# Methionine dependence in melanoma cell lines: Examining the role of *MMACHC*, an important gene in cobalamin metabolism

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## Abstract

Methionine dependence is the inability of many tumour-derived cell lines to proliferate in medium in which methionine is replaced by its precursor homocysteine (Met-Hcy+ medium), while retaining the ability to grow in medium containing methionine ($Met^{+}Hcy^{-}$). Methionine independence, the ability to proliferate in either medium, is typical of non-tumour-derived cell lines. The human melanoma cell line MeWo and its highly malignant derivative MeWoLC1 have been demonstrated to be methionine independent and dependent, respectively. *MMACHC* is a gene involved in vitamin $B_{12}$ (cobalamin) metabolism. Pathogenic variants in *MMACHC* result in the *cblC* defect, characterized by decreased synthesis of both methylcobalamin and adenosylcobalamin, and functional deficiency of both methionine synthase and methylmalonylCoA mutase. MeWoLC1 is a cellular phenocopy of *cblC*, showing decreased synthesis of both cobalamin cofactors and functional deficiencies of both cobalamin-dependent enzymes. Methionine dependence in MeWoLC1 appears to be due to hypermethylation of the *MMACHC* promoter. To investigate the extent of this phenomenon in tumour derived cell lines, we analysed the online Cancer Cell Line Encyclopedia, and found that high *MMACHC* DNA methylation and low *MMACHC* mRNA expression were disproportionately frequent in cell lines derived from melanomas compared to other types of cancer. We then studied the ability of seven melanoma lines with different *MMACHC* methylation and expression levels to grow in Met-Hcy+ and $Met^{+}Hcy^{-}$ media. MeWo (0.003/0.12, MMACHC methylation β-value/MMACHC expression transcript-per-million), A375 (0.023/10.2), and MeWoLC1 (high methylation/low expression [exact values unknown]) were previously described in literature as methionine independent, moderate dependent, and dependent, respectively. Of the additional melanoma lines investigated in this study, Colo829 (0.761/0.03), HMCB (0.748/2.29), and SH-4 (0.000/5.66)

demonstrated decreased ability to proliferate in Met-Hcy+ compared to $Met^{+}Hcy^{-}$, while SK-Mel-28 (0.192/1.94) was found to be methionine independent. Furthermore, of the cell lines showing methionine dependence, only MeWoLC1 showed alterations of cobalamin metabolism as determined by the ability to convert exogenous radiolabeled cobalamin to its active coenzyme derivatives and by assays of methionine synthase and methylmalonylCoA mutase function. These data show that, although *MMACHC* hypermethylation and decreased expression are relatively common among melanoma cell lines, these changes are not necessarily associated with methionine dependence in melanoma lines other than MeWoLC1. The reason for the overrepresentation of melanoma cell lines among cancer lines with hypermethylation and decreased expression of *MMACHC* remains to be determined.

## Résumé

La dépendance à la méthionine est l'incapacité de nombreuses lignées cellulaires dérivées de tumeurs à proliférer dans un milieu dans lequel la méthionine est remplacée par son précurseur l'homocystéine (milieu Met-Hcy+), tout en conservant la capacité de se développer dans un milieu contenant de la méthionine ($Met^{+}Hcy^{-}$). L'indépendance de la méthionine, la capacité à proliférer dans l'un ou l'autre milieu, est typique des lignées cellulaires non dérivées de tumeurs. Il a été démontré que la lignée cellulaire de mélanome humain MeWo et son dérivé hautement malin MeWoLC1 sont respectivement indépendants et dépendants de la méthionine. *MMACHC* est un gène impliqué dans le métabolisme de la vitamine $B_{12}$ (cobalamine). Les variantes pathogènes de *MMACHC* entraînent le défaut *cblC*, caractérisé par une diminution de la synthèse de la méthylcobalamine et de l'adénosylcobalamine, et un déficit fonctionnel de la méthionine synthase et de la méthylmalonylCoA mutase. MeWoLC1 est une phénocopie cellulaire de *cblC*, montrant une diminution de la synthèse des deux cofacteurs de la cobalamine et des déficiences fonctionnelles des deux enzymes dépendantes de la cobalamine. La dépendance à la méthionine dans MeWoLC1 semble être due à une hyperméthylation du promoteur *MMACHC*. Pour étudier l'étendue de ce phénomène dans les lignées cellulaires dérivées de tumeurs, nous avons analysé l'encyclopédie en ligne des lignées cellulaires cancéreuses et constaté que la méthylation élevée de l'ADN *MMACHC* et la faible expression de l'ARNm *MMACHC* étaient disproportionnellement fréquentes dans les lignées cellulaires dérivées de mélanomes par rapport aux autres types de cancer. Nous avons ensuite étudié la capacité de sept lignées de mélanome avec différents niveaux de méthylation et d'expression de *MMACHC* à se développer dans les milieux Met-Hcy+ et $Met^{+}Hcy^{-}$. MeWo (0,003/0,12; méthylation MMACHC valeur β/ expression MMACHC transcription-par-million), A375 (0,023/10,2) et MeWoLC1 (méthylation

élevé/expression faible [valeurs exactes inconnues]) ont été précédemment décrits dans la littérature comme indépendants de la méthionine, modérément dépendant et dépendant, respectivement. Parmi les lignées de mélanome supplémentaires étudiées dans cette étude, Colo829 (0,761/0,03), HMCB (0,748/2,29) et SH-4 (0,000/5,66) ont démontré une capacité réduite à proliférer dans Met-Hcy+ par rapport à $Met^{+}Hcy^{-}$, tandis que SK-Mel-28 (0,192/1,94) s'est avéré indépendant de la méthionine. De plus, parmi les lignées cellulaires montrant une dépendance à la méthionine, seule MeWoLC1 a montré des altérations du métabolisme de la cobalamine, déterminées par la capacité à convertir la cobalamine radiomarquée exogène en ses dérivés de coenzyme actifs et par des dosages de la fonction méthionine synthase et méthylmalonylCoA mutase. Ces données montrent que, bien que l'hyperméthylation et la diminution de l'expression de la *MMACHC* soient relativement courantes parmi les lignées cellulaires de mélanome, ces changements ne sont pas nécessairement associés à la dépendance à la méthionine dans les lignées de mélanome autres que MeWoLC1. La raison de la surreprésentation des lignées cellulaires de mélanome parmi les lignées cancéreuses présentant une hyperméthylation et une diminution de l'expression de *MMACHC* reste à déterminer.

# Table of Contents

## List of Abbreviations

1C – one-carbon

AdoCbl – adenosylcobalamin

ATCC – American Type Culture Collection

BCS – bovine calf serum

BSC – biological safety cabinet

Cbl(I) – cob(I)alamin

Cbl(II) – cob(II)alamin

Cbl(III) – cob(III)alamin

CCLE – Cancer Cell Line Encyclopedia

CNCbl – cyanocobalamin

Co – cobalt

dcSAM – decarboxylated S-adenosylmethionine

DepMap – Cancer Dependency Map Project

EMEM – Eagle's Minimum Essential Medium

EtOH – ethanol

FBS – fetal bovine serum

Hcy – homocysteine

$I_{m/e}$ – methylation-expression index

IQR – interquartile range

LOF – loss of function

MeCbl – methylcobalamin

$Met^{+}Hcy^{-}$ – cell culture medium containing methionine but not homocysteine

$Met^{-}Hcy^{+}$ – cell culture medium containing homocysteine but not methionine

MTA – methylthioadenosine

$N_D$ – number of cell population doublings

OHCbl – hydroxocobalamin

PBS – phosphate buffered saline

rMETase – recombinant L-methioninase

RRBS – reduced representation bisulfite sequencing

SAH – S-adenosylhomocysteine

SAM – S-adenosylmethionine

TC – transcobalamin

THF – tetrahydrofolate

TPM – transcript(s)-per-million

Genes/proteins discussed in this thesis and their abbreviations can be found in Appendix A.

# List of Figures

# List of Tables

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## Format of the Thesis

This thesis is written in accordance with traditional monograph format used by the Department of Human Genetics of McGill University, Montreal, QC, Canada, in 2022.

This thesis is written in the style of APA 7th edition, with the following three exceptions:

1. Section headings are numbered, to improve navigation.
2. Surnames and initials are provided for up to seven authors in reference entries (see Chapter 6), consistent with APA 6th edition.
3. Figure titles are listed below the figure, in accordance with most scientific journals.

## Contribution of Authors

William G. Bauer is the writer of this thesis. He analysed *MMACHC* data in the Cancer Cell Line Encyclopedia and selected the seven melanoma cell lines investigated in this thesis, performed tissue culture growth curves and designated proliferative ability and number of doublings within the cell lines, extracted cobalamin coenzyme forms for the [$^{57}$Co]cobalamin distributions, and performed the [$^{14}$C]5-MethylTHF incorporation and accompanying protein assay.

Dr. David Watkins is an editor of this thesis, extracted some of the cobalamin coenzyme forms, and performed the accompanying protein assay for [$^{14}$C]propionate incorporation.

Caitlin Zacharias performed the tissue culturing for the [$^{14}$C]propionate incorporation assay, and the fractionation of cobalamin coenzyme forms for the second Colo829, SH-4, SK-Mel-28 experiments.

Dr. Brian M. Gilfix is an editor of this thesis and was a thesis committee member.

Dr. William Foulkes was a thesis committee member.

Dr. Loydie Jerome-Majewska was a thesis examiner.

Dr. David S. Rosenblatt is the Principal Investigator and supervisor for this thesis project. He is also an editor of this thesis and was a thesis committee member.

# Chapter 1: Introduction

## 1.1 One-Carbon Metabolism

One-carbon (1C) metabolism refers to a network of intracellular metabolic pathways that traffic 1C units containing a single carbon atom, namely methyl ($CH_3$), formyl (CHO), and methylene ($CH_2$) (Clare *et al.,* 2019). Although these units are small, they are in constant high demand and necessary for biochemical and molecular biological processes including (but not limited to) DNA, RNA, and protein biosynthesis and methylation (Clare *et al.,* 2019; Pentieva, 2018). 1C metabolism is therefore a universal process, in that it is present in all living prokaryotic and eukaryotic organisms (Ducker & Rabinowitz, 2017). By extension, 1C metabolism is critical for human development and survival, as deficiencies in these pathways – whether nutritional or genetic – are associated with severe disease phenotypes such as neural tube defects and anemia (Ducker & Rabinowitz, 2017; Li *et al.,* 2016). This has led to longstanding and ongoing (*e.g.*, Chern *et al.* [2022]) research efforts to elucidate the many components of 1C metabolism.

The 1C pathways utilize both enzyme cofactors and substrates that are derived from a variety of vitamins including the "B-vitamins"; cobalamin (vitamin $B_{12}$), folate (vitamin $B_9$), pyridoxine (vitamin $B_6$), and riboflavin (vitamin $B_2$) (Pentieva, 2018). This thesis will only focus on the 1C metabolic pathways involving the former two. Although the pathways to process them are universal, only bacteria, yeasts, and plants can synthesise folate, while only prokaryotes can synthesise cobalamin (Ducker & Rabinowitz, 2017; Watanabe and Bito, 2018b). Animals must acquire folate and cobalamin externally to supplement 1C metabolism, and primarily do so through dietary sources such as green vegetables, citrus fruits, and meat and dairy products (Ducker & Rabinowitz, 2017; Lucock, 2000; Scott, 1999).

1C metabolism takes place in all cells and tissues, and is mostly compartmentalised in a cell's cytoplasm and mitochondria with a smaller nuclear component as well (Figure 1A; Clare *et al.,* 2019; Ducker & Rabinowitz, 2017; Pentieva, 2018,). The metabolism of cytoplasmic folate is the most extensively studied 1C metabolic pathway and consists of interdependent enzymes that convert folate into different derivatives required for purine and pyrimidine biosynthesis, methionine biosynthesis, and methylation of DNA, RNA, proteins, and other molecules (Fox & Stover, 2008). Conversely, the cytoplasmic cobalamin pathway is shorter, as its one function is to deliver cobalamin to be used as a cofactor for cobalamin-dependent methionine synthase (MTR, EC 2.1.1.13), a key enzyme also involved in the cytoplasmic folate pathway (Rush *et al.,* 2014). The mitochondrial folate pathway is essentially the reverse of its cytoplasmic counterpart, in that it contains enzymes that under normal conditions oxidise folate-derivatives (whereas cytoplasmic enzymes would reduce those same derivatives) to formate and glycine, which are substrates for the cytoplasmic reactions (Fox & Stover, 2008). In contrast to the cytoplasm, the mitochondrial cobalamin pathway is separate from any folate-dependent metabolism, but still has a similar function of delivering cobalamin to be used as a cofactor for methylmalonylCoA mutase (MMUT, EC 5.4.99.2) (Rush *et al.,* 2014). Nuclear 1C metabolism is comprised of only a second pyrimidine biosynthetic pathway, shown by Anderson *et al.* (2007) to be performed by small ubiquitin-like modifier-1 modification of the same enzymes used in the cytoplasmic folate pathway. This modification causes proteins to localize to the nucleus during S-phase, and is thought to provide a source for pyrimidines directly at the DNA replication fork, thereby minimising uracil misincorporation (Fox & Stover, 2008).

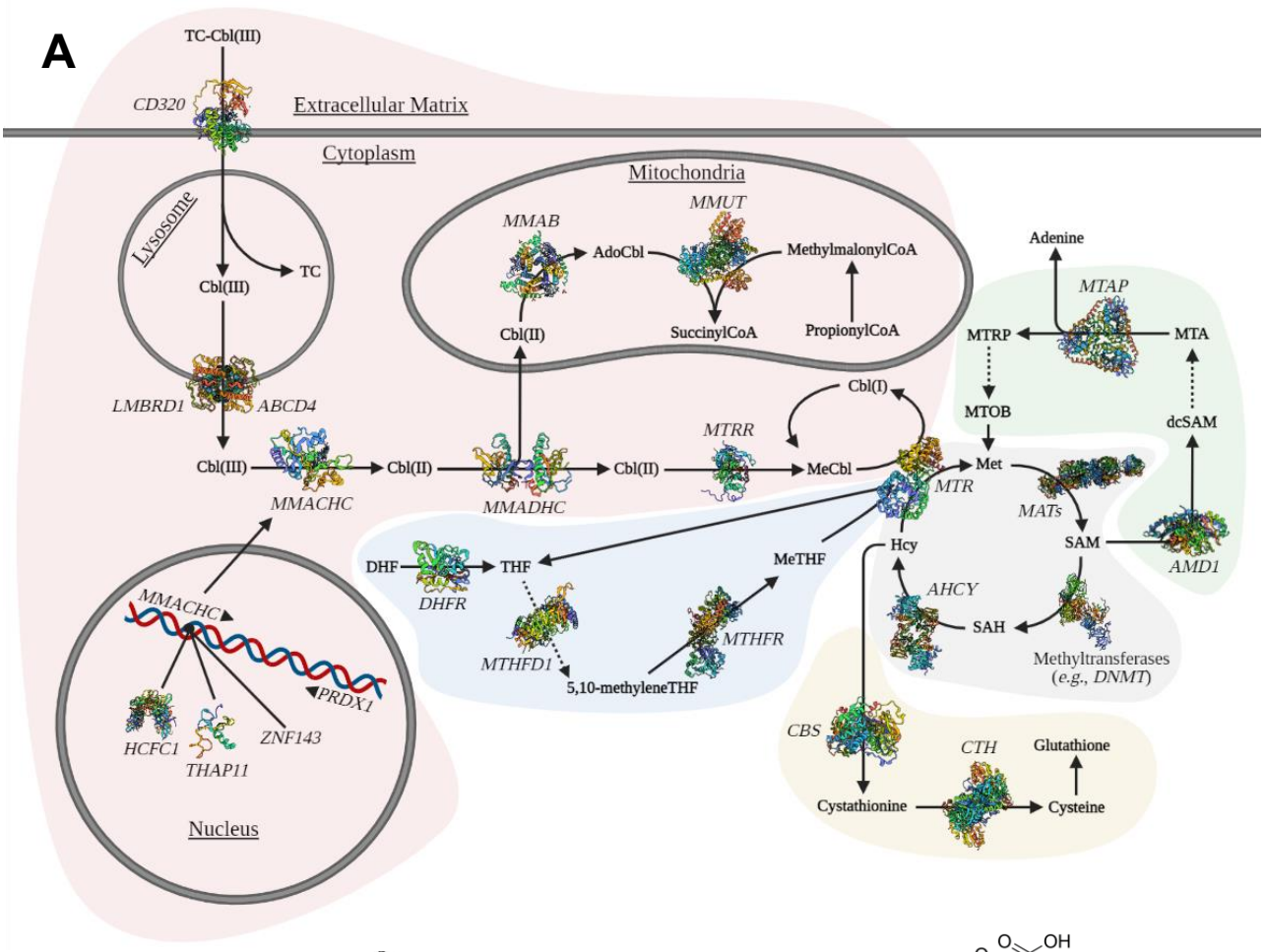
A
TC-Cbl(III)
CD320
Extracellular Matrix
Cytoplasm
Lysosome
TC
Cbl(III)
LMBRD1
ABCD4
Cbl(III)
MMACHC
Cbl(II)
MMADHC
Cbl(II)
Mitochondria
MMAB
AdoCbl
Cbl(II)
MMUT
MethylmalonylCoA
SuccinylCoA
PropionylCoA
MTRR
Cbl(I)
MeCbl
MTR
Met
MTOB
Adenine
MTAP
MTRP
MTA
dcSAM
MATs
SAM
AMD1
Hcy
AHCY
SAH
Methyltransferases
(e.g., DNMT)
DHF
DHFR
THF
MTHFD1
5,10-methyleneTHF
MTHFR
MeTHF
MMACHC
PRDX1
HCFC1
THAP11
ZNF143
Nucleus
CBS
CTH
Glutathione
Cystathionine
Cysteine
B
C

**Figure 1.** *1C Metabolic pathways and chemical structures of relevant vitamins.*

**A)** Components of 1C metabolism, including cobalamin transport (red), folate cycle (blue), transulfuration pathway (orange), methionine salvage pathway (green), and methionine cycle (grey). Proteins and/or enzymes denoted by their encoding gene (italicised), with corresponding names listed in Appendix A. Abbreviated intermediates (bolded) from left to right: **TC**, transcobalamin; **Cbl**, cobalamin; **DHF**, dihydrofolate; **THF**, tetrahydrofolate; **AdoCbl**, adenosylcobalamin; **MeTHF**, 5-methyltetrahydrofolate; **MeCbl**, methylcobalamin; **Hcy**, homocysteine; **MTRP**, 5-methylthioribose-1-phosphate; **MTOB**, 4-methylthio-2-oxobutanoic acid; Met, methionine; **SAH**, S-adenosylhomocysteine; **SAM**, S-adenosylmethionine; **MTA**, 5'-methylthioadenosine; **dcSAM**, decarboxylated S-adenosylmethionine. Three-dimensional structures of enzymes downloaded from protein data bank but are not to scale or in proper orientation. Created using BioRender.com. **B)** Cobalamin 3-dimensional structure, reproduced with permission (Appendix C; Watkins & Rosenblatt, 2011). **C)** Folate 2-dimensional structure, adapted from Laurieri *et al.* (2014).

### *1.1.1 Cobalamin*

Cobalamin is the largest B vitamin by molecular weight and refers to an organometallic molecule consisting of a planar corrin ring surrounding a central cobalt (Co) atom that is linked axially with a dimethylbenzimidazole base in the lower axial position, and one of several chemical entities in the upper position; the corrin ring is linked through one of its side chains to the same dimethylbenzimidazole base (Figure 1C; Banerjee & Matthews, 1990; Scott, 1999; Watkins & Rosenblatt, 2011). The bond between Co and the lower axial ligand can be broken, and the dimethylbenzimidazole will remain covalently bound only to the corrin ring side chain, known as a base-off conformation (Watkins & Rosenblatt, 2011). Chemical entities in naturally

occurring cobalamin are hydroxyl groups (hydroxocobalamin, OHCbl), water (aquocobalamin, AqCbl) adenosyl (5'-deoxyadenosylcobalamin, AdoCbl), methyl groups (methylcobalamin, MeCbl), and glutathione (glutathionylcobalamin), while synthetic cobalamin typically possesses a cyano group (cyanocobalamin, CNCbl) (Scott, 1999; Watkins & Rosenblatt, 2011). Natural cobalamins are more chemically labile than those CNCbl (the Co—C bond in alkylcobalamins AdoCbl and MeCbl will break when exposed to light) (Schrauzer *et al.,* 1968; Watanabe & Bito, 2018a). The Co nucleus of any cobalamin form can range from a fully oxidised to a fully reduced state, including $Co^{3+}$ (Cbl[III]), $Co^{2+}$ (Cbl[II]), and $Co^{+}$ (Cbl[I]) (Watkins & Rosenblatt, 2011). The majority of natural cobalamins found in the diet are AdoCbl and OHCbl; however, this can change if the manufacturing method of a food is reliant on specific microorganisms (Farquharson & Adams, 1976; Watanabe & Bito, 2018a).

Once digested and transported into the bloodstream, cobalamin is bound to transcobalamin (TC; previously known as transcobalamin II; Andres & Dali-Youcef, 2020; Fernandes-Costa *et al.,* 1982; Fràter-Schröder *et al.,* 1985; Yamani, 2008). The resulting cobalamin-TC complex binds to TC receptors and is taken up by cells through receptor-mediated endocytosis, where TC is degraded by lysosomes while cobalamin remains intact (Figure 1A; Andres & Dali-Youcef, 2020; Watkins & Rosenblatt, 2011). Separated, free cobalamin is transported out of the lysosomes by the membrane transporters ABCD4 (EC 7.6.2.8) and LMBRD1 (Coelho *et al.,* 2012; Rutsch *et al.,* 2011; Watkins & Rosenblatt, 2020). Cobalamin then binds to MMACHC (methylmalonic aciduria and homocystinuria type C protein; metabolism of cobalamin associated C; or, alkylcobalamin dealkylase, EC 2.5.1.151), which displaces the dimethylbenzimidazole into its base-off conformation, reduces the $Co^{3+}$ nucleus, and removes the upper axial ligand by respectively dealkylating or decyanating the

adenosyl/methyl or cyanide group of the natural or synthetic cobalamin, producing Cbl(II) (Plesa *et al.*, 2011; Watkins & Rosenblatt, 2011). Cbl(II) is directed towards the mitochondria or cytoplasm through a process that appears to involve MMADHC, which complexes with MMACHC and binds to Cbl(II) through a sulfur ligand (Froese *et al.*, 2015; Li *et al.*, 2020; Plesa *et al.*, 2011). How the complex is involved in partitioning Cbl(II) between the mitochondria and cytoplasm remains unknown (Watkins & Rosenblatt, 2011, 2022).

Cobalamin metabolism has two terminals, each involving enzymatic need for cobalamin as a cofactor (Figure 2A). If Cbl(II) is partitioned towards the mitochondria, it will be adenosylated by cobalamin adenosyltransferase (MMAB; EC 2.5.1.17) following reduction to Cbl(I) by an unidentified reductase enzyme, producing AdoCbl (Gherasim *et al.*, 2013). Methylmalonic aciduria type A protein (MMAA; EC 2.3.1.258) then supports transfer of AdoCbl from MMAB to MMUT (Watkins & Rosenblatt, 2022). AdoCbl is a necessary cofactor for MMUT, which catalyses the conversion of methylmalonylCoA to succinylCoA, a critical component in the citric acid cycle (Watkins & Rosenblatt, 2011). Occasionally during this reaction, the deoxyadenosine intermediate derived from AdoCbl may escape, leaving a Cbl(II) group bound to and inactivating MMUT; in this case, MMAA removes the inactive Cbl(II) and replaces it with new AdoCbl (Gherasim *et al.*, 2013). (Prior to a study by Dobson *et al.* [2002b], it was hypothesised that MMAA transported Cbl(II) into the mitochondria and reduced it to Cbl(I); however, this is no longer accepted [Moras *et al.*, 2007; Watkins & Rosenblatt, 2011]). If Cbl(II) is partitioned towards the cytoplasm, it will bind to MTR. MTR is complexed with another enzyme called MTR reductase (MTRR; EC 1.16.1.8), and this MTRR-MTR complex uses SAM as a methyl group donor to support reductive methylation of Cbl(II) to MeCbl (Watkins & Rosenblatt, 2011). MeCbl is a necessary cofactor for MTR, whose reaction cycles

cobalamin between MeCbl and Cbl(I), and occasionally erroneously produces Cbl(II) which MTRR re-reduces for further MTR activity (see 1.1.3; Watkins & Rosenblatt, 2011).

### *1.1.2 Folate*

The term "folate" is ambiguous, as it is commonly used interchangeably to describe related forms of vitamin $B_9$, despite the presence of biochemically distinct forms (Zheng & Cantley, 2019). Definitively, folate is a blanket term used to describe a molecule with a "2-amino-4-hydroxy-pteridine ring, linked by a $CH_2$ group to a p-aminobenzoyl moiety" (Figure 1C; Scaglione & Panzavolta, 2014; Zheng & Cantley 2019). This, in turn, is linked through the amide bond of an α-amino group of a single glutamate or glutamate chain, referred to as monoglutamate folates or polyglutamate folates, respectively (Scaglione & Panzavolta, 2014; Zheng & Cantley, 2019). Folic acid is a synthetic monoglutamate folate with a fully oxidised pteridine ring, used for pharmacological purposes due to its molecular stability compared to natural folates (Zheng & Cantely, 2019). Reduced folates refer to any form of tetrahydrofolate (THF) and its multiple derivatives including among others, 10-formylTHF, 5,10-methenylTHF, 5,10-methyleneTHF, and 5-methylTHF (Forssén *et al.*, 2000; Scaglione & Panzavolta, 2014; Visentin *et al.*, 2014). Reduced folates can also be monoglutamic or polyglutamic (Forssén *et al.*, 2000). The majority of natural dietary folates are polyglutamate methylTHF, although they are accompanied by small proportions of other combinations of the forms previously described (Scaglione & Panzavolta, 2014; Visentin *et al.*, 2014).

The metabolic role of folate is to deliver 1C units required for five principal processes within the cell: interconversion of serine and glycine, histidine catabolism, thymidylate synthesis, purine synthesis, and methionine synthesis (Lucock, 2000). Once inside a cell, folates interact with a series of enzymes, which are described in Fox and Stover (2008; Figure 1A).

Most folates enter the cytoplasm as 5-methylTHF and interact with MTR, the only enzyme capable of demethylating methylTHF, producing THF (see 1.1.3; Lucock, 2000). Other folates, such as THF, 10-formylTHF, 5,10-methenylTHF, and 5,10-methyleneTHF are interconverted by the trifunctional enzyme MTHFD1, which exhibits 5,10-methyleneTHF dehydrogenase (EC 1.5.1.5), 5,10-methenylTHF cyclohydrolase (EC 3.5.4.9), and 10-formylTHF synthetase (EC 6.3.4.3) activities (Burda *et al.*, 2015; Watkins & Rosenblatt, 2020). If needed, 10-formylTHF and 5,10-methyleneTHF can donate their 1C units towards purine biosynthesis and pyrimidine biosynthesis, respectively (Burda *et al.*, 2015). Otherwise, 5,10-methyleneTHF will primarily be reduced to 5-methylTHF in an irreversible reaction by 5,10-methyleneTHF reductase (MTHFR; EC 1.5.1.20) but may interact with cytoplasmic serine hydroxymethyltransferase (SHMT1; EC 2.1.2.1) to produce THF, a reversible reaction that simultaneously generates serine from glycine (Burda *et al.*, 2015; Fox & Stover, 2008,).

### *1.1.3 The Methionine Cycle*

Although the folate and cobalamin pathways in 1C metabolism are mostly separated (*i.e.*, produce metabolites independent of one another), they converge into, and their proper functions are thus each required for, the methionine cycle (Banerjee & Matthews, 1990). Indeed, the necessity of methionine cycle activity is consistently cited as one of the most important processes in 1C metabolism alongside thymidylate and purine synthesis. Methionine is an essential amino acid in humans, meaning that it cannot be biosynthesised *de novo* (Martinez *et al.*, 2017). Furthermore, while there is a limited ability to regenerate methionine from homocysteine (Hcy), the amino acid must be acquired exogenously to maintain cellular levels (Banerjee & Matthews, 1990; Martinez *et al.*, 2017). Methionine is involved in many critical processes of cellular metabolism, including protein biosynthesis (its RNA codon is the most common “start” codon,

which initiates ribosomal translation); however, the remainder of its widespread utility is its metabolic derivatives through the methionine cycle (Li *et al.*, 2007; Martinez *et al.*, 2017; Zhang, 2018).

The methionine cycle is centred on MTR, an enzyme whose highly conserved homology across all organisms exemplifies its biological importance (Banerjee & Matthews, 1990; Yamada *et al.*, 2006). While other methionine synthases exist in organisms such as prokaryotes and plants, they are not all cobalamin dependent. In mammals, the reaction of MTR requires presence of cobalamin, (hence "cobalamin-dependent methionine synthase"), methylTHF, and Hcy (Figure 1A; Banerjee, 1999). Cbl(II) binds to MTR, and is subject to a reductive methylation by the MTR-MTRR complex, producing MeCbl (Banerjee, 1999; Banerjee & Matthews, 1990; Wolthers & Scrutton, 2007). From then on, one catalytic cycle of MTR consists of (1) demethylation of MeCbl into Cbl(I) through reaction with bound Hcy, (2) Hcy receiving the methyl group and forming methionine, and (3) binding to methylTHF and transfer its 5-methyl group to Cbl(I), forming THF and reforming MeCbl (Banerjee, 1999; Banerjee & Matthews, 1990; Fox & Stover, 2008; Lucock, 2000; Solkolovskaya *et al.*, 2020; Watkins & Rosenblatt, 2011; Wolthers & Scrutton, 2007). Existence of Cbl(I) is transient, in that it will be remethylated to MeCbl almost instantly after MeCbl's demethylation, provided there is methylTHF. Sometimes (~1/2000 reactions), the Cbl(I) cofactor will be oxidised to Cbl(II) while bound to MTR, resulting in the enzyme's inactivation through inability to methylate Cbl(II) into MeCbl (Banerjee, 1999; Wolthers & Scrutton, 2007; Yamada *et al.*, 2006). For this reason MTRR is complexed with MTR: to efficiently correct this error by donating electrons from NADPH to reduce the central cobalt from $Co^{2+}$ to $Co^{+}$ and facilitating transfer of a methyl group from S-adenosyl methionine (SAM) to MTR-bound Cbl(II), restoring MeCbl (Wolthers &

Scrutton, 2007; Yamada *et al.,* 2006). SAM is used as a methyl donor only in initial catalysis of Cbl(II) to MeCbl and when MeCbl is accidentally oxidised to Cbl(II), whereas in proper MTR function, 5-methylTHF is the methyl donor to Cbl(I). As such, the function of MTR is sometimes referred to as the function of the MTRR-MTR enzyme complex (Wolthers & Scrutton, 2007).

Methionine – if not used in protein synthesis – will be adenylated to SAM by methionine adenosyltransferases (MAT; EC 2.5.1.6) consisting of a collection of isozymes MATI, MATII, and MATIII (Figure 1A; Kotb & Geller, 1993; Zhang, 2018). SAM is then demethylated by various methyltransferases (MTs), forming S-adenosylhomocysteine (SAH) (Clare *et al.,* 2019; Zhang, 2018). These reactions are the basis for DNA, RNA, and protein methylation, as the type of MT depends on the biochemical entity that will receive the methyl group (*e.g.*, DNMTs methylate DNA; Clare *et al.,* 2019; Zhang. 2018). SAH is then hydrolysed back to Hcy through SAH hydrolase, also known as adenosylhomocysteinase (AHCY; EC 3.3.1.1), which removes the adenosine group (Clare *et al.,* 2019; Scott, 1999; Zhang, 2018). This is the only route of Hcy production in vertebrates (Scott, 1999). Hcy can either be remethylated into methionine by MTR or enter the transsulfuration pathway which begins with condensation with serine to form cystathionine, by cystathionine beta synthase (EC 4.2.1.22) (Kotb & Geller 1993; Scott, 1999). Cystathionine is then converted to cysteine through cystathionine gamma lyase (EC 4.4.1.1), and following the transsulfuration pathway, will also comprise part of the antioxidant glutathione (Clare *et al.,* 2019; Kotb & Geller, 1993; Li *et al.,* 2007; Sanderson *et al.,* 2019; Schipper *et al.,* 2000).

Alternatively, in place of the MT-dependent SAM to SAH reaction, SAM decarboxylase (EC 4.4.1.50) can produce decarboxylated SAM (dcSAM) instead of SAH (Figure 1A; Clare *et al.,* 2019; Kotb & Geller, 1993; Schipper *et al.,* 2000). DcSAM is a critical component of

polyamine synthesis, as it acts as the aminopropyl donor to putrescine (forming spermidine) and spermidine (forming spermine) (Clare *et al.,* 2019; Schipper *et al.,* 2000). The by-product of polyamine synthesis, methylthioadenosine (MTA), is converted by MTA phosphorylase (MTAP, EC 2.4.2.28) into methylthioribose and adenine (Sanderson *et al.,* 2019). Methylthioribose will ultimately be recycled into methionine through the methionine salvage pathway (Sanderson *et al.,* 2019).

### *1.1.4 Metabolic Inborn Errors of Folate and Cobalamin*

Predictably, deleterious mutations within any gene coding for molecular components of 1C pathways can lead to severe disease phenotypes, as metabolic products of folate, cobalamin, and/or methionine are demonstrably involved in key cellular processes. Since folate and cobalamin pathways are interconnected, any error in metabolism that causes inability to sufficiently produce/metabolise compounds needed for the proper functioning of MTR (the enzyme connecting the folate and cobalamin pathways, see 1.1.3) will likely result in some disruption of both pathways (Green & Mitra, 2017). For example, if one is unable to convert Cbl(II) to MeCbl, MTR will not have ready access to its cofactor, and will be unable to demethylate 5-methylTHF to THF, leading deficiencies in biologically active folates other than 5-methylTHF. Disease due to nutritional folate deficiencies such as neural tube defects are no longer as prevalent in North America since both the United States and Canada have mandated the fortification of grains with folic acid; however, this has no effect on deficiencies caused by genetic inborn errors of folate metabolism.

In fact, there is one (sometimes many, *e.g.*, the multiple forms of *cblD*) disease(s) for nearly every enzyme in the cobalamin pathways, and several enzymes in the folate pathway (Watkins & Rosenblatt, 2011, 2016, 2020). Thus, inborn errors of both folate and cobalamin tend

to share symptoms. Among the most common are (1) megaloblastic anemia – the maturation in cytoplasm while nucleus remains immature within red blood cell precursors due to normal hemoglobin deposition alongside impaired DNA synthesis from lack of folates (*e.g.*, large cells reflect inability to divide normally while cytoplasmic protein synthesis continues), (2) methylmalonic aciduria/acidemia (limited to cobalamin deficiencies) – an increase of methylmalonic acid in urine/blood due to MMUT inactivity from lack of AdoCbl cofactor, and (3) homocystinuria and hyperhomocysteinemia – an increase of Hcy in urine and blood respectively due to MTR inactivity from lack of MeCbl cofactor (Green & Mitra, 2017; Watkins & Rosenblatt, 2011).

*CblC* is the most common (>1200 documented individuals worldwide) inborn error of cobalamin metabolism and is due to homozygous or compound heterozygous autosomal recessive mutations of *MMACHC* (Figure 1A; Lerner-Ellis *et al.,* 2006; Watkins & Rosenblatt, 2011, 2022). MMACHC among other actions reduces Cbl(III) to Cbl(II) by removal of the upper axial ligand (dealkylation or decyanation), where it can then be directed by MMADHC to ultimately be metabolised into a cofactor for MMUT (AdoCbl) or MTR (MeCbl) (see 1.1.2; Plesa *et al.,* 2011; Watkins & Rosenblatt, 2011). Loss of function (LOF) mutations in *MMACHC* therefore lead to decreased Cbl(III) reduction, and subsequent inactivity of MMUT and MTR due to lack of available cofactors. Lack of MTR enzymatic activity will lead to accumulation of 5-methylTHF (known as the methyl trap, or methylfolate trap), and decreased levels of methionine. Derivatives of folate and methionine include thymidylate and adenine which are required for DNA biosynthesis, and deficiencies can result in severe disease phenotypes including megaloblastic anemia, combined homocysteinuria/emia and methylmalonic aciduria/emia, cerebral atrophy, seizures, structural heart defects, retinopathy and maculopathy,

atypical hemolytic uremic syndrome, pulmonary hypertension, and dementia (Watkins & Rosenblatt, 2011, 2020). *CblC* is found in all populations studied, although some populations do have characteristic mutations. There is a noticeably higher frequency of *cblC* rate in some parts of China compared to the rest of the world (Watkins & Rosenblatt, 2011, 2022).

Epi-*cblC* is a disease first reported by Guéant *et al.* (2018). It is a phenocopy – in that it shares the same symptoms – of *cblC*. Interestingly, epi-*cblC* is not caused by two recessive mutations of *MMACHC*, rather one mutation in *MMACHC* along with a mutation in the gene *PRDX1* (or in one case, two *PRDX1* mutations); the *PRDX1* gene product has no role in 1C metabolism (Pollini *et al.,* 2020). *PRDX1* is physically adjacent in a tail-to-tail orientation to *MMACHC* on chromosome 1 (Guéant *et al.,* 2018, 2020; Pollini *et al.,* 2020). The epi-*cblC* mutation affects an intronic splice acceptor site which causes the skipping of the last exon and the polyA transcription termination signal of *PRDX1* (Guéant *et al.,* 2018). The transcript therefore continues into *MMACHC,* creating an antisense transcript which appears to induce hypermethylation of *MMACHC*'s promoter region located at the 5' end of *MMACHC*, effectively reducing expression to a point that its biochemically indistinguishable from conventional *cblC* (Guéant *et al.,* 2018; Pollini *et al.,* 2020). Epi-*cblC* is therefore considered the result of a secondary epimutation and is the first documented instance of a metabolic disease caused by such (Guéant *et al.,* 2018, 2020). Compared to regular *cblC*, epi-*cblC* is much less common, as only seventeen patients (16 heterozygotes and one homozygote) have been identified (Ousallah *et al.,* 2022; Pollini *et al.,* 2020). Most recently, Oussalah *et al.* (2022) have identified that in two epi-*CblC* patients, the antisense transcript also causes hypermethylation of the gene *TESK2,* which is located two genes away in the 5' direction from *MMACHC*.

*CblX* is also a recently discovered rare disease that shares many similarities to *cblC* and epi-*cblC* (Manoli *et al.,* 2016; Yu *et al.,* 2013). Compared to *cblC*, *cblX* patients show milder metabolic abnormalities in cobalamin metabolism, but have more severe neurological disorders, as well as a variety of metabolic and developmental disorders not seen in regular *cblC* patients (Watkins & Rosenblatt, 2016). *CblX* is caused by mutations of the *HCFC1* gene, which codes for a transcription coregulator of many genes, including *MMACHC* (Watkins & Rosenblatt, 2016). This explains why *cblX* patients exhibit phenotypes beyond those with other cobalamin inborn errors, as HCFC1's LOF would result in altered regulation of many genes in key processes. *HCFC1* is found on the X chromosome, making *cblX* the only X-linked error of cobalamin metabolism (Manoli *et al.,* 2016). As expected, all fifteen documented patients of *cblX* so far have been male (Manoli *et al.,* 2016; Watkins & Rosenblatt, 2016). HCFC1 requires presence of cotranscriptors THAP11 and ZNF143 to bind to *MMACHC*; patients with mutations in *ZNF143* are phenocopies of *cblX* (and epi-*cblC*), and a patient with mutations in *THAP11* shares symptoms of *cblX* but has a different overall cellular phenotype of less extreme AdoCbl and MeCbl conversion decrease (Guéant *et al.,* 2018; Quintana *et al.,* 2017).

*CblC*, epi-*cblC*, and *cblX* are not the only inborn errors of folate and cobalamin metabolism; Watkins and Rosenblatt (2011, 2016, 2020, 2022) and other colleagues have extensively published descriptions of all known genetic disorders (*e.g.,* Carmel *et al.,* 2003; Coelho *et al.,* 2008, 2012; Dobson *et al.,* 2002a, 2002b; Leclerc e*t al.,* 1996, 1998; Guéant *et al.,* 2018; Moras *et al.*, 2007; Rutsch *et al.*, 2011; Yu *et al.*, 2013). A shared phenotype of many 1C metabolic diseases, namely *cblC*, *cblD*, *cblE*, *cblF*, *cblG*, and MTHFR deficiency, is the inability to remethylate Hcy to methionine (in these cases, this phenotype is sometimes called methionine auxotrophy, but this is inaccurate as all human wild-type cells are technically methionine

auxotrophic since they cannot synthesise methionine *de novo*) (Carmel *et al.,* 2003; Garovic-Kocic & Rosenblatt, 1993). This also causes the aforementioned Hcy elevation in blood and urine (Garovic-Kocic & Rosenblatt, 1993; Green & Mitra, 2017; Moras *et al.*, 2007; Watkins & Rosenblatt, 2011). Ultimately all methionine is acquired exogenously (see 1.1.3), so when cells of folate and cobalamin metabolic disease patients are cultured *in vitro*, supplementation with Hcy is insufficient for cell survival and proliferation, and instead the cells must be supplemented with methionine (*sc.*, standard tissue culture medium contains methionine and not Hcy) (Garovic-Kocic & Rosenblatt, 1993).

**1.2 Epidemiology and Physiology of Melanoma**

Cancer can form from almost any tissue in humans, and many tissues are not restricted to only one type of cancer. Weinberg provides a detailed timeline of cancer progression towards malignancy, as well as a list of well-known proto-oncogenes (Weinberg, 1996). Skin cancer is the most common type of cancer in the USA, and third most common in the world (Cummins *et al.,* 2006; Dimitriou *et al.,* 2018; Gray-Schopfer *et al.,* 2007; Marks, 2000). Melanomas, or cutaneous melanomas, are rare (5%) forms of skin cancer tumours (Cummins *et al.,* 2006; Dimitriou *et al.,* 2018; Gray-Schopfer *et al.,* 2007; Marks, 2000). As such, melanoma specifically is the fifth most common cancer in the USA, and the twelfth most common in the world (Cummins *et al.,* 2006; Dimitriou *et al.,* 2018). Melanomas are considerably more common in populations of European ancestry, especially those in warm climates with high exposure to UV radiation, like Australia and New Zealand, and according to 2017 data are 60% more likely to arise in males compared to females (Dimitriou *et al.,* 2018).

Melanomas originate from melanocytes within the basal layer of the skin (Gray-Schopfer *et al.,* 2007). Although easily treatable through surgery if diagnosed soon after onset, melanomas

are dramatically more deadly upon reaching the circulatory and/or lymphatic systems and becoming malignant, accounting for 65% of skin cancer deaths with a 5% long-term survival rate (Cummins *et al.,* 2006; Gray-Schopfer *et al.,* 2007). The World Health Organisation divides melanomas into four subtypes: lentigo maligna (slow growing tumour that remains in epidermis), superficially spreading (the most common melanoma, primarily horizontally growing tumour), nodular (aggressive and primarily vertical growing tumour), and acral (flat patch on hands and feet) (Elder *et al.,* 2018; Hintsala *et al.,* 2015). Severity is measured by the depth the tumour penetrates into the epidermis, described by Breslow's index and Clark's classification, the latter of which is classified proportionally to tumour depth from levels I through V, with each level corresponding to a deeper epidermal layer (*e.g.*, level V melanomas have invaded subcutaneous fat) (Hintsala *et al.,* 2015). Additionally, melanomas are classified by nine pathways depending on the suspected cause of the melanoma; detailed explanations of these pathways are provided by Elder *et al.* (2018), but briefly, pathways I through III are those induced by UV radiation, while pathways IV through IX are those with no known association to UV radiation.

By far the most common cause of melanoma is excessive UV radiation exposure to one's skin (pathways I through III) (Cummins *et al.,* 2006). While mortality rates are beginning to plateau, the incidence of melanoma continues to increase (Gray-Schopfer *et al.,* 2007; Hintsala *et al.,* 2015). Susceptibility to developing melanoma is dependent on genetic factors, the most prominent being skin colour (Marks, 2000). Those with fair skin are more likely to develop melanoma from UV exposure due to low melanin content in epidermal cells, since melanin protects against DNA damage (Marks, 2000). Whole genome sequencing has revealed that melanomas are the most mutated cancers, with higher numbers of mutations corresponding to cumulative sun exposure (Dimitriou *et al.,* 2018; Elder *et al.,* 2018). Most melanomas arise

following accumulation of mutations in the genes for MAPK (EC 2.7.12.2), PI3K (EC 2.7.1.137), and Wnt signaling pathways, notably the genes *BRAF* and *NRAS* (both in MAPK pathway), which contribute to apoptotic processes (Dimitriou *et al.,* 2018; Gray-Schopfer *et al.,* 2007). Other common mutations are seen in the *NF1* tumour suppressor gene, and genes involved in the G1/S checkpoint (Dimitriou *et al.,* 2018; Elder *et al.,* 2018).

### 1.3 The Cancer Cell Line Encyclopedia

In 2005, an international research effort led to the observations that MITF and MEK had developmental roles in malignant melanoma and *BRAF*-mutated melanoma cell lines respectively (Garraway *et al.,* 2005; Solit *et al.,* 2006). The successful phase III therapeutic trials of MITF and MEK targeting in melanomas that followed suggested large-scale genetic characterization of cell lines across a variety of cancer types may lead to discovery of other exploitable "cellular perturbations", which could likewise be targeted by drugs to inhibit tumour proliferation (Broad Institute, n.d.). The Cancer Cell Line Encyclopedia (CCLE; https://sites.broadinstitute.org/ccle/) project was thus launched in 2008 as collaboration among several laboratories affiliated with Swiss pharmaceutical company Novartis and the Broad Institute of Harvard University (shared with the Massachusetts Institute of Technology). According to the CCLE's website:

> The overarching goals of [the CCLE] were: (1) to conduct a detailed genetic and pharmacologic characterization of a large panel of human cancer models; (2) to develop integrated computational analyses that link distinct pharmacologic vulnerabilities to characteristic genetic, gene expression, and cell lineage patterns; and, (3) to translate cell line integrative genomics into cancer patient stratification. (Broad Institute, n.d., Home section)

The CCLE was initially built with approximately 1000 cancer cell lines taken from repositories such as the American Type Culture Collection (ATCC; https://www.atcc.org/), Deutsche Sammlung von Mikroorganismen und Zellkulturen, and the Korean Cell Line Bank (Broad Institute, n.d.). The Broad Institute organises the CCLE's progression into three "phases" (Broad Institute, n.d.): Phase I (2008 – 2012) characterised the cell lines' global DNA copy numbers and point mutations, RNA expression of 6500 genes, and exon sequences of known or putative proto-oncogenes (Barretina *et al.*, 2012; Jaffe *et al.*, 2013); Phase II (2013 – 2019) involved next-generation sequencing methods, including (but not limited to) further characterisation of the cell lines' gene expression using RNA-seq, and DNA methylation using reduced representation bisulfite sequencing (RRBS) of transcription start sites (Cancer Cell Line Encyclopedia Consortium & Genomics of Drug Sensitivity in Cancer Consortium 2015; Ghandi *et al.*, 2019; Li, Ning, *et al.*, 2019; Stransky *et al.*, 2015); and, Phase III (2020 – ongoing as of 9 July 2022) shifted focus to proteomic data rather than genomic data, and has thus far characterised cell lines' protein abundance and phosphorylation events using tandem-mass tagging mass spectrometry and another unnamed method (as of February 2021), respectively (Nusinow *et al.*, 2020). This progression has been accompanied by an increase in sample size, and currently the CCLE contains data for over 1600 cancer cell lines; however, not every cell line possesses the same data (*e.g.*, during Phase II, RRBS was performed in 843 cell lines, while RNA-seq was performed in 1019 cell lines; Ghandi *et al.*, 2019).

Since its launch, the CCLE has been a valuable resource for many cancer researchers. The Broad Institute has published six principal studies (including three in *Nature*; Barretina *et al.*, 2012; Ghandi *et al.*, 2019; Stranski *et al.*, 2015) highlighting patterns observed across cancer lines when using CCLE data, which as of 9 July 2022 have been collectively cited over 8500

times. Notable medical advances and discoveries that were made possible through the CCLE are the development of hypoxia-targeted therapy, the role of *NSD2* mutations in development of acute lymphoblastic leukemia, and the role of alternatively-spliced *MDM4* in cancers with high *MDM2* (TP53 inhibitor) activity (Jaffe *et al.*, 2013; Li, Zhang, *et al.*, 2019). CCLE data has also aided in the improvement of personalised medicine; for example, Liu *et al.* (2016) used CCLE data to show how genes that respond to anti-cancer drugs differ with age and sex. The CCLE project is still ongoing, with new datasets periodically being added online for open access. While there are limitations to the CCLE, namely that most cell lines are cultured *in vitro* (sometimes for several decades) rather than recently obtained from tumour biopsies and originate mostly from patients of European ancestry, these will likely be rectified through acquisition of cancer lines from non-European populations and/or "fresh" tumours, as the CCLE grows in scale (Dutil *et al.*, 2019; Pavel & Korolev, 2017). Regardless, the CCLE remains as one of the primary open-access databanks that allows researchers to analyse panomic data across moderate to large sample sizes of cancer cell lines.

### 1.4 Methionine Dependence in Cancer Cells

Despite the heterogenic nature of tumour development, there are several key aspects, or "hallmarks", that are frequently observed in cancers (Bergers & Benjamin, 2003; Hanahan & Weinberg, 2011). Hanahan and Weinberg (2011) have defined ten "hallmarks of cancer", one of which is the reprogramming of energy metabolism, also known as the "Warburg effect" (first postulated and subsequently observed by Warburg and colleagues, in the 1920s). Cancer cells are under constant stress from molecular processes that negatively regulate cell proliferation, such as tumour suppressor proteins, reactive oxygen species production, and programmed cell death (Hanahan & Weinberg, 2011; Newman & Maddocks, 2017). Altered metabolism allows cancer

cells to circumvent such processes, allowing continued unrestricted proliferation and survival; however, these changes in metabolism often come with changes in metabolic/nutritional demand (Hanahan & Weinberg, 2011; Newman & Maddocks, 2017; Sanderson *et al.*, 2019).

1C metabolism (see 1.1) is critical for production of nucleotides and substrate methylation: processes which are heavily implicated in cancer (Figure 1A; Sanderson *et al.*, 2019; Sorin *et al.*, 2021). Sometimes, the metabolic pathways that are reprogrammed in a cancer line happen to be those of 1C and may result in the alteration of how the cell processes methionine, since derivatives of folate and cobalamin are required for methionine remethylation (see 1.1.3, Figure 1A). This is demonstrated by using intratumoural methionine uptake with positron emission topography imaging to measure therapeutic responses in certain cancer treatments (Guéant *et al.*, 2020). The change in ability to properly remethylate endogenous homocysteine into methionine can cause tumours to acquire an absolute requirement on exogenous methionine (Cavuoto & Fenech, 2012; Cellarier *et al.*, 2003). This usually does not affect the survival of cancer, as dietary methionine is more than enough to accommodate; however, when these cancers are cultured *in vitro*, they cannot maintain survival and growth if supplemented with Hcy and not methionine, similar to cell lines with inborn errors of folate and cobalamin. This phenomenon is known as methionine dependence.

### *1.4.1 Early History and Definition of Methionine Dependence*

Newman and Maddocks (2017) argue the earliest precursor to the discovery of methionine dependence occurred in 1948, when it was observed that (1) dietary folate deficiency reduced the leukemic cell number of children with leukemia and (2) use of antifolates achieved remission in 10 out of 16 patients. Independently, Sugimura *et al.* (1959) observed that growth of transplanted Walker-256 (W256) carcinoma in Sprague-Dawley rats was slowed in those that

were fed methionine-free diets (Cavuoto & Fenech, 2012; Epner, 2001; Guéant *et al.*, 2020). Several studies during the 1960s showed that malignant tumour cells had increased activity of tRNA methylase, which prompted a series of experiments on W256 cells throughout the following decade (Buch *et al.*, 1972; Cavuoto & Fenech, 2012). When determining the effectiveness of nicotinamide as a tRNA methylase inhibitor in W256, Buch *et al.* (1972) noted an inability of cells to survive and grow when methionine is replaced with Hcy, and a subsequent study by Halpern *et al.* (1974) confirmed these observations (Cavuoto & Fenech, 2012). In between these two studies, Chello and Bertino (1973) reported that L5178Y mouse leukemia cells "appear to have an absolute requirement for preformed methionine" (p. 1903) while observing that cell proliferation would also decline when folates were withheld. Thus, the discovery of methionine dependence in cancer cells should be attributed to the collective efforts of Buch *et al.* (1972), Chello and Bertino (1973), and Halpern *et al.* (1974).

A cancer cell line is deemed methionine dependent if it can proliferate in a standard cell medium that contains methionine ($Met^{+}Hcy^{-}$) but cannot proliferate in the same medium when methionine is replaced with its precursor homocysteine ($Met^{-}Hcy^{+}$) (Chello & Bertino, 1973; Halpern *et al.*, 1974; Sorin *et al.*, 2021). Conversely, cell lines that proliferate regardless if they are grown in $Met^{+}Hcy^{-}$ or $Met^{-}Hcy^{+}$ tissue culture media are known as methionine independent; this is characteristic of the vast majority of cell lines (including non-cancer). Some studies on methionine dependence determine if the proliferation of cells in $Met^{+}Hcy^{-}$ and cells in $Met^{-}Hcy^{+}$ is actually different using statistical analysis (*e.g.*, Lien *et al.* [2017], Morvan *et al.* [2006]), but many appear to classify them on visual inspection of comparative growth curves alone (*e.g.*, Lu and Epner [2000], Mecham *et al.* [1983]). Occasionally, classification includes a third group called methionine intermediate dependent or methionine moderate dependent (*e.g.*, Kaiser

[2020], Lu and Epner [2000]). Due to the limited data obtained in this thesis, cell lines will be only classified as methionine dependent or methionine independent, but parameters that encompass classification of moderate dependence in literature should still be mentioned. These parameters are not definitive, primarily due to (1) differences in medium and serum used among laboratories (*e.g.*, Mecham *et al.* [1983] used various media with 10%-20% fetal bovine serum [FBS], Lu and Epner [2000] used only RPMI 1640 medium with 10% FBS), and/or (2) degree of methionine dependence falling along a continuum that is arbitrarily divided into different classes; however, there are consistencies, such as cell lines proliferating more slowly (but still able to proliferate) and plateauing at lower cell density in $Met^{-}Hcy^{+}$ compared to $Met^{+}Hcy^{-}$ (Kaiser, 2020; Lu & Epner 2000; Mecham *et al.*, 1983). Kaiser (2020) used the arbitrary threshold of 20% to distinguish between dependence and moderate dependence: a cell line is methionine dependent if at a given time, its cell population in $Met^{-}Hcy^{+}$ is <20% of that in $Met^{+}Hcy^{-}$, and is methionine moderate dependent if its population in $Met^{-}Hcy^{+}$ is ≥20% that of $Met^{+}Hcy^{-}$ (P. Kaiser, personal communication, June 17, 2022). Likewise, P. Kaiser (personal communication, June 17, 2022) arbitrarily considered a cell line to be methionine independent if at a given time, its cell population in $Met^{-}Hcy^{+}$ was ≥50% that of $Met^{+}Hcy^{-}$. Using these parameters, as of 8 April 2020 a total of 47 commercially available cancer cell lines were described as either methionine dependent (n = 30), methionine moderate dependent (n = 8), or methionine independent (n = 9) (Kaiser, 2020). Despite this, the understanding of the underlying mechanisms of methionine dependence remains incomplete, because they are heterogenic (Sorin *et al.*, 2021; Watkins, 1998). Methionine dependence is hypothesised to be induced by several factors either alone or in tandem, including (but not limited to) gene deletions, somatic mutations, and abnormal gene expression (Guéant *et al.*, 2020).

### *1.4.2 Contributing Mechanisms of Methionine Dependence*

One mechanism recurrently involved in methionine dependence development is gene deletion of *MTAP* (OMIM #156540), which codes for a key component in the methionine salvage pathway that converts MTA – a product of polyamine synthesis – into methionine (see 1.1.3, Figure 1A). *MTAP* is physically proximal to the p16 tumour suppressor gene *CDKN2A* (OMIM #600160) on chromosome 9, and the two genes are commonly codeleted in cancers (Cavuoto & Fenech, 2012; Kaiser, 2020). *MTAP* deletion causes LOF in the entire methionine salvage pathway, removing the source of 10-20% (this is debated; Albers, 2009) of cellular methionine and causing accumulation of MTA (MTAP's substrate) (Cavuoto & Fenech, 2012; Kaiser, 2020; Sanderson *et al.,* 2019). Studies that attempted to correct methionine dependence through wild type *MTAP* transfection have shown the response is heterogeneous: it corrected methionine dependence in some cell lines, but had no effect in others, leading to doubts of *MTAP*'s effect on total cellular methionine metabolism (Cavuoto & Fenech, 2012; Cellarier *et al.,* 2003; Kaiser, 2020; Sanderson *et al.,* 2019). Still, *MTAP* transfection sometimes inhibited cancer growth, and evidence now shows it may also be a tumour suppressor since its deletion has been observed without *CDKN2A* codeletion in lung cancer and glioma cell lines (Cavuoto & Fenech, 2012).

Upregulated transmethylation reactions are the most frequently cited as potential contributors towards methionine dependence (*e.g.*, Hoffman [2015], Stern and Hoffman [1984], Stern *et al.* [1984]) by causing a requirement for exogenous methionine to feed both regular processes and increased methylation rate (it is for this reason methionine dependence has sometimes been referred to as "methionine addiction" since 2014; Sedillo & Cryns, 2022; Sorin

*et al.,* 2021). Halpern *et al.*'s (1974) study using W256, L1210, and J111 cancer cell lines and non-transformed controls led to a hypothesis that methionine dependence was due to diminished ability of MTR to remethylate Hcy. Hoffman and Erbe (1976) explored this hypothesis by measuring MTR activity in W256, two simian virus 40 transformed human cell lines, and non-transformed controls and found that the enzyme exhibited no metabolic defects in methionine dependent lines compared to independent lines. Follow up studies by Stern and Hoffman (1984), and Stern *et al.* (1984) evaluated: (1) the rate of transmethylation of the same cell lines by inhibiting AHCY and measuring resulting SAH accumulation, (2) the ability of the cells to incorporate methionine into proteins after supplementing cells with [$^{35}$S]methionine for 24 hours, and (3) level of free [$^{35}$S]methionine. Cultures derived from malignant tumours showed lower SAM:SAH ratios (reflecting the altered flux through the methionine cycle), no change in ability to incorporate exogenous methionine into proteins, and much lower free methionine, when compared to non-transformed fibroblasts (Stern & Hoffman, 1984; Stern *et al.,* 1984). (Hoffman and colleagues continue to extensively study the relationship between methionine dependence and increased transmethylation.) Methionine dependence in cancer is therefore not necessarily synonymous with absolute inability to remethylate methionine.

While more cell lines began to be identified as methionine dependent with increased transmethylation reactions, many also contradicted the predictions of Stern and Hoffman (1984), and Stern *et al.* (1984). Lien *et al.* (2017) have shown that decreased expression in *SLC7A11* (encodes a cysteine transporter) and missense mutations in *PIK3CA* (inhibits *SLC7A11*) likely have a role in the methionine dependence in ten cancer cell lines, by shifting intracellular homocysteine away from methionine remethylation towards the transsulfuration pathway (Figure 1A) and increasing cystine synthesis. Cavuoto and Fenech (2012) have produced a speculative

list of genes involved in 1C that may lead to methionine dependence if mutated (Appendix A). Some reviews mistakenly reference Cavuoto and Fenech's (2012) list as "approximately twenty somatic mutations" identified as contributors to methionine dependence (*e.g.,* Guéant *et al.* [2020]). Decreased MTR activity has been observed in methionine dependent cell lines, possibly due to (epi)mutations within genes coding enzymes involved in 1C metabolism, as they may lead to lack of 5-MethylTHF substrates and/or MeCbl cofactors (Cellarier *et al.,* 2003; Liteplo *et al.,* 1991); however, these (epi)mutations have not been elucidated. Indeed, the list of cancer cell lines that are known to be methionine dependent due to any kind of (epi)mutation is limited, but includes the melanoma and glioma lines MeWoLC1 and GaMg-P60 respectively, which are further described in 1.4.3.

### *1.4.3 MeWo and MeWoLC1*

Following the discovery of methionine dependence, Liteplo (1989) studied the relationship between methionine metabolism and cancer progression through the parent human cutaneous melanoma MeWo cell line and its highly malignant daughter lines. While the parent line MeWo (derived from a human lymph node) was found to be methionine independent, the malignant daughter line MeWoLC1 (derived from a tumour of a mouse inoculated with MeWo) showed inability to grow in $Met^{-}Hcy^{+}$ medium (Liteplo, 1989, 1990). Subsequent studies by Liteplo (1990) found that this was not primarily due to increased transmethylation rates, but rather an inability to synthesise methionine from Hcy and 5-methylTHF, and reduced capacity to generate MeCbl (see 1.1.3; Liteplo, 1990; Liteplo *et al.,* 1991). This metabolic pattern was akin to the methionine auxotrophy seen in inborn errors of folate and cobalamin metabolism, specifically *cblC* and *cblD* (see 1.1.4; Liteplo, 1990; Liteplo *et al.,* 1991; Watkins, 1998). Contemporaneously, Fiskerstrand *et al.* (1994) identified the GaMg-P60 glioma cell line as

methionine dependent, also due to altered cobalamin metabolism (Liteplo *et al.,* 1991; Sorin *et al.,* 2021; Watkins, 1998). Watkins (1998) chose fourteen cancer lines of various tissues (osteosarcoma, prostate, kidney, etc.) that were previously reported as methionine dependent, and compared them to lines with inborn cobalamin disorders using 5-methylTHF and propionate incorporation (measures of MTR and MMUT function, respectively, in intact cultured cells). The affected gene (unidentified at the time) in MeWoLC1 was confirmed to be identical to that affected in patients with *cblC* through cell complementation, but no other lines with similar defects in cobalamin metabolism were found (Watkins, 1998).

Lerner-Ellis *et al.* (2006) later identified the "*cblC* gene" as *MMACHC* (see 1.1.2), but follow-up studies by Loewy *et al.* (2009) found no LOF mutations of *MMACHC* in MeWoLC1. MeWo and MeWoLC1 are both heterozygous for *MMACHC* haplotypes AGC and GCG (defined by the genotypes for the *MMACHC* single nucleotide polymorphisms at rs2275276, rs11580609, and rs7903; Loewy *et al.,* 2009). MMACHC expression in MeWo was "almost exclusively" of the GCG allele, suggesting hypoexpression of the AGC allele (Loewy *et al.,* 2009). Infecting MeWoLC1 cells with wild-type *MMACHC* corrected all aspects of cobalamin metabolic defects, including the ability to grow in $Met^{-}Hcy^{+}$ medium, and quantitative analysis of DNA methylation using real-time PCR and bisulfite sequencing confirmed near complete methylation of the *MMACHC* 5'-CpG island in MeWoLC1 (Loewy *et al.,* 2009). The lack of functional MMACHC in MeWoLC1 was hypothesised by Loewy *et al.* (2009) to be due to the methylation of the AGC allele, which first occurred in MeWo, followed by a second methylation in the GCG allele when MeWoLC1 was derived from MeWo.

Recently, Guéant *et al.* (2018) have observed mutations in *PRDX1* – a gene adjacent to the 5' end of *MMACHC* – that caused an inborn cobalamin metabolic error called epi-*cblC* (see

1.1.4). Guéant *et al.* (2018) silenced the aberrant *PRDX1* antisense transcript that caused hypermethylation of *MMACHC* in epi-*cblC* cell lines using siRNA to negate mRNA synthesis, and found MMACHC expression increased in all cell lines, restoring proper cobalamin metabolism. When the same methods were used with MeWoLC1, MMACHC expression increased six fold (Guéant *et al.,* 2018). While data confirmed presence of the *PRDX1* splice site mutation in epi-*cblC* cells, no mutation was documented in MeWoLC1. Nonetheless, methionine dependence in MeWoLC1 tumour cells is believed to be caused by hypoexpression of MMACHC through *MMACHC* hypermethylation, with some relation to *PRDX1* (Guéant *et al.,* 2018; Pollini *et al.*, 2020; Sorin *et al.*, 2021).

### 1.5 *MMACHC* Expression and Methylation in Melanoma

Sorin *et al.* (2021) looked for other tumour cell lines that also exhibited low MMACHC expression or high *MMACHC* methylation on the CCLE, cBioPortal, and the Cancer Genome Atlas Wanderer online platforms, and noted melanomas represented a large proportion of both (Figure 2). Sorin *et al.* (2020) specifically found that, out of 25 cancer cell lines within the upper $97^{th}$ percentile of *MMACHC* methylation, 17 were melanoma cell lines (odds ratio 46.3; 95% CI 18.7 to 114.6), and CCLE melanomas in general had lower *MMACHC* expression compared to non-melanomas (odds ratio 5.75; 95% CI 1.99 to 16.65). High methylation is not synonymous with low expression; there are numerous genes whose hypermethylation in cancer cells increases their expression (Rauluseviciute *et al.*, 2020; Sanderson *et al.*, 2019). Indeed, those with high methylation values of *MMACHC* do not necessarily have low mRNA expression and vice versa, although an overall negative association is present. For example, while MeWo – a methionine independent cell line – is not included in the melanomas with high *MMACHC* methylation, it has the seventh-lowest MMACHC expression out of the 1019 cell lines with MMACHC expression

data in the CCLE. This may suggest that (1) low MMACHC expression is only partially affected by *MMACHC* methylation, and (2) *MMACHC* hypermethylation can confer methionine dependence while MMACHC hypoexpression does not necessarily. This is consistent with a study by Loewy *et al.* (2009), who showed that compared to control fibroblasts, the *MMACHC* promoter in MeWo cells was only ~50% methylated, while the *MMACHC* promoter in MeWoLC1 was nearly 100% methylated; however, since methylation data in Loewy *et al.*'s (2009) study was obtained with different methods than those of the CCLE, it may be inappropriate to compare specific values. Expression and methylation data for MeWoLC1 are not available in the CCLE.

With these observations, Sorin *et al.* (2021) hypothesised that decreased *MMACHC* expression through increased *MMACHC* methylation may be a recurrent mechanism for methionine dependence, particularly in melanomas, which may lead to specialised treatments for those with methionine dependent melanoma tumours. Although an additional prevalence of abnormal *MMACHC* expression and methylation data in gliomas was observed, Sorin *et al.* (2021) mainly focused on melanomas. Kaiser's (2020) list of available cell lines with known proliferation in $Met^{+}Hcy^{-}$ and $Met^{-}Hcy^{+}$ only contains two melanoma cell lines. It was not clear whether the melanomas highlighted by Sorin *et al.* (2021) are methionine dependent, and if so, whether it is due to low MMACHC expression and/or high *MMACHC* methylation. Sorin *et al.* (2021) concluded their review with:

> What causes the reduction in MMACHC expression and its hypermethylation in the cancer cell lines and patient tumors mentioned above? … Why is there such a large distribution in MMACHC expression and methylation profiles among melanoma patients? Is there any correlation with severity of disease? Would treatment of melanoma with methionine

restriction in patients with low MMACHC expression lead to improved survival as compared to patients not presenting with this genotype? (pp. 158-160)

Addressing these questions will not only contribute to the understanding of methionine dependence in cancer cells, but may lead to development of novel, effective therapies for specific cancers (Sorin *et al.*, 2021).

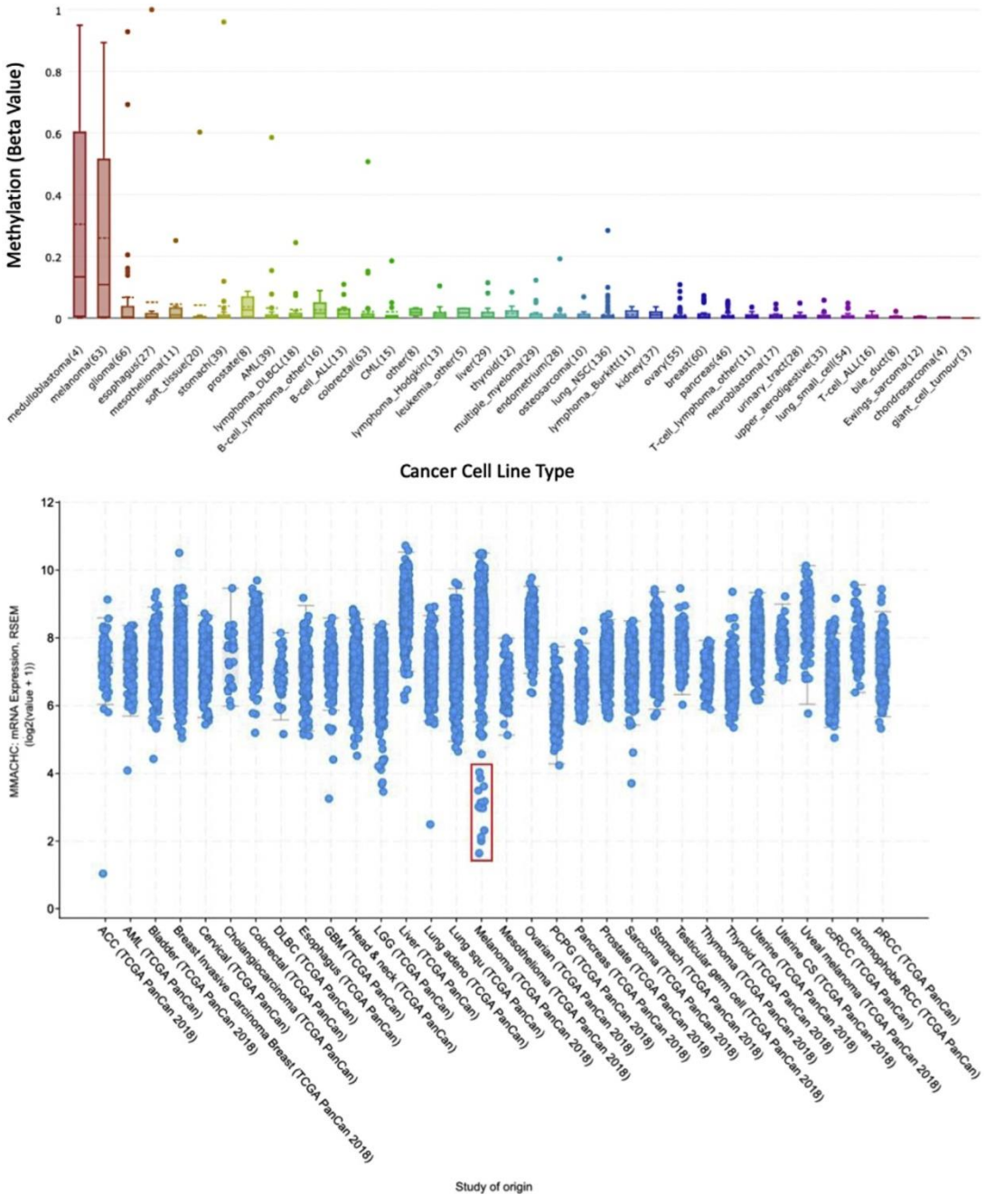
Methylation (Beta Value)
1
0.8
0.6
0.4
0.2
0
medulloblastoma(4)
melanoma(63)
glioma(66)
esophagus(27)
mesothelioma(11)
soft_tissue(20)
stomach(39)
prostate(8)
AML(39)
lymphoma_DLBCL(18)
lymphoma_other(16)
B-cell_lymphoma_other(13)
B-cell_ALL(13)
colorectal(63)
CML(15)
other(8)
lymphoma_Hodgkin(13)
leukemia_other(5)
liver(29)
thyroid(12)
multiple_myeloma(29)
endometrium(28)
osteosarcoma(10)
lung_NSC(136)
lymphoma_Burkitt(11)
kidney(37)
ovary(55)
breast(60)
pancreas(46)
T-cell_lymphoma_other(11)
neuroblastoma(17)
urinary_tract(28)
upper_aerodigestive(33)
lung_small_cell(54)
T-cell_ALL(16)
bile_duct(8)
Ewings_sarcoma(12)
chondrosarcoma(4)
giant_cell_tumour(3)
Cancer Cell Line Type
MMACHC: mRNA Expression, RSEM (log2(value + 1))
12
10
8
6
4
2
0
ACC (TCGA PanCan 2018)
AML (TCGA PanCan)
Bladder (TCGA PanCan 2018)
Breast Invasive Carcinoma (TCGA PanCan)
Cervical (TCGA PanCan 2018)
Cholangiocarcinoma (TCGA PanCan)
Colorectal (TCGA PanCan)
DLBC (TCGA PanCan)
Esophagus (TCGA PanCan)
GBM (TCGA PanCan)
Head & neck (TCGA PanCan)
LGG (TCGA PanCan)
Liver (TCGA PanCan)
Lung adeno (TCGA PanCan)
Lung squ (TCGA PanCan)
Melanoma (TCGA PanCan)
Mesothelioma (TCGA PanCan 2018)
Ovarian (TCGA PanCan 2018)
PCPG (TCGA PanCan 2018)
Pancreas (TCGA PanCan 2018)
Prostate (TCGA PanCan 2018)
Sarcoma (TCGA PanCan 2018)
Stomach (TCGA PanCan 2018)
Testicular germ cell (TCGA PanCan 2018)
Thymoma (TCGA PanCan 2018)
Thyroid (TCGA PanCan 2018)
Uterine (TCGA PanCan 2018)
Uterine CS (TCGA PanCan 2018)
Uveal melanoma (TCGA PanCan 2018)
ccRCC (TCGA PanCan)
chromophobe RCC (TCGA PanCan)
pRCC (TCGA PanCan)
Study of origin

**Figure 2.** *Melanoma cell lines appear to have abnormal MMACHC methylation and expression compared to other types of cancer, according to data from the CCLE and the Cancer Genome Atlas.*

Figures reproduced with permission (Appendix C; Sorin *et al.,* 2021). **Top)** *MMACHC* DNA methylation data of cancer cell lines derived from the CCLE. **Bottom)** *MMACHC* mRNA expression data of cancer cell lines derived from the Cancer Genome Atlas Wanderer, including a cluster of melanomas with low expression (red rectangle).

### *1.5.1 Thesis Objectives*

The principal objective of this thesis is to investigate if melanoma cell lines with *MMACHC* hypermethylation are unable to proliferate in $Met^{-}Hcy^{+}$ compared to $Met^{+}Hcy^{-}$. In other words, are there other melanoma cell lines that are methionine dependent as *cblC* phenocopies, like that of MeWoLC1? To begin to approach this, the metabolic activity of MMACHC within these cell lines must be investigated beyond online database meta-analyses. This includes (1) culturing melanoma-derived cell lines in $Met^{+}Hcy^{-}$ and $Met^{-}Hcy^{+}$, and comparing their ability to proliferate in both conditions, and (2) assays of melanoma cobalamin metabolism through cobalamin distributions, propionate incorporation, and methylTHF incorporation. High methylation is not necessarily synonymous with low expression in all genes; however, the results of Loewy *et al.* (2009) coupled with the existence of epi-*cblC*, suggests that *MMACHC* is a gene whose expression can be influenced by methylation (Guéant *et al.,* 2018; Sanderson *et al.,* 2019; Zhang *et al.,* 2015). It is therefore predicted that melanoma cell lines with *MMACHC* hypermethylation will show inability to proliferate in $Met^{-}Hcy^{+}$, accompanied by metabolic profiles that suggest defects in cobalamin expression like those of MeWoLC1.

A secondary objective of this thesis is to identify methionine metabolic properties (*e.g.*, methionine dependent, methionine independent) of previously undescribed melanoma cell lines, regardless if due to *MMACHC* epigenetic defects. Currently, 47 commercially available cell lines are known to be either methionine dependent, moderate dependent, or independent. However, 30 of these cell lines were described during the initial decade of research surrounding methionine dependence (Halpern *et al.,* 1974; Hoffman & Erbe, 1976; Kaiser, 2020; Mecham *et al.,* 1983; Stern & Hoffman, 1984; Stern *et al.,* 1984). Since 1984, the methionine metabolic growth properties of only 17 cell lines have been described, with 12 attributed to a 2017 study by Lien *et al.* (2017). Through literature review, one can find additional derivative cell lines (like MeWoLC1) with described ability to proliferate in $Met^{+}Hcy^{-}$ vs $Met^{-}Hcy^{+}$ (*e.g.*, glioma cell lines SWB-40 and GaMg-P60; Fiskerstrand *et al.,* 1994; Kokkinakis *et al.,* 1997). Many if not most of the previously described cell lines are no longer available. To allow researchers to more efficiently elucidate the mechanisms that lead to methionine dependence, the methionine metabolism of more commercially available cell lines across a broad variety of cancer types will need to be described. Only two of the aforementioned 47 cell lines are melanomas (Kaiser, 2020), and therefore this study presents an opportunity to contribute to that collection.

# Chapter 2: Methods

## 2.1 Literature Review

### *2.1.1 CCLE and DepMap Databases*

The data used in the review by Sorin *et al.* (2021) are no longer accessible through the same online platform and user interface. Instead of a dedicated CCLE website, the CCLE files have been and continue to be uploaded into the Broad Institute's Cancer Dependency Map Project (DepMap). Online links to these files can be found in Appendix B.

The methods of Sorin *et al.* (2021) were replicated, in that DNA methylation (measured in ß Values) and mRNA expression databases were downloaded from DepMap; however, whereas Sorin *et al.* (2021) used single-gene methylation and expression databases for *MMACHC* specifically, as of March 2021 the remaining databases on DepMap are comprehensive for all genes. Consequently, the DepMap DNA methylation database is organised into multiple studies/trials with varying RRBS reads per gene, therefore only the trial with the most reads was considered (the trial with the most reads for *MMACHC* appears to be identical to the data used by Sorin *et al.*, [2021]). Additionally, the DepMap expression data downloaded for this study were measured in raw transcripts-per-million (TPM), as opposed to Sorin *et al.* (2021), who used normalised expression data measured in $\log_2$(TPM+1).

Using R (ver. 4.0.3) through RStudio (R Core Team, 2020), the comprehensive expression and filtered methylation databases were combined. There are 843 and 1019 cell lines in the CCLE with general methylation and expression data respectively, as described above. The cell lines with expression data but no methylation data were omitted.

### *2.1.2 Trends Observed for MMACHC*

DNA methylation and mRNA expression data were filtered to only contain those for *MMACHC*. Descriptive statistics of *MMACHC* β Values and TPM for remaining CCLE cell lines (including melanomas) were calculated (Figure 3A). For future considerations, the shapes of the distributions of *MMACHC* β Values and TPM were determined using both a Shapiro-Wilk test for normality and a Sk-test for asymmetry (Figure 3B). To replicate the observations of Sorin *et al.* (2021) that melanoma cell lines exhibit disproportionally high and low *MMACHC* DNA methylation and mRNA expression, respectively, the *MMACHC* DNA methylation and mRNA expression data were organised by type of cancer (Figure 3C). Cell lines with methylation data but without expression data or vice-versa were omitted from the remainder of the study.

To determine if any of the 47 cell lines with known proliferative ability in $Met^{+}Hcy^{-}$ vs. $Met^{-}Hcy^{+}$ medium also may exhibit abnormal *MMACHC* DNA methylation and mRNA expression, the corresponding RRBS and RNA-seq CCLE data was cross-referenced with the cell lines listed in Kaiser (2020) (Figure 3D). Not all the 47 cell lines were included as they were omitted in earlier methods (see 2.1.1) for absence of *MMACHC* methylation data in the CCLE.

## 2.2 Tissue Culture

### *2.2.1 Selection of Cell Lines*

A total of seven cell lines are investigated in this thesis and are listed in Table 1. To select cell lines using a quantifiable threshold, a methylation-expression index ($I_{m/e}$) was created by dividing each cell line's *MMACHC* ß Value with its *MMACHC* TPM:

$$I_{m/e} = \frac{\beta\ Value}{TPM}$$

$I_{m/e}$ were then ranked low to high, with higher $I_{m/e}$ indicating cell lines with high *MMACHC* methylation and low *MMACHC* expression. The percentile ranking of $I_{m/e}$ was also considered,

to clarify status of $I_{m/e}$ compared to all CCLE cell lines. (*N.B.*, the only purpose of the $I_{m/e}$ is to organise data, it does not represent any genetic/epigenetic phenomena.) Cell lines were then filtered by cancer type to include only melanomas (SKIN; *e.g.*, Figure 3C).

Control melanoma cell lines were selected using previous reports as summarised by Kaiser (2020). Of the 47 tumour-derived (*i.e.*, cell lines not derived from the original tumour not included) commercially available cancer lines with known proliferative ability in Met+ Hcy vs. $Met^{-}Hcy^{+}$, two are human melanomas: MeWo, which is methionine independent, and A375, which is methionine moderate dependent (Figure 3D; Kaiser, 2020). There appear to be no tumour-derived commercially available methionine dependent melanomas (MeWoLC1 is known to be methionine dependent but was not derived from the original tumour and is not commercially available). MeWo was selected as positive methionine independent, and A375 and MeWoLC1 selected as positive methionine dependent, melanoma controls. MeWo has low *MMACHC* methylation (ß Value = 0.003), but also extremely low *MMACHC* expression (TPM = 0.12) despite being methionine independent; interestingly, Loewy *et al.* (2009) report ~50% *MMACHC* promoter methylation in MeWo, which seems to contradict CCLE data, albeit methylation was measured using different methods. A375 has moderate *MMACHC* methylation (ß Value = 0.023), despite having the second highest *MMACHC* expression (TPM = 10.20) among melanoma cell lines within the CCLE. High *MMACHC* methylation and little-to-no *MMACHC* expression was previously measured in MeWoLC1 (Loewy *et al.*, 2009).

Experimental cell lines were selected based on $I_{m/e}$ and availability from the American Type Culture Collection (ATCC; https://www.atcc.org/). SK-Mel-28, HMCB, and Colo829 are cell lines with high *MMACHC* methylation (ß Values = 0.192, 0.748, and 0.761 respectively) and variable *MMACHC* expression (TPM = 1.94, 2.29, and 0.03 respectively), with the latter two

among the highest 11 methylated CCLE cell lines for all cancer types. SH-4 is a cell line within the lowest $I_{m/e}$ percentile, with *MMACHC* methylation (ß Value = 0.000, meaning no methylation) and normal *MMACHC* expression (TPM = 5.66) that are within the interquartile range (IQR) of *MMACHC* ß Values and TPM respectively, for all CCLE cell lines (Figure 3A). Median and IQR were chosen as a more appropriate measure of central tendency instead of mean and standard deviation, as *MMACHC* ß Values and TPM are both skewed (Figure 3B).

MeWo and MeWoLC1 were originally obtained from Dr. Robert Liteplo. They were stored under liquid nitrogen in the Repository for Mutant Human Cell Strains at the McGill University Health Centre. All other cell lines were from the ATCC® (distributed by Cedarlane®). Identities of cell lines (except MeWo and MeWoLC1) were authenticated using short tandem repeat profiles obtained through e-mail correspondence with Cedarlane®.

MeWo is a metastatic cutaneous melanoma established by Y. Kodera and M. Bean in 1974, and derived from a lymph node of a 78-year-old male of European ancestry (ATCC, 2022d). Its tumorigenicity led to use in tumour inoculation in mice during a study by Kerbel *et al.* (1984). MeWoLC1 was derived from a primary tumour of one the mice in this study (Kerbel *et al.*, 1984). MeWoLC1 is therefore a human cell line passed through a mouse.

A375 is a malignant amelanotic melanoma established by D.J. Giard in 1973, and derived from the skin of a 54-year-old female of European ancestry (ATCC, 2022a).

Colo829 is a metastatic cutaneous melanoma established by G.E. Moore sometime before 1995, and derived from the skin of a 45-year-old male of European ancestry (ATCC, 2022b).

HMCB (Human Melanoma Cell Bowes) is a malignant melanoma which appears to have been established by E. Reich in 1975 and derived from the skin of a 57-year-old female of

European ancestry (ATCC, 2022c). HMCB is used by private companies such as Genentech® to produce pure human tissue plasminogen activator (ATCC, 2022c).

SH-4 is a metastatic melanoma established by Seman *et al.* (1975), derived from the pleural effusion of a 60-year-old female of European ancestry (ATCC, 2022e).

SK-Mel-28 is a malignant cutaneous melanoma established by T. Takahashi in 1976, and derived from the skin of a 51 year-old male of European and Asian ancestry (ATCC, 2022f). SK-Mel-28 happens to be a cell line in the NCI60 cell line panel, which can be considered the progenitor of the CCLE (Broad Institute, n.d.).

### *2.2.2 Culture Conditions*

All tissue culture was performed in sterile conditions, which includes (but is not limited to) proper use of a class 2A biological safety cabinet (BSC), frequently washing gloved hands and surfaces with ~70% ethanol, and 5min UV irradiation of the BSC surfaces when applicable after culturing. All applicable reagents were screened for mycoplasma contamination and likewise administered under sterile conditions.

Cell lines were maintained in modified Eagle's Minimum Essential Medium (EMEM) plus Earle's salts, L-glutamine, and non-essential amino acids, supplemented with 2.2g/L sodium bicarbonate, 10mg/L ferric nitrate, 1.5g/L glucose (Wisent Bioproducts, St. Bruno QC, 320-006-CL), 5% (v/v) FBS (Wisent, 080-150), 5% bovine calf serum (BCS; Wisent, 074-250), and no antibiotics. Cultures were incubated at 37°C with 5% $CO_2$.

### *2.2.3 Growth Curves*

Stock solutions of L-methionine (Sigma Aldrich Canada, Oakville ON, 63-68-3), L-homocysteine thiolactone (Sigma Aldrich, 31828-68-9), and folic acid (Sigma Aldrich, 59-30-3) were prepared by dissolving the corresponding reagents in phosphate buffered saline (PBS;

Wisent Bioproducts, 311-010-CL). To prepare $Met^{+}Hcy^{-}$ L-methionine (final concentration 0.1 mM) and folic acid (final concentration 26.5 µM) were added to EMEM lacking L-methionine and folic acid supplemented with 10% dialysed FBS (Wisent Bioproducts, 080-910), to match that of standard EMEM. $Met^{-}Hcy^{+}$ was prepared identically except with 0.1mM L-homocysteine thiolactone instead of methionine (standard EMEM does not contain homocysteine, so $Met^{-}Hcy^{+}$ was given the same concentration as that of methionine).

Following trypsinization, cells were plated in P60 tissue culture dishes at a density of 100,000 cells per dish and incubated at 37°C overnight. To harvest and count cells from a P60, a dish was washed twice with ~1mL PBS, trypsinized with ~1mL of 0.25% trypsin 2.21mM EDTA (Wisent, 325-240-EL), and resuspended in ~2mL of stock medium. Cell density of the resulting suspension was determined by average of two counts using a Countess3 Automated Cell Counter (Invitrogen). Countess3 size gating for live and dead cells was 9-25 for all cell lines except SK-Mel-28, which required 0-25 due to small size.

On the day after cells were plated (Day 0), six P60s were selected at random for each line, and cell number was determined using the Countess3. The remaining P60s were washed once with ~1mL PBS. Half of the remaining P60s were incubated in ~3mL $Met^{+}Hcy^{-}$, and the other half in ~3mL $Met^{-}Hcy^{+}$. Following day 0, three P60 cultures for each cell line and medium/condition were trypsinized and counted every 2 days for 8 days (save for SK-Mel-28 trial 2 day 6 and SH-4 trial 6 day 6: only two P60s counted due to contamination). P60 cultures were given ~3mL of fresh experimental medium on day 4 of every growth curve.

Growth curves were repeated in triplicate for each cell line (save for SH-4: repeated seven times due to variable results), for a total of at least eighteen day 0 counts and nine counts per 2-

day interval, for each of the two experimental conditions. Statistical significance of differences between the experimental conditions was assessed using an unpooled two-sided Student's t-test.

### *2.2.4 Number of Doublings*

To further quantify the effect of replacement of methionine with homocysteine in cell medium on cell lines, numbers of cell population doublings ($N_D$) over the 8-day period per condition per trial per cell line (*i.e.*, each individual growth curve) were determined using the following formula:

$$N_D = \log_2\left(\frac{t_8}{t_0}\right)$$

Where $t_8$ and $t_0$ represent the day 8 and day 0 cell counts in each curve, respectively. $N_D$ were favoured over cell population doubling times, as the latter are unreliable if cell number declined (*e.g.*, a doubling time of -0.01 days is a fast decline, while 0 days is instantaneous, and 1 day is slower positive) and it better represents changes in proliferation between conditions (*e.g.*, a cell population in $Met^-Hcy^+$ may be 50% that of $Met^+Hcy^-$ at day 8, suggesting methionine independence, but if $Met^-Hcy^+$'s population has been decreasing over time, then methionine dependence is a more appropriate designation). Like growth curves, statistical significance of differences between $N_D$ in both experimental conditions was assessed using a Student's unpooled two-sided t-test.

Designation of proliferative ability (dependent or independent) for cell lines was given using a modified version of the protocol used by Kaiser (2020). A cell line was considered methionine dependent if its average $N_D$ in $Met^-Hcy^+$ was ≤50% that of its average $N_D$ in $Met^+Hcy^-$, and methionine independent if >50% that in $Met^+Hcy^-$. Methionine moderate dependence was omitted as a classification as there was not enough data to differentiate among it, methionine dependence, and methionine independence.

## 2.3 Analysis of Cobalamin metabolism

### *2.3.1 [$^{57}$Co]Cobalamin Distribution*

Cell lines were each incubated for 96 hours in T175 tissue flasks containing filter-sterilised EMEM supplemented with 25pg/mL [$^{57}$Co]cyanocobalamin (total activity = 10.5µCi; MP Biomedicals LLC, 06B430002) that had been incubated with human serum (Innovative Research, ISERABOTC100ML) at 37℃ for 30 minutes prior to addition to medium to bind to TC. Following incubation, cells were trypsinised and cell suspensions were washed three times in PBS. For the final wash, the suspension was divided in two and the resultant pellets were frozen and stored at -80℃ after removal of PBS. Radioactivity of aliquots of the final suspension was measured by gamma counting using a Wizard™ 1470 Automatic Gamma Counter (PerkinElmer). Number of cells was determined by average of two counts using a Countess3 automated cell counter.

The procedures outlined in the following paragraph were performed under dim red light so as not to degrade photolabile cobalamin coenzymes. Radiolabelled [$^{57}$Co]cobalamin distributions were performed as outlined in Chern *et al.* (2022). Frozen cell pellets were each resuspended in HPLC-grade ethanol (EtOH) and cellular cobalamins were extracted at 80℃. The suspensions were centrifuged at ~1520g for 30min. Resultant supernatants were transferred to 50mL tubes to increase surface area, and completely evaporated under nitrogen. Prior to HPLC, dried samples were resuspended in 100µL HPLC-grade EtOH and vortexed for 60sec, then another 90sec after adding 1.5mL 0.05M phosphoric acid pH 3.0 and filtered into counting tubes containing unlabeled cobalamin standard (OHCbl, MeCbl, CNCbl, AdoCbl), which was analysed by gamma counting prior to injection onto the HPLC column.

Cobalamin coenzymes were separated with a LUNA 100 Å C8 HPLC column on a gradient of 13–35% acetonitrile in 0.05 M phosphoric acid buffer pH 3.0. Fifty 0.5 mL fractions were collected, and radioactivity was determined by gamma counting.

### *2.3.2 5-[$^{14}$C]MethylTHF and [$^{14}$C]Propionate Incorporation Assays*

Radiolabelled [$^{14}$C]propionate and 5-[$^{14}$C]methylTHF incorporation assays were performed as outlined in Chern *et al.* (2022).

To assess intact cell function of MTR, cell lines were plated in 1 six-well plate/cell line at ~600,000 cells/well, in ~1.5mL culture medium, and incubated for 1 day to allow attachment to the surface. Medium in three wells/cell line was replaced with EMEM lacking methionine and folic acid supplemented with 10% dialysed FBS, 0.1mM L-Hcy thiolactone, 0.1mM 5-[$^{14}$C]methylTHF (Pharmaron Custom Synthesis, UK) and 50µg/mL sodium ascorbate; and medium in the remaining three wells/cell line was replaced with the same modified medium additionally containing 3.75µM OHCbl. Cell lines were then incubated for 18h at 37℃ in 5% $CO_2$. The medium was removed and each well washed with PBS three times. Proteins were precipitated with 5% trichloroacetic acid (3 times for 15min each at 5℃) and the precipitate was resuspended in 0.2 N sodium hydroxide. Radioactivity was determined by liquid scintillation counting using a PerkinElmer Tri-Carb® 3110 TR, and protein concentration determined using the Lowry assay.

To assess MMUT function in intact cells, cell lines were plated the same way as the 5-methylTHF incorporation experiments, but incubated for 2 days. Culture medium in three wells/cell line was replaced with Puck's F medium supplemented with 15% FBS, 0.1mM [$^{14}$C]propionate, (Pharmaron) and 3.75µM OHCbl. Culture medium in the other 3 wells/cell line was replaced with the same modified medium but lacking OHCbl. Cell lines were then incubated

for 18h. The media were removed, and each well washed with PBS three times. Proteins were precipitated in 5% trichloroacetic acid and dissolved in 0.2 N sodium hydroxide as described for 5-methylTHF incorporation. Radioactivity was determined by liquid scintillation counting, and protein concentration determined using the Lowry assay.

# Chapter 3: Results

## 3.1 Literature Review

Out of 843 cell lines with global methylation data in the CCLE, only 19 lacked data for one of either *MMACHC* methylation or *MMACHC* expression (Figure 3A). Distributions for both *MMACHC* β Values and TPM were non-normal, and heavily right skewed and moderately right skewed, respectively (Figure 3B). This skewness prompted use of median and IQR instead of mean ± standard deviation when classifying and selecting cell lines for experiments (see 2.2.1). The majority of cell lines that occupy the highest β Values (meaning highest methylation) are melanomas (Figure 3B), emphasised by the shift in central tendency observed when comparing the IQR of β Values of all CCLE cell lines (0.000 – 0.013) to that of only the melanoma cell lines (0.011 – 0.531), considering the lower limit for β Values is 0 (Figure 3A). This disproportionate increase in melanoma lines with high *MMACHC* methylation (and to a lesser extent, low *MMACHC* expression) is especially noticeable when the CCLE cell lines are separated by type of cancer (Figure 3C), as previously highlighted by Sorin *et al.* (2021).

**A**

| | All Cancer Types | | Melanomas | |
|---|---|---|---|---|
| | DNA Methylation | mRNA Expression | DNA Methylation | mRNA Expression |
| Sample Size | 833 | 834 | 49 | 48 |
| Mean | 0.036 | 5.11 | 0.280 | 6.06 |
| Standard Deviation | 0.127 | 2.94 | 0.308 | 4.57 |
| Median | 0.002 | 4.48 | 0.153 | 5.76 |
| IQR* | 0.000 – 0.013 | 3.00 – 6.62 | 0.011 – 0.531 | 2.88 – 8.04 |
| Variance | 0.016 | 8.64 | 0.095 | 20.86 |
| Mode | 0.000 | N/A | 0.000 | N/A |
| Total Range | 0.000 – 1.000 | 0.00 – 19.64 | 0.000 – 0.893 | 0.01 – 19.09 |

*Interquartile Range

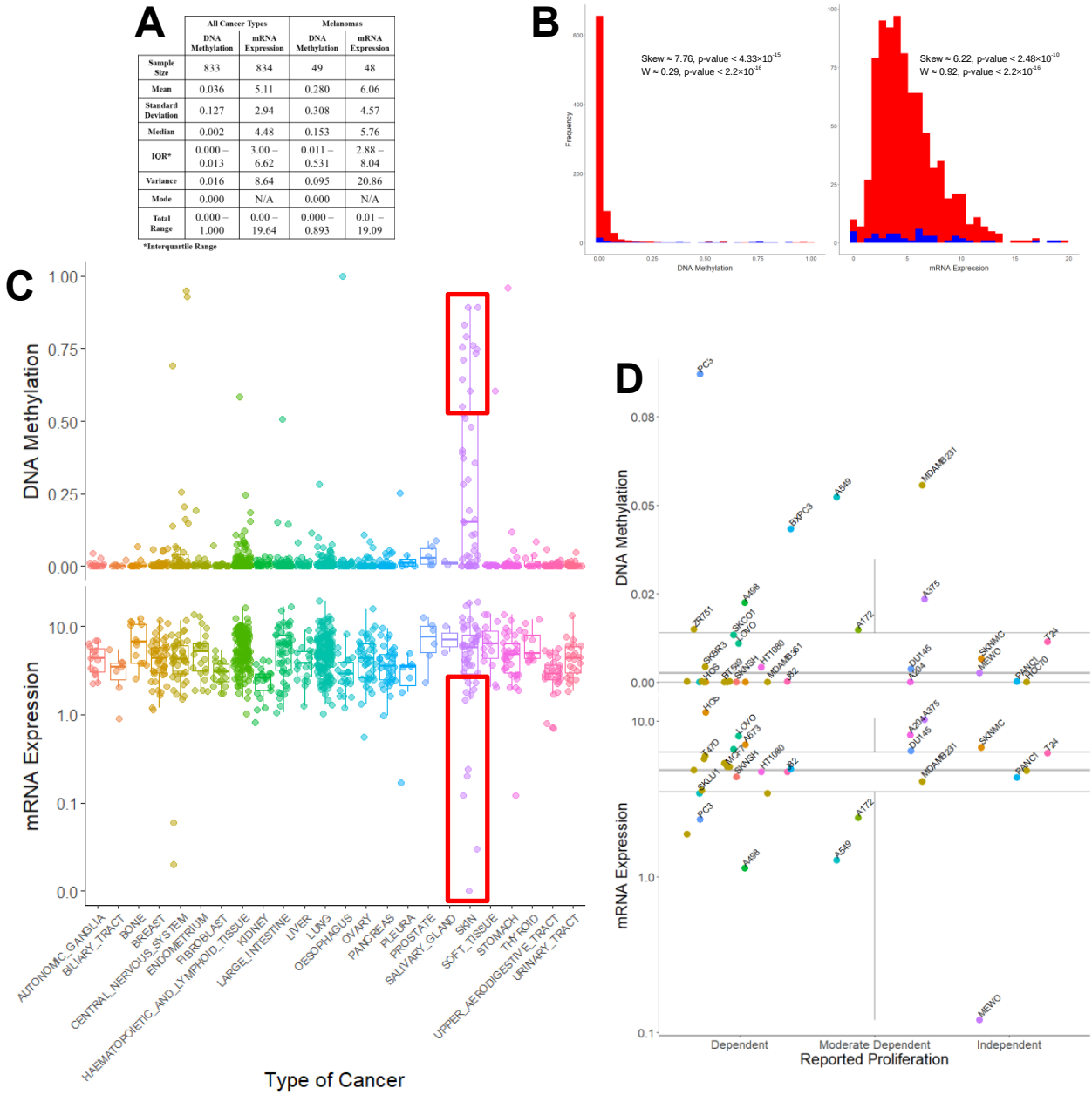

**Figure 3.** *MMACHC DNA methylation and mRNA expression data of cell lines in the CCLE.*

DNA Methylation and mRNA expression in the CCLE are measured by β Value and TPM, respectively. **A)** Panel of descriptive statistics for cell lines of all cancer types including melanomas, and melanomas alone. **B)** Frequency histograms of data distribution for all cancer types (red) including melanomas (blue), with Sk tests for asymmetry and Shapiro-Wilk tests for non-normality. **C)** Melanoma (SKIN) cell lines have abnormally (red rectangles) high methylation and low expression compared to other types of cancer; analogous to Figure 2 (Sorin *et al.*, 2021). **D)** Cell lines in CCLE format with previous reports of ability to proliferate in $Met^{+}Hcy^{-}$ vs. $Met^{-}Hcy^{+}$ medium, as described by Kaiser (2020); colour corresponds to type of cancer in **C**; boxes and whiskers represent distribution of all cell lines including those with unknown proliferative ability (Kaiser, 2020).

Several of the cell lines with known proliferative ability in $Met^{+}Hcy^{-}$ vs. $Met^{-}Hcy^{+}$ media also appeared to have abnormal *MMACHC* methylation and/or expression akin to that of MeWoLC1 (Figure 3D; Kaiser, 2020). MeWo has the lowest *MMACHC* expression of cell lines with known proliferative ability, despite being methionine independent.

### 3.2 Proliferation in $Met^{+}Hcy^{-}$ versus $Met^{-}Hcy^{+}$

Melanomas represent just 49 of 843 (5.8%) cell lines in the CCLE, but account for 28 of 83 (33.7%) of the cell lines above the $90^{th}$ percentile of *MMACHC* $I_{m/e}$. This is similar to the data of Sorin *et al.* (2020). The seven cell lines investigated in this thesis are listed in Table 1.

**Table 1.** *MMACHC DNA methylation and mRNA expression in melanoma lines used in this study.*

| Cell Line | Proliferation | ß Value | TPM | $I_{m/e}$ | $I_{m/e}$%$^{th}$ |
|---|---|---|---|---|---|
| SH-4 | Unknown | 0.000 | 5.66 | 0.000 | 48.3 |
| A375* | Dependent** | 0.023 | 10.2 | 0.002 | 71.8 |
| MeWo* | Independent | 0.003 | 0.12 | 0.021 | 92.5 |
| MeWoLC1* | Dependent | | N/A | | |
| SK-Mel-28 | Unknown | 0.192 | 1.94 | 0.099 | 96.1 |
| HMBC | Unknown | 0.748 | 2.29 | 0.327 | 98.5 |
| Colo829 | Unknown | 0.761 | 0.03 | 25.359 | 99.6 |

Methylation (β Value) and expression (TPM) data obtained from the CCLE. Cell lines are listed in ascending order of *MMACHC* $I_{m/e}$ and $I_{m/e}$%$^{th}$, with the exception of MeWoLC1 as it not included in the CCLE. *) ability to proliferate in Met+Hcy- vs. Met-Hcy+ medium previously described. **) previously described as methionine moderate dependent (Kaiser, 2020). $I_{m/e}$) *MMACHC* β Value / *MMACHC* TPM. %$^{th}$) percentile.

Most cell lines proliferated without issue in stock culture conditions; however, the SH-4 line appeared initially to have difficulty adhering to the treated plastic surface of the tissue culture flasks (based on high number of floating cells observed) and proliferating, and required approximately three weeks in culture to adapt to culture conditions (*i.e.,* once trypsinization would not lead to high numbers of floating cells). This may be an explanation for the variable results obtained with this cell line: in the first two experiments, using cells that had not adapted to culture conditions, there was little proliferation of SH-4 in either experimental medium,

making evaluation of methionine dependence difficult, but in subsequent experiments there was better proliferation in both media producing clear differences between conditions. Colo829 was also slow to multiply, but appeared to have no trouble adhering to treated plastic surface. All other lines multiplied quickly. Of note, MeWoLC1 and Colo829 appeared to produce more melanin (*i.e.*, the colour of the stock culture medium would darken faster) than other cell lines.

Previously studied lines MeWo, MeWoLC1, and A375 displayed growth curves consistent with those of methionine independent, methionine dependent, and methionine dependent cell lines, respectively (Figure 4; Kaiser, 2020; Mecham *et al.*, 1983). MeWo showed no significant difference between growth in $Met^{+}Hcy^{-}$ and $Met^{-}Hcy^{+}$, except on day 6 (Figure 4). SK-Mel-28 displayed growth curves consistent with those of methionine independent cell lines, with significant difference between growth in $Met^{+}Hcy^{-}$ and $Met^{-}Hcy^{+}$ (Figure 4). HMCB, SH-4, and Colo829 displayed growth curves consistent with those of methionine dependent cell lines, with significant differences between growth in $Met^{+}Hcy^{-}$ and $Met^{-}Hcy^{+}$ (Figure 4).

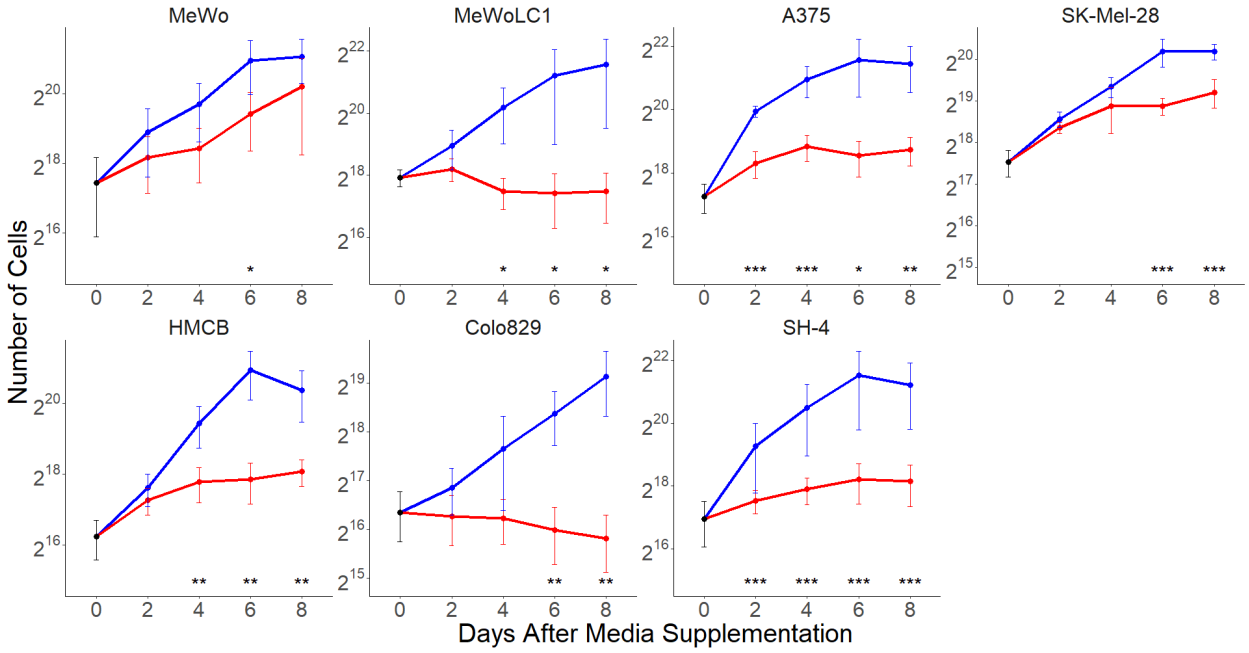


**Figure 4.** *Growth curves for seven melanoma cell lines supplemented with Met$^+$Hcy$^-$ or Met$^-$ Hcy$^+$ medium.*

Day 0 represents 1 day following plating in P60s containing EMEM + 10% FBS, replaced with either Met$^+$Hcy$^-$ (blue) or Met$^-$Hcy$^+$ (red). Points and error bars represent mean and standard deviation of triplicate cell counts in three separate trials (with the exception of SH-4: seven trials due to variable results), respectively. Statistical significance calculated using a Student's unpooled t-test, with *, **, and ***, indicating $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively above day of count.

Using the modified protocol of Kaiser (2020), MeWo, MeWoLC1, and A375 were classified as methionine independent, methionine dependent, and methionine dependent respectively, consistent with previous literature (Figure 5A; Liteplo *et al.*, 1991). Colo829, HMCB, and SH-4 were classified as methionine dependent, while SK-Mel-28 was classified as methionine independent (Figure 5A). MeWoLC1 and Colo829 not only were below the 50% $N_D$ threshold needed to be classified as dependent but declined in Met$^-$Hcy$^+$ medium (Figure 4, 5A,

5B). SK-Mel-28 was the cell line with the slowest proliferation in $Met^{+}Hcy^{-}$, even when compared to methionine dependent cell lines (Figure 5B).

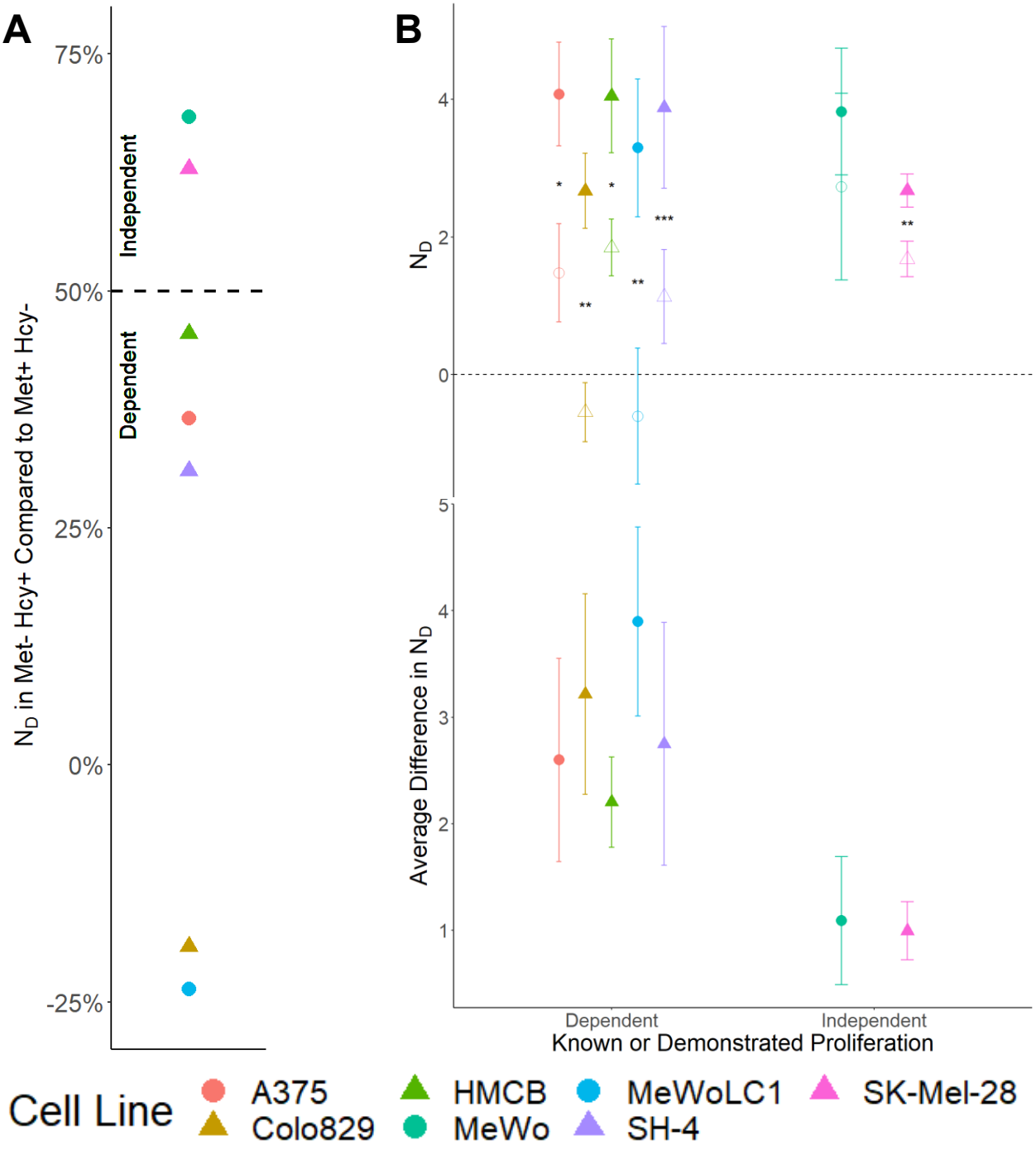

**Figure 5.** *Number of doublings over 8 days in $Met^{+}Hcy^{-}$ or $Met^{-}Hcy^{+}$ media, in three control and four experimental melanoma cell lines.*

Points represent mean of number of doublings ($N_D$) across across triplicate trials (with the exception of SH-4: seven trials due to variable results) of control (circles) and experimental (triangles) cell lines. **A)** Relative ability of cell lines to proliferate after plating in $Met^{-}Hcy^{+}$ medium compared to $Met^{+}Hcy^{-}$, and resultant classification (methionine dependent, independent). **B)** Top) cell lines plated in either $Met^{+}Hcy^{-}$ (filled) or $Met^{-}Hcy^{+}$ (empty) media; Bottom) difference between said experimental conditions; error bars represent standard deviation; statistical significance calculated using Student's unpooled t-test, with *, **, and ***, indicating $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

### 3.3 Cobalamin Coenzyme Synthesis

Cobalamin distributions were consistent across duplicate experiments, while the cobalamin uptake varied within and among cell lines. MeWoLC1 had extremely low MeCbl, low AdoCbl, and high CNCbl counts (Table 2), consistent with literature and indicative of a cobalamin metabolic defect. MeWo, A375, and SH-4 had profiles similar to each other save for an increase in AdoCbl in SH-4 (Table 2).

**Table 2.** *Cobalamin coenzyme synthesis in melanoma cell lines.*

| Cell Line | Cobalamin Uptake (pg / $10^6$cells) | % Cobalamin Distribution | | | | |
|---|---|---|---|---|---|---|
| | | OHCbl | CNCbl | AdoCbl | MeCbl | Other |
| MeWoLC1* | 1.71, 1.06 | 29.2, 40.8 | 31.2, 31.6 | 9.0, 11.4 | 0.5, 0.9 | 30.1, 15.3 |
| MeWo* | 3.46, 1.25 | 24.6, 18.6 | 5.7, 2.7 | 26.8, 27.0 | 28.9, 29.8 | 14.0, 21.9 |
| A375 | 7.26, 10.88 | 25.6, 26.7 | 5.9, 6.6 | 28.8, 23.4 | 25.6, 32.5 | 14.1, 10.8 |
| Colo829 | 2.85, 6.20 | 22.8, 22.9 | 17.0, 22.1 | 23.2, 22.0 | 17.7, 20.6 | 19.3, 12.4 |
| HMCB | 5.63, 3.04 | 13.9, 13.2 | 2.5, 3.8 | 14.0, 15.5 | 59.0, 55.8 | 10.6, 11.7 |
| SK-Mel-28 | 8.12, 16.29 | 9.8, 15.9 | 5.6, 9.9 | 20.0, 18.5 | 50.0, 49.2 | 14.6, 6.5 |
| SH-4 | 4.40, 17.36 | 18.6, 24.3 | 4.5, 6.2 | 38.4, 35.1 | 27.0, 25.9 | 11.5, 8.5 |

Experiments were performed in duplicate, following 96 hours of incubation in media containing [57Co]CNCbl. Results of individual experiments are separated by comma. *) control. OHCbl) hydroxocobalamin. CNCbl) cyanocobalamin. AdoCbl) adenosylcobalamin. MeCbl) methylcobalamin.

Propionate and 5-methylTHF incorporation are consistent with cobalamin distributions in that MeWoLC1 was the only cell line with data indicative of a cobalamin metabolic defect (Table 2, 3). MeWoLC1 showed near-double propionate incorporation and no increase in 5-methylTHF incorporation in presence of OHCbl compared to its absence. In contrast, all other cell lines show no clear difference in propionate incorporation between presence and absence of OHCbl, while at least more than double the incorporation of 5-methylTHF in presence of OHCbl compared to absence, apart from HMCB, which showed an increase in propionate incorporation

in presence of OHCbl compared to absence. Wild-type fibroblasts show negligible response to propionate incorporation with OHCbl which reflects maximally active MMUT, while cells (*e.g.*, fibroblasts with inborn errors of cobalamin metabolism) that do show a response suggest MMUT is functioning at a below-optimal capacity. HMCB and SK-Mel-28 had the highest propionate incorporation in both presence and absence of OHCbl. All cell lines besides MeWo show incorporations which parallel wild-type amniocytes (Morel *et al.*, 2005). Colo829 had the highest 5-methylTHF incorporation in presence of OHCbl. It should be noted that A375 had low protein yield in both assays, presumably because fewer cells were plated compared to other cell lines.

**Table 3.** *Propionate and 5-methylTHF incorporation assays of melanoma cell lines.*

| Cell Line | Propionate Incorporation (nmol/mg protein/18h) | | 5-MethylTHF Incorporation (pmol/mg protein/18h) | |
|---|---|---|---|---|
| | w/o OHCbl | w/ OHCbl | w/o OHCbl | w/ OHCbl |
| MeWoLC1* | 1.72 ± 0.03 | 3.18 ± 0.16 | 74.21 ± 7.82 | 80.01 ± 24.38 |
| MeWo* | 4.33 ± 0.06 | 4.83 ± 0.09 | 92.78 ± 13.34 | 306.20 ± 13.62 |
| A375 | 8.19 ± 0.37 | 8.29 ± 0.43 | 110.28 ± 12.81 | 238.62 ± 64.47 |
| Colo829 | 10.81 ± 0.82 | 10.23 ± 0.23 | 110.34 ± 29.42 | 626.48 ± 58.77 |
| HMCB | 11.32 ± 0.12 | 16.19 ± 1.65 | 137.40 ± 3.66 | 572.30 ± 71.08 |
| SK-Mel-28 | 11.49 ± 0.37 | 13.02 ± 0.42 | 113.48 ± 2.54 | 232.61 ± 11.11 |
| SH-4 | 5.62 ± 0.42 | 5.01 ± 0.42 | 85.79 ± 13.12 | 380.45 ± 118.48 |

Cell lines incubated in media containing [$^{14}$C]propionate or 5-[$^{14}$C]methylTHF respectively for 18 hours. Each assay was performed in presence and absence of OHCbl per cell line. ues reported are mean ± standard deviation of triplicate experiments. *) control. OHCbl) hydroxocobalamin.

# Chapter 4: Discussion

## 4.1 Interpretation of Results

The principal goal of this thesis is to determine if cell lines within the overrepresentation of *MMACHC* hypermethylation in melanomas (according to CCLE methylation data) are methionine dependent because of defective cobalamin metabolism akin to MeWoLC1. This was accomplished by (1) comparing growth of seven melanoma cell lines (including MeWoLC1) in $Met^{+}Hcy^{-}$ and $Met^{-}Hcy^{+}$, and (2) investigating aspects of cobalamin metabolism in those seven melanoma cell lines. Cell lines that show ≤50% proliferation (measured by $N_D$) in $Met^{-}Hcy^{+}$ compared to $Met^{+}Hcy^{-}$ are considered methionine dependent. Cell lines with a mix of low AdoCbl and/or MeCbl, propionate incorporation increase in response to OHCbl, decreased overall propionate incorporation, no 5-methylTHF incorporation increase in response to OHCbl, and/or decreased overall 5-methylTHF incorporation, are likely to have defects in cobalamin metabolism (Morel *et al.,* 2005), although there are no formal empirical thresholds. The results of these studies showed that while five out of seven of these melanoma lines are methionine dependent, only MeWoLC1 has defective cobalamin metabolism, and thus the phenomenon associated with MeWoLC1 remains unique. Moreover, melanoma lines with hypermethylation of *MMACHC* were not always found to be methionine dependent and/or have defects in cobalamin metabolism.

MeWo and MeWoLC1 were confirmed as methionine independent and methionine dependent, respectively (Figure 4, 5A; Kaiser, 2020). Liteplo *et al.* (1991) described the cobalamin distributions of both MeWo and MeWoLC1 in their original study which showed that the latter cell line was methionine dependent due to a defect in cobalamin metabolism (MeCbl < 1%, AdoCbl ≈ 1% of total cobalamin). Later, Loewy *et al.* (2009) produced similar results

(MeCbl = 0.95±0.07% [mean ± standard deviation], AdoCbl = 2.65±0.21% of total cobalamin). These distributions are similar to those of fibroblast lines from *cblC* and epi-*cblC* patients (Guéant *et al.,* 2018; Loewy *et al.,* 2009; Watkins, 1998). Conversely, cobalamin distributions for MeWo reported by Liteplo *et al.* (1991; MeCbl ≈ 23%, AdoCbl ≈ 7% of total cobalamin) and Loewy *et al.* (2009; MeCbl = 32.9%, AdoCbl = 39.5% of total cobalamin) are more akin to cell lines with no defects in cobalamin metabolism (the variability seen between reports may be due to different incubation conditions) (*e.g.*, amniocyte cell lines observed by Morel *et al.* [2005]). In his study of 14 methionine dependent cell lines, Watkins (1998) showed that MeWoLC1's propionate incorporation (w/o OHCbl = 110±26nmol/mg protein/18h [mean ± standard deviation], w/ OHCbl = 272±37nmol/mg protein/18h) and 5-methylTHF incorporation (w/o OHCbl ≈ 29.2±5.7pmol/mg protein/18h, w/ OHCbl ≈ 46.1±7.4pmol/mg protein/18h) were likewise consistent with those of *cblC* phenotypes. In unpublished data provided by Watkins (1996), MeWo's propionate incorporation (w/o OHCbl = 4.14±0.20nmol/mg protein/18h, w/ OHCbl = 5.59±0.17nmol/mg protein/18h) and 5-methylTHF incorporation (w/o OHCbl = 53.4±2.0pmol/mg protein/18h, w/ OHCbl = 107.3±3.0pmol/mg protein/18h) were consistent with those of cell lines with no defects in cobalamin metabolism (*e.g.*, amniocytes in Morel *et al.* [2005]). Likewise, the results outlined in Table 3 confirm that both propionate and 5-methylTHF incorporations of MeWo are consistent with cell lines without cobalamin metabolic defects, including those other than MeWoLC1 in Watkins' (1998) study.

A375 was one of two melanoma cell lines to be first described as methionine independent (later considered methionine moderate dependent; Kaiser, 2020) alongside MeWo in the study by Mecham *et al.* (1983), where the majority of current cell lines with known proliferative ability in $Met^{-}Hcy^{+}$ were identified. Its methionine dependence was confirmed in this thesis. While

CCLE does report higher *MMACHC* methylation in A375 than MeWo, it also reports nearly ten times more MMACHC expression than MeWo (Table 1). The cobalamin distributions, propionate incorporation and 5-methylTHF incorporation data collectively suggest no defects in cobalamin metabolism in A375, meaning the difference in proliferation seen between $Met^{+}Hcy^{-}$ and $Met^{-}Hcy^{+}$ is likely not due to a cobalamin metabolic perturbation induced by *MMACHC* methylation.

HMCB has the eleventh highest and seventh highest *MMACHC* methylation among all cell lines and melanomas respectively, in the CCLE. Conversely, its *MMACHC* expression is within the IQR (*i.e.*, close to the median) of both categories (Table 1, Figure 3A). HMCB was tested to determine if hypermethylation of *MMACHC* would still affect overall cobalamin metabolism including ability to remethylate Hcy, despite reported normal gene expression. HMCB was found to be methionine dependent (Figure 4, 5A), and cobalamin distributions do not suggest a defect in metabolism like that of MeWoLC1; whereas MeCbl synthesis is extremely low in MeWoLC1 compared to other cell lines, HMCB appears to have high MeCbl synthesis compared to the other cell lines except SK-Mel-28, and low CNCbl and AdoCbl compared to cell lines except MeWoLC1 and MeWo (Table 2). Although some cell lines, namely those of amniocytes with no cobalamin metabolic defects, have been shown to reach MeCbl levels of >50% of cellular cobalamin (Morel *et al.*, 2005), most of the other cell lines in this thesis had much lower levels. Furthermore, HMCB does show an increase in propionate incorporation in the presence of OHCbl compared to absence (Table 3), which may suggest some function of MMACHC or another enzyme in cobalamin metabolism may be inhibited (in control fibroblasts, presence of OHCbl does not elicit a response in propionate incorporation, implying MMACHC is functioning optimally); however, this is unlikely as all mutations causing *cblC* affect both MeCbl

and AdoCbl synthesis, and addition of OHCbl does not appear to affect MTR activity, as 5-methylTHF incorporation shows an increase in presence of OHCbl characteristic of wild-type metabolism and HMCB has the highest 5-methylTHF incorporation in absence of OHCbl among melanoma lines investigated in this thesis (Table 3).

Colo829 has the ninth highest *MMACHC* methylation and fourth-lowest *MMACHC* expression among all cell lines in the CCLE (fifth-highest *MMACHC* methylation and second-lowest MMACHC expression of melanomas in the CCLE), and as of January 2022, is the melanoma cell line with the highest *MMACHC* methylation and lowest MMACHC expression available in the ATCC. Colo829 was the only cell line besides MeWoLC1 to not only be methionine dependent, but to also decline in cell number after replacement of $Met^{+}Hcy^{-}$ with $Met^{-}Hcy^{+}$ (Figure 4, 5A). Conversely, cobalamin distributions, propionate incorporation, and 5-MethylTHF incorporation do not suggest a defect in cobalamin metabolism to the degree of MeWoLC1, although there appears to be a decrease in MeCbl and increase in CNCbl compared to the six cell lines (other than MeWoLC1) investigated in this thesis albeit with normal AdoCbl (Table 2, 3); however, wild-type amniocytes have been shown to have MeCbl counts as low as 7.9% (Morel *et al.*, 2005).

SK-Mel-28 is a cell line with relatively high *MMACHC* methylation and normal-to-low MMACHC expression. Growth curve patterns (Figure 4) and change in $N_D$ in $Met^{-}Hcy^{+}$ (Figure 5A), led us to classify SK-Mel-28 as methionine independent. SK-Mel-28 consistently had the highest cobalamin uptake of the seven cell lines, and the remaining cobalamin distributions, propionate, and 5-methylTHF incorporations collectively suggest no defect in cobalamin metabolism, save for an increase in MeCbl like that observed in HMCB (Table 2, 3).

SH-4 was selected due to both its standard *MMACHC* methylation and expression. SH-4 has a *MMACHC* β Value of 0, meaning the gene's transcription start site is not methylated, and a *MMACHC* TPM of 5.66, meaning the gene is being expressed normally (Table 1, Figure 3A). For these reasons, one might expect SH-4 to be methionine independent, but the cell line clearly displays growth curves consistent with those of methionine dependent cell lines (Figure 4, 5A). Moreover, the propionate and 5-methylTHF incorporation (Table 3) of SH-4 do not suggest any defect in cobalamin metabolism.

**4.2 Alternative Mechanisms for Methionine Dependence**

With the addition of SH-4, HMCB, and Colo829, there are now 41 (as opposed to 38) known commercially available methionine dependent cell lines including four melanomas (Kaiser, 2020). It is clear the extent of the methionine dependence phenomenon needs to be further characterised, especially since the majority of cancer cell lines that have been investigated in studies relating to methionine dependence have been found to be either methionine dependent or moderate dependent (Figure 3D; Kaiser, 2020; Mecham *et al.*, 1983). The creation of panomic databanks like the CCLE presents the opportunity to elucidate shared factors that confer the methionine dependent phenotype such as *MTAP* deletions, various deleterious mutations, or even factors that have not yet been implicated in methionine dependence, provided the proliferative ability in $Met^{+}Hcy^{-}$ and $Met^{-}$ $Hcy^{+}$ of more cancer cell lines are determined. Despite the limited number of cancer cell lines known to be methionine dependent, the causative mechanisms have not been determined for most of them; for example, those of MeWoLC1 have been largely determined, while those of A375 have not. Since none of the cell lines show cobalamin distributions, propionate incorporation or 5-methylTHF incorporation like those of MeWoLC1 (Table 2, 3), (epi)genetic defects of *MMACHC* are not

sufficient explanations for the decreased ability to proliferate in $Met^{-}Hcy^{+}$ (Figure 4, 5B). Using CCLE data for gene methylation, expression, copy number, and mutations (it is unclear if the mutations reported are homozygous), this section explores possible origins of the methionine dependence of the melanoma cell lines besides MeWoLC1 investigated in this thesis.

A375 has been described as having low transmethylation activity, despite also having a SAM:SAH ratio like those of methionine dependent cell lines (*i.e.*, typically with high transmethylation activity; Judde *et al.*, 1989; Stern *et al.*, 1984). Judde *et al.* (1989) also showed overall increased spermine and spermidine levels, as well as transient-yet-significant depletion of SAM and SAH following replacement of $Met^{+}Hcy^{-}$ with $Met^{-}Hcy^{+}$ in A375 cultures. Taken together, these suggest A375 may be methionine (moderate) dependent due to some defect within another component of 1C metabolism. A375 cells have a deleterious mutation in the *AHCY* gene (Figure 6A), which is responsible for encoding the enzyme that hydrolyses SAH to Hcy. If A375 does indeed have a homozygous (again, it is it is unclear if the mutations reported in the CCLE database are homozygous; Appendix B) LOF variant in this enzyme, it may explain both its (moderate) methionine dependence and patterns observed by Stern *et al.* (1984) and Judde *et al.* (1989). In mammals, SAH is only implicated in the production of Hcy and inhibiting SAM-dependent methyltransferases which favour SAH formation; it is not used for other molecular processes (as far as is known; Turner *et al.*, 2000). An inability to hydrolyse SAH may result in its build-up and subsequent inhibition of methyltransferases, leading to increased diversion of SAM into increased spermine and spermidine synthesis (Figure 1A). Eventually, the remainder of the methionine cycle may be slowed down; meaning replacement of $Met^{+}Hcy^{-}$ with $Met^{-}Hcy^{+}$ would hinder proliferation. The exogenous methionine in $Met^{+}Hcy^{-}$ would allow A375 to bypass any effect on methionine-necessary molecular processes expected of an inhibited

methionine cycle. A375 also has a non-deleterious yet non-conserved mutation in *DNMT1*, a methyltransferase (Carmel & Jacobsen 2001).

HMCB has a non-conserved *MTHFD1* variant (Figure 6A), which may explain a surplus of MeCbl, provided functionality is affected more than the data reports, although this is unlikely since decreased MTHFD1 activity has been consistently shown to result in decreased MeCbl (Field *et al.,* 2015). HMCB also has a non-conserved variant in *AHCY* (Figure 6A). *PRDX1* and *TESK2* are both hypermethylated alongside *MMACHC* in HMCB, as well as *HCFC1* (Figure 6B). (*N.B.*, *PRDX1* is not hypermethylated in MeWoLC1, but its expression does result in *MMACHC* hypermethylation through increased production of antisense transcripts [Guéant *et al.,* 2018; Oussallah *et al.*, 2022].) While *TESK2* hypermethylation is not thought to contribute to the cobalamin metabolic defects seen in epi-*cblC* patients, its presence in HMCB may suggest a similar phenomenon to the one described in some epi-*cblC* patients by Oussallah *et al.* (2022), and that the *MMACHC* hypermethylation observed in MeWoLC1 is only partially causative of its *cblC* phenocopy, that HMCB is able to compensate for a *MMACHC*-hypermethylation-induced metabolic defect through another molecular process, or that (like MeWo) only one of HMCB's *MMACHC* alleles is hypermethylated which would not lead to a cobalamin defect.

Colo829 is another cell line with a reported deleterious mutation in 1C metabolism, this case being in *ABCD4* (Figure 6A; Watkins & Rosenblatt, 2011); however, this would be expected to result in lysosomal cobalamin accumulation and deficit in coenzyme forms, which was not supported through biochemical data (Table 2, 3). *MMACHC* and *PRDX1* are also both hypermethylated in Colo829, with moderate *TESK2* methylation (*i.e.*, a lesser extent than HMCB; Figure 6B), further suggesting that the cobalamin distributions in MeWoLC1 may involve additional factors.

SK-Mel-28 is a methionine independent cell line, and since this can be considered a default phenotype – as in, most wild-type cells are able to proliferate well in $Met^{+}Hcy^{-}$ and $Met^{+}Hcy^{-}$ – it is difficult to propose confounding mechanisms surrounding 1C metabolism that may allow circumvention of *MMACHC* hypermethylation, apart from hypermethylation occurring in only one *MMACHC* allele (meaning there is nothing to circumvent). Furthermore, while CCLE data describes methionine independent cell line MeWo with low *MMACHC* methylation, Loewy *et al.* (2009) and Guéant *et al.* (2018) have shown ~50% *MMACHC* promoter methylation albeit using a different bisulfate sequencing method, and SK-Mel-28 may show similar results if tested in the same way. *HCFC1* is also hypermethylated in SK-Mel-28, a profile shared by two known methionine independent cell lines and HMCB (Figure 6C; Kaiser, 2020). *HCFC1* is an *MMACHC* regulator, and the former's methylation may affect the latter in a way that rectifies cobalamin metabolism and corrects methionine dependence (this may also be the compensatory mechanism in HMCB). *HCFC1* upregulation in tumours is associated with increased malignancy and severity (Glinsky *et al.*, 2005), and methionine dependent daughter cancer lines have been shown to be more malignant than their independent parent lines (Fiskerstrand *et al.* 1994; Liteplo, 1989). Interestingly, *MMACHC-PRDX1* hypermethylation appears to also be present in SK-Mel-28 but does not extend to *TESK2* (Figure 6B).

SH-4 is a cancer cell line with a deletion in chromosome 9 encompassing the *MTAP* gene (Figure 6D), as part of a commonly deleted genomic region in cancers (Cavuoto & Fenech, 2012; Kaiser, 2020). *MTAP* deletion has been shown to inhibit polyamine synthesis and consequently, block the methionine cycle through a build-up of dcSAM (Cavuoto & Fenech, 2012; Kaiser, 2020; Sanderson *et al.*, 2019). Again, *MTAP* deletion as a causative agent for methionine dependence varies among cell lines (Cavuoto & Fenech, 2012; Kaiser, 2020;

Sanderson *et al.*, 2019). No other cell lines investigated in this thesis reportedly have gene deletions of *MTAP* or those outlined in Appendix A. If no supporting evidence is found that SH-4's methionine dependence is due to *MTAP* deletion, another possible mechanism is that SH-4 is reported with a non-conserved variant of uncertain significance in association with *cblG* disease in the SAM-activation domain of *MTR* (Figure 6A), which may also contribute towards the dependent phenotype.

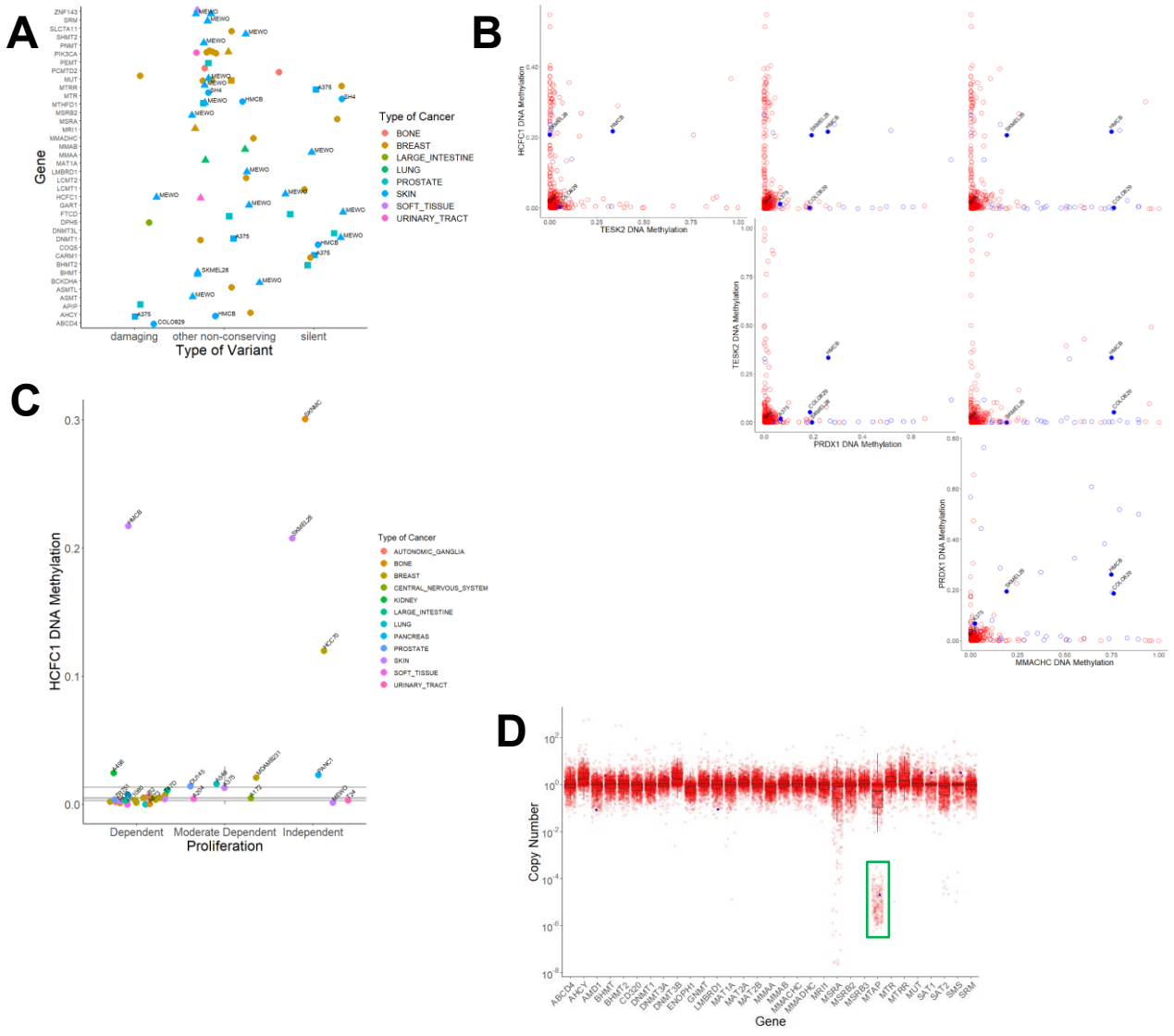

**Figure 6.** *Possible explanations for the development of altered methionine metabolism using databases from the CCLE.*

**A)** Mutations in genes involved in one carbon metabolism described in methionine dependent (circles) moderate dependent (squares) and independent (triangles) cell lines; named cell lines are those investigated in this thesis. **B)** A collection of melanoma (blue) cell lines share varying joint hypermethylation in genes *MMACHC*, *PRDX1*, *TESK2*, and *HCFC1*, which are known to interact in some capacity; filled and named points are cell lines investigated in this thesis. **C)** Three methionine independent cell lines and one dependent cell line exhibit *HCFC1* hypermethylation. **D)** SH-4 (blue) is a melanoma cell line with *MTAP* gene deletions (green rectangle).

### 4.3 Defining Methionine Dependence

Despite altered methionine metabolism being a known recurrent phenotype in cancer, the definition of what constitutes a methionine dependent, moderate dependent, or independent cell line is inconsistent across the literature. The study by Mecham *et al.* (1984) classified ability to proliferate in $Met^{+}Hcy^{-}$ and $Met^{-}Hcy^{+}$ through visual assessment, which appears to be the most common method. Kaiser (2020) classified through means previously discussed (see 1.4.1). Morvan *et al.* (2006) distinguished methionine independence and dependence using a Mann-Whitney-Wilcoxon sign test, which is a statistical test commonly used with experiments involving small sample sizes such as growth curves: a line or tumour was said to be methionine dependent if its proliferation in $Met^{+}Hcy^{-}$ was statistically significantly different than in $Met^{-}Hcy^{+}$. The more common statistical test used to separate methionine dependence from independence is the Student's t-test (*e.g.*, Lien *et al.* [2017]). Before proceeding with more studies aiming to elucidate the ability to proliferate in $Met^{+}Hcy^{-}$ and $Met^{-}Hcy^{+}$ of more cancer

cell lines, it may be beneficial to reach a consensus on how to distinguish among methionine (moderate) dependence, and methionine independence. In this thesis, this issue is best demonstrated with SK-Mel-28: using the modified Kaiser (2020) protocol, SK-Mel-28 was classified as methionine independent; however, if using Lien *et al.*'s (2017) protocol, this classification would be inappropriate, and SK-Mel-28 would likely be reclassified as methionine dependent.

Moreover, the term "methionine dependence" appears to be treated differently depending on the type of study. In studies focused on determining the ability of a cancer cell line to proliferate in $Met^{+}Hcy^{-}$ and $Met^{-}Hcy^{+}$ and/or the mechanisms underlying said ability (*e.g.*, this thesis, Mecham *et al.* [1983]), methionine dependence refers to lines that cannot proliferate in tissue culture medium where methionine is replaced with Hcy. In studies focused on exploring methionine restriction in cancer therapy, all cancer cell lines are considered methionine dependent as proliferation is always inhibited to some degree in Hcy (*e.g.*, Figure 5 shows SK-Mel-28 can be considered methionine independent while still doubling slightly fewer times in $Met^{+}Hcy^{-}$) (Hoffman, 2015). In the latter category, "methionine addiction" is usually used interchangeably with "methionine dependence" and all cancer cell lines are presumed to be methionine addicted since tumours require excess methionine to supplement the metabolic demand necessary to survival (Hoffman, 2015; Sedillo and Cryns, 2022). This may be why previously, only 47 commercially available cancer cell lines were definitively reported as methionine dependent, independent, or moderate dependent, because it is generally accepted that all cancer cell lines have altered methionine metabolism (Kaiser, 2020). This is supported by the original Mecham *et al.* (1984) study, where even cancer lines classified as methionine independent showed at least marginal inhibition in $Met^{-}Hcy^{+}$ (interestingly, MeWo is the

exception in that it was not inhibited at all in $Met^{-}Hcy^{+}$). It is reasonable to argue there should be a more clear distinction between the two terms: methionine addiction refer to the phenomenon of increased requirement of methionine observed in all cancer cells, while methionine dependence, moderate dependence, or independence refer to the specific ability of a cell line to proliferate in absence of methionine.

### 4.4 Methionine Metabolism in Cancer Treatment

Both tumours in danger of becoming malignant and those that are malignant warrant anticancer therapies that can target all tumours at once, which can be difficult given the variety seen among tumours overall, and the cellular variety within the tumours themselves (Vogt *et al.*, 2017). Even if treatment appears to be successful, some cancers can suddenly metastasize decades after a tumour has been apparently destroyed, so preferably anticancer treatments would be prolonged without causing harm to the patient (Hanahan & Weinberg, 2011). Prior to the discovery of methionine dependence, studies showed methionine-deficient or Hcy-replacing-methionine diets would extend survival and/or decrease metastases in tumour-bearing mice and rats (Hoffman, 2015). This was followed by use of recombinant L-methioninase (rMETase) – a bacterial methionine-degrading enzyme – to inhibit tumour growth in W256 rats (Hoffman, 2015). After methionine dependence was discovered, Stern and Hoffman (1986) investigated the efficacy of methionine deprivation when paired with chemotherapic agents by subjecting mixed cell cultures consisting of wild-type fibroblasts and one of several known methionine dependent or independent cancer cell lines to $Met^{-}Hcy^{+}$ and doxorubicin (DNA synthesis inhibitor) for ten days, then replacing with $Met^{+}Hcy^{-}$ and vincristine (mitotic spindle inhibitor), and successfully selectively killed the cancer cells. Kokkinakis *et al.* (2001) achieved similar success when pairing rMETase with chemotherapic agents in known methionine dependent cell lines.

The applications of methionine addiction exploitation are still being explored, and numerous reviews have been published on the history and overall success of its implication in cancer treatment (*e.g.*, Cavuoto & Fenech [2012], Cellarier *et al.* [2003], Chaturvedi *et al.* [2018], Hoffman [2015], Kaiser [2020], Sedillo and Cryns [2022]). The consensus is that methionine deficient diets in tandem with chemotherapies are effective (Cavuoto & Fenech, 2012; Cellarier *et al.*, 2003; Chaturvedi *et al.*, 2018; Sedillo & Cryns, 2022). While rMETase is not yet approved for use in treatment, it has been shown to be safe and effective *in vivo* in tumour-bearing mice, and in human tumour xenografts (Sedillo & Cryns, 2022). Currently, there are multiple clinical trials relating to cancer treatment through methionine restriction, which have been outlined by Sedillo and Cryns (2022). Of relevance to this thesis, is a series of clinical trials by Durando *et al.* (2007) and Thivat *et al.* (2009), which subjected patients with recurrent glioma and metastatic melanoma to a methionine-free diet (Cellarier *et al.*, 2003). Typically, chemotherapeutic agents only see a 20% size decrease in tumours when treating *in situ* melanoma and glioma, but the addition of a methionine-free diet was able to improve this response rate without increasing toxicity (Cellarier *et al.*, 2003; Durando *et al.*, 2007). When this was replicated in syngeneic mice, the methionine restriction led tumours to be more sensitive to chemotherapeutics such as cystemustine, resulting in increase in median survival (Thivat *et al.*, 2009). Phase I clinical trials found that optimal length of methionine restriction was one day, and Phase II found the one-day restriction paired with cystemustine treatment *in vivo* also produced amelioration in median survival and response rate (Thivat *et al.*, 2009).

Methionine restriction has been tested on some cell lines with known ability to proliferate in $Met^{+}Hcy^{-}$ and $Met^{-}Hcy^{+}$, namely PC-3, DU145, LNCaP, three prostate cancer lines described as methionine dependent, moderate dependent, and moderate dependent respectively, and MCF7,

a methionine dependent breast cancer line; however, the latter was tested for reasons unrelated to its ability to proliferate in $Met^{+}Hcy^{-}$ and $Met^{-}Hcy^{+}$ (Lamb *et al.,* 2015; Lu & Epner, 2000; Poirson-Bichat *et al.,* 1997). All cell lines showed inhibition of growth while subjected to a medium with restricted methionine, save for LNCaP which only was partially inhibited (Cellarier *et al.,* 2003; Lamb *et al.,* 2015; Lu & Epner, 2000; Poirson-Bichat *et al.,* 1997; Sorin *et al.,* 2021). It is unknown if in general, methionine dependent and/or moderate dependent cell lines are noticeably more sensitive to methionine restriction in tandem with chemotherapy, beyond that which has been explained by standard methionine addiction, especially considering the variable response between DU145 and LNCaP, two methionine moderate dependent cell lines (Lu & Epner, 2000). Similarly, it is unknown if in general, methionine independent cell lines are more resistant to methionine restriction in tandem with chemotherapy. If a greater effort is made to characterise the ability to proliferate in $Met^{+}Hcy^{-}$ and $Met^{-}Hcy^{+}$ in more cell lines (as discussed in section 4.2), it may lead to a better understanding on which metabolic alterations in cancers should be targeted by which therapies. Perhaps methionine restriction in tandem with chemotherapy will be reserved for patients whose tumour lines are methionine dependent, but this assumes that methionine dependence *in vitro* is indicative of methionine dependence *in situ,* and in fact there is evidence both for (*e.g.,* Guo *et al.* [1993]) and against (*e.g.,* Koziorowska *et al.* [1980]) the methionine dependence phenotype existing in tumours *in situ*.

**4.5 Current View of Methionine Dependence**

Methionine dependence in cancer has been studied for the past five decades, and although many cell lines have been found to be methionine dependent, there remains an absence of a comprehensive description of the underlying mechanisms. After the contrasting ability to proliferate in $Met^{+}Hcy^{-}$ and $Met^{-}Hcy^{+}$ was discovered in related melanoma cell lines MeWo and

MeWoLC1, Watkins (1998) found that the latter's inability was uniquely due to a *cblC* phenocopy (Liteplo, 1989, 1990). It was predicted that more cancer cell lines would be found to be methionine dependent due to defects in cobalamin metabolism, but this was not explored until Loewy *et al.* (2009) and Guéant *et al.* (2018) showed MeWoLC1's *cblC* phenocopy and certain patients with *cblC*-like phenotypes were induced by *MMACHC* hypermethylation. Sorin *et al.* (2021) found this hypermethylation was recurrent in melanomas as seen in sequencing databases, a resource that was unavailable during the first studies of MeWoLC1. The results of this thesis show that such described *MMACHC* hypermethylation does not always result in methionine dependence to the degree of, or cobalamin metabolic defects like, MeWoLC1. As such, another *cblC* phenocopy cancer line has yet to be found, and the reason for overrepresentation of melanomas among cancer cell lines with *MMACHC* hypermethylation remains unknown.

Methionine dependence is heterogenic, an observation with which the data described in this thesis corroborates. Because an "addiction" to methionine appears to be characteristic of all tumour lines, methionine dependence will likely only be investigated more frequently in the development of cancer treatments, especially with the advent of panomic databases like the CCLE. Simultaneously, more causative mechanisms of methionine dependence beyond increased transmethylation reactions, *MTAP* deletions, etc., will likely be elucidated.

# Chapter 5: Conclusions and Future Directions

## 5.1 Summary of Conclusions

The reason for the overrepresentation of melanoma cell lines among cancer lines with hypermethylation and decreased expression of *MMACHC* remains to be determined. *MMACHC* hypermethylation does not always result in methionine dependence as demonstrated by SK-Mel-28; however, this does not necessarily mean that the cell lines found to be methionine dependent are dependent because of hypermethylation of *MMACHC*. Similarly, *MMACHC* hypermethylation does not always result in cobalamin metabolic defects akin to those observed in MeWoLC1. The alternative hypothesis – that is, *MMACHC* hypermethylation in melanoma cell lines always confers methionine dependence through defects in cobalamin metabolism – can therefore be rejected.

## 5.2 Claims to Originality

The proliferative abilities in $Met^{+}Hcy^{-}$ vs. $Met^{-}Hcy^{+}$ of four melanoma cell lines were newly described. Colo829, HMCB, and SH-4 are methionine dependent. SK-Mel-28 is methionine independent.

The cobalamin metabolism of six melanoma cell lines has been elucidated through biochemical assays. Cobalamin distributions of A375, Colo829, HMCB, SK-Mel-28, and SH-4 have been newly described. The propionate incorporation and 5-methylTHF incorporation of MeWo, A375, Colo829, HMCB, SK-Mel-28, and SH-4 have been newly described.

The specificity of MeWoLC1's methionine dependence was upheld, in that six cancer cell lines (all melanomas) do not exhibit similar cobalamin metabolic profiles to that of MeWoLC1, albeit this was already confirmed in MeWo. This does not mean no cancer cell lines exist that are similar to MeWoLC1, only that if there are, they have not been identified.

### 5.3 Other Melanoma Cell Lines

Data from seven cell lines is not enough for conclusive evidence that *MMACHC* hypermethylation may or may not have a role in methionine (moderate) dependence. This section outlines some additional melanoma cell lines that may be considered in follow-up experiments. Additional factors that may contribute to a methionine (moderate) dependent phenotype are highlighted, in light of the unexpected results for SH-4 that was likely due to *MTAP* deletions (Figure 4, 5A, 6D).

Colo729 is the melanoma cell line with the highest *MMACHC* methylation and lowest MMACHC expression in the CCLE. However, it also has hypermethylation and hypoexpression of *MAT1A*.

WM983B and HS936T are the melanoma cell line with the second and third highest *MMACHC* methylation respectively in the CCLE. However, WM983B also has deletions in *MTAP* and *MSRA*, and both cell lines have extremely high expression of *PRDX1*.

MELHO is the melanoma cell line with the fourth highest *MMACHC* methylation in the CCLE. However, it has deletions of a variety of genes implicated in 1C metabolism.

A101D and LOXI-MVI have high methylation and low-to-moderate expression of MMACHC. However, they both have *MTAP* deletions. A101D was originally going to be studied in this thesis, but the cultures received by Cedarlane® were found to be contaminated.

WM2664 and Colo783 are the two melanoma cell lines with the highest MMACHC expression in the CCLE, and have low-to-moderate *MMACHC* methylation.

SK-Mel-1, WM793, and IGR39 have no methylation and normal expression of *MMACHC*. However, IGR39 is the melanoma cell line in the CCLE with the highest *MAT1A* methylation and lowest *MAT1A* expression.

### 5.4 Replicating the Methods of Loewy *et al.* (2009)

The availability of MeWo and MeWoLC1 presents an opportunity to evaluate *MMACHC* expression and methylation data of two closely related cell lines that exhibit differing metabolism in $Met^{-}Hcy^{+}$ medium, which may provide insight on the relationships among methylation, expression, and methionine dependence. Although comparing MeWo and MeWoLC1 in this regard would hardly produce a statistically significant conclusion, it may produce valuable preliminary data for future research.

It is unknown if the cell lines investigated in this thesis besides MeWo and MeWoLC1 have the AGC allele of *MMACHC* that according to Loewy *et al.* (2009) appears to be targeted for hypermethylation. Sequencing of *MMACHC* within A375, Colo829, HMCB, SH-4 and SK-Mel-28 may reveal that like MeWo, the cell lines only have one AGC allele, which is likely hypermethylated. This may also provide insight into how MeWoLC1 acquired the secondary hypermethylation necessary to induce a cobalamin metabolic defect.

Loewy *et al.* (2009) confirmed the absence of MMACHC due to hypermethylation as the cause of methionine dependence in MeWoLC1, by transfecting wild-type MMACHC into MeWoLC1 cells, and correcting the phenotype. While replication of this procedure was originally planned for any cell lines that were newly described in this thesis as methionine dependent, this was unfortunately not possible given the restrictions imposed by the COVID-19 pandemic. If any of the other melanoma cell lines in this thesis can be rescued by wild-type MMACHC transfection, this would definitively indicate that (1) epigenetic defects of *MMACHC* are involved in the cell lines' inability to remethylate methionine from Hcy, and (2) the cobalamin distributions, and propionate and 5-methylTHF incorporation of MeWoLC1 are caused by other factors in addition to *MMACHC* hypermethylation.

### 5.5 Methionine Independent Revertants

In rare cases, it has been observed that a methionine dependent cell line naturally begins to show signs of methionine independence, and more commonly, methionine dependent cell lines can be artificially selected to become methionine independent (Borrego *et al.*, 2019; Kaiser, 2020). These cell lines are called methionine independent revertants. Although not the first instance observed, Liteplo (1990) generated revertant cell lines from MeWoLC1 cultures to test a hypothesis that altered methionine metabolism contributed to progression of benign tumours to malignant. Another example of revertant incidence is that of the methionine dependent GaMg-P60 cell line while being cultured during experiments by Fiskerstrand *et al.* (1994). Their GaMg line originally began as absolutely methionine independent and became more dependent as the cancer progressed, leading to designation of a new line name: GaMg-P60. Fiskerstrand *et al.* (1994) then repeatedly exposed GaMg-P60 to 5-aza-2'-deoxycytidine – a compound known to reverse methylation (Jones & Baylin, 2002) and be toxic to methionine dependent tumours – and cultured the surviving cells until achieving a new independent line, called GaMg-P60R. Using a similar method without 5-aza-2'-deoxycytidine, Borrego *et al.* (2019) have developed a procedure to select and grow revertant daughter cells in methionine dependent cultures, thereby creating revertant cultures. Borrego *et al.* (2019)'s methods do not directly cause reversion, but rather provide an environment where the cells must become independent again to survive (Kaiser, 2020).

Revertants do share properties with non-revertant methionine independent lines. Of course, cell lines regain ability to grow in $Met^{-}Hcy^{+}$ medium, but in addition transmethylation rate appears to decrease (*i.e.*, SAM:SAH ratios increase) in those whose methionine dependent parent lines showed abnormal increases in transmethylation rate (Hoffman, 2015). Most interesting

however, is the reversion to a less malignant phenotype. Revertant cell lines may show increased anchorage dependence (can only grow when attached to a surface) in culture, suggesting that if transfected to *in vivo* models, they would not readily metastasise (Hoffman, 2015). Furthermore, cell morphology and density may return – or be similar – to those of regular tissues, and cultures may require more fetal calf serum for optimal growth, suggesting that many altered metabolic programs from malignancy are restored (Hoffman, 2015; Sorin *et al.,* 2021). Many of these changes happen without a change in MTR activity, supporting the belief that MTR has little to do with dependence. In contrast, some cell lines have shown increases (upwards of 31%) in MTR activity upon reversion, supporting that some dependencies are due to changes in MTR (Cellarier *et al.,* 2003). Again, reversion is likely heterogenic, and the mechanisms of the reversion itself are probably dependent on those that caused the metabolic alterations which led to methionine dependence in the first place.

If necessary, independent revertants may be generated from cell lines deemed methionine dependent using the protocol described by Borrego *et al.* (2019). As an alternative to wild-type MMACHC transfection, revertants could be generated from the three newly described methionine dependent cell lines in this thesis. Following this, RRBS of the transcription start sites and RNA-Seq (in accordance with CCLE protocol) of *MMACHC* in these revertant lines may show a reduction and/or increase in methylation and/or expression respectively or altered cobalamin metabolism. The absence of comparative methylation and expression profiles for *MMACHC* as well as other genes among methionine dependent cancer cell lines and their revertants presents an opportunity which may further elucidate the development of methionine dependence.

### 5.6 Relationship between Melanoma and Glioma

Gliomas are the most common (77%) primary tumours of the central nervous system in adults (Alfonso *et al.,* 2017; Schwartzbaum *et al.,* 2006). They originate from the four types of glial/supporting cells (oligodendrocytes, astrocytes, ependymal cells, and microglia) of the central nervous system, and cause 7% of all cancer deaths despite comprising only 2% of all cancer incidences (Davis, 2018). There are numerous types of gliomas, summarisations and lists of which can be found in the reviews by Ostram *et al.* (2014) and Louis *et al.* (2016) (Davis, 2018; Schwartzbaum *et al.,* 2006). Glioma case rates are highly variable across the world, with a fourfold increase in countries with predominantly populations of European descent compared to those without, although this may be due to socioeconomic factors like increases in detection from better healthcare (Davis, 2018; Ostram *et al.,* 2014, 2017; Schwartzbaum *et al.,* 2006). Regardless of region, gliomas tend to be diagnosed at later ages (median age is 59), apart from pilocytic astrocytomas occurring more commonly in children, and overall have a higher incidence in males (Davis, 2018; Schwartzbaum *et al.,* 2006), Over 95% of gliomas arise from environmental factors, the only firmly established of which is high doses of ionising radiation, usually therapeutic (Davis, 2018; Schwartzbaum *et al.,* 2006). Studies elucidating any underlying genetic factors that may affect one's degree of risk to environmentally induced glioma carcinogenesis, have been limited to children (Schwartzbaum *et al.,* 2006). Gliomas stemming purely from genetic factors (*e.g.*, Li-Fraumeni syndrome) are rare, but if found, behave as autosomal dominant genotypes in tumour suppressor genes (Davis, 2018). According to genome-wide association studies, genetic factors which affect environmental susceptibility are usually polygenic, as in the collective effect of many genes, as opposed to Mendelian disorders (single genes) with high penetrance (Ostrom *et al.,* 2014). The genes implicated in the polygenic

phenotypes include those for DNA repair and more interestingly, folate metabolism (Ostram *et al.,* 2014).

Recent research suggests there may be underlying factors that link melanomas and gliomas. As discussed in section 4.4, melanoma and glioma also seem to be similarly affected by methionine restriction (Cellarier *et al.,* 2003; Durando *et al.,* 2007; Thivat *et al.,* 2009). Melanoma incidence is more frequent than expected in patients with familial history of glioma, and patients who have had prior incidence of glioma appear prone to developing melanoma later in life (Endicott *et al.,* 2016; Scarbrough *et al.,* 2014). Environmental stressors which cause melanoma and glioma are mutually exclusive, and there is no evidence that treatment for glioma increases melanoma risk and vice versa (Scarbrough *et al.,* 2014). Both cancers arise from tissues of neuroectodermal origin and can become rapidly progressing malignancies (Endicott *et al.,* 2016). Furthermore, both cancers are seen in patients with melanoma-astrocytoma syndrome, demonstrating existence of shared etiology; however, this does not fully account for the general observed frequency of high co-occurrence of melanoma and glioma (Endicott *et al.,* 2016). Both melanoma and glioma risk are increased in those with certain alleles of the *TERT* gene, which codes for the protein subunit of telomerase (EC 2.7.7.49), an enzyme that elongates and manages telomeric DNA (Davis, 2018; Endicott *et al.,* 2016; Gray-Schopfer *et al.,* 2007; Hanahan & Weinberg 2011). Both genome-wide association studies and Mendelian randomization analyses through measurement of leukocyte telomere lengths support that longer telomeres increase risk of melanoma and glioma (Endicott *et al.,* 2016). *TERT* mutations are present in ~75% of glioblastomas and oligodendrogliomas, and its promoter is often mutated in the early stages of malignant melanoma (Davis, 2018; Gray-Schopfer *et al.,* 2007).

Sorin *et al.*'s (2021) review highlighted the disproportionately high incidence of hypermethylated and hypoexpressed *MMACHC* in melanomas, but also showed that a subset of gliomas shared this phenomenon (Figure 2 [LGG], 3C [CENTRAL_NERVOUS_SYSTEM]). Indeed, the 2nd-most common CCLE lines above the 90th percentile of *MMACHC* $I_{m/e}$ are gliomas (*e.g.,* A172 is a known moderate dependent glioma above the 83rd percentile of *MMACHC* $I_{m/e}$; Kaiser, 2020). One notable example is GaMg-P60, a GaMg subculture which had gone through 60 passages Fiskerstrand *et al.* (1994). According to Fiskerstrand *et al.* (1994), GaMg and GaMg-P60 shared a similar case to that of MeWo and MeWoLC1 respectively, in that the daughter/subculture line was methionine dependent and exhibited a cobalamin metabolic defect (Watkins, 1998). CCLE reports both low MMACHC expression (0.58) and *MMACHC* methylation (0.068) for GaMg, a similar profile to that of MeWo. Although the proliferative ability of GaMg in $Met^{-}Hcy^{+}$ has not been demonstrated in a way that allows it to be implicated in Kaiser's (2020) list of cell lines with known methionine metabolic properties, it is highly likely it is methionine independent based on data from Fiskerstrand *et al.* (1994) (it would be reasonable to add GaMg to Kaiser's [2020] list, bringing the total cell lines to 52). Furthermore, GaMg-P60 has a methionine independent revertant subculture, GaMg-P60R (see 5.5; Fiskerstrand *et al.,* 1994). Unfortunately, GaMg-P60 and GaMg-P60R have both been discontinued since the late 1990's (Fiskerstrand personal communication). Provided that the hypermethylation of *MMACHC* is found to induce methionine dependence regardless of cobalamin metabolic defects, investigating gliomas with abnormal *MMACHC* DNA methylation and mRNA expression may provide insight into a possible link between the developments of the two cancers.

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# Chapter 7: Appendices

## 7.1 Appendix A – Genes (including *MMACHC*) discussed in this thesis.

| HUGO Symbol | Name of Product |
|---|---|
| *ABCD4* | ATP binding cassette subfamily D member 4 |
| *AHCY** | adenosylhomocysteinase |
| *AMD1** | adenosylmethionine decarboxylase 1 |
| *APIP* | APAF1 interacting protein |
| *ASMT* | acetylserotonin O-methyltransferase |
| *ASMTL* | acetylserotonin O-methyltransferase like |
| *BCKDHA* | branched chain keto acid dehydrogenase E1 subunit alpha |
| *BHMT** | betaine--homocysteine S-methyltransferase |
| *BHMT2** | betaine--homocysteine S-methyltransferase 2 |
| *BRAF* | B-Raf proto-oncogene |
| *CARM1* | coactivator associated arginine methyltransferase 1 |
| *CBS* | cystathionine beta-synthase |
| *CD320* | transcobalamin receptor |
| *CDKN2A* | cyclin dependent kinase inhibitor 2A |
| *COQ5* | coenzyme Q5 |
| *CTH* | cystathionine gamma-lyase |
| *DHFR* | dihydrofolate reductase |
| *DNMT1** | DNA methyltransferase 1 |
| *DNMT3A** | DNA methyltransferase 3 alpha |
| *DNMT3B* | DNA methyltransferase 3 beta |
| *DNMT3L* | DNA methyltransferase 3 like |
| *DPH5* | diphthamide biosynthesis 5 |
| *ENOPH1** | enolase-phosphatase 1 |
| *FTCD* | formimidoyltransferase cyclodeaminase |
| *GART* | phosphoribosylglycinamide formyltransferase |
| *GNMT* | glycine N-methyltransferase |
| *HCFC1* | host cell factor C1 |
| *LCMT1* | leucine carboxyl methyltransferase 1 |
| *LCMT2* | leucine carboxyl methyltransferase 2 |
| *LMBRD1* | LMBR1 domain containing 1 |
| *MAPK* | mitogen-activated protein kinase |
| *MAT1A* (MAT)* | methionine adenosyltransferase 1A |
| *MAT2A* (MAT)* | methionine adenosyltransferase 2A |
| *MAT2B* (MAT)* | methionine adenosyltransferase 2B |
| *MDM2* | E3 ubiquitin-protein ligase mouse double minute 2 homolog |
| *MDM4* | mouse double minute 4 regulator of p53 |
| *MEK* | MAPK kinase |

| | |
|---|---|
| *MITF* | melanocyte inducing transcription factor |
| *MMAA* | metabolism of cobalamin associated A |
| *MMAB* | metabolism of cobalamin associated B |
| *MMACHC* | metabolism of cobalamin associated C |
| *MMADHC* | metabolism of cobalamin associated D |
| *MRI1** | methylthioribose-1-phosphate isomerase 1 |
| *MSRA* | methionine sulfoxide reductase A |
| *MSRB2** | methionine sulfoxide reductase B2 |
| *MSRB3** | methionine sulfoxide reductase B3 |
| *MTAP** | methylthioadenosine phosphorylase |
| *MTHFD1* | methylenetetrahydrofolate dehydrogenase 1 |
| *MTHFR* | methylenetetrahydrofolate reductase |
| *MTR** | methionine synthase |
| *MTRR** | methionine synthase reductase |
| *MMUT* | methylmalonyl-CoA mutase |
| *NF1* | neurofibromin 1 |
| *NRAS* | NRAS proto-oncogene, GTPase |
| *NRAS* | neuroblastoma RAS viral oncogene homolog |
| *NSD2* | nuclear receptor binding SET domain protein 2 |
| *PCMTD2* | protein-L-isoaspartate(D-aspartate)O-methyltransferase domain containing 2 |
| *PEMT* | phosphatidylethanolamine N-methyltransferase |
| *PI3K* | phosphoinositide 3-kinase |
| *PIK3CA* | phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha |
| *PNMT* | phenylethanolamine N-methyltransferase |
| *PRDX1* | peroxiredoxin 1 |
| *SAT1** | spermidine/spermine N1-acetyltransferase 1 |
| *SAT2** | spermidine/spermine N1-acetyltransferase family member 2 |
| *SHMT1* | serine hydroxymethyltransferase 1 |
| *SHMT2* | serine hydroxymethyltransferase 2 |
| *SLC7A11* | solute carrier family 7 member 11 |
| *SMS** | spermine synthase |
| *SRM** | spermidine synthase |
| *TERT* | telomerase reverse transcriptase |
| *TESK2* | testis associated actin remodelling kinase 2 |
| *THAP11* | ronin |
| *TP53* | tumour protein p53 |
| *ZNF143* | zinc finger protein 143 |

*) Described in Cavuoto and Fenech (2012) as likely to confer methionine dependence if mutated.

## 7.2 Appendix B – Online links to databases used in this thesis.

DNA Methylation:

https://depmap.org/portal/download/all/?releasename=Methylation+%28RRBS%29andfilename=CCLE_RRBS_TSS_CpG_clusters_20180614.txt

mRNA Expression:

https://depmap.org/portal/download/all/?releasename=CCLE+2019andfilename=CCLE_RNAseq_rsem_genes_tpm_20180929.txt.gz

Mutations:

https://depmap.org/portal/download/all/?releasename=DepMap+Public+22Q2andfilename=CCLE_mutations.csv

Copy Number Variation:

https://depmap.org/portal/download/all/?releasename=DNA+Copy+Numberandfilename=CCLE_copynumber_byGene_2013-12-03.txt

Cell Line Identification:

https://depmap.org/portal/download/all/?releasename=DepMap+Public+18Q3andfilename=DepMap-2018q3-celllines.csv

Gene Symbol Identification:

https://www.genenames.org/download/custom/

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