

Investigating Drug Response and Resistance to IDH1^{R132H} Inhibition and Hypomethylating Agents in AML

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Abstract

Mutations in epigenetic regulators frequently occur in AML. Furthermore, the epigenetic landscape is typically dysregulated in AML. Targeted epigenetic therapeutics that directly inhibit mutant epigenetic oncogenic drivers, such as AG120 inhibition of IDH1^{R132H}, have shown clinical success for the treatment of IDH1-mutant AML. Other epigenetic therapies target wildtype epigenetic regulators, such as DNMT1 inhibition by hypomethylating agents, with the aim of reprogramming the transcriptional networks driving malignant progression. Despite some patients achieving clinical remission, many AML patients do not respond to epigenetic therapies or relapse post-treatment. Furthermore, the contribution of mutant epigenetic regulators to leukaemogenesis is relatively poorly understood.

A clinically and pathophysiologically relevant murine model of IDH1^{R132H} driven AML co-expressing oncogenic DNMT3A and Nras was utilised to investigate the cellular and molecular consequences of IDH1^{R132H} expression in leukaemia initiation and progression. Results from this thesis suggest that IDH1^{R132H} governs distinct leukaemic properties within the different AML cell-types; IDH1^{R132H} drove the expression of genes that underpin HSC-like self-renewal and proliferation in leukaemic progenitor-like cells, whilst IDH1^{R132H} most profoundly impaired the differentiation capacity of leukaemic immature neutrophils. Furthermore, this thesis demonstrated that AG120 was a highly effective and on-target IDH1^{R132H} inhibitor for the treatment of IDH1^{R132H} driven *in vivo* AML, and induced neutrophil-skewed differentiation with distinct kinetics throughout the hierarchy of leukaemic cells.

Mechanisms of resistance to the hypomethylating agents AZA and GDAC were investigated utilising a genome-wide CRISPR/Cas9 screen *in vitro*. Members of the pyrimidine salvage pathway were implicated in the resistance to hypomethylating agents. Loss of DCK and UCK2 conferred resistance to GDAC and AZA, respectively, whilst SLC29A1 loss mediated resistance to both hypomethylating agents in both *in vitro* and *in vivo* models of haematological malignancies. This thesis demonstrated that DCK and UCK2 loss remained sensitive to AZA and GDAC, whilst cells with SLC29A1 loss were sensitive to inhibition of DHODH.

This research provided comprehensive insight of the impact of IDH1^{R132H} on distinct leukaemic cell subsets, transcriptional mechanisms underlying leukaemogenesis, and the transcriptional perturbations that may contribute to AG120 resistance. Furthermore, the results of this thesis

suggest that AZA and GDAC treatment are potential therapeutic avenues for AML resistance driven by DCK or UCK2 depletion or loss, respectively. Moreover, the results described herein suggest that targeting the *de novo* pyrimidine synthesis pathway, through DHODH inhibition, is a therapeutic strategy to overcome resistance to hypomethylating agents mediated by the depletion of pyrimidine salvage pathway enzymes.

Declaration

This declaration is to certify that:

- i. This thesis comprises only my original work towards the PhD 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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Preface

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List of Abbreviations

2-HG	D-2-hydroxyglutarate
5-caC	5-Carboxylcytosine
5-fC	5-Formylcytosine
5-hmC	5-Hydroxymethylcytosine
5-mC	5-Methylcytosine
α-KG	α -ketoglutarate
ACK	Ammonium-Chloride-Potassium (Lysis Buffer)
ALL	Acute Lymphoblastic Leukaemia
ALKBH	AlkB Homologue
AML	Acute Myeloid Leukaemia
Ara-C	Cytarabine
Arg	Arginine
Asp	Aspartate
ATP	Adenosine Triphosphate
AZA	Azacytidine
BBOX	γ -Butyrobetaine Hydroxylase
BCAA	Branched-Chain Amino Acid
BCAT	Branched-Chain Amino Transferase
BM	Bone Marrow
BSA	Bovine Serum Albumin
cat	Catalogue (Number)
CCLE	Cancer Cell Line Encyclopedia
CGI	CpG Islands
ChIP	Chromatin Immunoprecipitation
CHIP	Clonal Haematopoiesis of Indeterminate Potential
CLP	Common Lymphoid Progenitor
CMML	Chronic Myelomonocytic Leukaemia
CMP	Common Myeloid Progenitor
CpG	Cytosine-Guanine Dinucleotide
CPM	Counts Per Million
CTCF	CCCTC-Binding Factor
CTV	CellTrace Violet

DAC	Decitabine
DAPI	4',6-diamidino-2-phenylindole
DCK	Deoxycytidine Kinase
dATP	Deoxyadenosine Triphosphate
dCTP	Deoxycytidine Triphosphate
dGTP	Deoxyguanosine Triphosphate
DEGs	Differentially Expressed Genes
dH₂O	Distilled H ₂ O
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNMT	DNA Methyltransferase
DOX	Doxycycline
dNTPs	Deoxynucleotide Triphosphates
EDTA	Ethylene Diamine Tetra-Acetic Acid
Eo	Eosinophils
ERV	Endogenous Retrovirus
FAB	French-American-British Criteria
FACS	Fluorescence Activated Cell Sorting
FC	Fold Change
FCS	Foetal Calf Serum
FSC	Forward Scatter
FDA	Food and Drug Administration
FDR	False Discovery Rate
FIH	Factor Inhibiting HIF1 α
FL	Foetal Liver
GDAC	Guadecitabine
GFP	Green Fluorescent Protein
GMP	Granulocyte Macrophage Progenitor
GO	Gene Ontology
GvHD	Graft Versus Host's Disease
GSEA	Gene Set Enrichment Analysis
H3K4	Lysine 4 of Core Histone H3
H3K4me1	Mono-Methylated Lysine 4 of Core Histone H3

H3K4me3	Tri-Methylated Lysine 4 of Core Histone H3
H3K9	Lysine 9 of Core Histone H3
H3K9me3	Tri-Methylated Lysine 9 of Core Histone H3
H3K27	Lysine 27 of Core Histone H3
H3K27ac	Acetylated Lysine 27 of Core Histone H3
H3K27me3	Tri-Methylated Lysine 27 of Core Histone H3
H3K36	Lysine 36 of Core Histone H3
H3K79	Lysine 79 of Core Histone H3
H4K20	Lysine 20 of Core Histone H4
HIF	Hypoxia Inducible Factor
HPMCAS	Hypromellose Acetate Succinate
HSC	Haematopoietic Stem Cell
HSCT	Haematopoietic Stem Cell Transplant
ICE	Inference of CRISPR Edits
IDH	Isocitrate Dehydrogenase
IDN	<i>IDH1^{R132H}/DNMT3A^{R882H}/Nras^{G12D}</i>
IDN-TN	Treatment Naïve IDN Leukaemia
IDN-TE	Treatment Exposed IDN Leukaemia
IP	Immunoprecipitation
i.p.	Intraperitoneal
IRES	Internal Ribosomal Entry
JmjC	Jumonji C Domain Containing
KDM	Histone Lysine Demethylase
KL	Kit ⁺ /Lineage ⁻ /Sca1 ⁻
KMT	Histone Lysine Methyl Transferases
KO	Knockout
KSL	Kit ⁺ /Lineage ⁻ /Sca1 ⁺
LC-MS	Liquid Chromatography-Mass Spectrometry
LD₅₀	Lethal Dose to Kill 50%
LMPP	Lymphoid-Primed Multipotent Progenitor
LSC	Leukaemia Stem Cell
Luc	Luciferase
MBD	Methyl-CpG-Binding Domain
MDS	Myelodysplastic Syndrome

MDS Plot	Multidimensional Scaling Plot
MEP	Megakaryocyte-Erythroid Progenitor
Mo	Monocytes
MOI	Multiplicity of Infection
MPP	Multipotent Progenitor
MTase	Methyltransferase
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
Neu	Neutrophils
NK	Natural Killer Cells
ns	Not Significant
NSG	Non-Obese Diabetic Severe Combined Immunodeficiency Gamma (NOD.Cg- <i>Prkdc</i> ^{scid} <i>Il2rg</i> ^{tm1Wjl} /SzJ Mouse Strain)
p	p-value
PB	Peripheral Blood
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEI	Polyethylenimine
PHD	Protein Hydroxylase Domain Enzymes
PI	Propidium Iodide
PLT	Platelet
PMT	Post Translational Modification
PRC2	Polycomb Repressive Complex 2
Puro	Puromycine
QC	Quality Control
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
rAML	Relapsed AML
RBC	Red Blood Cell
RNA	Ribonucleic Acid
RSEM	RNAseq by Expectation Maximization
SAM	S-Adenyl-L-Methionine
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
Ser	Serine

SP	Spleen
T0	Time-point 0
TAD	Topologically Associated Domain
TBE	Tris-Borate-EDTA
TCA	Tricarboxylic Acid
TCGA	The Cancer Genome Atlas
TDG	Thymine-DNA Glycosylase
TE	Tris-EDTA
TET	Ten-eleven Translocation
TRE	Tetracycline-responsive element
tRNA	Transfer RNA
TSS	Transcriptional Start Site
tTA	Tetracycline-controlled Transactivator
UCK	Uridine Cytidine Kinase
VAF	Variant Allele Frequency
WBC	White Blood Cell
WHO	World Health Organisation

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Chapter 1: Literature Review

This chapter briefly summarises the inherent hierarchy of the haematopoietic compartment and discusses the genetic aetiologies and current treatment options for patients with myeloid malignancies. Theories in the literature regarding the molecular mechanisms of malignant transformation by mutant IDH and the mechanisms through which epigenetic regulators exert their anti-tumour effects, specifically hypomethylating agents and inhibitors of mutant IDH, will be reviewed.

1.1 The Haematopoietic Hierarchy

Haematopoiesis is the hierarchal process of producing mature blood cells from the rare multipotent haematopoietic stem cells (HSCs). Adult HSCs primarily exist in a quiescent state (Wilson et al., 2008), however, their multipotency and self-renewing capabilities allow them to regenerate all blood cell lineages (Spangrude et al., 1988). The mature cell compartment of the haematopoietic system is comprised of erythrocytes, leukocytes and platelets. Leukocytes, or white blood cells, can be further divided into lymphocytes (natural killer (NK) cells, B-cells, T-cells, lymphoid derived dendritic cells) and myeloid cells (macrophages, eosinophils, neutrophils, basophils and monocyte-derived dendritic cells) (Rieger and Schroeder, 2012). The evolution of the hierarchal map of the haematopoietic system has occurred over many years, and continues to evolve paralleling the advances in technologies and methodologies. The canonical model of the mammalian haematopoietic system defines the generation of different blood cell lineages through a series of discrete unidirectional and sequential differentiation steps (Figure 1.1). HSCs sit at the apex of this model and have the capacity to differentiate into multipotent progenitors (MPPs). MPPs are defined by the absence of self-renewing capabilities whilst maintaining multipotency with the ability to differentiate into the lineage-restricted progenitors: lymphoid-primed multipotent progenitors (LMPPs) or common myeloid progenitors (CMPs) (Rieger and Schroeder, 2012). CMPs can generate megakaryocyte-erythroid progenitors (MEPs) or granulocyte macrophage progenitors (GMPs), which can ultimately give rise to erythrocytes, megakaryocytes and platelets, or granulocytes, macrophages and monocyte-derived dendritic cells, respectively (Rieger and Schroeder, 2012). LMPPs maintain the capacity, albeit restricted, to generate both myeloid and lymphoid lineages by differentiating into common lymphoid progenitors (CLPs) or GMPs (Doulatov et al., 2010). CLPs will eventually give rise to T-, B- and NK-cells, and dendritic cell subsets. Each cell-type of the highly compartmentalised canonical model of haematopoiesis, whereby HSC differentiation proceeds through discrete intermediates, was largely defined by the repertoire of cell-surface markers as measured by flow cytometry (Oguro et al., 2013; Okada et al., 1992;

Spangrude et al., 1988). However, evidence arising in light of the advances in technologies, such as single-cell omics, are challenging the canonical model by providing evidence that the haematopoietic system is an assortment of hierarchically organised heterogeneous populations that exist on a continuous spectrum of differentiation (Hamey et al., 2017; Karamitros et al., 2018; Macaulay et al., 2016; Velten et al., 2017). Even though strong evidence exists supporting the revision of the model for haematopoiesis, the canonical model has been pertinent in furthering the haematopoietic stem cell biology field, and is still a valuable concept when studying the molecular pathways contributing to malignant haematopoiesis.

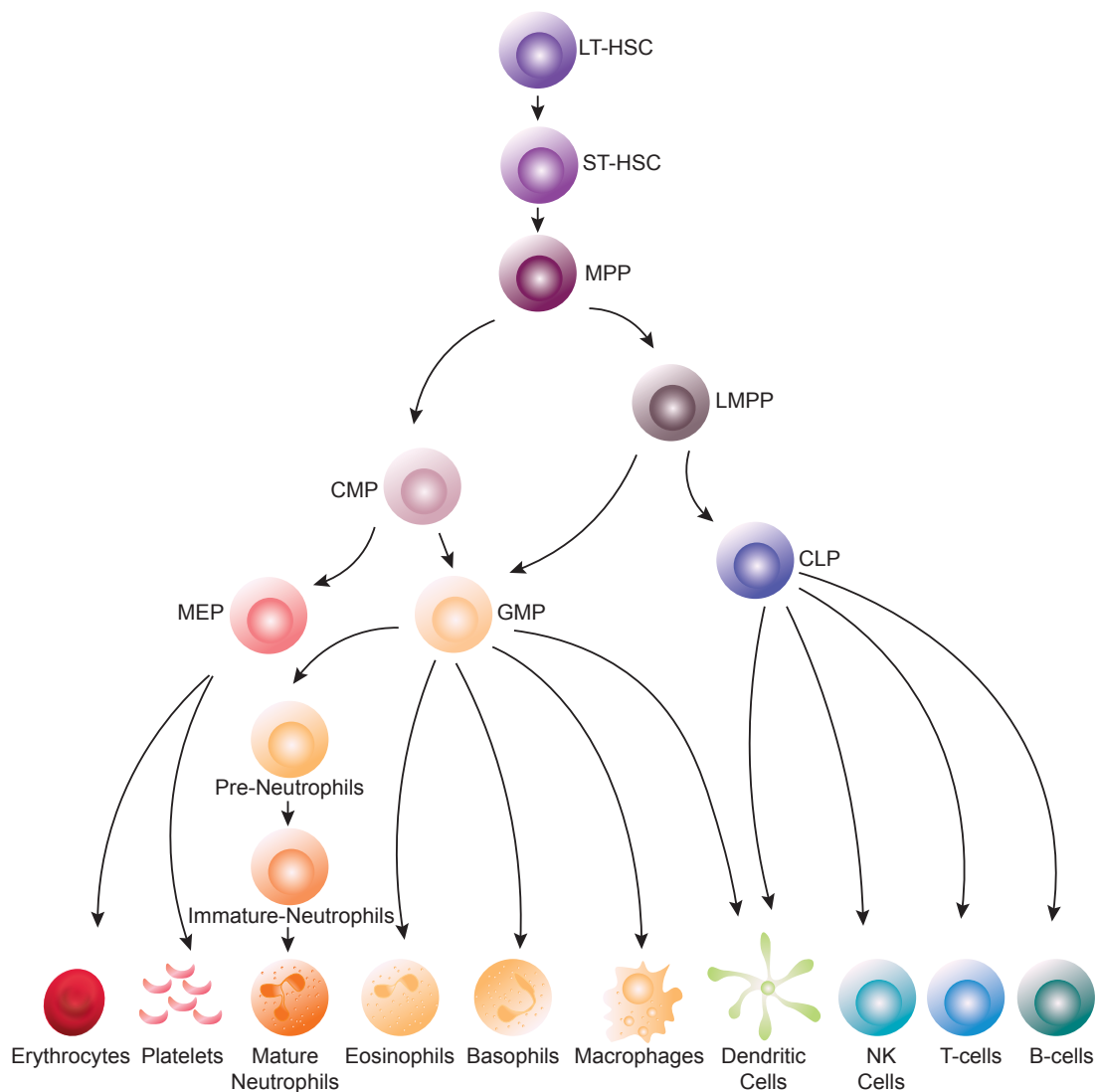


Figure 1.1 Canonical Model of Haematopoiesis

A simplified schematic of the canonical model of the haematopoietic hierarchy whereby haematopoietic differentiation occurs unidirectionally and sequentially through distinct intermediate cell types. HSC, haematopoietic stem cells; LT-HSC, long-term HSC; ST-HSC, short-term HSC; MPP, multipotent progenitor; LMPP, lymphoid-primed multipotent progenitor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocytic myeloid progenitor; MEP, megakaryocytic erythroid progenitor.

1.2 Haematopoietic Malignancies

Haematological malignancies encapsulate a plethora of diseases defined by the aberrant proliferation of haematopoietic cells, and accounts for approximately 9% of all cancer patients in Australia and the UK (Australian Institute of Health and Welfare, 2017; Smith et al. 2011). Leukaemia, lymphoma and multiple myeloma are distinct classes of haematopoietic malignancies, each comprised of a multitude of subtypes. Lymphomas originate in the lymphatic system and are predominantly characterised by the expansion of B-cells, and occasionally T- and NK-cells. Multiple myeloma is demarcated by the expansion of plasma cells in the bone marrow, and leukaemia presents with the uncontrolled proliferation of immature leukocytes within the bone marrow. Leukaemias can be further classified into four broad subsets based on cell lineage (myeloid or lymphoid) and rapidity of disease progression (acute or chronic).

1.2.1 Acute Myeloid Leukaemia

Acute myeloid leukaemia (AML) is a haematological malignancy characterised by the rapid clonal expansion of undifferentiated myeloid progenitors (Corces et al., 2017; De Kouchkovsky and Abdul-Hay, 2016). The abnormal acquisition of the self-renewal stem cell-like property, and often a defective capacity to differentiate into mature myeloid lineages, drives the proliferative expansion of the malignant myeloid precursors (Corces et al., 2017; De Kouchkovsky and Abdul-Hay, 2016). The accumulation of abnormal leukaemic blasts in the bone marrow impairs normal haematopoiesis and their immature state prevents many normal haematopoietic functions in these malignant cells. Inadequate numbers of healthy and functional mature haematopoietic cells eventually leads to anaemia, haemorrhage and/or infection.

In adults, AML is the most common acute leukaemia and disproportionately affects the elderly, with a median diagnosis age of 68.9 years (Australian Institute of Health and Welfare, 2017). According to the Australian Institute of Health and Welfare, the estimated occurrence of AML was 1143 people in Australia in 2018 (Australian Institute of Health and Welfare, 2017). Unfortunately, the 5-year survival rate of AML from diagnosis is 26.8%, which is substantially lower than that of all cancers combined (68.0%), keeping AML among the top 10 most fatal cancers in Australia (Australian Institute of Health and Welfare, 2017). Furthermore, over the past 40 years the mortality rate of AML has increased by approximately 20% with no

improvement in survival rate, despite the decline in mortality rate for all cancers combined (Australian Institute of Health and Welfare, 2017). This can be attributed to the ageing population, minimal advances in the treatment regimen for AML, a relatively recent discovery of the genomic diversity of AML (Cancer Genome Atlas Research Network, 2013; Papaemmanuil et al., 2016), and therapy-related AML arising from genetic aberrations induced by cytotoxic therapies, including chemotherapy and radiotherapy, used to treat primary malignant diseases (McNerney et al., 2017). Collectively, these data highlight the necessity to develop novel, well tolerated and effective therapeutics for the treatment of AML.

1.2.2 AML Diagnosis, Classification and Subtypes

According to the 2016 World Health Organization (WHO) and European LeukemiaNet (ELN), the diagnosis of AML requires a minimum of 20% myeloblasts in the bone marrow or peripheral blood as quantified by histology or flow cytometry (Arber et al., 2016; Döhner et al., 2017). However, the presence of mutated *NPM1*, specific cytogenetic abnormalities (t[8;21, inv[16], t[16;16]), or acute promyelocytic leukaemia are considered diagnostic of AML irrespective of the proportion of myeloid blasts in the bone marrow or blood (Arber et al., 2016). In addition to myeloblast counts, diagnostics of AML often include the assessment of the expression of specific cell-surface and cytoplasmic markers according to the recommendations from the ELN (Döhner et al., 2017). Malignant myeloblasts typically express myeloid lineage cell-surface markers, however, infrequently these cells may co-express lymphoid lineage markers and thus provide uncertainty in the classification of the malignancy as AML, ALL or a mixed haematological disease. Additionally, patients with acute undifferentiated leukaemia (those that satisfy the $\geq 20\%$ blast criterion but lack the expression of cell-surface markers) are often treated as AML (Arber et al., 2016).

Over the past 50 years, the classification of AML subtypes has been revised in order to correlate these subgroups with treatment regimen, response, and prognosis. The French-American-British (FAB) criteria of AML was the first attempt at classifying AML subtypes (Table 1.1) and utilised the morphological appearance of Romanowsky and cytochemical stained peripheral blood and bone marrow smears (Amadori et al., 1996; Bennett et al., 1976; Bennett et al., 1985). However, the lack of cytogenetic and genetic data, and the high susceptibility of discordant results as a consequence of inter-observer variation, resulted in a poor correlation between the FAB AML subtypes and clinical outcome (Arber et al., 2003).

The FAB criteria have since been replaced with AML classifications from the WHO and ELN, which supplement the morphological characteristics with clinical features, and cytogenetic, immunophenotypic and genetic data based on scientific and clinical studies, thus improving concordance in diagnosis and clinical outcome (Arber et al., 2016; Döhner et al., 2017). The molecular stratification of AML, as described by the WHO, is based on recurrent genetic aberrations including specific chromosomal translocations and inversions, and mutations in *NPM1*, *CEBPA* and *RUNX1* (Table 1.2). However, the WHO's most current classification fails to include many of the recurrent mutations present in cytogenetically normal AML, which accounts for approximately half of all adult AML cases (Cancer Genome Atlas Research Network, 2013; Papaemmanuil et al., 2016; Zeisig et al., 2012).

Advances in next-generation sequencing techniques have unearthed the multitude of genetic abnormalities associated with AML, thus informing disease classification. In 2016, Papaemmanuil and colleagues challenged the WHO classification of AML by incorporating data from targeted exon sequencing of 1,540 samples from AML patients (Papaemmanuil et al., 2016). Using a Bayesian statistical model informed by patterns of co-occurring mutations, Papaemmanuil *et al.* classified AML into 11 almost completely mutually exclusive genomic subtypes (Papaemmanuil et al., 2016). Their proposed genomic classification of AML extended the WHO classification to include AML with mutated chromatin and/or RNA-splicing genes, AML with *TP53* mutations and/or chromosomal aneuploidy, and AML with *IDH2*^{R172K} mutations and no other class-defining lesions (Papaemmanuil et al., 2016). The current WHO classification fails to categorise 48% of Papaemmanuil's cohort, whereas Papaemmanuil *et al.* reported successful stratification of 85% of patients, with 11% of patients possessing driver mutations (such as *FLT3*^{ITD} and mutant *DNMT3A*) but no detected class-defining lesions, and only 4% with no detected driver mutations. Additionally, they found comparable proportions within each AML subtype and improved stratification of The Cancer Genome Atlas (TCGA) Research Network's cohort of 200 AML patients, demonstrating the robustness and reproducibility of their genomic classification of AML. A thorough understanding of the mechanistic drivers of AML improves risk stratification and has the potential to inform the development of novel and effective therapies.

Table 1.1 FAB Classification of AML

M0	Minimally differentiated leukaemia
M1	Acute myeloblastic leukaemia without maturation
M2	Acute myeloblastic leukaemia with maturation
M3	Acute promyelocytic leukaemia
M4	Acute myelocytic leukaemia
M5	Acute monocytic leukaemia
M6	Acute erythroleukaemia
M7	Acute megakaryoblastic leukaemia

Table 1.2 2016 WHO Classification of AML with Recurrent Genetic Aberrations

AML with Chromosomal Abnormalities	
APL with PML-RARA	
AML with t(8;21)(q22;q22.1)	RUNX1-RUNX1T1
AML with t(9;11)(p21.3;q23.3)	MLLT3-KMT2A
AML with inv(3)(q21.3q26.1)/t(3;3)(q21.3;q26.2)	GATA2, MECOM
AML with inv(16)(p13.1q22)/t(16;16)(p13.1;q22)	CBFB-MHY11
Megakaryoblastic AML with t(1;22)(p13.3;q13.3)	RBM15-MKL1
AML with t(6;9)(p23;q34.1)	DEK-NUP214
AML with BCR-ABL1 (Provisional)	
AML with Normal Karyotype	
AML with mutated NPM1	
AML with biallelic mutations in CEBPA	
AML with mutated RUNX1 (Provisional)	

Adapted from the 2016 Revision to the World Health Organization Classification of Myeloid Neoplasms and Acute Leukemia (Arber et al., 2016).

1.2.3 Genetic Heterogeneity within Cytogenetically Normal AML

In 2000, Hanahan and Weinberg first proposed the six capabilities, termed hallmarks of cancer, which explain the genesis, progression and persistence of malignancies (Hanahan and Weinberg, 2000). Since then, these collections have been expanded upon to include additional hallmarks including genomic instability (Hanahan and Weinberg, 2016; Hanahan and Weinberg, 2011; Moses et al., 2018; Negrini et al., 2010). Genomic instability is the increased occurrence and accumulation of genetic aberrations during cell division, some of which provide a selective growth advantage, and is also considered a hallmark of aging (López-Otín et al., 2013). By age 70, approximately 10% of healthy individuals have acquired clonal mutations in haematopoietic cells, predisposing them to further genetic aberrations, thus increasing the likelihood of lesions in oncogenic drivers that promote transformation and progression into leukaemia (Jaiswal et al., 2014; Shlush et al., 2014; Xie et al., 2014).

Recently, next-generation sequencing studies have begun exposing the vast diversity in the mutational landscape of cytogenetically normal AML in an attempt to identify diagnostic and prognostic markers that will improve upon the current understanding of AML pathogenesis, classification and therapeutic outcome. AML patients have, on average, 13 coding mutations, which is significantly lower than the mutational burden of solid malignancies (Cancer Genome Atlas Research Network, 2013). Of these genes, 5 on average are recurrently mutated in AML (Cancer Genome Atlas Research Network, 2013), with 86% of patients harbouring mutations in 2 or more driver genes (Papaemmanuil et al., 2016). Despite the abundant inter-patient heterogeneity, non-stochastic patterns in the co-occurrence and mutual exclusivity of mutations exist between patients, highlighting the importance of the specific repertoire of mutations in leukaemogenesis (Cancer Genome Atlas Research Network, 2013; Papaemmanuil et al., 2016). Furthermore, whole genome deep sequencing and single-cell genotyping has revealed the complex clonal diversity, with the presence of coexisting and competing subclones, that develops during disease progression (Klco et al., 2014; Paguirigan et al., 2015) and facilitates the evolutionary plasticity when under therapeutic pressure (Ding et al., 2012). The clonal architecture defined by aberrant genetic and epigenetic alleles is proving AML to be a disease more complex and dynamic than can be detected by bulk allelic sequencing alone (Li et al., 2014b; Paguirigan et al., 2015). Collectively, these studies have highlighted the complexity of genetic and epigenetic aberrations that contribute to AML pathogenesis.

1.2.4 Epigenetic Regulators are Frequently Mutated in AML

The Cancer Genome Atlas project organised genes mutated in AML into nine functional classes (Table 1.3) based on data obtained by whole-exome (n = 150) or whole-genome (n = 50) sequencing of patients with *de novo* AML. Two of these functional classes encompass epigenetic pathways: DNA-methylation and chromatin modifying related genes. Epigenetic pathways are discussed in detail in section 1.2.1. In fact, a high proportion of patients with myeloid malignancies present with mutations in genes that deregulate epigenetic processes (Table 1.4) and frequently correspond with poor clinical prognosis (Shih et al., 2012).

Somatic mutations in pre-leukaemic haematopoietic stem cells have been detected in individuals decades prior to the onset of AML, suggesting that these are early founder events that provide a selective advantage for the clonal expansion of haematopoietic stem cells and probable progression into AML (Jaiswal et al., 2014). This phenomenon has recently been termed clonal haematopoiesis of indeterminate potential (CHIP) (Steensma et al., 2015), and has been identified in 10% of patients over 65 years of age with no diagnostic criteria for a haematological malignancy, and increases in prevalence with age (Jaiswal et al., 2014). Additionally, patients harbouring CHIP prior to cytotoxic therapy induction or autologous stem cell transplant for the treatment of the primary malignancy are at increased risk of developing therapy induced haematological malignancies (Ortmann et al., 2019; Takahashi et al., 2017).

Expanded clones containing mutations in epigenetic regulators, particularly in the DNA-methylation related genes *DNMT3A* and *TET2*, and in the chromatin modifying gene *ASXL1*, have been observed in peripheral blood and bone marrow samples from individuals with no clinical evidence of haematological malignancy (Jaiswal et al., 2014; Shlush et al., 2014). High variant allele frequencies of somatic mutations in *DNMT3A*, *TET2* and *ASXL1* have also been reported in AML patients, indicating their early acquisition during tumour evolution (Ding et al., 2012; Papaemmanuil et al., 2016; Rothenberg-Thurley et al., 2018). However, CHIP associated mutations alone are unlikely to be adequate for the induction of leukaemic transformation as evident in the low transformation rate of CHIP (Steensma et al., 2015), and inability to initiate leukaemia in a timely manner in the absence of additional mutations *in vivo* (Lu et al., 2016; Shih et al., 2015). Instead, the acquisition of and cooperation with subsequent lesions in genes, such as *NPM1* and *IDH1*, drive malignant transformation, as inferred from variant allele frequencies of mutations in AML patient samples (Ding et al., 2012; Papaemmanuil et al., 2016) and observed functionally in the induction of sequential mutations

in vivo (Loberg et al., 2019). Together these data suggest that mutations in epigenetic modifiers confer a selective advantage for the evolution of the preleukaemic clone. The prevalence of mutated epigenetic modifiers and the frequently irregular epigenetic state in AML, and the inherent plasticity of the epigenome has made epigenetic therapies attractive treatment avenues for myeloid malignancies.

Table 1.3 Categories of Related Genes Relevant for AML Pathogenesis

Classes of Mutations in AML	Percentage of cases
Transcription-factor fusions	18%
Mutant <i>NPM1</i>	27%
Tumour suppressor genes	16%
DNA-methylation related genes	44%
Activated signalling genes	59%
Chromatin modifying genes	30%
Myeloid transcription factor genes	22%
Cohesion-complex genes	13%
Spliceosome-complex genes	14%

Data extracted from The Cancer Genome Atlas Research Network, 2013.

Table 1.4 Epigenetic Modifiers Frequently Mutated in AML

Gene	Epigenetic Pathway	Frequency Mutated	Reference
<i>DNMT3A</i>	<i>De novo</i> DNA methylation	~ 20%	(Ley et al., 2010)
<i>TET2</i>	DNA hydroxymethylation	~5 – 30%	(Wang et al., 2019)
<i>IDH1/2</i>	DNA hydroxymethylation	~5 – 15%/ Histone demethylation	(Papaemmanuil et al., 2016) (Paschka et al., 2010)
<i>ASXL1</i>	Histone methylation	~5%	(Pratcorona et al., 2012)
<i>MLL-fusion</i>	Histone methylation	~3%	(Papaemmanuil et al., 2016)
<i>EZH2</i>	Histone methylation	~3 – 11%	(Zhang et al., 2019b)

1.2.5 Current Treatment for AML

Despite the efforts to elucidate the pathogenesis of AML and improve upon subtype classification and risk stratification, treatment options and outcomes have improved minimally. Frontline treatment for AML involves conventional 7+3 chemotherapy induction, consisting of 7 days cytarabine infusion with an anthracycline administered simultaneously for the first 3 days. Post remission therapies typically consist of allogeneic haematopoietic stem cell transplants (HSCT) or consolidation chemotherapy. However, the majority of adult AML patients are elderly and many cannot tolerate these intensive therapies (Short et al., 2018). Moreover, patients that receive the standard of care treatment regimen frequently relapse (Short et al., 2018). Hence, there is a strong impetus to develop novel targeted therapeutics with greater efficacy and lower toxicity.

Historically, therapeutic advances for AML have largely focused on optimising the use of cytotoxic agents, such as elevating the dose of daunorubicin for induction therapy (Fernandez et al., 2009) and administering high dose cytarabine for consolidation therapy (Löwenberg et al., 2011). Only recently have novel non-cytotoxic targeted therapeutics been approved by the Federal Drug Administration in the USA for the treatment of AML, with many agents in clinical trials. As such, the use of small molecule inhibitors to exploit molecular vulnerabilities dictated by distinct genetic aetiologies shows promise for the treatment of AML. Targeted small molecule inhibitors including Midostaurin and Gilteritinib (mutated *FLT3*), Enasidenib (*IDH2*^{R140Q}) and Ivosidenib (*IDH1*^{R132H}) inhibit oncogenic effectors encoded by recurrent AML associated mutations (Keiffer and Palmisiano, 2019; Short et al., 2018; Winer and Stone, 2019). Other targeted therapeutic agents including apoptotic regulators (Venetoclax), kinase inhibitors (Volasertib), and epigenetic modifiers (5-Azacytidine, Panobinostat) disrupt regulators or processes that leukaemic cells have become profoundly reliant upon for cell survival (Keiffer and Palmisiano, 2019; Short et al., 2018; Winer and Stone, 2019). However, despite these recent therapeutic developments, the number of treatment options approved or in clinical trials for the treatment of AML does not reflect the heterogeneity of the disease but rather the challenges of treating AML. Since the majority of patients harbour at least two driver mutations (Papaemmanuil et al., 2016) and AML shows evidence of genetic and epigenetic plasticity (Ding et al., 2012; Easwaran et al., 2014), targeting a single aberrant pathway or oncogenic effector is unlikely to result in long-term remission. A greater understanding of how co-occurring genetic lesions confer oncogenic capacity, predispose oncogenic dependencies, and

evade inhibition is required for the development of more efficacious targeted single and combination therapies.

1.3 Epigenetic Regulation in Normal and Malignant Haematopoiesis

Epigenetic regulation encompasses an array of processes that occur throughout the hierarchy of genomic organisation. The repertoire of modifications to DNA and histones, and the spatial organisation of chromatin, collectively influence DNA accessibility and gene expression. Moreover, the chromatin state is involved in the regulation of processes that guide cell identity and cell fate (Ji et al., 2010; Lara-Astiaso et al., 2014). As aforementioned, epigenetic dysregulation and mutations in epigenetic regulators frequently occur in haematological malignancies, signifying the necessity of tightly controlled epigenetic regulation in normal haematopoiesis. Hence, a thorough understanding of the differences in epigenetic regulation between normal and malignant haematopoietic cells can provide potential therapeutic avenues for the treatment of AML.

1.3.1 Mechanisms of Epigenetic Regulation

Each diploid cell in the human body contains ~6.4 billion base pairs of DNA, measuring approximately two metres if stretched end-to-end, which is condensed to fit within the nucleus measuring on average ~10 µm in diameter (McGinty and Tan, 2015). This degree of compaction is achieved through the hierarchal configuration of the interactions of DNA with histones, non-histone proteins and RNA to form the macromolecular complex, chromatin. In addition to condensing DNA to fit within the nuclear space, the genomic architecture is also important for transcriptional control. Epigenetics refers to heritable mechanisms that influence gene expression by regulating the access and utilisation of the information stored within DNA, rather than by altering the DNA code. Epigenetics encompasses a broad range of mechanisms that dynamically act to modulate transcription at various levels of the chromatin hierarchal structure. At increasing resolution (Figure 1.2), interphase chromosomes occupy discrete regions known as chromosome territories, within which long-range stretches of DNA physically interact to form topologically associated domains (TADs) thereby limiting the effect of regulatory elements to genes within the same TAD (Dixon et al., 2012). CCCTC-Binding Factor (CTCF) insulator proteins are enriched at the boundaries of TADs, and colocalise with cohesion within TADs, to mediate chromatin folding by facilitating the formation of loop domains (Dixon et al., 2012; Fudenberg et al., 2016; Zheng and Xie, 2019). CTCF-bound looped structures create insulated neighbourhoods that can either preclude or enable enhancer and gene promoter

interactions, thereby providing an additional level of transcriptional regulation (Hnisz et al., 2016; Zheng and Xie, 2019). Enhancer-promoter interactions are able to exert their effects on associated genes when all genomic elements are constrained within the same insulated neighbourhood, whilst these interactions are often impeded if CTCF loops partition genes from their regulatory sequences (Hnisz et al., 2016).

The most fundamental structural unit of eukaryotic chromosomes are the nucleosomes; 147 bp of DNA is wrapped around a histone octameric complex constituting H2A, H2B, and two molecules of both H3 and H4, connected by an H1 linker protein (McGinty and Tan, 2015). Nucleosomes not only serve as a scaffold to condense DNA, but also regulate transcription via nucleosome remodelling and the recruitment of transcription factors and various complexes through signalling by post translational modifications (PTMs) of histone tails (Sadakierska-Chudy and Filip, 2015). Chromatin accessibility is dynamically regulated by nucleosome assembly, eviction and sliding (Becker and Workman, 2013). Chromatin domains that do not associate with nucleosomes correlate with transcriptional activation (Elgin, 1981; Radman-Livaja and Rando, 2010). Additionally, there are over 500 PTMs encompassing at least 20 different functional groups at discrete residues on N-terminal histone tails (Zhao and Garcia, 2015), of which many have been implicated in transcriptional regulation. Finally, nucleotide residues can be chemically modified and impact gene expression (see section 1.2.2).

Functionally distinct classes of enzymes dynamically regulate the repertoire of epigenetic marks. Histone and DNA modifications are deposited and removed by epigenetic writer and eraser proteins, respectively. Epigenetic reader proteins decipher the functional consequence of these marks through association and recruitment of other complexes and transcription factors. The modulation of epigenetic marks facilitates the regulation of chromatin organisation and recruitment of transcription factors, thereby creating multifaceted and highly dynamic mechanisms for regulating gene expression (Sadakierska-Chudy and Filip, 2015). Thus, epigenetics governs cell identity and cell fate by directing transcriptional networks (Álvarez-Errico et al., 2015; Barrero et al., 2010).

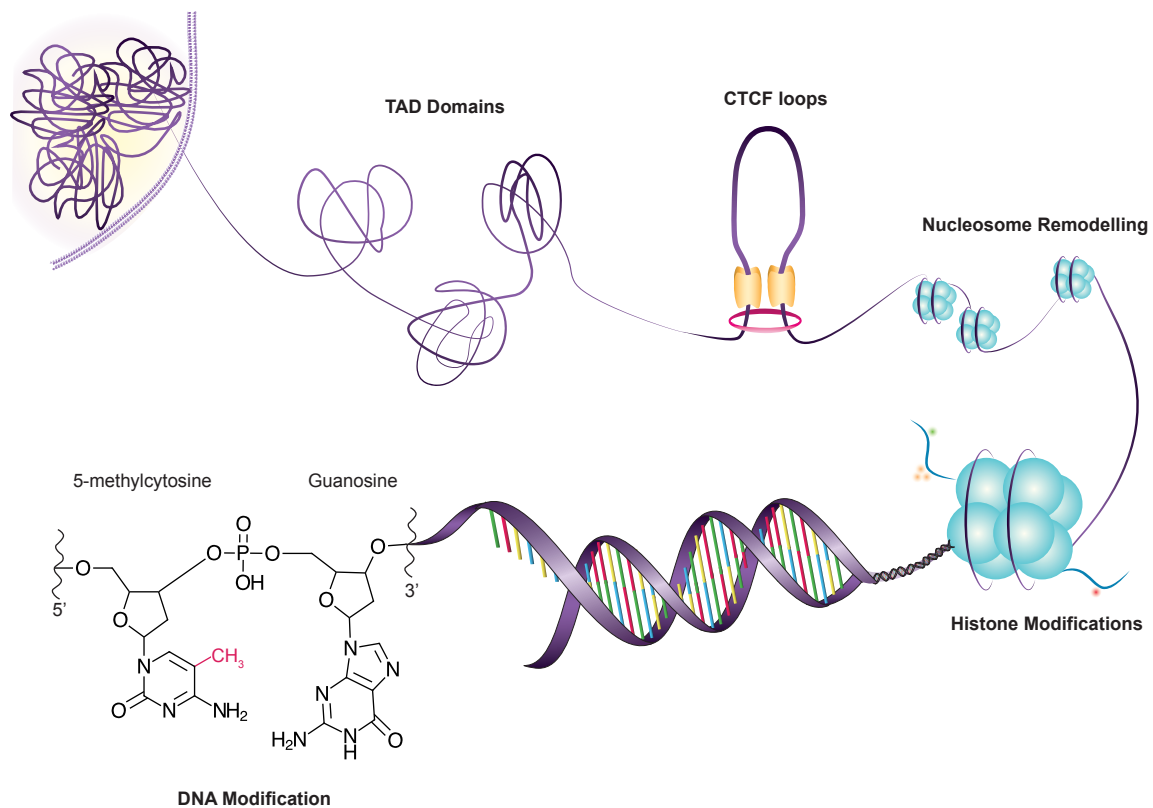


Figure 1.2 Hierarchy of Genomic Organisation and Epigenetic Regulation

At increasing resolution, interphase chromosomes occupy discrete regions known as chromosome territories within the nucleus. Topologically associated domains (TADs) comprise long-range stretches of DNA that physically interact, enabling regulatory regions to influence the expression of interacting genes. CTCF loops create insulated neighbourhoods that either partition or enable interactions between regulatory sequences and gene promoters. DNA is wrapped around nucleosome complexes comprising eight histone proteins. Nucleosome assembly, eviction and sliding, and histone post-translational modifications all contribute to chromatin accessibility and transcriptional control. Finally, methylation of cytosine residues at CpG dinucleotides in DNA provide an additional layer of epigenetic control.

1.3.2 DNA Methylation

DNA methylation is one of the most widely studied epigenetic modifications in mammals. DNA methylation predominantly refers to the establishment of 5-methylcytosine (5-mC), whereby a methyl moiety is covalently attached to the fifth carbon of the pyrimidine ring of a cytosine nucleotide that frequently precedes a guanine nucleotide (CpG) (Figure 1.3). 5-mC, now classified as an epigenetic modification, was first discovered in vertebrates in 1948 (Hotchkiss, 1948) and initially observed to influence gene expression and development 30 years later (Compere and Palmiter, 1981; Holliday and Pugh, 1975). Since then, 5-mC has been shown to play an essential role in imprinting (Li et al., 1993), X-chromosome inactivation (Norris et al., 1994), silencing of retroviral elements (Walsh et al., 1998), chromosomal stability (Rodriguez et al., 2006), and temporal and tissue specific gene expression during development and differentiation (Greenberg and Bourc'his, 2019).

Methylated CpG dinucleotides are the most abundant DNA modification in mammalian genomes. Although tissue specific differences exist, 70 – 80% of all CpG loci exhibit methylation in mammals (Greenberg and Bourc'his, 2019). Methylated cytosines adjacent to other nucleotides encompasses approximately one quarter of all 5-mC in murine and human embryonic stem cells (Lister et al., 2009; Ramsahoye et al., 2000), albeit become progressively infrequent during development and appearing sparsely in adult tissues other than the brain (Xie et al., 2012; Ziller et al., 2011). Additionally, emerging evidence suggests that the intermediates of active cytosine demethylation, 5-hmC and 5-fC, can serve as stable epigenetic marks with distinct binding partners (Iurlaro et al., 2013; Spruijt et al., 2013).

Methylation of cytosine nucleotides is catalysed by DNA methyltransferases (see section 1.2.2.1), whilst the ten-eleven translocation (TET) family of enzymes act to oppose the effects of DNMTs by catalysing the iterative oxidation of 5-mC to 5-hydroxymethylcytosine (5-hmC), then 5-formylcytosine (5-fC), and 5-carboxylcytosine (5-caC) (Ito et al., 2011; Wu and Zhang, 2017) (Figure 1.3). 5-fC and 5-caC can be excised by the thymine-DNA glycosylase (TDG) and base-excision repair pathway to restore the unmodified cytosine residue (He et al., 2011; Yin and Xu, 2016). Passive dilution of modified cytosines through DNA replication and decarboxylation of 5-fC and 5-caC also contribute to unmodified cytosine reversion (Schiesser et al., 2012; Wu and Zhang, 2017), although the extent of decarboxylation and the enzymes involved remains poorly understood.

There is mounting evidence that aberrant DNA methylation is important for the development and progression of many human diseases. Altered DNA methylation has been reported to influence the pathogenesis of autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus and multiple sclerosis (Sun et al., 2016), in addition to metabolic and neurological disorders (Andrews et al., 2018; Côté et al., 2016). Furthermore, irregular DNA methylation has also been observed in an array of cancer types including glioblastoma, breast, colon, bladder, liver, prostate, oesophageal cancer and leukaemia (Saghafinia et al., 2018). Mutations in *DNMT3A* and/or *TET2* occur in 25 – 35% of cytogenetically normal AML (Cancer Genome Atlas Research Network, 2013; Papaemmanuil et al., 2016), and therapeutic agents that alter the methylation state of DNA are frequently used in the clinic.

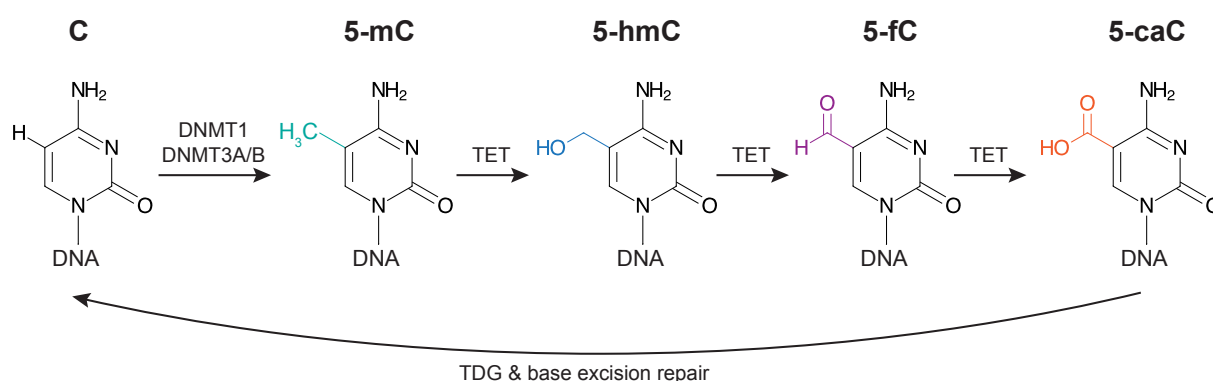


Figure 1.3 The Cycle of Methylation and Active Demethylation

Cytosine DNA methylation is catalysed by DNMT1 and DNMT3A/B. The TET family of enzymes catalyses the sequential oxidation of 5-mC to restore the unmethylated cytosine residue. C, cytosine; 5-mC, 5-methylcytosine; 5-hmC, 5-hydroxymethylcytosine; 5-fC, 5-formylcytosine; 5-caC, 5-carboxylcytosine; TDG, thymine-DNA glycosylase.

1.3.2.1 DNA Methyltransferases

DNA methyltransferases (DNMTs) catalyse the transfer of a methyl group from S-adenyl methionine (SAM) to the 5' carbon of a cytosine ring at CpG dinucleotides. Access to the 5' carbon of a cytosine ring is sterically hindered as it is internally positioned within the DNA double helix. To overcome this, DNMTs increase the distance between the phosphates flanking target cytosine, allowing the cytosine nucleotide to be rotated out of the double helix, thus exposing the 5' carbon of the pyrimidine ring for methylation (Klimasauskas et al., 1994).

Three enzymes within the human DNMT family of proteins possess the catalytic methyltransferase (MTase) domain required for 5-mC establishment, which is highly conserved across all catalytically active mammalian DNMTs (Jurkowska et al., 2011; Xie et al., 1999). DNMT1, the maintenance methyltransferase, methylates hemi-methylated DNA during DNA replication thus propagating existing methylation patterns onto the newly synthesised daughter strand (Hermann et al., 2004). Additionally, recruitment of DNMT1 to sites of DNA damage suggests a role for DNMT1 in restoring methylation during DNA repair (Mortusewicz et al., 2005). DNMT3A and DNMT3B are *de novo* methyltransferases, responsible for methylating unmodified cytosine residues, thereby establishing novel methylation patterns independent of DNA replication (Zhang and Xu, 2017). DNMT3A/B are not recruited to target CpGs through nearby DNA consensus sequences (Meissner et al., 2005), but rather through interactions with other complexes or transcription factors, or through signalling via histone marks, although these mechanisms are still poorly understood (Hervouet et al., 2018). DNMT3L, the catalytically inactive member of the DNMT3 family that is incapable of binding DNA, associates with the MTase domain of DNMT3A/B to stimulate and regulate methyltransferase activity (Jia et al., 2007; Suetake et al., 2004). DNMT2, prematurely and inappropriately named, was designated a methyltransferase by the presence of the DNA methyltransferase motif (Yoder and Bestor, 1998). However, DNMT2 possesses weak to no DNA methyltransferase activity (Hermann et al., 2003; Okano et al., 1998), but rather is capable of methylating tRNA (Goll et al., 2006).

1.3.2.2 DNA Methylation Regulates Transcription

It has long been known that DNA methylation can influence the transcriptional state. The concepts of methylated CpG inheritance during cell division and gene silencing by DNA methylation were first proposed in 1975 (Holliday and Pugh, 1975; Riggs, 1975). Almost a decade later, Venolia and Gartler (1983) were the first to experimentally demonstrate that

methyated CpGs could silence gene expression and were involved in X-chromosome inactivation. More recently, the advent of next-generation sequencing has enabled genome-wide studies of the methylome. Methyated DNA is not evenly distributed throughout the genome, nor uniformly dispersed between various genomic elements (Edwards et al., 2017; Lister et al., 2009). Transposon derived sequences are the most heavily methylated genomic features (Edwards et al., 2017) and are associated with the transcriptional repression of transposable elements (Walsh et al., 1998; Yoder et al., 1997). Although transposable elements and the inactivated X-chromosome exhibit consistent and persistent DNA methylation, the methylation state of CpGs throughout the rest of the genome is more variable and dynamic.

Sequences of high CpG density, termed CpG islands (CGIs), are enriched at promoters and regulatory features. CGIs are on average ~1 kb in length and are flanked by ~1 kb regions referred to as CGI shores (Jones, 2012). Most studies exploring differential DNA methylation during development and differentiation have predominantly focused on the association between the methylation state of CGIs that overlap with transcriptional start sites (TSS) and the expression of the associated gene (Baylin and Jones, 2011). Over two-thirds of mammalian promoters are embedded within CGIs (Greenberg and Bourc'his, 2019), particularly the promoters of housekeeping genes, and a proportion of developmentally regulated and tissue-specific genes (Gardiner-Garden and Frommer, 1987; Larsen et al., 1992). Promoter CGIs are highly conserved between human and mice, suggesting these regions are of functional importance (Illingworth et al., 2010). In normal cells, methylated CGIs that coincide with TSS correlate with transcriptional silencing and are often restricted to genes that require long-term repression, including genes located on the inactive X-chromosome, imprinted genes and germline specific genes (Greenberg and Bourc'his, 2019; Jones, 2012). Overall, the occurrence of methylated promoter CGIs in normal cells is infrequent; in most cases, methylated promoter CGIs largely exist in a hypomethylated state (Edwards et al., 2017), with cells employing other mechanisms to regulate the expression of proximal genes.

Several mechanisms by which DNA methylation can directly or indirectly induce transcriptional repression have been proposed. There is evidence to suggest that in some instances, transcription factors are sensitive to DNA methylation; methylated CGI promoters that encompass sequence-recognition motifs, sterically hinder the binding of transcription factors thereby impeding transcriptional activation (Iguchi-Ariga and Schaffner, 1989; Yin et al., 2017). Whilst 5-mC may obstruct the binding of particular transcription factors to their

recognition sites, other family of proteins exhibit a high affinity for methylated CpGs: methyl-CpG-binding domain (MBD) proteins, ubiquitin like with PHD and Ring finger domain (UHRF) proteins, and the zinc-finger proteins (Moore et al., 2013). MBD proteins can directly bind methylated DNA in a sequence independent manner (Hendrich and Bird, 1998; Meehan et al., 1989; Ohki et al., 2001). Studies have shown that MBD proteins recruit histone deacetylase and chromatin remodelling complexes, which can prompt the formation of heterochromatin thus establishing a transcriptionally repressed state (Harikrishnan et al., 2005; Hashimshony et al., 2003; Jones et al., 1998; Nan et al., 1998).

DNA methylation at TSS does not always correspond with transcriptional repression of proximal genes. Occasionally, methylated promoter CGIs are associated with the activation of gene expression (Bahar Halpern et al., 2014). Some transcriptional activators involved in cell-fate transitions exhibit a higher affinity to their recognition motifs in the methylated state; direct interactions between the transcription factor and the methyl moiety on 5-mC promotes gene expression (Bahar Halpern et al., 2014; Yin et al., 2017). There is also evidence to suggest that DNMT3A mediated *de novo* DNA methylation can result in the eviction of Polycomb repressive complex 2 (PRC2) from gene regulatory regions and hence result in the loss of the repressive epigenetic mark, H3K27me3, and transcriptional activation (Greenberg et al., 2017; Wu et al., 2010b). More frequently than methylated promoters and regulatory regions, DNA methylation within gene bodies, which is highly conserved across eukaryotes (Feng et al., 2010; Zemach et al., 2010), corresponds with increased gene expression particularly in dividing cells (Aran et al., 2011; Yang et al., 2014). Although the exact mechanisms through which gene body methylation exerts transcriptional control remains unclear, it has been proposed that gene body methylation may affect the processivity of PolIII, thereby influencing transcriptional elongation and cotranscriptional splicing (Greenberg and Bourc'his, 2019). At some gene loci, methylated CpGs can facilitate exon inclusion through the recruitment of MBDs (Maunakea et al., 2013; Shayevitch et al., 2018), which is further supported by the observation that, on average, constitutively expressed exons exhibit higher levels of 5-mC than alternative last exons (Gelfman et al., 2013).

Studies typically correlate the methylation state with the transcriptional state, so in many cases it remains unclear if DNA methylation precedes or is the consequence of transcriptional changes. Although it is typically presumed that changes in transcription follow DNA methylation, studies have demonstrated that CpG methylation can occur subsequent to

transcriptional repression (Epsztejn-Litman et al., 2008; Lock et al., 1987). For example, genes on the inactive X-chromosome are silenced prior to methylation of DNA, suggesting that DNA methylation is required for the maintenance of the transcriptionally repressed state on the inactive X-chromosome (Lock et al., 1987). Although many of the mechanisms through which DNA methylation exerts transcriptional control have not been fully elucidated, there is an abundance of evidence to suggest that DNA methylation, whether directly or indirectly, may contribute to the establishment, dynamic modulation, and maintenance of the transcriptional state.

1.3.3 DNA Methylation in Normal and Malignant Haematopoiesis

DNA methylation is important for the normal progression of haematopoiesis. Conditional depletion of Dnmt1 in murine HSCs significantly decreased HSC self-renewal capabilities and niche retention, and compromised the capacity for multi-lineage differentiation, biasing haematopoiesis down the myeloerythroid lineages (Bröske et al., 2009; Trowbridge et al., 2009). Conditional knockout of Dnmt3a in HSCs substantially increased their capacity for self-renewal. Whilst multi-lineage commitment was maintained, the quantity of mature haematopoietic cells did not reflect the proportion of HSCs in the bone marrow, suggesting a differentiation defect accompanied the increased self-renewal in Dnmt3a depleted HSCs (Challen et al., 2011). Next-generation sequencing technologies has enabled comprehensive mapping of the human and murine haematopoietic system's methylome, thus facilitating the identification of DNA methylation patterns that define stem cell identity, and the changes that occur throughout lineage commitment during differentiation (Farlik et al., 2016; Ji et al., 2010). Promoter regions of specific master transcription factors become increasingly hypermethylated as the cell progresses along the differentiation trajectory. For example, the promoter regions of the master transcription factors that regulate self-renewal properties of HSCs, *Meis1* and *Hoxa9* (Argiropoulos and Humphries, 2007; Unnisa et al., 2012), became progressively hypermethylated and transcriptionally silenced during differentiation (Ji et al., 2010).

Since DNA methylation is important for regular haematopoiesis, it is not surprising to find methylation frequently altered in diseases such as cancer (Baylin and Herman, 2000). Global DNA hypomethylation with focal promoter hypermethylation is often observed in cancer and was initially considered a hallmark (Baylin and Jones, 2011). Recent sequencing studies have provided a more extensive understanding of the aberrant methylation patterns observed in AML and the relationship with various genetic aetiologies and overall prognosis (Figueroa et al., 2010b; Glass et al., 2017). Sequencing of the methylome of AML patients, when compared to healthy individuals, revealed more hypermethylated CpGs than hypomethylated CpGs (Cancer Genome Atlas Research Network, 2013), and a subset of genes that were consistently aberrantly methylated (Figueroa et al., 2010b). These DNA methylation profiles have been used to cluster patients into distinct AML subtypes (Figueroa et al., 2010b; Glass et al., 2017). Interestingly, DNA methylation patterns at non-promoter regulatory elements, rather than CGI promoter regions, primarily dictated these AML subtypes (Glass et al., 2017). Naturally, distinct DNA methylation patterns were identified in AML patients with mutations in enzymes responsible for reading, writing and erasing DNA methyl marks (Glass et al., 2017). For example, distinct

hypomethylation signatures are seen in AML patients harbouring heterozygous DNMT3A^{R882} mutations (Qu et al., 2014). However, irregular DNA methylation signatures were also defined in AML patients with mutations in proteins that have no known role associated with DNA methylation (Glass et al., 2017), suggesting that the AML genetic background can influence the methylome. Whilst the popular view is one where aberrant DNA methylation precedes the transcriptional reprogramming required for leukaemic transformation, there is evidence to suggest that irregular promoter CpG island hypermethylation occurs as a result of cellular proliferation and is not a leukaemic specific phenomenon (Spencer et al., 2017). Collectively these data highlight the importance of DNA methylation in normal and irregular haematopoiesis, however, whether aberrant DNA methylation is the cause of leukaemogenesis or a consequence of AML progression continues to be disputed.

1.3.4 Epigenetic Therapies for the Treatment of Cancer

In recent years, there has been an emergence of an array of epigenetic therapies for the treatment of malignancies, with many advancing into clinical trials including inhibitors of histone methyltransferases (e.g. EZH2, DOT1L), histone demethylases (e.g. LSD1), arginine methyltransferases (e.g. PRMT5), Bromodomain and Extra-Terminal motif family of proteins (e.g. BRD4) and lysine acetyltransferases (e.g. CREBBP, EP300) (Wingelhofer and Somervaille, 2019). Whilst some small molecules directly inhibit oncogenic drivers, such as AG120 inhibition of IDH1^{R132H}, in order to reverse the consequential malignant epigenetic landscape, other therapeutics, such as hypomethylating agents, target wildtype epigenetic regulators with the aim of reprogramming the transcriptional networks driving malignant progression. The reversibility and plasticity of the epigenome, its role in transcriptional control and its influence on cancer biology, highlights the therapeutic promise for inhibitors of epigenetic regulators for the treatment of cancer.

1.3.4.1 Hypomethylating Agents for the Treatment of Haematological Malignancies

Hypomethylating agents, including 5-Azacytidine (AZA), Decitabine (DAC), and DAC derivatives, are azanucleoside analogues and were the first class of epigenetic drugs approved by the FDA for the treatment of MDS and AML. Originally, hypomethylating agents were utilised as classical cytostatic/cytotoxic agents over 40 years ago (Karon et al., 1973), but had soon evolved into an epigenetic therapy due to their ability to hypomethylate the genome at low doses (Wijermans et al., 1997).

5-Azacytidine and Decitabine are pyrimidine analogues of the nucleoside cytidine. The structure of AZA and DAC consists of a ribose or deoxyribose moiety, respectively, attached to a cytosine-like ring with a Nitrogen atom at the 5th position of the pyrimidine ring (Figure 1.4). AZA was the first hypomethylating agent to be approved in the clinic (Diesch et al., 2016). However, the majority of AZA is incorporated into RNA, with only 10 – 20% being incorporated into DNA where it is thought to exert its main anti-tumour effects. This observation prompted the development of DAC, a hypomethylating agent that is solely incorporated into DNA (Diesch et al., 2016). However, AZA and DAC are rapidly cleared from the plasma, with a mean elimination half-life of ~40 minutes (Derissen et al., 2013), owing to high levels of cytidine deaminase in the liver and spleen (Chabot et al., 1983). Guadecitabine (GDAC), a second-generation derivative of DAC is a dinucleotide consisting of the active

metabolite decitabine and deoxyguanosine (Figure 1.4) that is currently in phase 3 of clinical trials. GDAC was developed to prolong the exposure to hypomethylating agents and improve patient response; GDAC is relatively resistant to cytidine deaminase, owing to the deoxyguanosine moiety, and presents enhanced pharmacokinetics and pharmacodynamics (Issa et al., 2015).

The standard treatment of care for MDS and AML patients, comprising anthracycline and cytarabine induction, is associated with substantial morbidity, which is further amplified in patients over 65 years (Büchner et al., 2009; Kantarjian et al., 2010). Since adult myeloid malignancies disproportionately affect older individuals and hypomethylating agents are well tolerated at low doses (Navada and Silverman, 2017), this class of epigenetic therapy has become a front-line treatment option for patients unable to receive intensive chemotherapy induction (Tallman et al., 2019). Unfortunately, the use of the first-generation hypomethylating prodrugs as single agents was only effective in ~30% of patients with a survival benefit that was typically measured in months and relapse a common occurrence (Fenaux et al., 2009; He et al., 2017). However, the combination of hypomethylating agents and other therapeutics in order to improve patient outcomes is an area of active investigation as evident by the abundance of clinical trials (Craddock et al., 2019; Jilg et al., 2019; Onec et al., 2018; Swords et al., 2018). Despite the rapid expansion in our understanding of the genetic and epigenetic landscape of AML, researchers have not been able to identify common characteristics shared by patients that predict response to hypomethylating agents. It remains important to identify biomarkers that stratify between potential responders and non-responders. Furthermore, mechanisms through which resistance and subsequent relapse arises in patients remains unclear.

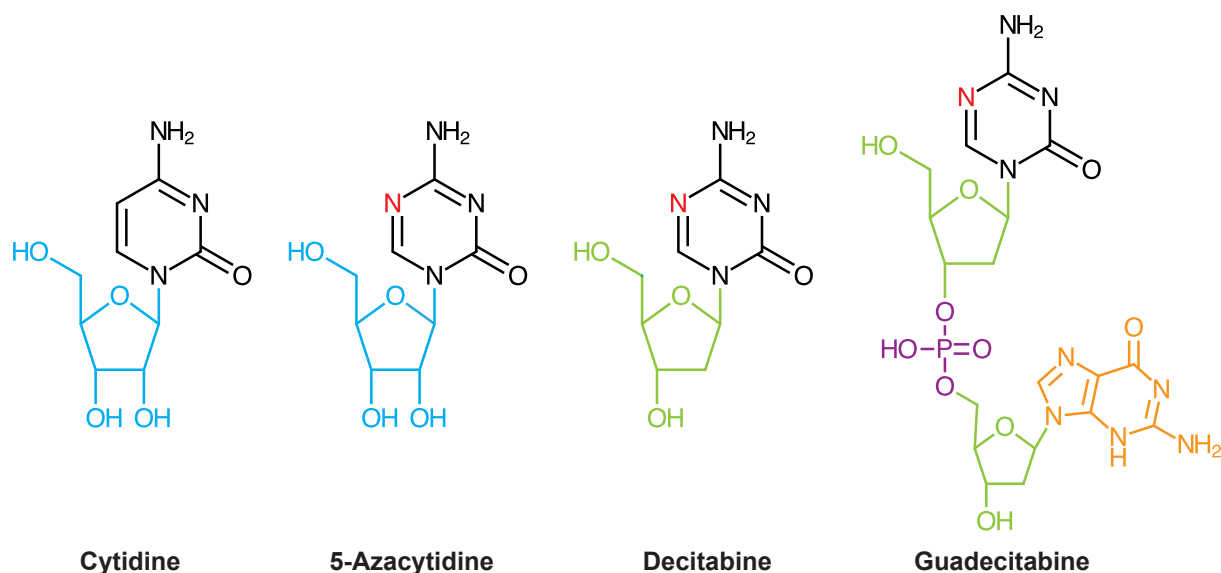


Figure 1.4 Azanucleoside Analogues of Cytidine

Chemical structures of cytidine and azanucleoside analogues of cytidine used as hypomethylating agents for the treatment of AML. Moieties are colour coded: blue, ribose sugar; green, deoxyribose sugar; purple, phosphate; orange, guanosine; black, cytosine ring; red, Nitrogen atom at the 5th position in the pyrimidine ring in the azanucleosides.

1.3.4.1.1 Mechanism of Action of Hypomethylating Agents

Hypomethylating agents proceed through a cascade of metabolic steps, by utilising the pyrimidine salvage pathway, prior to becoming the active metabolite capable of being incorporated into RNA/DNA. Equilibrative and concentrative nucleoside transporters facilitate the uptake of hypomethylating agents into the cell (Diesch et al., 2016). Cleavage of the phosphodiester bond in GDAC by phosphorylases releases guanosine and DAC. Uridine-cytidine kinase 2 (UCK2) and deoxycytidine kinase (DCK) catalyse the mono-phosphorylation of AZA and DAC, respectively. Biochemical assays quantifying isotopically labelled uridine and phosphorylated derivatives have demonstrated that the initial phosphorylation of uridine catalysed by UCK is the rate-limiting reaction in the pyrimidine salvage pathway (Anderson and Brockman, 1964). Hence, the first phosphorylation of azanucleosides is proposed to be the rate-limiting step in the metabolism of hypomethylating agents (Sripayap et al., 2014; Valencia et al., 2014), although has not been experimentally validated. The primary phosphorylation of hypomethylating agents by UCK2 and DCK instigates successive rounds of phosphorylation prior to the incorporation of the tri-phosphorylated hypomethylating agent into RNA or DNA, respectively. Approximately 10 – 20% of di-phosphorylated AZA is reduced to di-phosphorylated DAC by ribonucleotide reductase, where it is phosphorylated once more and subsequently incorporated into DNA (Diesch et al., 2016) (Figure 1.5).

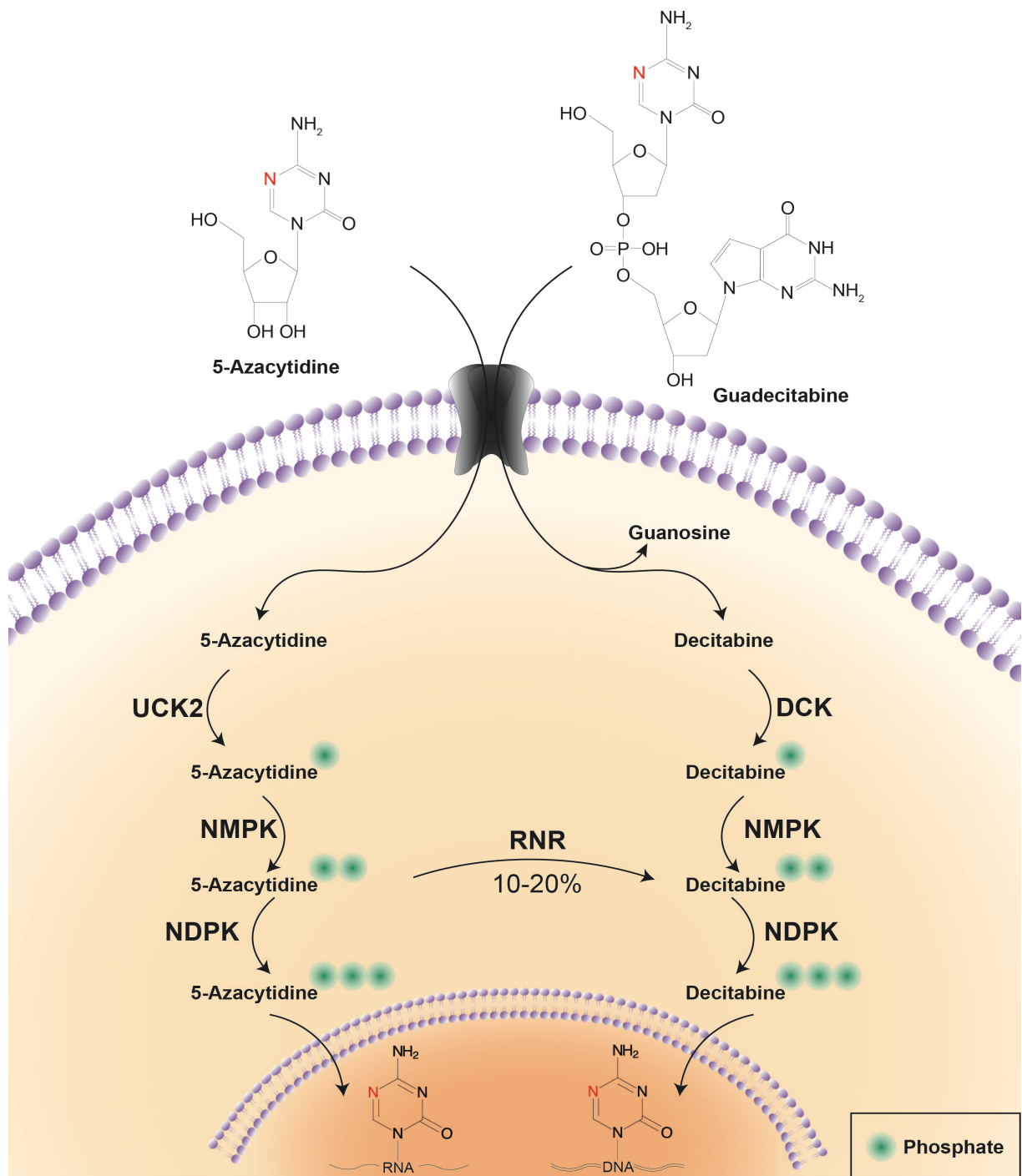


Figure 1.5 Metabolism of Azanucleosides

Azacytidine and Guadecitabine are imported into the cell by equilibrative and concentrative transporters where they undergo sequential phosphorylation before the tri-phosphorylated azanucleosides are incorporated into DNA or RNA. UCK2 and DCK catalyse the rate-limiting mono-phosphorylation of 5-Azacytidine and Decitabine, respectively. Nucleoside-monophosphate kinases (NMPKs) and nucleoside-diphosphate kinases (NDPKs) catalyse the secondary and tertiary phosphorylation of hypomethylating agents. Ribonucleotide reductase (RNR) converts ~10 – 20% of 5-Azacytidine diphosphate to Decitabine diphosphate.

Hypomethylating agents are frequently referred to as DNMT1 inhibitors due to their ability to bind and deplete DNMT1 at low doses, with DNMT3A and DNMT3B only affected at high doses (Ghoshal et al., 2005; Liu et al., 2003). Incorporation of azanucleosides into DNA occurs during S-phase and results in the irreversible covalent trapping of DNMT1 (Santi et al., 1984). Methylation by DNMTs proceeds through 3 sequential steps: the sulfhydryl group of cysteine in the catalytic domain of DNMTs form a covalent attachment to the 6' carbon of the cytosine ring, a methyl moiety is transferred from S-adenosyl-L-methionine (SAM) to the 5th carbon of the pyrimidine ring, and β -elimination releases a proton and DNMT (Chen et al., 1991; Santi et al., 1983) (Figure 1.6 A). The absence of a proton at the 5th position of the pyrimidine ring of azanucleosides prevents β -elimination, thereby irreversibly immobilising DNMT1 onto the azanucleoside adduct (Santi et al., 1984; Schermelleh et al., 2005) (Figure 1.6 B). Additionally, hypomethylating agents are capable of inducing proteasomal degradation of unbound DNMT1 (Ghoshal et al., 2005; Patel et al., 2010), although the mechanism through which this occurs remains unresolved. The loss of methyltransferase activity by azanucleoside induced trapping of DNMT1 and depletion of the cellular pool of free DNMT1, eventually results in the hypomethylation of the genome through the passive dilution of 5-mC during progressive rounds of DNA replication in proliferative cells (Creusot et al., 1982). It is widely accepted that treatment with hypomethylating agents reactivates tumour suppressor genes that were silenced by DNA methylation thus inducing differentiation or apoptosis in malignant cells (Bender et al., 1998) (Figure 1.6 B). However, this is likely an overly simplistic view of the anti-tumour effects that hypomethylating agents elicit. More recently, Tsai and colleagues (2012) reported that transient low-dose DAC exposure *in vitro* induced semi-sustained hypomethylation of promoter CGIs which were concordant with proximal gene expression changes, and reduced the aberrant self-renewal capacity without eliciting acute cytotoxicity from DNA damage, cell cycle alterations or apoptosis. However, unlike the gene expression changes induced by DAC exposure (Tsai et al., 2012), the perturbation to the transcriptome was subtle upon AZA treatment (Leung et al., 2019). Collectively, this suggests that epigenetic modulation of 5-mC contributes to the anti-leukaemic properties of hypomethylating agents, although the extent likely differs between distinct hypomethylating therapies.

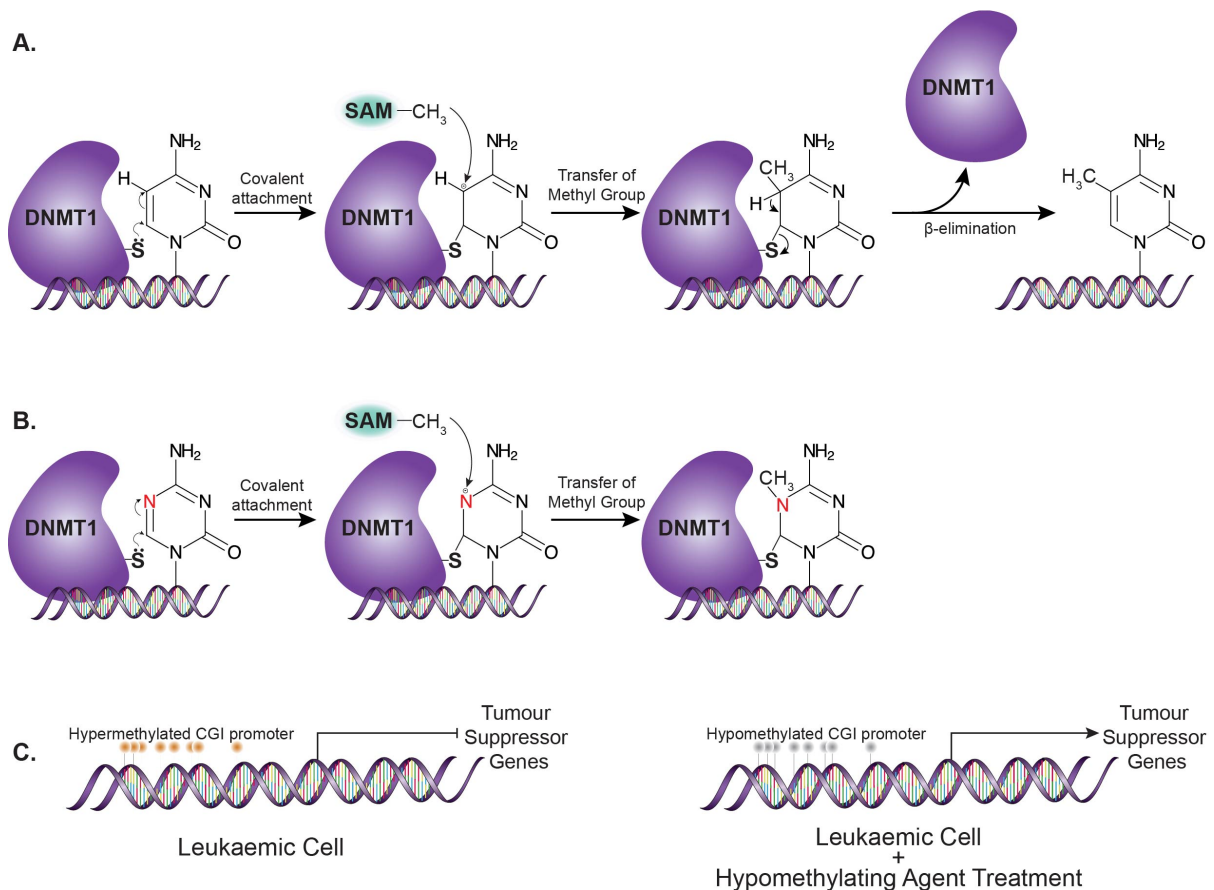


Figure 1.6 Mechanism of Action of Azanucleosides

(A) Mechanism of cytosine methylation by DNMT1: (1) the sulphydryl group of cysteine in the catalytic domain of DNMT1 covalently binds the 6' carbon of the cytosine ring, (2) a methyl moiety is transferred from S-adenosyl-L-methionine (SAM) to the 5th carbon of the pyrimidine ring, and (3) β -elimination releases a proton and DNMT1. **(B)** Mechanism of covalent trapping of DNMT1 by hypomethylating agents. Covalent attachment of DNMT1 to azanucleosides incorporated into DNA and the transfer of the methyl moiety from SAM occur as per the first 2 steps of cytosine methylation. The absence of a proton at the 5th position of the pyrimidine ring of azanucleosides prevents β -elimination, thereby irreversibly immobilising DNMT1 onto the azanucleoside adduct. **(C)** Reactivation of tumour suppressor genes by azanucleosides. Figure adapted from Schermelleh et al. (2005).

Studies investigating the mechanisms that drive the anti-leukaemic effects of hypomethylating agents have primarily focused on the association of hypomethylated CGI promoters and corresponding gene expression. However, DNMT1 has many interacting partners, including chromatin modifiers (Qin et al., 2011). Few studies have investigated the consequences of hypomethylating agent exposure on histone modifications and chromatin structure, and often only examine the histone modifications proximal to the gene of interest (Hesson et al., 2013; Si et al., 2010). Nonetheless, hypomethylating agents have been reported to induce changes in the abundance of specific histone marks, such as H3K27me3, H3K9ac and H3K4me3, that associate with proximal gene expression up-regulation (Hesson et al., 2013; Si et al., 2010). Furthermore, Tobiaasson and colleagues (2017) observed that AZA induced DNA hypomethylation associated with H3K9me3 changes, and weakly with up-regulated genes, in MDS patients. More research is needed to ascertain the scale at which histone modifications and chromatin structures are altered upon exposure to hypomethylating agents, and their contribution to therapy induced gene expression changes.

At higher doses, hypomethylating therapies cause direct cytotoxicity resulting from DNA damage, cell-cycle arrest and apoptosis. DNMT1-azanucleoside adducts in DNA induce genome rearrangements (Maslov et al., 2012) potentially through double-stranded DNA breaks (Palii et al., 2008). Increasing the dose of the hypomethylating agent resulted in the plateau of DNA methylation, cell-cycle arrest and the induction of apoptosis (Creusot et al., 1982; Hollenbach et al., 2010; Khan et al., 2006). Low dose AZA treatment with concurrent depletion of DNMT1 by RNA interference reduced the AZA induced DNA damage, suggesting that the DNMT1-azanucleoside adduct was the cause of the DNA damage and not hypomethylation (Vispé et al., 2015).

As aforementioned, the vast majority of AZA is incorporated into RNA, so it is not unreasonable to hypothesise that a proportion of the anti-tumour effects exerted by AZA are a consequence of the incorporation of AZA into RNA. Early studies demonstrated that the incorporation of AZA into RNA inhibited the methylation and processing of tRNA (Lee and Karon, 1976). Further evidence for the methylation of tRNAs was provided by Schaefer and colleagues, who demonstrated that AZA treatment inhibited the methylation of cytosine 38 on the DNMT2 tRNA substrates as determined by RNA bisulphite sequencing (Schaefer et al., 2009). AZA has also been utilised in the mapping of RNA methyltransferases to their target cytosine residues (Khoddami and Cairns, 2013). Furthermore, by exploiting the formation of

the covalent bond between AZA and an RNA methyltransferase (such as DNMT2 or NSUN2) for AZA mediated immunoprecipitation, Khoddami and Cairns (2013) were able to identify the distinct cytosine residues on specific types of RNA molecules whose methylation was catalysed by the RNA methyltransferase in question. Indirectly, this provided invaluable information on the specific RNA cytosines that are likely hypomethylated as a consequence of AZA treatment, and thus suggests that AZA inhibits DNMT2-mediated tRNA methylation. Most recently, proteomic quantitation following AZA treatment revealed changes to the nuclear proteome, although the mechanism by which AZA induced the altered nuclear proteome was not discussed (Bowler et al., 2019). Of note, this experiment was conducted under high doses of AZA whereby a significant difference in cell viability was observed. Despite these recent data, the implications of methylated RNA still remain poorly characterised. It remains to be seen whether low dose AZA induces aberrant splicing, impaired translation and affects the whole-cell proteome.

Recent evidence suggests that the effectiveness of hypomethylating agents is due, at least in part, to its impact on anti-tumour immunity. Gene expression analysis revealed that low dose AZA treatment in cell lines and patient biopsies induced upregulation of immunomodulatory pathways, including interferon signalling, suggesting an immune stimulatory role for hypomethylating agents and the rationale for the synergistic effect when combined with checkpoint blockade immunotherapies (Li et al., 2014a). Additionally, DNA hypomethylation, by AZA and DAC, and consequential transcriptional activation of endogenous retroviruses (ERVs) triggered the double-stranded RNA viral defence pathway that induced a type I interferon response (Chiappinelli et al., 2015; Jeong et al., 2015; Stone et al., 2017). These effects on tumour immunity elicited by low dose hypomethylating therapeutics has only been assessed in solid tumour models such as melanoma, breast, ovarian and non-small cell lung cancer, and has yet to be analysed in haematological malignancies. However, Gang et al. (2014) reported an AZA induced increase in the T-cell recognition of tumour cells in patients with myeloid malignancies. Overall, these data highlight the effect that hypomethylating agents elicit on tumour immunity, and the potential to enhance the therapeutic response when combined with relevant immunotherapies.

1.4 IDH in Normal and Malignant Cells

In normal cells, the IDH enzymes are responsible for the production of α -KG, a metabolite of the citric acid cycle and cofactor of ~60 enzymes involved in various cellular activities, and contribute to redox homeostasis by providing energy for the cells in the form of NADH/NADPH. Mutations in *IDH* occur frequently in normal karyotype AML and a variety of other cancers. Oncogenic IDH produces a novel oncometabolite, 2-HG, which can disrupt a multitude of pathways by inhibiting enzymes that utilise α -KG as a co-substrate. This section discusses the pathways that require α -KG, the implications of 2-HG inhibition and contribution to the malignant transformation, and the advances in mutant IDH targeted therapeutics.

1.4.1 IDH Protein Structure and Function

In eukaryotes, the isocitrate dehydrogenase (IDH) family comprises three metabolic enzymes that catalyse the oxidative decarboxylation of isocitrate to α -ketoglutarate (α -KG). These enzymes can be stratified into two structurally and evolutionarily distinct classes with cofactors of either nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP) (Ye et al., 2018). IDH1 and IDH2 are homodimeric NADP-dependent enzymes that share 70% amino acid sequence and exhibit highly conserved protein structure. IDH3 is a heterotetrameric NAD-dependent isoform, comprised of two α , one β and one γ subunit encoded by *IDH3A*, *IDH3B* and *IDH3G*, respectively (Bzymek and Colman, 2007; Ramachandran and Colman, 1980). Although distinct, both IDH2 and IDH3 localise to the mitochondrial matrix via N terminal mitochondrial targeting sequences (Waitkus et al., 2016), whereas eukaryotic IDH1 contains a C terminal peroxisomal targeting sequence (Nekrutenko et al., 1998) and localise to the cytosol and peroxisomes (Kim and Park, 2003).

The human NADP-dependent IDH1 enzyme functions as an asymmetric homodimer with each IDH1 subunit consisting of three domains: a large domain (residues 1 – 103 and 286 – 414), a small domain (residues 104 – 136 and 186 – 285), and a clasp domain (residues 137 – 185) (Figure 1.7) (Xu et al., 2004). The functional enzyme contains two active sites situated within the crevice between the large and small domains of one subunit and the small domain of the adjoining subunit (Xu et al., 2004). An NADP binding site and isocitrate-metal ion-binding site reside within each active site (Xu et al., 2004). In the open inactive conformation, substrate, cofactor and bivalent metal ion can access the exposed hydrophilic active site, and interact with residues that are highly conserved across eukaryotic NADP-IDHs (Xu et al., 2004). The active

state exists in a closed conformation bringing the bound isocitrate and NADP in close proximity thereby inducing catalysis. It was hypothesised that phosphorylation of a serine residue within the active site of NADP-dependent IDHs functioned to induce conformational change and hence regulate enzymatic activity, based on observations of Ser113 phosphorylation in *Escherichia coli* (Hurley et al., 1990) and Ser-104 *Bacillus subtilis* (Singh et al., 2001). However, no published evidence of serine phosphorylation of eukaryotic NADP-dependent IDHs has been reported. Rather, based on crystallisation and diffraction data, Xu and colleagues suggested a self-regulatory mechanism whereby the side chain of Asp-279 forms a hydrogen bond with Ser-94, thus competitively obstructing isocitrate from accessing the active site through steric hinderance and electrostatic repulsion (Xu et al., 2004). Furthermore, phosphorylation of specific tyrosine residues of human NADP-dependent IDHs has been reported to enhance dimerization of IDH monomers, and substrate and cofactor binding (Chen et al., 2019).

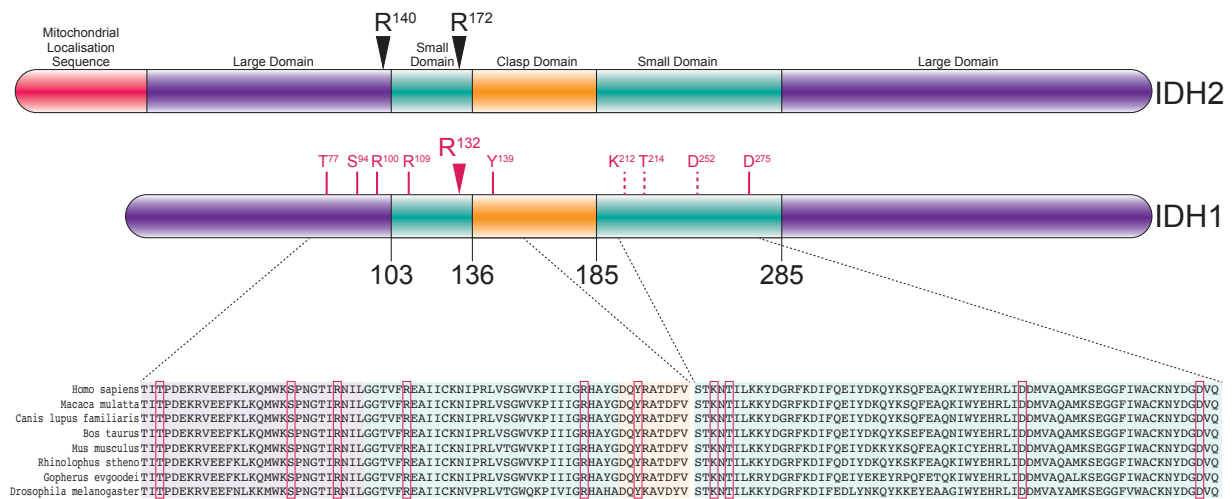


Figure 1.7 Structures of IDH1 and IDH2 Enzymes

Schematics of the domain structures of IDH1 and IDH2 are provided and regions containing evolutionarily conserved isocitrate binding residues highlighted. Arrows indicate residues frequently mutated in AML. Pink lines/arrows denote the residues within each of the catalytic sites of the IDH1 homodimer that interact with isocitrate; solid and dotted lines signify the two distinct IDH1 subunits.

1.4.2 The Role of α -KG in Cellular Metabolism

Alpha-ketoglutarate is an essential metabolite required for the second stage of cellular respiration, the Tricarboxylic Acid (TCA) cycle. In eukaryotes, The TCA cycle encompasses eight sequential reactions occurring in the mitochondrial matrix: the condensation of acetyl CoA and oxaloacetate to form citrate, isomerisation to isocitrate; oxidative decarboxylation to α -KG, oxidative decarboxylation to succinyl CoA, enzymatic conversion to succinate, oxidation to fumarate, hydration to malate, and finally oxidation to oxaloacetate (Figure 1.8) (Kumari, 2018). Each rotation of the TCA cycle yields 1 ATP molecule, and 1 FADH₂ and 3 NADH molecules whose energy potential is further converted into ATP along the electron transport chain (Kumari, 2018).

Due to differences in structure and subcellular compartmentalisation, the three IDH isoforms expressed in mammalian cells have non-redundant roles in cellular metabolism. Within mitochondria, IDH3 provides the primary source of α -KG in the TCA via an irreversible reaction (Losman and Kaelin, 2013), whilst IDH2 is believed to modulate energy metabolism by regulating the ratio of isocitrate and α -KG through the reversible interconversion between the two metabolites (Losman and Kaelin, 2013) (Figure 1.8). IDH1, present in the cytosol and peroxisomes, can reversibly interconvert between isocitrate and α -KG (Figure 1.8), and contributes to non-mitochondrial NADPH required for lipid biosynthesis and protection from oxidative stress (Jiang et al., 2016; Losman and Kaelin, 2013; Metallo et al., 2011).

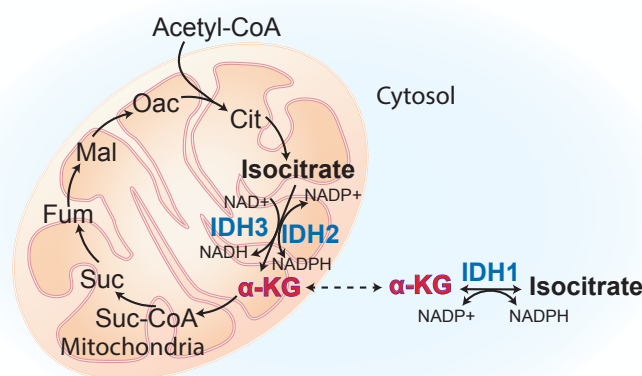


Figure 1.8 IDH in the TCA Cycle

Schematic of wildtype IDH localisation and function in the Tricarboxylic Acid Cycle. Oac, oxaloacetate; Cit, citrate; Suc-CoA, succinyl-CoA; Suc, succinate; Fum, fumarate; Mal, malate; α -KG, α -ketoglutarate.

1.4.3 Mutant IDH in Cancer

A missense mutation in *IDH1* was initially reported in a single colorectal cancer patient in a study investigating protein-coding exon mutations in a small cohort of breast and colorectal malignancies (Wood et al., 2007). Since then, several recurrent mutations in NADP-dependent IDHs have been implicated in a plethora of malignant diseases (Table 1.5) including AML (Mardis et al., 2009; Papaemmanuil et al., 2016; Paschka et al., 2010). Consequently, the number of mutant IDH associated publications rapidly increased, with mounting levels of evidence demonstrating the resultant metabolic and epigenetic reprogramming. Interestingly, mutations in the NAD-dependent isoform, *IDH3*, rarely occur in cancer patients (Remacha et al., 2017). However elevated wildtype IDH3 has been reported in glioblastoma patient samples (May et al., 2019) and demonstrated to promote tumour progression through metabolic reprogramming (May et al., 2019; Zeng et al., 2015).

Non-synonymous mutations occur almost exclusively in the codon of Arg-132 of IDH1, the equivalent IDH2 residue, Arg-172, and Arg-140 of IDH2 in *IDH* mutant malignancies, however the incidence of these mutant *IDH* alleles differs between cancer subtypes (Losman and Kaelin, 2013). These arginine residues are evolutionarily highly conserved in eukaryotes and are integral for isocitrate binding within the active site (Xu et al., 2004). Heterozygous arginine to histidine substitution at residue 132 is the most abundant genetic aberration in mutant IDH1 malignancies (Kang et al., 2009; Papaemmanuil et al., 2016; Yan et al., 2009), resulting in the formation of wildtype-wildtype ($IDH1^{R/R}$), wildtype-mutant ($IDH1^{R/H}$), and mutant-mutant ($IDH1^{H/H}$) dimers (Jin et al., 2011). The *IDH1^{R132H}* mutation disrupts the interaction of the self-regulatory segment, rendering the complex in an open or quasi-open conformation thus reducing isocitrate binding capabilities (Yang et al., 2010). Contrary to initial belief (Zhao et al., 2009), the mutant protein does not exert a dominant negative effect on the wildtype counterpart (Jin et al., 2011) but rather exhibits a greater affinity for α -KG and NADPH resulting in the neomorphic enzymatic activity proficient at reducing α -KG to D-2-hydroxyglutarate (2-HG) (Dang et al., 2009) (Figure 1.9 A). Ultimately, the supraphysiological accumulation of 2-HG in cells induces pleiotropic effects that drive malignant transformation or progression (Figure 1.9 B) (see section 1.3.2.2) (Losman et al., 2013).

Table 1.5 Frequency of NADP-dependent IDH Mutations in Cancer

Malignancy	Prevalence	Reference
Glioma	50 – 88%	(Amary et al., 2011) (Balss et al., 2008) (Kang et al., 2009) (Parsons et al., 2008) (Yan et al., 2009)
Solid papillary carcinoma with reverse polarity	78%	(Alsadoun et al., 2018)
Angioimmunoblastic T cell lymphoma	10 – 40%	(Cairns et al., 2012) (Nguyen et al., 2017) (Wang et al., 2017)
AML (cytogenetically normal)	10 – 30%	(Papaemmanuil et al., 2016) (Paschka et al., 2010)
Cholangiocarcinoma	10 – 20%	(Borger et al., 2012) (Farshidfar et al., 2017)
Myeloid dysplastic syndrome and/or myeloproliferative neoplasm	5 – 10%	(Kosmider et al., 2010)
Thyroid carcinoma	5 – 16%	(Hemerly et al., 2010) (Murugan et al., 2010)
Colorectal cancer	< 10%	(Wood et al., 2007) (Whitehall et al., 2014)
Melanoma	5%	(Linos and Tafe, 2018)
Prostate cancer	< 3%	(Ghiam et al., 2012) (Kang et al., 2009)
B-acute lymphoblastic leukaemia	< 2%	(Kang et al., 2009)
Paraganglioma	< 1%	(Gaal et al., 2010) (Fishbein et al., 2017)

1.4.3.1 2-HG is a Biomarker for Mutant IDH Malignancies

Errors in catalysis and promiscuous enzymatic activities result in low levels of 2-HG production (Dang et al., 2016; Fan et al., 2015). To date, 2-HG has no known metabolic functions under cellular homeostasis in normal cells, and is rapidly converted back to α -KG by enantiomer specific dehydrogenases (Losman and Kaelin, 2013). However, the acquisition of an oncogenic mutation in IDH1/2 elevates intracellular 2-HG to millimolar concentrations, at least 100 fold greater than IDH wildtype cells, thus overwhelming the cell's innate 2-HG clearance mechanism (Dang et al., 2009). Furthermore, heightened 2-HG concentration is detectable in the plasma and serum (DiNardo et al., 2013; DiNardo et al., 2018; Lombardi et al., 2015), and in the malignant tissue (Andronesi et al., 2012; Choi et al., 2012; Elkhaled et al., 2012) of mutant IDH patients. Elevated 2-HG has therefore been implicated as a diagnostic biomarker for mutant *IDH* malignancies. However, since 2-HG levels at baseline does not correlate with clinical outcome (Amatangelo et al., 2017), and 2-HG suppression has been observed during treatment with mutant IDH inhibitors in both responding and non-responding patients (Amatangelo et al., 2017; DiNardo et al., 2018), 2-HG is not a prognostic indicator of clinical response.

1.4.3.2 Pleiotropic Activities of α -KG and Oncometabolite 2-HG

TCA cycle metabolites can also serve as precursors for the biosynthesis of compounds including amino acids. For example, reductive amination of α -KG yields glutamate, which can be further converted to glutamine, proline and arginine (Walker and van der Donk, 2016). α -KG is also an intermediate for neurotransmitter synthesis in the brain (Leke et al., 2011). Furthermore, α -KG is an essential co-substrate of Fe(II)/ α -KG-dependent dioxygenases comprising more than 60 enzymes involved in a diverse array of cellular pathways including the regulation of epigenetic modifications of histone tails, genomic DNA and RNA, transamination of amino acids, and substrate hydroxylation.

2-hydroxyglutarate and α -ketoglutarate differ by a single bond; 2-HG contains a hydroxyl moiety in place of the ketone functional group present in α -KG (Figure 1.9 A). Consequently 2-HG is able to bind α -KG-dependent dioxygenases and disrupt their catalytic functions. Interestingly, α -KG-dependent dioxygenases have a significantly lower binding affinity for 2-HG relative to α -KG (Chang et al., 2019; Tarhonskaya et al., 2017; Xu et al., 2011). However, the abundant accumulation of 2-HG in mutant *IDH* cells increases the statistical likelihood of 2-HG occupying the substrate-binding pocket of α -KG-dependent dioxygenases and hence inhibiting its enzymatic function. Dang et al. (2009) and Ward et al. (2010) first proposed 2-HG as an oncometabolite whose accumulation resulted in the dysregulation of an array of pathways which contribute to oncogenic transformation. Since then, a plethora of research has further cemented 2-HG as an oncometabolite capable of facilitating leukaemic transformation and progression (Chaturvedi et al., 2013; Chen et al., 2013; Kats et al., 2014; Losman et al., 2013; Lu et al., 2012; Turcan et al., 2012).

Efforts to elucidate the mechanistic consequences of supraphysiological concentrations of 2-HG have predominantly focused on 2-HG inhibition of α -KG-dependent dioxygenases. Arguably, the most studied collection of α -KG-dependent dioxygenases inhibited by 2-HG are those involved in epigenetic processes. 2-HG mediated disruption of TETs, KDMs and ALKBHs is hypothesised to promote malignant transformation through epigenetic remodelling and consequential transcriptional dysregulation (Turcan et al., 2018; Xu et al., 2011).

1.4.3.2.1 2-HG Inhibition of KDMs Induces Histone Hypermethylation

Post-translational modifications of N-terminal histone tails are deposited, interpreted and removed by epigenetic “writers”, “readers” and “erasers”, respectively, to dynamically regulate chromatin organisation, DNA accessibility, recruitment of transcription factors and hence transcriptional activation and repression (Sadakierska-Chudy and Filip, 2015). The methylation state of specific histone lysine residues and the co-occurrence with other histone marks demarcate regions of active or silenced transcription (Hyun et al., 2017). For example, methylated H3K4, H3K36 and H3K79 are broadly considered marks of transcriptionally active euchromatin, whilst methylated H3K9, H3K27 and H4K20 are associated with transcriptionally repressed heterochromatin (Hyun et al., 2017). The co-existence of H3K27ac and H3K4me1 is indicative of an active transcriptional state, whilst H3K27me3 with H3K4me3 is suggestive of a transcriptionally poised state (Hyun et al., 2017). Adding to the complexity of the histone code, distinct methylation states of specific histone lysine residues are enriched within different genomic regions, for example, H3K4 monomethylation at enhancers, dimethylation near the 5' end of actively transcribing genes, and trimethylation at the promoters and transcriptional start sites of poised and actively transcribing genes (Hyun et al., 2017).

A family of histone lysine methyl erasers, the Jumonji C domain containing histone lysine demethylases (JmjC KDMs), are the most abundant class of α -KG-dependent dioxygenases, encompassing 33 enzymes in humans (Oh et al., 2019; Xu et al., 2011). JmjC KDMs mediate the demethylation of mono-, di- or trimethylated histone lysine residues by catalysing the hydroxylation of the methyl moiety, utilising α -KG as a co-substrate. The resultant hydrolytically labile carbinolamine moiety spontaneously fragments, releasing formaldehyde and the unmodified lysine residue (Oh et al., 2019; Walport et al., 2012). Disruption of histone lysine methyl transferases (KMTs) and JmjC KDMs can profoundly affect the transcriptome and has been implicated in the pathogenesis of various cancers (Cloos et al., 2008; McGrath and Trojer, 2015). 2-HG inhibition of many JmjC-domain containing KDMs results in the irregular methylation at specific histone tail lysine residues (Chowdhury et al., 2011; Lu et al., 2012; Xu et al., 2011), which is hypothesised to contribute to the pathogenesis of mutant *IDH* driven cancers (Lu et al., 2012). Alterations in H3K4, H3K27, H3K36 and H3K9 methylation abundance and patterns have been reported as a consequence of 2-HG inhibition of JmjC KDMs (Table 1.6). At least twelve JmjC KDMs have an affinity for methylated H3K4 or H3K27 substrates (Chang et al., 2019), and *in vitro* evidence has repeatedly shown that 2-HG inhibition of KDMs increased the abundance of these methylated marks (Table 1.6). Through the

correlation of the histone methylation status and transcriptional states of proximal genes, researchers have proposed that the increase in histone methylation observed in mutant *IDH* malignancies drives aberrant gene expression. However, irregular and profuse histone methylation has not yet been confirmed in mutant *IDH* AML patient samples, and nor has altered histone methylation been correlated with the aberrant expression of associated genes in this cohort. The dynamic reshaping of the histone methylome and the chromatin landscape by 2-HG has largely been studied in *in vitro* models ectopically expressing mutant *IDH*. Furthermore, conflicting evidence exists in the literature regarding the change in H3K9me3 and H3K36me3 abundance in the presence of 2-HG (Kernytsky et al., 2015; Su et al., 2018; Turcan et al., 2018).

In addition to histone lysine residues, JmjC KDMs have been reported to hydroxylate asparagine (Lando et al., 2002), arginine (Walport et al., 2016), histidine (Yang et al., 2011), RNA (Noma et al., 2010), and non-histone lysine residues (Han et al., 2019a), thereby instigating their demethylation. The functional consequences of these marks, however, remains largely uncharacterised. Unsurprisingly, the potential perturbation of these marks and subsequent cellular effects in mutant *IDH* expressing cells remains poorly understood.

Table 1.6 Consequences of 2-HG Inhibition of Histone Demethylases

α-KG-dependent dioxygenase	Increase in histone mark	Model system	Reference
KDM4A	H3K9me3	HeLa	(Chowdhury et al., 2011)
–	H3K4me1/2/3 H3K27me2 H3K79me2 H3K9me2	U-87MG	(Xu et al., 2011)
KDM4C	H3K9me2/3 H3K4me3 H3K27me3	HEK293T 3T3-L1	(Lu et al., 2012)
–	H3K79me2 H3K36me3 H3K4me3	Haematopoietic progenitor IDH1 ^{R132H} knock-in mouse model	(Sasaki et al., 2012)
–	H3K4me3 H3K9me3 H3K27me3 H3K36me3	TF-1	(Kernytsky et al., 2015)
–	H3K4me3 H3K36me3 H3K9me3 H3K27me3	Patient derived tumourspheres & Immortalised human astrocytes	(Turcan et al., 2018)
–	No increase: H3K9me3 H3K36me3	NOMO-1 U937 MA9.3ITD HEL K562 NB4	(Su et al., 2018)

1.4.3.2.2 2-HG Inhibition of TETs Induces DNA Hypermethylation

α -KG is a co-substrate of TETs, a class of enzymes involved in DNA demethylation. The TET family of enzymes catalyse the removal of the methyl moiety from 5-mC through the sequential oxidation of 5-mC. DNA methylation proximal to transcriptional start sites and the effect on the expression of the associated genes has been extensively studied (see section 1.2.2.2) (Jones, 2012). It is widely accepted that hypermethylation at CpG islands within promoter regions correlates with transcriptional repression of the downstream genes (Jones, 2012). The TET family of proteins are inhibited to varying degrees by 2-HG, thereby attenuating the sequential oxidation of 5-mC (Xu et al., 2011) (Figure 1.9 B). The loss of the dynamic regulation of cytosine methylation frequently leads to aberrant global 5-mC hypermethylation of the genome (Figuerola et al., 2010a; Flavahan et al., 2016; Noushmehr et al., 2010; Turcan et al., 2012). Given that different tissues and cell types can be distinguished by their methylomes (Zhang et al., 2013a), it is not surprising that the distribution of the average site-specific CpG methylation levels at different genomic regions in mutant relative to wildtype *IDH* tumours varied between different cancer subtypes (Unruh et al., 2019). Despite hypermethylation occurring at different genomic regions and features, research has predominantly focused on the aberrant CpG hypermethylation within promoters and the correlation with transcriptional repression of associated genes in mutant *IDH* cancers (Noushmehr et al., 2010; Turcan et al., 2012). Furthermore, a smaller proportion of differentially methylated regions have been reported to be hypomethylated in mutant *IDH* cancers (Glass et al., 2017), yet their occurrence and consequence remain largely unexplored.

DNA hypermethylation driven by TET2 inhibition is hypothesised to drive leukaemic transformation and promote a block in differentiation (Figuerola et al., 2010a; Losman et al., 2013). Indeed, genetic lesions in *TETs* and *IDHs* are typically mutually exclusive in haematological malignancies and differentially hypermethylated regions in mutant *TET2* AML partially overlapped with those in mutant *IDH* AML (Figuerola et al., 2010a; Gaidzik et al., 2012; Papaemmanuil et al., 2016; Patel et al., 2012), suggesting a level of redundancy in promoting leukaemogenesis in these AML subsets through DNA hypermethylation as a consequence of compromised TET2 catalytic function. However, hierarchical clustering of the CpG methylation status in AML patients did not enrich for aberrations in *TET2* within the clusters whereby *IDH* mutations were distinguishing molecular features (Glass et al., 2017). Moreover, global DNA hypermethylation is not always present in mutant *IDH* AML, and mutations such as DNMT3A^{R882H} that co-occur with mutant *IDH* can greatly influence the

methylome in haematological malignancies (Glass et al., 2017). Taken together, these data suggest that 2-HG inhibition of TET enzymes likely only partially contributes to leukaemic transformation in mutant *IDH* haematological malignancies.

DNA methylation can also influence the three-dimensional organisation of the genome, thereby regulating gene expression (Hnisz et al., 2016). CCCTC-binding factor (CTCF) is a highly conserved zinc-finger protein capable of binding insulator sequences and creating chromatin loops that partition topological domains and prevent the interaction between enhancers and gene promoters, thus repressing expression of the associated genes (Hnisz et al., 2016). Flavahan et al. (2016) reported hypermethylation at CTCF-binding sites in mutant *IDH1* gliomas. Although there was a strong correlation in CTCF binding patterns between wildtype and mutant *IDH1* tumours, a significant number of CTCF-binding loci exhibited increased DNA methylation and loss of CTCF binding in the mutant *IDH1* tumours, which associated with gene activation in the domains adjacent to the disrupted boundaries (Flavahan et al., 2016). Flavahan and colleagues specifically demonstrated that CTCF loss at a domain boundary allowed a constitutive enhancer to interact with and aberrantly activate a glioma oncogene. Loss of insulation and the subsequent abnormal interaction between enhancers and distal genes has not yet been validated in the context of mutant *IDH* AML.

2-HG inhibition of TET enzymes results in the accumulation of the epigenetic mark 5-mC by preventing the sequential oxidation of 5-mC and its derivatives during the active demethylation process (Figueroa et al., 2010a). The hydroxylation of 5-mC to 5-hmC by TET proteins was initially regarded as an indication of the occurrence of active demethylation. More recent research has recognised 5-hmC as a distinct epigenetic mark (Kozlenkov et al., 2018; Kriaucionis and Heintz, 2009; Spiers et al., 2017). Human astrocytes ectopically overexpressing *IDH1*^{R132H} exhibited decreased 5-hmC levels which was rescued by the concurrent overexpression of wildtype TET2 (Turcan et al., 2012), suggesting that 2-HG inhibits TET2 mediated hydroxylation of 5-mC. Decrease in 5-hmC has also been reported in HEK293 cells transiently transfected with mutant *IDH1/2* (Figueroa et al., 2010a; Xu et al., 2011). However, the change in 5-hmC levels and downstream effects remains poorly understood in mutant *IDH* AML.

1.4.3.2.3 2-HG Inhibition of ALKBHs Results in the Accumulation of m6A and Cytotoxic Lesions

In addition to DNA and histone methylation, α -KG has been implicated in the regulation of RNA modifications (Jia et al., 2011; Su et al., 2018). Over 100 chemical modifications of RNA have been identified in mammalian cells, although the functional significance of the majority of these modifications are yet to be elucidated (Fu et al., 2014). The most prevalent mRNA modification in eukaryotes is N6-methyladenosine (m6A) and is involved in splicing and translational control of gene expression (Fu et al., 2014; Zhang et al., 2019a). In mammals, members of the alkylated DNA repair AlkB homologue (ALKBH) family of proteins, such as FTO, utilise α -KG as a co-substrate for the demethylation of m6A residues (Figure 1.9 B). Based on prior knowledge that the m6A demethylase, FTO, was an α -KG-dependent dioxygenase, it was hypothesised that 2-HG could inhibit FTO resulting in aberrant m6A expression, thus providing another method of transcriptional deregulation in mutant *IDH* malignancies (Losman and Kaelin, 2013). Elkashef and colleagues (2017) were the first to demonstrate the presence of elevated m6A levels in mutant *IDH in vitro* and primary AML patient samples. This finding was validated by Su et al. (2018) in malignant haematological models and unequivocally attributed to FTO inhibition by 2-HG. However, 2-HG induced cell cycle arrest and apoptosis in most cell lines, and it was these cell lines where m6A global elevation was apparent (Su et al., 2018). Su and colleagues found that cells capable of proliferating in the presence of 2-HG displayed no 2-HG induced change in global m6A levels, a seemingly inconsistent result to those reported by Elkashef et al. (2017) who demonstrated elevated m6A levels in mutant *IDH*, but not wildtype *IDH*, primary AML samples. Su and colleagues concluded that 2-HG inhibition of FTO and consequential hyper-m6A led to suppression of *MYC* and *CEBPA* signalling, providing a potential therapeutic axis to target in 2-HG sensitive cell lines, whilst in 2-HG resistant cell lines, *MYC* hyper-activation diminished the anti-tumour effects of 2-HG thus conferring tolerance to 2-HG. However, Su and colleagues did not validate these findings in a model that carried an *IDH* mutation and inherently depended upon 2-HG for maintaining leukaemic capacity. Results obtained from cell lines that can tolerate 2-HG but did not display a proliferative advantage are not an analogous representation of mutant *IDH* driven malignancies, which likely explains the discrepancies between Elkashef et al. (2017) and Su et al. (2018). Consequently, the biological significance of m6A in mutant *IDH* driven cancers requires more investigation.

In addition to m6A demethylase activity, the ALKBH family utilise α -KG for the removal of methylated lysine residues in proteins, the repair of mutagenic and cytotoxic methylated lesions caused by alkylating agents including 1-methyladenine (1-meA), 3-methylcytosine (3-meC) and 1,N6-ethenoadenine, and the hydroxylation of specific tRNAs (Ougland et al., 2015) (Figure 1.9 B). 2-HG inhibits the ALKBH family from repairing alkylated DNA lesions, which is hypothesised to contribute to oncogenesis driven by mutant *IDH* (Wang et al., 2015). However, 2-HG inhibition of ALKBH mediated DNA repair also provides a potential therapeutic avenue that could be exploited for the treatment of mutant *IDH* malignancies; cells expressing mutant *IDH* are inherently more sensitive to alkylating-agent chemotherapy as a consequence of their inability to repair the therapy induced cytotoxic lesions (Wang et al., 2015).

1.4.3.2.4 2-HG Inhibition of Hydroxylases

In addition to enzymes required for epigenetic regulation, α -KG serves as a co-substrate for a range of protein hydroxylase domain enzymes (PHDs) (Figure 1.9 B). Prolyl-4-hydroxylases (P4HAs) utilise α -KG for collagen biosynthesis required for structural support by the extracellular matrix and connective tissues. Adaptation to hypoxic conditions requires α -KG and occurs through hypoxia inducible factor 1 α (HIF1 α) mediated transcriptional responses (Lando et al., 2002). Under normoxic conditions, factor inhibiting HIF1 α (FIH) hydroxylates HIF1 α , targeting it for proteasomal degradation thereby repressing HIF1 α regulated gene transcription (Lando et al., 2002). Additionally, γ -butyrobetaine hydroxylase (BBOX) catalyses the conversion of gamma butyrobetaine to carnitine utilising α -KG as a co-substrate, which facilitates long-chain fatty acid transport across the mitochondrial inner membrane, adjusts the ratio of coenzyme A /acetyl-coenzyme A to regulate glucose metabolism, and modulates the source of ATP production between the glucose oxidation and fatty acid β -oxidation pathways (Tars et al., 2014).

The impact of 2-HG on PHDs has been relatively poorly characterised, particularly in mutant *IDH* AML. Initial studies reported the expected 2-HG inhibitory effects on the PHD2 enzyme and consequent elevated HIF levels (Xu et al., 2011). However, conflicting evidence suggests 2-HG potentiates PHDs resulting in decreased HIF levels (Koivunen et al., 2012; Losman et al., 2013). Furthermore, biochemical assays have revealed that hydroxylases have a substantially lower affinity for 2-HG in comparison to other α -KG-dependent dioxygenases, suggesting that the pathological effects of 2-HG in mutant *IDH* malignancies are likely not largely mediated by inhibition of PHDs (Chowdhury et al., 2011).

1.4.3.2.5 Impacts of 2-HG on Non- α -KG-Dependent Dioxygenases

2-HG has also been implicated in perturbing cellular pathways that are independent of dysregulating α -KG-dependent dioxygenases. McBrayer et al. (2018) demonstrated that 2-HG inhibits the α -KG-dependent transaminases BCAT1 and BCAT2 (Figure 1.9). Catabolism of branched-chain amino acids (BCAA), including leucine, isoleucine and valine, is initiated by their transamination which is catalysed by branched-chain amino transferases (BCATs) (Brosnan and Brosnan, 2006). BCATs convert BCAA to branched-chain α -ketoacids (BCKA) by coupling the transfer of the amino moiety of BCAA to the simultaneous interconversion of α -KG to glutamate (Brosnan and Brosnan, 2006). Glioma cells expressing mutant-*IDH*

exhibited 2-HG inhibition of BCAT1/2, which resulted in the reduction of BCKAs and glutamate levels (McBrayer et al., 2018). Consequently, *IDH1^{R132H}* expressing glioma cells were highly dependent on the synthesis of glutamate through glutamine-dependent pathways (McBrayer et al., 2018). Exploiting the synthetically lethal interaction between glutaminase and mutant-IDH, through small-molecule inhibition of glutaminase (McBrayer et al., 2018), may also provide a therapeutic strategy for other IDH-mutant malignancies, as glutamine is required for the proliferation and survival of cancer cells (Jacque et al., 2015; Kroemer and Pouyssegur, 2008).

Oncogenic mutant-IDHs consume a large portion of intracellular NADPH for the synthesis of 2-HG (Badur et al., 2018). Cells require NADPH as a cofactor for the reductive biosynthesis of lipids and nucleic acids, and for the neutralisation of reactive oxygen species produced under oxidative stress (Lunt and Vander Heiden, 2011; Ren et al., 2017). 2-HG production competes with the NADPH-dependent redox homeostasis and reductive biosynthesis of lipids and nucleic acids, thereby increasing the sensitivity of cells to redox stress (Badur et al., 2018; Gelman et al., 2018). To maintain NADPH levels sufficient for cell survival, *IDH1^{R132H}* expression increases the production of NADPH through the pentose phosphate pathway (Gelman et al., 2018). These data suggest IDH-mutant cells may be acutely sensitive to the inhibition of the pentose phosphate pathway, and thus a potential therapeutic avenue for the treatment of IDH-mutant malignancies.

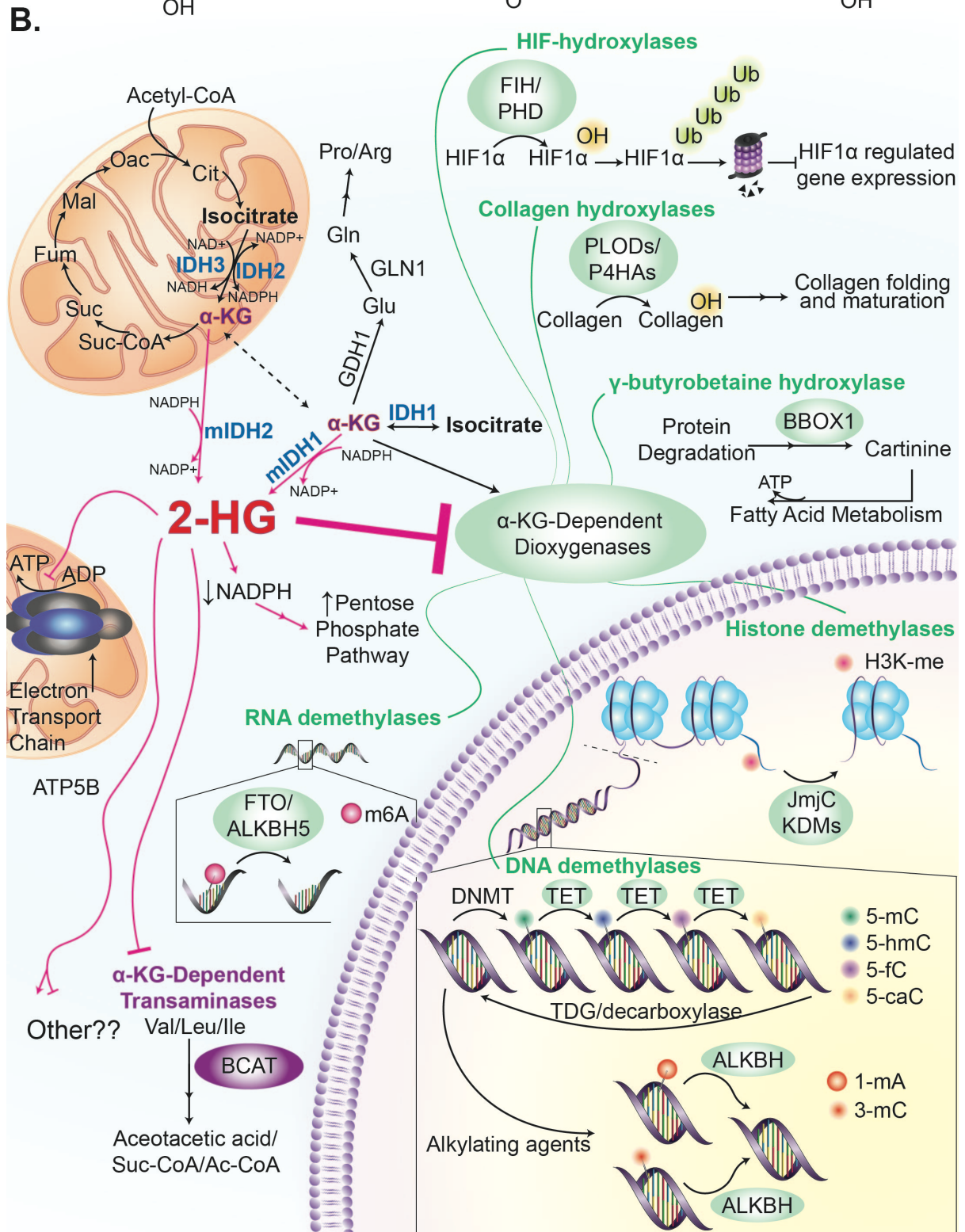


Figure 1.9 Pleiotropic Effects of α -KG and Oncometabolite 2-HG

(A) Chemical structures of isocitrate, α -KG and 2-HG. Wildtype IDHs catalyse the oxidative reduction of isocitrate to α -KG, and mutant IDH1/2 further convert α -KG to 2-HG. **(B)** Primary production of α -KG occurs in the mitochondria and is a metabolite of the TCA cycle. α -KG is a co-substrate required for the enzymatic activities of α -KG-dependent dioxygenases which encompass hydroxylases and DNA, histone and RNA demethylases. The accumulation of 2-HG in mutant *IDH* cells results in the dysregulation of an array of cellular processes, in particular through the inhibition of α -KG-dependent dioxygenases.

1.4.3.3 Mutant IDH Induces Aberrant Haematopoiesis

The aberrant acquisition of the HSC-like self-renewal capacity and accompanied block in differentiation are common characteristics of AML, and mutant *IDH* driven haematological malignancies are no exception. An erythroleukaemia cell line ectopically expressing mutant *IDH* displayed a partial differentiation block in cytokine-poor conditions (Losman et al., 2013), which was reversed upon treatment with a targeted mutant IDH inhibitor (Wang et al., 2013). Indeed, *IDH2^{R140Q}* mutant AML patients that responded to small-molecule inhibition of the mutant IDH isoform displayed therapy-induced myeloid differentiation of the malignant cells (Amatangelo et al., 2017). Since TET2 depletion mimicked the differentiation block, Losman et al. (2013) reasoned that the block in differentiation was the result of 2-HG inhibition of TET2 in the mutant *IDH* expressing cells. However, under cytokine-rich culture conditions, 2-HG did not provide this cell line with a proliferative advantage, but rather it induced cell death (Su et al., 2018). Indeed, 2-HG demonstrated potent anti-tumour properties in the majority of haematological cell lines that did not harbour genetic lesions in *IDH* (Su et al., 2018). This highlights the limitations of exogenously supplementing 2-HG or ectopically expressing mutant *IDH in vitro*, and the importance of studying the mechanisms of 2-HG driven leukaemogenesis in the appropriate pathophysiological context where mutant *IDH* is an oncogenic driver. Furthermore, perturbations of other pathways by 2-HG inhibition have been suggested to contribute to the block in differentiation observed in mutant *IDH* haematological malignancies (Lu et al., 2012). 2-HG inhibition of a single class of α -KG-dependent dioxygenases is unlikely to be the sole cause of the irregular haematopoiesis. Rather, it is likely that the cellular processes dysregulated by 2-HG synergise to promote leukaemic transformation and persistence of mutant *IDH* driven malignancies, with varying degrees of contribution from different pathways.

Despite the multitude of pathways affected by 2-HG inhibition, mutant *IDH* alone is insufficient to initiate leukaemia *in vivo*; conditional knock-in of *Idh1^{R132H}* and transgenic murine models expressing *IDH2^{R140Q}* in myeloid lineage progenitors eventually developed modest splenomegaly and mildly elevated numbers of early haematopoietic progenitors (Kats et al., 2014; Sasaki et al., 2012). The modest increase in the myeloid progenitor compartment in *IDH* mutant mice suggests that the acquisition of *IDH* mutations enables enhanced self-renewal properties and their eventual clonal expansion. In the context of an *IDH* mutant background, the acquisition of additional genetic lesions cooperated with mutant *IDH* to initiate leukaemic transformation *in vivo*, which rapidly led to AML driven mortality (Chen et al., 2013; Kats et al., 2017). These observations are further supported by the relatively high mutant *IDH* VAF in

AML patients implying that the acquisition of *IDH* mutations occurs early in leukaemogenesis (Papaemmanuil et al., 2016). Furthermore, the additional oncogenic driver mutations that co-occur with mutant *IDH* in AML suggest a cooperation between these genetic events are required for malignant transformation (Papaemmanuil et al., 2016) (Figure 1.10).

Although mutant *IDH* alone may be insufficient to initiate leukaemia, there are studies that indicate that mutant *IDH* driven AML exhibits an addiction to the oncometabolite 2-HG. Genetic deletion and targeted pharmacological inhibition of IDH2^{R140Q} in transgenic mouse models of AML harbouring multiple mutations induced differentiation and loss of leukaemia initiating capacity, decreased tumour burden and prolonged survival (Kats et al., 2017; McKenney et al., 2018; Quek et al., 2018; Shih et al., 2017). Furthermore, a large proportion of relapsed and refractory AML patients harbouring an *IDH* mutation, amongst other genetic lesions, enter remission following small molecule therapy targeting the mutant IDH isoform (DiNardo et al., 2018; Stein et al., 2017). Collectively, these data suggest that 2-HG is required for the maintenance of tumourgenicity in mutant *IDH* driven AML.

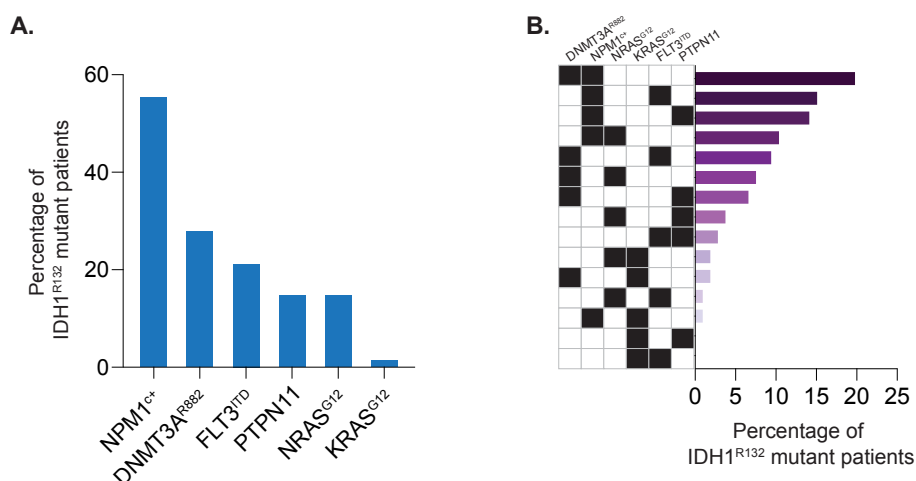


Figure 1.10 Mutations that Frequently Co-occur with *IDH1*^{R132} Lesions in AML

(A) The most frequently co-occurring and (B) triple combination of mutations in *IDH1*^{R132} AML patients. Black squares indicate the presence of a mutation at the corresponding loci. Data obtained from Papaemmanuil et al. (2016).

1.4.3.4 Small Molecule Inhibitors of Mutant IDH

The neomorphic activity and distinct catalytic site in mutant IDHs compared to their wildtype counterparts, coupled with the overall poor survival and limited therapeutic avenues for the treatment of AML (Yang et al., 2010), made inhibition of mutant IDHs a potential therapeutic option. Since the discovery of mutant *IDH* in cancers, pharmaceutical companies rapidly sought to develop on-target small molecule inhibitors against the mutant proteins (Table 1.7), many of which have advanced into clinical trials displaying high efficacy in mutant *IDH* haematological malignancies (Golub et al., 2019). Recently, two drugs, Ivosidenib (AG-120) and Enasidenib (AG-221), have been FDA approved for the treatment of relapsed and refractory *IDH1*-mutant and *IDH2*-mutant AML, respectively. Complete remission with or without haematological recovery was achieved in over 30% of relapsed or refractory AML patients harbouring *IDH1*^{R132} mutations following Ivosidenib treatment (DiNardo et al., 2018).

Despite the remarkable response in a subset of AML that is notoriously difficult to treat, ~ 70% of patients still do not enter complete remission and subsequent development of resistance and relapse is not uncommon following mutant *IDH* inhibition therapies (Amatangelo et al., 2017; DiNardo et al., 2018). Neither mutant *IDH* variant allele frequency nor plasma 2-HG concentration is a prognostic marker of response to small molecule inhibition (Amatangelo et al., 2017). However, targeted sequencing has implicated activating *RAS* pathway mutations as a potential prognostic marker of poor response, since such mutations were enriched in mutant *IDH* patients that did not respond to targeted small molecule inhibition of mutant IDH (Amatangelo et al., 2017). Moreover, mutant isoform switching and *cis* or *trans* dimer-interface mutations have been reported in instances of patient acquired resistance to mutant *IDH* targeted therapies (Harding et al., 2018; Intlekofer et al., 2018). However, these resistance pathways have only been reported in a few non-responding patients. Overall, the mechanisms of intrinsic and acquired resistance to small molecule inhibition of mutant IDH remain poorly understood. A comprehensive understanding of these resistance mechanisms will promote the development of combination therapies and ultimately improve patient outcome.

Table 1.7 Small molecule targeted therapeutics developed against mutant IDH

Drug	Target	Clinical Trial ID*	Used in Publications
Ivosidenib (AG-120)	IDH1 ^{R132H/C/G/S/L}	NCT02073994	
		NCT02074839	
		NCT02632708	
		NCT02677922	(Popovici-Muller et al., 2012)
		NCT02989857	(Dhillon, 2018)
		NCT03013998	(DiNardo et al., 2018)
		NCT03173248	(Popovici-Muller et al., 2018)
		NCT03282513	(Nassereddine et al., 2019)
		NCT03343197	(Polleya et al., 2019)
BAY-1436032	IDH1 ^{R132H/C/G/S/L}	NCT03564821	
		NCT03471260	
GSK321	IDH1 ^{R132H/C/G}	NCT02746081	(Chaturvedi et al., 2017)
		NCT03127735	(Pusch et al., 2017)
AGI-5198	IDH1 ^{R132H/C}	–	(Okoye-Okafor et al., 2015)
AGI-5198	IDH1 ^{R132H/C}	–	(Rohle et al., 2013)
IDH305	IDH1 ^{R132H/C}	NCT02381886	(Cho et al., 2017)
MRK-A	IDH1 ^{R132H/C}	–	(Kopinja et al., 2017)
Enasidenib (AG-221)	IDH2 ^{R140Q} IDH2 ^{R172K}	NCT01915498	(Amatangelo et al., 2017)
		NCT02273739	(Stein et al., 2017)
		NCT02989857	(Yen et al., 2017)
		NCT02632708	(Dogra et al., 2018)
AG-881	IDH1 ^{R132H/C/G/S/L}		(Stein, 2018)
	IDH2 ^{R140Q}	NCT02481154	(Ma and Yun, 2018)
	IDH2 ^{R172K}	NCT02492737	(Mellinghoff et al., 2018)
AGI-6780	IDH2 ^{R140Q}	NCT03343197	
		–	(Kernytsky et al., 2015)
			(Chen et al., 2017)

*Active or completed clinical trials that recruited mutant IDH cancer patient in the USA (clinicaltrials.gov).

1.5 Thesis Aims

Mutations in epigenetic regulators frequently occur in AML. Targeted therapeutics that directly inhibit epigenetic oncogenic drivers, such as AG120 inhibition of IDH1^{R132H}, have shown clinical success for the treatment of AML harbouring specific genetic lesion in epigenetic regulators. Other epigenetic therapies, such as hypomethylating agents, target wildtype epigenetic regulators with the aim of reprogramming the transcriptional networks driving malignant progression. Despite some patients achieving clinical remission, many AML patients do not respond to epigenetic therapies or relapse post-treatment. In order to provide insight into resistance mechanisms, it is important to understand the alterations in the epigenetic and transcriptional states in AML driven by mutations in epigenetic regulators, and the consequences of targeting mutant and wildtype epigenetic regulators for the treatment of AML.

This thesis will further review the literature regarding the use of hypomethylating agents for the treatment of haematological malignancies (Chapter 2), the consequences of IDH1^{R132H} on transcription, epigenetics and other cellular processes in IDH1-mutant AML (Chapter 3), and the use of targeted mutant IDH inhibitors (Chapter 3).

The overarching aims of this thesis were to:

- Chapter 3:** Identify mechanisms of resistance to low-dose hypomethylating agents and identify targetable therapeutic options in AMLs with acquired resistance to hypomethylating agents.
- Chapter 4:** Characterise the phenotype of an *IDH1^{R132H}* driven *in vivo* model of AML, and examine the progressive changes in immunophenotype, transcriptional state and epigenetic profile upon IDH1^{R132H} inhibition in established haematopoietic malignancies.

Specific aims are discussed in respective chapters.

Chapter 2: Methods

2.1 Cell Culture Methods and *In Vitro* Assays

2.1.1 Cell Lines and Culture Conditions

The THP-1, MV4-11 and OCI-AML3 cell lines, and their derivatives, were maintained in RPMI-1640 medium (Thermo Fisher Scientific, cat# 11875119) supplemented with 2 mM penicillin/streptomycin (Invitrogen, cat# 15140163) and 20% (v/v) heat-inactivated FCS (SAFC Biosciences, cat# 12003C), and cultured at 37 °C and 5% CO₂ in humidified incubators (Binder, Series C).

293Ts were incubated for 1 min at 37 °C to dissociate the cells from the flask prior to passaging. Unless stated otherwise, 293Ts were maintained in Anne Kelso modified DMEM (PMCC Research Division) supplemented with 2 mM penicillin/streptomycin (Invitrogen, cat# 15140122), 10% (v/v) heat-inactivated FCS (SAFC Biosciences, cat# 12003C) and 100 µM of L-glutamine (Invitrogen, cat # 21051040), and cultured at 37 °C and 10% CO₂ in humidified incubators (Binder, Series C).

Foetal liver cells (FL) were cultured in FL media (Table 2.1) at 37 °C and 10% CO₂ in humidified incubators (Binder, Series C).

Table 2.1 FL Media Formulation

Component (Concentration)	Manufacturer	Catalogue #
Anne Kelso modified DMEM	PMCC Research Division Media Kitchen	-
Heat inactivated FCS (20% v/v)	SAFC Biosciences	12003C
Penicillin/Streptomycin (2 mM)	Invitrogen Life Technologies)	15140122
β-mercaptoethanol (50 µM)	Sigma-Aldrich	M3148
L-asparagine (100 µM)	Sigma-Aldrich	A0884
L-glutamine (2 mM)	Sigma-Aldrich	35050061
Murine IL-3 (2 ng/mL)	Peprtech Asia	213-13
Murine IL-6 (2 ng/mL)	Peprtech Asia	216-16
Murine SCF (10 ng/mL)	Peprtech Asia	250-03

2.1.2 Chemical Compounds Used for *In Vitro* Studies

Chemical compounds used for *in vitro* experiments are provided in Table 2.2. Prior to *in vitro* use, 100 – 1000x drug concentrations were prepared in target cell media for 1/100 – 1/1000 dilution, respectively.

Table 2.2 Chemical Compounds Used for *In Vitro* Studies

Compound	Manufacturer	Vehicle
5-Azacytidine	Sigma (cat# A2385)	PBS
SGI-110 (Guadecitabine)	Astex Pharmaceuticals	DMSO
AG636	Agios Pharmaceuticals	DMSO

2.1.3 Generation of Cas9-Cherry Expressing Cells

Following a 30-minute incubation at room temperature, the transfection mix (pMDL (Addgene, Plasmid #12251) 7 µg, pRSV-REV (Addgene, Plasmid #12253) 3.5 µg, pVSVg (Addgene, Plasmid #12259) 4.2 µg, Cas9-mCherry (Addgene, Plasmid #70182) 14 µg, PEI (Sigma-Aldrich, cat# 9002-98-6) 118 µg/mL, in 1200 µL serum-free DMEM) was added to 12 x 10⁶ 293T cells plated the previous day in a T175 flask (Greiner Bio-One, cat# 660175). Following incubation overnight at 37 °C and 10% CO₂, the media was refreshed and the cells incubated for an additional 24 hours. Lentivirus supernatant was subsequently harvested, passed through a 0.45 µm filter (Pall Life Sciences, cat # 4614) to remove cellular debris, and concentrated to ~8 mL by centrifugation at 2000 x g in an Amicon® Ultra Centrifugal Filter with 100 kDa molecular weight cut-off (Merk Millipore, cat# UFC910024).

Twenty-four hours before transduction, one well of a non-treated 6-well plate (Sigma, cat# CLS3736) was coated with 1 mL of 3 µg/mL RetroNectin (TaKaRa, cat# T202) diluted in PBS. Following overnight incubation at 4 °C, RetroNectin coated wells were washed with PBS before transferring the concentrated Cas9-mCherry lentivirus supernatant and centrifuging at 2000 x g for 90 minutes at room temperature. Viral supernatant was replaced with 4 mL of target cells resuspended at 0.25 x 10⁶ cells/mL in target cell media containing 8 µg/mL Sequa-brene (Sigma, cat: S2667). Cells were centrifuged for 4 minutes at 450 x g and incubated overnight (37 °C, 5% CO₂) before undergoing a second round of transduction. Flow-cytometry was used to isolate mCherry⁺ cells 72 hours after the second round of transduction.

2.1.4 Generation of sgRNA Expressing Cells

Following a 30 minute incubation at room temperature, the transfection mix (pMDL (Addgene, Plasmid #12251) 1 µg, pRSV-REV (Addgene, Plasmid #12253) 0.5 µg, pVSVg (Addgene, Plasmid #12259) 0.6 µg, FgH1tUTG-sgRNA-GFP (Addgene, Plasmid #70182) 2 µg, PEI (Sigma, cat# 9002-98-6) 118 µg/mL, in 170 µL serum-free RPMI) was added to 2×10^6 293T cells in 10 mL target cell media plated the previous day in a T25 flask (Greiner Bio-One, cat# 690175). Following incubation overnight at 37 °C and 10% CO₂, the media was refreshed and the cells incubated for an additional 24 hours. Viral supernatant was subsequently harvested and passed through a 0.45 µm filter (Pall Life Sciences, cat# 4614) to remove cellular debris. Target cells were resuspended at 2.5×10^6 cells/mL and 200 µL transferred to the wells of a 24-well plate (Greiner Bio-One, cat# 662160) containing 2 mL of viral supernatant and 8 µg/mL Sequa-brene (Sigma, cat# S2667). Flow-cytometry was used to isolate mCherry⁺/GFP⁺ cells 72 hours after transduction.

2.1.5 Generation of MSCV-Luciferase-PuroR Expressing Cells

Following a 30-minute incubation at room temperature, the transfection mix (8.2 µg pCL-Ampho (PMCC, Johnstone Laboratory), 23.3 µg, MSCV-Luciferase-PuroR (generated in section 2.3.3), 1 µg/mL PEI (Sigma, cat# 9002-98-6), in 1200 µL serum-free DMEM per transfection) was added to each T175 flask containing 293T cells at ~50% confluence. Following incubation overnight at 37 °C and 10% CO₂, the media was refreshed and the cells incubated for an additional 24 hours. Retroviral supernatant was harvested, passed through a 0.45 µm filter to remove cellular debris, and concentrated to ~8 mL by centrifugation at 2000 x g in an Amicon[®] Ultra Centrifugal Filter with 100 kDa molecular weight cut-off (Merk Millipore, cat# UFC910024).

Twenty-four hours before transduction, one well of a non-treated 6-well plate (Sigma, cat# CLS3736) was coated with 1 mL of 3 µg/mL RetroNectin (TaKaRa, cat# T202) diluted in PBS. Following overnight incubation at 4 °C, RetroNectin coated wells were washed with PBS before transferring the concentrated MSCV-luciferase-puroR retroviral supernatant and centrifuging at 2000 x g for 90 minutes at room temperature. Viral supernatant was replaced with 4 mL of THP-1^{Cas9-mCherry/sgrNA-GFP} resuspended at 0.25×10^6 cells/mL in target cell media. Cells were centrifuged for 4 minutes at 450 x g and incubated overnight (37 °C, 5% CO₂) before undergoing a second round of transduction. Cells that successfully integrated the MSCV-

luciferase-puroR construct were selected by the culture in 1 µg/mL puromycin supplemented media for 1 week.

2.1.6 Generation of IDN Leukaemias

2.1.6.1 Isolation of Primary Murine Foetal Liver Cells

Embryonic day 13.5 foetuses were harvested under sterile conditions from euthanised wildtype C57BL/6 pregnant females. Each foetal liver (FL) was subsequently dissected and FL cells dissociated by pipetting in 1 mL PBS. FL cells were centrifuged at 450 x g for 4 min and washed with PBS prior to resuspension in 1 mL of 10% DMSO supplemented heat inactivated FCS and cryopreserved at -80 °C.

2.1.6.2 Transfection and Generation of Retrovirus

293Ts were incubated for 1 min at 37 °C to dissociate the cells from the flask prior to passaging. Unless stated otherwise, 293Ts were maintained in according to section 2.1.1.

293T cells, seeded at approximately 50% confluence 24 hrs prior to transfection, were transiently transfected using the calcium phosphate method. A solution of retroviral DNA plasmids (TRI-dsRED-IDH1^{R132H}, MSCV-GFP-DNMT3A^{R882H}, MSCV-tTA-Nras^{G12D} (PMCC Johnstone and Kats Laboratories)), pcl-Eco packaging vector (Addgene plasmid cat# 12371), 0.25 M CaCl₂, 1.25 mM HEPES buffered H₂O was added dropwise to an equivalent volume of 2x HEBS buffer (HEPES 50 mM, NaCl 180 mM, Na₂HPO₄ 1.5 mM pH 7) whilst air was bubbled through. The transfection mixture was incubated at room temperature for 20 min prior to the dropwise addition to 293T cells and overnight culture in humidified incubators at 37 °C and 10% CO₂. Media was replaced 24 hrs after transfection. Viral supernatant was harvested after a further 24 hrs, and media replenished on transfected 293T cells and incubated for an additional 24 hrs. Viral supernatant was filtered through a 0.45 µm filter (Pall Life Sciences, cat # 4614) to remove cellular debris.

2.1.6.3 Retroviral Transduction of Murine Foetal Liver Cells

Each well of a 6-well plate (Costar, Sigma Aldrich cat# CLS3736) was coated with 1 mL of 4 µg/mL RetroNectin (Takara, cat# T100B) / PBS solution and incubated overnight at 4 °C, prior to transduction. RetroNectin/PBS solution was removed and wells washed with PBS, prior to

the transfer of the IDH1^{R132H}, DNMT3A^{R882H} and Nras^{G12D} viral supernatants mixed in equal proportions and centrifugation at 2000 x g for 90 min. Viral supernatant was subsequently replaced with a suspension of murine foetal liver (FL) cells (1 x 10⁶ cells/mL) in FL media (Table 2.1). FL cells were centrifuged at 450 x g for 4 min and cultured overnight at 37 °C and 10% CO₂. FL cells were subject to a second round of transduction with the viral supernatant harvested 72 hrs after transfection, whereby viral-loaded RetroNectin-coated wells were centrifuged at 2000 x g for 90 min before the transfer of primary transduced FL cells and subsequent 450 x g for 4 min centrifugation. FL cells were cultured for 48 hrs prior to flow-cytometry validation of transduction efficiency and transplant into murine recipients.

2.1.7 Annexin / PI Assay

Cells were pelleted in a 96-well U-bottom plate by centrifugation at 450 x g for 4 minutes and resuspended in 100 µL of annexin-V binding buffer (HEPES 10 mM pH 7.4, NaCl 140 mM and CaCl₂ 5 mM) containing 0.01 µg/mL of propidium iodine (Sigma, cat# P4170) and 1:100 annexin V-APC (BD Pharmingen, cat# 550475) to measure DNA fragmentation and translocation of the phospholipid phosphatidylserine to the cell surface, respectively. Flow cytometry was used to quantify the proportion of annexin V⁻/PI⁻, annexin V⁺/PI⁻, annexin V⁻/PI⁺, and annexin V⁺/PI⁺.

2.1.8 Cell Proliferation Assay

Cells were resuspended at 1 x 10⁷/mL in PBS containing 5 µM CellTrace Violet (CTV) (Invitrogen, cat# C34557) and incubated for 20 minutes in a 37 °C water bath, protected from light, to allow CTV to covalently bind free amines on the surface and within the cells. Unbound dye was quenched with 5x the volume of RPMI-1640 supplemented with 10% FCS. Following centrifugation (450 x g, 4 °C, 4 min), cells were resuspended in PBS containing 2% FCS and a narrow region of CTV-positive cells sorted by flow cytometry. Cell proliferation was monitored daily by measuring the intensity of CTV-positive cells by flow cytometry.

2.1.9 Competitive Cell Proliferation Assay

Cas9 and sgRNA transduced cells were treated with 1 µg/mL doxycycline for 1 week to induce the expression of the sgRNA prior to incubation in a 1:1 ratio with Cas9 only expressing cells. 5-Azacytidine (300 nM), SGI-110 (150 nM) or DMSO (1:1000) was refreshed daily. The percentage of sgRNA expressing cells (GFP⁺ cells) was measured every 3 – 4 days by flow cytometry.

2.1.10 CRISPR Screen

2.1.10.1 Generation of Brunello Virus

Eight flasks each seeded with 12×10^6 293T cells in 25 mL of media were cultured overnight prior to transfection. Following 30 minutes incubation at room temperature, the transfection mix (pMDL (Addgene, Plasmid #12251) 56 µg, pRSV-REV (Addgene, Plasmid #12253) 28 µg, pVSVg (Addgene, Plasmid #12259) 33.6 µg, Brunello Human CRISPR Knockout Pooled Library in lentiGuide-Puro (Addgene, Pooled Library #73178) 112 µg, PEI (Sigma-Aldrich, cat# 9002-98-6) 118 µg/mL, in 9.6 mL serum-free DMEM) was equally divided amongst each flask of 293T cells. The cells were incubated overnight at 37 °C and 10% CO₂, the media refreshed and the cells incubated for an additional 24 hours. Lentivirus supernatant was subsequently harvested, passed through a 0.45 µm filter (Pall Life Sciences, cat# 4614) to remove cellular debris, and concentrated to ~16 mL by centrifugation at 2000 x g in an Amicon[®] Ultra Centrifugal Filter with 100 kDa molecular weight cut-off (Merk Millipore, cat# UFC910024). Virus was snap frozen in liquid N₂ and stored at -80 °C until required.

2.1.10.2 Titration of Virus

Viral supernatant was serially diluted 1:2 in target cell media ten times. Diluted viral supernatant and target cells (0.5×10^6 cells / mL) were mixed in a 1:1 ratio and seeded in 600 µL in a 24 – well plate in triplicate. Following incubation for 72 hours, 1 µg/mL puromycin was added to select for cells that had integrated the Brunello sgRNA constructs into their genome. Cells were incubated for 2 days, puromycin refreshed and the cells incubated for an additional 3 days prior to annexin/PI staining and quantification according to section 2.1.7. To ensure a maximum of one viral integration per cell, the viral concentration chosen for the subsequent CRISPR screen resulted in 30% live cells (annexin⁻/PI⁻) following 5 days of puromycin selection, corresponding to a MOI of ~0.3.

2.1.10.3 Transduction, Selection and Culture of Target Cells

To transduce target cells with Brunello Human CRISPR Knockout Pooled Library virus, the cells were incubated with 8 µg/mL of Sequa-brene and virus at an MOI of ~0.3 to ensure no more than one viral integration per cell. Following incubation for 72 hours, cells with viral integrations were selected for with 1 µg/mL puromycin for 5 days. A time-point zero pellet of 75×10^6 live cells was harvested by centrifugation at 450 x g for 4 minutes, snap frozen and stored at -80 °C until required.

To identify resistance to hypomethylating agents, the THP-1-Cas9 library transduced cells were split into 3 treatment conditions: 5-Azacytidine (300 nM), SGI-110 (150 nM), or Vehicle (1:1000 DMSO). Drug/vehicle was refreshed daily and cells passaged every 3 – 4 days, maintaining 1000x representation by seeding 75×10^6 cells in 300 mL every passage. Cells were harvested at the end-point by centrifugation at 450 x g for 4 minutes, snap frozen and stored at -80 °C until required.

To identify synthetically lethal targets in the SLC29A1 knockout cell lines, the THP-1-Cas9/sgSCR and THP-1-Cas9/sgSLC29A1 library transduced cells were passaged every 3 – 4 days, maintaining 1000x representation by seeding 75×10^6 cells in 300 mL every passage. Cells were harvested at the end-point by centrifugation at 450 x g for 4 minutes, snap frozen and stored at -80 °C until required.

2.2 *In vivo* Techniques

2.2.1 Ethics

In compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (N.H.M.R.C. 2016), all experimental procedures were approved by the Peter MacCallum Cancer Centre Animal Ethics Committee under Permit numbers E555 and E627.

2.2.2 Animal Housing and Mouse Strains

NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice were bred and housed at the Peter MacCallum Cancer Centre. Animals were group-housed in individually ventilated micro-isolator cages (6 mice per cage) on a 13-hour light / 11-hour dark cycle. Mice had continuous access to sterilised water and Barastoc irradiated mouse cubes (Ridley AgriProducts).

2.2.3 Preparation and Transplant of AML Cell Lines

THP-1^{Cas9/luc2/sgRNA} cells were pelleted at 450 x g for 4 min at 4 °C, washed twice with PBS (450 x g, 4 min, 4 °C) and resuspended in PBS at 0.5×10^6 cells/mL. NSG recipients were transplanted with 0.1×10^6 cells/mouse by tail-vein injection.

2.2.4 Transplant of IDN Cells

Transduced FL cells, and bulk or FACS isolated IDN tumour cells from the bone marrow and spleen were washed in PBS and resuspended at $0.5 - 1 \times 10^6$ cells/mL for tail vein injection of $\sim 0.1 - 0.2 \times 10^6$ cells/mouse. Ptpcr mice were sub-lethally irradiated with 6.5 Gy using Gammacell 40 caesium source (Atomic Energy of Canada Ltd., Ontario, Canada) prior to transplant, whilst NSG mice were not sub-lethally irradiated.

2.2.5 Drug Treatment

2.2.5.1 Administration of Hypomethylating Agents

Guadecitabine (20 mg/mL) and azacytidine (100 mM) were diluted in PBS to a final concentration of 0.1 mg/mL and 0.4 mg/mL, respectively. Drug was made fresh daily and administered by intraperitoneal (IP) injection in volumes relative to mouse body weight (10

μL/g) such that mice received 4 mg/kg azacytidine or 1 mg/kg guadecitabine. Treatment was administered once per day, in two cycles of 5 days on and 2 days off. Vehicle mice were administered PBS by IP injection according to body weight (10 μL/g).

2.2.5.2 Doxycycline Treatment

Mice assigned to doxycycline (DOX) treated cohorts were provided with DOX supplemented chow (600 mg/kg) (Speciality Feeds, cat# SF08-026) and DOX water (DOX 0.2% w/v, sucrose 2% w/v) for the duration defined by the experiment.

2.2.5.3 AG120 Formulation and Treatment

AG120 was administered by oral gavage of an amorphous solid dispersion with hypromellose acetate succinate (HPMCAS) as the carrier for the active pharmaceutical ingredient.

2.2.5.3.1 Vehicle Preparation

Vehicle was comprised of 1.5% (w/v) HPMCAS, 0.5% (w/v) methylcellulose and 0.2% (w/v) Tween80 in purified water. Purified water, equivalent to 50% of the final volume, was heated to 75 – 80 °C, whilst the remaining 50% was cooled to 2 – 8 °C. Methylcellulose (Sigma, cat# M0512) was slowly added to the hot purified water with continuous stirring. Once evenly dispersed, half of the cold purified water was added to the methylcellulose mixture and the pH adjusted to 11 – 12 with dropwise volumes of 1 M NaOH. Whilst continually stirring, HPMCAS was slowly added and pH subsequently adjusted with NaOH to lie between 6 – 7. Tween80 was subsequently added, followed by the remaining cold purified water was added to reach the final target volume. The vehicle formulation was stirred at 4 °C overnight or until visibly clear. pH was measured once more and adjusted with NaOH if needed. Vehicle was stored at 4 °C for a maximum of 1 week.

2.2.5.3.2 AG120 Preparation

AG120 was comprised of 3% (w/v) AG120-SDD, 0.5% (w/v) methylcellulose and 0.2% (w/v) Tween80 in purified water. AG120-SDD (Agiros Pharmaceuticals) contained the active pharmaceutical ingredient (AG120) in a 1:1 (w/w) ratio with HPMCAS. Purified water, equivalent to 50% of the final volume, was heated to 75 – 80 °C, whilst the remaining 50% was

cooled to 2 – 8 °C. Methylcellulose was slowly added to the hot purified water with continuous stirring. Once evenly dispersed, the cold purified water was added to the methylcellulose mixture prior to the addition of AG120-SDD. The mixture was subsequently sonicated for 30 min on ice, before Tween80 was added. If required, NaOH was used to adjust the pH to lie between 6 – 7. The amorphous solid dispersion was stored at 4 °C, with gentle spinning, in the dark for a maximum of 96 hours.

2.2.5.3.3 Oral Gavage Administration

Vehicle and AG120 were administered to NSG recipients by oral gavage twice daily at doses relative to body weight (10 mL/kg) for the duration of the experiment defined. Hence, AG120 treated mice received the active pharmaceutical ingredient at dosages relative to body weight (150 mg/kg).

2.2.6 Harvesting Samples for Analysis

2.2.6.1 Peripheral Blood and Plasma

Peripheral blood samples were acquired from transplanted mice throughout the experimental duration, to monitor disease progression, and at the ethical end-point. Approximately 75 – 100 µL of peripheral blood was obtained by tail vein incision into EDTA coated Microvette® capillary blood collection tubes (Sarstedt AG & Co, cat# 16.444). Peripheral blood was distributed for analyses including blood cell counts (section 2.2.8.1), flow-cytometry immunophenotyping (section 2.2.8.3) and plasma 2-HG quantification (section 2.2.8.2).

2.2.6.2 Bone Marrow Harvest

All experimental mice were euthanised by carbon-dioxide asphyxiation or cervical dislocation at specific timepoints during cross-sectional experiments, and at the ethical end-point reached when mice exhibited symptoms of poor health including hunched, ruffled, laboured breathing and/or weight loss. Leukaemic cells residing in the femurs, tibias, fibulas and hip bones were harvested for subsequent analyses including immunophenotyping (section 2.2.8.3), western blot (section 2.4), 3' mRNA Quantseq (section 2.3.4.1) and ChIP-seq (section 2.3.4.2).

A 10 mL single cell suspension of bone marrow in FACS buffer (2% heat inactivated FCS in PBS) from a single intact femur per mouse was obtained by mechanical dissociation, with a

mortar and pestle, which was subsequently filtered through a 70 µm cell-strainer (Greiner Bio-One, cat# 542070). One eighth of the single cell suspension obtained from a single femur per mouse was isolated for each flow-cytometry panel in order to quantify leukaemic immunophenotypes (section 2.2.8.3). A single cell suspension of the bone marrow from the remaining bones was obtained utilising the same procedure, and subsequently pooled with the residual cell suspension from one femur. Bone marrow cells were pelleted by centrifugation (450 x g, 4 min, 4 °C) and subjected to red cell lysis in ice cold ACK lysis buffer (150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA) for up to 10 min. Cells were pelleted by centrifugation and resuspended in FACS buffer for fluorescence activated cell sorting (section 2.2.7) for downstream analyses. Alternatively, cells were pelleted by centrifugation and resuspended in freezing mix (10% DMSO in heat inactivated FCS) and cryopreserved at -80 °C.

2.2.6.3 Spleen Harvest

Splenocytes were isolated by pressing the spleen through a 70 µm cell-strainer with a volume of FACS buffer relative to spleen weight (1 mg / 40 µL). 100 µL (equivalent to 2.5 mg spleen) of the splenocyte cell suspension was set aside for each flow-cytometry panel in order to quantify leukaemic immunophenotypes. Splenocytes were pelleted by centrifugation (450 x g, 4 min, 4 °C) and subjected to red cell lysis in ice cold ACK lysis buffer (150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA) for up to 10 min. Cells were pelleted by centrifugation and resuspended in FACS buffer for fluorescence activated cell sorting (section 2.2.7) for downstream analyses. Alternatively, cells were pelleted by centrifugation and resuspended in freezing mix (10% DMSO in heat inactivated FCS) and cryopreserved at -80 °C.

2.2.7 Fluorescence Activated Cell Sorting of IDN Cells

Cell suspensions of bone marrow and spleen were pelleted by centrifugation (450 x g, 4 min, 4 °C) and resuspended in 400 µL of FACS buffer containing the relevant antibody panel (Table 2.3). All antibodies in Table 2.3 were diluted 1/200, except for CD34 which was diluted 1/100. DAPI (5 mg/mL) was diluted 1/1000. Cells were stained for 15 min on ice or 5 min in a 37 °C water bath, then washed with FACS buffer and pelleted by centrifugation. Cells were resuspended in 0.5 – 1 mL of FACS buffer and sorted for the relevant population (Table 2.3) on the BD FACSAria Fusion 5 or BD FACSAria Fusion 3 (BD Biosciences).

Table 2.3 Antibodies for FACS

Experiment (Population sorted)	Source of Cells	Antibody	Fluorophore	Manufacturer (Catalogue #)
Isolating cells for western blot (GFP ⁺)	Bone marrow, spleen	NA	NA	NA
Isolating IDN myeloid progenitors for transplant (GFP ⁺ /CD11b ⁻ /CD117 ⁺ /Sca1 ⁻ /CD34 ^{mid-} high/FCγR-III ^{mid-} high)	Pooled bone marrow and spleen	CD117	APC	BD Pharmingen (553356)
		CD11b	APC-E780	Thermo Fisher Scientific (47-0112-82)
		CD34	BV421	BD Biosciences (562608)
		FCγR-III	BV786	BD Optibuild (740851)
		Sca1	Pe-Cy7	BD Pharmingen (558162)
Enriching for lymphoid negative cells (GFP ⁺ /DAPI ⁻ / TCRβ ⁻ /B220 ⁻ /NK1.1 ⁻)	Pooled bone marrow and spleen	TCRβ	APC-E780	eBioscience (47-5961-82)
		B220	APC-E780	Invitrogen (47-0452-82)
		NK1.1	APC-Cy7	BD Pharmingen (560618)
		DAPI*	NA	Biolegend 422801
Isolating IDN myeloid progenitors for molecular analyses (GFP ⁺ / CD11b ⁻ /CD117 ⁺)	Pooled bone marrow and spleen	CD117	BV650	BD Horizon (563553)
		CD11b	BV450	BD Horizon (560455)

*DAPI was added to cells after the FACS stain was removed and washed.

CD11b-Biotin was the primary stain. All other antibodies in this panel were added to the secondary stain.

2.2.8 Phenotyping IDN Tumour

Leukaemic cells residing in the bone marrow, spleen and peripheral blood were subjected to various assessments, mentioned below, in order to characterise the tumour model and response to IDH1^{R132H} inhibition.

2.2.8.1 Blood Cell Counts

20 µL of peripheral blood (isolated as per section 2.2.6.1) was diluted 1:26 in PBS and analysed on the Sysmex Cell Sorter (Sysmex Corporation, Kobe, Japan) to quantify the number of red blood cells, white blood cells and platelets in the peripheral blood.

2.2.8.2 2-HG Quantification

50 µL of peripheral blood (isolated as per section 2.2.6.1) was centrifuged at 2000 x g for 4 min at 4 °C to separate the plasma from the cells. 20 µL of the plasma supernatant was carefully transferred into a new eppendorf tube and immediately stored at -80 °C. The remaining 30 µL of blood was stored on ice until preparation for immunophenotyping by flow-cytometry (section 2.2.8.3).

Bone marrow from a single femur was isolated by pulse centrifugation into an eppendorf tube and stored at -80 °C. Plasma and bone marrow were sent to Agios Pharmaceuticals on dry-ice for 2-HG quantification by gas-chromatography/mass-spectrometry.

2.2.8.3 Flow Cytometry Immunophenotyping

The aliquots of bone marrow and splenocyte cell suspensions (section 2.2.6.2 – 2.2.6.3), and the blood remaining after plasma separation (section 2.2.6.1) were subjected to red cell lysis in ice cold ACK lysis buffer as described above. Cells were pelleted by centrifugation and resuspended in 100 µL of FACS buffer with the relevant antibody panel (Table 2.4 – 2.5). All antibodies were diluted 1/200, except for CD34, Siglec-F and CD115 which were diluted 1/100, and Ly-6G which was diluted 1/400. Cells were stained on ice for 15 min or in a 37 °C water bath for 5 min, then washed with FACS buffer and resuspended in 200 µL of FACS buffer and 10 µL of CountBright™ Absolute Counting Beads (Invitrogen, cat# C36950). Data was acquired on the BD LSR Fortessa X-20 or BD FACSymphony and analysed using FlowJo (v 10.4).

Table 2.4 Flow Cytometry Antibodies

Antibody *	Fluorophore	Manufacturer	Catalogue Number	FACS Panel #
B220	APC786	BD Horizon	563894	1, 5
B220	APC-780	Invitrogen	47-0452-82	2, 3, 4
#CD11b	Biotin	MACS Miltenyi Biotech	130-113-233	2
CD11b	BV711	Biolegend	101242	1, 3, 4
CD11b	BUV395	BD Horizon	563553	5
CD16/CD32 (FCγR-III)	BV786	BD Optibuild	740851	2
CD16/CD32 (FCγR-III)	BUV737	BD Horizon	565272	4, 5
CD34	BV421	BD Biosciences	562608	2, 4
CD34	E660	Invitrogen	50-0341-82	5
CD115	Superbright436	Invitrogen	62-1152-82	3,5
CD117	APC	BD Pharmingen	553356	1, 2
CD117	BV650	BD Horizon	563553	3, 4, 5
CD182	APC	Biolegend	149312	3, 4
CD182	APC/Cy7	Biolegend	149314	5
CD184	BUV395	BD Optibuild	740265	3, 4
CD184	BV711	Biolegend	146517	5
		BD Optibuild	740734	
Ly-6A/E (Sca1)	Pe/Cy7	BD Pharmingen	558162	2, 4, 5
Ly-6C	BV421	Biolegend	128032	3, 5
Ly-6C	BV785	Biolegend	128041	4
Ly-6G	BV605	Biolgened	127639	3, 4, 5
NK1.1	APC/Cy7	BD Pharmingen	560618	2, 3, 4
NK1.1	BV785	Biolegend	108749	5
Siglec-F	BV421	BD Horizon	562681	3, 5
Siglec-F	BV786	BD Optibuild	740956	4
Streptavidin	APC/Cy7	Biolegend	405208	2

TCR β	APC786	BD Optibuild	742484	5
TCR β	APC-E780	eBioscience	47-5961-82	2, 3, 4
TCR β	E450	eBioscience	48-5961-82	1

* All antibodies were reactive to mouse.

CD11b-Biotin was the primary stain. All other antibodies in panel 2 were added to the secondary stain.

Table 2.5 Flow Cytometry Panels

Panel Number	FACS Panel	Associated Figure
Panel 1	General Panel	4.6 – 4.10
Panel 2	HSC Panel	4.5, 4.16, 4.22 – 4.23, 4.25
Panel 3	Neutrophil Panel	4.5, 4.22, 4.24 – 4.25
Panel 4	HSC & Neutrophil Panel #1	4.3, 4.4
Panel 5	HSC & Neutrophil Panel #2	4.13, 4.30

2.2.9 Luciferase Imaging

Tumour burden was assessed by bioluminescent imaging prior to treatment initiation and at the conclusion of the treatment regimen. Mice were administered 50 mg/kg of D-luciferin (Promega, cat# P1041) by IP injection, and anaesthetised by isoflurane 5 min after injection. Bioluminescence was imaged using the IVIS100 imaging system (Perkin Elmer) over a 1-min and 5-min period. Living Image (v 4.5) was utilised to analyse images and quantify bioluminescence.

2.3 Molecular Techniques

2.3.1 DNA Isolation, Amplification and Sequencing of CRISPR Libraries

Genomic DNA was extracted using the Quick-DNA Midiprep Plus kit (ZYMO RESEARCH, cat# D4072) according to the manufacturer's instructions. Briefly, 25×10^6 cells were digested in 3 mL of BioFluid & Cell Buffer containing 2 mg of Proteinase K at 55 °C for 2 hours. Samples were lysed by the addition of 6 mL of Genomic Lysis Buffer and the lysates transferred to the Zymo-Spin™ V-E Column/Reservoir inserted in a 50 mL falcon tube (Greiner Bio-One, cat# 227261). Following centrifugation at 1000 x g for 10 minutes, the column was washed with 9 mL of DNA Pre-Wash Buffer then 7 mL of g-DNA Wash Buffer via centrifugation for a further 10 minutes at 1000 x g per wash. The Zymo-Spin™ V-E Column was transferred to a Collection Tube and residual wash buffer removed via centrifugation at 12,000 x g for 1 minute. The column was washed a final time with 200 µL of g-DNA Wash Buffer and centrifuged at 12,000 x g for 1 minute. The columns were subsequently incubated with 200 µL of DNA Elution Buffer at room temperature for 5 minutes and genomic DNA eluted via centrifugation at 12,000 x g for 1 minute. Genomic DNA concentration was quantified by nanodrop (Thermo Scientific, Nanodrop 2000) and stored at -20 °C until required. The sgRNA sequences were amplified by polymerase-chain reaction (PCR) from all genomic DNA in multiple reactions each containing 7.5 µg of DNA (Table 2.6) under the thermal cycler conditions given in Table 2.9. Separate samples within an experiment were amplified with unique P7 primers given in Table 2.8.

Following PCR amplification of sgRNA sequences, PCR products of the same sample were pooled libraries and purified using AMPure XP for PCR Purification (BECKMAN COULTER, cat# A63880). Briefly, 100 µL of pooled product was mixed with 100 µL of AMPure XP reagent and incubated at room temperature for 5 minutes to bind PCR products of at least 100 bp to the magnetic beads. Samples were placed on a DynaMag™-2 Magnet rack (Thermo Fisher Scientific, cat# 12321D) and supernatant subsequently removed. Beads were washed twice with 200 µL of 70% ethanol and supernatant discarded following placement on the magnetic rack. Beads were dried for 1 minute at room temperature on the magnetic rack prior to the elution of PCR product by the addition of 50 µL of TE buffer (10 mM Tris pH 7.5, 1 mM EDTA). Samples were placed on the magnetic rack and the PCR product supernatant transferred to a clean eppendorf tube and stored at -20 °C until required. Libraries were 75 bp single-end sequenced on the NextSeq500 (Illumina) at a depth of 10^7 reads per sample.

Table 2.6 PCR Mix

10x Ex Taq Buffer (TaKaRa, cat: RR001)	10 µL
2.5 mM dNTP mix	8 µL
100 µM P5 primer mix (primer sequences given in Table 2.7)	0.5 µL
5 µM P7 primer (primer sequences given in Table 2.8)	10 µL
Ex Taq [®] DNA Polymerase (TaKaRa, cat: RR001)	0.75 µL
Genomic DNA	7.5 µg
dH ₂ O	Total volume: 100 µL

Table 2.7 Sequences of P5 Primer Mix

AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTTCCG ATCTTTGTGGAAAGGACGAAACACCG
AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTTCCG ATCTCTTGTGGAAAGGACGAAACACCG
AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTTCCG ATCTGCTTGTGGAAAGGACGAAACACCG
AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTTCCG ATCTAGCTTGTGGAAAGGACGAAACACCG
AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTTCCG ATCTCAACTTGTGGAAAGGACGAAACACCG
AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTTCCG ATCTTGACCTTGTGGAAAGGACGAAACACCG
AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTTCCG ATCTACGCAACTTGTGGAAAGGACGAAACACCG
AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTTCCG ATCTGAAGACCCTTGTGGAAAGGACGAAACACCG

Table 2.8 Sequences of P7 Primers

Experiment	Sample	P7 primer sequence
Resistance to hypomethylating agents	Time-point 0	CAAGCAGAAGACGGCATACGAGAT CGGTTCAAGTGACTGGAGTTCAGACG TGTGCTCTTCCGATCTCCAATTCCCAC TCCTTTCAAACCT
Resistance to hypomethylating agents	Vehicle day 7	CAAGCAGAAGACGGCATACGAGATAT ACTCAAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTCCAATTCCCACCTC CTTTCAAGACCT
Resistance to hypomethylating agents	Vehicle day 42	CAAGCAGAAGACGGCATACGAGATG CTGGATTGTGACTGGAGTTCAGACGT GTGCTCTTCCGATCTCCAATTCCCACCT CCTTTCAGACCT
Resistance to hypomethylating agents	5-Azacytidine day 7	CAAGCAGAAGACGGCATACGAGATAT TGGATTGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTCCAATTCCCACCTC CTTTCAAGACCT
Resistance to hypomethylating agents	5-Azacytidine day 42	CAAGCAGAAGACGGCATACGAGATTA ACTCGGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTCCAATTCCCACCTC CTTTCAAGACCT
Resistance to hypomethylating agents	SGI-110 day 7	CAAGCAGAAGACGGCATACGAGATAT ACTCGGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTCCAATTCCCACCTC CTTTCAAGACCT
Resistance to hypomethylating agents	SGI-110 day 42	CAAGCAGAAGACGGCATACGAGATTA ACAGTTGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTCCAATTCCCACCTC CTTTCAAGACCT
Synthetic lethal targets in SLC29A1 knockout cells	sgSCR replicate 1 time-point 0	CAAGCAGAAGACGGCATACGAGATG CACGATTGTGACTGGAGTTCAGACGT GTGCTCTTCCGATCTCCAATTCCCACCT

		CCTTTCAAGACCT
Synthetic lethal targets in SLC29A1 knockout cells	sgSCR replicate 2 time-point 0	CAAGCAGAAGACGGCATACGAGATC GGTAGCCGTGACTGGAGTTCAGACGT GTGCTCTTCCGATCTCCAATTCCCCT CCTTTCAAGACCT
Synthetic lethal targets in SLC29A1 knockout cells	sgSCR replicate 1 day 35	CAAGCAGAAGACGGCATACGAGATTA TGGAGGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTCCAATTCCCCTC CTTTCAAGACCT
Synthetic lethal targets in SLC29A1 knockout cells	sgSCR replicate 2 day 35	CAAGCAGAAGACGGCATACGAGATC GCATCAAGTGACTGGAGTTCAGACGT GTGCTCTTCCGATCTCCAATTCCCCT CCTTTCAAGACCT
Synthetic lethal targets in SLC29A1 knockout cells	sgSLC29A1 replicate 1 time-point 0	CAAGCAGAAGACGGCATACGAGATTA GTTCTTGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCTCCAATTCCCCTCC TTTCAAGACCT
Synthetic lethal targets in SLC29A1 knockout cells	sgSLC29A1 replicate 2 time-point 0	CAAGCAGAAGACGGCATACGAGATTA CAAGTTGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTCCAATTCCCCTC CTTTCAAGACCT
Synthetic lethal targets in SLC29A1 knockout cells	sgSLC29A1 replicate 1 day 35	CAAGCAGAAGACGGCATACGAGATTA ACTCAAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTCCAATTCCCCTC CTTTCAAGACCT
Synthetic lethal targets in SLC29A1 knockout cells	sgSLC29A1 replicate 2 day 35	CAAGCAGAAGACGGCATACGAGATC GTGAGCCGTGACTGGAGTTCAGACGT GTGCTCTTCCGATCTCCAATTCCCCT CCTTTCAAGACCT

Table 2.9 Thermal Cycler Conditions

95 °C	1 minute	x 1
95 °C	30 seconds	x 28
53 °C	30 seconds	
72 °C	30 seconds	
72 °C	10 minutes	x 1

2.3.2 Cloning sgRNAs into FgH1tUTG or lentiGuide-Crimson

To anneal the forward and reverse sgRNA oligos given in Table 2.14, the annealing mix (Table 2.10) was incubated under conditions specified in Table 2.11. The vector backbone was digested by incubating the digestion mix (Table 2.12) at 55 °C for 2 hours and subsequently isolated using the NucleoSpin® Gel and PCR Clean-up kit (MACHERY-NAGEL, cat: 740609) according to the manufacturer's instructions. Briefly, Binding Buffer NTI was added to the digestion mix in a 2:1 ratio, to allow binding of the digested vector DNA to the Clean-up Columns during subsequent centrifugation at 11,000 x g for 30 seconds. The membrane in the column was washed twice with 700 µL of Wash Buffer NT3 via centrifugation at 11,000 x g for 30 seconds, dried by centrifugation for an additional 1 min at 11,000 x g and eluted in 30 µL of Elution Buffer NE via centrifugation at 11,000 x g for 1 minute. The concentration of digested vector DNA was quantified by nanodrop. To ligate the sgRNA into the digested vector, the ligation mix (Table 2.13) was incubated at room temperature for 15 minutes. Ligated constructs were heat shocked into 50 µL of STABL3 cells in a 1:20 (v/v) ratio by incubation on ice for 20 minutes, followed by 90 seconds at 42 °C then 2 minutes on ice prior to being plated on an agar plate containing 75 µg/mL of ampicillin and incubated overnight at 37 °C. One transformed colony was isolated via pipette tip and transferred to 3 mL of Lennox Luria Bertani (LB; 10 g/L tryptone, 5 g/L yeast extract, 0.5 g/L NaCl in H₂O) supplemented with 75 µg/mL of ampicillin and incubated overnight at 37 °C with agitation at 200 rpm. Plasmid DNA was subsequently extracted using the NucleoSpin® Plasmid kit (MACHERY-NAGEL, cat: 740588) according to the manufacturer's instructions. Briefly, 1.5 mL of bacterial culture was pelleted by centrifugation at 11,000 x g for 30 seconds and supernatant discarded prior to cell lysis in 250 µL of Buffer A1 and 250 µL of Buffer A2. Following incubation at room temperature for 4 minutes, the lysis reaction was neutralised with 300 µL of Buffer A3 and the mixture centrifuged at 11,000 x g for 5 minutes to remove cellular debris. The supernatant was transferred to a NucleoSpin® Column and centrifuged at 11,000 x g for 1 minute to allow the membrane to bind the plasmid DNA. The membrane was washed with 500 µL of Buffer AW, then 600 µL of Buffer A4 by centrifugation at 11,000 x g for 1 minute. The membrane was dried by centrifugation for 2 minutes at 11,000 x g and the DNA eluted in 50 µL of Buffer AE by centrifugation at 11,000 x g for 1 minute. Plasmid DNA concentration was quantified by nanodrop and submitted for Sanger sequencing (using the primer CCATGGAATTCGAACGCTGACG) at the Australian Genome Research Facility.

Table 2.10 Annealing Mix

Forward oligo (given in Table 2.14) 100 μ M	1.4 μ L
Reverse oligo (given in Table 2.14) 100 μ M	1.4 μ L
10x T4 DNA Ligase Reaction Buffer (New England BioLabs, cat: B0202)	5 μ L
dH ₂ O	42.2 μ L

Table 2.11 Annealing Incubation Conditions

85 °C	10 minutes
81 °C	2 minutes
77 °C	2 minutes
73 °C	2 minutes
69 °C	2 minutes
65 °C	2 minutes
61 °C	2 minutes
57 °C	2 minutes
53 °C	2 minutes
49 °C	2 minutes
45 °C	2 minutes
41 °C	2 minutes
37 °C	2 minutes
33 °C	2 minutes
29 °C	2 minutes
25 °C	2 minutes

Table 2.12 Digestion Mix

NEBuffer™ 3.1 10x (New England BioLabs, cat: B7203S)	4 μ L
BsmBI	2 μ L
FgH1tUTG or lentiGuide-Crimson	2 μ g
dH ₂ O	Total volume 40 μ L

Table 2.13 Ligation Mix

FgH1tUTG / lentiGuide-Crimson	50 ng
Annealed sgRNA oligos	0.75 µL
10x T4 DNA Ligase Reaction Buffer (New England BioLabs, cat: B0202)	2 µL
T4 DNA Ligase (New England BioLabs, cat: M0202)	1 µL
dH ₂ O	Total volume 20 µL

Table 2.14 sgRNA Oligo Sequences

sgRNA	Forward oligo sequence/ Reverse oligo sequence	Vector
sgARFGEF3 #1	TCCCACCGAAGCATGCATCAAGGG/ AAACCCCTTGATGCATGCTTCGGT	FgH1tUTG
sgARFGEF3 #2	TCCCTCTAGGGTGTGTGAATACGT/ AAACACGTATTACACACACCCTAGA	FgH1tUTG
sgARID1A #1	TCCCCAGCAGAACTCTCACGACCA/ AAACTGGTCGTGAGAGTTCTGCTG	FgH1tUTG
sgARID1A #2	TCCCCCTGTTGACCATAACCGCTG/ AAACCAGCGGGTATGGTCAACAGG	FgH1tUTG
sgBTG4 #1	TCCCGTCTGATGAGGCTCTACTAA/ AAACTTAGTAGAGCCTCATCAGAC	FgH1tUTG
sgBTG4 #2	TCCCTTGAAACATACAGAAGTCAC/ AAACGTGACTTCTGTATGTTTCAA	FgH1tUTG
sgCASC1 #1	TCCCAAATGTTACTCTGTATGTGT/ AAACACACATACAGAGTAACATTT	FgH1tUTG
sgCASC1 #2	TCCCTGTAGAAAAAGCAATCAGCA/ AAACTGCTGATTGCTTTTTCTACA	FgH1tUTG
sgCSN1S1 #1	TCCCCAGCATCAGTTCATCGAGTG/ AAACCACTCGATGAACTGATGCTG	FgH1tUTG
sgCSN1S1 #2	TCCCTATACCATTAGAATCAAGAG/ AAACCTCTTGATTCTAATGGTATA	FgH1tUTG
sgDCK #1	TCCCTCAAGAAAATCTCCATCGAA/ AAACTTCGATGGAGATTTTCTTGA	FgH1tUTG

sgDCK #2	TCCCTTCCTGAACCTGTTGCCAGA/ AAACTCTGGCAACAGGTTTCAGGAA	FgH1tUTG
sgEPC1 #1	TCCCGCAAATCCAATACACCTTTG/ AAACCAAAGGTGTATTGGATTTGC	FgH1tUTG
sgEPC1 #1	TCCCTATAATTTGGGCGACTACAA/ AAACTTGTAGTCGCCCAAATTATA	FgH1tUTG
sgGIF1B #1	TCCCGAAGCTAGGCTTGTAGAATG/ AAACCATTCTACAAGCCTAGCTTC	FgH1tUTG
sgGIF1B #2	TCCCGGGGTTCGGGACAGCACAATG/ AAACCATTGTGCTGTCCCGACCCC	FgH1tUTG
sgHNRNPUL2 #1	TCCCCAAGTGAGCAAAGACCGCTA/ AAACTAGCGGTCTTTGCTCACTTG	FgH1tUTG
sgHNRNPUL2 #2	TCCCTCTTACGGTTTCGATGGACG/ AAACCGTCCATCGAAACCGTAAGA	FgH1tUTG
sgKBTBD7 #1	TCCCTCAAGTCCTTCTACGACGCG/ AAACCGCGTCGTAGAAGGACTTGA	FgH1tUTG
sgKBTBD7 #2	TCCCGACTCTAGCAGATCTAACCC/ AAACGGGTTAGATCTGCTAGAGTC	FgH1tUTG
sgOR52B6 #1	TCCCAGGATTGTGACATATCGCAG/ AAACCTGCGATATGTCACAATCCT	FgH1tUTG
sgOR52B6 #2	TCCCTATTGATCTGGCAATAGTGC/ AAACGCACTATTGCCAGATCAATA	FgH1tUTG
sgPFKP #1	TCCCGATGTGTGTCAAACCTCTCGG/ AAACCCGAGAGTTTGACACACATC	FgH1tUTG
sgPFKP #2	TCCCGAAGTACGCCTACCTCAACG/ AAACCGTTGAGGTAGGCGTACTTC	FgH1tUTG
sgPRDX3 #1	TCCCACAAGAGTGCGATGTTTCATG/ AAACCATGAACATCGCACTCTTGT	FgH1tUTG
sgPRDX3 #2	TCCCGACAGCAGGTGCATGGCATG/ AAACCATGCCATGCACCTGCTGTC	FgH1tUTG
sgRBM12B #1	TCCAGTCTTATGTAAACTCAGAG/ AAACCTCTGAGTTTACATAAGACT	FgH1tUTG

sgRBM12B #2	TCCCCACCAACTGAGTATATTGGT/ AAACACCAATATACTCAGTTGGTG	FgH1tUTG
sgSCR	GCACTCACATCGGACATCA	FgH1tUTG / lentiGuide-Crimson
sgSDR16C5 #1	TCCCTCTGGGATATCAATAAGGAG/ AAACCTCCTTATTGATATCCCAGA	FgH1tUTG
sgSDR16C5 #2	TCCCGCACATCCGTATACACTCAG/ AAACCTGAGTGTATACGGATGTGC	FgH1tUTG
sgSLC29A1 #1	TCCCAAGTTGGACCTCATTAGCAA/ AAACTTGCTAATGAGGTCCAATT	FgH1tUTG
sgSLC29A1 #2	TCCCTACACGGCCCCCATCATGAG/ AAACCTCATGATGGGGGCCGTGTA	FgH1tUTG
sgSNRK #1	TCCCGGCATTGACGTACTCTACAA/ AAACTTGTAGAGTACGTCAATGCC	FgH1tUTG
sgSNRK #2	TCCCTGTCTTTACGGGTGAAAAGG/ AAACCCTTTTCACCCGTAAAGACA	FgH1tUTG
sgTXK #1	TCCCACCGTAGCTAAACCCAGCTG/ AAACCAGCTGGGTTTAGCTACGGT	FgH1tUTG
sgTXK #2	TCCCAGAACATCTATTGAGACAAG/ AAACCTTGTCTCAATAGATGTTCT	FgH1tUTG
sgUCK2 #1	TCCCTACTGTCTATCCCGCAGACG/ AAACCGTCTGCGGGATAGACAGTA	FgH1tUTG
sgUCK2 #2	TCCCTCTGCTCCGAGGTAAGGACA/ AAACTGTCCTTACCTCGGAGCAGA	FgH1tUTG
sgWDR17 #1	TCCCAAAGCCCTGTCAATTGCACC/ AAACGGTGCAATTGACAGGGCTTT	FgH1tUTG
sgWDR17 #2	TCCCGATCTGACTAGATTCTCAAG/ AAACCTTGAGAATCTAGTCAGATC	FgH1tUTG
sgZIM3 #1	TCCCGAAACGCTGACTACGCAGAA/ AAACTTCTGCGTAGTCAGCGTTTC	FgH1tUTG
sgZIM3 #2	TCCCTTCCAACCTCAAGATCACAT/ AAACATGTGATCTTGAGGTTGGAA	FgH1tUTG

2.3.3 Generation of MSCV-Luciferase-PuroR Construct

The puromycin coding domain sequence was amplified out of LentiGuide-PuroR (Addgene, cat# 52963) with primers that introduced XhoI and EcoRI restriction sites at the 5' and 3' ends of the sequence, respectively, in a reaction mix provided in Table 2.15 and under thermocycling conditions summarised in Table 2.16. The amplified puromycin-resistance (puroR) coding domain sequence was purified using the NucleoSpin® Gel and PCR Clean-up kit outlined in section 2.3.2. The puroR DNA eluate and 1 µg of MSCV-IRES-Luc2 plasmid (Addgene, cat# 18760) were digested with EcoRI-HF (NEB, cat# R3101S) and Xho-I (NEB, cat# R0146S) in a 40 µL reaction volume with 1x Cutsmart Buffer (NEB, cat# B7204S) for 30 min at 37 °C. After electrophoresis at 100 V for 30 min, the digested MSCV-IRES-Luc2 plasmid and puroR coding sequence were excised from a 1% (w/v) agarose gel cast in Tris-Borate-EDTA (TBE) buffer and gel purified using the NucleoSpin® Gel and PCR Clean-up kit according to the manufacturer's instructions. Purified DNA was quantified by nanodrop. Purified insert (25 ng) and backbone (100 ng) were ligated in a 20 µL reaction volume as described in section 2.3.2. Ligated constructs were heat shocked into 50 µL of TOP10F competent *E.coli* (Life Technologies, ThermoFisher, cat# C303003) as described in section 2.3.2. Plasmid DNA was extracted, quantified, and verified as described section 2.3.2.

Table 2.15 PCR Mix

5x Phusion HF Buffer*	5 µL
10 mM dNTPs	0.5 µL
LentiGuide-Puro	10 ng
Phusion DNA Polymerase*	0.25 µL
Forward primer 10 µM ATACTCGAGCACCATGACCGAGTACAAGCCCACG	1.25 µL
Reverse primer 10 µM ATAGAATTCTCAGGCACCGGGCTTGC	1.25 µL
H ₂ O	To a final volume of 15.75 µL

*(Thermo Scientific, cat# F530S)

Table 2.16 Thermal Cycler Conditions for PCR of Puromycin

98 °C	30 seconds	x 1
98 °C	30 seconds	
62 °C	20 seconds	x 30
72 °C	15 seconds	
72 °C	10 minutes	x 1

2.3.4.1 3' mRNA Quantseq

2.3.4.1.1 RNA Extraction

1 – 5 x 10⁵ cells sorted by FACS, per mouse, (section 2.2.7) were washed with PBS and pelleted by centrifugation (450 x g, 10 min, 4 °C) prior to being stored at -80 °C in TRIzol Reagent (Invitrogen, cat# 15596026). RNA was extracted with the Direct-zol RNA Microprep kit (Zymo Research, cat# R2060). Briefly, an equal volume of ethanol was added to the sample lysed in TRIzol Reagent, transferred into a Zymo-Spin IIICG Column in a collection tube and centrifuged at 11,000 x g for 30 sec to bind the RNA, washed with 400 µL of RNA Wash Buffer, and incubated for 15 min at room temperature with DNase I (30 U) added to 35 µL of DNA Digestion Buffer. Columns were subsequently washed by centrifugation (11,000 x g, 30 sec) twice with 400 µL of Direct-zol RNA PreWash, then 700 µL of RNA Wash Buffer. 12 µL of UltraPure DNase/RNase-Free Distilled Water (Invitrogen, cat# 10977015) was incubated on the column for 2 min at room temperature and centrifuged (11,000 x g, 30 sec) to elute RNA. To increase RNA yield, the eluate was transferred back onto the same column, incubated for 2 min at room temperature and centrifuged (11,000 x g, 30 sec). Purified RNA was quantified using the TapeStation 2200 system (Aligent) with RNA HS screentape (Aligent, cat# 5067-5576) according to the manufacturer's instructions. RNA was stored at -80 °C until required.

2.3.4.1.2 Library Preparation and Sequencing

The Molecular Genomics Core of the Peter MacCallum Cancer Centre used the QuantSeq 3' mRNA-seq Library Prep Kit for Illumina (Lexogen, cat# 015), according to the manufacturer's instructions, to generate the 3' libraries from the purified RNA (section 2.3.4.1.1). 75 base-pair single end reads with a depth of 6 – 10 million reads per sample were generated by sequencing the 3' mRNA libraries using the Illumina NextSeq500.

2.3.4.2 Low Cell Number ChIP-seq

2.3.4.2.1 Chromatin Immunoprecipitation

Approximately 1×10^6 FACS sorted cells (section 2.2.7), and drosophila S2 cells, were washed with PBS, centrifuged at 450 x g and 4 °C for 10 min and resuspended in 500 μ L of room temperature 10% FCS in PBS. An equal volume of cross-linking buffer (2% formaldehyde, 10% FCS in PBS) was added and the samples and incubated at room temperature for 10 min with rotation. Cross-linking was quenched with the addition of glycine (2.5 M, 1/10 dilution) and incubation for 5 min at room temperature with rotation. Cross-linked samples were washed in cold 5% (w/v) BSA in PBS, then resuspended in cold nuclear isolation buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% v/v NP-40, 1% v/v Triton X-100) and incubated on ice for 10 min. Samples were washed with 5% (w/v) BSA in PBS, pelleted at 800 x g and 4 °C for 5 min and snap frozen at -80 °C until all samples from the experiment were harvested.

All samples were thawed on ice and subsequently processed simultaneously. Samples were resuspended in 100 μ L of ChIP lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% v/v NP-40, 0.3% w/v sodium dodecyl sulphate, cOmplete Mini Protease Inhibitor Cocktail (Merk, cat# 4693159001). Samples were placed in a 100 μ L microtube (Covaris, cat# 520045) and sonicated for 15 min with an S220 focused ultrasonicator (Covaris) to obtain chromatin fragments of ~ 150 – 300 bp in size. Dilution buffer (20 mM Tris pH 8, 150 mM NaCl, 2 mM EDTA, 1% v/v Triton X-100, protease inhibitor) was added to the sample in a 1:1 ratio. 2% of the mixture was set aside as input, and reverse cross-linked as described below. DNA concentration was quantified using the Qubit dsDNA HS Assay Kit (Invitrogen, cat# Q32854) according to the manufacturer's instructions and drosophila DNA added to each sample at a ratio of 1:40 relative to the sample DNA concentration.

Protein A and G dynabeads (Invitrogen, cat# 10015D), mixed in a 1:1 ratio, were washed once with dilution buffer, resuspended in 150 μ L of dilution buffer containing 1 μ g of antibody (Table 2.17) per sample and incubated at 4 °C for 1.5 hrs with rotation. Sonicated chromatin was added to pre-coupled beads and incubated overnight at 4 °C with rotation. Chromatin immunoprecipitated (ChIP) lysates were washed in 200 μ L of dilution buffer, then 200 μ L of wash buffer 1 (20 mM Tris pH 8, 500 mM NaCl, 2 mM EDTA, 1% v/v Triton X-100, 0.1% w/v sodium dodecyl sulphate, cOmplete Mini Protease Inhibitor Cocktail), followed by 200 μ L of wash buffer 2 (20 mM Tris pH 8, 250 mM LiCl, 2 mM EDTA, 0.5% v/v NP-40, 0.5% w/v

deoxycholate, cOmplete Mini Protease Inhibitor Cocktail) for 5 min at 4 °C with rotation at each wash. ChIP lysates were washed twice with TE buffer (10 mM Tris pH 7.5, 1 mM EDTA) for 1 min at 4 °C with rotation per wash. Samples were resuspended in reverse cross-linking buffer (1% sodium dodecyl sulphate, 100 mM NaHCO₃, 200 mM NaCl, 300 µg / mL proteinase K), vortexed and incubated at 55 °C for 30 min. Supernatant was transferred to a new Eppendorf tube and incubated at 65 °C for 30 min. DNA was purified using the ChIP DNA Clean & Concentrate Kit (Zymo Research, cat# D5205) as per the manufacturer's instructions.

Table 2.17 ChIP Antibodies

Antibody	Manufacturer	Catalogue #
H3K27me3	Millipore	07449
H3K27ac	Abcam	AB4729

2.3.4.2.2 Library Preparation and Sequencing

The Molecular Genomics Core of the Peter MacCallum Cancer Centre used the KAPA Hyper Prep Kit for Illumina platforms (Kapa Biosystems, Roche, cat# 07962363001) and the SeqCap Adapter Kit (Roche, cat# 07962363001), according to the manufacturer's instructions, to generate the indexed libraries. High Sensitivity D1000 ScreenTape and reagents (Aligent, cat# 5067-5584, 5067-5585) with 2200 TapeStation Instrument (Aligent Technologies) were used for library quantification and QC. Libraries were size selected for 200 – 500 bp using Pippin Prep system (Sage Science). 15 – 20 × 10⁶ single-end 75 bp sequencing reads were generated per sample from pooled libraries sequenced on the NextSeq500 (Illumina).

2.4 Protein Analysis

2.4.1 Harvesting and Quantifying Whole Cell Lysate

Approximately $1 - 2 \times 10^6$ *in vitro* cells, or bone marrow and spleen derived IDN cells sorted by FACS (section 2.2.7), were pelleted by centrifugation (450 x g, 4 min, 4 °C). Pelleted cells were washed with PBS and lysed for 1 hr on ice in RIPA buffer (NaCl 150 mM, MgCl₂ 2 mM, Triton X-100 1% (v/v), Sodium Deoxycholate 0.5% (w/v), SDS 0.1% (w/v), Tris-Cl 50 mM pH 8.0) with 1 x protease inhibitor cocktail (Roche Diagnostics cat: 4693159001) and Bezonase 1.25 U/uL (Merk Millipore cat: 70746-3). Protein concentration was quantified using the Microplate Procedure with the Pierce BCA Protein Assay Kit (Thermo Scientific cat: 23225) according to the manufacturer's instructions. Briefly, 190 µL of 50-parts Reagent A and 1-part reagent B was added to triplicates of 10 µL of diluted lysate (1:10 in PBS) or 10 µL of BSA standards ranging from 62.5 – 2000 µg/mL (or to) in a 96-well flat-bottom plate. Samples were incubated at 37 °C for 15 min and absorbance at 595 nm measured using the Versamax microplate reader (Molecular Devices™). A quadratic curve was fitted to the average absorbance of the standards and used to calculate the concentration of the samples. Samples were stored at -20 °C until required.

2.4.2 Western Blot

20 – 40 µg of protein lysates were diluted to 1 – 2 µg/µL with PBS and 6 x SDS (Tris base 375 mM pH 6.8, SDS 12.3% (w/v), Glycerol 25.8% (v/v), bromophenol blue 600 µg/mL, 2-mercaptoethanol 60 µL/mL). Samples were incubated at 95 °C for 5 minutes prior to being resolved on a 4 – 15% Mini-PROTEAN TGX Precast Protein Gels (Bio-rad, cat# 4561084) gel alongside a pre-stained protein ladder (Thermo Fisher Scientific, cat# 26617) by gel electrophoresis at a constant voltage of 120 V for 90 minutes in SDS running buffer (Tris base 25 mM pH 8.3, glycine 0.192 M, SDS 0.1% (w/v)). Electroblothing in transfer buffer (Tris base 25 mM, glycine 1.92 M, methanol 10% (v/v)) for 60 minutes at 4 °C transferred the protein onto a methanol activated Immobilon-P PVDF membrane (Millipore, cat# IPVH00010). Membranes were blocked in 5% (w/v) skim milk powder in 1 x TBS/T (Tris base 50 mM pH 7.6, NaCl 150 mM, Tween20 0.1% (v/v)) for 1 hour at room temperature prior to incubation in primary antibody diluted in 5% (w/v) skim milk powder in 1 x TBS/T overnight at 4 °C. Membranes were washed with 1 x TBS/T for 15 min in 5 min intervals before incubation in HRP-conjugated secondary antibody diluted in 5% (w/v) skim milk powder in 1 x TBS/T for 1 hour at room temperature. Membranes were subsequently washed with 1 x TBS/T for 30 min

in 10 min intervals prior to incubation in ECL™ (GE Healthcare, cat# RPN2106) and exposure by film (FUJIFILM Super RX) using an AGFA CP1000 developer (AGFA).

Table 2.18 Antibodies for Western Blot

Antibody	Dilution	Targeting Species	Host Species	Manufacturer	Catalogue #
DCK	1/1000	Human	Rabbit	Abcam	ab96599
β-actin	1/3000	Mouse / Human	Polyclonal mouse	Sigma	A2228
IDH1	1/1000	Mouse / Human	Rabbit	Cell Signaling	8137s
DNMT3A	1/1000	Mouse / Human	Rabbit	Cell Signaling	2160s
NRAS ^{G12D}	1/1000	Mouse / Human	Rabbit	Cell Signaling	14429s
2° - anti Rabbit IgG/HRP	1/3000	Rabbit	Polyclonal swine	Dako	P0217
2° - anti Mouse IgG/HRP	1/3000	Mouse	Polyclonal rabbit	Dako	P0260

2.5 Bioinformatic Analyses

2.5.1 Analysis of CRISPR Screen Data

Sequencing reads were de-multiplexed and low-quality reads ($Q\text{-score} < 30$) filtered with bcl2fastq (v2.17.1.14). Cutadapt (v1.18) trimmed the reads to isolate the 20 bp sgRNA sequence. MAGeCK (v0.5.7) was subsequently used to count the sgRNA sequences and determine whether the median-normalised read count differences of each sgRNA was significantly different between the two conditions. Genes with at least 2 independent sgRNAs that were enriched/depleted and satisfied various statistical cut-offs (detailed in Section 3.3.2, Table 3.1, and Section 3.3.5, Table 3.3), were chosen for further validation. Figures were generated using R packages ggplot2 (v3.1.0), ggrepel (v0.8.0), and pheatmap (v1.0.12).

2.5.2 Analysis of Published RNA Sequencing Data

Sequencing reads downloaded from GEO: GSE76203 (Unnikrishnan et al., 2017) were aligned to the human genome (hg19) using HISAT2 (v 2.1.0), converted to a binary alignment map (BAM) file and indexed using SAMtools (v 1.4.1), and reads counted using Subread (v 1.6.3). Read normalisation was performed in R (v 3.6.2) using R packages LIMMA (v 3.42.2) and EdgeR (v 3.28.1).

2.5.3 Quantseq Analysis

Sequencing reads were demultiplexed using bcl2fastq (v 2.17.1.14), low quality ($Q < 30$) reads removed, and trimmed at the 5' and 3' ends using cutadapt (v 1.14) to remove adapter sequences and poly-A-tail derived reads, respectively. Sequencing reads were mapped to the mouse reference genome (mm10) using HISAT2 (v 2.1.0) and counted using the featureCounts command of the Subread package (v 1.6.3). Read normalisation and differential gene expression analysis was performed in R (v 3.6.2) using R packages LIMMA (v 3.42.2) and EdgeR (v 3.28.1). R packages pheatmap (v 1.0.12), ggplot2 (v 3.2.1), ggrepel (v 0.8.1), RColorBrewer (v 1.1-2) and LIMMA (v 3.42.2) were used to generate figures.

2.5.4 ChIPseq Analysis

Sequencing reads were demultiplexed using bcl2fastq (v 2.17.1.14), low quality ($Q < 30$) reads removed and aligned to a concatenated mm10/dm3 reference genome using Bowtie2 (v 2.3.3). Samtools (v 1.4.1) was used to convert between SAM and BAM files, remove duplicate reads,

split reads aligning to separate genomes, and index BAM files. MACS2 (v 2.1.1) was used to call peaks. Sequencing reads aligning to the mm10 and dm3 genomes were counted using the featureCounts command of the Subread package (v 1.6.3). R packages Csaw (v 1.20.0) and EdgeR (v 3.28.1) were used to quantify windows enriched for histone marks and perform differential enrichment analysis. During the analysis with Csaw, the mm10 reads were scaled by the ratio of the percentage of reads aligning to dm3 for the given sample and the mean percentage of dm3 reads in the vehicle treated cohort $\left(mm10\ reads \div \frac{\%dm3\ reads}{vehicle\ mean\ \%dm3\ reads} \right)$. Bedtools (v 2.27.1) was used to annotate the peaks and enriched windows to the nearest gene. Figures were generated using deeptools (v 3.3.0) and R packages ggplot2 (v3.2.1), ggrepel (v0.8.0), pheatmap (v1.0.12) and LIMMA (v 3.42.2).

Chapter 3: Investigating Mechanisms of Resistance to Hypomethylating Agents in AML

3.1 Introduction

Hypomethylating agents, including 5-Azacytidine (AZA) and Decitabine (DAC) are azanucleoside analogues of cytidine and were the first class of epigenetic drugs approved by the FDA for the treatment of MDS and AML. Originally, hypomethylating agents were utilised as classical cytostatic/cytotoxic agents over 40 years ago (Karon et al., 1973), but had soon evolved into an epigenetic therapy due to their ability to demethylate CpG dinucleotides across the genome at low doses (Wijermans et al., 1997).

AZA was the first hypomethylating agent to be approved in the clinic (Diesch et al., 2016). However, the majority of AZA is incorporated into RNA, with only 10 – 20% being incorporated into DNA where it is thought to exert its main anti-tumour effects. This observation prompted the development of DAC, a hypomethylating agent that is solely incorporated into DNA (Diesch et al., 2016). AZA and DAC are structurally similar hypomethylating agents, that can only be distinguished by their pentose-sugar moiety; AZA is a ribonucleoside analogue whereas DAC is a deoxyribonucleoside analogue. However, AZA and DAC are rapidly cleared from the plasma, with a mean elimination half-life of ~40 minutes (Derissen et al., 2013), owing to high levels of cytidine deaminase in the liver and spleen (Chabot et al., 1983). The development of next-generation hypomethylating agents aimed to improve the stability and potency of AZA or DAC through the attachment of additional ligands or moieties. Guadecitabine (GDAC) is a second-generation DAC derivative with pharmacokinetics and pharmacodynamics superior to DAC, that is currently undergoing phase 3 clinical trials (Issa et al., 2015).

Enzymes of the pyrimidine salvage pathway modify the hypomethylating agents into the active metabolite capable of being incorporated into RNA/DNA. Equilibrative and concentrative nucleoside transporters facilitate the uptake of hypomethylating agents into the cell (Diesch et al., 2016). Uridine-cytidine kinase 2 (UCK2) and deoxycytidine kinase (DCK) catalyse the rate-limiting mono-phosphorylation of AZA and DAC, respectively, (Anderson and Brockman, 1964; Sripayap et al., 2014), thus instigating successive rounds of phosphorylation prior to the incorporation of the tri-phosphorylated hypomethylating agent into RNA or DNA, respectively. Approximately 10 – 20% of di-phosphorylated AZA is reduced to di-phosphorylated DAC by ribonucleotide reductase, where it is phosphorylated once more and subsequently incorporated into DNA (Diesch et al., 2016). Incorporation of azanucleosides into DNA occurs during S-phase and results in the irreversible covalent trapping and proteasomal degradation of the

cellular pool of free DNMT1 (Ghoshal et al., 2005; Patel et al., 2010; Santi et al., 1984). DNMT1, the maintenance DNA methyltransferase, methylates hemi-methylated DNA during DNA replication thus propagating existing methylation patterns onto the newly synthesised daughter strand (Hermann et al., 2004). Additionally, recruitment of DNMT1 to sites of DNA damage suggests a role for DNMT1 in restoring methylation during DNA repair (Mortusewicz et al., 2005). Depletion of DNMT1 by hypomethylating agents eventually results in the demethylation of CpG sites across the genome through the passive dilution of 5-mC during progressive rounds of DNA replication in proliferative cells (Creusot et al., 1982). In turn, hypomethylation of the genome induces changes in the transcriptome unfavourable to malignant cell survival (Bender et al., 1998; Tsai et al., 2012).

Research into the mechanisms through which hypomethylating agents exert their anti-leukaemic effects have predominantly focused on the association of hypomethylated CGI promoters and proximal gene expression, particularly tumour suppressor genes (Bender et al., 1998; Leung et al., 2019; Tsai et al., 2012). More recently, the potency of hypomethylating agents has been partially attributed to its effect on tumour immunity; the AZA/DAC induced DNA hypomethylation and consequential transcriptional activation of endogenous retroviruses (ERVs) has been demonstrated to trigger the double-stranded RNA viral defence pathway and induce a type I interferon response (Chiappinelli et al., 2015; Jeong et al., 2015; Stone et al., 2017). These anti-tumour effects are ultimately a consequence of AZA/DAC incorporation into DNA. However, AZA is primarily incorporated into RNA and can also inhibit the methylation and processing of tRNA (Lee and Karon, 1976; Schaefer et al., 2009). Furthermore, proteomic quantitation following AZA treatment revealed changes to the nuclear proteome (Bowler et al., 2019). Taken together, AZA and DAC may elicit their anti-tumour effects through both overlapping and distinct mechanisms.

Adult AML disproportionately affects those over the age of 65, and hence a large proportion of patients are unable to tolerate the standard treatment of care, consisting of anthracycline and cytarabine induction (Büchner et al., 2009; Kantarjian et al., 2010). Hypomethylating agents have become a front-line life-prolonging treatment option for patients that do not qualify for intensive chemotherapy (Tallman et al., 2019), as this class of therapeutics is well tolerated at low doses (Navada and Silverman, 2017). However, first-generation hypomethylating prodrugs as single agents was only effective in ~30% of patients with a survival benefit that was typically measured in months and relapse a common occurrence (Fenaux et al., 2009; He et al., 2017).

In order to improve patient outcomes, the combination of hypomethylating agents with other therapeutics is an area of active investigation as evident by the abundance of clinical trials (Craddock et al., 2019; Jilg et al., 2019; Onec et al., 2018; Swords et al., 2018). However, researchers have not been able to identify common characteristics shared by patients that predict response to hypomethylating agents. Understanding mechanisms through which resistance to hypomethylating agents occurs and how subsequent relapse may arise is pertinent for the identification of biomarkers that stratify between potential responders and non-responders, and for the development of effective rationalised combination therapies.

3.2 Specific Aims

Low-dose hypomethylating agent therapy is considered a front-line treatment option for MDS and AML patients unable to receive intensive chemotherapy induction. However, hypomethylating agent therapy typically provides a survival benefit that is measured in months, with relapse an almost inevitable occurrence. Furthermore, mechanisms of acquired resistance remain largely unknown. In order to improve patient outcome, the combination of hypomethylating agents with additional therapeutics is actively being investigated, as evident by the abundance of clinical trials examining such combinations. Hence, this chapter aimed to identify mechanisms of resistance to low-dose hypomethylating agents, utilising unbiased experimental techniques, and identify targetable therapeutic options in AMLs with acquired resistance to hypomethylating agents.

The specific aims for Chapter 3 were to:

1. Identify genes whose loss mediates resistance to hypomethylating agents in AML *in vitro* models
2. Validate hypomethylating agent resistance in AML *in vivo* models
3. Elucidate targetable therapeutic options that demonstrate a synthetical lethal interaction with AML models resistant to hypomethylating agents

3.3 Results

3.3.1 Low Dose Exposure to Hypomethylating Agents Reduces Cell Proliferation

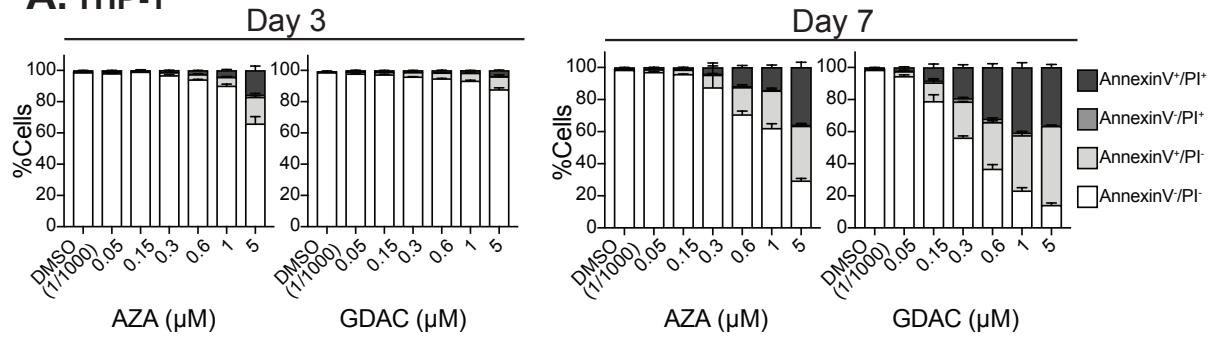
Prior to examining mechanisms of resistance to hypomethylating agents in the context of haematological malignancies, leukaemic cell lines were exposed to increasing concentrations of AZA and GDAC in order to characterise the phenotypic responses to these agents and identify a concentration that does not induce acute cytostatic effects. THP-1, MV4-11 and OCI-AML3 are human cell lines derived from myelomonocytic leukaemia patients and were utilised for the characterisation of the response to AZA and GDAC.

To establish the level of sensitivity in THP-1, MV4-11 and OCI-AML3 cell lines, titrations of AZA or GDAC were added to the culture media daily across a concentration gradient and the proportion of live and dead cells quantified by Annexin V / PI staining and flow-cytometry (Figure 3.1 A – C). After 3 days, all tested cell lines exhibited modest cell death (Annexin V⁺ / PI⁺) at high AZA concentrations and minimal changes in cell viability at equivalent molar concentrations of GDAC (Figure 3.1 A – C left). However, by day 7, GDAC had induced a substantially higher proportion of cell death than AZA at equivalent concentrations (Figure 3.1 A – C right); the 50% lethal-dose (LD₅₀) ranges for GDAC and AZA were 300 – 600 nM and > 1 μ M, respectively. These data suggest that long-term exposure of GDAC is more potent than AZA.

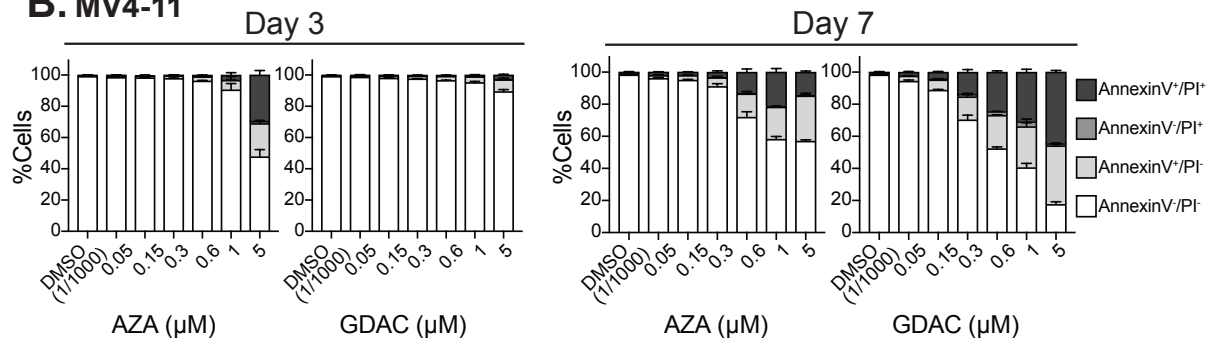
Since hypomethylating agents are administered to AML and MDS patients at low doses (15 mg/m² DAC, 75 mg/m² AZA) (Lübbert et al., 2011; Silverman et al., 2002), and 7-day low concentrations of AZA and GDAC induced minimal cell death *in vitro* (Figure 3.1 A – C right), it was hypothesised that low concentrations of AZA and GDAC could negatively influence the rate of cell division. CellTrace Violet (CTV) assays were utilised to investigate the impact hypomethylating therapies have on the proliferative capacity of AML cell lines *in vitro* (Figure 3.2). Cells were pre-stained with CTV, which covalently binds free intra-cellular and surface-bound amines, and sorted for a narrow range of evenly labelled cells. During each cell division, the dye is approximately evenly distributed amongst daughter cells, enabling tracking of cell proliferation (Figure 3.2 A). Low concentrations of AZA (50 – 600 nM) and GDAC (37.5 – 300 nM) were supplemented into the culture medium daily and the number of divisions quantified on day 3 (Figure 3.2 B) and day 5 (Figure 3.2 C – D). By day 3, 37.5 nM of GDAC induced a modest decrease in proliferation, whilst exposure to AZA $\geq$ 300 nM was required to

elicit the same effect (Figure 3.2 B). Pronounced proliferative defects were apparent by day 5; concentrations of AZA and GDAC as low as 50 nM and 37.5 nM, respectively, reduced the proportion of cells that had undergone 6 divisions by ~50% when compared to cells exposed to vehicle (Figure 3.2 C – D). Supporting the proposition that GDAC is more potent than AZA, lower nanomolar concentrations of GDAC induced similar growth defects to that of higher nanomolar concentrations of AZA (Figure 3.2 D – E). Collectively, these data suggest that non-cytotoxic concentrations of hypomethylating agents primarily reduce the proliferative capacity of cells *in vitro*, rather than initiating substantial cell death.

A. THP-1



B. MV4-11



C. OCI-AML3

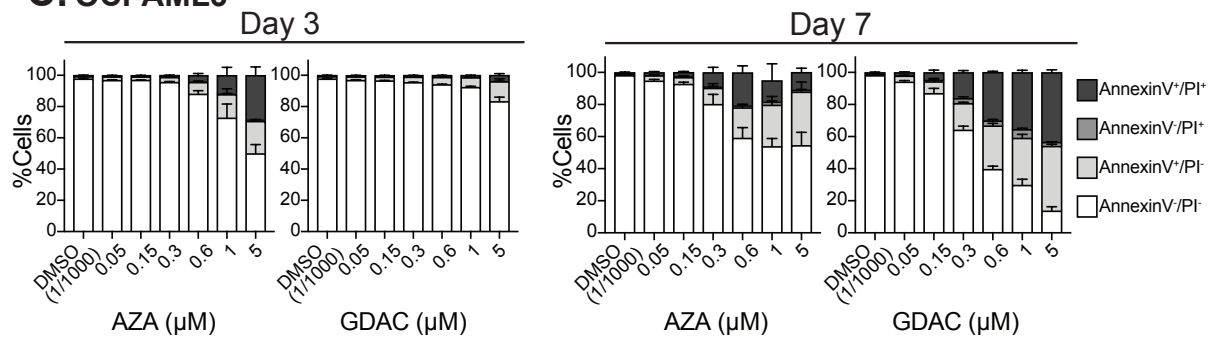


Figure 3.1 High AZA/GDAC Concentrations Induced Cell Death

(A – C) The percentage of cells classified as Annexin V⁻ / PI⁻, Annexin V⁺ / PI⁻, Annexin V⁻ / PI⁺, and Annexin V⁺ / PI⁺ by flow cytometry following 3 days or 7 days of daily AZA or GDAC treatment in (A) THP-1, (B) MV4-11 and (C) OCI-AML3 cells. The mean $\pm$ SD is represented for 3 biological replicates.

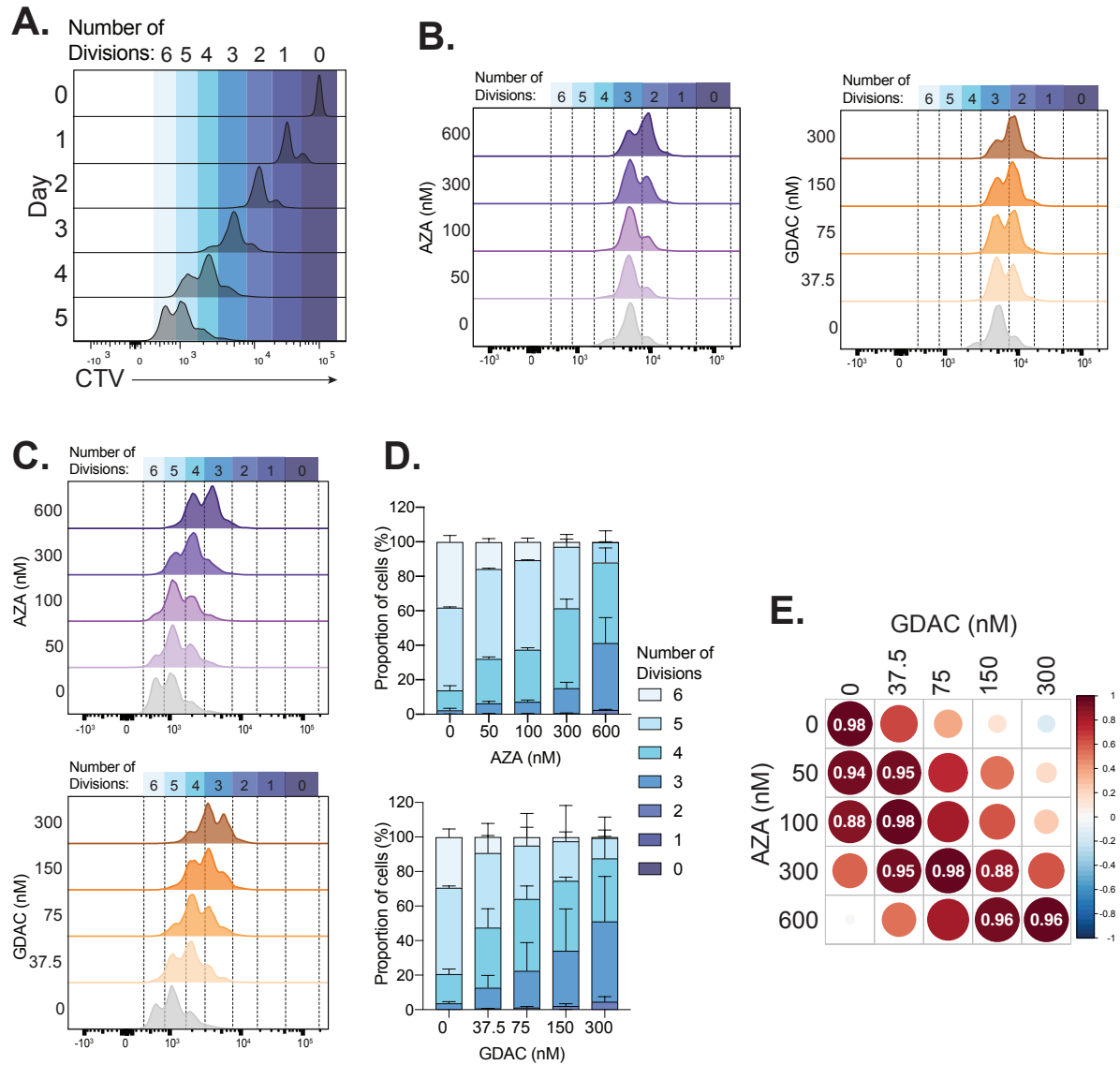


Figure 3.2 Low Concentrations of AZA and GDAC Reduced Proliferative Capacity

(A) CTV-labelled untreated THP-1 control cells used to set the division boundaries for B – E. (B – C) Representative CTV FACS histograms following (B) 3 days or (C) 5 days of daily drug treatment. (D) Quantitation of the proportion of cells within each division number following 5 days of daily drug treatment. The mean $\pm$ SD is represented for 2 biological replicates. (E) Correlation matrix comparing the distribution of cells within each division number of each treatment condition following 5 days of daily drug treatment. Pearson's correlation coefficients provided for the pairwise comparisons that pass statistical significance ($p < 0.01$).

3.3.2 Loss of Transporter or Cytidine/Uridine Kinase Activity Confers the Strongest Resistance to Hypomethylating Agents *In Vitro*

Responses to low-dose hypomethylating agents are rarely sustained and resistance frequently arises in MDS and AML patients (Dombret et al., 2015; Fenaux et al., 2009). The mechanisms that overcome the AZA and/or GDAC mediated anti-proliferative effects have been poorly characterised within the leukaemic patient population. To identify genes and molecular pathways that are important for AZA and/or GDAC to reduce the proliferative capacity of AML cells, an *in vitro* genome-wide knockout CRISPR screen utilising low concentrations of hypomethylating agent selection was employed (Figure 3.3 – 3.4). THP-1 cells, engineered to ectopically express Cas9, were transduced with the Brunello CRISPR knockout pooled library at a low multiplicity of infection (MOI) to ensure a maximum of 1 sgRNA incorporated per cell. Following puromycin selection of the cells that had successfully incorporated a sgRNA, a sample for an initial time-point 0 (T0) was harvested prior to selection with AZA (300 nM) or SGI (150 nM), or vehicle control (1/1000 DMSO) (Figure 3.3 A). These concentrations were chosen as they elicited comparable cell death and proliferation defects, and reflect clinically relevant range of plasma concentration (Derissen et al., 2013; Issa et al., 2015). Cell numbers were obtained every 3 – 4 days in order to track the cell proliferation rates (Figure 3.3 B). In order to identify gene knockouts that mediate either partial or complete resistance to the anti-proliferative effects of hypomethylating agents, cells were harvested and sequenced after 7 and 45 days of continuous exposure to AZA/GDAC, respectively. It was reasoned that after a short treatment period, sgRNAs that mediate resistance to differing degrees would be able to be detected since the cells transduced with sgRNAs that confer the strongest resistance would not yet encompass the majority of the population, as expected at the later time-point.

After 7 days of continuous exposure to AZA/GDAC, ~150 – 200 CRISPR/Cas9 mediated gene knockouts were positively enriched ($\log_2 \text{FC} \geq 1$, sgRNAs targeting gene ≥ 3 , p-value < 0.05) in the hypomethylated treated groups when compared to the DMSO control (Table 3.1). However, none passed the statistical significance cut-off (FDR < 0.05) once corrected for multiple testing (Table 3.1). Ten genes from the AZA/GDAC treated conditions were chosen for further validation that were positively enriched with respect to the DMSO treated control and time-point 0 ($\log_2 \text{FC} \geq 0.5$, sgRNAs targeting gene ≥ 2 , p-value < 0.05), and not in the DMSO treated control relative to time-point 0 ($\log_2 \text{FC} < 0.5$, sgRNAs targeting gene ≤ 2 , p-value ≥ 0.05) (Figure 3.3 C – E) (Table 3.2).

Next, the samples harvested after 45 days of continuous hypomethylating agent exposure were analysed. By day 45, the proliferation rates of the AZA and GDAC conditions paralleled that of the vehicle group, which was indicative that selection of AZA- and GDAC-resistant clones had occurred (Figure 3.3 B). Sequencing of the distribution of sgRNAs in each condition at the endpoint revealed only two genes per treatment condition that were positively enriched and represented by at least 2 sgRNAs, when compared to T0; *UCK2* and *SLC29A1* in the AZA-treated cells, and *DCK* and *SLC29A1* in the GDAC-treated cells (Figure 3.4 A, C). Importantly, sgRNAs targeting *SLC29A1*, *DCK* and *UCK2* were not enriched in the vehicle-treated sample (Figure 3.4 A – C), indicating that the knockout of these genes did not increase the proliferative capacity of THP-1 cells grown in the absence of hypomethylating agents. As mentioned in section 1.3.4.1.1, *SLC29A1*, *UCK2* and *DCK* are all known to be involved in the metabolism of hypomethylating agents (Diesch et al., 2016). *SLC29A1* is an equilibrative transporter that facilitates the uptake of hypomethylating agents into the cell, whilst *UCK2* and *DCK* catalyse the first rate-limiting phosphorylation of AZA and DAC/GDAC, respectively. sgRNAs targeting these 3 genes represented more than 50% of the total reads in the AZA and GDAC treated samples (Figure 3.4 B); the remaining reads were comprised of sgRNAs for which multiple independent sgRNAs targeting the same gene were not enriched. Genes represented by the enrichment of only one sgRNA could be explained by off-target effects of that sgRNA, or the cell attaining resistance to the continuous exposure of hypomethylating agents through the acquisition of endogenous mutations, independent of the sgRNA induced gene knockout.

Nucleoside transporters are comprised of 7 members that exhibit high levels of substrate redundancies (Pastor-Anglada et al., 2004). Furthermore, the uridine cytidine kinase family consists of two enzymes: *UCK1* and *UCK2* (Van Rompay et al., 2001). Multiple transporters and both uridine cytidine kinases have been reported to have an affinity for AZA to varying extents (Damaraju et al., 2012; Van Rompay et al., 2001). Thus, the results of the screen may suggest that AZA is primarily transported into the cell by *SLC29A1* and mono-phosphorylated by *UCK2* due to these enzymes displaying the highest affinity for AZA. However, the abundance of other transporters and enzymes may also explain, at least in part, why their loss did not confer resistance to hypomethylating agents to the same extent as *SLC29A1* and *UCK2* knockouts. To address this, RNAseq data for THP-1, MV4-11 and OCI-AML3 cells were obtained from the Cancer Cell Line Encyclopedia (CCLE), and z-scores calculated from the log-normalised RSEM values (Figure 3.4 D). This analysis revealed *SLC29A1* and *UCK2* to be the most abundantly expressed nucleoside transporter and uridine cytidine kinase, respectively,

amongst all three myeloid leukaemia cell lines tested (Figure 3.4 D). Thus, SLC29A1 and UCK2 may metabolise the greatest proportion of AZA, at least in part, by their relatively high expression when compared to their redundant counterparts, hence possibly explaining the outgrowth of only *SLC29A1* and *UCK2* knockout clones in the screen.

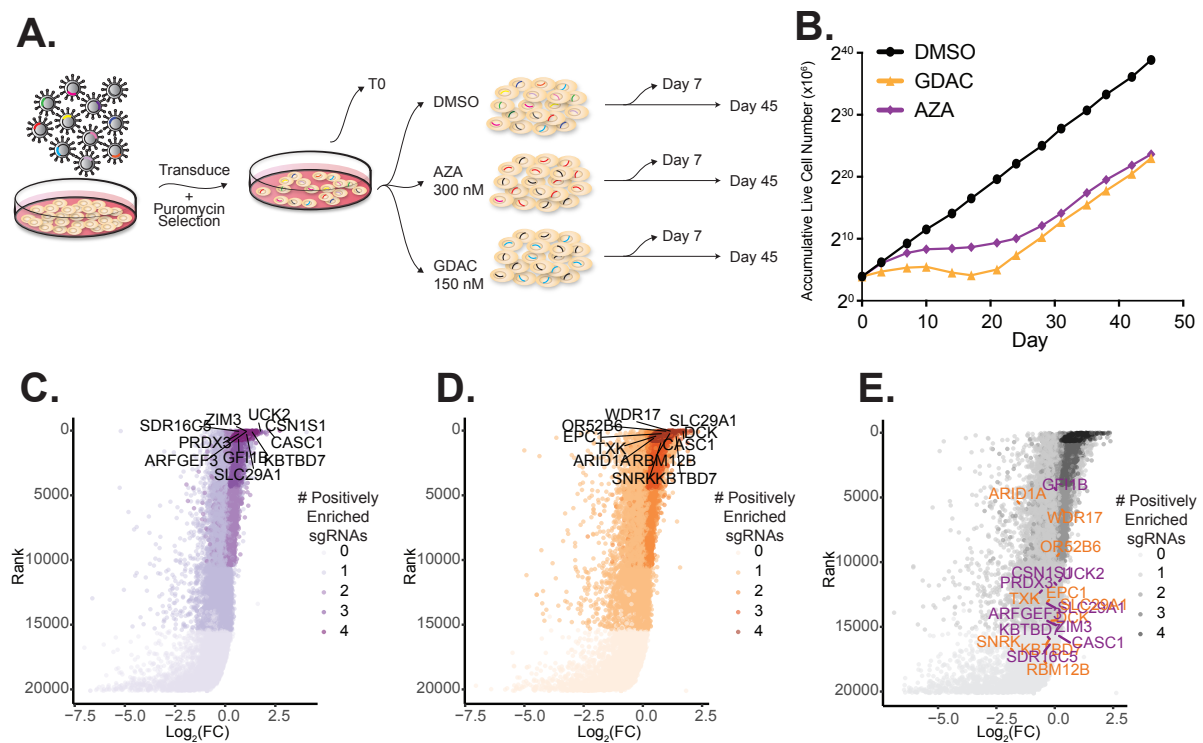


Figure 3.3 Day 7 CRISPR Screen Results

(A) Schematic of the genome-wide CRISPR screen. (B) Cumulative live cell number starting at T0. (C – E) Ranked positively enriched genes at day 7 of the genome-wide CRISPR screen under continuous exposure to (C) AZA, (D) GDAC, or (E) vehicle. (E) Purple; top 10 enriched genes in the AZA treated group, orange; top 10 enriched genes in the GDAC treated condition.

Table 3.1 Cut-off Statistics for CRISPR Screen Day 7

Comparison	logFC cut-off	Number sgRNAs targeting each gene	Statistic cut-off	Number of genes
AZA v DMSO	$\log_2 \text{FC} \geq 1$	sgRNAs ≥ 3	p-value < 0.05	158
AZA v DMSO	$\log_2 \text{FC} \geq 1$	sgRNAs ≥ 3	FDR < 0.05	0
GDAC v DMSO	$\log_2 \text{FC} \geq 1$	sgRNAs ≥ 3	p-value < 0.05	210
GDAC v DMSO	$\log_2 \text{FC} \geq 1$	sgRNAs ≥ 3	FDR < 0.05	0

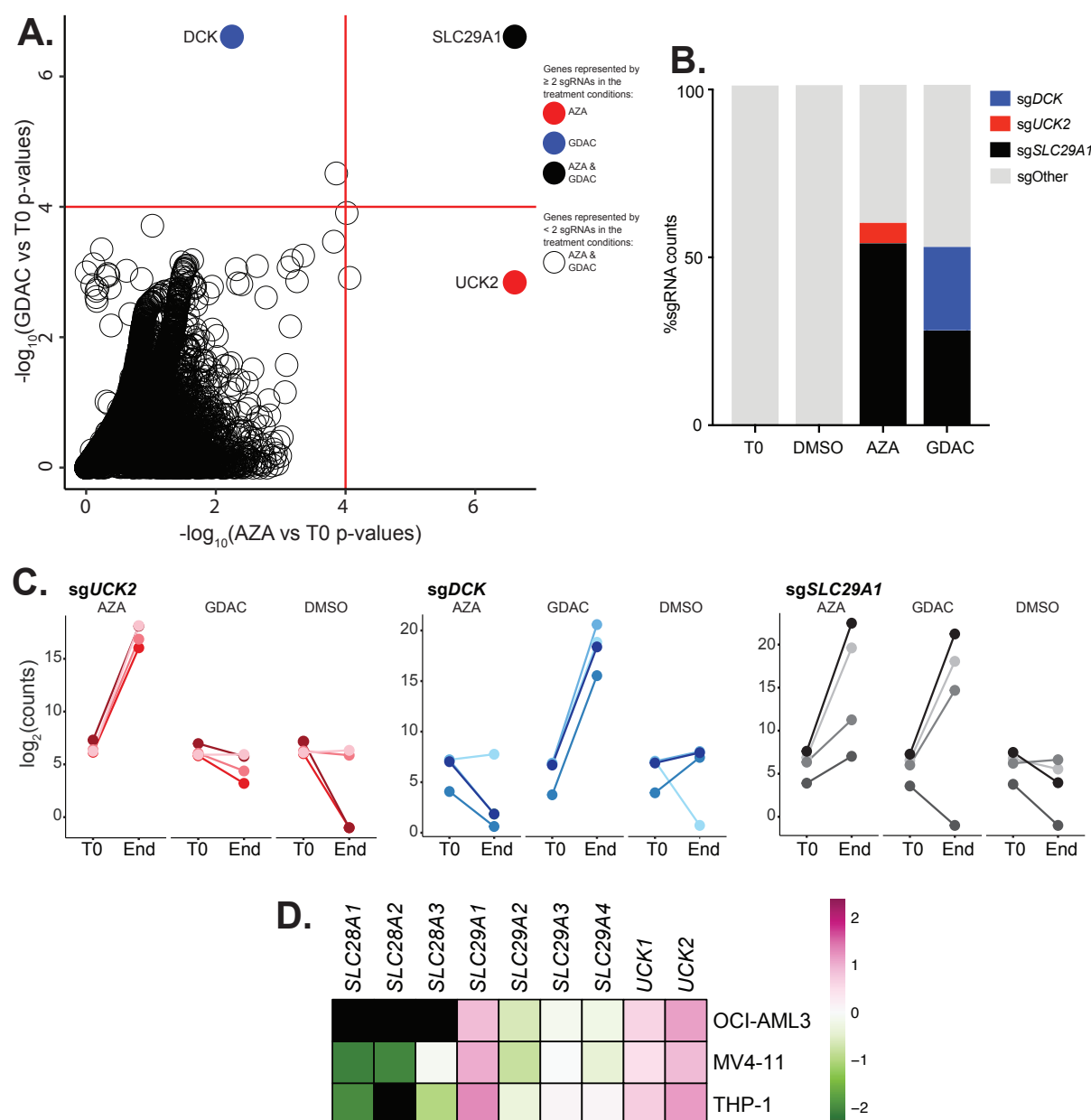


Figure 3.4 sgRNAs Directed Against *DCK*, *UCK2* and *SLC29A1* Confer the Strongest Resistance to Hypomethylating Agents

(A) Most significantly positively enriched sgRNAs in the AZA and GDAC treated samples at day 42 relative to T0. (B) The percentage of sequenced reads corresponding to the sgRNAs targeting *DCK*, *UCK2* and *SLC29A1* relative to all other sgRNAs in each sample. (C) Counts in the initial and final samples for sgRNAs targeting *DCK*, *UCK2* and *SLC29A1* across all treatment conditions. (D) Heatmap of standardised gene expression values for the family of nucleoside transporters and uridine cytidine kinases in THP-1, MV4-11 and OCI-AML3 cells. Black boxes signify genes that were not expressed. RNAseq data analysed from Cancer Cell Line Encyclopedia (CCLE).

A competitive growth assay in the presence or absence of hypomethylating therapies was utilised to validate 2 independent sgRNAs from each of the top 10 genes (Table 3.2) that passed the selection criteria aforementioned. THP-1^{Cas9} cells transduced with individual sgRNAs linked to a GFP reporter were seeded 1:1 with parental cells, continuously exposed to low dose hypomethylating agents or vehicle, and the proportion of GFP⁺ cells quantitated by flow cytometry (Figure 3.5 A). The THP-1 cells expressing sgRNAs against *DCK*, *UCK2* and *SLC29A1*, termed THP-1^{sgDCK}, THP-1^{sgUCK2} and THP-1^{sgSLC29A1}, respectively, provided a proliferative advantage in the presence of drug (Figure 3.5 B – C). The other THP-1^{sgRNA} cells tested were unable to outcompete the parental cells when exposed to hypomethylating agents (Figure 3.5 B – C), suggesting that these sgRNAs that demonstrated positive enrichment at day 7 in the screen did not truly provide any level of resistance to the hypomethylating agents, but were likely enriched in the screen due to sampling from cell passaging.

Resistance to the anti-proliferative effects of specific hypomethylating agents through CRISPR/Cas9 mediated knockout of *DCK*, *UCK2* and *SLC29A1* was further validated across MV4-11 and OCI-AML3 cells. Immunoblot assays confirmed knockout of *DCK* across all three cell lines, with sgDCK #1 providing the greatest loss of protein expression (Figure 3.5 D). Due to the lack of highly specific antibodies for *UCK2* and *SLC29A1*, inference of CRISPR edits (ICE) from Sanger sequencing trace data (Hsiau et al., 2018) was used to infer the editing and knockout efficiency in sg*SLC29A1* and sg*UCK2* transduced cell lines. By this method, high knockout efficiencies in the *SLC29A1* and *UCK2* knockouts were confirmed (Figure 3.5 E). As expected, across all cell lines, knockout of *SLC29A1* provided a proliferative advantage over untransduced cells when continuously exposed to low dose AZA or GDAC (Figure 3.5 F). Knockout of *DCK* and *UCK2* provided a competitive growth and/or survival advantage to these cells when exposed to GDAC and AZA, respectively, however did not enable cross-resistance to AZA and GDAC, respectively (Figure 3.5 F). Importantly, in culture conditions supplemented with DMSO, the initial ratio of *SLC29A1*, *DCK* or *UCK2* knockout cells (GFP⁺) to untransduced cells (GFP⁻) was maintained throughout the assay (Figure 3.5 F), indicating that loss of these genes did not change the inherent growth rates of the cell lines. Furthermore, loss of *SLC29A1* or *UCK2* increased the AZA LD₅₀ by at least 10-fold (Figure 3.6 A), whilst loss of *SLC29A1* or *DCK* elevated the GDAC LD₅₀ by at least 100-fold, as measured by the percentage of live (Annexin V⁻ / PI⁻) cells (Figure 3.6 B). Additionally, loss of *SLC29A1* and *DCK* increased the LD₅₀ of cytarabine by >100-fold (Figure 3.6 C). The correlation between the loss of *SLC29A1* or *DCK* and the significant reduction in sensitivity to cytarabine is

concordant with previously published data (Kurata et al., 2016) and the fact that cytarabine is a cytotoxic nucleoside analogue that utilises the same metabolic pathway as GDAC in order to achieve its active triphosphate form (Coleman et al., 1975).

Utilising unbiased and genome-wide techniques, these data were the first to demonstrate that CRISPR/Cas9-mediated knockout of *DCK*, *UCK2* or *SLC29A1* provides the strongest resistance to the AZA and/or GDAC induced anti-proliferative effects *in vitro*.

Table 3.2 Genes Chosen for Further Validation

Gene*	Condition	Treatment vs T0		DMSO vs T0	
		logFC	P-value	logFC	P-value
<i>SLC29A1</i>	AZA vs T0	1.07	1.38 x 10 ⁻²	-0.34	0.67
		8.81	2.46 x 10⁻⁷	-2.34	0.63
	SGI vs T0	1.72	2.80 x 10 ⁻⁴		
		10.08	2.46 x 10⁻⁷		
<i>DCK</i>	SGI vs T0	2.02	2.71 x 10 ⁻⁶	-0.14	0.74
		11.84	2.46 x 10⁻⁷	0.989	0.03
<i>UCK2</i>	AZA vs T0	1.35	7.84 x 10 ⁻⁴	0.20	0.58
		10.63	2.46 x 10⁻⁷	-3.21	0.68
<i>CASC1</i>	AZA vs T0	2.19	6.96 x 10 ⁻⁴	0.19	0.80
	SGI vs T0	1.70	2.02 x 10 ⁻³		
<i>CSN1S1</i>	AZA vs T0	1.76	1.90 x 10 ⁻³	-0.01	0.59
<i>ZIM3</i>	AZA vs T0	1.05	2.39 x 10 ⁻³	-0.04	0.75
<i>SDR16C5</i>	AZA vs T0	0.68	2.76 x 10 ⁻³	-0.22	0.83
<i>ARFGEF3</i>	AZA vs T0	1.06	3.83 x 10 ⁻³	-0.09	0.75
<i>GFI1B</i>	AZA vs T0	0.87	4.87 x 10 ⁻³	0.47	0.18
<i>KBTBD7</i>	AZA vs T0	1.35	4.91 x 10 ⁻³	-0.22	0.81
	SGI vs T0	1.41	2.09 x 10 ⁻²		
<i>PRDX3</i>	AZA vs T0	0.67	1.22 x 10 ⁻²	-0.56	0.62
<i>WDR17</i>	SGI vs T0	1.16	1.50 x 10 ⁻³	0.31	0.29
<i>OR52B6</i>	SGI vs T0	0.97	1.70 x 10 ⁻³	0.11	0.48
<i>EPC1</i>	SGI vs T0	0.78	9.83 x 10 ⁻³	-0.32	0.66
<i>TXK</i>	SGI vs T0	0.55	1.96 x 10 ⁻²	-0.78	0.66
<i>ARID1A</i>	SGI vs T0	0.45	2.74 x 10 ⁻²	-1.52	0.27
<i>SNRK</i>	SGI vs T0	0.76	4.38 x 10 ⁻²	-1.79	0.86

*Genes and statistics highlighted in bold indicate the genes enriched at day 42. All other genes were chosen from day 7 of the CRISPR screen.

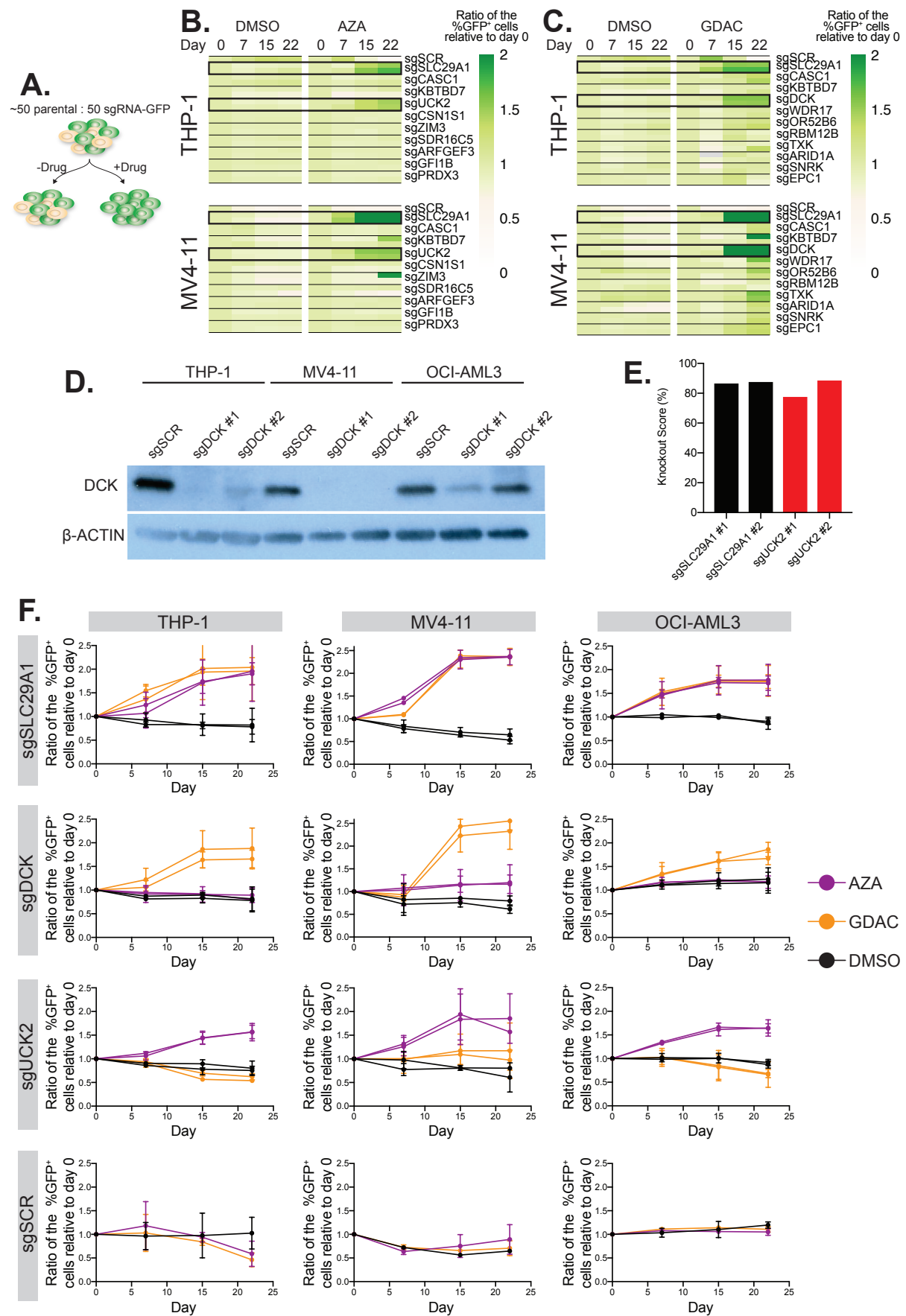


Figure 3.5 Competitive Growth Assay of *SLC29A1*, *DCK* and *UCK2* Knockouts

(A) Schematic of competitive growth assay. **(B – C)** Heatmap of competitive growth assay for the top 10 scoring genes from day 7 of the CRISPR screen under **(B)** Azacytidine and **(C)** Guadecitabine treatment conditions. Each gene is represented by two independent sgRNAs, with no replicates. **(D)** Immunoblot across 3 different leukaemic cell lines confirming loss of DCK in the *DCK* knockout clones derived from independent sgRNAs directed against *DCK*. **(E)** The proportion of cells with CRISPR-editing events predicted to result in a functional knockout. Knockout scores were generated utilising the ICE algorithm. **(F)** Competitive growth assay using AML cell lines transduced with independent sgRNAs (2 sgRNAs/gene) (GFP⁺ cells) and continuously exposed to AZA, GDAC or vehicle. Error bars represent mean $\pm$ SD from 2 experiments.

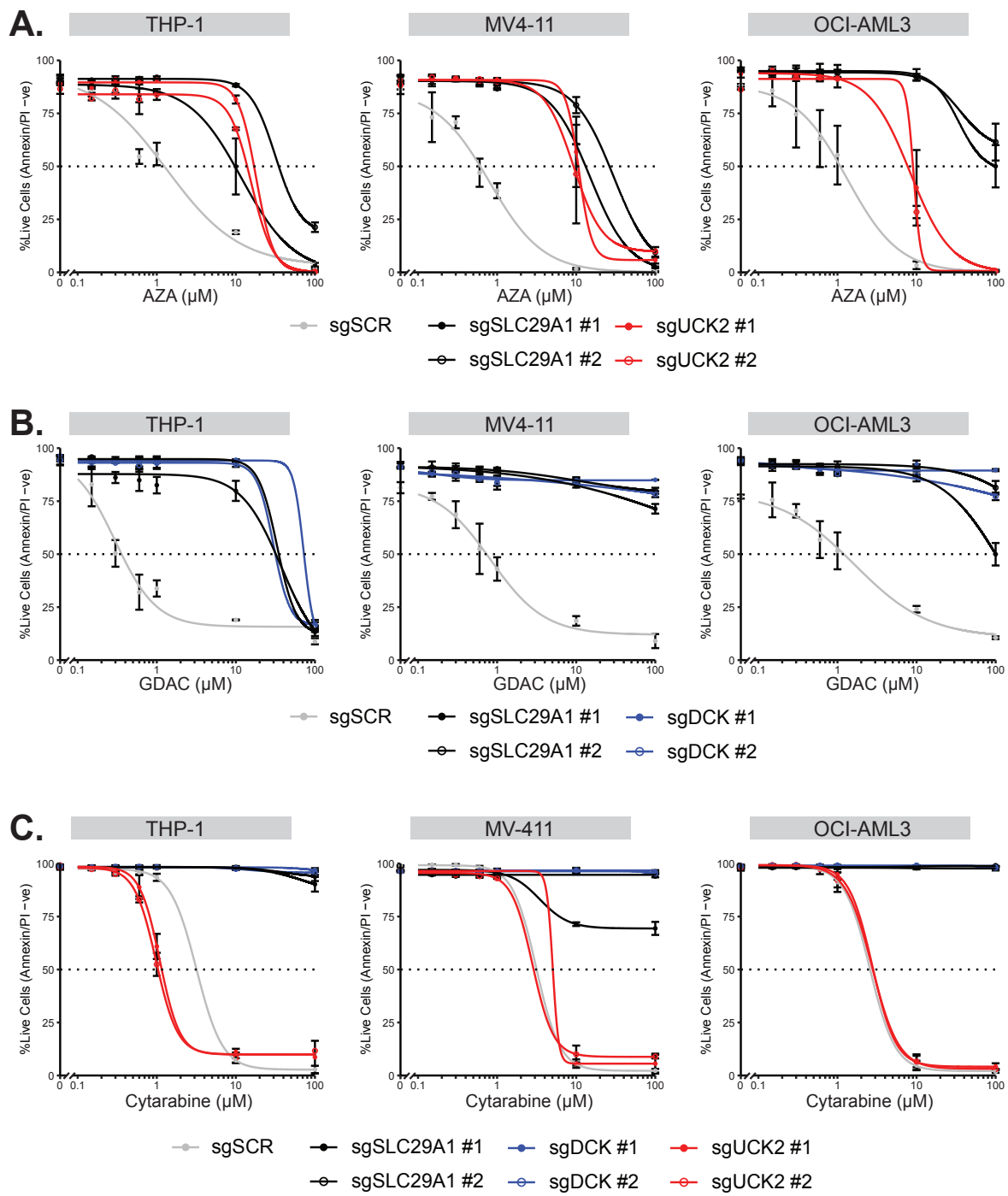


Figure 3.6 SLC29A1, DCK and UCK2 Loss Confer Decreased Sensitivity to Select Hypomethylating Agents

(A – C) Cell viability assessed by Annexin V / PI staining following 7 days of exposure to a titration of AZA, GDAC or cytarabine. Curves fitted using log-logistic regression. (A) SLC29A1 and UCK2 knockouts were treated AZA. (B) SLC29A1 and DCK knockouts were treated with GDAC. (C) SLC29A1, DCK and UCK2 knockouts were exposed to cytarabine. Error bars represent mean $\pm$ SD from 2 replicates.

3.3.3 Loss of SLC29A1, DCK or UCK2 Confers Resistance to Hypomethylating Agents *In Vivo*

To assess the significance of these findings *in vivo*, the THP-1^{sgRNA} cells were engineered to express firefly luciferase to enable systemic tumour detection *in vivo* by bioluminescence imaging. After an initial 14-day engraftment period of THP-1^{sgRNA/luc} cells, NSG recipients were administered two cycles of daily treatment by IP injection for 5 consecutive days followed by 2 days off (Figure 3.7 A). Engraftment was measured by bioluminescence imaging 10 days after transplant; no statistical difference in tumour burden was observed, indicating that the proliferation rates of *SLC29A1*, *DCK* and *UCK2* knockout cells were similar to control cells *in vivo* (Figure 3.7 B). In concordance with the *in vitro* observations (Figure 3.5 C (DMSO)), the comparable development in leukaemia burden of the THP-1^{sgRNA/luc} recipient mice further verified that loss of *SLC29A1*, *DCK* or *UCK2* were dispensable for tumour growth (Figure 3.7 B). Disease burden was further assessed post treatment with AZA (4 mg/kg), GDAC (1 mg/kg) or vehicle (PBS) (Figure 3.7 C – E). As expected, the disease burden of control cells was impaired by both hypomethylating agents when compared to vehicle (Figure 3.7 C – E). Tumour progression was delayed following AZA treatment in THP-1^{sgDCK/luc} recipient mice (Figure 3.7 C – E). GDAC treatment most significantly hindered tumour progression in mice transplanted with THP-1^{sgUCK2/luc} cells (Figure 3.7 C – E). THP-1^{sgSLC29A1/luc} cells were unresponsive to both AZA and GDAC therapies *in vivo* (Figure 3.7 C – E). Collectively, these data provide *in vivo* evidence that supports the association between *SLC29A1*, *DCK* or *UCK2* loss and the resistance to selective hypomethylating agents previously demonstrated *in vitro*.

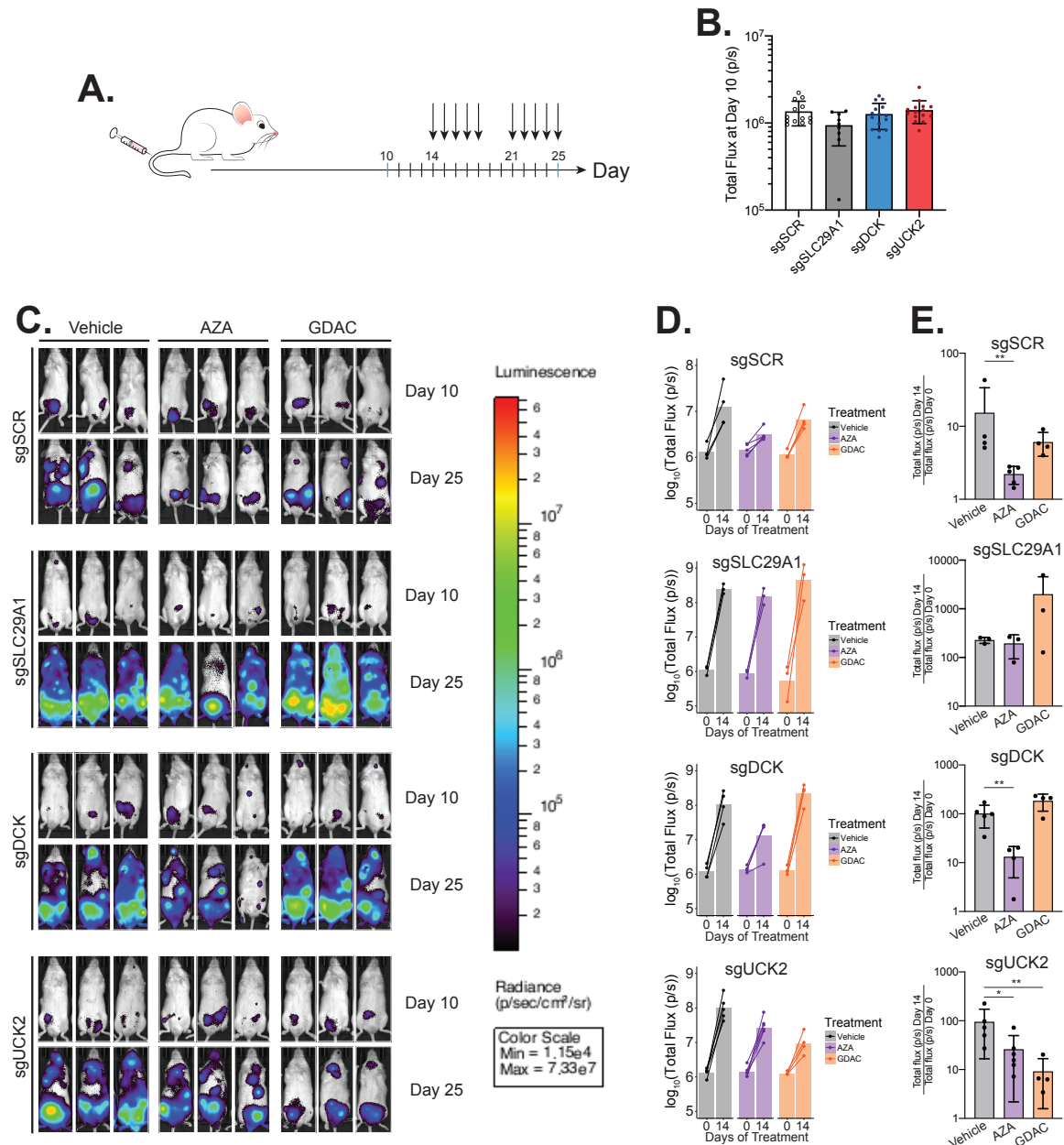


Figure 3.7 SLC29A1, DCK and UCK2 Loss Confer Decreased Sensitivity to Select Hypomethylating Agents *In Vivo*

(A) Schematic depicting the *in vivo* experimental design. Blue lines indicate the days where disease burden was assessed by bioluminescence imaging. Arrows indicate treatment days. **(B)** Day 10 tumour burden measured as total flux (p/s) by bioluminescence imaging. **(C)** Representative bioluminescence images from each cohort. **(D)** Total flux of each recipient before and after treatment. **(E)** The ratio between the tumour burdens, measured as total flux (p/s), before and after treatment. Error bars represent mean $\pm$ SD. Significance was analysed using non-parametric one-tailed student's t-test. * $p < 0.05$, ** $p < 0.01$.

3.3.4 Reduced *SLC29A1* and *UCK2* Expression was Observed in AZA-Treated Non-Responding AML Patients

The expression of *SLC29A1*, *DCK* and *UCK2* was analysed in patients with haematological malignancies that received hypomethylating agent therapy in order to determine whether the decreased expression of these genes was associated with a poor response and/or relapse in this patient population. The expression levels of *SLC29A1*, *DCK* or *UCK2* were analysed from previously published paired-end RNA-sequencing data of CD34⁺ HSPC cells isolated from the bone marrow of AZA responding and non-responding patients with haematological malignancies, before therapy and at cycle 6 day 28, (Unnikrishnan et al., 2017) (GEO: GSE76203). In this study, patients had not received therapy prior to AZA induction and were classified as AZA responders/non-responders according to the International Working Group criteria (Cheson et al., 2006). The pre-treatment expression of *UCK2* in patients with myelodysplastic syndrome (MDS) was statistically significantly lower in patients classified as non-responders when compared to responders (Figure 3.8 A middle). This trend was also apparent in the pre-treatment expression of *UCK2* in chronic myelomonocytic leukaemia patients (CMML), although did not reach statistical significance (Figure 3.8 A middle). A trend for reduced *SLC29A1* expression was observed in MDS and CMML non-responders prior to treatment initiation (Figure 3.8 A left), whilst pre-treatment *DCK* expression remained consistent between responders and non-responders (Figure 3.8 A right). These data suggest that lower *SLC29A1* and/or *UCK2* expression may be indicative of poor response to AZA, and thus warrants further investigation with larger cohorts.

AZA is typically administered to patients subcutaneously at 75 mg/m² per day, for 7 days every 28 days, and is repeated for at least 6 cycles (Fenaux et al., 2009). The use of hypomethylating prodrugs as single agents is often only effective in a small proportion of patients with a survival benefit that is typically measured in months and relapse a common occurrence (Fenaux et al., 2009; He et al., 2017). In the study by Unnikrishnan et al. (2017), patients classified as responders demonstrated an increase in median survival by ~17 months when compared to non-responders, but ultimately succumbed to disease suggesting the acquisition of resistance had occurred in this patient population. To ascertain whether attenuated expression of *SLC29A1* or *UCK2* are potential mechanisms of acquired resistance that occurs post AZA therapy, the expression levels of these genes were analysed in the aforementioned AZA responders (Unnikrishnan et al., 2017) (GEO: GSE76203) after receiving 6 cycles and 28 days of AZA treatment. No statistical difference in the mean expression of *SLC29A1* or *UCK2*, at the pre-

and post-treatment time-points, were observed in the MDS responders (Figure 3.8 B – C). Within the CMML AZA responder patient population, no statistical difference in the mean *SLC29A1* expression was observed from pre-treatment to cycle 6 day 28 of AZA treatment, however 4/5 individuals exhibited a decrease in *SLC29A1* expression (Figure 3.8 B). All CMML AZA responders exhibited a decrease in *UCK2* expression from pre-treatment to cycle 6 day 28 of AZA therapy, with the difference in mean expression between the two time-points reaching statistical significance ($p = 0.012$) (Figure 3.8 C). These data suggest that decreased *SLC29A1* and/or *UCK2* expression by cycle 6 day 28 of AZA therapy may contribute to acquired resistance in CMML patients that initially respond to AZA treatment.

It is important to note that the sample size of each cohort in the study by Unnikrishnan and colleagues (2017) was very small ($n = 2 - 6$ patients / cohort). Hence, it is difficult to draw conclusions regarding the relationship between the attenuated expression of *SLC29A1* and/or *UCK2* and the upfront or acquired resistance to AZA therapy. Nonetheless, analysis of this data suggests the presence of a trend between the decreased expression of *SLC29A1* and/or *UCK2* and the upfront resistance to AZA therapy in MDS and CMML patients, and the acquired resistance to AZA treatment in CMML patients. Thus, this analysis provides preliminary evidence that warrants further investigation into the relationship between resistance and the expression of *SLC29A1*, *UCK2* and *DCK* in larger cohorts of patients with haematological malignancies that received hypomethylating agent therapy.

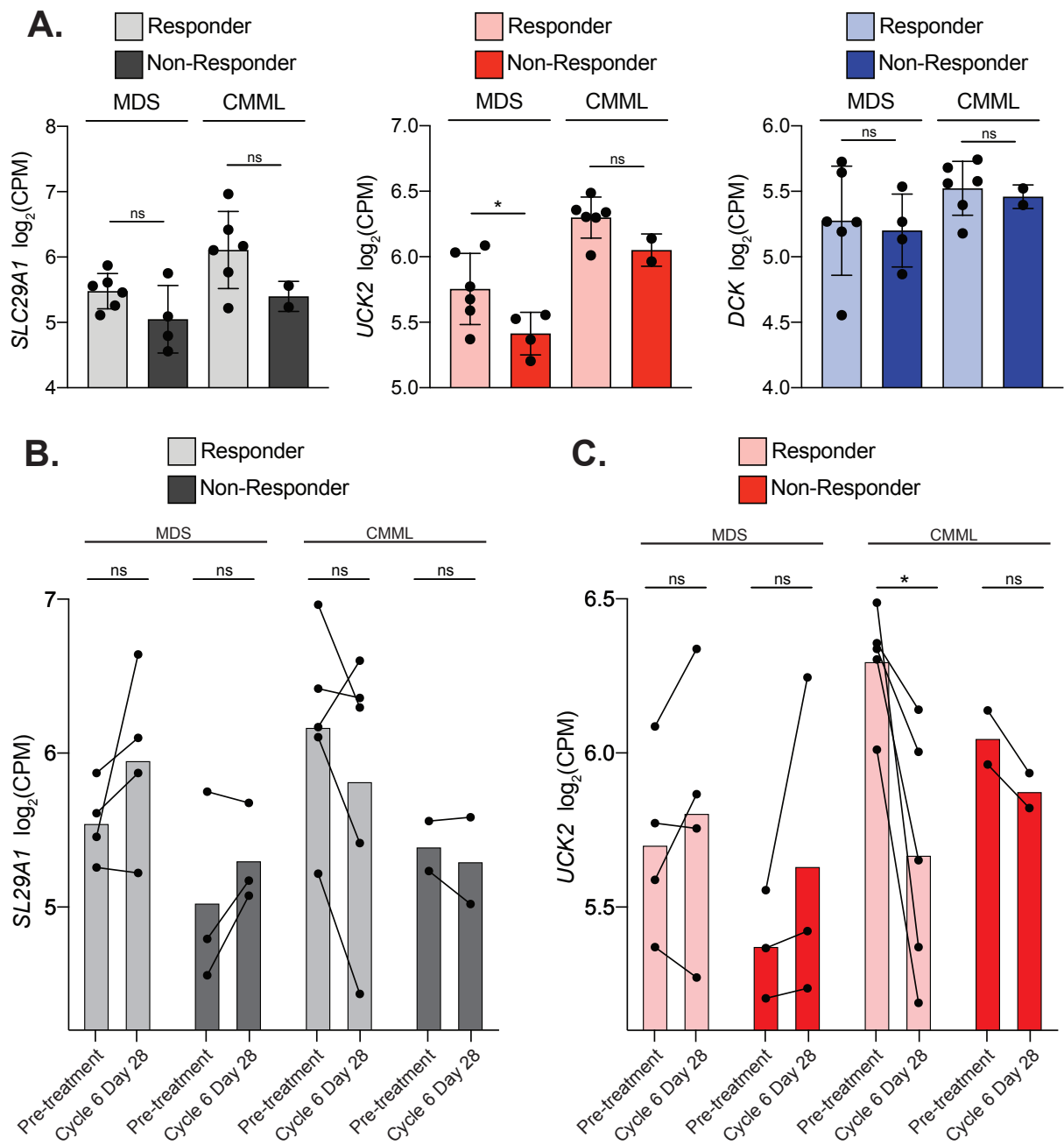


Figure 3.8 *SLC29A1* and *UCK2* Expression in AZA Treated Patients

(A) Normalised counts per million (CPM) for *SLC29A1*, *DCK* and *UCK2* at pre-treatment from myelodysplastic syndrome (MDS) and chronic myelomonocytic leukaemia (CMML) patients, classified as “Responders” or “Non-Responders” post AZA treatment. Each circle represents an individual patient. Error bars represent mean $\pm$ SD. **(B – C)** Trends in the expression of *SLC29A1* and *UCK2* for each patient from pre-treatment to cycle-6 day-28. Bars represents the mean expression at the given time-point and condition. Each line connecting two points represents the expression level at the different time-points of an individual patient. Differences in the mean expression was analysed using non-parametric two-tailed student’s t-test. * $p < 0.05$, ns; $p \geq 0.05$. Analysis was performed on data from Unnikrishnan et al. (2017) (GEO: GSE76203)

3.3.5 Identifying Dependencies in *SLC29A1* Knockout Cells

Data presented thus far demonstrated that loss of DCK and UCK2 did not provide cross-resistance to AZA and GDAC, respectively. In contrast, *SLC29A1* knockout cells were resistant to both AZA and GDAC, and the common chemotherapeutic, cytarabine, highlighting the need to elucidate effective therapeutic avenues for *SLC29A1* knockout cells.

Synthetic lethality occurs when the simultaneous perturbation of two genes induces cell death, whilst the disruption of either gene individually does not render the cells inviable (O’Neil et al., 2017). To identify synthetic lethal interactions between *SLC29A1* loss and a secondary gene knockout, a genome-wide CRISPR drop-out screen was executed. Prior to the initiation of the screen, THP-1^{sgSLC29A1} cells were treated with high dose AZA (1 μ M) for 7 days to ensure the population of cells were resistant to AZA. Subsequently, THP-1^{sgSLC29A1} and control cells (THP-1^{sgSCR}) were transduced with the genome-wide Brunello knockout library at ~500x fold representation in duplicate, selected with puromycin, and cultured for 36 days to enable sufficient dilution of sgRNAs whose gene knockouts are lethal to THP-1^{sgSLC29A1} cells (Figure 3.9 A). Samples were sequenced to identify genes that were negatively enriched in the THP-1^{sgSLC29A1} cells and not control cells (Figure 3.9 – 3.10).

Prior to analysing dropout in THP-1^{sgSLC29A1} cells, gene knockouts that were depleted in the THP-1^{sgSCR} cells were compared to essential genes identified in previously published genome-wide CRISPR knockout datasets (Dempster et al., 2019; Meyers et al., 2017) collated by DepMap, Broad (2020) (Figure 3.9 B – C). Indeed, sgRNAs directed against genes that were essential for the survival and/or proliferation of THP-1 cells in the previously published CRISPR dataset ($\log_2FC < -0.5$) were negatively enriched in the THP-1^{sgSCR} cells (Figure 3.9 B). The consistency between results of the CRISPR screen presented in this chapter and the previously published data, demonstrates that the results of the present CRISPR knockout screen were reliable.

Next, the data was analysed to identify genes that were negatively enriched in the THP-1^{sgSLC29A1} cells and not THP-1^{sgSCR} cells. Genome-wide CRISPR dropout screens require biological replicates in order to minimise the false positives due to sgRNA off-target effects, variable sgRNA efficiencies and sampling errors (Ong et al., 2017). Hypergeometric testing of the top significantly depleted genes ($\log_2FC \leq -1$, p-value ≤ 0.01 , represented by at least 2 individual sgRNAs) identified statistically significant overlap between biological replicates

(Figure 3.9 D), thus demonstrating concordant results between replicate conditions. In order to account for the variance between biological replicates and to subsequently make direct comparisons of the depleted genes in the THP-1^{sgSLC29A1} and THP-1^{sgSCR} cells, biological replicates were merged using the MAGeCK test command. Hypergeometric testing revealed significant overlap ($p < 5 \times 10^{-324}$) between the sgRNAs depleted ($\log_2FC \leq -1$, $p\text{-value} < 0.05$, represented by at least 2 individual sgRNAs) in the THP-1^{sgSLC29A1} and THP-1^{sgSCR} cells, whereby $> 50\%$ of negatively enriched genes in each cell-type overlapped (Figure 3.9 E). This indicates that the majority of genes essential for the proliferation and/or survival of THP-1^{sgSLC29A1} cells are shared by THP-1^{sgSCR} cells. To identify genes whose loss was synthetically lethal in THP-1^{sgSLC29A1} cells, the list of depleted genes in these cells ($\log_2FC \leq -1$, $p < 0.05$, represented by at least 2 individual sgRNAs) were filtered to exclude genes that were also negatively enriched in THP-1^{sgSCR} cells (Figure 3.9 F – G, Table 3.3). The sgRNAs for the top 100 ranked genes that were only negatively enriched in the THP-1^{sgSLC29A1} cells were subsequently analysed to ensure the directionalities of the individual sgRNAs were consistent between biological replicates (Figure 3.10 A). It is important to eliminate inconsistent sgRNAs that do not share similar directional and magnitude changes between biological replicates, as the representation of these sgRNAs are likely skewed by sampling during serial passaging of cells. Ten genes were chosen from this subset whereby at least 2 individual sgRNAs were depleted in both THP-1^{sgSLC29A1} biological replicates by at least 2-fold greater than in the control cells (Figure 3.10 B) for further validation.

Table 3.3 Number of Depleted Genes that Overlap in *SLC29A1* Knockout and Control Cells

Cells	Log ₂ FC	p-value	Number of depleted sgRNAs	Number of genes in overlap
THP-1 ^{sgSLC29A1}	$\log_2FC \leq -1$	-	-	1580
THP-1 ^{sgSCR}	$\log_2FC > -1$	-	-	genes
THP-1 ^{sgSLC29A1}	$\log_2FC \leq -1$	$p < 0.05$	sgRNAs ≥ 2	333
THP-1 ^{sgSCR}	$\log_2FC > -1$	$p \geq 0.05$	sgRNAs < 2	genes

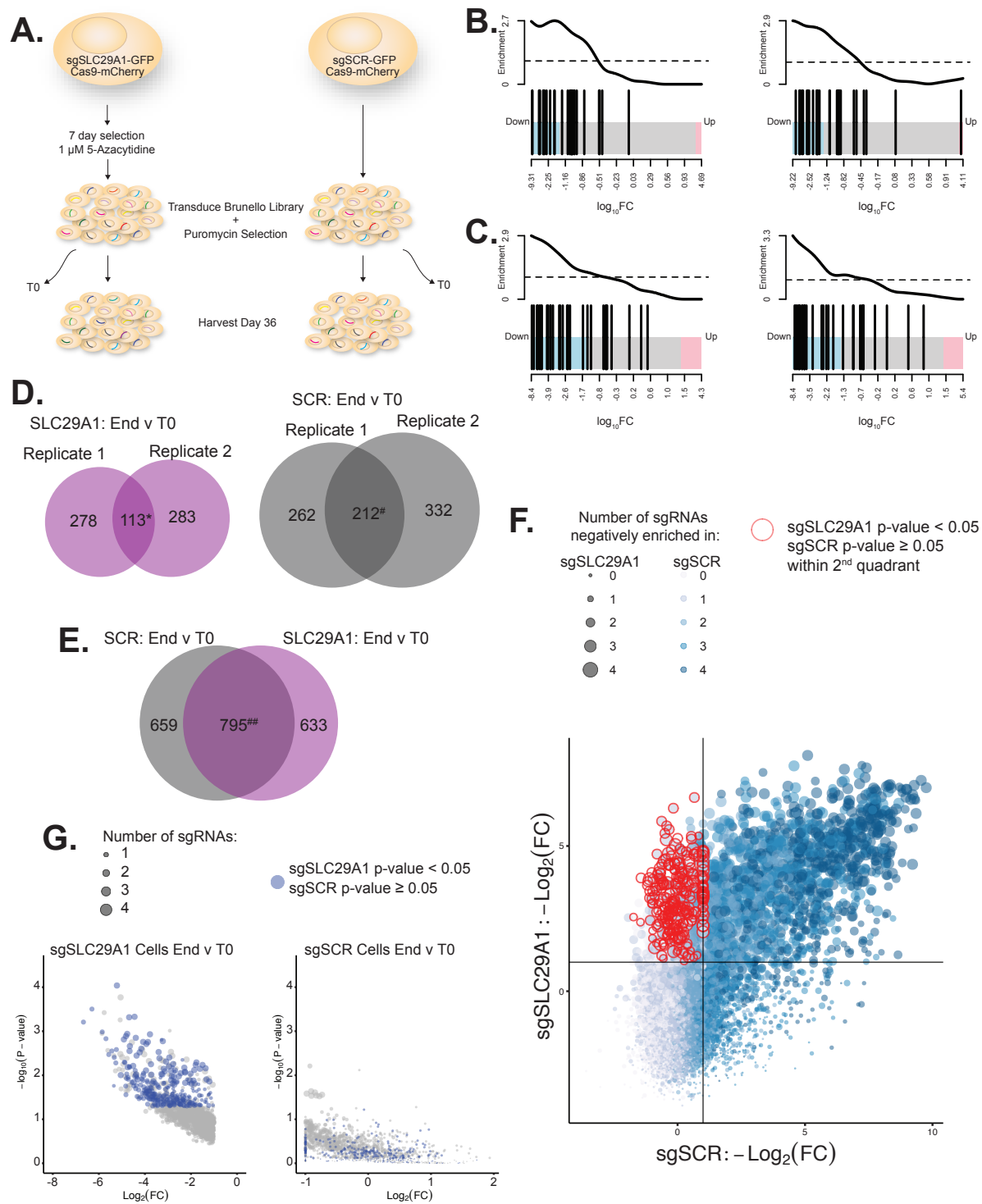


Figure 3.9 Whole-genome CRISPR Knockout Screen to Identify Synthetic Lethal Interactions with SLC29A1 Loss

(A) Schematic of whole-genome CRISPR dropout screen in THP-1 *SLC29A1* knockout cells. Prior to sgRNA library transduction, THP-1^{sgSLC29A1} cells were cultured in high dose AZA for 7 days to ensure the population was resistant to AZA. **(B – C)** Barcode plot of genes depleted in previously published THP-1 dropout screens were negatively enriched in **(B)** THP-1^{sgSCR} cells and **(C)** THP-1^{sgSLC29A1} cells. **(D)** Venn diagram of significantly depleted genes ($\log_2FC \leq -1$, $p\text{-value} \leq 0.01$, represented by at least 2 individual sgRNAs) in each of the biological replicates for THP-1^{sgSCR} and THP-1^{sgSLC29A1} cells. Significance of overlap was assessed using hypergeometric testing. * $p = 2.1 \times 10^{-108}$, # $p = 2.3 \times 10^{-227}$. **(E)** Venn diagram of significantly depleted genes ($\log_2FC \leq -1$, $p\text{-value} < 0.05$, represented by at least 2 individual sgRNAs) in the THP-1^{sgSCR} and THP-1^{sgSLC29A1} cells after accounting for variance amongst biological replicates. Significance of overlap was assessed using hypergeometric testing. ## $p < 5 \times 10^{-324}$. **(F)** Scatterplot of FC (End v T0) values of each gene in the THP-1^{sgSCR} and THP-1^{sgSLC29A1} cells. **(G)** Scatterplot of $\log_2FC$ and $-\log_{10}$ transformed p-values for genes with $\log_2FC \leq -1$ in THP-1^{sgSLC29A1} cells only.

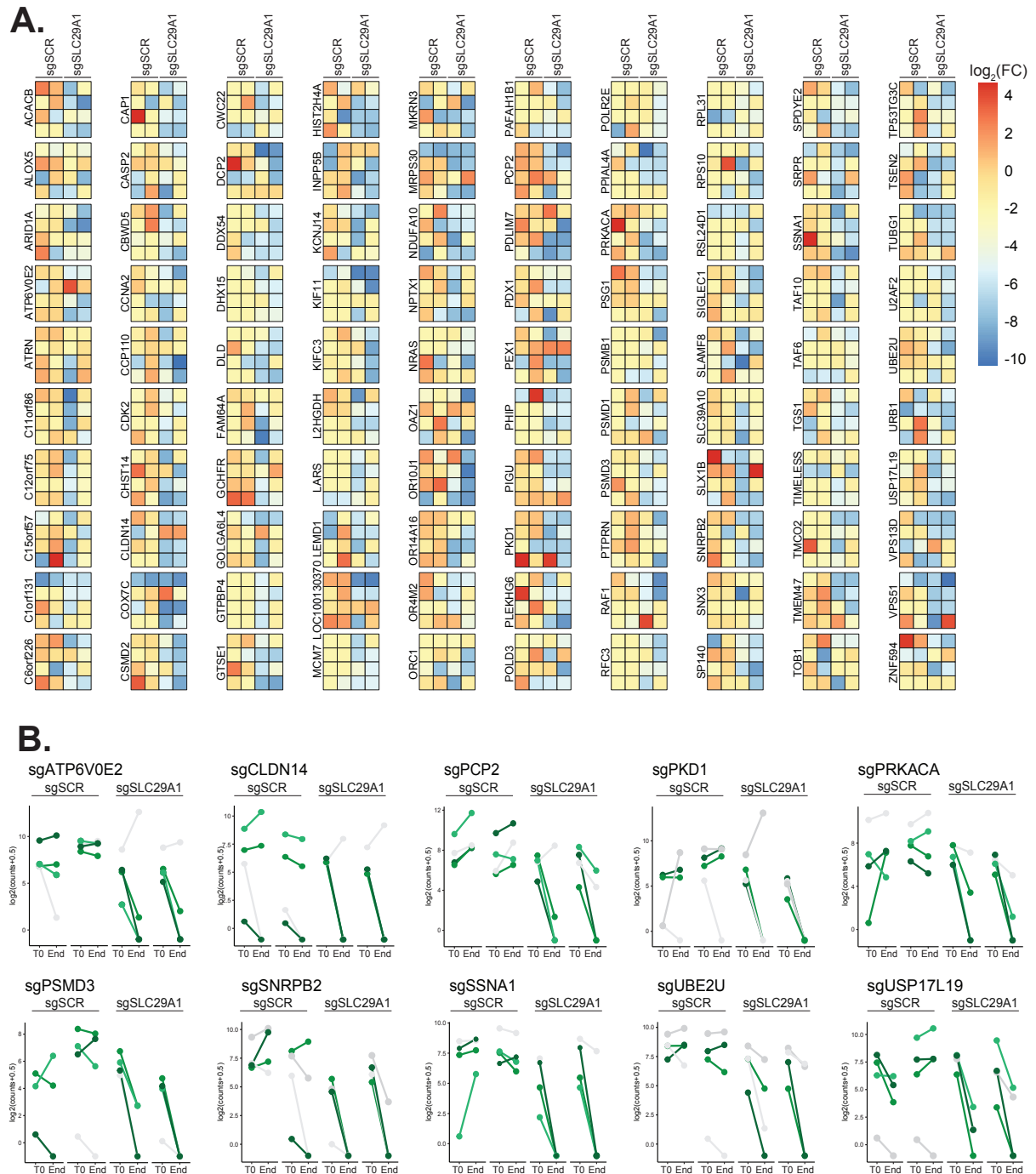


Figure 3.10 Top 10 Genes Depleted in THP-1^{sgSLC29A1} Cells

(A) Heatmap depicting the $\log_2(\text{FC})$ of the top 100 genes negatively enriched in THP-1^{sgSLC29A1} cells. Each row represents an independent sgRNA, with 4 independent sgRNA targeting each gene. **(B)** Normalised sgRNA counts for the top 10 genes which were negatively enriched in THP-1^{sgSLC29A1} cells and not THP-1^{sgSCR} cells. Each line represents an individual sgRNA. Green; sgRNAs negatively enriched in THP-1^{sgSLC29A1} cells and not THP-1^{sgSCR} cells, grey; otherwise.

To validate the 10 negatively enriched genes, THP-1^{sgSCR-GFP} and THP-1^{sgSLC29A1-GFP} cells were transduced with lentiviral constructs constitutively expressing the crimson reporter linked to sgRNAs targeted towards the selected genes, and mixed in a 1:1 ratio with untransduced cells to compare the proliferation rates in a competition growth assay (Figure 3.11 A). Each gene was represented by 2 individual sgRNAs. PSMD3 loss in THP-1^{sgSLC29A1} cells resulted in a proliferative disadvantage when compared to untransduced cells (Figure 3.11 B – C). However, THP-1^{sgSCR} cells transduced with sgRNAs targeting *PSMD3* were also outcompeted by untransduced cells (Figure 3.11 B – C), suggesting that THP-1 cells are innately sensitive to PSMD3 loss. sgRNAs targeting the other nine genes tested in the proliferation competition assay did not provide a proliferative disadvantage in the THP-1^{sgSLC29A1} cells, suggesting they were false positives identified in the screen. Indeed, none of the genes depleted in THP-1^{sgSLC29A1} cells ($\log_2\text{FC} \leq -1$, $p\text{-value} < 0.05$, represented by at least 2 individual sgRNAs) passed the $\text{FDR} < 0.01$ statistical cut-off once corrected for multiple testing. Depleted genes that passed the stringent significance cut-off ($\text{FDR} < 0.01$) in the THP-1^{sgSLC29A1} cells and THP-1^{sgSCR} cells, significantly overlapped as assessed by hypergeometric testing ($p = 1.9 \times 10^{-44}$). These data imply that genes essential for the proliferation and/or survival of THP-1^{sgSLC29A1} cells are shared with the THP-1^{sgSCR} cells.

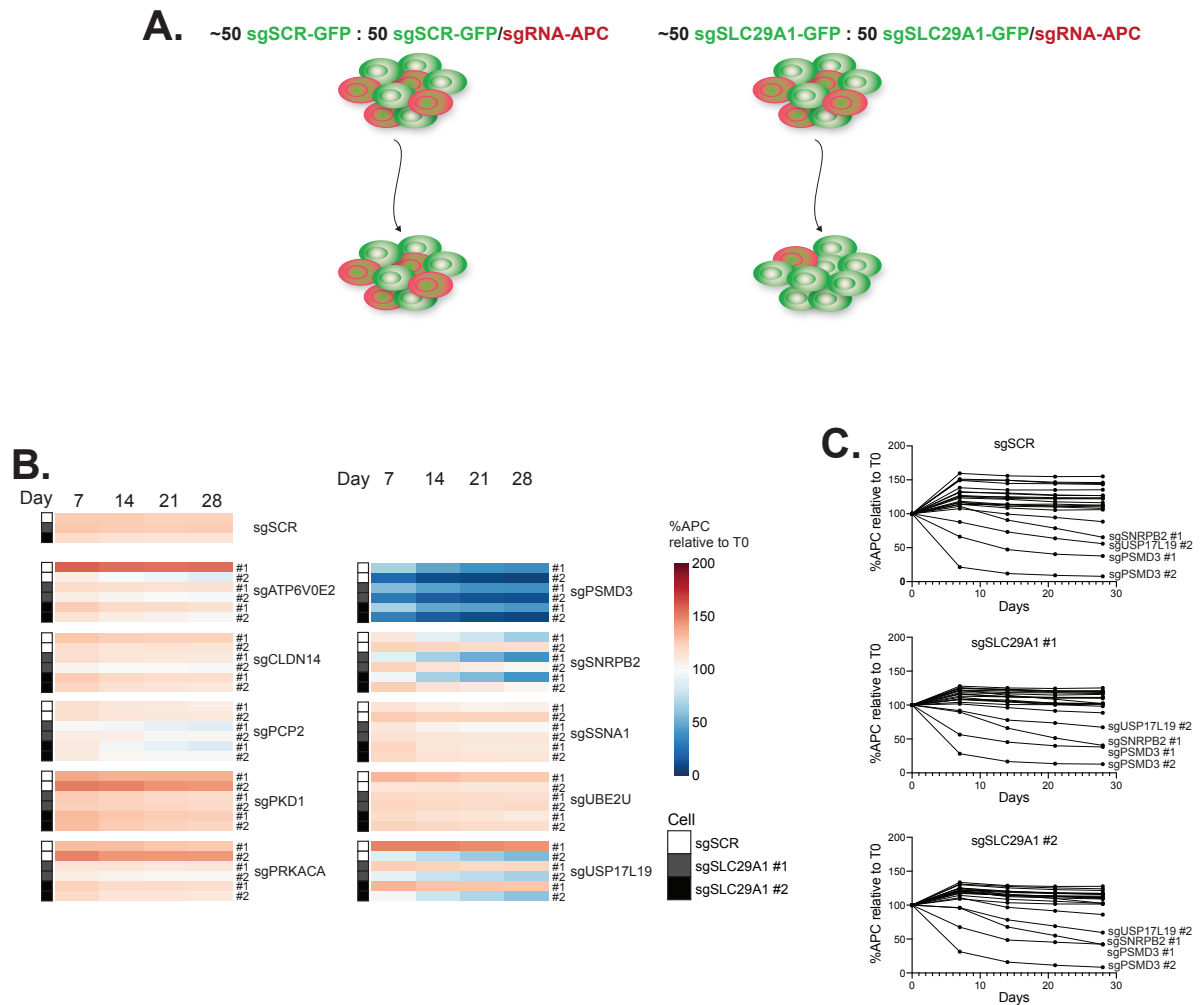


Figure 3.11 *SLC29A1* Knockout Does Not Predispose Cells to Synthetic Lethality by Secondary Gene Perturbation

(A) Schematic of competitive growth assay. (B) Heatmap and (C) dot-plot depicting the ratio, expressed as a percentage, of the proportion of APC at each time point relative to time-point 0 (T0). Two independent sgRNAs were directed towards each gene. Two independently derived *SLC29A1* knockout cell lines were utilised, containing distinct sgRNAs targeted towards *SLC29A1*.

3.3.6 Inhibiting *De Novo* Pyrimidine Synthesis Conferred Sensitivity to *SLC29A1* Knockout Cells

Pyrimidine can be synthesised through *de novo* biosynthesis and pyrimidine ribonucleotide or deoxyribonucleotide salvage pathways (Lane and Fan, 2015) (Figure 3.12 A). *SLC29A1*, *DCK* and *UCK2* are involved in the pyrimidine salvage pathways (Blázquez-Bermejo et al., 2019; Lane and Fan, 2015) (Figure 3.12 A). Hence it was hypothesised that *SLC29A1*, *DCK* and *UCK2* knockout cells would be more sensitive to the perturbation of the *de novo* pyrimidine synthesis pathway than wildtype cells. To test this hypothesis, THP-1^{sgRNA} cells were exposed to a titration of AG636 from 0.1 – 100 µM for 48 hrs and the proportion of live cells quantified by annexin V / PI staining and flow-cytometry. AG636 is a novel and potent inhibitor of DHODH (Zeng et al., 2019), an enzyme that catalyses the conversion of dihydroorotate or orotate in the *de novo* pyrimidine synthesis pathway (Lane and Fan, 2015). Pharmacological inhibition of DHODH resulted in a substantial reduction in the proportion of live THP-1^{sgSLC29A1} and THP-1^{sgDCK} cells after 48 hrs of exposure to AG636 at concentrations ≥ 500 nM (Figure 3.12 B – E). The greatest impact on viability was apparent at 10 µM of AG636, reducing the live population by ~ 50% to that of the control cells (Figure 3.12 E).

To understand why DHODH loss did not pass the criteria aimed at identifying synthetically lethal gene knockouts in THP-1^{sgSLC29A1} cells from the drop-out screen described in section 3.3.5, the representation of the individual sgRNAs directed against *DHODH* were evaluated at each timepoint and across both THP-1^{sgSLC29A1} and THP-1^{sgSCR} cells. This analysis revealed uniform loss of *DHODH* sgRNAs in both control THP-1^{sgSLC29A1} and THP-1^{sgSCR} cells (Figure 3.12 F), suggesting that DHODH is an essential enzyme in THP-1 cells, whereby complete loss is detrimental to cell survival.

Results from sections 3.3.2 – 3.3.3 demonstrated that *DCK* and *UCK2* knockout cells remained sensitive to AZA and GDAC therapy, respectively, whilst cells with *SLC29A1* loss were resistant to all hypomethylating agents and cytarabine. The data above provides preliminary evidence of the synthetically lethal interaction between *SLC29A1* or *DCK* loss and DHODH inhibition. These data provide the grounds for further validation in *in vivo* models and collectively provide potential therapeutic avenues to combat AZA resistance in patients.

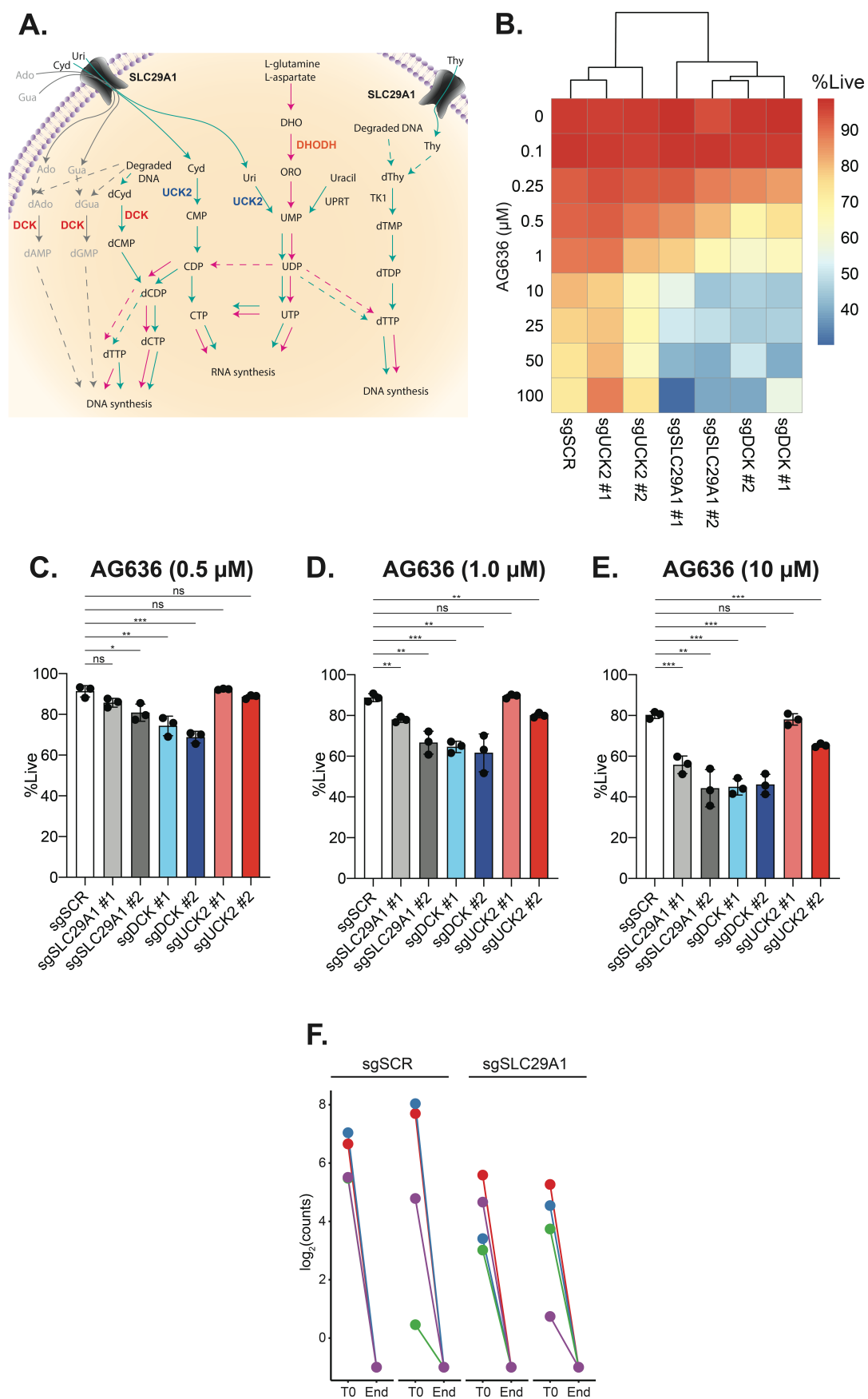


Figure 3.12 *SLC29A1* and *DCK* Knockout Cells are Sensitive to *DHODH* Inhibition

(A) Schematic of pathways that utilise *SLC29A1*, *DCK* or *UCK2*. Relevant enzymes are written next to the arrow. Arrow line type depict number of enzymatic steps: solid; single step, dashed; multistep. Arrow colours depict pathway: pink; pyrimidine *de novo* synthesis, green; pyrimidine salvage pathway, grey; other. Ado; adenosine, CMP; cytidine monophosphate, CDP; cytidine diphosphate, CTP; cytidine triphosphate, Cyd; cytidine, dAdo; deoxyadenosine, dAMP; deoxyadenosine monophosphate, dCMP; deoxycytidine monophosphate, dCDP; deoxycytidine diphosphate, dCTP; deoxycytidine triphosphate, dCyd; deoxycytidine, dGua; deoxyguanosine, dGMP; deoxyguanosine monophosphate, dThy; deoxythymidine, dTMP; deoxythymidine monophosphate, dTDP; deoxythymidine diphosphate, dTTP; deoxythymidine triphosphate, DHO; dihydroorotate, Gua; guanosine, ORO; orotate, Thy; thymidine, UMP; uridine monophosphate, UDP; uridine diphosphate, UTP; uridine triphosphate, Uri; uridine.

(B) Heatmap of the percentage of live (Annexin⁻/PI⁻) cells in each THP-1 sgRNA transduced cell line exposed to various AG636 concentrations. **(C – E)** Mean $\pm$ SD of the percentage live (Annexin⁻/PI⁻) population within each THP-1 sgRNA transduced cell line exposed to **(C)** 0.5 μ M, **(D)** 1.0 μ M and **(E)** 10.0 μ M of AG636. Means were compared using student's t-test. * 0.01 < p < 0.05, ** 0.001 < p $\leq$ 0.01, *** 0.0001 < p $\leq$ 0.001, **** p < 0.0001, ns; p $\geq$ 0.05.

(F) *DHODH* sgRNA representation at timepoint-0 (T0) and day-36 (End) in the sgSCR and sg*SLC29A1* transduced THP-1 cells from the genome-wide drop-out screen (see section 3.3.5).

3.4 Discussion

The aims of this chapter were to identify genes whose loss mediates resistance to hypomethylating agents in AML models and elucidate targetable therapeutic options for AZA/GDAC resistant cells. The identification of mechanisms that provide resistance to the various hypomethylating agents could help stratify patients into likely responders and non-responders for each class of hypomethylating agent. Furthermore, the identification of synthetic lethalties in AZA and/or GDAC resistant cells would help the development of therapeutic avenues for the treatment of patients with haematological cancers that present with, or acquire resistance to hypomethylating agents, thus improving patient outcomes. A genome-wide CRISPR-KO screen was utilised to identify overlapping and distinct gene knockouts that promote resistance to low-dose AZA and GDAC. The data presented in this chapter demonstrates that UCK2 and DCK loss provides strong resistance to AZA and GDAC, respectively, without cross-resistance to the alternate hypomethylating agent, whilst SLC29A1 knockouts conferred resistance to both hypomethylating therapies. Furthermore, preliminary *in vitro* evidence was presented demonstrating a heightened sensitivity to DHODH inhibition in *SLC29A1* and *DCK* knockout cells.

3.4.1 Characterisation of Low-dose and High-dose AZA and GDAC on Cell Proliferation and Survival *In Vitro*

Human cell lines derived from myelomonocytic leukaemia patients were utilised for the characterisation of the phenotypic response and mechanisms of resistance to hypomethylating agents. Consistent with previously published data (Hollenbach et al., 2010), high micromolar doses of hypomethylating agents were required for 50% cell death in AML cells *in vitro* following relatively short exposure periods. Short-term treatment with AZA was more potent in THP-1, MV4-11 and OCI-AML3 cells than the second-generation derivative of DAC (GDAC), further supporting the observations by Hollenbach and colleagues (2010), who demonstrated the same trends between AZA and DAC in haematological cell lines treated under the same timeframe. Since DAC and GDAC are solely incorporated into DNA, whilst only 10 – 20% of AZA is integrated into DNA (Diesch et al., 2016), and negligible cell death was observed across all cell lines after short-term GDAC treatment, the cytotoxicity observed at low-micromolar concentrations of AZA is unlikely a consequence of AZA integration into DNA. Rather, cell death following short-term low-micromolar concentrations of AZA is likely explained, at least in part, by the rapid incorporation of AZA into newly synthesised RNA.

Methylation and processing of tRNA is affected by AZA incorporation into tRNA (Lee and Karon, 1976; Schaefer et al., 2009), which likely contributes to the altered nuclear proteome (Bowler et al., 2019). Biochemical assays dissecting the effect that AZA incorporation into tRNA has on tRNA export, translation initiation and elongation, and splicing, need to be conducted utilising DAC/GDAC as controls to tease apart the contributions to cytotoxicity caused by azanucleoside integration into RNA and DNA.

Following a longer exposure period, AML cells were more sensitive to GDAC than AZA. The temporal differences in hypomethylating agent potencies may be rationalised by the differences between the types of nucleic acids into which AZA and GDAC are incorporated. DAC derivatives are entirely incorporated into DNA, whereas 80 – 90% of AZA is integrated into RNA with the minority incorporated into DNA (Diesch et al., 2016). Since the incorporation of hypomethylating agents into DNA occurs during S-phase, multiple rounds of cell cycle are required for the accumulation of cytotoxic levels of DNA-azanucleoside adducts. Hence, the rapidity of cell death induced by high-dose AZA is likely a consequence of its integration into newly transcribed RNA and thus, unlike GDAC, does not need successive rounds of cell cycling for its integration. Whilst the cytotoxic effects of AZA were more rapid, given a longer time-frame the potency of GDAC was superior to AZA suggesting that the accumulation of the DNA-azanucleoside adduct was more detrimental to cell survival than the RNA-azanucleoside adduct.

Although cytotoxic effects were negligible in AML cells after short-term exposure to low-nanomolar concentrations of hypomethylating agents, the proliferative potential was greatly reduced, consistent with previous reports of low-dose hypomethylating agents reducing colony formation capacity (Tsai et al., 2012). The proliferation defect was more pronounced under GDAC treatment, suggesting that the deoxyribose-azanucleoside contributed to a greater extent to the reduction in proliferative capacity than the ribose-azanucleoside. To date, this experiment provided the first direct comparison of the proliferative defects induced by AZA and GDAC. However, the reason for the disparities in proliferation potential between the various hypomethylating agents was not further investigated in this study. Quantification of the azanucleosides in the free cellular pool and integrated into DNA and RNA, in addition to the quantification of cell division at various titrations of AZA and GDAC, would provide correlative evidence of the azanucleoside type and cellular location associating the most with the induced anti-proliferative effects. Furthermore, differences in gene expression profiles have

been reported in AML cell lines exposed to various hypomethylating agents (Flotho et al., 2009). RNA-sequencing of AZA and GDAC treated cells would reveal whether the differences in cell-cycle progression between these hypomethylating agents were ultimately governed by differences in gene expression.

3.4.2 Loss of Pyrimidine Salvage Metabolic Enzymes Predisposed AML Cells to Hypomethylating Agent Resistance

Responses to low-dose hypomethylating agents are rarely sustained and resistance frequently arises in MDS and AML patients (Dombret et al., 2015; Fenaux et al., 2009). The mechanisms that overcome the AZA and/or GDAC mediated anti-proliferative effects have been poorly characterised within the leukaemic patient population. One of the aims of this chapter was to elucidate mechanisms that confer resistance to hypomethylating agents. The advent of CRISPR technology has enabled many researchers to identify mechanisms of resistance utilising reliable unbiased genome-wide screens (Hou et al., 2017; Kurata et al., 2016; Wei et al., 2019). To address the first aim of this chapter, a genome-wide CRISPR knockout screen was employed to identify gene knockouts that evaded the proliferative defect induced by hypomethylating agents. This experiment identified 3 enzymes integral to the pyrimidine salvage pathway, which are also utilised in the metabolism of hypomethylating agents, whose genetic depletion drove the strongest resistance to the anti-proliferative effects of AZA and/or GDAC. As mentioned in section 1.3.4.1.1, SLC29A1 is an equilibrative transporter with a high affinity for many nucleosides, whilst DCK and UCK2 are kinases responsible for the rate-limiting primary phosphorylation of nucleoside. These transporters and enzymes have previously been implicated in the resistance to cytidine nucleoside analogues, including the chemotherapeutic agent cytarabine (Ara-C) and hypomethylating agents (AZA and DAC) (Table 3.4). However, the majority of these studies utilised biased genetic approaches, whereby *SLC29A1*, *DCK* or *UCK2* were selectively knocked-down and cell survival subsequently measured in the presence of drug, or by targeted sequencing of these genes following long-term culture in drug supplemented medium (Table 3.4). To date, the CRISPR/Cas9 knockout screen executed to address the first aim of this chapter was the first study to assess genes that mediate resistance to hypomethylating agents utilising a genome-wide and unbiased approach. The results of this experiment highlighted that loss of SLC29A1 and either DCK or UCK2 provided the strongest level of resistance, when compared to all other genetic knockouts, for GDAC and AZA treated cells, respectively.

The *in vitro* and *in vivo* studies subsequently conducted, provided validation of the resistance to AZA and/or GDAC mediated by SLC29A1, DCK and UCK2 loss. In line with previously published data (Table 3.4), these results indicated the robust and reproducible consequences of SLC29A1, DCK and UCK2 loss in cells treated with hypomethylating agents. Furthermore, SLC29A1 and DCK loss conferred resistance to cytarabine (Ara-C), a cytidine analogue chemotherapeutic, also concordant with published data (Table 3.4).

SLC29A1, DCK and UCK2 loss provided resistance to the anti-proliferative effects induced by AZA and/or GDAC *in vitro* and *in vivo*. However, in the absence of low dose hypomethylating agent, the proliferation rate in *SLC29A1*, *DCK* and *UCK2* knockout cells was discordant between the *in vitro* and *in vivo* contexts. *In vitro*, under vehicle treatment, SLC29A1, DCK and UCK2 loss did not provide cells with a proliferative advantage over control cells as evident by the maintained proportion of knockout to parental cells throughout the duration of the competitive proliferation assay. In contrast, the leukaemia expansion between the two imaging timepoints was, on average, 10-fold higher in the vehicle-treated mice transplanted with knockout cells than control cells. This suggests that the effects of SLC29A1, DCK and UCK2 loss *in vivo* may differ from the *in vitro* setting. Of note, the cohort sizes for the *in vivo* experiment were relatively small. Furthermore, the ectopic expression of luciferase in the different cell lines was not assessed prior to conducting the *in vivo* experiment. Since the luciferase expressing cell lines were generated through separate transductions of the various knockout and control cell lines, differences in the number of luciferase vector integrations may exist between cell lines. Thus, cells with more luciferase vector integrations would exhibit greater bioluminescence upon luciferin exposure (Brennan et al., 2018). qRT-PCR could be utilised to ensure luciferase is homogenously expressed between the different cell lines. Alternatively, the knockout/luciferase expressing cell lines could be generated by reversing the order of transduction; first generate a population uniformly expressing Cas9/luciferase and subsequently transducing sgSCR or sgRNAs directed towards the target genes. Repeating this experiment with larger cohorts whilst additionally quantifying the absolute number of leukaemic cells (mCherry⁺/GFP⁺) in the periphery prior to therapy initiation and post-treatment in the peripheral blood, bone marrow, nodes and spleen by flow-cytometry, would provide a more thorough examination of the effects SLC29A1, DCK and UCK2 loss has on leukaemic cells in the presence and absence of hypomethylating agents *in vivo*.

The high level of resistance provided by SLC29A1, DCK or UCK2 loss was reflected by the dramatic increase in cytotoxic concentrations of AZA and/or GDAC required to kill 50% of cells *in vitro*. Given that SLC29A1, DCK and UCK2 are integral for the uptake and metabolism of hypomethylating agents and that loss of these transporters and kinases significantly increased the LD-50 of AZA and/or GDAC, loss of SLC29A1, DCK or UCK2 likely prevents DNA hypomethylation by AZA/GDAC. This could be validated with enhanced reduced representation bisulphite sequencing (ERRBS) (Meissner et al., 2005). Interestingly, the extent of resistance provided by SLC29A1, DCK or UCK2 loss differed between AZA and GDAC treatments; amongst all AML cell lines evaluated, when compared to control cells, the increase in the GDAC LD₅₀ was substantially greater in *SLC29A1* and *DCK* knockout cells, than the increase in AZA LD₅₀ in the *SLC29A1* and *UCK2* knockout cells. This suggests that there are likely redundancies in other transporters and enzymes capable of mediating AZA cellular uptake and mono-phosphorylation, although to a lesser extent than *SLC29A1* and *UCK2*, respectively.

Equilibrative and concentrative transporters are responsible for the uptake of nucleosides (Pastor-Anglada and Pérez-Torras, 2018). Collectively, these nucleoside transporter families are comprised of seven members, including SLC29A1, and provide a high level of redundancy between nucleoside substrates (Pastor-Anglada et al., 2004). Multiple nucleoside transporters are capable at facilitating the uptake of hypomethylating agents into the cell, albeit, with varying specificities and efficiencies (Damaraju et al., 2012). Damaraju and colleagues (2012) reported that human SLC29A1 and SLC28A1 have the highest affinity for AZA. Furthermore, both uridine cytidine kinases, UCK1 and UCK2, are capable of mono-phosphorylating AZA (Van Rompay et al., 2001). Although the exact enzyme efficiencies for the mono-phosphorylation of AZA have not been reported, the efficiency of UCK2 mediated mono-phosphorylation of cytidine and uridine is 15 – 20 fold higher than that of UCK1 (Van Rompay et al., 2001). These additional metabolic enzymes likely provide alternative routes for the first two steps in the metabolism of AZA, since high micromolar doses of AZA were still capable at inducing cell death in *SLC29A1* and *UCK2* knockout cells. However, these alternative routes likely only contribute to a small portion of AZA metabolism since these alternative enzymes were not identified in the resistance screen. Reduced expression of these alternative enzymes, when compared to *SLC29A1* and *UCK2*, and probable differences in AZA affinity between members of the same enzyme family, are likely contributing factors to the smaller proportions of AZA metabolised by the alternative pathways in THP-1, MV4-11 and OCI-AML3 cells.

Quantitating the AZA LD₅₀ in cells with concurrent knockout of these transporters or kinases with *SLC29A1* or *UCK2* loss, respectively, would potentially identify the alternate mechanisms through which AZA is metabolised. Alternatively, unbiased genome-wide CRISPR/Cas9 screens in *SLC29A1* or *UCK2* knockout cells cultured in high-micromolar concentrations of AZA, would enable the outgrowth of double-knockouts that confer strong resistance to high-dose hypomethylating agents and thus facilitate the identification of additional enzymes that contribute to AZA metabolism.

Alternative loss-of-function genetic events that facilitate a subtle resistance phenotype to hypomethylating agents *in vitro* may also be clinically relevant. The experimental design of the CRISPR/Cas9 screen preferentially selected for gene knockouts that mediate the highest proliferative advantage in the presence of hypomethylating agents. As such, loss-of-function genetic events that mediate partial resistance to hypomethylating agents were undetectable. Perhaps a shorter screening timeframe, whereby *SLC29A1*, *DCK* and *UCK2* knockout clones had not comprised the majority of the population, would enable the detection of additional genetic knockouts that mediate a subtle resistance phenotype. Alternatively, conducting a CRISPR screen utilising a library with no sgRNAs directed against *SLC29A1*, *DCK* and *UCK2*, under the selection pressure of low-dose AZA/GDAC, would enable the identification of additional resistance mechanisms to hypomethylating agents.

Resistance mechanisms do not only arise through reduction of gene expression or inactivating mutations. Drug resistance in cancer can occur through gene up-regulation, gene amplification, increase in drug efflux, secondary mutations that reactivate the target, interactions between the leukaemic stem cell population and the microenvironment, and diverse gene expression modulation as a consequence of perturbations to the epigenetic landscape and chromatin structure (Hu and Zhang, 2016; Mansoori et al., 2017; Rashidi and Uy, 2015). Recently, Gu and colleagues (2020) reported that AZA/DAC can induce gene expression changes that eventually promote resistance to hypomethylating agents, which may explain the transient effects seen in patients that respond to hypomethylating agent therapies (Fenaux et al., 2009; He et al., 2017). These adaptive responses are likely a consequence of AZA/DAC induced changes in DNA methylation, and could be verified by ERRBS (Meissner et al., 2005). Finally, hypomethylating agents have demonstrated tumour-extrinsic immunomodulatory effects (see section 1.3.4.1.1) (Chiappinelli et al., 2015; Jeong et al., 2015; Li et al., 2014a; Stone et al., 2017). Hence, further research is required in order to achieve a more comprehensive

understanding of the resistance mechanisms cells could employ to evade the anti-leukaemic effects of hypomethylating agents.

Despite the abundant *in vitro* data implicating pyrimidine salvage enzymes in the resistance to cytidine analogue therapeutics (Table 3.4), few studies have examined their contribution to the resistance in patients with haematological malignancies treated with hypomethylating agents (Qin et al., 2009; Wu et al., 2016; Wu et al., 2015). Studies investigating clinical resistance to hypomethylating agents are often comprised of small sample sizes and fail to distinguish between upfront and acquired resistance. In these studies, AZA and/or DAC patients are typically classified as responders and non-responders according to criteria that primarily focuses on bone marrow and peripheral blood evaluations. Often, a survival benefit is observed between these two cohorts, albeit typically measured in months. Thus, this classification system limits the investigation of clinical resistance to upfront mechanisms of resistance, providing no explanation to why patients classified as responders acquired resistance to hypomethylating agents and ultimately succumbed to the haematological malignancy. More recently, resistance to hypomethylating agents has been reported to arise from adaptive responses to AZA/DAC exposure, whereby AML murine models that relapse following AZA or DAC treatment demonstrated decreased *UCK2* or *DCK* expression, respectively (Gu et al., 2020). This study reflects the observation of the reduction in *UCK2* expression after 6 cycles of AZA treatment in CMML patients classified as responders, whom ultimately succumbed to disease with a median survival increase of ~17 months when compared to the non-responders. Collectively, these observations highlight the unmet need to investigate mechanisms of acquired clinical resistance to hypomethylating agents.

Table 3.4 Genes Implicated in Resistance to Cytidine Analogue Therapies

Drug	Gene	Description	Reference
DAC	DCK	HL60 cells acquired resistance through spontaneous mutations in DCK following long term passaging in Decitabine.	(Qin et al., 2009)
AZA	UCK2	HL60 and THP-1 cells acquired resistance through spontaneous mutations in the kinase domain of UCK2 following long term passaging in 5-Azacytidine. Although not measured, authors predict mutations diminished UCK2 activity.	(Sripayap et al., 2014)
DAC	DCK	HCT116 cells acquired resistance through a reduction in DCK protein levels, not mRNA, following long term passaging in Decitabine.	(Hosokawa et al., 2015)
Ara-C	SLC29A1	SLC29A1 knockdown in HEL and KG-1 cells confer survival advantage in the presence of Cytarabine.	(Kim et al., 2016)
Ara-C	DCK SLC29A1	CRISPR screen in U937 cells identified DCK and SLC29A1 knockouts as mechanisms of resistance to Cytarabine.	(Kurata et al., 2016)
Ara-C	DCK	K562 and Kasumi cells acquired resistance through deletion of the DCK locus or frameshift mutation in DCK resulting in loss of DCK expression following long term passaging in Cytarabine.	(Levin et al., 2019)

3.4.3 Overcoming Resistance to Hypomethylating Agents

Elucidating mechanisms of resistance to hypomethylating agents enables improved patient stratification based on the predictability of response, and novel research to be undertaken in search of therapies that are effective in the resistant cells, thus improving patient outcomes. Whilst DCK and UCK2 loss provided robust resistance to GDAC and AZA, respectively, strong cross-resistance was not observed *in vitro* nor *in vivo*. Concordant with these data, Gu et al. (2020) reported that the adaptive responses elicited by cells exposed to AZA or DAC, promoted resistance through the down-regulation of *DCK* or *UCK2* expression, respectively, such that alternating AZA and DAC periodically throughout the treatment regimen significantly prolonged survival *in vivo* (Gu et al., 2020). Collectively these data demonstrate that AZA and GDAC treatment may be efficacious in patients with decreased *DCK* and *UCK2* expression, respectively.

It is important to note that neither AZA nor GDAC treatment eliminated or resulted in tumour regression *in vivo*, but rather delayed and/or reduced the rate of leukaemia growth. Therefore, whilst mice transplanted with THP-1^{sgDCK/luc} or THP-1^{sgUCK2/luc} were sensitive to AZA or GDAC treatment, respectively, leukaemias from these mice were still expanding, albeit at a reduced rate, and thus the hypomethylating agent therapies these mice received were likely not curative but rather life-prolonging. Although hypomethylating agent therapies are not typically curative for MDS and AML patients, 3 – 6 months of treatment can prolong life in ~30% of patients (Fenaux et al., 2009; He et al., 2017). Given the aggressiveness of THP-1 xenografts, earlier AZA/GDAC treatment initiation with a longer treatment duration may enhance the transient anti-tumour effects of hypomethylating agents *in vivo*.

Whilst DCK and UCK2 loss did not facilitate cross-resistance to AZA and GDAC, respectively, SLC29A1 loss conferred resistance to both hypomethylating agents tested. The CRISPR-KO screen was designed to identify genes essential for the proliferation and/or survival of cells only when SLC29A1 expression is lost. Hence, the screen was performed over a relatively long time-frame to ensure such genes were non-essential in the control cells. However, perhaps this criterion was too stringent as no gene targets were identified. Reducing the length of time over which the screen was conducted could have identified gene knockouts whereby the rapidity in gene drop-out varied between *SLC29A1* knockout and control cells. Although this type of gene knockout would eventually be depleted in the control cells after an ample length of time, the difference in lethality may be sufficient to exploit as a therapeutic avenue for the treatment of

haematological malignancies that are resistant to hypomethylating agents via the down-regulation of *SLC29A1*.

The loss of *SLC29A1* predisposed THP-1 cells to a significant increase in sensitivity to pharmacological inhibition of DHODH. The depletion of all 4 independent sgRNAs directed towards *DHODH* across all samples in the CRISPR-KO screen suggests that DHODH is an essential enzyme in THP-1 cells. Indeed, homozygous knockout of DHODH is embryonic lethal in mice (Dickinson et al., 2016) and no viable DHODH-deficient animals have been reported (Sykes, 2018). Furthermore, Miller syndrome, an extremely rare condition in humans, occurs as a result of hypomorphic alleles rather than the absence of DHODH activity (Ng et al., 2010). Taken together, this emphasises the differences between complete loss versus decreased activity of DHODH, and likely explains the discrepancy between DHODH knockout and inhibition in the *SLC29A1* knockout cells. Moreover, this highlights the importance of conducting pharmacological inhibitor screens in parallel to CRISPR-KO screens, in order to increase the likelihood of identifying therapeutic targets.

Pyrimidine can be synthesised through *de novo* biosynthesis and pyrimidine ribonucleotide or deoxyribonucleotide salvage pathways (Lane and Fan, 2015) (Figure 3.12 A). *SLC29A1*, *DCK* and *UCK2* are involved in the pyrimidine salvage pathways (Blázquez-Bermejo et al., 2019; Lane and Fan, 2015), whilst DHODH is an enzyme that catalyses the conversion of dihydroorotate or orotate in the *de novo* pyrimidine synthesis pathway (Lane and Fan, 2015). In addition to *SLC29A1* knockout, *DCK* loss also amplified the sensitivity of THP-1 cells to DHODH inhibition. Interestingly, *UCK2* knockout cells were not as sensitive to DHODH inhibition when compared to the *SLC29A1* and *DCK* knockout cell lines. This could be explained, at least in part, by the likely differences in the perturbations of nucleoside pools induced by the various knockouts. It is widely understood that disruptions to the nucleoside pools can increase replication stress and be detrimental to cell survival (Austin et al., 2012; Ewald et al., 2008). Although substrate redundancies exist between nucleoside transporters, *SLC29A1* is the most abundant nucleoside transporter in many human cells (Leisewitz et al., 2011) and AML cell lines (Cancer Cell Line Encyclopedia), with high affinities for cytidine, thymidine, guanosine, adenosine, inosine and uridine (Pastor-Anglada and Pérez-Torras, 2018). The impact that *SLC29A1* reduction or loss has on nucleoside pools in the haematological context has not been investigated, however *SLC29A1*-null cardiomyocytes exhibit reduced cellular uptake of adenosine (Rose et al., 2011). *DCK* is responsible for the rate-limiting

monophosphorylation of deoxycytidine, and contributes to deoxyadenosine and deoxyguanosine mono-phosphorylation (Austin et al., 2012). Depletion in deoxycytidine triphosphate (dCTP) pools have been reported in *DCK*^{-/-} murine and AML models, with variable impact on deoxyadenosine triphosphate (dATP) and deoxyguanosine triphosphate (dGTP) pools amongst different haematopoietic lineages (Austin et al., 2012; Gu et al., 2020). Unlike SLC29A1 and DCK, which have been implicated in both purine and pyrimidine salvage pathways, UCK2 is only involved in the pyrimidine salvage pathway (Figure 3.12 A). In leukaemia cells, UCK2 loss was reported to decrease dTTP levels without perturbation of the dCTP pool (Gu et al., 2020). If DHODH inhibition perturbed the nucleoside pools to a greater extent in *DCK*- or *SLC29A1*-null cells, than in *UCK2*-null cells, then a more detrimental effect on the survival of *DCK*- or *SLC29A1*-null cells would be predicted. To faithfully address this hypothesis, liquid chromatography-mass spectrometry (LC-MS) could be utilised to quantitate the intracellular nucleoside levels from leukaemic cells harvested from mice transplanted with either control, *UCK2*-, *DCK*- or *SLC29A1*-knockout AML cells, that received either vehicle or AG-636 therapy.

The preliminary data demonstrating the significant sensitivity to DHODH inhibition that *SLC29A1* and *DCK* knockouts imparted on AML cells, requires further validation *in vivo* to ascertain whether AG-636 therapy is able to eliminate, or significantly reduce the proliferative capacity of the leukaemia, and hence significantly prolong survival. Furthermore, Gu and colleagues (2020) recently reported that resistance driven by the down-regulation of *DCK* expression in AML murine models was an adaptive response to DAC, and that periodically alternating between AZA and DAC was more efficacious than either hypomethylating agent alone *in vivo*, although not curative. Hence, *in vivo* studies whereby this treatment regime was adjusted by combining a DHODH inhibitor with DAC, whilst still cycling with AZA, may demonstrate an enhanced therapeutic outcome in AML murine models. Such experiments would demonstrate whether a more superior clinical response could be achieved in patients with haematological malignancies that do not qualify for intense chemotherapy.

3.5 Conclusions

The results presented in this chapter demonstrate that key members of the pyrimidine salvage pathway are integral to the metabolism of hypomethylating agents, such that SLC29A1, DCK or UCK2 loss provides resistance to AZA and/or GDAC. Furthermore, whilst loss of SLC29A1 provided resistance to all cytidine analogues, DCK and UCK2 loss did not provide cross-resistance to AZA and GDAC, respectively, thus highlighting a potential therapeutic avenue for AML resistance driven by DCK or UCK2 depletion or loss. DCK and SLC29A1 loss predisposed cells to a significant increase in sensitivity to pharmacological inhibition of DHODH, a member of the *de novo* pyrimidine synthesis pathway. Collectively, this research highlights genetic knockouts that drive strong resistance to hypomethylating agents, and alternate therapeutic avenues that remain efficacious or exploit a synthetic lethality in the resistant cells. Further clinical research is needed to examine whether SLC29A1, DCK or UCK2 loss, depletion or inactivation drives hypomethylating resistance in patients with haematological malignancies. Furthermore, understanding whether resistance is intrinsic, or driven by a sub-clone expansion, or acquired due to drug induced perturbations in transcription, would improve patient stratification, prognostic measures and treatment options.

Chapter 4: Investigating the Response and Resistance to IDH1^{R132H} Inhibition in AML

4.1 Introduction

In normal cells, the IDH enzymes produce α -KG, a metabolite of the Tricarboxylic Acid Cycle and cofactor of ~60 enzymes involved in various cellular activities, and contribute to redox homeostasis by providing energy for the cells in the form of NADH/NADPH. Mutations in *IDH1/2* occur frequently in normal karyotype AML and a variety of other cancers (Amary et al., 2011; Ghiam et al., 2012; Kang et al., 2009; Mardis et al., 2009; Papaemmanuil et al., 2016; Paschka et al., 2010; Wood et al., 2007; Yan et al., 2009). Oncogenic genetic aberrations in IDH1/2 are typically non-synonymous mutations that occur almost exclusively in the codon of Arg-132 of IDH1, the equivalent IDH2 residue, Arg-172, and Arg-140 of IDH2 in *IDH* mutant malignancies (Losman and Kaelin, 2013). These mutations confer a neomorphic activity in the malignant IDH1/2 enzyme, whereby mutant-IDH1/2 reduces α -KG to the structurally similar oncometabolite, 2-HG (Dang et al., 2009). *IDH1/2*-mutant malignancies exhibit a supraphysiologic accumulation of intracellular 2-HG (Dang et al., 2009). Furthermore, heightened 2-HG concentration is detectable in the plasma and serum (DiNardo et al., 2013; DiNardo et al., 2018; Lombardi et al., 2015), and in the malignant tissue (Andronesi et al., 2012; Choi et al., 2012; Elkhaled et al., 2012) of mutant IDH patients. Elevated 2-HG has therefore been implicated as a diagnostic biomarker for mutant *IDH* malignancies. However, since baseline 2-HG levels do not correlate with clinical outcome (Amatangelo et al., 2017), and 2-HG suppression has been observed during treatment with mutant IDH inhibitors in both responding and non-responding patients (Amatangelo et al., 2017; DiNardo et al., 2018), 2-HG is not a prognostic indicator of clinical response.

The supraphysiological accumulation of 2-HG in cells induces pleiotropic effects, typically through the inhibition of α -KG-dependent dioxygenases, that promote malignant transformation or progression (Losman et al., 2013). *In vitro* models ectopically expressing mutant-IDH1/2 or exogenously supplied with 2-HG have demonstrated that 2-HG inhibition of TETs and JmjC-domain containing KDMs results in DNA and histone hypermethylation, respectively, which is thought to contribute to the pathogenesis of mutant *IDH* driven cancers (Chowdhury et al., 2011; Figueroa et al., 2010a; Flavahan et al., 2016; Losman et al., 2013; Lu et al., 2012; Xu et al., 2011). Indeed, aberrant DNA hypermethylation has been reported in IDH-mutant AML patients (Figueroa et al., 2010a; Glass et al., 2017; Unruh et al., 2019). The α -KG-dependent m6A demethylase, FTO, is also inhibited by 2-HG (Su et al., 2018), which explains the elevated m6A levels in mutant *IDH in vitro* and primary AML patient samples

(Elkashef et al., 2017) and is hypothesised to provide another method of transcriptional deregulation in mutant *IDH* malignancies (Losman and Kaelin, 2013).

The aberrant acquisition of the HSC-like self-renewal capacity and accompanied block in differentiation are common characteristics of AML, including *IDH*-mutant AML (Losman et al., 2013). Murine models expressing *Idh1*^{R132H} or *IDH2*^{R140Q} in myeloid lineage progenitors exhibited a modest increase in the myeloid progenitor compartment suggesting that the acquisition of *IDH* mutations enables enhanced self-renewal properties and their eventual clonal expansion (Kats et al., 2014; Sasaki et al., 2012). The co-expression of additional oncogenes co-operated with mutant-IDH to initiate leukaemia transformation *in vivo*, which rapidly led to AML driven mortality (Chen et al., 2013; Kats et al., 2017). Concordant with these experimental data, *IDH*-mutant AML patients tend to have a relatively high mutant *IDH* VAF, implying that the acquisition of *IDH* mutations occurs early in leukaemogenesis (Papaemmanuil et al., 2016).

In addition to contributing to the HSC-like state, ectopic over-expression of mutant *IDH* has been reported to block differentiation of adipocytes and bone marrow derived cells *in vitro* (Figuerola et al., 2010a; Lu et al., 2012), and an erythroleukaemia cell line cultured under cytokine-depleted conditions (Losman et al., 2013). Furthermore, *in vivo* mutant-IDH AML cells are partially blocked in differentiation, as evident by the expression of mature myeloid cell-surface markers, namely Cd11b, on a portion of leukaemia cells (Chen et al., 2013; Kats et al., 2014; Kats et al., 2017).

Recently, two drugs, Ivosidenib (AG-120) and Enasidenib (AG-221), have been FDA approved for the treatment of relapsed and refractory *IDH1*- and *IDH2*-mutant AML, respectively. Complete remission with or without haematological recovery was achieved in over 30% of relapsed or refractory AML patients harbouring *IDH1*^{R132} mutations following Ivosidenib treatment (DiNardo et al., 2018). Experimental studies have demonstrated that small-molecule targeted inhibition of mutant IDH blocks the production of 2-HG and induces differentiation of cell lines and patient samples *in vitro* (Kernytsky et al., 2015; Rohle et al., 2013; Saha et al., 2014; Wang et al., 2013), and *IDH*-mutant *in vivo* models (Chen et al., 2013; Kats et al., 2017; Yen et al., 2017). However, the consequences of targeted inhibition of mutant-IDH1 on the aberrant self-renewal properties and the differentiation trajectory of the various AML haematopoietic cell-types remains poorly understood. Furthermore, whilst the efficacy of

IDH2-mutant inhibitors have been demonstrated *in vivo* (Kats et al., 2017; Shih et al., 2017), characterisation of the therapeutic efficacy of small-molecule inhibitors of IDH1^{R132H} has been restricted to cell lines and *ex vivo* patient samples (Chaturvedi et al., 2013; Popovici-Muller et al., 2018).

Despite the remarkable response in a subset of AML that is notoriously difficult to treat, ~ 70% of patients still do not enter complete remission and subsequent development of resistance and relapse is not uncommon following mutant *IDH* inhibition therapies (Amatangelo et al., 2017; DiNardo et al., 2018). Targeted sequencing has implicated activating *RAS* pathway mutations as a potential prognostic marker of poor response (Amatangelo et al., 2017). Moreover, mutant isoform switching and *cis* or *trans* dimer-interface mutations have been reported in instances of patient acquired resistance to mutant *IDH* targeted therapies (Harding et al., 2018; Intlekofer et al., 2018). However, these resistance pathways have only been reported in a few non-responding patients. Overall, the mechanisms of intrinsic and acquired resistance to small molecule inhibition of mutant IDH remain poorly understood. A comprehensive understanding of these resistance mechanisms could promote the development of combination therapies and ultimately improve patient outcome.

The context in which the oncogenic effects of mutant-IDH are studied is important. Su et al., (2018) reported that 2-HG elicited potent anti-tumour properties in the majority of haematological cell lines that did not harbour genetic lesions in *IDH*. This highlights the limitations of exogenously supplementing 2-HG or ectopically expressing mutant-*IDH* *in vitro*, and the importance of studying the mechanisms of 2-HG driven leukaemogenesis in the appropriate pathophysiological context where mutant-*IDH* is an oncogenic driver.

This chapter discusses the generation of a clinically and pathophysiologicaly relevant murine model of IDH1^{R132H} driven AML. This model is utilised to interrogate the contribution of IDH1^{R132H} on the transcriptional and epigenetic states, understand the impact of *IDH1^{R132H}* expression on the leukaemic properties within different AML cellular contexts, and the biological and molecular consequences of IDH1^{R132H} inhibition.

4.2 Specific Aims

Mutations in *IDH1/2* are present in ~10 – 30% of cytogenetically normal AML. The resultant supra-physiological accumulation of 2-HG dysregulates a plethora of cellular pathways contributing to aberrant self-renewal and block in differentiation. The distinct confirmation of mutant IDH, owing to its neomorphic capability, resulted in the rapid development of small molecule inhibitors targeting mutant IDH. Although this class of targeted epigenetic therapeutics has resulted in clinical remission of relapsed/refractory *IDH*-mutant AML patients, a large proportion of patients demonstrate either upfront or acquired resistance. Furthermore, understanding of the mechanisms of resistance to small molecule inhibition of mutant IDH is limited. Hence, this chapter aimed to comprehensively examine the effects of mutant *IDH* expression on haematopoietic stem/progenitor cells and the short- and long-term consequences of mutant IDH inhibition in established haematopoietic malignancies, in order to provide the foundations to identify mechanisms of resistance and promote the development of effective personalised therapies for mutant *IDH* AML, and ultimately improve patient outcome.

The specific aims for Chapter 4 were to:

1. Determine whether sustained IDH1^{R132H} activity was required for leukaemia maintenance and whether targeting IDH1^{R132H} was an effective therapeutic strategy *in vivo*
2. Understand the contribution of IDH1^{R132H}, and consequence of targeted inhibition, on the aberrant self-renewal properties and/or limited differentiation capacity within different AML haematopoietic cell-types
3. Elucidate key transcriptional pathways and epigenetic states contributing to the biological phenotype of *IDH1^{R132H}* driven AML and governing the response to targeted IDH1^{R132H} inhibition
4. Identify transcriptional programs that mediate resistance to IDH1^{R132H} inhibition

4.3 Results

4.3.1 IDH1^{R132H} Cooperated with DNMT3A^{R882H} and Nras^{G12D} to Initiate AML *In Vivo*

To investigate the role of IDH1^{R132H} in leukaemia initiation and maintenance, a tri-mutant allelic model of AML was engineered to concurrently express *IDH1*^{R132H}, *DNMT3A*^{R882H} and *Nras*^{G12D}, henceforth referred to as IDN, whereby rapid depletion of IDH1^{R132H} could be induced by the addition of doxycycline (DOX). The co-occurrence of these mutations is amongst the most prevalent triple-mutant combination in *IDH1*^{R132} AML, accounting for almost 10% of all mutant *IDH1*^{R132} AML patients (Papaemmanuil et al., 2016). Foetal liver cells (FLCs) harvested from day 13.5 C57BL/6 embryos were retrovirally transduced with 2 vectors encoding the constitutive expression of GFP-linked *DNMT3A*^{R882H} and tetracycline transactivator (tTA)-linked *Nras*^{G12D}, and an inducible dsRED-linked *IDH1*^{R132H} vector whereby expression is controlled by a tetracycline-responsive element (TRE-tight) promoter (Figure 4.1 A). Bulk transduced FLCs were transplanted into syngeneic Ptpcrp recipient mice to assess their ability to initiate leukaemia *in vivo* (Figure 4.1 B). Transplantation of IDN cells resulted in the development of a fully penetrant leukaemia characterised by splenomegaly and elevated white-blood cells (Figure 4.1 C – E), whilst transplantation of FLCs transduced with either *Nras*^{G12D} alone or *Nras*^{G12D}/*DNMT3A*^{R882H} resulted in a disease with an inconsistent phenotype between individual recipients, longer latency and incomplete penetrance (Kats et al., 2017). Furthermore, the expression of mutant IDH alone was insufficient in driving a lethal disease in a transgenic *in vivo* model (Kats et al., 2014). Collectively, these data indicate that IDH1^{R132H}, DNMT3A^{R882H} and Nras^{G12D} cooperate to yield a fully penetrant leukaemia in the *in vivo* IDN model.

IDN cells harvested from moribund recipients and subsequently transplanted into secondary and tertiary recipient mice, demonstrated a reduced latency, with median survival decreasing by 30 – 40 days (Figure 4.1 F). Immunoblot confirmed the overexpression of oncogenic IDH1^{R132H}, DNMT3A^{R882H} and Nras^{G12D} proteins in the bone marrow and spleen of moribund recipients (Figure 4.1 G). Furthermore, immune-compromised NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice transplanted with IDN tumour cells developed a lethal leukaemia with a phenotype and disease latency comparable to the Ptpcrp recipients (data not shown, Figure 4.1 H).

To validate the ‘Tet-Off’ system, NSG mice transplanted with IDN tumours were administered DOX-supplemented or normal food and water for 5 days following a 28-day engraftment period (Figure 4.2 A). Flow cytometry and immunoblotting confirmed that 5 days of DOX administration significantly reduced the expression of dsRED-linked IDH1^{R132H} cells in the bone marrow and spleen, while the expression of Nras^{G12D} and GFP-linked DNMT3A^{R882H} were unaffected (Figure 4.2 B – E).

Cell surface markers are frequently used to discriminate different haematopoietic cell-types. The immunophenotype of IDN cells (GFP⁺) from the bone marrow and spleens of moribund mice were characterised by flow cytometry and compared to wildtype C57BL/6 mice (Figure 4.3 – 4.4). Late-stage disease was marked by proportionally more mature myeloid cells (CD11b⁺) than HSCs / myeloid progenitors (CD117⁺) in the bone marrow and spleen of IDN recipients when compared to wildtype C57BL/6. The CD117⁺ IDN population primarily encompassed Kit⁺/Lineage⁻/Sca1⁻ (KL) cells (which are comprised of myeloid progenitors) with an almost negligible Kit⁺/Lineage⁻/Sca1⁺ (KSL) population (containing HSCs and MPPs) and an almost entirely absent CLP population. The principal IDN myeloid progenitor population present at late stage disease in the bone marrow and spleen were GMPs. The IDN CD11b⁺ population was predominantly marked by neutrophils, with substantially less monocytes and eosinophils when compared to wildtype controls. Neutrophils were classified into 3 subtypes: pre-neutrophils, immature neutrophils and mature neutrophils. There was a substantial increase in the proportion of IDN pre-neutrophils present at late-stage disease, which was most evident in the spleen. An almost complete block in differentiation from immature neutrophils to mature neutrophils was apparent in bone marrow and spleen of IDN recipient mice. Collectively, these data provide evidence that the IDN *in vivo* model is characteristic of a myeloid leukaemia.

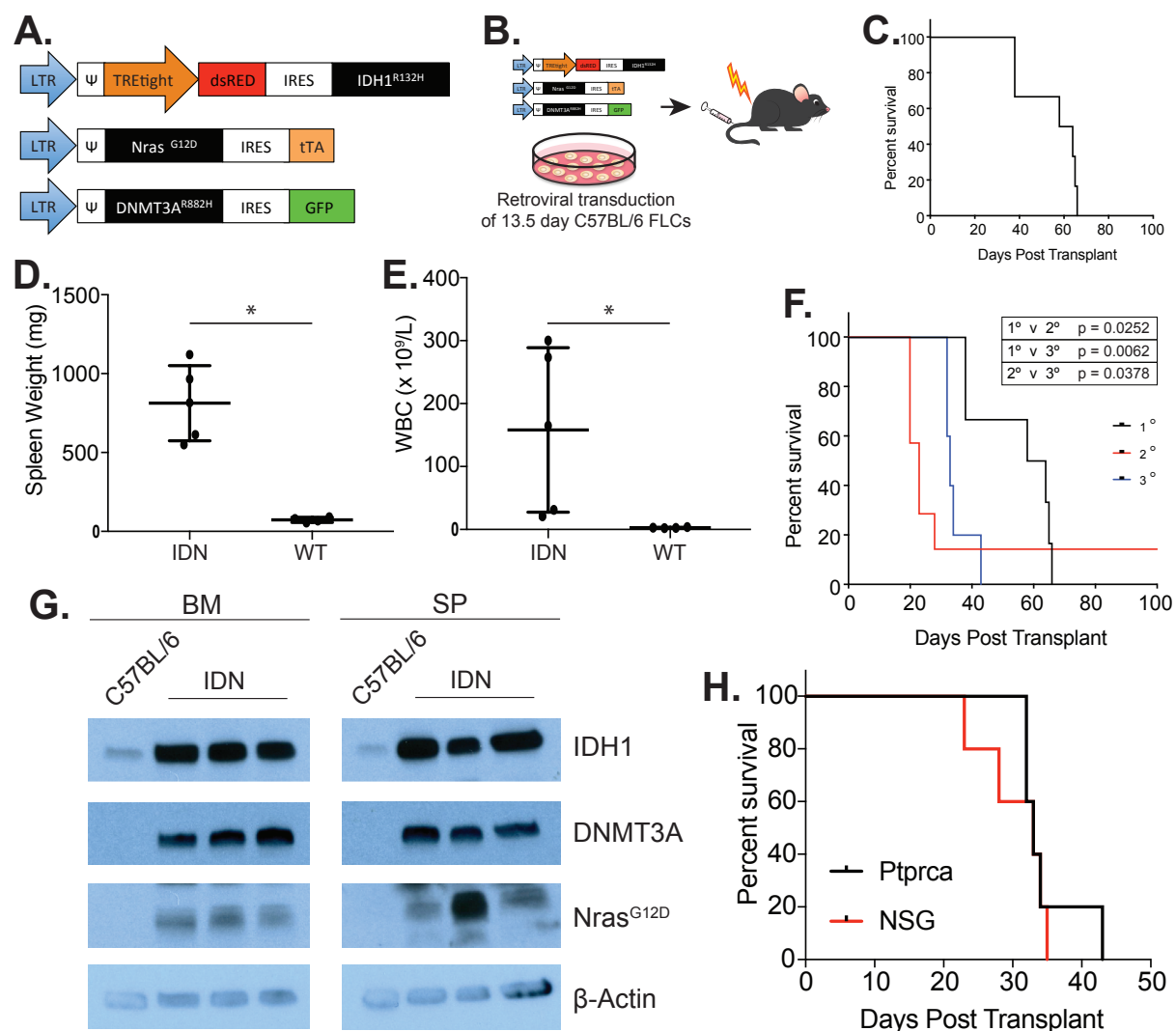


Figure 4.1 Generation of IDN *In Vivo* Model

(A) Schematic of the retroviral overexpression constructs used for the generation of the IDN triple mutant tumours. Top; tetracycline inducible *IDH1*^{R132H} linked to a dsRED reporter, middle; constitutive *Nras*^{G12D} linked to a tetracycline transactivator (tTA) protein, bottom; constitutive *DNMT3A*^{R882H} linked to a GFP reporter. (B) Schematic of the experimental protocol for the generation of primary tumours. FLCs; foetal liver cells. (C) Kaplan-Meier survival curve of Ptpcr recipient mice transplanted with primary IDN tumours. (D) Spleen weights of moribund primary recipients. (E) Peripheral white blood cell (WBC) counts of moribund primary recipients. (F) Kaplan-Meier survival curve of Ptpcr recipient mice transplanted with primary, secondary or tertiary IDN tumours. (G) Immunoblot of retrovirally over-expressed mutant proteins from the bone marrow (BM) and spleen (SP) of IDN mutant tumours. BM and SP from C57BL/6 mice were used as controls. (H) Kaplan-Meier survival curve of NSG and Ptpcr mice transplanted with IDN tumours. Means were compared using non-parametric two-tailed student's t-test. * p < 0.05.

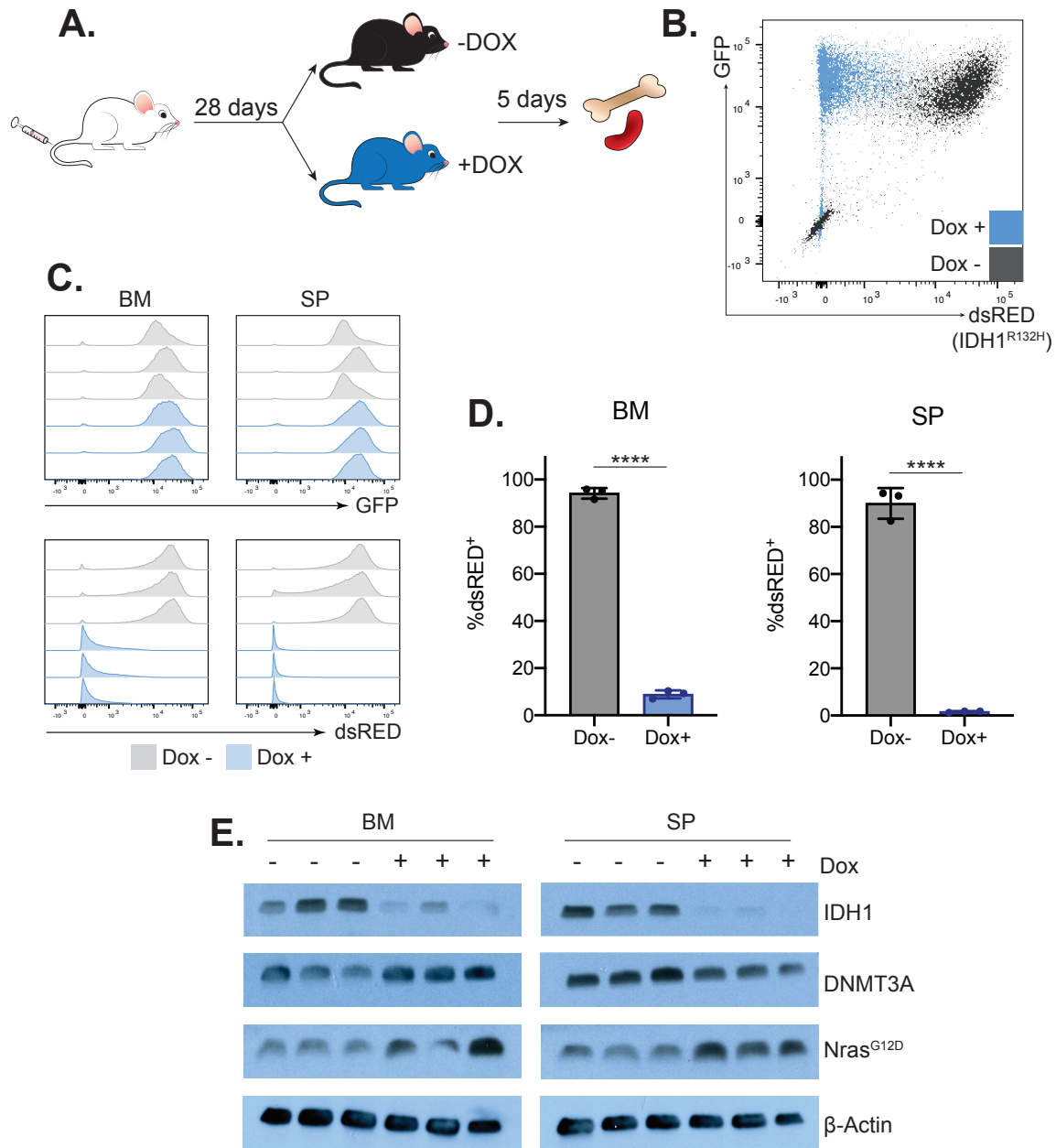
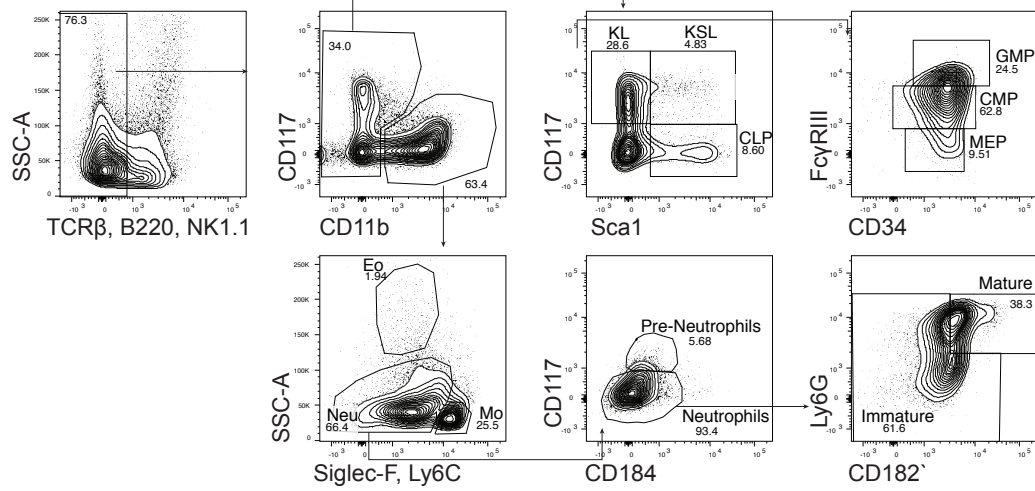


Figure 4.2 DOX Induced Repression of dsRED and *IDH1*^{R132H} Expression in IDN Cells

(A) Schematic of experimental procedure for *in vivo* dox de-induction (n = 3 / group). (B) Overlay of representative FACS plots from NSG recipients that received a DOX supplemented (DOX⁺) or normal (DOX⁻) diet. (C) Histograms of FACS sorted GFP⁺ IDN cells harvested from the bone marrow or spleen of recipient mice 5 days after +/- DOX supplementation. (D) Percentage of dsRED⁺ cells within the GFP⁺ compartment in the bone marrow and spleen. Means were compared using two-tailed student's t-test. **** p < 0.0001. (E) Immunoblots of FACS sorted GFP⁺ IDN cells harvested from the bone marrow or spleen of recipient mice 5 days after +/- DOX supplementation. BM; bone marrow, SP; spleen.

A. C57BL/6



B. IDN

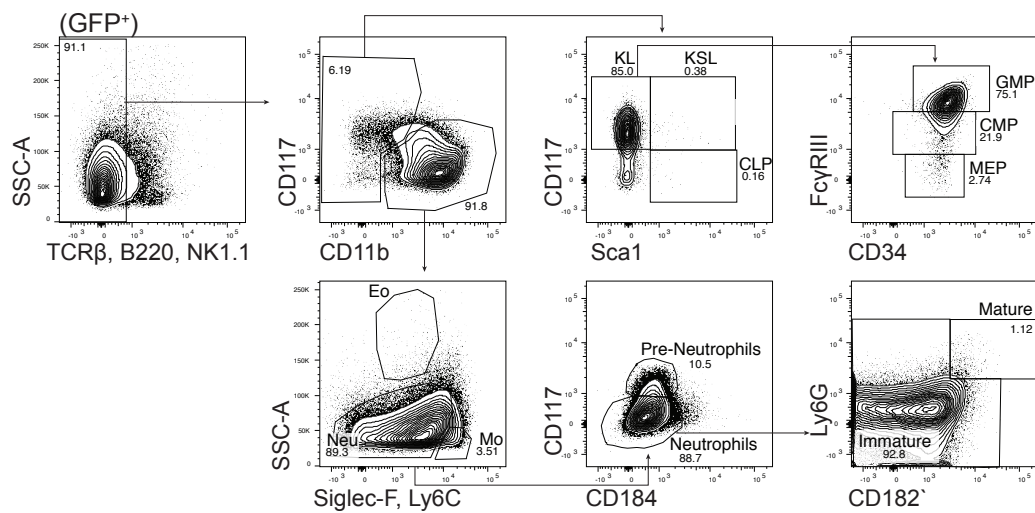
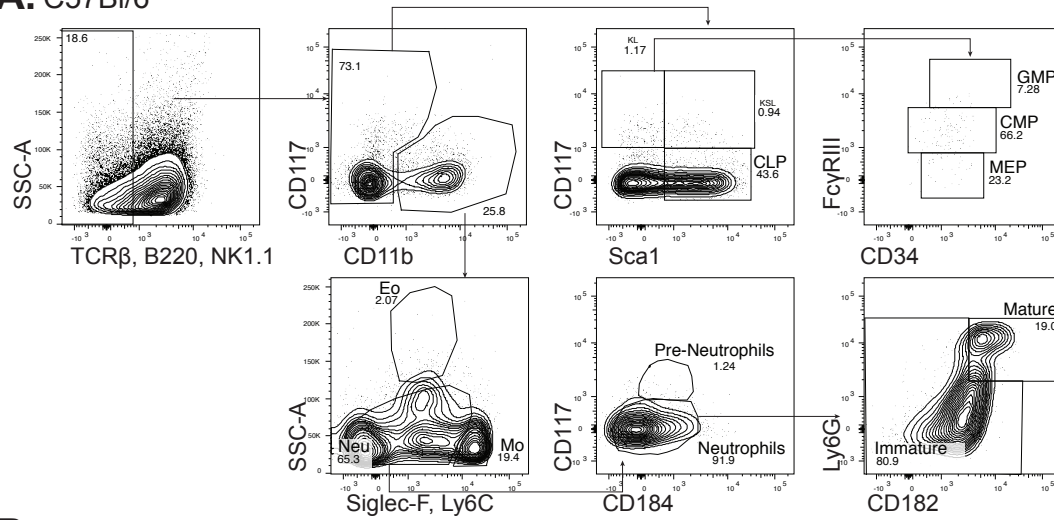


Figure 4.3 Gating Strategy for Immunophenotyping the Myeloid Lineage in the Bone Marrow

Representative FACS plots for the immunophenotyping along the myeloid haematopoietic lineage of cells from the bone marrow of (A) C57BL/6 mice and (B) IDN tumours harvested from moribund NSG recipients. Eo; eosinophils, Neu; neutrophils, Mo; monocytes.

A. C57BL/6



B. IDN

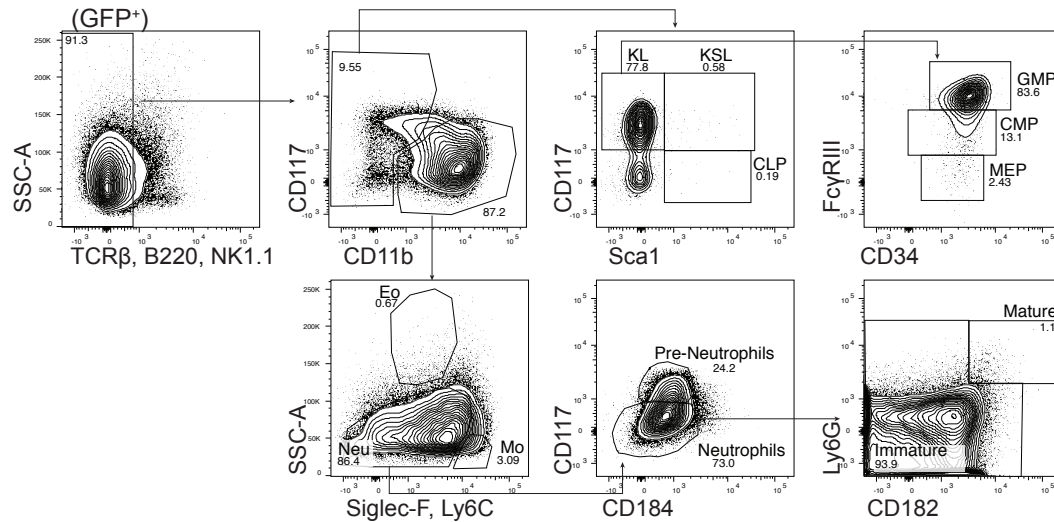


Figure 4.4 Gating Strategy for Immunophenotyping the Myeloid Lineage in the Spleen

Representative FACS plots for the immunophenotyping along the myeloid haematopoietic lineage of cells from the spleens of (A) C57BL/6 mice and (B) IDN tumours harvested from moribund NSG recipients. Eo; eosinophils, Neu; neutrophils, Mo; monocytes.

AML is characterised by the uncontrolled proliferation of immature myeloid progenitor cells (Corces et al., 2017; De Kouchkovsky and Abdul-Hay, 2016). A longitudinal limiting-dilution transplantation assay of the two most immature IDN cell-types, CMPs and GMPs, was utilised to identify the haematopoietic cell-type within the IDN model with the greatest leukaemia initiating potential (Figure 4.5 A). IDN CMP and GMP cells from the bone marrow of mice that previously succumbed to AML were isolated by FACS according to the gating strategy in Figure 4.5 B. NSG recipient mice were subsequently transplanted with 8×10^4 , 8×10^3 or 8×10^2 IDN myeloid progenitor cells. Mice transplanted with CMPs were the first to succumb to AML, with a median survival of 31, 35 and 46 days in order of decreasing transplanted cell number (Figure 4.5 C). IDN CMPs initiated disease in transplanted mice with a late-stage disease immunophenotype highlighted by the predominance of GMP cells in the KL compartment, and immature neutrophils within the mature myeloid lineage in the bone marrow and spleen. This immunophenotype was analogous to recipient mice transplanted with bulk-IDN tumours (Figure 4.5 D – E). Since mice transplanted with IDN GMP cells developed myeloid leukaemia with significantly longer latencies in comparison to IDN CMP transplanted recipients, it strongly suggested that IDN CMP cells possessed the greatest leukaemia initiating capacity (Figure 4.5 C).

Mice transplanted with 8×10^4 IDN GMP cells developed lethal myeloid leukaemia with a mean of 50 days and an analogous late-stage disease immunophenotype to that of IDN CMP transplanted recipients (Figure 4.5 F). Two NSG mice transplanted with 8×10^3 IDN GMP cells were culled prior to the onset of AML symptoms due to injuries sustained from murine conflict. Tumour burden was significantly lower in these recipients (Figure 4.5 G) and immunophenotyping unexpectedly revealed CMPs to be the principal cell type within the KL compartment (Figure 4.5 H). These observations further corroborated that the IDN CMPs were indeed the leukaemia initiating cell subtype with the capacity to produce all downstream lineages. It is likely the isolation of IDN CMPs and GMPs by FACS did not yield pure populations, but rather enriched for the respective population which contained a small fraction of the alternate cell-type. A strong linear relationship exists between the number of IDN CMP cells transplanted and the length of survival post-transplant (Figure 4.5 I). Extrapolation from this linear model predicts that the presence 0.1 – 0.3% IDN CMPs within the IDN GMP enriched population could account for the survival length in the 8×10^4 IDN GMP transplanted cohort; according to this model, only 160 ± 70 IDN CMPs within the 80,000 IDN GMP cells transplanted would result in a survival length ranging from 49 – 52 days (Figure 4.5 I). It is

important to note that predictions generally become increasingly unreliable when extrapolating and hence further experimental evidence would be required to definitively ascertain the leukaemia initiating potential of IDN GMP cells.

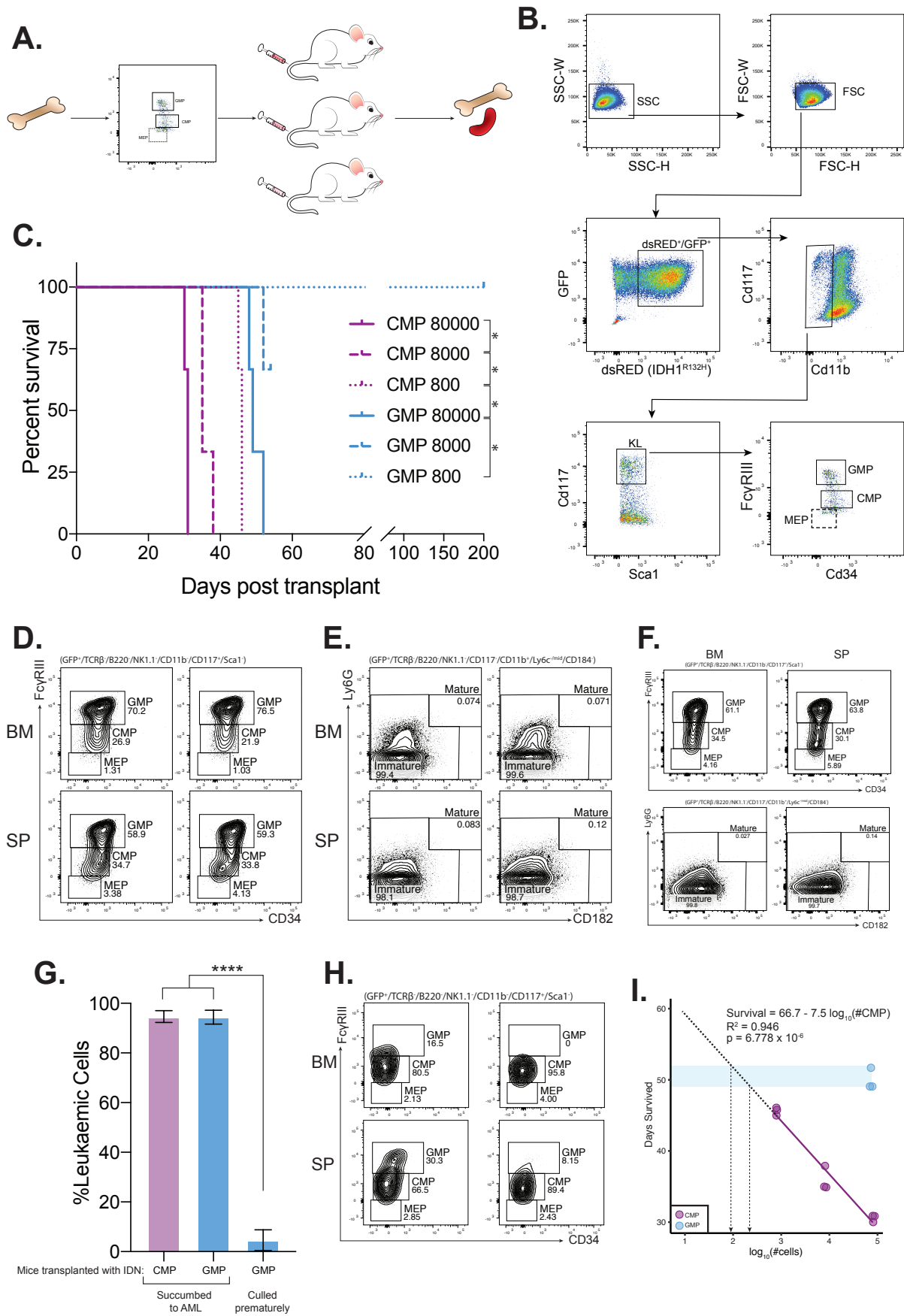


Figure 4.5 The IDN CMP Compartment Possessed the Greatest Leukaemia Initiating Capacity

(A) Schematic of experimental procedure for the serial dilution of IDN CMP and GMP cells. **(B)** Gating strategy for cell sorting IDN derived CMP and GMP cells. **(C)** Kaplan-Meier survival curve for NSG recipients transplanted with serially diluted CMP or GMP IDN tumour cells ($n = 3$ / group). Survival distributions were compared using log-rank Mantel-Cox test. * $p < 0.05$. **(D)** Representative FACS plots of the immature KL compartment within the BM and SP of mice transplanted with IDN CMP cells. **(E)** Representative FACS plots of the neutrophil compartment within the BM and SP of mice transplanted with CMP IDN cells. **(F)** Representative FACS plot of the immature KL compartment and neutrophil compartment within the BM and SP from mice transplanted with 8×10^4 IDN GMP cells. **(G)** Percentage of leukaemic cells (GFP⁺) within the bone marrow. Means compared using student's t-test. **** $p < 0.0001$. **(H)** FACS plots of the immature KL compartment within the BM and SP of two recipient NSGs transplanted with 8×10^3 IDN GMP cells, that were culled premature to the onset of AML symptoms, with wounds resulting from murine conflict. **(I)** Purple line represents the linear relationship between the number of IDN CMP cells transplanted and length of survival, and the continuation of this model (dashed line) represents the region of extrapolation. The highlighted blue rectangle depicts the survival length range for recipients transplanted with 8×10^4 IDN GMP cells. Dashed arrows indicate the predicted range of the numbers of transplanted IDN CMPs that would result in a survival length range comparable to the recipients transplanted with 8×10^4 IDN GMP cells. BM; bone marrow, SP; spleen.

4.3.2 Genetic Depletion and Pharmacological Inhibition of IDH1^{R132H} Conferred a Survival Advantage in the IDN *In Vivo* Model

It was previously demonstrated that the sustained expression of IDH2^{R140Q} was essential for maintaining the leukaemic phenotype in established IDH2^{R140Q} driven leukaemias (Kats et al., 2017). Hence, it was hypothesised that the continual expression of IDH1^{R132H} would be essential for maintaining the leukaemic phenotype in established IDN tumours. A longitudinal study was conducted whereby NSG recipients transplanted with a single IDN leukaemia were allocated into vehicle, DOX or AG120 administered cohorts following a 14-day engraftment period, allowing for the direct comparison between the genetic depletion (DOX) and pharmacological inhibition (AG120) of IDH1^{R132H} *in vivo* (Figure 4.6 A). Treatment was delivered for 28 days; the DOX cohort was provided with DOX supplemented food and water, whilst the remaining two cohorts received vehicle or AG120 (150 mg AG120 / kg body weight) administered by oral gavage twice daily in volumes proportional to body weight (10 mL / kg). Disease progression was monitored throughout the experiment through weekly analyses of peripheral blood samples (Figure 4.6 C – J). Tumour burden, assessed by flow-cytometry, was quantified as the proportion or absolute number of leukaemic cells (union of GFP⁺ and dsRED⁺ cells) per microlitre of peripheral blood (Figure 4.6 C – E). Following the engraftment period, the number of peripheral leukaemic cells exhibited exponential growth in the vehicle treated cohort, whilst the number of leukaemic cells within the periphery began to plateau in the second week of DOX and AG120 treatment (Figure 4.6 D – E) which corresponded with the first significant reduction in tumour burden relative to vehicle (Figure 4.6 E). The number of white blood cells present in the periphery paralleled the number of leukaemic cells in the peripheral blood in all cohorts (Figure 4.6 F). By 28 days post-transplant, the vehicle treated cohort had developed thrombocytopenia, marked by low platelet counts, whilst platelet counts for the DOX and AG120 treated cohorts remained relatively steady throughout the duration of the experiment (Figure 4.6 G). The vehicle-treated cohort displayed mild anaemia as the disease advanced, evident by the reduction in red blood cells (RBC) (RBC count of wildtype 6-month old NSG mice is $\sim 8 \times 10^{12}$ / L (Knibbe-Hollinger et al., 2015)) (Figure 4.6 H). Liquid chromatography/mass spectrometry confirmed the presence of 2-HG in the plasma of the vehicle cohort, which accumulated exponentially from week to week (Figure 4.6 I – J). Significant suppression of 2-HG in the DOX and AG120 treated cohorts was apparent 1 week after treatment was initiated (Figure 4.6 J). Furthermore, a strong linear relationship existed between the number of leukaemic cells per microlitre of peripheral blood and the 2-HG plasma concentration, in the vehicle treated cohort (Figure 4.6 K). The decrease in leukaemia cell

number, 2-HG suppression and lack of thrombocytopenia in response to DOX and AG120 treatment, was consistent with the significantly prolonged overall survival rates relative to the vehicle-treated cohort (Figure 4.6 B). This demonstrates that AG120 is highly effective as a single agent in the IDN *in vivo* model.

Despite the efficacy of AG120 as a single agent, two AG120 treated mice subsequently succumbed to myeloid leukaemias, hereafter referred to as the relapsed AML (rAML) leukaemias, 32 and 81 days after treatment cessation (Figure 4.7). Although AG120 therapy did not eradicate the disease in these two individuals, it significantly prolonged their survival, which may suggest the expansion of cells resistant to AG120 therapy. At the ethical cull-point, symptoms displayed by the rAML mice and vehicle treated cohort included loss of body weight, hunched posture, ruffled coat and slow movements. rAML mice exhibited similar phenotypic characteristics to that of vehicle-treated mice including: high tumour infiltration in the bone marrow and spleen (Figure 4.7 A – B), splenomegaly (Figure 4.7 C), supraphysiologic plasma 2-HG levels (Figure 4.7 D), elevated white blood cell counts (Figure 4.7 E) and thrombocytopenia (Figure 4.7 F). Additionally, the rAML mice exhibited significantly lower red blood cell counts (Figure 4.7 G), when compared to the vehicle treated cohort, indicative of pronounced anaemia. The leukaemic cells from rAML mice succumbed to myeloid leukaemia evident by the expression of CD117 and CD11b (Figure 4.7 H). Collectively, these data demonstrate that although AG120 is a highly effective therapeutic for the treatment of IDH1^{R132H} AML, 4-weeks of AG120 therapy does not eradicate the leukaemia in all IDN transplanted individuals. This may suggest that a longer treatment timeframe is required to eliminate all leukaemic cells, or that a proportion of leukaemic cells are resistant to AG120.

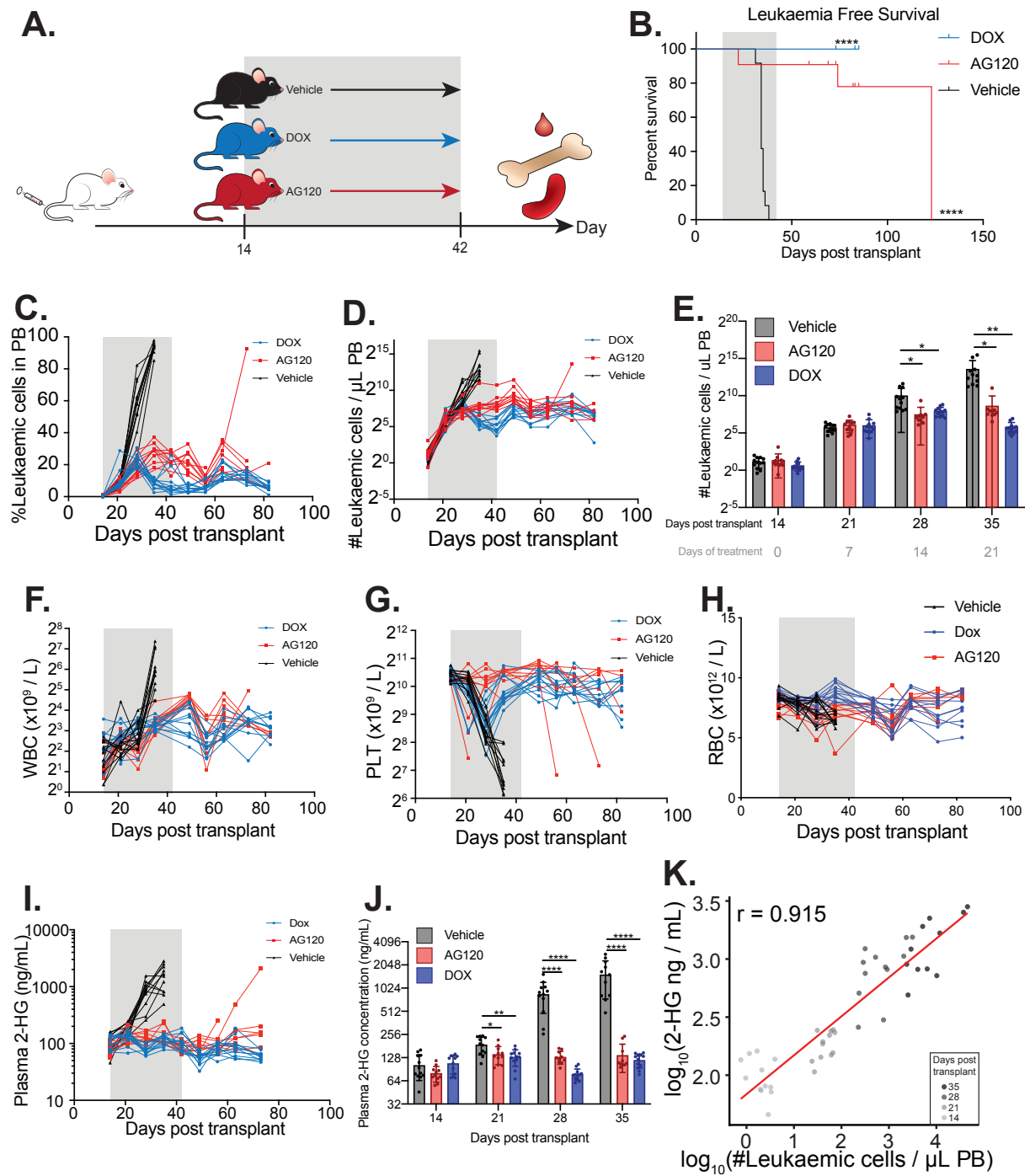


Figure 4.6 Genetic Depletion and Pharmacological Inhibition of IDH1^{R132H} Conferred a Survival Advantage for IDN Recipient Mice

(A) Schematic of the experimental procedure for DOX de-induction and pharmacological inhibition (AG120) of IDH1^{R132H} *in vivo*. N = 12 / group. **(B)** Kaplan-Meier disease free survival curve. Survival curves were compared with log-rank Mantel-Cox test. Longitudinal **(C)** percentage and **(D)** number of leukaemia cells (GFP⁺ ∪ dsRED⁺) per microlitre of peripheral blood (PB). Each line represents an individual mouse. **(E)** Mean ± SD of the number of leukaemic cells per microlitre of peripheral blood. **(F)** Longitudinal white blood cell (WBC) counts, **(G)** platelet (PLT) counts, **(H)** red blood cell (RBC) counts and **(I)** plasma 2-HG concentrations. Each line represents an individual mouse. **(J)** Mean ± SD of plasma 2-HG concentration. **(K)** Positive correlation between the number of leukaemia cells per microlitre of PB and plasma 2-HG levels from moribund vehicle treated mice. Means compared using student's t-test. * 0.01 < p < 0.05, ** 0.001 < p ≤ 0.01, *** 0.0001 < p ≤ 0.001, **** p < 0.0001.

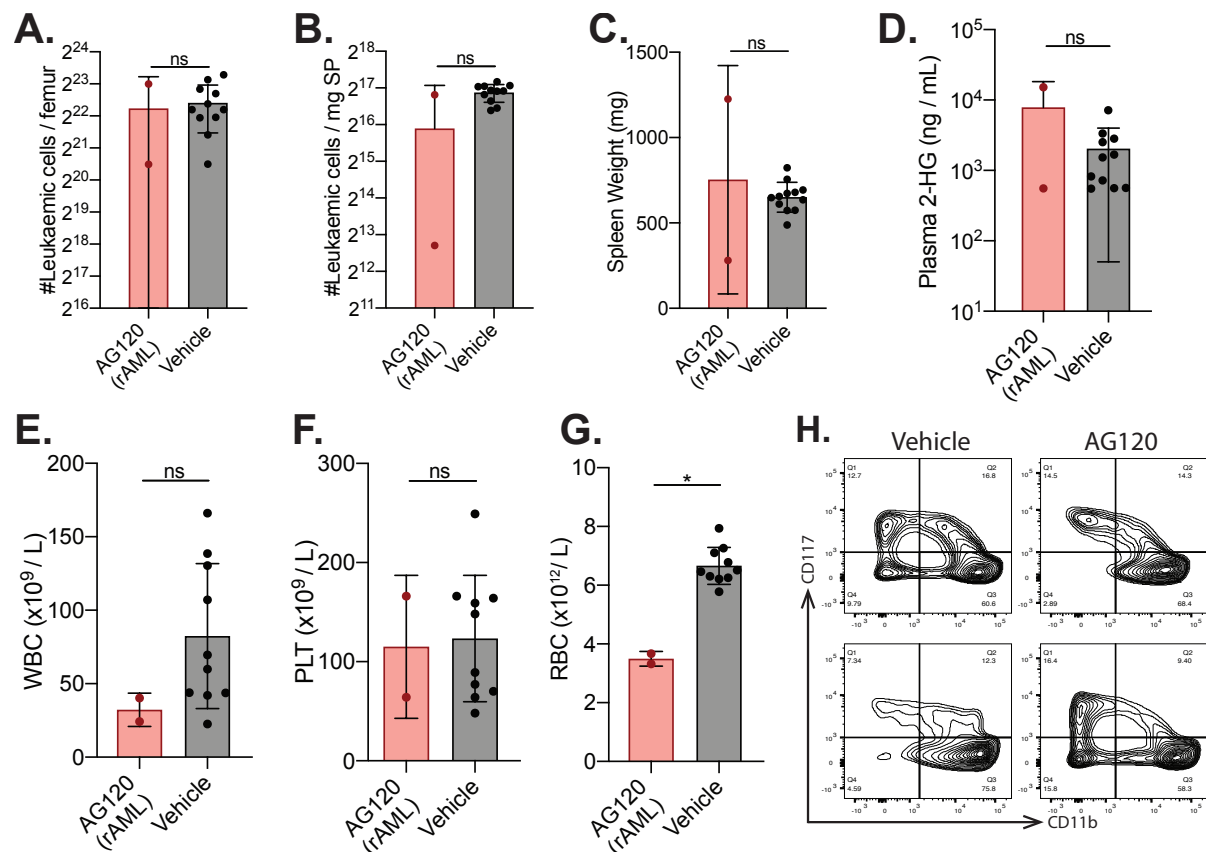


Figure 4.7 IDN Recipient Mice That Relapse Post-AG120 Therapy Succumb to a Myeloid Leukaemia

Number of leukaemic cells ($\text{GFP}^+ \cup \text{dsRED}^+$) per (A) femur and (B) milligram of spleen. (C) Spleen weight. (D) Mean plasma 2-HG concentration. (E) White blood cell (WBC) counts. (F) Platelet (PLT) counts. (G) Red blood cell (RBC) counts. (H) Representative FACS plot from the bone marrow of two vehicle and AG120 (rAML) recipient mice. Error bars represent mean $\pm$ SD. Statistical differences in the means were calculated using non-parametric unpaired student's t-test. * $0.01 < p < 0.05$, ** $0.001 < p \leq 0.01$, *** $0.0001 < p \leq 0.001$, **** $p < 0.0001$, not significant (ns) $p \geq 0.05$. rAML; relapsed AML.

4.3.3 Overcoming the Occurrence of GvHD in AG120 Treated IDN Recipient Mice

Although the majority of AG120 and DOX treated mice did not succumb to a myeloid leukaemia post-treatment cessation (Figure 4.6 A), 12/12 DOX and 9/12 AG120 treated mice subsequently developed symptoms consistent with chronic Graft versus Host Disease (GvHD) including loss of hair accompanied by dry and scaly skin (Figure 4.8 B), thickened and gassy gastrointestinal tract, diarrhoea, and other clinical features detailed in Table 4.1 (Ramachandran et al., 2019). These symptoms were not observed in the rAML mice (Figure 4.8 C). GvHD is a possible complication in leukaemia patients receiving allogenic haematopoietic stem cell transplants, arising when the donor-derived immune cells (specifically T-cells) attack the host's organs (Presland, 2016; Ramachandran et al., 2019).

Moribund DOX and AG120 treated mice that exhibited overt GvHD symptoms, displayed marked differences in tumour burden, spleen weight and blood cell counts when compared to mice that succumbed to symptoms of myeloid leukaemia (Figure 4.8). Overall leukaemia burden, indicated by IDN cell infiltration within the bone marrow and spleen, total white blood cell counts, spleen weight and plasma 2-HG concentration, was lower at the ethical cull time-point in GvHD symptomatic mice when compared to vehicle treated or rAML mice (Figure 4.8 D – H). Unlike the rAML recipient mice, GvHD symptomatic mice were not anaemic nor thrombocytopenic (Figure 4.8 I – J).

The IDN derived cells residing in the bone marrow and spleen of GvHD symptomatic mice were predominantly expressing the T-cell marker, TCR β , (Figure 4.9 A – B) with a smaller proportion of cells expressing markers of HSCs / myeloid progenitors (CD117) and myeloid maturation (CD11b) (Figure 4.9 C – F). Furthermore, IDN cells from GvHD symptomatic mice exhibited an expansion in the CLP compartment at the expense of the myeloid progenitor population (Figure 4.10 A – B). In contrast, the leukaemic cells from rAML mice primarily expressed CD117 and CD11b, with minimal TCR β expression (Figure 4.9) and no expansion of the CLP population (Figure 4.10 A – B). Of note, 3 mice within the AG120 treated and GvHD symptomatic group harboured dsRED⁺/GFP⁺ cells expressing CD117 and CD11b at proportions akin to the vehicle treated cohort, potentially signifying the coexistence of cells that could give rise to either myeloid leukaemia or GvHD. Collectively, these observations provide further evidence for the development of GvHD in these recipients.

Table 4.1 Symptoms of GvHD in AG120 and DOX Treated Mice

Skin	Loss of hair accompanied by dry and scaly skin around the periaural, periorbital, muzzle, chin, ventral abdomen, perianal, tail and lateral thigh areas. Severe diffuse crusting with hyperkeratosis infiltrations of mixed neutrophils and lymphocytes. The basal layer of the epidermis shows spongiosis with apoptosis.
Eyes	Dry eyes.
Liver	Periportal infiltration of mixed inflammatory infiltrates.
Pancreas	Mixed interstitial inflammatory infiltrates.
Stomach	Enlarged/obstructed.
Gastrointestinal tract	Thickened and gassy. Diarrhoea.
Lung	Marked diffuse perivascular and peribronchiolar lymphoplasmacytic infiltrates.

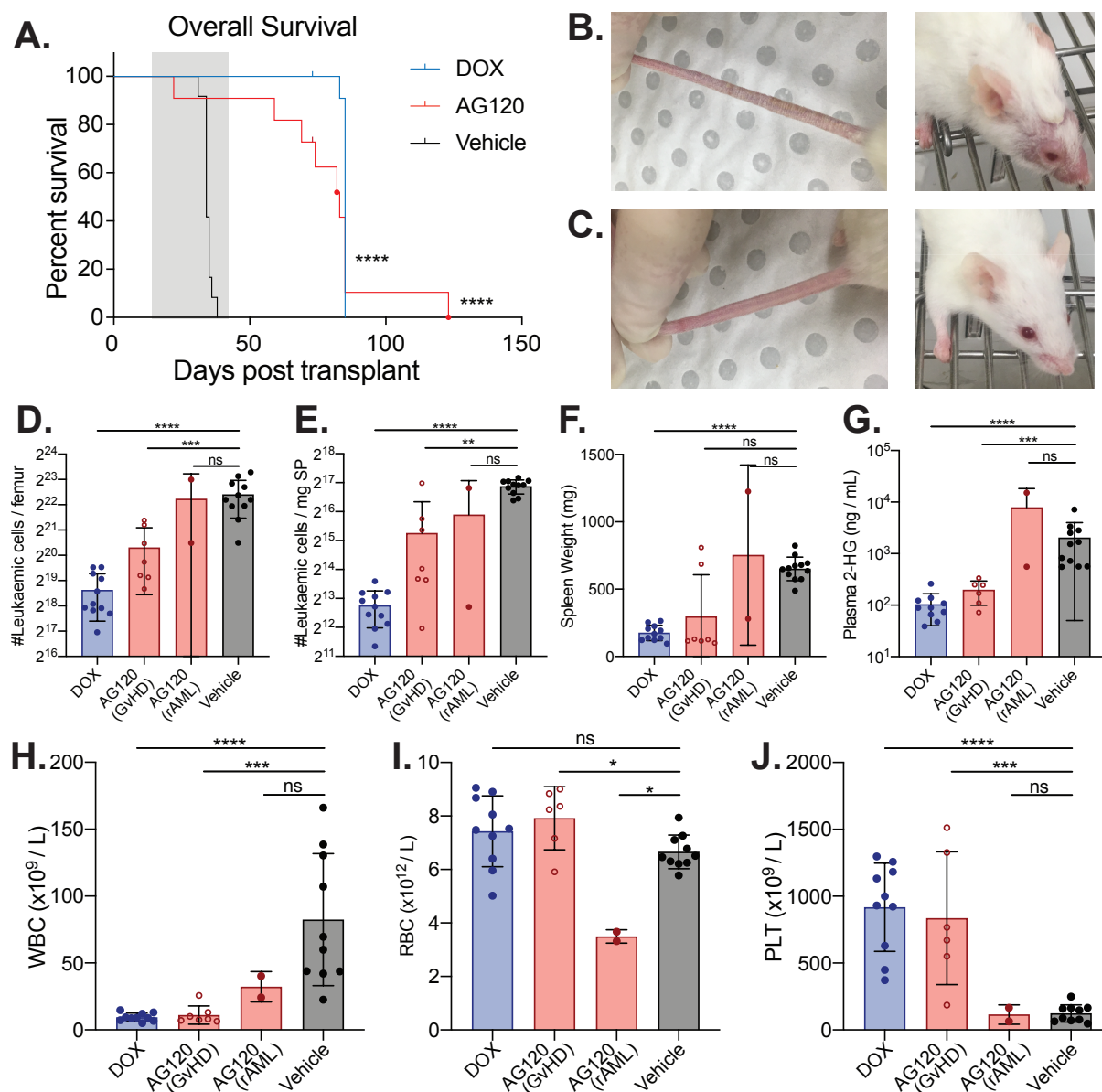


Figure 4.8 Characteristics of Moribund Mice

(A) Overall survival curve. Two mice that exhibited GvHD symptoms were sacrificed for pathophysiological analysis and were censored on the curve. Red circles correspond to the rAML mice. Survival curves were compared with log-rank Mantel-Cox test. (B) Representative photos of external physical symptoms of a recipient mouse with GvHD, in comparison to an (C) rAML mouse with low tumour burden. (D – E) Number of leukaemic cells per (D) femur and (E) milligram of spleen. (F) Spleen weight. (G) Mean plasma 2-HG concentration. (H) White blood cell counts. (I) Red blood cell counts. (J) Platelet counts. Error bars represent mean $\pm$ SD. Statistical differences in the means were calculated using non-parametric unpaired student's t-test. * $0.01 < p < 0.05$, ** $0.001 < p \leq 0.01$, *** $0.0001 < p \leq 0.001$, **** $p < 0.0001$, not significant (ns) $p \geq 0.05$. rAML; relapsed AML, GvHD; Graft versus Host Disease.

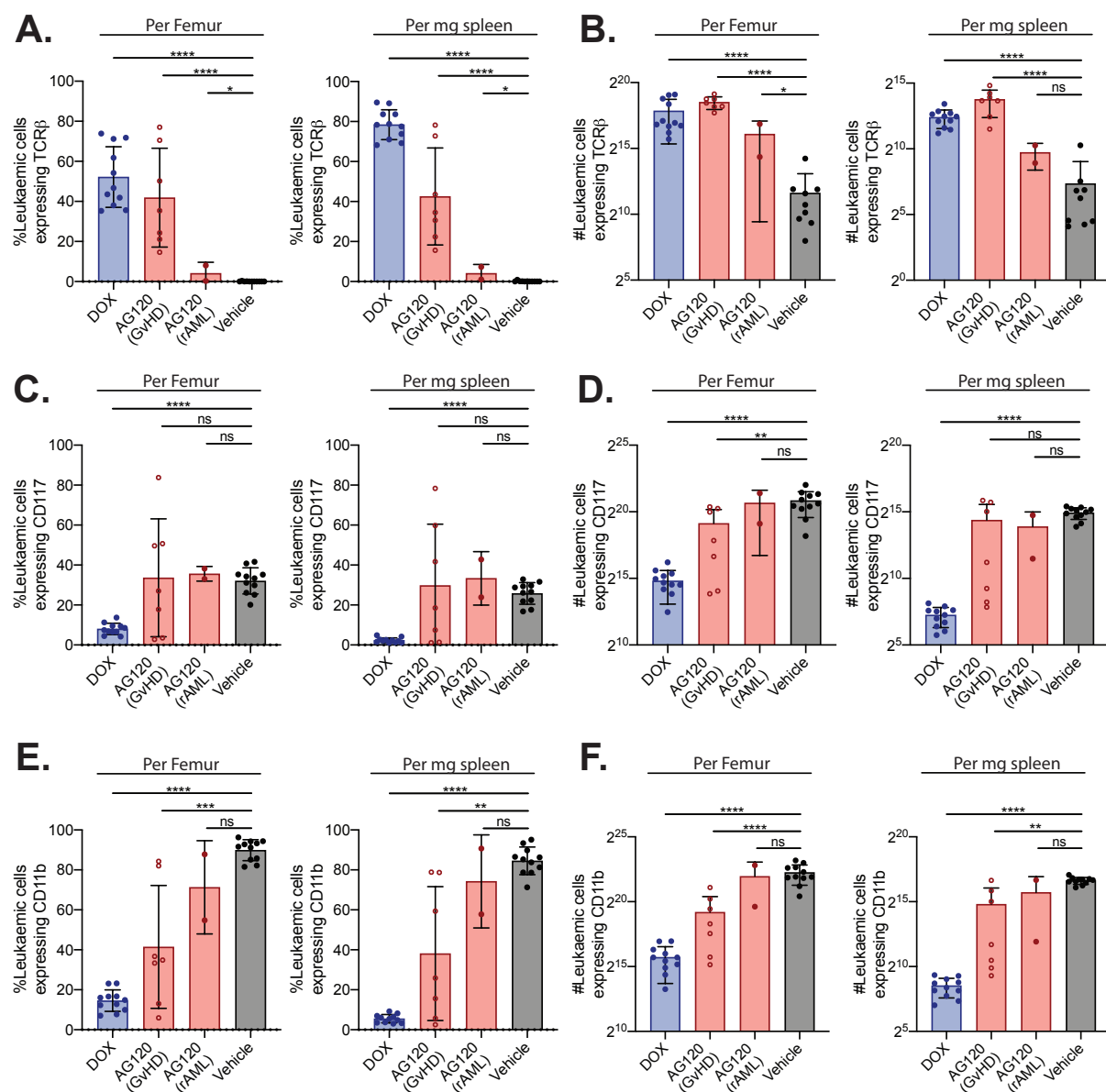


Figure 4.9 Immunophenotype of Moribund Mice

Mean (A) proportion and (B) number of leukaemic cells expressing TCRβ in the bone marrow and spleen. Mean (C) proportion and (D) number of leukaemic cells expressing CD117 in the bone marrow and spleen. Mean (E) proportion and (F) number of leukaemic cells expressing CD11b in the bone marrow and spleen. Error bars represent mean $\pm$ SD. Statistical differences in means were calculated using non-parametric unpaired student's t-test. * $0.01 < p < 0.05$, ** $0.001 < p \leq 0.01$, *** $0.0001 < p \leq 0.001$, **** $p < 0.0001$, ns; $p \geq 0.05$. rAML; relapsed AML, GvHD; Graft versus Host Disease.

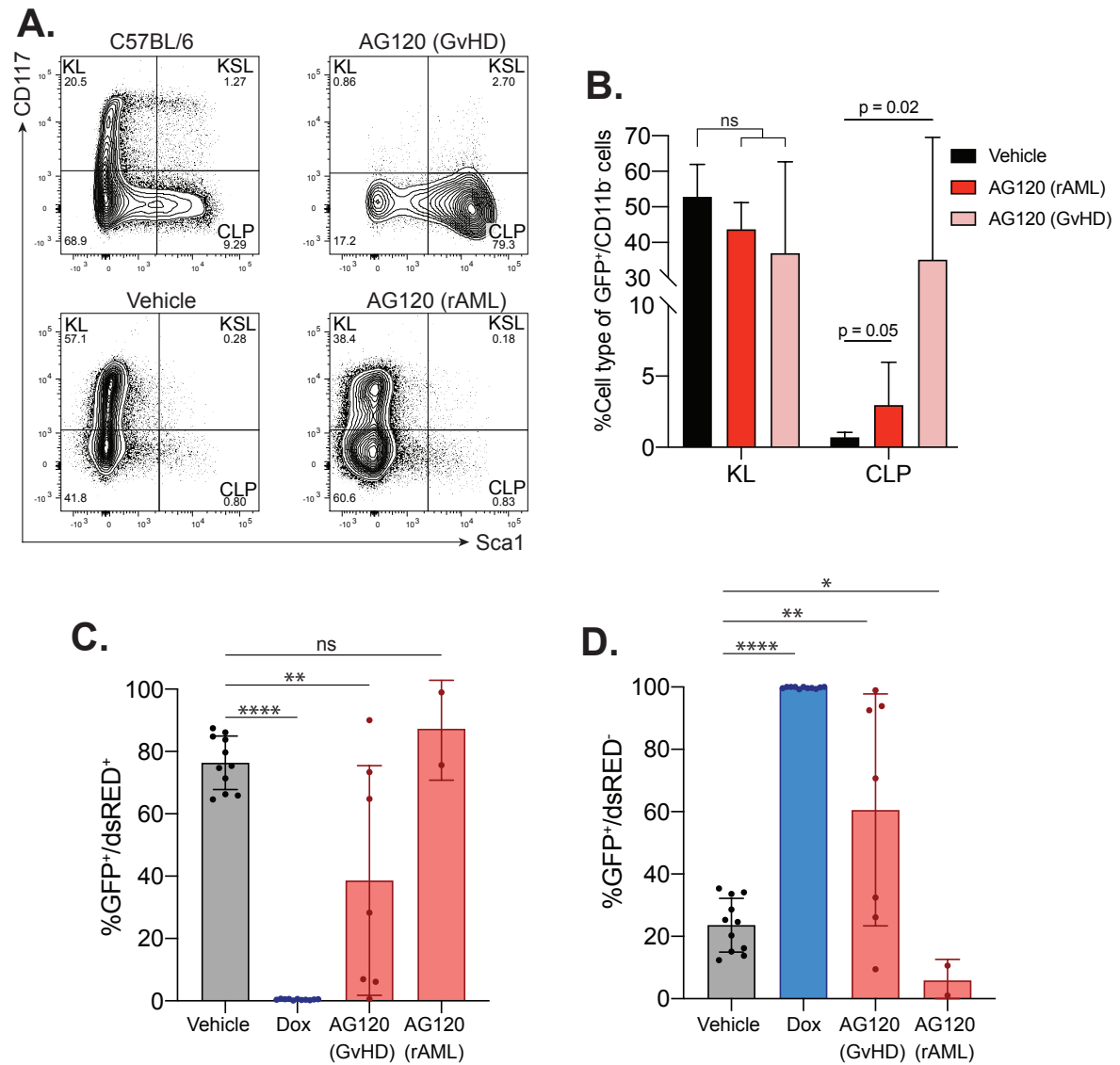


Figure 4.10 Expansion of CLP Compartment in GvHD Symptomatic IDN Mice

(A) Representative bone marrow FACS plots gated on CD11b⁺ cells for wildtype (C57BL/6) and GFP⁺/CD11b⁺ cells for IDN mice. (B) Mean proportion of KL and CLP cells in the GFP⁺/CD11b⁺ population within the bone marrow of IDN recipient mice. (C) Percentage of GFP⁺/dsRED⁺ and (D) GFP⁺/dsRED⁻ cells as a fraction of all IDN cells in moribund mice. Error bars represent mean ± SD. Statistical differences in mean proportions was calculated using student's t-test. * 0.01 < p < 0.05, ** 0.001 < p ≤ 0.01, *** 0.0001 < p ≤ 0.001, **** p < 0.0001, ns; p ≥ 0.05. rAML; relapsed AML, GvHD; Graft versus Host Disease.

The development of GvHD in the majority of DOX and AG120 treated mice was likely a result of the methods used to generate the IDN model. Although the retrovirally transduced foetal liver cells, used to generate the IDN model, were cultured in cytokine supplemented media to promote the outgrowth of haematopoietic stem cells and myeloid progenitors, it is possible that a small proportion of cells survived with the capacity to produce the lymphoid lineage. This is supported by the small proportion (< 1%) of CLPs present in the vehicle treated IDN cohort (Figure 4.10 A – B). Since the genetic depletion and pharmacological inhibition of IDH1^{R132H} were both extremely effective at depleting the leukaemic population (GFP⁺/dsRED⁺) (Figure 4.6 C), it is possible that, under DOX and AG120 treated conditions, the CLP population was able to expand and produce T-cells that would induce an autoimmune response. Of note, at the ethical cull-point, the predominant transplanted cell type in the vehicle and rAML mice were the triple mutant IDN cells (dsRED⁺/GFP⁺), whilst an expansion in the dsRED⁻/GFP⁺ population was observed in GvHD recipients (Figure 4.10 C – D), suggesting that GvHD initiating cells largely lacked the expression of IDH1^{R132H}, and were mostly driven by the expression of *DNMT3A*^{R882H} and/or *Nras*^{G12D}/*DNMT3A*^{R882H}.

To reduce the incidence of GvHD, the viable dsRED⁺/GFP⁺/lymphoid⁻ cells were isolated by FACS from a single vehicle-treated IDN leukaemia (termed IDN-AML-veh cells) and transplanted into NSG recipients (Figure 4.11 A – B). Following the 14-day engraftment period, recipients were administered either AG120 or vehicle, as previously described. The AG120-treated cohort were monitored for characteristics of GvHD. IDN-AML-veh cells were isolated by FACS and were re-transplanted into NSG recipients. The second round of IDN-AML-veh cell enrichment resulted in an ~85% reduction in the incidence of GvHD in the AG120-treated cohort (Figure 4.11 C). IDN-AML-veh cells from this cohort were cryopreserved and used in subsequent experiments. These experiments provided further evidence that the occurrence of GvHD was linked to the presence of *DNMT3A*^{R882H} or *DNMT3A*^{R882H}/*Nras*^{G12D} expressing lymphoid-lineage cells, and that negatively enriching this population was effective at reducing the prevalence of GvHD in the AG120 treated cohort.

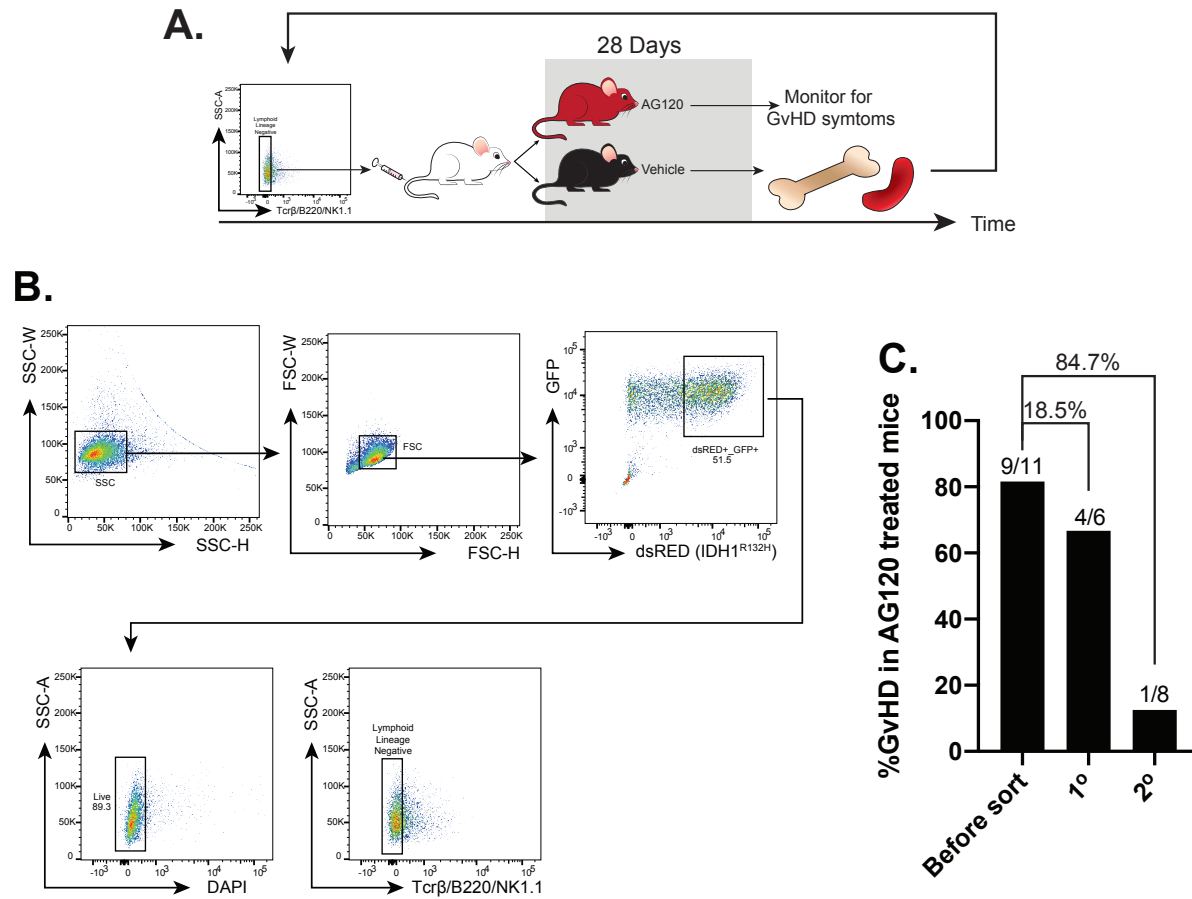


Figure 4.11 Experimental Procedure to Reduce the Occurrence of GvHD

(A) Schematic of the experimental procedure to reduce the occurrence of GvHD in IDN recipients treated with AG120. **(B)** Gating strategy for cell sorting IDN cells lacking the expression of lymphoid lineage cell-surface markers. **(C)** Percentage of AG120 treated recipients that succumbed to GvHD following successive rounds of enrichment and transplant of IDN cells devoid of lymphoid lineage cell-surface markers. The number of mice with GvHD within each AG120 treated cohort are labelled above each column. The percentage reduction in the incidence of GvHD is provided.

4.3.4 Pharmacokinetics of AG120 *In Vivo*

To understand the pharmacokinetics of AG120 *in vivo*, NSG recipients were transplanted with an IDN leukaemia followed by a 28-day engraftment period before bone marrow and plasma concentrations of AG120 and 2-HG quantified 1, 4, and 12 hours after a single dose of AG120 was administered. From 1 to 12 hrs after AG120 was administered, the bone marrow and plasma levels of AG120 decreased by 61% and 50%, respectively (Figure 4.12 A, B). Although not statistically different, the mean number of leukaemic cells in the peripheral blood was consistently lower in the AG120 treated mice, when compared to vehicle (Figure 4.12 C). Given that prior experiments demonstrated that a reduction in tumour burden in the peripheral blood was first evident after 2 weeks of twice daily AG120 treatment (Figure 4.6 C – E), the number of leukaemic cells in the blood is expected to remain relatively constant during the first 12 hrs after a single dose of AG120. Hence, the variation in leukaemia burden between the vehicle- and AG120-treated cohorts over the 12-hours needed to be accounted for in order to reliably compare the differences in plasma 2-HG concentration. Since a strong linear relationship existed between the leukaemic cell number and plasma 2-HG concentration, the ratio of these two variables would remain relatively constant over time; as the leukaemic cell number increased, a proportionate increase would be seen in the level of 2-HG (Figure 4.6 K). However, acute AG120 treatment periods, whereby 2-HG reduction is observed prior to the depletion of the leukaemic population, would result in a decrease in the ratio between 2-HG concentration and leukaemic cell number. As such, normalisation to leukaemia burden revealed that a single dose of AG120 reduced the plasma 2-HG concentration by 14% when compared to the vehicle treated cohort (Figure 4.12 D).

The pharmacokinetics of AG120 in the bone marrow was subsequently assessed over the first 4 days of twice daily treatment. Mice were randomised amongst the two treatment cohorts. Although no statistical difference in tumour burden was observed prior to treatment initiation, the majority of the AG120 cohort had a pre-treatment tumour burden that was less than the mean of the vehicle cohort (Figure 4.13 A). To control for the variation observed in leukaemic cell number, the ratio of the 2-HG concentration and leukaemic cell number was plotted against the AG120 levels in the bone marrow (Figure 4.13 B). As expected, the ratio remained relatively constant for the vehicle treated cohort (Figure 4.13 B). In the AG120-treated cohort, as the concentration of AG120 increased over time, the ratio of 2-HG to tumour burden decreased (Figure 4.13 B), demonstrating that a decrease in 2-HG occurs prior to a reduction in leukaemia burden. Indications of AG120 induced differentiation were noticeable 3 days after

treatment initiation (Figure 4.13 C – D) despite no statistical reduction in overall tumour burden (Figure 4.13 E); by day 3, the leukaemic KL compartment was comprised of higher proportions of GMPs relative to CMPs in the AG120 cohort (Figure 4.13 C – D).

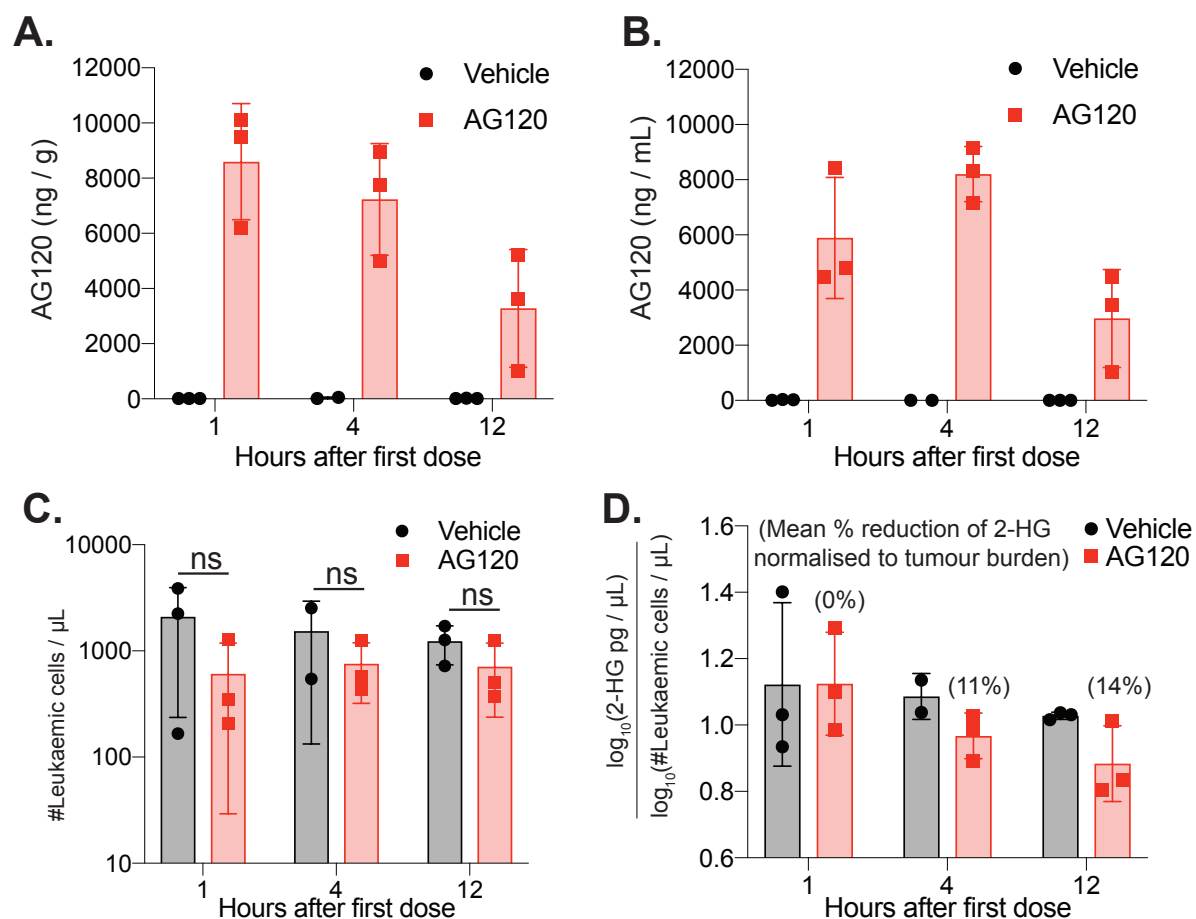


Figure 4.12 Pharmacokinetics of AG120

(A – B) Mean concentration of AG120 in the **(A)** bone marrow and **(B)** plasma. **(C)** Mean number of leukaemic cells (GFP⁺ $\cup$ dsRED⁺) per microlitre of peripheral blood. **(D)** Ratio between 2-HG concentrations and number of leukaemic cells per microlitre of peripheral blood. Means were compared using non-parametric unpaired student's t-test. ns; $p \geq 0.05$. Error bars represent mean $\pm$ SD of 2 – 3 replicates per group.

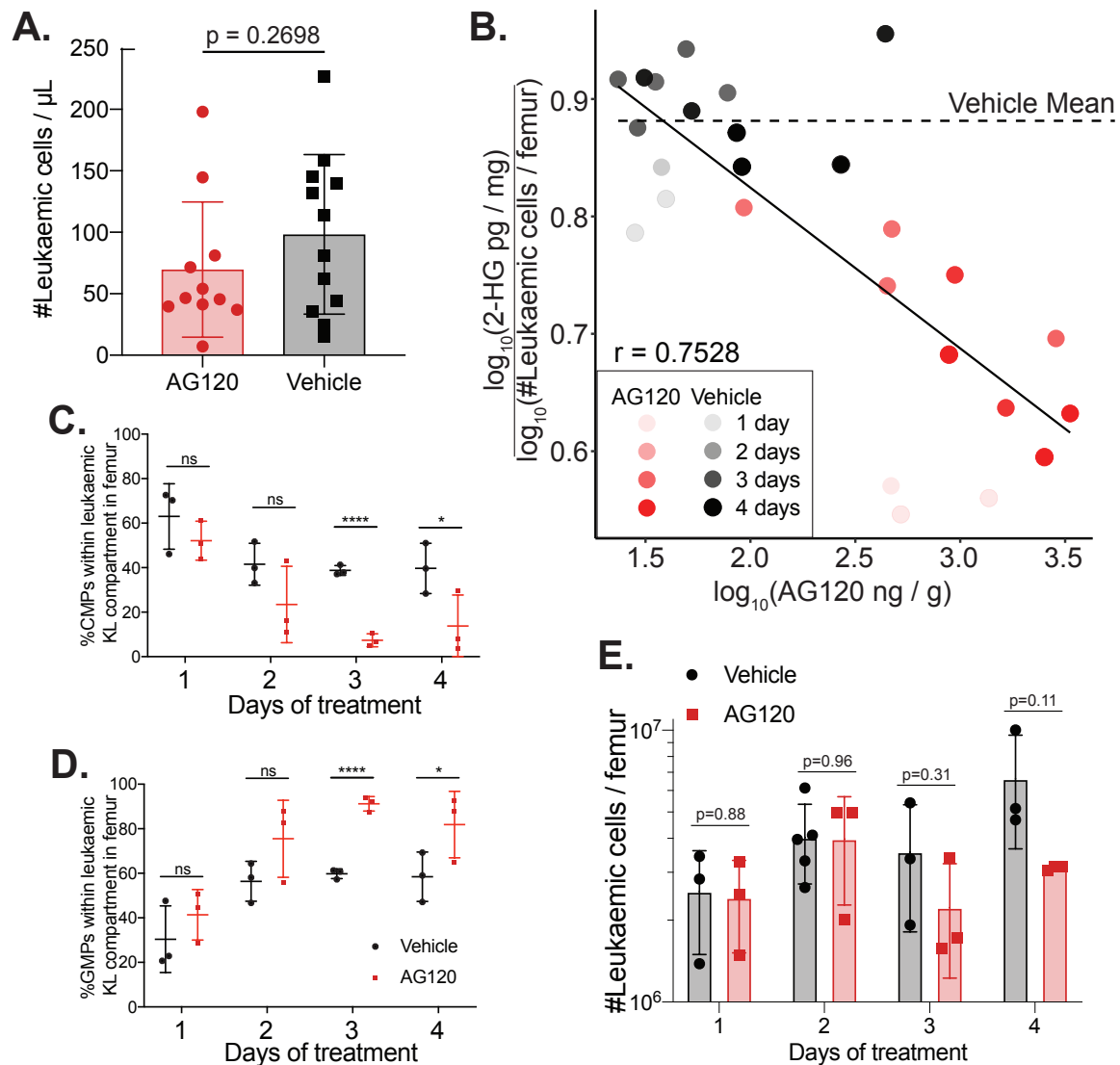


Figure 4.13 Pharmacokinetics of AG120 Over 4 Days

(A) Number of leukaemic cells ($\text{GFP}^+ \cup \text{dsRED}^+$) in the peripheral blood prior to treatment initiation. (B) Scatterplot of AG120 concentration relative to bone marrow weight versus the ratio between the 2-HG concentrations and number of leukaemic cells in the bone marrow. The greater the deviation below the vehicle mean line, the greater reduction in 2-HG relative to leukaemic cell number. Percentage of (C) common myeloid progenitors (CMPs) and (D) granulocytic myeloid progenitors (GMPs) within the leukaemic $\text{cKit}^+/\text{Lin}^-$ (KL) compartment in the bone marrow. (E) Average number of leukaemic cells per femur. Error bars represent mean $\pm$ SD of 2 – 3 replicates per group. Means were compared using two-tailed unpaired student's t-test. * $0.01 < p < 0.05$, ** $0.001 < p \leq 0.01$, *** $0.0001 < p \leq 0.001$, **** $p < 0.0001$, ns; $p \geq 0.05$.

4.3.5 Pharmacological Inhibition of IDH1^{R132H} Reversed Malignant Transcriptional Profiles

Distinct transcriptional states are associated with mutant IDH expression in both solid and blood cancer patients (Unruh et al., 2019). Hence, the following series of experiments were undertaken to determine whether the disruption of IDH1^{R132H} expression in IDN established tumours, by either genetic depletion or pharmacological inhibition, would elicit transcriptional changes that underpin the effects of these interventions on leukaemia burden. RNA sequencing was executed on the IDN myeloid progenitor population following 5 days of vehicle, DOX or AG120 administration (Figure 4.14 A) as this population encompasses the leukaemic stem cell (LSC) (Figure 4.5). Investigating the transcriptional perturbations induced by depletion of IDH1^{R132H} in the LSC would enable the deduction of pathways dysregulated by IDH1^{R132H} that are required for the maintenance of the leukaemia. The progenitor-like leukaemic population (GFP⁺/CD117⁺/CD11b⁻) residing within the bone marrow was isolated by FACS. Due to limited cell numbers, and hence lower RNA yields, QuantSeq 3' mRNA sequencing was performed as it requires fewer sequencing reads than total RNA sequencing.

The multi-dimensional scaling (MDS) plot in Figure 4.14 B is a visual representation of the variation in expression profiles between each sample across the two principal attributes that account for the largest variability between samples, such that the distances between samples proportionally reflect the degree of variation (Ritchie et al., 2015). The greatest amount of variation amongst the samples (Leading logFC dimension 1) is likely attributed to the treatment condition, whereby the greatest separation was between vehicle mice and the genetic depletion or pharmacological inhibition of IDH1^{R132H} (Figure 4.14 B). Moreover, unsupervised hierarchical clustering of the pair-wise Pearson's correlation coefficients, calculated from the normalised counts of the top 500 most variably expressed genes, demonstrated an analogous separation of samples with AG120 and DOX treated recipients clustering together yet distinct from the vehicle cohort (Figure 4.14 C – D). Differential gene expression analysis revealed 532 and 218 significantly differentially expressed genes (DEGs) ($FDR \leq 0.05$, $|\log_2(FC)| \geq 0.5$) in the AG120 and DOX treated cohorts, respectively, relative to vehicle, with significantly high overlap between the two datasets according to hypergeometric testing (188 overlapping genes, $p = 7 \times 10^{-213}$) (Figure 4.14 E). Furthermore, a strong and positive correlation existed between the fold-changes of the significantly DEGs elicited in response to depletion and inhibition of IDH1^{R132H}, signifying that the directionality of the DEGs were consistent between DOX and AG120, even amongst non-overlapping DEGs (Figure 4.14 F). Collectively, these results

indicate a high degree of similarity in the transcriptional profiles of the IDH1^{R132H} genetically depleted and pharmacologically inhibited cohorts, which appear distinct from the unimpeded IDN tumours.

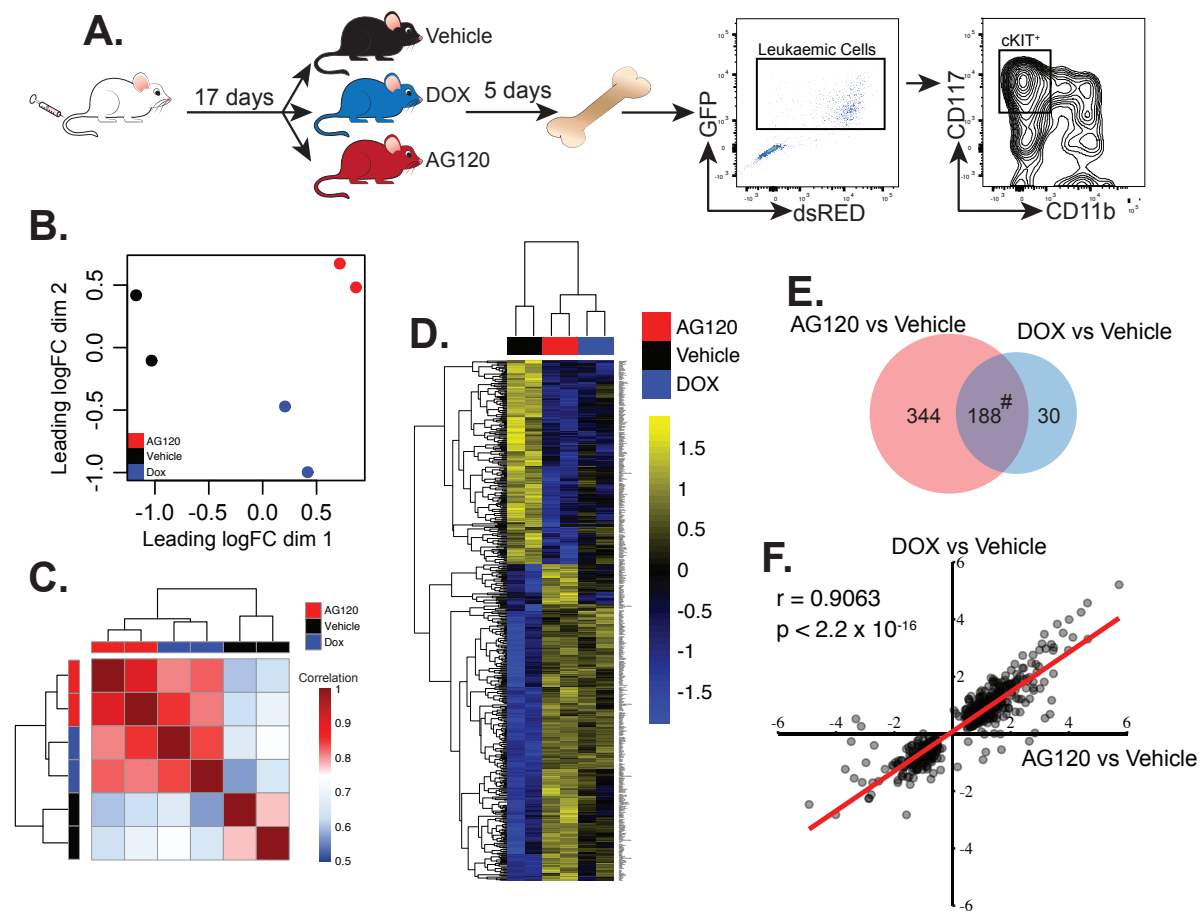


Figure 4.14 Genetic Depletion and Pharmacological Inhibition of IDH1^{R132H} Induced Parallel Transcriptional Changes

(A) Schematic of experimental procedure for RNAseq of CD117⁺ leukaemic cells isolated from the bone marrow of mice treated for 5 days with vehicle, DOX or AG120. N = 2 per cohort. (B) MDS plot of the first two dimensions accounting for the most inter-sample variation from the QuantSeq 3' mRNA sequencing data. (C) Heatmap of the pair-wise Pearson's correlation coefficients calculated on the top 500 most variable genes across all samples. (D) Heatmap of the top 500 differentially expressed genes between the AG120 and vehicle treated cohorts. (E) Venn diagram of significantly DEGs (FDR < 0.05, $|\log_2(\text{FC})| \geq 0.5$) in AG120 or DOX treated conditions, when compared to vehicle. Hypergeometric testing was used to assess the significance of overlap, # $p = 7 \times 10^{-213}$. (F) Scatter plot between the fold-change (FC) values of the union of genes significantly enriched in AG120 or DOX treated conditions (FDR < 0.05, $|\log_2(\text{FC})| \geq 0.5$), when compared to vehicle.

Some of the significantly DEGs with the greatest magnitude changes in the AG120 cohort, that were shared amongst the DOX DEGs, were genes involved in normal haematopoiesis and hematopoietic cell function, and frequently dysregulated in leukaemia (Table 4.2). *Meis1*, *Sox4* and *Slpr1* were amongst the shared down-regulated DEGs, whilst *Prg2*, *CD177* and *Ncam1* were shared up-regulated DEGs. *Meis1* and *Sox4* encode transcription factors that regulate the self-renewal properties of HSCs (Argiropoulos et al., 2007; Zhang et al., 2013b). Expression of *Meis1* and *Sox4* are commonly dysregulated in AML and contribute to the increased self-renewal capacities that drive uncontrolled proliferation. Frequently, down-regulation of these transcription factors in AML abrogates the augmented self-renewal and restores differentiation (Argiropoulos et al., 2007; Zhang et al., 2013b). *Slpr1*, expressed in murine HSCs, facilitates the egress of lineage committed HSC progenitors from the bone marrow into the blood (Juarez et al., 2012). The expression of *Prg2* and *Ncam1* increase during myeloid differentiation (Paul et al., 2016), whilst *CD177* is required for neutrophil function (Bai et al., 2017). Taken together, alterations in the expression of these genes suggested that depletion and inhibition of IDH1^{R132H} impeded the elevated self-renewal properties of the IDN tumours whilst encouraging differentiation along the myeloid lineage.

Table 4.2 Top Shared DEGs in AG120 and DOX Treated IDN Recipients

Gene	AG120 v Vehicle		Dox v Vehicle	
	logFC	FDR	logFC	FDR
<i>Slpr1</i>	-2.881	0.0049	-2.253	0.0258
<i>Sox4</i>	-1.904	0.0002	-1.062	0.0101
<i>Meis1</i>	-1.854	0.0001	-0.936	0.0097
<i>Prg2</i>	3.152	0.0002	1.813	0.0161
<i>CD177</i>	2.652	0.0002	1.978	0.0048
<i>Ncam1</i>	1.943	0.0028	1.738	0.0125

With respect to vehicle-treated cohort, of the 532 genes with significant changes in expression following 5 days of AG120 treatment, 327 genes (61.5%) were upregulated whilst 205 genes (38.5%) were down-regulated (Figure 4.15 A). Similarly, a larger proportion of the significantly DEGs were upregulated (72.0%) in the DOX cohort, relative to the vehicle-treated cohort. Since the transcriptional profile of the IDN model was substantially altered in response to inhibition and depletion of IDH1^{R132H}, gene set enrichment analysis (GSEA) was performed to determine whether specific transcriptional pathways were enriched amongst the DEGs. Pathway enrichment was analysed using hallmark, chemical and genetic perturbation, oncogenic, and immunogenic gene sets from the Molecular Signatures Database (MSigDB). A large number of gene sets were enriched (FDR q-value < 0.1) upon depletion and inhibition of IDH1^{R132H} (Figure 4.15 B – D). Hypergeometric testing identified significant overlap between the dysregulated pathways induced by AG120 and DOX (up: $p < 5 \times 10^{-324}$, down: $p = 6.8 \times 10^{-194}$) (Figure 4.15 B), an expected result given the previously described high overlap in DEGs. Furthermore, the AG120 and DOX cohorts shared directionalities of the dysregulated pathways (Figure 4.15 C), which was predicted given the parallel directionalities of DEGs between these cohorts. Utilising the hallmarks collection of gene sets, GSEA identified gene sets often implicated in cancer that were negatively enriched upon IDH1^{R132H} perturbation, such as the MYC- and E2F-targets gene sets (Figure 4.15 C). The abundance of dysregulated pathways as a consequence of DOX and AG120 treatment, suggested that the maintained expression and oncogenic function of IDH1^{R132H} was required in order to sustain the transcriptional profiles driving the leukaemia in established IDN tumours.

The chemical and genetic perturbation collection of 3302 gene sets was curated from experimental data of published expression profiles following a disturbance to the cells. Hence, these experimentally derived datasets provide an unbiased analysis of perturbed pathways, in contrast to datasets curated based on prior knowledge (Subramanian et al., 2005). Utilising the chemical and genetic perturbation dataset, in response to pharmacological inhibition of IDH1^{R132H}, GSEA revealed a strong positive enrichment (NES > 2 and FDR q-value < 0.005) of gene sets that govern myeloid differentiation (Ivanova: Haematopoiesis Mature Cell, Brown: Myeloid Cell Development Up), and a strong negative enrichment (NES < -2 and FDR q-value < 0.005) of gene sets down-regulated in myeloid differentiation or underpin properties of HSC and progenitor cells (Brown: Myeloid Cell Development Down, Ivanova: Haematopoiesis and Stem Cell) (Figure 4.15 D – E). Moreover, these gene sets were also significantly enriched in response to genetic depletion of IDH1^{R132H} by DOX (Figure 4.15 F – G). Furthermore, Cbfa2t3,

a transcriptional corepressor of several haematopoietic transcription factors (Engel et al., 2010; Steinauer et al., 2017) which is highly up-regulated in HSCs and progenitor cells (Lindberg et al., 2005), was down-regulated upon IDH1^{R132H} inhibition (Figure 4.15 H). Collectively these data demonstrate that IDH1^{R132H} contributes to the aberrant stem-like properties of the IDN progenitors. Interestingly, although neutrophil-lineage gene sets were up-regulated upon IDH1^{R132H} inhibition (Figure 4.15 I – J), they were highly expressed in the unperturbed (vehicle treated) IDN leukaemias when compared to the average gene expression (Figure 4.15 K).

The production of the oncometabolite 2-HG is considered the primary mechanism through which mutant-IDH elicits its tumorigenic effects (Losman et al., 2013). Thus, it was predicted that the reduction in 2-HG as a consequence of IDH1^{R132H} inhibition would induce transcriptional changes similar to those elicited by the inhibition of IDH2^{R140Q}. The transcriptional profile elicited in response to 5 days of AG221 (IDH2^{R140Q} inhibitor) treatment, in an *in vivo* model of *IDH2^{R140Q}/DNMT3A^{R882H}/Nras^{G12D}* driven AML had previously been published (Kats et al., 2017). Rotating gene set testing (ROAST) (Wu et al., 2010a) identified significant enrichment of the DEGs that were down-regulated (FDR < 0.05, log₂FC < -0.5) upon 5 days of AG221 treatment, within the down-regulated DEGs in the AG120 treated IDN model (Table 4.3, Figure 4.15 L). Although not statistically significant, a large proportion of the up-regulated DEGs (FDR < 0.05, log₂FC > 0.5) in the AG221 cohort were also upregulated in the AG120 treated cohort (Table 4.3, Figure 4.15 L). DEGs elicited by AG221 in the *IDH2^{R140Q}/DNMT3A^{R882H}/Nras^{G12D}* driven AML were also enriched in gene sets that govern myeloid differentiation and properties of HSC and progenitor cells (Kats et al., 2017). Collectively, these results suggest that the altered transcriptional profiles in response to mutant-IDH inhibition are largely driven by the reduction in 2-HG.

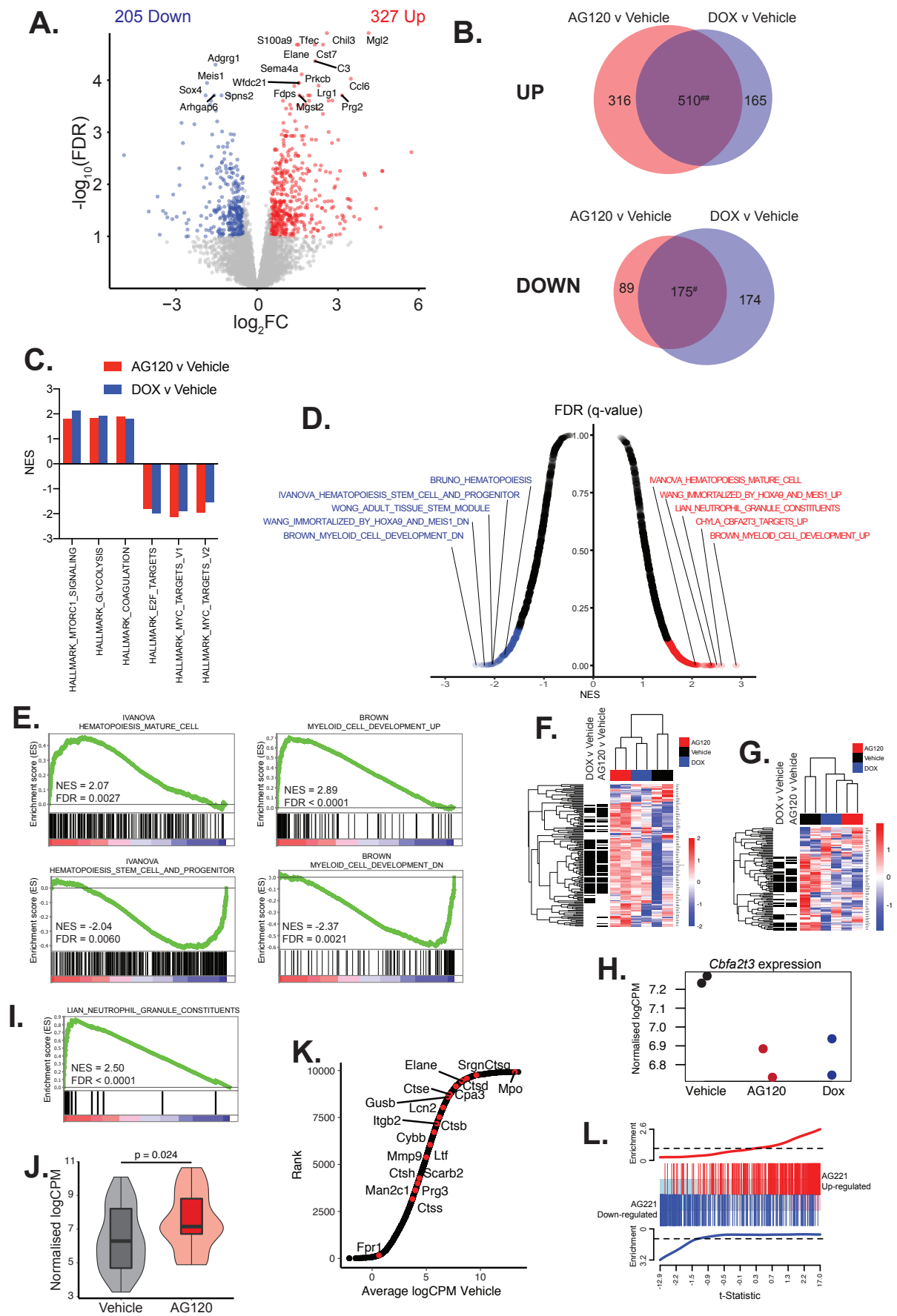


Figure 4.15 Depletion and Inhibition of IDH1^{R132H} Promoted Transcriptional Programs Driving Myeloid Differentiation

(A) Volcano plot highlighting the most significant DEGs ($FDR \leq 0.05$, $|\log_2(FC)| \geq 0.5$) between the AG120 and vehicle treated cohorts. (B) Venn diagram of the gene sets positively (UP) and negatively (DOWN) enriched (FDR q-value < 0.1) upon depletion and inhibition of IDH1^{R132H}. Hypergeometric testing identified significant overlap, $## p < 5 \times 10^{-324}$, $\# p = 6.8 \times 10^{-194}$. (C) Consistent enrichment directionalities between the AG120 and DOX treated cohorts. (D) Normalised enrichment scores (NES) versus FDR scores, computed from GSEA analysis, of gene sets enriched in the AG120 treated cohort relative to vehicle. (E) GSEA enrichment plots negatively enriched (right 2 plots) and positively enriched (left 2 plots) in the AG120 treated cohort relative to vehicle. Heatmap of (F) Downregulated and (G) Upregulated Myeloid Cell Development (Brown) gene sets. Black barcode indicates genes in the leading edge for the corresponding comparisons. (H) Normalised gene expression of *Cbfa2t3*. (I) GSEA enrichment plot of the AG120 treated cohort relative to vehicle. (J) Normalised counts of enriched genes from (I). (K) Average normalised counts of the vehicle treated IDN cells. Red circles indicate genes from the gene set in (I). (L) Barcode plot demonstrating enrichment of DEGs induced by AG120 in the *in vivo* IDN model, and the positive association with either significantly up-regulated (red) ($FDR < 0.05$, $\log_2(FC) > 0.5$) or significantly down-regulated (blue) ($FDR < 0.05$, $\log_2(FC) < -0.5$) genes post AG221 treatment of *IDH2^{R140Q}/DNMT3A^{R882H}/Nras^{G12D}* transplanted recipient mice.

Table 4.3 ROAST Enrichment Analysis of AG120 DEGs in the Significantly Differentially Expressed Gene Sets Elicited by AG221 in IDH2^{R140Q}/DNMT3A^{R882H}/NRAS^{G12D} driven AML

Gene Set	Number of Genes*	Proportion Down	Proportion Up	Direction Change	P-value	FDR
AG221 Up	267	0.0487	0.3857	Up	0.124	0.124
AG221 Down	323	0.4861	0.0867	Down	0.0015	0.0015

*Number of genes in the AG221 gene set that overlapped with genes in the expression matrix of the 3' mRNA Quantseq data.

Immunophenotyping of the leukaemic cells residing in the bone marrow of IDN recipients whose transcriptional profiles were sequenced, revealed an increase in the ratio of GMPs:CMPS upon depletion and inhibition of IDH1^{R132H} (Figure 4.16 A). A larger cohort of IDN recipients, transplanted and treated alongside the mice whose tumours were RNA-sequenced, demonstrated a decrease in the proportion of leukaemic cells that reside within the KL compartment (Figure 4.16 B) and an increase in the proportion of GMP cells within the leukaemic KL compartment (Figure 4.16 C). Taken together, these data demonstrate an important role for mutant IDH in driving transcriptional programs required for maintaining stem-cell like properties, such as the capacity to self-renew, and preventing differentiation in IDN established leukaemias *in vivo*.

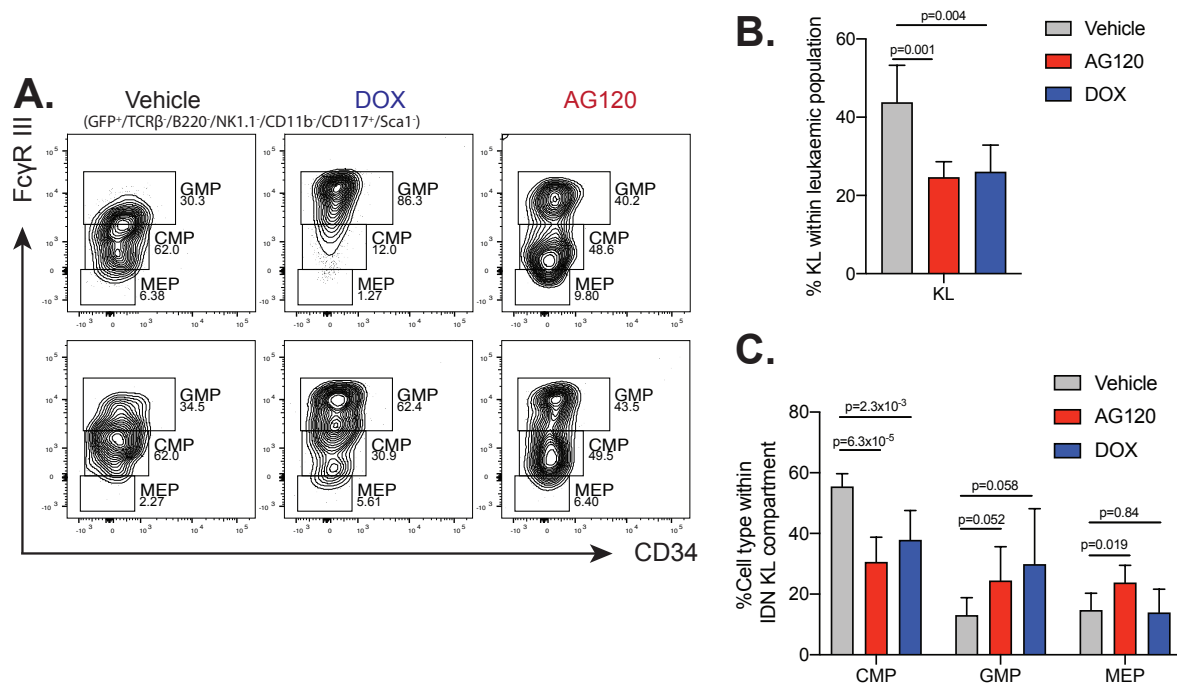


Figure 4.16 Differentiation Within the KL Compartment in Response to Genetic Depletion and Pharmacological Inhibition of IDH1^{R132H}

(A) FACS plots of the KL leukaemic population within the bone marrow of mice whose tumour was harvested for RNAseq. **(B)** Percentage of KL cells within the leukaemic population residing in the bone marrow of IDN mice following 5 days of treatment. **(C)** Percentage of the myeloid progenitors within the leukaemic KL population residing in the bone marrow of IDN mice following 5 days of treatment. Mean $\pm$ SD of 6 IDN recipients is represented. Means were compared using student's t-test.

4.3.6 Demethylation of H3K27me3 in Response to Mutant-IDH Inhibition

2-HG has been shown to inhibit α -kg-dependent dioxygenases, including the family of JmJc histone demethylases (section 1.4.3.2.1). However, histone hypermethylation, in the context of mutant IDH myeloid leukaemia, has largely been observed in haematopoietic cell lines ectopically expressing mutant *IDH* and whose survival was not reliant upon the continual presence of mutant IDH. The IDN *in vivo* model provides a tractable and clinically relevant system that is inherently reliant upon the maintained expression of *IDH1^{R132H}*, in which to study the consequence of *IDH1^{R132H}* inhibition on the epigenetic landscape and associated transcriptional profiles.

A cross-sectional study was performed to characterise the extent of differentiation, transcriptional and epigenetic changes throughout the course of AG120 treatment. Previous experiments had shown that IDN recipient mice would not survive the full course of AG120 treatment if the peripheral tumour burden was greater than 3% at the point of therapy initiation (Figure 4.17 A). Within 1 week of AG120 treatment, the peripheral blood leukaemia burden was significantly higher in mice whose peripheral tumour burden was > 3% at therapy induction (Figure 4.17 B). It was suspected that AG120 treated mice whose therapy began when the peripheral tumour burden higher than 3%, succumbed to differentiation syndrome as a result of drug induced differentiation of myeloblasts into mature cells, a known side effect in IDH mutant AML patients receiving targeted inhibitors (Fathi et al., 2018). In contrast, if treatment initiation occurred when the tumour burden was too low, the leukaemic cell yield would be insufficient for downstream analyses. To optimise the tumour yield whilst preventing the onset of premature drug induced death of experimental mice, the cross-sectional study was designed with two different therapy induction start dates (Figure 4.18). The “cross-sectional cohort” encompassed recipient mice whose treatment began following a 14-day engraftment period and were utilised for the cross-sectional immunophenotyping (see section 4.3.7), analysis of day-14 AG120-induced transcriptional and epigenetic changes, and characterisation of mice that succumbed to AML post AG120 therapy (see section 4.3.8) (Figure 4.18). Within this cohort, the vehicle-treated group was only extended for 14-days post treatment initiation since the ethical cull-point would have been reached soon after, as informed from previous experiments. To ensure adequate cell numbers for transcriptional and epigenetic analyses, the treatment for the other recipients was delayed until 21 days post-transplant and were harvested following 5 days of treatment (“delayed 5-day treatment cohort”) (Figure 4.18).

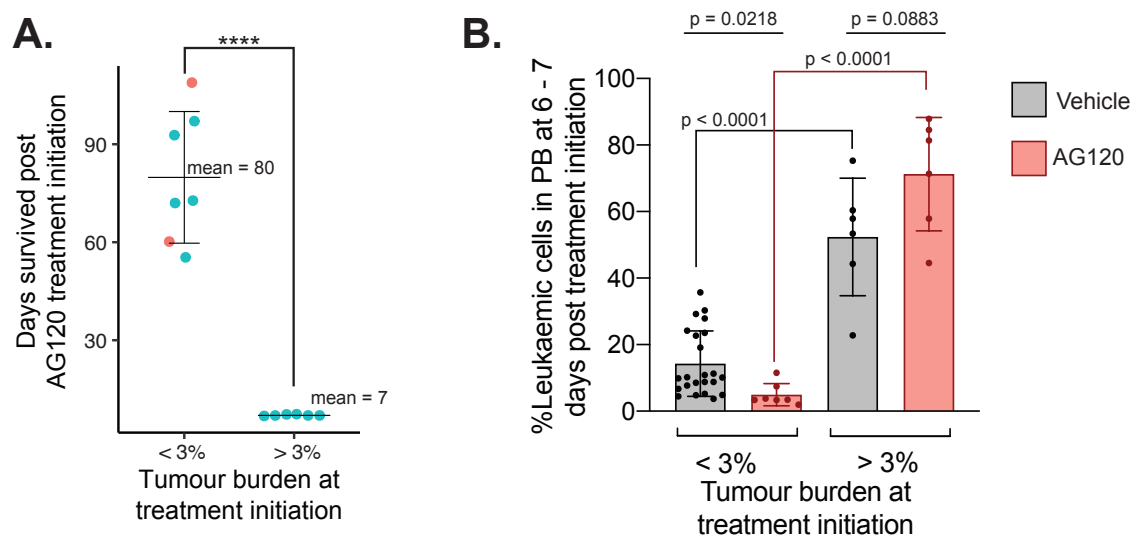


Figure 4.17 AG120 Treatment Needs to be Initiated at Tumour Burden Levels Less than 3% in the PB

(A) Relationship between tumour burden at treatment initiation in the PB and the days survived post AG120 therapy induction. Different colours depict mice from separate experiments. **(B)** Relationship between tumour burden at treatment initiation and 6 – 7 days post. Mean $\pm$ SD is represented. Statistical differences in the means was calculated using student's t-test. **** $p < 0.0001$. PB; peripheral blood.

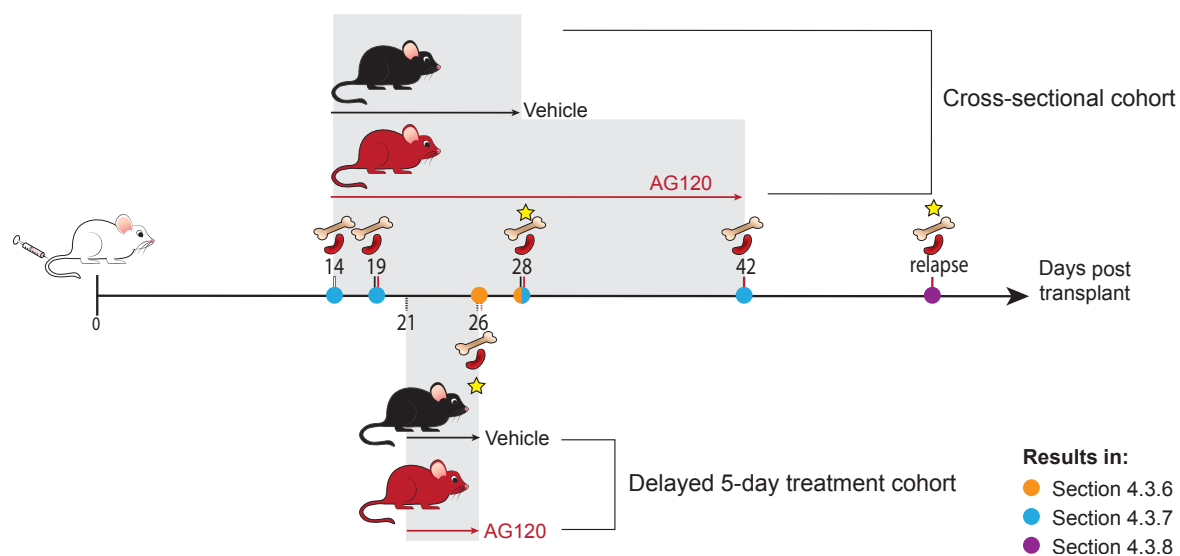


Figure 4.18 Schematic of Cross-Sectional and Delayed 5-Day Experimental Cohorts

Schematic of the experimental procedure for the cross-sectional immuno-phenotyping, RNAseq and ChIPseq analysis. Therapy began for the cross-sectional mice following a 14-day engraftment period. In order to obtain sufficient cell numbers for the molecular analysis of 5 day treated mice, the delayed 5-day treatment cohort began therapy after a 21-day engraftment period. Star denotes samples used for molecular analyses. Bone and spleen denote samples harvested for immuno-phenotyping by FACS.

ChIP-seq was performed for H3K27me3 on the IDN myeloid progenitor (CD117⁺/CD11b⁻) population following 5-days of AG120 or vehicle treatment (n = 6 / treatment group) (Figure 4.18). FACS isolated IDN myeloid progenitor cells were pooled from 2 recipient mice of the same treatment group, resulting in 3 biological replicates for each condition. Pooled cells were subsequently evenly distributed between the two immunoprecipitation (IP) reactions. Of note, the IDN cell yield from the 14-day AG120 treated mice of the cross-sectional cohort was insufficient to proceed with ChIP-seq. H3K27me3 was chosen for ChIP analysis as this histone methyl modification is removed by the JmJc KDMs and is typically associated with transcriptional repression (Hyun et al., 2017). Given the cell yield, a more comprehensive assessment of the alterations in the epigenetic landscape, through the detection of additional histone marks, was not possible and highlights a limitation of using *in vivo* sorted samples.

To detect any potential global H3K27me3 changes as a consequence of AG120 treatment, drosophila DNA was spiked into each IP reaction (Orlando et al., 2014). Only 192 differentially enriched 1 kb windows were identified between the 5-day AG120- and vehicle-treated IDN leukaemias, and no regions passed the false discovery rate threshold (FDR < 0.01) once corrected for multiple testing (Figure 4.19 A). This suggests that 5 days of IDH1^{R132H} inhibition did not result in global changes to the distribution of H3K27me3. Nonetheless, the differentially enriched windows (p < 0.05) were largely hypomethylated upon AG120 treatment (Figure 4.19 B). In addition to ChIP-seq, QuantSeq 3' mRNA sequencing was performed on the CD117⁺/CD11b⁻ IDN population isolated from the same recipients, allowing for the direct comparisons between the alterations in epigenetic and transcriptional states in response to IDH1^{R132H} inhibition. Genes associated with the hypomethylated 1 kb windows (p < 0.05, log₂FC < -0.5), were transcriptionally up-regulated in response to IDH1^{R132H} inhibition (Figure 4.19 C). These data are consistent with previous analysis of histone hypermethylation in mutant IDH expressing cells (Kernytsky et al., 2015), and associations between the presence of H3K27me3 modifications and transcriptional repression (Wiles and Selker, 2017).

Previously, the Kats laboratory performed a similar experiment in the IDH2^{R140Q}/Nras^{G12D}/DNMT3A^{R882H} *in vivo* model, whereby ChIP-seq was performed for the H3K27me3 modification in the CD117⁺/CD11b⁻ leukaemic population following 14 days of *in vivo* IDH2^{R140Q} de-induction with DOX. Overlap in the changes to the H3K27me3 landscape induced by IDH2^{R140Q} depletion and IDH1^{R132H} inhibition was predicted to exist, since the transcriptional profiles in response to IDH2^{R140Q} depletion (DOX) paralleled that of IDH2^{R140Q}

inhibition (AG221) in the IDH2^{R140Q}/Nras^{G12D}/DNMT3A^{R882H} *in vivo* model (Kats et al., 2017), and the DEGs induced by AG120 in the IDN *in vivo* model were enriched and shared directionalities with the DEGs elicited by AG221 in the *in vivo* IDH2^{R140Q}/Nras^{G12D}/DNMT3A^{R882H} model (Figure 4.15 L). To test this hypothesis, sequencing reads from the 3 biological replicates of the IDH2^{R140Q} de-induction experiment were processed as per the IDN samples.

A vast reduction in the occurrence of H3K27me3 was evident at the TSS in response to 14 days of IDH2^{R140Q} depletion (Figure 4.19 D). This was reflected in the large number of statistically differentially enriched windows, which were predominantly hypomethylated (Table 4.4, Figure 4.19 E). When H3K27me3 peaks residing at the TSS were subset on the significantly differentially methylated regions (FDR < 0.01) elicited after 14 days of IDH2^{R140Q} depletion, a reduction in the average abundance of H3K27me3 was apparent in response to 5 days of IDH1^{R132H} pharmacological inhibition in the IDN tumours (Figure 4.19 F). Although not statistically significant, a large proportion of the differentially hypomethylated (FDR < 0.01, log₂FC < -2) H3K27me3 regions upon 14-days of IDH2^{R140Q} depletion were negatively enriched amongst the differentially methylated H3K27me3 regions in response to 5 days of IDH1^{R132H} inhibition in the *in vivo* IDN model (Figure 4.19 G, Table 4.6). Furthermore, genes associated with the H3K27me3 regions that were hypomethylated as a consequence of IDH2^{R140Q} depletion, were statistically enriched for up-regulated transcriptional states in response to IDH1^{R132H} inhibition (Figure 4.19 H, Table 4.6). Taken together, these data are highly suggestive of H3K27me3 hypomethylation upon IDH1^{R132H} inhibition in established IDN tumours.

Table 4.4 Number of Differentially Enriched H3K27me3 Windows Upon IDH2^{R140Q} Depletion

Bin-size	Cut-off statistic	Number of differential windows
1 kb	p < 0.05	103 Up
		12658 Down
	FDR < 0.01	43 Up
		5621 Down

To ensure that the discrepancy in the number of differential H3K27me3 peaks that passed statistical significance upon mutant-IDH depletion in the IDN and IDH2^{R140Q} *in vivo* models was a real biological outcome and not due to a difference in enrichment-to-background signal, the fingerprint plots were compared. Fingerprint profiles depict the relationship between the cumulative proportion of all genomic bins (x-axis) and the fraction of the genomic bins that contain the highest coverage (y-axis), such that enrichment is present if the IP curve lies below the input curve (Diaz et al., 2012; Ramírez et al., 2016). The fingerprint plots of the H3K27me3 immunoprecipitated IDN and IDH2^{R140Q}/Nras^{G12D}/DNMT3A^{R882H} leukaemia samples both demonstrated enrichment over input (Figure 4.19 I – J). Indeed, the H3K27me3 enrichment over input was greater in the IDN samples than the IDH2^{R140Q}/Nras^{G12D}/DNMT3A^{R882H} leukaemias (Figure 4.19 I – J). The fingerprint plots demonstrated a shallow and gradual increase in the cumulative proportion of genomic reads (Figure 4.19 I – J), which is characteristic of modifications, such as H3K27me3, that span broad domains (Diaz et al., 2012). These plots demonstrate that the discrepancy in the number of differential H3K27me3 peaks that passed statistical significance upon mutant-IDH depletion in the IDN and IDH2^{R140Q} *in vivo* models was not due to a difference in enrichment-to-background signal.

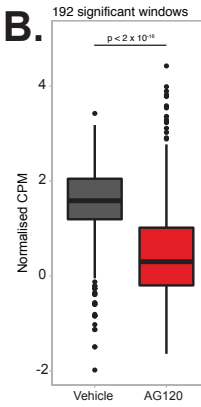
A.

Number of Differentially Enriched H3K27me3 Windows

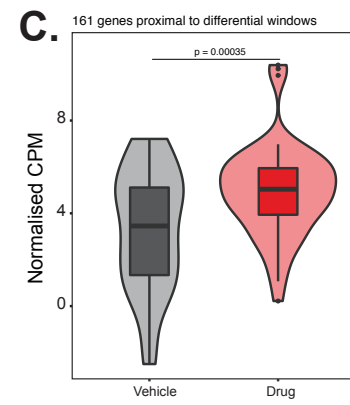
Cut-off statistic*	Number of differential windows	Number of associated genes
$p < 0.05$	192	161
FDR < 0.01	0	0

*In addition to $|\log_2FC| > 0.5$

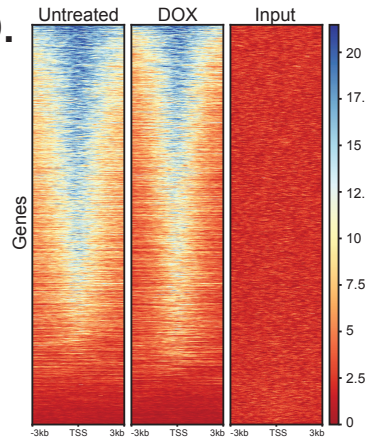
B.



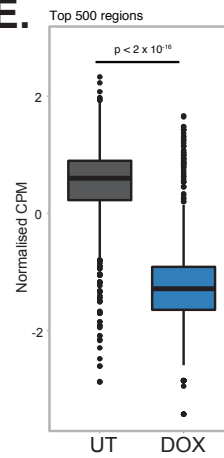
C.



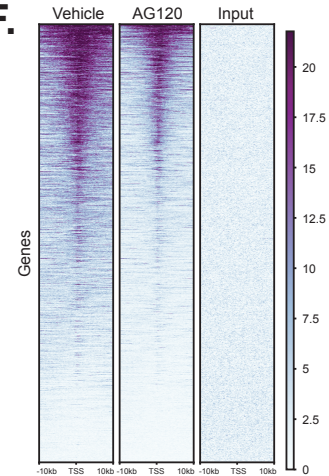
D.



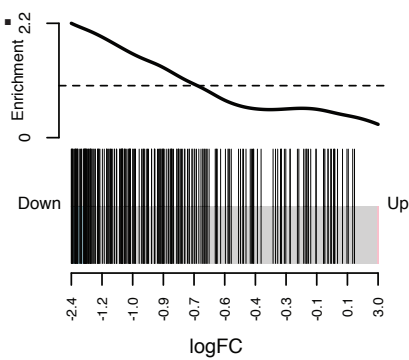
E.



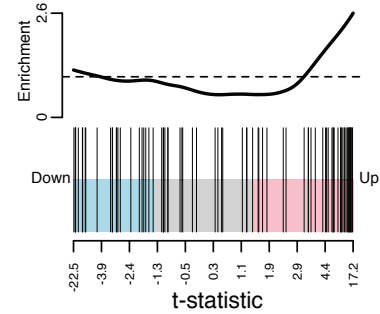
F.



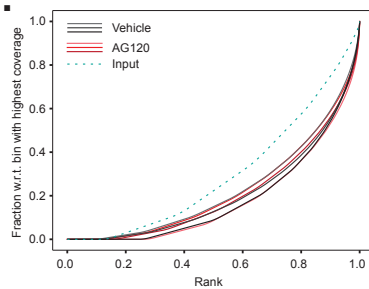
G.



H.



I.



J.

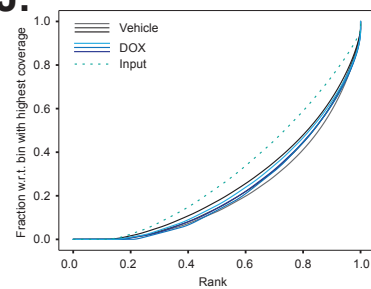


Figure 4.19 Hypomethylated H3K27me3 Peaks Associated with Transcriptional Up-Regulation Upon Mutant-IDH Inhibition

(A) Table summarising the number of differentially enriched 1 kB windows between 5-day AG120- and vehicle-treated IDN *in vivo* leukaemias. **(B)** Boxplots of the normalised counts for the differentially methylated H3K27me3 windows ($p < 0.05$) from (A). **(C)** Expression of genes proximal to differentially enriched H3K27me3 windows from (A) between AG120 and vehicle cohorts. **(D)** Heatmap of the union of all H3K27me3 peaks residing at the TSS in the DOX and untreated leukaemia cells from an IDH2^{R140Q}/Nras^{G12D}/DNMT3A^{R882H} *in vivo* model. 3 biological replicates were merged. **(E)** Boxplot of the top 500 differentially enriched H3K27me3 1 kB windows in the DOX treated IDH2^{R140Q}/Nras^{G12D}/DNMT3A^{R882H} *in vivo* model. **(F)** Heatmap of H3K27me3 peaks residing at the TSS of vehicle and AG120 treated IDN samples, subset on DOX-induced differentially methylated regions ($FDR < 0.01$) in the IDH2^{R140Q}/Nras^{G12D}/DNMT3A^{R882H} *in vivo* model. 3 biological replicates were merged. **(G)** Barcode plot demonstrating genes associated with significant H3K27me3 hypomethylation ($FDR < 0.01$, $\log_2FC < -2$) upon 14-days of IDH2^{R140Q} depletion (DOX treated IDH2^{R140Q}/Nras^{G12D}/DNMT3A^{R882H} recipients) were negatively enriched amongst the differentially methylated H3K27me3 regions in response to 5 days of IDH1^{R132H} inhibition in the *in vivo* IDN model. **(H)** Barcode plot demonstrating genes associated with H3K27me3 regions that were hypomethylated as a consequence of IDH2^{R140Q} depletion (DOX treated IDH2^{R140Q}/Nras^{G12D}/DNMT3A^{R882H} recipients), were enriched for up-regulated transcriptional states in response to IDH1^{R132H} inhibition. **(I – J)** Fingerprint plots of **(I)** IDN leukaemias after 5-days of vehicle or AG120 treatment *in vivo* and **(J)** IDH2^{R140Q}/Nras^{G12D}/DNMT3A^{R882H} leukaemias after 14-days of +/- DOX treatment *in vivo*. Statistical differences in the means for (B) and (E) were calculated using Student's t-test.

Although, few differential H3K27me3 peaks passed statistical significance following 5 days of AG120 treatment, an abundance of gene expression changes had occurred by this time (Table 4.5). Of note, gene expression changes in response to AG120 in section 4.3.5 were enriched, and shared directionalities, with those induced by IDH1^{R132H} inhibition in the delayed 5-day treatment cohort (Figure 4.20 A), indicating transcriptional responses elicited as a consequence of IDH1^{R132H} inhibition in the IDN *in vivo* model were robust and reproducible. 3' mRNA Quantseq was also performed on the IDN myeloid progenitors from recipients of the cross-sectional experiment that received 14 days of treatment. Due to limiting leukaemic cell numbers after 14 days of AG120 treatment, IDN CD117⁺/CD11b⁻ sorted cells were pooled from two AG120 treated recipients prior to RNA extraction and sequencing. All other samples are representative of an individual recipient mouse. High concordance in the transcriptional profiles of the leukaemic myeloid progenitors within each treatment condition was observed, regardless of treatment duration (Figure 4.20 B). Leukaemia cells from vehicle treated recipient mice, whose transcriptional states were unperturbed, demonstrated the highest intra-cohort correlation (Figure 4.20 B). Significant gene expression changes (FDR < 0.01, |log₂FC| > 0.5) induced by 5 and 14 days of AG120 treatment were highly positively correlated ($r = 0.9019$, $p < 2.2 \times 10^{-16}$) (Figure 4.20 C). Taken together with the similar number in differentially expressed genes after 5 and 14 days of treatment (Table 4.5), these data indicate that 5 days of IDH1^{R132H} pharmacological inhibition was a sufficient length of time to induce primary and consequential transcriptional responses.

Taken together, the H3K27me3 ChIP-seq and 3' mRNA Quantseq data were suggestive of transcriptional alterations preceding epigenetic remodelling in response to IDH1^{R132H} pharmacological inhibition of IDN *in vivo* tumours. Genes associated with H3K27me3 peaks in the 5-day vehicle treated IDN tumours were significantly enriched for suppressed genes in this cohort (Table 4.6, Figure 4.20 D), however, displayed significant up-regulation in expression upon 5-days of AG120 treatment ($p = 8.5 \times 10^{-5}$) (Figure 4.20 E) despite the statistically insignificant reduction in H3K27me3. Furthermore, the expression of genes associated with significantly hypomethylated H3K27me3 regions (FDR < 0.01, log₂FC < -2) as a consequence of the 14-day IDH2^{R140Q} depletion, were significantly up-regulated by 5 days of IDH1^{R132H} inhibition (Figure 4.20 F) despite not reaching statistical significance in H3K27me3 reduction.

The data presented in this section demonstrates that aberrant H3K27me3 is largely hypomethylated upon depletion of mutant-IDH in AML. Furthermore, these data suggest that the transcriptional changes in response to IDH1^{R132H} inhibition precede AG120-induced alterations in the distribution of H3K27me3.

Table 4.5 Number of Differentially Expressed Genes

Comparison	Number Genes	Number Genes
	Up-regulated (FDR < 0.05, log₂FC > 0.5)	Down-regulated (FDR < 0.05, log₂FC < -0.5)
Day 5 AG120 v Vehicle	1970	1672
Day 14 AG120 v Vehicle	1823	1441

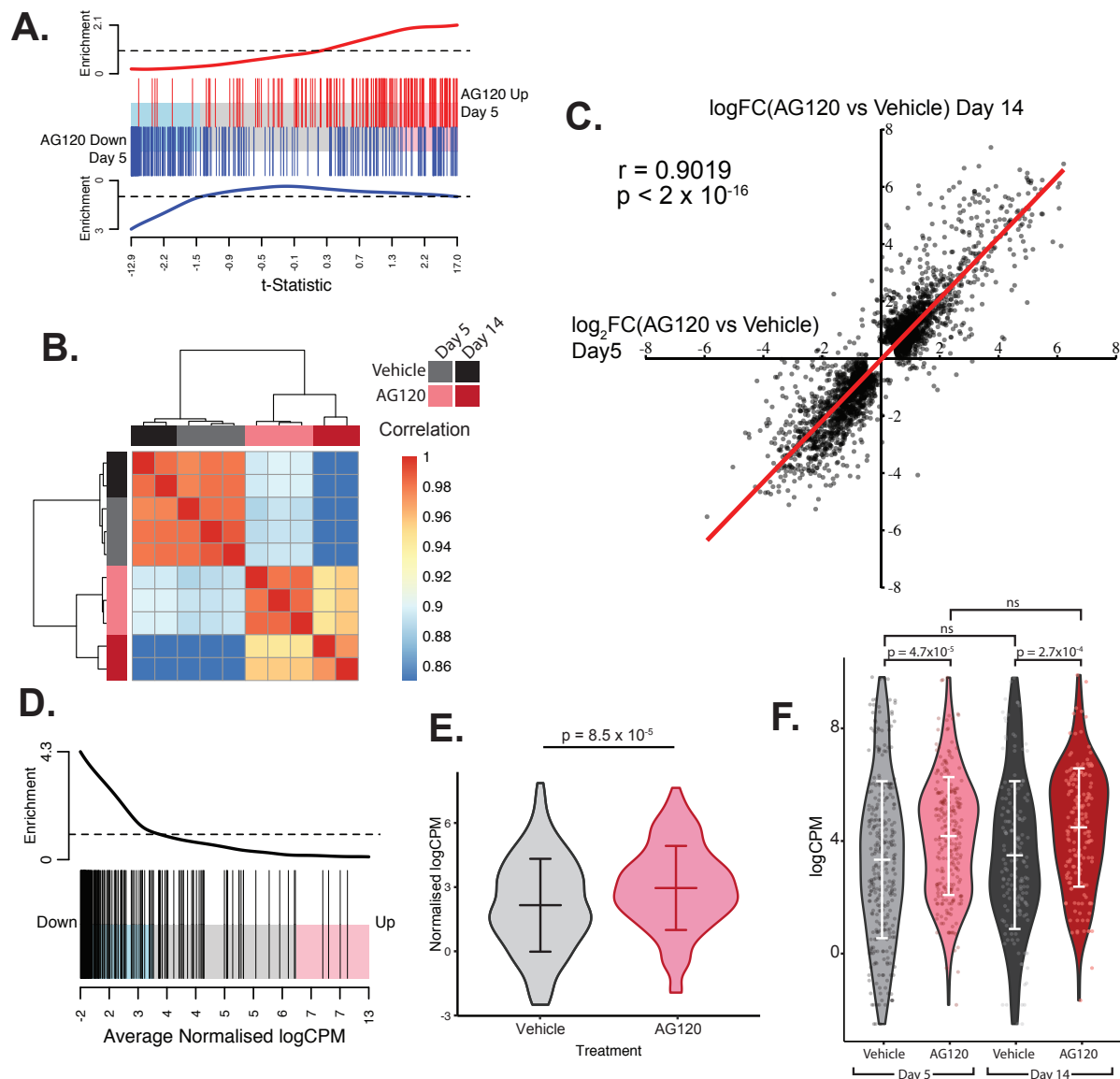


Figure 4.20 Transcriptional Changes Precede Global H3K27me3 Alterations

(A) Barcode plot demonstrating gene expression changes in response to AG120 in the cohort described in section 4.3.5 were enriched, and shared directionalities, with those induced by IDH1^{R132H} inhibition in the delayed 5-day treatment cohort (FDR < 0.01, $|\log_2FC| > 2$). **(B)** Heatmap of Pearson's correlation coefficients for 3' mRNA Quantseq performed on IDN myeloid progenitors isolated from 5 and 14-day AG120 and vehicle treated IDN recipients. **(C)** Correlation plot of the log-transformed fold changes in gene expression in response to IDH1^{R132H} inhibition for 5 days (x-axis) and 14 days (y-axis). **(D)** Barcode plot demonstrating genes associated with H3K27me3 peaks in at least 2 out of 3 vehicle replicates were enriched for below average gene expression in the vehicle cohort. **(E)** Violin plot of the normalised gene expression values after 5 days of AG120 treatment, for the genes associated with H3K27me3 peaks in at least 2 out of 3 vehicle replicates. Mean $\pm$ SD is represented for 3 biological replicates. **(F)** Violin plot of the normalised gene expression values for IDN transplanted mice +/- AG120 subset on the genes associated with significant H3K27me3 hypomethylation (FDR < 0.01, $\log_2FC < -2$) upon 14-days of IDH2^{R140Q} depletion (DOX treated IDH2^{R140Q}/Nras^{G12D}/DNMT3A^{R882H} recipients). Statistical differences in the means was calculated using student's t-test

Table 4.6 ROAST Enrichment Analysis

Associated Figure	Gene Set	Dataset Testing for Enrichment	Number of Genes*	Proportion Up / Down (Direction Δ)	P - value
Figure 4.19 G	H3K27me3 down in response to IDH2 ^{R140Q} depletion (FDR < 0.01, log ₂ FC<-2)	log ₂ FC of H3K27me3 peaks in response to AG120 in IDN <i>in vivo</i> model	217	0 / 0.6037 (Down)	0.1545
	H3K27me3 down in response to IDH2 ^{R140Q} depletion (FDR < 0.01, log ₂ FC<-2)	log ₂ FC of gene expression in response to AG120 in IDN <i>in vivo</i> model	81	0.4691 / 0.2840 (Up)	5 × 10 ⁻⁴
Figure 4.20 D	H3K27me3 peaks associated with genes in vehicle treated IDN <i>in vivo</i> model	Normalised log ₂ CPM gene expression in vehicle treated IDN <i>in vivo</i> model	129	0.0788 / 0.8727 (Down with respect to average normalised log ₂ CPM)	5 × 10 ⁻⁴

* Number of genes that overlap between the gene set and test set.

4.3.7 Pharmacological Inhibition of IDH1^{R132H} Induced Progressive Differentiation Along the Neutrophil Axis

In order to understand the disease evolution and response to IDH1^{R132H} inhibition, flow-cytometry was utilised to monitor tumour burden, immunophenotype and hence quantitate the IDN hematopoietic cell subsets of the cross-sectional cohort (Figure 4.18, 4.21 – 4.25). As expected, in the absence of inhibitor the IDN cells proliferated continuously, demonstrating rapid expansion in all compartments examined (Figure 4.21 A – B, D). By day-14 of vehicle administration, the number of leukaemic cells in the peripheral blood, bone marrow and spleen had increased by >100-fold since treatment initiation (Figure 4.21 B). Gross splenomegaly, a common symptom in AML patients and observed in the late-stages of the IDN disease *in vivo* (Figure 4.1 D, 4.7 C), was not apparent until day 28 post-transplant (14 days post treatment initiation) in the vehicle group of the cross-sectional cohort (Figure 4.21 E – F), which reflected the time-point at which the IDN cells accounted for > 50% of all cells residing in the bone marrow and spleen (Figure 4.21 B).

Overall, the most prominent IDN cell-type present within the bone marrow at the commencement of therapy were CMP cells (4.25 A left, C left), an expected result given that IDN CMPs possessed the greatest leukaemic initiating capacity demonstrated in Figure 4.5. Immature neutrophils were the second most prominent IDN cell type in the bone marrow, following closely behind CMPs at this time-point (4.22 A left, C left). On the contrary, immature neutrophils were the predominant leukaemic cell subset in the spleen after the 14-day engraftment period (4.22 B left, D left). Over time, tumour burden continued to increase throughout all organs (Figure 4.21 A – B, 4.22 C – D left), however the rates at which the different IDN haematopoietic cell populations increased were not uniform (4.22 E – H left), and resulted in a progressive change in the distribution of the proportion of cell-types that encompassed the leukaemic population (Figure 4.22 A – B left, 4.23 A, C – D). Within the IDN KL compartment residing in the bone marrow, the percentage of CMPs declined whilst the GMP fraction increased over time (Figure 4.23 D top). However, throughout disease progression, the myeloid progenitors encompassed a decreasing proportion of all bone marrow residing leukaemic cells (Figure 4.22 A top-left, 4.23 C). In contrast, the proportion of IDN KL cells within the splenic compartment, and the ratio of myeloid progenitors, remained relatively stable between day-14 and day-28 post-transplant in the vehicle cohort (Figure 4.22 B top-left, 4.23 C – D bottom).

As the disease advanced, the immature neutrophil fraction within the IDN population became the most abundant leukaemic cell type present in the spleen and bone marrow (Figure 4.22 A – B left). Although the IDN immature neutrophil population was initially highly abundant in the bone marrow, this cell-type exhibited the largest fold-change increase in the absolute number and proportion relative to all leukaemic cells, from pre-treatment to 14 days of vehicle exposure (Figure 4.22 E left, G left). Interestingly, even though IDN immature neutrophils were also the primary cell-type in the spleen (Figure 4.22 B left), this leukaemic population did not exhibit the greatest expansion within the spleen over the 2 weeks of vehicle treatment (Figure 4.22 F left, H left). In the splenic compartment, mature neutrophils appeared to have the largest increase in proportional fold-change (Figure 4.22 H), potentially suggesting that a small percentage of immature neutrophils were capable of maturing as the disease approached lethal tumour burden levels. However, this result was likely amplified by calculating the fold-change ratio with very small proportions; by 14 days of therapy exposure, the percentage of mature neutrophils within all spleen-residing leukaemic cells increased from 0.0189 ± 0.163 to 0.720 ± 0.225 (mean $\pm$ SD), the means of which were not statistically different when compared using the nonparametric student's t-test ($p = 0.0571$). Hence, it is difficult to conclude whether or not this elevated fold-change in the proportion of mature neutrophils reflected important biology of the *in vivo* IDN model.

The trajectory of the proportions of distinct IDN cell types over the course of disease progression were converging towards the previously observed ratios of the late-stage disease haematopoietic cell types (Figure 4.3 B, 4.4 B). Since the IDN leukaemia that was transplanted into the recipients of this experiment, was harvested from a moribund mouse with late-stage disease, its immunophenotype was used as a surrogate for end-point disease in Figures 4.23 A (right column) and 4.24 A (right column). These data, in conjunction with the previously characterised late-stage disease cell subsets (Figure 4.3 – 4.4), provided insight into the evolution of leukaemia in the *in vivo* IDN model. CMPs, with their leukaemia initiating capabilities, populated the bone marrow and spleen and retain the capacity to differentiate into GMPs. As disease progressed, the proportion of GMPs increased within the KL compartment as a consequence of their limited differentiation potential, evident by the preservation in the capacity of GMPs to differentiate into cell types along the neutrophil axis, whilst blocked in eosinophil and monocyte differentiation. The IDN immature neutrophils were almost completely incapable at differentiating into mature neutrophils, hence they eventually became the most abundant leukaemic cell type occupying the bone marrow and spleen. Whether the

IDN immature neutrophils acquire aberrant self-renewal properties has not been experimentally determined.

Previously published data provides evidence that inhibition of mutant-IDH stimulates the differentiation of the leukaemic population (Amatangelo et al., 2017; Losman et al., 2013; Wang et al., 2013). Preliminary evidence of this was seen by the maturation of myeloid progenitors within the KL population (Figure 4.16) and by the altered transcriptional profiles elicited as a consequence of depletion and inhibition of IDH1^{R132H} in the IDN *in vivo* model (Figure 4.15, 4.20 B – C). To characterise the extent of differentiation throughout the course of treatment, the leukaemic cell subsets residing in the bone marrow and spleen of the cross-sectional AG120 treated cohort were analysed. There was no statistical difference in the mean peripheral tumour burden between the different cohorts prior to treatment initiation (Figure 4.21 C), which suggested that any subsequent differences observed between vehicle and AG120 treated mice was a result of IDH1^{R132H} inhibition, rather than discrepancies in pre-treatment tumour burden.

Interestingly, whilst the rate of tumour burden in the peripheral blood of AG120 treated mice increased proportionally to the vehicle cohort following 5 days of AG120 administration (Figure 4.21 A, D left), the mean overall tumour burden in the bone marrow and spleen was higher in the AG120 treated cohort after 5 days of therapy, although not statistically significant (Figure 4.21 B, E). Moreover, in both the cross-sectional and delayed treatment cohorts, 5 days of AG120 therapy induced a rapid expansion in the size of the spleen with macroscopically visible regions of dense blast cell infiltrate into the medulla of the spleen, suggestive of an initial rapid increase in the IDN cells followed by splenic clearance (Figure 4.21 E, F). Together with the initial increase in overall tumour burden when compared to the tumour growth of the vehicle cohort (Figure 4.21 B), these data were suggestive of an initial expansion in the IDN population in response to IDH1^{R132H} inhibition. After this initial increase in leukaemic cell number, tumour burden within the bone marrow and spleen began to decrease in response to AG120 therapy, whilst continuing to slowly increase in the peripheral blood (Figure 4.21 A – B, D).

Characterisation and quantification of the IDN subsets revealed a rapid spike across all leukaemic cell types in the bone marrow and spleen over the first 5 days of AG120 treatment (Figure 4.22 C – D right), except for the leukaemic initiating CMPs in the bone marrow (Figure

4.22 C top-right, E right). As a proportion of all IDN cells within the bone marrow, the immature neutrophils expanded the most over the first 5 days of IDH1^{R132H} inhibition (Figure 4.22 A bottom-right, E right). Although the magnitude of immature neutrophil expansion was not observed in the spleen upon AG120 treatment (Figure 4.22 F right), this haematopoietic cell subset remained the most abundant IDN cell-type residing within the spleen (Figure 4.22 B right, D right). When compared to the vehicle treated cohort, AG120 induced rapid differentiation as evident by the significant decrease in the fraction of CMPs, and increase in GMPs, within the KL compartment of the bone marrow (Figure 4.23 B, D). However, the extent of differentiation at this time point was limited to the myeloid progenitors; with respect to the vehicle controls, inhibition of IDH1^{R132H} did not elicit a statistical difference in the proportion of immature and mature neutrophils relative to all neutrophil subsets, indicating negligible neutrophil maturation after 5 days of therapy (Figure 4.24 B, C).

As treatment progressed for an additional 11 days, total tumour burden in the bone marrow and spleen remained relatively stable (Figure 4.21 B), however, most leukaemic cell subsets had declined by this time-point, except for the absolute numbers in IDN CMPs and mature neutrophils (Figure 4.22 C – D right). The number of leukaemic CMPs persisted relatively unchanged in the bone marrow and spleen between 5 and 14 days of AG120 administration (Figure 4.22 C – D top-right). Since the number of IDN GMPs were in rapid decline by day 14 of therapy, CMPs regained their predominant myeloid progenitor status within the IDN KL compartment of the bone marrow at this time-point (Figure 4.22 C – D top-right). The absolute number of mature IDN neutrophils continued to increase throughout the first half of the treatment regime, peaking by day 14 of AG120 exposure (Figure 4.22 C – D bottom-right, E – F right).

At the conclusion of AG120 treatment, tumour burden had diminished to ~ 0.45% and ~ 5% in the bone marrow and spleen, respectively, (Figure 4.21 B). Consistent with the decrease in tumour burden, a regression in spleen size was observed (Figure 4.21 E). Overall, mature neutrophils and immature neutrophils encapsulated the greatest percentage of leukaemic cells in the bone marrow and spleen, respectively, (Figure 4.22 A – B right, G – H right, 4.24 D). The KL population accounted for ~ 0.87% and ~ 0.44% of the entire leukaemic population within the bone marrow and spleen, respectively. Within the leukaemic KL population, CMPs and GMPs existed in an approximately 1:1 ratio within the bone marrow, whilst CMPs were almost the exclusive IDN myeloid progenitor present in the spleen (Figure 4.23 E).

Collectively, these observations provided insight into the progressive differentiation elicited in response to IDH1^{R132H} inhibition. Initially, AG120 induced rapid differentiation of the IDN myeloid progenitors, evident by the increase in the GMP:CMP ratio within the leukaemic KL population (Figure 4.23 D), which was consistent with the transcriptional pathways perturbed in response to IDH1^{R132H} inhibition (Figure 4.15). This was accompanied by an overall decrease in the KL compartment (Figure 4.23 C) and a prompt increase in the proportion of immature neutrophils within the leukaemic population (Figure 4.22 A – B bottom-right). Of note, > 90% of the IDN CD11b⁺ cells were consistently comprised of neutrophil-lineage cells in both treatment conditions within the cross-sectional cohort (data not shown, flow-cytometry gating as per Figure 4.3 – 4.4), suggesting that AG120 did not restore the capacity of GMPs to differentiate into monocytes and eosinophils. Differentiation of IDN immature to mature neutrophils lagged behind the differentiation of the myeloid progenitors, with IDN mature neutrophils becoming the most abundant leukaemic cell-type within the bone marrow by therapy conclusion. Taken together, these data suggest that inhibition of IDH1^{R132H} down-regulated the aberrant self-renewal transcriptional programs acquired by the myeloid progenitors, and promoted the differentiation of myeloid progenitors and immature neutrophils along the neutrophil axis, albeit, with distinct kinetics between various IDN haematopoietic cell-types.

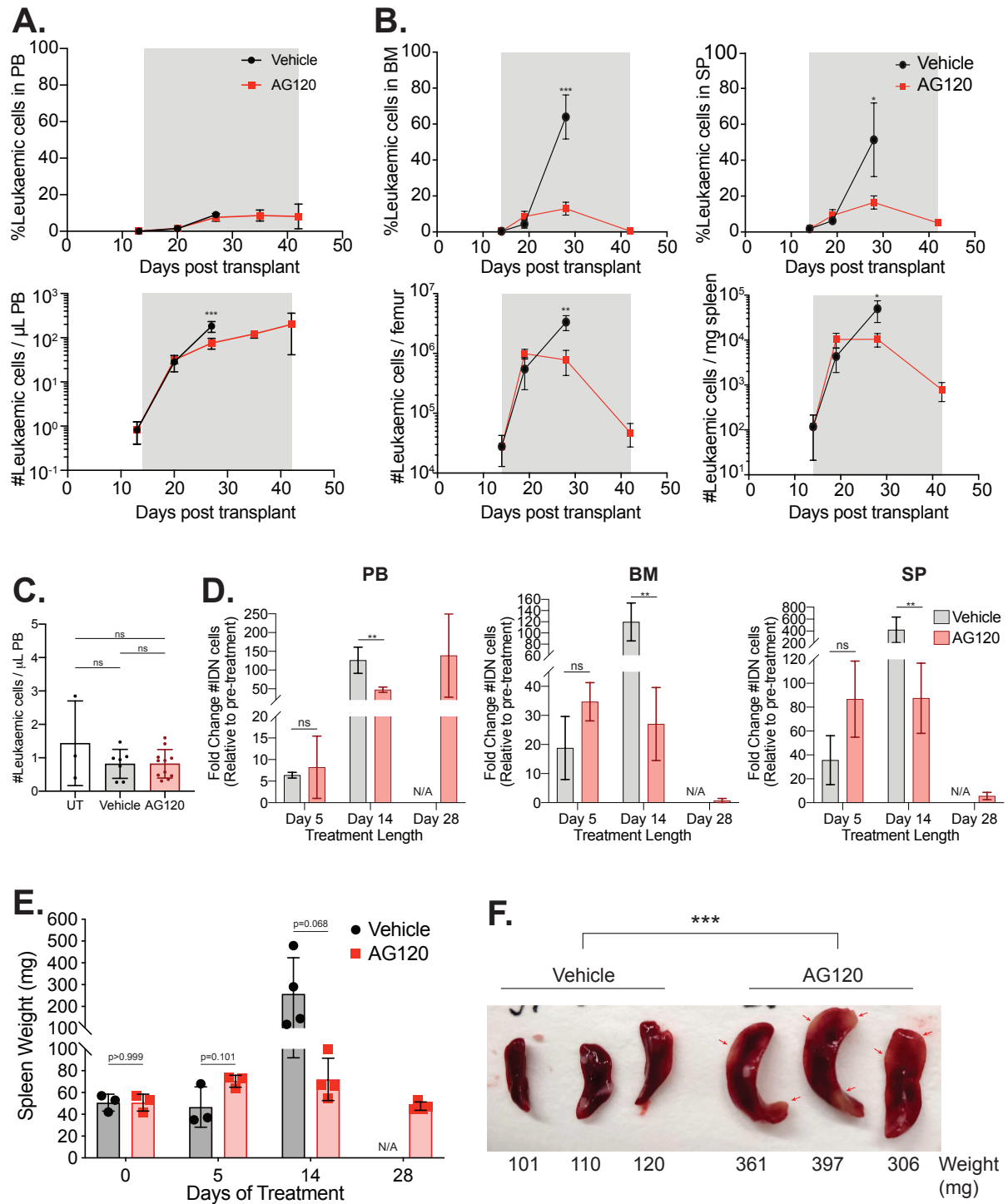


Figure 4.21 AG120 Induced Rapid Expansion in IDN Cells Followed by Tumour Regression

(A) Percentage (top) and number (bottom) of leukaemic cells in the peripheral blood of mice remaining in the cross-sectional study at each time-point. Error bars represent mean $\pm$ SD of 3 – 4 biological replicates. **(B)** Percentage (top) and number (bottom) of leukaemic cells in the femur (left) and spleen (right). Error bars represent mean $\pm$ SD of 3 – 4 biological replicates harvested at each time-point. **(C)** Mean $\pm$ SD of the number of leukaemic cells in per microlitre of peripheral blood. UT (untreated); mice harvested prior to treatment initiation. **(D)** Fold change in the number of leukaemic cells, relative to the mean number of leukaemic cells prior to treatment initiation, in the peripheral blood (left), bone marrow (middle) and spleen (right). Error bars represent mean $\pm$ SD of 3 – 4 biological replicates harvested at each time-point. **(E)** Mean $\pm$ SD of spleen weights of the cross-sectional samples. **(F)** Rapid spleen enlargement after 5 days of AG120 therapy in the treatment delayed cohort. Arrows indicate macroscopically visible regions of dense blast cell infiltrate into the medulla of the spleen. Statistical difference in the means were calculated using the student's t-test. * $0.01 < p < 0.05$, ** $0.001 < p \leq 0.01$, *** $0.0001 < p \leq 0.001$, **** $p < 0.0001$, ns; $p \geq 0.05$. PB; peripheral blood, BM; bone marrow, SP; spleen.

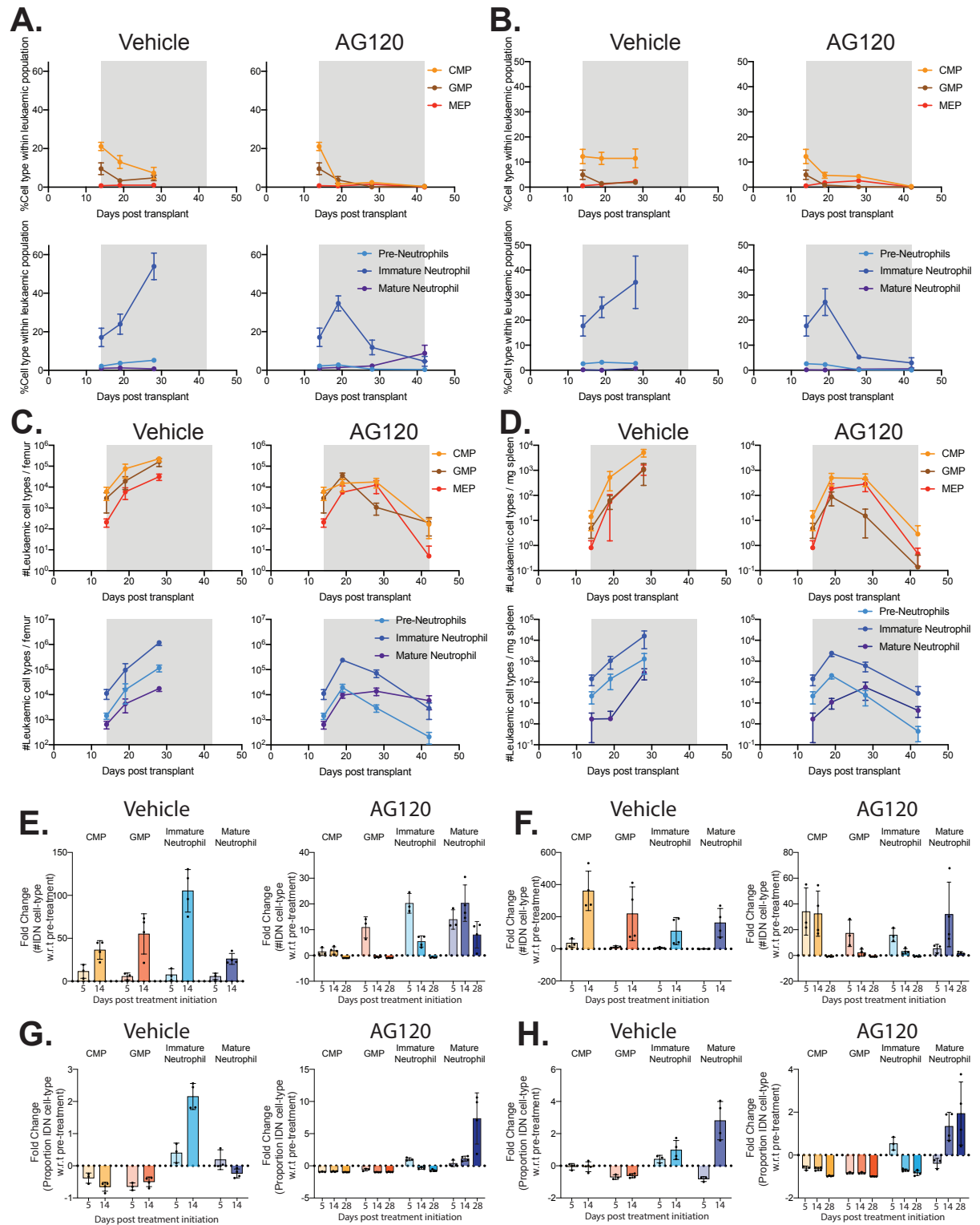


Figure 4.22 Quantitation of IDN Cell Subtypes During Disease Progression and in Response to AG120 Treatment

(A – B) Percentages of immature (top) and mature (bottom) cell types within the leukaemic population in the (A) bone marrow and (B) spleen. (C – D) Numbers of immature (top) and mature (bottom) cell types within the leukaemic population per (C) femur and (D) spleen. (E – F) Fold-change in the number of leukaemic cell subsets, with respect to average pre-treatment IDN cell-type number, in the (E) bone marrow and (F) spleen. (G – H) Fold-change in the IDN cell subsets as a proportion of all leukaemic cells, with respect to the average pre-treatment IDN cell-type proportion, in the (G) bone marrow and (H) spleen. The mean $\pm$ SD is shown for 3 – 4 replicates.

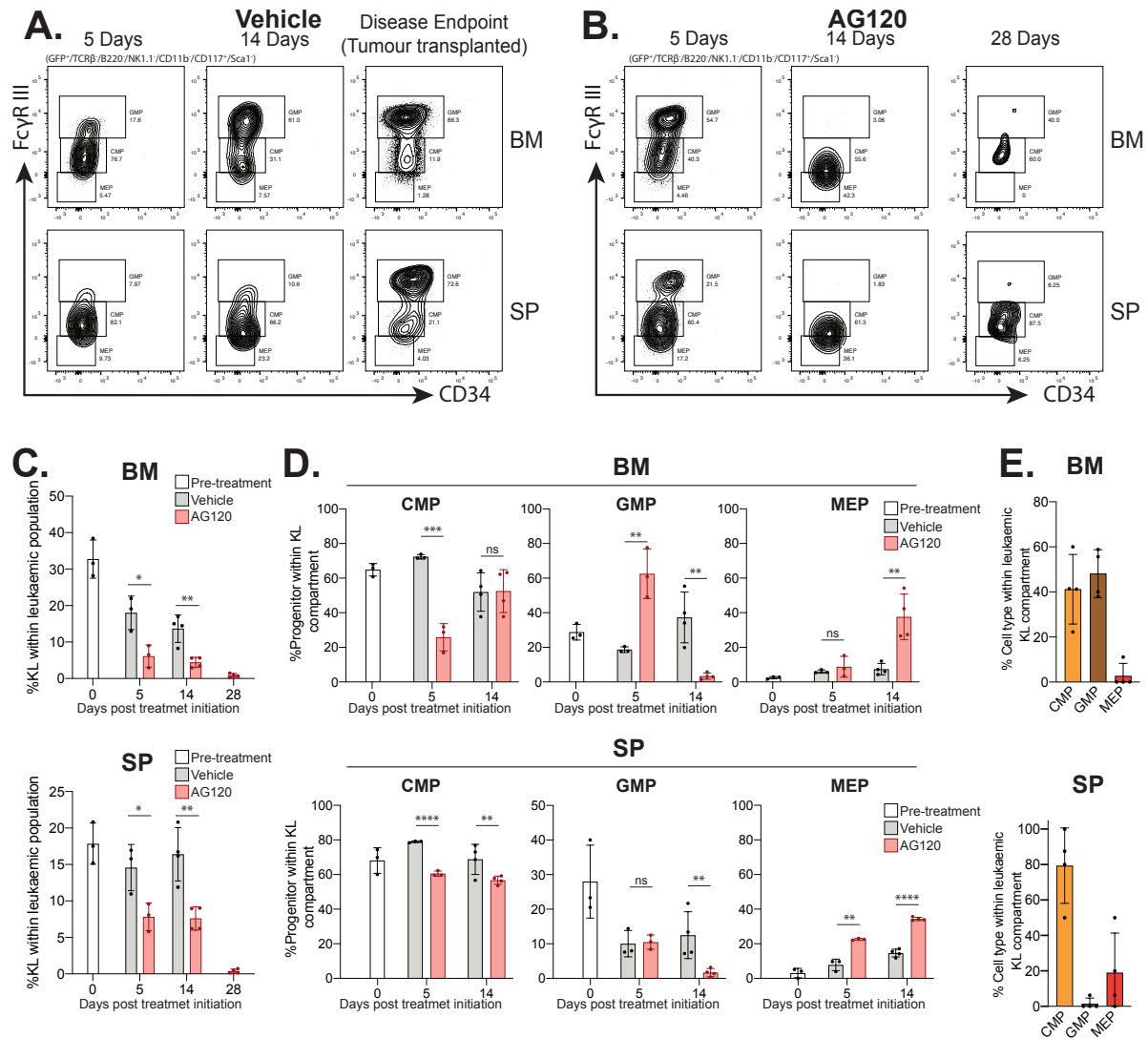


Figure 4.23 Characterisation of IDN KL Compartment During Disease Progression and in Response to AG120 Treatment

(A – B) Representative FACS plots of the leukaemic KL compartments within the bone marrow (top) and spleen (bottom) in the (A) vehicle and (B) AG120 treated cohorts. (C) Proportion of KL cells within the leukaemic population in the bone marrow (top) and spleen (bottom). (D) Proportion of myeloid progenitors within the leukaemic KL compartment in the bone marrow (top) and spleen (bottom). (E) Proportion of myeloid progenitors within the IDN KL compartment in the bone marrow (top) and spleen (bottom) at 28 days of AG120 treatment. The mean $\pm$ SD is shown for 3 – 4 replicates from the cross-sectional cohort. The statistical difference in the means were calculated using the student's t-test. * $0.01 < p < 0.05$, ** $0.001 < p \leq 0.01$, *** $0.0001 < p \leq 0.001$, **** $p < 0.0001$, ns; $p \geq 0.05$. BM; bone marrow, SP; spleen.

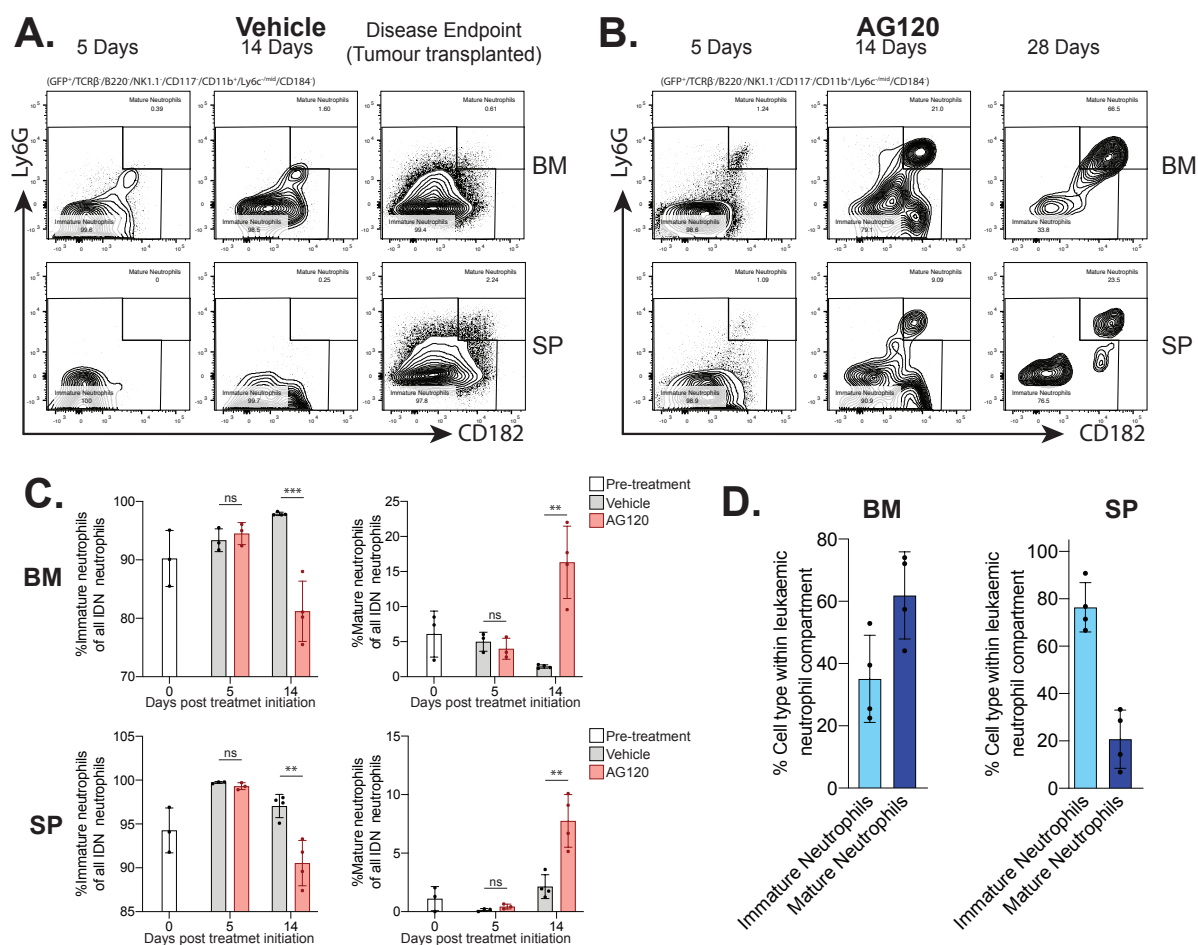


Figure 4.24 Characterisation of IDN Neutrophil Compartment During Disease Progression and in Response to AG120 Treatment

(A – B) Representative FACS plots of the leukaemic neutrophil compartments within the bone marrow (top) and spleen (bottom) in the (A) vehicle and (B) AG120 treated cohorts. (C) Proportion of immature (left) and mature (right) neutrophils within the neutrophil compartment in the bone marrow (top) and spleen (bottom). (D) The proportion of immature and mature neutrophils within the IDN neutrophil compartment, in the bone marrow (left) and spleen (right) at 28 days of AG120 treatment. The mean $\pm$ SD is shown for 3 – 4 replicates from the cross-sectional cohort. The statistical difference in the means were calculated using the student's t-test. * $0.01 < p < 0.05$, ** $0.001 < p \leq 0.01$, *** $0.0001 < p \leq 0.001$, **** $p < 0.0001$, ns; $p \geq 0.05$. BM; bone marrow, SP; spleen.

4.3.8 Leukaemias Resistant to IDH1^{R132H} Inhibition Have Distinct Transcriptional Profiles

The cross-sectional experiment from (Figure 4.18) section 4.3.6 – 4.3.7, also included recipient mice that received the complete 28-day AG120 treatment regimen and were subsequently monitored for an increase in the IDN cells in the peripheral blood and the onset of AML symptoms including hunched and ruffled coats. Flow-cytometry, 3' mRNA Quantseq and ChIPseq were performed on the IDN leukaemias harvested from these “relapsed” mice, herein referred to as IDN treatment exposed (IDN-TE) leukaemias, in order to elucidate the mechanisms that contribute to acquired resistance of mutant IDH inhibitors. Tumour burden in the peripheral blood was measured weekly by flow-cytometry (Figure 4.25 A). Relapsed mice were culled and IDN-TE leukaemias harvested once the percentage of leukaemic cells in the peripheral blood exceeded 3% and physical AML symptoms, such as hunched posture and ruffled coats, were first observed. It is important to note that the 4 relapsed mice were harvested on separate days between 47 – 53 days post treatment cessation (Figure 4.25 B). At cull, these mice presented with a myeloid leukaemia whose disease-stage was comparable to that observed by the cohort that received 14 days of vehicle treatment; no statistical difference was observed in the total tumour burden nor number of different leukaemic cell types between the 14-day vehicle treated cohort and the relapsed mice (Figure 4.25 C – G). Once culled, the leukaemic progenitors (GFP⁺/CD117⁺/CD11b⁻) were isolated by FACS and either stored at -80°C in TRIzol for RNAseq, or cross-linked with 1% (v/v) formaldehyde for ChIP-seq. Downstream processes such as RNA extraction, H3K27me3 immunoprecipitation, and next-generation sequencing were performed as a single batch comprised of all samples isolated from the cross-sectional cohort and delayed 5-day treatment cohort (see section 4.3.6 – 4.3.7) (Figure 4.18 A) in order to minimise batch effects.

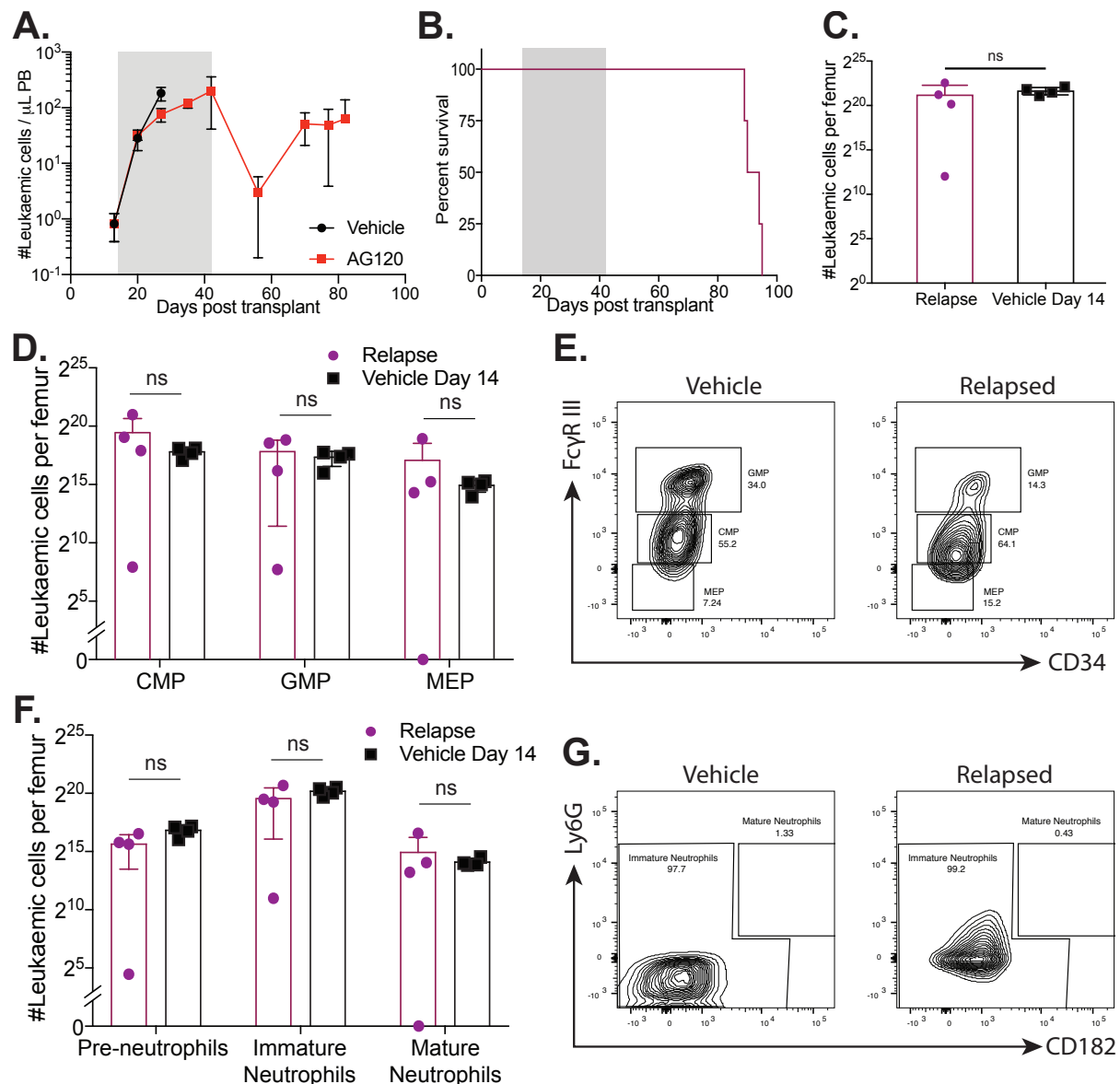


Figure 4.25 Mid-stage Disease Immunophenotype of AG120 Relapsed Mice

(A) Mean $\pm$ SD of the number of leukaemic cells (GFP⁺) per microlitre of peripheral blood (PB) isolated from the cross-sectional study. (B) Kaplan-Meier survival curve of the AG120-treated relapsed mice. Mice were culled when peripheral tumour burden was greater than 3% and physical AML symptoms, such as hunched posture and ruffled coats, were first observed. (C) Mean $\pm$ SD of the number of leukaemic cells per femur. (D) Mean $\pm$ SD of the number of leukaemic myeloid progenitors per femur. (E) Representative FACS plot of the leukaemic KL compartment from a relapsed mouse and a 14-day vehicle treated mouse. (F) Mean $\pm$ SD of the number of leukaemic neutrophil subsets per femur. (G) Representative FACS plot of the neutrophil compartment from a relapsed mouse and a 14-day vehicle treated mouse. Statistical differences in the means were compared using non-parametric student's t-test; ns, $p \geq 0.05$.

Next, the transcriptional states of the IDN-TE leukaemias were examined and compared to the vehicle-treated leukaemias in order to identify any gene expression changes that could mediate resistance to AG120. The primary attribute contributing the greatest inter-sample variation appeared to be whether or not the IDN cells had been exposed to AG120 at the time of harvest (Figure 4.26 A x-axis); along the primary dimension, IDN cells harvested from vehicle and relapsed recipients clustered together, and away from IDN cells harvested from day-5 and day-14 AG120 treated mice. This observation was further supported by the higher correlation between the transcriptional profiles of the IDN-TE and vehicle-treated leukaemias, than with the day-5 and day-14 AG120 treated leukaemias (Figure 4.26 B). Interestingly, the second-highest leading dimension of the MDS plot separated the IDN-TE leukaemias into two distinct clusters (Figure 4.26 A y-axis), henceforth referred to as IDN-TE cluster 1 (IDN-TE1) or IDN-TE cluster 2 (IDN-TE2).

The high correlation in transcriptional states between the vehicle and relapsed mice, which appeared distinct from the day-5 and day-14 AG120 treated groups, was highly suggestive of the restoration of a large proportion of the transcriptional networks previously perturbed by IDH1^{R132H} inhibition. This was evident in the heatmaps of the top 500 most variably expressed genes amongst all samples (Figure 4.26 C), and the significantly differentially expressed genes ($\text{FDR} < 0.05$, $|\log_2\text{FC}| > 0.5$) induced by 5 and/or 14 days of AG120 treatment relative to vehicle (Figure 4.26 D). Hence, gene sets of transcriptional pathways once perturbed by AG120 treatment, such as those driving myeloid maturation (Ivanova's haematopoiesis mature cell gene set) and aberrant self-renewal properties (eg. Ivanova's stem cell and progenitor gene set), were reversed upon the removal IDH1^{R132H} inhibitor (Figure 4.26 E). Furthermore, the distribution of H3K27me3 in the relapsed recipients demonstrated a higher correlation with the vehicle treated leukaemias, than the day-5 AG120 treated leukaemias (Figure 4.26 F). H3K27me3 genomic regions significantly ($p < 0.05$) differentially methylated in the day-5 AG120 treated group were largely reversed upon AG120 withdrawal (Figure 4.26 G). Collectively, these data imply a reversal in the global transcriptional state and epigenetic landscape upon withdrawal of IDH1^{R132H} targeted therapeutics in the IDN *in vivo* model.

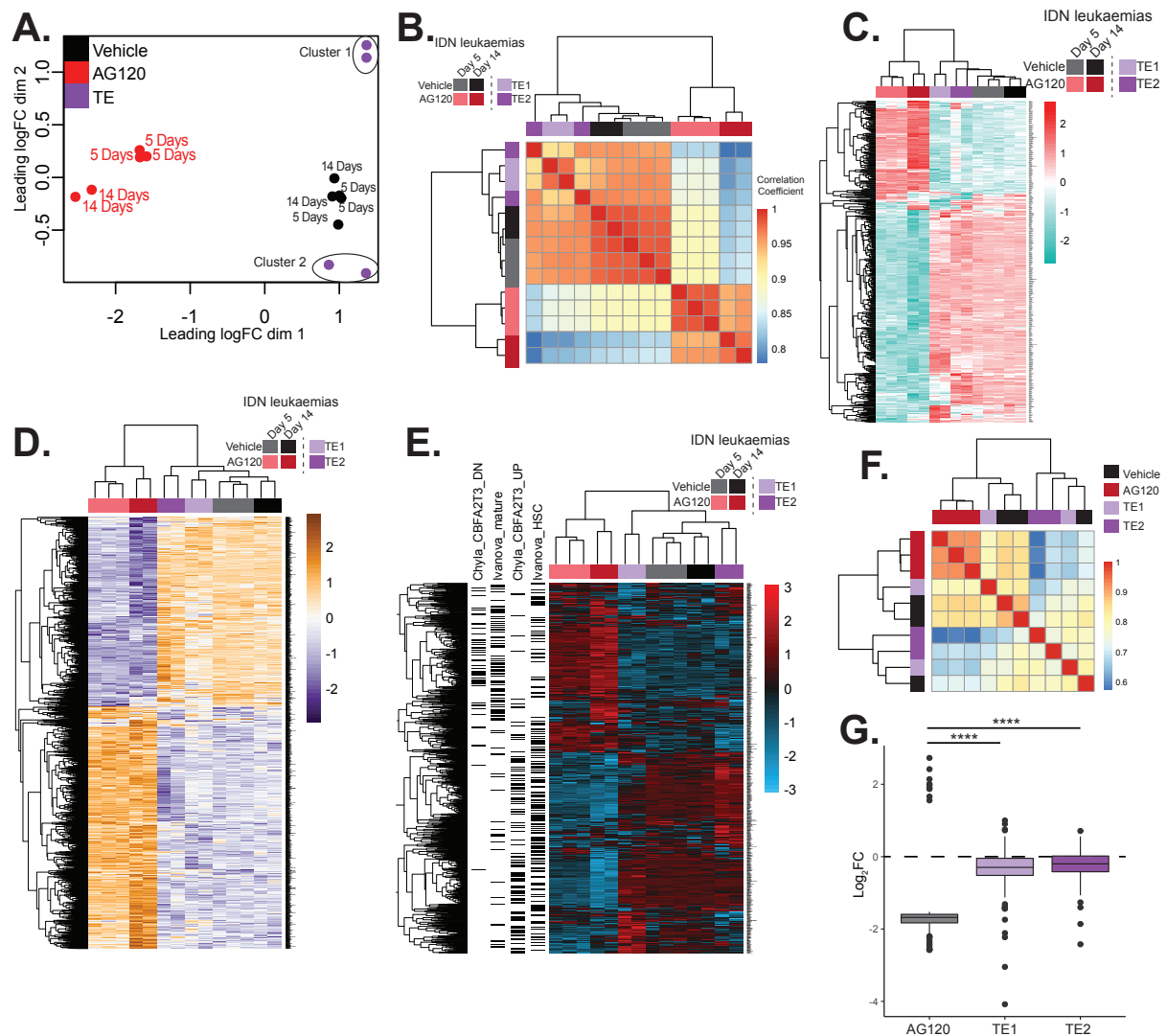


Figure 4.26 Reversal in Transcriptional and Epigenetic States Upon AG120 Withdrawal

(A) MDS plot of RNAseq samples from all mice of the cross-sectional cohort. (B) Heatmap of the pairwise Pearson's correlation coefficients for the RNAseq samples from all mice of the cross-sectional cohort. (C) Heatmap of the top 500 most variably expressed genes amongst all samples. (D) Heatmap of the significantly differentially expressed genes (FDR < 0.05, $|\log_2FC| > 0.5$) induced by 5 and/or 14 days of AG120 treatment relative to vehicle. (E) Heatmap of a sample of gene sets significantly enriched in AG120 treated mice relative to vehicle. Barcode indicates the genes within the indicated gene set. (F) Pearson's correlation plot of the trimethylated H3K27 peaks enriched in 1 kb genomic windows. (G) Boxplot of the fold-change of H3K27me3 significantly ($p < 0.05$) differentially methylated 1 kb genomic windows, with respect to vehicle. Means were compared using student's t-test; **** $p < 0.0001$. TE; treatment exposed IDN leukaemias.

Although the vast majority of transcriptional changes induced by IDH1^{R132H} inhibition were reversed upon the cessation of AG120 therapy, the distinct IDN-TE clusters observed in MDS plot (Figure 4.26 A) were suggestive of the existence of distinct transcriptional states between the two IDN-TE clusters. In accordance with this observation, no correlation existed between the fold-changes in gene expression, relative to vehicle treated leukaemias, of the significant DEGs (FDR < 0.05, $|\log_2FC| > 0.5$) in each IDN-TE cluster (Pearson's $r = 0.0153$, $p = 0.5478$) (Figure 4.27 A). Furthermore, high correlation existed between the fold-changes, relative to vehicle treated leukaemias, of the significant DEGs (FDR < 0.05, $|\log_2FC| > 0.5$) in each IDN-TE leukaemia within the same cluster (Cluster 1: Pearson's $r = 0.8101$, $p = 2.2 \times 10^{-16}$, Cluster 2: Pearson's $r = 0.6015$, $p = 2.2 \times 10^{-16}$) (Figure 4.27 B). Hence, these results warranted further investigation into whether any deviations from the treatment naïve transcriptional state contributed to post-therapy relapse.

To ascertain whether AG120 induced any transcriptional changes that persisted beyond the conclusion of therapy, overlap between the significant DEGs (FDR < 0.05, $|\log_2FC| > 0.5$) in each IDN-TE cluster and AG120 (day-14), relative to vehicle was calculated (Figure 4.27 C – D). As expected, the AG120 (day-14) treated cohort exhibited the largest number of gene expression changes relative to vehicle (Figure 4.27 C – D, Table 4.7). IDN-TE1 and IDN-TE2 shared 5.8% and 7.4%, respectively, of their up-regulated DEGs with the AG120 group, whilst 27.3% and 13.1% of their down-regulated DEGs were shared with AG120 repressed DEGs (Figure 4.27 C – D), suggesting the persistence of a small fraction of AG120 induced transcriptional changes beyond treatment duration. Assessing the magnitude of the DEGs shared between relapsed clusters and AG120 revealed the extent of the fold changes in gene expression, relative to vehicle, to be smaller in the relapsed clusters (Figure 4.27 E – F). For example, the mean normalised expression counts for the down-regulated DEGs shared between AG120 and either of the IDN-TE clusters, were significantly lower in the AG120 (day-14) treated cells than either IDN-TE cluster, relative to vehicle, (Figure 4.27 E right). This indicated that the extent of transcriptional repression was greater in the AG120 treated cohort. The converse was apparent for up-regulated genes, whereby the mean of the normalised gene expression values was higher in the AG120 treated cohort, although did not always meet the statistical significance cut-off (Figure 4.27 E left). Furthermore, pair-wise comparisons of the fold-change of each “persistent” DEG, between the AG120 group and IDN-TE clusters, provided further evidence that the magnitude of perturbation from the innate state (vehicle

cohort) was greater in the 14-day AG120 treated group than the IDN-TE clusters (Figure 4.27 F). In the scatterplots depicted in Figure 4.27 F, the majority of shared DEGs lie in the shaded region above the line $y = x$, indicating that the magnitude of the fold-change was greater in the 14-day AG120 treated leukaemias than the IDN-TE clusters, relative to vehicle treatment. Collectively, these data may suggest that the small portion of DEGs, whose transcriptional state persisted beyond treatment cessation, were converging back to the treatment naïve state, albeit at a slower rate.

Although the IDN-TE clusters did not demonstrate a large perturbation in their global transcriptional profiles relative to the vehicle treated leukaemias, ~ 500 – 1000 genes deviated from the original transcriptional state (Table 4.7, Figure 4.28 A). GSEA and gene ontology analyses were performed to identify pathways enriched amongst these DEGs. Metabolic processes, including carbohydrate, xenobiotic and drug metabolism gene sets, were significantly positively enriched in relapsed cluster 1 relative to vehicle (Figure 4.28 B, C, E), whilst relapsed cluster 2 DEGs were negatively enriched for nucleotide metabolism and cell-cycle related gene sets (Figure 4.28 B, D, F).

Table 4.7 Number of Differentially Expressed Genes

Comparison	Up-regulated DEGs	Down-regulated DEGs
	FDR < 0.05, $\log_2\text{FC} > 0.5$	FDR < 0.05, $\log_2\text{FC} < -0.5$
AG120 day 14 vs Vehicle day 14	1822	1442
IDN-TE1 vs Vehicle day 14	275	239
IDN-TE2 vs Vehicle day 14	570	566

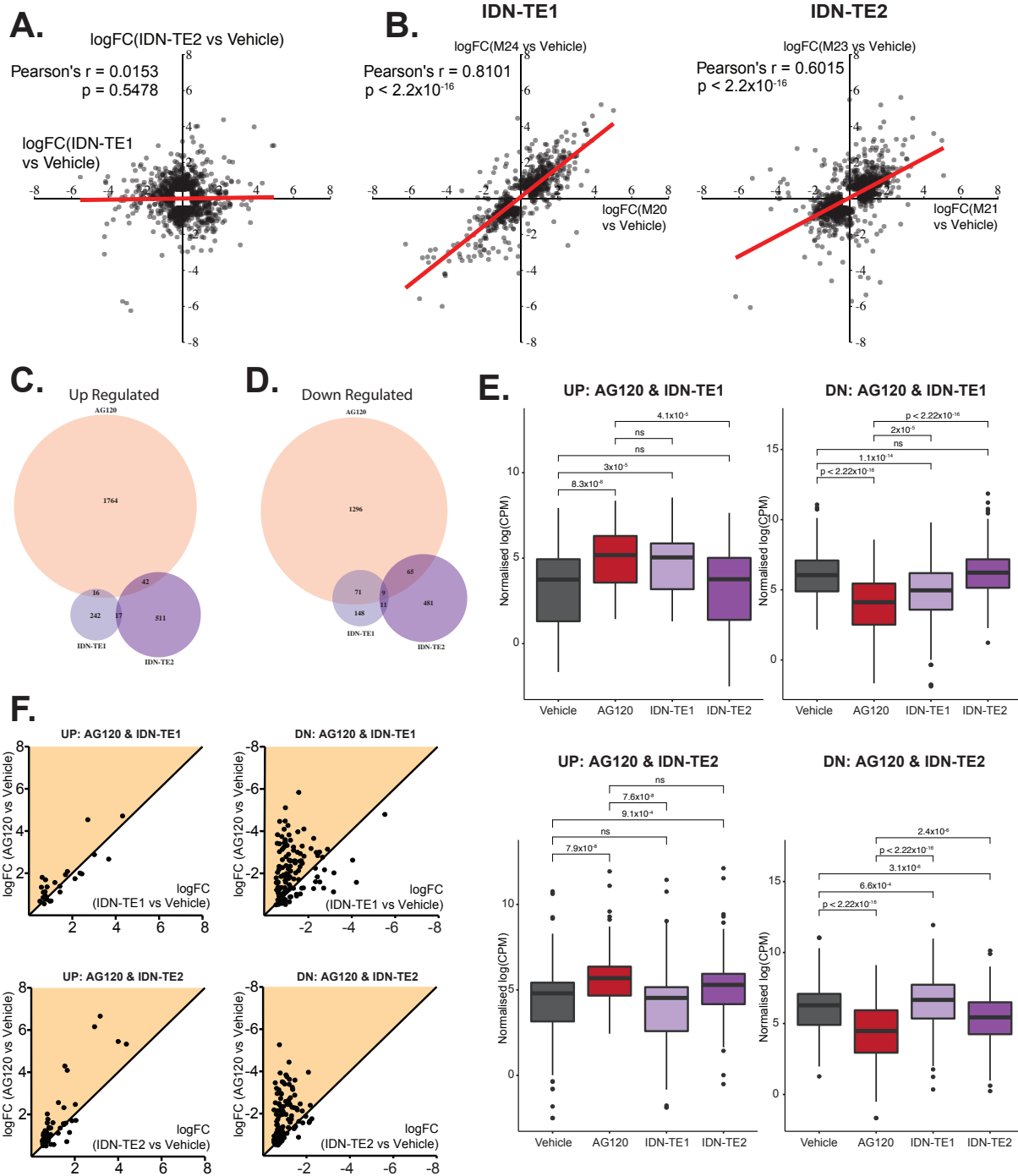


Figure 4.27 DEGs that Persisted Post-Treatment

(A) Scatterplot of the gene expression fold changes of each relapsed cluster relative to vehicle day-14. (B) Scatterplot of the gene expression fold changes of each mouse within each relapsed cluster relative to vehicle day-14. (C – D) Venn diagram of the significantly (C) up-regulated DEGs ($\text{FDR} < 0.05$, $\log_2\text{FC} > 0.5$) or (D) down-regulated DEGs ($\text{FDR} < 0.05$, $\log_2\text{FC} < -0.5$) for AG120 day-14 treated or each relapsed cluster relative to vehicle day-14. (E) Boxplots of the overlapping DEGs between AG120 and each relapsed cluster. Means were compared using student's t-test. * $0.01 < p < 0.05$, ** $0.001 < p \leq 0.01$, *** $0.0001 < p \leq 0.001$, **** $p < 0.0001$, ns; $p \geq 0.05$. (F) Scatterplot of the gene expression fold changes, relative to vehicle day-14, of the overlapping DEGs between AG120 and each relapsed cluster. The shaded region (above the line $y = x$) depicts where the magnitude of gene expression fold change is greater in the AG120 day-14 cohort.

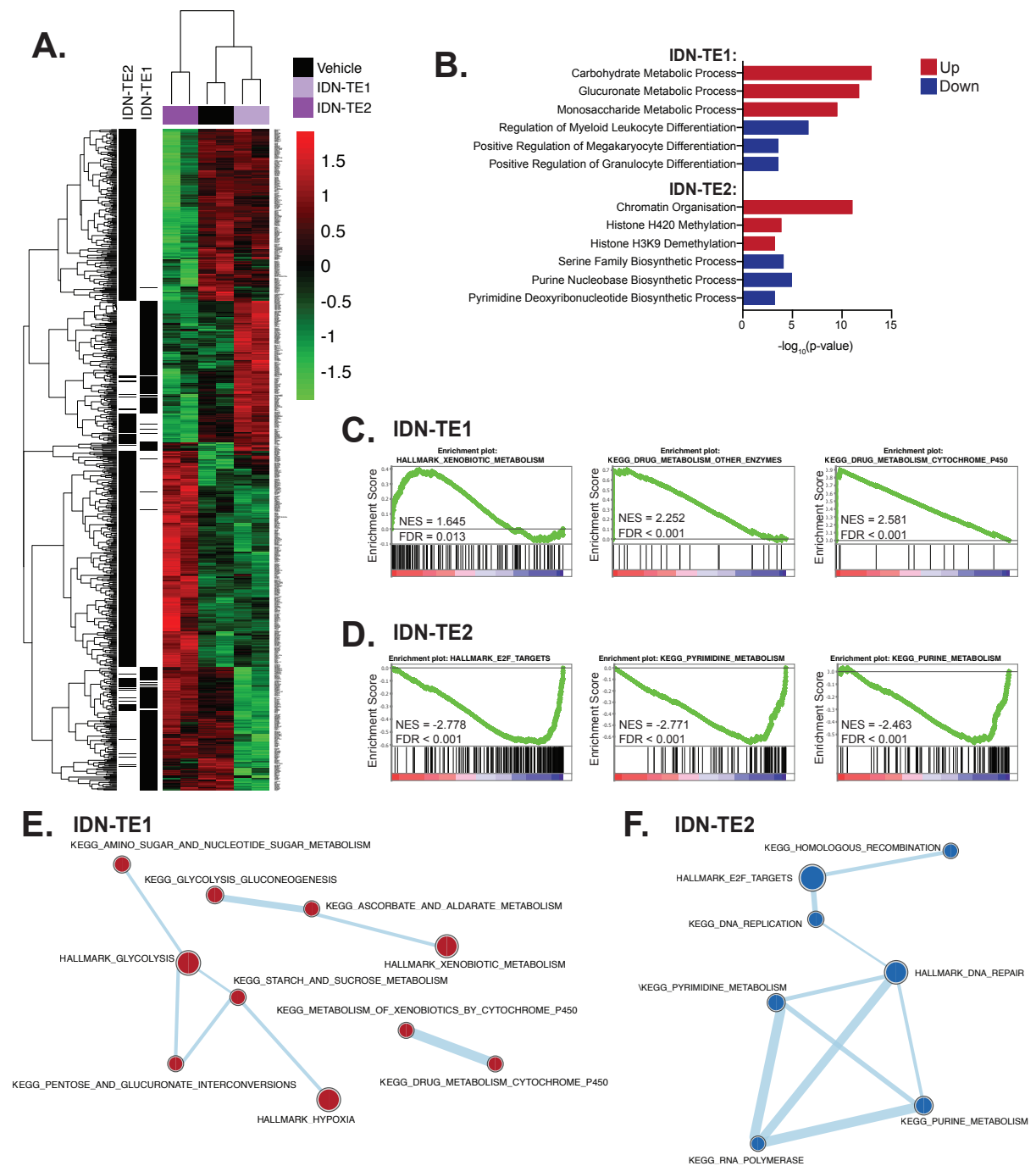


Figure 4.28 Pathways Enriched in Relapsed Clusters

(A) Heatmap of the significant DEGs in each relapsed cluster, relative to vehicle day-14 (FDR < 0.05 , $|\log_2FC| > 0.5$). **(B)** GO terms enriched within the significant DEGs in each TE cluster. **(C – D)** GSEA enrichment plots of gene sets enriched in the DEGs in TE **(C)** cluster 1 and **(D)** cluster 2. **(E – F)** Enrichment maps of gene sets enriched amongst **(E)** cluster 1 and **(F)** cluster 2 DEGs. Node colour represents enrichment directionality: positively enriched (red), negatively enriched (blue). Width of connecting lines is proportional to the number of genes shared by the gene set at each node.

Next, the IDN-TE leukaemias were assessed for the retention of leukaemia initiating capacity and resistance to further IDH1^{R132H} inhibition. Two of the IDN-TE leukaemias, one from each cluster, were cryopreserved for future use as these two samples provided a surplus of cells required for the molecular analyses aforementioned (Figure 4.26 – 4.28). The live leukaemic (DAPI/GFP⁺) population was isolated by FACS from the cryopreserved IDN-TE leukaemias and from the parental IDN treatment naïve (IDN-TN) leukaemia, and subsequently transplanted into NSG mice (1×10^5 cells / mouse) (Figure 4.29 A). After engraftment, mice either received the complete therapy regime for survival analysis or were treated for 5 days prior to the isolation of the leukaemic progenitor population for 3' mRNA QuantSeq (Figure 4.29 A).

Mice transplanted with the IDN-TN leukaemia demonstrated a significant increase in the median survival upon IDH1^{R132H} inhibition with AG120 (vehicle median survival = 48 days, AG120 median survival = 215 days, $p = 0.001$) (Figure 4.29 B). However, pharmacological IDH1^{R132H} inhibition of the IDN-TE leukaemias prolonged the median survival by 0.5 – 3 days, demonstrating a significant difference in the survival advantage provided by AG120 between the treatment naïve and exposed leukaemias (Figure 4.29 B). Of note, although the median survival of all vehicle treated cohorts occurred within a 7-day window, a significant difference in survival was detected between vehicle administered IDN-TN and IDN-TE transplanted recipients (Figure 4.29 B), suggesting that IDN-TE leukaemias proliferate more rapidly. As mentioned in section 4.3.6, when the proportion of leukaemic cells within the peripheral blood exceeded 3%, it was suspected that AG120-treated IDN recipient mice rapidly succumbed to drug induced differentiation syndrome. Although a higher mean tumour burden in the peripheral blood was observed in the IDN-TE cohorts in comparison with the IDN-TN cohort prior to the commencement of AG120 therapy, the mean tumour burden in the peripheral blood, expressed as a percentage or absolute number of leukaemic cells per microlitre of peripheral blood, for each cohort resided within the 95% confidence interval of the mean pre-treatment peripheral tumour burden that previously resulted in AG120 response (Figure 4.29 C). The 95% confidence intervals were derived from prior experiments whereby AG120 treated recipients responded to drug and did not develop GvHD nor drug induced differentiation syndrome. This suggests that the tumour burden at treatment initiation in the IDN-TE cohorts was likely below the threshold required to elicit therapy-induced differentiation syndrome. Taken together, these results suggest that the IDN-TE leukaemias were resistant to AG120 treatment.

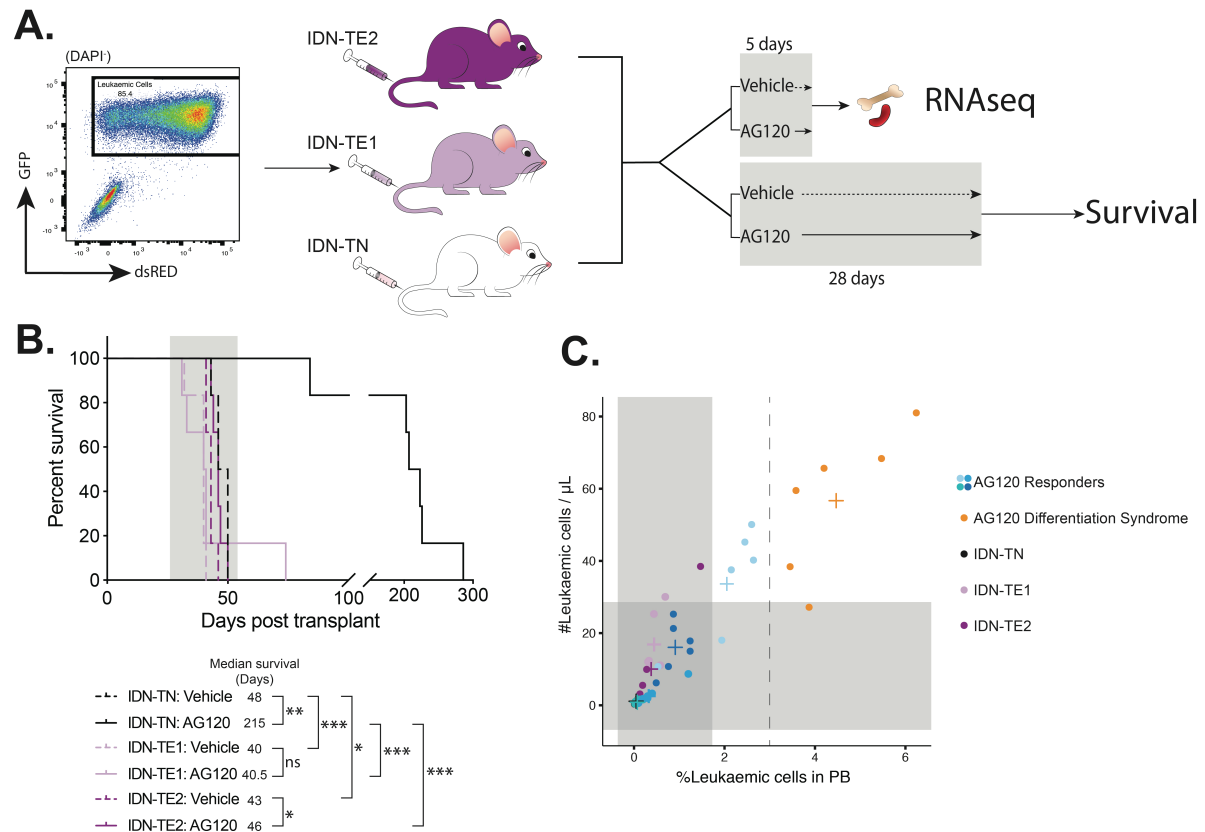


Figure 4.29 AG120 Provided No Survival Benefit to Treatment Exposed Leukaemias

(A) Schematic of experimental procedure. **(B)** Kaplan-Meier Survival Curve (n = 6 / group). Survival distributions were compared using log-rank Mantel-Cox test. * 0.01 < p < 0.05, ** 0.001 < p ≤ 0.01, *** 0.0001 < p ≤ 0.001, **** p < 0.0001, ns; p ≥ 0.05. **(C)** Scatterplot of the percentage and absolute number of leukaemic cells (GFP+) per microlitre of peripheral blood (PB) at the commencement of AG120 treatment. Circles represent individual mice. Crosses denote the mean percentage and number of leukaemic cells per group. Grey shaded areas indicate the 95% confidence intervals for the mean peripheral tumour burden, expressed as a percentage and absolute number, for prior experiments whereby AG120 treatment induced tumour regression (AG120 Responders). The dashed line denotes the previously defined 3% peripheral tumour burden cut-off, whereby, if exceeded, recipients would succumb to AG120-induced differentiation syndrome.

In accordance with the survival data, no statistical differences were observed in the spleen sizes (Figure 4.30 A) and tumour burden (Figure 4.30 B – C) between the AG120- and vehicle-treated groups of the IDN-TE transplanted cohorts. Although no difference in absolute tumour burden was observed in the IDN-TN transplanted recipients, a statistically significant increase in the spleen size was observed upon 5 days of AG120 administration in the IDN-TN cohort (Figure 4.30 A), characteristic of the IDN model. Within the myeloid progenitor compartment (KL) in the bone marrow (Figure 4.30 D) and spleen (Figure 4.30 E) of the IDN-TN transplanted recipients, the proportion of leukaemic GMPs increased at the expense of CMPs upon 5-days of AG120 treatment, consistent with prior experiments (section 4.3.7). The rapid increase in the leukaemic GMP:CMP ratio was not apparent following 5-days of AG120 administration in mice transplanted with IDN-TE2 (Figure 4.30 D – E), and nor in the bone marrow of IDN-TE1 recipient mice (Figure 4.30 D). However, within the spleen of AG120-treated IDN-TE1 recipient mice, an increase in the leukaemic GMP:CMP ratio was observed, although did not reach statistical significance (Figure 4.30 E). Of note, the distribution of leukaemic cell-types within each treatment exposed cohort differed between clusters and with respect to the treatment naïve leukaemia (Figure 4.30 F). IDN-TE1 leukaemic cells were marked by a very high proportion of immature neutrophils, whilst IDN-TE2 cells was comprised of ~ 50% myeloid progenitors (Figure 4.30 F). Minimal changes in the distribution of leukaemic cell-types was observed upon 5 days of IDH1^{R132H} inhibition in both IDN-TE clusters (Figure 4.30 F). An increase in the proportion of immature neutrophils and overall decrease in myeloid progenitors was observed upon 5 days of AG120 induction in the IDN-TN transplanted cohort (Figure 4.30 F), consistent with prior experiments (see section 4.3.7). Collectively, these data demonstrate that AG120 treatment does not induce rapid differentiation of the IDN-TE leukaemias, unlike the IDN-TN leukaemias, thus providing further evidence that the IDN-TE leukaemias are resistant to IDH1^{R132H} inhibition.

To assess whether AG120 induced transcriptional changes in the IDN-TE clusters, 3' mRNA Quantseq was performed on the leukaemic progenitor population (GFP⁺/CD117⁺/CD11b⁻) as per earlier experiments (see section 4.3.5 – 4.3.6). Unfortunately, insufficient leukaemic progenitor cells were isolated from the AG120-treated recipients transplanted with an IDN-TN leukaemia, hence the RNA yield was inadequate for sequencing. In order to compare the AG120-induced transcriptional perturbations of the IDN-TE transplanted cohorts with the previously acquired 3' mRNA Quantseq data from cross-sectional study (see section 4.3.6) (Figure 4.18, 4.20, 4.26), excess RNA extracted from various samples of the cross-sectional

study were included in the generation of libraries and sequencing. After removing sequencing batch effects with LIMMA, the MDS plot showed high concordance between re-sequenced control samples (Figure 4.30 G), indicating high reproducibility between experiments. IDN-TE leukaemias maintained two distinct clusters based on transcriptional states (Figure 4.30 G). Collectively, these data demonstrate that the aberrant transcriptional state of the IDN-TE leukaemias was stable upon retransplant. AG120-treated IDN-TE leukaemias clustered tightly with their respective vehicle-treated controls in the MDS plot (Figure 4.30 G). Analysis of differential gene expression revealed minimal AG120-induced significant gene expression changes in the IDN-TE leukaemias (Figure 4.30 H – I). Taken together, these data suggest that IDH1^{R132H} inhibition did not greatly perturb the transcriptional states of the IDN-TE leukaemias. Taken together, the minimal differences in survival, immunophenotype and gene expression between AG120 and vehicle treated groups for each IDN-TE leukaemia, suggests these leukaemias are resistant to IDH1^{R132H} inhibition.

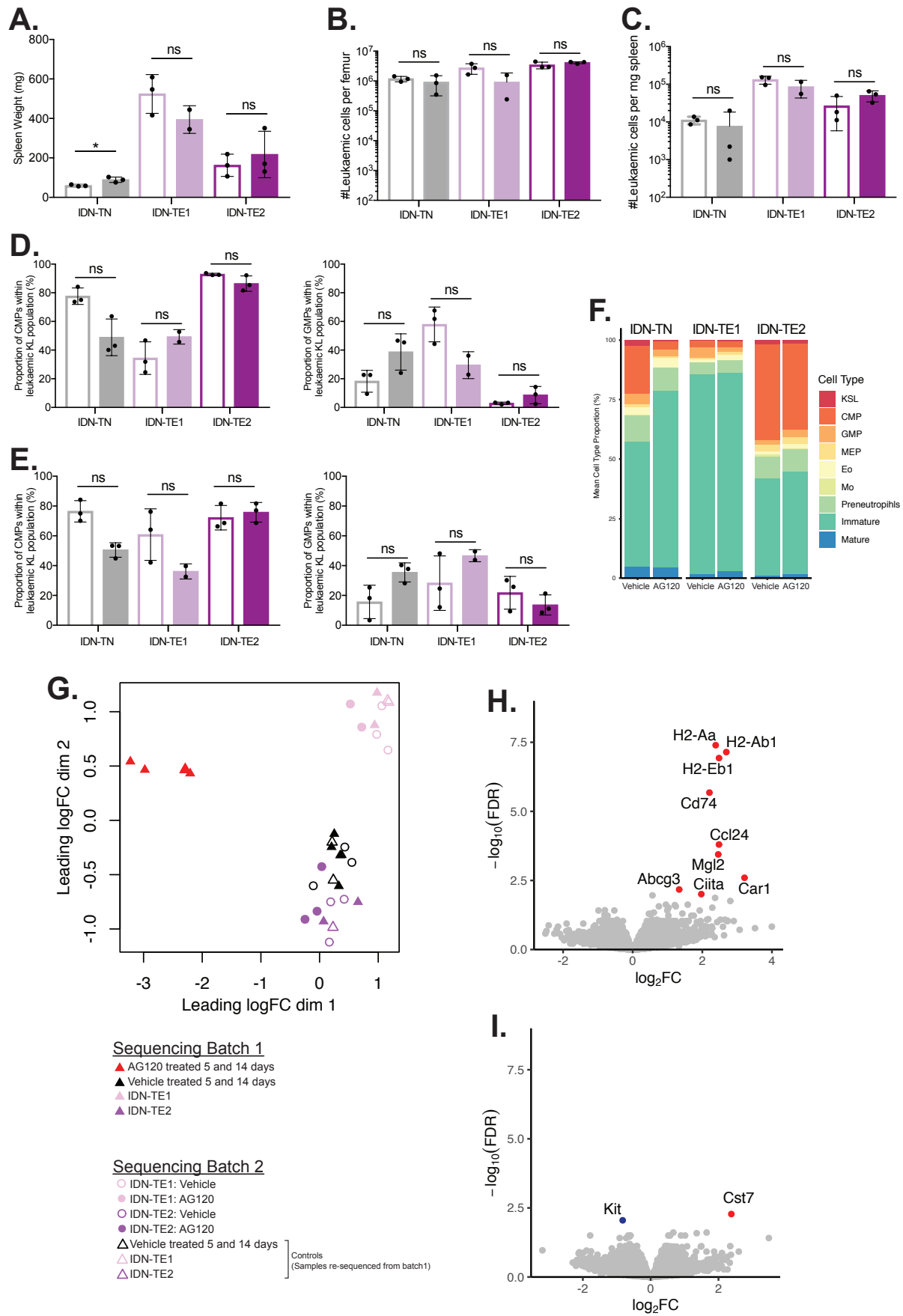


Figure 4.30 AG120 Induced No Immunophenotype Nor Transcriptional Changes in Treatment Exposed Leukaemias

(A) Mean spleen weight after 5 days of treatment. **(B – C)** Number of leukaemic cells after 5 days of treatment per **(B)** femur and **(C)** milligram of spleen. **(D)** Proportion of CMPs (left) and GMPs (right) within the myeloid progenitor (KL) compartment of the leukaemic cells in the bone marrow. **(E)** Proportion of CMPs (left) and GMPs (right) within the myeloid progenitor (KL) compartment of the leukaemic cells in the spleen. **(F)** Proportion of different cell types within the leukaemic (GFP⁺) CD117⁺ and/or CD11b⁺ population residing in the bone marrow after 5 days of treatment. **(G)** MDS plot of 3' mRNA Quantseq data. Sequencing batch 1 refers to the samples in section 4.3.6, Figure 4.26. **(H – I)** Volcano plot highlighting significantly differentially related genes ($\text{FDR} < 0.01$ & $|\log_2\text{FC}| > 0.5$) between AG120 and vehicle in **(H)** treatment exposed cluster 1 and **(I)** cluster 2.

To establish whether the IDN-TE leukaemias had acquired endogenous mutations that could explain the resistance to IDH1^{R132H} inhibition, whole exome sequencing was performed. Mutations with variant allele frequencies (VAF) greater than 0.75 in the IDN-TE leukaemias, which were absent in the IDN-TN cells, were not predicted to have a detrimental effect on protein function as determined by the Variant Effect Predictor (VEP) functional consequence rank (McLaren et al., 2016) (Table 4.8, Figure 4.31 A – B). Comparisons of the mutations in each of the IDN-TE leukaemias, that were absent in the IDN-TN leukaemia, revealed mutations that co-occurred in both leukaemias, suggesting the two IDN-TE leukaemias shared a common ancestor (Figure 4.31 C). However, the majority of mutations in each of the IDN-TE leukaemias, that were absent in the IDN-TN leukaemia, were non-overlapping between the transcriptionally distinct clusters, suggesting divergence from their shared common ancestor.

Mutant isoform switching mutations have been observed in a proportion of patients that acquired resistance to IDH inhibitors (Harding et al., 2018). Close examination of the endogenous *IDH* loci revealed no mutations within the coding domain sequences of these genes, including the recurrently mutated hotspot codons (Figure 4.31 D – E).

Table 4.8 High Consequence Mutations in Treatment Exposed Leukaemia

Gene	Treatment Exposed Leukaemia	VAF Treatment Exposed (TE)	VAF Treatment Naïve (TN)	Chromosome, Position (TN → TE)	Consequence
Tmem5	Cluster 1	0.67	0.00	10, 122096459 (C → A)	Stop gained, nonsense mediated decay
Lrr1	Cluster 2	0.51	0.00	10, 103202426 (G → A)	Stop gained
Ablm1	Cluster 2	0.50	0.00	19, 57059115 (AG → A)	Frameshift
Hspb1	Cluster 2	0.50	0.00	5, 135888276 (C → T)	Missense

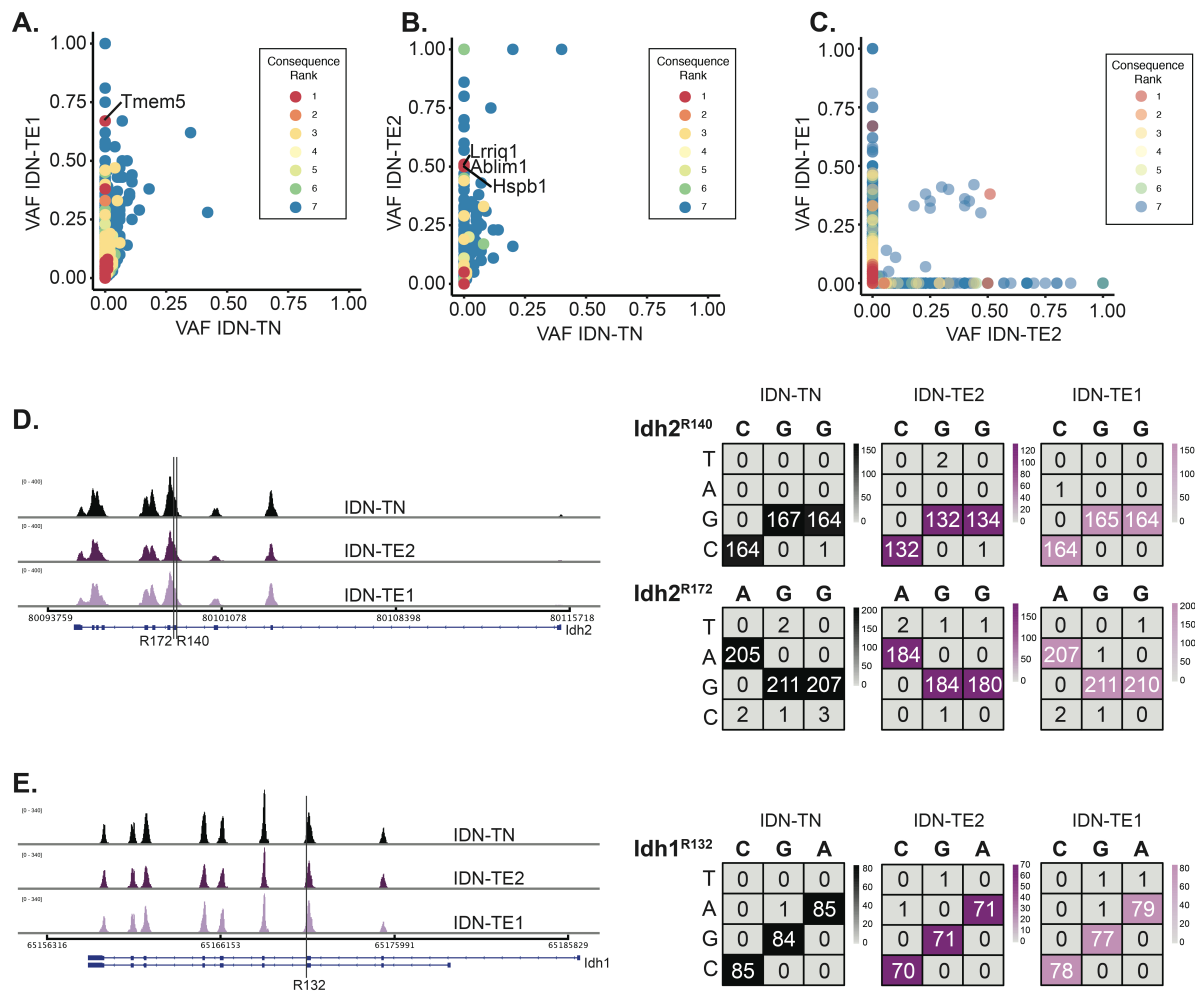


Figure 4.31 Absence of Endogenous Mutation in the CDS of Treatment Exposed Leukaemias

(A – B) Variant allele frequencies of mutations in treatment exposed (A) cluster 1 or (B) cluster 2 relative to treatment naïve. (C) Scatterplot of variant allele frequencies of mutations in treatment exposed leukaemias. (D – E: left) Coverage of whole exome sequencing across the (D) *IDH2* and (E) *IDH1* loci. (D – E: right) Coverage of (D) *IDH2* and (E) *IDH1* codons that are recurrently mutated in AML.

To establish whether the IDN-TE leukaemias remained reliant upon 2-HG for survival, IDN-TE and IDN-TN leukaemias were transplanted into NSG recipients and placed on DOX supplemented food and water in order to genetically deplete IDH1^{R132H} (Figure 4.32 A). A significant survival advantage was provided to the IDN-TE2 transplanted recipients upon IDH1^{R132H} depletion (Figure 4.32 B). However, the increase in median survival in the IDH1^{R132H} depleted cohorts, relative to the DOX⁻ group, was 62.5 days for the IDN-TE2 transplanted recipient mice, in comparison to >170 days for the IDN-TN transplanted recipients (Figure 4.32 B). This may suggest that although the IDN-TE2 maintained reliance upon 2-HG, it was not as dependent on 2-HG for survival when compared to the IDN-TN leukaemia. The mean pre-treatment leukaemia burden for the IDN-TE1 transplanted recipient mice lay outside the 95% confidence interval of the mean pre-treatment peripheral blood tumour burden that previously resulted in AG120 response (Figure 4.32 C). This likely indicates that the pre-treatment tumour burden of the IDN-TE1 transplanted mice was too high for DOX to elicit a therapeutic effect. Although this experiment would need to be repeated for the IDN-TE1 leukaemia, this experiment demonstrated that the IDN-TE2 leukaemia retained dependence on 2-HG for survival albeit at a reduced reliance in comparison to the IDN-TN leukaemia.

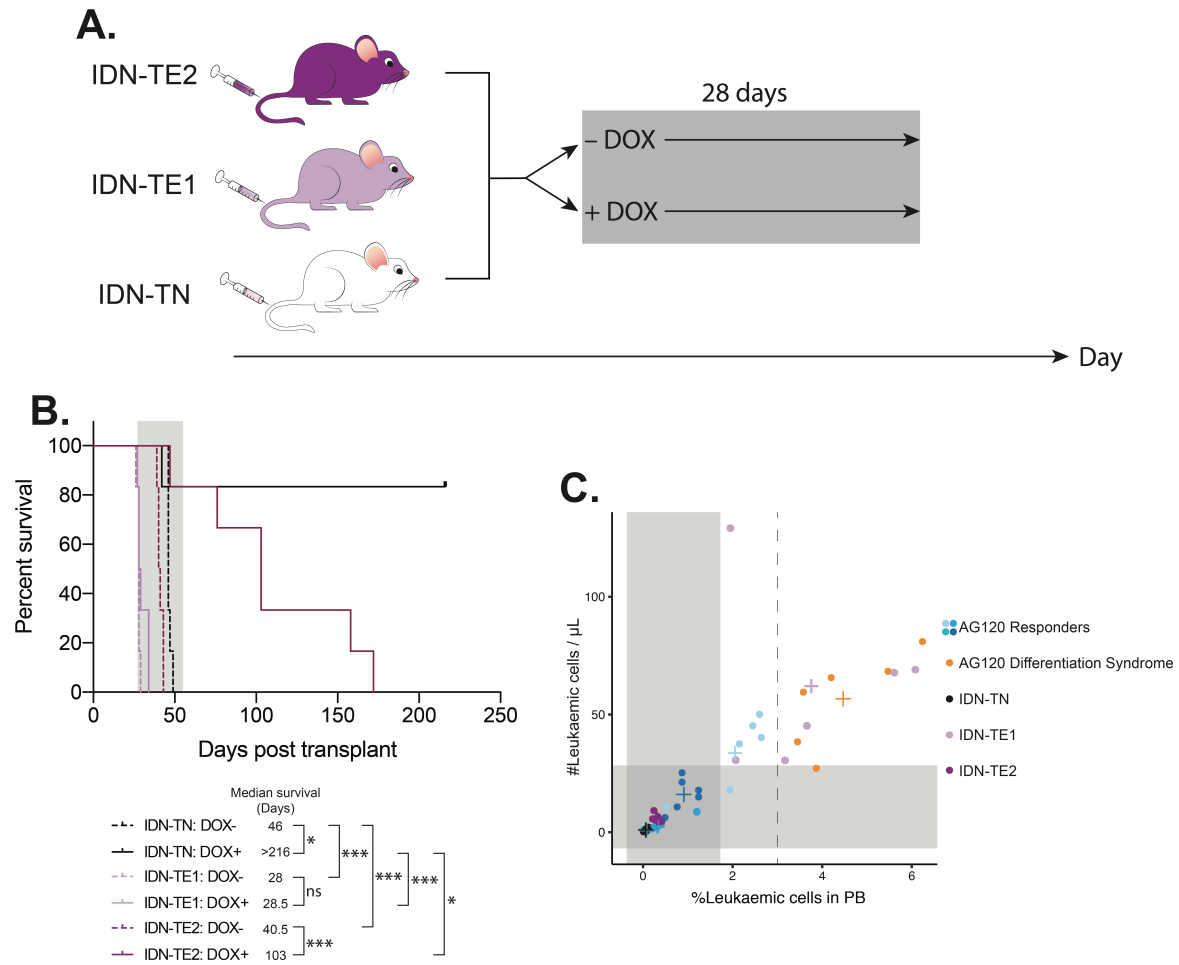


Figure 4.32 Genetic Depletion of IDH1^{R132H} Prolongs Survival of Treatment Exposed Cluster 2 Recipient Mice

(A) Schematic of experimental procedures. **(B)** Kaplan-Meier survival curve of recipient mice. Survival distributions were compared using log-rank Mantel-Cox test. * $0.01 < p < 0.05$, ** $0.001 < p \leq 0.01$, *** $0.0001 < p \leq 0.001$, **** $p < 0.0001$, ns; $p \geq 0.05$. **(C)** Scatterplot of the percentage and absolute number of leukaemic cells (GFP⁺) per microlitre of peripheral blood (PB) at the commencement of DOX/AG120 treatment. Circles represent individual mice. Crosses denote the mean percentage and number of leukaemic cells per group. Grey shaded areas indicate the 95% confidence intervals for the mean peripheral tumour burden, expressed as a percentage and absolute number, for prior experiments whereby AG120 treatment induced tumour regression (AG120 Responders). The dashed line denotes the previously defined 3% peripheral tumour burden cut-off, whereby, if exceeded, recipients would succumb to AG120-induced differentiation syndrome.

4.4 Discussion

Mutations in *IDH1/2* are present in ~10 – 30% of cytogenetically normal AML (Papaemmanuil et al., 2016; Paschka et al., 2010) and result in the production of 2-HG which accumulates to supraphysiologic levels. 2-HG dysregulates a multitude of cellular pathways that contribute to leukaemia onset and growth (Losman et al., 2013). Whilst small molecule inhibitors have demonstrated clinical response in relapsed/refractory *IDH*-mutant AML patients, a large proportion of these patients demonstrate either upfront or acquired resistance. Utilising a tractable murine model of *IDH1^{R132H}* myeloid leukaemia, the data presented in this chapter demonstrated the effects of *IDH1^{R132H}* expression in leukaemic stem/progenitor cells, the temporal consequences of mutant IDH inhibition in established haematopoietic malignancies and provided preliminary evidence of AG120 resistance and models in which to further study the resistance to targeted mutant *IDH* inhibition.

4.4.1 Co-expression of 3 Oncogenic Drivers was Required for the Initiation and Maintenance of IDN Leukaemias *In Vivo*

Next-generation sequencing studies have revealed that AMLs typically harbour at least 2 driver mutations, and the co-occurrence of *DNMT3A*^{R882H} and *NRAS*^{G12D} in *IDH*-mutant driven AML are frequent (Papaemmanuil et al., 2016). Co-expression of *IDH1*^{R132H}, *DNMT3A*^{R882H} and *Nras*^{G12D} drove the development of an aggressive experimental leukaemia (termed IDN) with indefinite transplant potential. Transplanted recipient mice succumbed to a disease with pathologies characteristic of AML including splenomegaly, thrombocytopenia, elevated white blood cell counts and weight loss. 2-HG accumulated in the plasma and bone marrow proportionally to the degree of IDN infiltration, and by late-stage disease 2-HG had accumulated to supraphysiologic concentrations. Thus, the murine model of AML co-expressing *IDH1*^{R132H}, *DNMT3A*^{R882H} and *Nras*^{G12D} reported in this chapter represents a physiologically relevant model that is concordant with the previously reported murine model of myeloid leukaemia co-expressing *IDH2*^{R140Q}, *DNMT3A*^{R882H} and *Nras*^{G12D} (Kats et al., 2017).

The transplant of bulk transduced FLCs allowed for the direct comparison of the proliferative capacity between single-, double- and triple-mutant cells *in vivo*. Since dsRED⁺/GFP⁺ cells were consistently the predominant population at late-stage disease, this implied that the co-expression of all 3 mutant genes provided a substantial proliferative advantage over the single- and double-mutant transduced FLCs transplanted into the same mouse. These observations are further supported by the studies that demonstrated that transplantation of FLCs transduced with either *Nras*^{G12D} or *Nras*^{G12D}/*DNMT3A*^{R882H} results in a leukaemic disease with an inconsistent phenotype between individual recipients, longer latency and incomplete penetrance (Kats et al., 2017). Furthermore, the expression of mutant IDH alone is insufficient in driving a lethal disease in a transgenic *in vivo* model (Kats et al., 2014). Whilst IDH-mutant leukaemias harbouring one additional mutation have been reported to induce a fully penetrant myeloid leukaemia, the median survival is ~ 60 days longer than the IDN transplanted recipients, despite transplanting 5x more leukaemic cells (Chen et al., 2013). Collectively, these data suggest that IDH-mutant leukaemic cells require at least one additional oncogenic driver to initiate leukaemia, and the greater the number of co-occurring driver mutations, the more aggressive the disease. Furthermore, these data demonstrate that oncogenic-IDH cooperates with mutant-*DNMT3A*/*NRAS* to yield a fully penetrant and rapidly lethal myeloid leukaemia.

4.4.2 The Effects of IDH1^{R132H} in AML are Cell Context Dependent

Leukaemic stem cells and myeloblasts are typically capable of differentiating, although with reduced and/or limited capacity (Agarwal et al., 2019; Meacham and Morrison, 2013; Quek et al., 2018). One of the aims of this study was to understand the contribution of IDH1^{R132H} on the aberrant self-renewal properties and differentiation capacities within the different AML haematopoietic cell types. This study demonstrated that IDH1^{R132H} provided greater leukaemia initiating potential to leukaemic CMP cells than GMP cells. Furthermore, IDH1^{R132H} skewed leukaemic CMP differentiation along the GMP-to-neutrophil lineage, with intermediates demonstrating varying reductions in differentiation capacities.

4.4.2.1 IDH1^{R132H} Provides a Greater Leukaemia Initiating Capacity to CMP than GMP Cells

Transformation of normal HSCs or progenitor cells into leukaemic stem cells (LSCs) occurs by the acquisition of genetic aberrations (Vetrie et al., 2020). Furthermore, the leukaemia initiating cell-type has been associated with distinct tumour subtypes and disease potency (George et al., 2016; van Rhenen et al., 2005; Visvader, 2011). This chapter demonstrated that *IDH1^{R132H}*, *DNMT3A^{R882H}* and *Nras^{G12D}* co-expressing CMP cells expressed gene pathways that promote HSC-like self-renewal and possessed the greatest leukaemia initiating potential. This finding is concordant with the longer disease latencies reported in mice transplanted with MLL-AF9 retrovirally transduced murine GMPs than CMPs (George et al., 2016). Indeed, the more “immature” the haematopoietic cell-type, the higher the proportion of leukaemia initiating cells within the population, which is associated with decreased disease latency (George et al., 2016; Krivtsov et al., 2013; Ye et al., 2015). Thus, the greater leukaemia initiating capacity of the IDN CMP-like cells, in comparison to IDN GMP-like cells, is likely attributed to its more immature state. Taken together, these data demonstrate that the co-expression of IDH1^{R132H}, DNMT3A^{R882H} and Nras^{G12D} can endow leukaemia initiating capacity to haematopoietic progenitors.

4.4.2.2 IDH1^{R132H} AML Have Skewed and Reduced Differentiation Capacities

For experimental purposes, AML is frequently considered as the accumulation of primitive myeloblasts which are completely blocked in differentiation (Chopra and Bohlander, 2019; Olsson et al., 1996). In the context of mutant-IDH, ectopic over-expression of mutant *IDH* has been reported to block differentiation of adipocytes and bone marrow derived cells *in vitro*

(Figuerola et al., 2010a; Lu et al., 2012), and an erythroleukaemia cell line cultured under cytokine-poor conditions (Losman et al., 2013). However, the notion that AML is characterised by myeloblasts that are completely blocked in differentiation is not entirely correct (Agarwal et al., 2019; Meacham and Morrison, 2013; Quek et al., 2018). Utilising mathematical modelling of haematopoiesis, Agarwal and colleagues (2019) reported that skewing of the differentiation rates, as opposed to a complete block, can produce an AML phenotype. Interphase fluorescent in situ hybridisation (FISH) and sequencing of purified haematopoietic cell populations from AML patients confirmed the presence of known genetic drivers of AML in various haematopoietic sub-types including mature cells (Agarwal et al., 2019). Collectively, this indicates that the leukaemic stem cells and myeloblasts are not completely blocked in differentiation but rather are capable of differentiating along their lineage trajectories, albeit with reduced or limited capacity.

The extensive immunophenotypic characterisation of the IDN AML murine model reported in this chapter is concordant with the results reported by Agarwal and colleagues (2019). Over time, total IDN leukaemia burden continued to increase, however, the proportions of different IDN haematopoietic cell populations changed. This suggests that IDH1^{R132H} had differing impacts on the proliferation and/or differentiation potentials of the various leukaemic cell-types. (Figure 4.33 B). IDH1^{R132H} driven AML was not characterised by a complete block in differentiation at the immature myeloblast-like state, but rather the leukaemic CMP population retained limited differentiation potential that was skewed along the neutrophil axis, with intermediates demonstrating various differentiation capabilities (Figure 4.33 B). These observations are concordant with the hierarchical cellular organisation observed in AML patient samples, with leukaemia initiating cells representing a minor fraction in mid-to-late stage disease (Bonnet and Dick, 1997; Thomas and Majeti, 2017). Furthermore, the immunophenotypically defined leukaemic cell-types in the IDN *in vivo* AML model were consistent with the expansion in haematopoietic compartments in AML patients harbouring IDH2^{R140Q} prior to targeted inhibition of the mutant IDH2 protein (Quek et al., 2018). CMP- and GMP-like cells encompassed the majority of the haematopoietic stem/progenitor cell compartment in the bone marrow of most IDH-mutant AML patients (Quek et al., 2018). Consistent with the IDN model, progenitor cells or immature myeloid-committed precursors encompassed a larger proportion of the total bone-marrow residing cells in IDH-mutant AML patients when compared to healthy individuals (Quek et al., 2018). Hence, the IDN model presented in this chapter represents a clinically relevant *in vivo* model of IDH1^{R132H} driven

AML that can be utilised in the study of IDH1^{R132H} biology and molecular response to IDH1^{R132H} inhibition. Moreover, the data presented in this chapter, in conjunction with Quek and colleagues, demonstrate that IDH1^{R132H} has varying impacts on the aberrant self-renewal properties and reduced differentiation capacities within different AML cellular contexts.

Whilst immunophenotyping throughout disease progression provided evidence that the various malignant cell-types possessed limited and skewed differentiation capacities, the rates of differentiation were never strictly determined. Furthermore, whether IDN non-progenitor-like cells exhibited the capacity for self-renewal was never formerly assessed. Colony formation assays and limiting dilution transplantation assays of FACS isolated IDN populations could provide insight into the self-renewing and leukaemia initiating potentials of the various IDH1^{R132H} driven AML subpopulations. Whilst the cross-sectional study (Figure 4.18) reported in this chapter analysed the infiltration and progression of leukaemia cells within the bone marrow and spleen of distinct recipient mice at each time-point, intravital microscopy has the advantage of tracking the tumour evolution within an individual recipient, and thus can elucidate the rates of cell proliferation and differentiation (Ahn et al., 2017; Hawkins et al., 2016). Coupling the detection of the leukaemic cells (GFP⁺/dsRED⁺) with fluorescently labelled antibodies that detect cell-surface markers (Ahn et al., 2017; Hawkins et al., 2016) that discern immature (e.g. CD117) and mature (e.g. CD11b, CD182, Ly-6G) cell-types, would enable the detection of the hierarchy of malignant maturation. Such experiments would elucidate the frequency in which the various IDH1^{R132H} driven AML cell-types proliferate and differentiate, and would enable the direct comparison to the normal haematopoietic system in the bone marrow niche.

4.4.3 AG120 is an Effective On-Target Inhibitor of IDH1^{R132H}

Small-molecule targeted inhibition of mutant IDH blocks the production of 2-HG and induces differentiation of cell lines and patient samples *in vitro* (Kernytsky et al., 2015; Wang et al., 2013). Whilst the efficacy of IDH2-mutant specific inhibitors has been demonstrated *in vivo* (Kats et al., 2017; Shih et al., 2017), characterisation of the therapeutic efficacy of small-molecule inhibitors of IDH1^{R132H} has been restricted to cell lines and *ex vivo* patient samples (Chaturvedi et al., 2013; Popovici-Muller et al., 2018). One of the aims of this study was to determine whether AG120, an IDH1^{R132H} inhibitor, was an effective therapeutic strategy for IDH1^{R132H} AML *in vivo*.

The IDH1^{R132H}-driven AML *in vivo* studies presented in this chapter demonstrated that the continual expression of IDH1^{R132H} and retention of neomorphic activity were vital for sustaining the leukaemia phenotype. AG120 treatment reduced plasma and bone marrow 2-HG levels, suppressed HSC-like self-renewal gene signatures in the LSCs, activated transcriptional pathways that promoted differentiation, reduced overall leukaemia burden and provided a significant disease-free survival advantage. These data are the first to demonstrate that AG120 is a highly effective therapeutic strategy for the treatment of IDH1^{R132H} AML *in vivo*. Moreover, the strong and positive correlation in the transcriptional changes elicited in response to the genetic depletion and pharmacological inhibition of IDH1^{R132H} in the IDN *in vivo* model infers that AG120 is an on-target inhibitor.

4.4.4 IDH1^{R132H} Inhibition Promoted Myeloid Differentiation

This study aimed to understand the consequence of targeted IDH1^{R132H} inhibition on the aberrant self-renewal and differentiation properties of IDH1^{R132H}-driven AML cell types. Furthermore, this thesis aimed to delineate the key transcriptional pathways and epigenetic states contributing to the response of targeted IDH1^{R132H} inhibition. The transcriptional state of the immature myeloid progenitor-like IDN cells were analysed as this population encompassed the LSCs. Acute depletion and inhibition of IDH1^{R132H} induced extensive gene expression changes. The most significantly dysregulated genes upon depletion and inhibition of IDH1^{R132H} were associated with the suppression of HSC-like self-renewal and proliferation properties, and the promotion of myeloid differentiation pathways. Furthermore, extensive characterisation of the response to AG120 *in vivo* demonstrated that inhibition of IDH1^{R132H} promoted progressive differentiation of leukaemia cells with distinct kinetics; AG120 promoted rapid differentiation of the LSC, whilst overcoming the block in differentiation of leukaemic immature to mature neutrophils took substantially longer to occur.

4.4.4.1 IDH1^{R132H} Inhibition Promotes Progressive Differentiation Along the Neutrophil Axis

The kinetics of AG120-induced differentiation varied between leukaemic cell-types; AG120 induced progressive differentiation along the neutrophil axis, rather than simultaneous differentiation of all leukaemic cell-types (Figure 4.33). Differentiation by AG120 was rapidly induced in the LSCs, whereas differentiation of leukaemic immature- to mature-like neutrophils lagged behind the differentiation of the malignant myeloid progenitors. Overall, these data are concordant with the reports that targeted small-molecule inhibition of mutant-IDH overcomes the 2-HG induced differentiation block *in vitro* (Kernytsky et al., 2015; Rohle et al., 2013; Saha et al., 2014; Wang et al., 2013). However, *in vitro* leukaemia models are often immunophenotypically uniform (Stockholm et al., 2007), and thus do not necessarily reflect the disease phenotype. Hence, studying the impact of differentiation therapies *in vitro* likely limits the therapeutic effects to a proportion of malignant cell-types that make up a leukaemia. Although differentiation induced by mutant-IDH inhibitors has been reported in IDH-mutant *in vivo* models, characterisation of the differentiation trajectory was not extensive (Chen et al., 2013; Kats et al., 2017; Yen et al., 2017). The differences in the kinetics of AG120-induced differentiation in the various IDH1^{R132H}-driven AML cell contexts could be due to differences in AG120 up-take, reliance on 2-HG and/or pathways dysregulated by 2-HG between the

different leukaemia cell-types. Assessing the AG120-induced transcriptional response, and intracellular AG120 and 2-HG levels across the various IDN cell-types in a cross-sectional *in vivo* study would provide insight into mechanisms underlying the differences in the kinetics of AG120-induced differentiation. Such investigations may identify that longer treatment regimens are required to eliminate all leukaemic populations, and may also identify co-targetable pathways that enhance the therapeutic response of mutant-IDH inhibitors in specific leukaemic cell-types.

The data presented in this chapter is also concordant with the reestablishment in the proportions of cells within the haematopoietic hierarchy in IDH2^{R140Q} AML patients who achieved complete response to targeted inhibition of the mutant IDH protein (Quek et al., 2018). The proportion of haematopoietic cell-types within the bone marrow were immunophenotyped in most patients prior to treatment initiation and upon entering complete remission. However, these cell-types were also assessed throughout AG221 therapy for one patient, revealing an initial decrease in haematopoietic stem/progenitor population with concurrent increase in the proportion of immature myeloid-committed precursors, prior to differentiation into mature myeloid cells (Quek et al., 2018). Such assessments need to be conducted amongst a larger cohort of IDH-mutant patients in response to targeted therapies, however, these observations by Quek and colleagues provide evidence that the IDN *in vivo* model of AML reported in this chapter responds to AG120 in a clinically relevant manner.

AG120 did not restore the capacity of CMPs to differentiate into MEPs, nor the potential of GMPs to differentiate into monocytes and eosinophils, but rather increased the capacity to differentiate along the neutrophil axis (Figure 4.33). The AG120-induced neutrophil-skewed differentiation of the IDN progenitors was also reflected in the transcriptional changes elicited in response to IDH1^{R132H} inhibition; haematopoietic stem-cell signatures were down-regulated (Ivanova et al., 2002) whilst neutrophil-lineage differentiation pathways were up-regulated (Brown et al., 2006; Ivanova et al., 2002; Lian et al., 2001). Interestingly, many genes associated with neutrophil differentiation and function (Lian et al., 2001) were inherently highly expressed in the IDN leukaemias, concordant with the immunophenotypic data demonstrating limited neutrophil-lineage differentiation in the IDN leukaemia. These neutrophil associated genes were further up-regulated upon IDH1^{R132H} inhibition, consistent with the AG120-induced rapid increase in the differentiation of CMPs to immature neutrophils and subsequent maturation to mature-neutrophils. Furthermore, the myeloid translocation gene on chromosome

16 (*MTG16*), also known as *CBFA2T3*, which is a transcriptional corepressor of several haematopoietic transcription factors (Engel et al., 2010; Steinauer et al., 2017) required for haematopoietic cell fate decisions (Chyla et al., 2008; Engel et al., 2010), was down-regulated upon IDH1^{R132H} inhibition. *Cbfa2t3* is highly up-regulated in HSCs and progenitor cells (Lindberg et al., 2005), and *Cbfa2t3*^{-/-} mice are deficient in megakaryocyte/erythroid differentiation, but possess increased granulocytic/monocytic differentiation potential (Chyla et al., 2008). Genes up-regulated in HSCs and progenitor *Cbfa2t3*^{-/-} cells were positively enriched upon IDH1^{R132H} inhibition, suggesting that the reduction in *Cbfa2t3* expression upon AG120 treatment may also contribute to the drug-induced neutrophil-skewed differentiation.

Furthermore, clinical differentiation and differentiation syndrome, marked by the predominance of mature neutrophils in the peripheral blood, have been reported in IDH1^{R132H}-mutant relapsed/refractory AML patients receiving AG120 monotherapy (Birendra and DiNardo, 2016). Taken together, these data demonstrate that IDH1^{R132H} targeted inhibition of *IDH1^{R132H}/DNMT3A^{R882H}/NRAS^{G12D}*-driven AML promotes the expression of genes that govern granulocytic/neutrophil differentiation. Furthermore, the results presented in this chapter suggest that the kinetics of IDH1^{R132H} inhibition differs according to the AML cellular context.

4.4.4.2 IDH1^{R132H} Inhibition Represses Self-Renewal Transcriptional Networks in IDN LSCs

Concordant with the IDN CMP cells possessing leukaemia initiating potential, analysis of the transcriptome revealed that the phenotypically immature IDN cells up-regulated HSC signatures that govern self-renewal properties. DNA binding transcription factors *Meis1* and *Sox4*, were highly expressed in this population of IDN cells and were amongst the top shared down-regulated DEGs upon IDH1^{R132H} depletion and inhibition. *Meis1* and *Sox4* regulate the self-renewal properties of HSCs, and are often aberrantly expressed in AML thus contributing to the increased self-renewal capacities that drive uncontrolled proliferation (Argiropoulos et al., 2007; Zhang et al., 2013b). Established leukaemias harbouring mutations in IDH have previously been shown to up-regulate *Meis1* expression (Ogawara et al., 2015) and other genes regulating HSC-like properties (Kats et al., 2017). Although mutant *IDH* alone is insufficient to initiate leukaemia *in vivo*, conditional knock-in of *Idh1^{R132H}* and transgenic murine models expressing *IDH2^{R140Q}* in myeloid lineage progenitors eventually developed modest splenomegaly and mildly elevated numbers of early haematopoietic progenitors, suggesting

that the acquisition of *IDH* mutations enables enhanced self-renewal properties and their eventual clonal expansion (Kats et al., 2014; Sasaki et al., 2012). Taken together, these findings indicate that $IDH1^{R132H}$ dysregulates genes that promote a stem-like state.

MYC and E2F target gene signatures were negatively enriched upon $IDH1^{R132H}$ depletion and inhibition in IDN leukaemias. Myc and E2f transcription factors regulate genes involved in cell cycle, DNA replication, DNA damage repair, differentiation and apoptosis (Bracken et al., 2004; Chen et al., 2018). Myc, E2f and their targets have been implicated in the regulation of haematopoiesis and in promoting cancer properties (Delgado and León, 2010; Hao et al., 2016; Kikuchi et al., 2007; Su et al., 2018). In the context of mutant-IDH malignancies, up-regulation of MYC and E2F targets was reported in human AML cell lines that were not inherently driven by mutant-IDH but could proliferate in 2-HG supplemented culture conditions, and in primary AML cells that harboured *IDH* mutations when compared to *IDH* wildtype samples (Su et al., 2018). Furthermore, primary *IDH*-mutant AML cells were more sensitive to inhibition of MYC signalling than the *IDH*-wildtype cells (Su et al., 2018). Moreover, inhibition of MYC signalling in *IDH2^{R172K}/Nras^{G12D}* recipient mice increased the median survival by 18 days (Chen et al., 2013). Taken together, these data suggest that MYC and E2F targets are key pathways through which mutant-IDH elicits its oncogenic effects. Targeted small-molecule inhibitors of mutant-IDH, used as single agents, are only effective in ~ 30% of *IDH*-mutant AML patients (DiNardo et al., 2018). No direct small-molecule inhibitors of MYC have been approved for cancer treatment (Han et al., 2019b). However, indirect targeting of MYC, such as through BRD4 or CDK7 inhibition, negatively influences MYC signalling thereby inducing potent anti-cancer effects in malignant *MYC* over-expressing *in vitro* and *in vivo* models (Chen et al., 2018; Delmore et al., 2011). Furthermore, targeting the E2F pathway by using CDK4/6 inhibitors has been demonstrated to re-establish cell cycle control (Johnson et al., 2016). Since the MYC and E2F signalling pathways are key pathways through which mutant-IDH elicits its oncogenic effects, combining mutant-IDH inhibitors with inhibitors of MYC signalling or the E2F pathway may improve overall survival in mutant-*IDH* cancer patients.

Analysis of the temporal transcriptional changes within the progenitor-like IDN cells elicited in response to $IDH1^{R132H}$ inhibition revealed a strong positive linear association between 5 and 14 days of AG120 treatment, with a similar number of significantly DEGs between the two treatment cohorts relative to vehicle. Immunophenotypically defined differentiation within the IDN progenitor-like compartment was evident by 3-days of AG120 therapy, with a reduction

in the total progenitor-like leukaemic population by 5 days. Taken together, these data suggest that the transcriptional perturbations in response to 5 days of AG120 treatment *in vivo* encompassed both primary and secondary consequences of IDH1^{R132H} inhibition. This makes it difficult to identify the genes whose transcriptional state is directly reliant upon the maintained production of 2-HG, and hence altered upon IDH1^{R132H} inhibition. Furthermore, the transcriptional changes induced by IDH1^{R132H} inhibition were also likely convoluted by the heterogeneity of the IDN progenitor-like population that was isolated for RNA sequencing, highlighting a limitation of bulk RNA sequencing techniques. The progenitor-like leukaemic population encompassed different haematopoietic populations, namely CMP and GMP-like cells, whose proportions significantly differed following 5 days of AG120 treatment when compared to the vehicle cohort. Hence, some of the transcriptional differences between AG120 and vehicle treated leukaemias may be attributed to the induction of differentiation and/or differences in transcriptional states between the various leukaemic cell-types (Brown et al., 2006; Ivanova et al., 2002; Lara-Astiaso et al., 2014).

4.4.4.3 IDH1^{R132H} Inhibition Promotes H3K27me3 Hypomethylation

2-HG has been reported to elicit transcriptional changes through epigenetic perturbations (Chowdhury et al., 2011; Glass et al., 2017; Kernytsky et al., 2015; Unruh et al., 2019). The family of JmjC histone demethylases are α -kg-dependent dioxygenases that are inhibited by 2-HG (Chowdhury et al., 2011; Lu et al., 2012; Xu et al., 2011). However, histone hypermethylation in AML as a consequence of mutant IDH has typically been studied via the ectopic expression of oncogenic IDH in AML *in vitro* models whose survival was not reliant upon the continual presence of mutant IDH (Su et al., 2018; Urban et al., 2017). In contrast, the IDN *in vivo* model is inherently reliant upon the maintained expression of IDH1^{R132H}. The results presented in this study were suggestive of broad hypomethylation of the repressive H3K27me3 mark in response to mutant-IDH depletion, which was consistent with previous reports (Kernytsky et al., 2015; Lu et al., 2012). Furthermore, this study provided preliminary evidence suggesting that upon IDH1^{R132H} inhibition, the transcriptional response precedes the global hypomethylation of H3K27me3 in mutant-IDH dependent AML. Extensive perturbations to the transcriptome were evident by 5 days of AG120 treatment in IDN transplanted recipients, which were not reflected by the number of changes in H3K27me3 peaks at the same timepoint. However, in a similar IDN *in vivo* model driven by IDH2^{R140Q} rather than IDH1^{R132H}, genetic depletion of mutant-IDH2 for 14 days resulted in an abundance of

differential H3K27me3 peaks relative to untreated. Genes proximal to the hypomethylated H3K27me3 regions following 14 days of IDH2^{R140Q} depletion were predominantly upregulated following 5 days of AG120 treatment in the IDH1^{R132H} driven model. Given the high transcriptional correlation between 5-day IDH2^{R140Q} and IDH1^{R132H} inhibition, these data were suggestive that transcriptional alterations precede epigenetic remodelling in response to pharmacological inhibition of IDH1^{R132H}. Such results are concordant with reports that the patterns in histone modifications can maintain the transcriptional state (Henikoff and Shilatifard, 2011; Kimura, 2013), however, further experimental validation is needed to definitively conclude a causative relationship between the epigenetic and transcriptional state of IDH1-mutant AML.

This study provided extensive characterisation of the effects of IDH1^{R132H} inhibition in a triple-mutant *in vivo* model of AML, demonstrating that mutant-IDH inhibition abrogated the HSC-like self-renewal properties exhibited by the immature IDN cells and initiated progressive differentiation through the IDN haematopoietic hierarchy with various rates. The differences in self-renewal and differentiation potentials within the IDN hierarchy, in addition to the progressive differentiation that is induced by IDH1^{R132H} inhibition, suggests that 2-HG likely elicits distinct phenotypes driven by differing transcriptional networks in the various IDN haematopoietic cell-types. To further complement these data, interrogation of the transcriptional profiles within the different IDN cell-types at the single-cell resolution, and the changes in response to IDH1^{R132H} inhibition, would provide insight into the baseline transcriptional states, the transcriptional pathways perturbed in response to AG120 therapy and the kinetics of AG120 therapy throughout the IDN hierarchy. RNA-sequencing of FACS isolated IDN subpopulations or cellular indexing of transcriptomes and epitopes (CITE-seq) of IDN cells would elucidate the similarities and differences in the aberrant transcriptional pathways driving the malignant phenotype within the IDN hierarchy of cell-types. CITE-seq is a method of assessing the transcriptome and surface protein levels of a single cell (Stoeckius et al., 2017), which would enable the dissection of the transcriptional networks perturbed within the various immunophenotype-defined IDN subpopulations identified in this chapter. Furthermore, since the oncometabolite 2-HG produced by mutant-IDH has been implicated in driving aberrant DNA and histone methylation (Chowdhury et al., 2011; Glass et al., 2017; Kernysky et al., 2015; Unruh et al., 2019), coupling such experiments with single-cell epigenomic assays, such as scChIP-seq (Grosselin et al., 2019) or scNMT-seq (Clark et al., 2018), would provide a comprehensive understanding of the temporal trajectories of the

epigenome in response to IDH1^{R132H} inhibition within the IDN haematopoietic hierarchy. This would provide further insight as to whether the epigenetic remodelling is the cause or consequence of the transcriptional perturbations in response to IDH1^{R132H} inhibition.

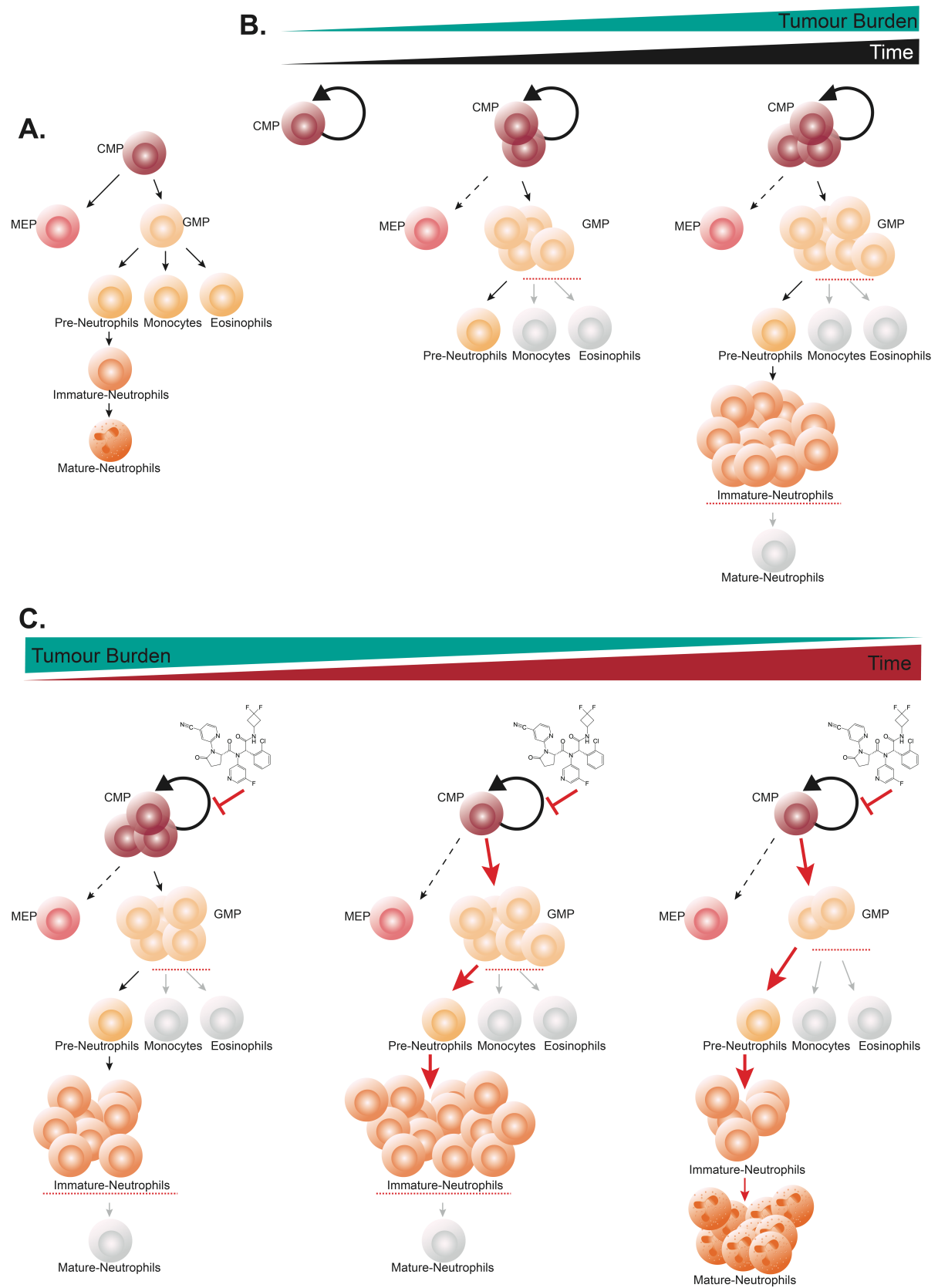


Figure 4.33 Schematic of Haematopoietic Hierarchy of the IDN AML Model Throughout Disease Progression and in Response to AG120

(A) Schematic of normal haematopoiesis. **(B)** Schematic of IDN disease evolution. The IDN driven AML model is not characterised by a complete block in differentiation at the immature myeloblast-like state, but rather the IDN CMP population retained limited differentiation potential that was skewed along the neutrophil axis, with intermediates demonstrating various differentiation capabilities. IDN CMP cells possessed the greatest leukaemia initiating capacity and represented a major leukaemic cell-type subpopulation at early-stage disease. As disease progressed, the relative proportion of leukaemic GMPs to CMPs increased, a likely consequence of the limited differentiation potential of IDN GMP-like cells as evident by their retention to promote differentiation along the neutrophil axis, whilst blocked in eosinophil and monocyte maturation. The IDN immature neutrophils were almost completely incapable of differentiating into mature neutrophils, which explains their continual accumulation throughout leukaemia progression to the point where they become the most abundant leukaemic cell-type occupying the bone marrow and spleen. **(C)** Schematic of IDN leukaemia in response to IDH1^{R132H} inhibition. AG120 induced progressive differentiation along the neutrophil axis, with distinct kinetics between the various IDN haematopoietic cell-types. Initially, AG120 induced rapid differentiation of the IDN myeloid progenitors, evident by the increase in the GMP:CMF ratio within the leukaemic KL population. This was accompanied by an overall decrease in the absolute numbers of progenitor-like IDN cells, with concurrent increase in IDN cells immunophenotypically resembling the neutrophil-lineage. AG120 did not restore the capacity of GMPs to differentiate into monocytes and eosinophils, but rather increased the capacity to differentiate along the neutrophil axis. Differentiation of IDN immature- to mature-like neutrophils lagged behind the differentiation of the myeloid progenitors. IDN mature-like neutrophils became the most abundant leukaemic cell-type within the bone marrow by therapy conclusion.

4.4.5 Emergence of Distinct Relapsed Leukaemias

Despite the clinical success of mutant IDH inhibitors in patients with relapsed/refractory AML harbouring *IDH* mutations, ~ 70% of these patients do not enter complete remission (DiNardo et al., 2018) or relapse after an initial response (Amatangelo et al., 2017). Hence, the identification of mechanisms through which inherent or acquired resistance to mutant-IDH inhibitors arises is important (Amatangelo et al., 2017; Harding et al., 2018; Intlekofer et al., 2018). Whilst many studies have investigated the consequences of mutant-IDH inhibition (Chen et al., 2013; Kats et al., 2014; Kats et al., 2017; Kernysky et al., 2015; Rohle et al., 2013; Saha et al., 2014; Wang et al., 2013), few have investigated the transcriptional and epigenetic reprogramming and/or persistence upon removal of targeted mutant-IDH therapy (Turcan et al., 2018). This thesis revealed that the majority of the transcriptional networks and H3K27me3 distribution previously perturbed by IDH1^{R132H} inhibition, were subsequently restored following treatment cessation. These data are concordant with the gradual reversion of the transcriptional landscape upon genetic depletion of mutant-IDH in an *in vitro* glioma model ectopically expressing TET-inducible IDH1^{R132H} (Turcan et al., 2018). The results presented in this chapter were the first to demonstrate the reversion of the transcriptional and epigenetic landscapes upon the cessation mutant-IDH inhibition, in IDH1^{R132H} dependent AML.

Despite the high correlation between the transcriptional states of the IDN-TE and IDN-TN leukaemias, differences in transcriptional profiles were observed. The IDN-TE leukaemias separated into two transcriptionally distinct clusters, which was maintained upon retransplant, suggesting that the transcriptional perturbations were not transient. Relative to the treatment naïve leukaemias, the IDN-TE1 leukaemia transcriptionally activated pathways involved in the metabolism of sugars, xenobiotics and drugs. Xenobiotics are molecules foreign to the cell. Drug and xenobiotic metabolising enzymes have been broadly categorised into two classes: phase I drug-metabolising enzymes introduce reactive functional groups that are typically subsequently conjugated by phase II enzymes to yield a hydrophilic moiety that enables the compound to be easily excreted by the cell (Kaur et al., 2020). Cytochrome P450s are a large group of phase I enzymes whose elevated expression has been implicated in cancer drug resistance by resulting in the rapid turnover and removal of the therapeutic agent before reaching its target (Kaur et al., 2020). Prakash et al. (2019) reported that cytochrome P450s are involved in the metabolism of AG120. Hence, elevated expression of cytochrome P450s may explain why survival and transcription states were not affected upon subsequent IDH1^{R132H} inhibition by AG120 in the recipients retransplanted with the IDN-TE1 leukaemia. Further

analysis of the RNAseq data would enable the identification of the cytochrome P450s that are up-regulated in the IDN-TE1 leukaemia with respect to IDN-TN. To address whether elevated expression of specific cytochrome P450s facilitate AG120 resistance, the IDN-TN leukaemia could be transduced with retroviral constructs permitting the overexpression of specific cytochrome P450s, or an empty vector control, and subsequently transplanted into murine recipients. Following an initial engraftment period, the recipients would be challenged with AG120 therapy and survival monitored to see whether a diminished survival benefit is observed following IDH1^{R132H} inhibition in the mice transplanted with IDN leukaemias concurrently overexpressing various cytochrome P450s. Assessing whether the suppression of various cytochrome P450s re-sensitises the IDN-TE1 leukaemia to AG120 therapy, would provide further evidence for the role of elevated cytochrome P450 expression in AG120 resistance. This could be achieved by transducing IDN-TE1 cells with shRNAs directed against specific cytochrome P450s, or a control hairpin, and subsequently transplanting the cells into mice and treating with AG120. Furthermore, the concentration of plasma AG120 levels could be monitored to assess whether elevated cytochrome P450 expression corresponds with a reduction in plasma AG120. Whether the IDN-TE1 leukaemia was sensitive to genetic depletion of IDH1^{R132H} remains inconclusive, as the DOX treatment was initiated too late in disease and mice transplanted with IDN-TE1 cells likely succumbed to differentiation syndrome. Hence, the DOX de-induction experiment needs to be repeated with the IDN-TE1 leukaemia.

GSEA and gene ontology analyses identified nucleotide metabolism and cell-cycle related gene sets to be suppressed in the IDN-TE2 leukaemia, relative to IDN-TN. How these pathways may contribute to AG120 resistance is unclear, however, experiments such as those aforementioned could be undertaken to see whether genes in these pathways are truly responsible for the resistance to AG120 in the IDN-TE2 leukaemia. The IDN-TE2 leukaemia appeared to maintain its dependency on the production of 2-HG, as genetic depletion of IDH1^{R132H} provided a significant survival advantage. Since small-molecule inhibition of IDH1^{R132H} did not confer a survival advantage, these observations suggest that the resistance in these cells may be governed by the inability of AG120 to reach and/or effectively inhibit IDH1^{R132H}. Unlike IDN-TE1, the transcriptional up-regulation of genes involved in the metabolism and elimination of drugs and other foreign molecules from the cell was not observed in IDN-TE2. However, the regulation may not necessarily be at the gene expression level. Post-translational modifications including phosphorylation, glycosylation, ubiquitination, acetylation and palmitoylation all

modulate the activity and function of cell-membrane transporters (Czuba et al., 2018). Hence, post-translational modifications of specific transporters may be contributing to AG120 resistance by reducing the influx of the IDH1^{R132H} inhibitor into the cell. Alternatively, the ectopically expressed IDH1^{R132H} may have acquired a mutation that prevents drug binding whilst maintaining 2-HG production capabilities. Indeed, *cis* and *trans* dimer-interface mutations have been shown to confer resistance *in vitro* and have been observed in a few patients that acquired resistance to IDH inhibitors (Intlekofer et al., 2018). Such mutations in the retrovirally introduced *IDH1^{R132H}* transgene would confer resistance to AG120, yet still maintain sensitivity to DOX-induced suppression of IDH1^{R132H} expression. Targeted sequencing of the transgene would determine whether the IDN-TE leukaemias harboured additional genetic aberrations in mutant-IDH, since exome sequencing did not capture the transgene.

It is important to note that the transcriptional states of the IDN-TE leukaemias were only assessed in the progenitor-like leukaemic population. Although this population encompassed the LSC in the treatment naïve IDN population, that may not necessarily be the case for the IDN-TE leukaemias. Differences in the proportions of immunophenotypically defined haematopoietic IDN cell-types were observed between the cohorts transplanted with the IDN-TN and IDN-TE leukaemias (Figure 4.30 F). Hence, examining the transcriptome of the progenitor-like leukaemic population in the IDN-TE leukaemias may not necessarily be informative. CITE-seq analysis of the various haematopoietic cell-types within the IDN-TN and IDN-TE leukaemias would provide insight into the different transcriptional networks perturbed within the haematopoietic hierarchy and whether specific cell-types are the source of resistance to AG120 therapy. Furthermore, limiting dilution transplantation assays of FACS isolated CMP-, GMP- and immature neutrophil-like leukaemic cells from the treatment naïve and IDN-TE leukaemias would provide insight into whether the LSC differs between the different leukaemias. Such experiments would also identify whether differences in sensitivity to AG120 exist between the different haematopoietic IDN cell-types, and whether a small neutrophil precursor population may be able to evade AG120 therapy by de-differentiating into a progenitor-like cell. This concept has been demonstrated in a murine AML model driven by *PU.1* depletion, whereby restoration of *PU.1* expression induced differentiation of the immature myeloblasts into morphologically defined mature neutrophils (McKenzie et al., 2019). The authors reported that subsequent suppression of *PU.1* in a minor population of differentiated AML cells resulted in the reversion of transcriptional profiles, immunophenotype and

morphology to an immature state, capable of re-establishing a fully penetrant disease (McKenzie et al., 2019). The capacity of mature differentiated cancer cells to revert to an immature state has been further demonstrated in solid cancer models (Shimokawa et al., 2017).

The IDN-TE leukaemias may not be the result of the outgrowth of a single resistant IDN cell, but rather multiple distinct resistant cells, thus presenting a challenge when identifying the mechanism through which AG120 resistance is occurring. The triple retroviral overexpression system used in the generation of the IDN model produces a heterogenous population with multiple viral integrations that occur in different locations between individual cells. Sequencing the viral integration sites would aid in the delineation of the clonal architecture of the IDN-TN and IDN-TE leukaemias (Niederer and Bangham, 2014).

4.4.6 Limitations of the IDN Leukaemia Model

IDH1^{R132H} was expressed at supraphysiologic levels in the IDN model, which may impact disease biology. Although the production of the oncometabolite, 2-HG, is the recurrent feature in malignancies harbouring an IDH1/2 hotspot mutations (Lemonnier et al., 2016; Losman et al., 2013; Ward et al., 2010), the incidence of these mutant *IDH* alleles differs between cancer subtypes (Losman and Kaelin, 2013). Some studies have reported that the differences in 2-HG accumulation between *IDH* mutants may contribute to the differing prevalence in *IDH* mutations amongst various cancers (Lemonnier et al., 2016; Ward et al., 2013). The use of overexpression systems likely results in the accumulation of 2-HG that may not accurately reflect the levels produced by the heterozygous knock-in of *Idh1*^{R132H} in HSCs and myeloid progenitors. However, it is difficult to compare the plasma 2-HG accumulation produced by the IDN model presented in this chapter and other *in vivo* models in the literature, since published studies tend to report serum 2-HG concentrations (Lemonnier et al., 2016), and also report the levels of 2-HG in “arbitrary units” relative to controls rather than precise measurements per millilitre of plasma (Sasaki et al., 2012).

Differences between endogenous and supraphysiological expression of the oncogenic proteins is likely not only limited to mutant-IDH1. Indeed, the expression of DNMT3A^{R882H} promotes cytokine-independence in TF-1 cells in a dose-dependent manner (Lu et al., 2019). Furthermore, studies have reported that gene-dosage effects of mutant-Nras and Dnmt3a loss of function drive different haematopoietic malignancies (Wang et al., 2010; Wang et al., 2011;

Yang et al., 2016). Heterozygous mutations predisposed HSCs to an AML disease whilst homozygous genetic aberrations in *Dnmt3a* and *Nras* promoted transformation of a T-cell malignancy (Wang et al., 2010; Wang et al., 2011; Yang et al., 2016). Hence, ectopic overexpression of *DNMT3A^{R882H}* and/or *Nras^{G12D}/DNMT3A^{R882H}*, in the absence of *IDH1^{R132H}*, may predispose the development of a lymphoid malignancy and highlights a limitation of the overexpression system. Furthermore, mice transplanted with the *DNMT3A^{R882H}* and/or *Nras^{G12D}/DNMT3A^{R882H}* leukaemias exhibited longer disease latencies, incomplete penetrance and inconsistent haematological malignancies expressing either lymphoid or myeloid immunophenotypic markers in separate recipients (Kats et al., 2017). These data are consistent with the outgrowth of lymphoid-like cells ectopically expressing *DNMT3A^{R882H}* and/or *DNMT3A^{R882H}/Nras^{G12D}* (dsRED⁻/GFP⁺) in the GvHD-symptomatic mice. Given that the IDN leukaemias were generated in an immune-competent C57BL/6 background and initially contained a small fraction of dsRED⁻/GFP⁺ cells, upon elimination of the triple-mutant IDN cells with DOX or AG120 treatment, the lymphoid-like *DNMT3A^{R882H}* and/or *DNMT3A^{R882H}/Nras^{G12D}* cells were able to expand and attack the immune-deficient NSG recipient, thus resulting in GvHD.

Despite these limitations, the IDN model utilised in this chapter has provided insight into *IDH1^{R132H}* driven AML and the consequences of *IDH1^{R132H}* inhibition. However, endogenous mutant protein expression levels could be achieved utilising genetically engineered mouse models with the heterozygous knock-in of the *Idh1^{R132H}*, *Nras^{G12D}* and *Dnmt3a^{R878H}* (murine equivalent of *DNMT3A^{R882H}*) alleles.

4.5 Conclusions

The data presented in this chapter demonstrated that continuous activity of IDH1^{R132H} was required to sustain the production of the oncometabolite 2-HG and maintain the transcriptional states, epigenetic programs and leukaemia phenotype in AML driven by the co-expression of IDH1^{R132H}, DNMT3A^{R882H} and Nras^{G12D}. IDH1^{R132H} contributed to the expression of transcriptional programs governing the leukaemia phenotype in the LSCs, including the gene pathways that underpin HSC-like self-renewal and heightened proliferation properties. Leukaemias co-expressing IDH1^{R132H}, DNMT3A^{R882H} and Nras^{G12D} demonstrated restricted neutrophil-lineage differentiation, however, the impact on the differentiation capacity varied within the different AML cellular contexts. IDH1^{R132H} expression most profoundly inhibited the maturation of immature neutrophils.

This study demonstrated that targeted inhibition of IDH1^{R132H} with AG120 was a highly effective therapeutic strategy for the treatment of *in vivo* AML. IDH1^{R132H} inhibition reduced 2-HG levels, suppressed HSC-like self-renewal gene signatures in the LSCs, activated transcriptional pathways that promoted differentiation, reduced overall leukaemia burden and provided a significant disease-free survival advantage. IDH1^{R132H} inhibition promoted progressive differentiation along the neutrophil axis with kinetics dependent on the AML cell type. IDH1^{R132H} inhibition did not restore the capacity of IDH1^{R132H}, DNMT3A^{R882H} and Nras^{G12D} AML cells to differentiate into other lineages such as the megakaryocyte/erythroid lineage. Further research utilising single-cell transcriptome and epigenome techniques would provide valuable insight into the influence that IDH1^{R132H} has in different malignant haematopoietic cell-types, and the progressive temporal consequences of IDH1^{R132H} inhibition throughout the malignant haematopoietic hierarchy. Finally, the strong correlation in the transcriptional changes elicited in response to genetic depletion and pharmacological inhibition of IDH1^{R132H} *in vivo*, indicates that AG120 is an on-target inhibitor.

Despite the clinical efficacy of AG120, ~ 70% of patients with relapsed/refractory AML harbouring IDH mutations do not enter complete remission or relapse after an initial response. This study identified two transcriptionally distinct leukaemias resistant to IDH1^{R132H} inhibition. Analysis of the transcriptional states suggested that AG120 resistant leukaemias may transcriptionally activate pathways involved in the metabolism of xenobiotics and drugs. However, further characterisation and investigation into the resistant leukaemias are needed to definitely validate the mechanisms through which AG120 resistance is occurring.

Chapter 5: Thesis Discussion and Conclusions

5.1 Epigenetic Therapies for the Treatment of AML

Until recently, therapeutic avenues for the treatment of AML were limited to cytotoxic chemotherapies. The last few years has seen the approval of novel therapeutic agents including regulators of apoptosis, kinase inhibitors, antibody-based therapies and epigenetic therapies (Keiffer and Palmisiano, 2019; Short et al., 2018; Williams et al., 2019; Winer and Stone, 2019). The identification of efficacious epigenetic therapies for the treatment of AML has gained traction due to the reversibility and plasticity of the epigenome (Flavahan et al., 2017), the role of epigenetics in transcriptional control and normal haematopoiesis (Flavahan et al., 2017), and since the chromatin landscape is typically dysregulated in AML, with epigenetic regulators frequently mutated in AML (Papaemmanuil et al., 2016; Shih et al., 2012). Whilst some small-molecule epigenetic therapies directly inhibit oncogenic drivers, such as AG120 inhibition of IDH1^{R132H}, in order to reverse the consequential malignant epigenetic landscape, other therapeutics, such as hypomethylating agents, target wildtype epigenetic regulators with the aim of reprogramming the transcriptional networks driving malignant progression. By exploiting the transcriptional plasticity governed by the epigenome, epigenetic therapies reverse the transcriptional pathways that underpin aberrant self-renewal and abrogated differentiation.

DNA methylation patterns can define distinct AML subtypes with associated prognostic outcomes and can indicate the presence of recurring mutations in specific epigenetic regulators (Bullinger et al., 2010; Figueroa et al., 2010a; Figueroa et al., 2010b; Glass et al., 2017; Jiang et al., 2020; Shih et al., 2017). Furthermore, both global hypo- and hypermethylation have been reported in AML, suggesting that aberrant DNA methylation may be important for oncogenesis (Figueroa et al., 2010b; Schoofs et al., 2014). For example, *TET2* inactivating mutations and *IDH1/2* mutations are generally associated with broad DNA hypermethylation whilst *DNMT3A*^{R882H} is associated with global hypomethylation (Figueroa et al., 2010a). Hypomethylating agents were thought to elucidate their anti-leukaemia effects primarily through the activation of tumour-suppressor genes that were previously silenced by aberrant CpG methylation (Bender et al., 1998). However, patients with haematological malignancies harbouring mutations in *TET2* or *IDH1/2* are not associated with an increased overall survival following treatment with hypomethylating agents (Bejar et al., 2014; DiNardo et al., 2014). Indeed, hypomethylating agents as monotherapies for the treatment of AML are rarely curative. This raises the question as to how hypomethylating agents exert their anti-tumour effects. An increasing body of evidence is suggesting that hypomethylating agents induce pleiotropic

effects, including tumour-extrinsic immunomodulatory effects (see section 1.3.4.1.1) (Chiappinelli et al., 2015; Jeong et al., 2015; Li et al., 2014a; Stone et al., 2017). However, most studies focus on the consequences arising from AZA/DAC incorporation into DNA, with few studies investigating the anti-tumour effects of AZA incorporation into RNA (see section 1.3.4.1.1) (Schaefer et al., 2009). A comprehensive understanding of the similarities and differences through which AZA and DAC elicit their anti-tumour effects is imperative for the identification of resistance mechanisms, stratification of patients and the development of rationalised combination therapies in order to improve patient outcomes. Furthermore, understanding how treatment with hypomethylating agents increase the cell's dependency on specific pathways will enable the identification of rationalised combination therapies. Given that Gu and colleagues (2020) reported that decreased expression of *UCK2* and *DCK* occurred as an adaptive response to AZA or DAC exposure, respectively, the findings of this thesis would suggest that combining inhibitors of DHODH with hypomethylating agents may provide a synergistic effect by simultaneously targeting the pyrimidine *de novo* synthesis and salvage pathways.

Despite the success of epigenetic therapies under experimental conditions, few epigenetic monotherapies have resulted in a significant curative response in AML patients. Instead the majority of patients do not enter complete remission, and/or subsequent resistance and relapse is a common occurrence (Amatangelo et al., 2017; DiNardo et al., 2018; Navada and Silverman, 2017). Indeed, the lack of high cure rates is not limited to single-agent epigenetic therapies. Monotherapies for the treatment of AML that target apoptotic machinery, cell-surface antigens, tyrosine kinases and cell-cycle regulators have also resulted in a minor proportion of patients achieving complete remission (Antar et al., 2020; Castaigne et al., 2012; Konopleva et al., 2016; Winer and Stone, 2019). This highlights that drug resistance is a major challenge for the treatment of AML.

Genetic heterogeneity can contribute to the fitness of cancer cells throughout disease evolution and in response to therapeutic pressures (Turajlic et al., 2019). However, AML has one of the lowest mutational burdens of all cancers (Lawrence et al., 2013; Cancer Genome Atlas Research Network, 2013), despite the vast genetic heterogeneity of the disease (Papaemmanuil et al., 2016). Hence, the mutational status of AML is likely not the only contributing factor to therapeutic resistance. Next-generation sequencing studies have revealed that the complexity of the clonal architecture in AML is attributed to both genetic and epigenetic heterogeneity (Li

et al., 2016). Epigenetic heterogeneity is more dynamic than genetic heterogeneity, as the epigenome can rapidly respond to genetic, metabolic and environmental stimuli, thereby regulating the transcriptional plasticity of the cancer cells (Dawson, 2017; Flavahan et al., 2017). Thus, the overall poor response to cancer monotherapies is hypothesised to be largely governed by the epigenetic regulation of transcriptional plasticity in response to therapeutic pressures (Dawson, 2017; Flavahan et al., 2017). Indeed, adaptive responses to therapeutic pressures, not driven by the acquisition of additional genetic aberrations, has been reported for hypomethylating agents (Gu et al., 2020), BET inhibitors (Bell et al., 2019) and FLT3 inhibitors (Melgar et al., 2019), providing further evidence of the transcriptional plasticity of AML cells.

The contribution of intratumour heterogeneity in cancer evolution and in response to therapeutic pressures are not yet fully understood. Next-generation single-cell sequencing techniques are providing insight into the clonal architecture throughout disease progression and in response to therapeutic pressures. Single-cell sequencing of recurring genomic mutations has enabled the assessment of the clonal dynamics of AML from diagnosis through to relapse. Such studies have identified distinct patterns in clonal evolution through which AML relapse can occur, including the outgrowth of a minor subclone present at diagnosis, persistence of the major clone present at diagnosis, or with further clonal evolution through the acquisition of additional genetic mutations (Corces-Zimmerman et al., 2014; Ediriwickrema et al., 2020; McMahon et al., 2019; Quek et al., 2018). Single-cell sequencing techniques that interrogate both the genome and transcriptome have provided evidence that differentiated malignant cells also contribute to AML biology with distinct roles to that of the primitive AML cells (van Galen et al., 2019). Van Galen and colleagues demonstrated that HSC- and progenitor-like leukaemic cells expressed genes that promote stemness and self-renewal properties whilst differentiated monocyte-like AML cells expressed immunomodulatory factors that suppress T cells (van Galen et al., 2019). Taken together with studies by McKenzie et al. (2019), who demonstrated therapy resistance can arise from the dedifferentiation of mature-like AML cells, these data highlight the importance of studying and modelling the entire leukaemia population and not just the cancer stem cells (van Galen et al., 2019). Single-cell sequencing techniques that interrogate the genome, transcriptome, epigenome and proteome are rapidly evolving, and will likely provide further insight into the AML development hierarchies, the contribution of different malignant haematopoietic cell-types to AML biology, the response of AML haematopoietic cell-types to therapeutic pressures and the molecular states that predict drug resistance.

Given the transcriptional plasticity of cancer cells, the efficacy of epigenetic therapies is hypothesised to be most advantageous in combination with other therapeutics (Dawson, 2017). Furthermore, most AML patients harbour 2 – 4 mutations, thus selectively targeting a single oncogene may be insufficient to attain durable remission, as the co-occurring genetic lesions may provide a permissive environment for the cell to adapt to therapeutic pressures. Given the immunomodulatory effects elicited by hypomethylating agents (Jones et al., 2019), hypomethylating agents are currently being investigated in combination with immunotherapies that stimulate the anti-tumour response (Daver et al., 2018; Daver et al., 2019). Clinical trials are also investigating the combination of hypomethylating agents with therapies targeting mutant-IDH (NCT03683433), cell-surface antigens (NCT02632721), tyrosine kinases (NCT02752035) and apoptotic machinery (NCT03404193).

A comprehensive understanding of the intratumour heterogeneity throughout disease progression and in response to therapeutic pressure, will provide valuable information regarding resistance mechanisms, facilitate the identification of molecular signatures that act as biomarkers for the prediction of therapeutic efficacy, and will ultimately guide the development of rational combination therapies to improve the outcome for AML patients.

5.2 Thesis Summary and Conclusions

Mutations in epigenetic regulators frequently occur in AML. Furthermore, the epigenetic landscape is typically dysregulated in AML, regardless of the mutational status of genes that encode epigenetic regulators. Recently there has been an emergence of an array of epigenetic therapies for the treatment of haematological malignancies, with many advancing into clinical trials (Wingelhofer and Somervaille, 2019). Targeted therapeutics that directly inhibit epigenetic oncoproteins, such as AG120 inhibition of IDH1^{R132H}, have shown clinical success for the treatment of AML harbouring specific genetic lesions in epigenetic regulators (Wingelhofer and Somervaille, 2019). Other epigenetic therapies, such as hypomethylating agents, target wildtype epigenetic regulators with the aim of reprogramming the transcriptional networks driving malignant progression. Despite some patients achieving clinical remission, many AML patients do not respond to epigenetic therapies or relapse post-treatment. Furthermore, the contribution of mutant epigenetic regulators to leukaemogenesis remains relatively poorly understood. This thesis aimed to understand the contribution of IDH1^{R132H} to the leukaemic phenotype in different AML haematopoietic cellular contexts, and the key transcriptional pathways regulated by IDH1^{R132H} in the leukaemia initiating cells. Moreover, this thesis aimed to delineate the transcriptional and epigenetic consequences of IDH1^{R132H} inhibition, understand the effects of AG120 within the AML hierarchy of cell-types, and elucidate transcriptional pathways that mediate AG120 resistance. Work detailed herein also aimed to elucidate mechanisms that mediate resistance to the anti-proliferative effects of low-dose hypomethylating agents, and identify therapeutic avenues for AZA/GDAC resistant cells.

This thesis demonstrated that key members of the pyrimidine salvage pathway are integral to the metabolism of hypomethylating agents, such that SLC29A1, DCK or UCK2 loss provided the greatest proliferative advantage to cells cultured in the presence of hypomethylating agents. Whilst many studies have reported findings concordant with these results (Hosokawa et al., 2015; Qin et al., 2009; Sripayap et al., 2014), this study was the first to assess genes that mediate resistance to hypomethylating agents utilising an unbiased genome-wide approach. Concordant with the genome-wide screen, the results in chapter 3 outlined trends for down-regulated expression of *UCK2* and *SLC29A1* in a small cohort of MDS and CMML patients refractory to AZA treatment, relative to AZA responsive patients (Unnikrishnan et al., 2017). Taken together, these results suggest that SLC29A1, DCK and UCK2 depletion may be a prognostic marker of therapeutic efficacy and clinical resistance to nucleoside analogue therapies. However, further research into the expression of *SLC29A1*, *DCK* and *UCK2* throughout the

treatment regimen through serial biopsy of larger patient cohorts is required. Furthermore, cellular mechanisms that impart partial resistance to the anti-proliferative effects of hypomethylating agents, which were undetectable in the screen, may be clinically relevant and require further investigation. Finally, given the growing body of evidence demonstrating the tumour-extrinsic immunomodulatory effects of hypomethylating agents (Chiappinelli et al., 2015; Jeong et al., 2015; Li et al., 2014a; Stone et al., 2017), further research is required utilising *in vivo* models with an intact immune system in order to elucidate if tumour-extrinsic mechanisms could promote resistance to hypomethylating agents.

Chapter 3 demonstrated that SLC29A1 loss mediated resistance to both AZA and GDAC, however, significantly increased the sensitivity of cells to pharmacological inhibition of DHODH, a member of the pyrimidine salvage pathway. Furthermore, this thesis demonstrated that DCK and UCK2 loss did not provide cross-resistance to AZA and GDAC, respectively. Recently, a study reported that the adaptive responses elicited by cells exposed to AZA or DAC, promoted resistance through the down-regulation of *DCK* or *UCK2* expression, respectively, such that alternating AZA and DAC periodically throughout the treatment regimen significantly prolonged survival *in vivo* (Gu et al., 2020). Taken together, these results indicate that AZA and GDAC treatment are potential therapeutic avenues for AML resistance driven by *DCK* or *UCK2* depletion or loss. Moreover, results from chapter 3 suggest that targeting the *de novo* pyrimidine pathway, through DHODH inhibition, is a potential therapeutic strategy to overcome resistance to hypomethylating agents mediated by the depletion of pyrimidine salvage enzymes.

Pre-clinical studies have demonstrated that small-molecule inhibition of mutant *IDH1/2* can block 2-HG production and induce differentiation of cell lines and patient samples *in vitro* (Chaturvedi et al., 2013; Kernytsky et al., 2015; Popovici-Muller et al., 2018; Wang et al., 2013). These results have been further verified *in vivo* for small-molecules targeting mutant-IDH2 (Kats et al., 2017; Shih et al., 2017), however, to date, detailed characterisation of the cellular consequences and therapeutic efficacy of AG120 had not been investigated *in vivo*. Utilising a murine model of AML co-expressing *IDH1^{R132H}*, *DNMT3A^{R882H}* and *Nras^{G12D}*, chapter 4 revealed that *IDH1^{R132H}* contributed to the expression of transcriptional programs governing the leukaemia phenotype in the LSCs, including the genetic pathways that underpin HSC-like self-renewal and heightened proliferation properties. Furthermore, *IDH1^{R132H}* was found to variably impact the differentiation capacity within the different AML cellular contexts,

suggesting that IDH1^{R132H} governs distinct properties within the different AML cell-types. Chapter 4 demonstrated that inhibition of IDH1^{R132H} with AG120 was a highly effective therapeutic strategy for the treatment of *in vivo* IDH1^{R132H} driven AML, which is concordant with the recent clinical trials reporting the efficacy of AG120 for the treatment of IDH1^{R132H} driven AML (DiNardo et al., 2018). This study revealed that AG120 induced differentiation of the leukaemia cells with distinct kinetics dependent on the AML cellular context, such that differentiation was most rapid in AML progenitor-like cells. Thus, combining targeted mutant-IDH inhibitors with therapies that promote differentiation, such as ATRA, or target cell death machinery, such as Venetoclax, may enhance the differentiation or induce cell death of the IDH1^{R132H} expressing cells that are less responsive to AG120 treatment. Further research utilising single-cell transcriptome and epigenome techniques would provide valuable insight into the influence that IDH1^{R132H} has in different malignant haematopoietic cell-types, the progressive temporal consequences of IDH1^{R132H} inhibition throughout the malignant haematopoietic hierarchy. Such studies may also provide insight into whether a small population of hierarchically mature leukaemia cells are able to evade AG120 therapy by de-differentiating into progenitor-like cells, a concept described by McKenzie and colleagues as a resistance mechanism to differentiation therapies in AML (McKenzie et al., 2019). Finally, chapter 4 identified gene pathways involved in the metabolism of xenobiotics and drugs that may promote AG120 resistance, although further experimental validation is required.

Collectively, this thesis provided valuable insights into IDH1^{R132H} AML biology, transcriptional pathways perturbed and leukaemia properties reversed by AG120, and mechanisms that promote resistance to hypomethylating agents.

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