**Adapted from the 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia (Arber et al., 2016).*

In 2017, the European LeukaemiaNet (ELN) consortium performed systemic analysis of the available clinical literature with regards to the WHO AML subtypes, integrating relative rates of complete remission (CR), disease-free survival (DFS) and overall survival (OS) to arrive at three distinct prognostic risk groups for each of the AML subtypes (Döhner et al., 2017). AMLs that include *RUNX1-RUNX1T1*, *CBFB-MYH11*, AML with mutated *NPM1* (without *FLT3-ITD* or with low allelic-burden *FLT3-ITD*) and AML with biallelic mutations in *CEBPA* are classified as favourable risk, and thus patients who harbour these mutations should be treated with high dose induction and

consolidation chemotherapy with the intent to cure. AMLs harbouring *MLLT3-KMT2A* translocations, mutant *NPM1* with high allelic-burden *FLT3-ITD*, wild-type *NPM1* without *FLT3-ITD* or with low allelic-burden *FLT3-ITD*, and AMLs with cytogenetic abnormalities not classified as favourable or adverse are classed as intermediate risk. *DEK-NUP214*, *GATA2*, *MECOM*, *t(v;11q23.3)*; *KMT2A*-rearranged, AML with *BCR-ABL1*, complex/monosomal karyotype and wild-type *NPM1* with high allelic-burden *FLT3-ITD* are all classified as high risk subtypes of AML, and typically present with a chemoresistant phenotype and poor prognosis (Grimwade et al., 2010). Patients carrying these mutations should be considered for HCT post induction therapy and remission.

Whilst these evidence-based classifications allow for the efficient stratification of patient therapy based on inherent genetic risk, they do not account for the fact that up to 60% of *de novo* AML patients present with normal karyotype at the time of diagnosis, severely limiting the prognostic information these classifications are able to provide in a large cohort of patients (Marcucci et al., 2005; Santamaría et al., 2009). In what stands as perhaps the most comprehensive analysis of the AML genome to date, Papaemmanuil and colleagues sequenced the genomes of 1,540 AML patients and integrated this with cytogenetic and clinical data (Papaemmanuil et al., 2016). In addition to currently defined subgroups by the WHO, three previously unappreciated heterogenous recurrent genetic events were discovered. 18% of patients possessed mutations within genes encoding chromatin-cohesin regulators, RNA splicing regulators or both; a further 13% of patients possessed mutations within the tumour suppressor *TP53*, chromosomal aneuploidy or both; and, provisionally, 1% of patients possessed R172 mutations in the mitochondrial enzyme *IDH2*. Within this 1,540-patient cohort, 736 patients (48%) were cytogenetically normal or non *NPM1/CEBPA* mutant, and thus would not be able to be classified in accordance to the WHO guidelines despite 96% of patients recording at least one driver mutation (Papaemmanuil et al., 2016). Thus, this study was among the first to comprehensively highlight the complex, heterogenous nature of AML.

1.3.4 Recurrent genetic mutations in AML

While the impact of cytogenetics on AML prognostication is well established in the WHO/ELN guidelines, the Papaemmanuil study also revealed recurrent mutations in 76 distinct genes or genomic regions independent of cytogenetic aberrations. Some, such as

mutations in the *FLT3* gene, have been previously demonstrated to co-occur with certain cytogenetic events and *NPM1* mutations (Meyer and Levine, 2014) while others were novel. Using a Bayesian statistical model to compartmentalise AML into mutually exclusive subtypes with respect to the presence of co-occurring mutations, the authors broadly grouped recurrently mutated genes as epigenetic modifiers; chromatin-cohesin-mediating genes; genes which mediate RNA splicing; and receptor tyrosine kinase-RAS signalling genes (Papaemmanuil et al., 2016). These subtypes are discussed briefly in the sections below. Since 2017, a number of novel therapeutic agents has been approved for the treatment of AML, primarily driven by translational findings linked to the genomic landscape of AML, and greater understanding of resistance mechanisms to traditional therapies. Thus, any relevant advancements in therapy are also highlighted in the sections below.

1.3.4.1 Mutations in epigenetic modifiers

Among the most frequently mutated genes in AML are epigenetic modifiers including the DNA (cytosine-5) methyltransferase 3A (*DNMT3A*), ten-eleven translocation-2 methylcytosine dioxygenase 2 (*TET2*), the isocitrate dehydrogenase (*IDH*) family of metabolic enzymes, and regulators/recruiters of the Polycomb-group repressor complex (PRC) such as additional sex combs-like 1 (*ASXL1*). Mutations in these genes are often acquired early in leukaemogenesis in a founder haematopoietic stem cell; the acquisition of further mutations in these clones thus greatly increases the chance of developing myeloid malignancies, such as myelodysplastic syndromes (MDS) or overt acute myeloid leukaemia (AML) (Martignoles et al., 2018). Indeed, it has been demonstrated that between 10-20% of the elderly >70 years of age harbour mutations in *DNMT3A*, *TET2* or *IDH1/2* without frank malignancy, a term referred to as clonal haematopoiesis of indeterminate potential (CHIP) (Jaiswal and Ebert, 2019).

DNMT3A is an epigenetic regulator mediating the methylation of cytosine-5 residues within DNA. Sequencing of AML genomes revealed 20-25% of patients possess *DNMT3A* mutations, of which 50% harbour recurrent hotspot mutations at residue R882 (Ley et al., 2010). The exact mechanisms that underpin leukaemic progression are unclear. Patients with *DNMT3A*^{R882} mutations exhibit global hypomethylation, particularly at promoters and CpG islands (Qu et al., 2014; Russler-Germain et al., 2014),

though there has been some reports of promotor hypermethylation in the literature (Yan et al., 2011). The homeobox (*HOX*) family of transcription factors, which are established drivers of AML in other contexts such as *MLL*- and *NPM1*-driven AML, are observed to be hypomethylated in the context of *DNMT3A*^{R882} mutations, however the significance of these findings is unknown (Qu et al., 2014; Yan et al., 2011). From a therapeutic perspective, hypomethylating agents have been utilised in AML for many years, particularly in frailer patients unable to tolerate high-dose chemotherapy. Azacitidine, and its deoxy derivative decitabine, are chemical analogues of cytosine used in the treatment of myelodysplastic syndromes (MDS). Following uptake, azacitidine and decitabine are converted to mono, di, and tri-phosphate forms. At low doses, 5-aza-2'-deoxycytidine triphosphate is incorporated into DNA, forming adducts between DNA and DNMT1. These adducts are subsequently proteasomally degraded, after which DNA synthesis resumes in the absence of DNMT1 (Sato et al., 2017). Thus, global hypomethylation is achieved, inducing expression of previously silenced genes that may be relevant to myeloid differentiation or apoptosis (Christman, 2002). This effect is not specific to DNMT3A and to date, no DNMT3A-specific inhibitors have been developed. Several small molecule candidate compounds have been developed in recent years, such as quinoline-based inhibitors that compete with *S*-adenosyl methionine (SAM) in methylation reactions (SGI-1027) (Datta et al., 2009) and dual G9a/DNMT inhibitors (CM-272) (San José-Enériz et al., 2017), however no small molecule currently has DNMT3A-specific activity, nor a high degree of potency against this target.

The *TET2* family of enzymes catalyse the conversion of 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) in an α -ketoglutarate-dependent fashion to promote DNA demethylation (Moran-Crusio et al., 2011). *TET2* has been defined as a tumour suppressor gene, and *TET2* deletion induces myeloid and lymphoid malignancies in mice (Li et al., 2011a). This phenotype is reflected in the mutational landscape of this gene in myeloid malignancies such as AML, wherein loss-of-function in-frame deletions, frame shifts, generated stop codons and amino acid substitutions are often detected (Weissmann et al., 2012). Mechanistically, conditional depletion of *Tet2* in mouse models resulted in expansion of the *Sca1*⁺/*cKit*⁺ (LSK) cell compartment, and the adoption of an increased self-renewal phenotype concomitant with induction of a chronic myelomonocytic

leukaemia (CMML) (Ko et al., 2011; Li et al., 2011a; Moran-Crusio et al., 2011; Quivoron et al., 2011; Shide et al., 2012).

IDH1 and *IDH2* encode enzymes that convert isocitrate to α -ketoglutarate as part of the citric acid cycle in both cytosolic and mitochondrial intracellular locales respectively. Approximately 20% of AML patients possess mutations in either of these enzymes, and they are mutually exclusive in *de novo* AML (Papaemmanuil et al., 2016). *IDH1* mutations were originally identified in malignant gliomas and subsequently in AML (Mardis et al., 2009). *IDH2* mutations, particularly at residue R140, are unique to leukaemias (Ward et al., 2010a). Mutations in either enzyme results in the acquisition of neomorphic function, wherein α -ketoglutarate is converted to the oncometabolite 2-hydroxyglutarate (2-HG). 2-HG inhibits *TET2*, resulting in a hypermethylation phenotype and outgrowth of malignant *IDH*-mutant cells (Figueroa et al., 2010). Oral, small molecule inhibitors of both *IDH1* and *IDH2* have been developed and have demonstrated clinical efficacy. AG-120 (ivosidenib), an inhibitor of mutant *IDH1*, demonstrated significant efficacy in a phase I study of 258 relapsed/refractory *IDH1*-mutant AML patients, with an overall response rate of 41.6%, of which 21.6% of patients exhibited a complete response. Median overall survival was 8.8 months in this cohort (Rosnet et al., 1993). Likewise, a phase I trial of AG-221 (enasidenib), an inhibitor of R140Q and R172K-mutant *IDH2*, demonstrated an overall response rate of 40.3% and a complete response rate of 20.6% with a median overall survival of 9.3 months in relapsed/refractory *IDH2*-mutant patients (Stein et al., 2017). Based on these results, both compounds were approved by the United States (US) Food and Drug Administration (FDA) in 2018 in relapsed/refractory and, in some cases, frontline therapy.

ASXL1 encodes an epigenetic scaffold protein involved in transcriptional repression through its interaction with PRC complex 2 (PRC2) (Katoh, 2013). Mutations in *ASXL1* have been identified in a number of myeloid haematological malignancies (such as AML, MDS and MPN) and are universally associated with poor prognosis (Gelsi-Boyer et al., 2012). While genetic depletion experiments have shown loss of *ASXL1* induces an MDS-like disease phenotype, this is not reproduced clinically; most *ASXL1* mutations in patients with haematological diseases harbour frameshift/nonsense mutations at the 5' end of exon 12, producing a truncated protein (Abdel-Wahab et al., 2012, 2013; Wang et al.,

2014a). As *ASXL1* mutations are always heterozygous, this suggests their contribution to leukaemogenesis is via gain-of-function mechanisms. Indeed, C-terminal truncation of ASXL1 was sufficient to induce MDS-like disease in mice (Inoue et al., 2016), and, more recently it was characterised that truncated ASXL1 acts as a gain-of-function mutant, driving leukaemogenesis by increasing chromatin instability and directly binding to and recruiting BRD4 to genes such as *Fos* and *Prdm16* (Yang et al., 2018). Notably, WT ASXL1 does not have the capability to bind and recruit BRD4, further supporting the gain-of-function role of ASXL1 mutations in leukaemogenesis.

Several other epigenetic modifiers, such as BCL6 corepressor (*BCOR*) and Enhancer of Zeste Homolog 2 (*EZH2*), which are members of PRC1 and 2 respectively, have been shown to undergo loss-of-function mutations in AML and other haematological malignancies; however these mutations are relatively rare, with *BCOR* mutations found in approximately 4% of AML patients and *EZH2* mutations in 12% of MDS/MPN patients respectively (Ernst et al., 2010; Zhang et al., 2020).

1.3.4.2 Mutations in chromatin-cohesin complex genes

The chromatin-cohesin complex is involved in the regulation of chromatid separation during mitosis (Michaelis et al., 1997), three-dimensional genome organisation (Merkenschlager and Nora, 2016) and in the transcriptional regulation of cellular differentiation (Mazumdar et al., 2015; Mullenders et al., 2015; Viny et al., 2015). Mutations in cohesin complex members such as stromal antigen 2 (*STAG2*), double-strand-break repair protein 21 (*RAD21*), structural maintenance of chromosomes protein 1A (*SMC1A*), and structural maintenance of chromosomes 3 (*SMC3*) occur in 6-13% of AML cases, as well as other myeloid malignancies such as MDS, MPN, and CML (Cancer Genome Atlas Research Network et al., 2013). Mutations in cohesin complex genes are mutually exclusive and heterozygous, and overexpression of mutant forms of cohesin factors *in vitro* substantially alters assembly of the cohesin complex, suggesting dominant-negative activity (Mazumdar et al., 2015; Thol et al., 2014). Whilst impaired cohesin function has been shown to result in premature sister chromatid separation (Peters and Nishiyama, 2012; Peters et al., 2008), AML is typically not characterised by aneuploidy, and indeed approximately 50% of all AML patients present with normal karyotypes highlighting that overt aneuploidy is likely not the primary mechanism of

leukaemogenesis (Kon et al., 2013). Instead, one of the primary mechanisms by which cohesin complex mutations contribute to leukaemogenesis is via chromatin remodelling (Faure et al., 2012; Yan et al., 2013). Loss of cohesin complex activity in haematopoietic stem cells results in genome-wide alterations in chromatin accessibility which broadly alters gene expression, leading to induction of aberrant transcription factor programs that result in abnormal differentiation and increased self-renewal of myeloid progenitors (Galeev et al., 2016; Mazumdar et al., 2015; Mullenders et al., 2015; Viny et al., 2015). A potential specific mechanism underpinning this phenotype is the upregulation of the homeobox (*HOX*) family of transcription factors (particularly *HOXA7* and *HOXA9*) that increase self-renewal capability upon loss of the cohesin complex (Fisher et al., 2017).

1.3.4.3 Mutations in genes mediating RNA splicing

Mutations in members of the spliceosome machinery have been identified in myeloid malignancies and are particularly prevalent in myelodysplastic syndromes. Approximately 60% of MDS patients (Papaemmanuil et al., 2011; Yoshida et al., 2011) and approximately 50% of secondary AML patients (Lindsley et al., 2015) harbour mutations in genes encoding components of the spliceosome. The most common mutations occur in splicing factor 3b subunit 1 (*SF3B1*), serine and arginine rich splicing factor 2 (*SRSF2*) and U2 small nuclear RNA auxiliary factor 1 (*U2AF1*), which collectively constitute the core components of the spliceosome (Taylor and Lee, 2019). The reason why spliceosome mutations confer selective advantage during MDS progression and in the context of AML is not completely understood, and isogenic mouse models have been developed in an attempt to understand the fundamental biology underpinning the contribution of spliceosome mutations in neoplastic transformation. Competitive bone marrow transplant experiments with wild-type haematopoietic stem cells and progenitor cells harbouring spliceosome mutations in *Sf3b1*, *Srsf2* and *U2af1* uniformly demonstrated impaired reconstitution in mutant progenitors versus wild type cells (Kim et al., 2015; Lee et al., 2018; Obeng et al., 2016). In addition, it has been experimentally demonstrated that spliceosome mutant cells are dependent on the wildtype allele for survival, hence providing an attractive prospect for pharmacological intervention (Fei et al., 2016; Lee et al., 2016; Seiler et al., 2018; Shirai et al., 2017). In 2018, an oral small molecule modulator of the SF3b complex, H3B-8800, was developed with high potency in spliceosome-mutant solid and haematological malignancies (Seiler

et al., 2018). Similarly, indirect targeting of the spliceosome, such as via inhibition of protein arginine methyltransferases (PRMTs) which reduces splicing fidelity in spliceosome-mutant haematological malignancies has been demonstrated to be a specific form of therapeutic intervention in this mutational subset (Fong et al., 2019). An important limitation of many models of spliceosome-mutant haematological cancers is the fact that none of the models progress to overt acute leukaemia, suggesting that additional co-occurring mutations are required to model the molecular steps leading to leukemogenesis. A very recent study demonstrated that *IDH2* and *SRSF2* mutations commonly co-occur in AML patients, wherein co-expression of mutant forms of both genes fundamentally altered the splicing effects of *SRSF2* and resulted in more profound impact on splicing changes than either mutation alone (Yoshimi et al., 2019). Thus, it is likely that complex crosstalk with other mutations is necessary to cooperate with spliceosome mutations and thus drive the development of acute leukaemia beyond MDS.

1.3.4.4 Mutations in receptor tyrosine kinase/RAS-signalling genes

Mutations in signal transduction pathway members have been appreciated for decades, and formed a central tenant of the original “two-hit model” of AML development that arose in the late 1990s and early 2000s (Gilliland, 2001; Kelly and Gilliland, 2002). In this model, class I mutations in receptor tyrosine kinases (RTKs) (such as FMS-like tyrosine kinase 3 (*FLT3*), tyrosine-protein kinase KIT (*C-KIT*) or downstream signalling mediators (such as the rat sarcoma family of oncogenes (*RAS*)) results in uncontrolled cellular proliferation and enhanced survival of malignant clones. These cooperate with class II mutations (chromosomal translocations), as described in chapter 1.3.3, which were thought to inhibit cellular differentiation. While our understanding of the complexities of the AML genome has greatly increased and thus rendered the “two-hit model” as over-simplified, mutations in RTKs and RAS signalling genes are still highly relevant in the development of AML. Large scale sequencing studies have revealed that contrary to the “two-hit model”, in which these class I mutations were thought to be essential for the *initiation* of leukaemogenesis, RTK/RAS mutations are typically acquired relatively late in AML progression and act to induce complete transformation into a malignant leukaemic clone which then gives rise to subsequent, more genetically diverse clones (Cancer Genome Atlas Research Network et al., 2013; Papaemmanuil et al., 2016; Welch et al., 2012).

The RAS family of guanine nucleotide binding proteins, encompassing *NRAS*, *KRAS* and *HRAS*, are downstream of receptor tyrosine kinases and are an important scaffold in the induction of signal transduction through the mitogen-activated protein kinase (MAPK) pathway (Hobbs et al., 2016). Constitutive activation of RAS can be induced via autophosphorylation of upstream receptor tyrosine kinases (such as *FLT3* and *C-KIT*) as a result of mutation or overexpression, or in mutations in RAS itself. Approximately 30% of AML patients harbour some form of RAS mutation (typically in *NRAS* or *KRAS*) and point mutations in codons 12, 13 and 61 result in the inactivation of intrinsic GTPase activity, thus resulting in constitutive downstream signalling (Stalneck and Der, 2020). Mutant RAS hyperactivates members of the MAPK pathway (RAF/MEK/ERK) and phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/mechanistic target of rapamycin (mTOR) pathway, thereby driving malignant proliferation (Schubbert et al., 2007). Though approximately 24% of all human cancers harbour RAS mutations, the development of potent and specific small molecule inhibitors of RAS has historically proven challenging. The three-dimensional structure of RAS does not display attractive potential for specific small molecule inhibitor binding, and inhibition of MAPK members downstream of RAS (such as MEK, particularly in AML) has proven disappointing due to resistance by compensatory reactivation of ERK (Jain et al., 2014). Attempts to target RAS directly, via inhibition of farnesyltransferases (FTase) which act to transfer a farnesyl group to pre-RAS protein and thus allow attachment of RAS to the cell membrane showed encouraging *in vitro* results – but these did not translate to *in vivo* efficacy due to unexpected compensatory activation of the related enzyme geranylgeranyltransferase-I (GGTase I) which restored *KRAS* and *NRAS* membrane association (Whyte et al., 1997). Use of farnesyltransferase inhibitors in RAS-driven AML also proved inefficacious (Lancet et al., 2007). Recently, the discovery of specific inhibitors of *KRAS*^{G12C} has provided an exciting prospect for directly targeting mutant RAS itself; whether these agents provide durable responses in cancer, including AML, remains to be seen (Canon et al., 2019).

C-KIT is receptor tyrosine kinase (RTK) that acts as the receptor for stem cell factor (SCF). Mutations in *C-KIT* occur in 20-30% of patients with translocation t(8;21) (*RUNX1-RUNX1T1*; formerly known as core binding factor AML or CBF-AML) and are associated with poor prognosis (Paschka et al., 2006). Mutations in the codon 816 of the C-KIT activation loop result in ligand-independent activation of signal transducer and

activator of transcription 3 and 5 (STAT3/STAT5) and PI3K/AKT pathways, while mutations in exon 8 of extracellular C-KIT activate MAPK and PI3K/AKT signalling pathways (Malaise et al., 2009). Whilst no C-KIT-specific small molecule inhibitors have been developed, the promiscuous nature of several tyrosine kinase inhibitors has been exploited in C-KIT-driven disease. For instance, use of the BCR-ABL1 inhibitor dasatinib, which also inhibits C-KIT, has seen significant interest in core binding factor (CBF; t(8;21) and inv(16))-driven AML, which commonly co-occurs with C-KIT mutations (Marcucci et al., 2020; Paschka et al., 2018).

Mutations in the *FLT3* gene, encoding for another RTK of the same family as C-KIT, represent one of the most common mutations in AML, found in approximately 30% of patients (Papaemmanuil et al., 2016). FLT3 has emerged as one of the most intensely studied mutations in the context of AML, due to its widespread prevalence, and the 2017 approval of the multi-kinase inhibitor midostaurin as frontline therapy in patients harbouring FLT3 mutations, the first novel therapy in AML in over a decade (Stone et al., 2017). The next section of this review discusses in detail the structure, function, and mutational landscape of FLT3; the biology underpinning FLT3 signalling in normal and malignant haematopoiesis; and the clinical and therapeutic implications of FLT3 mutations in AML.

1.4 Mutations in FLT3

Binding of exogenous ligands to receptor tyrosine kinases (RTKs) is an essential means by which cells control proliferation, by inducing downstream effectors to initiate transcription and thus stimulate cell growth. The FMS-like tyrosine kinase 3 (*FLT3*) gene encodes a membrane-bound RTK that has a central role in the regulation of normal haematopoiesis (Stirewalt and Radich, 2003). As detailed above, large scale sequencing efforts in AML patients have revealed mutations in FLT3 to be among the most common in this patient cohort, highlighting the relevance of establishing both the fundamental biology underpinning the role of FLT3 in normal and malignant haematopoiesis, and the clinical implications of FLT3 mutations on disease progression, prognosis and therapy.

1.4.1 Structure and role of FLT3 in normal haematopoiesis

The *Flt3* gene, also known as *Flk2* (foetal liver tyrosine kinase 2), *Stk-1* (stem cell tyrosine kinase 1) and *Cd135*, was first cloned from mouse foetal liver and placenta cells in the early 1990s, with the human *FLT3* gene cloned not long after (Matthews et al., 1991; Rosnet et al., 1991, 1993a; Small et al., 1994). The human *FLT3* gene is located on chromosome 13q12 and consists of 24 exons covering approximately 96 kilobases (kb) (Agnès et al., 1994; Rosnet, 1999). The protein is largely homologous with mouse Flt3, itself located on chromosome 5 (Rosnet et al., 1993b). In the human context, the protein encoded by *FLT3* is a transmembrane receptor of 933 amino acids and with a molecular weight of 155-160 kilo Daltons (kDa) (Rosnet et al., 1993a). Like all type III RTKs, FLT3 is composed of 5 immunoglobulin-like domains in the N-terminal extracellular region (the 3 most distal to the plasma membrane are involved in binding of FLT3 ligand (FLT3L), while the 2 proximal domains are involved in receptor dimerization), a transmembrane domain, a juxtamembrane domain, and two intracellular split-kinase tyrosine kinase domains (TKDs) at the C-terminal region (denoted TKD1 and TKD2), connected by an interkinase domain (IK) (Agnès et al., 1994) (Figure 1.1). Two forms of FLT3 have been characterised. The extracellular FLT3L binding region is heavily glycosylated at *N*-linked glycosylation sites, whilst the non-glycosylated, immature, isoform is not associated with the plasma membrane and has a molecular weight of 130-143 kDa (Carow et al., 1996; Lyman et al., 1993). In normal haematopoiesis, FLT3 is expressed in early myeloid and lymphoid progenitors, but not in other cells of the myeloid compartment (such as megakaryocytes, erythrocytes or basophils) (Gabbianelli et al., 1995; Ratajczak et al., 1996; Rosnet et al., 1996). Many leukaemias express FLT3 independent of activating FLT3 mutations. This is usually a consequence of the fact that many malignancies arise in the context of arrested differentiation in progenitor cells (Carow et al., 1996). A good example of this phenomenon is illustrated in chronic myeloid leukaemia (CML), which arises from myeloid progenitor cells. As a population of cells in CML retain the ability to differentiate, FLT3 expression is selectively lost in this population of cells during this process (Uckun et al., 1997). Alternatively, specific oncogenic mutations can induce expression of wild-type (WT) FLT3. Translocation of the *MLL* gene in ALL and AML has been shown to transcriptionally upregulate *FLT3*, and this effect was exacerbated by mutations in the activation loop of FLT3 (Armstrong et al., 2003). Subsequent analysis revealed this phenotype was a result of upregulated *MEIS1*, a canonical target gene of *MLL*-driven malignancies.

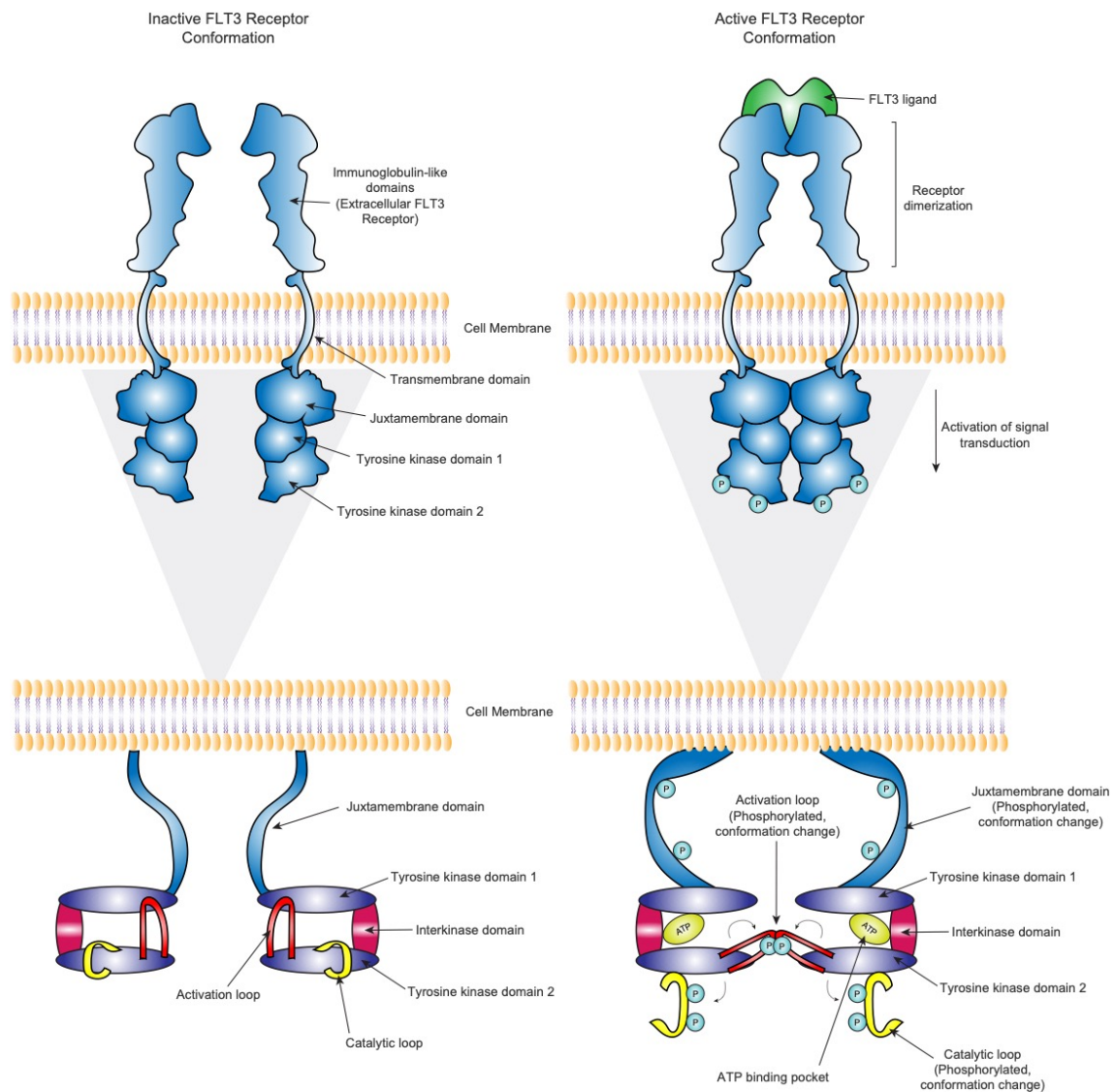


Figure 1.1 Structure of the inactive and active FLT3 receptor.

The FLT3 receptor is composed of an extracellular domain (itself composed of five immunoglobulin-like domains), a transmembrane domain, a juxtamembrane domain, and two tyrosine kinase domains (TKD1 and TKD2). When FLT3 ligand (FLT3L) binds to the extracellular domain, the FLT3 receptor undergoes dimerization, and conformational changes in several functional domains (right panel). The juxtamembrane domain is phosphorylated and changes conformation to a convex shape, thus releasing the activation loop from its ‘locked’ position; the activation loop (that rests in-between tyrosine kinase 1/2 domains) is also phosphorylated and ‘flips’ outwards, providing access to the adenosine triphosphate (ATP) binding pocket. The catalytic loops are also phosphorylated and ‘flip’ outwards, allowing for binding with intracellular proteins and induction of downstream signalling to occur. Conformation diagrams (bottom) adapted from (Grafone et al., 2012).

Inhibition of the Menin-MLL interaction within the MLL fusion complex transcriptionally repressed *MEIS1*, and its transcriptional target gene *FLT3* (Dzama et al., 2020).

1.4.2 Translation, modification, and activation of FLT3

Newly translated FLT3 protein is glycosylated at the endoplasmic reticulum and Golgi apparatus by the addition of fucose, mannose, galactose and N-acetylglucosamide to the N-terminal portion of the protein, which enables membrane association. While the signalling pathways employed by FLT3 to promote its proliferative effects are discussed in more detail in subsequent sections, it is important to note that activating FLT3 mutations still retain signal transduction capabilities intracellularly, prior to FLT3 glycosylation. Oncogenic FLT3 internal tandem duplication (ITD) has been shown to aberrantly activate the transcription factor STAT5 whilst localised at the endoplasmic reticulum, but not other canonical FLT3 downstream effectors such as the MAPK or PI3K pathway (Choudhary et al., 2009). This provides compelling evidence that oncogenic activation of RTKs intracellularly may provide a means of evading normal negative regulatory feedback loops associated with membrane bound RTKs.

Wild-type FLT3 is normally found in its inactive monomeric form, but undergoes dimerization after binding to FLT3L, thus promoting phosphorylation of the tyrosine kinase domain resulting in downstream effector activation. Upon FLT3L binding and dimerization of FLT3, phosphorylated acceptor sites are exposed in the tyrosine kinase domain. The two kinase domains, TKD1 and TKD2, are connected by a flexible peptide that imparts three-dimensional flexibility to the FLT3 kinase domain. Rotation of TKD1 away from TKD2 results in inactive catalytic conformation, whilst TKD1 rotation toward TKD2 results in alignment of catalytic regions of both kinase domains and thus an active conformation (Griffith et al., 2004). The activation loop, a long, thin peptide segment that rests between TKD1 and TKD2 and contains tyrosine residues that can undergo phosphorylation, acts as a physical barrier in the absence of phosphorylation, folding into the space between TKD1 and TKD2 and thus preventing binding of adenosine triphosphate (ATP) and other substrates to the kinase domain. When these residues are phosphorylated, the activation loop adopts an open conformation and thus allows substrates to bind the kinase domain. Apart from the activation loop, the juxtamembrane

domain also possesses two tyrosine residues that can undergo phosphorylation. When FLT3 is in an inactive state, the juxtamembrane region adopts a concave conformation that prevents TKD1 rotation toward TKD2, while also stabilising the activation loop in its closed conformation. Upon binding of FLT3L and dimerization, the tyrosine residues on the juxtamembrane domain are phosphorylated and thus adopt a convex conformation that releases the activation loop from its closed position, allowing for the binding of ATP and other substrates and phosphorylation and activation of downstream proteins (Grafone et al., 2012). After dimerization, the FLT3L-bound receptor is internalised and degraded rapidly to minimise prolonged downstream signalling, in a process that takes approximately 20 minutes. The complex chain of events required to initiate FLT3 signalling, and the rapid turnover of FLT3 post activation is indicative of the necessity to carefully control the potent pro-growth machinery induced by this RTK, and this lifecycle is captured in Figure 1.2.

1.4.3 Downstream effector pathway activation by FLT3

Activation of FLT3 broadly initiates signal transduction via the PI3K-AKT-mTOR, RAS, and MAPK pathways (Scholl et al., 2008). Activated FLT3 binds the linker protein growth factor receptor bound protein 2 (GRB2) via Src homology 2 containing protein (SHC), which allows for binding to other proteins, such as guanine nucleotide exchange factor (SOS). SOS binds to RAS-GTPases and forces their release of guanosine diphosphate (GDP), thereby facilitating the subsequent binding of guanosine triphosphate (GTP) to RAS, activating downstream effector pathways such as MAPK (Simon et al., 1993). These linker/adaptor proteins have also been experimentally demonstrated to be important in PI3K pathway induction. FLT3/SHC forms complex associations with one or more Src homology 2 domain containing inositol polyphosphate 5-phosphatase (SHIP) proteins, along with GRB2, GRB2-associated binding protein 2 (GAB2), Src homology 2 domain containing tyrosine phosphatase 2 (SHP2) and the Casitas B-lineage lymphoma (CBL) family of E3 ubiquitin ligases that act on the p85 subunit of PI3K (Lavagna-Sévenier et al., 1998; Zhang and Broxmeyer, 1999, 2000; Zhang et al., 1999). Activation of the p85 subunit of PI3K results in downstream stimulation of factors such as AKT, which phosphorylates the mechanistic target of rapamycin (mTOR). mTOR is an important component of the nutrient sensory apparatus of the cell, and thus controls a variety of key metabolic systems essential for the maintenance of cellular and

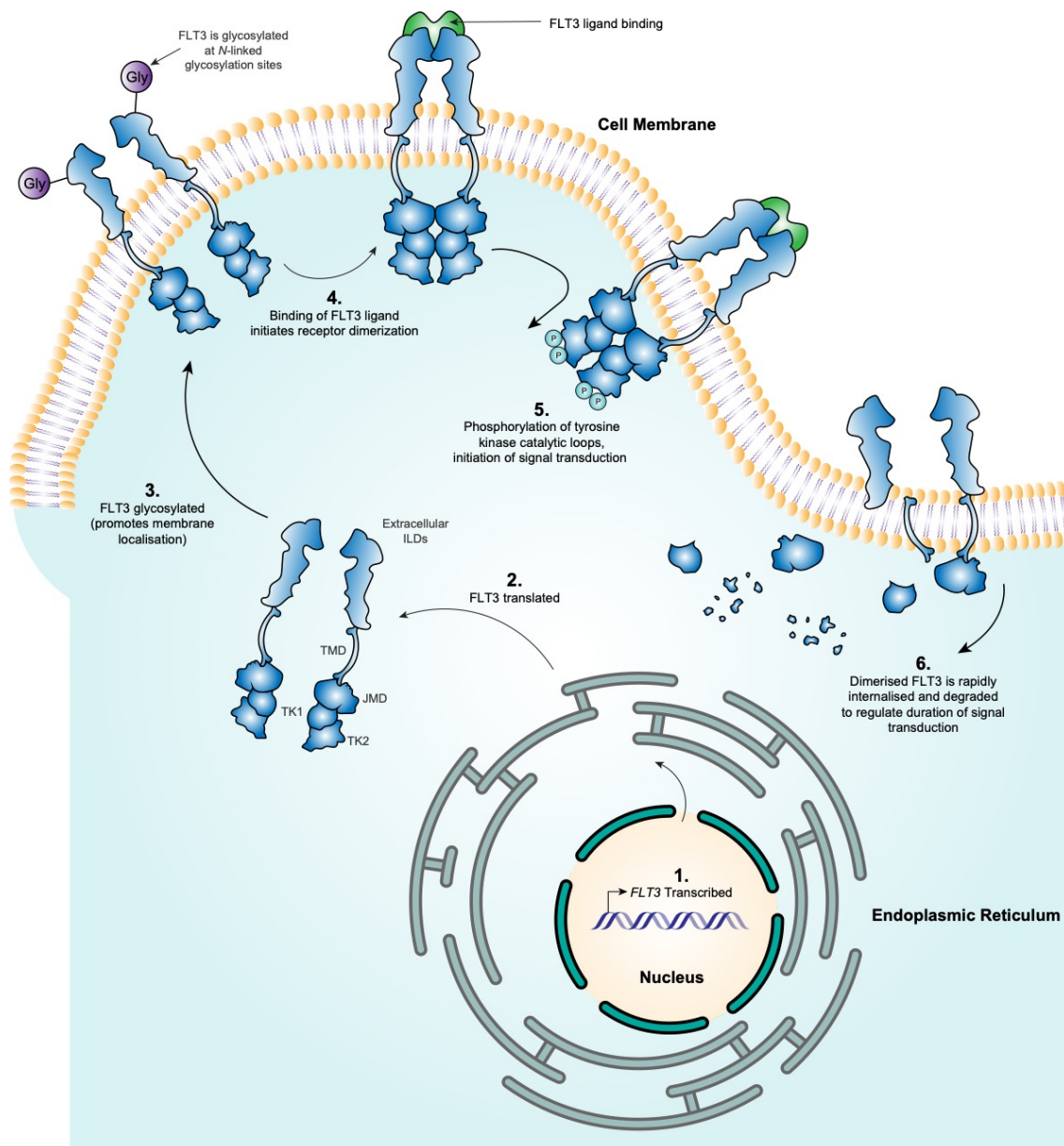


Figure 1.2 Lifecycle of the FLT3 receptor.

After transcription, the FLT3 protein is translated by the endoplasmic reticulum. The ‘immature’ FLT3 protein then undergoes *N*-linked glycosylation which promotes membrane localisation. Upon binding of FLT3L, the FLT3 receptor undergoes dimerization, conformational changes in several key functional domains, and is phosphorylated at the TKDs, thus initiating downstream signal transduction. Active FLT3 is rapidly internalised and degraded to regulate the duration of FLT3-induced signalling.

physiological homeostasis (Liu and Sabatini, 2020). mTOR thus activates the transcription of key metabolic cellular genes, such as those involved in protein synthesis and autophagy, in part through activation of the p70 S6-kinase (p70S6K) and inhibition of eukaryotic transcription initiation factor 4E-binding protein (4E-BP1). As these two proteins are critically important in the mediation of protein translation, activation of p70S6K results in phosphorylation of Ribosomal protein S6 (S6), a component of the 40S ribosomal subunit, thus stimulating protein synthesis and cell survival (Altman et al., 2011; Martelli et al., 2010).

FLT3 activation modulates key transcription factors, such as STAT5A, but generally not the Janus kinases (JAK) family of kinases and other STAT family members, such as STAT1-4, 5B or 6 (Zhang et al., 2000). STAT5 is a key transcriptional regulator of proteins important in the modulation of cell death, such as the B-cell lymphoma 2 (BCL-2) family of anti-apoptotic proteins, and indeed promotes expression of BCL-2 and suppresses transcription of the apoptotic initiator gene BCL-2-associated X protein (BAX) upon FLT3 induction (Debierre-Grockiego, 2004; Lisovsky et al., 1996). Thus, in the context of haematological malignancies wherein FLT3 expression is constitutively activated, pro-survival signals are enhanced whilst pro-apoptotic mediators are suppressed. These biological observations are consistent with clinical responses seen in patients treated with the BCL-2 inhibitor venetoclax, wherein FLT3-ITD mutations conferred either primary or secondary resistance to BCL-2 inhibition (Chyla et al., 2018). Thus, dual inhibition of FLT3 and BCL-2 may prove effectual in overcoming this inherent resistance mechanism in FLT3-driven malignancies (Mali et al., 2020).

1.4.4 *FLT3* mutations in haematological malignancies

Somatic mutations in *FLT3* represent one of the most common oncogenic drivers in AML patients, with some 30% of all patients harbouring *FLT3* mutations either at initial presentation or at relapse (Papaemmanuil et al., 2016). The occurrence of *FLT3* mutations with other oncogenic drivers in AML has been an active area of investigation; it is now relatively well defined that *FLT3* mutations have distinct patterns of co-mutation and mutual exclusivity. For instance, in patients with mutant *NPM1*, *FLT3-ITD* mutations co-occur in 39% of cases (Papaemmanuil et al., 2016). Likewise, *FLT3* mutations are commonly found to co-occur with *PML-RARA* mutations (35%), and AML with

t(6;9)(p23;q34) *DEK-NUP214* translocations (80%). In contrast, *FLT3* mutations are relatively rare in the context of inv(3) and *TP53*-aneuploidy-driven AML, suggesting that only specific genomic events result in molecular “synergy” with mutant *FLT3*.

In AML, two primary classes of *FLT3* mutations arise; internal tandem duplication (ITD, referred to as *FLT3-ITD*) generally within the juxtamembrane domain, defined by the insertion of repeated amino acid sequences, and point mutations in the tyrosine kinase domain (TKD) that result in amino acid substitutions typically in the activation loop of the TKD (referred to as *FLT3-TKD*). In both instances, mutation results in constitutive activation of the FLT3 kinase, and activation of downstream effectors. The following sections discuss these two mutational events in greater detail and outline their implications on downstream signalling effectors.

1.4.4.1 *FLT3* internal tandem duplication (ITD)

Internal tandem duplication of the *FLT3* juxtamembrane region arises as a result of insertion of a variable number of bases (always in multiples of three and can range from anywhere between 3 to >400 base pairs (bp) in exons 14 and 15 of the *FLT3* gene, upstream of the open reading frame (Nakao et al., 1996). During ITD events, a region of the juxtamembrane domain becomes replicated and inserted in a direct head-to-tail orientation, typically at the 5'-end of the juxtamembrane domain (exon 14) (Yokota et al., 1997) (Figure 1.3). However, up to 30% of cases of FLT3-ITD occur in the tyrosine kinase domain itself (Breitenbuecher et al., 2009a), and rare instances of point mutation within the juxtamembrane domain have also been identified (Gianfelici et al., 2011; Reindl et al., 2006). How ITD mutations arise has not been completely elucidated, however it is suggested that DNA replication errors are key contributors, and that subsequent selection of these clones occurs due to the growth advantage imparted by ITD-induced constitutive activation of the FLT3 kinase (Kiyoi et al., 1998).

ITD mutations result in ligand-independent phosphorylation and thus dimerization of the FLT3 receptor, including with WT FLT3 expressed in the same cell (Kiyoi et al., 1998). Mechanistically how ITDs constitutively activate FLT3 has been characterised through use of X-ray crystallography. Strong evidence exists that the juxtamembrane domain region of most RTKs possesses negative regulatory capacity, and crystallography of the

WT FLT3 receptor revealed that the juxtamembrane region extensively contacts the amino- and carboxy-terminal regions of the tyrosine kinase, which acts to stabilise its inactive configuration (Griffith et al., 2004). In the context of ITD, the insertion of extra amino acids prevent association of the juxtamembrane domain with the tyrosine kinase domain by physically lengthening it, thus exposing the kinase to constitutive activation and a catalytically active state in the absence of ligand (D. Kottaridis et al., 2003).

1.4.4.2 *FLT3* tyrosine kinase domain mutations (TKD)

Whilst a majority of newly diagnosed patients that present with mutations in FLT3 harbour ITD mutations, a smaller subset constituting approximately 7-10% of patients harbour amino acid residue replacements caused by missense mutations in the TKD (Nagel et al., 2017). These point mutations primarily occur within the activation loop (residues D835, I836 and Y842) of TK2 (constituting approximately 90% of all FLT3-TKD mutations) (Bacher et al., 2008; Papaemmanuil et al., 2016; Smith et al., 2012), while point mutations in TK1 (N676 and F691) have been reported but are significantly rarer (<10%) (Opatz et al., 2013). Point mutations in other FLT3 domains, such as in extracellular and juxtamembrane domains have also been reported in ~2% of AML patients (Chatain et al., 2015; Fröhling et al., 2007; Reindl et al., 2006). A majority of the mutations discussed above occur in the activation loop, which acts to block ATP and substrate binding to the kinase domain when the receptor is inactive. These missense mutations interfere with the activation loop's negative-regulatory function and result in constitutive kinase activation, independent of FLT3L binding (Abu-Duhier et al., 2001; Xu et al., 2003). Similar to ITD, TKD mutations constitutively activate downstream effector pathways such as PI3K/AKT/mTOR, RAS/MAPK, however unlike ITDs, FLT3-TKD mutations cannot inhibit the function of the C/EBP and PU.1 transcription factors nor activate the STAT5 signalling pathway (Choudhary et al., 2005). A comprehensive summary, and comparison, of the FLT3 mutational landscape is shown in Figure 1.4.

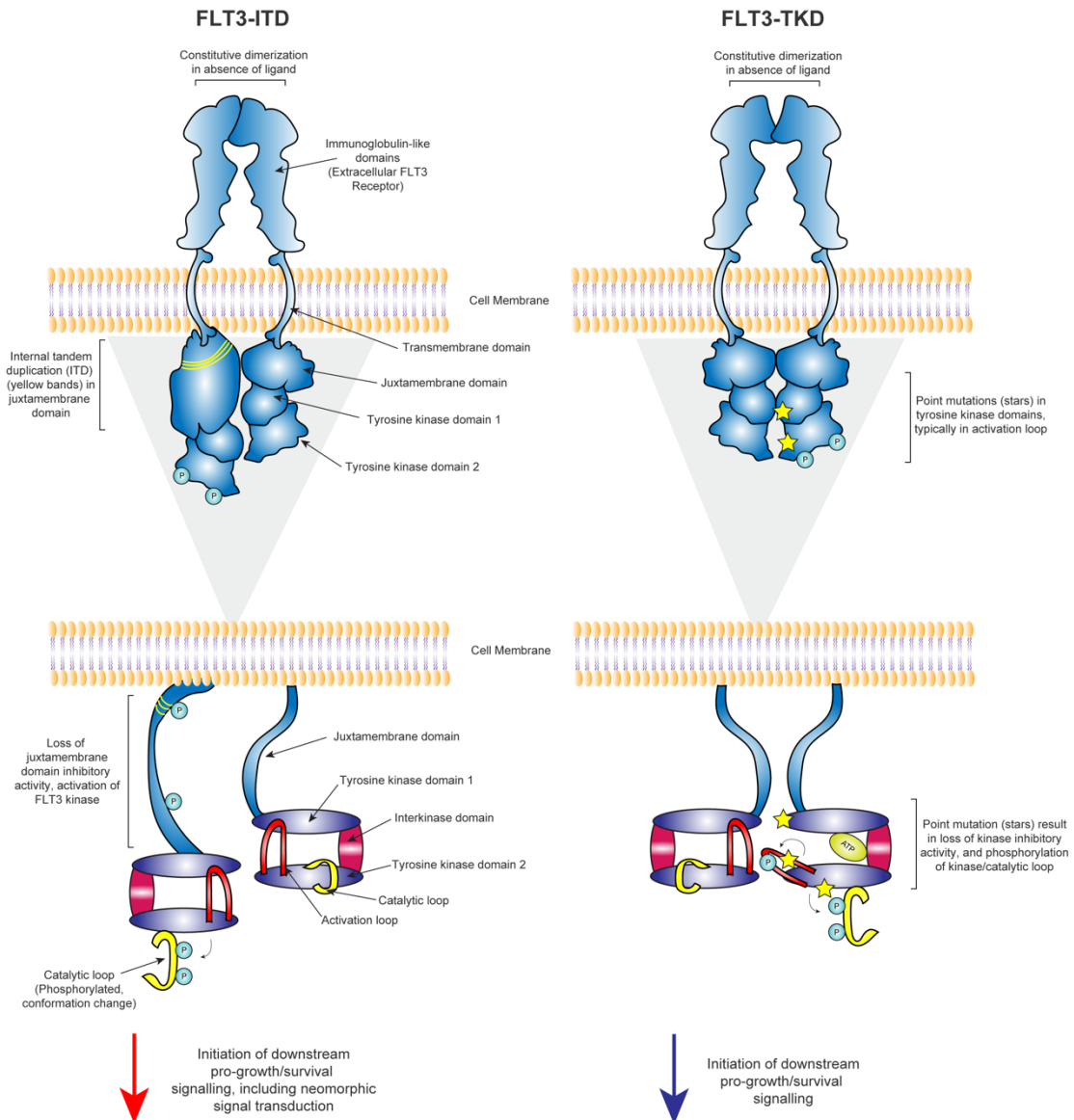


Figure 1.3 FLT3 receptor conformational changes induced by FLT3 mutations

Internal tandem duplication (ITD, left panel) involves 3 to >400bp insertions in (typically) the juxtamembrane domain of FLT3, resulting in constitutive phosphorylation of the FLT3 receptor (left panel). The elongated juxtamembrane domain results in loss of ability to stabilise the activation loop spanning TKD1/2, and thus phosphorylation of the tyrosine kinase domains and activation of downstream signalling, including gain-of-function neomorphic signal transduction. Tyrosine kinase domain (TKD, right panel) mutations typically occur in TKD2 of the FLT3 receptor, particularly in the activation loop spanning TKD1/2. These mutations result in constitutive phosphorylation of the tyrosine kinase domains and activation of downstream signalling transduction. Interestingly, TKD mutations cannot initiate some of the neomorphic signal transduction events triggered by FLT3-ITD mutations.

1.4.4.2.1 Aberrant signalling pathways induced by mutant FLT3

Carefully regulated induction of signalling effectors downstream of WT FLT3, such as RAS, MAPK, PI3K and STAT are required during normal haematopoiesis. FLT3-ITD mutations constitutively activate the RAS/MAPK and PI3K pathways which contribute to the growth advantage leukaemic clones carrying these mutations possess (Chen et al., 2010; Takahashi, 2006). However, the role of STAT5 in FLT3-ITD is particularly intriguing. Upon dimerization and phosphorylation post FLT3L binding, WT FLT3 phosphorylates STAT5, but it does not possess DNA binding activity; in FLT3-ITD phosphorylation of STAT5 is markedly increased and gains DNA binding capacity (Hayakawa et al., 2000). This STAT5 gain-of-function is independent of SRC, JAK and tyrosine kinase 2 (TYK2), and *in vitro* kinase assays revealed STAT5 is a direct target of FLT3-ITD (Choudhary et al., 2007). STAT5 is a transcriptional activator of the cyclin D1 cell cycle regulator, the anti-apoptotic regulator B-cell lymphoma-extra-large (BCL-xL) and the proto-oncogene c-MYC, all of which contribute to enhanced proliferation and anti-apoptotic signalling induced by FLT3-ITD (Nosaka et al., 1999; Yonemura et al., 1997). Besides direct transcriptional activation of the above effectors, more recent studies have demonstrated that FLT3-ITD-induced STAT5 expression contributes to the increased generation of reactive oxygen species (ROS) via RAS-related C3 botulinum toxin substrate 1 (RAC1), a component of the ROS-producing NADPH oxidases. Increased ROS results in double-stranded DNA breaks (DSBs) and repair errors, an effect that was abrogated upon FLT3 inhibition. This data suggests that FLT3 signalling may regulate DSB repair pathways (Fan et al., 2010; Sallmyr et al., 2008).

Other transcription factors have been shown to be aberrantly regulated in the context of FLT3-ITD mutations. The myeloid maturation transcription factors PU.1 and CCAAT/enhancer-binding protein (C/EBP) are both repressed, suggesting FLT3-ITD contributes to the differentiation block seen in AML (Zheng et al., 2004). Similarly, FLT3-ITD can enhance AML survival and proliferation by suppressing the transcription factor forkhead box O3 (FOXO3)-mediated apoptosis function (via upregulation of pro-survival effectors such as p27Kip1 and BCL-2-like protein 11 (BIM) (Scheijen et al., 2004), and via FOXO3-mediated upregulation of the histone deacetylase 8 (HDAC8) to inactivate p53 (Long et al., 2020).

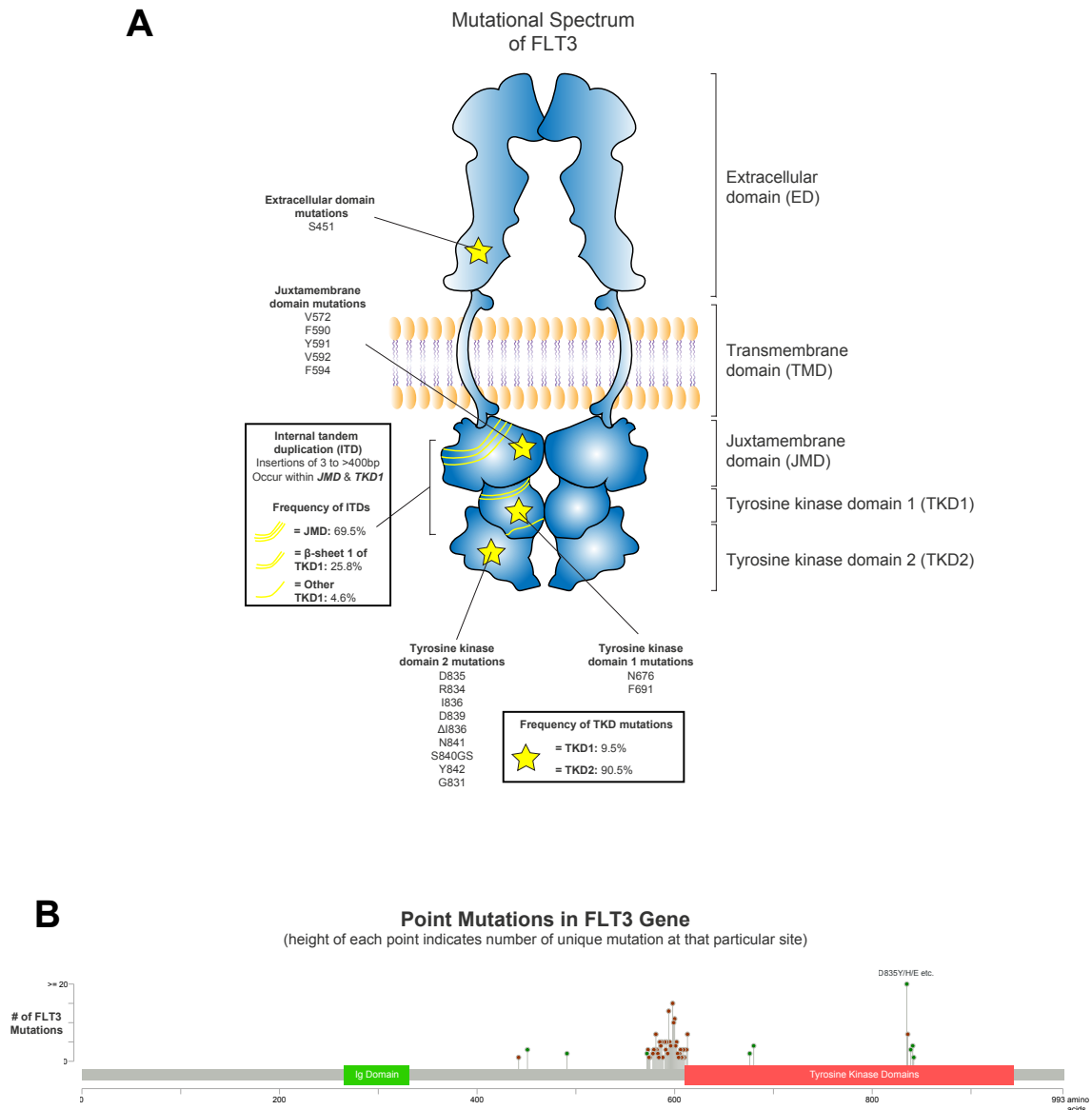


Figure 1.4 Mutational landscape of FLT3 receptor mutations.

A. Graphical representation of mutations in different subunits of the FLT3 receptor. While point mutations can occur in most subunits of the receptor, ITDs occur primarily in the juxtamembrane domain (JMD, 69.5%) and β -sheet 1 of tyrosine kinase domain 1 (TKD1). A majority of point mutations that affect the tyrosine kinase domains occur in TKD2 (90.5%), particularly in the activation loop guarding the ATP-binding pocket. **B.** Graphical representation of the number of unique point mutations occurring at each amino acid sequence of the FLT3 receptor. The higher the point, the more unique mutations known to occur at this site. Panel A data adapted from (Patnaik, 2018).

In recent years, FLT3-ITD-induced reprogramming events, particularly in cellular metabolism, have been described. Experiments in murine BaF3 cells expressing FLT3-ITD revealed enhanced glycolytic pathway activity via AKT/mTOR-mediated phosphorylation of hexokinase 2 (HK2), a key glycolytic enzyme (Ju et al., 2017). Mitochondrially-localised HK2 was found to favour ATP transfer from oxidative phosphorylation (OXPHOS) to enhance glycolysis, an observation that additionally suppressed mitochondrial apoptotic processes by preventing release of cytochrome c from the mitochondrial permeability transition pore. Combination therapy of glycolytic inhibitors along with FLT3 inhibitors showed encouraging results (Huang et al., 2016). These data support previously published results illustrating that use of the non-functional glucose isomer 2-deoxy-D-glucose (2-DG) displayed favourable anti-leukaemic activity in FLT3-ITD-driven AMLs via inhibition of N-linked glycosylation (Larrue et al., 2015). Several other studies have explored various mechanisms of therapeutically leveraging this glycolytic dependence; synthetic lethality screens designed to elucidate mechanisms of sensitisation to FLT3 inhibitors revealed that several of the top hits were associated with metabolic processes, such as the ataxia telangiectasia mutated (ATM) gene, which was demonstrated to activate glucose-6-phosphate dehydrogenase (G6PD) to maintain redox homeostasis (Gregory et al., 2016). In addition, inhibiting FLT3 itself with FLT3-specific inhibitors has been demonstrated to suppress this glycolytic dependence, whilst concomitantly reducing glutathione metabolism resulting on increased dependence on exogenous glutamine, a mechanism of resistance to FLT3 inhibition. This phenomenon can be therapeutically leveraged by utilising FLT3 inhibitors in combination with glutaminase inhibitors (Alvarez-Calderon et al., 2015; Gallipoli et al., 2018; Gregory et al., 2018).

Besides glycolytic metabolism, mutant FLT3 has been implicated in other metabolic processes necessary for proliferation. Whilst the one-carbon/folate cycle has been targeted for decades in haematological malignancies through means of antifolate chemotherapy (Farber et al., 1948), more recently the mitochondrial enzyme methylenetetrahydrofolate dehydrogenase-cyclohydrolase 2 (MTHFD2), which catalyses conversion of methylene-tetrahydrofolate (THF) to formyl-THF and has important roles in purine metabolism, OXPHOS and redox homeostasis, has been described as a metabolic vulnerability in FLT3-ITD AMLs (Pikman et al., 2016). In *IDH2*^{R140Q}/FLT3-ITD-mutant AML, combined inhibition of FLT3 and the IDH2 inhibitor AG-221

promoted induction of normal haematopoiesis, and reduction of mutant allele burden greater than AG-221 alone, via reversal of dysregulated DNA methylation and transcription (Shih et al., 2017). Similarly, in a recent study, both WT FLT3 and FLT3-ITD were found to enhance activation of mutant IDH1 via activation of JAK2, providing rationale to combine FLT3 inhibition with IDH1 inhibition irrespective of FLT3 mutation status (Chen et al., 2019a). Metabolic rewiring events in haematological malignancies will be revisited later in this chapter. A summary of signalling events initiated by both WT and mutant FLT3 are shown in Figure 1.5.

1.4.5 FLT3 targeted therapy

Therapeutic inhibition of FLT3 has become one of the most intensely studied aspects of AML biology in recent years, underpinned by the frequency of FLT3 mutations in disease and historical success targeting other RTKs (Daver et al., 2019). Along with improved supportive care, the integration of FLT3 inhibitors and increased use of allogeneic haematopoietic stem cell transplantation (alloHSCT) has greatly increased clinical outcomes in patients, particularly in the relapsed/refractory context. This was demonstrated in a recent retrospective study investigating differences in FLT3-ITD-mutant patient outcome from 2000-2014, where it was seen that in later years as FLT3 inhibitors and alloHSCT become more widespread clinical outcome vastly improved (Badar et al., 2015). In this section, we will discuss the current status of FLT3 inhibitors for the treatment of AML.

1.4.5.1 First-generation tyrosine kinase inhibitors

The first generation of tyrosine kinase inhibitors (TKIs) used for the treatment of FLT3 mutations in AML were ‘broad-spectrum’, and displayed off-target activity against other RTKs due to the structural homology of the tyrosine kinase ATP binding site, which they competitively inhibit (Sawyers, 2002). Several TKIs have been explored in FLT3 mutant AML, and include lestaurtinib (CEP-701), sunitinib (SU11248), sorafenib and midostaurin (PKC412) (Fiedler et al., 2015; Pratz et al., 2009; Smith et al., 2004; Stone et al., 2005). Weak monotherapy activity of first generation TKIs was generally observed, with mixed responses when combined with standard of care chemotherapy and increased toxicity in certain contexts (Fiedler et al., 2015; Röhlig et al., 2015).

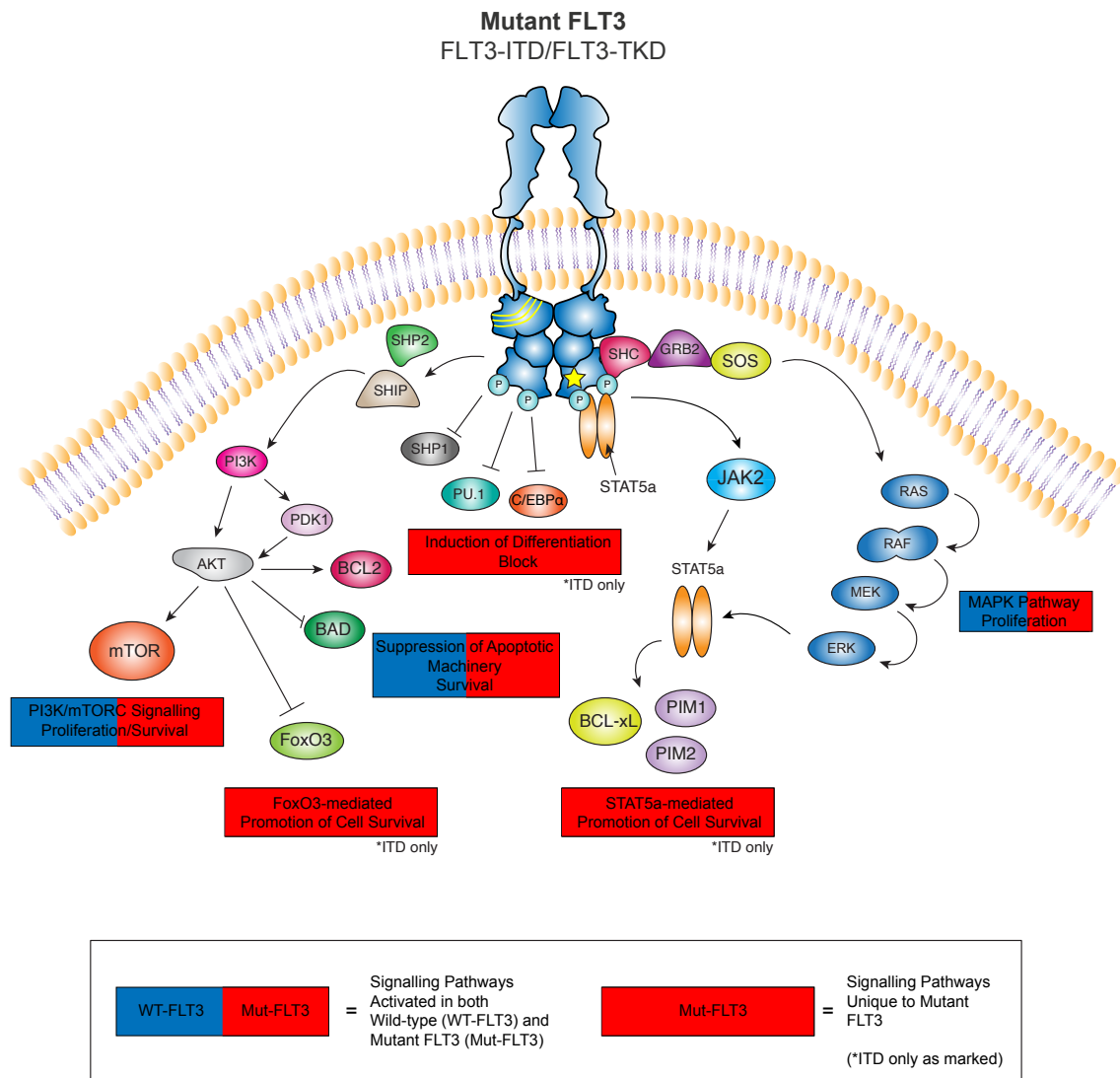


Figure 1.5 Signal transduction cascades initiated by mutant FLT3.

Schematic representation of a selection of signalling pathways downstream of mutant FLT3 (either FLT3-ITD or FLT3-TKD). In the context of wild type (WT) FLT3 signalling, the activation of the PI3K/AKT/mTOR and MAPK pathways in appropriate circumstances is necessary for the proliferation and survival of haematopoietic cells (denoted as blue pathways in the diagram). Mutant FLT3 results in constitutive activation of these pathways (denoted by blue/red pathways in the diagram) as well as unique neomorphic signalling cascades, particularly the activation of STAT5A with DNA-binding capacity in FLT3-ITD mutations (denoted by red pathways in the diagram). Interestingly, FLT3-TKD mutations cannot activate some FLT3-ITD-induced signalling cascades; these are denoted as ‘ITD only’ on the diagram.

Lestaurtinib treatment, in either post-salvage chemotherapy or in combination with high-dose chemotherapy did not result in improved response rates in either case (Knapper et al., 2017; Levis et al., 2011). Similarly, sunitinib monotherapy induced only partial, and transient, responses (Fiedler et al., 2005), whilst sunitinib with standard of care induction chemotherapy resulted in significant response – 50% of patients with FLT3-ITD mutations and 38% of patient with FLT3-TKD mutations achieved complete response (Fiedler et al., 2015). On this vein, single-agent administration of sorafenib displayed weak anti-leukaemic activity in a phase 1 study of relapsed/refractory FLT3-driven AML (in which 10% of patients achieved complete remission/complete remission with incomplete haematological recovery) (Borthakur et al., 2011), while combination with induction chemotherapy resulted in extended event free survival (EFS) but not overall survival in a younger AML patient cohort (<60 years) (Röllig et al., 2015). In frailer, elderly patients, induction chemotherapy and sorafenib resulted in no improved survival benefit, and was associated with significant toxicity (Serve et al., 2013). As seen with other multi-kinase inhibitors, single-agent midostaurin treatment in relapsed/refractory FLT3-mutant patients showed limited, albeit well-tolerated activity (Fischer et al., 2010). However, in a pivotal phase 3 study (the RATIFY study), midostaurin in combination with standard of care (7+3) chemotherapy, was demonstrated to lead to significantly improved event-free and overall survival compared to chemotherapy alone (Stone et al., 2017). Based on this landmark study, the US FDA and the European Commission approved midostaurin in combination with induction chemotherapy in newly diagnosed *FLT3*-mutant AML in April and September 2017 respectively.

An interesting observation in the clinical trial run by Stone and colleagues is that midostaurin and chemotherapy resulted in increased overall survival in all *FLT3* mutation subtypes, including *FLT3-ITD* (low and high allelic burden) and *FLT3-TKD*, suggesting that midostaurin's off-target RTK inhibitory function may contribute to the phenotype observed, by inhibiting multiple kinases (Daver et al., 2019). Indeed, even though midostaurin combination therapy is clinically approved, significant debate remains on its role in overall survival responses and in maintenance therapy; several ongoing studies are currently underway examining these issues (Maziarz et al., 2016; Schlenk et al., 2015).

1.4.5.2 Next-generation specific FLT3 inhibitors

A key limitation of first-generation multi-kinase inhibitors was their lack of specificity for mutant FLT3, which resulted in reduced inhibition of FLT3 kinase activity and the potential for concomitant increases in toxicity associated with inhibition of multiple signalling pathways (Daver et al., 2019). In recent years, extensive research has yielded several highly specific and potent inhibitors of mutant FLT3, that have progressed to various stages of clinical development. Type I inhibitors, such as gilteritinib (ASP2215) and crenolanib (CP-868), are able to bind both the active and inactive conformations of FLT3. Type II inhibitors, such as quizartinib (AC220), are only able to bind the inactive kinase conformation (Daver et al., 2015) (shown in Figure 1.6).

Unlike their first-generation counterparts, highly specific FLT3 inhibitors have been demonstrated to possess significant single-agent activity. A phase 1/2 trial of gilteritinib in relapsed/refractory AML elicited a 40% overall response rate, inclusive of patients harbouring WT FLT3 (Perl et al., 2017). The overall response rate was increased to 52% in patients harbouring FLT3 mutations. Interestingly, in patients previously treated with the multi-kinase inhibitor sorafenib, an overall response of 49% was achieved. A subsequent pivotal phase 3 trial (the ADMIRAL study), examining either gilteritinib monotherapy or salvage chemotherapy in relapsed/refractory FLT3-mutant AML demonstrated increased overall survival (9.3 months versus 5.6 months) with less adverse events recorded in the gilteritinib-treated group (Perl et al., 2019). Based on this data, the US FDA approved gilteritinib for the treatment of relapse/refractory FLT3-mutant AML in November 2019. Similarly, the type I inhibitor crenolanib has demonstrated encouraging efficacy in relapsed/refractory FLT3-mutant AML. An early-stage phase 2 trial elicited a complete remission (with incomplete blood count recovery) in 23% of patients previously untreated with FLT3 inhibitors; this was reduced to 5% in patients previously exposed to FLT3 inhibition, however this study had relatively small patient numbers ($n = 38$) (Randhawa et al., 2014). A larger phase 2 study in 69 patients elicited a complete remission (with incomplete blood count recovery) in 39% of patients, and a partial response of 11% in FLT3 inhibitor-naïve patients; and combined complete remission (with incomplete blood count recovery) and partial response rate of 28% in patients previously treated with FLT3 inhibitors (Cortes et al., 2016). Trials in larger cohorts of patients are still ongoing.

The type II FLT3 inhibitor quizartinib was the first potent and specific FLT3 inhibitor developed and has undergone extensive preclinical and clinical study (Chao et al., 2009; Zarrinkar et al., 2009). As mentioned above, all FLT3 inhibitors interact with the ATP-binding pocket of the intracellular tyrosine kinase, thus preventing autophosphorylation and downstream effector activation; however, there are differences in binding modalities based on the conformation of receptor itself. Upon binding to FLT3L, a conserved Asp-Phe-Gly motif (referred to as the DFG motif) in the activation loop is ‘flipped’. The phenylalanine (Phe) residue of the DFG motif in particular determines conformation of the activation loop; when the phenyl group of the Phe residue is angled away from the ATP-binding pocket, the active form of the receptor is activated (referred to as DFG-in). Similarly, when the phenyl group is angled toward the ATP-binding pocket, the inactive (DFG-out) conformation is achieved (Ke et al., 2015). In contrast to type I inhibitors, which directly bind the ATP-binding pocket while the receptor is in active conformation, type II inhibitors such as quizartinib bind a hydrophobic region directly adjacent to the ATP-binding pocket that is accessible only when the FLT3 receptor is in its inactive conformation (Angiolini, 2011). As a result, type II inhibitors are efficacious only in FLT3-ITD mutations, as tyrosine kinase point mutations (discussed above) result in a constitutively active receptor conformation. In several phase 2 studies, quizartinib elicited promising results, with high rates of response particularly in the relapsed/refractory patient cohort. Single agent quizartinib was demonstrated to induce an overall response rate of 74-77% with good tolerance and safety profile in relapsed/refractory patients (Cortes et al., 2018a). A follow up phase 2 study examining the efficacy of lower dose quizartinib in a similar patient cohort yielded promising results, with an overall composite complete remission rate of 47%, illustrating that lower doses than originally examined in the first phase 2 trial may be similarly, or equally, efficacious (Cortes et al., 2018b). A large phase 3 trial, examining the efficacy of single-agent quizartinib versus salvage chemotherapy in relapsed/refractory patients recently reported superior overall survival in the quizartinib treated group versus chemotherapy (median survival 6.2 versus 4.7 months) with good safety profile and an overall response rate of 69% versus 30% in the quizartinib and chemotherapy arms respectively (Cortes et al., 2019). However, the US FDA rejected approval for quizartinib, citing the modest survival results, high proportion of randomly assigned but subsequently untreated patients, and general lack of robust data. Further clinical trials utilising quizartinib are underway.

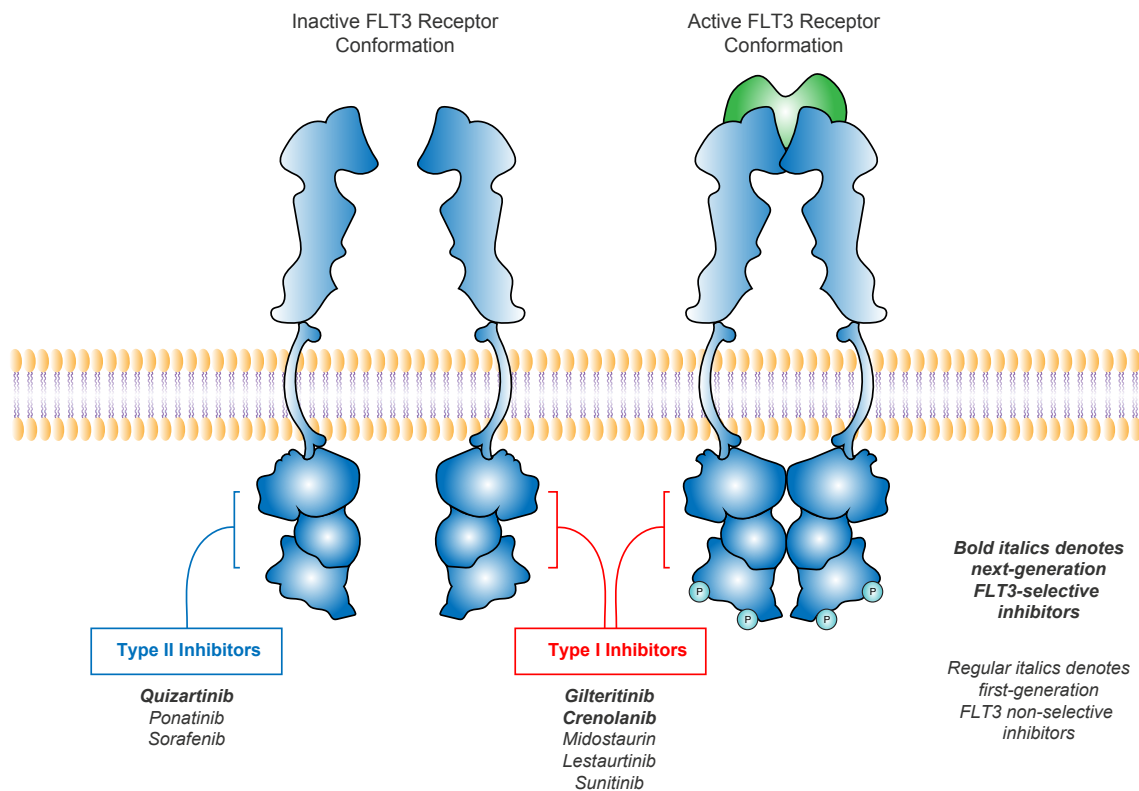


Figure 1.6 Summary of FLT3 inhibitors currently under pre-clinical and clinical investigation.

Schematic illustration of small molecule inhibitors currently under investigation for treatment of FLT3 mutations in haematological malignancies. Type I inhibitors are able to bind both the active and inactive conformations of the FLT3 kinase domain (shown in red in the diagram). Type II inhibitors are only able to bind the inactive kinase conformation (shown in blue in the diagram). In both families of inhibitors, bold italics denotes next-generation compounds with high specificity for the FLT3 receptor; regular italics denotes first-generation inhibitors that are non-selective for FLT3 and thus inhibit multiple kinases (also known as pan-kinase inhibitors). Figure adapted from (Daver et al., 2019).

1.4.5.3 Mechanisms of resistance to FLT3 inhibitors

FLT3 inhibitor monotherapy elicits significant responses in AML patients harbouring FLT3-mutant disease; however, these responses are in general transient, and eventually patients relapse. The resistance mechanisms to FLT3 inhibition have been well characterised and include both cell intrinsic and extrinsic factors. These are briefly summarised below.

1.4.5.3.1 Primary intrinsic resistance mechanisms

Among the most important primary intrinsic resistance mechanisms to targeted FLT3 inhibitor therapy is inherent lack of addiction of FLT3 signalling within the AML blast population, as a result of multiple leukaemic clones or low allelic burden for mutant FLT3 (Larrosa-Garcia and Baer, 2017). As several studies of AML population dynamics in response to chemotherapy/targeted therapy have demonstrated, clones harbouring FLT3 mutations tend to emerge during relapse, suggesting that maximal clinical efficacy can be obtained when allelic burden for mutant FLT3 is highest (Pratz et al., 2010). Additionally, the type of FLT3 inhibitor used can have significant implications on therapeutic efficacy, as use of type II inhibitors such as quizartinib in FLT3-TKD disease will prove inefficacious (Smith et al., 2012).

Other cell intrinsic mechanisms of resistance, such as rewiring of apoptotic regulatory pathways, have been reported. A non-juxtamembrane ITD integrated into the first kinase domain (denoted FLT3-ITD^{627E}) was demonstrated to confer resistance to midostaurin by upregulating the pro-survival protein MCL1 via the adapter protein GBR2 (Breitenbuecher et al., 2009b). Additionally, whilst FLT3-ITD and -TKD mutations are considered to be mutually exclusive in FLT3 inhibitor-naïve patients, 1-2% of patients harbour both mutations, dubbed FLT3-ITD-TKD. These mutations were shown to confer resistance to FLT3 inhibitors via upregulation of the pro-survival protein BCL-xL (Bagrintseva et al., 2005). Finally, inherent upregulation of the pro-survival protein BCL-2 in a FLT3-independent manner was further demonstrated to confer resistance to FLT3 inhibition (Kohl et al., 2007).

1.4.5.3.2 Secondary (acquired) resistance mechanisms

Among the most frequent and well-characterised mechanisms of secondary resistance to FLT3 inhibition is the acquisition of TKD mutations in the context of FLT3-ITD mutant AML. As class II inhibitors such as quizartinib possess efficacy only in FLT3-ITD-driven AML where the FLT3 receptor is in its inactive state, acquired point mutations, typically D835 and F691, confer resistance to class II inhibitors and cross-resistance to the first generation inhibitor sorafenib (Baker et al., 2013; Man et al., 2012; Smith et al., 2015). The D835 point mutation occurs in the activation loop of FLT3, serving to stabilise the loop in a “DFG-in” conformation, thus preventing binding of quizartinib and sorafenib. The F691 mutation occurs in the binding pocket of ATP and is thus a conserved gatekeeper residue; substitution of larger amino acids for smaller ones prevents binding of the inhibitor to the ATP binding site. Of considerable interest is the observation that F691 mutations appear to occur exclusively in patients previously treated by FLT3 inhibitors; to date, the F691 mutation has not been described in FLT3 inhibitor-naïve patients (Nguyen et al., 2017).

Aside from acquisition of novel mutations within FLT3 itself, several other secondary resistance mechanisms to FLT3 inhibitor therapy have been described. A comprehensive analysis of patients who relapsed following single agent gilteritinib therapy revealed acquired mutations in the RAS/MAPK signalling pathway as among the most common mechanism of resistance, particularly activating mutations in *NRAS* and *KRAS* (40% of patients) (McMahon et al., 2019a). A further 12.2% of patients acquired mutations in the F691 gatekeeper residue as aforementioned and interestingly, 5% of patients acquired *BCR-ABL* fusion mutations. In addition to these activating mutations in canonical FLT3 downstream effector pathways, the Proviral Integration site for Moloney murine leukaemia virus (PIM) family of kinases, which promote growth and survival in AML, have been demonstrated to be transcriptionally upregulated downstream of FLT3-ITD, and to potentiate FLT3 signalling (Green et al., 2015; Natarajan et al., 2013). In these studies, upregulation of PIM-1 and PIM-2 was uncovered in patients with acquired resistance to sorafenib, and ablation of PIM signalling either genetically or pharmacologically re-sensitized cells to FLT3 inhibition (Green et al., 2015). Additionally, increased phosphorylation of the AXL receptor tyrosine kinase is observed in FLT3-ITD mutant AML when treated with FLT3 inhibitors, such as midostaurin and quizartinib, and constitutive phosphorylation of AXL can occur resulting in acquired

resistance to these compounds (Park et al., 2015). These data provide a molecular rationale for co-targeting the AXL kinase concurrently with FLT3, which can be achieved using the type I inhibitor gilteritinib (Ueno et al., 2014). Finally, the spleen tyrosine kinase (SYK), a cytoplasmic kinase that transactivates FLT3 by directly binding to it has been demonstrated to act as a resistance mechanism to FLT3 inhibitors *in vivo* – however, the relevance of this phenomenon clinically in broad terms has not been characterised (Puissant et al., 2014).

1.4.5.3.3 Extrinsic resistance mechanisms

As myeloid malignancies originate from within the bone marrow, the microenvironmental niche of this unique site can further contribute to the resistance of FLT3 targeted therapy. Indeed, it has been anecdotally noted in the clinic that FLT3 inhibitors induce rapid clearance of AML blasts in peripheral blood, a phenomenon not seen in the bone marrow – suggesting location-specific resistance mechanisms may be at play (Minson et al., 2017). For instance, in response to chemotherapy, increased FLT3L secretion by the bone marrow microenvironment further stimulates blasts harbouring FLT3 mutations, thus promoting resistance to FLT3 inhibition (Levis et al., 2011; Sato et al., 2011). Despite carrying mutations that constitutively activate the kinase activity of FLT3, these cells still remain sensitive to FLT3L. In addition, microenvironmental secretion of fibroblast growth factor 2 (FGF2) in response to chemotherapy binds to fibroblast growth factor receptor (FGFR), thus further stimulating blasts (Poulain et al., 2017; Tamburini et al., 2009; Xu et al., 2003, 2016). In both of the above contexts, FLT3L and FGF2 mechanistically blocks ERK inhibition in response to FLT3 inhibitors, a phenomenon that is potentially mediated by WT FLT3 co-expressed in the cell (Chen et al., 2016). The CXCL12-CXCR4 axis, a pathway critical for mediating homing of progenitor cells to the bone marrow and their subsequent utilisation in response to stress, has also been implicated as a resistance mechanism to FLT3 therapy. Deletion of *Pim1* in FLT3-ITD cells prevented induction of disease in *in vivo* bone marrow reconstitution assays, by decreasing surface expression of Cxcr4 and thus abrogating Cxcl12-Cxcr4 homing which intrinsically activate compensatory pro-growth pathways that overcome FLT3 inhibition (Grundler et al., 2009; Onish et al., 2014). Finally, hepatic enzymatic inactivation of midostaurin by cytochrome P450 3A4 (CYP3A4) has been reported

(Duttreix et al., 2013), including, importantly, in the bone marrow microenvironment where CYP3A4 is highly expressed in stromal cells (Chang et al., 2017).

In summary, significant advances in the understanding of FLT3 biology and therapeutic interventions have occurred in the last two decades. Whilst the canonical FLT3 signalling pathways have been relatively well characterised, several emerging facets of FLT3 biology are only now being discovered including the role of mutant FLT3 in mediating immune system interactions, epigenetic regulation of mutant FLT3, and, importantly, metabolic rewiring events induced by mutant FLT3. FLT3-ITD-induced metabolic reprogramming events have been demonstrated to contribute to resistance to FLT3 inhibitor therapy; however, other metabolic reprogramming events mediated by FLT3 mutations are only now being characterised. The following section of this chapter briefly examines metabolic reprogramming events, particularly in the context of haematological cancers.

1.5 Cancer metabolism

Tumour cells reprogram cellular metabolic pathways to meet heightened biosynthetic and bioenergetic demands associated with uncoupled cellular growth. Indeed, as many of these reprogramming events are observed across a broad spectrum of tumours, this phenomenon is acknowledged as an emerging Hallmark of Cancer (Hanahan and Weinberg, 2011) and considerable work in recent years has revealed the complex rewiring that occurs to drive these proliferative events. The earliest discoveries in the field of cancer metabolism, namely the observation by Otto Warburg in the 1920s that cancer cells favour fermentation (wherein pyruvate is converted to lactate) in preference to aerobic respiration processes utilised in normal cells (Alfarouk et al., 2014), predates the discovery of tumour suppressors and oncogenes by approximately 50 years (DeBerardinis and Chandel, 2016). Indeed, folate antimetabolites which inhibit the activity of dihydrofolate reductase (DHFR) formed the basis of the first chemotherapy agents ever utilised in patients in the 1940s, when Sidney Farber used aminopterin to induce tantalising (yet transient) remissions in childhood ALL (Farber et al., 1948). Increased understanding of the plethora of metabolic rewiring events has in recent years revealed exciting new therapeutic modalities, including targeting of disease-specific mutations (such as *IDH* mutations in AML and aggressive brain tumours such as glioblastoma) and

the potential for dietary modifications to inhibit cancer progression (Kanarek et al., 2020; Tajan and Vousden, 2020).

While most metabolic rewiring events broadly occur across multiple distinct tumour types, many are also context specific and a complete discussion of the spectrum of metabolic reprogramming events threatens to overwhelm any review. Thus, the remainder of this chapter shall focus on metabolic rewiring phenomena occurring primarily in leukaemias or other haematological malignancies.

1.5.1 Aberrant glucose and aerobic glycolytic metabolism in leukaemia

Glycolysis converts glucose into pyruvate and in so doing yields two molecules of ATP. Downstream of this process, pyruvate is converted to lactate by aerobic glycolysis or shunted into the tricarboxylic acid (TCA) cycle, where it is converted to acetate by pyruvate dehydrogenase (PDH) in a process that yields nicotinamide adenine dinucleotide hydrogen (NADH), which subsequently undergoes oxidative phosphorylation within the mitochondria and yields 36 molecules of ATP (Fadaka et al., 2017). Paradoxically, tumours prefer to utilise pyruvate for the less efficient fermentation reaction to lactate (referred to as the Warburg Effect, as alluded to above) in preference to oxidative phosphorylation.

Aside from the observations by Otto Warburg almost a century ago, considerable effort has been dedicated to determining how cancer cells utilise glucose, and particularly whether this process can be exploited for therapeutic gain. In AML, some evidence exists that patients harbour high glycolytic metabolism at diagnosis (which was suggested to associate with positive outcome), but this study did not stratify patients based on mutational status (and thus the molecular driver of glycolytic metabolism was not elucidated), and the total number of patients studied was small (Herst et al., 2011). Another study utilised a 6-metabolite signature (all involved in glucose metabolism) and patient serum to demonstrate prognostic value in cytogenetically normal AML (Chen et al., 2014). The mechanistic relevance of these findings was characterised in detail by Scadden and colleagues, who showed that deletion of the glycolytic enzymes M2 pyruvate kinase (PKM2) significantly inhibited leukaemia initiation while sparing normal

haematopoiesis, and lactate dehydrogenase A (LDHA) which broadly inhibited both leukaemic and steady-state haematopoiesis *in vivo* (Wang et al., 2014b).

Similarly, inhibition of glucose metabolism has been demonstrated to mediate antileukaemic effects. 2-deoxy-D-glucose (2-DG), a glucose analogue and glycolytic inhibitor, induces apoptosis in paediatric precursor-B acute lymphoblastic leukaemia cells; indeed, the authors show that this subtype of malignancy transcriptionally upregulates genes involved in glycolysis, with concomitant downregulation of TCA cycle genes compared to normal CD34⁺ progenitor cells suggesting disease-specific dependency (Boag et al., 2006). Similarly, inhibition of the hexokinase family of enzymes, which catalyse the first step of glycolysis, depletes ATP pools in AML cells and synergises with inhibition of mechanistic target of rapamycin complex 1 (mTORC1) to trigger apoptosis (Xu et al., 2005). Along the same vein, impairment of glucose uptake via genetic depletion of solute carrier family 2 member 1 (*SLC2A1*), shifts cells to catabolic metabolism (in preference to anabolic metabolism) and results in cell growth arrest in *BCR-ABL1* fusion B-ALL cells. Inhibition of glucose metabolism via 2-DG thus synergises with dasatinib, a next-generation multi-kinase inhibitor (Liu et al., 2014b). This phenomenon of aberrant tyrosine kinase-mediated glucose addiction has also been demonstrated in the context of FLT3-ITD mutations in AML (as mentioned above in chapter 1.4.4.1.1), where constitutive FLT3 kinase activity was shown to induce glucose dependence of sensitize cells to 2-DG (Huang et al., 2016; Ju et al., 2017). It is important to consider however, that 2-DG more broadly alters global glycosylation processes and thus may impair the shuttling of newly-glycosylated FLT3-ITD or oncogenic KIT tyrosine kinase to the cell surface (Larrue et al., 2015).

If oxidative phosphorylation results in greater ATP yield from a single molecule of glucose than does fermentation, why do cancer cells prefer to acquire their energy sources in this manner? A prevailing hypothesis is that the glycolytic intermediates generated during aerobic glycolysis support multiple important downstream metabolic processes including metabolism of amino acids and, downstream of the pentose phosphate pathway (PPP), nucleotide metabolism. In AML, activation of the master metabolic regulator mTORC1 (downstream of oncogenic tyrosine kinase signalling such as FLT3-ITD or KIT) results in enhanced protein translation, cell growth, and increased glucose flux through the PPP; this results in dependence on glucose 6 phosphate dehydrogenase

(G6PD), the first and rate-limiting enzyme of the PPP and raises the possibility for therapeutic intervention via inhibition of G6PD (Poulain et al., 2017; Tamburini et al., 2009; Xu et al., 2003, 2016). Indeed, inhibition of G6PD sensitizes haematological malignancies to standard of care agents such as cytarabine (Poulain et al., 2017), FLT3 inhibitors (Bhanot et al., 2017) and interestingly, the antimalarial agent dihydroartemisinin (Elf et al., 2017). Aside from the generation of pentose and ribose 5-phosphate necessary for the synthesis of nucleotides, the PPP additionally plays a key role in the generation of cellular redox potential via reduction of nicotinamide adenine dinucleotide phosphate (NADP⁺) to nicotinamide adenine dinucleotide phosphohydrogen (NADPH) for protection against oxidative stress and to act as a co-factor for several biosynthetic processes (Ge et al., 2020). In B-ALL, the serine/threonine-protein phosphatase 2A (PP2A), a well-characterised tumour suppressor gene commonly downregulated in cancer, has been shown to divert glucose to the PPP as a preventative against oxidative stress (Xiao et al., 2018). This diversion was shown to act as a compensatory mechanism against the activity of the prototypic B-cell transcription factors paired box 5 (*PAX5*) and ikaros family zinc finger 1 (*IKZF1*) which normally act to suppress G6PD and other PPP enzymes. The authors conclude by demonstrating that inhibition of PP2A with the inhibitor LB-100 blocks the diversion of glucose-derived carbons to the PPP and thus results in antitumour effects (Xiao et al., 2018).

Aberrant glycolysis has also been implicated in resistance to a wide variety of anti-leukaemic therapies. For instance, primary resistance to the glucocorticoid steroid prednisolone, the standard of care therapy in ALL, has been demonstrated in blasts that transcriptionally upregulate glycolytic enzymes (Holleman et al., 2004). Perturbation of glycolytic metabolism with 2-DG can re-sensitize these cells to prednisolone (Hulleman et al., 2009). In AML, a fascinating recent study has suggested that myeloblasts maintain the ability to manipulate the leukaemic microenvironment to enhance glycolytic uptake and thus drive proliferation. AML blasts were shown to systemically secrete insulin-like growth factor binding protein 1 (IGFBP1), a protein that desensitises tissues to insulin, thus promoting further uptake of glucose (Ye et al., 2018). This positive feedback loop thus provides blasts with the capability to self-regulate glucose uptake based on relative systemic factors such as availability of glucose in the leukaemic microenvironment. In summary, while glucose is undoubtedly required for the survival of all cells (malignantly transformed or otherwise), certain genetic or non-genetic events can create neo-

dependencies on enhanced glycolytic metabolism. These metabolic vulnerabilities can then be therapeutically exploited in the context of primary therapy or re-sensitization to therapy upon relapse.

1.5.2 Aberrant amino acid metabolism in leukaemia

Amino acids are important precursors necessary for protein biosynthesis, but they also serve as less-appreciated donors of carbon and nitrogen atoms for a wide-range of biosynthetic pathways such as nucleotide metabolism (Rashkovan and Ferrando, 2019). Autophagy, and other degradation pathways can further supply amino acid-derived precursors for these biosynthetic processes. In the section below, key amino acids that support leukaemogenesis are discussed.

1.5.2.1 Glutamine

Glutamine, an important non-essential glycogenic amino acid, plays an important role in the generation of pyruvate and TCA cycle intermediates (Willems et al., 2013). Indeed, intracellular glutamine levels control the uptake of the amino acid leucine (an important ketogenic amino acid which contributes to co-enzyme A (CoA) biosynthesis) as the SLC7A5/SLC3A2 transporters exchange intracellular glutamine for leucine, an important step in the Rheb-mediated activation of mTOR (Kim et al., 2008; Nicklin et al., 2009). The conversion of glutamine to glutamate (referred to as glutaminolysis) and then subsequently to α -ketoglutarate (α -KG) is an important TCA cycle carbon source and intermediate; α -KG plays further important roles in systemic metabolism, notably by acting as an important nitrogen transporter (DeBerardinis et al., 2007). Glutamine is critically important in the maintenance of AML, as it has been demonstrated to play important roles in oxidative phosphorylation; depletion of glutamine induces cell death (Jacque et al., 2015; Willems et al., 2013). Much of the interest in perturbing glutamine metabolism has centred on the enzyme glutaminase (GLS), which mediates the conversion of glutamine to glutamate, although abrogating import of glutamine via the neutral amino acid transporter SLC1A5 has also been shown to impair tumour growth *in vivo* (Willems et al., 2013). Indeed, several studies have explored GLS inhibition as a therapeutic modality in AML, including, interestingly, in FLT3 inhibitor monotherapy resistance (Gallipoli et al., 2018; Gregory et al., 2018; Matre et al., 2016). Global glutamine depletion results in compensatory upregulation of members of the *de novo*

serine synthesis pathway – phosphoglycerate dehydrogenase (*PHGDH*) and phosphoglycerate aminotransferase 1 (*PSAT1*), and GLS inhibition synergises with serine deprivation (Polet et al., 2016). Serine is a non-essential amino acid that serves as an important carbon donor to the one-carbon/folate cycle and can be generated *de novo* via the redirection of glucose-derived carbons down the serine biosynthesis pathway. This pathway will be examined in greater detail later in this chapter. Finally, glutamine metabolism has been shown to be relevant in the context of T-ALL therapy and resistance. Following the discovery that Notch homolog 1 (NOTCH1), a single-pass transmembrane receptor and member of the evolutionarily conserved NOTCH signalling pathway in T-cells, can acquire gain-of-function mutations in up to 50% of all T-ALLs (including across a broad range of molecular oncogenic subtypes, principally in the extracellular heterodimerization domain/C-terminal PEST domain of NOTCH1) (Weng et al., 2004), significant effort has been dedicated to developing therapies that abrogate NOTCH signalling (Li and von Boehmer, 2011). In an important study, the authors demonstrated that inhibition of NOTCH signalling, via the γ -secretase inhibitor dibenzazepine (DBZ, which blocks NOTCH cleavage) dramatically inhibits glutaminolysis and triggers the induction of autophagy as a salvage mechanism for maintaining leukaemic growth. Thus, triple inhibition of GLS and autophagy strongly synergise to enhance the anti-leukaemic effects of DBZ (Herranz et al., 2015).

1.5.2.2 Asparagine

Asparagine is a non-essential amino acid that is involved in metabolism of the toxic by-product ammonia as well as the biosynthesis of proteins, particularly glycoproteins, where it provides key residues for *N*-linked glycosylation (Larkin and Imperiali, 2011). In most tissues it is *de novo* generated in a transamination reaction which converts aspartate to asparagine and glutamine to glutamate in a reaction catalysed by asparagine synthetase (ASNS). However, it has long been appreciated that T-ALL and other ALL cells exhibit low levels of ASNS expression, thus rendering these cells auxotrophic for asparagine (Balasubramanian et al., 2013; Richards and Kilberg, 2006). Thus, these cells are uniquely dependent solely on exogenous asparagine acquired via the diet. Bacterially derived L-asparaginase treatment, now a backbone component of modern T-ALL therapy, thus degrades systemic asparagine pools and depletes the capacity of leukaemic cells to import extracellular asparagine necessary for the maintenance of proliferation (Broome,

1963a, 1963b; Capizzi et al., 1971; Haskell and Canellos, 1969; Kidd, 1953; Miller et al., 1969; Ohnuma et al., 1970). It is important to note that given ASNS' roles in concurrently mediating glutamine to glutamate conversion, asparagine degradation in T-ALLs also reduces intracellular glutamine pools and inhibits other related metabolic processes such as glycolysis and translation, while enhancing fatty-acid oxidation (Hermanova et al., 2016). Finally, a whole genome CRISPR-Cas9 screen was recently performed to determine means of re-sensitizing asparaginase-resistant T-ALLs to asparaginase treatment. The authors discovered that inhibition of the Wnt pathway, via glycogen synthase kinase 3 (GSK3), sensitized cells to asparaginase treatment (Hinze et al., 2019).

1.5.2.3 Arginine

Arginine is a non-essential amino acid that plays an important role in a wide variety of biologic systems, including nitric oxide (NO) production, creatine production and polyamine synthesis (Husson et al., 2003; Wu and Morris, 1998). Though in normal physiologic conditions arginine can be *de novo* synthesised, under catabolic stress or situations in which proliferation is required, cells rely heavily on exogenous dietary-derived arginine, rendering this amino acid “semi-essential” (Feun et al., 2008). Arginine biosynthesis primarily occurs in the renal tubule via the urea cycle but can also take place in a wide variety of other cell types. In the urea cycle, L-citrulline and aspartic acid are converted to argininosuccinate by argininosuccinate synthase (ASS1), which is then further catalysed to become L-arginine and fumaric acid by argininosuccinate lyase (ASL). Recycling of these metabolic intermediates results in L-arginine being degraded to L-ornithine by the enzyme arginase (ARG1), and L-ornithine subsequently converted back to L-citrulline in a reaction mediated by ornithine transcarbamyl transferase (OTC) (Feun et al., 2008). Thus, the relative abundance of arginine in the cell can be carefully regulated by this feedback cycle mediated by ASS1, ASL, ARG1 and OTC.

In leukaemias, particularly in AML and T-ALL, the heightened metabolic demands that drive the proliferation of these aggressive diseases result in these cells displaying particular dependence on exogenous arginine. In a similar vein to T-ALLs and ASNS expression, many AML and T-ALL subtypes additionally harbour defects in the arginine recycling pathway, particularly in *ASS1* and *OTC* – thus rendering these leukaemias solely dependent on exogenous arginine imported by the amino acid transporters SLC7A1 and

SLC7A2. Thus, in both AML and T-ALL, systemic degradation of arginine with pegylated recombinant arginase rapidly depletes intracellular and extracellular arginine pools, induces blast cell apoptosis and reduces engraftment potential of transplanted blasts *in vivo* (Hernandez et al., 2010; Morrow et al., 2013; Mussai et al., 2015). Interestingly, and somewhat surprisingly, a study also demonstrated that AML blasts exogenously release arginase II, an alternative isoform of arginase, to suppress the adaptive immune response by impeding T-cell proliferation; a phenotype that could paradoxically be reversed with small-molecule arginase inhibitors (Mussai et al., 2013). The relative efficacies of both of these therapeutic modalities *in vivo* – either that of leveraging the auxotrophic nature of leukaemias to arginine with arginase therapy or re-activating suppressed immune signalling by blocking arginase activity has not yet been definitely examined but appears to be context-specific.

1.5.2.4 Cysteine

Cysteine is a semi-essential amino acid that performs critically important roles to maintain cellular proliferation. Besides the diet, cysteine can be *de novo* synthesised from the precursor amino acids serine and methionine (itself a downstream product of serine and one-carbon/folate metabolism) via the transsulfuration pathway. Methionine firstly receives an adenosine group from ATP (catalysed by *S*-adenosyl-methionine synthetase, MAT), yielding *S*-adenosylmethionine (SAM). As SAM acts as the global methyl donor, SAM undergoes demethylation, yielding *S*-adenosyl-L-homocysteine (SAH). SAH itself is converted to homocysteine in a hydrolysis reaction that yields L-homocysteine and adenosine, mediated by the enzyme adenosylhomocysteinase (AHCY). Homocysteine and serine then undergo a condensation reaction catalysed by cystathionine β -synthase (CBS) to yield cystathionine, in a reaction that uses pyridoxine (more commonly known as Vitamin B6) as a cofactor. Interestingly, Vitamin B6 was recently characterised as an AML-specific metabolic vulnerability, the loss of which, besides abrogating cysteine metabolism, also altered nucleotide precursor and polyamine metabolites (Chen et al., 2020). Finally, cystathionine γ -lyase (CTH) cleaves cystathionine to yield cysteine, α -ketobutyrate and ammonia (Serpa, 2020).

Besides contributing to protein biosynthesis, cysteine is also an important precursor for the synthesis of glutathione (GSH), an antioxidant pivotal to maintaining intracellular

redox potential (Orlowski and Meister, 1971; Wu et al., 2004). The heightened proliferation characteristic of many leukaemias (such as CLL and B-ALLs) results in high levels of reactive oxygen species (ROS). Thus, these cells are particularly dependent on cysteine abundance to generate GSH and maintain redox balance, a requirement primarily fulfilled via extracellular import through the channel transporter SLC7A11 (Zhang et al., 2012a). As a result, treatment of CLL-bearing mice with cysteinase enzymes selectively induced cell cycle defects and apoptosis in blast cells, principally via depletion of intracellular GSH and subsequently elevated ROS levels. Interestingly, chronic treatment of mice with cysteinase therapy did not result in any obvious toxicities, affirming that dysregulated redox balance is an induced metabolic vulnerability in cancer cells (Cramer et al., 2017). Of note, glutamine also contributes to GSH generation via glutamate, the first product of the glutaminolysis pathway. A recent study demonstrated that AML cells treated with the FLT3 inhibitor quizartinib utilise glutamine to maintain mitochondrial function and redox homeostasis in response to treatment-induced cellular stress, primarily via the generation of GSH; glutamine starvation potently reduced GSH levels in quizartinib treated cells and increased ROS levels (Gallipoli et al., 2018).

While the amino acids described above are by no means exhaustive, they provide examples of specific amino acids that in certain genetic, non-genetic or microenvironmental contexts can act as metabolic ‘choke-points’ for leukaemia maintenance. Serine, an important non-essential amino acid referred to briefly above and examined in detail in this thesis, is an important one-carbon donor to the folate cycle which supports a myriad of other biologic functions such as nucleotide biosynthesis, methylation reactions, generation of other amino acids such as glycine and cysteine, the production of phospholipids, and generation of NADPH for redox balance (Yang and Vousden, 2016). In this section, the various metabolic roles of serine will be examined in greater detail.

1.5.3 Serine and one-carbon metabolism

Though non-essential amino acids are, by definition, able to be synthesised *de novo*, the metabolic burden imparted by the requirement to rapidly proliferate results in cancer cells relying on both endogenous and exogenous sources of amino acids for optimal growth (Yang and Vousden, 2016). These metabolic dependencies are complex. In many

contexts, the redirection or recycling of metabolites to *de novo* synthesise amino acids often is not sufficient to solely meet the cells' proliferative requirements, and in itself can induce further complications such as increased oxidative stress; mutations in *de novo* synthesis pathways (such as in the case of asparagine and arginine in leukaemias described above) can further tilt cell dependency to exogenous sources. Conversely, specific cellular metabolic rewiring events can induce dependencies on *de novo* amino acid synthesis offering potentially attractive therapeutic opportunities.

The discovery that exogenous serine depletion can hamper tumour growth both *in vitro* and *in vivo* has ignited a slew of research into this important non-essential amino acid, further encouraged by the fact that a subset of tumours selectively upregulate the *de novo* serine synthesis pathway (Maddocks et al., 2013). In the sections below we examine the systemic role of serine, the *de novo* serine biosynthesis pathway, the interplay between serine and the one-carbon/folate cycle, and the implications of perturbed serine metabolism in cancer.

1.5.3.1 Serine availability *in vitro* and *in vivo*

Cellular serine requirements are met by three key modalities: the import of serine into the cell via neutral amino acid transporters (such as SLC1A4 and SLC1A5), the redirection of glucose-derived carbons down a dedicated *de novo* biosynthetic pathway, and via degradation/recycling of other proteins (via autophagy), amino acids (such as glycine) and other metabolites. In physiological conditions, exogenous serine is typically sufficient to meet cellular requirements; although in certain microenvironmental niches *de novo* serine synthesis can contribute substantially to intracellular serine pools (Kalhan and Hanson, 2012). A good example of this is in brain neurons, where the D-enantiomer of serine acts as an important neurotransmitter that is almost exclusively sourced from *de novo* serine synthesis due to the difficulty of dietary-derived serine crossing the blood-brain-barrier (BBB) (Furuya, 2008). In *in vitro* culture, exogenous serine is heavily utilised to drive proliferation, and exogenous serine depletion induces profound proliferative defects which concomitantly rewire cells to increase *de novo* serine synthesis pathway flux (Jain et al., 2012; Maddocks et al., 2013).

1.5.3.2 *De novo* serine biosynthesis

The *de novo* serine synthesis pathway diverts glucose-derived carbons into a dedicated biosynthetic pathway to generate serine. Although glucose is the primary source of carbons for this pathway, under glucose-limited conditions alternative cellular processes, such as the generation of glucose from pyruvate, lactate or other amino acids (gluconeogenesis) can make up for this shortfall (Kalhan et al., 2011). To generate serine, the glycolytic intermediate 3-phosphoglycerate (3PG) is diverted from glycolysis/gluconeogenesis and oxidised to form 3-phosphohydroxypyruvate (3PHP) by phosphoglycerate dehydrogenase (PHGDH), the first, rate limiting enzyme of the *de novo* serine synthesis pathway in a NAD⁺ dependent manner. 3PHP is transaminated to 3-phosphoserine (3PS) by phosphoserine aminotransferase 1 (PSAT1), in a reaction that utilises glutamate-derived nitrogen and produces α -ketoglutarate. Finally, 3PS undergoes phosphate ester hydrolysis by phosphoserine phosphatase (PSPH) to yield serine (Yang and Vousden, 2016). A summary of serine metabolism is included in Figure 1.7. The physiological triggers that regulate this reaction include depleted intracellular pools of glutamine, glucose, and serine itself; increased abundance of 2-phosphoglycerate (2PG), the next glycolytic intermediate downstream of 3PG, which has been shown to activate PHGDH (Hitosugi et al., 2012); and by a feedback loop mediated by the pyruvate kinase M2 isoform (PKM2) whereby reduced serine levels induce PKM2 to throttle the conversion of phosphoenolpyruvic acid (PEP) to pyruvate, thus slowing glycolytic flux and promoting 3PG to 3PHP conversion and increased *de novo* serine synthesis pathway activity (Chaneton et al., 2012; Ye et al., 2012). Additionally, other sensory pathways exist that can detect reduced intracellular serine pools such as via the master metabolic regulator activating transcription factor 4 (ATF4), which can directly transcriptionally activate PHGDH to stimulate serine synthesis (Reina-Campos et al., 2020).

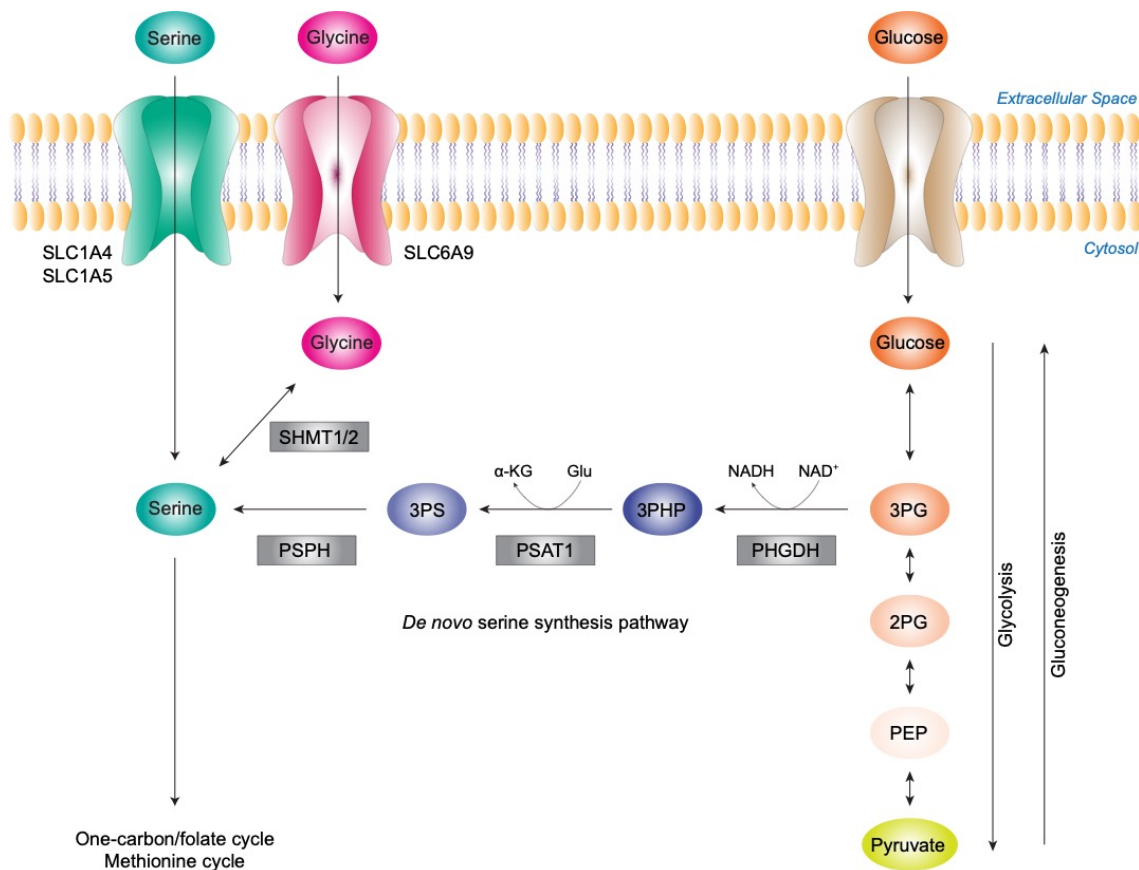


Figure 1.7 Summary of serine metabolism.

The cellular requirements of the non-essential amino acid serine can be achieved via import into the cell through neutral amino acid transporters (SLC1A4 and SLC1A5). Glycine, which serine is converted to, can also be directly imported via amino acid transporters (SLC6A9). Alternatively, glucose-derived carbons can be redirected from the glycolytic intermediate 3-phosphoglycerate (3PG) down the *de novo* serine biosynthesis pathway to generate serine, which is then used for downstream metabolic pathways such as the one-carbon/folate cycle, and methionine cycle. PHGDH, phosphoglycerate dehydrogenase; PSAT1, phosphoserine aminotransferase 1; PSPH, phosphoserine phosphatase; 3PHP, 3-phosphohydroxypyruvate; 3PS, 3-phosphoserine; NADH, nicotinamide adenine dinucleotide hydrogen; NAD⁺, nicotinamide adenine dinucleotide; Glu, glutamate; α -KG, α -ketoglutarate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvic acid; SHMT1/2, serine hydroxymethyltransferase 1/2.

1.5.3.3 Serine as a one-carbon donor to the one-carbon/folate cycle

One-carbon metabolism supports multiple physiological processes including nucleotide biosynthesis, amino acid homeostasis, epigenetic maintenance, and redox balance (Ducker and Rabinowitz, 2017). Serine, choline, betaine and other amino acids such as glycine, histidine and catabolised tryptophan are all one-carbon donors to the one-carbon/folate cycle (Allen and Moskowitz, 1978; Brosnan et al., 2015; Davis et al., 2004; Rowe et al., 1985; Tibbetts and Appling, 2010). One-carbon/folate metabolism is compartmentalised, occurring in both the cytosol and mitochondria. In the mitochondria, the β -carbon of serine is transferred to tetrahydrofolate (THF) by serine hydroxymethyltransferase 2 (SHMT2), generating the folate intermediate 5,10-methylene-THF. The bifunctional methylenetetrahydrofolate dehydrogenase/cyclohydrolase 2 (MTHFD2) enzyme then hydrolyses 5,10-methylene-THF to yield 10-formyl-THF, a folate intermediate that carries a one-carbon unit. Mitochondrially-produced one-carbon units are principally used in nuclear and cytosolic reactions, or are secreted from the cell (Brosnan et al., 2015; Ducker et al., 2016).

Folate intermediates carrying one-carbon units are unable to pass the mitochondrial membrane, and so must undergo processing to facilitate transition from mitochondria. Monofunctional tetrahydrofolate synthase 1-like (MTHFD1L) cleaves mitochondrial 10-formyl-THF to generate formate, which is then exported to the cytosol. Cytosolic formate is then reconstituted by the cytoplasmic trifunctional enzyme methylenetetrahydrofolate dehydrogenase 1 (MTHFD1) which also re-establishes cytosolic 5,10-methylene-THF pools. The cycle is completed with the conversion of glycine to serine by serine hydroxymethyltransferase 1 (SHMT1) (Yang and Vousden, 2016). Several of these cytosolic folate cycle intermediates are important one-carbon donors for downstream metabolic reactions; 10-formyl-THF acts as a carbon donor for the generation of *de novo* purine nucleotides, specifically the assembly of the purine ring (Fan et al., 2019). Similarly, cytosolic 5,10-methylene-THF is a one-carbon donor for thymidylate synthase (TYMS), which methylates 2-deoxy-uridine-5-monophosphate (dUMP) to 2-deoxy-thymidine-5-monophosphate (dTMP) (Field et al., 2016). Other outputs of the one-carbon/folate cycle that are important for the maintenance of optimal proliferation and have garnered significant research interest is the contribution of the folate cycle to redox homeostasis, and global methylation via the closely linked methionine cycle.

1.5.3.4 One-carbon/folate metabolism in the maintenance of redox homeostasis

Nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) is a cofactor utilised for many metabolic reactions where it acts as a reducing agent. NADPH further acts as an antioxidant protecting against ROS-induced damage and replenishes GSH pools (Marty et al., 2009). Cytosolic NADPH is typically produced by the PPP, as well as by malate dehydrogenase (MDH), which oxidises malate to oxaloacetate and produces NADPH. The IDH family of enzymes, IDH1 (cytosolic) and IDH2 (mitochondrial), which catalyse the oxidative carboxylation of isocitrate to 2-oxoglutarate in the TCA cycle, can also generate and consume NADPH (Fan et al., 2014). Whilst the one-carbon/folate cycle is not typically acknowledged as a key contributor to NADPH homeostasis, emerging evidence suggests that NADPH derived from one-carbon/folate metabolism may be important in the context of malignantly transformed cells. For instance, the one-carbon/folate cycle directly alter NADPH pools via oxidation of 5,10-methylene-THF by methylenetetrahydrofolate dehydrogenase/cyclohydrolase 2-like (MTHFD2L) in the mitochondria, or MTHFD1 in the cytosol (Bolusani et al., 2011; MacFarlane et al., 2008). The importance of these steps in the cancer context are questionable however, as MTHFD2L is not expressed in cancer cells, and most cells utilise mitochondrial MTHFD2 to catalyse this step (Mejia and MacKenzie, 1988; Nilsson et al., 2014). Indeed, in recent years the observation that MTHFD2 is over-expressed in a wide variety of malignancies including solid cancers (Ju et al., 2019; Lin et al., 2018; Liu et al., 2014a, 2016b; Nilsson et al., 2014) and AML (Pikman et al., 2016) has renewed the interest in the tumour-propagating roles of this enzyme. Another means by which the folate cycle can alter NADPH pools is mediated by 10-formyltetrahydrofolate dehydrogenases 1-like 1 ALDH1L1 (in the cytosol) and -like 2 ALDH1L2 (in the mitochondria). These enzymes catalyse the conversion of 10-formyl-THF to NADPH and CO₂. As this process essentially results in loss of carbon units for subsequent processing in the one-carbon/folate cycle, this mechanism could act as a regulatory ‘check’ on the relative abundances of different one-carbon/folate cycle metabolite pools (Brosnan et al., 2015). Studies have demonstrated that ALDH1L2 is upregulated in many cancers, ostensibly to provide a source NADPH for regulation of redox homeostasis in the mitochondria, where oxidative phosphorylation in cancer cells is a major source of ROS (Davis et al., 2004).

Bioinformatic approaches examining gene expression profiling and flux analysis of the serine/glycine/one-carbon metabolic network across a wide variety of cancers demonstrated that while pathways influencing methylation and redox metabolism were heterogeneously expressed across cancers, nucleotide biosynthesis, glutathione and NADPH metabolism was common across all cancer cell types, suggesting universal importance for the maintenance of proliferation (Mehrmohamadi et al., 2014). Besides acting as a precursor for the generation of GSH via glycine, serine also contributes to antioxidant defence in other ways. Catabolism of serine in the mitochondria has been shown to reduce mitochondrial redox imbalance in hypoxic conditions (Ye et al., 2014) and increased mitochondria stress via elevated ROS can induce serine metabolism to provide the necessary precursors for generation of antioxidants (Ost et al., 2015).

1.5.3.5 Serine and one-carbon/folate metabolism as inputs for the methionine cycle

Serine, via the one-carbon/folate cycle, is an important input into the methionine cycle (Figure 1.8). Besides producing SAM, the methionine cycle also produces homocysteine which contributes the production of GSH as described above, via the transsulfuration pathway. SAM is generated in a process catalysed by *S*-adenosyl-methionine synthetase (MAT) wherein methionine receives an adenosine group from ATP and acts as a substrate for methyltransferases and a global methyl-group donor for proteins, lipids and other metabolites, as well as histones and DNA, thus intricately linking methionine metabolism to epigenetic regulation (Kinnaird et al., 2016). 5,10-methylene-THF, a one-carbon/folate cycle intermediate for which serine contributes one-carbon units, is catalysed to become 5-methyl-THF in a reaction mediated by methylenetetrahydrofolate reductase (MTHFR). 5-methyl-THF acts as a co-substrate for homocysteine re-methylation to methionine, a process catalysed by methionine synthase (MS) in a reaction that required Vitamin B12 as a cofactor. The importance of serine in the re-methylation of homocysteine has been demonstrated *in vivo*, with serine acting as the primary source of methyl groups (Davis et al., 2004). Choline and betaine can also contribute to the re-methylation of homocysteine via betaine-homocysteine *S*-methyltransferase (BHMT).

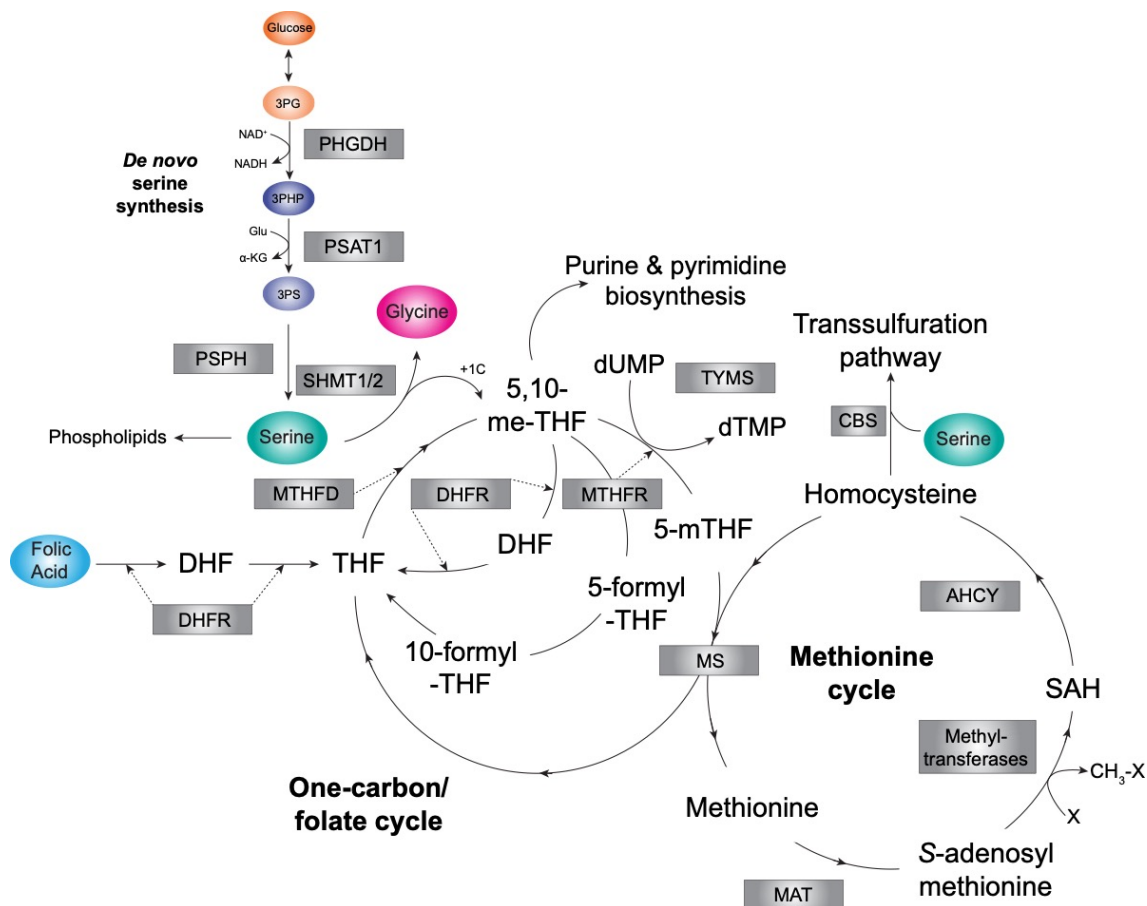


Figure 1.8 Serine as an input into the one-carbon/folate and methionine cycles.

Serine acts as a one-carbon donor to the one-carbon folate cycle, which generates metabolites necessary for purine nucleotides, and the pyrimidine species deoxythymidine monophosphate (dTMP). The methionine cycle interfaces with the one-carbon/folate cycle and generates *S*-adenosyl methionine (SAM), the universal methyl-donor from methionine. Methyl-group depleted SAM (*S*-adenosylhomocysteine (SAH)) is converted to homocysteine which acts as the immediate precursor metabolite for the transsulfuration pathway, before recycling back to methionine. 1C, 1-carbon unit; DHF, dihydrofolate; THF, tetrahydrofolate; DHFR, dihydrofolate reductase; MTHFD, methylenetetrahydrofolate dehydrogenase; MTHFR, methylenetetrahydrofolate reductase; TYMS, thymidylate synthase; MS, methionine synthase; MAT, *S*-adenosylmethionine synthetase; AHCY, adenosylhomocysteinase; cystathionine beta-synthase.

In vitro methionine requirements are primarily met via replete abundance of methionine in culture medium, thus placing less emphasis on serine/one-carbon/folate-derived carbon and methyl groups (Maddocks et al., 2016; Mentch et al., 2015). Serine tracing experiments in these contexts nevertheless demonstrated that while serine-derived one-carbon units are not utilised for homocysteine re-methylation, they did contribute to the synthesis of ATP, which is a required cofactor for the conversion of methionine to SAM (Maddocks et al., 2016). Thus, these experiments suggest that besides the contribution of serine to the maintenance of nucleotide pools as described above, serine-derived ATP is required to sustain a broader array of metabolic reactions.

Aside from serine, the methionine cycle also intersects with polyamine metabolism. Polyamines (such as putrescine, spermidine and spermine) are polycationic alkylamines involved in cell growth, regulation of nucleotide and protein metabolism, regulation of chromatin structure, redox homeostasis and regulation of ion channels (Casero et al., 2018). During polyamine synthesis, L-ornithine, a non-proteinogenic amino acid, is converted to putrescine via the holoenzyme ornithine decarboxylase (ODC). Putrescine is then converted to spermine using decarboxylated *S*-adenosylmethionine (SAM) as a cofactor. The decarboxylation of SAM is catalysed by *S*-adenosylmethionine decarboxylase proenzyme 1 (AMD1). Spermidine is then converted to spermine, yielding 5'-*S*-methyl-5'-thioadenosine (MTA) as a biproduct of this reaction. The methionine salvage pathway, a pathway comprised of six reactions, regenerates methionine from MTA. MTAP, the first and rate-limiting enzyme in this pathway, converts MTA to 5'-methylthio ribose-1-phosphate and adenine. 5'-methylthio ribose-1-phosphate then undergoes a series of catalytic reactions (involving the enzymes APIP, ENOPH1 and GOT1) to generate methionine (Sanderson et al., 2019).

1.5.4 Serine and one-carbon metabolism in cancer

Cancer cells rewire metabolic pathways to sustain the elevated biosynthetic and bioenergetic demands associated with malignant proliferation. This is especially important in the tumour microenvironment, where malignant cells encounter gradients of metabolite availability (such as oxygen and nutrients) which necessitate active adaptation. This section will discuss current literature concerning serine pathway reprogramming in cancer.

1.5.4.1 Dysregulation of *de novo* serine synthesis

Tissue-specific utilisation of serine biosynthesis has long been appreciated, as evidenced by almost exclusive reliance of neurons on *de novo* synthesis to generate D-serine for neurotransmitter use. In recent years significant evidence has emerged that *de novo* serine synthesis is upregulated – either via genomic amplification or overexpression of *PHGDH* in breast cancers, melanoma, and colorectal cancers (Gylfe et al., 2013; Locasale et al., 2011; Mullarky et al., 2011; Pollari et al., 2011; Possemato et al., 2011; Yoon et al., 2015). A computational study examining gene expression in breast cancers demonstrated that higher expression of key *de novo* serine synthesis enzymes was associated with poorer overall outcome in patients (Antonov et al., 2014). The role of PHGDH in haematological malignancies is less clear; conditional deletion of *Phgdh* in a *MYC*-driven lymphoma mouse model demonstrated that *de novo* serine synthesis is dispensable for tumour development, as was *Shmt1* and lactate dehydrogenase A (*Ldha*). However, a recent manuscript which derived a 4-gene signature to determine survival in AML as an independent prognostic parameter and within the ELN-classification system identified *PHGDH* as one of the genes in which high expression correlated with poor outcome (Nguyen et al., 2019). These findings suggest that in haematological cells, *de novo* serine synthesis may be dispensable for steady-state haematopoiesis but may be required in the context of haematological malignancy.

A series of studies in the last several years have characterised the metabolic consequences of enhanced *de novo* serine biosynthesis pathway activity. As expected, amplification or upregulation of PHGDH results in greater flux through the *de novo* serine biosynthesis pathway and a concomitant decrease in reliance on exogenous serine. Interestingly however, and analogous to more classical oncogene addiction, genetic or pharmacological inhibition of PHGDH results in proliferative defects and decreased survival in cancer cells in both the *in vitro* and *in vivo* context (Locasale et al., 2011; Pacold et al., 2016; Possemato et al., 2011). These findings are even more interesting if one considers the fact that these cells were grown in serine replete conditions – raising the possibility that glucose-derived *de novo* serine synthesis supports other metabolic processes independent of simply contributing to intracellular serine pools. The mechanistic basis of these observations was partially explained in a landmark paper that comprehensively surveyed the metabolomic landscape in response to chemical PHGDH inhibition. The authors discovered that inhibition of PHGDH reduced incorporation of

both glucose-derived serine and exogenous serine into nucleotides (Pacold et al., 2016). SHMT1, the cytosolic enzyme that catalyses the conversion of glycine and 5,10-methylene-THF to serine (but also acts to regulate the 5,10-methylene-THF pool by favouring thymidylate synthesis (Herbig et al., 2002)) was shown to be repressed by *de novo* serine synthesis pathway flux (or an intermediate of this pathway). Pharmacological inhibition of PHGDH thus removes the negative-regulatory capacity of the *de novo* serine synthesis pathway on SHMT1, which catalyses the redundant conversion of glycine and 5,10-methylene-THF to serine – and thus depleting 5,10-methylene-THF pools required for generation of dTMP (Pacold et al., 2016). Deletion of SHMT1 rescued the incorporation of 5,10-methylene-THF into dTMP in the presence of PHGDH inhibition, an effect that was not reproduced upon SHMT2 deletion providing further specificity to the SHMT1-mediated nature of this phenotype (Pacold et al., 2016). Another study suggested that PHGDH itself supports other metabolic processes beyond serine biosynthesis. Locasale and colleagues demonstrated that inhibiting PHGDH affected nucleotide synthesis independent of serine utilisation (Reid et al., 2018). Indeed, by driving *de novo* serine synthesis pathway flux, PHGDH may additionally drive the anabolic reactions which supply central carbon metabolism which feeds into both the PPP and TCA cycle (Reid et al., 2018). Another proposed PHGDH-mediated process that could contribute to oncogenesis includes the production of the oncometabolite 2-HG (typically associated with oncogenic *IDH* mutations in AML) (Fan et al., 2015).

1.5.4.2 Regulation of *de novo* serine synthesis and one-carbon/folate metabolism

De novo serine synthesis and the one-carbon/folate cycle as a whole are controlled by several complementary regulatory mechanisms, including nutrient sensory apparatus (which modulate activity of these pathways to meet the energetic requirements of the cell); posttranslational modification of metabolites and enzymes in these pathways (which drive feedback loops and allow for availability of cofactors that drive enzymatic reactions); and transcriptional and epigenetic activation of enzymes (that trigger biosynthetic processes and regulate the network as a whole) (Reina-Campos et al., 2020).

1.5.4.2.1 Nutrient sensor-mediated transcriptional and epigenetic induction of *de novo* serine synthesis and one-carbon/folate metabolism

In nutrient insufficient conditions, cells possess several mechanisms that allow them to compensate for reduced nutrient availability; these include diverting essential metabolites to processes that promote cell survival (but render the cell ‘stressed’) or transcriptionally activating biosynthetic pathways to replenish diminished metabolite pools. For example, serine/glycine deficiency triggers p53 and cyclin-dependent kinase inhibitor 1A (CDKN1A) activation and a concomitant induction of cell-cycle arrest. This subsequently allows for remaining serine/glycine/one-carbon/folate metabolite pools to be redirected – towards generation of GSH to maintain redox balance, and to the TCA cycle to support oxidative phosphorylation (Maddocks et al., 2013). The sensory apparatus that detects reduced intracellular serine pools centres around general control nonderepressible 2 (GCN2), an enzyme that monitors the relative balance of unloaded/loaded tRNA. When intracellular serine pools are reduced, GCN2 triggers the translation of ATF4 which then transcriptionally activates the *de novo* serine synthesis pathway to divert glucose-derived carbons to generate serine (Ye et al., 2010). Beyond a reduction in serine pools, other events can also trigger ATF4 activation, such as inhibition of pathways that contribute to serine metabolism (such as lactate metabolism via LDHA) (Pathria et al., 2018).

ATF4 is a member of the basic leucine-zipper (bZIP) family of transcription factors that form heterodimers in complex with other ATF transcription factors, as well as the JUN/FOS and CCAAT enhancer-binding protein (C/EBP) transcription factor families (Chevray and Nathans, 1992; Hai and Curran, 1991; Vallejo et al., 1993). ATF4 binds to the cAMP responsive element (CRE) or C/EBP-ATF4 response element (CARE) on DNA to induce gene expression (Hai and Curran, 1991; Vallejo et al., 1993). As a stress-induced transcription factor, ATF4 controls the expression of a wide range of genes necessary to endure periods of stress, such as amino acid biosynthesis enzymes, amino acid transporters, enzymes involved in redox homeostasis, and endoplasmic reticulum chaperones (Harding et al., 2003; Novoa et al., 2003). It is upregulated in the many cancers to drive generation of amino acids and other metabolites to sustain heightened proliferation. In solid cancers, nuclear factor erythroid 2-related factor 2 (NRF2), another bZIP transcription factor that regulates redox homeostasis and is a potent activator of ATF4, has been shown to redirect glucose and glutamine to pro-anabolic pathways to drive proliferation and redox protection (Mitsuishi et al., 2012). This NRF2-ATF4 axis has been further demonstrated to drive the expression of enzymes in the *de novo* serine

synthesis pathway and one-carbon/folate cycle (and subsequently, nucleotide metabolism) in a subset of poor-prognosis non-small-cell lung carcinomas (NSCLC) (DeNicola et al., 2015).

One of the canonical ATF4 target genes is ASNS, and in liposarcoma and breast cancer cells it has been demonstrated to drive the conversion of aspartate to asparagine, which is then exchanged for extracellular amino acids such as serine, arginine and histidine to maintain mTORC1 activation (Krall et al., 2016). The link between ATF4 and mTORC1 has been appreciated for some time. ATF4 was first discovered to be important in the regulation of age and diet-related obesity and glucose homeostasis in both mammals and *drosophila melanogaster*, suggesting at least some of its metabolic-regulatory capacity was evolutionarily conserved (Seo et al., 2009). Further, insulin was shown to induce ATF4 expression in an mTORC1-mediated manner, by coupling amino acid uptake and synthesis with mTORC1's well-established mRNA translation roles (Adams, 2007). In recent years, more mechanistic evidence has emerged characterising the interaction between mTORC1 and ATF4. Aside from mediating the stability of ATF4 mRNA transcripts, mTORC1 also controls ATF4 translation via three upstream open reading frames (uORFs) in the 5'-untranslated region (UTR) of ATF4 (Park et al., 2017). The mTORC1-ATF4 axis was further shown to transcriptionally upregulate MTHFD2 to directly drive purine nucleotide biosynthesis (Ben-Sahra et al., 2016). Finally, a recent study reported that loss of protein kinase C (PKC) λ/ι in neuroendocrine prostate cancer (NEPC) resulted in increased expression of *de novo* serine synthesis pathway enzymes via the mTORC1/ATF4 axis (Reina-Campos et al., 2019). Increased serine biosynthesis pathway flux elevated SAM levels in cells, which drove epigenetic changes such as DNA methylation on genes associated with development/stemness and cancer progression (Reina-Campos et al., 2019).

In serine deprived conditions, the histone 3, lysine 9 (H3K9) methyltransferase euchromatic histone-lysine N-methyltransferase 2 (EHMT2), more commonly known as G9a, is essential for maintaining activity of H3K9-monomethylated (H3K9me1) PHGDH and PSAT1 (Ding et al., 2013). Thus, in many cancers where epigenetic regulators such as G9a are upregulated, inhibition of this methyltransferase may prove to be a therapeutic opportunity, by reversing epigenetic marks on genes necessary to sustain metabolic

reprogramming events. In a similar vein, the histone lysine-specific demethylase 4C (KDM4C), which is often overexpressed in cancer primarily via gene amplification, was demonstrated to epigenetically activate the *de novo* serine synthesis pathway by removing the repressive H3K9 trimethylation mark (H3K9me3) from these genes in an ATF4-dependent manner in both steady-state and serine deprived conditions (Zhao et al., 2016).

Other transcription factors can activate the *de novo* serine synthesis pathway, or one-carbon/folate cycle enzymes. Members of the orphan nuclear receptors, such as estrogen-related receptor alpha (ERR α) together with peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α) act as central transcriptional regulators of energy metabolism in normal cells and have been demonstrated to play important roles in neoplastic-induced metabolic reprogramming. For instance, the PGC-1 α /ERR α axis was shown to repress one-carbon/folate enzymes downstream of 5' AMP-activated protein kinase (AMPK) which renders ERR α -positive (ER-positive) breast cancer cells sensitive to methotrexate, an anti-folate chemotherapy agent that targets dihydrofolate reductase (DHFR) (Audet-Walsh et al., 2016). In another study, the authors utilised single-cell transcriptomics in ER-positive breast cancer cells to demonstrate estrogen promotes the mitochondrial one-carbon/folate pathway, thereby driving heightened polyamine and purine biosynthesis (Zhu et al., 2019). Interestingly, mis-splicing, or aberrant splicing events, have also been shown to be relevant in the regulation of *de novo* serine synthesis. Knockdown of epithelial splicing regulatory protein 1 (ESRP1) in ER-positive breast cancer induced growth arrest and downregulated PHGDH expression (Gökmen-Polar et al., 2019). In MDS, hotspot mutations in the splicing regulator SF3B1 resulted in mis-splicing-associated downregulation of mitochondrial respiratory genes, and the *de novo* serine synthesis pathway – in particular PHGDH (Dalton et al., 2019). Mechanistically, SF3B1^{K700E} mutations were shown to induce cryptic splicing events in PHGDH, thus inducing heightened dependence on exogenous serine *in vitro* and *in vivo*. The authors followed up this finding with the discovery that SF3B1^{K666N} mutations in otherwise lower-risk MDS, induce the same splicing patterns as SF3B1^{K700E} mutations in PHGDH, and accelerate disease progression (Dalton et al., 2020).

Finally, several other transcription factor-independent regulatory mechanisms of the *de novo* serine synthesis pathway/one-carbon/folate cycle have been identified. Mouse

double minute 2 (MDM2), the canonical negative regulator of the p53 tumour suppressor, was shown to regulate a transcriptional program involved in amino acid synthesis (such as *de novo* serine synthesis and redox homeostasis) and was tethered to chromatin by ATF3/ATF4 (Riscal et al., 2016). These phenotypes were independent of p53. Interestingly, a very recent study investigating liposarcoma (wherein *MDM2* is genomically amplified) found that treatment of this class of disease with Nutlin-3A, a pharmacological inhibitor of the MDM2-p53 interaction stabilised p53 but enhanced MDM2-mediated *de novo* serine synthesis pathway activity, by increasing MDM2 recruitment to chromatin (Cissé et al., 2020). Other genes relevant for cancer progression in different contexts have been identified to regulate the *de novo* serine synthesis pathway. *MYCN*, an oncogene commonly mutated in neuroblastoma was shown to upregulate *de novo* serine synthesis directly via ATF4 (Liu et al., 2016). Similarly, epidermal growth factor receptor (EGFR), but not fibroblast growth factor receptor (FGFR) was shown to activate the *de novo* serine synthesis pathway directly via ATF4 (Jin et al., 2019). Interestingly, a recent study identifying tumour suppressors in small cell lung carcinoma (SCLC) identified MAX, the obligate heterodimerization partner of the oncogene MYC. MAX deletion increased the growth of SCLC cells, primarily via the upregulation of the *de novo* serine synthesis pathway on which functional MAX normally acts as a repressive check (Augert et al., 2020). Collectively, this section describes the complex, and indeed, context-specific regulation of *de novo* serine synthesis through both direct transcription and epigenetic activation, or via downstream activation of oncogenes/tumour suppressors.

1.5.4.2.2 Post-translational modifications of enzymes driving *de novo* serine synthesis and one-carbon/folate metabolism

Post-translational modifications are important regulatory mechanisms of the *de novo* serine synthesis pathway and one-carbon/folate cycle. PKC ζ was identified as an important metabolic tumour suppressor in colon cancer, by repressing PHGDH and PSAT1 and directly phosphorylating PHGDH to inhibit its enzymatic activity (Ma et al., 2013). Another study showed that the protein stability and degradation of PHGDH is controlled by the deubiquitinating enzyme Josephin domain-containing 2 (JOSD2) (Zhang et al., 2017). In the first study characterising a chemical PHGDH inhibitor, Cantley and colleagues noted that treatment of cells with the compound CBR-5884 de-

stabilised the tetrameric form of PHGDH in favour of a dimerised species (Mullarky et al., 2016). These findings are noteworthy, as they suggest that PHGDH flips between its di- and tetrameric forms, which may be important for its enzymatic activity. Important evidence supporting this contention was derived from tagging studies, where *N*-terminal polyhistidine-tagging of PHGDH resulted in altered enzymatic structure and a concomitantly reduced capacity of PHGDH to convert 3-PG to 3-PHP (Mattaini et al., 2015). Finally, a very recent study examined the post-translational mechanism of PHGDH upregulation in breast cancer cells. The authors identified ring finger protein 5 (RNF5), an E3 ubiquitin ligase, as a degrader of PHGDH whose action is blocked by acetylation of PHGDH at K58 by the histone acetyltransferase KAT5 (more commonly known as Tip60) (Wang et al., 2020). The high availability of glucose was identified as the key mechanism by which Tip60-mediated acetylation of PHGDH occurred.

While not strictly an enzyme involved in *de novo* serine biosynthesis or the one-carbon/folate cycle, it is worth highlighting that ATF4, a key transcriptional activator of these pathways is extensively regulated at the post-translational level. While some modifications are known to influence the transcriptional activity ATF4 and its stability, the role of other modifications remains unclear. High throughput mass spectrometry revealed ATF4 is ubiquitinated at multiple lysine residues (Hornbeck et al., 2015; Kim et al., 2011). It is known that nuclear-translocated ATF4 is recognised by F-box/WD repeat-containing protein 1A (FBXW1A), more commonly known as β TrCP1, which recognises casein kinase (CK)-placed Ser219 phosphorylation marks on ATF4, thereby resulting in its ubiquitination and degradation (Maddocks et al., 2013). Phosphorylation marks around the β TrCP1 recognition site (such as Ser224, 231, 235 and 248 and Thr213) further promotes β TrCP1-mediated degradation (Pons et al., 2008). Interestingly, an enzymatic activity-independent role for the histone acetyltransferase p300 in mediating ATF4 stability was also reported (Lassot et al., 2005). In this study, p300 was shown to associate with ATF4 at its *N*-terminal region, possibly blocking the lysine residues and thus preventing subsequent ubiquitination of these residues and degradation of the ATF4 protein.

1.5.5 Targeting *de novo* serine synthesis/one-carbon/folate metabolism

Whilst our understanding of the transcriptional and contextual mechanisms driving *de novo* serine synthesis and one-carbon/folate metabolism have greatly improved in recent years, the standard-of-care in perturbing this metabolic network remains antifolate therapy, first utilised by Sidney Farber in the 1940s to treat aggressive childhood ALL (Farber et al., 1948) (Figure 1.9). Antifolates such as aminopterin (now largely phased out due to its toxicity) and its closely related cousin methotrexate, competitively inhibit dihydrofolate reductase (DHFR), an enzyme that converts dihydrofolic acid (DHF) to tetrahydrofolic acid (THF), key intermediates in the one-carbon/folate cycle. Indeed, high-dose methotrexate is still a backbone of ALL therapy (Sakura et al., 2018). The opportunity afforded by increased understanding of this metabolic pathway and, importantly, the contexts in which upstream genetic events result in greater dependence on pathway activity, could allow for targeted exploitation of these metabolic vulnerabilities without associated toxicity.

Specific inhibition of enzymes mediating the *de novo* serine synthesis and the one-carbon/folate cycle has been the focus of a considerable number of studies in recent years. Several first generation PHGDH inhibitors have been developed that induce cytotoxic and antiproliferative effects in cells in which PHGDH is overexpressed, either through genomic means or upstream transcriptional activation (Mullarky et al., 2016; Pacold et al., 2016; Reid et al., 2018; Wang et al., 2017). A key limitation of these ‘first generation’ compounds is their poor *in vivo* characteristics, typified by IC₅₀ values of approximately 10 µmol/L; second generation inhibitors, which exhibit higher potency and increased pharmacokinetics and pharmacodynamics *in vivo* have recently been characterised (Ngo et al., 2020; Rodriguez et al., 2019). However, compensatory reprogramming in response to inhibition of PHGDH, as well as often-replete extracellular serine availability suggests that PHGDH inhibition should be used as a targeted therapy rather than an indiscriminate antifolate therapy. Other enzymes that mediate the one-carbon/folate cycle have also received attention as potential therapeutic targets. SHMT2 has been identified as a metabolic vulnerability in B-cell lymphoma due to impaired import of glycine, and small molecule inhibitors have been generated with cytotoxic properties in this context (Ducker et al., 2017). A very recent study further demonstrated that *SHMT2* cooperates with the anti-apoptotic gene *BCL-2* to induce B-cell lymphoma via epigenetic tumour suppressor

silencing; loss of SHMT2 via chemical or genetic means abrogates lymphoma development (Parsa et al., 2020). Similarly, MTHFD2, which is often upregulated in many cancers, has been identified to be important for AML maintenance (Pikman et al., 2016) and for restoring sensitivity to tyrosine kinase therapy in resistant lung cancer cells (Nishimura et al., 2019). Small molecule inhibitors of MTHFD2 have also recently been developed (Gustafsson et al., 2017). Importantly, inactivation of one-carbon/folate cycle enzymes has also been shown to be a negative predictor of therapy response. A recent article elucidated that loss of methylenetetrahydrofolate reductase (MTHFR) in patients harbouring reduced-function polymorphisms (~10% of Caucasians) renders them resistant to Bromodomain and Extra-Terminal motif (BET) and cyclin-dependent kinase 7 (CDK7) inhibitors by reducing H3K27/K9 histone methylation and activating transcriptional programs which counter BET inhibitors (Su et al., 2020).

An important therapeutic modality currently under investigation is the combination of metabolic interventions with standard of care therapy. For example, dietary supplementation of histidine increases flux through the histidine degradation pathway and histidine catabolism drains intracellular pools of tetrahydrofolate, thus synergising with methotrexate therapy (Kanarek et al., 2018). Similarly, inhibition of glycine decarboxylase (GLDC) prevents catabolism of glycine from regenerating THF species, thus increasing sensitivity of NSCLC to methotrexate (Zhang et al., 2012b). Another study demonstrated that SHMT2-specific inhibition synergised with methotrexate in a T-ALL model *in vivo* (García-Cañaveras et al., 2020). Downstream products of the *de novo* serine synthesis pathway and one-carbon/folate cycle, such as SAM, can also be therapeutically leveraged. For instance, methotrexate therapy in Wnt-driven cancers reduces SAM levels and prevents cytoplasmic arginine methylation mediated by protein arginase methyltransferase 1 (PRMT1) (Albrecht et al., 2019).

Finally, the modulation of dietary-sourced amino acids and nutrients as mechanisms for cancer therapy has seen intense research in recent years, and has recently been reviewed in a series of excellent papers (Bose et al., 2020; Kanarek et al., 2020; Lien and Vander Heiden, 2019; Tajan and Vousden, 2020). Restriction of serine in the diet was shown to be sufficient in inducing tumour regression in several *in vivo* models of cancer by impairing redox homeostasis and thus inducing DNA damage (Maddocks et al., 2017) or in p53-null tumours where cells lose the ability to respond to metabolic stress (Maddocks

et al., 2013). Interestingly, tumours harbouring *KRAS* mutations were shown to be largely resistant to dietary serine withdrawal, suggesting *KRAS* mutations have the capacity to drive *de novo* serine synthesis (Maddocks et al., 2017). While the principle of dietary amino acid modulation is sound (that is, cells must adapt to loss of exogenous amino acids, thus placing increased importance to intracellular biosynthesis processes that can then be therapeutically perturbed) several important limitations and caveats exist. Serine deprivation may cause off-target toxicity in healthy cells which are dependent on *de novo* serine synthesis – such as neurons, for example (Furuya, 2008). This caveat is especially important in the context of essential amino acids, whereby no meaningful *de novo* synthetic capability exists *in vivo*. Other biological processes independent of cellular proliferation/homeostasis may also be negatively affected. For instance, serine and the one-carbon/folate cycle are important for activation of naïve T-cell cells, by driving purine and thymidine synthesis (Ron-Harel et al., 2016). Conversely, a recent study found the serine-derived metabolite GSH plays an important role in regulating serine import and synthesis in T-regulatory (T-reg) cells, such that reduced GSH boosts the proliferation of T-reg cells and anti-tumour immunity (Kurniawan et al., 2020). Thus, the conflicting nature of biological processes in requiring certain metabolites in certain contexts, the need to avoid off-target toxicity in healthy cells, and the requirement to avoid compromising important biological functions such as the immune system renders any therapeutic intervention in the *de novo* serine synthesis/one-carbon/folate cycle, whether chemical or dietary, quite challenging. Presumably, the key to leveraging this therapeutic modality will be centred primarily around discovering metabolic vulnerabilities in certain genetic contexts.

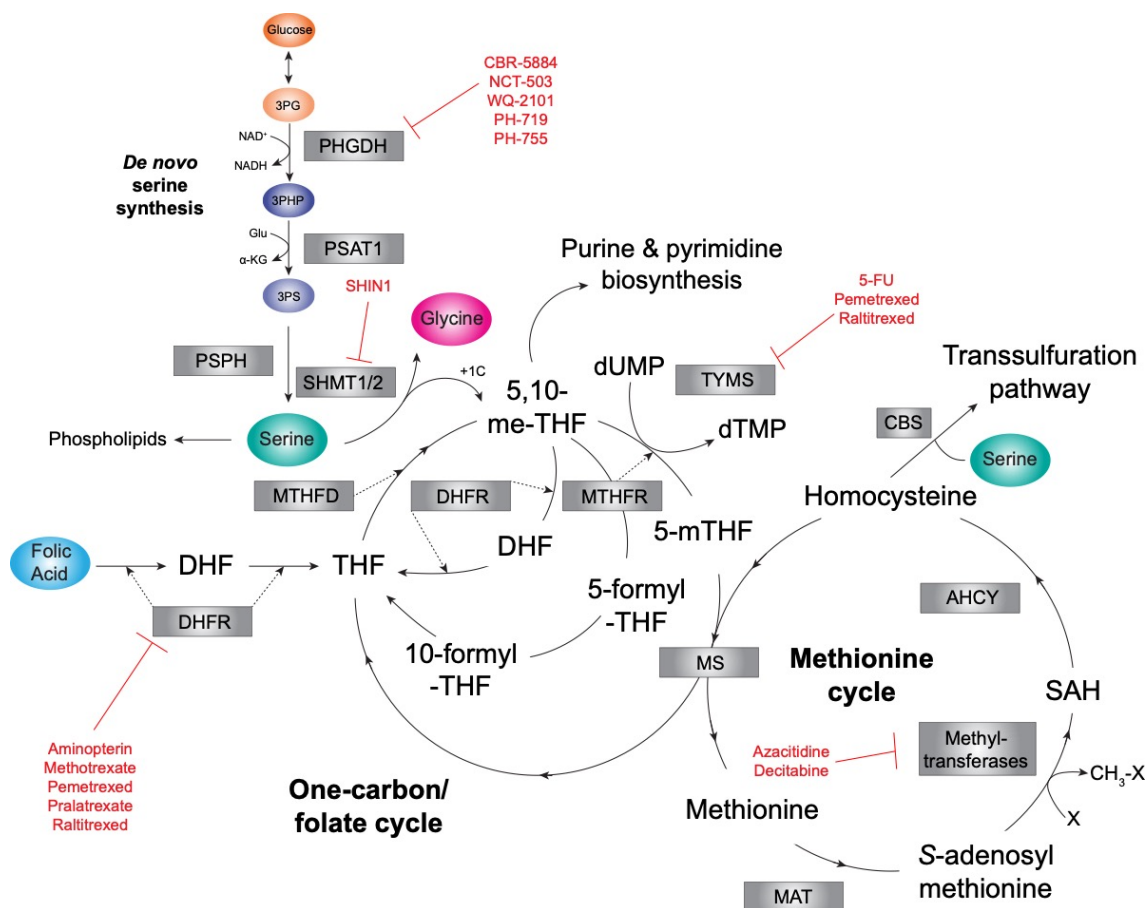


Figure 1.9 Targeting the *de novo* serine synthesis/one-carbon/folate/methionine cycles.

Several compounds targeting the *de novo* serine synthesis/one-carbon/folate/methionine cycles have been developed and are in pre-clinical and clinical use. These include inhibitors targeting phosphoglycerate dehydrogenase (PHGDH); serine hydroxymethyltransferase (SHMT); dihydrofolate reductase (DHFR); thymidylate synthase (TYMS); and various methyltransferases. All inhibitors and their targets are denoted by red arrows.

1.6 Interplay between serine/one-carbon/folate metabolism and epigenetics in cancer

In this chapter, the interplay between epigenetic control of the metabolome (and vice-versa) has been recurrently highlighted – for example, the critical roles SAM plays in methylation reactions, and the lysine methyltransferase G9a-mediated methylation of key enzymes in the *de novo* serine synthesis pathway. The intricate mechanisms underpinning epigenetic regulation of the metabolome are becoming increasingly clearer. In this section, a brief characterisation of some of these mechanisms (with particular emphasis on serine/one-carbon/folate metabolism) will be provided, relevant to some of the research questions explored in this thesis.

In cancer, the crosstalk between epigenetics and metabolism can be defined by four separate interactors. Metabolic reprogramming events could, firstly, modulate the relative availability of cofactor pools required for epigenetic modifications; secondly, they could generate excessive oncometabolites which could act as inhibitors or potentiators of epigenetic reactions; thirdly, by aberrant epigenetic events directly modulating metabolic gene expression; and lastly, by altering signal transduction pathways which drive metabolic processes (Wong et al., 2017). Thus, these observations make it clear that while metabolic reprogramming events can have downstream consequences on the epigenome, the opposite also holds true; epigenetic changes can influence, or even rewire, the metabolome.

1.6.1 DNA and histone methylation

DNA and histone methylation are often dysregulated in cancer; promotor DNA methylation at CpG islands suppresses gene expression by preventing RNA Polymerase II (RNA Pol II) access (Watt and Molloy, 1988), while methylation of residues on histone tails regulates chromatin structure and subsequent gene transcription (Zhang and Reinberg, 2001). DNA is methylated by DNA methyltransferases (DNMTs) which add methyl groups to cytosine to form 5-methylcytosine (5-mC); these include DNMT1, DNMT2 and DNMT3 as previously mentioned in chapter 1.3.4.1. Interestingly, all of these enzymes are known to be overexpressed in cancers (Robertson et al., 1999). Similarly, histone methyltransferases, which exist as either lysine-specific Su(var)3-9, Enhancer of Zeste, Trithorax-domain (SETD) containing, lysine-specific non-SETD

containing (e.g., disrupter of telomeric silencing 1-like, DOT1L) and arginine-specific methyltransferases (Greer and Shi, 2012) act to mono-, di- or tri-methylate multiple lysine or arginine residues on histone tails, resulting in inhibitory or promotive chromatin and gene expression conformations to be adopted. In cancer, these epigenetic events can result in aberrant activation of normally silenced genetic programs that drive malignant proliferation.

DNA methylation acts directly on metabolic genes, by either direct regulation of transcription or upstream pathway modulation. For instance, fructose-1,6-bisphosphate 1 and 2 (FBP1 and FBP2), gluconeogenesis pathway enzymes, are silenced via promotor methylation in breast cancer, thus promoting increased glucose uptake, increased flux through the PPP and *de novo* serine synthesis pathway, and ATP production under hypoxic conditions (Chen et al., 2011; Dong et al., 2013). Heightened glucose uptake can in itself be epigenetically controlled; promotor CpG island hypermethylation of Derlin-3 (*DERL3*), a component of the ER-associated protein degradation pathway, can result in overexpression of the glucose transporter SLC2A1 in several cancers (Lopez-Serra et al., 2014). Importantly, multiple enzymes that negatively regulate the central sensory apparatus that mediates systemic metabolism, the PI3K/AKT/mTOR pathway, can be inactivated by promotor hypermethylation, such as PTEN (Kang et al., 2002; Soria et al., 2002), the liver kinase B1/AMPK (LKB1/AMPK) axis (Trojan, 2000) and Von Hippel-Lindau tumour suppressor (VHL) (Vanharanta et al., 2013), resulting in greater PI3K/AKT/mTOR pathway activity.

DNMTs and histone methyltransferases utilise methyl groups derived from the universal methyl donor *S*-adenosylmethionine (SAM) as previously described. Methyl-depleted SAM becomes *S*-adenosyl-homocysteine (SAH); the ratio of SAM:SAH is carefully regulated as SAH is a potent negative regulator of DNMTs and histone methyltransferases. As such, intracellular SAH pools are quickly recycled back to homocysteine and methionine, thus preventing accumulation of SAH. However, in the cancer context, this regulation can be perturbed, resulting in irregular DNA/histone methylation patterns, stemming from aberrant SAM:SAH ratios (Mentch et al., 2015). For instance, excessive SAM has been shown to hypermethylate DNA and induce aberrant gene silencing in human patients and mouse models where glycine *N*-methyltransferase (*Gnmt*), a critical regulator of SAM/SAH levels was deleted (Mudd et

al., 2001). Indeed, *Gnmt* deletion has been shown to result in methylation, and therefore suppression, of the tumour suppressors *Rassf1* and *Socs2*, with increased incidence of hepatocellular carcinoma (Martínez-Chantar et al., 2007). Upstream signalling promoting greater methionine levels and thus SAM have also been described. For instance, cells can increase extracellular methionine uptake via the channel transporters *SLC7A5* and *SLC43A2* (Haase et al., 2007), and can utilise glucose-derived serine to increase intracellular SAM pools, or via increasing abundance of cofactors required for SAM generation. For example, serine-derived ATP is an important co-factor in the conversion of methionine to SAM (Maddocks et al., 2016). In the above examples SAM is in excess, however, the opposite can also occur. Nicotinamide *N*-methyltransferase (NNMT) converts nicotinamide to 1-methylnicotinamide (1-MNA), in a process that uses SAM as a methylation donor. NNMT has been shown to be overexpressed in several types of cancers (Tang et al., 2011; Wu et al., 2008); in this context, NNMT consumes significant amounts of SAM, greatly perturbing SAM:SAH balance (Ulanovskaya et al., 2013). As such, tumours that overexpress NNMT show decreased global methylation marks, such as H3K4, H3K27, H3K9 and H4K20, inducing transcriptional programs favouring tumour proliferation. Depletion of NNMT reversed this phenotype (Ulanovskaya et al., 2013).

1.6.2 DNA and histone demethylation

DNA and histone methylation dynamics are actively regulated by the countering activity of DNA and histone demethylases. DNA demethylases oxidise 5-mC to become 5-hydroxymethylcytosine (5-hmC), which is then converted back to cytosine by thymine DNA glycosylase (TDG). The Ten-Eleven Translocation (TET) family of DNA demethylases catalyse the first step of the demethylation process converting 5-mC to 5-hmC and are regarded as putative tumour suppressors (Guo et al., 2011; Ito et al., 2010; Kohli and Zhang, 2013; Tahiliani et al., 2009). Indeed, as mentioned previously in chapter 1.3.4.1, *TET2* is often mutated in haematological malignancies (particularly AML) resulting in promotor DNA hypermethylation in cancers as a result of loss of DNA demethylation capability (Abdel-Wahab et al., 2009). The role of histone demethylases in tumour initiation and progression is less clear; histone demethylases include lysine specific protein demethylases (KDMs) and jumonji C-domain-containing (JMJD)

enzymes that in certain contexts have been shown to be downregulated or upregulated (Kooistra and Helin, 2012).

Both TETs and JMJDs are dependent on the TCA cycle metabolite α -KG as a cofactor, and other TCA cycle metabolites, such as succinate and fumarate, can inhibit TET and JMJD activity (Xiao et al., 2012). The dependence TETs and JMJDs on α -KG as a cofactor are particularly important in cancer. Mutations in TCA metabolic enzymes, such as isocitrate dehydrogenase 1/2 are common in AML (Paschka et al., 2010; Ward et al., 2010b) and glioblastomas (Balss et al., 2008). In normal cells, the IDH family of enzymes catalyse the reversible oxidative carboxylation of isocitrate to α -KG (Hurley et al., 1991); however, point mutations in the substrate binding sites of IDH1/2 can result in neomorphic capacity to generate the oncometabolite 2-hydroxyglutarate (2-HG) concomitant with a loss of capacity in converting α -KG to isocitrate (Dang et al., 2010). The result of these mutations is high intracellular levels of 2-HG, which act to competitively antagonise reactions in which α -KG acts as a cofactor. Indeed, high 2-HG levels inhibit JMJDs, resulting in hypermethylated histones and increased proliferation of haematopoietic progenitor cells (Sasaki et al., 2012). 2-HG has been shown to inhibit JMJD2A, JMJD2C, JHDM1A and KDM7A to a greater extent than other histone demethylases (Chowdhury et al., 2011; Xu et al., 2011). In one study, enforced overexpression of IDH1^{R132H} in haematopoietic cells resulted in increased methylation at several lysine residues; in particular, H3K9 trimethylation (H3K9me3) was associated with the induction of stem-like and suppressed differentiation transcriptional programs (Lu et al., 2012). 2-HG can also act as a competitive inhibitor of the TET mediated conversion of 5-mC to 5-hmC *in vitro* (Chowdhury et al., 2011; Xu et al., 2011). Importantly, DNA and histone hypermethylation as a result of elevated intracellular 2-HG is reversible, suggesting inhibition of mutant IDH1/2 enzymes may be sufficient to alleviate the inhibitory block on TETs and JMJDs, and inhibitors of mutant IDH1/2 have been developed as discussed in chapter 1.3.4.1.

Other enzyme mutations can induce TCA-cycle mediated epigenetic reprogramming. Activity of Fumarate hydratase (FH, which catalyses the reversible hydration/dehydration of fumarate to malate) and succinate dehydrogenase (SDH, which catalyses the oxidation of succinate to fumarate) is lost in a subset of gastrointestinal tumours (Janeway et al.,

2011; Ricketts et al., 2012). Thus, increased intracellular abundances of succinate and fumarate behave as inhibitors of TETs and JMJDs, demonstrated by inhibition of TET-mediated hydroxylation of 5-mC and the histone demethylases KDM2A and KDM4A (Xiao et al., 2012) (Figure 1.10). Global hypermethylation was further observed in paragangliomas and gastrointestinal stromal tumours harbouring SDH mutations (Killian et al., 2013; Letouzé et al., 2013).

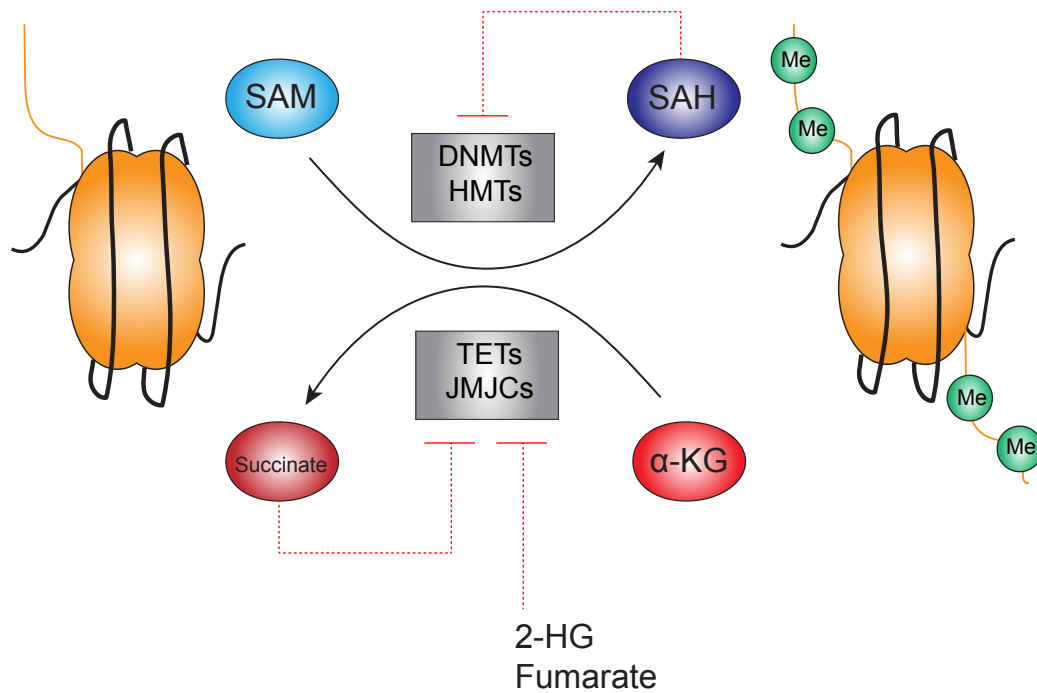


Figure 1.10 Methylation and demethylation of histones and DNA.

S-adenosylmethionine (SAM) acts as the universal methyl-group donor, and DNA methyltransferases (DNMTs) and histone methyltransferases (HMTs) utilise SAM-derived methyl groups to methylate DNA and histones (denoted by Me groups). Methyl-group depleted SAM, referred to as *S*-adenosylhomocysteine (SAH) can in itself inhibit the activity of DNMTs and HMTs. Methyl marks can be removed from DNA and histones through the action of the Ten-Eleven Translocation (TET) family of demethylases, as well as the jumonji C-domain-containing family of demethylases (JMJs), in a process that consumes α -ketoglutarate (α -KG) and yields succinate. As with SAH, succinate can inhibit the activity of DNA and histone demethylases. Similarly, the oncometabolite 2-hydroxyglutarate (2-HG), which accumulates as a result of mutations in isocitrate dehydrogenases (IDHs) and the tricarboxylic acid (TCA) cycle intermediate metabolite fumarate can also inhibit the activity of TETs and JMJs.

1.7 Thesis Aims

Activating FMS-like tyrosine kinase 3 (*FLT3*) mutations occur in approximately 30% of all acute myeloid leukaemias (AMLs) and are associated with poor prognosis. While the transcriptional programs initiated by FLT3 mutations are known to drive proliferative and pro-survival signalling, the mechanism by which these signals reprogram the metabolome to sustain proliferation are not completely understood. Moreover, the interplay between epigenetic regulation of metabolic processes in mutant FLT3-driven AML is also poorly characterised. For instance, the master metabolic regulator, mTORC1, is known to be hyperactivated downstream of mutant FLT3; however, the epigenetic mechanisms mediating mTORC1 activity remain largely unknown.

This work therefore aims to delineate previously unappreciated metabolic reprogramming events induced by mutant FLT3 to sustain malignant AML proliferation, as well as to characterise the interplay between epigenetics and metabolic regulatory pathways in maintaining AML.

Chapter 3 **Aim 1:** Develop a mouse model of FLT3-ITD-driven AML to identify metabolic signalling pathways downstream of FLT3-ITD;

Aim 2: Characterise metabolic signalling pathways regulated by FLT3-ITD and identify novel metabolic vulnerabilities that can be therapeutically exploited.

Chapter 4 **Aim 1:** Perform a small-molecule screen to determine epigenetic-modifying compounds that potentiate inhibition of mTORC1, the master metabolic regulator in a mouse model of FLT3-ITD-driven AML;

Aim 2: Characterise how dual epigenetic/mTORC1 inhibition collectively alters gene expression and affects leukaemic maintenance.

Chapter 2: Materials and Methods

2.1 Human cell lines and cell culture

The following human cell lines and culture conditions were utilised in this thesis. Generation of genetically engineered murine cell lines and relevant culture conditions are outlined in the following section. All cells used in this study were tested regularly for *mycoplasma* via PCR validation and were validated via short-tandem repeat (*STR*) profiling prior to use.

Table 2.1 Summary of human cell lines

DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures).

Cell Line	Disease	Growth Properties/ Conditions	Media	Source
MV4-11	MLL-AF4, FLT3-ITD Acute myeloid leukaemia	37°C and 5% CO ₂ Suspension	Complete RPMI, 10% FBS	ATCC, Manassas, VA, USA
Kasumi-1	AML1-ETO Acute myeloid leukaemia	37°C and 5% CO ₂ Suspension	Complete RPMI, 20% FBS	ATCC, Manassas, VA, USA
HL-60	<i>c-MYC</i> , NRAS ^{Q61R} Acute promyelocytic leukaemia	37°C and 5% CO ₂ Suspension	Complete RPMI, 20% FBS	ATCC, Manassas, VA, USA
HEK-293T	Human embryonic kidney	37°C and 10% CO ₂ Adherent	Complete DMEM 10% FBS	ATCC, Manassas, VA, USA
MOLM-13	MLL-AF9, FLT3-ITD Acute myeloid leukaemia	37°C and 5% CO ₂ Suspension	Complete RPMI, 10% FBS	Leibniz-Institut DSMZ Braunschweig, Germany
OCI-AML3	NPM1 DNMT3A ^{R882C} Acute myeloid leukaemia	37°C and 5% CO ₂ Suspension	Complete RPMI, 20% FBS	Leibniz-Institut DSMZ Braunschweig, Germany

PL-21	FLT3-ITD Complex karyotype Acute myeloid leukaemia	37°C and 5% CO ₂ Suspension	Complete RPMI, 10% FBS	Leibniz-Institut DSMZ Braunschweig, Germany
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2.1.1 Human cell culture medium

The following *in vitro* culture medium was used for the maintenance of the human cell lines listed in **Table 2.1**.

Table 2.2 Complete RPMI Composition

Component	Concentration	Manufacturer
RPMI (Roswell Park Memorial Institute) 1640	–	Gibco, Grand Island, NY, USA
Heat-inactivated foetal bovine serum (FBS)	10% or 20% (v/v)	Invitrogen Life Technologies, Carlsbad, CA, USA
Penicillin/streptomycin	2 mM 1% (v/v)	Invitrogen Life Technologies, Carlsbad, CA, USA
L-glutamine (GlutaMax)	2 mM 1% (v/v)	Sigma-Aldrich, St Louis, MO, USA

Table 2.3 Complete DMEM Composition

Component	Concentration	Manufacturer
Low-glucose DMEM (Dulbecco's Modified Eagle Medium)	–	Gibco, Grand Island, NY, USA
Heat-inactivated foetal bovine serum (FBS)	10% (v/v)	Invitrogen Life Technologies, Carlsbad, CA, USA
Penicillin/streptomycin	2 mM 1% (v/v)	Invitrogen Life Technologies, Carlsbad, CA, USA

L-glutamine (GlutaMax)	2 mM 1% (v/v)	Sigma-Aldrich, St Louis, MO, USA
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2.2 Generation of murine cell lines

2.2.1 Retroviral generation of MLL-AF9/iFLT3-ITD cells and foetal liver transductions

For generation of murine MLL-AF9/iFLT3-ITD cells, FLT3-ITD was cloned into a TRE-tight-DsRED-IRES-Empty vector and MLL-AF9 was cloned into an MSCV-Luc2-IRES-Empty vector using standard cloning techniques, and as previously described by our laboratory (Ghisi et al., 2016). All transfections were performed in HEK-293T cells using polyethylenimine (PEI) reagent (Sigma-Aldrich), and the pCL-Ampho retroviral packaging vector. Viral supernatant was collected at 48- and 72-hours post transfection and spun onto Retronectin (Takhara)-coated tissue culture plates at 2000g for 1 hour in either in MLL-AF9 alone or 1:1 MLL-AF9/FLT3-ITD virus conditions. 1×10^6 13.5 embryonic mouse day foetal liver cells were harvested from mice endogenously expressing the CAG-rtTA3 transgene, and subsequently spun onto viral-coated plates at 500g for 4 minutes and allowed to colocalise with virus for 48 hours. *Ptprca* mice were then sub-lethally irradiated with 6.5 Gy gamma irradiation, allowed to rest for 4 hours, and then IV injected via tail vein with transduced cells. Leukaemias were allowed to engraft and monitoring of disease was performed via luciferase bioluminescence imaging as described. At endpoint, mice were ethically euthanised, and AML blasts extracted from bone marrow and spleen. Cells from two independent clones, #21/0 (used in the primary manuscript) and #34/0 (used for validation experiments), were isolated from bone marrow and cultured in Anne Kelso modified DMEM as described, in 1 μ g/mL doxycycline to maintain expression of the iFLT3-ITD transgene. In all genetic depletion experiments of iFLT3-ITD, cells were cultured for a minimum of 24 hours in doxycycline to stabilise transgene expression, prior to 2 washes with PBS to remove doxycycline from medium.

2.2.2 Murine cell line tissue culture

Table 2.4 Anne Kelso Modified DMEM

Component	Concentration	Manufacturer
Low-glucose DMEM (Dulbecco's Modified Eagle Medium)	–	Gibco, Grand Island, NY, USA
Glucose	4 g/L	Sigma-Aldrich, St Louis, MO, USA
Folic Acid	36 mg/mL	Sigma-Aldrich, St Louis, MO, USA
L-arginine HCl	116 mg/L	Sigma-Aldrich, St Louis, MO, USA
L-glutamine	216 mg/L	Sigma-Aldrich, St Louis, MO, USA
NaHCO ₃	2 g/L	Sigma-Aldrich, St Louis, MO, USA
HEPES	10 mM	Sigma-Aldrich, St Louis, MO, USA

Table 2.5 Complete Anne Kelso DMEM Composition

Component	Concentration	Manufacturer
Anne Kelso Modified DMEM	–	In House
Heat-inactivated foetal bovine serum (FBS)	20% (v/v)	Invitrogen Life Technologies, Carlsbad, CA, USA
Penicillin/streptomycin	2 mM 1% (v/v)	Invitrogen Life Technologies, Carlsbad, CA, USA
L-asparagine	100 µM	Sigma-Aldrich, St Louis, MO, USA

2.3 Serine/glycine-free RPMI medium

To generate serine/glycine free RPMI 1640 otherwise identical to the commercial medium utilised in all other experiments in this study (Gibco Cat #11875), we synthesised medium as per manufacturer's protocol at the URL below and omitted the addition of serine and glycine in the final formulation. At the time of culture, we added 10% or 20% dialysed FBS prepared as previously described (Cantor et al., 2017). All other medium

conditions, such as antibiotic and Glutamax supplementation were identical to regular culture medium (discussed above).

URL: <https://www.thermofisher.com/au/en/home/technical-resources/media-formulation.114.html>

2.4 Small molecule inhibitors

Small molecule inhibitors used in this thesis are listed in the table below in table 2.6. For *in vitro* experiments, quizartinib, everolimus, rapamycin, UNC0379, AZD5576, JQ1, and AZD6244 were reconstituted in DMSO at a concentration of 10 mM and stored at -20°C. WQ-2101 was reconstituted in DMSO at a concentration of 6 mM and stored at -20°C. Clinical stocks of cytarabine (411 mM) were stored at room temperature and directly diluted in media prior to use. For *in vivo* dosing, WQ-2101 was purchased from Glxxx Labs and reconstituted in 10% DMSO, 20% Kolliphor HS-15 and 70% sterile water supplemented with 0.1% Tween 80 prior to dosing daily.

Table 2.6 Small molecule inhibitors utilised for *in vitro* and *in vivo* experiments.
PMCC, Peter MacCallum Cancer Centre.

Compound	Class	Source
Quizartinib (AC220)	FLT3 inhibitor	Selleck Chemicals, Houston, TX, USA
WQ-2101 (<i>In vitro stock</i>)	PHGDH inhibitor	Sigma-Aldrich, St Louis, MO, USA
WQ-2101 (<i>In vivo stock</i>)	PHGDH inhibitor	Glxxx Laboratories, Hopkinton, MA, USA
Everolimus (RAD001)	mTORC1-specific inhibitor	Selleck Chemicals, Houston, TX, USA
Rapamycin	mTORC1-specific inhibitor	Selleck Chemicals, Houston, TX, USA
UNC0379	SETD8 inhibitor	Selleck Chemicals, Houston, TX, USA

AZD5576	CDK9 inhibitor	AstraZeneca, Cambridge, UK
JQ1	BET inhibitor	Bradner Lab, Dana Farber Cancer Institute, Boston, MA, USA
AZD6244	MEK inhibitor	AstraZeneca, Cambridge, UK
Cobimetinib (GDC-0973)	MEK inhibitor	Selleck Chemicals, Houston, TX, USA
Pictilisib (GDC-0941)	PI3K α/δ	Selleck Chemicals, Houston, TX, USA
Cytarabine (cytosine arabinoside)	Chemotherapeutic agent	PMCC Cytotoxic Suite

2.5 *In vitro* cell death and proliferation assays

2.5.1 Cell death assays

For assessment of cell viability via Annexin V staining, cell suspension was stained with 1:100 dilution Annexin V conjugated to an APC fluorophore (BD Pharmingen, Cat #550475) in binding buffer (10 mM HEPES, 5 mM CaCl₂, 140 mM NaCl₂) prior to flow cytometry analysis. For assessment of cell viability in response to WQ-2101, 8,000 AML cells were plated into 384-well plates and exposed to escalating concentrations of WQ-2101 for 48 hours. At endpoint, 1:2 dilution Cell Titer Glo (CTG) was added to each well and cells lysed for 10 minutes prior to assessment of absorbance with a Cytation 3 spectrophotometer (BioTek).

2.5.2 Cell proliferation assays

For determination of proliferation in the presence/absence of serine and WQ-2101, 50,000 cells were seeded in 6-well plates and cultured for 3 or 9 days respectively. Cells were counted using a cellometer, and viability determined by trypan-exclusion assay. For Cell Trace Violet (CTV) analysis of cell proliferation in response to WQ-2101 or OCI-AML3 cells in response to serine/PHGDH deprivation, 1×10^7 cells were washed in PBS, and stained for 20 minutes at 37°C with agitation with Cell Trace Violet label (Thermo Fisher). A narrow peak of cells was then sorted using a FACS Aria Fusion 5 (BD) to

synchronise cell cycle phase and enable defined peak analysis. Cells were then cultured for 72 hours in 3 μ M WQ-2101 and proliferation assessed via flow cytometry. For OCI-AML3 cells in response to serine deprivation, sgSCR or sgPHGDH-transduced cells were cultured for 48 hours in either full serine/glycine or serine/glycine deficient RPMI and proliferation assessed via flow cytometry.

2.5.3 *In vitro* and *In vivo* proliferative competition experiments

For *in vitro* competition experiments, Tet-inducible sgRNAs were activated by addition of 1 μ g/mL doxycycline to cell culture medium 5 days prior to the assay. 50:50 mixes of parental and GFP-tagged sgSCR or sgPHGDH-transduced cells were then cultured in 6-well plates and the ratio of parental to GFP-positive cells assessed via flow cytometry at timepoint 0 and over 14 days. For *in vivo* competition assays, Tet-inducible sgRNAs were activated by addition of 1 μ g/mL doxycycline to cell culture medium 5 days prior to the assay. 2.5×10^6 parental Cas9-mCherry-expressing cells were mixed in a 50:50 ratio with 2.5×10^6 sgSCR-GFP or sgPHGDH-GFP/Cas9-mCherry cells such that all transplanted cells were mCherry-tagged and approximately 50% of the population contained GFP-tagged sgRNAs. This 5×10^6 mixture of cells was then IV-transplanted into each NSG recipient ($n = 4-6$ mice per group). At ethical endpoint of any mouse in any group, all animals across the experiment were culled to draw direct comparisons (i.e., within each cell line). Spleens were extracted from mice, mechanically homogenised, and red blood cells (RBCs) lysed with RBC lysis buffer (155 mM NH_4Cl , 12 mM NaHCO_3 , 0.1 mM EDTA). Remaining white blood cells were washed twice with PBS, filtered, and analysed via flow cytometry.

For flow cytometry analysis of the representation of sgRNA-GFP-tagged cells at endpoint, all single, live cells in the sample were gated, and the mCherry-tagged population further gated (representing all transplanted cells). The % GFP-tagged cells of this mCherry-tagged population was then recorded and presented for each mouse for Figure 3D of the manuscript in chapter 3.2.

2.5.4 *In vitro* synergy assays and analysis

For analysis of *in vitro* synergy with MV4-11, MOLM-13 and PL-21 cells, 8,000 cells were seeded per well in a 384-well plate and incubated with escalating concentrations of

WQ-2101 and cytarabine (AraC) in a checkerboard format for 48 hours with a D300e Digital Dispenser (Tecan Technologies). At endpoint, viability was assessed using Cell Titer Glo as described above. Raw absorbance values were converted to % cell death for each condition and normalised to DMSO controls. Synergy was determined by utilising the SynergyFinder R package (v1.4.2) (Ianevski et al., 2017a) using the Bliss synergy model, for which a Bliss Synergy Index was computed for each assay. To ensure synergistic effects were not confounded by the ATP-based readout of Cell Titer Glo, we cultured MV4-11 cells in 1 μ M cytarabine + 3 μ M WQ-2101 for 48 hours *in vitro* and used Annexin V flow cytometry as an alternative method for quantifying cell viability.

For primary patient cell *in vitro* synergy analyses, 50,000 AML cells were plated in each well of a 96-well plate and cultured with escalating concentrations of WQ-2101 and cytarabine (AraC) to a maximum of 100 μ M for 5 days in medium conditions as described above. At endpoint, cells were stained with 5 μ M Sytox Blue (Invitrogen) and cell viability assessed via flow cytometry. Synergy analysis was performed as above.

For *in vitro* synergy analysis on FLT3-WT HL-60 and KASUMI-1 cells, 20,000 cells were seeded per well of a 96-well plate and incubated with escalating concentrations of WQ-2101 and cytarabine (AraC) in a checkerboard format for 48 hours with a D300e Digital Dispenser (Tecan Technologies). At endpoint, cells were stained with 1 mg/mL 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen) and cell viability assessed via flow cytometry. Synergy analysis was performed as above.

2.6 Small molecule epigenetic compound screen

2,500 MLL-AF9/iFLT3-ITD cells were seeded into 40 mL of Complete Anne Kelso DMEM in 384-well plates using an automated liquid handler (BioTek, Winooski, VT, USA). Cells in one half of the plates were cultured in Complete Anne Kelso medium containing 300 nM of everolimus. Plates were pulsed at 500g to pellet cells and allowed to rest for 4 hours. At this point, half of the plates received pre-treatment with everolimus, while the other half remained untreated. After 4 hours, lyophilised compounds (representing 181 distinct epigenetic targeting compounds) at three concentrations (0.1 mM, 1 mM and 10 mM) supplied by Compounds Australia (Brisbane, QLD, Australia) were rehydrated in 25 μ L of Complete Anne Kelso DMEM. 10 μ L of each compound

was then transferred to plates containing MLL-AF9/iFLT3-ITD cells incubated in Complete Anne Kelso DMEM or Complete Anne Kelso DMEM plus 300 nM everolimus (this condition thus representing the combination therapy treated arm) using a Sciclone ALH3000 robot (Perkin Elmer, Waltham, MA, USA). Appropriate control wells, containing media and DMSO controls, as well as compound controls (AZD5576, JQ1, AZD6244, everolimus) were seeded into columns 23 and 24 of each plate. Plates were again pulsed at 500g to pellet cells and incubated with epigenetic library compounds, or epigenetic library compounds and everolimus, for 48 hours.

At endpoint, 20 mL of Cell Titer Glo (CTG) was dispensed into each well, and incubated for 2 minutes with agitation, followed by 10 further minutes of incubation. Plate luminescence was then imaged using a Cytation 3 or Cytation 5 cell imager (BioTek, Winooski, VT, USA). Details regarding analysis of data, quality control checks and determination of synergy between epigenetic compounds and everolimus are provided in greater detail in chapter 4.

2.7 Western immunoblotting

After appropriate treatment, whole-cell protein lysate was obtained by suspension of cells in Laemmli lysis buffer at room temperature for 10 minutes, followed by boiling of samples at 95°C for 10 minutes. Protein lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes by electroblotting. The membranes were blocked in 5% skim milk and incubated with primary antibody overnight. Membranes were subsequently washed in TBS-T and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody as appropriate. Blots were developed using enhanced chemiluminescence (ECL) or ECL Prime (GE Healthcare). For blots probing for phosphorylated and total targets, a master mix of each lysate was prepared, and an equal volume of protein run on separate blots, with each blot probed with appropriate primary antibody. Loading controls were developed with representative examples shown in each figure. A complete list of primary antibodies and secondary antibodies is described below.

Table 2.7 Primary antibodies utilised for western immunoblotting and ChIP-sequencing

WB, Western immunoblot; ChIP, chromatin immunoprecipitation

Antibody	Manufacturer	Catalogue #	Dilution	Application
Phospho-FLT3 (Tyr842) (10A8) Rabbit mAb	Cell Signaling	4577S	1:500	WB
FLT3 (8F2) Rabbit mAb	Cell Signaling	3462S	1:500	WB
Phospho-STAT5 (Tyr694) Rabbit mAb	Cell Signaling	9351S	1:1000	WB
STAT5 Rabbit mAb	Cell Signaling	9363S	1:1000	WB
PARP Rabbit mAb	Cell Signaling	9542L	1:1000	WB
Caspase-3 Mouse Ab	BD Biosciences	611048	1:1000	WB
PHGDH Rabbit Ab	Sigma-Aldrich	HPA021241	1:1000	WB
PSAT1 Rabbit Ab	Novus Biologicals	NBP1-55368	1:1000	WB
ATF4 (D4B8) Rabbit mAb	Cell Signaling	11815S	1:1000 13 µL/ChIP	WB ChIP
RNA Pol II (CTD4H8) Mouse mAb	Merck Millipore	05-623	6 µL/ChIP	ChIP
Phospho-S6 Ribosomal Protein (Ser240/244) Rabbit Ab	Cell Signaling	2215S	1:1000	WB
S6 Ribosomal Protein (54D2) Mouse mAb	Cell Signaling	2317S	1:1000	WB
Phospho-Histone H2A.X (Ser139) (20E3) Rabbit mAb	Cell Signaling	9718S	1:1000	WB
H4K20me1 ChIP-Grade Rabbit Ab	Abcam	Ab9051	1:1000	WB
β-Actin (AC-74) Mouse mAb	Sigma-Aldrich	A2228	1:5000	WB
HSP90 (AC88) Mouse mAb	Enzo Life Sciences	ADI-SPA-830	1:3000	WB

Table 2.8 Secondary antibodies utilised for western immunoblotting

WB, Western immunoblot

Antibody	Manufacturer	Catalogue #	Dilution	Application
Swine Anti-Rabbit Immunoglobulin HRP Ab	Dako	P0127	1:3000	WB
Rabbit Anti-Mouse Immunoglobulin HRP Ab	Dako	P0260	1:3000	WB

2.8 Metabolic techniques

2.8.1 Metabolic rescue experiments

For serine rescue experiments in sgPHGDH cells, 2.5×10^5 cells were seeded in 24-well plates, and escalating concentrations of L-serine (Sigma-Aldrich) was supplemented into serine/glycine-free RPMI at seeding. Cells were incubated for 48 hours prior to cell death analysis via Annexin V staining as described in the main text.

For metabolite rescue experiments in response to FLT3-ITD inhibition with quizartinib, 2.5×10^5 exponentially growing MV4-11 cells were cultured in 24-well plates for 48-72-hours as described and cultured in the presence of 3 nM of quizartinib. Metabolites were then supplemented into medium at time of seeding as follows: For nucleoside rescue experiment, 150 μ M purine cocktail mix (Adenine, Guanine in sterile water, heated to 37°C prior to use – Sigma-Aldrich); 150 μ M pyrimidine cocktail mix (Cytosine, Thymine, Uridine in sterile water – Sigma-Aldrich); 150 μ M EmbryoMax Nucleosides (Merck-Millipore, Cat # ES-008-D) and 286 μ M L-serine (Sigma-Aldrich). For metabolite rescue experiments, 10 mM sodium pyruvate (Sigma-Aldrich); 15 mM sodium L-lactate (Sigma-Aldrich); 150 μ M hypoxanthine (6-hydroxypurine – Sigma-Aldrich); 572 μ M glycine (Sigma-Aldrich); 10 mM sodium formate (Sigma-Aldrich); and 286 μ M L-serine (Sigma-Aldrich). Cell viability was assessed via Annexin V flow cytometry as described above. In these experiments, sodium pyruvate, sodium lactate, serine, and hypoxanthine rescue experiments were performed at the same time, and thus utilise the same DMSO and Quizartinib + Vehicle conditions/data. Likewise, glycine and sodium formate experiments were performed at the same time and utilise the same DMSO and Quizartinib + Vehicle conditions/data.

2.8.2 LC/MS-MS and steady-state metabolomics analysis

MV4-11 cells were maintained in full growth medium and fresh medium (full or serine/glycine-free) was added at the time cells were treated with quizartinib or WQ-2101. Following a 24-hour treatment, 3×10^6 MV4-11 cells were harvested by centrifugation, washed with normal saline and cell pellets were snap-frozen. For metabolite extraction, cell pellets were resuspended in 500 μ L ice-cold MeOH:H₂O (80:20) containing internal standards (¹³C-AMP, ¹³C-UMP, ¹³C Sorbitol, and ¹³C Valine) and vortexed. Samples were incubated on ice for 5 minutes, vortexed and debris was pelleted by centrifugation at 16,000 g for 10 minutes. The resulting supernatants were injected and analysed by hydrophilic interaction liquid chromatography (HILIC) and high-resolution mass spectrometry (Agilent 6545 LC/Q-TOF). Quality control checks were performed using QTOF MassHunter Quant software (Agilent) and metabolite peak calling was conducted using EI-MAVEN analysis software (Agrawal et al., 2019). Data are presented as fold change in metabolite abundance relative to control.

2.8.3 U-¹³C-glucose labelling metabolomics analysis

MV4-11 and OCI-AML3 cells were maintained in full RPMI growth medium and fresh medium was added at the time cells were pre-treated with quizartinib or Everolimus for 4 hours. Cells were then pelleted, washed twice with PBS and resuspended in RPMI in which U-¹³C-glucose (Sigma-Aldrich, Cat # 389374) replaced the unlabelled glucose component. Following an additional 20-hour treatment with quizartinib or Everolimus (for a total 24-hour exposure to drug), 3×10^6 cells were harvested and pelleted by centrifugation, washed with normal saline and cell pellets were snap-frozen. For metabolite extractions, cell pellets were resuspended in 500 μ L ice-cold MeOH:H₂O (80:20) and vortexed. Samples were incubated on ice for 5 minutes, vortexed and debris was pelleted by centrifugation at 16,000 g for 10 minutes. The resulting supernatants were injected and analysed by high-resolution mass spectrometry (Thermo Scientific Orbitrap ID-X Tribrid Mass Spectrometer). Metabolite peak calling was conducted using EI-MAVEN analysis software as described above (Agrawal et al., 2019).

2.8.4 Tritiated serine labelling

MV4-11, MOLM-13, and OCI-AML3 cells were cultured in full-serine/glycine RPMI with DMSO, 5 nM quizartinib, or 5 μ M WQ-2101 for 24 hours. 2×10^6 cells were

harvested per condition and washed twice with PBS, prior to resuspension and labelling in serine-free RPMI supplemented with 250 nM of L-[³H(G)]-Serine (Perkin Elmer, Cat # NET248250UC) for 30 mins. Post labelling, cells were washed twice with PBS, and a whole-cell lysate was obtained by resuspending cells in Laemmli lysis buffer. After boiling the lysate for 5 minutes at 95°C, an equivalent volume of tritiated lysate was resuspended in Optiphase HiSafe 3 liquid scintillation cocktail (Perkin Elmer), and beta decay emissions detected with a Tri-Carb 2910 TR liquid scintillation analyser (Perkin Elmer). Data was analysed using QuantaSmart v4.02 software (Perkin Elmer). All experiments were performed using three biological replicates, and background absorbance was subtracted from each sample as appropriate.

2.9 Molecular biology techniques

2.9.1 Retroviral/lentiviral transductions and plasmid cloning

For generation of Tet-inducible sgRNAs, two independent sgRNA sequences were utilised from the Brunello library (Doench et al., 2016) against *PHGDH*:

sgPHGDH-1: 5'-GATGACATCAGCGGTCACCT

sgPHGDH-2: 5'-GACACACCTACCTGTCGTGG

sgRNAs were cloned into the FgH1t_UTG Tet-inducible sgRNA vector (Addgene ID # 70183). HEK-293T cells were transfected using polyethyleneimine (PEI) reagent (Sigma-Aldrich) and the pMDLg/pRRE, pRSV-Rev and pVSV.G packaging vectors, and viral supernatant harvested 48- and 72-hours post transfection. Spin infections of constitutively expressing Cas9 MV4-11, MOLM-13 and OCI-AML3 cells (FUCas9-Cherry vector; Addgene ID # 70182) with harvested virus were performed at 1500g for 30 minutes with 4 mg/mL Sequa-brene reagent (Sigma-Aldrich) at room temperature. For generation of inducible shRNAs, two independent shRNA sequences were utilised against *ATF4*:

shATF4-1: GCCTAGGTCTCTTAGATGATTCTCGAGAATCATCTAAGAGACCTAGGC

shATF4-2: CCACTCCAGATCATTCTTTACTCGAGTAAAGGAATGATCTGGAGTGG

shRNAs were cloned into the Tet-pLKO-puro Tet-inducible shRNA vector (Addgene ID # 21915) and transduced into MV4-11 cells as described above. shRNA-expressing MV4-11 cells were selected by culturing in 1 µg/mL puromycin (Sigma-Aldrich) for 5 days prior to experimental use and induced by the addition of 200 ng/mL doxycycline to deplete ATF4.

2.9.2 Real-time quantitative PCR analysis (RT-qPCR)

Cells were cultured/harvested as described, and RNA extracted using TRIzol reagent (Thermo Fisher Scientific) and the Direct-Zol RNA Miniprep kit (Zymo Research) as per manufacturer's instructions. RNA was reverse-transcribed with oligo-dT and random hexamers using the Transcriptor First Strand cDNA Synthesis Kit (Roche) as per manufacturer's instructions. Quantitative real-time polymerase chain reaction (RT-qPCR) was performed with this cDNA and the LightCycler 480 SYBR Green Kit (Roche) as per manufacturer's instructions. Briefly, 100 ng of cDNA template was combined with forward and reverse primers at a final concentration of 1.25 μ M and 5 μ L SYBR Green, for a total reaction volume of 10 μ L per well of a 364-well plate. Quantification of gene expression fold changes were analysed using the $\Delta\Delta$ Ct method normalised to the *18s rRNA* housekeeping gene (for murine RT-qPCR) or *RPL0* (for human RT-qPCR). Forward and reverse primers used in this study are provided in the table below:

Table 2.9 Primer pairs used for quantitative real-time PCR

Gene	Species	Direction	Sequence
Phgdh	Mouse	Forward	TGGCCTCGGCAGAATTGGAAG
		Reverse	TGTCATTCAGCAAGCCTGTGGT
Psat1	Mouse	Forward	GATGAACATCCCATTTCGCAT
		Reverse	GCGTTATACAGAGAGGCACG
Slc1a4	Mouse	Forward	TGCTCTGGCGTTCATCATCA
		Reverse	AGTGAATGCGGCAACCACAA
Slc1a5	Mouse	Forward	TGGCCAGCAAGATTGTGGAGAT
		Reverse	TTTGCGGGTGAAGAGGAAGT
PHGDH	Human	Forward	CTTGGATTGGTCTGGCAGAAGC
		Reverse	GCTTAGGCAGTTCCCAGCATTC
PSAT1	Human	Forward	TCCTCAAACCTCCTGTCCAAGC
		Reverse	CAGCAGGTCATCACGGACAATC
SLC1A4	Human	Forward	TGTGGTTGCAGCTTTCCGTACG
		Reverse	CCAGAGCAAACAGGACCAATCC
SLC1A5	Human	Forward	GTGTCCTCACTCTGGCCATC
		Reverse	TACAGGACCGGTCGACTAGC

Table 2.10 House-keeping gene primer pairs used for quantitative real-time PCR

Gene	Species	Direction	Sequence
18s rRNA	Mouse	Forward	GAGGTTCGAAGACGATCAGA
		Reverse	TCGCTCCACCAACTAAGAAC
RPLO	Human	Forward	TCGACAATGGCAGCATCTAC
		Reverse	GCCTTGACCTTTTCAGCAAG

2.10 Molecular genomics techniques

2.10.1 QuantSeq 3'-RNA sequencing and analysis

Cells were cultured/harvested as described, and RNA extracted using TRIzol reagent (Thermo Fisher Scientific) and the Direct-Zol RNA Miniprep kit (Zymo Research) as per manufacturer's instructions. The QuantSeq 3'RNA-seq Library Prep Kit for Illumina (Lexogen) was then utilised from 500 ng of RNA to generate libraries as per manufacturer's instructions. Libraries were then sequenced on the Illumina NextSeq 500, with single end 75bp reads to a depth of 15M reads per sample. Sequencing reads were subsequently trimmed at the 5'-end using CutAdapt (v1.14) software (Martin, 2011) to remove random primers introduced during library preparation. 3' ends were also trimmed to remove poly-A-tail derived reads. Reads were then mapped to reference genomes (mm10 for all mouse sequencing, hg19 for human) using HISAT2 (v2.1.0) software (Kim et al., 2019). Counting of reads was conducted using featureCounts, a component of the subread package (v1.5.2) (Liao et al., 2014). Differential gene expression was performed using the Voom-Limma methodology to determine statistical significance (Law et al., 2014, 2016). All RNA-seq analysis figures were generated using R (v3.6.1) software. GO Term analysis was performed using ToppGene software using term sets as described (Chen et al., 2009). Gene Set Enrichment Analysis (GSEA) software (v3.0) was used for identification of enriched gene sets, using the MSigDB KEGG and Hallmarks datasets (Liberzon et al., 2015).

2.10.2 Chromatin immunoprecipitation library preparation and sequencing analysis

MV4-11 and OCI-AML3 cells (60×10^6 per condition) were cultured in the presence or absence of 5 nM quizartinib for 24 hours, and pellets washed in ice-cold PBS. Cells were crosslinked with 1% formaldehyde for 10 minutes at room temperature and subsequently

quenched with 1.25 M glycine. Cells were washed three times with ice-cold PBS and lysed in ChIP lysis buffer (20 mM Tris-HCl, 150 mM NaCl₂, 2 mM EDTA, 1% IGEPAL CA-630 (Sigma-Aldrich) and 0.3% SDS in water) prior to sonication on a Covaris ultrasonicator at maximum power for 16 minutes to achieve an average DNA fragment size of 300-500bp. Immunoprecipitation reactions were performed in ChIP dilution buffer (20 mM Tris-HCl, 150 mM NaCl₂, 2 mM EDTA, 1% Triton-X, and phosphatase/protease inhibitors in water) overnight at 4°C. A 1:1 ratio of protein A and G magnetic beads (Life Technologies) were used to bind crosslinked protein/DNA. Antibodies used in ChIP-seq experiments are outlined in the '*Primary and secondary antibodies*' section of this chapter. Beads were then washed in increasingly high-salt concentration wash buffers and reverse crosslinked in the presence of Proteinase K (Zymo Research) prior to purification and concentration with the ChIP DNA Clean and Concentrator Kit (Zymo Research) per manufacturer's specifications.

Post elution and concentration of DNA, quantification was performed using the Qubit dsDNA HS Assay kit (Thermo Fisher), and ChIP-Seq libraries were prepared using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England BioLabs) as per manufacturer's instructions. Libraries were then size selected between 200 bp and 500 bp using a Pippin Prep 1.5% agarose cassette (Sage Science). Final library QC was performed on a Tape Station High Sensitivity DNA Analysis Kit (Agilent Technologies). For sequencing analysis of DNA immunoprecipitated with anti-ATF4 and anti-RNA Pol II antibodies, libraries were then pooled and sequenced on an Illumina NextSeq 500 and 20-25 million single-end 75 bp reads were generated for each sample.

Raw sequencing reads were demultiplexed using bcl2fastq (v2.17.1.14) and FASTQ files generated and quality controlled with FASTQC (v0.11.5). Adaptor sequences were removed and reads aligned to the human reference genome (GRCh37/hg19) using Bowtie2 (v2.3.5). Samtools (v1.4.1) was used for processing of SAM and BAM files, from which Model-based Analysis of ChIP-Seq (MACS2, v2.2.5) peak calling was performed (Zhang et al., 2008). TDF files were generated with IGVTools (v2.5.3) and ChIP-Seq tracks were visualised and figures generated using IGV software (v2.6.2). HOMER (v4.10) was used for quantification and annotation of the ChIP-Seq datasets, for determining peak scores of ATF4 target genes, and for *de novo* motif discovery on ATF4 bound sites. BigWig files were generated from sorted, indexed BAM files using deeptools

(v3.3.1). ATF4 genome-wide binding in MV4-11 and OCI-AML3 cells was visualised using the heatmap and profile functions within deeptools (v3.3.1) software.

2.10.3 TCGA SingScore/Differential gene expression analysis

For differential gene expression analysis of FLT3 mutant versus FLT3 wild type (WT) samples in the LAML_TCGA dataset, FLT3 mutation status of each of the LAML samples was downloaded from cBioPortal and any patients with loss of function, deletion, or no FLT3 mutation annotation were removed from the analysis. The Broad Institute’s GDAC Firehose tool was used to download mRNA count files for each of the LAML samples, which were subsequently annotated with FLT3 mutation status and normalised prior to statistical determination of DEG (FLT3-mutant versus FLT3-WT samples) via the Voom-Limma method (Law et al., 2014, 2016).

To generate the serine synthesis pathway – one-carbon metabolism – purine signature (SSP-OC-Purine Signature), we utilised canonical genes associated with these pathways that were selected from our own transcriptional analyses in this paper and supplemented from a study by Manning and colleagues (Ben-Sahra et al., 2016). Importantly, we selected the most differentially expressed purine biosynthesis genes from this study, while excluding those with minimal differential expression. A complete list of the genes utilised from each pathway are summarised in the table below. For interrogation of the LAML-TCGA dataset, we used the TCGAbiolinks R package (v2.15.3) to download and normalise the mRNA counts file using the edgeR method (Law et al., 2016). FLT3 mutation status of each of the LAML samples was downloaded from cBioPortal. Samples were stratified based on FLT3 mutation vs FLT3 WT, and the singscore method was subsequently utilised to plot *FLT3* mRNA LogRPKM values against the signature score (Foroutan et al., 2018).

Table 2.11 Genes used for generation of the Serine Synthesis Pathway – One-Carbon Cycle Pathway – Purine Biosynthesis Pathway (SSP-OC-Purine) genetic signature

Serine Synthesis Pathway (SSP)	One-Carbon Cycle Pathway (OC)	Purine Biosynthesis Pathway (Purine)

<i>PHGDH</i>	<i>DHFR</i>	<i>PRPS1</i>
<i>PSAT1</i>	<i>MTHFD1</i>	<i>PRPS2</i>
<i>PSPH</i>	<i>MTHFD1L</i>	<i>PPAT</i>
<i>SLC1A4</i>	<i>MTHFD2</i>	<i>GART</i>
<i>SLC1A5</i>	<i>SHMT1</i>	
<i>SLC6A9</i>	<i>SHMT2</i>	
	<i>GLDC</i>	
	<i>TYMS</i>	

2.11 *In vivo* experimentation

2.11.1 *In Vivo* analysis

All *in vivo* experiments performed in this study were approved by the Peter MacCallum Cancer Centre (PMCC) Animal Ethics Committee under ethics approval numbers E555 and E627. *Ptprca*, NSG, and C57BL/6J mice endogenously expressing the CAG-rtTA3 transgene were bred in-house (PMCC). For primary *Ptprca* recipient transplants, 4-6-week-old females were sub-lethally irradiated with 6.5 Gy whole-body gamma-ray irradiation, using a Gammacell 1000 Cs-137 irradiator (AECL). 1×10^6 retrovirally-transduced foetal liver cells were then IV tail-vein injected, and mice placed on doxycycline-containing chow (Specialty Foods) and water (0.2% doxycycline hyclate (Sigma-Aldrich), 2% sucrose) to maintain transgene expression until ethical endpoint (hunched, ruffled, impaired movements). For secondary recipients, 4-6-week-old female NSG mice were IV-injected with MLL-AF9/iFLT3-ITD cells as described above. At endpoint, blast cells were harvested from femoral bone. For *in vivo* bioluminescence imaging, mice were injected with 50 mg/kg D-luciferin and imaged in an IVIS bioluminescence imager (Perkin-Elmer) to assess disease burden. Analysis and normalization of *in vivo* luminescence was performed using Living Image v3.2 and v4.2 software.

2.11.2 *In Vivo* therapy experiments

For *in vivo* combination therapy experiments, 2.5×10^6 MV4-11 cells transduced with an MSCV-Luc2-IRES-mCherry reporter were IV-injected into 6-8-week-old female NSG recipients. Therapy commenced after blast content of peripheral blood reached a threshold of $\geq 1\%$ mCherry-positive cells. For *in vivo* combination assays, 5 mg/kg WQ-

2101 was i.p. injected into mice daily for 12 days, for a total of 13 doses of compound. 40 mg/kg cytarabine (AraC) was i.p. injected on alternate days for a total of 6 doses of compound, with appropriate single-agent controls for each treatment condition ($n = 8$ mice per group). After completion of therapy, mice were culled at ethical endpoint as described above. No censored events were recorded in this study, and all mice who received treatment were included in survival analysis.

2.11.3 Steady-state haematopoiesis analysis

For assessment of the effect of WQ-2101 on steady-state haematopoiesis, healthy non tumour-bearing *PTPRCA* mice were chronically treated with either 5 mg/kg (dose used for combination therapy as above) or 20 mg/kg of WQ-2101 (highest dose tested in original characterisation study (Wang et al., 2017)), or vehicle controls, for 14 days ($n = 5$ mice per group). At the conclusion of the treatment period, a blood sample was obtained from each mouse via retroorbital bleeding, mice were culled, and cells isolated from bone marrow (extracted via crushing from a single femur) and spleen (homogenised and RBC lysed as above).

To interrogate the effect of WQ-2101 on various haematopoietic compartments, we devised a flow cytometry-based protocol to quantify the relative number of various stem and progenitor cells (HSPC), as well as more differentiated myeloid and lymphoid cells (Mature), in response to both doses of WQ-2101 or vehicle control in the bone marrow and spleen. Cell suspensions of bone marrow and spleen were pelleted by centrifugation (450g, 4 min, 4°C), and resuspended in 100 μ L of antibody cocktail diluted in FACS buffer (2% FCS in PBS). Note, to assess the hematopoietic stem and progenitor compartment, cells were stained with sequential antibody cocktails, washing the cells with FACS buffer between incubations. Cells were stained on ice for 15 min, washed by centrifugation and resuspended in 100 μ L of FACS buffer with 10 μ L of CountBright™ Absolute Counting Beads (Invitrogen, Cat # C36950). Data was acquired on the BD FACSymphony and analysed using FlowJo software (v10.4). The antibodies used, including relevant dilutions and identification of which panel they correspond to is included below.

Table 2.12 List of primary antibodies for flow cytometry

Antibody	Fluorophore	Dilution	Supplier	Catalogue #	Panel
Lineage Cell Depletion (Biotin)	N/A	1:200	Miltenyi Biotec	130-090-858	HSPC (part 1)
Streptavidin	BUV805	1:200	BD Horizon	564923	HSPC (part 2)
CD117	BUV395	1:200	BD Horizon	564011	HSPC (part 2)
Scal	PeCy7	1:200	BD Pharmingen	558162	HSPC (part 2)
FcγRIII	BUV737	1:200	BD Horizon	565272	HSPC (part 2)
CD34	EF660	1:100	BD Pharmingen	560233	HSPC (part 2)
CD48	BV605	1:200	BioLegend	103441	HSPC (part 2)
CD150	BV711	1:200	BioLegend	115941	HSPC (part 2)
CD19	BV711	1:200	BioLegend	115555	Mature
CD3	BV605	1:200	BioLegend	100237	Mature
NK1.1	BB700	1:200	BD Horizon	566503	Mature
CD71	BV421	1:200	BioLegend	113813	Mature
Ter119	APC	1:200	BioLegend	116212	Mature
CD117	BUV395	1:200	BD Horizon	564011	Mature
CD11b	BUV737	1:200	BD Horizon	612800	Mature
F4/80	PE	1:200	BioLegend	123110	Mature
SiglecF	BV786	1:100	BD Optibuild	740956	Mature
Ly6C	BV785	1:200	BioLegend	128041	Mature
CD71	BV421	1:200	BioLegend	113813	Mature
CD182	BV650	1:200	BD Optibuild	747813	Mature
Ly6G	BUV563	1:400	BD Horizon	565707	Mature

Analysis of gross peripheral haematology cells (white blood cells, WBCs; red blood cells, RBCs; haemoglobin, HGB; and platelets, PLT) in response to either WQ-2101 or vehicle was performed by diluting whole blood in PBS (1:25) and running the sample on a Sysmex automated haematology analyser (Sysmex).

2.12 Primary patient samples

Primary patient samples used for *in vitro* synergy studies were obtained from the Alfred Hospital (Melbourne, Australia) after obtaining written informed consent under Alfred Health Ethics Committee-approved guidelines. These studies were performed under

Alfred Heath Ethics Approval Number 42/20. All use of human tissue was performed in accordance with the Declaration of Helsinki. Primary AML blasts were cultured in StemSpan SFEM (Stemcell Technologies) supplemented with 10 ng/mL IL-3, 10 ng/mL IL-6, 50 ng/mL SCF and 50 ng/mL FLT3L (all Peprotech) and 35 nM UM171 and 500 nM stemreginin (StemCell Technologies). No patient data other than age, sex and genomic profile were made available to non-clinical/authorised authors.

2.13 Statistical analyses

GraphPad Prism v8.0 and R version 3.6.1 software were used for statistical analysis. Statistical tests performed for each experiment are highlighted in the figure legends. Unless otherwise specified, error bars are indicative of plus/minus standard deviation ($\pm$ s.d.) and threshold for significance was $P < 0.05$.

2.14 Data availability

RNA-seq and ChIP-seq data relevant to chapter 3 was deposited in the Gene Expression Omnibus (GEO) under accession number GSE163932.

**Chapter 3: Serine biosynthesis is a metabolic vulnerability
in FMS-like tyrosine kinase 3-internal tandem duplication
(*FLT3-ITD*)-driven acute myeloid leukaemia**

3.1. Introduction

Mutations in the FMS-like tyrosine kinase 3 (*FLT3*) gene are among the most common recurrent somatic mutations in acute myeloid leukaemia (AML) with several large-scale sequencing studies revealing that some 30% of all AML patients harbour either internal tandem duplication (termed FLT3-ITD) or point mutations in the tyrosine kinase domain of FLT3 (termed FLT3-TKD) (Metzeler et al., 2016; Papaemmanuil et al., 2016; Patel et al., 2012; Tyner et al., 2018). During haematopoiesis, FLT3 coordinates a ligand-dependent signalling cascade that regulates the proliferation and maturation of the progenitor pool, but is largely silenced during mature haematopoiesis, remaining expressed on stem/progenitor cell populations (Kenins et al., 2010). In malignant haematopoiesis, FLT3 mutations result in ligand-independent autophosphorylation of the FLT3 kinase and activation of downstream signalling pathways that promote proliferation and suppress apoptosis. The frequency of FLT3 mutations in AML, coupled with the fact that they represent a subset of disease associated with poor prognosis, has resulted in significant efforts to develop small molecule inhibitors that inhibit FLT3 and its downstream pathways. Indeed, in 2017 the pan-kinase inhibitor midostaurin was approved as frontline therapy in combination with the chemotherapeutic agent cytarabine in patients carrying FLT3 mutations at diagnosis (Stone et al., 2017). In addition, several small molecule inhibitors with greater specificity for FLT3 have also been tested in clinical trials (as discussed in depth in chapter 1). In all examples, whilst these inhibitors have shown initial promise in clinical trials, the rapid development of resistance limits their widespread use and efficacy, highlighting the need to better understand the pathways downstream of mutant FLT3 that contribute to disease maintenance and resistance to therapy (McMahon et al., 2019a). As a receptor tyrosine kinase, the downstream signalling cascades of mutant FLT3 include the STAT5, MAPK and PI3K/mTOR pathways (Choudhary et al., 2007; Lindblad et al., 2016; Takahashi, 2006; Zhang et al., 2000). These pathways are known to mediate transcriptional regulation of pro-survival factors, suppress apoptotic signalling, and provide leukaemic cells with proliferative advantage; indeed, inhibition of these pathways has been shown to induce antiproliferative, and in some cases, apoptotic effects in cells harbouring FLT3-ITD mutations. The fundamental limitation of all of these therapeutic approaches however is the development of secondary resistance, typically via circumvention of the inhibited pathway. However, inhibition of MEK and mTOR, two pathways that allow for development of secondary resistance, has not advanced clinically beyond early stage

clinical trials (Larrosa-Garcia and Baer, 2017). In recent years, novel biological roles for mutant FLT3 independent of these pathways have been reported in the literature, including the reprogramming of cellular metabolism. Recent studies have demonstrated that oncogenic FLT3-ITD promotes glycolysis, primarily via sustaining glycolytic flux through the pentose phosphate pathway (Ju et al., 2017; Poulain et al., 2017). Another study demonstrated glutaminolysis mediates resistance to FLT3 targeted therapy, and that glutaminase (GLS), the enzyme that catalyses the hydrolysis of glutamate to glutamine, synergises with FLT3 inhibition (Gallipoli et al., 2018; Gregory et al., 2018). These insights provide mechanistic context to many long-held observations regarding the role of FLT3 mutations in AML. For example, rewiring of metabolic processes to drive greater glycolytic flux through the pentose phosphate pathway (as described by Ju and Poulain above) allows AMLs to promote nucleotide biosynthesis due to increased availability of biomass, thus driving increased cellular proliferation. These observations thus provide the basis for the argument that FLT3 mutations in AML promote proliferation via defined, metabolic reprogramming events.

In this chapter, we sought to elucidate the molecular mechanisms that underpin FLT3-ITD signalling in AML. Utilising a novel transgenic mouse model of tetracycline-inducible FLT3-ITD in MLL-AF9-rearranged AML, we demonstrate that FLT3-ITD transcriptionally promotes serine synthesis via the *de novo* serine biosynthesis pathway, as well as serine uptake by the neutral amino acid transporters *SLC1A4* and *SLC1A5* in both *in vitro* and *in vivo* contexts. We show that human AML cells which harbour FLT3-ITD mutations are dependent on *de novo* serine biosynthesis, and that depletion of *PHGDH*, the first rate-limiting enzyme of the *de novo* serine biosynthesis pathway, impairs proliferation and survival of these cells *in vitro* and *in vivo*. FLT3-ITD-mediated reprogramming of serine metabolism was dependent on the mTORC1-ATF4 axis, which promoted RNA-Pol II occupancy at key *de novo* serine biosynthesis pathway (*PHGDH*, *PSATI*) and amino acid transporter (*SLC1A4*, *SLC1A5*) genes. We reveal that FLT3-ITD mutations play a role in promoting nucleotide synthesis, wherein glucose-derived serine from the *de novo* serine biosynthesis pathway contributes to the production of purine nucleotides. Finally, we demonstrate that this dependency on *de novo* serine biosynthesis can be exploited therapeutically, by sensitizing FLT3-ITD mutant AMLs to cytotoxic chemotherapy via exacerbation of DNA damage. This work was accepted for publication in the journal *Cancer Discovery*. Collectively, the following manuscript, supplementary

and addendum data reveal key insights into metabolic reprogramming induced by FLT3-ITD mutations in AML and reveal a novel combinatorial therapeutic strategy to enhance the activity of standard of care chemotherapy in FLT3-mutant AML.

3.2. Publication in *Cancer Discovery*



ABSTRACT

Internal tandem duplication of the FMS-like tyrosine kinase 3 gene (*FLT3-ITD*) occurs in 30% of all acute myeloid leukemias (AML). Limited clinical efficacy of FLT3 inhibitors highlights the need for alternative therapeutic modalities in this subset of disease. Using human and murine models of FLT3-ITD-driven AML, we demonstrate that FLT3-ITD promotes serine synthesis and uptake via ATF4-dependent transcriptional regulation of genes in the *de novo* serine biosynthesis pathway and neutral amino acid transport. Genetic or pharmacologic inhibition of PHGDH, the rate-limiting enzyme of *de novo* serine biosynthesis, selectively inhibited proliferation of FLT3-ITD AMLs *in vitro* and *in vivo*. Moreover, pharmacologic inhibition of PHGDH sensitized FLT3-ITD AMLs to the standard-of-care chemotherapeutic cytarabine. Collectively, these data reveal novel insights into FLT3-ITD-induced metabolic reprogramming and reveal a targetable vulnerability in FLT3-ITD AML.

SIGNIFICANCE: FLT3-ITD mutations are common in AML and are associated with poor prognosis. We show that FLT3-ITD stimulates serine biosynthesis, thereby rendering FLT3-ITD-driven leukemias dependent upon serine for proliferation and survival. This metabolic dependency can be exploited pharmacologically to sensitize FLT3-ITD-driven AMLs to chemotherapy.

INTRODUCTION

Recent large-scale genome sequencing studies have revealed the remarkable heterogeneity that underpins acute myeloid leukemia (AML; refs. 1–4). Although new therapies for AML have recently been approved (5), the backbone of induction therapy in newly diagnosed patients remains a high-dose chemotherapy regimen of the cytosine analogue cytarabine, in conjunction with an anthracycline such as daunorubicin. Long-term survival is relatively poor, with a five-year survival rate of approximately 30% (6).

Activating mutations in receptor tyrosine kinases (RTK), including FMS-like tyrosine kinase 3 (FLT3), are frequently

observed in AML and are associated with dismal prognosis (7, 8). FLT3 is primarily expressed on hematopoietic progenitor cells, and during early hematopoiesis coordinates a ligand-dependent signaling cascade that regulates the proliferation and maturation of the progenitor pool (9). FLT3 internal tandem duplication (ITD), the most common type of FLT3 mutation, promotes constitutive FLT3 tyrosine kinase activity and hyperactivation of downstream signaling pathways, including STAT5, PI3K/mTOR, and MAPK pathways (10–13). A number of FLT3 inhibitors have been developed and are in clinical trials. First-generation inhibitors, such as midostaurin (currently approved as first-line therapy in patients with AML with FLT3-ITD mutations), sorafenib, and lestaurtinib, were developed as broad-spectrum kinase inhibitors. The inability of these agents to induce durable clinical responses resulted in the development of highly specific second-generation inhibitors (quizartinib and gilteritinib) with potent binding affinity for FLT3. Unfortunately, the efficacy of FLT3 inhibitors is limited by the rapid development of acquired resistance (14).

Extensive reprogramming of cellular metabolism is required to fulfill the increased energetic and biosynthetic demands associated with unrestrained proliferation (15). Metabolic reprogramming events can also introduce new vulnerabilities that can be exploited for cancer therapy (16, 17). In line with this notion, recent studies have demonstrated that FLT3-mutant AML cells are dependent on glycolysis and the pentose-phosphate pathway (18, 19). Moreover, glutamine metabolism has been shown to promote resistance to FLT3-targeted therapy (20, 21). Emerging evidence also suggests that many cancer cells are highly dependent on the non-essential amino acid serine (22, 23). Serine is a major source of one-carbon units to the folate cycle and therefore supports numerous metabolic pathways including nucleotide synthesis (24). The functional importance of serine metabolism in FLT3-mutant AML has not been defined.

¹Cancer Research Division, Peter MacCallum Cancer Centre, Melbourne, Australia. ²The Sir Peter MacCallum Department of Oncology, The University of Melbourne, Melbourne, Australia. ³Centre for Cancer Research, The University of Melbourne, Melbourne, Australia. ⁴Department of Clinical Pathology, The University of Melbourne, Melbourne, Australia. ⁵Human Oncology and Pathogenesis Program, Memorial Sloan Kettering Cancer Center, New York, New York. ⁶Australian Centre for Blood Diseases, Monash University, Melbourne, Australia. ⁷Department of Clinical Haematology, The Alfred Hospital, Melbourne, Australia. ⁸Department of Pathology, The Alfred Hospital, Melbourne, Australia. ⁹School of Clinical Sciences at Monash Health, Monash University, Clayton, Australia. ¹⁰Department of Biochemistry and Pharmacology, The University of Melbourne, Melbourne, Australia.

Note: Supplementary data for this article are available at Cancer Discovery Online (<http://cancerdiscovery.aacrjournals.org/>).

K.K. Brown and R.W. Johnstone are co-senior authors of this article.

Corresponding Authors: Ricky W. Johnstone, Gene Regulation Laboratory, Peter MacCallum Cancer Centre, 305 Grattan Street, Melbourne, 3000, Victoria, Australia. Phone: 61-3-8559-7133; E-mail: ricky.johnstone@petermac.org; and Kristin K. Brown, Level 12, Cancer Research Division, Peter MacCallum Cancer Centre, 305 Grattan Street, Melbourne, 3000, Victoria, Australia. Phone: 61-3-8559-5457; E-mail: kristin.brown@petermac.org

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Herein, we developed a physiologically relevant mouse model of MLL-rearranged, FLT3-ITD-driven AML and used a multiomics approach to identify exploitable vulnerabilities in this disease. We demonstrate that FLT3-ITD reprograms serine biosynthesis and uptake in an mTORC1-ATF4-dependent manner and show that FLT3-ITD AML cells are selectively sensitive to inhibition of serine biosynthesis. Importantly, this metabolic dependency can be exploited to sensitize FLT3-ITD AML cells to cytotoxic chemotherapy *in vitro* and *in vivo*. Collectively, these data reveal key insights into metabolic reprogramming events driven by FLT3 mutations in AML and reveal a novel combinatorial therapeutic strategy to enhance the efficacy of standard-of-care chemotherapy in this aggressive subtype of AML.

RESULTS

A Genetically Engineered Mouse Model of MLL-Rearranged, FLT3-ITD AML Reveals FLT3-ITD Is Essential for Leukemia Survival

Although FLT3-ITD mutations play a critical role in the pathogenesis of AML, FLT3-ITD alone is insufficient to induce leukemic transformation (25–27). We therefore generated a genetically engineered mouse model of doxycycline-inducible FLT3-ITD expression in MLL-AF9-rearranged AML (ref. 28; Fig. 1A). Mice injected with hematopoietic progenitor cells coexpressing MLL-AF9 and doxycycline-inducible FLT3-ITD (denoted as MLL-AF9/iFLT3-ITD) developed fully penetrant disease, detectable seven days after transplantation via luciferase bioluminescence imaging (Fig. 1B and C). In contrast, detectable disease was observed only 35 days after mice were transplanted with MLL-AF9-expressing hemopoietic progenitor cells, although all transplanted mice did eventually succumb to disease (Fig. 1B and C). MLL-AF9/iFLT3-ITD cells expressing the dsRed reporter were harvested from bone marrow of mice at endpoint and used for subsequent experiments. We confirmed harvested cells faithfully recapitulated AML via immunophenotyping and histologic analysis (Supplementary Fig. S1A).

To investigate the consequences of FLT3-ITD loss *in vitro*, MLL-AF9/iFLT3-ITD cells were propagated in doxycycline-free culture medium for 24 hours (denoted MLL-AF9/iFLT3-ITD-OFF). Doxycycline withdrawal rapidly abolished FLT3-ITD expression and resulted in decreased phosphorylation of the canonical FLT3 target STAT5 (Fig. 1D). Importantly, FLT3-ITD depletion induced a time-dependent decline in cell viability, as determined by Annexin V staining (Fig. 1E; Supplementary Fig. S1B), immunoblot analysis of PARP/caspase-3 cleavage (Fig. 1F), and reduction in dsRed expression (Supplementary Fig. S1C). To determine if loss of FLT3-ITD expression impairs *in vivo* disease progression, secondary NSG recipient mice (with no prior irradiation) were injected with MLL-AF9/iFLT3-ITD cells and placed on doxycycline water and chow for three days prior to removal (Supplementary Fig. S1D). Doxycycline withdrawal (and thus depletion of FLT3-ITD) dramatically reduced tumor burden (Fig. 1G) and enhanced overall survival (Fig. 1H). Depletion of FLT3-ITD following removal of doxycycline from leukemia-bearing mice was confirmed in bone marrow, spleen, and peripheral blood using dsRed as a marker of transgene expression (Sup-

plementary Fig. S1E). Collectively, these data demonstrate that MLL-AF9 and FLT3-ITD cooperate to drive onset and progression of AML, and genetic depletion of FLT3-ITD alone is sufficient to induce apoptosis of MLL-AF9/iFLT3-ITD cells and extend overall survival in mice transplanted with these leukemias.

Transcriptomics and Metabolomics Analysis Reveal That *De Novo* Serine Biosynthesis and Serine Uptake Are Regulated by FLT3-ITD

To elucidate the pathways involved in FLT3-ITD signaling in AML, we performed 3' RNA sequencing (RNA-seq) comparing MLL-AF9/iFLT3-ITD-OFF cells cultured for 24 or 48 hours in the absence of doxycycline with MLL-AF9/iFLT3-ITD-ON cells maintained in doxycycline-supplemented medium (Fig. 2A). We identified a total of 1,009 differentially expressed genes (DEG), of which 575 were downregulated and 434 upregulated ($P < 0.05$ and $-1 > \log_{2}FC > 1$) 24 hours post FLT3-ITD depletion (Supplementary Table S1). We further identified a total of 1,972 DEGs (1,057 downregulated, 915 upregulated, $P < 0.05$ and $-1 > \log_{2}FC > 1$) 48 hours post FLT3-ITD depletion (Supplementary Fig. S2A; Supplementary Table S2). Interestingly, among the most suppressed genes 24 hours post FLT3-ITD depletion were genes encoding components of the *de novo* serine biosynthesis pathway (*Phgdh* and *Psat1*), neutral amino acid transporters involved in serine and glycine uptake (*Slc1a4*, *Slc1a5*, and *Slc6a9*), the mitochondrial serine transporter (*Sfxn1*), and one-carbon metabolism (*Mthfd2*, *Shmt1*, and *Shmt2*; Fig. 2B). Indeed, terms that defined gene sets involved in serine biosynthesis, one-carbon metabolism, and nucleotide metabolism were significantly suppressed following depletion of FLT3-ITD in MLL-AF9/iFLT3-ITD cells (Fig. 2C), and gene set enrichment analysis (GSEA) further showed marked suppression of the KEGG pathway gene sets "glycine, serine, and threonine metabolism," and "one-carbon pool by folate," consistent with Gene Ontology (GO) term analysis and individual gene changes observed (Fig. 2D). Examination of gene-expression changes 48 hours post FLT3-ITD depletion generally showed exacerbation of loss of mRNA in all of the genes examined (Supplementary Fig. S2B).

To confirm that the transcriptional response to FLT3-ITD depletion was not secondary to changes in cell viability, FLT3-ITD was depleted for 24 hours in MLL-AF9/iFLT3-ITD cells cultured in the presence or absence of the pan-caspase inhibitor QVD-OPH. QVD-OPH treatment reduced apoptosis (Supplementary Fig. S2C) but did not affect changes in *Phgdh*, *Psat1*, *Slc1a4*, and *Slc1a5* expression induced upon FLT3-ITD depletion, confirming repression of these genes to not be a consequence of apoptosis that occurs following depletion of FLT3-ITD (Supplementary Fig. S2D). Consistent with our RNA-seq data (Fig. 2E), immunoblot analysis confirmed loss of *Phgdh* and *Psat1* expression 24 hours following FLT3-ITD depletion (Fig. 2F). It has been acknowledged that the use of tetracyclines, including doxycycline, can affect mitochondrial translation and cellular metabolism (29, 30). To discount the possibility that differential gene expression was a confounding result of doxycycline withdrawal, we performed RNA-seq in our previously published Tet-inducible MLL-AF9/NRAS^{G12D} AML leukemias

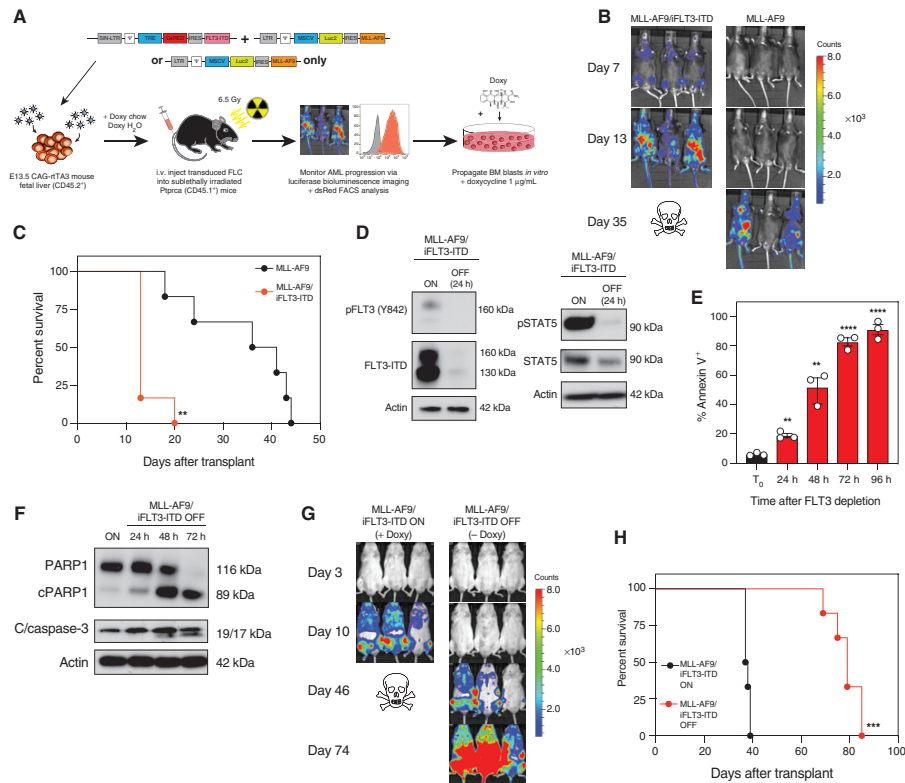


Figure 1. A genetically engineered mouse model of MLL-rearranged, FLT3-ITD AML reveals FLT3-ITD is essential for leukemia survival. **A**, Schematic depicting generation of a Tet-inducible iFLT3-ITD mouse model. E13.5 murine fetal liver cells endogenously expressing the CAG-rTA3 transgene were retrovirally transduced with Tet-DsRED-IRES-FLT3-ITD, MSCV-Luc2-IRES-MLL-AF9, or both vectors, and injected into sublethally irradiated Ptpcr^{fl} recipients. Leukemia development was monitored via luciferase bioluminescence imaging and FACS analysis for dsRed, and at endpoint, bone marrow (BM) was harvested and blasts were cultured in vitro in 1 μ g/mL doxycycline (Dox). **B**, Primary transplant recipients were injected with 50 mg/kg D-luciferin and imaged in an IVIS bioluminescence imager to assess disease burden. **C**, Kaplan-Meier survival analysis of MLL-AF9/iFLT3-ITD or MLL-AF9 alone primary recipients. **, $P < 0.01$ as determined by Mantel-Cox test, with $n = 6$ mice per condition. **D**, MLL-AF9/iFLT3-ITD cells were cultured in vitro with 1 μ g/mL doxycycline and then washed twice in PBS with subsequent culture in doxycycline-free medium to deplete FLT3-ITD transgenic expression (MLL-AF9/iFLT3-ITD-OFF). Phosphorylated and total FLT3 and STAT5 expression was probed 24 hours post FLT3-ITD depletion via Western blot. β -actin served as the loading control. **E**, FLT3-ITD was depleted in MLL-AF9/iFLT3-ITD cells as described above over a period of 96 hours, and percentage of apoptotic cells was assessed every 24 hours via Annexin V staining and flow cytometry. **, $P < 0.01$; ****, $P < 0.0001$ by Student's t test relative to T_0 . Error bars, $\pm$ SD and three independent biological replicates. **F**, Apoptotic markers for cleaved/total PARP1 and cleaved/total caspase-3 were assessed over 72 hours post FLT3-ITD depletion via Western blot in MLL-AF9/iFLT3-ITD cells. β -actin served as the loading control. **G**, 1×10^6 MLL-AF9/iFLT3-ITD cells were intravenously injected into secondary NSG recipients, and mice were placed on doxycycline-containing water and chow. After 72 hours, one arm of the study was placed on normal water and chow (MLL-AF9/iFLT3-ITD-OFF, -Dox) and disease burden was assessed via bioluminescence imaging as described above. **H**, Kaplan-Meier survival analysis of secondary recipients from **G**. ***, $P < 0.001$ determined by Mantel-Cox test, with $n = 6$ mice per condition.

(31). Analysis of RNA-seq data at 24 hours post MLL-AF9 depletion revealed minimal overlap with our FLT3-ITD depletion data. Importantly, many of the genes involved in serine biosynthesis and one-carbon metabolism that respond to FLT3-ITD depletion were not differentially expressed in this system (Supplementary Fig. S2E; Supplementary Table

S3). A complementary leukemia model, wherein doxycycline withdrawal results in depletion of MLL-AF9 (referred to as iMLL-AF9/FLT3-ITD), revealed that loss of MLL-AF9 did not affect the expression of *Phgdh*, *Pstat1*, *Slc14a4*, or *Slc1a5* (Supplementary Fig. S2F). These data provide important insights into the divergent molecular pathways regulated

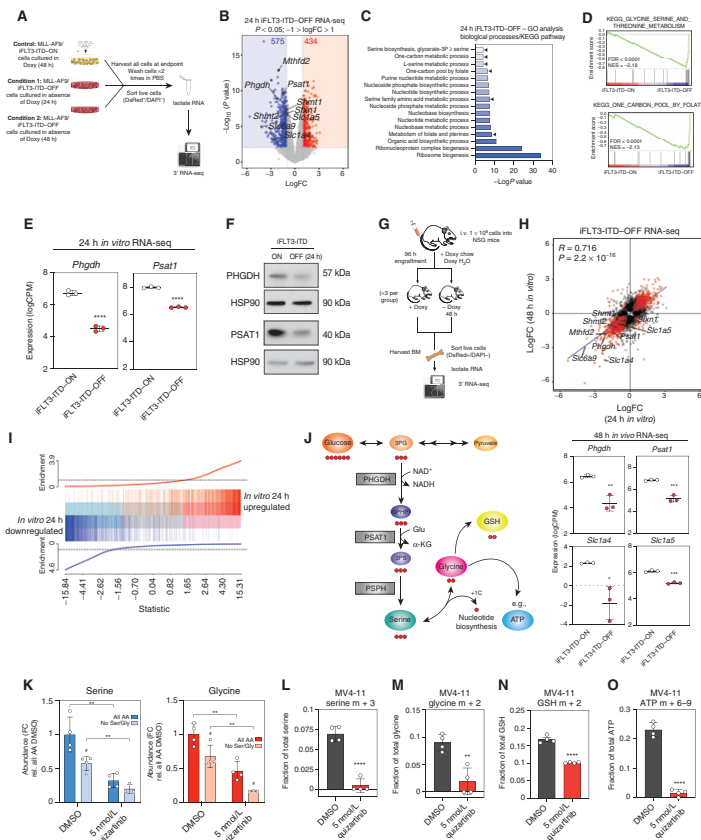


Figure 2. Transcriptomics and metabolomics analysis reveal *de novo* serine biosynthesis and serine uptake are regulated by FLT3-ITD. **A**, Schematic of RNA-seq analysis of MLL-AF9/FLT3-ITD cells post FLT3-ITD depletion. **B**, Volcano plot of DEGs 24 hours post FLT3-ITD depletion, highlighting genes involved in *de novo* serine biosynthesis, serine and glycine uptake, and one-carbon/folate metabolism. Suppressed DEGs (575) are denoted by blue shading and enriched genes (434) by red shading. Significance was defined as $P < 0.05$ and $-1 > \log_{2}FC > 1$. **C**, KEGG pathway and biological process GO term analysis of DEGs 24 hours post FLT3-ITD depletion. **D**, Enrichment score plots from GSEA using the KEGG signature for glycine, serine, and threonine metabolism and one-carbon pool by folate. FDR, false discovery rate; NES, normalized enrichment score. **E**, Dot plots of normalized log counts-per-million (CPM) gene expression values 24 hours post FLT3-ITD depletion for *Phgdh* and *Psat1*. Error bars, $\pm$ SD, and each condition was composed of three independent biological replicates. **F**, Immunoblot analysis of PHGDH and PSAT1 protein expression 24 hours after FLT3-ITD depletion. HSP90 served as a loading control. **G**, Schematic depicting in vivo depletion of FLT3-ITD in transplanted MLL-AF9/FLT3-ITD cells. **H**, Correlation plot of DEGs post FLT3-ITD depletion at 24 hours (in vitro) and 48 hours (in vivo), highlighting genes relevant to *de novo* serine biosynthesis, serine uptake, and one-carbon/folate metabolism. Pearson correlation coefficient $R = 0.716$, $P = 2.2 \times 10^{-18}$. Genes highlighted in red are representative of an FDR < 0.05 . **I**, Barcode plot demonstrating enrichment of DEGs following 48-hour FLT3-ITD depletion in vivo, and the positive association with either significantly increased (red; FDR < 0.05 , $\log_{2}FC > 0.5$) or significantly decreased (blue; FDR < 0.05 , $\log_{2}FC < -0.5$) genes 24 hours post FLT3-ITD depletion in vitro. **J**, Schematic representation of the glucose-derived *de novo* serine biosynthesis pathway. ^{13}C -labeled carbon atoms are denoted in red for each intermediate. **K**, Fold changes in serine and glycine abundance, as measured by LC/MS-MS, in DMSO-treated MV4-11 cells versus MV4-11 cells treated with 5 nmol/L quazantinib for 24 hours in complete RPMI, or serine/glycine-depleted RPMI. Asterisks (*) are indicative of statistical comparisons between DMSO and treatment conditions as indicated. Hashes (#) are indicative of statistical comparisons within the same treatment condition, i.e., complete medium versus serine/glycine-depleted medium for each condition. #, $P < 0.05$; **, $P < 0.01$ as determined by Student t test as indicated. **L**, Fractional abundance of ^{13}C -glucose-derived: **L**, m + 3 serine; **M**, m + 2 glycine; **N**, m + 2 glutathione (GSH); and **O**, m + (6–9)-labeled ATP in MV4-11 cells treated with either DMSO or 5 nmol/L quazantinib for 24 hours. Error bars, $\pm$ SD of three to four independent biological replicates. **, $P < 0.01$; ****, $P < 0.0001$ by Student t test relative to DMSO.

by FLT3-ITD and MLL-AF9. Our data indicate a role for FLT3-ITD in the transcriptional regulation of important metabolic processes, including serine and one-carbon/folate metabolism, whereas MLL-AF9 transcriptionally regulates a well-characterized network of genes involved in cellular differentiation (32). These observations are consistent with the divergent biological consequences of FLT3-ITD and MLL-AF9 depletion, namely, induction of apoptosis (Fig. 1E) and myeloid differentiation, respectively (33).

To determine if our *in vitro* results were conserved *in vivo*, mice transplanted with MLL-AF9/iFLT3-ITD leukemias were divided into two cohorts, one with and one without doxycycline supplementation for 48 hours (Fig. 2G). Leukemias were subsequently harvested from bone marrow for 3'-RNA-seq. Global transcriptome analysis showed a high degree of correlation between DEGs derived from 24-hour *in vitro* and 48-hour *in vivo* FLT3-ITD depletion (Fig. 2H; Supplementary Table S4), with strong overlap in the direction and magnitude of DEGs observed (Fig. 2I). Finally, to determine if acute FLT3-ITD depletion in human AML cell lines phenocopies genetic FLT3-ITD depletion in our murine model, we treated MV4-11 cells (which harbor FLT3-ITD and an MLL translocation) with quizartinib, a potent FLT3 inhibitor (34, 35), for 24 hours and performed 3' RNA-seq. Strikingly, *PHGDH*, *PSAT1*, *SLC1A4*, and *SLC1A5* were among the most highly suppressed genes upon FLT3-ITD inhibition in this human leukemia cell line (Supplementary Fig. S2G; Supplementary Table S5).

Although cellular serine requirements are largely fulfilled via uptake of dietary serine, cells retain the capacity to generate serine endogenously via the redirection of glycolytic intermediates into the *de novo* serine biosynthesis pathway (36). PHGDH, the first and rate-limiting enzyme of the *de novo* serine biosynthesis pathway, catalyzes oxidation of the glycolytic intermediate 3-phosphoglycerate (3-PG) to 3-phosphohydroxypyruvate (3-PHP) in a NAD⁺-dependent manner (Fig. 2J). 3-PHP is transaminated to 3-phosphoserine (3-PS) by PSAT1 in a reaction that utilizes glutamate-derived nitrogen and produces α -ketoglutarate (α -KG). Finally, 3-PS undergoes phosphate ester hydrolysis by PSPH to yield serine. Serine is essential for the biosynthesis of proteins and other metabolites that sustain cellular proliferation, including nucleotides (23). Consistent with the observed changes in the expression of genes involved in serine/glycine uptake and *de novo* serine biosynthesis, a dramatic reduction in intracellular serine and glycine abundance was observed when MV4-11 cells were treated with quizartinib (Fig. 2K). To specifically demonstrate that *de novo* serine biosynthesis was suppressed upon FLT3-ITD inhibition, we examined the fate of glucose-derived serine in MV4-11 cells and FLT3 wild-type (FLT3-WT) OCI-AML3 cells treated with quizartinib for 24 hours. FLT3-ITD inhibition dramatically reduced the incorporation of U-¹³C-glucose into serine (Fig. 2L) and glycine (Fig. 2M) in MV4-11 cells. This subsequently reduced ¹³C incorporation, via serine, into the antioxidant glutathione (GSH; Fig. 2N) and ATP (Fig. 2O). Importantly, quizartinib treatment of FLT3-WT OCI-AML3 cells did not alter ¹³C incorporation into any of these metabolites (Supplementary Fig. S2H–S2K). Collectively, these data suggest

that FLT3-ITD regulates serine uptake, *de novo* serine biosynthesis, and one-carbon metabolism.

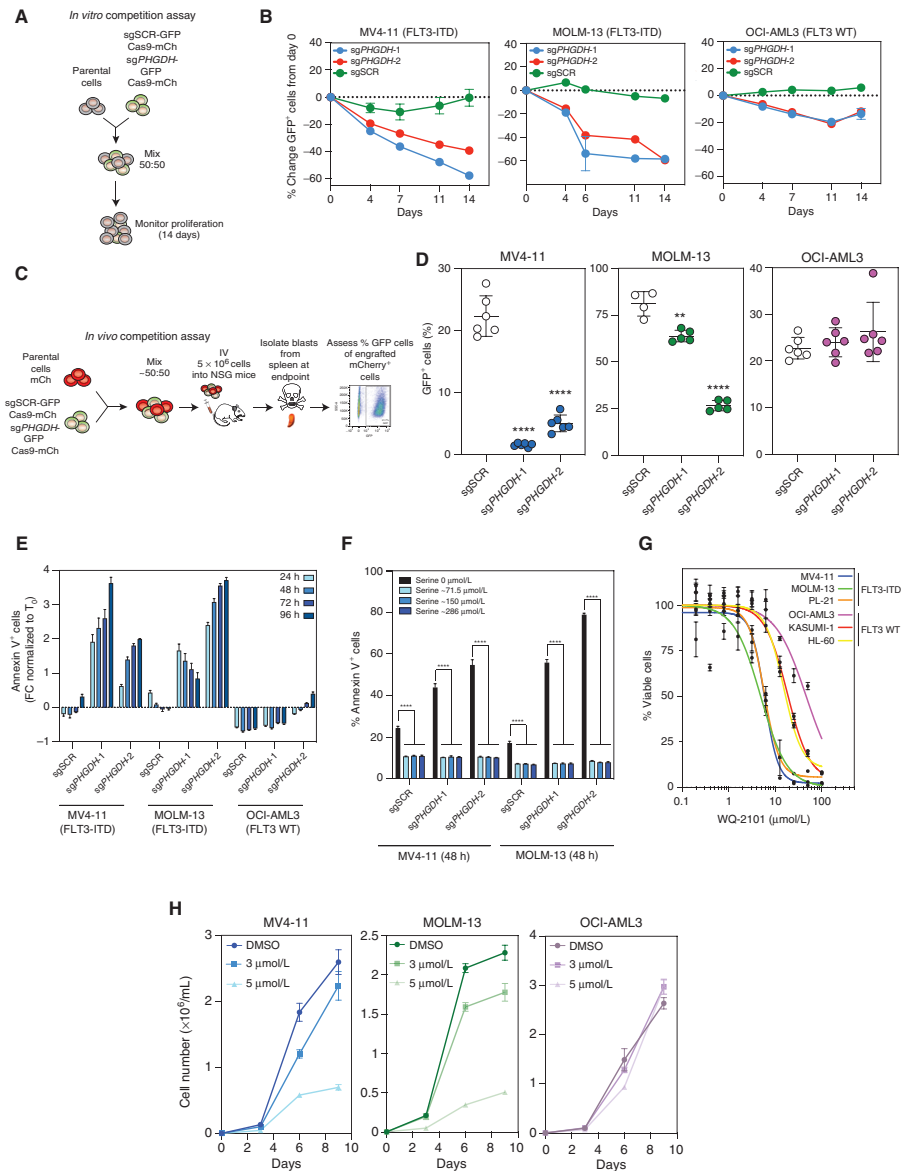
Serine Biosynthesis Is a Metabolic Vulnerability in FLT3-ITD-Driven AML

To determine the functional role of serine metabolism in FLT3-ITD-driven human AML, we generated MV4-11, MOLM-13 (MLL-rearranged, FLT3-ITD expressing), and OCI-AML3 AML cell lines in which PHGDH was deleted using CRISPR/Cas9 gene editing (Supplementary Fig. S3A). A competition assay in which PHGDH-deleted cells were cocultured with WT cells and proliferation was monitored over 14 days was then performed (Fig. 3A). Deletion of PHGDH in MV4-11 and MOLM-13 cells, using two independent single-guide RNAs (sgRNA), resulted in a loss of representation of these cells over time *in vitro*. In contrast, deletion of PHGDH in FLT3-WT OCI-AML3 cells had minimal effect (Fig. 3B). To address the broader implications of these findings, we performed an *in vivo* competition assay, in which a fixed 50:50 ratio of parental Cas9-mCherry cells was cotransplanted with cells expressing Cas9-mCherry and GFP-tagged sgRNAs targeting PHGDH into NSG recipients. The percentage of splenic GFP-positive cells was subsequently assessed at the ethical endpoint (Fig. 3C; Supplementary Fig. S3B). Deletion of PHGDH in MV4-11 and MOLM-13 cells resulted in marked loss of representation *in vivo* relative to nontargeting scramble controls (Fig. 3D). Importantly, PHGDH depletion in FLT3-WT OCI-AML3 cells did not affect representation relative to nontargeting controls (Fig. 3D). Collectively, these data provide strong evidence that inactivation of the *de novo* serine biosynthesis pathway *in vitro* and *in vivo* is selectively detrimental to the maintenance of FLT3-ITD-mutant but not FLT3-WT AMLs, even in contexts where exogenous serine/glycine is available.

Given our results showing that transporters involved in serine and glycine uptake are regulated by FLT3-ITD (Fig. 2B), we next sought to determine the contribution of exogenous serine/glycine to AML maintenance. Withdrawal of exogenous serine and glycine from RPMI medium suppressed the proliferation of leukemia cells expressing WT FLT3 or FLT3-ITD with minimal effects on cell viability (Supplementary Fig. S3C). However, serine/glycine withdrawal triggered apoptosis in PHGDH-depleted FLT3-ITD mutant cell lines (MV4-11 and MOLM-13; Fig. 3E; Supplementary Fig. S3D). Although PHGDH deletion exacerbated the antiproliferative impact of serine/glycine withdrawal in FLT3-WT cells (OCI-AML3), the apoptotic effect in these cells was significantly less than that observed in MV4-11 and MOLM-13 cells (Supplementary Fig. S3E). Previous studies have shown that serine, but not glycine, supports one-carbon metabolism and proliferation of cancer cells (37). Therefore, a rescue experiment was performed to determine if serine alone could reverse the loss of viability observed in MV4-11 and MOLM-13 cells following PHGDH depletion and serine/glycine withdrawal. Serine supplementation at three concentrations (~286 μ M/L, the concentration of serine in RPMI culture medium; ~150 μ M/L, the concentration of serine found in human serum; ref. 38; and 71.5 μ M/L, to mimic a low serine state) was sufficient to reverse the apoptotic phenotype (Fig. 3F), suggesting serine is essential for the survival of FLT3-ITD mutant AMLs.

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Collectively, these experiments illustrate that, while exogenous serine is uniformly required for the maximal proliferation of AML cells, depletion of *de novo* and exogenous serine is exquisitely lethal to FLT3-ITD mutant AMLs.

To determine the effect of pharmacologic inhibition of PHGDH in the context of AML, an allosteric small-molecule inhibitor, WQ-2101 (39), was applied across a panel of FLT3-ITD mutant and FLT3 WT human AML cell lines. Potent induction of apoptosis was observed in cell lines harboring FLT3-ITD mutations (MV4-11, MOLM-13, and PL-21) at low micromolar ranges of WQ-2101, compared with FLT3 WT cell lines (OCI-AML3, Kasumi-1, HL-60; Fig. 3G). WQ-2101 did not induce apoptosis in PHGDH-depleted MOLM-13 cells (Supplementary Fig. S3F), thereby confirming the on-target specificity of the compound. Additionally, a profound antiproliferative effect was observed in FLT3-ITD mutant cells exposed to sublethal concentrations of WQ-2101 (Fig. 3H; Supplementary Fig. S3G). Collectively, these data provide genetic and pharmacologic evidence that disruption of the *de novo* serine biosynthesis pathway is detrimental to the maintenance of FLT3-ITD-mutant AMLs *in vitro* and *in vivo*.

An FLT3-ITD/mTORC1/ATF4 Axis Transcriptionally Modulates Serine Biosynthesis and Serine Transporters in FLT3-ITD-Driven AML

We next sought to identify the molecular pathways downstream of FLT3-ITD that regulate expression of genes involved in the *de novo* serine biosynthesis pathway and amino acid transporters involved in serine/glycine uptake. Differentially repressed genes from quizartinib-treated MV4-11 cells, from the RNA-seq data set described in Fig. 2, were compared with differentially repressed genes from MLL-AF9/iFLT3-ITD cells wherein iFLT3-ITD had been depleted for 24 hours (Fig. 4A). A total of 84 genes overlapped in both data sets and included *PHGDH*, *PSAT1*, *SLCIA4*, *SLCIA5*, *SLC6A9*, *SHMT2*, and *MTHFD2* (Supplementary Table S6). Consistent with the observation that genetic depletion of FLT3-ITD from MLL-AF9/iFLT3-ITD cells resulted in reduced expression of PHGDH (Fig. 2E), a similar reduction in PHGDH protein expression was observed following quizartinib treatment of MV4-11 cells (Supplementary Fig. S4A). To identify potential transcription factors mediating FLT3-ITD-dependent gene expression, the i-cisTarget motif analysis platform was used to interrogate the 84 overlapping genes (40). Strikingly, an ATF4 binding motif was identified as the most highly enriched

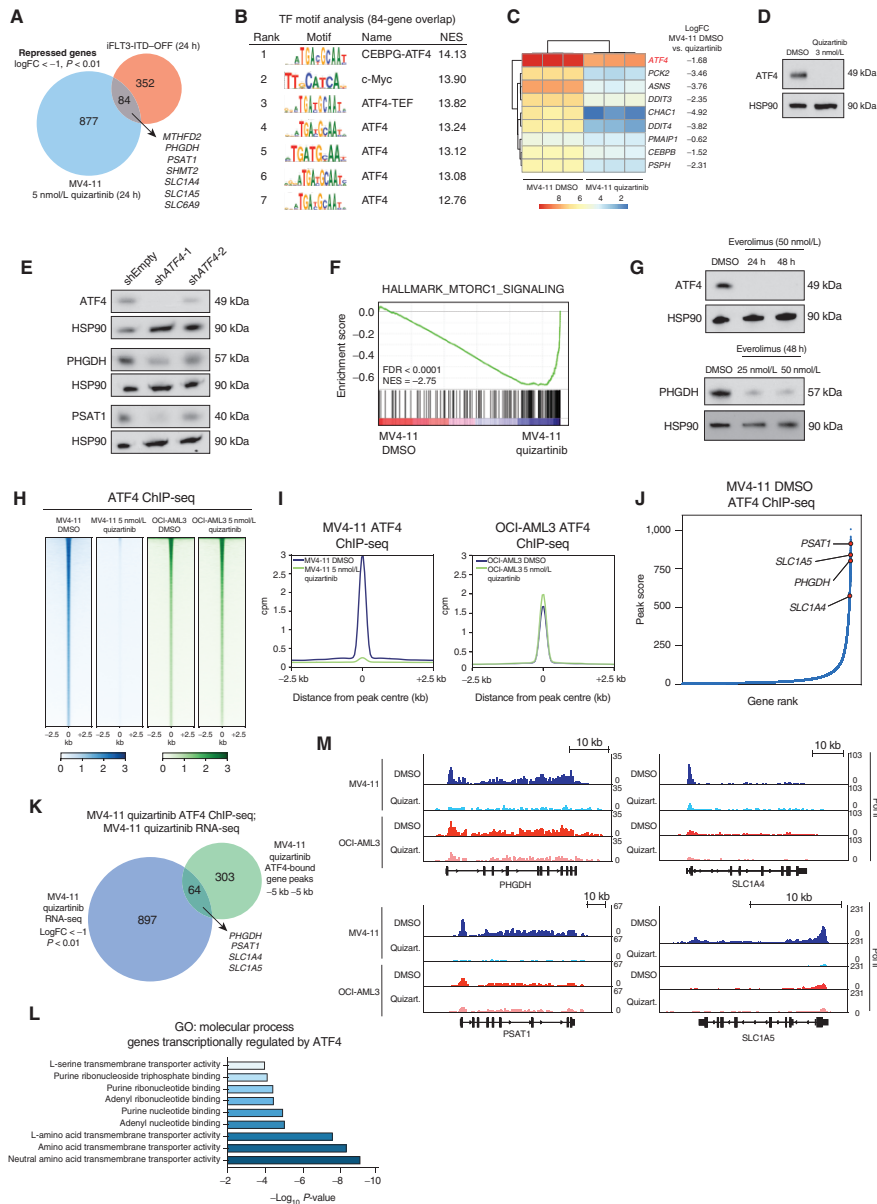
(Fig. 4B). Indeed, ATF4 and several canonical ATF4 target genes (*ASNS*, *DDIT3*, and *CHAC1*) were transcriptionally repressed following pharmacologic inhibition of FLT3-ITD in MV4-11 cells (Fig. 4C), as well as in MLL-AF9/iFLT3-ITD cells 24 hours post FLT3-ITD depletion (Supplementary Fig. S4B) with complete loss of ATF4 expression observed at the protein level (Fig. 4D). ATF4 is a stress-induced master transcriptional regulator of amino acid metabolism and has previously been linked with regulation of serine metabolism (41). Importantly, ATF4 knockdown in MV4-11 cells resulted in a reduction in PHGDH and PSAT1 expression, indicating ATF4 regulates the *de novo* serine biosynthesis pathway in FLT3-ITD mutant AML (Fig. 4E). GSEA of the transcriptional data set from MV4-11 cells treated with quizartinib revealed mTORC1 signaling to be among the most suppressed gene sets (Fig. 4F), a finding that was recapitulated in MLL-AF9/iFLT3-ITD cells 24 hours post FLT3-ITD depletion (Supplementary Fig. S4C). The link between FLT3-ITD and mTORC1 has been previously studied (42), and mTORC1 has been shown to regulate both the stability of ATF4 mRNA transcripts and ATF4 translation through three upstream open reading frames (uORF) in the 5' UTR of ATF4 (43). Consistent with these observations, genetic or pharmacologic inhibition of FLT3-ITD reduced phosphorylation of ribosomal protein S6 (RPS6), a canonical downstream target of mTORC1 (Supplementary Fig. S4D). Moreover, treatment of MV4-11 cells with the mTORC1-specific inhibitor everolimus reduced ATF4 and PHGDH expression (Fig. 4G).

To determine how ATF4 modulates *de novo* serine biosynthesis and serine uptake, chromatin immunoprecipitation sequencing (ChIP-seq) was performed in MV4-11 and OCI-AML3 cells using an anti-ATF4 antibody. A 24-hour treatment of MV4-11 cells with quizartinib resulted in global loss of ATF4 occupancy across the genome (Fig. 4H and I), whereas ATF4 occupancy was not substantially altered in quizartinib-treated FLT3-WT OCI-AML3 cells (Fig. 4H and I). Peak calling analysis of ATF4 ChIP-seq data and subsequent annotation of peaks to genes in DMSO-treated MV4-11 cells identified 11,869 unique genes (5 kb either side of TSS), while in quizartinib-treated MV4-11 cells this was reduced to 367 genes. Both conditions maintained enrichment for the ATF4 binding motif (Supplementary Fig. S4E). Ranking of all genes according to their ATF4 occupancy in DMSO-treated MV4-11 cells revealed that ATF4 binding was enriched at key components of the *de novo* serine biosynthesis pathway (*PHGDH* and

Figure 3. Serine biosynthesis is a metabolic vulnerability in FLT3-ITD-driven AML. **A**, Schematic depicting the *in vitro* competition assay protocol. **B**, Competitive proliferation assays in scramble control (sgSCR) or PHGDH knockout (sgPHGDH) MV4-11, MOLM-13, and OCI-AML3 cells. Error bars, $\pm$ SD and three independent biological replicates. **C**, Schematic depicting the *in vivo* competition assay protocol. **D**, Percentage GFP-positive sgPHGDH MV4-11, MOLM-13, and OCI-AML3 cells in spleen at endpoint following an *in vivo* competitive proliferation assay. Error bars, $\pm$ SD and $n = 4$ –6 mice per group. **, $P < 0.01$; ****, $P < 0.0001$ by Student *t* test relative to sgSCR. **E**, MV4-11, MOLM-13, and OCI-AML3 cells transduced with either of two independent sgRNAs against PHGDH (sgPHGDH-1 or -2) or scramble DNA (sgSCR) were cultured in serine/glycine-deprived media for 96 hours, and cell viability was determined via Annexin V flow cytometry every 24 hours. Data presented as fold change relative to time point zero (T_0); error bars, $\pm$ SD and three independent biological replicates. **F**, PHGDH-depleted MV4-11 and MOLM-13 cells were cultured in serine/glycine-deprived media for 48 hours. In each condition, three separate biologically relevant concentrations of serine (~ 286 μ M/L, serine concentration in commercial RPMI; ~ 150 μ M/L, serine concentration in peripheral blood; and ~ 71.5 μ M/L serine, representing a serine-insufficient state) were supplemented into culture medium at the time of seeding. Cell viability was assessed at 48 hours via Annexin V flow cytometry. ****, $P < 0.0001$ by multiple Student *t* test relative to serine 0 μ M/L for each condition. Error bars, $\pm$ SD and three independent biological replicates. **G**, Escalating doses of a small-molecule inhibitor of PHGDH, WQ-2101, were screened against a panel of FLT3-ITD-mutant (MV4-11, MOLM-13, and PL-21) and FLT3 WT (OCI-AML3, Kasumi-1, HL-60), but otherwise genetically diverse, AML cell lines in complete RPMI. Viability was determined via CellTiter-Glo. **H**, MV4-11, MOLM-13, and OCI-AML3 cells were treated with DMSO or two low concentrations of WQ-2101 (3 or 5 μ M/L) and cell number assessed over a period of 9 days (time points at days 3, 6, and 9). All error bars, $\pm$ SD, and three independent biological replicates.

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PSATI) and serine transporters (*SLC1A4* and *SLC1A5*), suggesting these genes are particularly strongly bound by ATF4 (Fig. 4J). The ATF4 peaks for *PHGDH*, *PSATI*, *SLC1A4*, and *SLC1A5* were almost completely eliminated in quizartinib-treated MV4-11 cells (Supplementary Fig. S4F). Quizartinib treatment had negligible effects on ATF4 expression in OCI-AML3 cells relative to MV4-11 cells (Supplementary Fig. S4G).

To determine target genes directly transcriptionally regulated by ATF4, the RNA-seq data from Fig. 4A were integrated with the ChIP-seq data from Fig. 4H and I. This analysis yielded 64 genes that were most highly enriched for ATF4 binding and were significantly repressed following treatment of MV4-11 cells with quizartinib (Fig. 4K; Supplementary Table S7). GO term analysis revealed a recurrent amino acid transmembrane transporter activity signature that included the L-serine pathway, as well as a purine nucleotide binding signature (Fig. 4L). To confirm that FLT3-ITD functionally promotes transcriptional activity at genes involved in serine biosynthesis and serine uptake, ChIP-seq using an anti-RNA polymerase II (Pol II) antibody was performed in MV4-11 and OCI-AML3 cells cultured in the presence or absence of quizartinib for 24 hours. In all instances, MV4-11 cells demonstrated greater Pol II occupancy in transcribed regions of *PHGDH*, *PSATI*, *SLC1A4*, and *SLC1A5* genes than corresponding regions in OCI-AML3 cells (Fig. 4M). Strikingly, quizartinib treatment almost completely ablated Pol II occupancy at these regions in MV4-11 cells, suggesting FLT3-ITD inhibition effectively silences active transcription of these genes. Importantly, quizartinib treatment did not induce global reduction in Pol II occupancy (Supplementary Fig. S4H). Finally, to directly link ATF4 to transcriptional regulation of *PHGDH*, *PSATI*, *SLC1A4*, and *SLC1A5*, we performed RT-qPCR analysis on ATF4-depleted MV4-11 cells and confirmed reduced mRNA expression (Supplementary Fig. S4I). Collectively, these data suggest that FLT3-ITD regulates *de novo* serine biosynthesis and serine uptake in an ATF4-dependent manner.

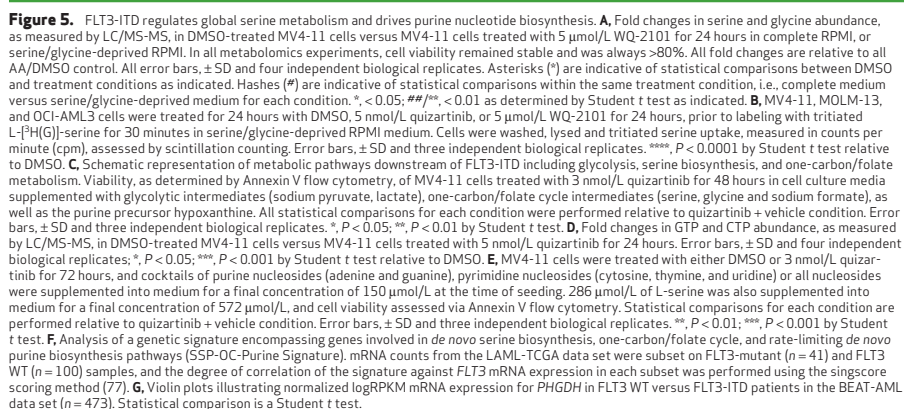
FLT3-ITD Regulates Global Serine Metabolism and Drives Purine Nucleotide Biosynthesis

We next sought to investigate the downstream metabolic consequences of both FLT3-ITD and PHGDH inhibition. Although quizartinib treatment of MV4-11 cells greatly

reduced intracellular serine and glycine pools (Fig. 2K), inhibition of *de novo* serine biosynthesis with WQ-2101 had little impact on global serine/glycine abundance (Fig. 5A). Critically, however, serine/glycine levels in cells exposed to WQ-2101 and deprived of exogenous serine/glycine largely phenocopied the results seen following treatment of MV4-11 cells with quizartinib, providing further evidence that FLT3-ITD inhibition suppresses both *de novo* serine biosynthesis and serine uptake (Fig. 5A). Importantly, the abundance of other nonessential (Supplementary Fig. S5A) and essential (Supplementary Fig. S5B) amino acids remained largely unaltered, demonstrating that pharmacologic inhibition of FLT3-ITD or PHGDH did not induce widespread changes in amino acid abundance. Quizartinib treatment induced changes in metabolites associated with glycolysis, namely, increased glucose-6-phosphate and fructose-6-phosphate, coupled with decreased fructose-1,6-bisphosphate (Supplementary Fig. S5C), suggesting a block in glycolytic metabolism. Although this phenomenon is not necessarily related to specific effects on serine metabolism, it is indicative of disruption of central carbon metabolism as previously described (18, 19). Furthermore, FLT3-ITD inhibition with quizartinib or inhibition of PHGDH with WQ-2101 resulted in dramatic alterations in the steady-state abundance of TCA cycle intermediates (Supplementary Fig. S5D). Of note, PHGDH inhibition significantly increased the abundance of citrate/isocitrate and succinate, consistent with previous reports (44). To functionally examine serine uptake dynamics in AML cells, MV4-11, MOLM-13, and OCI-AML3 cells were pretreated with quizartinib or WQ-2101 for 24 hours before the uptake of tritium-radiolabeled L-[³H(G)]-serine was measured. FLT3-ITD inhibition potently reduced serine uptake in FLT3-ITD-driven MV4-11 and MOLM-13 cells, whereas no change in serine uptake was observed in FLT3-WT OCI-AML3 cells (Fig. 5B).

To examine the relative importance of metabolic pathways perturbed upon FLT3-ITD inhibition on AML survival, a series of metabolite rescue experiments were performed. Supplementation with the glycolytic intermediates pyruvate and lactate had little impact on quizartinib-induced changes in MV4-11 cell viability (Fig. 5C). In contrast, supplementation with glycine or the one-carbon/folate intermediate formate

Figure 4. An FLT3-ITD/mTORC1/ATF4 axis transcriptionally modulates serine biosynthesis and serine transporters in FLT3-ITD-driven AML. **A**, Venn diagram of suppressed DEGs from MLL-AF9/FLT3-ITD-OFF cells at 24 hours and human MV4-11 suppressed DEGs in response to 5 nmol/L quizartinib for 24 hours. A logFC > -1, $P < 0.01$ statistical cutoff was applied. **B**, Transcription factors with enriched binding sites in genes differentially suppressed upon FLT3-ITD depletion in genetic murine and human pharmacologic cell contexts (84-gene overlap from **A**). Analysis is ranked using normalized enrichment score (NES) and was performed using the i-cisTarget motif analysis platform. **C**, Heat map of ATF4 target gene suppression post treatment of MV4-11 cells with 5 nmol/L quizartinib or DMSO for 24 hours. All gene-expression changes are denoted as logFC. **D**, Western blot depicting rapid loss of ATF4 in MV4-11 cells post 24 hours of treatment with low-dose (3 nmol/L) quizartinib. HSP90 acted as the loading control. **E**, Two independent shRNAs against ATF4 (denoted shATF4-1 and shATF4-2) and an empty vector control were lentivirally transduced into MV4-11 cells and induced in the presence of doxycycline for 72 hours prior to assessment of ATF4, PHGDH, and PSAT1 protein expression via Western blot. HSP90 served as the loading control. **F**, GSEA plots depicting suppressed mTORC1 signaling after treatment of MV4-11 cells with 5 nmol/L quizartinib for 24 hours, FDR < 0.0001, NES = -2.75. **G**, MV4-11 cells were treated with 50 nmol/L of the mTORC1 inhibitor everolimus for 24 and 48 hours, and ATF4 expression was assessed via Western blot. Cells were subsequently treated with 25 or 50 nmol/L of everolimus for 48 hours and PHGDH expression assessed via Western blot. HSP90 served as the loading control in both experiments. **H**, Occupancy heat maps of genome-wide ATF4 peaks in the presence and absence of 5 nmol/L quizartinib for 24 hours in MV4-11 and OCI-AML3 cells. **I**, Average profile of genome-wide ATF4 peaks in the presence and absence of 5 nmol/L quizartinib in MV4-11 and OCI-AML3 cells. **J**, Plot ranking genes by peak score as determined by MACS2 and HOMER software. Data depict MV4-11 DMSO-treated cells, and genes relevant to *de novo* serine biosynthesis and serine/glycine uptake are highlighted. **K**, Venn diagram depicting ATF4 transcriptionally regulated genes. MV4-11 cells were treated with 5 nmol/L quizartinib for 24 hours and suppressed genes from 3' RNA-seq (logFC > -1, $P < 0.01$) were overlapped with remaining ATF4-bound peaks (-5 to 5 kb) post quizartinib treatment. **L**, GO term analysis (Molecular Process) of ATF4 transcriptionally regulated genes from the Venn diagram union of **J**. **M**, ChIP-seq reads for RNA Pol II mapped to the PHGDH, PSAT1, SLC1A4, and SLC1A5 loci in MV4-11 and OCI-AML3 cells treated with 5 nmol/L quizartinib or DMSO for 24 hours.



conferred a small but significant protection against quizartinib treatment (Fig. 5C). Supplementation with serine or hypoxanthine, a purine nucleobase derivative that undergoes conversion into the purine nucleotide inosine monophosphate (IMP), was sufficient to significantly reduce the apoptotic effects of quizartinib treatment on MV4-11 cells (Fig. 5C). Indeed, the steady-state abundance of the purine nucleotide guanosine triphosphate (GTP) was significantly reduced upon quizartinib treatment, whereas, interestingly, the abundance of the pyrimidine nucleotide cytosine triphosphate (CTP) increased in response to quizartinib treatment (Fig. 5D). Given that nucleotide imbalance is genotoxic to proliferating cells (45, 46), we hypothesized that suppression of one-carbon/folate cycle activity and thus purine biosynthesis might contribute to the cytotoxicity-associated with FLT3-ITD inhibition. Supplementation of purine nucleosides, but not pyrimidine nucleosides or a cocktail of purine and pyrimidine nucleosides, significantly reversed the apoptotic effects of quizartinib treatment (Fig. 5E). Of note, increasing the exogenous supply of serine (from 286 to 572 $\mu\text{mol/L}$) largely phenocopied the effects of purine supplementation (Fig. 5E), further supporting the idea that serine contributes to purine biosynthesis in FLT3-ITD AML. Given that purine biosynthesis in particular has been shown to be heavily dependent on the mTORC1-ATF4 axis (47, 48), we examined the impact of the mTORC1-specific inhibitor everolimus on U- ^{13}C -glucose incorporation into purine nucleotide species. Everolimus reduced ^{13}C incorporation into ATP in MV4-11 cells, but not OCI-AML3 cells, supporting the notion that downstream of FLT3-ITD, mTORC1 directly contributes to purine biosynthesis (Supplementary Fig. SSE).

To determine if our findings could be recapitulated in primary human samples, a genetic signature encompassing genes involved in *de novo* serine biosynthesis, the one-carbon/folate cycle, and key rate-limiting enzymes of the *de novo* purine synthesis pathway was devised (ref. 47; see Supplementary Materials and Methods). As FLT3-mutant AMLs have been demonstrated to upregulate *FLT3* mRNA expression (49), stratification of The Cancer Genome Atlas (TCGA) LAML data set by FLT3 mutation status showed a greater, and significant, positive correlation between this gene signature and *FLT3* mRNA expression in FLT3-mutant patient samples (Fig. 5F). Furthermore, examination of the BEAT-AML data set of 473 FLT3-ITD versus FLT3 WT leukemias (4) verified *PHGDH* is more highly expressed in FLT3-ITD-mutant tumor cells (Fig. 5G). Collectively, these data are consistent with the notion that FLT3-ITD inhibition reduces global serine availability and induces nucleotide insufficiency by suppressing both *de novo* serine biosynthesis pathway activity and serine uptake.

Inhibition of *De Novo* Serine Biosynthesis Sensitizes FLT3-ITD-Driven AMLs to Cytarabine

Having demonstrated that PHGDH inhibition is a metabolic vulnerability in FLT3-ITD-mutant AML, strategies by which this could be exploited for the treatment of AML were explored. Cytarabine, the standard-of-care chemotherapeutic agent used as first-line therapy in patients with AML, is a deoxycytosine analogue that interferes with DNA synthesis by damaging DNA during the S-phase of replication. Given

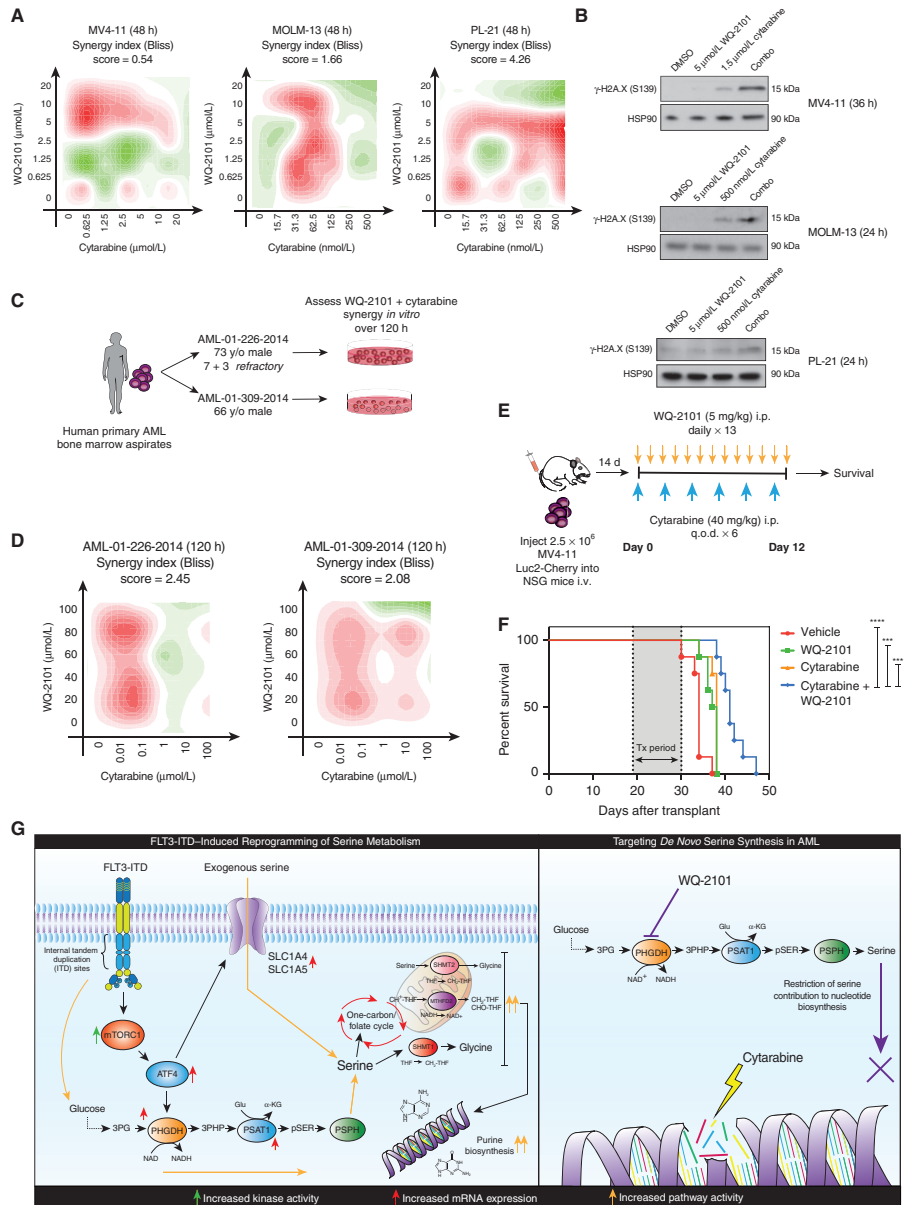
our findings demonstrating purine nucleotide insufficiency upon FLT3-ITD inhibition (Fig. 5D and E), we hypothesized that simultaneous treatment of FLT3-ITD-positive AMLs with cytarabine and a PHGDH inhibitor would exacerbate DNA damage by restricting serine, and thus one-carbon unit incorporation for purine nucleotide synthesis and subsequent DNA-repair processes.

Three FLT3-ITD-positive AML cell lines—MV4-11, MOLM-13, and PL-21—were used for these studies. Of note, although MV4-11 and MOLM-13 cells display an *MLL*-translocated karyotype, PL-21 cells display a complex non-*MLL* karyotype (50). Exposure of all three cell lines to increasing concentrations of WQ-2101 in combination with cytarabine revealed positive synergistic interactions (highlighted in red), as determined using the SynergyFinder computational package and the Bliss synergy index (ref. 51; Fig. 6A; Supplementary Fig. S6A). The combinatorial effect of cytarabine and WQ-2101 in MV4-11 cells was confirmed by Annexin V staining (Supplementary Fig. S6B). Importantly, the combination of cytarabine and WQ-2101 did not induce synergistic cell death in FLT3-WT cell lines (HL-60 and KASUMI-1) as determined by a negative Bliss synergy index (Supplementary Fig. S6C). Consistent with the hypothesis that PHGDH inhibition would limit the availability of purine nucleotide species for DNA repair, WQ-2101 exacerbated cytarabine-induced phosphorylation of the DNA damage marker histone variant H2A.X in all three FLT3-ITD-mutant AML cell lines (Fig. 6B). To determine if this combinatorial strategy has potential clinical implications, two FLT3-ITD-mutant primary AMLs obtained from patient bone marrow aspirates, termed AML-01-226-2014 and AML-01-309-2014, were sourced (Fig. 6C, clinical data available in Supplementary Fig. S6D). Of note, AML-01-226-2014 was refractory to standard-of-care 7+3 therapy with cytarabine and daunorubicin. Consistent with data shown using FLT3-ITD-mutant human AML cell lines, a combinatorial effect between cytarabine and WQ-2101 was evident in both primary AML samples (Fig. 6D; Supplementary Fig. S6E).

We next sought to determine the *in vivo* efficacy of combining cytarabine with WQ-2101. Initially, the effects of PHGDH inhibition on steady-state hematopoiesis in non-tumor-bearing PTPRCA mice treated with two doses of WQ-2101 (5 and 20 mg/kg; ref. 39) for 14 days was assessed. WQ-2101 had negligible effects on hematopoietic progenitor and more differentiated cells of the myeloid and lymphoid lineages in both the bone marrow and spleen of treated mice (Supplementary Fig. S6F). In addition, WQ-2101 treatment did not alter gross peripheral white and red blood cell counts or hemoglobin and platelet counts (Supplementary Fig. S6G). We subsequently transplanted MV4-11 cells into NSG recipient mice and treated them with single-agent cytarabine, WQ-2101, or a combination of both (Fig. 6E). Single-agent WQ-2101 or cytarabine provided limited survival advantage to leukemic mice relative to vehicle treatment, whereas the combinatorial arm displayed significant survival advantage relative to vehicle and both WQ-2101 and cytarabine single-agent arms (Fig. 6F). These *in vitro* data in human AML cell lines and primary patient specimens, in conjunction with evidence of *in vivo* activity in an aggressive disseminated model of AML, provide a strong rationale for targeting dysregulated serine

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biosynthesis in conjunction with standard-of-care chemotherapy in FLT3-ITD mutant AML (Fig. 6G).

DISCUSSION

The recent approval of the first-generation pan-RTK inhibitor midostaurin, as first-line therapy for patients harboring FLT3-ITD mutations, has resulted in significant interest in targeting mutant FLT3 in the context of AML (7). Although FLT3 inhibitor monotherapy initially yielded encouraging results, the development of acquired resistance has reduced the efficacy of this approach. Here, we reveal key insights into metabolic reprogramming events driven by FLT3 mutations and identify novel therapeutic strategies to treat this aggressive subtype of AML.

A key finding from this study is that FLT3-ITD modulates serine metabolism by upregulating mRNA expression of genes involved in serine synthesis (including PHGDH and PSATI) and serine uptake (including SLC1A4 and SLC1A5). Although the cellular demand for serine is often met by uptake of exogenous serine, genomic and non-genomic events can divert glucose-derived carbons to the *de novo* serine biosynthesis pathway. Indeed, genomic analyses in primary breast cancer, melanoma, and prostate cancers identified copy-number amplification in enzymes of the *de novo* serine biosynthesis pathway, most notably PHGDH, which encodes the first and only rate-limiting enzyme of this pathway (52–55). Importantly, transcriptional mechanisms that mediate activation of the *de novo* serine biosynthesis pathway have also been discovered. ATF4, a transcription factor that responds to cellular stressors such as amino acid starvation and hypoxia, and in this study was identified as central to FLT3-ITD-mediated regulation of the serine biosynthesis pathway, has been previously demonstrated to activate key enzymes of the *de novo* serine biosynthesis pathway in other disease contexts (41, 56). Other transcription factors such as MYC (57, 58) and NRF2 via ATF4 (59) have also been demonstrated to induce the *de novo* serine biosynthesis pathway. Interestingly, RAS pathway activation has been shown to enhance serine biosynthesis. KRAS activation and concurrent loss of the tumor suppressor gene LKB1 enhances proliferative capacity in pancreatic cancer models, primarily via stimulation of serine biosynthesis (60). A second study demonstrated lack of sensitivity to restriction of dietary serine restriction

in pancreatic and intestinal tumors, due to the inherent ability of KRAS mutations to activate *de novo* serine biosynthesis (61). Thus, the work herein raises the possibility that concurrent inhibition of PHGDH and dietary modulation of serine/glycine may prove to be particularly detrimental to the maintenance of FLT3-ITD-positive AML. Indeed, the modulation of amino acid pools via diet is an emerging area of interest in the field (62–65). Whether FLT3-ITD inhibition would prove synergistic with a serine- and glycine-deprived diet remains an open question, particularly as our data suggest loss of FLT3-ITD hampers both the *de novo* generation of serine and exogenous serine uptake.

The data from our study and others show that the mTORC1–ATF4 axis is pivotal to the induction of serine biosynthesis. The link between FLT3-ITD and mTORC1 has been previously defined (42), with mTORC1 modulating ATF4 via two distinct mechanisms: by regulating the stability of ATF4 mRNA transcripts and via regulation of ATF4 translation through three upstream uORFs in the 5′ UTR of ATF4 (43). In the context of FLT3-ITD mutations in AML, we provide a model whereby mTORC1 activates ATF4 to drive transcriptional regulation of both the *de novo* serine biosynthesis pathway and several key amino acid transporters associated with serine/glycine import into the cell. Previous studies have demonstrated that FLT3-ITD-driven AMLs are preferentially sensitive to mTOR/PI3K inhibitors (66–69), and it is possible that at least part of their efficacy can be attributed to suppression of the mTORC1/ATF4 axis and disruption of downstream metabolic pathways such as the *de novo* serine biosynthesis pathway.

There has been considerable interest in the development of small-molecule inhibitors to target the *de novo* serine biosynthesis pathway, and small-molecule PHGDH inhibitors have been investigated in the context of solid malignancies such as breast and prostate cancers (39, 70–73). Our analysis provides evidence that PHGDH inhibitors can be applied to exploit the dependency of FLT3-ITD-driven AML on sustained serine biosynthesis. Why tumors can exhibit sensitivity to PHGDH inhibition even when exogenous serine is available is poorly understood. One possible explanation is that *de novo*-generated serine may ensure availability of one-carbon units for nucleotide biosynthesis by reducing cytoplasmic SHMT1 activity (71). Inhibition of PHGDH, and subsequent activation of SHMT1, even in contexts in which exogenous serine

Figure 6. Inhibition of *de novo* serine biosynthesis sensitizes FLT3-ITD-driven AMLs to cytarabine. **A**, MV4-11, MOLM-13, and PL-21 cells were treated with escalating concentrations of WQ-2101 and cytarabine for 48 hours to determine the effect on viability, as determined by CellTiter-Glo. The presence of treatment synergy was determined using the SynergyFinder computational package and the Bliss synergy index and is denoted as regions of red in the graphs. The mean of three biological replicates was used to determine synergy for each data point. **B**, MV4-11 cells were treated with either DMSO, 5 μmol/L WQ-2101, 1.5 μmol/L cytarabine, or a combination of both, for 36 hours and DNA damage assessed via induction of γ-H2AX, as determined by Western blot. MOLM-13 and PL-21 cells were treated with either DMSO, 5 μmol/L WQ-2101, 500 nmol/L cytarabine, or a combination of both for 24 hours and DNA damage assessed as above. HSP90 served as the loading control in all experiments. **C**, Schematic representation of experiment testing combination therapy of WQ-2101 and cytarabine *in vitro* in primary patient AML bone marrow aspirates. **D**, Cells from two independent primary AMLs, AML-01-226-2014 and AML-01-309-2014, were treated with escalating concentrations of WQ-2101 and cytarabine for 120 hours to determine the effect on cell viability, as determined by Sytox Blue flow cytometry. The presence of treatment synergy was determined using the SynergyFinder computational package and the Bliss synergy index and is denoted as regions of red in the graphs. Background death of AML-01-226-2014 and AML-01-309-2014 upon thaw was 10.4% and 15.5%, respectively. Data are indicative of one experiment. **E**, Schematic diagram of *in vivo* combination therapy experimental protocol, using an aggressive human MV4-11 patient-derived xenograft (PDX) disseminated model in NSG mice. **F**, Kaplan–Meier survival analysis of the MV4-11-disseminated PDX model in NSG mice. Mice were treated with vehicle, 5 mg/kg WQ-2101 daily, 40 mg/kg cytarabine every second day, or a combination of both for 12 days. All statistical comparisons are combination therapy relative to all other treatment conditions as indicated. ****P* < 0.001; *****P* < 0.0001 as determined by the Mantel–Cox test, with *n* = 8 mice per condition. **G**, Schematic outline of this study. FLT3-ITD transcriptionally modulates *de novo* serine biosynthesis and serine uptake, as well as downstream one-carbon/folate cycle activity. This results in heightened purine biosynthesis. Pharmacologic inhibition of PHGDH sensitizes AML cells to standard-of-care cytarabine-induced DNA damage by exacerbating the DNA damage response.

is abundant, reduces the incorporation of one-carbon units into nucleotides which may result in the inability of exogenous serine to rescue the loss of PHGDH. Another study investigating WQ-2101 discovered that PHGDH inhibition affects nucleotide synthesis in a manner that is independent of serine utilization and instead involves defects in central carbon metabolism, the pentose-phosphate pathway, and TCA cycle (44). As we have demonstrated, FLT3-ITD inhibition triggers purine insufficiency. The *de novo* serine biosynthesis pathway donates carbon units to the folate cycle, which produces cytosolic one-carbon formyl units that are critical for purine ring assembly (47, 48). Inhibition of purine metabolism has long been explored as a therapeutic modality in leukemia (74), and more recently several groups have investigated the therapeutic implications of perturbing enzymes in the *de novo* purine synthesis pathway (such as PAICS) in the context of AML (75). We showed that inhibition of PHGDH sensitized FLT3-ITD AML to cytarabine, and posit that this is through enhanced DNA damage resulting from nucleotide insufficiency. Our biochemical studies demonstrating greatly enhanced phosphorylation of γ H2A.X signal following combined cytarabine and WQ-2101 treatment is consistent with this hypothesis. Moreover, a very recent study in the context of brain metastasis has shown that serine deprivation, resulting from PHGDH inhibition and the low serine/glycine state of cerebrospinal fluid, is in itself sufficient to reduce purine nucleotide pools and induce DNA damage (76).

A potential limitation of this study is the difficulty in performing systemic investigation of metabolic changes induced by FLT3-ITD or PHGDH inhibition *in vivo*. Although our competitive proliferation experiments provide important functional evidence that PHGDH is required for the maintenance of FLT3-ITD-driven AML blasts *in vivo*, the abundance of metabolites in the bone marrow and spleen (two physiologic niches relevant to AML) in response to FLT3-ITD or PHGDH inhibition was not interrogated in this study. The process of isolating AML blasts or interstitial fluid from these niches, without altering metabolite abundances during purification, is technically challenging but warrants further investigation.

Collectively, our study reveals new mechanistic insights into FLT3-ITD-induced metabolic reprogramming events in AML and reveals serine biosynthesis as a unique vulnerability in this disease. Our work also highlights the therapeutic potential of combining small-molecule PHGDH inhibitors with standard-of-care chemotherapy for the treatment of FLT3-ITD AML.

METHODS

Cell Lines and Cell Culture

MLL-AF9/iFLT3-ITD murine cells were generated via retroviral transduction of E13.5-day fetal liver cells, and cultured in Anne Kelso Modified Dulbecco's Modified Eagle Medium (AK-DMEM—low-glucose DMEM; Invitrogen Life Technologies, 4 g/L glucose, 36 mg/mL folic acid, 116 mg/L L-arginine HCl, 216 mg/L L-glutamine, 2 g/L NaHCO₃ and 10 mmol/L HEPES), supplemented with 20% FBS (Invitrogen Life Technologies), 1% penicillin/streptomycin (PenStrep; Invitrogen Life Technologies), and 1% L-asparagine (Sigma-Aldrich) at 37°C and 10% CO₂. Cells at baseline were cultured in 1 µg/mL of doxycycline (Sigma-Aldrich) to maintain transgene expression. MV4-11, Kasumi-1, HL-60, and HEK-293T cells were purchased from the

ATCC. MOLM-13, OCI-AML3, and PL-21 cells were purchased from the DSMZ. MV4-11, MOLM-13, and PL-21 cells were cultured in RPMI-1640 (Gibco) supplemented with 10% FBS, 1% PenStrep, and 1% Glutamax (Thermo Fisher). Kasumi-1, HL-60, and OCI-AML3 cells were cultured in the same conditions, with the exception of 20% FBS content. HEK-293T cells were cultured in DMEM (Gibco), supplemented with 10% FBS, 1% PenStrep, and 1% Glutamax. All human lines were cultured at 37°C and at 5% CO₂. For serine/glycine deprivation experiments, we synthesized RPMI-1640 and omitted inclusion of serine and glycine during preparation of medium. Exact composition of media is supplied in Supplementary Materials and Methods. All cells used in this study were tested regularly for *Mycoplasma* via PCR validation. All cell lines were validated via short-tandem repeat (STR) profiling prior to use.

Retroviral/lentiviral Transductions and Plasmid Cloning

MLL-AF9/iFLT3-ITD murine cell lines were generated via retroviral transduction of E13.5 fetal liver cells from a mouse endogenously expressing the CAG-rTA3 transgene as described in Supplementary Materials and Methods. For lentiviral transductions for CRISPR/Cas9-mediated depletion of *PHGDH*, and lentiviral transductions for shRNA-mediated silencing of *ATF4*, see Supplementary Materials and Methods.

In Vivo Analysis

All *in vivo* experiments performed in this study were approved by the Peter MacCallum Cancer Centre (PMCC) Animal Ethics Committee under ethics approval numbers E555 and E627. *Ptpcr*, NSG, and C57BL/6J mice endogenously expressing the CAG-rTA3 transgene were bred in-house (PMCC). For primary *Ptpcr* recipient transplants, 4- to 6-week-old females were sublethally irradiated with 6.5 Gy whole-body gamma-ray irradiation, using a Gammacell 1000 Cs-137 irradiator (AECL). 1×10^6 retrovirally transduced fetal liver cells were then intravenously (i.v.) tail-vein injected, and mice were placed on doxycycline-containing chow (Specialty Foods) and water [0.2% doxycycline hyclate (Sigma-Aldrich), 2% sucrose] to maintain transgene expression until ethical endpoint (hunched, ruffled, impaired movements). For secondary recipients, 4- to 6-week-old female NSG mice were i.v. injected with MLL-AF9/iFLT3-ITD cells as described above. At endpoint, blast cells were harvested from femoral bone. For *in vivo* bioluminescence imaging, mice were injected with 50 mg/kg D-luciferin and imaged in an IVIS bioluminescence imager (Perkin-Elmer) to assess disease burden. Analysis and normalization of *in vivo* luminescence was performed using Living Image v3.2 and v4.2 software.

Primary Patient Samples

Primary patient samples used for *in vitro* synergy studies were obtained from the Alfred Hospital (Melbourne, Australia) after obtaining written informed consent under Alfred Health Ethics Committee-approved guidelines. These studies were performed under Alfred Health Ethics Approval Number 42/20. All use of human tissue was performed in accordance with the Declaration of Helsinki. Primary AML blasts were cultured in StemSpan SFEM (STEMCELL Technologies) supplemented with 10 ng/mL IL3, 10 ng/mL IL6, 50 ng/mL stem cell factor, and 50 ng/mL FLT3 L (all PeproTech) and 35 nmol/L UM171 and 500 nmol/L stemreginin (STEMCELL Technologies). No patient data other than age, sex, and genomic profile were made available to nonclinical/authorized authors in this article.

Compounds and Chemicals

Quizartinib, WQ-2101, and everolimus small-molecule inhibitors were purchased from Selleckchem and reconstituted in DMSO for *in vitro* use. Fresh cytarabine in saline was acquired from the PMCC cytotoxic suite. *In vivo* quantities of WQ-2101 were purchased from Glixx Labs and reconstituted in 10% DMSO, 20% Kolliphor HS-15, and 70% sterile water supplemented with 0.1% Tween 80 as previously

described (39). For *in vivo* dosing, WQ-2101 was reconstituted daily. Cytarabine was diluted in PBS as required.

Cell Death Assays

For the assessment of cell viability via Annexin V staining, cell suspension was stained with 1:100 dilution Annexin V conjugated to an APC fluorophore (BD Pharmingen; cat. #550475) in binding buffer (10 mmol/L HEPES, 5 mmol/L CaCl₂, 140 mmol/L NaCl) prior to flow cytometry analysis. For assessment of cell viability in response to WQ-2101, 8,000 AML cells were plated into 384-well plates and exposed to escalating doses of WQ-2101 for 48 hours. At endpoint, 1:2 dilution CellTiter-Glo was added to each well, and cells were lysed for 10 minutes prior to assessment of absorbance with a Cytation 3 spectrophotometer (BioTek).

Competition Assays and Assessment of Cell Proliferation

For *in vitro* and *in vivo* competition assay protocols, see Supplementary Materials and Methods. For determination of proliferation in the presence/absence of serine and WQ-2101, 50,000 cells were seeded in 6-well plates and cultured for 3 or 9 days, respectively. Cells were counted using a cellometer, and viability was determined by the trypan-exclusion assay. For CellTrace Violet analysis of cell proliferation in response to WQ-2101 or OCI-AML3 cells in response to serine/PHGDH deprivation, 1×10^7 cells were washed in PBS, and stained for 20 minutes at 37°C with agitation with CellTrace Violet label (Thermo Fisher). A narrow peak of cells was then sorted using a FACS Aria Fusion 5 (BD) to synchronize cell-cycle phase and enable defined peak analysis. Cells were then cultured for 72 hours in 3 μ mol/L WQ-2101 and proliferation assessed via flow cytometry. For OCI-AML3 cells in response to serine deprivation, sgSCR or sgPHGDH-transduced cells were cultured for 48 hours in either full serine/glycine or serine/glycine-deficient RPMI, and proliferation was assessed via flow cytometry.

QuantSeq 3'RNA-seq

Details of 3'-RNA-seq RNA preparation, sequencing, and analysis pipelines can be found in Supplementary Materials and Methods.

ChIP-seq

Details of ChIP-seq protocols, library preparation, sequencing, and analysis pipelines can be found in Supplementary Materials and Methods.

Steady-State and U-¹³C-Labeling Metabolomics

MV4-11 and/or OCI-AML3 cells were treated as described, harvested, and processed for steady-state metabolomics and ¹³C-glucose labeling analysis as described in Supplementary Materials and Methods.

Metabolic Rescue Experiments

Detailed methods for serine rescue experiments in sgPHGDH cells and metabolite rescue experiments in response to FLT3-ITD inhibition with quizartinib can be found in Supplementary Materials and Methods.

Tritiated Serine Labeling

Detailed methods for the assessment of exogenous serine uptake post FLT3-ITD and PHGDH inhibition with quizartinib and WQ-2101 can be found in Supplementary Materials and Methods.

Combination Therapy Analysis In Vitro and In Vivo

Experimental protocols and analysis for combination therapy with WQ-2101 and cytarabine in AML cell lines, primary patient samples, and *in vivo*, can be found in Supplementary Materials and Methods.

Statistical Analyses

GraphPad Prism v8.0 and R version 3.6.1 software were used for statistical analysis. Statistical tests performed for each experiment are highlighted in the figure legends. Unless otherwise specified, error bars are indicative of $\pm$ SD, and threshold for significance was $P < 0.05$.

Data Availability

RNA-seq and ChIP-seq data were deposited in the Gene Expression Omnibus under accession number GSE163932. Outputs from RNA-seq DEG analysis and other relevant data are included in the Supplementary Data Tables. Any other relevant raw data are available from the corresponding authors at reasonable request.

Authors' Disclosures

L. Kats reports personal fees and other support from Agios Pharmaceuticals and Celgene outside the submitted work. A. Wei reports grants and personal fees from Novartis, AbbVie, Servier, Celgene/BMS, Amgen, and AstraZeneca and personal fees from Astellas, Pfizer, MacroGenics, Genentech, and Janssen outside the submitted work; in addition, A. Wei has a patent for Venetoclax with royalties paid. G.P. Gregory reports personal fees from Roche, Novartis/Sandoz, and Gilead, grants and personal fees from Janssen, grants from AbbVie, BeiGene, and MSD outside the submitted work. R.W. Johnstone reports grants from AstraZeneca during the conduct of the study; grants from Roche and BMS, grants and personal fees from MecRx outside the submitted work. No other disclosures were reported.

Authors' Contributions

S. Bjelosevic: Conceptualization, data curation, formal analysis, validation, investigation, visualization, methodology, writing—original draft, project administration, writing—review and editing. **E. Gruber:** Resources, data curation, software, formal analysis, investigation, and visualization. **A. Newbold:** Formal analysis and investigation. **C. Shembrey:** Conceptualization, resources, software, formal analysis, investigation, and visualization. **J.R. Devlin:** Resources, formal analysis, and investigation. **S.J. Hogg:** Resources and investigation. **L. Kats:** Conceptualization, resources, formal analysis, investigation, and methodology. **I. Todorovski:** Resources, data curation, software, formal analysis, and investigation. **Z. Fan:** Resources and investigation. **T.C. Abreghart:** Investigation. **G. Pomilio:** Resources, formal analysis, and investigation. **A. Wei:** Resources, supervision, writing—original draft, writing—review and editing. **G.P. Gregory:** Conceptualization, supervision, funding acquisition, writing—original draft, writing—review and editing. **S.J. Vervoort:** Conceptualization, data curation, software, formal analysis, supervision, validation, investigation, writing—original draft. **K.K. Brown:** Conceptualization, resources, supervision, funding acquisition, investigation, methodology, writing—original draft, project administration, writing—review and editing. **R.W. Johnstone:** Conceptualization, resources, supervision, funding acquisition, investigation, methodology, writing—original draft, project administration, writing—review and editing.

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Serine Biosynthesis Is a Metabolic Vulnerability in FLT3-ITD-Driven Acute Myeloid Leukemia

Stefan Bjelosevic, Emily Gruber, Andrea Newbold, et al.

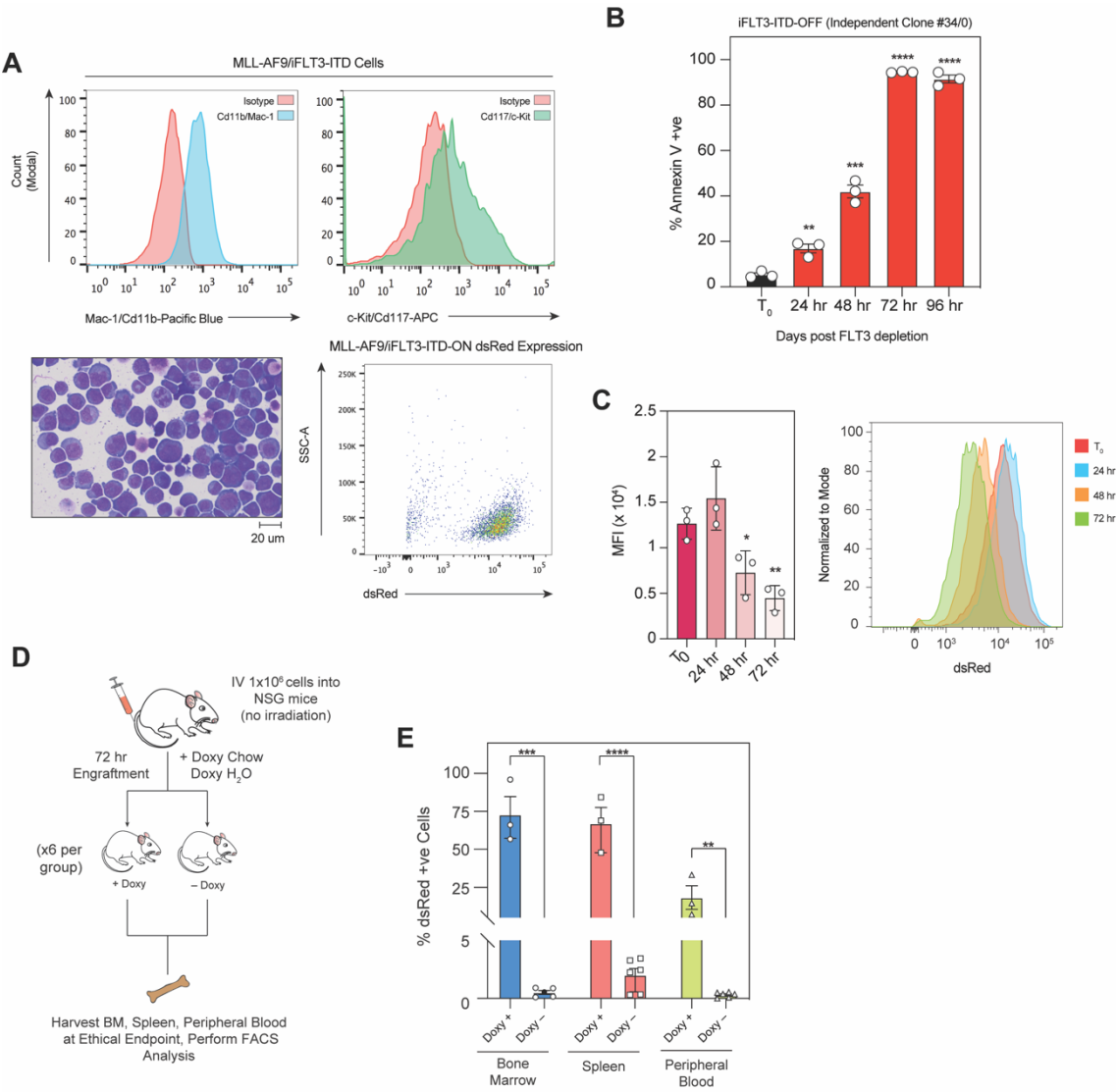
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3.3 Supplementary information associated with *Cancer Discovery* publication

Please note that supplementary materials and methods relevant to this publication are provided in chapter 2 of this thesis and are not repeated in this section to avoid redundancy. Supplementary Tables may be accessed on the *Cancer Discovery* website.



Supplementary Figure 1

Supplementary Figure S1. A genetically engineered mouse model of MLL-rearranged, FLT3-ITD AML reveals FLT3-ITD is essential for leukaemia survival.

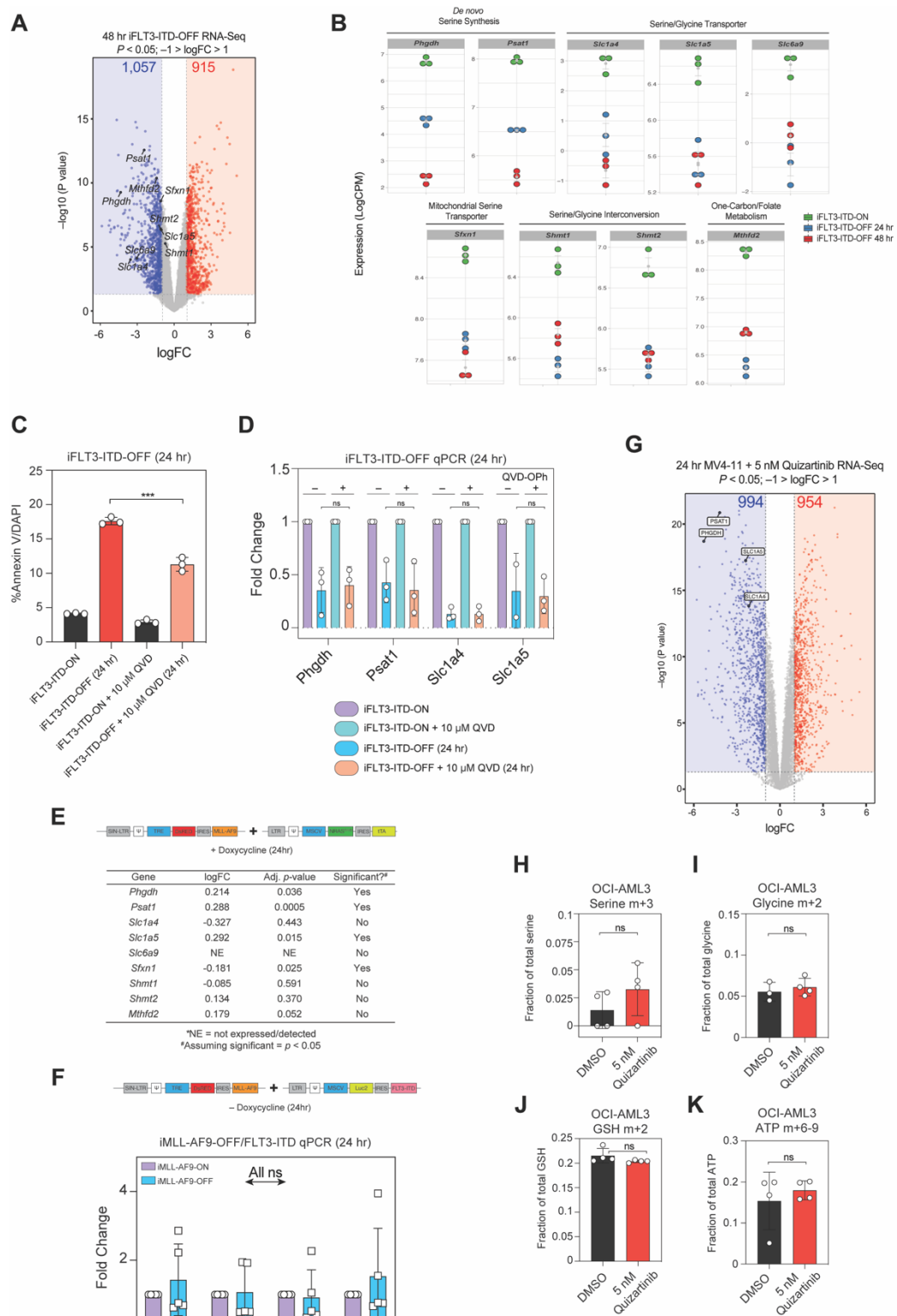
A. Characterisation of primary MLL-AF9/iFLT3-ITD AML cells. Flow cytometry-based analysis demonstrated blasts expressed Mac-1/Cd11b and c-Kit/Cd117, consistent with MLL-rearranged AML as previously characterised (1). Isotype controls used in each experiment are denoted with red shading. May-Grünwald-Giemsa staining demonstrating histology consistent with AML blast cells. Scale is indicative of 20 μ m. MLL-AF9/iFLT3-ITD cells can be monitored by dsRed expression via flow cytometry.

B. A second independent primary MLL-AF9/iFLT3-ITD clone was harvested from mouse #34/0 and blasts were cultured *in vitro* in 1 μ g/mL doxycycline. Cells were then washed and cultured in doxycycline-free medium to deplete iFLT3-ITD expression for 96 hours, and percent apoptotic cells subsequently assessed via Annexin V flow cytometry every 24 hours. ** denotes $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ by Student's *t*-test relative to T_0 . Error bars are representative of $\pm$ s.d. and three independent biological replicates.

C. dsRed expression post iFLT3-ITD depletion was assessed at 24-hour intervals up to 72 hours via flow cytometry and presented as mean fluorescence intensity (MFI). Error bars are representative of $\pm$ s.d. and three independent biological replicates. * denotes $P < 0.05$, ** denotes $P < 0.01$ by Student's *t*-test relative to T_0 .

D. Schematic representation of *in vivo* iFLT3-ITD depletion. Mice were IV injected with 1×10^6 MLL-AF9/iFLT3-ITD cells and placed on doxycycline containing chow and water. 72 hours post injection, half of the cohort was placed on normal chow and water to deplete FLT3-ITD transgene expression ($n = 6$ mice per group).

E. At endpoint, MLL-AF9/iFLT3-ITD-dsRed-positive leukaemic blast burden was assessed in bone marrow, spleen and peripheral blood compartments via flow cytometry. ** denotes $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ as determined by Student's *t*-test relative to Doxy + condition. Error bars are representative of $\pm$ s.d. and 3-6 independent biological replicates.



Supplementary Figure 2

Supplementary Figure S2. Transcriptomics and metabolomics analysis reveal *de novo* serine biosynthesis and serine uptake are regulated by FLT3-ITD

A. Volcano plot of differentially expressed genes 48 hours post iFLT3-ITD depletion, highlighting genes involved in mediation of *de novo* serine biosynthesis, serine/glycine channel transporters, and one-carbon/folate metabolism in MLL-AF9/iFLT3-ITD cells. Suppressed differentially expressed genes (1,057) are denoted by blue, and enriched genes (915) by red shading. Significance was defined as $P < 0.05$ and $-1 > \log FC > 1$.

B. Dot plots of normalised log counts-per-million (CPM) gene expression values 24 and 48-hours post iFLT3-ITD depletion of genes highlighted in Supplementary Figure S2A. Genes are grouped under biological/molecular function. Error bars denote $\pm$ s.d. and each condition was composed of 3 independent biological replicates.

C. Annexin V viability staining of MLL-AF9/iFLT3-ITD cells with and without iFLT3-ITD depletion and in the presence and absence of the pan-caspase inhibitor QVD-OPh. Error bars are representative of $\pm$ s.d. and three independent biological replicates and *** denotes $P < 0.001$, as determined by Student's *t*-test.

D. Real-time quantitative PCR for the expression of key *de novo* serine biosynthesis genes *Phgdh* and *Psat1* and neutral amino acid transporters *Slc1a4* and *Slc1a5* upon depletion of iFLT3-ITD in MLL-AF9/iFLT3-ITD cells in the presence and absence of the pan-caspase inhibitor QVD-OPh. All data is normalised to the *18s rRNA* housekeeping gene and is expressed as relative fold change versus non-depleted iFLT3-ITD. Error bars are representative of $\pm$ s.d. and three independent biological replicates. Statistical test performed is a Student's *t*-test.

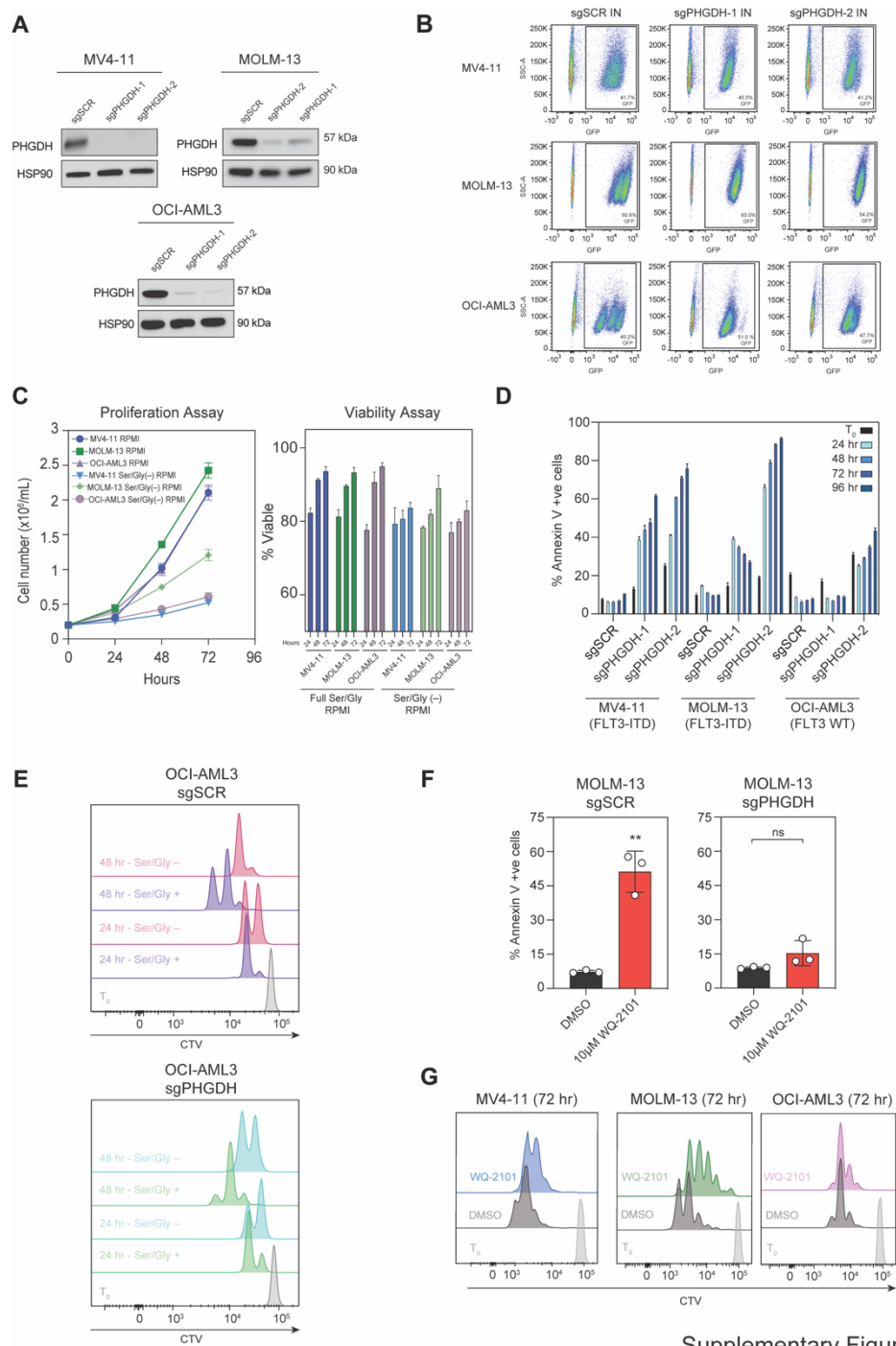
E. Table depicting logFC, adjusted *P*-value and significance of key genes suppressed upon MLL-AF9 depletion in a Tet-inducible model of MLL-AF9. Tet-inducible MLL-AF9/NRAS^{G12D} cells were cultured in 1 μ g/mL doxycycline to suppress expression of MLL-AF9 for 24 hours, and RNA extracted and 3'-RNA-sequenced. NE, not expressed/detected; significance $P < 0.05$.

F. Real-time quantitative PCR for the expression of *Phgdh*, *Psat1*, *Slc1a4* and *Slc1a5* upon depletion of iMLL-AF9 in a murine model of Tet-inducible MLL-AF9 with constitutive FLT3-ITD expression (iMLL-AF9/FLT3-ITD). All data is normalised to the *18s rRNA* housekeeping gene and is expressed as relative fold change versus non-depleted iMLL-AF9. Error bars are

representative of $\pm$ s.d. and five independent biological replicates. Statistical test performed is a Student's *t*-test, calculated between iMLL-AF9-ON and iMLL-AF9-OFF for each gene.

G. Volcano plot of differentially expressed genes 24 hours post FLT3-ITD inhibition with 5 nM of quizartinib, highlighting genes involved in mediation of *de novo* serine biosynthesis and serine channel transporters in MV4-11 cells. Suppressed differentially expressed genes (994) are denoted by blue, and enriched genes (954) by red shading. Significance was defined as $P < 0.05$ and $-1 > \log FC > 1$.

H-K. Fractional abundance of ^{13}C -glucose-derived: **H**, m+3 serine; **I**, m+2 glycine; **J**, m+2 glutathione (GSH); and **K**, m+(6-9)-labelled ATP in OCI-AML3 cells treated with either DMSO or 5 nM quizartinib for 24 hours. Error bars are representative of $\pm$ s.d. of 3-4 independent biological replicates and statistical comparisons are determined by a Student's *t*-test.



Supplementary Figure 3

Supplementary Figure S3. Serine biosynthesis is a metabolic vulnerability in FLT3-ITD-driven AML.

A. Efficiency of sgRNAs against PHGDH (sgPHGDH-1 or -2) or Scramble DNA (sgSCR) was validated via Western blotting in MV4-11, MOLM-13 and OCI-AML3 cells. HSP90 served as the loading control in all experiments.

B. MV4-11, MOLM-13 and OCI-AML3 cells expressing GFP-tagged sgRNAs against PHGDH and Cas9-mCherry were mixed in a 50:50 ratio with Cas9-mCherry expressing parental cells and transplanted into NSG mice. % GFP-expressing cells prior to transplantation at timepoint T_0 were measured via flow cytometry.

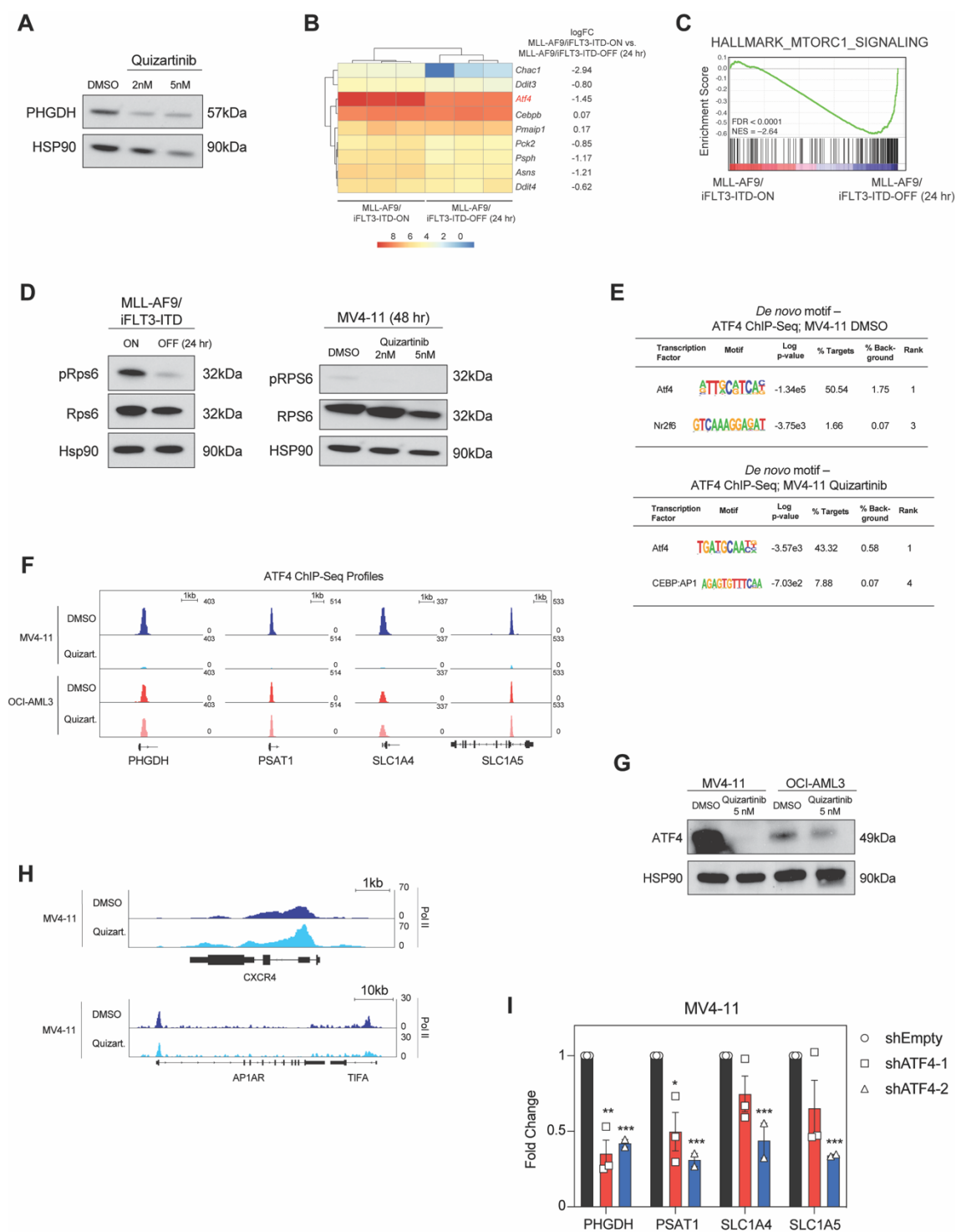
C. Cell counts assessing proliferation of two FLT3-ITD-driven cell lines (MV4-11 and MOLM-13) and FLT3-WT OCI-AML3 cells in either complete or serine/glycine-deprived RPMI over 72 hours. Cell viability was concurrently determined via trypan-exclusion assay over the same period of time. Error bars are representative of $\pm$ s.d. and 3 independent biological replicates.

D. MV4-11, MOLM-13 and OCI-AML3 transduced with either two independent sgRNAs against PHGDH (sgPHGDH-1 or -2) or Scramble DNA (sgSCR) and were cultured in serine/glycine deprived medium for 96 hours, and cell viability determined via Annexin-V flow cytometry every 24 hours. Raw viability data of each timepoint is shown, and error bars are representative of $\pm$ s.d. and 3 independent biological replicates.

E. OCI-AML3 cells expressing either Scramble DNA (sgSCR) or sgRNA against PHGDH, were cultured in serine/glycine depleted or complete RPMI medium for 48 hours and proliferation, as measured by Cell Trace Violet (CTV) assay, measured every 24 hours. Experiment is representative of two independent experiments.

F. MOLM-13 cells expressing either Scramble DNA (sgSCR) or sgRNA against PHGDH were treated with 10 μ M WQ-2101 for 48 hours and cell viability assessed via Annexin V flow cytometry. ** denotes $P < 0.01$ as determined by Student's *t*-test relative to DMSO. Error bars are representative of $\pm$ s.d. and three independent biological replicates.

G. MV4-11, MOLM-13 and OCI-AML3 cells were treated with DMSO, or 3 μ M WQ-2101 and proliferation assessed via Cell Trace Violet 72 hours post dosing. Data is representative of 3 independent experiments.



Supplementary Figure 4

Supplementary Figure S4. A FLT3-ITD/mTORC1/ATF4 axis transcriptionally modulates serine biosynthesis and serine transporters in FLT3-mutant AML.

A. MV4-11 cells were treated with DMSO, or two doses of quizartinib (2 nM or 5 nM) for 48 hours, and PHGDH expression assessed via Western blot. HSP90 served as the loading control.

B. Heatmap of *Atf4* target gene suppression post 24-hour iFLT3-ITD depletion in murine MLL-AF9/iFLT3-ITD cells. All gene expression changes are denoted as logFC.

C. Gene set enrichment (GSEA) plots depicting suppressed mTORC1 signalling after 24 hours of iFLT3-ITD depletion in murine MLL-AF9/iFLT3-ITD cells, FDR < 0.0001, NES = -2.64.

D. Murine MLL-AF9/iFLT3-ITD cells were cultured in the absence of doxycycline for 24 hours to deplete iFLT3-ITD, and phospho- and total Rps6 assessed via Western blot. The experiment was repeated in MV4-11 cells treated with either DMSO, or two doses of quizartinib (2 nM or 5 nM) for 48 hours. HSP90 served as the loading control in both experiments.

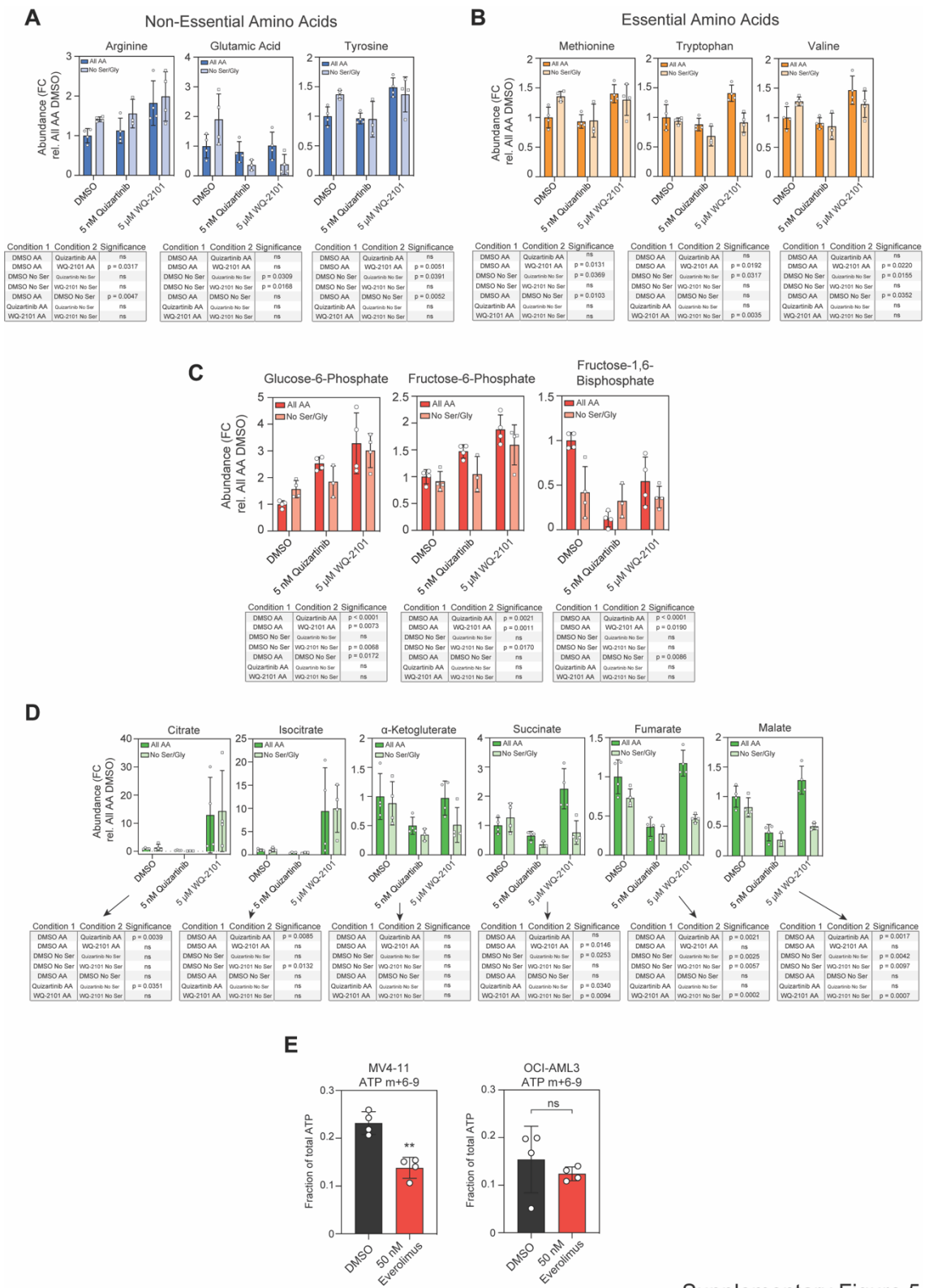
E. *De novo* motif discovery on ATF4 bound sites in DMSO and quizartinib-treated conditions in MV4-11 cells.

F. ChIP-seq reads for ATF4 mapped to the PHGDH, PSAT1, SLC1A4 and SLC1A5 loci in MV4-11 and OCI-AML3 cells treated with 5 nM quizartinib or DMSO for 24 hours.

G. MV4-11 and OCI-AML3 cells were treated with 5 nM quizartinib or DMSO for 24 hours, and ATF4 expression assessed via Western blot. HSP90 served as the loading control.

H. ChIP-seq reads for RNA Pol II mapped to the CXCR4, AP1AR, and TIFA loci in MV4-11 cells treated with 5 nM quizartinib or DMSO for 24 hours.

I. Real-time quantitative PCR for the expression of *PHGDH*, *PSAT1*, *SLC1A4* and *SLC1A5* upon 48-hour depletion of *ATF4* via two independent shRNA hairpins in MV4-11 cells. All data is normalised to the *RPLO* housekeeping gene and is expressed as relative fold change versus empty vector control (shEmpty). Error bars are representative of $\pm$ s.d. and three independent biological replicates. * denotes $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ by Student's *t*-test relative to shEmpty.



Supplementary Figure S5. FLT3-ITD regulates global serine metabolism and drives purine nucleotide biosynthesis.

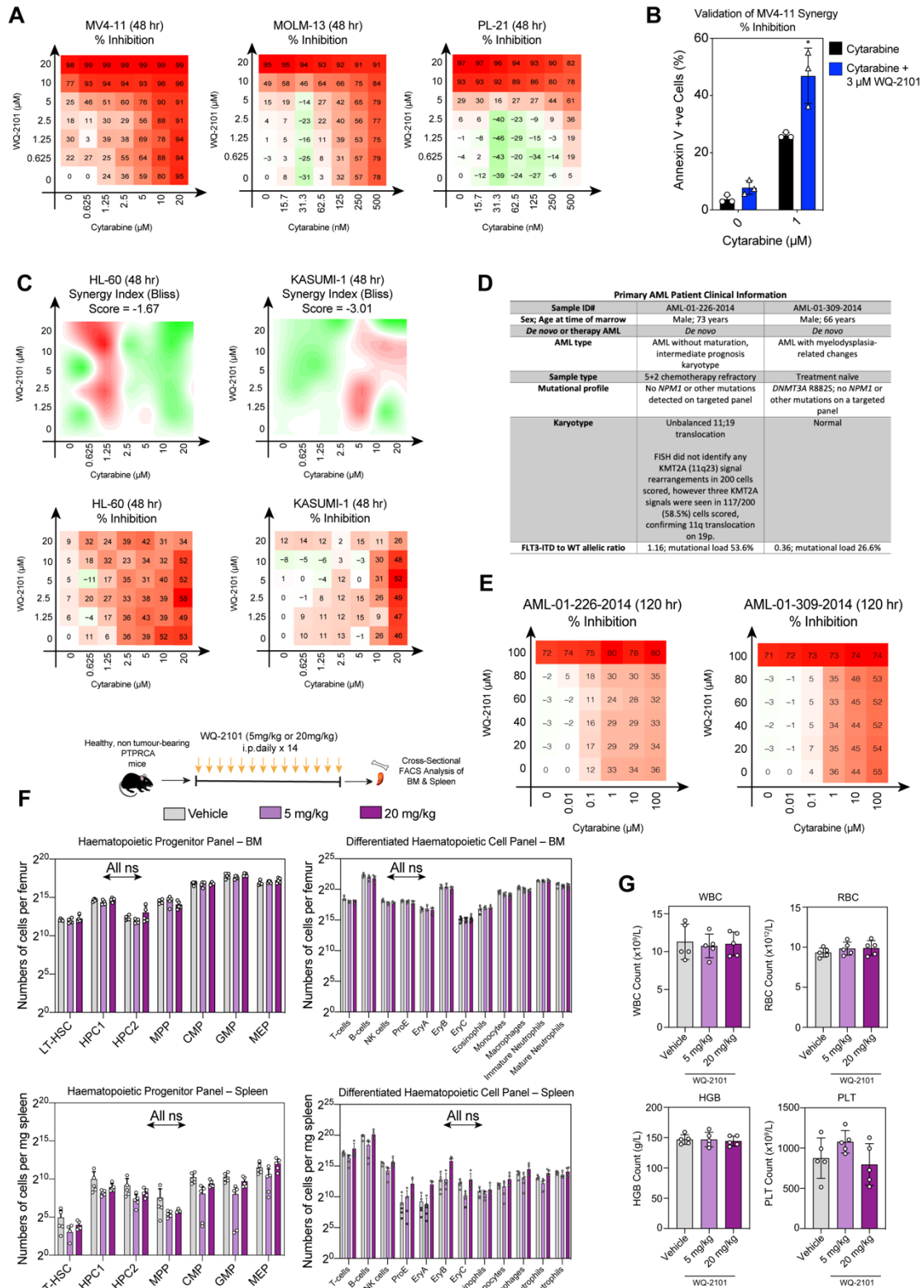
A. Fold changes of non-essential amino acids arginine, glutamic acid and tyrosine abundances, as measured by LC/MS-MS, in DMSO-treated MV4-11 cells versus MV4-11 cells treated with 5 nM quizartinib or 5 μ M WQ-2101 for 24 hours in all amino acid (AA)-containing RPMI medium, or serine/glycine-depleted medium.

B. Fold changes of essential amino acids methionine, tryptophan and valine abundances, as measured by LC/MS-MS, in DMSO-treated MV4-11 cells versus MV4-11 cells treated with 5 nM quizartinib or 5 μ M WQ-2101 for 24 hours in all amino acid (AA)-containing RPMI medium, or serine/glycine-depleted medium.

C. Fold changes in glucose-6-phosphate, fructose-6-phosphate and fructose-1,6-bisphosphate abundances, as measured by LC/MS-MS, in DMSO-treated MV4-11 cells versus MV4-11 cells treated with 5 nM quizartinib or 5 μ M WQ-2101 for 24 hours in all amino acid (AA)-containing RPMI medium, or serine/glycine-depleted medium.

D. Fold changes in citrate, isocitrate, α -ketoglutarate, succinate, fumarate and malate abundances, as measured by LC/MS-MS, in DMSO-treated MV4-11 cells versus MV4-11 cells treated with 5 nM quizartinib or 5 μ M WQ-2101 for 24 hours in all amino acid (AA)-containing RPMI medium, or serine/glycine-depleted medium. For all figures **S5A-S5D**, tables containing statistical analyses for each condition are below each graph and calculated via Student's *t*-test with comparisons as indicated. Error bars are indicative of $\pm$ s.d. and 4 biological replicates.

E. Fractional labelling of intracellular U- 13 C-glucose-derived m+6-9 labelled ATP in MV4-11 and OCI-AML3 cells treated with either DMSO or 50 nM of the mTORC1 inhibitor everolimus for 24 hours. Error bars are indicative of $\pm$ s.d. and 4 biological replicates. ** denotes $P < 0.01$ by Student's *t*-test relative to DMSO.



Supplementary Figure 6

Supplementary Figure S6. Inhibition of *de novo* serine biosynthesis sensitizes FLT3-ITD-driven AMLs to cytarabine.

A. MV4-11, MOLM-13 and PL-21 cells were treated with escalating concentrations of WQ-2101 and cytarabine for 48 hours to determine the effect on viability as determined by Cell Titer Glo. The mean of three biological replicates was used to determine each data point and was normalized to DMSO controls.

B. MV4-11 cells were treated with 1 μ M of cytarabine and/or 3 μ M WQ-2101 for 48 hours, and cell viability assessed using a non-ATP-based readout (Annexin V flow cytometry). Error bars are indicative of $\pm$ s.d. and 3 biological replicates. * denotes $P < 0.05$ as determined by Student's *t*-test relative to cytarabine alone.

C. FLT3-WT HL-60 and KASUMI-1 cells were treated with escalating concentrations of WQ-2101 and cytarabine for 48 hours to assess the effect on viability, as determined by DAPI exclusion flow cytometry. The presence of treatment synergy was determined using the SynergyFinder computational package and the Bliss synergy index and is denoted as regions of red in the graphs. The mean of two biological replicates was used to determine synergy/each data point and was normalized to DMSO controls. Negative synergy score is indicative of greater degree of antagonism between two tested compounds.

D. Clinical information summary of primary AML patient samples.

E. Cells from two independent primary AMLs, AML-01-226-2014 and AML-01-309-2014, were treated with escalating concentrations of WQ-2101 and cytarabine for 120 hours to determine the effect on cell viability as determined by Sytox Blue exclusion flow cytometry. Data is indicative of one experiment.

F. Non-tumour bearing PTPRCA mice were chronically treated with either 5 mg/kg or 20 mg/kg WQ-2101 for 14 days as per the schema. At endpoint, all mice were sacrificed and both bone marrow (BM) and Spleen compartments assessed for haematopoietic cell composition and abundance via flow cytometry (haematopoietic progenitor panel for both compartments, left; differentiated haematopoietic cell panel for both compartments, right). All error bars are indicative of $\pm$ s.d. and 5 biological replicates.

G. At the conclusion of treatment as outlined in **F**, gross haematology analysis was performed from a retroorbital blood sample using an automated haematology analyser. WBC, white blood cell count; RBC, red blood cell count; HGB, haemoglobin count; PLT, platelet count.

SUPPLEMENTARY REFERENCES

1. Somervaille TCP, Cleary ML. Identification and characterization of leukemia stem cells in murine MLL-AF9 acute myeloid leukemia. *Cancer Cell* **2006**;10(4):257-68 doi <https://doi.org/10.1016/j.ccr.2006.08.020>.

3.4 Addendum

3.4.1 Interrogation of cryptic splicing of PHGDH in haematological malignancies

Recent studies have reported that *SF3B1* mutant AML cells are sensitive to exogenous serine and glycine depletion due to cryptic mis-splicing of PHGDH which results in loss of function of the *de novo* serine biosynthesis pathway (Dalton et al., 2019). In addition, *SF3B1* loss of function may also cause sensitivity to serine deprivation (Dalton et al., 2020). Though our MLL-AF9/iFLT3-ITD mouse model was not engineered to express other mutations such as *Sf3b1*, there remained a possibility that these, or the MV4-11/MOLM-13 cells used in this study, had acquired this mutation *in vitro* or *in vivo*, thus providing a potential mechanistic explanation as to why cells are sensitive to exogenous serine depletion. To interrogate this hypothesis, we took advantage of human-specific primers reported by Dalton and colleagues and designed complementary murine-specific primers spanning the exon 6/7 splice site identified to give rise to cryptic (mis-spliced) PHGDH (**Figure 3.4.1A**). RNA was extracted from the MV4-11, MOLM-13 and MLL-AF9/iFLT3-ITD cells utilised in this study and subsequently converted to cDNA. The exon 6/7 splice site was amplified via polymerase chain reaction (PCR) using the above primers and the products visualised on an agarose gel. As per the study by Dalton and colleagues, the presence of cryptic PHGDH splicing would result in two bands, signifying mis-splicing on the PHGDH transcripts. We confirmed the presence of cryptic PHGDH in *SF3B1*^{K700E}-mutant HNT-34 cells (used as a positive control in this and the Dalton, et al. study), and no evidence of PHGDH mis-splicing in MV4-11, MOLM-13 or MLL-AF9/iFLT3-ITD cells (in both iFLT3-ITD-ON and iFLT3-ITD-OFF conditions) used in this study (**Figure 3.4.1B**).

Collectively, these data demonstrate that the cell lines utilised in this chapter have not acquired *SF3B1* mutations which result in mis-splicing, and thus loss of function of, PHGDH. The sensitivity of these cells to exogenous serine/glycine withdrawal is thus due to the mechanisms proposed in this chapter, and not as a result of mis-splicing of PHGDH transcripts.

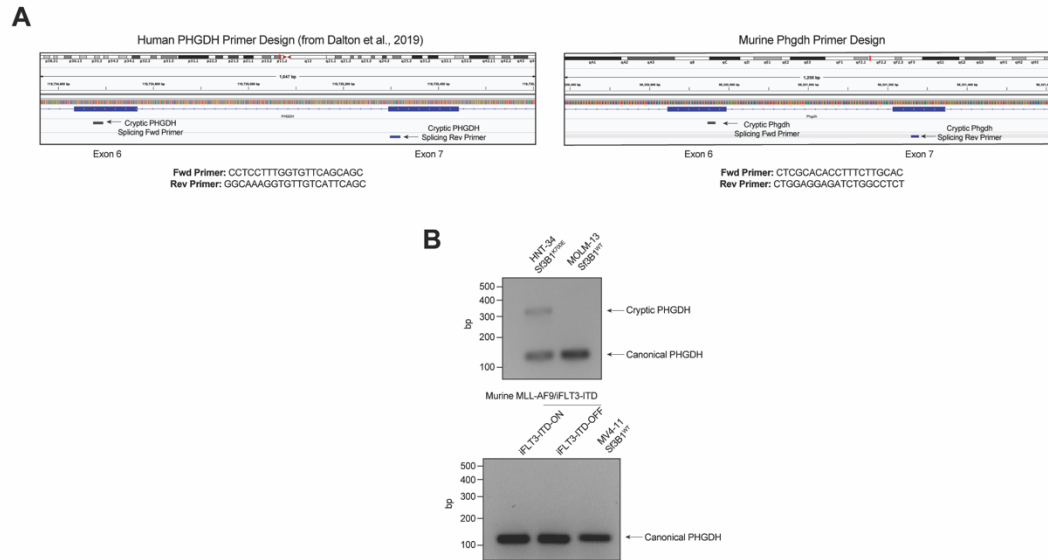


Figure 3.4.1 Detection of aberrant PHGDH splicing in SF3b1 mutant AML and MLL-AF9/iFLT3-ITD cells

A. Design of primers spanning exon 6/7 of PHGDH where aberrant splicing of PHGDH is detected as per Dalton, et al., 2019. Left pane denotes primer design for human PHGDH, and right pane denotes our own primer design for murine Phgdh. **B.** RNA was extracted from HNT-34 (SF3B1^{K700E} mutant), MOLM-13 (SF3B1 WT), MV4-11 (SF3B1 WT) and MLL-AF9/iFLT3-ITD cells in both FLT3-ITD depleted and expressed state and converted to cDNA. Primers from **A.** were used to amplify the exon 6/7 region of PHGDH/Phgdh, and the product run on a 2% agarose gel. Cryptic PHGDH was detected in SF3B1^{K700E} mutant HNT-34 cells, a phenotype not detected in any of our other cell lines.

3.4.2 Enforced PHGDH does not rescue cell proliferation upon quizartinib treatment

Given the data in this chapter has provided evidence of glucose-derived serine incorporation into nucleotides such as ATP, and suppression of FLT3-ITD results in downregulation of *de novo* serine biosynthesis (namely *PHGDH*), an important question is whether enforced PHGDH is sufficient to rescue the anti-proliferative effects of quizartinib. As PHGDH expression is lost following ablation of FLT3-ITD signalling via suppression of mTORC1 and rapid loss of ATF4, ectopic PHGDH expression might thus provide the glucose-derived serine one-carbon units necessary for the maintenance of proliferation. To address this question, wild-type PHGDH was subcloned into an MSCV-PHGDH-IRES-GFP vector either with or without a V5 C-terminal tag, given that some reports have indicated N-terminal tagging of PHGDH results in loss of function (Mattaini et al., 2015). Stable over-expression of PHGDH was achieved in MV4-11 and MOLM-13 cells (**Figure 3.4.2A**). A detailed analysis of proliferation dynamics (**Figure 3.4.2B**) and mean division number (**Figure 3.4.2C**) demonstrated that PHGDH over-expression was not sufficient to rescue the antiproliferation response to FLT3-ITD inhibition. Similarly, PHGDH over-expression was not sufficient to rescue changes in cell viability resulting from FLT3-ITD inhibition (**Figure 3.4.2D**). These findings are not entirely unsurprising and do not detract from the overall impact/mechanism of our findings. It has been well-characterised that FLT3-ITD, and indeed, receptor tyrosine kinases in general, regulate a wide variety of cellular pathways that drive proliferation and pro-survival signalling (Grafone et al., 2012).

Thus, it is likely that enforced expression of PHGDH simply is not sufficient to overcome the broad signalling changes induced by loss of FLT3-ITD.

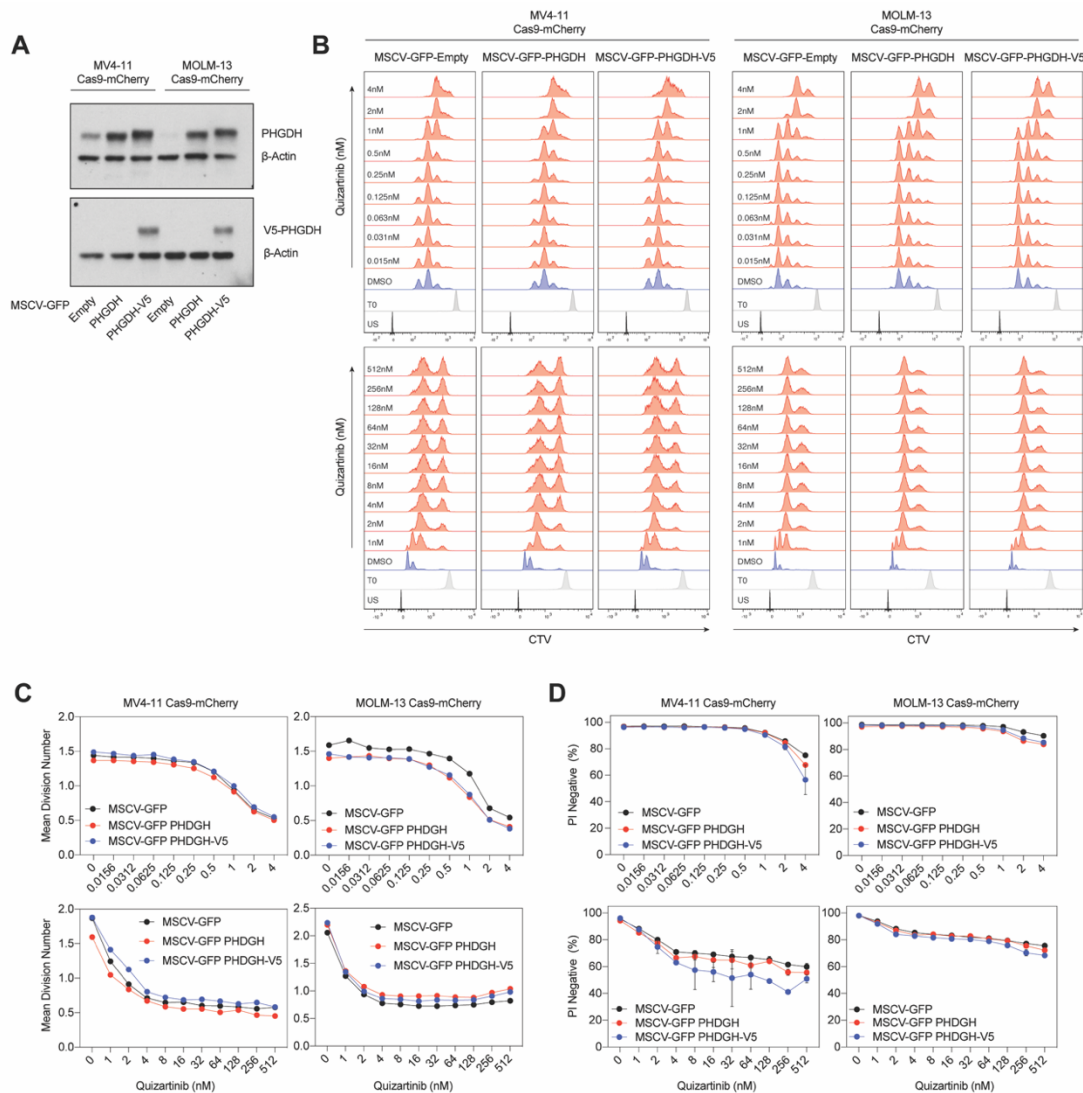


Figure 3.4.2 Ectopic over-expression of PHGDH does not rescue FLT3-ITD inhibition

A. Western blot depicting MV4-11 and MOLM-13 cells bearing over-expression of PHGDH after transduction with either an MSCV-PHGDH-IRES-GFP or MSCV-PHGDH-V5-IRES-GFP vector. Actin served as the loading control. **B.** MV4-11 and MOLM-13 cells, expressing either MSCV-PHGDH-IRES-GFP, MSCV-PHGDH-V5-IRES-GFP or MSCV-GFP-Empty Vector were exposed to increasing concentrations of the FLT3 inhibitor quizartinib and proliferation assessed for 48 hours via Cell Trace Violet (CTV) assays. **C.** Mean Division Number curves of MV4-11 and MOLM-13 cells, expressing either MSCV-PHGDH-IRES-GFP, MSCV-PHGDH-V5-IRES-GFP or MSCV-GFP-Empty Vector, in response to increasing concentrations of the FLT3 inhibitor quizartinib for 48 hours. **D.** MV4-11 and MOLM-13 cells, expressing either MSCV-PHGDH-IRES-GFP, MSCV-PHGDH-V5-IRES-GFP or MSCV-GFP-Empty Vector were exposed to increasing concentrations of the FLT3-ITD inhibitor quizartinib for 48 hours, and cell viability assessed via PI exclusion assay.

3.4.3 FLT3-ITD-mutant cell lines are not preferentially sensitive to glucose depletion

FLT3-ITD has been demonstrated to promote glycolysis, primarily via sustaining glycolytic flux through the pentose phosphate pathway, thus promoting the Warburg effect (Liu et al., 2016a). This data would thus suggest that glucose deprivation would reveal FLT3-ITD cells are more sensitive to glucose deprivation. To test this hypothesis, FLT3-ITD-driven MV4-11 and MOLM-13 cells, and FLT3-WT OCI-AML3 cells were incubated with glucose-free media and cell proliferation was monitored via Cell Trace Violet staining (**Figure 3.4.3A**). All cell lines demonstrated an anti-proliferative response to glucose deprivation (**Figure 3.4.3B**). This data is not surprising given that there are diverse mechanisms regulating glycolysis, a multitude of metabolic pathways responsive to glucose deprivation, and the use of non-isogenic cell lines.

Thus, these results suggest that while FLT3-ITD promotes glycolysis, global depletion of glucose universally induces proliferative defect regardless of FLT3-ITD status. There is some supporting evidence of this observation in the original papers which investigated FLT3-ITD-mediated glycolytic promotion. Poulain, et al. reported that MOLM-14 (FLT3-ITD-driven), and OCI-AML2, OCI-AML3 and HL-60 (FLT3-WT) cells all displayed decreased cell viability in response to exogenous glucose depletion (Poulain et al., 2017). Similarly, Ju, et al. utilised isogenic BaF3 cell lines transduced with FLT3-ITD over-expression vectors to interrogate glucose uptake dynamics, demonstrating FLT3-ITD enhanced glucose uptake (Ju et al., 2017). Further, the authors did not investigate proliferation in the context of global glucose depletion.

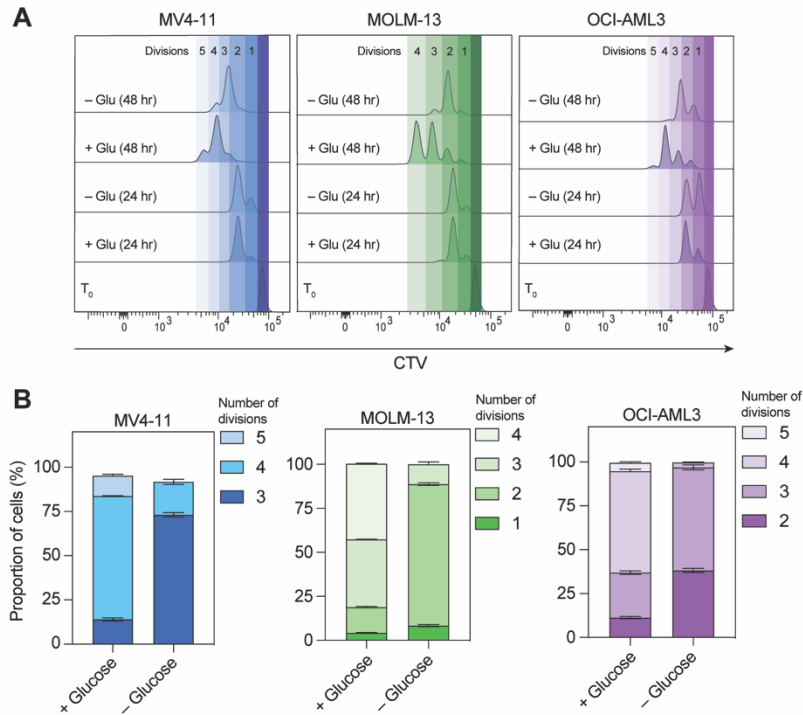


Figure 3.4.3 Depletion of glucose from culture medium does not exclusively affect FLT3-ITD-driven AML proliferation

A. MV4-11 and MOLM-13 (FLT3-ITD-driven), and OCI-AML3 (FLT3-WT) cells were cultured in full glucose or glucose depleted medium for 48 hours, and proliferation dynamics assessed every 24 hours via Cell Trace Violet (CTV) assay. Approximate division number is highlighted by increasingly faint shading for each cell line. **B.** Division number for each of the cell lines in **A.** was quantified in glucose replete or depleted conditions and presented as fraction of cells in each division number/condition. Error bars are indicative of 3 biological replicates for each condition.

3.5 Discussion

This chapter has provided important insights into the metabolic reprogramming events induced by mutant FLT3 in AML. Indeed, these observations provide mechanistic context to long-observed hallmarks of FLT3-ITD mutations; namely, that they promote increased generation and utilisation of amino acids, such as serine, as important sources of biomass to fuel downstream processes that enable increased proliferation. As seen in this chapter, glucose-derived serine one-carbon units are incorporated into ATP and other nucleotides to drive proliferation. The publication in chapter 3.2 discussed some of the key implications of these findings; here, this discussion shall be extended by considering additional questions generated by the manuscript and extending the findings in the context of recent literature regarding metabolic reprogramming events driving serine metabolism that has emerged since this article was published. The broader implications of serine biosynthesis as a common metabolic ‘node’ and means of exploiting serine biosynthesis therapeutically shall also be addressed.

As mentioned in the discussion section of the manuscript in chapter 3.2, the *in vivo* dependence of FLT3-ITD-driven AML cells on *de novo* serine biosynthesis is critically important. Our *in vivo* competition experiments provide strong rationale that genetic depletion of PHGDH impairs AML cell proliferation and results in potent depletion of the leukaemic cell pool. However, this result raises several questions. Chemical inhibition of PHGDH with WQ-2101 in mice bearing a disseminated model of MV4-11 AML cells had only modest impact on survival (Chapter 3.2, Figure 6F). As WQ-2101 is a “first generation” allosteric inhibitor of PHGDH (Ngo et al., 2020), it is likely that this discrepancy can be explained by suboptimal pharmacokinetic/pharmacodynamic properties (indeed, in the original manuscript characterising WQ-2101, doses of >10 mg/kg elicited the most potent anti-tumour effects, a dose which we were unable to utilise due to toxicity in the combination therapy arm (Wang et al., 2017). Another interesting question which was not directly addressed in chapter 3.2 is whether supraphysiological concentrations of exogenous serine are sufficient to rescue the effects of WQ-2101. The answer is likely to be context specific, and there is evidence supporting the contention that serine is and is not sufficient to rescue PHGDH inhibition. For instance, high serine concentrations in liquid culture were sufficient to partially rescue the effects of quizartinib treatment on MV4-11 cells (Chapter 3.2, Figure 5E). Conversely, it is generally accepted that cancer cells which respond to PHGDH inhibition are physiologically dependent on

the contribution of *de novo* serine biosynthesis to maintain proliferation even in serine replete conditions – a phenomenon somewhat akin to the concept of classic oncogene addiction (Pacold et al., 2016; Reid et al., 2018; Reina-Campos et al., 2020). Additionally, WQ-2101 elicits profound changes in the abundance of intermediates in the TCA cycle and the PPP, which are not induced by quizartinib treatment, further supporting the notion that serine supplementation may not be sufficient to reverse these alterations. Thus, whether FLT3-ITD-driven AMLs inhibited with WQ-2101 can be rescued by exogenous serine is an open question.

Since this chapter was initiated and eventually published in *Cancer Discovery*, several studies exploring important roles for serine in tumorigenesis in various disease contexts have been reported. A very recent study showed that in high fructose (versus high glucose) environments, leukaemia cells display increased dependence on the *de novo* serine biosynthesis pathway (Jeong et al., 2021). This metabolic reprogramming event is mediated by increased *de novo* serine biosynthesis pathway flux, which increases the rate of generation of α -ketoglutarate from glutamine, thus allowing cells to proliferate in the absence of glucose. Depletion of PHGDH reduced leukaemia engraftment even in the presence of high fructose, highlighting the specific dependency on *de novo* serine biosynthesis in maintaining this phenotype. Whilst not a specific focus of this chapter, the importance of serine-derived α -ketoglutarate production has become increasingly acknowledged. Another paper showed that breast cancer metastases in the lung, but not the primary tumours themselves, upregulate *de novo* serine biosynthesis pathway and mTORC1 activity to drive proliferation (Rinaldi et al., 2020). In this study, pyruvate uptake promoted mTORC1 signalling by driving *de novo* serine-derived α -ketoglutarate production, and, interestingly, heightened expression of PHGDH was required to sensitize cells to mTORC1 inhibitors. Finally, serine is an important contributor to sphingolipid synthesis, a metabolic role that has not been examined in this chapter. A recent study reported that restriction of serine results in serine palmitoyltransferase (SPT), an enzyme that catalyses the *de novo* biosynthesis of sphingolipids, to use alanine instead of serine as a substrate resulting in generation of noncanonical and toxic 1-deoxysphingolipids (Muthusamy et al., 2020). Among other observations, the authors showed that dietary serine and glycine deficiency, in addition to inhibition of PHGDH, promoted this metabolic switch to the use of alanine by SPT, thus resulting in

accumulation of toxic 1-deoxysphingolipids and decreased tumour growth. Collectively, these data highlight the intense research interest in determining the multi-faceted metabolic roles of serine, as well as the pathways that modulate its uptake and *de novo* generation. The wide array of cancers now implicated in the reprogramming of serine metabolism suggest that microenvironmental factors contribute to this phenomenon. In the data presented in this chapter, *in vivo* depletion of PHGDH in AML blasts localised to the spleen resulted in profound loss of representation of these cells in FLT3-ITD-driven disease. Thus, an interesting area for further research could be to examine the relationship between FLT3-ITD mutations and disease locale; that is, does FLT3-ITD-mediated promotion of *de novo* serine biosynthesis provide fitness to leukaemic clones as a means of overcoming low exogenous serine conditions in disease-relevant microenvironments? To the best of our knowledge, this question has not been interrogated in the bone marrow or spleen niche. We were not able to fully interrogate this effect in the bone marrow due to poor AML cell engraftment (in 2 of 3 cell lines used in the experiment), potentially due to the fact that all mice in the cohort (including those harbouring PHGDH depletion) were culled at onset of clinical signs of AML, thus not allowing for sufficient engraftment of AML cells. Additionally, the isolation of bone marrow interstitial fluid without altering fundamental metabolite composition during handling and purification is technically challenging. In other diseases, heightened *de novo* serine biosynthesis pathway activity has been demonstrated to overcome limited environmental serine in brain and lung cancer metastases (Ngo et al., 2020; Rinaldi et al., 2020) as well as other physiological niches in which exogenous serine is limiting (Sullivan et al., 2019). Given FLT3 mutations are typically developed late in leukaemogenesis (Kiyoi et al., 2020), it is possible that FLT3-ITD-mediated stimulation of *de novo* serine biosynthesis might provide leukaemic clones with the proliferative advantage necessary to overcome potentially serine-limited microenvironments.

A wide repertoire of genetic events that drive *de novo* serine biosynthesis pathway activity have now been characterised, and include transcription factors such as *MYC*, *NRF2* via *ATF4*, and mutations in *KRAS* (DeNicola et al., 2015; Maddocks et al., 2017; Nilsson et al., 2012; Sun et al., 2015). Given *MYC* is overexpressed in many haematological cancers, it is possible that *MYC*-mediated reprogramming of serine metabolism may occur in other haematological diseases aside from AML (Stine et al., 2015). Further, given that mutations in RAS occur in approximately 20-30% of AML patients, it is also possible

that promotion of *de novo* serine biosynthesis pathway activity may represent a common metabolic ‘node’ that provides one-carbon units for downstream processes such as nucleotide synthesis. As a result, in AMLs driven by RAS mutations inhibition of PHGDH may be a viable therapeutic modality, and the combinatorial chemotherapy/PHGDH strategy proposed in this chapter may be similarly efficacious. Whether RAS-mediated activation of the *de novo* serine biosynthesis pathway is also dependent on mTORC1/ATF4 signalling is not known, and the original publication which reported KRAS mutations drive *de novo* serine biosynthesis did not propose a specific mechanism for this effect (Maddocks et al., 2017). Regardless, it is likely that RAS-mediated activation of *de novo* serine biosynthesis is context/disease specific; for instance, OCI-AML3 cells, which were used as a non-responsive comparator to FLT3-ITD-driven cells in this chapter, harbour an NRAS^{Q61L} mutation (Chen et al., 2018). Whether NRAS mutant AMLs inherently promote *de novo* serine biosynthesis, or do so only in the context of drug treatment as a resistance mechanism (as recently demonstrated in melanoma cells exposed to MAPK inhibitors) remains unanswered (Nguyen et al., 2020). Finally, whilst the manuscript in chapter 3.2 focussed on the ability of FLT3-ITD mutations to promote *de novo* serine biosynthesis, some 7-10% of FLT3-mutant AMLs harbour mutations in the tyrosine kinase domain of FLT3 (FLT3-TKD) (Daver et al., 2019). Given that FLT3-TKD mutations are known to constitutively activate downstream pro-growth signalling via the RAS/MAPK and PI3K/AKT/mTOR pathways (Abu-Duhier et al., 2001; Xu et al., 2003), there is evidence that the mTORC1/ATF4/PHGDH axis identified in chapter 3.2 would potentially be relevant in the context of FLT3-TKD. Whether any other commonly occurring AML mutations also have the capacity to mediate serine metabolism is currently unknown and worth investigation.

Finally, this chapter provided evidence that genetic or pharmacological inhibition of the *de novo* serine biosynthesis pathway in FLT3-ITD-driven AML induces antiproliferative and apoptotic responses. As many environments AML cells are localised in have serine in replete conditions (at least *in vitro*; as discussed above, serine abundance in bone marrow and spleen is unknown), adaptive reprogramming to loss of PHGDH is likely to rapidly occur. Thus, the importance of inhibition of *de novo* serine biosynthesis as a means of sensitizing cells to other antineoplastic agents is the most likely therapeutic modality to be explored when implementing PHGDH inhibitors in the clinic. In this chapter, it was demonstrated that PHGDH inhibition sensitized AML cells to

chemotherapeutic agents, thus exacerbating DNA damage. Inhibition of PHGDH has been used to sensitize cells to mTORC1 inhibition in breast cancer metastases in the lung (Rinaldi et al., 2020), and to re-sensitize NSCLC cells to the epidermal growth factor receptor (EGFR) inhibitor erlotinib (Dong et al., 2018). Thus, further work examining synthetic lethal interactions with PHGDH inhibition should be performed to maximise the potential clinical utility of *de novo* serine biosynthesis inhibitors.

Overall, the findings in this chapter describe previously uncharacterised biological roles for FLT3-ITD mutations in AML. Through the generation of a novel mouse model of inducible FLT3-ITD in *MLL*-rearranged AML, we show that FLT3-ITD mutations promote *de novo* serine biosynthesis and serine uptake to drive proliferation. Perturbation of the *de novo* serine biosynthesis pathway is sufficient to impair proliferation and survival of FLT3-ITD-driven AMLs. Importantly, inhibition of *de novo* serine biosynthesis synergises with chemotherapy to exacerbate DNA damage. Thus, these results provide the conceptual basis for harnessing these metabolic vulnerabilities as novel therapeutic targets in cancer and encourage further investigation into other FLT3-ITD-induced metabolic reprogramming events in AML.

Chapter 4: Epigenetic potentiation of mTORC1 inhibition in acute myeloid leukaemia

4.1. Introduction

In chapter 3, important insights into how FLT3-ITD mutations co-opt metabolic machinery within the cell were revealed, and thus give rise to neomorphic metabolic vulnerabilities. While FLT3-ITD mutations have long been known to utilise PI3K/AKT/mTOR signalling for proliferative advantage (Chen et al., 2010), a crucial finding from chapter 3 was the role FLT3-ITD-induced hyperactivation of mTORC1 plays in activating components of the integrated stress response, including transcription factors such as *ATF4*. Co-opting *ATF4* to transcriptionally induce amino acid biosynthetic pathways, neutral amino acid transporters, and the downstream generation of purine nucleotides is thus a key mechanism by which FLT3-ITD, via mTORC1, remodels the metabolic landscape of the AMLs. As has been discussed in chapter 1, the multi-faceted roles of mTORC1, which additionally include coordinating cell responses to oncogenic growth factor signals and sensing nutrient abundance, make mTORC1 a “master metabolic regulator” in cancer (Laplane and Sabatini, 2009).

Significant effort has been dedicated to developing small molecule inhibitors of the mTOR complexes (mTORC1/mTORC2) in the hope that inhibition of this signalling axis might suppress the pro-growth machinery downstream of mTOR. In FLT3-ITD-driven AML, use of the PI3K and pan-mTOR inhibitor BEZ-235 resulted in potent induction of apoptotic responses in an *MLL-AF9/FLT3-ITD* model similar to the model described in this thesis (Sandhöfer et al., 2015). Moreover, the Johnstone laboratory has used BEZ-235 in aggressive *MYC*-driven lymphomas, where BEZ-235 induced apoptosis via induction of the pro-apoptotic BH3-only protein BMF, in a p53-independent manner (Shortt et al., 2013). The potent apoptotic responses observed in these two divergent genetic models is most likely a result of the ability of BEZ-235 to inhibit mTOR compensatory loops, such as the phosphorylation of AKT upon mTORC1-specific inhibition via insulin receptor substrate-1 (IRS-1) and PI3K-RAS activation of the MAPK pathway (Carracedo et al., 2008; Sarbassov et al., 2006). However, as *in vivo* experiments in both pre-clinical settings and in early-phase clinical trials revealed, broadly inhibiting the PI3K/AKT/mTOR pathway carries significant toxicity risk (manifesting as stomatitis, non-infectious pneumonitis, rash, hyperglycaemia and immunosuppression in clinical trials (Chia et al., 2015)) and, as of 2016, development of BEZ-235 has ceased (Carlo et al., 2016). mTOR inhibitors have been well characterised, but, in contrast to broad-spectrum PI3K/mTOR, have seen limited success as anti-neoplastic agents (Wander et

al., 2011). First generation mTOR inhibitors, based chemically on the canonical mTOR inhibitor rapamycin (known as ‘rapalogs’, e.g. everolimus), which bind the cytosolic protein FK-binding protein 12 (FKBP12) and thus indirectly bind mTORC1, have been FDA-approved for certain classes of cancers such as renal-cell carcinomas (Thorat et al., 2017). In general, however, these compounds have proved to be anti-proliferative, most likely because they do not completely inhibit 4E-BP-dependent translation, and do not silence the AKT-mTORC2 axis, which modulates pro-survival responses within the cell (Hsieh et al., 2012; Tabernero et al., 2008). The activation of autophagy responses upon mTORC1-specific inhibition is a further mechanism by which cells can supply themselves with nutrients, particularly tumour cells with high metabolic rates (Palm et al., 2015). Second-generation inhibitors of mTOR, such as Torin1 and PP242, compete with ATP to bind to the kinase active site, thus inhibiting both the mTORC1 and mTORC2 complexes. Despite this broad inhibitory activity, and in contrast to inhibitors such as BEZ-235 (which also inhibit the PI3K/AKT complexes), evidence suggests that these compounds can be tolerated in the clinical setting (Feldman et al., 2009). An unresolved issue with the chronic use of second generation mTOR inhibitors however is the ability for metabolic reprogramming to occur, by activating AKT in an mTORC2-independent manner and thereby resulting in resistance (Rodrik-Outmezguine et al., 2011). Thus, it is clear that much work remains to be done to interrogate how mTOR complex inhibition can be leveraged in cancer.

Metabolic signals play important roles in determining chromatin structure and the epigenetic state of the cell is closely regulated by metabolic processes (Lu and Thompson, 2012). A classic mechanism previously discussed in chapter 1 is the methylation of DNA and histones by DNA methyltransferases and histone methyltransferases. Here, a methyl group is transferred from *S*-adenosylmethionine (SAM), and a by-product, *S*-adenosyl homocysteine (SAH) is generated (Lu and Thompson, 2012). SAM is generated from the essential amino acid methionine, through a catalytic reaction mediated by methionine adenosyltransferase (MAT). Interestingly, serine-derived one-carbon units are important for the remethylation of homocysteine, and are thus intricately linked in methionine metabolism (Yang and Vousden, 2016). Thus, it is clear that amino acid abundance, either through dietary intake or via biosynthetic mechanisms regulated by axis such as mTORC1/ATF4, is intricately tied with global methylation state. This has been

demonstrated empirically, where cellular availability of SAM was found to be a major factor of aggressive prostate cancer initiation (Reina-Campos et al., 2019).

mTORC1 has been shown to be specifically involved in epigenetic processes including histone acetylation and deacetylation. In yeast, mTORC1 promotes binding of the histone acetyltransferase *Esa1* to ribosomal protein promoters (Rohde and Cardenas, 2003). Silencing of mTORC1 with rapamycin reduced histone H4 acetylation and releases *Esa1* from chromatin, thus ablating transcription of these ribosomal proteins. More broadly, the ability of mTORC1 to regulate transcriptional responses, particularly in nutrient deficient contexts, is an active area of investigation. mTORC1 inhibition has been shown to increase ribosomal DNA (rDNA) binding of the tumour suppressor ING-1, which actively recruits HDAC1 to suppress RNA Pol I transcription via deacetylation of rDNA chromatin (Rajarajacholan et al., 2017). The above findings suggest that mTORC1 can utilise epigenetic mechanisms such as histone acetyltransferase and deacetylase activity to intricately regulate the generation of ribosomal protein mRNA and ribosomal DNA in response to cellular stressors and nutrient abundance.

SETD8, also known as SET8, Pr-SET7 or KMT5A is the only known lysine-specific methyltransferase that catalyses the monomethylation of lysine 20 on histone H4 (denoted as H4K20me1), a epigenetic mark implicated in the regulation of DNA replication, the DNA damage response, and cell cycle/transcriptional regulation (Milite et al., 2016). Aside from the monomethylation of H4K20, SETD8 has been shown to methylate the lysine residues of p53 (p53K382me1) and proliferating cell nuclear antigen (PCNA, at PCNAK248me1) (Shi et al., 2007; Takawa et al., 2012). SETD8-mediated methylation of p53 has been shown to reduce the cell-cycle arrest and pro-apoptotic roles of p53; thus, inhibition of SETD8 relieves this silencing effect and results in increased p53 protein levels and induction of cell death (Veschi et al., 2017).

H4K20me1 levels are carefully choreographed during the cell cycle as well as in response to DNA damage. For instance, SETD8 expression is highest during the early G1 and G2/M phase of mitosis (where the H4K20me1 mark is required for the formation of the pre-replication complex) but is largely lost during S phase (Oda et al., 2009; Tardat et al., 2010; Wu et al., 2010). At double-stranded DNA-breaks (DSBs), PCNA-bound SETD8 is recruited, methylates histones adjacent to the DSB site, and thus acts as a scaffold for

the repair molecule p53-binding protein 1 (53BP1) (Dulev et al., 2014). Genetic depletion of SETD8 prior to induction of DSBs prevents the association of 53BP1, suggesting SETD8-mediated H4K20 monomethylation is critical for DNA repair.

The role SETD8 plays in the regulation of cellular metabolic processes is largely unknown, though there are some examples in the literature. For instance, it has been reported that SETD8 depletion with UNC0379 upregulates cholesterol biosynthesis and the related mevalonate pathway, though the authors did not suggest a mechanism in this context (Veschi et al., 2017). SETD8 has also been implicated in the promotion of glycolytic metabolism by binding and inactivating KLF4 and thus suppressing SIRT4, a known regulator of glycolysis (Chen et al., 2019b). Perhaps most interestingly, SETD8 has been shown to prevent senescence in cancer cells by suppressing nucleolar and mitochondrial oxidative phosphorylation (OXPHOS) via regulation of ribosomal RNA expression (Tanaka et al., 2017). In this context, SETD8 regulated H4K20me1 levels at key ribosomal proteins such as *RPL5* and *RPL27A*, and loss of SETD8 resulted in upregulation of these genes.

In this chapter, potentiation of the modest anti-neoplastic effects of mTORC1 inhibition was addressed by modulating the epigenome. Given that FLT3-ITD was shown to strongly activate mTORC1, bioinformatic approaches were initially used to transcriptionally dissect FLT3-ITD mediated pathways and validate that mTORC1 is hyperactivated in primary AML patient samples in the LAML-TCGA dataset. The efficacy of different types of mTOR/PI3K/AKT inhibitors in FLT3-ITD-positive primary patient samples was examined, as well as in AML cell lines and in the MLL-AF9/iFLT3-ITD mouse model used extensively in this thesis. The utilization of a small molecule compound screen consisting of 181 epigenetic-targeting compounds in combination with the rapalog everolimus in our MLL-AF9/iFLT3-ITD mouse model was implemented. Several small molecule inhibitors that synergise with everolimus were identified and the mechanisms by which these may functionally intersect with the mTOR pathway were assessed.

4.2. Results

4.2.1. FLT3-ITD transcriptionally regulates mTORC1 target genes in patient samples, but inhibition of mTORC1 in this AML subset is therapeutically ineffectual.

In chapter 3 how genetic and pharmacological depletion of FLT3-ITD suppresses key mTORC1 target genes in both murine models of AML and human AML cell lines was described. To further dissect these findings in the context of primary human samples, a bioinformatics approach was utilized to characterise key differentially expressed genes (DEGs) in FLT3-mutant samples versus FLT3-wildtype (WT) samples in the LAML-TCGA dataset. FLT3 mutation status of each of the LAML samples was downloaded from cBioPortal and any patients with loss of function, deletion, or no FLT3 mutation annotation were removed from the analysis. The Broad Institute's GDAC Firehose tool was used to download mRNA count files for each of the LAML samples, which were subsequently annotated with FLT3 mutation status and normalised prior to statistical determination of DEG (FLT3-mutant versus FLT3-WT samples) via the Voom-Limma method (Law et al., 2014, 2016).

Initial analysis yielded 460 downregulated genes, and 176 upregulated genes ($-1 > \log_{2}FC > 1$; $P = 0.01$) (**Figure 4.1A**). Closer investigation of gene ontology (GO) terms associated with upregulated genes revealed a high degree of overlap with well-established mTORC1 functions (**Figure 4.1B**). For instance, in the LAML-TCGA dataset FLT3-ITD expression was concordant with upregulation of important pathways such as central carbon metabolism and metabolism of nucleotides (denoted in blue in the GO-Terms-UP bubble plot), as well as biological processes such as purine deoxyribonucleotide metabolic processes and cellular amino acid metabolic processes. These findings are consistent with the novel roles identified in chapter 3 in which FLT3-ITD drives a metabolic program of heightened one-carbon/folate cycle activity via mTORC1/ATF4-mediated activation of the serine synthesis pathway to drive purine biosynthesis. Further evidence that these GO-terms were primarily associated with mTORC1 activity arises from other enriched processes observed in the analysis, such as intracellular protein transport, protein-containing complex localisation and organelle assembly. These are hallmark characteristics of mTORC1-mediated translation and nutrient utilisation processes (Cuéllar et al., 2019; Liu and Sabatini, 2020).

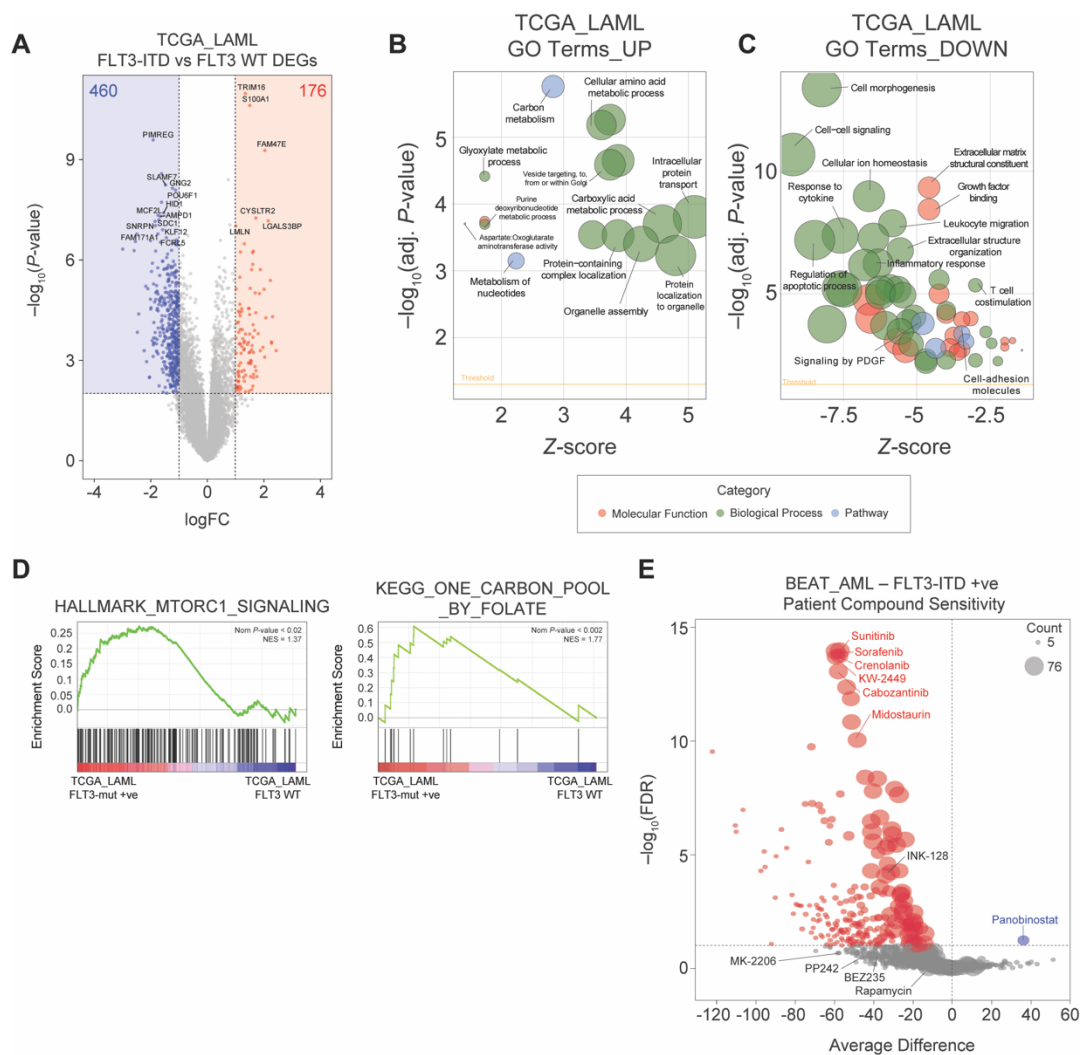


Figure 4.1 FLT3-ITD-mediated hyperactivation of mTORC1 transcriptionally upregulates mTORC1 targets but does not confer sensitivity to mTOR inhibitors.

A. Volcano plot of differentially expressed genes (DEGs) in FLT3-mutant versus FLT3-WT samples in the LAML-TCGA dataset. Shading and number of DEGs is representative of a statistical cut-off of $P < 0.01$, and a $\log_{2}(\text{FC})$ cut-off of $-1 > \log_{2}(\text{FC}) > 1$. **B.** GOBubble plots of upregulated GO-Terms. Bubbles are plotted against a Z-score indicating directionality of the terms, and the negative logarithm of the adjusted P -value on the y-axis. The area of circles is proportional to the number of genes assigned to the term, and colours indicative of category as per the key. **C.** GOBubble plots of downregulated GO-Terms. **D.** Gene set enrichment analysis of DEGs in FLT3-mutant versus FLT3-WT samples in the LAML-TCGA dataset, showing enrichment of mTORC1 signalling targets in the HALLMARK gene set and enrichment of the one-carbon pool by folate KEGG gene set. **E.** Volcano plot of differential compound sensitivities in FLT3-ITD-positive patient samples in the BEAT-AML program. Counts denote number of patients screened.

Examination of GO-Terms associated with FLT3-ITD-mediated gene suppression identified several processes associated with dysregulated cell-to-cell signalling, response to cytokine and growth factor binding, as well as suppression of genes involved in cell morphogenesis, extracellular structure organization, and general downregulation of apoptotic processes (**Figure 4.1C**). These GO-terms can thus be explained by FLT3-ITD-supported transcriptional processes maintaining an undifferentiated cell state; indeed, studies have shown that inhibition of FLT3-ITD using compounds such as quizartinib (used in chapter 3) and gilteritinib can induce terminal differentiation of AML blasts in some contexts (McMahon et al., 2019b; Nybakken et al., 2016).

Gene set enrichment analysis (GSEA) was next performed on the FLT3-ITD DEGs. mTORC1 as well as one-carbon pool by folate signalling was found to be significantly enriched (**Figure 4.1D**) as previously demonstrated in the murine MLL-AF9/FLT3-ITD model. These data are consistent with key findings in chapter 3. Given that mTORC1 signalling was shown to be activated in murine AML models, AML cell lines and primary patient samples, the relative sensitivity of FLT3-ITD mutant AML patient samples to a wide range of inhibitors targeting various components of the mTOR complexes was assessed. Data acquired in the BEAT-AML program, a large multi-centre project in which 562 primary patient samples were screened *ex vivo* with a wide variety of targeted therapies (Tyner et al., 2018) was utilised. Drug sensitivity data was filtered for FLT3-ITD positive patients only, and relative sensitivity to approximately 100 small molecule inhibitors plotted (**Figure 4.1E**). As expected, FLT3/multi-kinase inhibitors such as sunitinib, sorafenib and the FDA-approved multi-kinase inhibitor midostaurin (currently approved for frontline therapy in combination with standard-of-care chemotherapy for FLT3-ITD positive patients (Stone et al., 2017)) were among the highest scoring compounds (denoted in red; average difference refers to difference in sensitivity relative to FLT3-WT patients). Interestingly, a panel of diverse mTOR complex inhibitors showed minimal cytotoxic efficacy in FLT3-ITD-driven patient samples. First generation rapalogs such as rapamycin had almost no effect, while the PI3K/pan-mTOR inhibitor BEZ-235, second-generation ATP-competitive inhibitor of mTORC1/mTORC2 PP242, and pan-AKT inhibitor MK-2206 all showed modest responses. Only the ATP-competitive inhibitor of mTORC1/mTORC2 INK-128 featured more robust clinical responses, however this was an isolated case. These findings demonstrate that although FLT3-ITD mutations potentially activate transcriptional signatures consistent with

mTORC1 hyperactivation, this does not result in greater sensitivity to mTORC1 inhibition.

4.2.2. Pharmacologically diverse PI3K/AKT/mTOR inhibitors display weak cytotoxic responses in human and mouse FLT3-ITD mutant AML cell lines.

Given the analyses above illustrated mTORC1 and its targets are transcriptionally hyperactivated in FLT3-mutant AMLs, but this does not translate to enhanced sensitivity to mTOR inhibitors, further examination of this data in human AML cell lines and the MLL-AF9/iFLT3-ITD mouse model was performed. The Genomics of Drug Sensitivity in Cancer (GDSC) database (Garnett et al., 2012; Iorio et al., 2016; Yang et al., 2013) was used to interrogate the differential sensitivities of several FLT3-ITD-driven human AML cell lines utilised in chapter 3 of this thesis (MV4-11, MOLM-13 and PL-21) to a diverse panel of inhibitors targeting various complexes in the PI3K/AKT/mTOR pathway. Sensitivity plots illustrated most cell lines exhibited only mild sensitivity to these compounds, with complex-specific inhibitors (such as mTORC1-specific rapamycin and temsirolimus) illustrating significantly less cytotoxic potency compared to next-generation multi-complex inhibitors (such as the mTORC1/mTORC2 inhibitor AZD8055 and OSI-027) (**Figure 4.2A**). In these figures, an IC₅₀ Z-score of +2 denotes compounds cells are resistant to, while a score of -2 denotes sensitivity. This data is consistent with previous analyses of primary patient cell sensitivities to this class of inhibitors shown in **Figure 4.1E**.

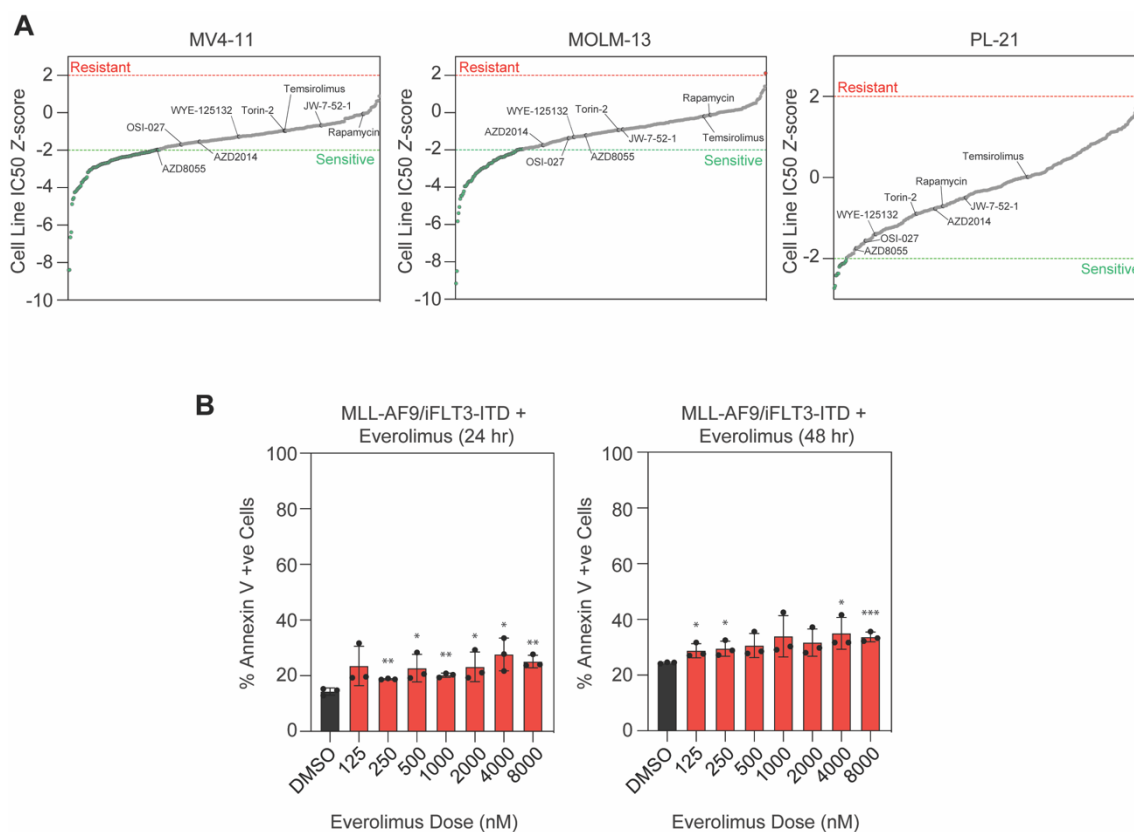


Figure 4.2 Pharmacologically diverse PI3K/AKT/mTOR inhibitors display weak cytotoxic and antiproliferative responses in human and mouse AML cell lines.

A. Sensitivity profiles of the MV4-11, MOLM-13 and PL-21 FLT3-ITD-mutant AML cell lines, ranking relative sensitivity to a panel of anti-neoplastic compounds. IC50 Z-scores of +2 denote resistance to screened compound, while scores of -2 denote sensitivity. Data is sourced from the Genomics of Drug Sensitivity in Cancer (GDSC) database and was originally acquired with resazurin or Syto60 as a viability marker, 72 hours post dosing with compound. Candidate drugs targeting the PI3K/AKT/mTOR pathway are highlighted in each figure. **B.** Murine MLL-AF9/iFLT3-ITD cells were treated with escalating concentrations of everolimus for 24- and 48-hours, and cell viability was determined using Annexin V flow cytometry. Error bars are indicative of $\pm$ s.d. and three biological replicates. Significance was determined by Student's *t*-test relative to DMSO, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Given that many of the screened compounds are in pre-clinical stages of development, and that transcriptional analyses specifically identified mTORC1 as a key target of FLT3-ITD hyperactivation, the clinically approved mTORC1-specific compound everolimus was selected as the inhibitor on which to base further experiments. Leukemia cells derived from the MLL-AF9/iFLT3-ITD were exposed to increasing concentrations of everolimus for 24- and 48-hours (**Figure 4.2B**). At both time points cell death did not exceed ~35%, including at relatively high concentrations approaching 8 μ M even though statistically significant cell death greater than DMSO controls was observed at several individual concentrations. Collectively, these experiments confirm the previous bioinformatically-derived results, in that mTORC1 inhibition does not result in potent cytotoxic responses in FLT3-ITD mutant AML human and mouse cell lines.

4.2.3. Design and optimization of a targeted epigenetic small-molecule sensitization screen to mTORC1 inhibition.

The data obtained to date demonstrated the limited therapeutic benefit of mTORC1 inhibition in FLT3-ITD-driven primary patient samples, human and mouse AML cell lines. Accordingly, methodologies by which FLT3-ITD-driven leukemia cells could be sensitized to mTORC1 inhibition in a synthetically lethal, unbiased fashion were assessed. The intersection between epigenetics and metabolic regulation was of particular focus given that mTORC1 is considered a master metabolic regulator and many downstream pathways, including some directly mediated by mTORC1 such as transcriptional regulation of ribosomal proteins, are dependent on the acetylation status of histone H4 (Rajarajacholan et al., 2017).

A sensitization screen was designed, wherein a library of small molecule inhibitors targeting epigenetic regulators was combined with the mTORC1 inhibitor everolimus to identify agents that sensitize cells to mTORC1 inhibition. As mTORC1 inhibition results in minimal impact on cell viability as demonstrated above, compounds that elicited the greatest difference between single agent and combination treatment were posited to be ideal candidates for subsequent validation. A small molecule screen workflow utilising our MLL-AF9/iFLT3-ITD cells was designed, whereby cells were plated into duplicate 384-well plates, with one arm receiving the epigenetic compound library alone, and the other arm receiving a 4-hour pre-treatment with a fixed concentration of everolimus,

followed by co-culture with the epigenetic library compounds. Cells were then incubated for 48 hours, and endpoint determined using Cell Titer Glo (CTG), an ATP-based viability readout. Candidates for further validation were then determined, as shown in the schematic (**Figure 4.3A**).

The Compounds Australia platform of 181 epigenetic targeting compounds was used, which featured a distribution of clinically available as well as pre-clinical compounds, covering a diverse range of biological functions (**Figure 4.3B**; exact list of compounds can be found in Appendix). The library contained several well characterised epigenetic-targeting compounds (such as DNA methyltransferase, as well as histone acetyl/deacetylase and methyl/demethylase inhibitors), in addition to compounds targeting pathways with emerging epigenetic regulatory properties, such as JAK/STAT signalling, and PIM/Aurora kinases (Liu et al., 2016a). To account for variable target affinity/potency of compound, three concentrations of each compound (0.1 μ M, 1 μ M, 10 μ M) were used for analysis.

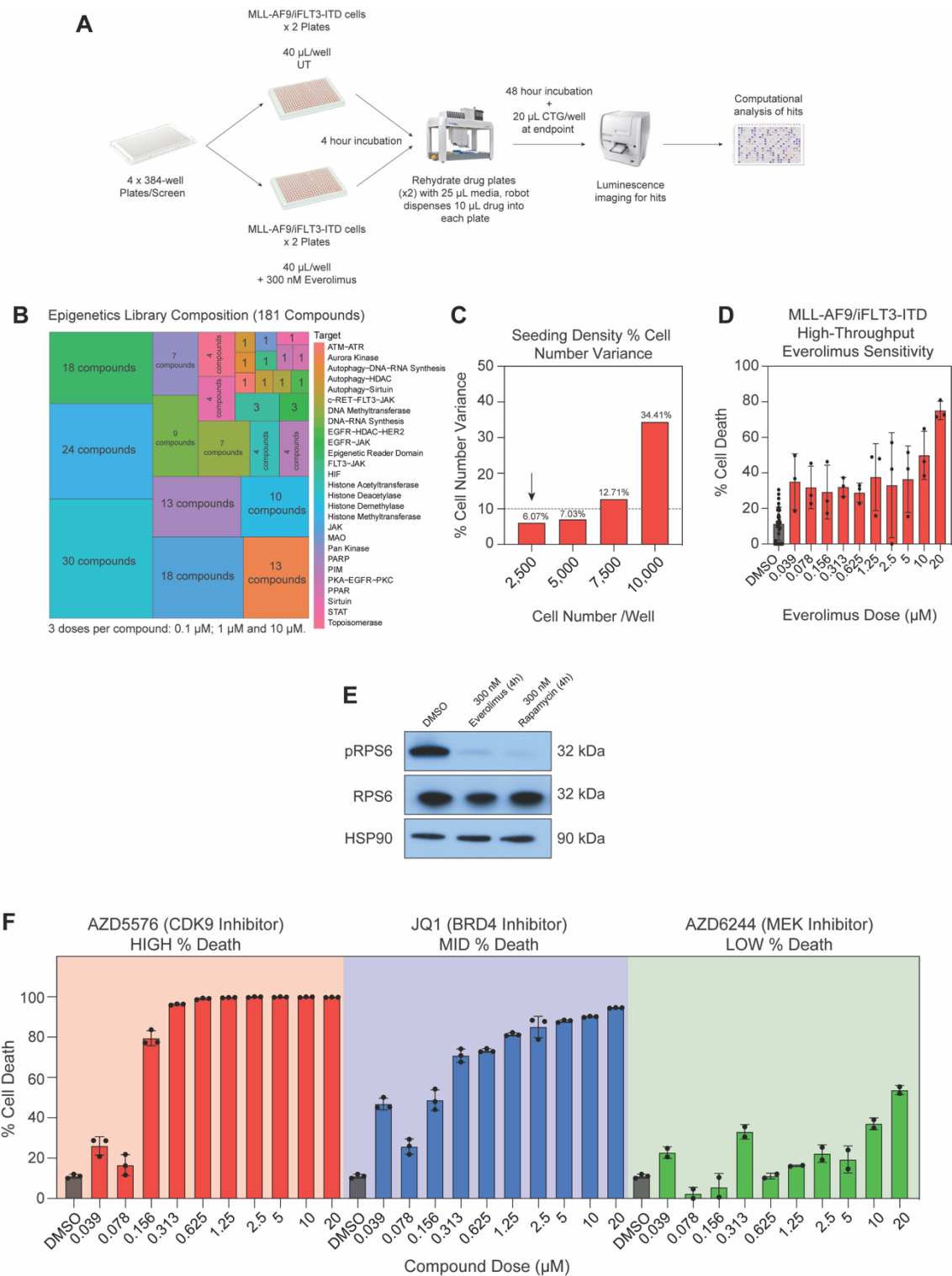


Figure 4.3 Design and optimization of a targeted epigenetic small-molecule sensitization screen to mTORC1 inhibition.

A. Schema of combinatorial epigenetic compound screen approach. 2,500 MLL-AF9/iFLT3-ITD cells were seeded into each well of a 384-well plate in duplicate, with one arm receiving compound library alone, and the other compound library and a fixed dose of everolimus. Plates were incubated for 48-hours, and cell viability determined via Cell Titer Glo (CTG) at endpoint.

B. Heatmap depicting composition of epigenetic compound screen library. 181 compounds were utilised, spanning clinical/pre-clinical compounds and known epigenetic targets, as well as targets with emerging epigenetic regulatory functions. 3 independent concentrations (0.1 μ M, 1 μ M, 10 μ M) of each compound were utilised in the screen.

C. Plot depicting percentage seeding variance of MLL-AF9/iFLT3-ITD cells in replicate wells, and in increasing cell number. Each variance calculation is based on $n = 16$ wells.

D. High-throughput cytotoxicity assay of MLL-AF9/iFLT3-ITD cells exposed to escalating concentrations of everolimus. Cells were incubated in drug for 48-hours prior to viability determination via CTG at endpoint. Error bars denote $\pm$ s.d. and 3 biological replicates for each everolimus dose replicate and 30 biological replicates for DMSO controls.

E. MLL-AF9/iFLT3-ITD cells were treated with DMSO, 300 nM of everolimus, or 300 nM rapamycin for 4 hours, and the phosphorylation status of the canonical mTORC1 target RPS6 determined via Western blot. HSP90 served as the loading control.

F. Cytotoxicity assays of increasing concentrations of AZD5576 (CDK9 inhibitor), JQ1 (BRD4 inhibitor) and AZD6244 (MEK inhibitor) in MLL-AF9/iFLT3-ITD cells. Cells were treated with compound for 48-hours, and viability determined via CTG at endpoint. Error bars denote $\pm$ s.d. and 3 biological replicates.

Excessive initial numbers of cells can result in higher background apoptotic rates, and consequently increased cell number variability (Wright Muelas et al., 2018) highlighting the need to determine optimum seeding density of MLL-AF9/iFLT3-ITD cells for the screen. Escalating numbers of cells were seeded in 384-well plates over 48-hours (low of 2,500 and high of 10,000 cells) and cell viability at endpoint was determined with CTG (**Figure 4.3C**). Calculation of cell number variance showed approximately 34% variability in the 10,000-cell condition, compared to 6% in the 2,500-cell condition, suggesting lower cell numbers per well was the optimum seeding density in this context. Cell number variance > 24% was deemed unacceptable in this screen, as per Compounds Australia specifications. To determine the fixed-dose of everolimus to be utilised in the screen, the previously performed everolimus sensitivity experiments were repeated in a high-throughput format. 2,500 MLL-AF9/iFLT3-ITD cells per well were exposed to escalating concentrations of everolimus in the 384-well format. Consistent with previous experiments detailed in **Figure 4.2**, everolimus treatment resulted in a relatively low degree of cell death with higher concentrations of compound elicited greater cytotoxic responses (**Figure 4.3D**). As relatively low concentrations of everolimus are sufficient to dephosphorylate canonical mTORC1 targets such as ribosomal protein S6 (RPS6) and 4E-BP1 (Breuleux et al., 2009), a concentration of everolimus that did not greatly affect cell viability, while potently suppressing mTORC1 activity was preferred. Thus, 300 nM was selected as an appropriate fixed concentration for combinatorial studies. Validation of this selection via treatment of MLL-AF9/iFLT3-ITD cells with 300 nM everolimus for 4 hours showed complete loss of phosphorylated RPS6 via immunoblot, as did equivalent concentrations of rapamycin, thus confirming this concentration as appropriate in this context (**Figure 4.3E**).

When performing compound-based screening, it is often beneficial to include compounds known to elicit cytotoxic responses in cells of interest to allow for troubleshooting or subsequent comparisons (Hughes et al., 2011). Compounds with validated high, mid-range and low cytotoxic properties in MLL-AF9/iFLT3-ITD cells were used as "compound controls". CDK9 inhibitors have been demonstrated by our lab and others to be potent in MLL-rearranged leukaemias, due to the recruitment of the super elongation complex (which contains CDK9) by MLL fusion complexes (Baker et al., 2016; Boffo et al., 2018). Hence, MLL-AF9/iFLT3-ITD cells were treated with the CDK9 inhibitor AZ5576 (Cidado et al., 2016) and almost complete induction of cell death at a

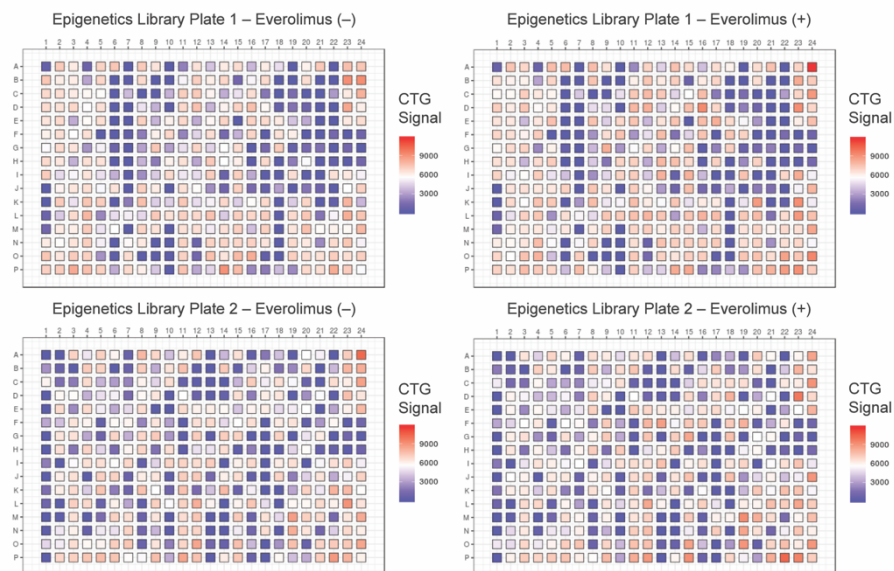
concentration of $\sim 1 \mu\text{M}$ was observed (**Figure 4.3F**). Similarly, bromodomain and extra terminal domain (BET) inhibitors such as JQ1 have shown efficacy in MLL-rearranged leukaemias, after the discovery that BRD4, a BET family member, is recruited by the MLL fusion complex and is required for AML survival (Zuber et al., 2011a). In contrast to AZD5576, which potently induces apoptosis, treatment of MLL-AF9/iFLT3-ITD cells with $\sim 1 \mu\text{M}$ of JQ1 resulted in approximately 70% cell death, and so was utilised as a mid-efficacy control of cell death in our screen. As JQ1 is in itself an epigenetic-targeting compound, JQ1 and other control compounds were dispersed in separate, defined control wells (columns 23 and 24 on all 384-well plates) so as to not confound JQ1 utilised as part of the screen itself. Finally, though FLT3-ITD mutations are known to activate MAPK signalling, kinase inhibitors of this pathway, such as MEK, have shown poor single-agent responses and are thus often combined with other compounds such as tyrosine kinase inhibitors in the context of FLT3-ITD-mutant AML (Bruner et al., 2017). In alignment with those observations, $\sim 1 \mu\text{M}$ of the MEK inhibitor AZD6244 induced negligible cell death over DMSO controls in MLL-AF9/iFLT3-ITD cells. A high concentration ($1 \mu\text{M}$) of everolimus was included as a further control. Collectively, the optimization and utilisation of three compounds of varied potency is an important part of pre-screen planning, providing useful ‘references’ during post-screen analysis and troubleshooting.

4.2.4. Validation and normalization of a targeted epigenetic small-molecule sensitization screen to mTORC1 inhibition

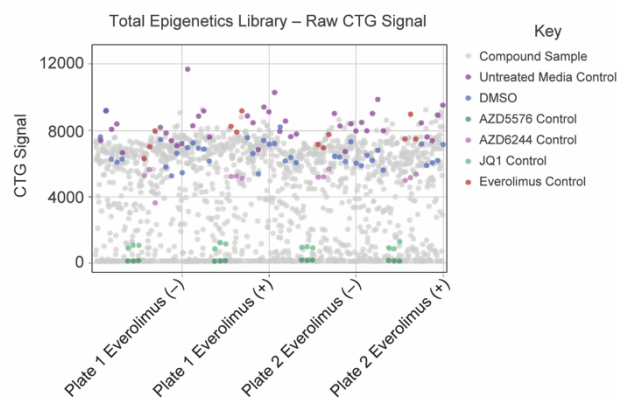
After ensuring adequate cell seeding density, concentration ranges of tested compounds and appropriate control compounds, the screen was performed using MLL-AF9/iFLT3-ITD cells. Heatmaps depicting raw CTG signals for each plate utilised in the screen were generated (**Figure 4.4A**). Given the target epigenetic compounds were seeded randomly and in escalating concentration, the patterns of low/high CTG signals in each plate should be, theoretically, relatively random. Examination of the screen plate heatmaps suggested that none of these phenomena had occurred (**Figure 4.4A**) in either the epigenetic single agent or epigenetic compound + everolimus conditions, suggesting no instrumentation or technical issues that could potentially confound the screen results.

Statistical quality control analysis (mean, standard deviation (SD) and % cell variance (% CV) was next performed across all plates to test the robustness of the data acquired in the screen. After firstly normalising raw CTG signals to the mean of DMSO control wells on a per-plate basis, these calculations were performed on the control wells of each plate. As mentioned previously, a % CV of $> 24\%$ across the screen was deemed unacceptable, with the exception of positive controls (i.e., AZD5576 and or/JQ1; as these compounds induce a high degree of cell death and thus small CTG signal numbers, greater variability in the context of these small numbers is expected). Negative controls, such as media controls, or DMSO controls, should ideally have % CV of $< 15\%$. These calculations are shown in the table below.

A



B



C

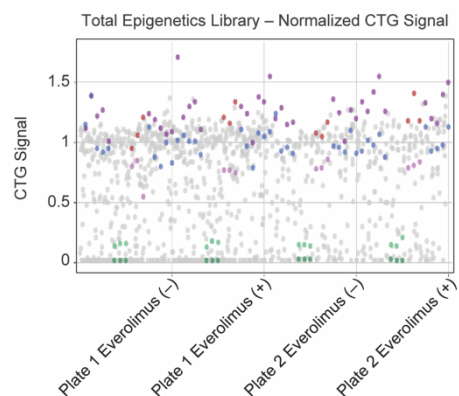


Figure 4.4 Validation and normalization of a targeted epigenetic small-molecule sensitization screen to mTORC1 inhibition.

A. Heatmaps of CTG signal recorded for each well of screened 384-well plates, in the context of single-agent epigenetic compound (left panels) and epigenetic compound in combination with 300 nM everolimus (right panels). Each well is indicative of a different epigenetic compound and dose. CTG signal scale is normalised across all plates. Red denotes higher percentage of live cells; blue denotes greater percentage of dead cells. **B.** Dot plots of all compound raw CTG signals, including controls shaded as per supplied key. Each vertical rectangle is indicative of samples recorded on a single plate with conditions as described on the *x*-axis. **C.** Dot plot of CTG signals from **B.** normalised to DMSO controls.

Table 4.1. Quality control metrics of control wells of epigenetic compound screen.

Treatment indicates whether everolimus was co-cultured with the control drug of interest (Everolimus +) or not (Everolimus –). Normalised CTG mean is calculated by normalising the CTG signal of each well to the mean of the DMSO control wells on each plate. SD, standard deviation; % CV, percent cell variance.

TREATMENT	CONTROL COMPOUND	CONTROL TYPE	CONCENTRATION	NORMALISED CTG MEAN	NORMALISED CTG SD	NORMALISED % CV
Everolimus +	Media Control	Negative	–	1.32	0.16	11.96
Everolimus –	Media Control	Negative	–	1.21	0.13	10.78
Everolimus +	DMSO	Negative	0.01 μ M	1	0.09	8.79
Everolimus –	DMSO	Negative	0.01 μ M	1	0.14	14.4
Everolimus +	AZD5576	Positive	1 μ M	0.02	0	18.84
Everolimus –	AZD5576	Positive	1 μ M	0.02	0.01	21.91
Everolimus +	JQ1	Positive	1 μ M	0.16	0.03	18.02
Everolimus –	JQ1	Positive	1 μ M	0.15	0.01	5.96
Everolimus +	AZD6244	Positive	1 μ M	0.79	0.03	4.12
Everolimus –	AZD6244	Positive	1 μ M	0.77	0.11	14.69
Everolimus +	Everolimus	Positive	1 μ M	1.25	0.1	8.27
Everolimus –	Everolimus	Positive	1 μ M	1.09	0.09	8.53

All negative and positive controls were under the required variability thresholds – interestingly, even positive controls for which CV values > 24% are expected were below this threshold, suggesting the data is robust and statistical variability among samples should not confound the results of the screen.

Raw CTG signals of compound samples and controls in a dot plot format for each plate in the screen were next plotted (**Figure 4.4B**). Importantly, controls all yielded expected results, with positive controls universally inducing potent cell death (AZD5576 and JQ1, shown as shades of green at the bottom of the plot) and negative controls, including the weakly cytotoxic control compound AZD6244, clustering with media and DMSO controls. These trends were further maintained when all samples were normalised to DMSO as described above (**Figure 4.4C**). Interestingly, greater than half of the screened epigenetic compounds induced minor, or indeed negligible, cytotoxic effects even when combined with everolimus (**Figure 4.4B** and **Figure 4.4C**).

4.2.5. A targeted epigenetic small-molecule sensitization screen to mTORC1 inhibition identifies synergistic and antagonistic interactions.

To determine synergistic interactions between an epigenetic compound and everolimus, the % delta (percent difference, Δ) between normalised CTG of epigenetic compound + everolimus versus epigenetic compound alone was calculated on a well-by-well basis. A threshold of $\pm \Delta 20\%$ was applied to the screen, such that interactions $< 20\%$ were deemed synergistic and $> 20\%$ deemed antagonistic. 8 compounds were deemed to act synergistically with everolimus, while 46 compounds were deemed to act antagonistically when combined with everolimus (**Figure 4.5A**). Interestingly, 3 of the top 5 synergistic hits were compounds belonging to the histone deacetylase (HDAC) family of inhibitors which have recently been demonstrated to synergise with mTORC1 inhibition by greatly increasing oxidative stress via the thioredoxin antioxidant pathway and co-operative effects on chromatin and transcription (Malone et al., 2017) (**Figure 4.5B**). This combination is currently also under investigation in phase 1 clinical trials in the context of clear cell renal cell carcinoma (Wood et al., 2020). Additionally, another high scoring compound hit, SP2509, a specific inhibitor of the lysine-specific histone demethylase 1A (KDM1A, more commonly known as LSD1) was recently shown to synergise with mTORC1 inhibition by transcriptionally potentiating cell differentiation programs in *MLL*-driven AML (Deb et al., 2020). These examples provide further evidence of the reliability of the screen, given several of the top hits of the screen had been validated previously as synergising with mTORC1 inhibition. Moreover, examination of the top 10 most synergistic compounds revealed two primary classes of inhibitor that scored more than once: histone deacetylase inhibitors (3 compounds) as described above, and inhibitors of the Janus kinase (JAK) family (2 compounds) (**Figure 4.5C**).

Interestingly, several members of the epigenetic reader domain family of BET inhibitors demonstrated antagonistic interactions when combined with everolimus, including BI-7273, a bromodomain containing protein 9 (BRD9)-targeting compound, and MS436, a pan BRD2/4 inhibitor (**Figure 4.5B**). A recent study reported that PIK3CA (the catalytic subunit of PI3K and a member of the PI3K/AKT/mTOR pathway) and KRAS mutations upregulate BRD9 and MYC target genes in breast epithelial cancers (Bell et al., 2019). Another study showed that BET inhibitors can disassociate BRD4 from chromatin and key PI3K-regulated genes, including insulin receptor and EGFR family RTKs

(Stratikopoulos et al., 2015). However, in both of these studies, dual PI3K and BET inhibition proved synergistic and elicited antiproliferative/apoptotic responses. Thus, it is likely that the antagonistic interactions identified in the screen are cell and context dependent.

As three independent concentrations were used for each compound in the screen (10 μ M, 1 μ M and 0.1 μ M) and each point of **Figure 4.5A** denoted a single concentration of a compound, it was determined if multiple concentrations of the same compound demonstrated similar synergistic interactions. This analysis would allow for determination of optimal concentration ranges for a subsequent validation screen. Among the synergistic compounds, 10 μ M exhibited greatest synergy with everolimus in all of the compounds, with the exception of trichostatin A (TSA, a HDAC inhibitor), in which the 0.1 μ M concentration scored (**Figure 4.6A**). As many of the compounds utilised in the screen are in pre-clinical development and do not exhibit optimised pharmacodynamic/pharmacokinetic properties *in vitro*, this result was not greatly surprising. The scoring concentrations among the compounds which demonstrated antagonistic interactions with everolimus were more varied, with 0.1 μ M scoring in 2 compounds, 1 μ M scoring for 1 compound, and 10 μ M scoring for 2 compounds (**Figure 4.6B**). As the aim of this chapter was to discover interactions which potentiate mTORC1 inhibition, subsequent analysis concentrated on further characterising the top scoring synergistic hits by means of a secondary validation screen.

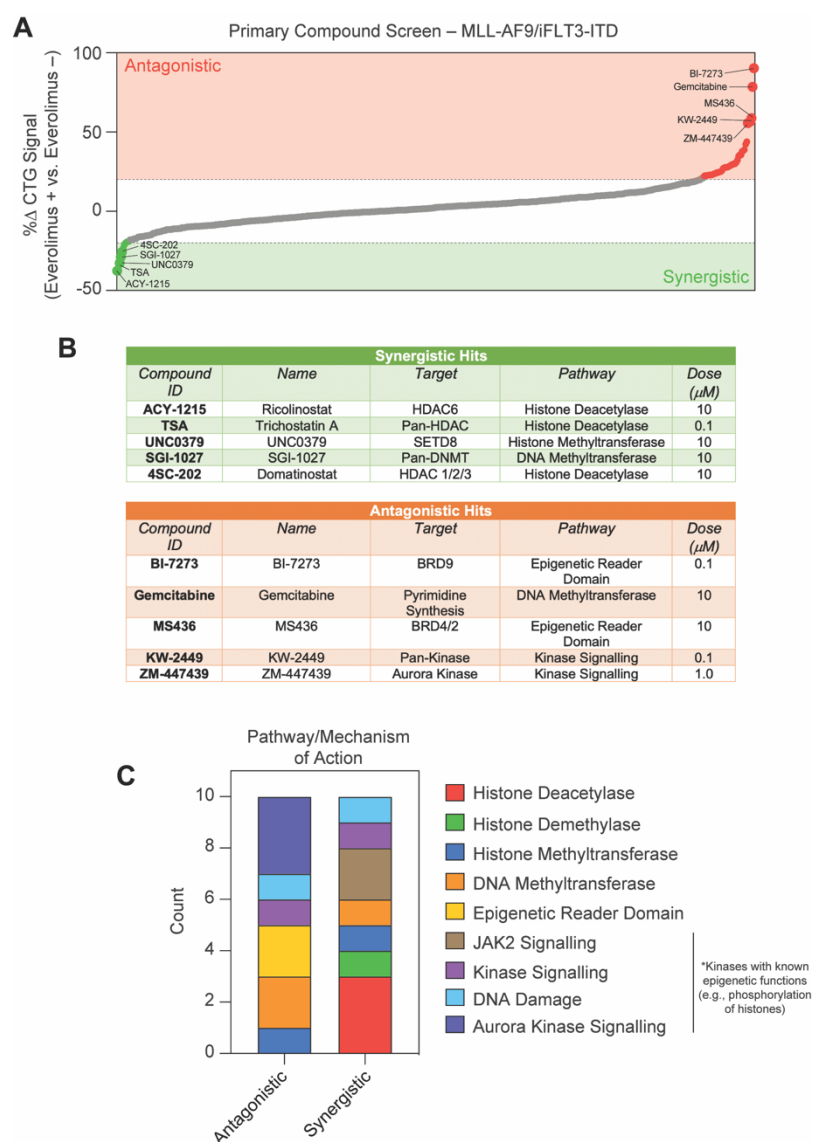


Figure 4.5 A targeted epigenetic small-molecule sensitization screen to mTORC1 inhibition identifies synergistic and antagonistic interactions.

A. Ranking of epigenetic compounds according to % Δ of CTG signal (combination of epigenetic compound with everolimus minus epigenetic compound alone). Each dot is representative of one dose of a specific inhibitor. Interactions that yielded a % Δ of < 20% were deemed synergistic ($n = 8$ total; green shading, top 5 compounds labelled) and interactions that yielded a % Δ of > 20% were deemed antagonistic ($n = 46$ total; red shading, top 5 compounds labelled). **B.** Tables of top 5 scoring synergistic/antagonistic compounds, including compound ID, name, molecular target, pathway, and scoring dose. **C.** Cigar plots depicting pathways/mechanisms of action associated with top 10 scoring antagonistic and synergistic compounds.

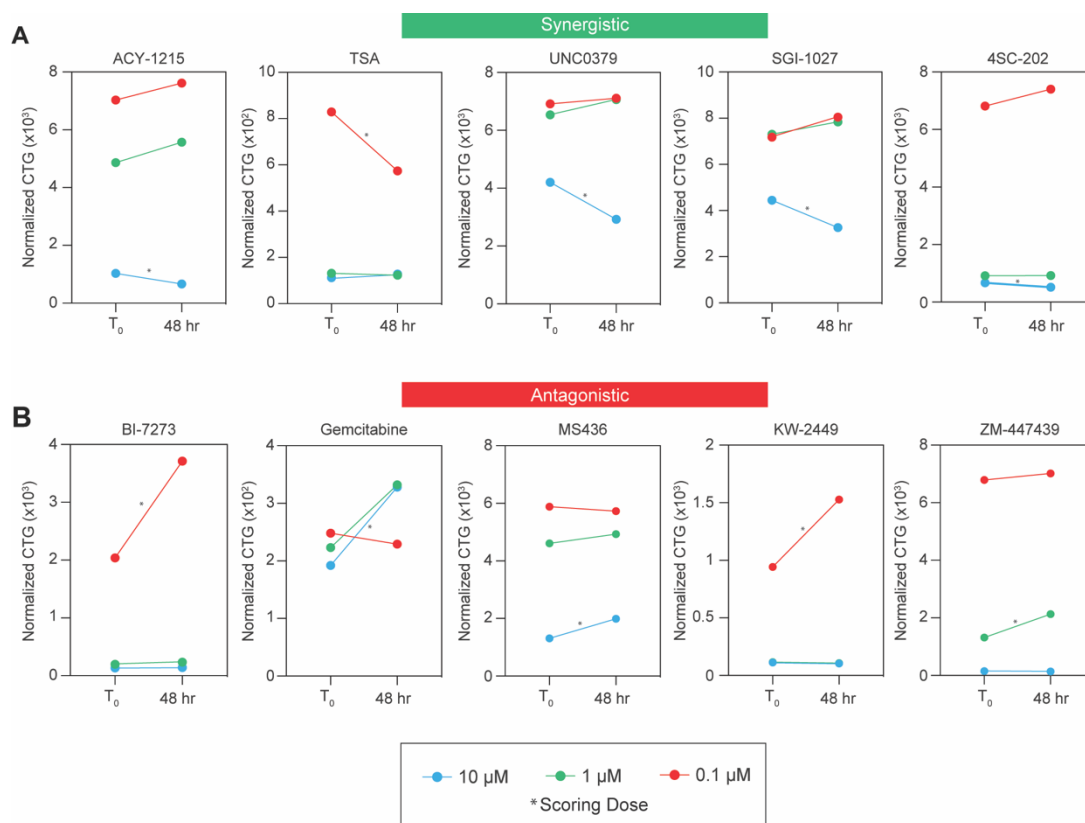


Figure 4.6 Analysis of top 5 most synergistic/antagonistic hits.

A. Normalized CTG at start (T₀) and endpoint (48 hr) of screen, depicted for each individual dose: 10 μM (blue), 1 μM (green) and 0.1 μM (red), of the top 5 most highly scoring synergistic compounds. The dose that scored most highly in the compound screen is marked *. **B.** Normalized CTG at start (T₀) and endpoint (48 hr) of screen, depicted for each individual dose as described above for the top 5 most highly scoring antagonistic compounds. The dose that scored most highly in the compound screen is marked *.

4.2.6. Validation screen of epigenetic small molecules that sensitize to mTORC1 inhibition.

Given that several of the highest-scoring synergistic compounds, including HDAC and LSD1 inhibitors have been previously reported to synergise with mTORC1 inhibition, the secondary screen focussed on novel compounds where no synergy with mTORC1 inhibition had been previously reported. 5 compounds, including UNC0379 (an inhibitor of lysine-specific methyltransferase 5A [KMT5A], more commonly known as SETD8); SGI-1027 (a pan-DNA methyltransferase inhibitor); NVP-BSK805 (a JAK2 inhibitor); Fedratinib (a JAK2 inhibitor); and Pirarubicin, a classic DNA damaging chemotherapy agent were selected for secondary screening. Given that many of these compounds

exhibited optimal synergy at higher concentrations the secondary screen was designed to capture a greater range of drug concentrations (12.6 μ M, 7.2 μ M, 3.6 μ M, 1.5 μ M, 0.7 μ M). The screen itself was otherwise performed in exactly the same format as previously described in the schematic in **Figure 4.3A**, using MLL-AF9/iFLT3-ITD cells. Given extensive validation of the optimal dose of everolimus prior to the first screen, the fixed 300 nM concentration was retained to ensure consistency in the validation screen.

The secondary screen revealed potent synergistic interactions between everolimus and UNC0379 (**Figure 4.7A**), SGI-1027 (**Figure 4.7B**) and NVP-BSK805 (**Figure 4.7C**), and weaker synergistic interactions with Fedratinib and Pirarubicin (**Figure 4.7D-E**) in MLL-AF9/iFLT3-ITD cells. Interestingly, in the single agent UNC0379 context, none of the screened compound concentrations, with the exception of the highest (12.6 μ M) elicited any antitumour effects (**Figure 4.7A**). However, the addition of 300 nM everolimus at all concentrations significantly reduced cell viability, even at low, sub 5 μ M ranges (**Figure 4.7A**). Similarly, single agent SGI-1027 treatment elicited minor cell death at low concentrations that did not increase with escalating drug concentration; the addition of 300 nM everolimus subsequently sensitized cells to SGI-1027 (**Figure 4.7B**). MLL-AF9/iFLT3-ITD cells were sensitive to single agent JAK2 inhibition with NVP-BSK805/Fedratinib and subsequent everolimus combination therapy further induced potent cell death (**Figure 4.7C** and **Figure 4.7D**). While less synergy was observed between Fedratinib and Pirarubicin with everolimus, both of these compounds had potent single agent effects, thus reducing the dynamic range in which synergistic interactions could be observed (**Figure 4.7D** and **Figure 4.7E**). Repeating this experiment with lower concentrations of these two compounds would potentially yield greater synergistic interactions. These data support the findings of the initial compound screen, and suggest that UNC0379, SGI-1027, NVP-BSK805 and potentially Fedratinib and Pirarubicin synergise with mTORC1 inhibition to induce anti-tumour responses.

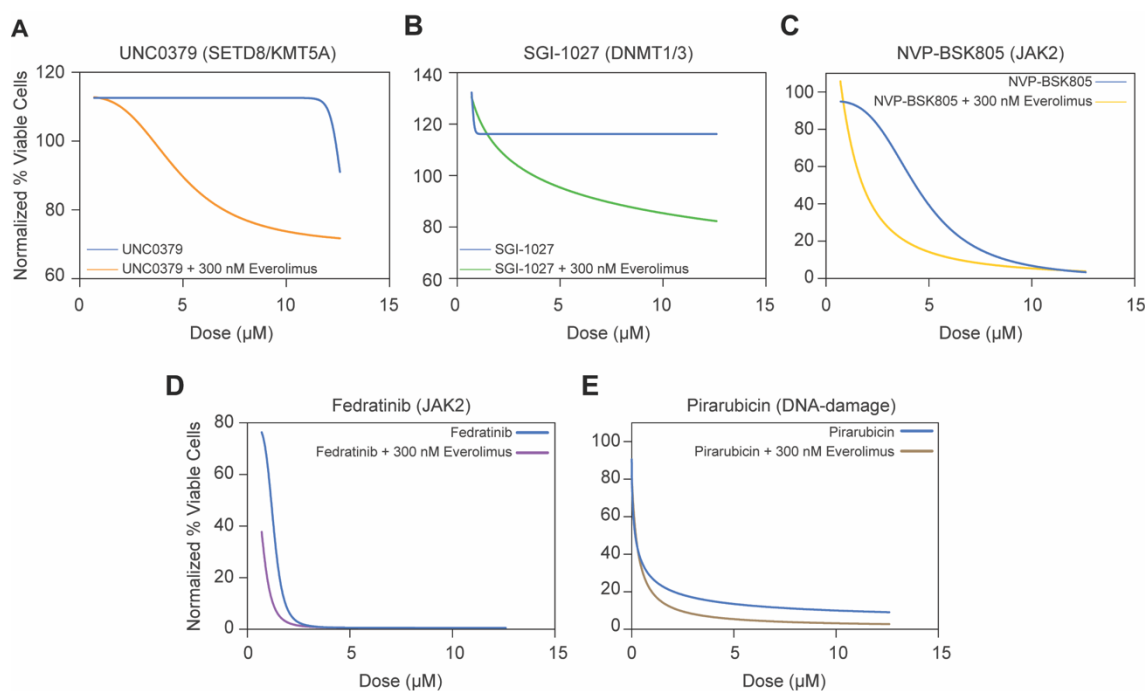


Figure 4.7 Secondary validation screen dose-response relationships between epigenetic compounds and everolimus-mediated mTORC1 inhibition.

A-E. Dose-response curves for highest-scoring compounds deemed to synergise with mTORC1 inhibition in the primary validation screen described above. In all graphs, blue is indicative of epigenetic single agent compound efficacy. Dose (μM) refers to concentration of single agent compound used at five dose points ($12.6 \mu\text{M}$, $7.2 \mu\text{M}$, $3.6 \mu\text{M}$, $1.5 \mu\text{M}$, $0.7 \mu\text{M}$). In all instances, combination therapy (colour as indicated in the legend of each graph) is determined by the addition of 300 nM everolimus to each dose of the single agent compound. Viability is shown as normalised % viable cells. **A.** UNC0379. **B.** SGI-1027. **C.** NVP-BSK805. **D.** Fedratinib. **E.** Pirarubicin.

4.2.7. Inhibition of SETD8 potentiates mTORC1 inhibition.

The potent combinatorial responses observed following dual UNC0379/everolimus treatment and the relative lack of literature on the molecular target of UNC0379, SETD8, provided a rationale for subsequent analyses on the mechanistic basis underlying the synergy between SETD8 and mTORC1 inhibition. As discussed in chapter 4.1, mTORC1 is a master regulator of ribosome biogenesis (Gentilella et al., 2015) and SETD8 has been shown to suppress ribosomal genes such as *RPL5* and *RPL27A*. Why ribosomal proteins, and other mTORC1-regulated pathways (such as cholesterol metabolism) are specifically dependent on SETD8/H4K20me1 remains unknown.

Additional validation experiments were performed to strengthen the initial observation regarding synergy between inhibition of mTOR and inhibition of SETD8. In the initial compound screen and subsequent validation experiments, only one mTORC1 inhibitor, everolimus, was used. Combinatorial assays *in vitro* using a fixed dose of several chemically distinct mTOR inhibitors were subsequently performed to validate synergistic effects with escalating concentrations of UNC0379. Treatment of MLL-AF9/iFLT3-ITD cells with the prototypical mTORC1 inhibitor rapamycin and UNC0379 largely phenocopied the combinatorial effects seen using everolimus (**Figure 4.8A**). PP242, a selective ATP-competitive inhibitor of mTORC1 and mTORC2 demonstrated similarly potent combinatorial responses with UNC0379 (**Figure 4.8A**). Interestingly, culture of MLL-AF9/iFLT3-ITD cells with GDC-0941, a PI3K α/δ inhibitor or cobimetinib, an inhibitor of MEK, demonstrated significantly less combinatorial responses with UNC0379, suggesting the synergistic effects seen between dual SETD8/mTORC1 inhibition are mTORC1-specific. Given both the primary and secondary validation screen used fixed concentrations of everolimus (300 nM), further synergy assays using UNC0379 at different concentrations were performed. MLL-AF9/iFLT3-ITD cells were cultured in escalating concentrations of UNC0379 (to a maximum of 20 μ M) and everolimus (to a maximum of 1 μ M) either alone or in combination for 48 hours. At endpoint, cells were harvested and stained with Annexin V to determine cell death via flow cytometry, and the SynergyFinder computational package was used to determine synergistic interactions between the two compounds (Ianevski et al., 2017b) (**Figure 4.9A**).

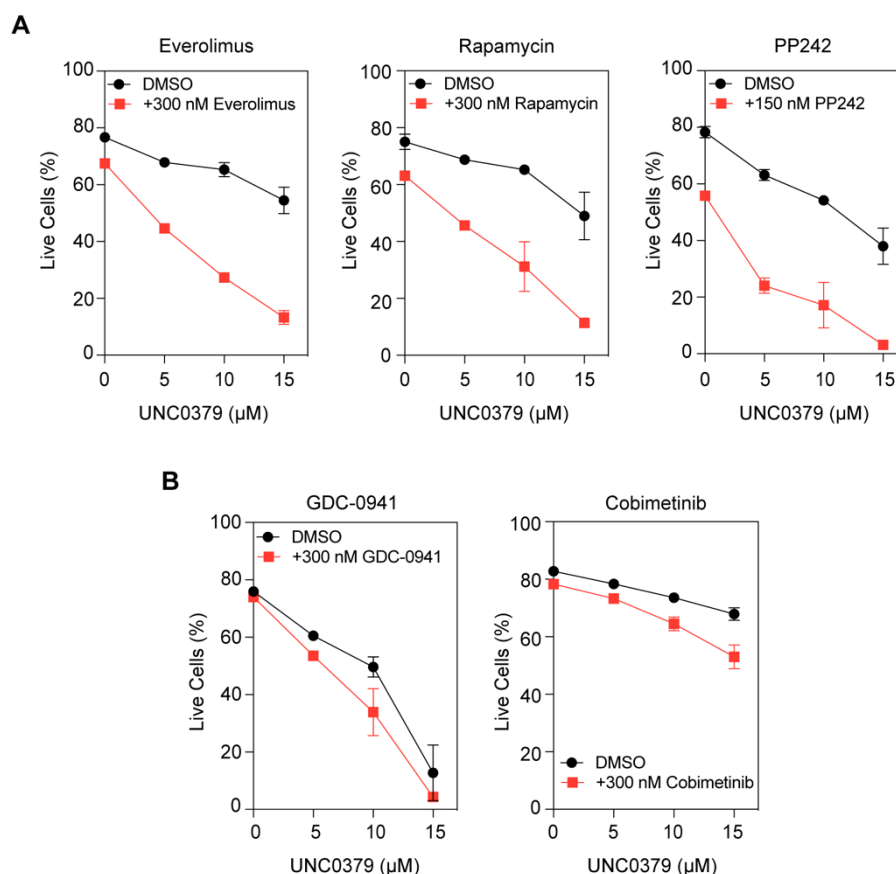


Figure 4.8 Combinatorial effects of UNC0379 and chemically distinct mTOR, PI3K and MEK inhibitors.

A. Escalating concentrations of UNC0379 and a fixed dose (as indicated) of the chemically distinct mTOR inhibitors everolimus, rapamycin and PP242 were cultured with MLL-AF9/iFLT3-ITD cells for 48 hours and cell death assessed via Annexin V flow cytometry. Y-axis depicted as % live cells (Annexin V -ve). **B.** Escalating concentrations of UNC0379 and a fixed dose (as indicated) of the PI3K inhibitor GDC-0941 or MEK inhibitor cobimetinib were cultured with MLL-AF9/iFLT3-ITD cells for 48 hours and cell death assessed via Annexin V flow cytometry. Error bars represent $\pm$ s.d., and 3 biological replicates at each dose point.

This analysis revealed potent synergistic interactions using the Zero Interaction Potency (ZIP) computational model (Yadav et al., 2015) (ZIP synergy index = 15.8) at 300 nM of everolimus and both low (1.25 μ M) and high (10 μ M) concentrations of UNC0379 (**Figure 4.9B**, observed as regions of red), consistent with the dose-response curves generated during secondary validation screening (**Figure 4.7A**). Whilst discrete regions of synergy were detected at higher concentrations of everolimus (notably at 1000 nM),

the remaining experiments were performed using the 300 nM concentration to avoid potential off-target effects of the compound. Thus, these experiments provide strong evidence that the combinatorial effects observed between UNC0379 and everolimus are mTOR-specific and confirm that the 300 nM concentration of everolimus used during primary and secondary screening is suitable for further experiments.

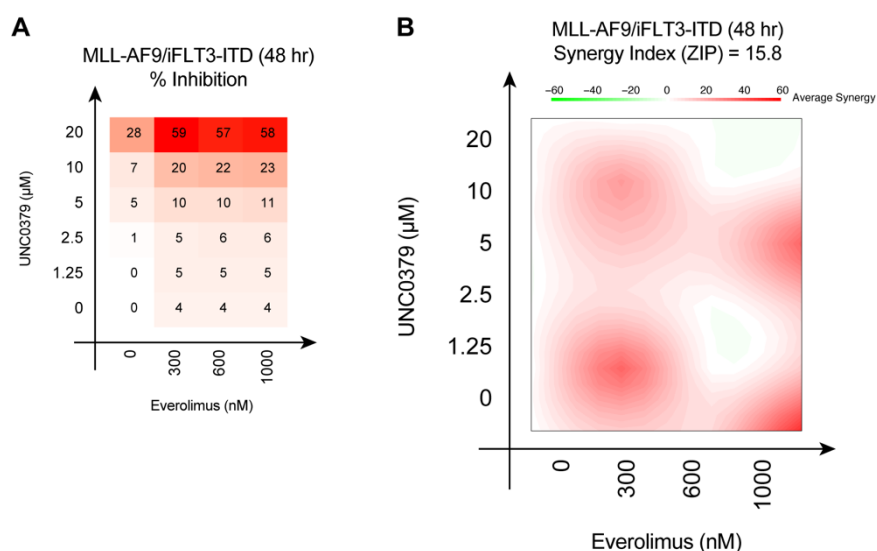


Figure 4.9 Synergy analysis between UNC0379 and everolimus.

A. MLL-AF9/iFLT3-ITD cells were treated with escalating concentrations of UNC0379 and everolimus for 48 hours to determine the effect on viability, as determined by Annexin V flow cytometry. The mean of two biological replicates was used to determine each data point and was normalised to DMSO (0,0) controls. **B.** The presence of treatment synergy was determined using the SynergyFinder computational package and the Zero Interaction Potency (ZIP) synergy index (ZIP = 15.8) and is denoted by regions of red on the graphs.

4.2.8. Optimisation of conditions for transcriptional profiling in MLL-AF9/iFLT3-ITD cells exposed to UNC0379 and everolimus.

To determine the transcriptional effects of dual UNC0379 and everolimus treatment in MLL-AF9/iFLT3-ITD cells, preliminary experiments were initially performed to optimise the conditions for subsequent RNA-sequencing, such as optimal concentration of compound and duration of drug treatment. MLL-AF9/iFLT3-ITD cells were treated with escalating concentrations of UNC0379 for 24 and 48 hours and Western immunoblot analysis was performed for the H4K20me1 mark catalysed by SETD8. This analysis

revealed that at the 24-hour time point, 10 μ M was the lowest concentration of UNC0379 that potently ablated H4K20me1 levels; at 48 hours, this was increased to 20 μ M, suggesting UNC0379 efficacy reduces with time *in vitro* (**Figure 4.10A**). Given transcriptional analysis should be performed at earlier time points to negate the effects of off-target or secondary transcriptional events, the 10 μ M concentration dynamics were further interrogated by performing a time course experiment. MLL-AF9/iFLT3-ITD cells were treated with 10 μ M UNC0379, and protein lysates harvested at 1, 2, 4, 6, 8, 16- and 24-hours post treatment. H4K20me1 levels were rapidly depleted 1-hour post treatment, almost completely abolished at 4-6 hours, and increased 8-24 hours post treatment (**Figure 4.10B**). These biochemical experiments suggested that 10 μ M of UNC0379 almost completely depletes H4K20me1 levels at 4-6 hours, and, while there is some increase in H4K20me1 levels at the 24-hour time point, this is significantly less than in DMSO-treated controls.

A series of experiments were designed to interrogate the transcriptomic response in MLL-AF9/iFLT3-ITD cells to either DMSO, 10 μ M UNC0379, 300 nM everolimus, or a combination of both UNC0379 and everolimus for either 4 hours (representing ‘acute’ treatment) or 24 hours (representing ‘chronic’ treatment). At the appropriate endpoint, cells were harvested, washed, and RNA extracted with TriZol reagent prior to preparation of 3’-RNA QuantSeq libraries. Given that acute 4-hour treatment with UNC0379 and everolimus is likely an insufficient timeframe to trigger apoptotic responses that can confound transcriptional analyses, viable cells were not selected at this time point; however, cells treated for 24 hours were sorted for viable cells to minimise the impact of apoptotic responses confounding subsequent RNA-sequencing analysis, despite the fact that cell viability remained relatively high post combination therapy at 48 hours at this concentration (approximately 70%) (**Figure 4.9A**).

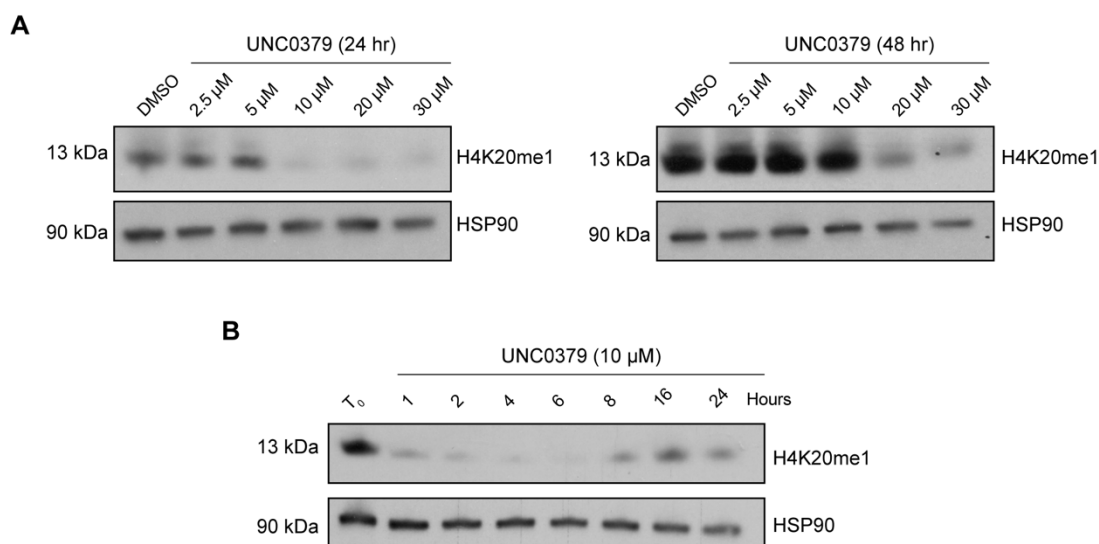


Figure 4.10 Biochemical investigation of UNC0379-induced H4K20me1 dynamics.

A. MLL-AF9/iFLT3-ITD cells were treated with escalating concentrations of the SETD8 inhibitor UNC0379 as indicated for 24 hours (left panel) and 48 hours (right panel), and H4K20me1 levels assessed via Western blot. HSP90 served as the loading control in both experiments. **B.** MLL-AF9/iFLT3-ITD cells were treated with 10 μM of UNC0379 and protein lysates harvested at the indicated time points prior to assessment of H4K20me1 levels by Western blot. HSP90 served as the loading control.

4.2.9. Dual SETD8 and mTORC1 inhibition suppresses mTORC1 target genes and pathways.

To evaluate the effect of combined SETD8 and mTORC1 inhibition, 3'-RNA sequencing data from MLL-AF9/iFLT3-ITD cells treated with DMSO, 10 μM UNC0379, 300 nM everolimus, or a combination of both UNC0379 and everolimus for 4 hours or 24 hours was analysed (**Figure 4.11A**). A total of 48 differentially expressed genes (DEGs) (12 downregulated and 36 upregulated, $-1 > \log_{2}FC > 1$ and $P < 0.05$) were detected after 4 hours of single-agent everolimus treatment, whilst no DEGs which passed this statistical cut-off were determined after 4 hours of single-agent UNC0379 treatment. Interestingly, the combination of UNC0379 and everolimus yielded 213 DEGs (79 downregulated and 134 upregulated, $-1 > \log_{2}FC > 1$ and $P < 0.05$), a significantly greater number than either single agent alone. Among the downregulated genes, 7 were differentially expressed in both everolimus and UNC0379 + everolimus treated combination treatment, whilst 72 genes were unique to the combination of UNC0379 and everolimus (**Figure 4.11B**). Among upregulated genes, 31 were differentially expressed in both everolimus and

UNC0379 + everolimus conditions, whilst 103 genes were unique to the combination condition (**Figure 4.11B**). Hierarchical cluster analysis of genes that passed the $-1 > \log_{2}FC > 1$ and $P < 0.05$ statistical cut-off in at least one of the three treatment groups ($n = 310$, duplicates removed) revealed distinct clusters of genes both downregulated and upregulated following culture in UNC0379 and everolimus to a greater extent than the effects of either single agent (**Figure 4.11C**). Interestingly, among the genes suppressed in the combination treatment group were branched chain amino acid transaminase 1 (*Bcat1*), Chac glutathione specific gamma-glutamylcyclotransferase 1 (*Chac1*), and DNA damage inducible transcript 4 (*Ddit4*) (**Figure 4.11D**). The expression of *Bcat1*, the first enzyme in the branched-chain amino acid degradation pathway which catalyses the transamination of branched chain amino acids, has been linked to mTORC1 activity in several cancer types, though the precise mechanisms appear to be context specific (Hattori et al., 2017; Zhang and Han, 2017). *Chac1* and *Ddit4*, as discussed in chapter 3, are known target genes of the master metabolic transcriptional regulator *Atf4*. As all three of these genes do not pass the statistical cut-off for DEGs in either single-agent treated condition but do in the combination-treated group, dual SETD8/mTORC1 inhibition was proposed to specifically suppresses mTORC1 target genes and pathways, resulting in enhanced transcriptional repression of these genes and potentially greater rates of protein turnover. This contention is supported by the fact that *Atf4* itself is a known target of mTORC1 and is primarily regulated at the post-translational level with a half-life of approximately 30-60 minutes (Ameri et al., 2004; Frank et al., 2010). Thus, 4-hour dual SETD8/mTORC1 inhibition may result in a faster rate of *Atf4* turnover, and subsequently greater transcriptional repression of *Chac1* and *Ddit4*. As alluded to earlier in this chapter, this effect could thus be H4K20me1-dependent.

These findings were extended by examining the effect of ‘chronic’ SETD8/mTORC1 inhibition for 24 hours. MLL-AF9/iFLT3-ITD cells were treated with DMSO, 10 μ M UNC0379, 300 nM everolimus, or a combination of both UNC0379 and everolimus for 24 hours and viable cells sorted at endpoint prior to RNA extraction to remove dead cells (**Figure 4.12A**). As expected, a significantly greater number of differentially expressed genes were observed at this timepoint compared to the 4-hour timepoint. A total of 186 DEGs (50 downregulated upregulated and 136 upregulated, $-1 > \log_{2}FC > 1$ and $P < 0.05$) were detected after 24 hours of single agent everolimus treatment, whilst a total of 111

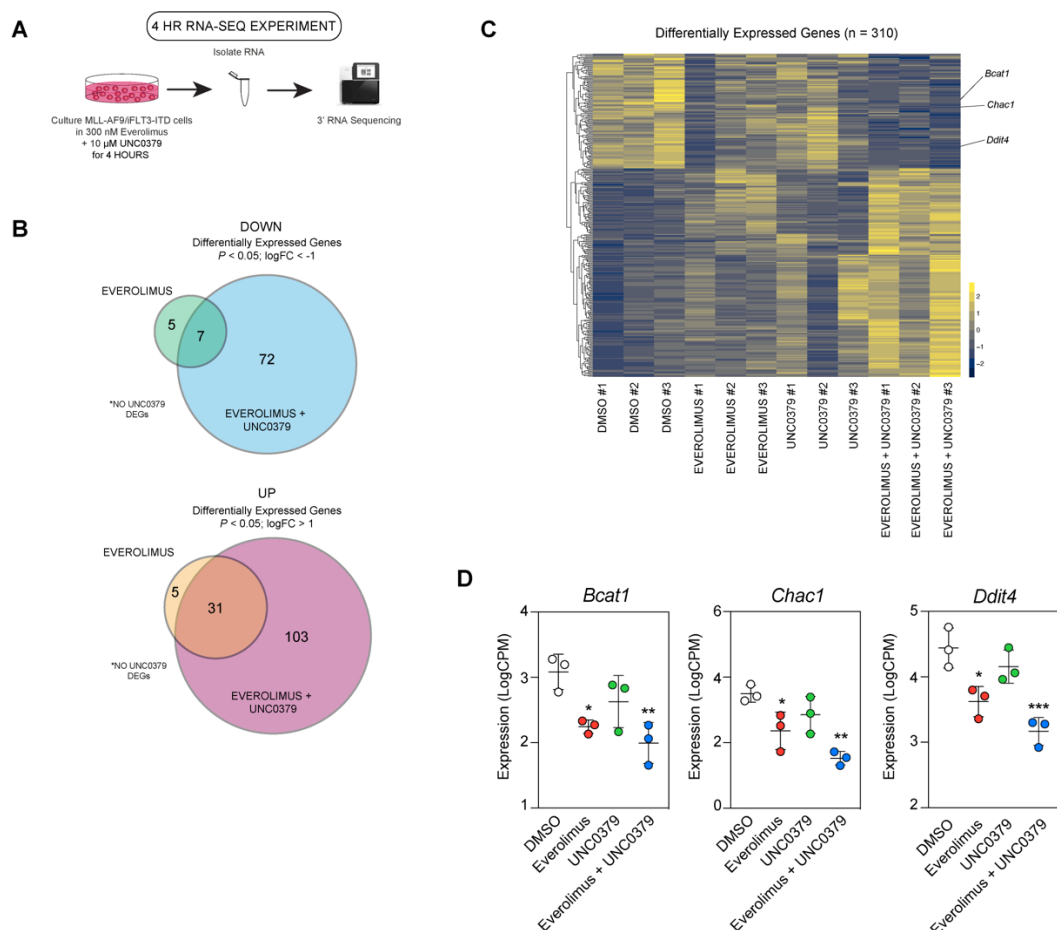


Figure 4.11 Acute SETD8 and mTORC1 inhibition combinatorially suppresses mTORC1 target genes and pathways.

A. Schematic of experiment. MLL-AF9/iFLT3-ITD cells were treated with either 10 μ M UNC0379, 300 nM everolimus, or a combination of both, for 4 hours. Cells were harvested, washed with PBS, and RNA extracted prior to 3'-RNA QuantSeq. **B.** Venn diagrams of downregulated (top panel) and upregulated (bottom panel) DEGs in response to UNC0379, everolimus or a combination of both. A statistical cut-off of $-1 > \log FC > 1$ and $P < 0.05$ was applied. There were no DEGs that met this statistical cut-off for the UNC0379 treatment group at this timepoint. **C.** Heatmap of all genes that pass the $-1 > \log FC > 1$ and $P < 0.05$ statistical cut-off in at least one treatment condition ($n = 310$). Duplicates are removed. *Bcat1*, *Chac1* and *Ddit4* are highlighted. **D.** Dot plots of log counts per million (CPM) gene expression values for *Bcat1*, *Chac1* and *Ddit4* in response to DMSO, everolimus, UNC0379 or a combination treatment of both. * denotes $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ as determined by one-way ANOVA with Dunnett's multiple comparisons test relative to DMSO. Each drug condition is representative of 3 biological replicates.

DEGs (75 downregulated and 36 upregulated, $-1 > \log FC > 1$ and $P < 0.05$) were detected after 24 hours of single agent UNC0379 treatment. The combination of UNC0379 and everolimus yielded 637 DEGs (307 downregulated and 330 upregulated, $-1 > \log FC > 1$ and $P < 0.05$) a significantly greater number than either single agent alone. Among downregulated genes, 2 DEGs were suppressed in all three datasets (*Cth* and *Lox12*), 37 in both single agent everolimus and UNC0379 + everolimus conditions, 50 in single agent UNC0379 and UNC0379 + everolimus conditions, and 222 genes unique to the UNC0379 + everolimus condition (**Figure 4.12B**). Among upregulated genes, 12 DEGs were enriched in all three datasets, 107 in both single agent everolimus and UNC0379 + everolimus treated conditions, 26 in single agent UNC0379 and UNC0379 + everolimus conditions, and 209 genes unique to the UNC0379 + everolimus condition (**Figure 4.12B**). As in the 4-hour RNA-seq dataset, hierarchical cluster analysis ($n = 726$, duplicates removed) revealed distinct clusters of genes both downregulated and upregulated following exposure of cells to UNC0379 and everolimus to a greater extent than either single agent alone (**Figure 4.12C**). Among the most potently suppressed genes were once again known *Atf4* target genes (*Asns*) and mTORC1/*Atf4* target genes mediating the *de novo* serine biosynthesis pathway and one-carbon/folate metabolism (*Mthfd2*, *Phgdh* and *Psat1*) (see chapter 3). These results support the hypothesis that dual SETD8/mTORC1 inhibition potently suppresses mTORC1 target genes and pathways to a greater extent than either single agent alone. This contention was further supported by consideration of the top 1000 most variable genes in a multidimensional scaling (MDS) plot, which revealed discrete clustering of each of the treatment conditions (**Figure 4.12D**).

4.2.10. SETD8 and mTORC1 inhibition combinatorially suppresses amino acid and polyamine metabolic pathways and methionine salvage.

As 24 hours treatment resulted in a larger number of differentially expressed genes and thus greater chance of detecting multiple genes associated with particular biological processes, this dataset was used to perform pathway analysis on DEGs that were suppressed upon single agent and combination treatment. This analysis focussed on suppressed DEGs, to identify pathways/genes which underwent “combinatorial suppression” relative to single agent controls.

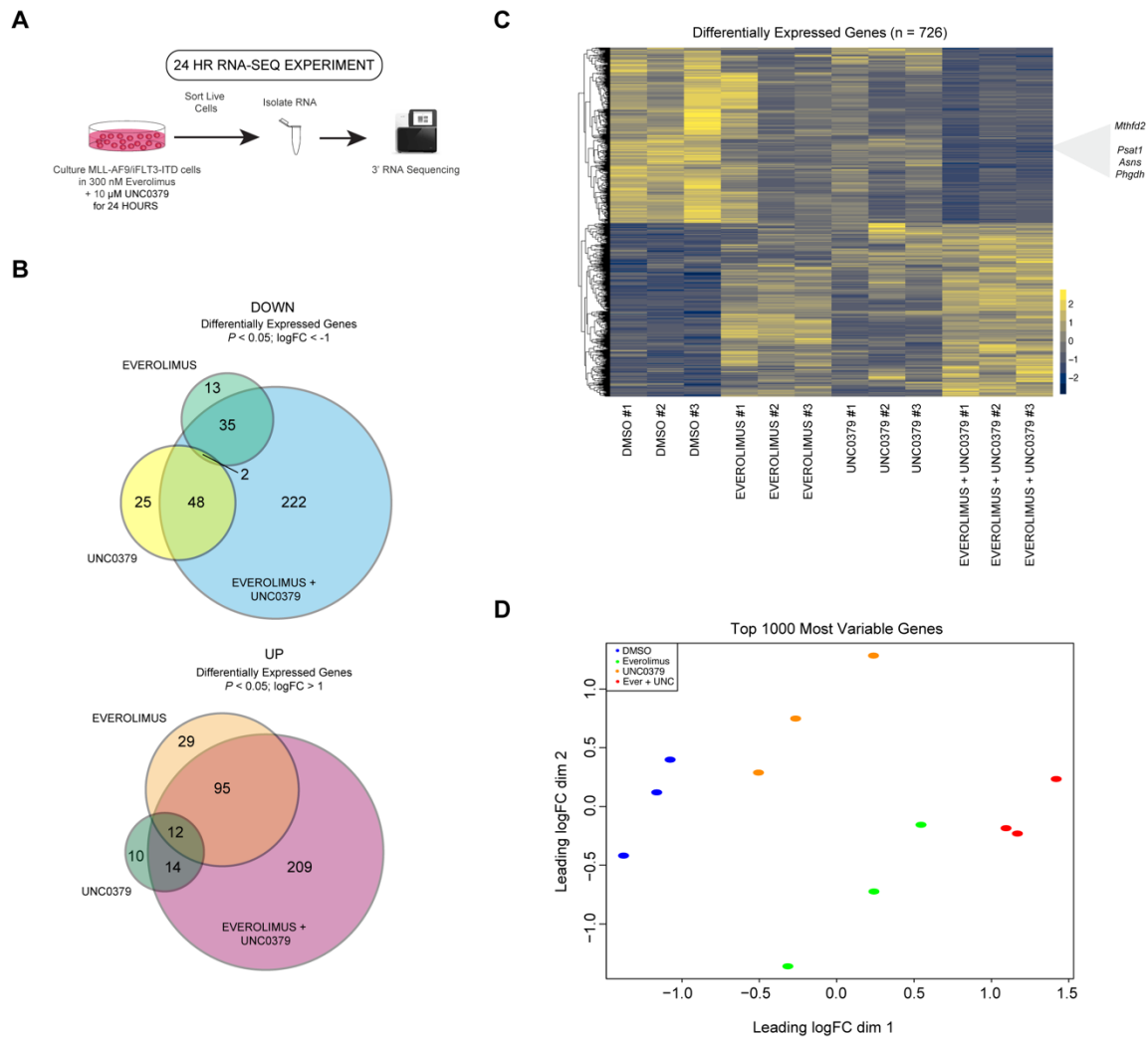


Figure 4.12 Chronic SETD8 and mTORC1 inhibition combinatorially suppresses mTORC1 target genes and pathways.

A. Schematic of experiment. MLL-AF9/iFLT3-ITD cells were treated with either 10 μ M UNC0379, 300 nM everolimus, or a combination of both, for 24 hours. Cells were harvested, washed with PBS, viable cells sorted via flow cytometry, and RNA extracted prior to 3'-RNA QuantSeq. **B.** Venn diagrams of downregulated (top panel) and upregulated (bottom panel) DEGs in response to UNC0379, everolimus or a combination of both. A statistical cut-off of $-1 > \logFC > 1$ and $P < 0.05$ was applied. **C.** Heatmap of all genes that pass the $-1 > \logFC > 1$ and $P < 0.05$ statistical cut-off in at least one treatment condition ($n = 726$). Duplicates are removed. *Mthfd2*, *Psat1*, *Asns* and *Phgdh* are highlighted. **D.** MDS plot of top 1000 most variable genes in response to DMSO, everolimus, UNC0379 or a combination of both everolimus + UNC0379 (Ever + UNC).

Pathway analysis of genes common to both everolimus single agent and combination treatment demonstrated enrichment for amino acid biosynthetic pathways (including serine biosynthesis) and amino acid transamination (**Figure 4.13**). No specific pathways were enriched in the intersection of single agent UNC0379 and combination treatment (**Figure 4.13**). Interestingly however, pathway and biological process analysis of DEGs unique to the combination treatment group revealed a striking and recurring enrichment (across multiple gene ontology datasets) of genes mediating ribosome biogenesis (a hallmark of mTORC1 inhibition) methionine salvage and polyamine synthesis (**Figure 4.13**). Indeed, the genes *Enoph1*, *Got1*, *Amd1*, and *Apip*, all key enzymes in the polyamine synthesis and methionine salvage pathway were uniquely enriched in the combination treatment group (that is, surpassed the statistical cut-off of $-1 > \log FC > 1$ and $P < 0.05$ in the combination treatment group, but neither single agent group alone). MTAP, the first enzyme of the methionine salvage pathway, was not enriched in the combination treatment dataset as it did not pass the required $\log FC > -1$ statistical cut-off, though it was very close ($\log FC < -0.92$, $P < 0.0001$). Moreover, MTAP did not pass the P -value threshold of $P < 0.05$ in any single agent arm.

These transcriptional analyses suggested that dual SETD8/mTORC1 inhibition suppresses key mTORC1-mediated amino acid biosynthetic pathways to a greater extent than single agent SETD8/mTORC1 inhibition alone. As indicated by analysis of the 4-hour RNA-seq dataset which was enriched for *Atf4* target genes such as *Chac1* and *Ddit4*, this may be an mTORC1/Atf4 mediated mechanism due to the short half-life of Atf4. Interrogation of the 24-hour RNA-seq dataset, which examined chronic exposure to UNC0379, everolimus, or both, further supported this contention by enriching pathways relevant to amino acid biosynthesis and transamination. Indeed, transcriptional examination of branched chain amino acid transaminases 1 and 2 (*Bcat1* and *Bcat2*), enzymes mediating *de novo* serine biosynthesis (*Phgdh*, *Psat1*, and *Psph*), the canonical Atf4 target *Asns* and one-carbon folate metabolism gene *Mthfd2* were all largely suppressed to a greater extent in combination-treatment conditions relative to single agents at 24 hours (**Figure 4.14A**). Intriguingly, combined SETD8/mTORC1 inhibition suppressed polyamine and methionine salvage pathways uniquely in the combination treated group (via key genes that mediate intermediary steps in polyamine and methionine salvage such as *Enoph1*, *Got1*, *Amd1*, *Apip* and *Cth*), and not in the context

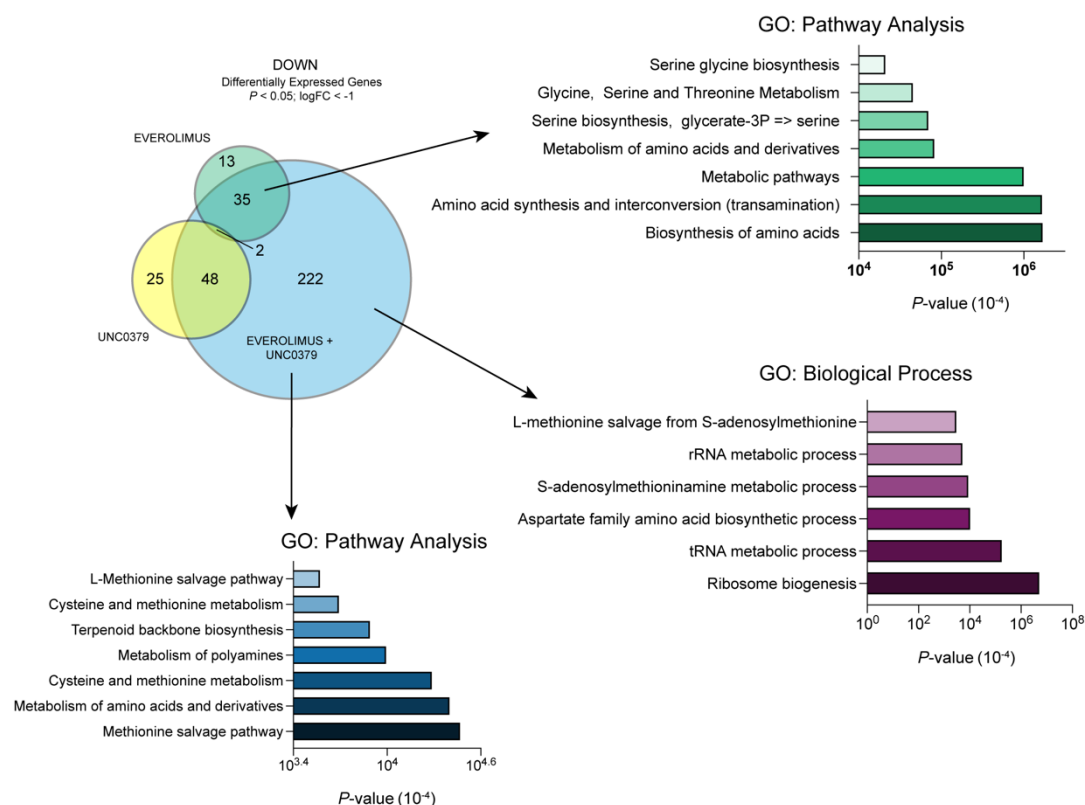


Figure 4.13 Pathway and biological process gene ontology (GO) analysis reveals SETD8/mTORC1 inhibition suppresses amino acid biosynthetic processes and polyamine/methionine salvage pathway.

Venn diagram of differentially suppressed genes ($\log_{2}FC < -1$, $P < 0.05$) unique to each treatment condition (everolimus alone, UNC0379 alone, UNC0379 + everolimus alone) and enriched in both single agent and combination therapy. Gene ontology (GO) pathway analysis was performed on genes enriched in both everolimus single agent and UNC0379 + everolimus combination therapy (green) and enriched exclusively in the combination therapy arm (blue) using ToppGene Suite analysis tools. GO biological process analysis was additionally performed on genes enriched exclusively in the combination therapy arm (purple).

of single agent SETD8/mTORC1 inhibition suggesting these pathways are regulated by, or dependent on, SETD8 and mTORC1 (**Figure 4.14B**).

4.2.11. Downregulation of amino acid and polyamine biosynthetic pathways and methionine salvage is not related to cell viability or suppressed global transcription and metabolism.

A potential explanation for the increased number of DEGs observed in the UNC0379 + everolimus treatment condition could be that combination therapy induces secondary response genes due to induction of apoptotic processes or a loss of active transcription. To rule out this idea, cell viability analysis was performed via Annexin V staining on MLL-AF9/iFLT3-ITD cells treated with DMSO, 10 μ M UNC0379, 300 nM everolimus or a combination of both for 24 hours. This analysis revealed that dual treatment with UNC0379 and everolimus only marginally increased cell death relative to single agent treatment or DMSO (approximately 30%) (**Figure 4.15A**). In addition, viable cells were enriched via fluorescence activated cell sorting (FACS) prior to RNA extraction, providing a further means of limiting this confounding variable. To verify that combination therapy did not suppress cellular transcription, the log fold changes relative to DMSO control of 3'-RNA-seq signals (LogCPM) across all expressed genes for all treatment groups. This analysis revealed no difference in global mRNA transcription relative to DMSO, supporting the contention that combined UNC0379 and everolimus treatment does not induce a defect in global transcription (**Figure 4.15B**).

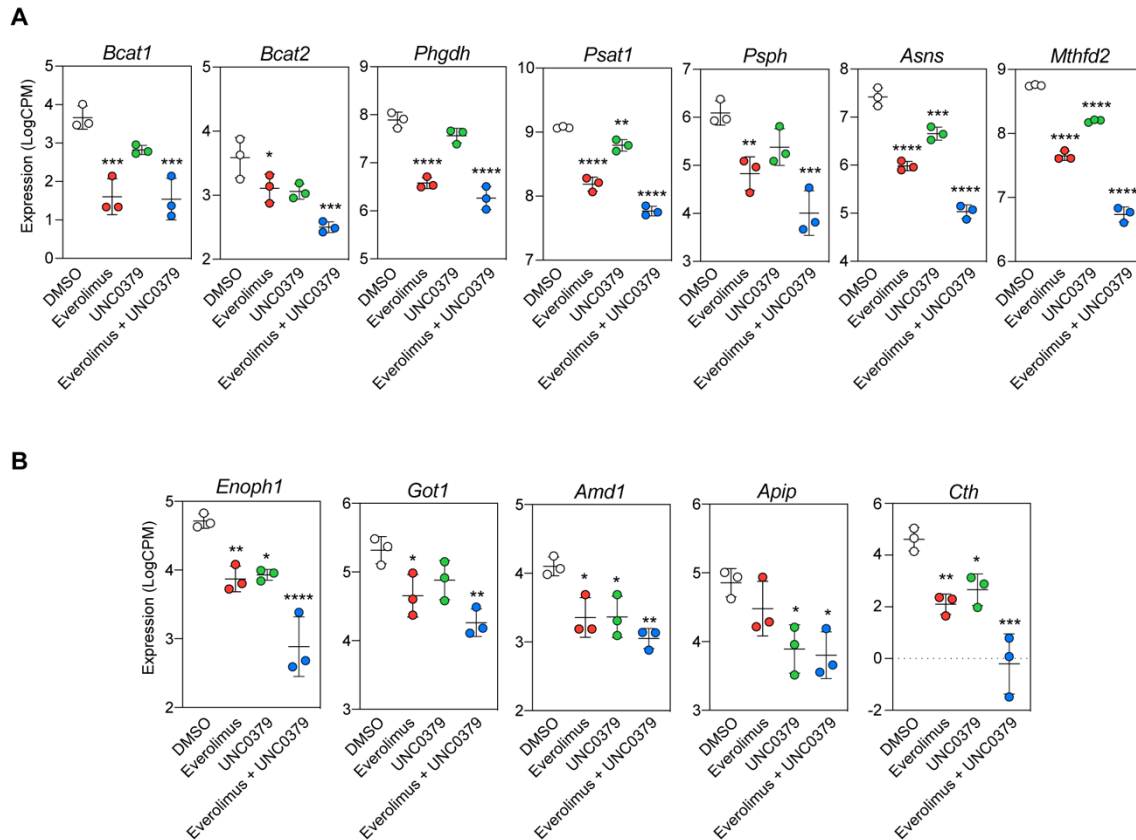


Figure 4.14 SETD8 and mTORC1 inhibition induces combinatorial gene suppression on key genes mediating amino acid and polyamine biosynthesis and methionine salvage.

A. Dot plots of log counts per million (CPM) gene expression values for enzymes mediating transamination of branched chain amino acids (*Bcat1* and *Bcat2*), *de novo* serine biosynthesis (*Phgdh*, *Psat1*, and *Psph*), the canonical Atf4 target *Asns* and one-carbon folate metabolism gene *Mthfd2* in response to DMSO, everolimus, UNC0379 or a combination treatment of both. **B.** Dot plots of log counts per million (CPM) gene expression values for enzymes mediating polyamine synthesis (*Amd1*) and methionine salvage (*Enoph1*, *Got1*, *Apip* and *Cth*) in response to DMSO, everolimus, UNC0379 or a combination treatment of both. * denotes $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ and **** $P < 0.0001$ as determined by one-way ANOVA with Dunnett's multiple comparisons test relative to DMSO. Each drug condition is representative of 3 biological replicates.

Finally, to examine the specificity of the metabolic gene expression changes induced by treatment with UNC0379, everolimus or a combination of both, the expression of hexokinase 1 and 2 (*Hk1* and *Hk2*), two genes involved in primary glycolytic metabolism was examined. In the cytosol, glucose binds to and is phosphorylated by Hk1/2 (Leprivier and Rotblat, 2020) and in the context of glucose starvation Hk2 has been shown to bind mTORC1 to inhibit its downstream signalling and induce autophagy (Durán et al., 2012). Interestingly, these two metabolic enzymes were not suppressed to a greater extent following dual treatment with UNC0379 and everolimus, and indeed, *Hk1* displayed no significant expression changes in response to any of the treatment conditions, whilst *Hk2* expression was significantly repressed only in the everolimus-alone treated condition (**Figure 4.15C**). These data therefore suggest that combination treatment with UNC0379 and everolimus does not induce secondary response genes, nor suppress global mRNA transcription as a result of reduced cell viability or impact on the transcriptional machinery of the cell. The analysis also suggests that the gene expression changes elicited, namely in the context of suppressed amino acid, polyamine and methionine salvage metabolism, are specifically repressed following dual SETD8/mTORC1 inhibition and this is not a global response induced in all metabolic regulatory enzymes in the cell.

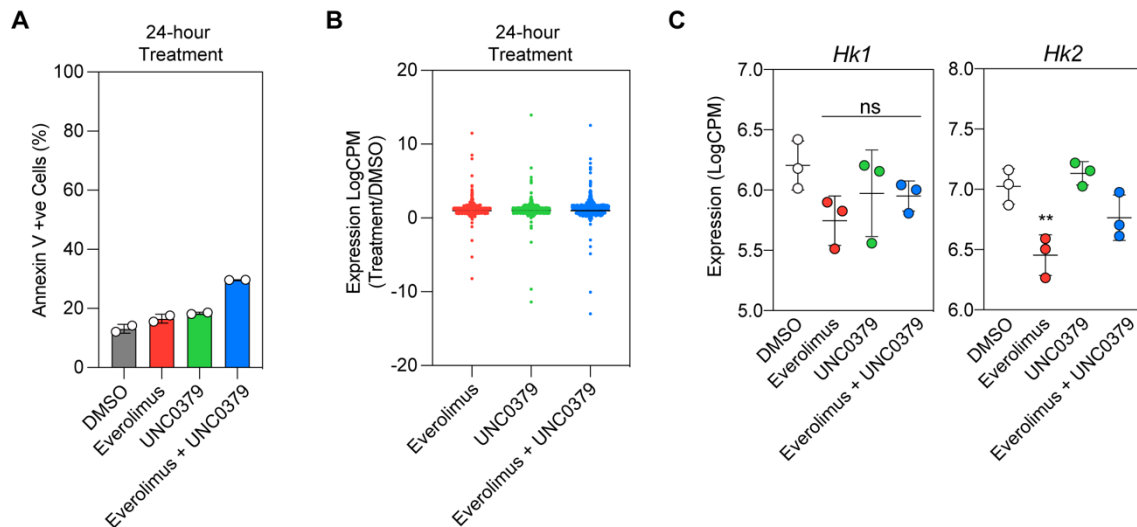


Figure 4.15 Combined SETD8 and mTORC1 inhibition is not as a result of low cell viability or impaired global mRNA transcription and does not induce global suppression of metabolic regulatory genes.

A. MLL-AF9/iFLT3-ITD cells were treated with DMSO, 300 nM everolimus, 10 μ M UNC0379 or a combination of both everolimus and UNC0379 for 24 hours and cell viability assessed via Annexin V flow cytometry. Data is indicative of two biological replicates. **B.** Log fold change relative to DMSO control of 3'-RNA-seq signal (LogCPM) across all expressed genes in MLL-AF9/iFLT3-ITD cells as indicated for 24 hours. Outlier genes ($\pm 1.5 \times \text{IQR}$) were removed from the analysis. **C.** Dot plots of log counts per million (CPM) gene expression values for the glycolytic enzymes *Hk1* and *Hk2* in response to DMSO, everolimus, UNC0379 or a combination treatment of both.

4.3. Discussion

In this chapter, a small molecule compound screen of 181 epigenetic inhibitors was performed to identify chemical combinations that sensitize cells to the mTORC1 inhibitor everolimus. AMLs, including those harbouring FLT3-ITD mutations which are known to upregulate mTORC1 signalling (further supported by the findings in chapter 3) display cytostatic rather than apoptotic responses when treated with mTORC1 inhibitors. Some of these effects are known to be induced by compensatory responses upon mTORC1 loss, such as phosphorylation of AKT and activation of MAPK signalling (Carracedo et al., 2008), while mTORC2 can also play a role in pro-survival signalling. Thus, inhibitors of both mTORC1/mTORC2 have seen greater anti-tumour responses in the clinic (Feldman et al., 2009). Here, UNC0379, an inhibitor of lysine-specific methyltransferase SETD8, was shown to cooperate with mTORC1 inhibition to kill MLL-AF9/iFLT3-ITD cells. These data suggest synergy with UNC0379 is not everolimus specific and can be induced with mTOR inhibition via chemically and structurally distinct compounds. Transcriptomic analysis of cells exposed to single agent UNC0379 or everolimus treatment – or a combination of both – revealed profound gene expression changes in cells treated with dual UNC0379/everolimus combination treatment. Acute (4 hour) combination treatment suppressed expression of target genes of the metabolic transcriptional regulator Atf4 (*Chac1*, *Ddit4*), itself a target of mTORC1. Chronic (24 hour) combination therapy suppressed many mTORC1-mediated pathways, such as amino acid biosynthesis and transamination reactions, as well as, surprisingly, methionine salvage pathways to a greater extent than either single agent treatment alone. Importantly, these effects were independent of gene expression changes induced by impaired cellular viability or global transcriptional suppression, as other metabolic-regulatory genes did not respond to combination treatment in the same manner as the pathways above. SETD8 is the only known methyltransferase that mediates monomethylation of H4K20, an epigenetic mark critical for DNA replication, DNA damage responses, and cell cycle and transcriptional regulation. Given the data presented in this chapter suggest transcriptional responses to dual SETD8/mTORC1 inhibition appear to be limited to mTORC1 target genes or related downstream metabolic pathways rather than global combinatorial suppression of all genes, a model wherein SETD8-mediated H4K20me1 is critical for the activation/suppression or maintenance of mTORC1 signalling or its target genes could be presented.

Several studies employing genetic or chemical approaches similar to those detailed herein have identified loss of SETD8 as a vulnerability in cancer cells, namely in neuroblastoma (Veschi et al., 2017) and recently, in *MYC*-driven medulloblastoma (Veo et al., 2019). This study is the first to suggest dual SETD8 and mTORC1 inhibition is a synthetic lethal interaction in AML or indeed, any other cancer setting. Some evidence linking known mTORC1-mediated biological processes to SETD8 have been suggested in the literature. Namely, a study using normal lung fibroblast cells discovered SETD8, and thus SETD8-catalysed H4K20me1, repressed gene sets associated with ribosome biogenesis, p53 signalling and cytokine-cytokine receptor interactions (Tanaka et al., 2017). Genetic depletion of SETD8 with shRNAs resulted in induction of cellular senescence and transcriptional upregulation of genes involved in these pathways, such as *RPL5*, *RPS24*, and *RPS18*. Given mTORC1 is a master regulator of ribosome biogenesis, mTORC1 inhibition post SETD8 inhibition may act to counteract the upregulation of *RPL* genes induced by loss of SETD8. However, analysis of transcriptional responses in AML cells did not recapitulate upregulation of *RPL5*, *RPS24*, and *RPS18*. Single agent UNC0379 treatment for 24-hours did not result in significant changes in *RPL5*, and *RPS24* and *RPS18* gene expression was only marginally suppressed and not upregulated ($\log\text{FC} < -0.57, P < 0.047$; $\log\text{FC} < -0.43, P < 0.024$ respectively). Thus, it is possible that responses to SETD8 loss are likely context, disease and potentially cell-type dependent. Moreover, there is contradictory evidence in the field regarding the role of H4K20me1 in gene expression with some studies associating H4K20me1 with active transcription (Barski et al., 2007; Karlić et al., 2010; Li et al., 2011b; Wakabayashi et al., 2009) and others, such as the study above, with gene repression (Congdon et al., 2010; Kalakonda et al., 2008; Karachentsev et al., 2005; Liu et al., 2010). Thus, why *RPL* genes in particular were enriched for H4K20me1, and whether this phenomenon extends, firstly, beyond *RPL* genes to other metabolic pathways and secondly, to other cell types and disease contexts beyond senescence is not adequately addressed in the above study by Tanaka and colleagues. In a paper that performed ChIP-seq analysis for H4K20me1 in human cervical adenocarcinoma cells (HeLa), several genes on human chromosome 21 and 22 were found to contain multiple H4K20me1 peaks with high statistical stringency ($P < 0.001$) associated with gene repression, including ribosomal proteins (*RPL3*, *RRP1*), *ATF4*, enzymes encoding Vitamin B6 metabolism (*PDXP*, *PDXK*), glycolytic metabolism (*PGK1*, *PFKL*) and phospholipid biosynthesis (*CHKB*) (Congdon et al., 2010). Another study reported that UNC0379 treatment in neuroblastoma cells upregulated

lipid/cholesterol metabolism and vitamin/mineral metabolism, however the authors did not offer a mechanistic explanation as to why this effect occurred (Veschi et al., 2017). It is thus clear from the literature that SETD8-mediated H4K20me1 plays a role in the suppression of certain key metabolic pathways and genes in certain contexts, and deletion of SETD8 results in upregulation of these pathways. However, several questions remain unanswered. Firstly, chapter 3 demonstrated that FLT3-ITD-mediated upregulation of mTORC1 and ATF4 activates *PHGDH* and other metabolic pathway enzymes in AML cells. What role, and indeed whether H4K20me1 is a transcriptionally repressive mark in this particular context remains unknown, particularly given in HeLa cells *ATF4* was among the genes found to score highly in repressive H4K20me1 peak calling (Congdon et al., 2010). Secondly, whether H4K20me1 is enriched at the same genomic regions as discussed above (and thus whether the specificity of *RPL* genes and other metabolic genes/pathways for the H4K20me1 mark is maintained) in FLT3-ITD-driven AML has not been explored. Lastly, the mechanistic basis of how dual SETD8 and mTORC1 inhibition remodels the H4K20me1 landscape and synergises to induce cell death (though partially explored via transcriptional profiling) has not been fully elucidated.

Interrogation of these questions was hampered by the ongoing worldwide COVID-19 pandemic in 2020. Thus, performing several relatively simple experiments may begin to unravel some of these issues. Firstly, global interrogation of H4K20me1, such as through ChIP-sequencing should be performed in the context of UNC0379, everolimus, or a combination treatment of both to elucidate the effect on H4K20me1 levels and in genomic regions associated with mTORC1 and its target genes, such as amino acid biosynthetic enzymes. These experiments should be performed in MLL-AF9/iFLT3-ITD cells, as well as in non-engineered human cell lines driven by a genetically diverse spectrum of driver mutations; this is important to interrogate the relevance of FLT3-ITD-mediated chronic upregulation of mTORC1 signalling, and thus the broader implications of these findings beyond FLT3-ITD-mutant AML. Next, the interaction between SETD8 and its binding partners could be investigated globally via mass spectrometry; again, this should be performed in a number of AML cell lines with varied genetic drivers. This analysis should reveal whether the binding of SETD8 to certain proteins is consistent or is context specific, such as in AMLs driven by FLT3-ITD mutations for instance. A key hypothesis in this chapter has been that SETD8/H4K20me1 activity is particularly important for mTORC1 target genes, such as those involved in amino acid biosynthesis and

transamination, as well as for the methionine salvage pathway. Whether H4K20me1 occupancy is higher/lower at these genomic regions or if SETD8 associates with proteins relevant for these pathways is another question that can further be explored by the above experiments. Lastly, it is possible that its interactions with SETD8, or dependency on SETD8-mediated H4K20me1, is directly required for the function of mTORC1 itself. While the biochemical mechanisms underlying mTORC1 regulation are not completely understood, mTORC1 is known to be post-translationally modified. Several mTORC1 phosphorylation sites (S1261, T2446, S2448, S2481) have been identified (among others) (Acosta-Jaquez et al., 2009; Cheng et al., 2004; Navé et al., 1999; Peterson et al., 2000), and activation of mTORC1 signalling by P300-mediated acetylation of the mTORC1-complex component Raptor (at K1097) has also been reported (Son et al., 2019, 2020). Methylation of mTORC1 or any of its complex components has not been explored in detail to date. Some evidence of the hypothesis that SETD8/H4K20me1 is required for mTORC1 function can be derived from the work detailed in **Figure 4.14A** and **Figure 4.14B**; many transcriptional responses on direct mTORC1 target genes/mediated pathways were suppressed to a greater degree in dual SETD8/mTORC1-inhibited contexts, a phenomenon that was not necessarily universal and was independent of general effects on cell viability and global transcriptional repression (**Figure 4.15**). Further, *Chac1* and *Ddit4*, two canonical targets of Atf4 (itself a direct target of mTORC1) were suppressed to a greater following dual UNC0379/everolimus treatment in the 4-hour RNA-seq dataset (**Figure 4.11D**). Given that Atf4 is primarily regulated post-translationally and has short half-life, these observations provide circumstantial evidence that SETD8/H4K20me1 could be required for mTORC1 function.

From a technical perspective, several additional experiments can be performed to further validate the findings presented so far in this chapter. For instance, as described in chapter 3, steady-state culture of MLL-AF9/iFLT3-ITD cells requires continual supplementation of 1 µg/mL doxycycline into liquid culture to maintain iFLT3-ITD transgene expression. Given doxycycline is known to inhibit mitochondrial protein synthesis (Luger et al., 2018), further experiments, particularly utilising human cell lines not dependent on doxycycline supplementation (e.g., MV4-11, MOLM-13, PL-21 and other non-FLT3-ITD-driven commercial cell lines), should be performed to exclude the possibility of confounding doxycycline-induced effects on gene expression/translation. Some evidence

that doxycycline does not contribute to the apoptotic effects seen with dual UNC0379/everolimus treatment can be found in experiments utilising the PI3K inhibitor GDC-0941 and cobimetinib (**Figure 4.8B**). As these experiments were performed using MLL-AF9/iFLT3-ITD cells cultured in the presence of doxycycline, and they did not induce cell death to the same extent as everolimus, rapamycin or PP242 treatment, it is likely that the synergistic effects seen in the context of everolimus, rapamycin or PP242 treatment with UNC0379 are independent of doxycycline. Next, given that a majority of the work presented thus far has been performed using chemical small molecule inhibitors, genetic depletion of SETD8 using shRNAs or CRISPR-Cas9-mediated sgRNAs should be performed to determine if doing so sensitizes cells to everolimus treatment. This important control experiment provides additional information regarding the specificity of UNC0379 and ensures that the synergistic effects observed in the screen are not due to off-target specificity of the compound. Finally, it is of note that the design of the screen – whereby MLL-AF9/iFLT3-ITD cells were exposed to epigenetic compounds for 48 hours – may in itself have contributed to the induction of false negatives. For instance, EZH2, DOT1L and EED inhibitors can require several days of continuous culture to induce alterations in cell viability (Bisserier and Wajapeyee, 2018; Daigle et al., 2013; Zhu et al., 2020). Thus, it is possible that additional synergistic interactions with everolimus could be uncovered with longer compound incubation times.

In summary, this work provides data suggesting a potential synthetic lethality between the lysine-specific methyltransferase SETD8 and mTORC1 inhibition in FLT3-ITD-driven AML. This chapter presents evidence that key mTORC1-mediated target genes and pathways undergo greater transcriptional repression in dual SETD8/mTORC1-inhibited contexts (such as amino acid biosynthesis and transamination genes, and polyamine and methionine salvage pathways), potentially suggesting the presence or absence of SETD8 or its catalysed H4K20me1 epigenetic mark are required for expression or function of these processes, or indeed, mTORC1 itself. Several key future experiments that can enhance the broad applicability, specificity and impact of these findings are described herein.

Chapter 5: Discussion

Since the 1940s, perturbation of cellular metabolic pathways has been utilised as anti-cancer therapy, when Sidney Farber employed antifolates to induce remissions in childhood ALL. Despite the fact that the next-generation sequencing age has revealed fundamental insights into the nature of the recurrent somatic mutations and molecular pathways that drive leukaemogenesis, non-specific chemotherapeutic agents, not unlike those employed by Farber 80 years ago, still form the backbone of frontline standard of care therapy in newly-diagnosed cases of AML. Since their characterisation in the early 1990s, the capacity of FLT3 mutations to promote proliferation and survival advantage in leukaemic blasts was a key property of activated tyrosine kinase signalling. Indeed, early models of leukaemogenesis centred around the so-called “Two-Hit” model of leukaemic development, wherein class I mutations, occurring in RTKs, conferred proliferative and survival advantage to haematopoietic progenitors (Peterson et al., 2007; Stirewalt and Radich, 2003). These mutations were insufficient to induce overt leukaemia in the absence of class II mutations, typically chromosomal translocations in genomic regions encoding transcription factors that induced a block in haematopoietic differentiation. While large-scale sequencing projects of primary AML samples have since demonstrated that this model is highly simplistic and the actual picture is much more complex, subsequent data have supported the original notion that FLT3 mutations profoundly remodel downstream signalling pathways to provide proliferative and survival advantage to AML blasts by characterising these downstream signalling events. This thesis adds to this extensive body of work by providing significant insight into metabolic reprogramming events induced by mutant FLT3-ITD. FLT3-ITD-mediated reprogramming of global serine metabolism, broadly, promotes increased exogenous uptake of serine and re-direction of glucose to *de novo* generate serine as a means of providing one-carbon units for downstream biosynthetic pathways (such as nucleotides), which drive proliferation. Beyond identifying a novel metabolic vulnerability that can be exploited for anti-cancer therapy, these data provide important mechanistic insight into how FLT3-ITD mutations promote proliferation. This thesis also provided preliminary data towards establishing how perturbing the epigenome can potentiate mTORC1 inhibition. Upregulated mTORC1 signalling downstream of FLT3-ITD has long been appreciated, however inhibition of mTORC1 itself yields transient antiproliferative responses. Herein, it was identified that inhibition of SETD8 – an enzyme that catalyses monomethylation of H4K20 – synergises with mTORC1 inhibition and preferentially suppresses key mTORC1 target genes to a greater extent than SETD8 or mTORC1

inhibition alone. Importantly, this effect is independent of global transcriptional suppression and transcriptional changes in response to loss of cellular viability. This work thus lays the foundation for further research into the importance of SETD8/H4K20me1 for the function of mTORC1, or vice-versa.

In chapter 3, a novel mouse model of tetracycline inducible FLT3-ITD in *MLL*-driven AML was generated to interrogate the transcriptional response to genetic depletion of FLT3-ITD. Tetracycline inducible AML mouse models, which model leukaemia development *in vivo* and allow for genetic depletion of key genes that drive leukaemogenesis, have yielded seminal molecular discoveries such as MLL-AF9-driven AML relying on a Myb-coordinated transcriptional program that aberrantly drives self-renewal (Zuber et al., 2009, 2011b). In this study, the use of a Tet-On system, which requires continual doxycycline presence to bind the reverse tetracycline transactivator (rtTA) and thus allow it to bind to the TRE-tight promotor, presented the possibility that the metabolic effects observed upon FLT3-ITD depletion were a direct consequence of doxycycline withdrawal. The antibacterial effect of tetracyclines on bacteria primarily involves impairment of protein translation, and this effect is also observed on mammalian cells exposed to doxycycline (Chatzispyrou et al., 2015; Moullan et al., 2015). Several key experiments, examining transcriptional changes in PHGDH, PSAT1, SLC1A4 and SLC1A5 in response to MLL-AF9 depletion in the Tet-On iMLL-AF9/FLT3-ITD mouse model and the Johnstone Lab's previously characterised Tet-Off iMLL-AF9/NRAS^{G12D} system (Ghisi et al., 2016) provided strong evidence that gene expression changes in enzymes relevant to serine biosynthesis and uptake were independent of doxycycline effects. Other technical challenges in this study limited the ability to more broadly interrogate the metabolic changes induced by loss of FLT3-ITD or PHGDH. The QTOF metabolomics platform used for steady-state LC-MS/MS employed targeted coverage of approximately 200 metabolites and did not capture many pathway intermediary metabolites. For instance, in publication Figure 5D, 24-hour quizartinib treatment reduced the abundance of the purine nucleotide guanosine triphosphate (GTP), whilst concomitantly increasing pyrimidine cytidine triphosphate (CTP) abundance (Bjelosevic et al., 2021). Whilst we postulated that this nucleotide imbalance may contribute to the cytotoxic and antiproliferative effects of quizartinib (Kunz et al., 1994), this analysis was restricted to only a handful of nucleotide triphosphate species. The effects on other nucleotide species, and indeed on upstream nucleotides such the purine precursor inosine-

5'-monophosphate (IMP) is unknown. Further, greater metabolite coverage might also suggest a mechanistic basis for the increase in pyrimidine abundance. As the one-carbon cycle provides intermediates for pyrimidine synthesis (the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) is mediated by thymidylate synthase (TYMS) and requires one-carbon cycle intermediates as 1-carbon donors), it can be assumed that this effect is independent of quizartinib's effects on one-carbon metabolism. Interestingly, whilst not specifically addressed in the manuscript, the abundance of the amino acid aspartate increased upon quizartinib treatment; as aspartate provides carbons 4-6 and nitrogen 1 to the pyrimidine ring, this may be mechanistic explanation for increased pyrimidine species abundance post FLT3 inhibition (Shuvalov et al., 2017), however many of the other metabolites and precursors necessary for pyrimidine generation, and indeed why aspartate abundance increased in response to FLT3 inhibition, was not captured by the LC-MS/MS analysis.

In chapter 3, it was demonstrated that FLT3-ITD-mediated promotion of *de novo* serine biosynthesis and serine uptake was dependent on the mTORC1-ATF4 axis. Whilst ATF4 has been previously described to transcriptionally activate *PHGDH* and *PSAT1* (DeNicola et al., 2015) this is the first study to link upregulated mTORC1 signalling in FLT3-ITD-driven AML with control of ATF4 to drive these genes. Quizartinib treatment potently suppressed mTORC1 target genes, an effect phenocopied by iFLT3-ITD depletion in mouse MLL-AF9/iFLT3-ITD cells (Figure 4F, Supplementary Figure 4C in manuscript). Additionally, everolimus treatment reduced ATF4 and PHGDH expression at the protein level, further linking mTORC1 to ATF4. Of interest, whilst everolimus treatment potently suppressed PHGDH at the protein level at 48 hours, it is well known from literature (and the analyses in Figure 4.2) that mTORC1 inhibition yields only cytostatic *in vivo* responses and initiates substantial feedback responses, which can serve to reactivate MAPK, PI3K-AKT and RTK signalling (Li et al., 2014). Thus, it is likely that mTORC1 inhibition alone induces transient reduction in PHGDH levels that are reversed upon induction of feedback loops.

In this study, the exact mechanism by which mTORC1 acts on ATF4 was not determined in this study. Quizartinib treatment rapidly abolishes ATF4 at the protein level within 30 minutes to 1-hour post treatment (unpublished, data not shown) suggesting that post-translational modifications potentially mediate the rapid turnover of ATF4 post FLT3

inhibition. Indeed, it is also known that mTORC1 kinase activity is rapidly suppressed upon pharmacological inhibition with rapamycin (Kleinert et al., 2014). In previous studies, proteasome inhibitors failed to prevent ATF4 degradation upon mTORC1 inhibition, suggesting ATF4 protein stability is not regulated by mTORC1 (Ben-Sahra et al., 2016; Park et al., 2017). Instead, mTORC1 controls ATF4 translation via three upstream ORFs in the 5'-UTR of ATF4 (Park et al., 2017). In addition, it is well characterised that ATF4 is regulated via other post-translational modifications. Mass spectrometry high throughput screening revealed that ATF4 undergoes ubiquitination at several lysine residues (Hornbeck et al., 2015; Kim et al., 2011), as well as phosphorylation at multiple serine, threonine and tyrosine residues; these have been associated with a wide array of biological roles including protein destabilisation and inactivation of NOXA and PUMA (Bagheri-Yarmand et al., 2015), interaction with β TrCP1 (Lassot et al., 2001; Pons et al., 2008), and activation of CHOP and ATF3 (Ampofo et al., 2013). Interestingly, roles for epigenetic enzymes in mediating ATF4 stability have been described. An enzymatic activity-independent role for the histone acetyltransferase p300, which was shown to associate with ATF4 at its *N*-terminal region, acted to block lysine residues involved in ubiquitination, thus promoting ATF4 stability (Lassot et al., 2005). The protein arginine methyltransferase PRMT1 has also been shown to methylate ATF4 at Arg239, resulting in transcriptional activation of pro-apoptotic targets (Yuniati et al., 2016). Further detailed mechanistic analysis, potentially by mutating key validated ATF4 residues required for phosphorylation, ubiquitination, acetylation or methylation and then exposing cells to quizartinib and interrogating ATF4 protein stability, would reveal the post-translational events responsible for ATF4 degradation following FLT3-ITD inhibition.

Given mTORC1 is upregulated downstream of FLT3-ITD and mTORC1 inhibitors have poor clinical anti-cancer efficacy, we sought to characterise epigenetic regulators that potentiate mTORC1 inhibition. In chapter 4, an epigenetic small molecule compound screen was performed, revealing that inhibition of SETD8, a lysine-specific methyltransferase that catalyses the monomethylation of H4K20 synergised with mTORC1 inhibition. Transcriptional analysis of cells in which both SETD8 and mTORC1 was inhibited revealed suppression of key mTORC1-mediated pathways (such as amino acid biosynthesis and transamination, as well as polyamine and methionine

salvage pathways) to a greater extent than either single agent alone. These observations therefore suggested that SETD8/H4K20me1 may be required for the expression of mTORC1 and/or its target genes, or alternatively, mTORC1 itself may regulate SETD8 and thus global H4K20 monomethylation. Interplay between mTORC1 and the epigenome is an emerging area of interest; in the early 2000s, the budding yeast histone acetyltransferase Esa1 was shown to bind to ribosomal protein gene promoters, thus acetylating histone H4 and promoting RNA-Pol II transcription in an mTORC1-dependent manner (Larabee and Weisman, 2020; Rohde and Cardenas, 2003). The link between mTORC1 and histone methylation is less defined, however several recent studies have suggested that mTORC1 signalling is required for global methylation. For instance, in hepatocellular carcinoma cell lines, rapamycin treatment reduced 5-mC levels and concurrently suppressed DNMT1 expression, in a transcriptional and post-translational modification-independent manner (Zeng et al., 2018). Instead, mTORC1 modulated DNMT1 expression via regulating initial translational efficiency, a mechanism akin to mTORC1-mediated control of ATF4 translation. Similarly, another study demonstrated that Raptor (a key mTORC1 complex subunit) -deficient pancreatic beta cells exhibited reduced expression of DNMT3A, and aberrant DNA hypomethylation (and thus upregulation) of genes typically suppressed in beta cells; here, the authors again suggested that mTORC1 directly regulates DNMT3A levels via translational control (Ni et al., 2017). Collectively, these observations provide evidence for a model whereby combination therapy with the SETD8 inhibitor UNC0379 and the mTORC1 inhibitor everolimus potentially reduces SETD8 expression by mTORC1-mediated control of translation, and simultaneous inhibition of already translated SETD8 catalytic activity. Several other examples of mTORC1 interplay with methyltransferases have been reported. A genetic screen in budding yeast revealed that loss of Set2, the only lysine-specific methyltransferase that catalyses H3K36 trimethylation, sensitized cells to rapamycin treatment by promoting cryptic transcription (McDaniel et al., 2017). Further, in pre-adipocytes, it was discovered that mTORC1 activated S6-kinase 1 (S6K1), which phosphorylated histone H2B; this phosphorylation event controls Polycomb methyltransferase Ezh2-dependent H3K27 trimethylation at key *Wnt* target genes, resulting in transcriptional suppression (Yi et al., 2016). More recently, mTORC1 was found to phosphorylate the Jumonji demethylase JMJD1C, which removes H3K9 methylation marks; phosphorylated JMJD1C subsequently demethylates H3K9me2 at genes which drive lipogenesis in the liver (Viscarra et al., 2020).

Summary

FLT3 mutations are among the most common recurrent mutations in AML and are associated with poor prognosis, highlighting the pressing need to comprehensively unravel the repertoire of signalling modalities these aggressive mutations employ to drive proliferation. In this thesis, FLT3-ITD-induced metabolic reprogramming which promotes both uptake and *de novo* generation of the amino acid serine from glucose to drive proliferation was described. This work proposes that perturbation of the *de novo* serine biosynthesis pathway via genetic or pharmacological depletion of PHGDH, the first rate-limiting enzyme of this pathway, is a novel metabolic vulnerability in FLT3-ITD-driven AML. Thus, these data provide the clinical rationale for further developing PHGDH inhibitors and employing them in this subset of AML.

Given upregulated serine uptake and *de novo* biosynthesis was identified to be mTORC1/ATF4 dependent, but mTORC1 inhibitors have traditionally displayed poor anti-cancer responses in the clinic, the second aim of this thesis was to discover synthetic lethal interactions which potentiate mTORC1 inhibition. Analysis revealed inhibition of the lysine specific methyltransferase SETD8 synergised with mTORC1 inhibition, with dual SETD8/mTORC1 inhibition suppressing canonical mTORC1 target genes to a greater extent than either single agent alone. This work thus provides the basis for further studies to determine the exact mechanistic basis for this effect, and suggests a model whereby SETD8/H4K20me1 is required for the function of mTORC1 target genes, or mTORC1 regulates SETD8 translation in a manner similar to DNA methyltransferases.

Chapter 6: Appendices

CA Sample #	Compound Name	Target
SN01730747	Veliparib (ABT-888)	PARP
SN01730748	Roxadustat (FG-4592)	HIF
SN01730749	Panobinostat (LBH589)	HDAC
SN01730750	Trichostatin A (TSA)	HDAC
SN01730751	Vorinostat (SAHA or MK0683)	Autophagy-HDAC
SN01730752	Tozasertib (VX-680 or MK-0457)	Aurora Kinase
SN01730753	Entinostat (MS-275)	HDAC
SN01730754	Olaparib (AZD2281 or Ku-0059436)	PARP
SN01730755	Belinostat (PXD101)	HDAC
SN01730756	Iniparib (BSI-201)	PARP
SN01730757	Abexinostat (PCI-24781)	HDAC
SN01730758	Dacinostat (LAQ824)	HDAC
SN01730759	Quisinostat (JNJ-26481585) 2HCl	HDAC
SN01730760	Rucaparib	PARP
SN01730761	MLN8054	Aurora Kinase
SN01730762	ZM 447439	Aurora Kinase
SN01730763	Danuserib (PHA-739358)	Aurora Kinase-Bcr-Abl-c-RET-FGFR
SN01730764	Mocetinostat (MGCD0103)	HDAC
SN01730765	SRT1720 HCl	Sirtuin
SN01730766	INO-1001 (3-Aminobenzamide)	PARP
SN01730767	Alisertib (MLN8237)	Aurora Kinase
SN01730768	AT9283	Aurora Kinase-Bcr-Abl-JAK
SN01730769	AG-490 (Tyrphostin B42)	EGFR-JAK
SN01730770	Barasertib (AZD1152-HQPA)	Aurora Kinase
SN01730771	SNS-314 Mesylate	Aurora Kinase
SN01730772	Valproic acid sodium salt	GABA Receptor-HDAC-Autophagy
SN01730773	CYC116	Aurora Kinase-VEGFR
SN01730774	ENMD-2076	Aurora Kinase-FLT3-VEGFR
SN01730775	CUDC-101	EGFR-HDAC-HER2
SN01730776	Decitabine	DNA Methyltransferase
SN01730777	PFI-1 (PF-6405761)	Epigenetic Reader Domain
SN01730778	2-Methoxyestradiol (2-MeOE2)	HIF
SN01730779	JNJ-7706621	Aurora Kinase-CDK
SN01730780	Ellagic acid	Topoisomerase
SN01730781	Ruxolitinib (INCB018424)	JAK
SN01730782	Pirarubicin	Topoisomerase
SN01730783	Resveratrol	Autophagy-Sirtuin
SN01730784	Droxinostat	HDAC
SN01730785	Aurora A Inhibitor I	Aurora Kinase
SN01730786	PHA-680632	Aurora Kinase

SN01730787	Ofloxacin	Topoisomerase
SN01730788	MC1568	HDAC
SN01730789	Norfloxacin	Topoisomerase
SN01730790	Pracinostat (SB939)	HDAC
SN01730791	Hesperadin	Aurora Kinase
SN01730792	Selisistat (EX 527)	Sirtuin
SN01730793	Azacitidine	DNA Methyltransferase
SN01730794	PCI-34051	HDAC
SN01730795	ENMD-2076 L-(+)-Tartaric acid	Aurora Kinase-FLT3-VEGFR
SN01730796	KW-2449	Aurora Kinase-Bcr-Abl-FLT3
SN01730797	AZD1480	JAK
SN01730798	Givinostat (ITF2357)	HDAC
SN01730799	AG-14361	PARP
SN01730800	Gandotinib (LY2784544)	JAK
SN01730801	SGI-1776 free base	Pim
SN01730802	AZ 960	JAK
SN01730803	Momelotinib (CYT387)	JAK
SN01730804	AR-42	HDAC
SN01730805	Quercetin	Src-Sirtuin-PKC-PI3K
SN01730806	Daphnetin	PKA-EGFR-PKC
SN01730807	Tubastatin A HCl	HDAC
SN01730808	NVP-BSK805 2HCl	JAK
SN01730809	TG101209	c-RET-FLT3-JAK
SN01730810	Resminostat	HDAC
SN01730811	TAK-901	Aurora Kinase
SN01730812	AMG-900	Aurora Kinase
SN01730813	Fedratinib (SAR302503 or TG101348)	JAK
SN01730814	GSK1070916	Aurora Kinase
SN01730815	CUDC-907	HDAC-PI3K
SN01730816	MK-5108 (VX-689)	Aurora Kinase
SN01730817	M344	HDAC
SN01730818	Tofacitinib (CP-690550 or Tasocitinib)	JAK
SN01730819	WP1066	JAK
SN01730820	Sirtinol	Sirtuin
SN01730821	CEP-33779	JAK
SN01730822	Tacedinaline (CI994)	HDAC
SN01730823	RG108	DNA Methyltransferase-Transferase
SN01730824	Baricitinib (LY3009104 or INCB028050)	JAK
SN01730825	WHI-P154	EGFR-JAK
SN01730826	PJ34	PARP

SN01730827	S-Ruxolitinib (INCB018424)	JAK
SN01730828	IOX2	HIF
SN01730829	Clevudine	DNA-RNA Synthesis
SN01730830	Entacapone	Histone Methyltransferase
SN01730831	Sodium Phenylbutyrate	HDAC
SN01730832	Tranlycypromine (2-PCPA) HCl	MAO
SN01730833	Procainamide HCl	DNA Methyltransferase-Sodium Channel
SN01730834	Tofacitinib (CP-690550) Citrate	JAK
SN01730835	AZD2461	PARP
SN01730836	XL019	JAK
SN01730837	CX-6258 HCl	Pim
SN01730838	Pinometostat (EPZ5676)	Histone Methyltransferase
SN01730839	GSK J4 HCl	Histone Demethylase
SN01730840	SGC 0946	Histone Methyltransferase
SN01730841	UNC1215	Epigenetic Reader Domain
SN01730842	AZD1208	Pim
SN01730843	(+)-JQ1	Epigenetic Reader Domain
SN01730844	Zebularine	DNA Methyltransferase
SN01730845	3-deazaneplanocin A (DZNeP) HCl	Histone Methyltransferase
SN01730846	C646	Histone Acetyltransferase
SN01730847	I-BET-762	Epigenetic Reader Domain
SN01730848	RGFP966	HDAC
SN01730849	GSK2801	Epigenetic Reader Domain
SN01730850	Bromosporine	Epigenetic Reader Domain
SN01730851	IOX1	Histone Demethylase
SN01730852	OG-L002	Histone Demethylase
SN01730853	NVP-TNKS656	PARP
SN01730854	SGC-CBP30	Epigenetic Reader Domain
SN01730855	MM-102	Histone Methyltransferase
SN01730856	SGI-1027	DNA Methyltransferase
SN01730857	JIB-04	Histone Demethylase
SN01730858	RG2833 (RGFP109)	HDAC
SN01730859	PFI-2 HCl	Histone Methyltransferase
SN01730860	RVX-208	Epigenetic Reader Domain
SN01730861	ML324	Histone Demethylase
SN01730862	PJ34 HCl	PARP
SN01730863	CPI-203	Epigenetic Reader Domain
SN01730864	MS436	Epigenetic Reader Domain
SN01730865	PFI-3	Epigenetic Reader Domain
SN01730866	TMP269	HDAC
SN01730867	EPZ004777	Histone Methyltransferase
SN01730868	OTX015	Epigenetic Reader Domain

SN01730869	UNC669	Epigenetic Reader Domain
SN01730870	ME0328	PARP
SN01730871	Nexturastat A	HDAC
SN01730872	MG149	Histone Acetyltransferase
SN01730873	Decernotinib (VX-509)	JAK
SN01730874	4SC-202	HDAC
SN01730875	UNC0379	Histone Methyltransferase
SN01730876	A-366	Histone Methyltransferase
SN01730877	GSK-LSD1 2HCl	Histone Demethylase
SN01730878	GSK J1	Histone Demethylase
SN01730879	Anacardic Acid	Histone Acetyltransferase
SN01730880	BRD4770	Histone Methyltransferase
SN01730881	Filgotinib (GLPG0634)	JAK
SN01730882	UNC0631	Histone Methyltransferase
SN01730883	EI1	Histone Methyltransferase
SN01730884	CPI-169	Histone Methyltransferase
SN01730885	MI-2 (Menin-MLL Inhibitor)	Histone Methyltransferase
SN01730886	MI-3 (Menin-MLL Inhibitor)	Histone Methyltransferase
SN01730887	GSK1324726A (I-BET726)	Epigenetic Reader Domain
SN01730888	Niraparib (MK-4827) tosylate	PARP
SN01730889	Remodelin	Histone Acetyltransferase
SN01730890	CPI-360	Histone Methyltransferase
SN01730891	SP2509	Histone Demethylase
SN01730892	OF-1	Epigenetic Reader Domain
SN01730893	EPZ015666(GSK3235025)	Histone Methyltransferase
SN01730894	AZ6102	PPAR
SN01730895	ORY-1001 (RG-6016) 2HCl	Histone Demethylase
SN01730896	GSK2879552 2HCl	Histone Demethylase
SN01730897	GSK503	Histone Methyltransferase
SN01730898	EPZ011989	Histone Methyltransferase
SN01730899	SGC707	Histone Methyltransferase
SN01730900	I-BRD9	Epigenetic Reader Domain
SN01730901	Ricolinostat (ACY-1215)	HDAC
SN01730902	ZM 39923 HCl	JAK
SN01730903	SMI-4a	Pim
SN01730904	BIX 01294	Histone Methyltransferase
SN01730905	UPF 1069	PARP
SN01730906	Scriptaid	HDAC
SN01730907	Tubastatin A	HDAC
SN01730908	Lomeguatrib	DNA Methyltransferase
SN01730909	Pacritinib (SB1518)	FLT3-JAK
SN01730910	Mirin	ATM-ATR

SN01730911	GSK591	Histone Methyltransferase
SN01730912	MS023	Histone Methyltransferase
SN01730913	Mitomycin C	DNA-RNA Synthesis
SN01730914	BI-7273	Epigenetic Reader Domain
SN01730915	PF-CBP1 HCl	Epigenetic Reader Domain
SN01730916	Oclacitinib	JAK
SN01730917	HLCL-61 HCL	Histone Methyltransferase
SN01730918	ITSA-1 (ITSA1)	HDAC
SN01730919	Gemcitabine HCl	Autophagy-DNA-RNA Synthesis
SN01730920	Carboplatin	DNA-RNA Synthesis
SN01730921	Daptomycin	DNA-RNA Synthesis
SN01730922	Mizoribine	DNA-RNA Synthesis
SN01730923	Cytarabine	DNA-RNA Synthesis
SN01730924	Nedaplatin	DNA-RNA Synthesis
SN01730925	Procarbazine HCl	DNA-RNA Synthesis
SN01730926	Blasticidin S HCl	DNA-RNA Synthesis
SN01730927	APTSTAT3-9R	STAT

Appendix A. Compounds Australia Epigenetics Library used for small molecule compound screen in Chapter 4.

CA Sample # refers to the internal Compounds Australia compound identification number, used to identify lyophilized compounds when plated onto drug plates to be used for subsequent screening. Compound Name refers to the chemical or developmental names of compounds. Where appropriate, alternative names for compounds are provided. Target refers to the molecular pathways/processes perturbed by compounds.

Chapter 7: References

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