Université de Toulouse

# THÈSE

**En vue de l'obtention du**

## DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE

**Délivré par :**
Université Toulouse III Paul Sabatier (UT3 Paul Sabatier)

**Discipline ou spécialité :**

Cancérologie

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**Présentée et soutenue par :**
Sarah J. Scotland

**le :** mardi 9 juillet 2013

**Titre :**

Manipulation du métabolisme énergétique dans les leucémies aiguës myéloïdes: Mitochondrie, apoptose et mécanisme d'action de la metformine

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**Ecole doctorale :**
Biologie, Santé, Biotechnologies (BSB)

**Unité de recherche :**
INSERM U1037

**Directeur(s) de Thèse :**
Pr. Christian Récher
Dr. Jean-Emmanuel Sarry

**Rapporteurs :**

Dr. Frédéric Bost
Dr. Rodrigue Rossignol

**Membre(s) du jury :**

Dr. Martin Carroll
Pr. Louis Casteilla
Pr. Thierry Levade
Dr. Laurent Le Cam

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*For my Mom*

Your everlasting strength and sheer determination, even in the face of terrible odds, inspired me to join this field and keeps me fighting every day for a cure.

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# Abbreviations

## A

AA – Antimycin A
abn – Abnormal
ACC – Acetyl-CoA carboxylase
ACLY – ATP citrate lyase
ADP – Adenosine diphosphate
AIF – Apoptosis-inducing factor
AKT – Protein Kinase B
ALAT – Alanine aminotransferase
ALDO-A – Aldolase A
ALL – Acute lymphoid leukemia
AML – Acute myeloid leukemia
AMPK – AMP-kinase
AMP – Adenosine monophosphate
AnV– AnnexinV APC
APL – Acute promyelocytic leukemia
AraC – Cytosine arabinoside
ARNT – Aryl hydrocarbon receptor nuclear translocator
ASCT2 – Anti-neutrak amino acid transporter
ASK1 – Apoptosis signal-regulating kinase 1
ATM – Ataxia telangiectasia mutated
ATP – Adenosine triphosphate
ATRA – All trans-retinoic acid
APL – Acute promyelocytic leukemia

## B

BAALC – Brain and acute leukemia, cytoplasmic
Bad – Bcl-xL/Bcl-2 associated death promoter
Bak – Bcl-2 homologous antagonist/killer
Bax – Bcl-2-associated x protein
BEZA – Bezafibrate
Bcl-2 – B cell leukemia-2
Bcl-xL – B cell leukemia-x long
BID – BH3 interacting-domain death agonist
BIM – Bcl-2 interacting mediator
BM – Bone marrow
BSO – L-buthionine-S,R-sulfoximine

## C

CAAT – Controlled amino acid therapy
CAD – Carbamoyl-phosphate synthetase
CAIX – Carbonic anhydrase IX
CaMKKβ – $Ca^{2+}$/calmodulin-dependent protein kinase kinaseβ
cAMP – cyclic adenosine monophosphate
CaMK – Calcium/calmodulin-dependent kinase
CAR – Coxsackievirus and adenovirus receptor
CAT – Carnitine acyl transferase
CBP – Core-binding factor or CREBP-binding protein
CDK – Cyclin-dependent kinase
CDP – Cytidine diphosphate
cFLIP – Cellular FLICE (Caspase-8) inhibitory protein
Chk2 – Checkpoint kinase 2
Chmp4c – Chromatin modifying protein 4C
ChREBP – Carbohydrate response element binding protein
CI – Confidence interval
CML – Chronic myeloid leukemia
CMP – Cytidine monophosphate
CN – cytogenically normal
CoA-SH – Coenzyme A with sulfhydryl functional group
COX10 – homolog, cytochrome c oxidase 10
$CO_2$ – Carbon dioxide
CPT1 – Carnitine acyl transferase I
CREB – cyclic AMP response element binding protein
CRTC2 – CREB-regulated
CR – Complete Remission
CSF – Colony stimulating factor
CS – Citrate synthase
CTP – Cytidine triphosphate
CVAD – cyclophosphamide, vincristine, adriamycin (doxorubicin), dexamethasone
C/EBPα – CAAT enhancer biding protein α
c-Myc – Avian myelocytomatosis virus oncogene cellular homolog

## D

DAPK-1 – Death-associated protein kinase 1
DCA – Dichloroacetate
DDR – DNA damage response
DEK-NVP –
del – deleted
DHAP – Dihydroxyacetone phosphate
DHEA – Dehydroepiandroesterone
DNA – Deoxyribonucleic acid
DNMT3A – DNA methyltransferase 3A
DON – 6-diazo-5-oro-L-norleucine
DRAM – Damage-regulated autophagy modulator
DXG – 2-Deoxyglucose

## E

EIF4E – Eukaryotic translation initiation factor 4E
ELK1 – ETS domain-containing like kinase
ENO2 – Enolase 2
EPO – Erythropoietin
ERG – Low v-etc erythroblastosis virus E26 oncogene homolog
ERK – Extracellular signal-regulated kinase
ESF1 – Nucleolar pre-rRNA processing protein
ETC – Electron transport chain
ETO – Etomoxir
EVI1 – Ecotropic virus integration site-1

## F

FAB – French-American-British
FAO – Fatty acid β-oxidation
FAS – Fatty acid synthesis
FASN – Fatty acid synthase
FBP – Fructose 2,6-bisphosphate
FBPase – Fructose 2,6-bisphosphatase
FBS – Fetal bovine serum
FCCP – Carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone
FGP – Fetal-type glycogen phosphorylase
FH – Fumarate hydrogenase
FIH – Factor inhibiting HIF-1
FLT3 – FMS-like tyrosine kinase 3
FOXO3 – Forkhead box protein O3
FYVE – Fab1, Yotb, Vac1, EEA1
F-1,6-BP – Fructose 1,6-bisphosphate

## G

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase
GA3P (or G3P) – Glyceraldehyde-3-phosphate
GCL – Glutathione cysteine ligase
GDH – Glutamate dehydrogenase
GDP – Guanosine-diphosphate
GLDC – Glycine decarboxylase
GLUT1/4 – Glucose transporter 1/4
GLS1/2 – Glutaminase 1 or 2
GLY-3P – 1,3-phosphodiglycerate
GMP – Guanosine monophosphate
GM – Granulocyte-macrophage
GOT1/2 – Glutamate oxaloacetate transaminase
GPAT – Glycerol phosphate acyl transferase
GPCR – G protein-coupled receptor
GPI – Glycophosphatidylinositol
GSH – Glutathione
GSL – Glycosphingolipid
GS – Glutamine synthetase
GTP – Guanosine-triphosphate
G – Granulocyte
G1P – Glucose 1 phosphate
G6PDH – Glucose 6 phosphate dehydrogenase
G6P – Glucose 6 phosphate

## H

HAT – Histone acetyltransferase
HBSS – Hank's balanced salt solution
HDAC – Histone deacetylation
Her2 – Human epidermal growth factor receptor 2
HIF-1α – Hypoxia-Inducible Factor 1α
HK-I/II – Hexokinase I/II
hMC – 5-hydroxymethylcytosine
HMGR – 3-hydroxy-3-methylglutaryl CoA reductase
HNF4 – Hepatocyte nuclear factor 4
HRE – Hypoxia response element
HR – Hazard risk
HSC – Hematopoietic stem cell
HSL – Hormone-sensitive lipoase
HSP70 – Heat shock protein 70kDa
hTERT – Human telomerase reverse transcriptase

## I

IDH1/2 – Isocitrate dehydrogenase 1 and 2
IF – Immunofluorescence
IMP – Inosine monophosphate
IODO - Iodoacetate
inv – inversion
IP - intraperitoneal
IRS-1/IR – Insulin receptor substrate 1 / insulin receptor
ITD – Internal tandem duplication

## K

KLS – c-Kit positive, Lineage negative, Sca-1 positive
Kit – v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog

## L

LC3 – Protein light chain 3
LDHA – Lactate dehydrogenase A
Lin - Lineage
LKB1 – Liver kinase B1
LND – LT-HSC – Long-term hematopoietic stem cell
L-NAME – NG-Nitro6L-arginine methyl ester

## M

Man6P – Mannose 6 dephosphorylation of lysosomal protein
Mcl-1 – Myeloid cell leukemia sequence 1
MCT1/4 – Monocarboxylate transporter 1/4
MDH – Malate dehydrogenase
mdm2 – Mouse double minute 2 homolog
MEK – Mitogen-activated protein kinase kinase
MET – Metformin
ME – Malic enzyme
mir-23a/b – Micro RNA 23a/b
MLLT3 – Mixed lineage leukemia, translocated to 3
MLL – Mixed lineage leukemia
mLST8 – 6 mTOR associated protein, LST8 homolog
MMP – Mitochondrial membrane permeabilization
Mo25 – Drosophila melanogaster
MSC – Mesenchymal stem cell
mtDNA – Mitochondrial DNA
mTORc1/2 – Mammalian target of rapamycin complex ½

## N

NAD/NADH – Nicotinamide adenine dinucleotide
NADP/NADPH – Nicotinamide adenine dinucleotide phosphate
ND4 – NADH dehydrogenase subunit 4
NFKβ - Nuclear factor kappa B
NF1 – Neurofibromin 1
NHE1 – Na/H exchanger 1
NNK – Nicotine-derived nitrosamine ketone
NOD-SCID – Severe combined immunodeficiency
NOXA – Phorbol-12-myristate-13-acetate-induced protein 1
NPM1 – Nucleophosmin 1
NSG – NOD scid gamma

## O

OCR – Oxygen consumption rate
OCT1 – October 1
ODDD – Oxygen-dependent degradation domain
OH - hydroxylation
OLIGO – Oligomycin
ORLI – Orlistat
OR – Odds ratio
OS – Overall Survival
OXPHOS – Oxidative phosphorylation
OXY – Oxythiamine

## P

PARP – Poly ADP-Ribose Polymerase
PBS – Phosphate-buffered saline
PCK2 – Phosphoenolpyruvate carboxykinase 2
PDH-E1α – Pyruvate dehydrogenase, E1α subunit
PDK1 – Phosphoinositide-dependent kinase 1
PED/PEA15 – Phosphoprotein enriched in astrocytes 15
PET – Positron emission tomography
PFKFB – 6-phosphofructo-2 kinase/fructose-2,6-bisphosphate
PFK1/2 – Phosphofructokinase 1/2
PGAM1 – Phosphoglycerate Mutase 1
PGC-1α – PPARγ coactivator 1 alpha
PGK1 – Phosphoglycerate kinase 1
PHD – Prolyl hydroxylase
PHGDH – Phosphoglycerate dehydrogenase
PIP3 – Phosphatidylinositol (3,4,5)-trisphosphate
PI3K – Phosphoinositide 3-kinase
PKA – Protein kinase A
PH – Pleckstrin homology
PKM2 – Pyruvate kinase Type M2
PML-RARα – Promyelocytic leukemia protein-retinoic acid receptor α
PPARα - Peroxisome proliferator-activated receptor alpha
PPP – Pentose phosphate pathway
PP242 – [2-(4-Amino-1-isopropyl-1H-pyrazolo[3,4-d]pyrimidin-3-yl)-1H-indol-5-ol]
pRB – Retinoblastoma protein
PRAS40 – Proline rich Akt substrate of 40 kDa
PRPP – Phosphoribosyl pyrophosphate
PSAT1 – Phosphoserine-aminotransferase 1
PTD – Partial tandem duplicate
PTEN – Phosphatase and tensin homolog protein
PUMA – p53-modulated upregulator of apoptosis
p53 – Protein 53

## R

Raf – Rapidly accelerated fibrosarcoma
Rap/Rapa - Rapamycin
Ras – Rat sarcoma
REDD1 (DDIT4/DIG2/RTP801)
RHEB – Ras homolog enriched brain
Ribu5P – Ribulose-5-phsophate
RNAi – RNA interference
ROS – Reactive oxygen species
ROT – Rotenone
RPN1 – Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 1
RP-HPLC – Reversed-phase high-performance liquid chromatography
RR – Risk ratio
Rsk – Ribosoma S6 kinase
RTK – Receptor tyrosine kinase
RUNX-1 – Runt-related transcription factor 1
R1P – Ribulose-1-phosphate
R5P – Ribulose-5-phosphate

## S

SASP – Senescence-associated secretory phenotype
Sca-1 – Stem cell antigen-1
SCD – Stearoyl-CoA desaturase
SCF – Stem cell factor
SCO2 – synthesis of cytochrome c oxidase 2
SCT – Stem cell transplant
SDF-1 – Stromal cell-derived factor 1
SDH – Succinate dehydrogenase
SDHD – Succinate dehydrogenase complex subunit D
SED7P – Sedoheptulose 7-phosphate
Ser – Serine
shRNA – Short hairpin RNA
siRNA – Small interfering RNA
SIRT1/3 – Sirtuin 1 or 3
SLAM – Signaling lymphocyte activation molecule
SLC1A5 – Solute carrier family 1 member 5
SLC7A5 – Solute carrier family 7 member 5
SPT – Serine palmitoyltransferase
STZ – Streptozotocin
SU – Sulphonylureas
S6 – Ribosomal protein S6

## T

TAK1 – TGFβ-activated kinase-1
TCA – Tricarboxylic Acid
TDP – Thiamine diphosphate
TFAM – Transcription factor A, mitochondrial
TGF-β1 – Transforming growth factor B1
Thr – Threonine
TIGAR – TP53-induced glycolysis and apoptosis regulator
TKI – Tyrosine kinase inhibitor
TKT/TKTL1 – Transketolase 1
TMP – Thymidine monophosphate
TNF-α – Tumor necrosis factor-alpha
TN – Triple-negative
TPI1 – Triosephosphate isomerase
TPO – Thrombopoietin
TSC1/2 – Tuberous sclerosis complex protein 1 or 2
Tyr – Tyrosine

## U

Ub - Ubiquitin
UDP – Uridine diphosphate
ULK1 – Serine/threonone-protein kianse 1
UMP – Uridine monophosphate
UTP – Uridine triphosphate
UTR – untranslated region
UVRAG – UV radiation resistance-associated gene protein
UCP2 – uncoupling protein 2
UPR – Unfolded protein response

## V

VDAC – Voltage-dependent anion channel
VLACD – Very long-chain Acetyl-CoA Synthetase
VHL – von Hippel-Lindau
VPS34 – Class III PI 3-kinase
V-ATPase – Vacuolar-type H+-ATPase

## W

WHO – World Health Organization
WTI – Wilms tumor 1

## X

XBP-1 – Xbox Binding Protein 1
Xylu5P – Xyulose-5-phosphate

## Numbered

α-KG – Alpha-ketoglutarate
1-3,BPGA (or 1,3PGA) – 1,3-Biphosphoglycerate
2DG – 2-deoxyglucose
2-HG – 2-hydroxyglutarate
2-PGA (or 2PG) – 2-phosphoglycerate
3BrPa – 3-bromopyruvate
3-PGA (or 3PG) – 3-phosphoglycerate
4E-BP1 – Eukaryotic translation initiation factor 4E binding protein
5mC – methylcytosine
6-ANA – 6-aminonicotinamide
7AAD – 7 Amino-actinomycin D

# – Introduction –

# I Introduction

## 1 Normal hematopoiesis

Hematopoiesis is the hierarchical process of blood cell formation, which is initiated from a pool of hematopoietic stem cells (HSC) located in the bone marrow (BM) (Figure 1). HSCs constitute a rare population, averaging only 1 in 10,000 to 1,000,000 cells in BM. Although HSCs are normally quiescent, they can also re-enter the cell cycle and proliferate in order to produce blood cells. This is strictly controlled by numerous growth factors, which are also listed in Figure 1.

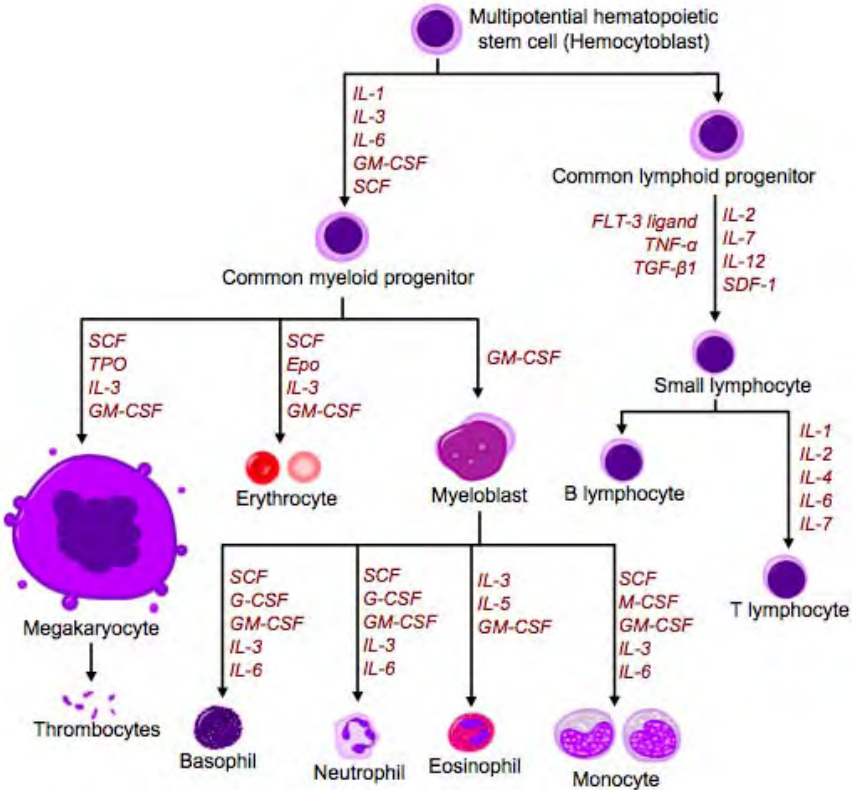



**Figure 1. Overview of normal hematopoiesis.** Normal hematopoiesis is the hierarchical process of blood cell formation, which is initiated from a pool of multipotential hematopoietic stem cells (HSC). HSCs branch into either common myeloid or lymphoid progenitors. Myeloid progenitors will then produce a range of blood cells, including megakaryocytes, erythrocytes, and myeloblasts, which further branch into basophils, neutrophils, eosinophils and monocytes. Lymphoid progenitors, on the other hand, produce B and T lymphocytes. At each step in this process, different growth factors signal the type of differentiation to occur.

HSCs are thus characterized by the ability to self-renew and differentiate into mature and functional blood cells of both myeloid and lymphoid lineages. As such, HSCs give long-term reconstitution of the hematopoietic system, as evidenced by reconstitution after myeloablative irradiation (Weissman 2000). Additionally, physical separation methods have shown that even within the HSC population, there are two main sub-populations, one of which consists of cells with long-term repopulating ability, which are responsible for maintaining sustained multi-lineage repopulation, and a second

consisting of cells with only transient, short-term, repopulating abilities (Morrison and Weissman 1994; Morrison et al. 1997).

HSCs can be further characterized by cell surface makers using immunophenotyping (Figure 2). Mouse BM cells with HSC activities are characterized as positive for stem cell factor c-kit and Sca-1 and negative for all the lineage specific antigens (lin-), collectively referred to as KLS cells. A single murine BM KLS cell can reconstitute donor hematopoiesis in lethally irradiated mice (Osawa et al. 1996). Within the KLS population, the most primitive long-term self-renewing HSCs (LT-HSCs) are negative or express very low levels of Thy1.1 ($Thy1.1^{-/lo}$), fms-like tyrosine kinase 3 ($Flt3^{-/lo}$) and CD34 ($CD34^{-/lo}$) (Morrison and Weissman 1994; Osawa et al. 1996; Goodell et al. 1997; Adolfsson et al. 2001). On the other hand, KLS cells, which are $Thy1.1^{-/lo}CD34^{+}Flt3^{+}$ are only capable of short-term hematopoietic reconstitution (ST-HSCs) (Morrison and Weissman 1994; Osawa et al. 1996; Adolfsson et al. 2001; Weissman et al. 2001). More recently, the KLS $Thy1.1^{-/lo}$ population was further sub-fractionated by the integrin-α2 (CD49B) surface marker, as only the integrin-α2 population supports long-term hematopoietic reconstitution (Wagers and Weissman 2006). In addition, the cell surface receptors of the signaling lymphocyte activation molecule (SLAM) family have also been used to characterize murine HSCs (Kiel et al. 2005; Yilmaz et al. 2006a). In particular, murine BM $CD150^{+}CD244^{-}CD48^{-}$ cells are highly enriched with HSCs, whereas the $CD150^{-}CD244^{+}CD48^{-}$ cells are more enriched with downstream progenitors (Kiel et al. 2005; Yilmaz et al. 2006a).

On the other hand, the surface phenotypes of human HSCs have not been fully elucidated. A group of hematopoietic cells, which can provide both myeloid and lymphoid lineages, are defined by the expression of the transmembrane glycoprotein CD34 (Ghosh et al. 1996). This suggests CD34 might be a marker for activated stem cells. Early studies in the 1980's showed that in baboons, autologous $CD34^{+}$ enriched BM cells could engraft lethally irradiated animals, whereas recipients of $CD34^{-}$ cells did not engraft and developed prolonged cytopenia (Berenson et al. 1988), confirming the presence of HSCs in the $CD34^{+}$ population. HSCs can then be further purified by CD38 or lineage marker expression (Bhatia et al. 1997). More stringent phenotyping also includes $CD117^{+}$ (Wognum et al. 1996) and $CD90^{+}$ (Baum et al. 1992). This subset, however, contains not only HSCs, but also heterogeneous groups of progenitors and committed cells that are different in functions and phenotypes. Alternative surface phenotypes, such as the transmembrane glycoprotein CD133 (Yin et al. 1997), are also used to purify human HSCs to high homogeneity. Finally, human LT-HSCs were differentiated from the more downstream multipotent progenitors (MPP) using the combination of CD90 and CD45RA (Majeti et al. 2007). LT-HSC activities were identified in $CD34^{+}CD38^{-}Lin^{-}CD90^{+}CD45RA^{-}$ cells of human umbilical cord blood and the $CD34^{+}CD38^{-}Lin^{-}CD90^{-}CD45RA^{-}$ fraction is enriched with MPPs.

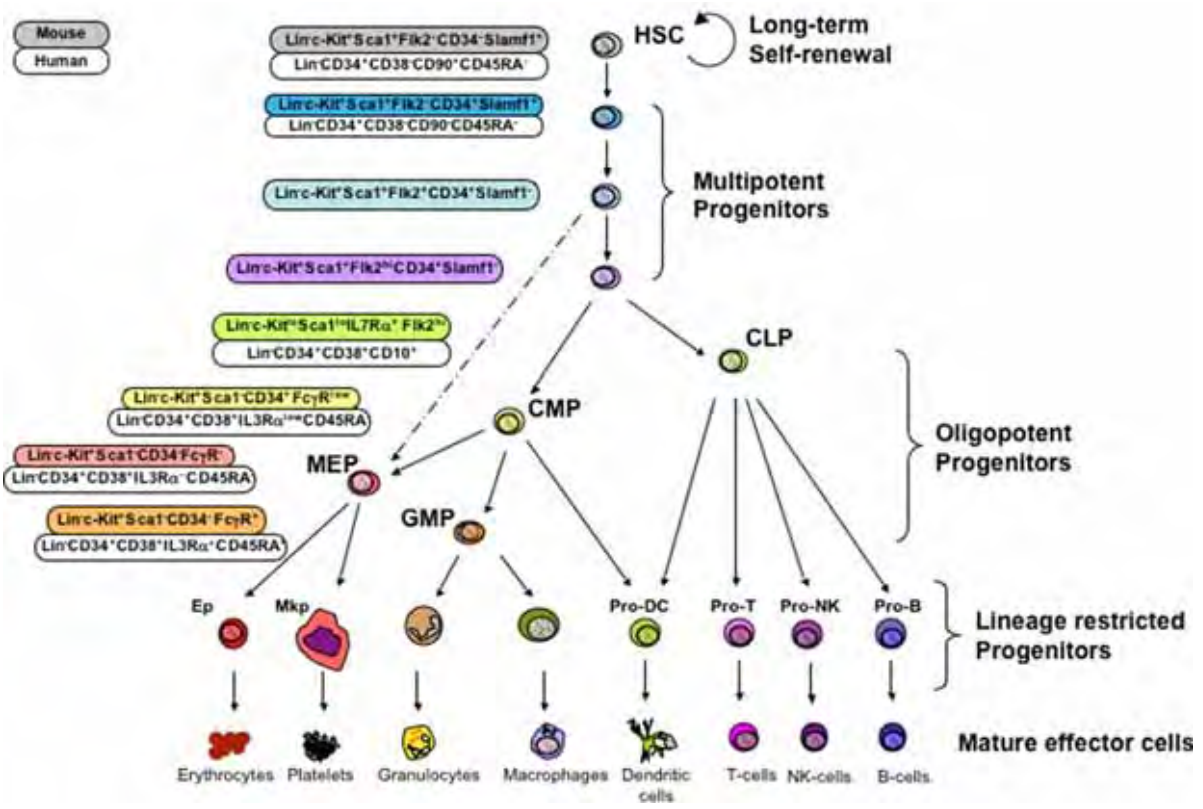



**Figure 2. Surface markers used for the isolation of murine and human hematopoietic cells.** This schema provides an overview of surface markers used for the isolation of hematopoietic cells in humans (top) and mice (bottom) for each stem and progenitor cell. Figure adapted from Weissman and Shizuru (2008).

# 2 Acute myeloid leukemia

## 2.1 Overview of disease

Acute myeloid leukemia (AML) is the most common adult acute leukemia and is characterized by clonal expansion of immature myeloblasts, initiating from rare leukemic stem or progenitor cells. The accumulation of these immature cells leads to deregulation of normal hematopoiesis and bone marrow failure (Lowenberg et al. 1999; Estey 2012). AML is a heterogeneous disease, or rather a set of diseases, in terms of morphology, cytogenetics and prognosis. In 1976, the French-American-British (FAB) group subdivided AML into eight different subtypes (M0-M7), which can be found in Table 1 (Bennett et al. 1976). These classifications rely on the morphology, degree of maturity, cellularity, blast percentage and cytochemistry of the leukemic cells. However, this classification system has little correlative value to prognosis as patients still have highly variable responses to treatments. In 2001, the World Health Organization (WHO) introduced a new classification, which was based off the FAB criteria but was expanded to include all available information, including genetic phenotyping, biological features and clinical characteristics (Vardiman et al. 2002). The WHO classification was again revised in 2008 (Vardiman et al. 2009) and described in Table 2.

**Table 1. French-American-British (FAB) AML subtypes.**

| FAB Subtype | Morphological Description |
|---|---|
| M0 | Undifferentiated acute myeloblastic leukemia |
| M1 | Myeloblastic, without maturation |
| M2 | Myeloblastic, with maturation |
| M3 | Promyelocytic (APL) |
| M4 | Myelomonocytic |
| M5 | Monocytic |
| M6 | Erythroid |
| M7 | Megakaryoblastic |

**Table 2. 2008 WHO classification of AML and related neoplasms.**

| **WHO Classification: Acute myeloid leukemias and related neoplasms** |
|---|
| **Acute myeloid leukemia with recurrent genetic abnormalities** |
| AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1* |
| AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11* |
| APL with t(15;17)(q22;q12); *PML-RARA* |
| AML with t(9;11)(p22;q23); *MLLT3-MLL* |
| AML with t(6;9)(p23;q34); *DEK-NUP214* |
| AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1* |
| AML (megakaryoblastic) with t(1;22)(p13;q13); *RBM14-MKL1* |
| Provisional entity: AML with mutated *NPM1* |
| Provisional entity: AML with mutated *C/EBP*α |
| **Acute myeloid leukemia with myelodysplasia-related changes** |
| Includes AML with a previous history of myelodysplastic syndrome (MDS) or myeloproliferative disorder (MPD) |
| **Therapy-related myeloid neoplasms** |
| Includes AML with previous history of chemotherapy and/or radiation treatment |
| **Acute myeloid leukemia, not otherwise specified** |
| AML with minimal differentiation |
| AML without maturation |
| AML with maturation |
| Acute myelomonocytic leukemia |
| Acute monoblastic/monocytic leukemia |
| Acute erythroid leukemia |
| Pure erythroid leukemia |
| Erythroleukemia, erythroid/myeloid |
| Acute megakaryoblastic leukemia |
| Acute basophilic leukemia |
| Acute panmyelosis with myelofibrosis |
| **Myeloid sarcoma** |
| **Myeloid proliferations related to Down syndrome** |
| Transient abnormal myelopoiesis |
| Myeloid leukemia associated with Down syndrome |
| **Blastic plasmacytoid dendritic cell neoplasm** |
| **Acute leukemias of ambiguous lineage** |
| Acute undifferentiated leukemia |
| Mixed phenotype acute leukemia with t(9;22)(q34;q11.2); *BCR-ABL1* |
| Mixed phenotype acute leukemia with t(v;11q23); *MLL* rearranged |
| Mixed phenotype acute leukemia, B-myeloid, NOS |
| Mixed phenotype acute leukemia, T-myeloid, NOS |
| Provisional entity: natural killer (NK) cell lymphoblastic leukemia; lymphoma |

Diagnosis of AML requires a blast count of greater than 20% in the bone marrow, with the exception of patients carrying special recurring genetic aberrations (Vardiman et al. 2009). In patients younger than 60 years old, 70-80% enter complete remission (CR) following one or two induction treatments (Fernandez et al. 2009; Mandelli et al. 2009; Burnett et al. 2010b), with CR being defined as less than 5% blasts in the bone marrow with recovery of peripheral blood cell counts (Cheson et al. 2003). However, a high percentage of patients will relapse, the rate of which correlates to overall survival (OS). Thus, additional treatments usually follow a CR and typically include high-dose chemotherapy or stem cell transplants (SCT) for the highest risk patients. These post-induction therapies reduce the risk of relapse to 50-55% giving a final 45-50% of patients that will be cured. While the course of treatments has not changed drastically over the past 30 years, there has been a steady improvement in overall survival. Much of the improvement can be attributed to better understanding of AML as a disease as well as improved patient care following diagnosis. However, this survival improvement occurs primarily in patients younger than 60 years old, whereas there has been little improvement in older patients (Figure 3). In these older patients, 40-65% will achieve CR following initial treatments, but 85% relapse within 2-3 years for various reasons (Lowenberg et al. 1989; Goldstone et al. 2001; Buchner et al. 2009; Lowenberg et al. 2009; Burnett et al. 2010b).

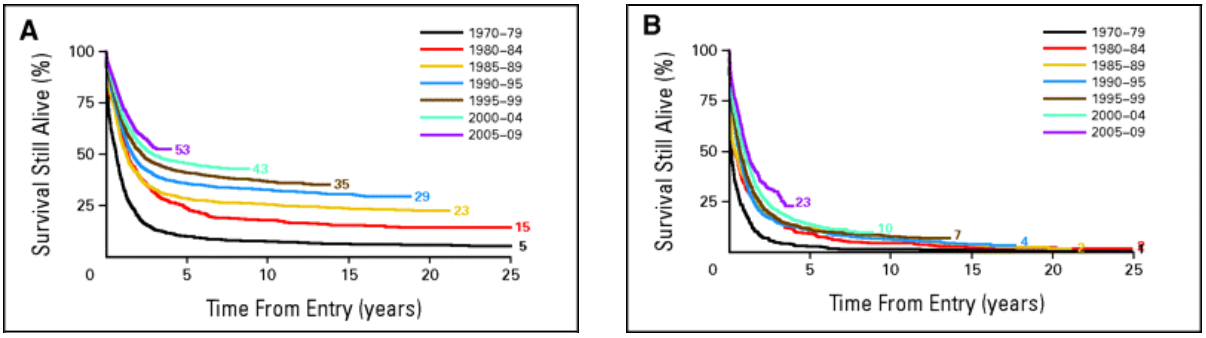



**Figure 3. Changes in overall survival of AML patients with time.** (A) Age 15 to 59 years (B) 60 or more years. Figure adapted from Burnett et al. (2011).

## 2.2 Risk factors

Prognostic risk factors can be subdivided into three categories, which include patient-related, therapy-related and leukemia-related factors (Cheson et al. 2003; Dohner et al. 2010).

### 2.2.1 Patient-related risk factors

Age is one of the most defining characteristics in terms of overall survival, which is due to the fact that many elderly patients often display chemotherapy resistance or high-risk cytogenetics. Furthermore, older patients have significant co-morbidities, such as secondary disease (Appelbaum et al. 2006). Due to co-morbidities and the overall health of elderly patients at diagnosis, a number of these patients will not even be treated with intensive chemotherapy either by choice or because it is considered unsuitable. Thus, overall survival of this particular cohort is extremely low (Kolb 2008).

### 2.2.2 Therapy-related risk factors

Patient response to initial treatment and achievement of CR are strong prognostic factors for survival. Patients with greater than 15% blast counts after the first treatment or those who require more than two courses of chemotherapy to enter CR have impaired long-term survival (Kern et al. 2003; Wheatley et al. 2009). A remaining minimal residual disease ($\geq$ 0.1%) detectable by flow cytometry has also been linked to higher relapse rates and shorter survival (Venditti et al. 2000; Buccisano et al. 2012).

### 2.2.3 Leukemia-related risk factors

Cytogenetic information of patients also has major prognostic value (Grimwade and Mrozek 2011; Kuhnl and Grimwade 2012). Patients are generally categorized into three groups based on their cytogenetic factors, including favorable, intermediate and adverse (Dohner et al. 2010; Kuhnl and Grimwade 2012) as summarized in Table 3. Patients in the favorable group have a CR rate above 90% with 55-85% overall survival. Of the patients in the adverse group, however, only 60% enter CR following initial treatment with 10-20% overall survival, which decreases to about 5% in elderly patients (Lowenberg et al. 1999; Slovak et al. 2000; Grimwade et al. 2010). The remaining intermediate patients make up the largest and most heterogeneous group of the three, consisting of 60-70% of patients with normal cytogenetics and most others with non-conclusive karyotypes.

**Table 3. Cytogenetic-based risk group classification**

| Risk Group | Cytogenetic Abnormality |
|---|---|
| Favorable | t(15;17)(q22;q21)<br>t(8;21)(q22;q22); *RUNX1-RUNX1T1*<br>inv(16)(p13q22)/t(16;16)(p13;q22); *CBFB-MYH11*<br>Mutated *NPM1* without *FLT3*-ITD (normal karyotype)<br>Mutated *C/EBP*α (normal karyotype) |
| Intermediate-I | Mutated *NPM1* and *FLT3*-ITD (normal karyotype)<br>Wild-type *NPM1* and *FLT3*-ITD (normal karyotype)<br>Wild-type *NPM1* without *FLT3*-ITD (normal karyotype) |
| Intermediate-II | t(9;11)(p22;q23); *MLLT3-MLL*<br>Cytogenetic abnormalities not classified as favorable or adverse |
| Adverse | In the absence of favorable risk cytogenetic abnormalities:<br>inv(3)(q21q26) or t(3;3)(q21;q26.2); *RPN1-EVI1*<br>t(6;9)(p23;q34); *DEK-NUP214*<br>t(v;11)(v;q23); *MLL* rearranged<br>-5 or del(5q); -7; abn1(17p); complex karyotype |

Currently, more than 90% of AML patients can be categorized based on either cytogenetic or molecular genetic features (Marcucci et al. 2010) as summarized in Table 4. In 25-30% of AMLs, *FLT3* is commonly expressed at high levels in leukemic blasts. This can be due to internal tandem duplications in the juxtaposition membrane domain (*FLT3*-ITD), leading to constitutive kinase activation (Kottaridis et al. 2001), but also from tyrosine kinase domain point mutations (*FLT3*-TKD) (Mead et al. 2007). The majority of these mutations are *FLT3*-ITD, which gives a proliferative advantage, increases anti-apoptotic signals and is thereby associated with an increased relapse rate and reduced overall survival (Gale et al. 2008). This information may push patient towards undergoing allogeneic SCT. There are also randomized trials for the combination of chemotherapy with tyrosine kinase inhibitors that inhibit *FLT3*. A number of additional mutations give unfavorable prognoses, which include *KIT*, *MLL*, *EVI1* and *WT1* (Gaidzik and Dohner 2008; Mrozek 2008; Schlenk et al. 2008; Lugthart et al. 2010).

There are also cytogenetic features that give favorable prognoses and help to determine post-induction therapy options. Approximately 10% of cytogenetically normal (CN)-AML patients have mutations in CCAAT/enhancer binding protein α gene (*C/EBP*α), for which half are biallelic and have favorable outcomes (Wouters et al. 2009; Dufour et al. 2010). This may help identify a group of young AML patients who do not need allogeneic stem cell transplantation in first CR. Core-binding factor (*CBF*) leukemias comprise 15-20% of patients with favorable prognosis, as long as there is no parallel *KIT* mutation (Mrozek 2008). The presence of a *KIT* mutation increases the risk of relapse from 40-50% to 70% in these patients (Mrozek 2008). 50% of CN-AML, and some patients with intermediate prognoses, have nucleophosmin 1 (*NPM1*) mutations that result in its delocalization into the cytoplasm, which, in the absence of *FLT3-ITD*, confers a favorable outcome to young patients (Falini et al. 2009). Treatment for these patients consists of chemotherapy without SCT in the first CR. There are have also been suggestions that *NPM1* mutated-patients should receive all-trans

retinoic acid therapy, but this has yet to be confirmed (Schlenk and Dohner 2009; Burnett et al. 2010a). Additionally, young CN-AML patients with low expression of *BAALC*, without association with *FLT3*, *NPM1* and *C/EBPα*, also have favorable prognoses (Langer et al. 2008; Santamaria et al. 2010). This prognosis is even more favorable if present with low erythroblastosis virus E26 oncogene homolog (ERG) expression, with which patients average 70% survival at 5 years and do not require SCT in first CR (Dufour et al. 2010).

Recently, the *IDH1/2* mutation was discovered to be present in 33% of CN-AML patients (Abbas et al. 2010; Boissel et al. 2010; Marcucci et al. 2010; Paschka et al. 2010) and 15-20% of overall AML patients (Mardis et al. 2009; Chou et al. 2010; Gross et al. 2010; Ward et al. 2010). Multiple studies have demonstrated that *IDH1/2* mutations are frequently associated with both normal karyotypes and *NPM1* mutations without *FLT3*-ITD, the latter of which normally provides a favorable prognosis (Abbas et al. 2010; Boissel et al. 2010; Marcucci et al. 2010; Paschka et al. 2010). However, these studies have suggested that *IDH1/2* mutations may give an adverse prognostic value in these conditions, allowing refined risk stratification of AML subsets (Abbas et al. 2010; Boissel et al. 2010; Marcucci et al. 2010; Paschka et al. 2010). An additional study identified 2-hydroxyglutarate (2HG), the oncometabolite produced by this mutation, as an adverse prognostic tool because IDH-mutant patients with 2HG levels greater than 200 ng/mL at complete remission had shorter overall survival compared to patients with lower levels (Dinardo et al. 2013). Conversely, two independent cohort studies on AML patients observed no association of *IDH1/2* mutations with CR, remission duration, OS and event-free survival (Ravandi et al. 2012; Koszarska et al. 2013). Additionally, Patel et al. (2012) demonstrated that *C/EBPα* and *IDH2* mutations were associated with improved overall survival (P=0.05 for *C/EBPα* and P=0.01 for *IDH2*). In this study, the favorable effect of *NPM1* mutations in *FLT*3-WT patients was restricted to those with co-occurring *IDH1/2* mutations, which was detected from a mutational analysis of 18 genes in 398 AML patients younger than 60 years of age who were randomly assigned to receive induction therapy with high-dose or standard-dose daunorubicin (Patel et al. 2012). Thus, the prognostic impact of *IDH1/2* mutations in AML cannot be concluded at this time and requires further study. Finally, the *IDH2* R172 mutations were observed to be associated with a novel subset of CN-AML patients lacking other prognostic mutations and associated with unique gene- and microRNA-expression profiles, which may lead to the discovery of novel, therapeutically targetable leukemogenic mechanisms regardless of the prognostic value of these mutations (Marcucci et al. 2010).

There are also several other leukemia-related risk factors that can occur, often in covariance, which makes them difficult to evaluate as independent variables. Secondary and therapy-related AML are reportedly correlated to reduced CR rate and impaired OS (Larson 2007; Kayser et al. 2011). Furthermore, hyperleukocytosis is a risk factor for early complications and death in remission induction (Greenwood et al. 2006).

**Table 4: Prognostic impact of molecular aberrations in AML implications** (Burnett et al. 2011).

| Aberration | Prognostic Impact | Possible Therapeutic Considerations | Standard Testing |
|---|---|---|---|
| *FLT3*-ITD | Unfavorable, especially with high allelic ratio, larger size and C-terminal location | Allogeneic SCT or FLT3 inhibitor-containing clinical trial | Yes* |
| *KIT* mutations in *CBF* AML | Unfavorable | Allogeneic SCT or TKI-containing clinical trial | Optional |
| *MLL*-PTD | Unfavorable | Allogeneic SCT or clinical trials with DNA methyltransferase or histone deacetylase inhibitors | Optional |
| High *EVI1* expression or mutations | Unfavorable | Allogeneic SCT or clinical trials with DNA methyltransferase or histone deacetylase inhibitors | Optional |
| *WT1* mutation | Unfavorable | Undecided | Investigational |
| Biallelic *C/EBPα* mutations | Favorable | Consolidation chemotherapy | Yes* |
| *NPM1* mutations but no *FLT3*-ITD | Favorable | Consolidation chemotherapy | Yes* |
| Low *BAALC* expression | Favorable, especially those with low *ERG* | Consolidation chemotherapy | Optional |
| *IDH1* and *IDH2* mutations | - | Undecided | Optional |
| Low global DNA methylation | - | Undecided | Investigational |
| Increased genome-wide promoter methylation | - | Undecided; Clinical trials with demethylating therapies | Investigational |

Abbreviations: AML, acute myeloid leukemia; *CBF*, core-binding factor; SCT, stem-cell transplantation; TKI, tyrosine kinase inhibitor; *FLT3*, FMS-like tyrosine kinase 3; ITD, internal tandem duplication; *MLL*, mixed lineage leukemia; PTD, partial tandem duplication; *EVI1*, ecotropic virus integration site-1; *IDH*, isocitrate dehydrogenase; *NPM1*, nucleophosmin1; *C/EBPα*, CCAAT/enhancer binding protein α; *ERG*, ets erythroblastosis virus E26 oncogene homolog (avian); *WT1*, Wilms tumor 1.
*In normal cytogenetics AML

## 2.3 Treatment of patients

### 2.3.1 Treatment of patients under 60 years of age

Despite advanced patient stratification, the initial induction therapy for AML is still very uniform. The exception is acute promyelocytic leukemia (APL), which is treated with differentiation agent, all-trans retinoic acid (ATRA) and/or arsenic. In general, however, AML treatment is a combination of cytotoxic agents, traditional anthracyclines, such as daunorubicin or idarubicin, and cytarabine (Dohner et al. 2010), a full description of which can be found in Table 5. The standard induction treatment is currently a combination of 3 days of daunoribicin and 7 days of cytarabine (3 + 7), which has remained the most effective treatment for the past 30 years (Burnett et al. 2011).

Once patients have reached CR through induction therapy, further treatment is required to decrease the risk of relapse. Currently, this treatment consists of various combinations of standard chemotherapy. Large studies from the United Kingdom Medical Research Council have determined that a total of four years, including induction, are needed to prevent relapse (Burnett et al. 2010b). Thus, the most common post-induction treatment is three doses of high-dose cytarabine given every other day for a total of four courses (Mayer et al. 1994). An alternative therapy is stem cell transplant (SCT) from a well-matched sibling donor, if possible (Cornelissen et al. 2007). SCTs depend on the so-called “graft-versus-leukemia” effect, which is an immunological response from donor T-cells against remaining leukemic cells. However, this benefit is limited by the potential “graft-versus-host” reaction in which donor T-cells actually attack normal tissues of the recipient (Kolb 2008; Cornelissen et al. 2012). Additionally, it is difficult to determine when to perform the SCT and which patients will benefit with an increased OS. The final decision is determined by the risk of relapse. If the risk is high, the chance of responding to induction therapy following relapse is low, which pushes for SCT following the first CR. It is the opposite case for low risk patients, who could respond well following relapse and would therefore revisit the option of a SCT following a second CR. Making this decision for intermediate-risk patients, however, is still difficult and must be first evaluated by a quantified risk score (Sorror et al. 2005).

### 2.3.2 Treatment for patients above 60 years of age

The majority of AML patients are older than 60 years with a median age of 70, which limits the intensity of treatment options (Menzin et al. 2002). For this reason, and as described above, there is a substantial number of elderly patients who are not treated either by choice or because it is considered unsuitable (Menzin et al. 2002; Kantarjian et al. 2006). For those that are able to undergo treatment, age still gives an unfavorable prognosis due to independent health-related factors, but also because elderly patients have reduced anthracycline sensitivity, chemotherapy resistance and an overall increase in unfavorable cytogenetics with fewer favorable cytogenetic factors (Kantarjian et al. 2006). The resulting induction therapy is typically 45 to 50 mg/m$^2$ of daunorubicin for 3 days and 100 to 200 mg/m$^2$ of cytarabine for 7-10 days, which are lower doses than those given to younger patients and induce CR in only 45-65% of patients (Lowenberg 1997). Different doses and schedules of cytarabine do not appear to have therapeutic benefits, while using high-dose cytarabine is too toxic for patients older than 60 years (Mayer et al. 1994). Additionally, alternative treatments of mitoxantrone and etoposide or combinations with idarubicin (12 mg/m$^2$) or mitoxantrone (12 mg/m$^2$) do not appear more effective than daunorubicin and cytarabine (Lowenberg et al. 2009). Finally, while few patients are fit to undergo SCT following CR, there is a small subgroup of patients that appear to have some success from reduced-intensity allogeneic SCT (Hegenbart et al. 2006). Despite this potential positive result, however, the fact remains that there is no accepted post-remission therapy for elderly AML patients, who have particularly rapid relapse rates with only 10% overall survival 5 years following diagnosis (Buchner et al. 2009; Lowenberg et al. 2009). It is for these patients especially that new drugs must be developed to specifically target leukemic blasts and progenitor cells without causing too much cytotoxicity in patients.

**Table 5: Standard AML chemotherapeutic agents** (Burnett et al. 2011).

| Chemotherapeutic Agent | Drug Class | Mechanism of Action |
|---|---|---|
| Daunorubicin | Anthracycline, cytotoxic antibiotic | Inhibitor of DNA synthesis by interacalation between base pairs. Topoisomerase II inhibition. Single and double strand breaks. |
| Idarubicin | Anthracycline, cytotoxic antibiotic | Inhibitor of DNA synthesis by intercalation between base pairs. Topoisomerase II inhibition. Single and double strand breaks. |
| Cytarabine | Antimetabolite, pyrimidine analogue | Enters target cell and undergoes phosphorylation reaction as the physiological nucleoside. Incorporated in both RNA and DNA but main cytotoxic action through inhibition of DNA polymerase. |
| Fludarabine | Antimetabolite, purine analogue | Metabolized to triphosphate. Inhibition of DNA synthesis similar to cytarabine. |
| Etoposide | Topoisomerase II inhibitor | DNA damage, inhibition of mitochondrial function and nucleoside transport. Topoisomerase II inhibition. |
| Mitoxantrone | Topoisomerase II inhibitor | DNA binding causes strand break. RNA reaction. Topoisomerase II inhibition. |
| Amsacribe | DNA-synthesis inhibitor | Not entirely clear, inhibits DNA synthesis, not RNA. |
| Azacitidine* | Hypermethylating agent | Inhibition of DNA, RNA and protein synthesis. |
| *MDS-AML with 20-30% blast counts | | |

# 3 Cancer cell metabolism

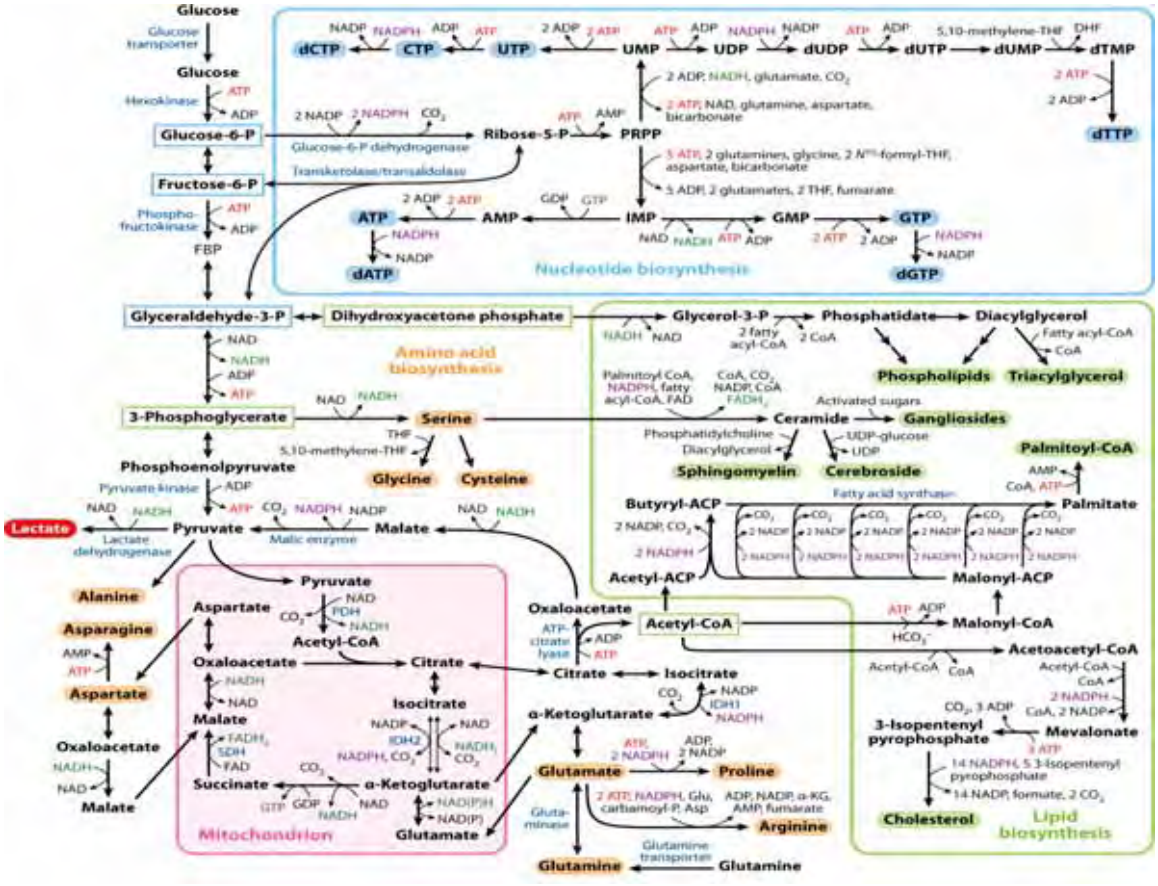



**Figure 4. Overview of cell metabolism.** This schematic represents the current understanding of how glycolysis, oxidative phosphorylation, the pentose phosphate pathway and glutamine metabolism contribute to biomass precursors. Enzymes that control critical steps and are often overexpressed or mutated in cancer cells are shown in dark blue. Nucleotides that can be incorporated into DNA and RNA are highlighted in light blue, representative lipids are highlighted in green and non-essential amino acids are highlighted in orange. Some key metabolites that serve as important precursors for biomass production are boxed. This figure provides a roadmap and reference for the cancer cell metabolism that will be discussed in this introductory section. Figure adapted from Lunt and Vander Heiden (2011).

## 3.1 Overview

In the 1920s, Otto Warburg reported that even in the presence of ample oxygen, cancer cells exhibit higher rates of glucose uptake and lactate production compared to normal cells. This discovery, which completely revolutionized the understanding of cancer cells, was deemed the Warburg effect (Warburg et al. 1927). Metabolism, summarized in Figure 4 for reference throughout this introduction, gained a new role in cancer research. The impact has been observed starting *in vitro* and continuing up through clinical and diagnostic tests, such as the development of the positron emission tomography (PET) scan to measure increased uptake of 2-deoxy-2($^{18}$F)-fluoro-D-glucose and allow for tumor identification in patients (Gambhir 2002).

Despite the steps medicine has taken since Warburg's discovery of cancer cell metabolism, however, the understanding of why cancer cells exhibit this metabolic switch is still under debate

today. Warburg and others suggested the effect was due to mutations or defects in mitochondrial respiration, which cells adapt to by using glycolysis as an alternative energy route (Warburg 1956). However, more recent discoveries have indicated that most cancer cells have functional mitochondria (Moreno-Sanchez et al. 2007) and that the glycolytic switch is prompted by oncogenes and inhibited by tumor suppressors, suggesting that the Warburg effect occurs at the forefront of cancer development (DeBerardinis et al. 2008; Jones and Thompson 2009; Munoz-Pinedo et al. 2012). These and other works suggest that although glycolysis has a lower efficiency for ATP production, the fact that it occurs at a faster rate than oxidative phosphorylation allows for rapid cell proliferation. This increased glycolysis provides not only ATP, but important biosynthetic precursors, such as nucleotides, amino acids and lipids, which are necessary for proliferation and whose production helps differentiate metabolism in normal *versus* cancer cells (Lunt and Vander Heiden 2011; Romero-Garcia et al. 2011) (Figure 5). It is important to understand how and why these metabolic adaptations occur in order to aid in future therapeutic developments. Therefore, this introduction will detail the theories on how and why the Warburg effect occurs in cancer cells and give a thorough explanation of current knowledge of cancer cell metabolism.

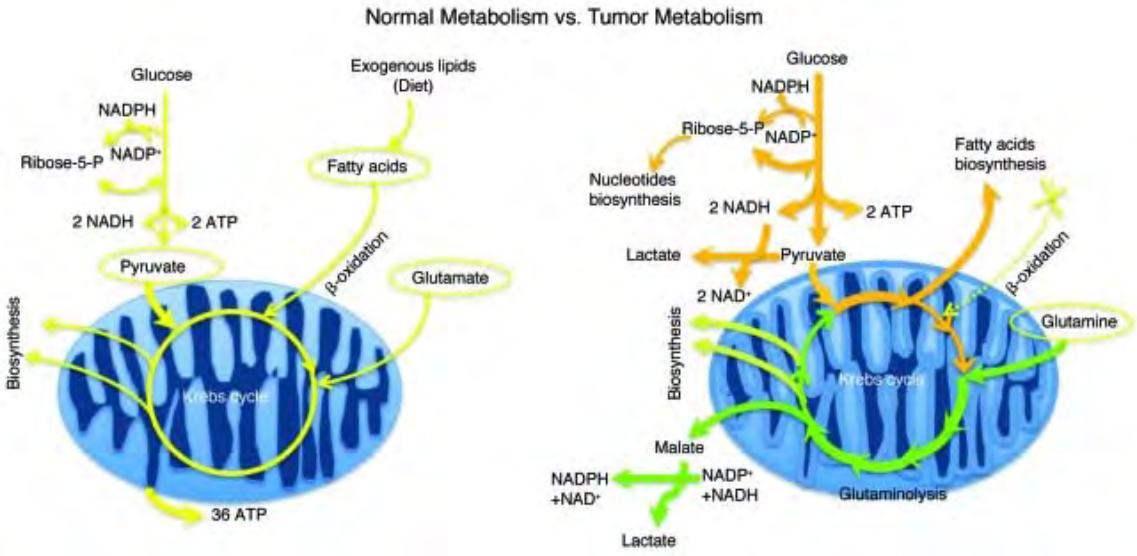



**Figure 5. Comparison of metabolism in normal *versus* tumoral cells.** In tumor cells, glucose supports cellular growth through nucleotide and lipid biosynthesis. About 90% of R5P and 60% of fatty acids are glucose derived. Glutamine supports cells via anaplerosis of the Krebs cycle and NADPH regeneration, accompanied by lactate production, the latter of which is about 60% derived from glutamine. Figure adapted from Romero-Garcia et al. (2011).

### 3.2 Mitochondrial-related enzymatic mutations

Warburg's theory that mitochondrial defects induce the metabolic switch in cancer cells is supported by increased mutations in the mtDNA of cancer cells compared to normal cells. These mutations are typically missense mutations and are expected to develop due to ROS exposure, since mtDNA lack histones for DNA protection (Piruat and Lopez-Barneo 2005; Bonora et al. 2006).

However, most mtDNA mutations are inconsistent and silent, leading to functional mitochondria in many cancer cells (Brandon et al. 2006). Despite this fact, there is a subset of human cancers that have mutations leading to impaired mitochondrial function.

### 3.2.1 SDH and FH family mutations

One of the most well known of these mutations occurs in the succinate dehydrogenase (*SDH*) and fumarate hydratase (*FH*) families (Tomlinson et al. 2002; Gottlieb and Tomlinson 2005) (Figure 6). Mutations in the *SDH* families have been identified primarily in head and neck paragangliomas (Baysal 2007) while *FH* mutations have been observed in several cancers, including uterine leiomyatosis, cerebral cavernomas, breast cancer and renal cell cancer (Tomlinson et al. 2002). The *SDH* and *FH* families convert succinate into fumarate and reversibly convert fumarate into malate, respectively, as part of the TCA cycle. With these mutations, succinate and fumarate will accumulate in the cytosol where they impair α-ketoglutarate-dependent dioxygenases (Selak et al. 2005), whose role is to hydroxylate HIF-1α, resulting in its ubiquination and subsequent degradation through the Hippel-Lindau (VHL)-mediated pathway (Martin-Puig et al. 2004). Normally, these dioxygenases are functional during normoxia and only allow for HIF-1α stabilization under hypoxic conditions. By inhibiting dioxygenases, the *SDH* and *FH* mutations allow for the stabilization of HIF-1α under normoxia, which is a characteristic of many cancers and can enhance the downstream metabolic changes of the Warburg effect (Weidemann and Johnson 2008).

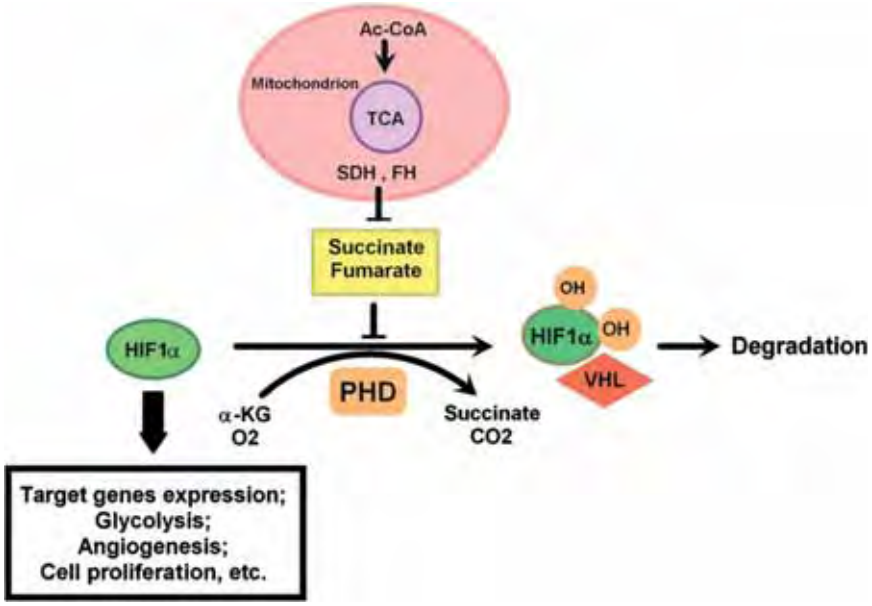



**Figure 6. Function of *SDH* and *FH* mutations in cancer cell metabolism.** *SDH* and *FH* mutations increase the accumulation of succinate and/or fumarate, both of which can inhibit the enzymatic activity of PHD, resulting in the reduced degradation of HIF-1α and increased expression of some specific genes involved in glycolysis, angiogenesis and cell proliferation, etc. Figure adapted from Wu and Zhao (2013).

### 3.2.2 IDH mutations

It was also discovered that 70% of grade II-III gliomas and most of secondary glioblastomas (Dang et al. 2009; Yan et al. 2009), 15-20% of acute myeloid leukemias (Mardis et al. 2009; Chou et al. 2010; Gross et al. 2010; Ward et al. 2010) and chondrosarcomas (Amary et al. 2011) express mutations in *IDH1* and *IDH2* (isocitrate dehydrogenase). IDH1 and IDH2 proteins, located in the cytosol and mitochondria, respectively, may play roles in redox metabolism as they use NADP+ as a cofactor, thereby producing NADPH. Additionally, while the wild-type IDH enzyme converts isocitrate into alpha-ketoglutarate (α-KG), the mutant IDH enzyme produces oncometabolite, 2-hydroxyglutarate (2-HG), from α-KG (Dang et al. 2009). The result is a major disruption of mitochondrial oxidative phosphorylation as total levels of α-KG are decreased due to increased 2-HG production. Additionally, as 2-HG is an analog of α-KG, it can negatively inhibit enzymatic reactions that could increase α-KG levels (Xu et al. 2011). As α-KG promotes activity of α-KG-dependent dioxygenases as well as activity of PHD for HIF-1α degradation, its loss can stabilize HIF-1α and have widespread biological impacts (Xu et al. 2011; Wu and Zhao 2013) (Figure 7).

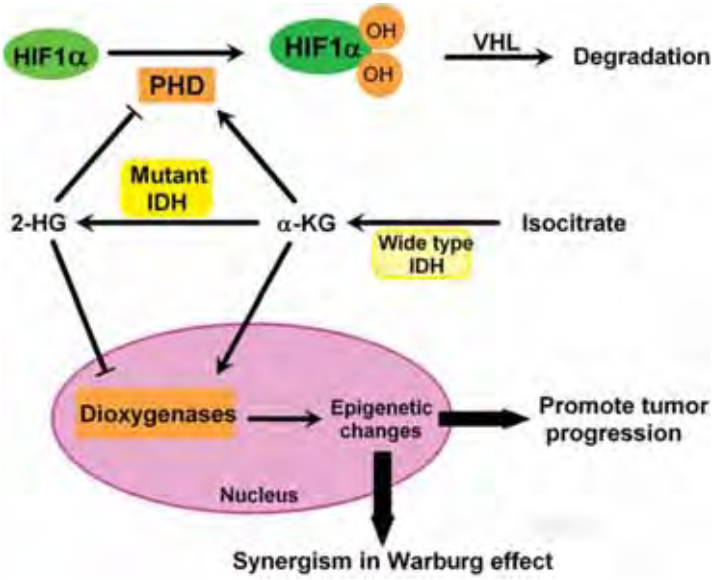



**Figure 7. Function of *IDH* mutations in cancer cell metabolism.** Mutant IDH can convert α-KG into 2-HG. The increased 2-HG inhibits the activities of PHD, leading to increased HIF-1α stabilization, and of α-KG-dependant dioxygenases, leading to wide biological effects promoting tumor progression and Warburg metabolism. Figure adapted from Wu and Zhao (2013).

*IDH1* and *IDH2* mutations are also associated with abnormal chromatin and DNA methylation profiles, which can induce a cell differentiation arrest (Figueroa et al. 2010; Lu et al. 2012; Sasaki et al. 2012) (Figure 8). This effect is likely due to the increased 2-HG and decreased α-KG, which can prevent histone demethylase activity (Chowdhury et al. 2011). Additionally, 2-HG will inhibit TET2, which is an enzyme catalyzing the conversion of methylcytosine (5mC) to 5-hydroxymethylcytosine (hmC). This seems to lead to base-excision repair and *de novo* production of un-methylated cytosine (Figueroa et al. 2010). Although *IDH1/2* mutations were not the first mutations discovered in oncogenesis, they were the first to specifically link metabolite production to oncogenesis. More importantly, these mutations have potential as clinical markers as both glioma and AML patients with *IDH1/2* mutations had 2-HG levels that were 100-fold higher than non-mutated patients (Dang et al. 2009; Ward et al. 2010) and 2-HG levels in blood serum predict *IDH* mutations and clinical outcome in AML (Dinardo et al. 2013).

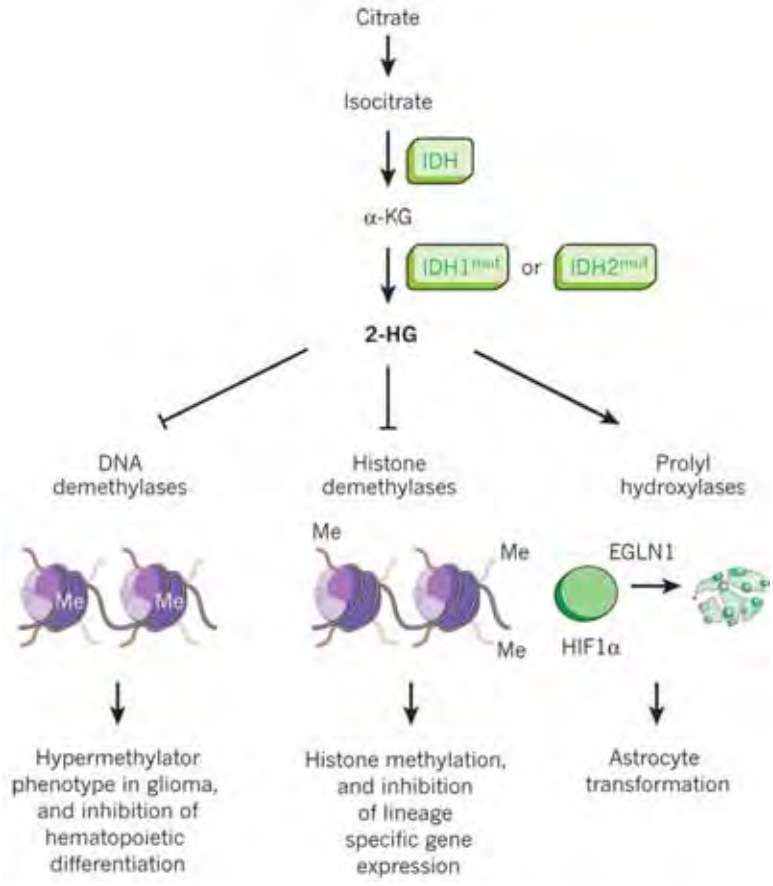



**Figure 8. The effect of *IDH* mutations on methylation profiles.** Increased 2-HG as a result of *IDH* mutations leads to decreased DNA and histone demethylase activity as well as increased prolyl hydroxylase activity. Altogether, these effects decrease differentiation and lineage specific gene expression, while increasing astrocyte transformation through HIF-1α stabilization. Figure adapted from Schulze and Harris (2012).

### 3.2.3 Other mutations

Renal onocytomas, which are benign tumors, have increased mitochondria accumulation, mitochondrial DNA and catalytic activities of several oxidative phosphorylation complexes (Simonnet et al. 2003). However, their mitochondrial complex I activity, as measured by NADH dehydrogenase activity and protein content, were found to be decreased. Surprisingly, other malignant, renal tumors have decreases of all other respiratory chain complexes. Thus, it is possible that non-functional complex I in oncocytomas might be the early event in the loss of mitochondrial function, leading to increased mitochondrial biogenesis in an attempt to compensate the loss (Simonnet et al. 2003). Mutations in ATP production have also been linked to decreased mitochondrial activity (Koppenol et al. 2011). Specifically, there are mutations in the gene encoding mitochondrial $H^+$-ATPase in human kidney, lung, breast and colon carcinomas as well as depletion of cellular mitochondrial content in human liver carcinomas (Cuezva et al. 2002; Lopez-Rios et al. 2007). Both mutations decrease mitochondrial cellular activity, which can limit the mitochondrial-apoptotic potential of tumor cells and support the switch to Warburg metabolism. Additionally, there is evidence of defects in *SCO2* (synthesis of cytochrome c oxidase 2), as re-expression of *SCO2* reverses the Warburg effect (Matoba et al. 2006). Lastly, the mitochondrial proton gradient can be affected by overexpression of uncoupling proteins, like *UCP2*, which could be due to increased HIF-1α stability and used to combat increases in ROS by impairing mitochondria (Derdak et al. 2008; Samudio et al. 2009).

However, not all mitochondrial mutations are associated with a negative outcome on patient survival or cancer risks. Mutations in NADH dehydrogenase subunit 4 (*ND4*), a mitochondrial encoded transmembrane component of ETCI, has been identified in approximately 6.4% of AML patients and, when somatically mutated, correlates with significantly longer relapse-free survival and overall survival than wild type patients (Damm et al. 2012). Patients with mutated, somatic *ND4* also had a higher prevalence of concomitant *DNMT3A* mutations and a higher percentage of the *NPM1/FLT3*-ITD low-risk genotype. Thus, in this case, the decrease in mitochondrial activity was linked to increased patient survival.

### 3.3 Metabolic switch regulated by oncogenic transformation

While mitochondrial mutations do occur in cancer cells, the majority of cancer cells with high proliferation have functional mitochondria and oxidative phosphorylation (Moreno-Sanchez et al. 2007). On the other hand, while many cancers exhibit *LDHA* overexpression, including gastric and lung cancers and head and neck carcinomas, cancer cells with knockdown of *LDHA* still produce ATP through oxidative phosphorylation (Fantin et al. 2006). Altogether, recent data suggests that increased glycolysis in the presence of oxygen is due to both activation of oncogenes and loss of tumor suppressors, as summarized in Figure 9, which leads to metabolic reprogramming that provides energy and molecular building blocks for cancer cell growth and proliferation (Romero-Garcia et al. 2011). In this metabolic introduction, I will outline the specific metabolic reprogramming of cancer cells, including the oncogenic transformations occurring in parallel, and the therapeutic potential this metabolic reprogramming has for numerous cancers.

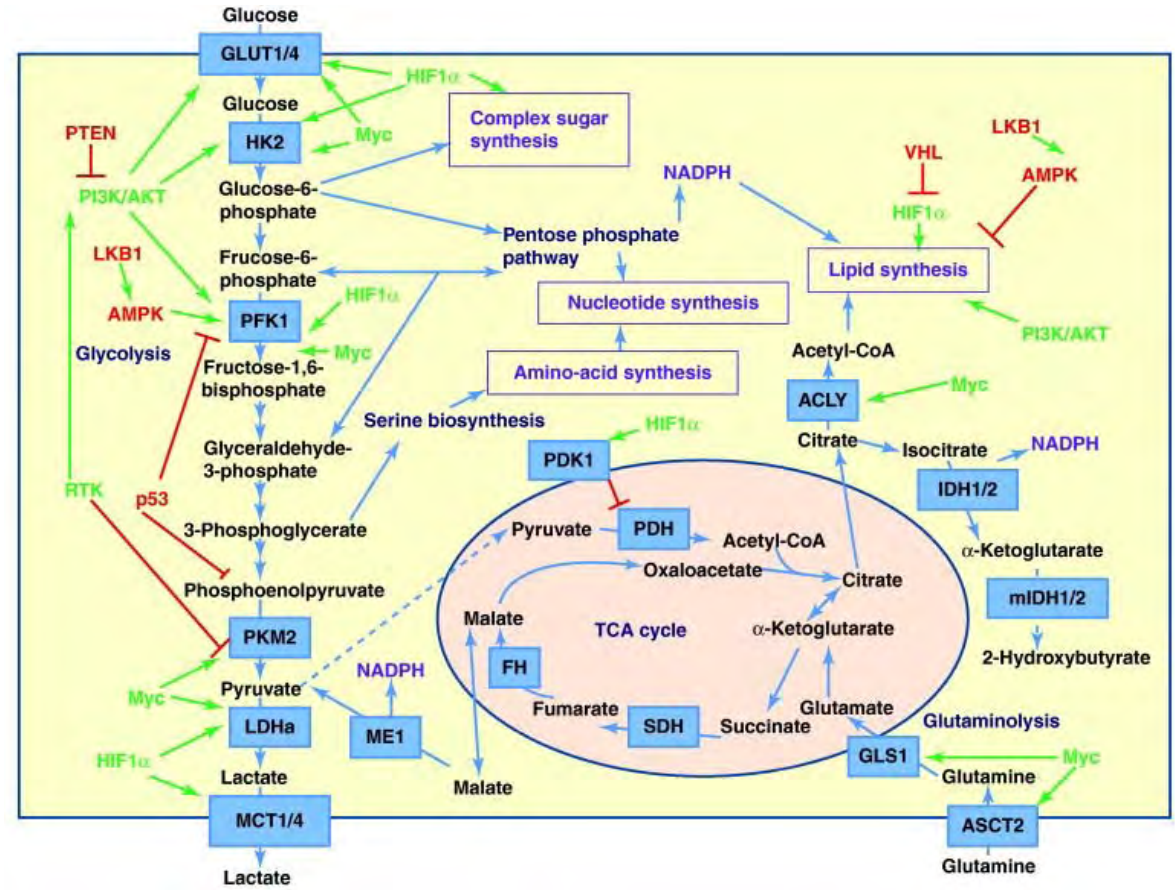



**Figure 9. Overview of oncogenic manipulation of cancer cell metabolism.** This schematic shows the current understanding of how metabolic pathways active in proliferating cells are directly controlled by signaling pathways involving oncogenes and tumor suppressor genes. PI3K activation stimulates glucose uptake and flux through the early part of glycolysis *via* AKT. Tyrosine kinase signaling negatively regulates flux through the late steps of glycolysis, providing glycolytic intermediates for macromolecular synthesis and NADPH production. Myc drives glutamine metabolism and supports NADPH production. LKB1/AMPK signaling and p53 manipulate metabolism to decrease proliferation and energy-consuming pathways during periods of low energy availability or oxidative stress. Tumor suppressors are in red, oncogenes are in green, key metabolic pathways are labeled boxes and enzymes controlling critical metabolic steps are in blue. Figure adapted from Vander Heiden et al. (2009).

## 3.4 Summary of specific metabolic pathways

### 3.4.1 Glycolysis

To fully understand the complex nature of cancer cell glycolysis, it is important to understand the overall pathway of glycolysis and its branches as overviewed in Figure 4 and summarized for specifically in Figure 10. Glucose is imported by the cell through glucose transporters (GLUTs) and then phosphorylated by hexokinase-II (HK-II) to become glucose-6-phosphate (G6P). G6P will then either continue through glycolysis, converted to fructose-6-phosphate (F6P) by phosphoglucose isomerase (PGI), or branch off into the pentose phosphate pathway (PPP). Entry into the PPP is determined by glucose-6-phosphate dehydrogenase (G6PDH) and glucose carbons entering this pathway are used primarily for nucleotide biosynthesis. Continuing though the glycolytic flux, phosphofructokinase-1 (PFK-1) will convert F6P to fructose-1,6,-bisphosphate (F-1,6-BP), which will then be converted to either glyceraldehyde-3-phosphate (GA3P) to continue through glycolysis or to dihydroxyacetone phosphate (DHAP), which is a critical branch involved in lipid synthesis. GA3P will then be converted to 1,3-bisphosphoglycerate (1,3-PGA) by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and subsequently converted to 3-phosphoglyceric acid (3-PGA) by phosphoglycerate kinase (PGK), producing ATP in the process. 3-PGA can then branch off to produce amino acids, such as serine and glycine, or be converted to 2-phosphoglyceric acid (2-PGA) by phosphoglycerate mutase (PGAM) and subsequently converted to phosphoenol pyruvate (PEP) by enolase 1 (ENO1). Pyruvate kinase (PK) then converts PEP into pyruvate with the production of ATP. NADH produced throughout glycolysis will then be oxidized either by oxidative phosphorylation in the mitochondria or by lactate dehydrogenase-A (LDHA), which is just downstream of glycolysis and converts pyruvate to lactate to regenerate NAD+ and allow for continued glycolysis (Zhang and Yang 2013).

While non-cancerous cells are also reliant on glucose metabolism for survival, there are many modulations in cancer cells that increase glucose dependence and provide potential therapeutic targets. Microarray studies showed that most of the genes involved in glucose transport and glycolysis are upregulated in several different tumor types (Altenberg and Greulich 2004; Lu et al. 2007). Glycolytic genes like *PKM2, TPI1*, *PGK1*, *ALDO-A, ENOα*, and *GPI* were significantly upregulated in most of the 20 different types of common cancer examined, regardless of tissue origin (Lu et al. 2007). Additionally, glucose transporters are overexpressed in hepatocarcinomas, breast cancer, neuroendocrine carcinomas, lymphoblastic leukemia and many others (Romero-Garcia et al. 2011), with GLUT1 as the dominant glucose transporter in most cancers (Younes et al. 1997; Kang et al. 2002). Inhibition of glucose transporters by drugs like phloretin can sensitize cancer cells to chemotherapy agents (Cao et al. 2007).

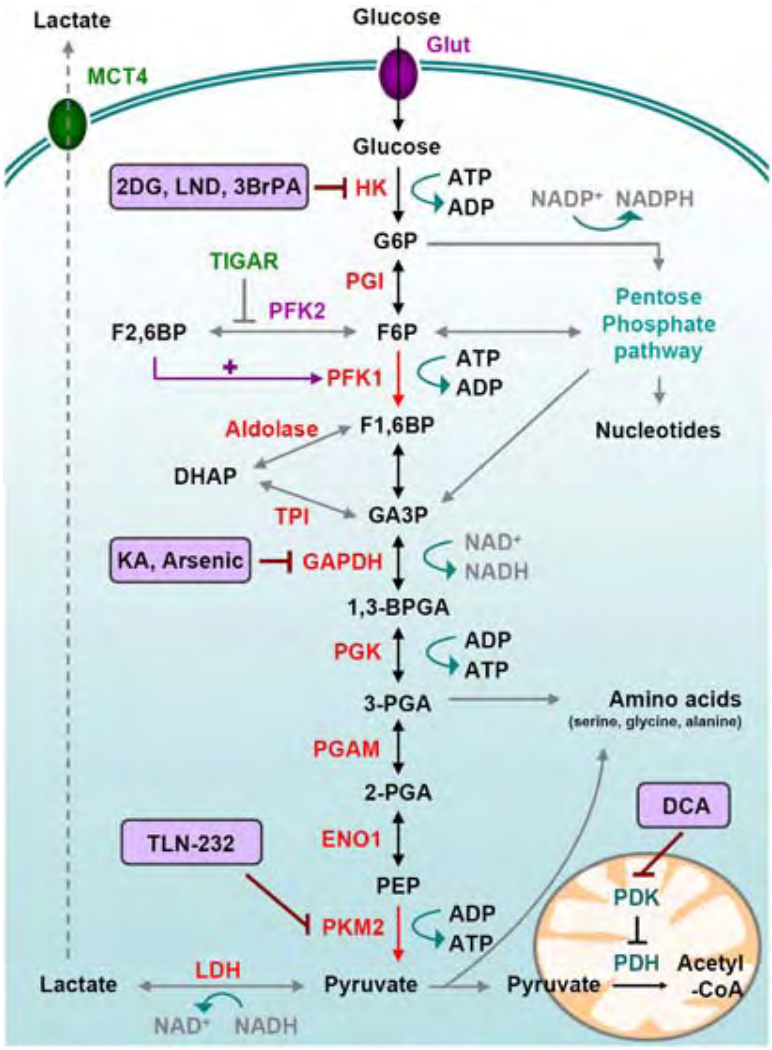



**Figure 10. Overview of glycolysis.** Glycolysis is a series of metabolic processes, driven by nine specific enzymes, by which one mole of glucose is catabolized to two moles of pyruvate, producing two moles of NADH and a net gain of two ATP. As indicated, several intermediates can fuel the pentose phosphate pathway or lead to amino acid production. Accumulation of those intermediates is favored by the rate-limiting activity of PKM2. In cancer cells, pyruvate is further converted into lactate, thereby generating NAD+ from NADH. Pyruvate can be also imported into the mitochondrial matrix to feed the TCA cycle. This entry is controlled by pyruvate dehydrogenase kinase (PDK), which can inactivate pyruvate dehydrogenase (PDH), therefore limiting the conversion of pyruvate into acetyl-CoA and entry into the TCA cycle. This schema provides a summary of glycolysis in cancer cells as well as the molecular targets of several chemical inhibitors of glycolysis, including HK (2DG, LND, 3BrPA), GAPDH (KA, Arsenic), PKM2 (TLN-232) and PDK (DCA). Figure adapted from Munoz-Pinedo et al. (2012).

### 3.4.2 Hexokinase

The key glycolytic enzyme catalyzing the first step of glycolysis, HK-II, is overexpressed in many cancer types and correlates to poor prognosis (Porporato et al. 2011). The overexpression of HK-II, as compared to other hexokinase isoforms, is promoted by active HIF-1α (Mathupala et al. 2001). Additionally, HK-II can be phosphorylated, which promotes binding to the mitochondrial membrane at the protein porin and increases its interaction with the voltage-dependent anion channel (VDAC) (Bustamante and Pedersen 1977; Nakashima et al. 1986; Nakashima et al. 1988; Gottlob et

al. 2001). This interaction allows HK-II to have direct access to ATP leaving the mitochondria through VDAC and prevents negative feedback inhibition by G6P (Bustamante and Pedersen 1977). Importantly, this binding of HK-II to VDAC also prevents pro-apoptotic proteins Bax and Bak from binding to VDAC and initiating the release of cytochrome c and its subsequent activation of caspase-dependent apopotic pathways (Pastorino et al. 2002; Pedersen 2008). Depletion of HK-II in malignant glioma cells stimulates ROS production and leads to cell death (Wolf et al. 2011). Additionally, some drugs like 3-bromopyruvate (3-BrPA) and clotrimazole can detach HK-II from the mitochondria, which thereby eliminates the advantages and induces cancer cell death (Ko et al. 2001).

### 3.4.3 Pentose phosphate pathway

In cancer cells, the pentose phosphate pathway (PPP), branching off of glycolysis, provides key biosynthetic intermediates and consists of both an oxidative and non-oxidative branch (Tong et al. 2009) (Figure 4, 11). The oxidative branch, entrance of which is dependent on the enzyme glucose 6-phosphate dehydrogenase (G6PDH), converts glucose-6-phosphate (G6P) from glycolysis to ribose-5-phosphate (R5P) and produces NADPH and $CO_2$ in the process. R5P will eventually be converted to phosphoribosyl pyrophosphate (PRPP) and carried through nucleotide bioysnthesis while NADPH can provide antioxidants through GSH reduction and is also required for lipid synthesis. The non-oxidative branch also produces R5P from fructose-6-phosphate (F6P) or glyceraldehyde-3-phosphate (G3P), the latter of which occurs in a reversible process that can also convert R5P back to G3P and then G6P to re-enter the oxidative PPP branch. In situations where cancer cells require a great deal of NADPH, coupling of the two branches of this pathway can provide up to six molecules of NADPH from only one molecule of glucose (Tong et al. 2009).

Many cancer cell lines exhibit increased flux through the PPP and one of the enzymes that allows this increase, G6PDH, is overexpressed in gastric, colorectal and kidney cancers (Langbein et al. 2008; Kekec et al. 2009). Additional data has shown that G6PDH is negatively regulated by wild-type, but not mutant, p53 (Jiang et al. 2011). This implies that cancer cells expressing the mutant or null form of p53 would have increased G6PDH activity and increased flux through the PPP, which allows specific targeting of G6PDH in these cancer cells. This has already been accomplished in several cancer cell lines with RNA interference of G6PDH, specific inhibitors of G6PDH, such as dehydroepianodrosterone (DHEA) or 6-aminonicotinamide (6-ANA), and NO synthase inhibitor, L-NAME (Jiang et al. 2011). There have also been several links between G6PDH activity and DNA repair as DNA-damage-sensing kinase ATM appears to activate G6PDH through HSP70 (Cosentino et al. 2011). Considering that many chemotherapies function by inducing DNA damage and ROS production, G6PDH may be a response tool that provides chemotherapy resistance and could therefore be an especially interesting target to improve chemotherapy efficacy.

Studies have also focused on the importance of the non-oxidative PPP branch as certain cancers, including head and neck carcinomas, metastatic tumors and colon, urothelial and lung cancers exhibit overexpression of transketolase (Langbein et al. 2006; Frederiks et al. 2008; Langbein et al. 2008; Schultz et al. 2008; Vizan et al. 2009). In the non-oxidative branch, TKT reversibly converts both fructose-6-phosphate and glyceraldehyde-3-phosphate to xylulose-5-phosphate, which is reversibly converted to R5P by transaldolase 1 (TALDO1). Targeting TKT with RNA interference impairs growth in cancer cells exhibiting high TKT expression. In colon carcinoma cells, targeting TKT also decreases glucose consumption and lactate production (Coy et al. 2005; Langbein et al. 2006; Foldi et al. 2007; Langbein et al. 2008; Xu et al. 2009). Additional studies have shown that the non-oxidative arm of the PPP is the principal pathway for R5P synthesis in tumor cells (Boros et al. 2000; Tong et al. 2009). It is possible that the reason this non-oxidative branch is predominant is that G6PDH activity can be suppressed by the accumulation of fructose-1,6-bisphosphate (F-1,6-BP). Thus, future studies may focus on other aspects of the non-oxidative branch of the PPP for cancer cell dependence and as a potential therapeutic target.

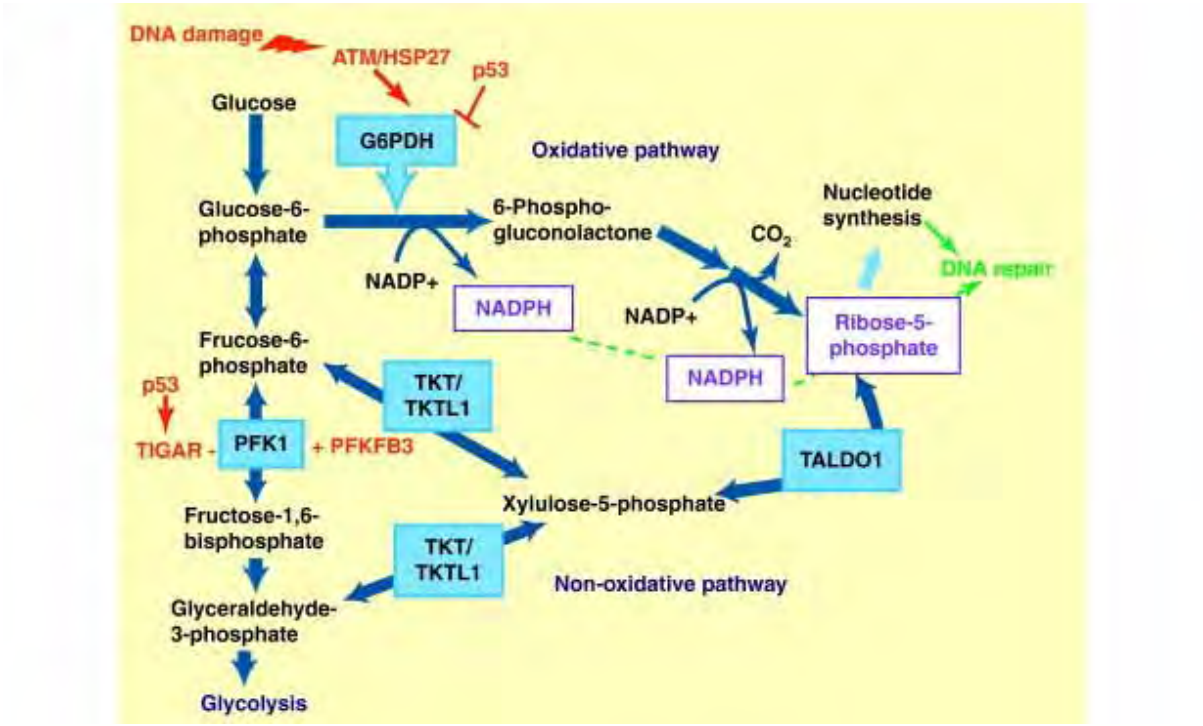



**Figure 11. Overview of the pentose phosphate pathway.** The PPP consists of both an oxidative and a non-oxidative branch. Both branches result in the production of ribose-5-phosphate, but only the oxidative branch produces NADPH in the process. The non-oxidative branch, *via* transketolase enzymes (TKT/TKTL1), reversibly converts either fructose-6-phosphate or glyceraldehyde-3-phosphate to xylulose-5-phosphate. The reversible nature of this branch can convert ribose-5-phosphate back into glycolysis allowing NADPH production and carbon recycling. Figure adapted from Schulze and Harris (2012).

### 3.4.4 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase family

Another family of enzymes that is partially responsible for the increased glycolytic flux in cancer cell metabolism, and is also implicated in PPP activity, is the family of homodimeric enzymes known as 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB). PFKFBs are bifunctional enzymes catalyzing either ATP-dependent phosphorylation of fructose-6-phosphate (F6P) to fructose-2,6-bisphosphate (F-2,6-BP) (PFK activity) or the dephosphorylation of F-2,6-BP to F6P (FBPase activity). The family consists of four isoforms, including PFKFB1 and PFKFB2, which display equal PFK2 and FBPase activities, PFKFB3, which has high PFK2 and almost no FBPase activity, and PFKFB4, which has high FBPase activity and low PFK2 activity (Okar and Lange 1999; Okar et al. 2001; Ros et al. 2012). All four members are induced by hypoxia, but only PFKFB3 is directly targeted by HIF-1α (Minchenko et al. 2002; Minchenko et al. 2003). PFKFB3 supports hypoxic cells by sustaining high-rate glycolysis through increased PFK2 activity, which leads to production of F-2,6-BP, an allosteric activator of PFK1. This is especially important considering high citrate and ATP levels inhibit PFK1 activity, so constitutively high PFKFB3 activity, and subsequent F-2,6-BP levels, counteract the negative feedback to promote glycolysis (Yalcin et al. 2009). Not surprisingly, PFKFB3 is overexpressed in many human cancers (Atsumi et al. 2002; Minchenko et al. 2005a; Minchenko et al. 2005b; Kessler et al. 2008). PFKFB4, on the other hand, has high FBPase activity, which converts F-1,6-BP back to F6P. The result is a decrease in glycolytic activity through decreased PFK1 activity, which increases flux through the PPP and leads to high antioxidant production (Ros et al. 2012). Thus, the preferential activation of a PFK isoform by hypoxia supports a balance between the need for glycolytic ATP *versus* the need for antioxidants to counteract ROS production. A summary of these activities and their regulation can be found in Figure 12.

TIGAR is a p53-regulated protein that possesses a F-2,6-BP domain that is also found in the glycolytic regulator PFK2 (Bensaad et al. 2006). Similar to PFKFB4, TIGAR has high FBPase activity to convert F-2,6-BP back to F6P thereby negatively regulating PFK1, slowing the glycolytic rate and promoting G6P entry into the PPP (Bensaad et al. 2006; Bensaad et al. 2009). TIGAR can be activated by p53 to respond to DNA damage and high levels of ROS (DeBerardinis et al. 2008; Bensaad et al. 2009). Positive regulation by p53 could suggest a tumor suppressor function of TIGAR, but knockdown of TIGAR actually sensitizes cancer cells to p53-induced apoptosis (Bensaad et al. 2006). Thus, it is possible that TIGAR responds to p53 in an effort to produce NADPH and antioxidants to decrease ROS, inhibit p53-induced apoptosis and support nucleotide biosynthesis to compensate DNA damage. Additionally, TIGAR can be activated independently of p53 to promote biosynthetic production of antioxidants, which are essential for cancer cell growth and proliferation (Bensaad et al. 2006; Bensaad et al. 2009). Thus, the role of TIGAR as a tumor suppressor downstream of p53 is not obvious. Overall, TIGAR and PFKFB enzymes are key regulators for

metabolic activity in cancer cells, but it is important to identify the activity in each cancer type, and even within cancers, to determine possible therapeutic strategies as both directions can benefit cancer cells in different capacities.

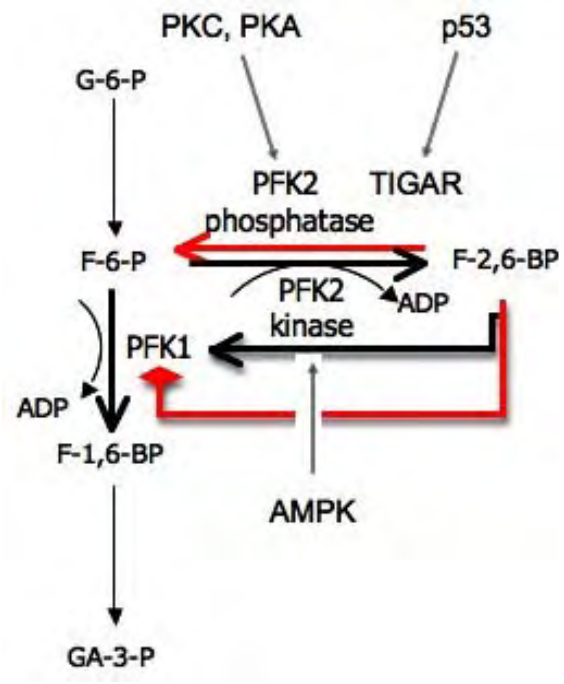



**Figure 12. PFKFB activity in cancer cell metabolism.** PFKFBs are bifunctional enzymes catalyzing either PFK activity, which involves ATP-dependent phosphorylation of fructose-6-phosphate (F6P) to fructose-2,6-bisphosphate (F-2,6-BP) or FBPase activity in which F-2,6-BP is dephosphorylated to F6P. High PFK activity, increased by AMPK activity, promotes the formation of F-2,6-BP, which increases the glycolytic flux due to positive feedback on PFK1. High FBPase activity, increased by PKC and PKA, promotes the alternative direction, by decreasing positive feedback on PFK1, and subsequently pushes carbons into biosynthetic precursor pathways, such as PPP. TIGAR is a related protein that carries a PFKFB domain with increased FBPase activity.

### 3.4.5 Serine and glycine biosynthetic pathway

Another branch diverting from glycolysis, and recently implicated in cancer cell metabolism, is the serine biosynthesis pathway, which converts glycolytic metabolite 3-phosphoglycerate (3PG) into serine (Figure 4, 13). Serine is a non-essential amino acid and can provide fuel for the synthesis of other amino acids, like cysteine and glycine, folates, phospholipids and nucleotides. During the conversion of 3PG to serine, one of the intermediate steps, the conversion of 3-phospho-hydroxypyruvate to phosphoserine, by phosphoserine-aminotransferase 1 (PSAT1), also produces another key metabolic intermediate, α-KG, from glutamate breakdown. α-KG can then enter the TCA cycle and exported for energetic or biosynthetic pathways, such as lipid synthesis and NADPH production (Snell 1984; Possemato et al. 2011; King et al. 2012).

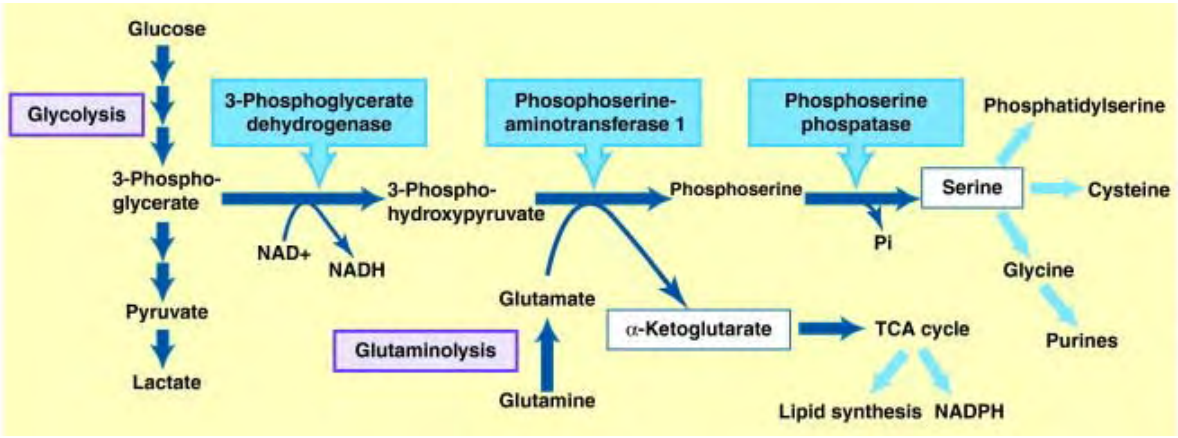



**Figure 13. Overview of the serine pathway in cancer cell metabolism.** The serine biosynthetic pathway converts glycolytic intermediate, 3-phosphoglycerate, to serine through a three-step process involving enzymes 3-phosphoglycerate dehydrogenase, phosphoserine-aminotransferase 1 and phosphoserine phosphatase, which produce NADH and convert glutamate to α-KG in the process. Serine is then incorporated in the production of the phospholipid, phosphatidylserine, and amino acids, cytseine and glycine, the latter of which is involved in purine biosynthesis. Figure adapted from Schulze and Harris (2012).

The three components of the serine biosynthesis pathway are often overexpressed in cancers, but especially phosphoglycerate dehydrogenase (*PHGDH*), which encodes the enzyme involved in the first step in catalyzing 3PG towards serine biosynthesis (Snell et al. 1988; Locasale and Cantley 2011; Pollari et al. 2011; Possemato et al. 2011). *PHGDH* is amplified in 16% of melanomas and 6% of breast cancers, while elevated in 70% of estrogen receptor-negative breast cancers and associated with a poor percentage of five-year overall survival (Possemato et al. 2011). Furthermore, PHDGH-dependent cancers, like melanoma and breast cancer, use the serine biosynthesis pathway for up to 50% of net α-KG production from glutamate and shuttle a significant portion of glucose through the serine pathway compared to non-dependent cancers (Locasale and Cantley 2011; Possemato et al.

2011). Additionally, shRNA depletion of PHDGH in ER-negative breast cancer and melanoma cells dependent on PHDGH decreased cell growth, while overexpression in MCF10α human breast epithelial cells caused morphological changes that resemble breast cancer oncogenic transformations (Locasale and Cantley 2011; Possemato et al. 2011).

In addition to melanoma and breast cancer cells, another group has shown that cancer stems cells from non-small cell lung cancer also show increased expression of genes, like glycine decarboxylase (*GLDC*) involved in serine and closely associated glycine pathways (Zhang et al. 2012; Pecqueur et al. 2013). Subsequent silencing of *GLDC* decreased tumor characteristics, while overexpression of this gene transformed NIH 3T3 cells *in vitro* while increasing tumor formation *in vivo* (Zhang et al. 2012; Pecqueur et al. 2013).

### 3.4.6 Pyruvate kinase 2

Pyruvate kinase (PK) appears to play a key role in allowing highly proliferative cancer cells to produce both energy and macromolecules. PK is the final enzyme of the glycolytic pathway that converts phosphoenolpyruvate (PEP) into pyruvate with the production of ATP. PK exists in four forms, which include L, R, M1 and M2. PKL is found mainly in the liver and kidney, while PKR is mainly in erythrocytes. PKM1 is the form found in most adult tissues, while PKM2, normally expressed in embryonic and rapidly dividing cells (Gupta et al. 2010), has recently been implicated in tumor development (Christofk et al. 2008). Selective expression of PKM1 or PKM2 is due to alternative splicing, as the two enzymes are located on the same gene. Expression of PKM2 is mediated by three heterogeneous nuclear ribonucleoproteins, hnRNP1/A1/A2, which are under the regulation of c-Myc (David et al. 2010).

PKM2 activity is determined by its conformation, which is regulated by the binding of allosteric and signaling molecules that induce protein modifications (Gupta et al. 2010). Allosteric molecules that increase PKM2 activity include some metabolic intermediates, such as fructose-2,6-bisphosphate and fructose-1,6-bisphophate (F-1,6-BP), which promote their consumption through the glycolytic pathway (Gupta et al. 2010). On the other hand, PKM2 can be controlled post-translationally by phosphorylation at the tyrosine residue 105, which is mediated by fibroblast growth factor receptor type 1. Studies show that this phosphorylation can actually disrupt allosteric activation by F-1,6-BP (Hitosugi et al. 2009). In human lung cancer cells, PKM2 activity is also decreased through oxidation of Cys358 due to increases in intracellular ROS production (Anastasiou et al. 2011). This decreased activity pushes glycolytic intermediates into the PPP to promote the generation of antioxidants to combat the oxidative stress.

The increased expression of PKM2 in cancer cells is interesting considering that PKM1 is actually the more efficient of the two, as it is constitutively active and induces high pyruvate

conversion. PKM2, on the other hand, has either high or low activity, and can thereby respond to the needs of a cancer cell. Low activity of PKM2 is useful for cancer cells, because it regulates metabolism to use glycolytic intermediates for biosynthetic pathways, rather than energy production. However, the ability for PKM2 to change into its high activity state allows cancer cells to switch to high glycolytic flux if glycolytic ATP production is necessary, such as in severe hypoxia where oxidative phosphorylation of alternative carbon sources, like glutamine, is not possible. Additionally, HIF-1α shifts PKM2 into its high activity form through increased mTOR activity (Sun et al. 2011) and PKM2 can actually relocate into the nucleus to promote *HIF-1α* transcription through direct binding (Luo et al. 2011). Therefore, it is likely that the activity of PKM2 depends on cellular needs, in which low PKM2 activity supports biosynthetic growth, but can be balanced by HIF-1α activity to promote glycolysis, as summarized in Figure 14.

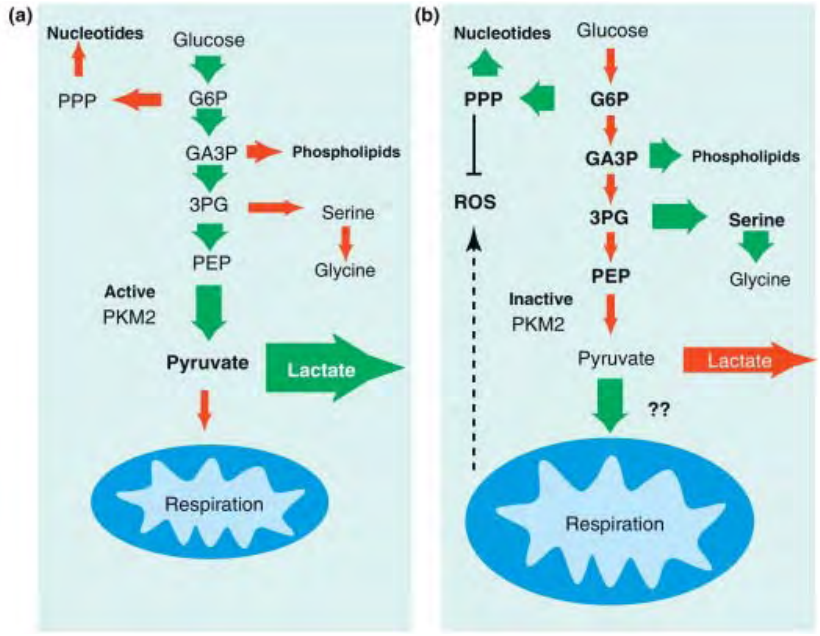



**Figure 14. The effect of PKM2 activity on glycolytic metabolism in cancer cells.** (A) Active PKM2 results in high conversion of PEP to pyruvate and subsequently leads to high overall glycolysis and lactate production with decreased production of biosynthetic precursors, such as nucleotides, phospholipids and amino acids. (B) Inactive PKM2, however, leads to a block in the conversion of PEP to pyruvate in parallel with an overall decrease in glycolysis and lactate production and an increase in production of biosynthetic precursors, including nucleotides, phospholipids and serine as well as antioxidants to combat ROS production. Its effect on oxidative phosphorylation and entrance of pyruvate into the TCA cycle, however, is still unknown. Figure adapted from Chaneton and Gottlieb (2012).

When PKM2 is expressed, and especially in its low state, glycolytic intermediates, like phosphoenolpyruvate (PEP) accumulate in the cytosol. Accumulation of PEP causes negative feedback on triose phosphate isomerase (TPI). This inhibition leads to equal conversion of F-1,6-BP to dihidroxyacetone phosphate (DHAP), by aldolase, as well as glyceraldehyde-3-phosphate (GA3P).

DHAP feeds glycerol formation for triglyceride synthesis and GA3P can be shunted through the PPP for nucleotide biosynthesis (Gruning et al. 2010). Converting GA3P into its downstream components of 1,3-BPGA and then 3PG sustains serine biosynthesis, as 3PG is shunted off into this pathway (Lincet et al. 2012). However, an alternative method of converting intermediates to pyruvate was discovered that could keep PKM2 activity low while continuing to support glycolysis for energy production. Rather than a direct conversion of PEP to pyruvate, PEP can be converted to pyruvate through the transfer of the phosphate group from PEP to the glycolytic protein phosphoglycerate mutase 1 (PGAM1), which increases its mutase activity while also providing a supply of pyruvate and eliminating the intermediate backup (Vander Heiden et al. 2010; Hitosugi et al. 2012). This redirection is completely independent of PK activity, which means the production of pyruvate does not produce ATP as a byproduct. This is an important point, because ATP generation leads to negative feedback loops on upstream glycolytic steps. Additionally, pyruvate can be obtained from alanine by transamination or from malate decarboxylation by malic enzyme (ME) (DeBerardinis et al. 2008), which will be discussed in an introductory section, “Pyruvate and the TCA cycle” on page 44. Altogether, this data provides a significant rationale for expressing the less efficient PKM2 isoform as it allows the dissociation between ATP production and anabolic production of biosynthetic precursors required for rapid proliferation in cancer cells.

Finally, the most recent study of PKM2 showed that upon endothelial growth factor receptor activation, PKM2 directly binds to and phosphorylates histone 3 at threonine 11. This phosphorylation dissociates HDAC3 from the *CCDN1* and *MYC* promoter regions, which is required for acetylation of histone H3 at K9, and thereby increases expression of cyclin D1 and c-Myc and promotes tumor cell proliferation, cell-cycle progression and brain tumorigenesis (Yang et al. 2012). Research has shown that knockdown of PKM2 expression using RNA interference can increase apoptosis *in vitro* and limit tumorigenesis *in vivo* (Goldberg and Sharp 2012), which could be especially effective against cancer cells in severe hypoxic environments that actually depend on high PKM2 activity for ATP production. On the other hand, overactivation of PKM2 could also be useful for inducing oxidative damage by ROS in cancer cells existing in only slightly hypoxic or normoxic conditions. Thus, the therapeutic potential of PKM2 exists, but is dependent on multiple factors, including microenvironment and energetic needs of the cell. Overall, it is evident that PKM2 has multiple metabolic and signal regulatory functions that promote tumorigenesis. Yet, it is likely that we have only just begun to breach the extent of changes PKM2 elicits in cancer cells.

### 3.4.7 Lactate metabolism

Many cancer cells are characterized by an overproduction of lactate, which can come from pyruvate produced by glycolysis but also other sources, such as the conversion of TCA cycle intermediates (malate) to pyruvate, proteolysis or glutaminolysis. For lactate overproduction, much evidence points to the lactate dehydrogenase (LDH) family and more specifically HIF-1α target, LDHA, which converts pyruvate to lactate and reestablishes NAD+ to maintain the glycolytic flux. Inhibition of LDHA by FX11 reduces ATP levels, increases oxidative stress and inhibits human lymphoma and pancreatic cancer xenograft progression (Le et al. 2010).

Lactate must also be exported from the cell, which leads to microenvironment acidification that favors tumor invasion, suppresses immune effectors of normal cells (Stern et al. 2002; Gatenby and Gawlinski 2003; Fischer et al. 2007; Swietach et al. 2007; Robey et al. 2009) and leads to tumor recurrence and poor survival (Walenta et al. 2000). Cells export lactate using the monocarboxylate transporter 4 (MCT4), also regulated by HIF-1α (Ullah et al. 2006). MCT4 is overexpressed in renal cancers whereas depletion of MCT4 leads to accumulation of lactate, internal acidification and cell death (Gerlinger et al. 2012). Acidification of the microenvironment also occurs due to excretion of protons, which restrict ribosomal biogenesis (Mekhail et al. 2006) and lead to cell cycle arrest if retained within the cell (Smallbone et al. 2007). Protons can be transported by systems like the V-ATPase (Forgac 1989), sodium-proton exchanger 1 (NHE1) (Shimoda et al. 2006) and carbonic anhydrase IX (CAIX) (Wykoff et al. 2000). These groups also suggest that the three proton export systems are implicated in oncogenic activity, especially because NHE1 and CAIX are HIF-1α targets. A summary of export mechanisms can be found in Figure 15.

Finally, there is evidence that lactate produced by tumors can be taken up and oxidized by stromal cells (Koukourakis et al. 2006), providing oxygenated areas to hypoxic tumors (Sonveaux et al. 2008). Additional groups have shown that oxygenated cancer cells can also take up lactate with MCT1 and use this lactate to produce pyruvate to feed the TCA cycle, which can produce 18 ATP per lactate molecule (Semenza 2008b; Sonveaux et al. 2008; Feron 2009; Porporato et al. 2011; Dhup et al. 2012). The use of lactate as a respiratory substrate for oxygenated cells saves glucose for hypoxic cells and allows for maximal cell cooperation and adaptation to the microenvironment (Laconi 2007; Cardenas-Navia et al. 2008; Semenza 2008b; Sonveaux et al. 2008; Dhup et al. 2012) (Figure 16).

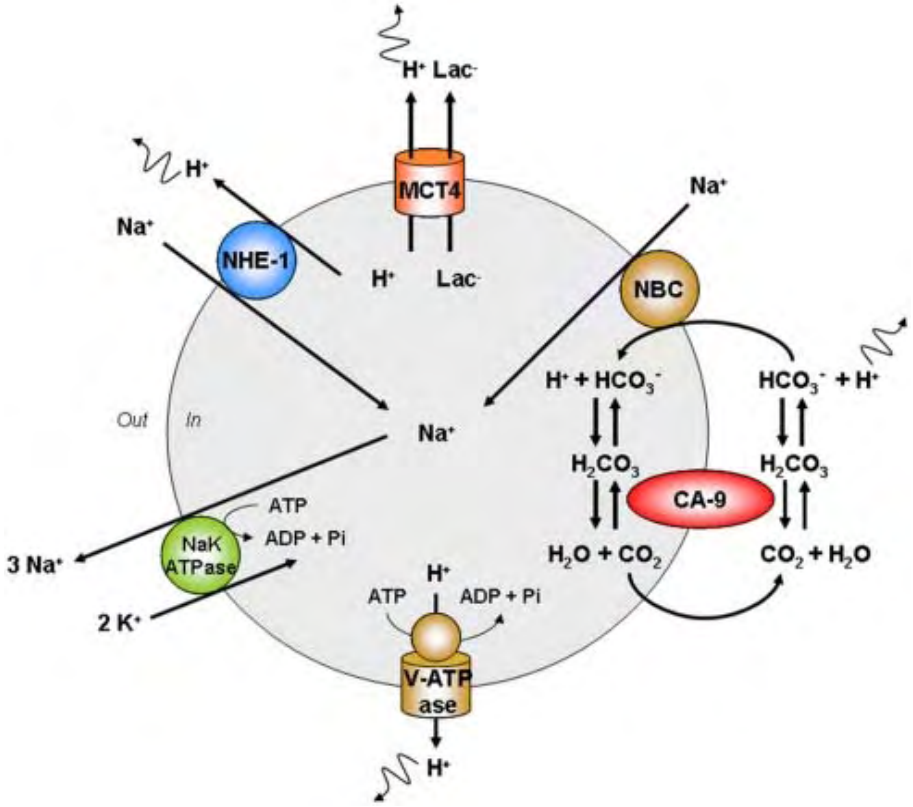



**Figure 15. Overview of lactate and proton export.** Lactate can be exported from cancer cells via MCT4 while harmful protons can be exported through NHE-1, V-ATPase and CA-9. These exports are also balanced by ion exchange through Na-K ATPases and NBC. Figure adapted from Porporato et al. (2011).

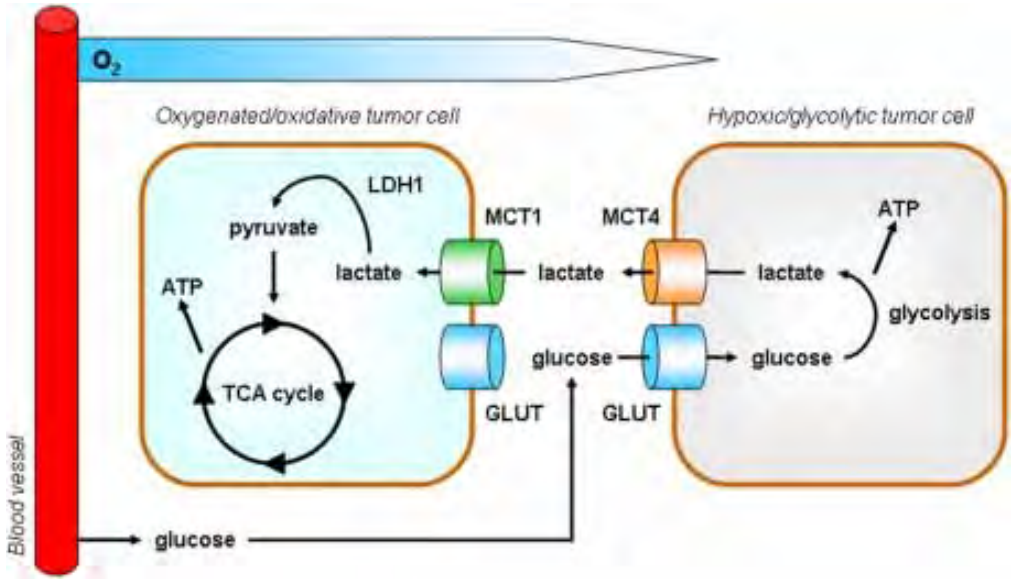



**Figure 16. Symbiotic use of lactate as an oxidizable fuel in tumor cells.** Oxidative tumor cells can import lactate produced by hypoxic or glycolytic tumor cells as a fuel to produce pyruvate and feed the TCA cycle, which saves glucose for less-oxidative tumors. Figure adapted from Porporato et al. (2011).

### 3.4.8 Pyruvate and the TCA cycle

Up to this point, we have primarily discussed glycolysis, which is responsible for the production of a majority of macromolecules required for rapidly proliferating cancer cells. However, while most pyruvate from glycolysis is converted to lactate by LDHA, there is a small percentage that will enter the mitochondria and continue through the TCA cycle, summarized in Figure 4, to supply energy, produce biosynthetic precursors, including amino acids, and function as a reducing agent of NADH. While the percentage of total pyruvate that enters the TCA cycle is low, the high glycolytic flux of cancer cells provides substantial amounts of mitochondrial pyruvate. Thus, we must examine the role mitochondria play in cancer cells as related to production of energy for both survival and continued biosynthetic production.

Once pyruvate enters the mitochondria, it can be converted to either oxaloacetate (OAA) by pyruvate carboxylase (PC) or converted to acetyl-CoA by pyruvate dehydrogenase (PDH). Acetyl-CoA will then condense with OAA to generate citrate. While cells operating under more "normal" metabolism will continue pushing citrate through the complete TCA cycle, many cancer cells export this metabolite to the cytosol where it is converted back to acetyl-CoA by ATP citrate lyase (ACLY) and then consumed for lipid and amino acid biosynthesis. This has been proven by the fact that ACLY inhibition in lung, pancreatic and ovarian cancer cell lines decreases glucose-dependent lipid synthesis and impairs cell proliferation (Hatzivassiliou et al. 2005).

The loss of citrate for lipid synthesis results in a net loss of TCA intermediates, which need to be replenished by alternate sources to keep the TCA cycle viable. Once source of pyruvate is alanine aminotransferase (ALAT), which produces glutamate and pyruvate. Additionally, when citrate is reconverted to acetyl-CoA in the cytosol by ACLY, OAA is reproduced and can be converted to malate by malate dehydrogenase (MDH), also producing NAD+. Malate can re-enter the mitochondria through the malate-aspartate shuttle or it can be transformed into pyruvate by malic enzyme (ME), also producing NADPH (DeBerardinis et al. 2008). In hypoxic cancer cells, however, it is likely that the malate-aspartate shuttle is unprimed, as mitochondrial function is decreased, and that the conversion of malate to pyruvate represents a large source of lactate. In normoxia, it appears that the TCA cycle is replenished by glutamine, which can be oxidized to produce α-KG to be metabolized by the TCA cycle (DeBerardinis et al. 2007). This alternative source also helps to explain why cancer cells require glutamine for proliferation (Griffiths and Keast 1990).

In cancer cells, activity of pyruvate dehydrogenase (PDH), the enzyme converting pyruvate to acetyl-CoA, can also help explain both the need for other sources of TCA intermediates and the uncoupling of glycolysis and mitochondria. PDH activity is primarily controlled by pyruvate dehydrogenase kinase (PDK). When activated, PDK will phosphorylate PDH at the α subunit of the E1 complex to inhibit the activity of PDH, which can be reversed by decreasing PDK activity

(Korotchkina and Patel 2001). PDK is regulated by phosphorylation but also by allosteric molecules. PDK is stimulated by acetyl-CoA, ATP and NADH, but inhibited by CoA-SH, pyruvate, alanine, ADP and NAD+ (Sugden and Holness 2003). The activity of PDK is typically higher in cancer cells compared to normal cells, which suggests that PDH activity, and the conversion of pyruvate to acetyl-CoA, is low in cancer cells (Board et al. 1990). Additionally, PDK1 is regulated by HIF-1α (Koukourakis et al. 2005; Kim et al. 2006) and has increased activity in hypoxic cancer cells to help decrease mitochondrial ROS production and support the switch to glycolytic metabolism. The importance of the link between PDK and HIF-1α is evidenced by the fact that overexpression of PDK1 in HIF-1α null cells in the presence of hypoxia is enough to rescue these cells from hypoxia-induced apoptosis, while increasing ATP levels and decreasing ROS (Kim et al. 2006). This is a key study to show that hypoxia, and HIF-1α, play a direct role in the metabolic switch from mitochondrial to glycolytic metabolism. This kinase also offers therapeutic potential as it can be inhibited by dichloroacetate (DCA) to increase mitochondrial ROS production and shift metabolism back to the mitochondria (Michelakis et al. 2008).

### 3.4.9 Glutaminolysis

Glutamine plays an extremely important role in the oxidative mitochondrial metabolism of proliferating cells and cancer cells. Without it, cancer cell lines could not support the Warburg effect or maintain high rates of proliferation (Eagle 1956; Kovacevic and Morris 1972). Under oxidative stress, glutamine can be directly converted into antioxidant, GSH, by glutathione cysteine ligase (GCL) (Wise and Thompson 2010). However, most glutamine is taken up into mitochondria from which it is converted by glutaminase (GLS) into glutamate and then transformed by glutamate dehydrogenase (GDH) into α-KG (Figure 4). Through α-KG, glutamine plays many important roles in the cell. Some cancer cells actually generate a significant proportion (50%) of ATP by completely oxidizing α-KG in the TCA cycle. Lymphoma cells, for example, are able to fully support ATP production from the TCA cycle even in the absence of glucose (even in c-Myc cells) or under hypoxia (Le et al. 2012). In lymphoma cells and proliferating glioblastoma cells, $^{13}C$ labeling of glutamine and glucose showed that glutamine is the major source of oxaloacetate (OAA) while glucose is the predominant source of acetyl-CoA in the TCA cycle (Portais et al. 1993; Portais et al. 1996; DeBerardinis et al. 2007). Interestingly, the role of glutamine in supporting TCA cycle function is essential for glutamine-addicted cancer cells, such as those expressing c-Myc. These cells die following glutamine withdrawal, but can be rescued with the addition of TCA cycle intermediates like pyruvate, OAA or α-KG (Yuneva et al. 2007; Wise et al. 2008; Gao et al. 2009). Functioning as a source of OAA, glutamine fuels citrate metabolism, fatty acid synthesis and general lipid synthesis (Hatzivassiliou et al. 2005).

Once glutamine is converted to α-KG in the mitochondria, α-KG can undergo a mechanism known as reductive carboxylation, in which IDH1 converts α-KG to isocitrate, producing NADP+, and isocitrate is then converted to citrate (Scott et al. 2011; Wise et al. 2011; Metallo et al. 2012; Mullen et al. 2012). This pathway is especially important under hypoxia or mitochondrial dysfunction, as cells depend on reductive metabolism of α-KG to produce acetyl-CoA and support lipogenesis (Scott et al. 2011; Wise et al. 2011; Metallo et al. 2012; Mullen et al. 2012). In fact, while many cancer cell types, like proliferating glioblastoma cells, depend on glutamine as the major source of OAA and glucose for acetyl-CoA, during hypoxia, glutamine is responsible for up to 80% of acetyl-CoA for lipogenesis (Metallo et al. 2012). Interestingly, HIF-1α can drive reductive carboxylation even under normoxic conditions (Wise et al. 2011; Metallo et al. 2012), but this has to be considered in relation to c-Myc, which drives the oxidation of glutamine even under hypoxia (Le et al. 2012). When citrate levels are high, reductive carboxylation can also work in reverse where citrate can produce isocitrate and eventually α-KG. The stock of α-KG can either feed the TCA cycle or be diverted to produce aspartate by aspartate aminotransferase (AAT) or to produce glutamate and pyruvate from alanine aminotransferase (ALAT) (DeBerardinis et al. 2007; DeBerardinis et al. 2008).

Glutamine carbon can also be exported from the mitochondria as a carbon source. For instance, glutamine can be exported as malate and subsequently converted to pyruvate by malic enzyme, also producing NADPH, and eventually to lactate (DeBerardinis et al. 2007). This occurs in parallel with the portion of malate not sent through the malate-aspartate shuttle following conversion of OAA to malate by malate dehydrogenase (MDH). Additionally, the pyruvate produced from glutamine can re-enter the TCA cycle to produce acetyl-CoA and form another direct source for lipid synthesis (Wellen et al. 2010; Le et al. 2012). The subsequent production of NADPH from glutamine is extremely important to cancer cells. NADPH maintains the antioxidant pool of reduced glutathione (GSH) and prevents ROS damage. Additionally, NADPH is crucial for maintaining the cytochrome c redox state of mitochondria and thereby preventing apoptosis (Vaughn and Deshmukh 2008). NADPH is also required for fatty acid, amino acid and nucleotide biosynthesis (Eagle 1956; Feron 2009; Gruning et al. 2010).

Glutamine also serves as a nitrogen source by providing amido and amino groups for the synthesis of non-essential amino acids, nucleotide biosynthesis, hexosamines and ammonia (DeBerardinis et al. 2007). Just as previous groups showed the supplementation of TCA cycle intermediates in glutamine-addicted and c-myc driven cells rescues viability, other groups have shown that supplementing glutamine-starved Hep3B cells with nitrogen sources like alanine or asparagine could rescue cell proliferation (Meng et al. 2010). Supplementation of both alanine and α-KG together produced a synergistic rescue effect (Meng et al. 2010). Additionally, glutamine-deprived K-Ras transformed fibroblasts exhibit reduced cell proliferation and cell cycle, which can be restored by adding deoxyribonucleotides (Gaglio et al. 2009). Finally, some groups have shown that

transamination, rather than glutamine uptake from the media, is the main source of glutamine-derived carbon (Moreadith and Lehninger 1984). While glutamine inhibition at the transporter level, such as inhibition of SLC1A5 by L-γ-glutamyl-p-nitroanilide (GPNA), can reduce glutamine uptake and suppress glutamine-dependent mTOR activation (Nicklin et al. 2009) it may also have a toxic effect on non-cancerous cells. Thus, an alternative option is inhibition of aminotransferase to induce cytotoxicity in cancer cells without effecting whole-organism glutamine metabolism.

### 3.4.10 Citrate metabolism

Once citrate is produced and relocated from the mitochondria to the cytosol, citrate can be restored into OAA and acetyl-CoA by ACLY (Figure 4). Acetyl-CoA feeds fatty acid synthesis, while OAA is converted into pyruvate through malate, as described above. Elevation of ACLY expression and activity has been reported in lung, prostate, bladder, breast, liver, stomach and colon tumors (Halliday et al. 1988; Varis et al. 2002; Turyn et al. 2003; Yancy et al. 2007; Migita et al. 2008; Zaidi et al. 2012). In human lung adenocarcinoma, phosphorylated ACLY expression correlates to the stage, differentiation grade, and poor prognosis (Migita et al. 2008). In cancer cells, phosphorylation and activity of ACLY can be indirectly and directly regulated by AKT. Indirectly, AKT can upregulate ACLY mRNA levels *via* activation of SREBP-1, a protein involved in cholesterol and fatty acid synthesis. However, in most cancer cells, ACLY protein levels are independent of SREBP-1 and it is more likely that AKT itself directly phosphorylates ACLY (Sato et al. 2000; Migita et al. 2008). Despite this data, lung cancer cells treated with PI3K inhibitors did not show significant decreases in ACLY (Migita et al. 2008), thus this enzyme could be regulated by other pathways and kinases, such as nucleoside diphosphate kinase (Wagner and Vu 1995) and cyclic AMP-dependent protein kinase (Pierce et al. 1981).

ACLY inhibition provides an effective target for cancer therapy. Some pharmacological inhibitors of ACLY, such as SB-204990, have anti-proliferative effects on lung cancer cells, both *in vitro* and *in vivo* (Hatzivassiliou et al. 2005). However, this therapy is still new and quite limited, requiring further study of existing inhibitors and discovery of new, more efficient inhibitors. What seems to be more effective is the use of RNA interference (RNAi) knockdown of ACLY, which can result in growth arrest in tumor cells, both *in vitro* and *in vivo* (Hatzivassiliou et al. 2005; Migita et al. 2008). These anti-proliferative effects are due to a cell cycle arrest (Hatzivassiliou et al. 2005; Migita et al. 2008) and apoptosis (Hanai et al. 2012). The chronic myelogenous leukemia cell line, K562, was also shown to undergo erythroid differentiation in response to ACLY inhibition (Hatzivassiliou et al. 2005). An interesting new study has also suggested that the anti-proliferative activity of ACLY inhibition correlates to the glycolytic phenotype of the tumor. Cancer cells displaying a high rate of glucose metabolism are more severely affected by ACLY inhibition, whereas those displaying a low rate of aerobic glycolysis are unaffected (Hatzivassiliou et al. 2005), allowing patient stratification.

The breakdown of OAA to pyruvate regenerates NAD+, which is crucial for the continued function of glycolysis, but also for poly ADP-ribose polymerase (PARP) function, which participates in nucleotide synthesis (Gruning et al. 2010). Additionally, the increased mitochondrial citrate synthase activity increases flux towards lipid and triglyceride synthesis while repressing ketone body formation, which is observed in many cancers (Schlichtholz et al. 2005). The large supply of acetyl-CoA provides increased lipid synthesis due, in part, from increasing activity of acetyl-CoA carboxylase (ACC), which is the first enzyme involved in fatty acid synthesis. One result of this favored route is decreased β-hydroxybutyrate, which is a histone deacetylation (HDAC) inhibitor, thus allowing increased HDAC activity. The level of acetyl-CoA produced from citrate also seems to regulate histone acetyltransferase (HAT) activity (Rice et al. 2007). Together, HDAC and HAT activity can target regions of chromatin to regulate specific gene transcription (Sterner and Berger 2000; Kurdistani and Grunstein 2003; Li and Greene 2007), and especially genes involved in glucose metabolism, including *HK-II*, *PFK1* and *LDHA* (Wellen et al. 2009). These effects may be downstream of ATP-citrate lyase (ACLY) activity, as most cytosolic acetyl-CoA comes from citrate exported from mitochondria in a reaction catalyzed by ACLY. In support of this hypothesis there was a profound defect in histone acetylation and expression of genes regulating glucose metabolism in cells where ACLY was knocked down (Wellen et al. 2009).

Finally, citrate plays an extremely important role in regulating different metabolic pathways, a summary of which can be found in Figure 17. High citrate production leads to negative feedback on glycolysis and the TCA cycle while stimulating glucagon and lipid synthesis. More specifically, citrate can completely inhibit PFK1 (Lehninger 1975) and PFK2 (Chesney 2006), the latter of which is responsible for producing F-2,6-BP, an allosteric activator of PFK1 in cancer cells. This could be one explanation for the fact that many cancer cells have constitutive overexpression of PFK2, which would allow these cells to override the inhibition of PFK1 by ATP and citrate. By decreasing PFK1, citrate decreases the total level of F-1,6-P, which leads to inhibition of PKM2. Citrate also inhibits the TCA cycle by inhibiting SDH (Hillar et al. 1975) and by decreasing PDH activity, which decreases the conversion of pyruvate to acetyl-CoA (Taylor and Halperin 1973). Finally, citrate can inhibit fatty acid β-oxidation, because citrate production leads to fatty acid synthesis. The first step of fatty acid synthesis produces malonyl-CoA, which inhibits carnitine acyl transferase I (CPTI), a mitochondrial transporter of fatty acids for subsequent oxidation (Lehninger 1975; Paumen et al. 1997). Furthermore, excess malonyl-CoA can form malonate, which also inhibits SDH (Lehninger 1975).

Due to the regulatory capabilities of citrate, its level in cancer cells is extremely well balanced. Citrate levels are maintained high enough to promote cell growth, but also rapidly consumed by ACLY or the reverse production of α-KG by IDH1/2 to protect cancer cells from consuming ATP and biosynthetic precursors too quickly. Excessive citrate may therefore offer a novel therapeutic option as various cancer cells administered with 10 mM of citrate exhibited cell

growth inhibition and extremely high levels of cell death (Zhang et al. 2009c; Lu et al. 2011). Additionally, citrate sensitized cancer cells to chemotherapy agent, cisplatin (Zhang et al. 2009c).

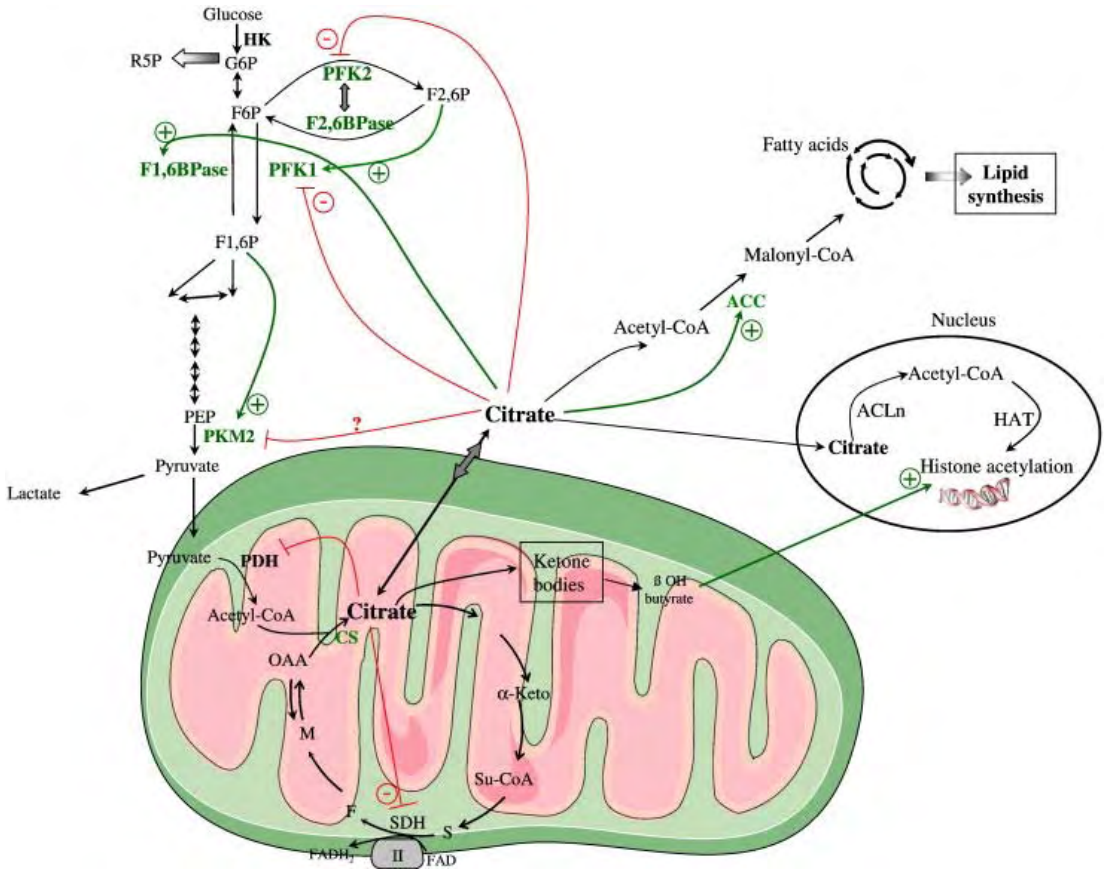



**Figure 17. The regulation of metabolism by citrate.** Citrate has numerous regulatory activities on cancer cell metabolism, including increased ACC activity to promote fatty acid metabolism, F-1,6-BPase activity to decrease the glycolytic flux and histone acetylation activity. Citrate will also negatively impact PDH and SDH to decrease the TCA cycle as well as PFK2, PFK1 and PKM2 to further decrease the glycolytic flux. Figure adapted from Icard et al. (2012).

### 3.4.11 Lipid & fatty acid metabolism

Most non-cancerous human cells use lipids acquired from food for the synthesis of new structural lipids, such as fatty acids, sphingolipids, phospholipids, cholesterol and isoprenoids (Figure 4). Cancer cells, however, have enhanced *de novo* fatty acid synthesis (FAS), which leads to fatty acid accumulation. These fatty acids can be oxidized for energy production or converted to triglycerides for storage or phospholipids for membrane production. In proliferating cells, fatty acids are directed especially towards lipid synthesis, which contributes to both membrane production and post-translational lipid modification of proteins (Bauer et al. 2005). Most of the acetyl-CoA that fuels this fatty acid synthesis comes from the citrate exported from mitochondria (DeBerardinis et al. 2007; Hoyos et al. 2012).

Fatty acid synthesis involves three enzymes, which include aforementioned ACLY as well as acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN). ACLY converts citrate to OAA and acetyl-CoA, the latter of which is converted by ACC to produce malonyl-CoA. FASN then catalyzes the NADPH-dependent condensation of acetyl-CoA and malonyl-CoA to produce long-chain fatty acids (Mashima et al. 2009). Tumor cells exhibit overexpression of all three enzymes. In particular, several human cancers, including lung, prostate, ovary, breast, endometrium and colon cancer exhibit overexpression of FASN as compared to non-cancerous cells (Sebastiani et al. 2004; Tsuji et al. 2004; Visca et al. 2004; Kawamura et al. 2005; Zhao et al. 2006; Knowles and Smith 2007; Grunt et al. 2009; Kearney et al. 2009; Migita et al. 2009; Ueda et al. 2010). This overexpression has been linked to tumorigenesis, tumor growth and poor survival (Kearney et al. 2009; Mashima et al. 2009). FASN overexpression also protects cells from death by inhibiting apoptotic pathways (Migita et al. 2009). High fatty acid synthesis in cancer cells is also promoted by the PI3K/AKT/mTOR pathway, which stimulates lipogenic gene expression by increasing nuclear localization of SREBP-1, a transcription factor that targets *ACLY*, *ACC*, *FASN* and *ME1* (DeBerardinis et al. 2008; Mashima et al. 2009).

While much data has been obtained on the preferential synthesis of lipids in cancer cells, the consumption of lipids by cancer cells remains to be studied. Fatty acid β-oxidation (FAO) is supposedly suppressed in tumor cells due to the fact that most proliferating cancer cells promote glycolysis and lipid synthesis, often due to PI3K/AKT activation, which inhibits β-oxidation (Bird and Saggerson 1984; Wang and Sul 1998). However, it is important to remember that cancer cells often carry out glycolysis for biosynthetic rather than energy production, with decreased PKM2 and PDH activity. Additionally, FAO produces acetyl-CoA, NADH and ATP, which also inhibit PDH and PKM2 (Lehninger 1975). Thus, it is possible that glycolysis and FAO could occur at the same time, with FAO providing acetyl-CoA for citrate synthesis while glycolysis produces pyruvate directed towards lactate production. Additionally, FAO can produce acetyl-CoA to be transformed into carnitine by carnitine palmitolytransferases (CPT), which happen to be upregulated in many cancers, such as lung cancer, and leads to increased FAO, ATP production and resistance to hypoxia and glucose deprivation (Zaugg et al. 2011). FAO can produce large amounts of ATP. For example, complete oxidation of palmitate, a common 16-carbon fatty acid, produces 106 molecules of ATP.

Cancer cells can obtain fatty acids by uptake from the extracellular media or by inducing lipolysis in adipose tissues (Tisdale 2007). In support of this, ovarian cancer cells can obtain fatty acids from surrounding adipocytes (Nieman et al. 2011) and FAO rescues cells from matrix-detachment and promotes anchorage-independent survival *in vitro* (Schafer et al. 2009). Leukemia cells can also be supported by stromal interactions that promote FAO (Samudio et al. 2008). AKT-transformed cells can use FAO in response to glucose-starvation to protect against apoptosis (Buzzai et al. 2005). Finally, fatty acids can also be obtained from within cells by neutral hydrolysation of triglycerides in cytoplasmic lipid droplets or acidic hydrolysation of lipids through a novel autophagy pathway, lipophagy (Singh and Cuervo 2012; Carracedo et al. 2013).

High lipid synthesis, however, directly inhibits β-oxidation because fatty acid synthesis produces malonyl-CoA, which inhibits mitochondrial import of fatty acids by CPT1. Thus, it is unknown whether FAO and FAS could occur at the same time. It is suggested that the preferential use of oxidation could be an important characteristic of cancer-initiating or resting cancer stem cells (Wang et al. 2011a; Le et al. 2012). In primary AML cells, inhibition of FAO decreased quiescent leukemic progenitor cells (Samudio et al. 2010). Ito et al (2012) also demonstrated that PML-driven FAO regulates HSC maintenance (Ito et al. 2012). Regardless, both fatty acid synthesis and oxidation offer therapeutic opportunities for specific targeting of cancer cell metabolism. Targeting ACC and ACLY diminishes cellular proliferation, tumor size and cellular viability (DeBerardinis et al. 2008; Zhang et al. 2009a; Beckner et al. 2010). FASN inhibitors, such as cerulenin, C75, C93 and orlistat, and siRNA increase apoptosis in several cancer cell lines (Zhao et al. 2006; Orita et al. 2007; Migita et al. 2009; Uddin et al. 2009). Inhibition of fatty acid oxidation in human leukemia cells through the loss of CPT1 by shRNA or etomoxir treatment also sensitized these cells to chemotherapy (Samudio et al. 2010). Inhibition of FAO also leads to palmitate-induced apoptosis in murine hematopoietic stem cell line, LyD9 (Paumen et al. 1997). However, to understand the maximum potential of such drugs would first require cancer stratification to determine the relative dependence on either synthesis or oxidation to determine the appropriate treatment. Altogether, these interesting discoveries encourage more research on fatty acid synthesis and oxidation in cancer cells for novel therapies.

### 3.4.12 Nucleotide biosynthesis

Nucleotide biosynthesis represents the unification of multiple metabolic networks producing individual components for a final product (Figure 4). For pyrimidine synthesis, aspartate is consumed by aspartate transcarbamylase in combination with carbamyl-phosphate derived from glutamine breakdown by carbamyl-phosphate synthase. Aspartate is derived from transamination of OAA or from L-asparagine derived from food and proteolysis by asparaginase, which is one of the main drugs used to treat ALL and certain non-Hodgkin lymphomas (van den Berg 2011). Glutamine, on the other hand, is derived from proteolysis, except under glutamine depletion where it can be synthesized from glutamate and ammonia by glutamine synthetase (GS) (Tong et al. 2009). For purine synthesis, R5P from the PPP is converted to 5-pyrophosphate (PRPP), which is an allosteric activator of the carbamyl-phosphate synthase. Glycine is derived from serine favored by 3-PG accumulation above the PKM2 bottleneck. Thymidine synthesis, which requires NADPH for dihydrofolate reductase functioning, is coupled with the folate cycle, which uses NADPH and serine (Nijhout et al. 2004). Ribonucleosides are transformed into deoxyribonucleosides by thioredoxine, also consuming NADPH. All of these branches come together in the production of nucleotides for DNA synthesis and cell proliferation, a limiting step in cancer cell progression, which depends on cancer metabolism.

# 4 Oncogenic signaling pathways regulate metabolic reprogramming

## 4.1 Hypoxia and hypoxia-inducible factors

Reduced oxygen availability, also known as hypoxia, is a metabolic stress experienced by many types of cancer cells and results in a metabolic adaptation of increased anaerobic glycolysis and a subsequent increase in glucose consumption and lactate production. The major player that carries out this metabolic switch is hypoxia-inducible factor, HIF-1α, which is a transcription factor complex that is activated by oxidative stress (Gordan and Simon 2007; Semenza 2007). Under normoxia, and in non-cancerous cells, HIF-1α accumulation is suppressed through modification by prolyl hydroxylation, which leads to ubiquitination by the von Hippel-Lindau (VHL) tumor suppressor and subsequent degradation. Under low oxygen, prolyl hydroxylation of HIF-1α is reduced, resulting in HIF-1α stabilization, increased HIF-1α transcription and HIF-1α association with HIF-1β, which is ubiquitously expressed in all cells, including non-cancerous cells (Martin-Puig et al. 2004; Semenza 2012) (Figure 18).

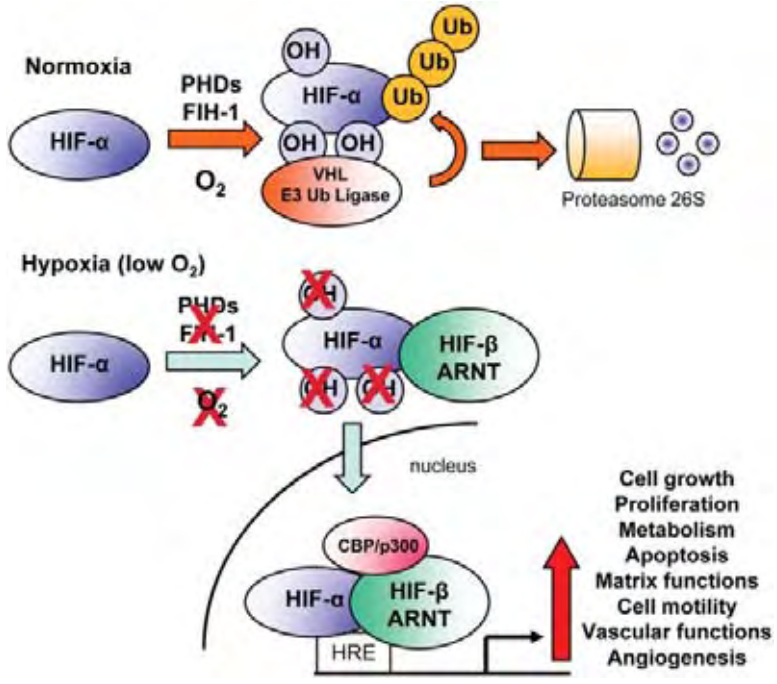



**Figure 18. Regulation of HIF-1α by oxygen levels.** Under normoxic conditions, HIF-α subunits are polyubiquitinated at 2 proline residues within the oxygen-dependent degradation domain (ODDD) by a family of enzymes known as prolyl hydroxylases (PHDs). This promotes recognition by the VHL E3 ubiquitin ligase complex and subsequent degradation of HIF-α via the 26S proteasome. In addition, hydroxylation of a C-terminal asparagine residue of HIF-α by factor-inhibiting HIF-1 (FIH-1) prevents binding of cofactors required for HIF activity. Hypoxia inhibits the activity of the PHD and FIH-1 enzymes, allowing HIF-α proteins to escape recognition by VHL, stabilize, and translocate to the nucleus. There, they dimerize with HIF-1β/ARNT and bind hypoxia response elements (HREs) within the promoters of target genes. Together with the co-activator proteins p300 and CBP, the HIF complex activates the transcription of a panel of genes required for the response to hypoxia. Figure adapted from Krock et al. (2011).

HIF-1α can play a major role in cancer cells, due to increased intratumoral hypoxia, which is associated with a larger risk of local spread, metastasis, treatment failure and patient morality (Gort et al. 2008; Semenza 2008a; Heddleston et al. 2010), but also because HIF-1α in cancer cells can be stabilized under normoxic conditions to promote the metabolic switch. In fact, HIF-1α is overexpressed in a broad range of human cancer types and this activity is often associated with increased tumor aggressiveness and therapeutic resistance (Weidemann and Johnson 2008; Semenza 2010a). As described above in the section "Mitochondrial-related enzymatic mutations" on page 25, mutations in the SDH and FH families can also stabilize HIF-1α, because accumulation of succinate and fumarate following these mutations impairs α-ketoglutarate-dependent dioxygenases and allow for increased HIF-1α (Isaacs et al. 2005). Direct inhibition of the prolyl hydroxylase activity by endogenous 2-oxoacid oxaloacetate can also occur (Dalgard et al. 2004). Additionally, HIF-1α can be stabilized by increases in ROS, which also inhibit prolyl hydroxylase activity (Brunelle et al. 2005; Guzy et al. 2005; Selak et al. 2005). In fact, ROS from complex III can inactivate PHD2 and thereby stabilize HIF-1α (Chandel et al. 2000; Guzy et al. 2007). Mitochondrial sirtuin 3 (SIRT3) also regulates HIF-α through mitochondrial ROS production (Bell et al. 2011). Finally, the activation of AKT in cancer cells may also increase HIF-1α levels under normoxia through an mTORC1-mediated increase of HIF-1α RNA translation (Robey and Hay 2009).

HIF-1α stabilization leads to a multitude of metabolic changes in cancer cells, some of which have already been discussed throughout this introduction. The overall effect functions to uncouple glycolysis from mitochondrial oxidative phosphorylation to use glycolysis as the main source of ATP (Semenza 2003) (Figure 19). Hypoxia also decreases radiation-induced, but not TRAIL-induced, apoptosis in cancer cells (Weinmann et al. 2004). Most notably, HIF-1α controls the expression of approximately 450 genes involved in glycolysis, lactate production and extrusion, angiogenesis, metastasis and iron metabolism. In terms of metabolism, HIF-1α has control over glycolytic enzymes (HKI, HKII, PFK-1, ALDO-A, ALDO-C, PGK1, ENO-α, PKM2, PFKFB3), glucose transporters (GLUT1, GLUT3), and enzymes related to lactate production and extrusion (LDHA, MCT4) (Marin-Hernandez et al. 2009; Semenza 2010b; Zancan et al. 2010). HIF-1α activation of PKM2 also increases HIF-1α activity, as PKM2 can serve as a co-transcriptional activator of HIF-1α through enhanced binding (Luo et al. 2011; Luo and Semenza 2011).

Hypoxia also affects mitochondria by inducing the expression of pyruvate dehydrogenase kinases (PDK1/3), which are involved in the phosphorylation and subsequent inactivation of pyruvate dehydrogenases (PDH) that convert pyruvate to acetyl-CoA (Korotchkina and Patel 2001). Decreasing PDH activity decreases oxidative stress derived from mitochondrial metabolism and also favors lactate production along with regeneration of $NAD^+$. HIF-1α also activates mitophagy to degrade mitochondria (Hervouet et al. 2008; Mazure and Pouyssegur 2010). HIF-1α upregulates *miR-210* transcription, which inhibits expression of genes for subunits of complex I (*NDUFA4*), complex

II (*SDHD*) and complex IV (*COX10*) as well as the *ISCU1/2* genes that encode proteins involved in iron sulfur center synthesis, thus effecting both mitochondria and iron metabolism (Devlin et al. 2011). Additionally, HIF-1α promotes overexpression of UCP2 to help reduce ROS levels (Derdak et al. 2008; Samudio et al. 2009) and of anti-apoptotic proteins like Mcl-1 and Bcl-xL (Zhang et al. 2009b; Varin et al. 2010).

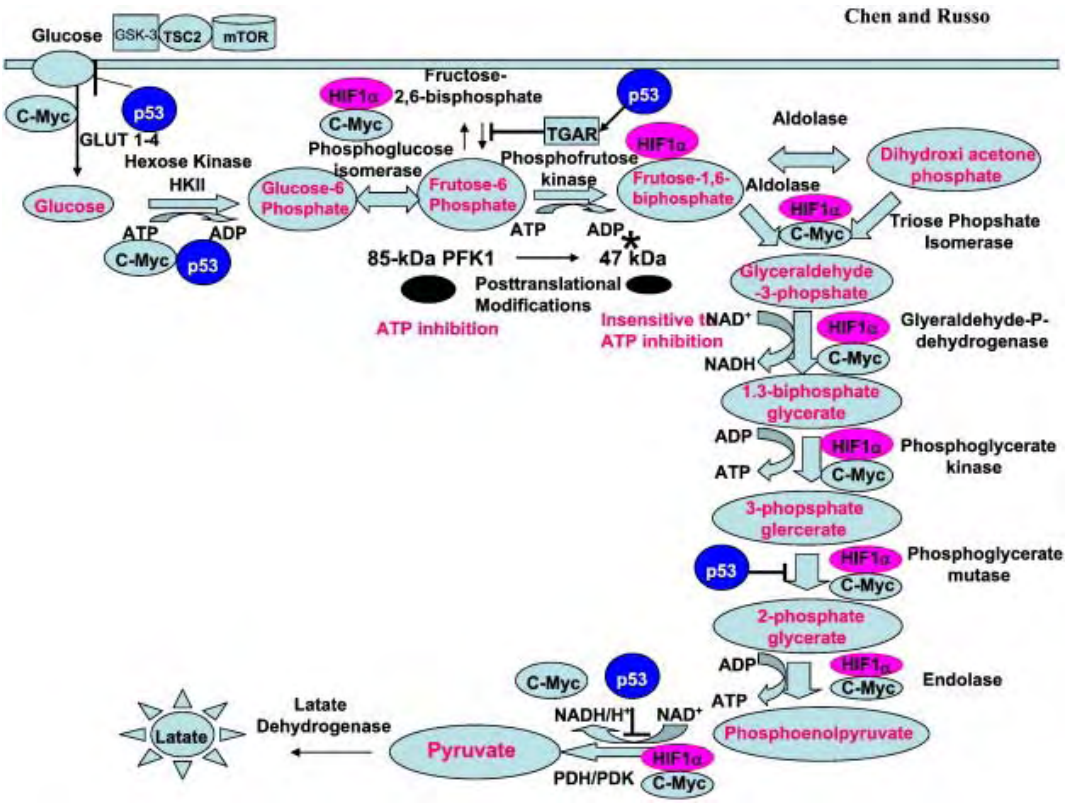



**Figure 19. Overview of p53, HIF-1α and c-Myc control of glycolytic metabolism.** p53, HIF-1α and c-Myc control glycolytic metabolism at several points through both transcriptional and direct regulation of key glycolytic enzymes involved in both glucose uptake and the glycolytic flux. Overall, HIF-1α and c-Myc induce a transcriptional program to support glucose metabolism, especially as an adaptation to a hypoxic environment. p53, however, manipulates glycolytic metabolism under times of oxidative stress and DNA damage to promote flux through the pentose phosphate pathway, leading to antioxidant formation and nucleotide biosynthesis. Figure adapted from Chen and Russo (2012).

The inhibition of HIF-1α in cancer cells could be an effective therapeutic strategy. Inhibition of HIF-1α inhibits glycolysis and promotes oxidative phosphorylation in tumor cells, leading to decreased proliferation (Semenza 2003; Welsh et al. 2004). Cardiac glycosides have been shown to inhibit HIF-1α translation, which reduces expression of HIF-1α target genes like *HK* and *GLUT* (Rapisarda et al. 2002; Rapisarda et al. 2004; Madhok et al. 2011). In mouse lymphoma and human AML cells, HIF-1α was selectively activated in normoxia (Wang et al. 2011b). More specifically, HIF-1α transcription levels were highest in AML leukemic stem cell population, CD34+CD38- cells. When targeted with specific HIF-1α inhibitor, echinomycin, mouse lymphoma and human AML

xenografts were eradicated through the elimination of cancer stem cells (Wang et al. 2011b). Similar *in vivo* experiments were performed with PR104, which is a drug that is active only in hypoxia and targets DNA cross-linking of hypoxic cells. NOD-SCID mice injected with 2 different ALL patient samples had significantly delayed onset of ALL in response to PR104 (Wellmann et al. 2004). The drug was also tested against human AML cells and was found to prolong survival, decrease circulating human CD45 positive cells to an almost undetectable level and significantly decrease tissue-infiltrating leukemic cells. This correlated with less hypoxic areas in the bone marrow of treated *versus* vehicle mice (Wellmann et al. 2004).

In some cases, direct targeting of HIF-1α eradicated in cancer cells is not possible or is too toxic due to downstream targets of HIF-1α required even in non-cancerous cells. However, it is possible to target HIF-1α without severe toxicity through targeting downstream of HIF-1α, which includes metabolic targets. Therapeutic treatment of these metabolic targets has already been covered in earlier sections. Additionally, many anticancer drugs used for one action also inhibit HIF-1α either as part of or in addition to their known function. For example, proteasome inhibitor, bortezomib, and mTOR inhibitors, such as rapamycin, temsirolimus and everolimus, block translational activity, which includes that of HIF-1α (Laughner et al. 2001; Majumder et al. 2004; Kaluz et al. 2006; Thomas et al. 2006). Inhibition of PI3K and ERK also inhibits HIF-1α (Pages and Pouyssegur 2005). Finally, BCR/ABL inhibitor imatinib also inhibits HIF-1α, which is responsible for its anti-angiogenic effect (Mayerhofer et al. 2002). Since HIF-1α activates major glycolytic enzymes, inhibition of HIF-1α protein directly by HIF-1α inhibitors or indirectly by those above-mentioned drugs will also decrease the aerobic glycolysis in cancer cells.

Inhibitors of HIF-1α can also be used in combination with some common therapies, including radiation and chemotherapy, to target cancer cells more potently and to kill resistant cells. Radiation can induce HIF-1α activity, which leads to the production of VEGF and other angiogenic cytokines that protect endothelial tumor cells from radiation-induced death. Targeting of these resistant cells is possible by simultaneously treating patients with HIF-1α inhibitors, such 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1), which down-regulates HIF-1α and HIF-2α post-translationally and *via* FIH-dependent CAD inactivation, as well as PX-478, which inhibits HIF-1α through decreased translation (Koh et al. 2008; Li et al. 2008; Schwartz et al. 2009). Some chemotherapy agents are also potent HIF-1α inhibitors, including anthracyclines, doxorubicin and daunorubicin, topoisomerase I inhibitors and microtubule-targeting drugs, such as taxotere (Rapisarda et al. 2002; Escuin et al. 2005; Lee et al. 2009). The effectiveness of using chemotherapy agents that also inhibit HIF-1α in HIF-1α-expressing tumors is that they can be used at lower doses to prevent bone marrow toxicity (Hahnfeldt et al. 2003; Lee et al. 2009).

## 4.2 c-Myc

*MYC* is an oncogene that encodes transcription factor, c-Myc, which is often deregulated in human cancers (Dang et al. 2008; Albihn et al. 2010). c-Myc induces gene expression and microRNAs that regulate the cell cycle as well as glucose and glutamine metabolism (Dang 2010) (Figure 19). One of the ways in which c-Myc manipulates glycolysis is to promote preferential PKM2 splicing at the transcriptional level. In many human cancers, like gliomas, overexpression of c-Myc correlates to PKM2 expression (David et al. 2010). c-Myc also induces expression of LDHA (Shim et al. 1997) potentially to metabolize pyruvate generated by enhanced glycolytic flux in proliferating cells (DeBerardinis et al. 2007) as it has been suggested that the TCA cycle is saturable (Helmlinger et al. 2002). Additionally, c-Myc upregulates PDK1 to increase glycolysis and decrease PDH activity, thereby preventing glycolytic pyruvate from entering the TCA cycle. Not surprisingly, c-Myc metabolism mimics hypoxic metabolism, but during normoxic conditions. c-Myc also promotes *HIF* expression and inhibits HIF-1$\alpha$ degradation, which not only leads to a positive feedback on c-Myc but also promotes hypoxic metabolism (Dang et al. 2008). Finally, and unlike hypoxic metabolism, c-Myc activation promotes increased oxygen consumption and mitochondrial biogenesis, which is associated with transcriptional induction of transcription factor A, mitochondrial (TFAM), complex I subunits, uncoupling proteins, mitochondrial membrane proteins and genes involved in intermediary metabolism (Li et al. 2005). c-Myc promotes mitochondria to support glutaminolysis and its downstream pathways. Glutamine provides many crucial functions for cancer cells, as outlined above in the section "Glutaminolysis" on page 45, and c-Myc activation is one of the oncogenic switches that supports these changes.

More recently, the importance of c-Myc in directly stimulating glutamine uptake and metabolism has been explored (Wise et al. 2008; Gao et al. 2009). High c-Myc activity leads to an increase in glutamine transporters, ASCT2, SLC1A5 and SLC7A1, expression of mitochondrial GLS1, which converts glutamine to glutamate, and glutamine metabolism (Wise et al. 2008; Gao et al. 2009; Mates et al. 2009). To increase glutaminase expression, c-Myc inhibits the expression of microRNAs, miR-23a/b, which are translational inhibitors of GLS1. Interestingly, GLS1 downregulation inhibits oncogenic transformation and cancer cell proliferation (Lobo et al. 2000; Seltzer et al. 2010; Wang et al. 2010; Le et al. 2012) and RNAi-mediated GLS1 suppression increases ROS levels and cell death, which is linked to diminished glutathione levels (Gao et al. 2009). Not surprisingly, carbon derived from glutamine in c-Myc-driven cells is preferentially used for glutathione synthesis (Le et al. 2012). Altogether, c-Myc-transformed cells are addicted to glutamine and, subsequently, depletion of glutamine induces apoptosis (Yuneva et al. 2007; Wise et al. 2008). Thus, glutamine metabolism provides a novel and effective target for c-Myc-transformed cancer cells.

### 4.3 p53

The p53 protein is coded by the *TP53* gene, which is the most frequently mutated gene in human tumors (Sermeus and Michiels 2011). In its wild-type form, p53 is a tumor suppressor as it plays a significant role in normal growth and development, apoptosis, the cell cycle, DNA repair and genome stability (Vousden and Ryan 2009). p53 is also activated by stress, such as intracellular ROS, hypoxia, DNA damage and metabolic stress (Nakano et al. 2000; Bensaad et al. 2009). For example, ROS is continuously released during both normal and stressful conditions and cells respond with p53 depending on the level of ROS. When ROS is low, p53 directs cells to proliferate, while cells will be directed towards apoptosis under high ROS conditions (Polyak et al. 1997).

More recently, the role of p53 in regulating both glycolytic and oxidative phosphorylation has become an important topic in cancer cell metabolism (Figure 19). p53, functioning as a tumor suppressor, can decrease glucose uptake and subsequent glycolysis by decreasing gene expression of *GLUT1* and *GLUT4* (Schwartzenberg-Bar-Yoseph et al. 2004). p53 can also regulate glycolytic genes with p53-responsive elements that exist in the promoters of genes like *HK-II* (Vousden and Ryan 2009). Furthermore, p53 can increase levels of TIGAR, which is involved in apoptosis induction and, as described in the section "6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase family" on page 36, degrades F-2,6-BP, an allosteric regulator of PFK1, to slow the glycolytic rate and promote G6P entry into the PPP to increase NADPH production (Bensaad et al. 2006; Bensaad et al. 2009). p53 also negatively regulates phosphoglycerate mutase and AKT, further inhibiting glycolysis and upregulating ETCIV by the induction of the synthesis of cytochrome c oxidase (COX) copper chaperone, SCO2 (Matoba et al. 2006). Loss of p53 or its regulation of SCO2 impairs the mitochondrial electron transport chain and promotes the glycolytic switch (Teodoro et al. 2006).

In addition, p53 promotes glutamine utilization through upregulating mitochondrial glutaminase 2 (GLS2). Increased GLS2 activity correlates to increased glutathione production to combat redox stress and protect against p53-driven apoptosis (Gao et al. 2009; Hu et al. 2010; Suzuki et al. 2010). It is important to note that increasing GLS2, as compared to mitochondrial GLS1 through c-Myc, correlates to tumor suppression (Hu et al. 2010; Suzuki et al. 2010).

As a whole, inactivation of p53 should decrease oxidative phosphorylation in favor of glycolysis, while also decreasing antioxidant production and inhibiting apoptosis. However, while many cancers exhibit p53 mutations, there are also cancers that have wild-type p53 and can benefit from this response tool in times of stress. In p53 wild-type cancer cells, p53 is activated under metabolic or hypoxic stress (Sermeus and Michiels 2011), while many cells that do not have p53 cannot handle metabolic stress, like irradiation, and induce cell death as a result (Jones et al. 2005a). Additionally, p53 may be indirectly activated by AMP-activated protein kinase (AMPK) through the ATM/ATR response, which will lead to phosphorylation of p53 on the Ser15 subunit following certain metabolic stresses, such as glucose deprivation (Assaily et al. 2011). Overall, this activation

promotes cell survival in response to metabolic stress by inducing a cell cycle arrest, down-regulating anabolic pathways and up-regulating catabolic pathways to compensate and re-balance the energetic status (Jones et al. 2005b). However, it is important to point out that AMPK activator, liver kinase B1 (LKB1), is absent in many tumors, which makes these cells sensitive to nutrient deprivation because they don't have a metabolic stress response (Shaw et al. 2004). AMPK will be discussed in full in the next section "AMPK," however, this example shows that while p53 inactivation can promote the cancer metabolism profile, it also renders cancer cells more sensitive to stress.

### 4.4 AMP-activated protein kinase

AMP-activated protein kinase (AMPK), like p53, is a tumor suppressor for cancer cells as it regulates metabolism and cell growth in response to cellular energetic activity. However, like p53, AMPK activity in cancer cells gives cells an advantage in stress or conditions when energy demand is higher than energy produced. During energetic stress, such as ischemia, hypoxia and hypoglycemia, there is an increase in the AMP:ATP ratio and a subsequent activation of AMPK (Young 2008). AMPK can also be activated by signals in the extracellular environment, including hormones and cytokines. Activation of AMPK can occur through phosphorylation of AMPKαThr172 by LKB1 (Hawley et al. 2003), $Ca^{2+}$/calmodulin-dependent protein kinase kinase beta (CaMKKβ) (Hawley et al. 2005; Hurley et al. 2005; Woods et al. 2005) and transforming growth factor-β (TGFβ)-activated kinase-1 (TAK1) (Momcilovic et al. 2006; Xie et al. 2006). Not only does the AMP:ATP ratio activate these upstream kinases, but AMP can bind directly to the AMPK γ subunit to change the conformation of AMPK, providing allosteric activation and preventing inhibition of dephosphorylation of the Thr172 subunit (Davies et al. 1995). While having AMPK in times of stress could help cancer cells survive, AMPK activation at any time could still lead to decreased tumorogenic activities. It is for this reason that many cancer cells have non-functional LKB1 and/or TSC2 activities, which are also tumor suppressors upstream and downstream of AMPK, respectively (Tiainen et al. 2002; Boudeau et al. 2003; Jimenez et al. 2003; Carretero et al. 2004; Ji et al. 2007; Contreras et al. 2008; Gurumurthy et al. 2008; Lesma et al. 2012). Altogether, this data supports the use of AMPK activation for cancer prevention or therapy.

Once activated, AMPK stimulates catabolic pathways, such as glycolysis and fatty acid oxidation, while inhibiting anabolic pathways like protein, lipid, sterol and glycogen synthesis (Figure 20). AMPK activates glycolysis by first increasing glucose uptake in skeletal muscles through activation of glucose transporters, GLUT1 (Barnes et al. 2002) and GLUT4 (Frosig et al. 2010). AMPK also phosphorylates and activates PFKFB2 in cardiac myocytes and PFKFB3 in monocytes and macrophages, to produce F-2,6-BP, which activates PFK1 and subsequent glycolysis (Marsin et al. 2000). AMPK increases fatty acid oxidation through phosphorylation and inactivation of acetyl-

CoA carboxylase (ACC). The inhibition of ACC1 leads to acute inhibition of fatty acid synthesis (Corton et al. 1995), while inactivation of ACC2 leads to a decrease in malonyl-CoA, which releases its downstream inhibition of mitochondrial fatty acid transporter, CPT1, to allow for an increase in fatty acid β-oxidation (Merrill et al. 1997). AMPK also increases fatty acid uptake through translocation of the transporter CD36 to the plasma membrane (Habets et al. 2009).

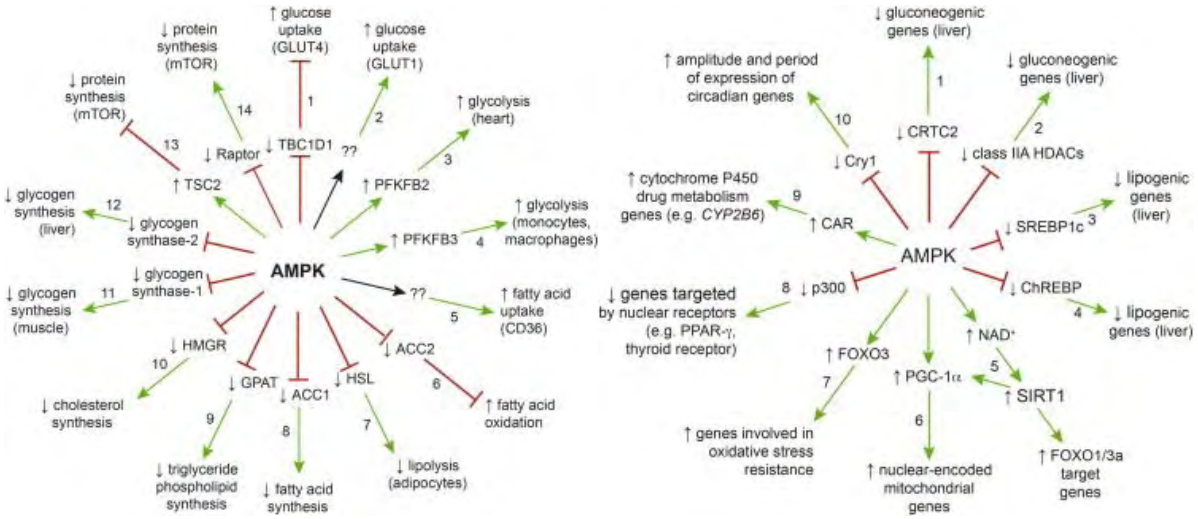



**Figure 20. Overview of AMPK regulation of metabolism.** AMPK regulates multiple target proteins to increase catabolic pathways, such as glycolysis and fatty acid oxidation, and decrease anabolic pathways, such as protein, lipid, sterol and glycogen synthesis, in response to energetic stress in cancer cells. Figure adapted from Hardie (2003) and Hardie and Alessi (2013).

While simultaneously activating glucose transport, AMPK inhibits anabolic energy-consuming pathways, such as glycogen synthesis through inhibition of glycogen synthase-1 in muscles (Jorgensen et al. 2004) and glycogen synthase-2 in the liver (Bultot et al. 2012). Additionally, AMPK inhibits lipolysis in rodent and human adipocytes through phosphorylation of hormone-sensitive lipase (HSL), which antagonizes its activation through cyclic AMP-dependent protein kinase and its translocation to the surface of the lipid droplet (Garton et al. 1989; Daval et al. 2005; Bourron et al. 2010). Although lipolysis is a catabolic pathway, limiting this pathway decreases the release of free fatty acids, whose accumulation would normally lead to induction of the ATP-consuming triglyceride synthesis pathway (Gauthier et al. 2008). Consistent with this theory, AMPK controls the use of fatty acyl CoA by inhibiting triglyceride and phospholipid synthesis by inactivating glycerol phosphate acyl transferase (GPAT) (Muoio et al. 1999; Park et al. 2002) and serine palmitoyltransferase (SPT) (Blazquez et al. 2001). AMPK also inhibits cholesterol synthesis by direct phosphorylation and inactivation of 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) (Clarke and Hardie 1990). Finally, AMPK inhibits mTORC1-dependent protein synthesis through two mechanisms. The first mechanism is AMPK activation of tuberous sclerosis complex protein-2 (TSC2), which is a GTPase activating protein that hydrolyzes the GTP of the Rheb-GTP complex,

converting it to the inactive Rheb-GDP complex. Only the active Rheb-GTP activates mTORC1, thus conversion to Rheb-GDP leads to inactivation of mTORC1 (Inoki et al. 2003a). Additionally, AMPK directly phosphorylates Raptor, an mTORC1 subunit, to inhibit its activity (Gwinn et al. 2008).

AMPK also exhibits metabolic regulation through transcriptional changes, such as inhibition of cyclic AMP response element binding protein (CREB) (Koo et al. 2005) and sterol response element binding protein-1c (SREBP-1c) (Li et al. 2011). This inactivation inhibits downstream gluconeogenic and lipogenic gene transcription, including ACC1 and FASN, with lipogenic activity specific to SREBP-1c inhibition (Ruderman and Prentki 2004; Li et al. 2011). AMPK also decreases transcription of additional lipogenic genes, such as the liver isoform of pyruvate kinase, by phosphorylating and inactivating carbohydrate response element binding protein (ChREBP) (Kawaguchi et al. 2002). Additionally, AMPK regulates mitochondrial functions in response to cell stress. AMPK activates mitochondrial biogenesis by indirectly phosphorylating peroxisome proliferator-activated protein gamma (PPARγ) (Jager et al. 2007). AMPK helps cells respond to oxidative stress by phosphorylating forkhead box O3 (FOXO3), thus increasing transcription of many genes involved in this stress relief process (Greer et al. 2007). Additionally, AMPK phosphorylates unc-51-like kinase 1 (ULK1), which is a protein kinase that triggers autophagy. This process could be critical under times of energetic stress, as it allows the breakdown of cellular macromolecules for energy production (Egan et al. 2011). Finally, AMPK induces cell cycle arrest by activating $p27^{KIP1}$ and $p21^{WAF1}$, which are cyclin-dependent kinase inhibitors that trigger G1-S phase cell cycle arrest (Imamura et al. 2001; Jones et al. 2005b; Liang et al. 2007). Due to these downstream activities, activation of AMPK in cancer cells offers an advantageous therapy to change the overall energetic and translational status of a cell, leading to cell death in certain cell types (Hardie et al. 2012).

### 4.5 The mammalian target of rapamycin

The mammalian target of rapamycin (mTOR) coordinates protein translation and proliferation and is deregulated in many cancers (Guertin and Sabatini 2007). mTOR is the central kinase in two distinct complexes, known as complex 1 and 2. mTORC1 increases the initiation of cap-dependent translation by altering activity of translation components, including activation of ribosomal S6 kinase and inhibition of the translational inhibitor 4E-BP1, which in turn promotes initiation of translation by the EIF4F complex. EIF4F is an oncogene upregulated in a number of cancers (Ruggero and Pandolfi 2003) and its overexpression is sufficient to promote resistance to mTORC1 inhibitor, rapamycin, (Wendel et al. 2006) and increase cyclin D1 (Averous et al. 2008). While mTORC1 is sensitive to cellular nutrient levels, mTORC2 is insensitive and irresponsive to rapamycin (Guertin and Sabatini 2007; Garcia-Martinez and Alessi 2008). For this introduction, mTORC1 will be the focus.

mTORC1, along with other signaling players, senses the nutritional and metabolic status of the cell and can then control cellular activities using its translational control (Guertin and Sabatini 2007). mTORC1 responds to changes in oxygen concentration, insulin, glucose and amino acid availability and mitochondrial function. When nutrients are low, mTORC1 will be decreased so that cells do not synthesize proteins and therefore decrease growth and proliferation. However, mTORC1 hyperactivation, as seen in many cancer cells, leads to overactivation of translation and increased cell proliferation. More specifically, mTORC1 controls protein translation of Mcl-1, cyclin D1, HIF-1α and c-Myc, to promote cell cycle progression, cell growth, glycolysis and angiogenesis, thereby increasing tumorogenesis (Guertin and Sabatini 2007). However, if cells are hyperactivated in mTORC1, mTORC1 cannot mediate translation in response to stress in order to help cells survive, which can lead to mTORC1-mediated energetic stress and cell death (Choo et al. 2010).

mTORC1 can be regulated by a number of different oncogenes and tumor suppressors in cancer cells (Figure 21). AKT, which is overactivated in many cancers, regulates mTORC1 function by phosphorylating and inhibiting TSC2, which disrupts the TSC1/TSC2 complex and promotes mTORC1 activation (Inoki et al. 2002). However, mTORC1 is also controlled by tumor suppressors, TSC1/TSC2 and LKB1/AMPK, which function to block mTORC1 activation through inhibition of the small GTPase Rheb. Thus, mutations of these genes leads to elevated mTORC1 activity (Inoki et al. 2003a; Corradetti et al. 2004; Shaw et al. 2004). Finally, in response to hypoxia, DNA damage, energy stress and ROS, mTORC1 can also be inhibited by hypoxia-inducible gene, REDD1/RTP801, in a TSC1-TSC2-dependent mechanism (Ellisen et al. 2002; Shoshani et al. 2002; Brugarolas et al. 2004; Reiling and Hafen 2004; Lin et al. 2005; Sofer et al. 2005; DeYoung et al. 2008).

REDD1 (regulated in development and DNA damage responses; also known as RTP801/Dig2/DDIT4) is a protein that is down-regulated in a subset of human cancers (DeYoung et al. 2008), and as such has potential as a tumor suppressor. REDD1 can inhibit downstream mTORC1 activity through activation of TSC2. Specifically, REDD1 directly binds to 14-3-3 proteins to disassociate these proteins from TSC2 and allow for TSC2-dependent mTORC1 inhibition (DeYoung et al. 2008). REDD1 is also a direct transcriptional target of p53 as the REDD1 promoter contains a consensus p53 family-binding element required for p53-induced REDD1 activation (Ellisen et al. 2002). Following p53 activation, REDD1 may play a special role in exerting negative feedback control on p53 by inhibiting mTORC1-dependent p53 translation. This was shown in REDD1 knockout mice who exhibited enhanced sensitivity to radiation and chemotherapy treatment due to abnormally increased p53 protein expression and activity. Thus, p53 activates REDD1 to decrease cellular stress and also decrease p53 activity to limit the apoptotic response following DNA damage and subsequent p53 activation (Vadysirisack et al. 2011). As a stress response tool, REDD1 can be either stress relieving or toxic to cells depending on the cell context and level of stress.

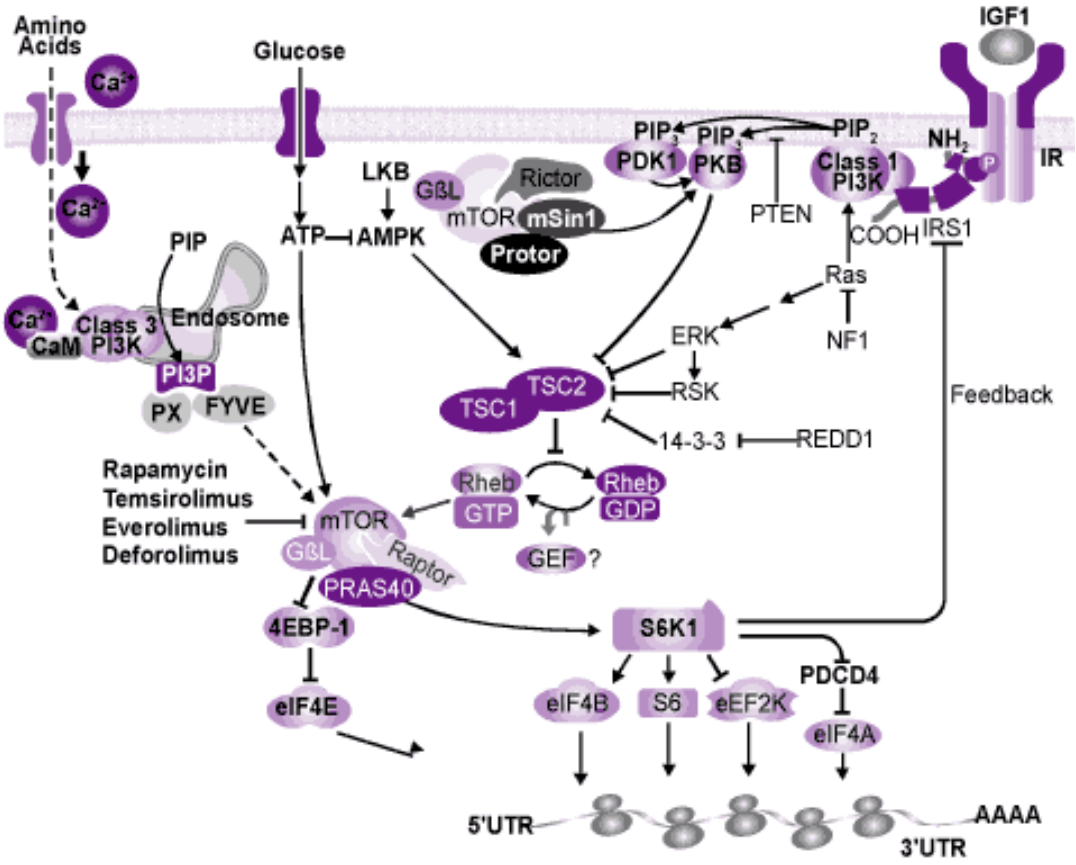



**Figure 21. Overview of the mechanisms activating and inhibiting mTORC1.** mTORC1 activity is promoted by amino acid and ATP levels as well as PI3K/AKT, PI3K/Ras/ERK/RSK and insulin signaling pathways. However, mTORC1 can be inhibited by AMPK and REDD1. Many signaling pathways control mTORC1 activity through upstream regulation by TSC1/2 and Rheb GTPase activity. Downstream, mTORC1 activity regulates S6K1 and 4EBP-1/eIF4E activity, which are both involved in translational regulation.

REDD1 is activated by many stresses, including DNA damage, hypoxia, reactive oxygen species, endoplasmic reticulum stress and energy depletion (Ellisen et al. 2002; Brugarolas et al. 2004; Sofer et al. 2005; Jin et al. 2009; Regazzetti et al. 2010; Kimball and Jefferson 2012). In head and neck squamous cell carcinoma, hypoxia and subsequent DNA damage activates REDD1 to block mTORC1, which requires normal TSC1/2 activity (Brugarolas et al. 2004; Schneider et al. 2008). In humans, REDD1 overexpression is enough to potently inhibit mTORC1 activity, while loss of REDD1 reduces the ability of hypoxia to inhibit mTORC1 (Brugarolas et al. 2004; Corradetti et al. 2004; Sofer et al. 2005). REDD1 can be expressed by HIF-1α activation in adipocytes (Regazzetti et al. 2010), neuronal cells (Shoshani et al. 2002) and breast cancer cells (Shoshani et al. 2002; Aprelikova et al. 2006). Like p53, increased REDD1 by HIF-1α actually leads to a decrease in HIF-1α (Horak et al. 2010). REDD1 appears to decrease HIF-1α by translocating to mitochondria and directly decreasing mitochondrial ROS leading to destabilization of HIF-1α (Horak et al. 2010). Additional evidence of REDD1 function in the regulation of cellular ROS is that the restoration of either REDD1 or p63 expression in TP63 null MEFs increases ROS induction and corrects oxidative

stress sensitivity (Adler et al. 1999; Finkel 2000; Shoshani et al. 2002). REDD1 is also upregulated by activating transcription factor-4 in response to endoplasmic reticulum stress (Jin et al. 2009; Whitney et al. 2009). REDD1 may be activated by energy and environmental stress, such as ATP depletion (Sofer et al. 2005), starvation (Reiling and Hafen 2004) and high cell density (Jin et al. 2007). Finally, environmental stress-induced REDD1 occurs downstream of Elk-1 and CCAAT/enhancer-binding protein following arsenic treatment in keratinocytes (Lin et al. 2005).

Therefore, activation of REDD1 and its anti-tumoral activities may be an effective cancer therapy. REDD1 may act as a tumor suppressor through apoptotic effects (Shoshani et al. 2002; Bakker et al. 2007) and as a direct and indirect target of tumor-suppressor FOXO (Bakker et al. 2007; Harvey et al. 2008). As mentioned above, REDD1 works downstream of tumor suppressor p53 during cancer DNA damage (Ellisen et al. 2002). In neuronal PC12 cells, REDD1 expression induced cytotoxicity by increasing sensitivity to ischemic injury and oxidative stress (Shoshani et al. 2002). There is also low expression of REDD1 in 40% of HER2 overexpressing and TNBC breast cancer types, which correlates to increased tumor cell proliferation and survival in a hypoxic tumor environment by disinhibition of the mTOR pathway and HIF-1$\alpha$ stabilization (Koo and Jung 2010). Interestingly, REDD1 expression is extremely low in highly invasive H1299 non-small cell lung cancer cells as compared to H460 cells with lower invasive activity. Overexpression of REDD1 inhibited the invasiveness of H1299 cells, while suppression of REDD1 with siRNA enhanced the invasiveness of H460 cells and the invasiveness of non-small cell lung cancer cells. This activity was through downstream mTOR activity (Jin et al. 2011). In a parallel study, it was also shown that cells with nonfunctional REDD1 are defective in downregulation of S6K, S6 and 4E-BP1 phosphorylation following energy depletion (Sofer et al. 2005). Additionally, REDD1 can suppress downstream effects following overexpression of PI3K proteins PKB and PDK-1, such as increased cell growth (Reiling and Hafen 2004). Finally, genetic inhibition of REDD1 increases proliferation and growth under hypoxic conditions (DeYoung et al. 2008).

In addition to protein synthesis, mTORC1 regulates other metabolic pathways such as fatty acid, lipid and pyrimidine biosynthetic pathways. Amino acid availability also has an important relationship with mTORC1 and protein translation. Signal transduction by mTORC1 increases amino acid uptake by increasing and maintaining surface expression of amino acid transporters (Rosario et al. 2013). Amino acids can then directly influence protein synthesis in the reverse through mTORC1 stimulation. This activity works through the Rag GTPase family, whose members interact with mTORC1 and promotes its activation in response to amino acids signals (Kim et al. 2008; Sancak et al. 2008). The Rag GTPase control over mTORC1 is especially interesting in cancer cells with mutations in the TSC1/2 complex, because the Rags mediate mTORC1 directly and also control its localization within the cell (Sancak et al. 2008). Thus, amino acid starvation can decrease mTORC1 even in cancer cells with TSC1/2 mutations or constitutively active AKT.

mTORC1 also plays a role in lipid metabolism. Firstly, mTORC1 activates SREBP1 in rat hepatocytes, which activates enzymes involved in lipogenesis, including ACC, FAS and SCD (Peng et al. 2002; Brown et al. 2007; Mauvoisin et al. 2007). Supporting this data, inhibition of mTORC1 with rapamycin blocks SREBP1 and these downstream enzymes (Peng et al. 2002; Brown et al. 2007; Laplante and Sabatini 2009). mTORC1 also appears to have control over PPARγ activity and its control over fatty acid uptake, synthesis, esterification and storage in the liver (Kim et al. 2012). Interestingly, blocking mTORC1 with rapamycin in human BJAB B-lymphoma cell lines and murine CTLL-2 T lymphocytes increased β-oxidation and catabolism of free fatty acids due to increased very long chain acylCoA dehydrogenase (VLACD) and carnitine acyl transferase (CAT) expression (Peng et al. 2002). This appears to be dependent on the S6K1 activity downstream of mTOR as mice deficient in S6K1 show increased β-oxidation and lipolysis compared to normal mice (Um et al. 2004). Additionally, knockdown of 4EBP1/2 leads to decreased lipolysis (Le Bacquer et al. 2007).

Due to all the cellular activities of mTORC1, there has been a lot of research in recent years to find drugs to target mTOR. Rapamycin, which has been mentioned throughout this review, is a natural product known to inhibit mTORC1 (Vezina et al. 1975). Rapamycin induces G1 cell cycle arrest in some tumor cell lines along with inhibition of mTORC1 (Neshat et al. 2001). In some tumor cell lines, rapamycin induced apoptosis both *in vitro* and *in vivo* (Huang et al. 2004; Majumder et al. 2004; Wendel et al. 2004). Rapamycin also reduced tumor growth *in vivo* in mice xenografted with human tumor cells (Podsypanina et al. 2001). In AML, treatment of PTEN-deficient mice with rapamycin not only blocks leukemogenesis, but restores normal stem cell function (Yilmaz et al. 2006b). As a monotherapy in Phase II trials, rapamycin induced significant clinical response in 4 out of 9 patients with either refractory or relapsed AML (Recher et al. 2005). However, the effect of these drugs is only cytostatic *in vitro* (Park et al. 2010). Additionally, more recent clinical studies have demonstrated that rapamycin may only be effective against some cancers, which include mantle cell lymphoma, endometrial cancer and renal cell carcinoma (Guertin and Sabatini 2007). In fact, the response to rapamycin is variable, which demands the need for a better understanding of all mTOR activities and a more complete mechanism of action of rapamycin. It also creates a demand for novel mTOR inhibitors. One option is a second generation of mTOR inhibitors known as TORK inhibitors. Their most interesting attribute is inhibition of both mTORC1 and mTORC2 complexes, which may offer a more efficient inhibition of protein translation and anti-cancer activity (Feldman et al. 2009; Garcia-Martinez et al. 2009).

## 4.6 Protein kinase B

The serine/threonine protein kinase AKT, also known as protein kinase B (PKB) plays a major role in regulating metabolism and is one of the most frequently activated oncoproteins in human cancers, including acute myeloid leukemia (Luo et al. 2003; Recher et al. 2005; Sujobert et al. 2005; Dos Santos et al. 2006; Tamburini et al. 2007). AKT exists in three forms (AKT1-3) in mammalian cells, sharing more than 80% of the same amino acid sequence. AKT activation starts with binding of phosphatidylinositol 2,4,5-trisphosphate (PIP3), generated by phosphatidylinositol 3-kinase (PI3K), to its pleckstrin homology (PH) domain, which allows AKT to be translocated to the plasma membrane in its rate-limiting activation step (Vanhaesebroeck and Waterfield 1999; Katso et al. 2001). AKT can also be activated by Ras through its physical activation of PI3K at the p10 catalytic subunit (Rodriguez-Viciana and Downward 2001; Vergez et al. 2012). AKT is then phosphorylated and activated at the Thr308 and Ser473 domains by PDK1 and PDK2 (Alessi et al. 1998). Active AKT then goes on to either activate or deactivate a broad range of downstream proteins to control cell proliferation, apoptotic resistance and metabolism, a summary of which can be found in Figure 22.

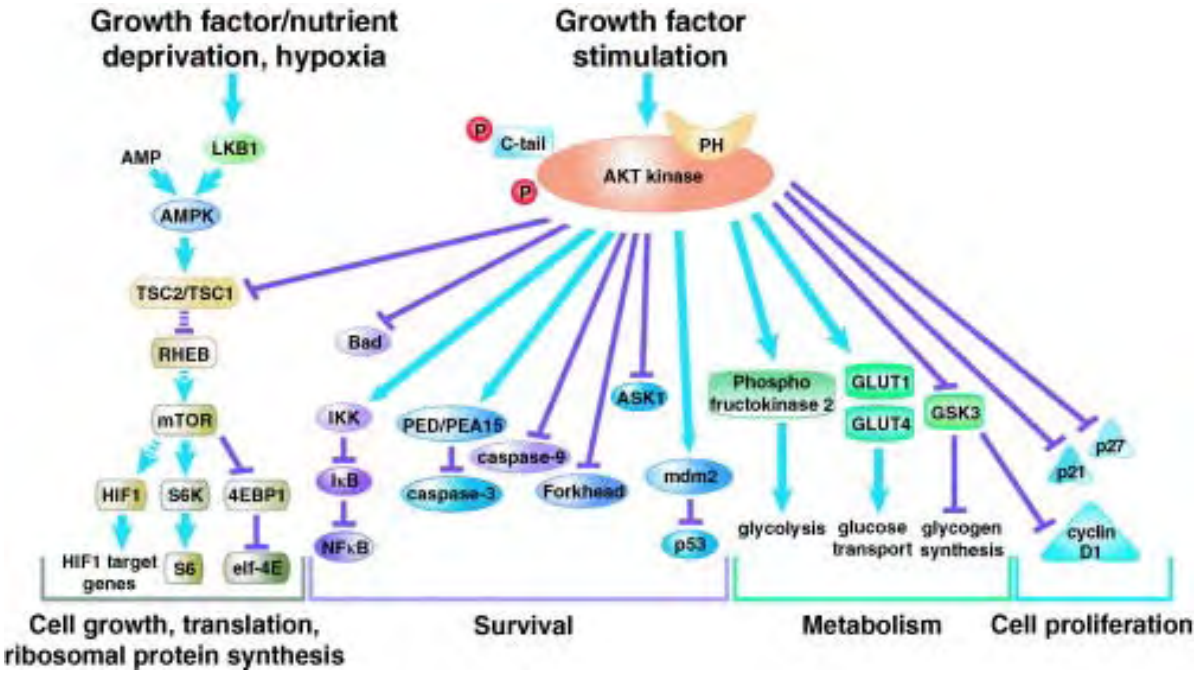



**Figure 22. Overview of downstream AKT effectors.** AKT affects pathways involved in cell proliferation, targeting p21, p27, GSK3 and cyclin D1 to maintain cell cycle progression. AKT also impacts survival by regulating pro- and anti-apoptotic proteins and other key players, including Bad, the IKK/$I_KB$/$NF_KB$ pathway, PED/PEA15, caspase-3/9 activation, the forkhead family, ASK1 and p53 through mdm2 activity. AKT is also involved in cell growth, translation and ribosomal protein synthesis through promotion of mTORC1 activity *via* inhibition of TSC1/2. Finally, AKT impacts metabolism in multiple ways, which can include increased glycolysis (PFK2) and glucose transport (GLUT1/4), and by blocking glycogen synthesis through GSK3 inhibition. Figure adapted from Bellacosa et al. (2005)

Inhibition of AKT occurs through tumor suppressor, phosphatase and tensin homolog deleted on chromosome 10 (PTEN), which dephosphorylates PIP3 to inhibit AKT localization to the cell membrane (Myers et al. 1997; Maehama and Dixon 1998; Cantley and Neel 1999). However, PTEN mutations or deletions occur frequently in several human cancers, including prostate and endometrial cancers, gliobastoma and melanoma, leading to constitutive activation of AKT (Cantley and Neel 1999). Hyperactivation of AKT can also be a result of constitutive activation or overexpression of PI3K, which is observed in many human cancers (Woenckhaus et al. 2002). Finally, activating RAS mutations can increase AKT activity, as observed in 30% of epithelial tumors (Downward 2003).

Although AKT regulates many cellular processes, here we will focus on AKT's role in cancer cell metabolism. First, AKT has an extremely prominent role in glycolytic metabolism, as evidenced by the fact that AKT hyperactivation is correlated to increased glucose metabolism in tumor cells (Kohn et al. 1996; Elstrom et al. 2004; Robey and Hay 2009). In glioblastoma cells, for example, AKT activity is directly correlated to the level of glycolysis, while increasing AKT activity in these cells increased the level of aerobic glycolysis without increasing oxygen consumption (Elstrom et al. 2004). This activation can occur in a number of ways, such as increased localization of glucose transporter, GLUT1, to the plasma membrane (Clarke et al. 1994; Okada et al. 1994; Barthel et al. 1999). AKT also regulates hexokinase expression and activity via direct phosphorylation and induced mitochondrial interaction (Miyamoto et al. 2008). Additionally, AKT increases glycolytic flux by phosphorylating and activating PFK2, which produces F-1,6-BP, an allosteric activator of rate-controlling enzyme, PFK1 (Deprez et al. 1997). Hyperactive AKT also phosphorylates and inactivates FOXO, which is a transcription factor involved in the suppression of glycolytic gene expression (Zhang et al. 2006). Finally, AKT hyperactivity increases mTORC1 activity, which thereby increases HIF-1$\alpha$ levels to induce the Warburg effect in cancer cells (Brugarolas et al. 2003).

AKT can also influence mitochondrial oxidative phosphorylation. AKT-promoted HK activity increases the coupling efficiency between glycolysis and respiration through ATP production and utilization. AKT indirectly promotes oxidative phosphorylation by increasing the production of glycolysis-derived intermediates for entry into the TCA cycle, such as pyruvate and NADH (Gottlob et al. 2001). Increased TCA cycle activity provides increased citrate levels needed for lipid biosynthesis to maintain high proliferation rates. Additionally, ACLY is an AKT target (Berwick et al. 2002) and required for AKT-mediated tumorigenesis (Bauer et al. 2005). The elevated oxidative metabolism in hyperactivated AKT cancer cells can also lead to increased ROS production, which is enhanced by AKT's inhibition of ROS scavengers (Nogueira et al. 2008). Increased ROS can actually contribute to tumorigenesis by activating signaling pathways through oxidation of catalytic cysteine residues within the active sites of certain phosphatases (Tonks 2006). Even more importantly is the increased mutation rates of both genomic and mitochondrial DNA following increased ROS production (Nogueira et al. 2008). However, this also leads us to AKT's Achilles' heel, which is susceptibility to ROS-induced cell death (Nogueira et al. 2008).

Altogether, this suggests AKT as one of the potential proteins that initially induces the Warburg effect in cancer cells. However, it also leads us into a new direction in cancer cell metabolism, which is that glycolysis and oxidative phosphorylation, rather than glycolysis alone, can be combined to promote tumorigenesis, with AKT at the forefront of this metabolic syndrome (Gottlob et al. 2001; Nogueira et al. 2008). Mitochondrial-dependence in this sense must also include fatty acid β-oxidation, which can protect cells that are constitutively activated in AKT during glucose deprivation. This survival mechanism, downstream of AMPK activation, interconnects fatty acid oxidation, glycolysis, AKT and AMPK in cancer cell metabolism and survival (Buzzai et al. 2005). In AML specifically, the PI3K/AKT/mTOR pathway has been of particular interest as these pathways are highly deregulated and contribute to high cell proliferation, insensitivity to apoptotic pathways and drug resistance (Martelli et al. 2006). As such, it has been shown that AKT inhibitor, AKTi-1/2, inhibited cell proliferation and clonogenic properties and induced apoptosis in AML cells with high-risk cytogenetics (Gallay et al. 2009). From a metabolic perspective, combining AKT inhibitors with metabolic drugs offers an enormous therapeutic potential in many types of cancer, including AML.

## 4.7 Regulation of apoptotic pathways by energetic and mitochondrial metabolism

Mitochondrial outer membrane permeabilization (MOMP) is often the decisive event through which cancer cells undergo mitochondrial-dependent apoptosis and cell death (Kroemer et al. 2007). Following MOMP, the release of apoptogenic factors, such as cytochrome C, from the mitochondria will induce cell death. A number of pro- and anti-apoptotic Bcl-2 family members and proteins of the mitochondrial permeability transition pore complex determine whether or not MOMP will occur in response to apoptotic signaling (Kroemer et al. 2007). Additionally, the Warburg effect not only gives a metabolic advantage to cancer cells by providing biosynthetic molecules and energy to proliferate, but also protects these cells against mitochondrial-dependent apoptosis (Weinmann et al. 2004). There are multiple mechanisms through which this can occur. One of the most studied mechanisms is the high association of hexokinases (HKs) with the voltage-dependent channel protein (VDAC) during high glycolytic metabolism (Figure 23). When HK-II is bound to VDAC, it competes with anti-apoptotic proteins, such as Bax and Bak, thereby preventing cytochrome c release, activation of downstream caspases and subsequent apoptosis (Danial et al. 2003; Pastorino and Hoek 2008). As discussed above, AKT phosphorylates HK-II, driving its association with VDAC (Robey and Hay 2005; Robey and Hay 2006). Additionally, overexpression of both HK and GLUT1 can phosphorylate and inhibit GSK3 through increased glycolysis (Zhao et al. 2007). Inhibition of GSK3 is key because this protein can directly phosphorylate VDAC, to inhibit HK-binding, (Pastorino et al. 2005) and anti-apoptotic protein, Mcl-1, to induce destabilization (Maurer et al. 2006).

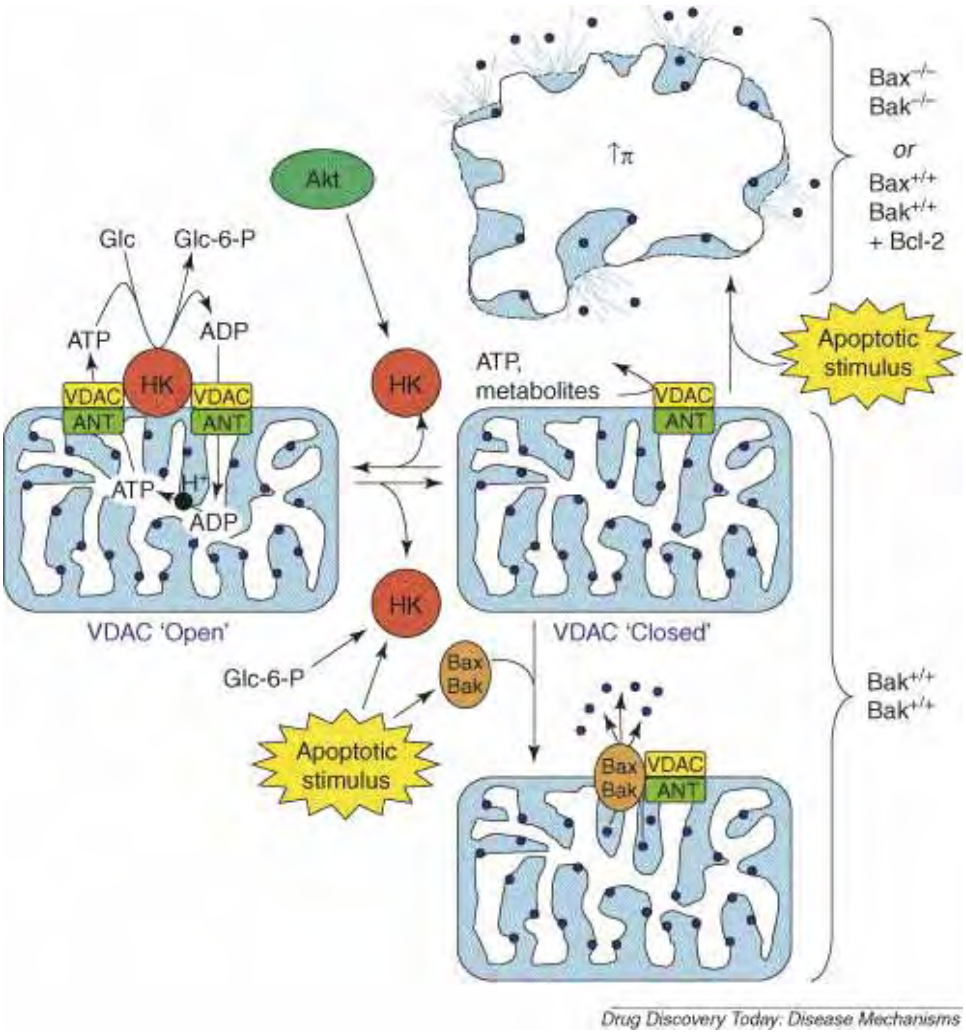



**Figure 23. HK blocks mitochondrial-dependent apoptosis through interactions with VDAC.** HK, phosphorylated by AKT, binds to the mitochondrial port, VDAC, to efficiently acquire ATP for glucose oxidation. HK binding to VDAC also inhibits apoptosis by blocking signaling of pro-apoptotic signals, such as Bak and Bax. Figure adapted from Robey and Hay (2005).

In addition to HK-II association to VDAC, hyperactivated AKT plays other roles in preventing apoptosis in cancer cells. The first mechanism is phosphorylation of Bad, which dissociates Bad from Bcl-2 or Bcl-xL and allows these proteins to carry out their anti-apoptotic functions (Datta et al. 1997; Nechushtan et al. 1999; Datta et al. 2000; Masters et al. 2001). AKT may also phosphorylate Bax, which induces a conformational change and inhibits Bax from translocating to the mitochondrial membrane and inducing apoptosis (Yamaguchi and Wang 2001). Additionally, Rathmell et al. (2003) showed that AKT-induced expression of HK-I and GLUT1 promotes increased NADH and NADPH levels during growth factor withdrawal, which prevents Bax activation and downstream apoptosis (Rathmell et al. 2003). High AKT levels are also enough to prevent BID-mediated Bax and Bak activation and subsequent apoptosis (Scorrano et al. 2003; Majewski et al. 2004b). Hyperactivation of AKT leads to increased protein translation of anti-apoptotic proteins like Mcl-1, cFLIP and Bcl-xL through increased mTORC1 activity (Peponi et al. 2006; Mills et al. 2008; Pradelli et al. 2010; Coloff et al. 2011). Finally, AKT also phosphorylates members of the forkhead

transcription factors (Burgering 2008). Their phosphorylation prevents them from activating the extrinsic, mitochondrial-independent apoptotic pathway, but also from increasing expression of BIM, another pro-apoptotic protein (Dijkers et al. 2000; Obexer et al. 2007).

High glycolysis can also prevent mitochondrial-dependent apoptosis by mobilization of members of the anti-apoptotic Bcl-2 family, including Bcl-xL, to VDAC (Ponnala et al. 2012). Bcl-xL regulates mitochondrial respiration and metabolism and appears to block apoptosis by reducing citrate-derived acetyl-CoA production (Gottlieb et al. 2000). This decrease prevents the N-acetylation process, which is required for activation of multiple proteins, including caspases (Zhao et al. 2010). It is for this reason that adding citrate to media restores N-alpha-acetylation in Bcl-xL-expressing cells and blocks Bcl-xL's anti-apoptotic effect (Yi et al. 2011). High glycolysis may also inactivate BAD through JNK-mediated phosphorylation, which is required for maintaining normal glycolysis for cell survival through activation of PFK-1 (Deng et al. 2008). Bim and PUMA, two other pro-apoptotic proteins, are also down-regulated by high glucose metabolism (Zhao et al. 2008; Concannon et al. 2010).

Thus, targeting cancer cell metabolism not only affects a cell's energetic status, but can manipulate apoptotic effects as well. This may be an especially interesting therapy for leukemic stem cells, which have higher levels of anti-apoptotic proteins, such as Bcl-2 (Tallman 2005; Blum and Marcucci 2008; Fathi et al. 2010) and glioma stem cells, which have high levels of Bcl-2 and Bcl-xL (Jiang et al. 2003). In AML, specifically, this high Bcl-2 activity is linked to drug resistance and poor prognosis (Moon et al. 2010; Cluzeau et al. 2012). Anti-apoptotic abilities of cancer cells can be targeted in a number of ways. A competitive inhibitor of HK, 2-deoxyglucose (2DG), not only decreases ATP levels through inhibition of glycolysis, but also inhibits the anti-apoptotic binding of HK to VDAC (Maher et al. 2005). This compound seems to be effective in combination with other agents and actually potentiates the effect of radiotherapy in brain tumor patients (Singh et al. 2005). It may also be possible to directly target the HK-II/VDAC interaction in cancer cells with clotrimazole and bifanazole (Penso and Beitner 1998), methyl jasmonate (Goldin et al. 2008) and peptide sequences that correspond to the HK-II N-terminal (Majewski et al. 2004a; Chiara et al. 2008). In all cases, apoptosis was induced in target tumors and especially so in highly glycolytic tumors. Dichloroacetate (DCA) is another molecule that downregulates glycolysis by inhibiting PDK and forcing pyruvate into mitochondria (Bonnet et al. 2007). DCA, in combination with chemotherapy or radiation, induces a Bax-dependent apoptotic cascade (Morfouace et al. 2012).

There are additional metabolic enzymes and pathways that influence cell survival and apoptosis. Glycolytic enzyme, GAPDH, promotes survival against caspase-independent cell death in cells that have undergone mitochondrial permeabilization, with a parallel blockage of caspase activity, by increasing glycolysis and initiating autophagy (Colell et al. 2007; Tarze et al. 2007). An additional mechanism of apoptotic protection in cancer cells is upregulation of UCP2, which inhibits ROS production and apoptosis in hypoxic conditions (Dalla Pozza et al. 2012; Deng et al. 2012). In

pancreatic adenocarcinoma cells, UCP2 inhibition triggers ROS-dependent nuclear translocation of GAPDH, formation of autophagosomes and increased expression of autophagy marker LC3-II. Furthermore, UCP2 inhibition induces a ROS-dependent cell death, which is decreased in the presence of radical scavenger, NAC, or inhibition of autophagy by chloroquine or 3-methyladenine (Dando et al. 2013). Interestingly, leukemic cells, which are extremely sensitive to ROS, also have a high dependence on FAO-dependent NADPH production for survival (Carracedo et al. 2013). Additionally, TIGAR, a p53 target that inhibits glycolysis by reducing FBP levels, redirects glucose intermediates into the PPP and increases antioxidants. Thus, TIGAR decreases sensitivity of cells to p53 and ROS-induced apoptosis (Bensaad et al. 2006). However, under severe DNA damage, p53 will bypass the effects of TIGAR to induce apoptosis through induction of PUMA and NOXA (Oda et al. 2000; Nakano and Vousden 2001; Jeffers et al. 2003). Increased PFK expression also increases resistance to oxidative stress in a similar mechanism to TIGAR (Boada et al. 2000). Overall, there are a number of interactions between metabolism and apoptosis in cancer cells. Thus, drugs targeting effectors of these processes could have a high therapeutic index due to this multi-targeted approach.

## 5. New identified role of metabolism in AML

Based on the growing importance of metabolism in AML, we expect that metabolism plays a role in leukemogenesis and may therefore offer potential therapy options. Previous works have suggested that energetic metabolism plays a role in cellular differentiation and chemoresistance in AML *in vitro* (Ahmed et al. 1993; Samudio et al. 2008; Samudio et al. 2009). However, this activity is not always straight forward, because AML cells have different metabolic phenotypes due to the large heterozygous nature of AML cells in general. For example, a recent publication treated AML cell lines with glycolytic inhibitor, 2-deoxyglucose (2DG), and found that AML cell lines respond differently depending on their metabolic phenotype. NB4 cells consume mainly glucose as an energy source, processed through glycolysis and oxidative phosphorylation in mitochondria, as evidenced by increased glycolytic and TCA cycle intermediates in response to 2DG. THP-1 cells, however, did not exhibit this response as they appear to depend on fatty acid β-oxidation (FAO) as an energy source, supported by high levels of carnitine and acetylcarnitine (Miwa et al. 2013). Thus, while these unique metabolic dependencies can provide new targets for AML treatment, true efficacy of metabolic drugs will require biomarkers to stratify patients at diagnosis and determine the metabolic dependency and appropriate targets of cell types.

However, it is possible to induce a specific metabolic phenotype in AML cells in order to stimulate drug sensitivity. For example, immortalized HL60 cells can be differentiated with DMSO and exhibit different metabolic characteristics from undifferentiated cells. While both types of HL60 cells exhibit high glucose and glutamine consumption, undifferentiated cells use this metabolism primarily for biosynthetic precursors. Differentiated HL60 cells, on the other hand, exhibit increased oxidative PPP flux and reactive oxygen intermediate synthesis, suggesting increased mitochondrial oxidative phosphorylation (Ahmed et al. 1993). Another group showed that differentiated HL60 cells had significantly decreased lactate production, which was greater than the decrease in glucose utilization and suggested that glucose was being used in other pathways (Schumacher et al. 1974). This suggests that forcing HL60 cells into mitochondrial oxidative dependency may lead to differentiation and potentially cell death, especially following drug-induced inhibition of mitochondria and increased oxidative stress.

Several groups have indicated that FAO dependency may be a potential metabolic therapy in AML, as pharmacological inhibition of FAO with etomoxir or ranolazine inhibited proliferation and sensitized human leukemic cells to Bcl-2 inhibitor, ABT-737 (Samudio et al. 2010). Inhibition of fatty acid synthesis by orlistat also sensitized cells to ABT-737. Thus, the release of pro-apoptotic proteins, like Bak, from inhibitory anti-apoptotic proteins of the Bcl-2 family appears to be inhibited by fatty acids. Furthermore, FAO regulates activity of Bak-dependent mitochondrial permeability transition and downstream apoptosis. This provides a potential target for AML cells, as etomoxir decreased

quiescent leukemic progenitor cells in 50% of primary AML samples and provided a therapeutic benefit in combination with ABT-737 or cytarabine in a murine model of leukemia (Samudio et al. 2010).

In AML cell lines, co-culture with mesenchymal stromal cells (MSC) promotes the Warburg effect, as observed with lactate accumulation and reduced mitochondrial membrane potential (Samudio et al. 2008). This was induced through increased UCP2 expression, which is a mitochondrial uncoupling protein that inhibits mitochondrial ATP synthesis by decreasing the mitochondrial outer membrane potential (MOMP). This effect was observed in OCI-AML3 cells, while siRNA-mediated suppression of UCP2 reversed mitochondrial uncoupling and aerobic glycolysis elicited by MSC co-culture. Additionally, HL60 cells, which do not exhibit increased UCP2 expression when co-cultured with MSCs, also fail to induce uncoupling or increased aerobic glycolysis (Samudio et al. 2008). This group went on to demonstrate that through UCP2 induction, glucose is consumed but blocked from entering the mitochondrial TCA cycle, while non-glucose carbon sources, like fatty acids, are oxidized to support mitochondrial integrity and function. Altogether, this extends our current understanding of the role of FAO in leukemic cell survival and expands to include decreased mitochondrial ATP production as a method of chemoresistance and a potential target for AML therapy (Samudio et al. 2009).

Considering the importance of FAO, and therefore mitochondria, it can be no surprise that targeting mitochondrial respiration has developed as a new strategy to increase drug-induced apoptosis in human leukemic cells. For example, inhibition of mitochondrial electron transport chain complex I with rotenone not only induces a metabolic and energetic stress in AML cells, but also increases superoxide $O_2$ radical generation, leading to apoptosis. Additionally, this inhibition sensitized cells to anti-cancer agents whose mechanism also involves superoxide generation (Pelicano et al. 2003).

Furthermore, and as described above (Figure 7), several groups (Mardis et al. 2009; Chou et al. 2010; Gross et al. 2010; Ward et al. 2010) found that 15-20% of AML patients harbor mutations in isocitrate dehydrogenase (*IDH1* or *IDH2)* (Figueroa et al. 2010; Levine and Puzio-Kuter 2010). These findings have changed our vision about the importance of cell metabolism in leukemia. IDH1 and IDH2 proteins, located in the cytosol and mitochondria, respectively, may play roles in redox metabolism as well as lipid and sterol biosynthetic pathways as they use NADP+ as a cofactor, thereby producing NADPH. Additionally, while wild-type IDH converts isocitrate into alpha-ketoglutarate (α-KG), mutant IDH produces oncometabolite, 2-hydroxyglutarate (2-HG), from α-KG (Dang et al. 2009). As described above, the result is a major disruption of mitochondrial oxidative phosphorylation, stabilization of HIF-1α and increased Warburg metabolism (Xu et al. 2011) (Figure 7). *IDH1* and *IDH2* mutations are also associated with abnormal chromatin and DNA methylation profiles, which can induce cell differentiation arrest (Figueroa et al. 2010; Lu et al. 2012; Sasaki et al. 2012) (Figure 8). This is especially key in AML, which is characterized by clonal expansion of

immature myeloblasts that are blocked in completing differentiation and maintaining normal hematopoiesis. The accumulation of these immature cells leads to deregulation of normal hematopoiesis and bone marrow failure (Lowenberg et al. 1999; Estey 2012). Thus, metabo-oncogenic mutations, like *IDH1/2*, are the current focus and future of new therapeutic options for AML. With further research and development, we can expect that new targets related to these mutations with be discovered and provide pointed targets for molecular inhibition and treatment.

## 6. Metabolic targets and drugs

Throughout this introduction, we have identified several metabolic targets that are deregulated in cancer cell metabolism. A summary of these biomarkers and the drugs/compounds currently available to target them can be found in Table 6.

**Table 6: Summary of potential drugs/compounds targeting cancer cell metabolism.** An overview of metabolic targets in cancer cells and the known drugs, and their respective mechanisms of action, used to target them (Jones and Schulze 2012; Upadhyay et al. 2013; Zhang and Yang 2013).

| Mechanism of Action | Molecular Target | Drug/Compound |
|---|---|---|
| Blocks glucose uptake | GLUT1/4 | Imatinib |
| | | Phloretin |
| | | Fasentin |
| Blocks glycolytic flux | HK | 2-Deoxyglucose |
| | | Lonidamine |
| | | 3-Bromopyruvate |
| | | Mannoheptulose |
| | | Clotrimazole |
| | | Methyl Jasmonate |
| | ENO | Fluoride |
| Inhibits conversion of F6P to F-1,6-BP to inhibit PFK1 | PFK2 (PFKFB3) | 3-PO |
| Blocks pyruvate formation *via* the PK route | PKM2 | Cap-232/TLN-232 |
| | | Flourophosphates |
| | | Creatine phosphate |
| | | Oxalate |
| | | L-Phospholactate |
| Promotes glycolytic flux, reducing the synthesis of biosynthetic intermediates | PKM2 Activator | (Agios Pharmaceuticals) |
| Blocks PPP (non-oxidative and oxidative, respectively) | TKTL1 | Oxythiamine |
| | | Tarvagenix |
| | G6PD | 6-Aminonicotinamide |
| | | Buthionine S'R'-sulfoximine |
| | | DHEA |
| Promotes pyruvate entry into mitochondria | PDK1 | Dichloroacetate |
| Blocks metabolic flux pathways | LDHA, Aspartate aminotransferase | FX11 |
| | | Oxamate |
| | | Amino oxyacetate |
| Mitochondrial energy metabolism | PDH (complex and/or kinase) | CPI-613 |
| | ETCI/AMPK | Metformin |
| | ETCI | Rotenone |
| | ETCII | α-TOS |
| | ETCIII | Benzylisothiocyanate |
| | ETCIV | Oligomycin |
| | | Resveratrol |

| | | |
|---|---|---|
| Blocks mIDH alternative catalytic functions | Mutant IDH1/2 | (Agios Pharmaceuticals) |
| Blocks NAD production and reduces glycolysis | Nicotinamide phosphoribosyltransferase | MPC-9528 |
| Depletes glutamine uptake | Glutamine uptake | L-asparaginase |
| Blocks glutamine uptake and inhibits mTOR activation | SLC1A5 (Myc target) | L-γ-glutamyl-*p*-nitroanilide (GPNA) |
| Blocks glutaminolysis (glutamine conversion to glutamate) | GLS | 6-Diazo-5-oxo-L-norleucine |
| | | 968 |
| | | BPTES |
| Blocks transamination | Glutamine aminotransferase | Aminoxyacetic acid (AOA) |
| Blocks fatty acid synthesis | FASN | GSK837149A |
| | | Orlistat |
| | | C75 |
| | | C93 |
| | | FAS31 |
| | ACLY | SB-204990 |
| Blocks HIF-1α Activity | HIF-1α | Topotecan |
| | | Digoxin |
| | | YC-1 |
| | | GA |
| | | 2ME2 |
| | | PX-478 |
| | | Echinomycin |

# 7 Metformin

## 7.1 Metformin activity in type 2 diabetes

Type 2 diabetes is a disease that has reached epidemic proportions worldwide and is characterized by insulin resistance, hyperinsulinemia and hyperglycemia (Martin et al. 1992). Metformin (1,1-dimethylbiguanide) is the most commonly recommended and used oral normoglycemic agent for this disease (Kirpichnikov et al. 2002). Metformin is a member of the biguanide family and was first tested in clinical trials in 1957 (Sterne 1957) before being approved by the Federal Drug Administration in 1995 for diabetes treatment. Phenformin is another biguanide that is chemically and mechanistically similar to metformin, but is not approved for diabetic treatment as it can induce lactic acidosis in patients (DeFronzo and Goodman 1995; Stumvoll et al. 1995).

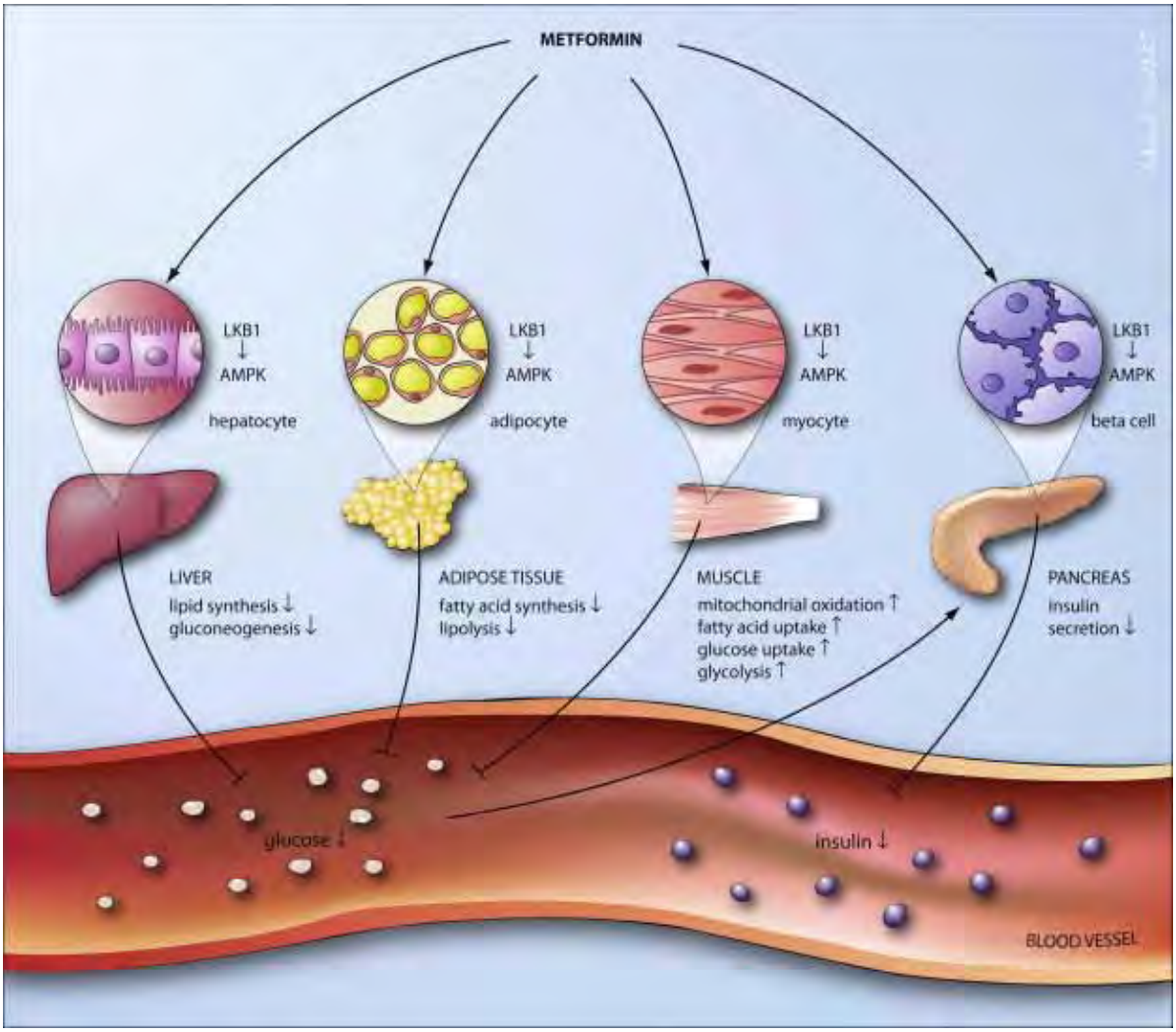



**Figure 24. Overview of metformin's mechanism of action in type 2 diabetics.** In type 2 diabetics, metformin's activation of the LKB1/AMPK pathway leads to decreased lipid synthesis and gluconeogenesis in hepatocytes, decreased fatty acid synthesis and lipolysis in adipocytes, increased mitochondrial oxidation, fatty acid uptake, glucose uptake and glycolysis in muscles and decreased insulin secretion in the pancreas. Figure adapted from Jalving et al. (2010).

Metformin is preferred in most cases over other type 2 diabetic treatments, such as sulfonylureas and insulin, because metformin does not induce weight gain, hypoglycemia or hyperinsulinemia (Bailey and Turner 1996). In type 2 diabetics, metformin decreases circulating glucose levels by decreasing hepatic gluconeogenesis and increasing muscle glucose uptake, glycolysis and sensitivity to insulin (Stumvoll et al. 1995), as well as a variety of other downstream effects, including, but not limited to, decreased hepatic lipid synthesis, decreased fatty acid synthesis and lipolysis in adipose tissue and decreased insulin secretion in the pancreas (Jalving et al. 2010) (Figure 24). Despite this data, metformin's mechanism of action is not entirely known.

What is currently understood is that metformin decreases insulin levels and insensitivity by increasing the phosphorylation and tyrosine kinase activity of insulin receptors while simultaneously increasing insulin binding to these receptors (Bailey and Turner 1996). Metformin works preferentially in hepatocytes due to the predominant expression of metformin transporter, organic cation transport 1 (OCT1) (Shu et al. 2007). By increasing hepatic insulin sensitivity, metformin reduces gluconeogenesis and hepatic glucose output (Stumvoll et al. 1995; Scarpello and Howlett 2008). Additionally, metformin causes increased skeletal muscle glucose uptake without increasing lactate levels (Bailey and Turner 1996). It appears that muscle cells can increase glucose uptake partially through PKC activation, but also through AMPK activation (Turban et al. 2012). Finally, metformin functions as an AMPK agonist (Zhou et al. 2001; Shaw et al. 2005) and an inhibitor of electron transport chain complex I (El-Mir et al. 2000; Owen et al. 2000).

In rat hepatocytes, metformin activates AMPK in both a time- and dose-dependent, which is responsible for many of the drug's anti-diabetic effects (Zhou et al. 2001). AMPK, as described above, is activated by increases in the intracellular AMP:ATP ratio. AMP acts as an allosteric activator at the gamma subunit of AMPK, which leads to conformational changes and increased phosphorylation at the Thr172 subunit by LKB1 and CaMKKβ (Viollet et al. 2009). Hawley et al. (2010) demonstrated that AMPK activation by metformin is inhibited in cell lines that stably express a mutant, AMP-insensitive gamma2 AMPK subunit, proving that metformin-induced accumulation of AMP triggers activation of AMPK (Hawley et al. 2010). Additionally, metformin appears to activate AMPK activation downstream of LKB1 as mice lacking hepatic LKB1 did not exhibit metformin-induced activation of AMPK (Shaw et al. 2005). Mitochondrial reactive nitrogen species can also lead to AMPK activation as Zou et al. (2004) showed *in vitro* and *in vivo* that metformin dose-dependently activated AMPK in aortic endothelial cells in parallel with increased reactive nitrogen species. Furthermore, depletion of mitochondria, adenoviral overexpression of superoxide dismutases and inhibition of nitric-oxide synthase abolished metformin-induced AMPK phosphorylation and downstream activities (Zou et al. 2004). AMPK can also be activated independently of adenine nucleotide changes by other stresses (Hawley et al. 2003), including $Ca^{2+}$, mitochondrial matrix pH and transmembrane potential, magnesium ions, inorganic phosphate, and cyclophilin D levels (Owen et al. 2000; Guigas et al. 2004; Shaw et al. 2005; Kisfalvi et al. 2009; Hawley et al. 2010).

AMPK activation leads to multiple changes in the cell, which, generally speaking, decrease anabolic activities and increase catabolic activities to generate energy and decrease the AMP:ATP ratio. Metformin-induced AMPK decreases gluconeogenesis through phosphorylation of CREBP binding protein (CBP), which dissociates the CREBP-CBP-TORC2 transcription complex and reduces gluconeogenic enzyme gene expression (Koo et al. 2005). In mouse skeletal muscles, AMPK increases intracellular $NAD^+$ levels, which activates sirtuin 1, also known as NAD-dependent deacetylase sirtuin-1 (SIRT1). This increased activity leads to deacetylation and inhibition of downstream SIRT1 targets, including peroxisome proliferators-activated receptor-γ coactivator 1α (PGC-1α), which is responsible for gluconeogenic genes, and the forkhead box O1 and O3 (FOXO1 and FOXO3) transcription factors (Jager et al. 2007; Canto and Auwerx 2009). Additionally, deletion of LKB1 in mice livers results in severe hyperglycemia as well as increased gluconeogenic and lipogenic gene expression. Metformin treatment of these mice was unable to activate AMPK or reduce any of the resulting effects of LKB1 deletion, which further demonstrates the upstream role of LKB1 in metformin's anti-diabetic activity (Shaw et al. 2005).

Metformin-induced AMPK also reduces hepatic lipid synthesis by inhibiting fatty acid synthase and acetyl-CoA carboxylase (ACC) by direct phosphorylation of transcription factors carbohydrate response element binding protein (ChREBP) and hepatocyte nuclear factor 4 (HNF4) (Foretz et al. 1998; Leclerc et al. 2001; Kawaguchi et al. 2002; Hong et al. 2003; Foretz et al. 2005). The inhibition of ACC releases the negative feedback on mitochondrial fatty acid transport, CPT1, which allows increased fatty acid β-oxidation to produce ATP, as observed in rat hepatocytes (Zhou et al. 2001). AMPK also inhibits downstream mTORC1 activity through phosphorylation of the TSC1/TSC2 complex (Inoki et al. 2003b). mTORC1 inhibition can manipulate metabolism as described above in the metabolic section "The mammalian target of rapamycin" on page 60 and includes, but is not limited to, decreased glycolysis, decreased SREBP translation, which decreases FAS, and increased FAO (Peng et al. 2002; Brown et al. 2007; Guertin and Sabatini 2007; Laplante and Sabatini 2009; Foretz et al. 2010; Miller and Birnbaum 2010).

However, as metformin does not directly activate AMPK, activation of this kinase is likely to be secondary to its inhibition of electron transport chain complex I (ETCI) (Hardie 2006) Figure 25. Metformin decreases gluconeogenesis and increases glycolysis in rat hepatocytes in a time- and dose-dependent manner, which correlates to an indirect inhibition of ETCI, decreased oxygen consumption and an increase in the AMP:ATP ratio (El-Mir et al. 2000; Owen et al. 2000; Stephenne et al. 2011). The time-dependency of metformin is compatible with the slow membrane-potential-driven accumulation of the positively charged drug within the mitochondrial matrix (El-Mir et al. 2000; Owen et al. 2000). Evidence of metformin ETCI inhibition was also shown in MIN6 β-cells and primary rat β-cells, in which methyl succinate, a complex II substrate, appeared to bypass metformin's inhibition of ETCI and repressed phosphorylation of AMPK (Hinke et al. 2007). While

the exact by which metformin inhibits ETCI is unknown, El-Mir et al. (2000) eliminated the possibilities of metformin as an insulin-signaling pathway inhibitor, nitric oxide precursor or inhibitor, or intra- or extracellular $Ca^{2+}$ chelator (El-Mir et al. 2000).

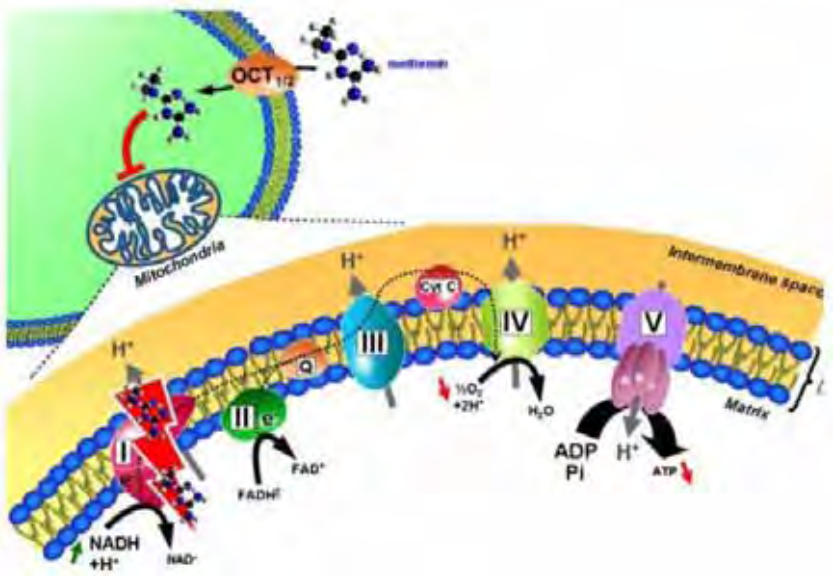



**Figure 25. Metformin inhibits the electron transport chain.** Metformin is taken up in the cell through transporters OCT1/2 and then induces a block in the electron transport chain at complex I, although the exact mechanism by which it blocks complex I is currently unknown. Figure adapted from Viollet et al. (2009).

One of the harmful effects of hyperglycemia is increased glycolysis, which can lead to increased oxidative phosphorylation, as diabetic cells do not necessarily exhibit Warburg-like metabolism, and subsequent superoxide overproduction by the electron transport chain. While rotenone both increased and decreased ROS production at complex I, depending on forward (glutamate-malate) or reverse (succinate) electron flux, metformin did not increase ROS production and selectively blocked the reverse electron flow or respiratory $O_2$ flux in mitochondria isolated from rat livers and skeletal muscle of obese rats (Batandier et al. 2006; Kane et al. 2010). Additionally, metformin decreased NADPH oxidase activity in diabetic podocytes, leading to reduced ROS production (Piwkowska et al. 2010). Finally, metformin can normalize mitochondrial ROS production by induction of MnSOD and promotion of mitochondrial biogenesis through activation of the PGC-1$\alpha$ pathway (Fujisawa et al. 2009).

Finally, and in support of metformin's inhibition of ETCI as the primary and most important effect, it appears that metformin may be able to inhibit gluconeogenesis without activation of AMPK. Foretz et al. (2010) demonstrated that a reduction in the hepatic energy status alone could regulate hepatic glucose output (Foretz et al. 2010). In addition, increased AMP could also inhibit gluconeogenesis through allosteric regulation of key enzymes in this pathway, such as fructose-1,6-bisphosphate (Miller and Birnbaum 2010). Additionally, metformin can decrease gluconeogenesis independent of transcriptional repression of genes in this pathway. Forced expression of

gluconeogenic genes through overexpression of PGC-1α did not reduce metformin reduction of glucose output, which was instead associated with a depletion of energy stores (Foretz et al. 2010). In insulin-resistant rats fed a high-fat diet, repression of hepatic glucose production was dependent on inhibition of the flux through G6Pase rather than gene expression (Mithieux et al. 2002). Supporting this data, addition of metformin to isolated rat hepatocytes led to stimulation of pyruvate-kinase activity due to the decrease in cellular ATP levels, an allosteric inhibitor of PK, and inhibition of gluconeogenesis (Argaud et al. 1993). Overall, metformin induces a number of metabolic changes in diabetic cells, a summary of which can be found in Figure 26. However, while these changes effectively counteract the negative impacts of type 2 diabetes, there are still several effects of metformin that are not entirely understood, such as the exact mechanisms by which metformin inhibits ETCI and activates AMPK. Additionally, due to the broad range of changes metformin induces in diabetic cells, it is likely that there are other impacts that have not yet been discovered. Thus, metformin's activity in diabetic cells still requires further investigation.

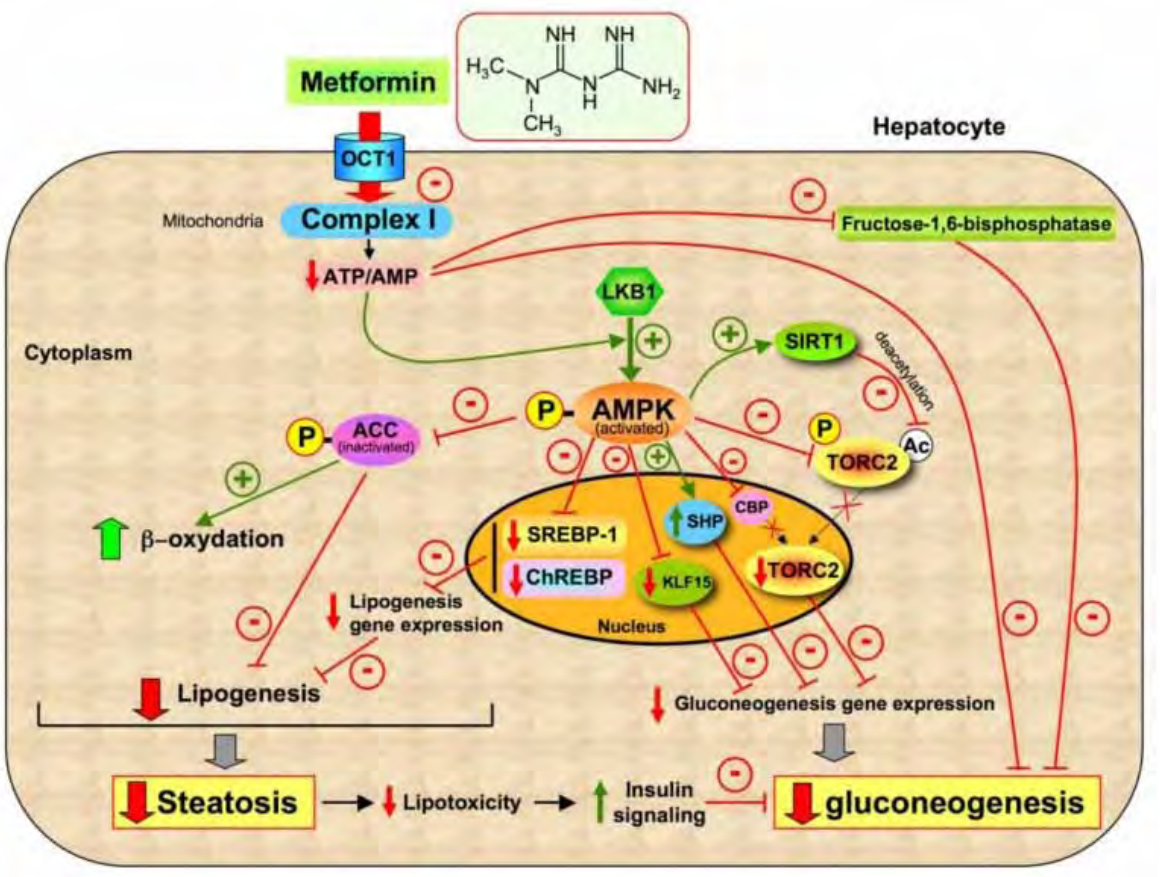



**Figure 26. Overview of metformin's metabolic activity in type 2 diabetics.** Metformin uptake in the cell depends on expression of transport protein, OCT1. Metformin then inhibits ETCI leading to decreases in the ATP/AMP ratio, which directly blocks fructose-1,6-bisphosphate and gluconeogenesis, but also activates the LKB1/AMPK pathway. AMPK activation leads to phosphorylation and inactivation of ACC, which decreases FAS in favor of FAO. AMPK also decreases gluconeogenesis gene expression through increased SIRT1 and SHP as well as decreased mTORC and KLF15 activity. AMPK also decreases lipogenesis and its gene expression through down-regulation of SHREBP-1 and ChREBP. Altogether, decreased lipogenesis leads to decreased steatosis, decreased lipotoxicity and increased insulin signaling, which also blocks gluconeogenesis. Figure adapted from Viollet et al. (2009).

## 7.2 Epidemiological studies

There are many risk factors for cancer, which include, but are certainly not limited to, hyperglycemia and hyperinsulinemia, two characteristics of type 2 diabetes (Giovannucci and Michaud 2007; Grote et al. 2010). Although not everyone with hyperglycemia, hyperinsulinemia or type 2 diabetes is diagnosed with cancer, there is a higher prevalence of cancer in these groups compared to non-diabetics (Vigneri et al. 2009; Giovannucci et al. 2010). The increased risk associated with diabetes is about 2-fold or more for cancers of the liver, pancreas and endometrium and about 1.2-1.5-fold for colon, rectum, breast and bladder cancers. Other cancers like lung cancer do not seem to be associated with diabetes while prostate cancer is actually reduced (Giovannucci et al. 2010). Epidemiological studies have also shown that cancer patients with diabetes have increased mortality rates (Barone et al. 2008).

It is still unknown exactly how diabetes increases the risk for cancer. A number of possible associations are hyperglycemia, hyperinsulinemia, insulin resistance and inflammation. Specifically, cancer cells can benefit from the increased glucose and insulin levels, which can switch cancer cell metabolism in the glycolytic direction to support increased proliferation and growth (Vigneri et al. 2009; Giovannucci et al. 2010). Additionally, increased insulin levels can affect hormone-dependent pathways by increasing active estrogen levels, which increases the risk of breast and endometrial cancers. However, while these factors are associated with increased cancer risk, they may just define diabetes as a marker for upstream metabolic and hormonal factors that are either linked to cancer or due to the causes of diabetes, such as obesity (Renehan et al. 2008; Basen-Engquist and Chang 2011).

Recent studies have also suggested an association between some of the medications used to treat hyperglycemia, such as sulfonylureas, insulin and metformin, and their effect on cancer risk (Giovannucci et al. 2010; Currie et al. 2012). In 2005, Evans et al. first reported that type 2 diabetics treated with metformin had a decreased overall cancer incidence with an odds ratio (OR) of 0·86 and 95% confidence interval (CI between 0·73-1·02) (Evans et al. 2005). Since then, multiple retrospective studies have shown that diabetic patients treated with metformin, as compared to other drugs, had a significantly lower risk of developing cancer and a lower mortality rate if diagnosed with cancer, a summary of which can be found in Table 7. Libby et al. (2009) showed that cancer incidence in a 10 year follow-up period was 7.3% in metformin-treated diabetics but 11.6% in diabetics treated with other drugs. Additionally, patients had a higher median time to cancer when treated with metformin (Libby et al. 2009). Metformin has also been shown to improve the prognosis of cancer, with a standardized cancer mortality ratio of 0·88 for metformin and 1·625 for other diabetic drugs (Landman et al. 2010). Several groups have performed retrospective analyses to demonstrate that metformin, compared to insulin and sulphonylurea, also lowers the rate of mortality in type 2 diabetics (Bowker et al. 2006; Libby et al. 2009). The most recent meta-analysis study has

shown a 31% reduction in overall cancer incidence or mortality in diabetics when treated with metformin (Decensi et al. 2010) (Figure 27).

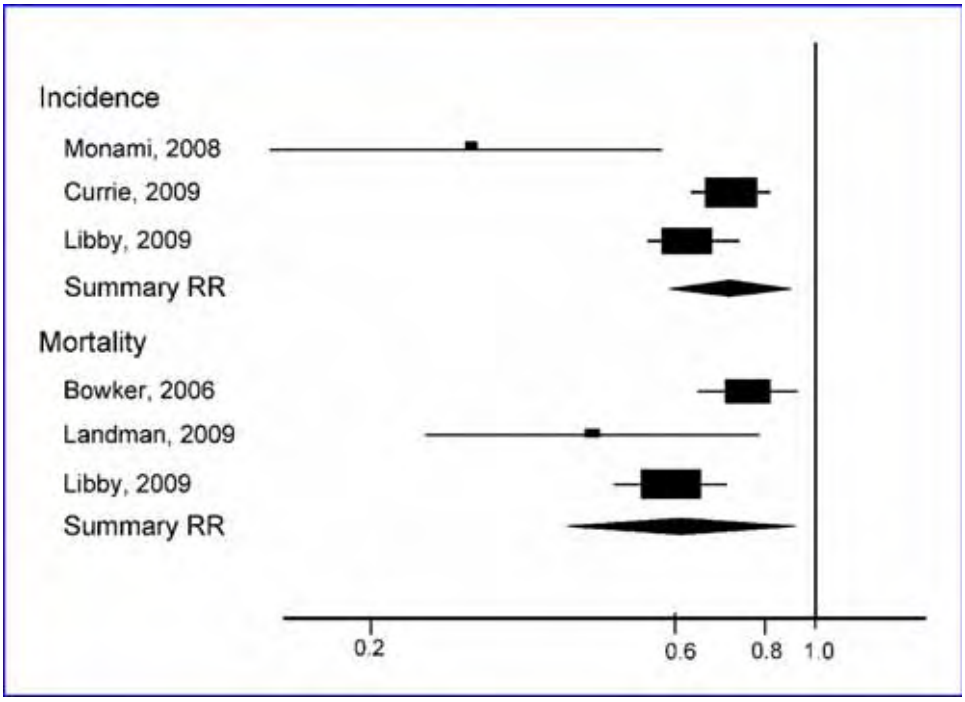



**Figure 27. Metformin decreases overall cancer incidence and mortality in type 2 diabetics.**
Figure adapted from Decensi et al. (2010).

Specifically, metformin reduced the risk of colorectal, pancreatic, hepatocellular, breast, ovarian, prostate, liver and lung cancer (References summarized in Table 7). Metformin's preventative effects against colorectal cancer and breast cancer are especially strong as both cancers are associated with obesity. Patients with colorectal and pancreatic carcinomas treated with metformin also showed a 30% improvement in survival compared to diabetic patients treated with other drugs (Lee et al. 2011). In terms of hepatocellular carcinomas, it was recently shown that every incremental year of metformin treatment induces a 7% reduction in the risk of this cancer in type 2 diabetics (Chen et al. 2013). Additionally, type 2 diabetics with hepatocellular carcinomas who are treated with radiofrequency have a lower survival rate than non-diabetics, but metformin treatment actually increased survival outcomes in this population (Chen et al. 2011). Although prostate cancer is inversely associated with type 2 diabetes, hyperglycemia and hyperinsulinemia are still risk factors of higher mortality once prostate cancer is diagnosed. One retrospective study has suggested that Caucasian men with diabetes and treated with metformin have a 44% lower risk of prostate cancer, while another study showed that metformin was a significant predictor of improved survival (Wright and Stanford 2009; He et al. 2011). A second, independent study also provided restrospective analysis that treatment with thiazolidinedione and metformin were significant predictors of improved survival

(He et al. 2012). However, a third group has actually reported that metformin has no beneficial effect on survival in prostate cancer (Patel et al. 2010).

Thus, while many of these findings are very promising, there are still some conflicting results. One of the reasons for this is that most data is retrospective and was not collected in a standardized experiment. Thus, there are no controls to compare to metformin and the observed differences could be due to the cancer-promoting effects of other drugs, such as sulphonylureas and insulin, as compared to cancer-preventing effects of metformin (Bowker et al. 2010; Home et al. 2010; Currie et al. 2012). Thus, every experiment should be analyzed with caution, while simultaneously taking confounding factors into consideration. Some groups have attempted to clinically test metformin in a controlled setting. Two small, randomized trials showed no cancer-protective effect of metformin (Home et al. 2010), but other studies that accounted for the nature of other antidiabetic treatments actually observed a protective effect of metformin (Libby et al. 2009; Monami et al. 2009; Landman et al. 2010; Lee et al. 2011; Monami et al. 2011). One issue with these types of analyses is that within the diabetic field, it would be unethical to give some diabetic patients metformin while withholding treatment from other patients to test whether metformin is actually beneficial or just reducing risks associated with insulin and other treatments. More likely, future trials will test metformin in non-diabetic patients to determine if there is an effect on cancer risk or precaution.

**Table 7: Summary of epidemiological metformin studies** (Decensi et al. 2010; Aljada and Mousa 2012; Andujar-Plata et al. 2012; Dowling et al. 2012; Pierotti et al. 2013).

| Reference/ Year | Design, Population, Outcome | Site | Comparison | Outcome HR/OR/RR (95% CI) |
|---|---|---|---|---|
| Yang et al. (2004); UK | Nested case-control; TIID | Colon | Met vs Non Met<br>Met + SU | OR: 1·0 (0·6-1·7)<br>OR: 1·2 (0·7-2·2) |
| Evans et al. (2005); Scotland | Cohort; TIID; Incidence | Multiple | Met vs Non Met | OR 0·86 (0·73-1·02)<br>RR 0·77 (0·64-0·92) |
| Bowker et al. (2006); Canada | Retrospective cohort; TIID; Mortality | Multiple | Met<br>Insulin<br>SU | HR 1·0<br>HR 1·9 (1·5-2·4)<br>HR 1·3 (1·1-1·6) |
| Oliveria et al. (2008); USA | Cohort, TIID; Incidence | Colorectal<br>Liver<br>Pancreas | Met vs Non Met | RR: 0·67 (0·52-0·85)<br>RR: 0·73 (0·34-1·56)<br>RR: 1·26 (0·80-1·99) |
| Currie et al. (2009); UK | Retrospective Cohort; TIID; Incidence | Breast<br>Colorectal<br>Pancreas<br>Prostate | Relative to Met:<br>SU<br><br><br><br>Met+SU<br><br><br><br>Insulin-based<br><br><br><br><br>Met vs SU<br>Insulin<br>Not in patients under Insulin TX | HR:<br>B: 0·98 (0·69-1·41)<br>C: 1·80 (1·29-2·53)<br>Pa: 4·95 (2·74-8·96)<br>Pr: 1·07 (0·76-1·49)<br>B: 0·90 (0·67-1·21)<br>C: 1·43 (1·05-1·94)<br>Pa: 0·38 (0·13-1·12)<br>Pr: 1·18 (0·89-1·57)<br>B: 1·07 (0·79-1·44)<br>C: 1·69 (1·23-2·33)<br>Pa: 4·63 (2·64-8·10)<br>Pr: 1·10 (0·79-1·52)<br><br>RR: 0·74 (0·65-0·84)<br>RR: 0·70 (0·63-0·79)<br>RR: 0·54 (0·43-0·66) |
| Donadon et al. (2009); Italy | Case-control; Incidence | Liver | Insulin or SU<br>Met | OR: 2·99 (1·34-6·65)<br>OR: 0·33 (0·1-0·7) |
| Libby et al. (2009); Scotland, UK | Retrospective Cohort; TIID; Incidence, Mortality | Colorectal<br>Lung<br>Breast<br>Overall | Met vs Non Met | RR:<br>C: 0·6 (0·38-0·94)<br>L: 0·7 (0·43-1·15)<br>B: 0·6 (0·32-1·10)<br>O: 0·63 (0·53-0·75) |
| Li et al. (2009); TX, USA | Case-Control; Pancreas Cancer *versus* Controls; Incidence | Pancreas | Insulin<br>Insulin secret.<br>Thiasolidinediones<br>Metformin | OR: 5·04 (2·38-10·7)<br>OR: 1·74 (0·80-3·77)<br>OR: 1·65 (0·71-3·87)<br>OR: 0·41 (0·19-0·87) |
| Jiralerspong et al. (2009); TX, USA | Retrospective Cohort; Invasive breast cancer with neoadjuvant chemotherapy | Breast | Thiazolidinediones;<br>Insulin | pCR:<br>Met: 24%<br>NonMet: 8%<br>Nondiabetic: 16% |
| Monami et al. (2009); Italy | Case-Control; Incidence | Multiple | Met vs Non Met | RR: 0·28 (0·13-0·57) |
| Wright and Stanford (2009); USA | Case-control; Prostate vs Control; Incidence | Prostate | Met vs Non Met | OR: 0·56 (0·32-1·00) |

| | | | | |
|---|---|---|---|---|
| Bodmer et al. (2010); UK | Nested Case-Control; TIID; Incidence | Breast | Met vs Non Met | OR: 0·44 (0·24-0·82) |
| Bowker et al. (2010); Canada | Cohort; Mortality | Multiple | Met vs SU | RR: 0·80 (0·65-0·98) |
| Hassan et al. (2010); TX, USA | Case-Control; Incidence | Liver | Biguanides<br>Thiasolidinediones<br>SU<br>Insulin | OR: 0·3 (0·2-0·6)<br>OR: 0·3 (0·1-0·7)<br>OR 7·1 (2·9-16·9)<br>OR: 1·9 (0·8-4·6) |
| Home et al. (2010); Multi | Randomized control trials; Incidence | Multiple | Met vs<br>SU<br>Glibenclamide<br>Met+SU vs<br>Rosiglitazone+SU | <br>RR: 0·92 (0·63-1·35)<br>RR: 0·78 (0·53-1·14)<br>RR: 1·22 (0·86-1·74) |
| Landman et al. (2010); Netherlands | Prospective Cohort; TIID; Mortality | Multiple | Met vs Non Met | HR: 1·47 (1·22-1·76)<br>RR: 0·43 (0·23-0·80) |
| Azoulay et al. (2011); UK | Nested Case-Control; Incidence | Prostate | Met vs Non Met | RR: 1·23 (0·99-1·52) |
| Bodmer et al. (2011); UK | Case-Control, TIID; Incidence | Ovarian | Metformin<br>SU<br>Insulin | OR: 0·61 (0·30-1·25)<br>OR: 1·26 (0·65-2·44)<br>OR: 2·29 (1·13-4·65) |
| Bosco et al. (2011); Denmark | Nested Case-Control, TIID; Incidence | Breast | Met vs Non Met | OR: 0·81 (0·63-0·96) |
| He et al. (2011); TX, USA | Diabetic cancer patients; Mortality | Prostate | Thiasolidinediones<br>Metformin<br>Insulin | HR: 0·45 (0·21-0·97)<br>HR: 0·55 (0·32-0·96)<br>HR: 0·85 (0·44-1·62) |
| Lee et al. (2011); Taiwan | Prospective Cohort, TIID; Incidence | Colorectal<br>Liver<br>Pancreas<br>Esoph.<br>Stomach<br>Overall | Met vs Non Met | HR: 0·36 (0·13-0·98)<br>HR: 0·06 (0·02-0·16)<br>HR: 0·15 (0·03-0·79)<br>RR: 0·44 (0·07-2·61)<br>RR: 1·41 (0·42-4·73)<br>HR: 0·12 (0·08-0·19) |
| Monami et al. (2011); Italy | Case-Control; Incidence | Multiple | Metformin<br>SU<br>Met vs Non Met | OR: 0·46 (0·25-0·85)<br>OR: 0·75 (0·39-1·45)<br>RR: 0·46 (0·25-0·85) |
| Bodmer et al. (2012b); Bodmer et al. (2012a); USA | Case-Control, TIID; Incidence | Colorectal<br>Pancreas | Met vs Non Met | RR: 1·43 (1·08-1·90)<br>RR: 0·87 (0·59-1·29) |
| Bo et al. (2012); Italy | Retrospective Cohort; Mortality | Multiple | Metformin<br>Insulin | HR: 0·56 (0·34-0·94)<br>HR: 1·56 (1·22-1·99) |
| Lee et al. (2012); Korea | Retrospective Cohort; Mortality | Colorectal | Met vs Non Met | HR: 1·45 (1·08-1·93) |
| Abbreviations: TIID, Type 2 diabetes; Met, Metformin; SU: Sulphonylureas; B, Breast Cancer; C, Colorectal Cancer; L, Lung Cancer; Pa, Pancreatic Cancer; Pr, Prostate Cancer; O, Overall; HR, Hazard Ratio, RR, Risk Ratio; OR, Odds Ratio; TX, treatment | | | | |

## 7.3 Metformin effects in cancer cells

### 7.3.1 Overview

While many continue to study the role of metformin in cancer prevention in diabetic patients, others have begun to focus on metformin's potential as a cancer therapy for non-diabetics through a number of mechanisms, summarized in Figure 28. In breast cancer, a large clinical study has shown that complete remission is more frequent from neoadjuvant chemotherapy in diabetic patients under metformin treatment (24%) than in diabetics not treated with metformin (8%) or even non-diabetic patients (16%) (Jiralerspong et al. 2009). Additionally, a small prospective clinical trial in 26 non-diabetic patients with aberrant crypt foci (ACF), a marker for colon carcinoma, showed that 1 month of metformin treatment reduced ACF number and rectal epithelial cell proliferation (Hosono et al. 2010a). We now know that, *in vitro* and *in vivo*, metformin inhibits the growth of AML, ALL, melanoma, hepatocellular carcinoma and endometrial, pancreatic, colon, prostate, ovarian, lung, glioma and breast cancer cells (Isakovic et al. 2007; Algire et al. 2008; Hirsch et al. 2009; Algire et al. 2010; Cantrell et al. 2010; Green et al. 2010; Kalender et al. 2010; Algire et al. 2011; Ben Sahra et al. 2011; Iliopoulos et al. 2011; Rattan et al. 2011a; Rattan et al. 2011b; Tomic et al. 2011; Pan et al. 2012; Shi et al. 2012; Xiong et al. 2012).

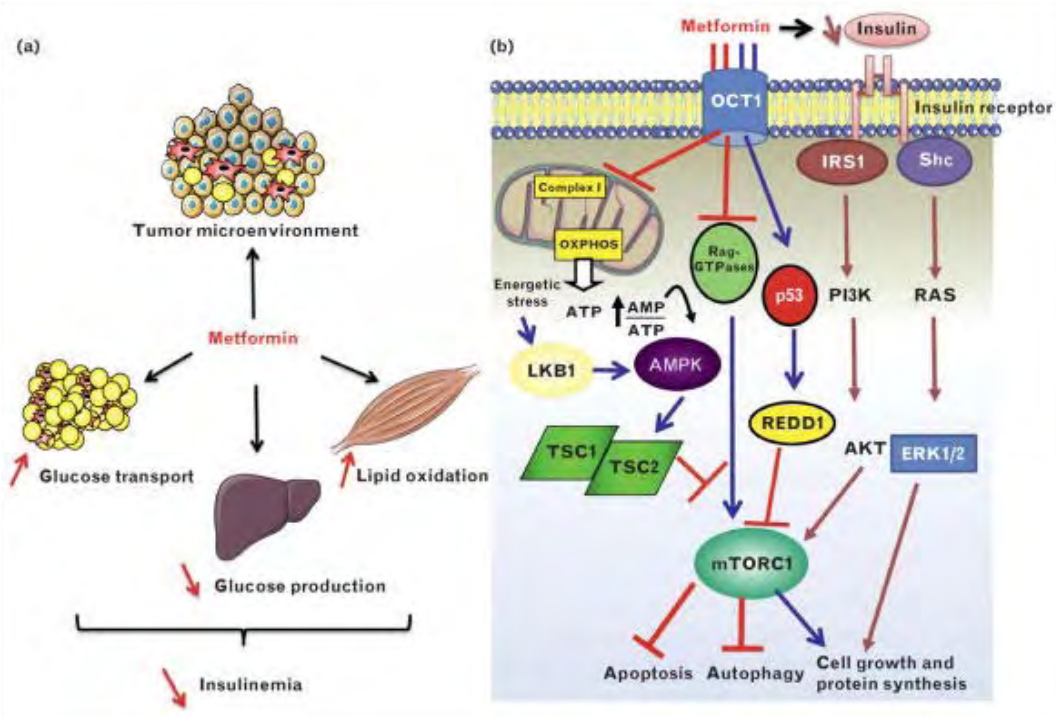



**Figure 28. Molecular and physiological effects of metformin in cancer.** (a) Indirect effects: metformin increases glucose transport and lipid oxidation in adipocytes and skeletal muscle, respectively, and decreases hepatic glucose production, thereby reducing insulinemia. (b) Direct effects: metformin inhibits ETCI and mTORC1 in an AMP-activated kinase-dependent or -independent manner (through REDD1 or Rag GTPase). Metformin indirectly decreases insulinemia and, therefore, indirectly modulates the proliferative pathways: AKT and extracellular signal-regulated kinases (ERK). Figure adapted from Bost et al. (2012).

### 7.3.2 Breast cancer

*In vitro*, *in vivo* and clinical data has demonstrated a strong anti-cancer effect of metformin in breast cancer cells. Metformin inhibits breast cancer growth *in vitro* and *in vivo*, with an especially strong effect in cells overexpressing the human epidermal growth factor receptor 2 (HER2), as metformin reduced HER-2 expression (Zakikhani et al. 2006; Vazquez-Martin et al. 2009; Zakikhani et al. 2010). Metformin reduces proliferation and colony formation in four breast cancer cell lines by decreasing cyclin D1 and ESF1 expression to induce a G1 cell cycle arrest (Alimova et al. 2009). In several breast cancer cell lines, metformin induces a cytotoxic effect through both caspase-dependent and -independent mechanisms, including poly (ADP-ribose) polymerase (PARP) activation associated with mitochondria enlargement (Zhuang and Miskimins 2011). *In vitro* and *in vivo,* metformin also inhibits the growth of triple negative (TN) breast cancer, a very aggressive subtype, by inducing G1 cell cycle arrest and apoptosis (Liu et al. 2009). Finally, in a genome-wide analysis of mTOR translational targets in MCF7 breast cancer cells, metformin has been shown to control mRNA translation as comparable to specific mTOR inhibitors, rapamycin and PP242 (Larsson et al. 2012).

Additional breast cancer studies have shown that metformin can specifically inhibit cellular transformation and kill cancer stem cells in four genetically different breast cancer cell lines (Hirsch et al. 2009). The targeting of breast cancer stem cells by metformin was also reproduced using the JIMT-1 breast cancer cell line. This group demonstrated that metformin actually targeted the progenitor $CD44^{+}CD24^{-}$ cells 10-fold more than non-$CD44^{+}CD24^{-}$ cells and was enough to overcome *in vivo* resistance to trastuzumab, a monoclonal antibody designed to target HER2 receptors (Cufi et al. 2012). Both *in vitro* and *in vivo*, metformin also increased the efficacy of chemotherapy drug, doxorubicin, blocking tumor growth and preventing relapse (Hirsch et al. 2009; Iliopoulos et al. 2011). Hirsch et al. (2009) and Iliopoulos et al. (2011) also showed that doxorubicin and metformin could work in combination to prevent relapse for prostate and lung cancer xenografted mice. Finally, metformin not only increases cell death in response to chemotherapy, but also caused massive apoptosis in combination with glucose withdrawal in breast cancer cells (Menendez et al. 2012).

### 7.3.3 Ovarian cancer

Metformin also induces anti-proliferative effects and a G1 cell cycle arrest in ovarian cancer cell lines, without having any effect on primary ovarian cells. However, while metformin did target chemoresistant cell lines, metformin had a lower impact on more aggressive cell lines (Rattan et al. 2011a; Rattan et al. 2011b). Another study showed that metformin induced changes in Bcl-2 and apoptosis through increased activation of pro-apoptotic proteins, Bad and Bax, while decreasing the levels of anti-apoptotic proteins, Bcl-2, Bcl-xL, and Mcl-1 (Yasmeen et al. 2011).

### 7.3.4 Colon cancer

In colon cancer cells, metformin reduces cell growth *in vitro* (Zakikhani et al. 2008) and reduces the development of intestinal polyps and colorectal aberrant crypt foci at the protein-synthesis level *in vivo* in non-diabetic mice (Tomimoto et al. 2008; Hosono et al. 2010b). Buzzai et al. (2007) showed *in vitro* and *in vivo* that metformin suppressed tumor growth and induced apoptosis of colon cancer cell line HCT116, but only when p53 was knocked out (Buzzai et al. 2007). Mice xenografted with colon carcinoma and fed a high-energy diet had tumors of approximately twice the volume of mice on normal diets, which was due to increased insulin levels, phosphorylated AKT and fatty acid synthase (FASN) expression. Treatment with metformin blocked the effect of the high-energy diet, but had no effect on mice fed a normal diet (Algire et al. 2010). Additionally, metformin only induced apoptosis in high-energy diet mice through upregulation of Bcl-2 family member, BNIP-3, and subsequent PARP cleavage (Algire et al. 2010). Unfortunately, these data suggest that the effect of metformin in colon cancer may depend on the diabetic and energetic status of patients at both the cell and whole organism levels.

### 7.3.5 Pancreatic cancer

Pancreatic cell lines exhibit a novel response to metformin, which is a block in the cross talk between insulin and G protein-coupled receptors (GPCR), which inhibits insulin-induced increases of $Ca^{2+}$ signaling, DNA synthesis and anchorage-independent proliferation *in vitro* and *in vivo*. Furthermore, this effect was not viewed in AsPC-1 cells, which do not express LKB1 (Kisfalvi et al. 2009). This suggests a role of the LKB1-AMPK pathway in the response to metformin in these cancer cell lines. Kisfalvi et al. (2009) also observed a decrease in the growth of MIAPaCa-2 and PANC-1 cells xenografted in flanks of nude mice and treated intraperitoneally with 250 mg/kg/day metformin.

### 7.3.6 Glioblastoma

Metformin exhibits anti-glioma activity through a block in G0/G1 cell cycle progression and, in confluent cultures, a massive induction of caspase-dependent cell death associated with JNK activation, mitochondrial membrane depolarization and oxidative stress. This apoptosis was inhibited by cyclosporine A, which blocked the mitochondrial permeability transition pore and n-acetylcysteine, which blocked oxygen radical production (Isakovic et al. 2007). Additionally, metformin may target tumor-initiating stem cell-like glioma cells through AMPK-dependent inhibition of FOXO3 and subsequent differentiation as observed *in vivo* within the brain, in developed tumors and in terms of overall survival (Sato et al. 2012).

### 7.3.7 Endometrial cancer

In endometrial cancer cells, metformin inhibits growth through a G1 cell cycle arrest and induces apoptosis at high doses. Additionally, metformin decreases hTERT mRNA expression, which is a rate-limiting determinant of telomerase activity (Cantrell et al. 2010). Finally, metformin decreased the *in vitro* invasion of endometrial cancer cells (Tan et al. 2011).

### 7.3.8 Lung cancer

As previously observed in colon cancer, mice with lung LLC1 carcinoma had increased cancer growth when fed a high-energy diet, which was almost completely reversed by metformin. In high-energy diet mice, this effect seemed to be occurring through reduced insulin levels (Algire et al. 2008). Additionally, metformin prevented tobacco-specific NNK-induced lung cancer by inhibiting mTORC1 (Memmott et al. 2010).

### 7.3.9 Leukemia and lymphoma

Several groups have tested metformin as an anti-leukemic agent. Green et al. (2010) first demonstrated that metformin reduced *in vitro* AML cell proliferation in parallel with decreased mTORC1 activity, mRNA translation and expression of cyclin D1, Bcl-xL and c-Myc. Metformin also decreased clonogenic growth of leukemic progenitors without impacting growth of normal $CD34^+$ cells. Finally, metformin significantly reduced AML tumor growth in nude mice (Green et al. 2010). In acute lymphoid leukemia (ALL), while pharmacologically achievable concentrations of some anti-diabetic drugs increased total ALL cell numbers, metformin significantly decreased cell proliferation by blocking the mTOR pathway. Additionally, metformin sensitized Reh B-ALL cells to daunorubicin-induced apoptosis, while other anti-diabetic drugs increased chemoresistance (Pan et al. 2012). For patients diagnosed with ALL, these findings are crucial, because hyper-CVAD (cyclophosphamide, vincristine, adriamycin [doxorubicin] and dexamethasone) chemotherapy often induces hyperglycemia, which is associated with poor outcomes (Weiser et al. 2004).

### 7.3.10 Clinical trials

Currently, 154 clinical trials are investigating the effects of metformin in cancer patients (www.clinicaltrials.gov). Metformin is being tested in combination with adjuvant and conventional chemotherapies and anti-tumoral drugs in leukemia, lymphoma, breast, colorectal, pancreatic, prostate and head and neck cancers, with additional trials on metabolic and polycystic ovarian syndromes.

## 7.4 Metformin mechanisms of action in cancer cells

### 7.4.1 Overview

While it is very intriguing that metformin has multiple effects on many cancer cell types, the issue that remains is the exact mechanism through which metformin targets these cells. Specifically, what is the role of the most commonly studied metformin target, AMPK? This question is extremely important for several reasons. First, metformin is not a single-action drug. By impacting cellular and energetic metabolism, multiple cell signaling pathways and other off-target sites, there are a number of potential mechanisms through which metformin could induce anti-cancer activity. Second, it appears that metformin can affect cancer cell types in different ways. Thus, articles focusing on metformin's mechanism of action should use both negative and positive controls to clearly prove whether an observed cellular change directly induces a downstream anti-cancer activity. Many of the observed effects, such as increased AMPK activity, may occur in parallel to anti-cancer effects. However, without performing the siRNA or AMPK inhibitor/activator experiments to confirm this data, these effects can only be described as correlative. If not, this can mislead readers to unsupported conclusions, when in fact there are multiple other mechanisms that could be responsible.

In the above-mentioned literature describing anti-tumoral effects of metformin in cancer cells, there are multiple responses to this mechanistic question on the role of AMPK. In colon cancer, all groups cited above observed increased AMPK activity/phosphorylation in parallel with *in vitro* and *in vivo* decreases in cell proliferation and occasional apoptosis, with two of these groups showing AMPK-dependence with either siRNA or an AMPK activator, AICAR, as a positive control (Buzzai et al. 2007; Tomimoto et al. 2008; Zakikhani et al. 2008; Algire et al. 2010; Hosono et al. 2010b). Pancreatic cells and glioma cells, like colon cancer, also showed AMPK-dependent effects that were supported by an AMPK inhibitor, Compound C, and AICAR (Isakovic et al. 2007; Kisfalvi et al. 2009; Sato et al. 2012). In AML and ALL, metformin increased pAMPK levels, which correlated to decreased proliferation both *in vitro* and *in vivo*. However, only Green et al. (2010) performed Compound C studies confirming AMPK-dependent inhibition of mTORC1 (Green et al. 2010).

In breast, ovarian, lung and endometrial cancers, the responses were not quite as clear. In breast cancer, two previously mentioned groups observed AMPK-dependent decreases in proliferation (Zakikhani et al. 2006; Zakikhani et al. 2010) and increases in apoptosis (Zhuang and Miskimins 2011), supported by siRNA data. Two other groups observed increases in pAMPK that correlated to anti-tumoral effects, but did not provide siRNA or AMPK inhibitor/activator experiments to support this data (Alimova et al. 2009; Liu et al. 2009). Four other groups did not provide AMPK data (Hirsch et al. 2009; Iliopoulos et al. 2011; Cufi et al. 2012; Menendez et al. 2012). Finally, Vazquez-Martin et al. (2009) provided data demonstrating that metformin decreases proliferation in HER-2 over-

expressing breast cancers independently of AMPK (Vazquez-Martin et al. 2009). In endometrial cancer, anti-proliferative and pro-apoptotic metformin effects have been correlated to increased pAMPK levels without any further experimental evidence (Cantrell et al. 2010), while additional studies demonstrated that metformin suppressed endometrial cell invasion independently of AMPK (Tan et al. 2011). Effects in ovarian cancer were proven to be both AMPK-dependent and -independent (Gotlieb et al. 2008; Rattan et al. 2011a; Yasmeen et al. 2011), while some lung cancer experiments showed increased pAMPK while others did not (Algire et al. 2010; Memmott et al. 2010). This data only covers what was previously mentioned above in "Metformin mechanisms of action in cancer cells: Overview," but it effectively demonstrates the current debate in the literature. Thus, we will next discuss mechanisms of action of metformin that are AMPK-dependent, AMPK-independent and those involving mitochondrial inhibition of electron transport chain complex I.

### 7.4.2 AMPK-dependent mechanisms of action in cancer

Much research has demonstrated that metformin is an AMPK agonist in normal, diabetic and cancer cells. As the LKB1/AMPK pathway is suggested to have tumor suppressor activities, it is this metformin target that has been the primary focus of much research on the anti-cancer role of metformin. Activation of AMPK can target cancer cells through a number of mechanisms, including inhibition of the mTOR pathway, cell cycle proteins, the AKT pathway and ATM activity. AMPK may also play a role downstream of LKB1 or p53 and in autophagy, as will be described below.

#### 7.4.2.1 AMPK-dependent mTORC1 inhibition

Most studies on metformin in cancer cells have focused on metformin's activation of AMPK to inhibit downstream mTORC1 kinase activity, which decreases protein synthesis, cell growth and proliferation processes (Zakikhani et al. 2006; Gotlieb et al. 2008; Zakikhani et al. 2008; Zakikhani et al. 2010; Song et al. 2012) as summarized in Figure 29. In fact, many articles clearly prove that AMPK-dependent inhibition of mTORC1 is required for multiple anti-cancer effects.

Zakikhani et al. (2008, 2010) demonstrated that metformin activates AMPK in prostate, colon and breast cancer leading to downstream inhibition of mTORC1 through AMPK-dependent activation of TSC2, which led to decreases in p70S6 kinase activation and pS6 (Zakikhani et al. 2006; Zakikhani et al. 2008; Zakikhani et al. 2010). Gotlieb et al. (2008) showed similar results in two ovarian cancer cell lines, OVCAR-3 and OVCAR-4. However, while Gotlieb et al. (2008) showed the growth inhibitory effect was only partially AMPK-dependent, because Compound C only slightly abrogated the effect, Zakikhani showed AMPK was primarily responsible, as demonstrated with siRNA of AMPK (Zakikhani et al. 2006; Gotlieb et al. 2008; Zakikhani et al. 2008; Zakikhani et al. 2010).

Additionally, Song et al. (2012) verified that AMPK activation by metformin led to inhibition of mTORC1 and suppression of S6K1 and 4EBP1 activity, leading to cytotoxicity in breast and fibroscarcoma cancer cells. This activity was especially effective against cancer stem cells and even enhanced radiosensitivity in both cancer types (Song et al. 2012).

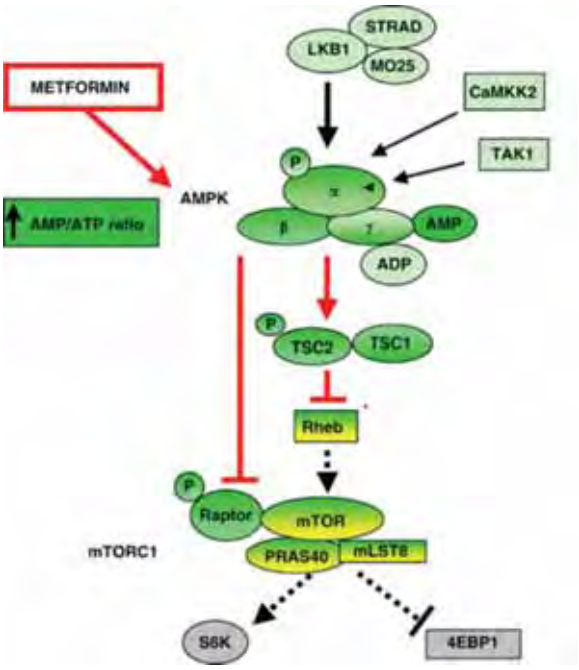



**Figure 29. Metformin can decrease mTORC1 through activation of AMPK in cancer cells.** Metformin can increase AMPK activity, potentially through an increase in the AMP/ATP ratio, which leads to both a direct block on the Raptor subunit of mTORC1 and increased phosphorylation and activation of TSC2, which blocks Rheb GTPase activation of mTORC1. The result is decreased mTORC1 activity, including decreased activation of S6K and inhibition of 4EBP1. Figure adapted from Pierotti et al. (2013).

The LKB1/AMPK pathway has also developed increasing interest in hematological malignancies for its tumor suppressor activities. Metformin effectively targets mTORC1 and cancer activity in multiple leukemic subtypes (Grimaldi et al. 2012). Green et al. (2010) demonstrated that metformin significantly decreased mTORC1 activity and downstream mRNA translation as well as cell growth and cell cycle progression in AML cells. Additionally, inhibition of AMPK with Compound C, shRNA of TSC2 and a constitutively activated form of mTORC1 abrogated metformin-induced inhibition of mTORC1 downstream targets, p4EBP1 and pS6 (Green et al. 2010). In BCR-ABL-expressing cells, like chronic myeloid leukemia and Ph(+) acute lymphoblastic leukemia, metformin significantly activates AMPK, decreases mTORC1 activity and causes potent suppression of leukemic precursors and BCR-ABL-dependent cells (Vakana et al. 2011). Additionally, metformin has been shown to upregulate the LKB1/AMPK pathway in T-ALL and B-ALL cell lines and patient samples, leading to inhibition of mTORC1 activity and mRNA translation (Grimaldi et al. 2012; Shi et al. 2012). Interestingly, the anti-mTORC1 and translational activity was stronger than that seen with rapamycin. Metformin also targeted a leukemia-initiating subpopulation ($CD34^+/CD7^-/CD4^-$) in

these T-ALL patient samples (Grimaldi et al. 2012; Shi et al. 2012). In lymphoma cells, metformin increases the activity of chemotherapy agent, doxorubicin, and mTOR inhibitor, temsirolimus. Pharmacological and molecular inhibition of AMPK decreased metformin-mediated lymphoma cell growth inhibition and drug sensitivity. *In vivo*, metformin activated AMPK, inhibited mTOR and blocked tumor growth in murine lymphoma xenografts (Shi et al. 2012).

#### 7.4.2.2 LKB1, p53 and autophagy

In considering the role of AMPK in the metformin effect, it is also important to consider LKB1, an upstream kinase that activates AMPK. Cervical cancer cells are especially sensitive to metformin *in vitro* when LKB1 is expressed. In fact, ectopic expression or inducible expression of LKB1 in LKB1-deficient cells increases the response to metformin, AMPK activation, mTOR inhibition and downstream apoptosis and autophagy (Xiao et al. 2012). A second group also demonstrated *in vitro* dependence on LKB1 for AMPK activation and downstream phosphorylation of ACC and stimulation of fatty acid β-oxidation, as confirmed with LKB1 siRNA in ovarian cells. This group also presented a novel result, which was that siRNA of LKB1 reversed the block in cell cycle progression (Rattan et al. 2011a).

However, Algire et al. (2011) recently presented contrasting *in vivo* data, performed with MC38 colon carcinoma cells, demonstrating the effect of both LKB1 expression and diet on tumor growth in response to metformin. Metformin inhibited tumor growth and reduced insulin receptor activity in tumors of mice with diet-induced hyperinsulinemia, independent of LKB1 expression. However, in the absence of hyperinsulinemia, metformin only inhibited growth of tumors transfected with short hairpin RNA against LKB1. In the chow diet mice, this growth inhibition could not be attributed to either host insulin levels or AMPK activation. Additional *in vitro* studies showed that cells with lower LKB1 levels were more sensitive to metformin-induced ATP depletion due to an impaired ability to activate the LKB1-AMPK-dependent energy-conservation pathways. Further investigation suggested that mice with normal LKB1/AMPK could increase p53 and autophagy to survive cell stress and metformin-induced growth inhibition. Thus, loss of LKB1 can accelerate proliferation, but also sensitize cells to metformin where LKB1 would actually benefit cells (Algire et al. 2011). Similar results were observed *in vivo* with non-small lung cancer cells, which were sensitive to phenformin-induced apoptosis only when LKB1 was mutated or lost. Again, this scenario capitalizes on the fact that LKB1-deficient tumors cannot sense and respond to metabolic stress (Shackelford et al. 2013). However, these types of effects are observed primarily *in vivo*.

In some cancer cell types, metformin induces p53 activation as a survival mechanism to ETC1 inhibition and AMPK activation, leading to multiple downstream effects, including decreased IGF1/IR signaling, AKT activity and mTORC1 activity, the latter occurring potentially through

TSC1/2 or REDD1 activation, as summarized in Figure 30. Metformin-induced p53 activation was observed in breast cancer cells, which was downstream of ERK signaling and in parallel with Bax activation and cell apoptosis (Malki and Youssef 2011). Buzzai et al. (2007) demonstrated *in vivo* that metformin suppressed tumor growth and induced apoptosis of colon cancer cell line HCT116, but only when p53 was knocked out. Similar to Algire's LKB1 studies, this suggests that wild-type p53 can be used for stress survival to a certain extent. In fact, Buzzai et al. (2007) demonstrated that only p53 wild-type cells were able to switch to glycolysis and/or fatty acid β-oxidation to survive metformin's ETC1 inhibition. Of note, the use of fatty acid β-oxidation was not as high in metformin-treated cells compared to AICAR-treated cells. Additionally these p53 wild-type cells were able to induce autophagy in response to metformin as summarized in Figure 31 (Buzzai et al. 2007).

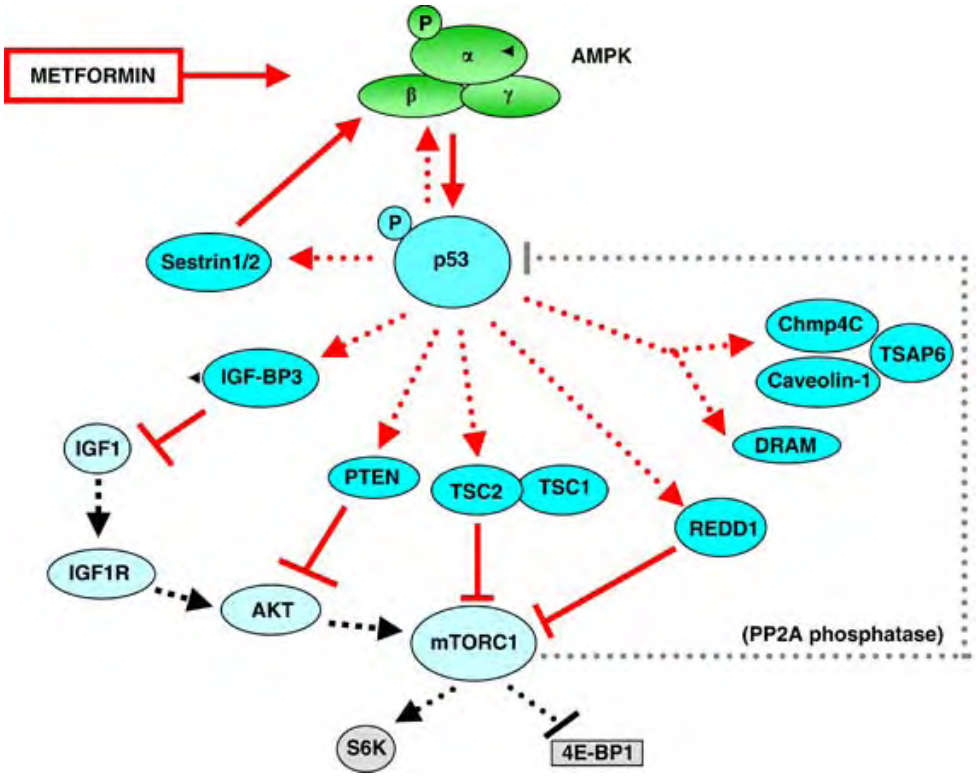



**Figure 30. Metformin action on the p53-controlled pathways in cancer cells.** Through the indirect AMPK-mediated p53 phosphorylation on Ser15, metformin induces cell-cycle arrest in p53-proficient cells and transactivation of a wide number of genes, including AMPK through a positive feedback loop, leading to downregulation of each related loop/pathway. Figure adapted from Pierotti et al. (2013).

Ben Sahra et al. (2010) presented another interesting work on metformin in combination with 2-deoxyglucose (2DG) *in vitro* in prostate cancer cells. Together, the two drugs induce significant ATP depletion, which leads to synergistic cell death. The combination induces higher activation of p53 and pAMPK and total inhibition of pS6 (as compared to each drug alone) as well as AMPK- and p53-dependent apoptosis within 48 hours of treatment. Inhibition of AMPK by siRNA and compound C prevented inhibition of pS6 and caspase-3 cleavage. While, unlike Buzzai et al. (2007), only cells with wild-type p53 could induce caspase-3 activity upon treatment. The combination also led to a

G2/M cell cycle arrest independent of p53. Finally, 2DG alone induced autophagy as a pathway for cell survival under energetic stress. However, in combination, metformin increases the energetic stress on the cell to the point where autophagy is inhibited and the cells induce apoptosis and cell death (Ben Sahra et al. 2010).

Grimaldi et al. (2012) also observed metformin-induced autophagy in combination with AMPK-dependent inhibition of mTORC1 and mRNA translation in T-ALL cells. Metformin-induced autophagy in these cells was confirmed by increased LC3 cleavage and cytoplasmic vacuoles containing degraded organelles, as observed with a transmission electron microscopy (Grimaldi et al. 2012). In a second study, both *in vitro* and *in vivo,* B- and T-lymphoma cells also exhibited an induction of autophagy, as evidenced by LC3 cleavage, following treatment with metformin and mTOR inhibitor, temsirolimus. Interestingly, autophagy was significantly reduced in lymphoma cells transfected with AMPK-α siRNA. Additionally, autophagy inhibitor, 3-methyladenine, abrogated the lymphoma cell growth inhibition by metformin alone and in combination with temsirolimus (Shi et al. 2012). Shi et al. (2012) is a key study in describing metformin's mechanism of action in lymphoma cells, as the activation of AMPK, inhibition of mTORC1 and subsequent autophagy were all interconnected and associated with cell growth inhibition (Shi et al. 2012). This has also been previously described by Lee et al. (2010), in which metformin activated AMPK, which induced AMPK to bind to ULK1 and activate ULK1-mediated autophagy in U-2OS osteosarcoma cells. This effect was dependent on simultaneous AMPK-dependent phosphorylation of raptor, also leading to increased ULK1 activity (Lee et al. 2010). Additionally, AMPK can induce FOXO3A-dependent autophagy by interaction with the protein of the human UV radiation resistance-associated gene (UVRAG) (Chiacchiera and Simone 2010). This increased FOX03A activity could also occur downstream of AMPK-dependent decreases in IRS-1 and AKT activity (Pierotti et al. 2013), which other studies have previously observed in lung cancer, glioma and squamous cell carcinoma cells (Memmott et al. 2010; Chaudhary et al. 2012; Wurth et al. 2013). These metformin-induced autophagy effects are summarized in Figure 31. However, not all autophagy-related metformin effects depend on AMPK, which will be discussed below on page 103.

Altogether, this data brings to light an extremely important issue, especially in studying cancer cell metabolism, which is the conflicting data between *in vitro* and *in vivo* studies. It appears that while LKB1 and p53 may be necessary and/or enhance the anti-cancer effects of metformin *in vitro*, they can actually inhibit metformin effects *in vivo* by allowing these cells to effectively respond to the cell stress induced by metformin. It will be very interesting to compare this pre-clinical data to patient studies in order to identify the appropriate physiological requirements to test metformin in cancer cells. It will also be interesting to link this LKB1/AMPK and p53 data to autophagy downstream of these tumor suppressors. Autophagy as a pro-survival pathway *versus* a cell death-inducing process requires fine-tuned balancing in cancer cells. Cancer cells may induce autophagy in times of stress as a survival pathway, but the fact that many cancer cells exhibit a basal level of

autophagy to support growth and proliferation suggests that a shift in either direction could be detrimental. The role of autophagy and its impact on anti-tumoral metformin effects *in vitro*, *in vivo* and in clinical studies is one of the most interesting and novel focuses of metformin in cancer cells today.

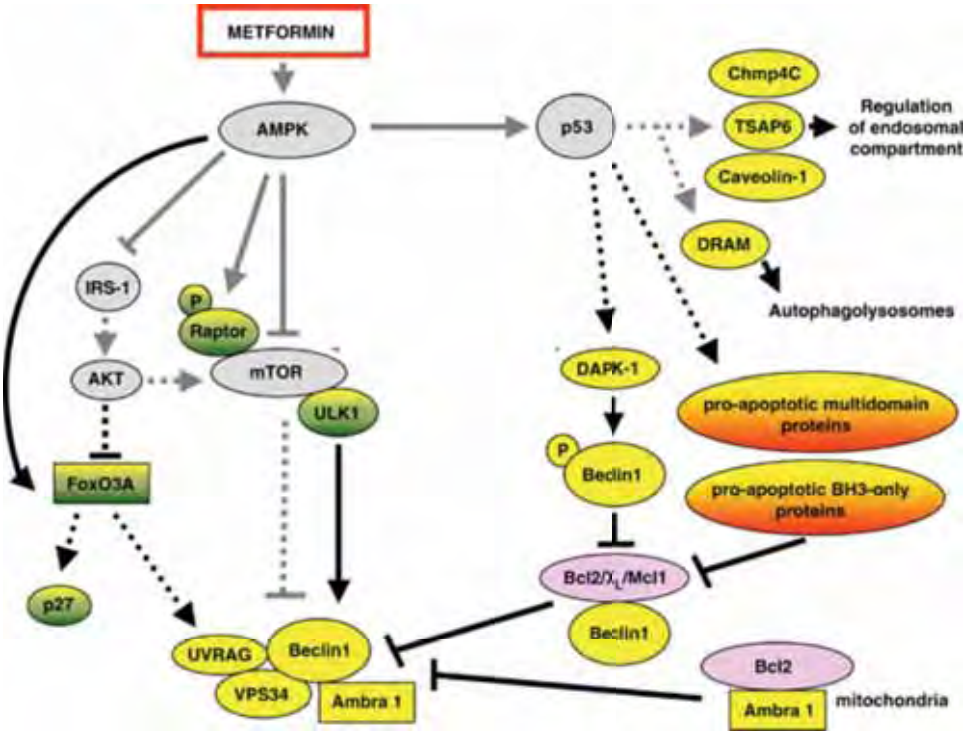



**Figure 31. Overview of mechanisms of autophagy-induction by metformin in cancer cells.** Following metformin treatment, multiple pathways may contribute to an induction of autophagy in cancer cell types. Metformin-induced nutrient starvation, sustained either by AMPK, Rag GTPase or REDD1 mechanisms, induces phosphorylation of raptor in the raptor-ULK1 complex, favoring autophagy. Additionally, decreased insulin and AKT signaling can decrease the negative feedback on FoxO3A, which may also regulate genes involved in autophagy, such as UVRAG. LKB1/AMPK-dependent p27 phosphorylation can also trigger autophagy. Metformin-induced p53 activation may also play a major role in autophagy. p53 activation can induce DAPK-mediated beclin-1 activation leading to beclin-1/Bcl-2/Bcl-xL complex dissociation and to high levels of beclin-1 *versus* Bcl-2/Bcl-xL/Mcl-1 favoring autophagy, whereas the reverse (high level of Bcl-2/Bcl-xL/Mcl-1 *vs* beclin-1) favors survival. AMBRA can also compete with Bcl-2 for binding to beclin-1, which induces autophagy if Bcl-2 is located in the endoplasmic reticulum or induces survival if the location is mitochondrial. p53 can also control the generation of autophagosomes through genes involved into vesiculation processes, such as Chmp4C, TASP6 and caveolin-1. At the latest stage, p53-induced damage-regulated autophagy modulator (DRAM) induces the formation of autophagosomes for degradation. Figure adapted from Pierotti et al. (2013).

#### 7.4.2.3 ATM

ATM (ataxia telangiectasia, mutated) is a tumor suppressor gene implicated in DNA repair and cell cycle control. A region in the ATM gene modulates the response to metformin in type 2 diabetics and is apparently required for the full anti-glycemic activity of metformin (Glazer 2011; Zhou et al. 2011). It was recently discovered that metformin promotes *in vitro* activation of ATM and its targets, such as protein kinase, Chk2 (Vazquez-Martin et al. 2011). ATM can then phosphorylate AMPK through LKB1-dependent and independent mechanisms (Suzuki et al. 2004; Sun et al. 2007; Alexander et al. 2010). This was previously demonstrated in breast cancer cells and occurred in parallel with alteration of the carbon flow through the folate-related one-carbon metabolic pathways required for purine, pyrimidine and glutathione de novo synthesis. As such, addition of thymidine and hypoxanthine for purine salvaging decreased metformin's activation of ATM and AMPK and thereby protected against cytotoxic effects (Corominas-Faja et al. 2012). Thus, ATM is another mechanism through which metformin targets AMPK and elucidates its anti-cancer activity.

Activation of the ATM-mediated DNA damage response (DDR) has also been suggested in metformin's ability to prevent cancer in type 2 diabetics by acting as a "tissue sweeper" of pre-malignant cells (Menendez et al. 2011). By decreasing oxidative phosphorylation, metformin decreases the oxidative stress and ROS that can induce downstream oncogenic mutations. Additionally, metformin's activation of ATM may create a pseudo-DDR that accelerates onset of cellular senescence. In this way, metformin can activate a senescence-associated secretory phenotype (SASP) to reinforce the growth arrest and trigger an immune-mediated clearance of senescent cells, including those that are pre-malignant and tumorous (Menendez et al. 2011). This mechanism could be preferentially responsible for the preventative anti-cancer effect of metformin, but more research is required to determine the effect of this pathway when the cancer is already established.

#### 7.4.2.4 Cell cycle inhibition

Metformin's AMPK-dependent inhibition of mTORC1 activity can decrease certain cell cycle factors, like cyclin D1, to induce cell cycle arrest. However, several other groups have shown that metformin can induce AMPK-dependent cell cycle arrest independently of mTORC1 inhibition. Metformin inhibits *in vitro* breast cancer cell proliferation independent of estrogen receptor, HER2 or p53 status. This growth inhibition was correlated to AMPK activation, downregulation of cyclin D1 and a G0/G1 cell cycle arrest. The reduction in cyclin D1 led to a release of CDK inhibitors, $p27^{Kip1}$ and $p21^{Cip1}$, which associated with cyclin E/CDK2 complexes and decreased cell cycle progression. MDA-MB-23 cells, which exhibited AMPK activation and decreased cyclin D1 levels without a block in cell cycle, had significantly lower levels of $p27^{Kip1}$ and $p21^{Cip1}$. These cells only exhibited a

cell cycle arrest when $p27^{Kip1}$ and $p21^{Cip1}$ were overexpressed (Zhuang and Miskimins 2008). Additional works have demonstrated that MDA-MB-23 cells do not exhibit decreased mTORC1 in response to metformin, which suggests AMPK can reduce the cell cycle without decreasing mTORC1 in breast cancer cells (Dowling et al. 2007). The same mechanism of action was also observed in hepatocellular carcinoma cell lines and was independent of p53 (Chen et al. 2013). However, in most cases mTORC1 and cell cycle inhibition occur in parallel, which makes it difficult to determine the exact roles of AMPK and mTORC1 in this anti-tumoral effect.

#### 7.4.2.5 AKT inhibition

As opposed to rapamycin, which decreases mTORC1 activity while activating AKT through a release of the mTOR-related negative feedback loop on AKT, metformin has been shown to decrease AKT activity in breast cancer cells through insulin-receptor signaling (Zakikhani et al. 2010). Metformin phosphorylation of AMPK led to increased phosphorylation of IRS-1 at Ser789, which has been reported to inhibit downstream signaling of AKT. siRNA of AMPK led to decreased phosphorylation of IRS-1 and decreased the inhibition of AKT as described in Figure 32.

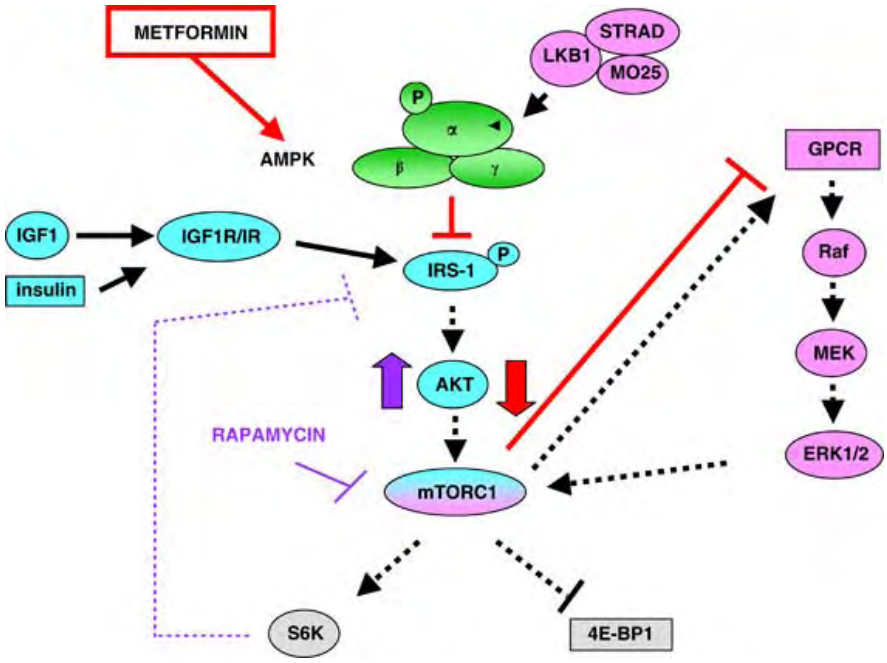



**Figure 32. Metformin action on the IRS-1 and GPCR pathways in cancer cells.** Metformin-induced AMPK-dependent inhibition of the IGF-1/insulin pathway is mediated by phosphorylation of IRS-1 at Ser 794, which, at variance with IGF1R/IR-mediated phosphorylation, leads to a decreased activation of IRS-1, AKT and mTORC1. Of note, metformin and rapamycin have opposing effects on AKT as only rapamycin leads to a release of the negative feedback loop on AKT. Through LKB1-mediated silencing of mTORC1, metformin also disrupts the cross talk between the IGF-1/insulin and GPCR pathways and shuts down ERK1/2. Figure adapted from Pierotti et al. (2013).

An AKT inhibitory mechanism could help explain metformin's anti-neoplastic activity as compared to rapamycin (Zakikhani et al. 2010). Similar results have been observed in cutaneous squamous cell carcinoma (Chaudhary et al. 2012) and in human lung cancer xenografts (Tsakiridis 2011 AACR Abstract), although the latter is currently unpublished. In pancreatic cancer cells, metformin abolished mTORC1 activity without increasing pAKT activity and actually had a more potent inhibition of cell proliferation than rapamycin. Metformin also prevented mitogen-stimulated ERK activation in these cells, which can be linked to decreased mTORC1 activity and a subsequent block on the GPCR-Raf-Mek-ERK1/2 pathway (Soares et al. 2013) (Figure 32). In glioblastoma cells, we described above that metformin inhibits cell proliferation of tumor-initiating cells more potently than more differentiated glioma cancer cells (Sato et al. 2012). Interestingly, this selective anti-cancer activity was correlated to the fact that metformin inhibited AKT only in glioma stem cells (Wurth et al. 2013).

### 7.4.3 AMPK-independent mechanisms of action in cancer

Despite the fact that metformin is known as an AMPK agonist, there are many studies that have demonstrated that metformin induces anti-cancer effects through mechanisms independent of AMPK. Some of these studies have focused on metformin's anti-diabetic actions, which can be correlated to anti-cancer activity. Other studies have observed very specific cellular effects. Thus, future research on the anti-tumoral effects of metformin will focus on whether metformin's mechanism of action occurs at the cellular or whole organism level (Birsoy et al. 2012). Importantly, this question will have to be approached on a cell- and tissue-dependent level in each cancer type.

#### 7.4.3.1 Insulin-dependent mechanisms

Goodwin et al. (2008) found that women with early-stage breast cancer who were treated with metformin for 6 months following diagnosis had a 22.4% decrease in fasting insulin levels and a 25.6% increase in insulin sensitivity, while also reducing total cholesterol, low-density cholesterol and body weight (Goodwin et al. 2008). As these authors and others have suggested that women with obesity, and especially hyperinsulinemia, may be at higher risk for breast cancer, it could help explain the anti-cancer effect of metformin in this cancer subtype (Goodwin et al. 2002; Borugian et al. 2004).

Insulin may also play a role in metformin's ability to inhibit tobacco-carcinogen-induced lung tumorogenesis. Mice treated either orally or intraperitoneally (IP) with metformin after exposure to the tobacco carcinogen NNK had reduced lung tumors by up to 53% and 72%, respectively. Additionally, while metformin activated AMPK and inhibited mTOR in the liver tissue of these mice, mTOR was decreased (more so by IP than oral metformin) in the lung tumors while AMPK activation was not observed. Rather, metformin inhibited phosphorylation of growth factor-I receptor/insulin

receptor (IGF-IR/IR) and AKT upstream of mTOR in lung tissues, suggesting an insulin-dependent mechanism (Memmott et al. 2010; Liu et al. 2011). Metformin also impaired tumor-initiating glioma cells and squamous cell carcinomas cells, which occurred downstream of insulin signaling through AKT inhibition and was associated with a strong inhibition of AKT-dependent cell survival pathways (Chaudhary et al. 2012; Wurth et al. 2013).

#### 7.4.3.2 AMPK-independent mTORC1 inhibition through upregulation of REDD1

Many studies on metformin focus on its anti-proliferative effects through AMPK-dependent inhibition of mTORC1 and its downstream translational effects. However, several groups have established that metformin can decrease mTORC1 through mechanisms other than AMPK activation, as summarized in Figure 33. One such group has suggested REDD1 (also known as DDIT4 and RTP801) as an alternative upstream protein in prostate cancer cells. In 2008, Ben Sahra et al. first published an article demonstrating, *in vitro* and *in vivo*, that metformin inhibited proliferation, decreased viability and induced a G0/G1 cell cycle arrest in prostate cancer cell lines. While metformin did activate the AMPK pathway, inhibition of AMPK with siRNA did not prevent the anti-proliferative effects of metformin (Ben Sahra et al. 2008). In their second article published in 2011, Ben Sahra et al. demonstrated that p53-dependent activation of REDD1 is the upstream target through which metformin inhibits mTORC1 (Figure 33). Inhibition of REDD1 using siRNA or REDD1-/- cells abrogated metformin's inhibition of mTOR, cell cycle arrest and proliferation. They also observed increased REDD1 expression in response to metformin in breast (MCF7) and lung (A549) cancer cell lines (Ben Sahra et al. 2011), suggesting it could be a mechanism in multiple cancer cell types. The fact that REDD1 activation is p53-dependent in prostate cancer cells is of particular importance as this data was observed *in vitro*, which again supports the idea that, whether AMPK-dependent or -independent, p53 may be necessary for *in vitro* metformin mechanisms of action. It would be interesting to determine if p53 inhibits metformin effects *in vivo* in this cell type, as previously observed by Buzzai et al. (2007). Either result would support the need to identify mechanisms independently in each cell type as well as both *in vitro* and *in vivo*.

### 7.4.3.3 AMPK-Independent mTORC1 Inhibition through RAG GTPases

It has been previously demonstrated that mTORC1 can sense nutrient, and especially amino acid availability, through Rag GTPase signaling (Kim et al. 2008; Sancak et al. 2008). In the presence of amino acids, Rag GDP will be converted to Rag GTP, which activates mTOR. During amino acid starvation, Rag GTPases will not be activated and mTORC1 activity is subsequently decreased (Kim et al. 2008; Sancak et al. 2008). The ability of Rag GTPases to mediate mTORC1 activity is through its ability to translocate mTORC1 to a perinuclear intracellular compartment occupied by Rheb (Sancak et al. 2008). Upon removal of amino acids, mTOR diffuses through the cytoplasm and its activity is inhibited (Kalender et al. 2010).

Kalender et al. (2010) showed that metformin inhibits mTORC1 independent of both AMPK and TSC1/2 through a Rag GTPase-dependent manner (Figure 33). Like amino acids, metformin causes diffusion of mTOR throughout the cytosol, but without inducing any changes in amino acid levels. Additionally, transfection of constitutively active Rag B abrogated the metformin effect on mTORC1 (Kalender et al. 2010). This is an especially interesting mechanism as it directly correlates upstream cell metabolism to mTORC1 levels and does not require functional TSC1/2 to inhibit mTORC1.

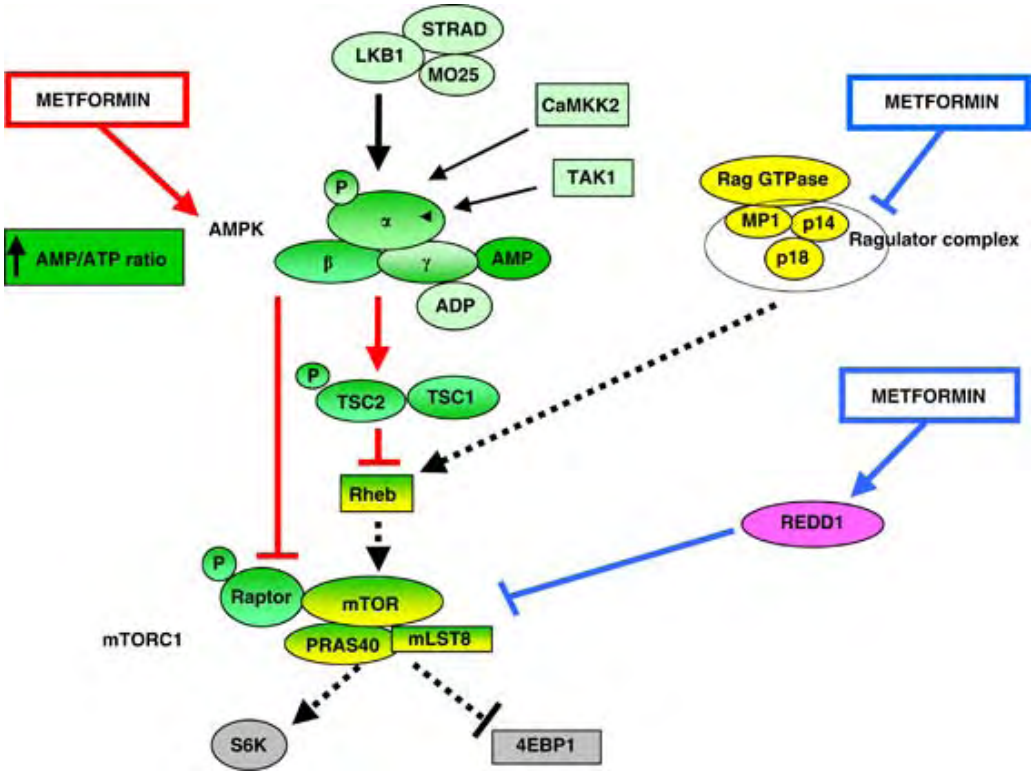



**Figure 33. Metformin can decrease mTORC1 independently of AMPK in cancer cells.** Metformin suppression of mTORC1 signaling occurs through AMPK-dependent and AMPK-independent mechanisms. Two AMPK-independent mechanisms are known: the first is regulated by the Rag proteins through a trimeric complex named the "Ragulator complex", which can translocate mTORC1 from its perinuclear location, where its activator Rheb is located, to the cytoplasm, to suppress mTORC1 signaling, and the second is REDD1-dependent. Figure adapted from Pierotti et al. (2013).

### 7.4.3.4 Cell differentiation and cyclic AMP

While metformin induces apoptosis in non-M3 AML cell lines, metformin induced both caspase-3-activated apoptosis and differentiation in acute promyelocytic M3 leukemic cell line, NB4 (Huai et al. 2012). Additional data elucidated that metformin caused differentiation through hyperphosphorylation of extracellular signal-regulated kinase (ERK) in APL cells. Additionally, a specific MEK/ERK activation inhibitor, U0126, inhibited metformin-induced differentiation. Metformin also induced a strong synergistic effect in combination with differentiation agent, all-trans retinoic acid (ATRA), which was not surprising considering the discovery that metformin also induced degradation of oncoproteins, PML-RARα and c-Myc (Huai et al. 2012).

A recent paper showed that cyclic AMP (cAMP) elevating agents promote APL progression and protect blasts against cell death induced by anti-leukemic agents like anthracyclines, including daunorubicin. Additionally, cAMP protected against 2-deoxyglucose, rotenone, a proteasome inhibitor and a BH3-only mimetic. Interestingly, while cAMP increases ATRA-induced terminal APL cell differentiation, cAMP actually protects against ATRA-based cell death (Gausdal et al. 2013). However, a recent study demonstrated that metformin reduces the cAMP levels normally induced by glucagon in primary hepatocytes, which was correlated to reduced phosphorylation of PKA substrates. Reduced PKA led to a release of the block on PFKFB1, allowing this enzyme to increase F-2,6-BP levels to promote the glycolytic flux and decrease gluconeogenesis (Figure 34).

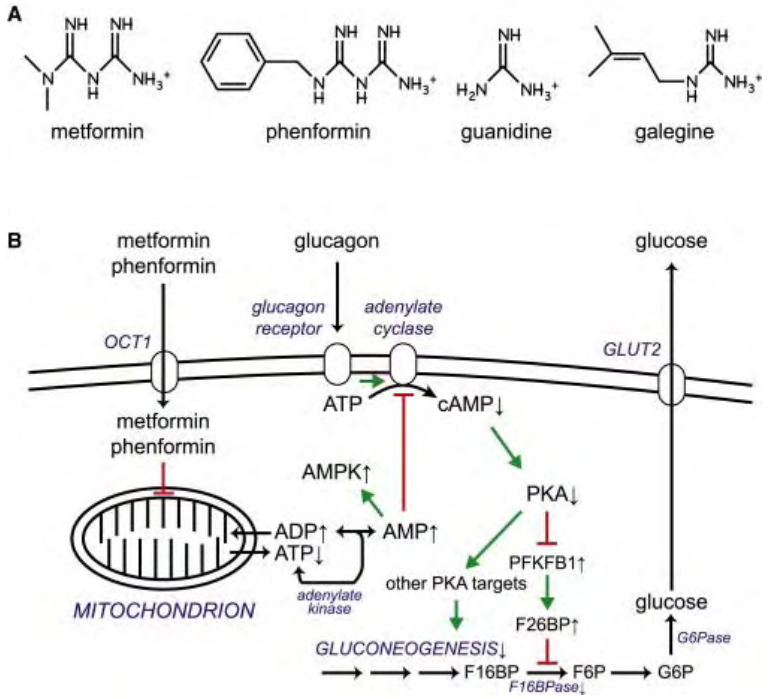



**Figure 34. Metformin can induce a block in cyclic AMP and downstream gluconeogenesis in cancer cells.** By increasing the AMP/ATP ratio, metformin blocks the production of cAMP by adenylate cyclase due to the need to conserve ATP. Decreased cAMP leads to decreased PKA, which subsequently releases the block on PFKFB1. The result is increased PFKFB1 activity and increased F-2,6-BP, which promotes glycolysis and inhibitions gluconeogenesis. Figure adapted from Hardie and Alessi (2013).

Lowering cAMP would therefore inhibit the switch from glycolysis to gluconeogenesis triggered by glucagon. Additionally, AMPK activation was not required to see metformin's decrease of cAMP (Miller et al. 2013). Rather, the mechanism by which metformin could be lowering cAMP is *via* a direct increase of AMP (Miller et al. 2013), which inhibits adenylate cyclase and in turn decreases cAMP production (Blume and Foster 1975). Considering that metformin induces apoptosis and differentiation in APL cells in parallel with a decrease in cAMP levels, which normally protect against APL cell death, metformin could be a strong therapy against APL.

#### 7.4.3.5 Metformin-dependent decreases in HER-2 expression

Vazquez-Martin et al. (2009) showed that metformin inhibited cell proliferation in HER-2 overexpressing human breast cancer cells independently of AMPK. This effect was dependent on metformin's inhibition of HER-2, which was downregulated by up to 85%. This effect appears to occur through direct inhibition of p70S6K1 activity, which was independent of AMPK as neither Compound C nor siRNA could reverse the effect. Additionally, HER-2 breast cancer cells transfected with siRNA against p70S6K1 did not exhibit metformin-induced HER-2 suppression. The HER-2 inhibitory effect was augmented when metformin was combined with N-acetylcysteine, which blocks reactive oxygen species (Vazquez-Martin et al. 2009).

One possible explanation relates back to a previously discussed paper on page 63, which showed that REDD1 is downregulated in 40% of HER-2 and triple-negative breast cancers (TNBC) (Koo and Jung 2010). These breast cancer types are especially sensitive to metformin (Liu et al. 2009; Vazquez-Martin et al. 2009). As described above, Vazquez-Martin et al. (2009) demonstrated AMPK was not responsible for metformin's growth inhibition and while Liu et al. (2009) showed increases in pAMPK in TNBC in parallel with a G1 cell cycle arrest and apoptosis, this group provided no evidence that pAMPK was necessary for these effects. Thus, it is highly possible that increased REDD1 by metformin could be responsible for the anti-cancer effects in these breast cancer subtypes.

#### 7.4.3.6 Autophagy

While metformin-induced activation of AMPK can induce autophagy in cells (Buzzai et al. 2007; Algire et al. 2011; Grimaldi et al. 2012; Lee et al. 2012; Shi et al. 2012), other studies have proven that metformin activation of AMPK is not necessary for downstream activation of autophagy. Tomic et al. (2011) showed that autophagy was primarily responsible for the anti-proliferative and apoptotic effects of metformin in melanoma cells, as knocking down autophagy components, LC3 or ATG5, decreased these effects. Additionally, they demonstrated that siRNA of AMPK$\alpha$1/2 partially prevented cell death and PARP cleavage induced by 5 and 10 mM metformin, but only partially

decreased LC3 cleavage at 5 mM with no effect at 10 mM metformin. This suggests that metformin induces autophagy and anti-cancer effects in melanoma cells with both AMPK-dependent and AMPK-independent pathways (Tomic et al. 2011). A second group studying B16 mouse melanoma cells showed similar anti-cancer effects of metformin, including a cell cycle arrest and apoptosis (Janjetovic et al. 2011). These anti-tumoral effects were associated with oxidative stress, mitochondrial membrane depolarization, increased p53, decreased anti-apoptotic Bcl-2 mRNA levels and, finally, increased autophagy. Similar to Tomic et al. (2011), while autophagy inhibitors partly restored viability of metformin-treated melanoma cells, AMPK inhibitor, Compound C, completely failed to restore viability of metformin-treated B16 cells (Janjetovic et al. 2011).

#### 7.4.3.7 AMPK-independent but not clearly elucidated

In ovarian cancer cells, the question of AMPK involvement in the anti-cancer effects of metformin is under high debate. Gotlieb et al. (2008) first showed that AMPK could have a partial role in the inhibition of *in vitro* cell proliferation of two ovarian cell cancer lines, because Compound C slightly abrogated the effect (Gotlieb et al. 2008). However, another group then demonstrated that metformin could inhibit cell proliferation and mTORC1 translational signaling in both AMPK-null MEFs and AMPK-silenced ovarian cancer cells (Rattan et al. 2011a). Like many papers, this group showed that metformin could still activate pAMPK and pACC while stimulating fatty acid β-oxidation, but that this AMPK effect was not necessary for anti-proliferative activity. They did demonstrate, however, that siRNA of LKB1 rescued these AMPK-induced metabolic changes. Additionally, siRNA of LKB1 inhibited the cell cycle block induced by metformin (Rattan et al. 2011a). This is a very novel result in metformin anti-cancer activities and, as the mechanism is currently unknown, requires further investigation to understand the exact mechanism by which LKB1, without AMPK, could be directly related to an arrest in the cell cycle.

Gotlieb's group later tested the role of AMPK in the apoptotic effect of metformin in ovarian cancer cells, finding it to be AMPK-independent (Yasmeen et al. 2011). Instead of a role of AMPK, metformin activated caspase-3/7, down-regulated Bcl-2 and Bcl-xL, and up-regulated Bax and Bad expression. Metformin apoptosis was also enhanced by chemotherapy agent, cisplatin, but unrelated to changes in Bcl-2 family expression (Yasmeen et al. 2011). Concurrent with apoptosis, cells exhibited an arrest in the S and G2/M phases of the cell cycle, which correlated to cyclin D1, A and B levels (Yasmeen et al. 2011). However, the exact mechanism by which this occurs was not entirely elucidated.

An additional group studying metformin in prostate cancer cell lines demonstrated that metformin inhibited cell proliferation and viability by inducing a G0/G1 cell cycle block (Ben Sahra et al. 2008). This block was in parallel with increases in p27 protein levels, decreases in cyclin D1 and

phosphorylation of pRb. While metformin activated the AMPK pathway, siRNA against AMPK did not prevent anti-proliferative effects. *In vivo*, this group also observed decreases in tumor growth in mice xenografts of LNCaP prostate cancer cell line in parallel with a reduction of cyclin D1 levels (Ben Sahra et al. 2008).

In hepatocellular carcinoma cells, like ovarian cells, metformin induces mitochondrial-mediated apoptosis independent of AMPK (Xiong et al. 2012). In parallel with PARP activation and cytochrome c release with subsequent activation of caspase-3, metformin decreased levels of Bcl-2 and Bcl-xL while increasing Bax. Additionally, the Hep-G2 cells exhibited decreased proliferation, inhibition of the mTOR translational pathway and a G1 cell cycle arrest, which were also independent of AMPK. AMPK independency was determined based on the fact that anti-cancer effects of metformin were still observed following Compound C inhibition of AMPK. Finally, this group also showed decreased colony forming abilities and inhibited *in vivo* tumor growth of Hep-G2 cells following metformin treatment (Xiong et al. 2012).

In brief, we propose that changes induced by metformin, and especially increases in AMPK activation, are not necessarily correlated to apoptosis, proliferation or other anti-cancer effects. Control experiments must be performed to describe causative effects of metformin. Additionally, in this section, on AMPK-independent anti-tumoral effects without clearly elucidated mechanisms, there are multiple mitochondrial-related effects, including mitochondrial-mediated apoptosis and changes in pro- and anti-apoptotic proteins. As these activities are currently unexplained by known signaling pathways, we suggest that the effect of ETCI inhibition and metabolic changes should be further analyzed as a potential mechanism. As such, we have detailed potential mechanisms below.

### 7.4.4 ETCI inhibition and metabolic control

Metformin decreases gluconeogenesis in rat hepatocytes in a time and dose-dependent manner, (El-Mir et al. 2000; Owen et al. 2000), which correlates to decreased oxygen consumption and an indirect inhibition of ETCI. Several works have demonstrated that anti-tumoral effects, including AMPK phosphorylation, are likely to be secondary to ETCI inhibition (Hardie 2006). For example, depletion of mitochondria, adenoviral overexpression of superoxide dismutases (SOD1/2) and inhibition of nitric-oxide synthase abolished metformin-induced AMPK phosphorylation and downstream activities (Zou et al. 2004). As such, it is also probable that inhibition of ETCI could have direct anti-cancer effects. Even though cancer cells are very dependent on glycolysis for ATP production, there is still a percentage of pyruvate that enters the mitochondrial TCA cycle and gives mitochondrial ATP production through rapid turnover. As such, inhibition of mitochondrial oxidative phosphorylation by metformin may eliminate a major ATP source in some cancer cells, which was previously confirmed by work presented by Buzzai et al. (2007).

Cells that are dependent on mitochondria could be extremely sensitive to metformin inhibition of ETCI. Epithelial breast cancer cells, for instance, have exhibited hyperactivation of oxidative mitochondrial metabolism. This activity was identified in frozen sections of tumors by analyzing *in situ* enzymatic activity of cytochrome C oxidase (COX), also known as ETCIV, NADH staining to measure Complex I and succinate dehydrogenase (SDH) activity to measure Complex II (Whitaker-Menezes et al. 2011). Some breast cancer patients have also exhibited transcriptional upregulation of mitochondrial oxidative phosphorylation, which correlated with metastasis. Metformin has the ability to block the COX and NADH activities as well as total oxidative phosphorylation, supporting metformin as an anti-cancer agent in breast cancer due entirely to inhibition of ETC1 (Whitaker-Menezes et al. 2011). Interestingly, inhibition of COXII and iNOS were also inhibited by metformin in squamous cell carcinoma due to downstream NFkB inhibition (Chaudhary et al. 2012). Another group on epithelial breast cancer cells showed that these cells are dependent on mitochondria for generation of high-energy fuels, such as ketone bodies. The inhibition of mitochondria with metformin thereby reduced cell growth and induced autophagy in cancer cells due to the loss of an important energy source (Sanchez-Alvarez et al. 2013). Thus, it is possible that certain cancers could be effectively targeted with metformin as a single agent drug that inhibits mitochondrial activities and induces anti-tumoral downstream effects.

Many groups studying metformin in type 2 diabetics have indicated that metformin decreases mitochondrial ROS over-production and increases PPP-related NADPH formation through G6PDH activity (Leverve et al. 2003; Fujisawa et al. 2009; Piwkowska et al. 2010). However, the redox and ROS status in cancer cells is not always affected in the same way by metformin as in diabetic cells. Metabolic hyperactivity in most cancer cells leads to intrinsic oxidative stress and requires fine-tuned mechanisms to balance ROS and antioxidants. Pelicano et al. (2003) demonstrated that inhibition of complex I in human leukemia cells caused increased electron leakage across the ETC, leading to increased ROS generation and sensitization of leukemic cells to anti-cancer agents (Pelicano et al. 2003). A second group showed that, in HL60 AML cells, inhibition of Complex I with rotenone, which is a potent ETCI inhibitor, induced ROS production that led to cytochrome c release and caspase-3-dependent apoptosis. This apoptosis was inhibited by treatment with antioxidants that combated increases in ROS (Li et al. 2003).

The question that remains is whether or not metformin, as a mild ETCI inhibitor, could have the same effect on ROS production as rotenone. While both compounds inhibit ETCI, rotenone has been shown to both increase and decrease ROS production at complex I in rat liver and skeletal muscle cells, depending on forward (glutamate-malate) or reverse (succinate) electron flux, while metformin did not increase ROS production and selectively blocked the reverse electron flow (Batandier et al. 2006; Kane et al. 2010). Despite this fact, metformin has been shown to increase ROS in several cancer cells. In ovarian cancer cells, metformin increased total cellular and mitochondrial ROS while also decreasing cell growth. The growth inhibitory effects of metformin

were then reversed by methyl succinate supplementation at ETCII, confirming both ETCI as metformin's site of action and the role of ROS in these anti-cancer effects of metformin. In white adipose tissues, metformin causes oxidative stress and increases the level of ROS (Anedda et al. 2008). Additionally, in an unpublished, AACR abstract, Ahn et al. (2011) demonstrated that phenformin, a more potent form of metformin, causes inhibition of ETCI, overproduction of ROS, mitochondrial DNA damage, severe apoptosis and PARP-dependent cell death in colon cancer cell line, CT26 (Ahn 2011 AACR Abstract). While phenformin is known to be more toxic than metformin, as it leads to lactic acidosis, and could therefore have larger impacts on cell metabolism and ROS, it is possible that high doses of metformin could mimic the effects of phenformin.

The anti-glioma activity of metformin through a block in G0/G1 cell cycle progression and, only in confluent cultures, a massive induction of caspase-dependent cell death is likely to be caused by mitochondrial inhibition of ETCI. Cell death was associated with significant increases in ROS, mitochondrial membrane depolarization and JNK activation. This apoptosis was inhibited by cyclosporine A, which blocked the mitochondrial permeability transition pore and n-acetylcysteine, which blocked oxygen radical production (Isakovic et al. 2007). This is an especially interesting finding considering metformin has been shown to inhibit the mitochondrial permeability pore opening at the same level as cyclosporine A in a human carcinoma-derived KB cell line (Guigas et al. 2004), again suggesting that mechanisms must be identified on a cell by cell basis as different cell types exhibit opposing effects. In addition to ROS, metformin has been shown to increase mitochondrial reactive nitrogen species in aortic endothelial cells both *in vitro* and *in vivo*, again suggesting a likely role of mitochondrial inhibition in anti-tumor effects in numerous cancer cell types.

Metformin's anti-cancer activity solely through inhibition of ETCI has been the least studied mechanism in metformin literature. However, the growing importance of cancer cell metabolism stresses the need for more research focusing on this mechanism of action induced by metformin. This research would not only be helpful for identifying the potential role of metformin as a therapeutic agent, but it would allow us to understand exactly how mitochondrial changes could affect cancer directly and through downstream pathways. These downstream pathways would include the changes metformin elicits on all downstream metabolic pathways, but especially glycolysis and fatty acid metabolism.

# – Hypothesis and Objectives –

## II Hypothesis and objectives

Of the multitude of studies on metformin as an anti-tumoral agent, a vast majority of articles have focused on metformin's role as an AMPK agonist and signaling agent. However, there are few studies that have strived to understand the effect of metformin on cancer cell metabolism and the impact on survival, particularly in AML. Our hypothesis was that in AML cells, metformin's inhibition of electron transport chain complex I could have multiple effects on energetic and cellular metabolism, which affect cell survival alone and through downstream effects on cell metabolism, including activation of the LKB1/AMPK pathway, inhibition of mTORC1 and changes in translation and proliferation (Figure 35). We proposed that AML cells would be cytotoxically sensitive to metformin depending on their basal cell metabolism and ability to activate downstream pathways to inhibit translation and proliferation and activate apoptotic pathways. **We suggested that basal cell metabolism would be the major determinant, because unlike other indirect AMPK agonists (e.g. AICAR), metformin inhibits ETCI, thereby preventing fatty acid oxidation and restricting glucose oxidation by mitochondria. The therapeutic potential of metformin might stem from this dual mechanism of action, which increases catabolism but limits substrate availability and oxidation.** Importantly, both the effect of and sensitivity to metformin between and within cancer cells varies, primarily due to metformin's multitude of effects on cellular and energetic metabolism. Thus, this study would provide information on metformin's effect in AML cells, providing a rationale for a therapeutic index for use of metformin in cancer therapy and pushing others to perform similar research in other cancer types.

***Specific objectives:***

- To determine if metformin could affect AML proliferation and cell survival.
- To determine if metformin could affect AML cell growth in a xenograft model.
- To determine changes in mitochondrial functions and signaling pathways in response to metformin
- To determine the metabolic signature of AML cells in response to metformin.
- To correlate the metabolic signature to the anti-tumoral activity of AML cells.
- To elucidate metformin's mechanism of action in AML cells.

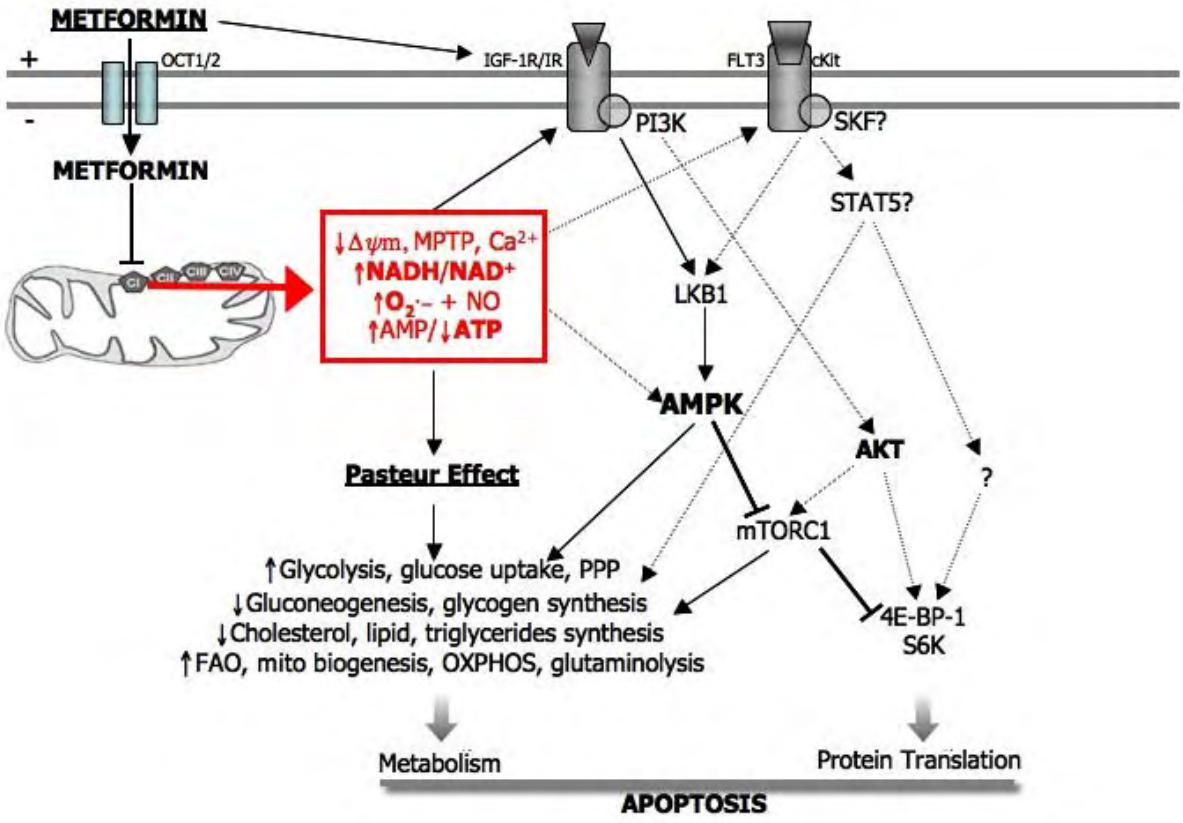



**Figure 35. Initial hypothesis.** This schema represents a putative multi-pathway metabolic mechanism of action of metformin in cancer cells. We hypothesize that in AML cells, metformin could induce cellular changes in signaling and metabolism either directly through insulin receptor signaling or through inhibition of electron transport chain complex I (ETCI). Inhibition of ETCI can disrupt the cellular mitochondrial membrane potential, permeabilization and calcium levels, NADH/NAD+ and AMP/ATP ratios, and ROS levels, which can impact metabolism either directly or indirectly through signaling pathways. Overall, we expect that metformin will induce changes in metabolism and protein translation that could induce apoptosis in AML cells. Our goal was to determine which, if any, of these targets were responsible for anti-tumoral effects of metformin in AML.

# – Materials and Methods –

## III Materials and methods

### Reagents

1,1-dimethylbiguanide hydrochloride (Metformin or Met), rotenone (ROT), antimycin A (AA), 2-deoxy-D-glucose (2DG), oligomycin A (oligo), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), sodium iodoacetate (iodo), orlistat (ORLI), etomoxir (ETO), 6-aminonicotinamide (6-ANA), oxythiamine (OXY), bezafibrate (BEZA), 6-diazo-5-oro-L-norleucine (DON), dichloroacetate (DCA) and buthionine sulfoxide (BSO) were obtained from Sigma-Aldrich. AKT inhibitor VIII, isozyme-selective AKTi-1/2 was obtained from Calbiochem.

### Cell culture

All AML cell lines, HL60, U937 and MOLM14, were maintained in MEM-α medium (Invitrogen; either standard with 5.6 mM glucose or with 20 mM glucose added for a total of 25.6 mM glucose). Cell lines were incubated at 37°C with 5% CO2. The cultured cells were split every 2-3 days and maintained in an exponential growth phase. We also used 6 AML patient samples for colony-forming assays. The clinical and mutational features of our AML cell lines and patient samples are described in table 8.

**Table 8:** Clinical and mutational features of AML cell lines and primary samples in this study

| Name | FAB | Karyotype | Sex | FLT3 | | NPM1 | IDH1, IDH2 | CEBPα | Kit | N/K Ras | WT1 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | ITD | TKD | | | | | | |
| **HL60** | M2 | Complex; amplified c-Myc | F | WT | WT | WT | WT | WT | WT | **+,WT** | + |
| **U937** | M5 | t(10;11)(p13; q14) | M | WT | WT | WT | WT | WT | WT | WT | + |
| **MOLM14** | M5 | Complex | M | **ITD** | WT | WT | WT | WT | WT | WT | + |
| **878** | M5a | No mitoses | Unk | WT | WT | WT | WT | WT | Unk | WT | WT |
| **983** | M5a | 46,XY[4] | M | WT | **D835** | | | | | | |
| **1526** | M1 | 46,XY[25] | M | **ITD** | WT | | | | | | |
| **1892** | M4/5 | 46,XY[4] | M | Unk | Unk | | | | | | |
| **1932** | NOS | 46,XX[16] (2 months prior) | F | Unk | Unk | | | | | | |
| **2093** | M4 | 46,XX[3] | F | **ITD** | WT | | | | | | |

### Western blots

Cells were boiled in lysis buffer and proteins (amount calculated with Bradford assay) from $10^6$ cells were resolved onto Invitrogen NuPage 4-12% gels by electrophoresis, transferred to nitrocellulose membranes, and probed with primary antibodies, including p-AMPKα T172 (40H9), AMPKα, pAKT (S473), pAKT (T308), AKT, pPTEN, PTEN, HK-II, pPKM2, PKM2, pLDHA, LDHA, Caspase-3, pS6 (Ser240-242), 4EBP1, Mcl-1, Bcl-2, Bcl-xL, Bak, Bax, AIF, PUMA, BIM and LC3 from Cell Signaling Technology, pPDHE1α from Calbiochem, PDHE1α from Invitrogen, CPT1, cyclin D1, cyclin E, IDH1, S6 and p4EBP1 from Santa Cruz, IDH2, pGSK3β, VDAC1 from Abcam, GSK3β

from BD Bioscience, REDD1 from Protein Tech and β-actin from Chemicon. Proteins were visualized using secondary antibodies, anti-rabbit IgG-HRP (Promega), anti-mouse IgG-HRP (Promega) and donkey anti-goat IgG-HRP (Santa Cruz), and Super Signal West Pico Chemiluminescent Substrate. The images were captured on Genesee Scientific Blue Devil Autoradiography Film using a KODAK X-OMAT 1000 Processor.

**Cell proliferation and apoptosis**

Cell proliferation was calculated by measuring the cell density using a Nexcelom cellometer with trypan blue dye. The cytotoxic response was determined by measuring apoptosis by flow cytometry after staining with AnnexinV-APC (Invitrogen) and 7-AAD (BD Pharmingen). Samples were washed with cold HBSS (Invitrogen) and resuspended in 100 uL 2X Annexin-V-Binding Buffer (BD). Cells were stained, on ice and in the dark, with Annexin V-APC for 15 minutes followed by 7-AAD for another 15 minutes. 200 uL of 1X Annexin-V-Binding Buffer were then added for a final volume of 300 uL. Samples were analyzed using a FACS Calibur flow cytometer and CELL Quest-Pro software.

**Analysis of metformin concentration in plasma of mice**

Metformin concentrations in plasma of NOD-SCID mice (n = 12 mice) were detected in two experiments over a two-week period. The first week, mice were injected intraperitoneally at time 0 with 200 mg/kg metformin, with 3 mice been bled retro-orbitally 15 minutes prior to provide a control group. Groups of 2-3 mice were then bled retro-orbitally 0.5, 1, 2 and 24 hours after injection. The following week, the experiment was repeated except mice were bled 2, 4, 8 or 24 hours after injection. Blood was collected in heparin-treated tubes. After collections, blood was homogenized and centrifuged at 10,000 x g for 10 minutes to extract the plasma. 20 uL of mouse plasma was added to falcon tubes with 180 uL human plasma, to provide an accurate testing volume and avoid a matrix effect, and frozen at -80°C. Human plasma was previously prepared by thawing a vial of human plasma from -80°C, which was then homogenized and centrifuged at 10,000 x g for 10 minutes. A volume of 180 uL of plasma was resuspended in falcon tubes and temporarily frozen at -80°C. Finally, plasma metformin concentrations were analyzed at the Laboratoire de Pharmacocinétique et Toxicologie, IFB – 330 Avenue de Grande Bretagne TSA 40031, 31059 Toulouse. In summary, this assay was performed using a validated reversed-phase high-performance liquid chromatographic (RP-HPLC) method. 200 uL of sample were spiked by 40 uL of a 10mg/L 1-Phenylbiguanide solution as an internal standard. After extraction, 150 uL of supernatant were injected in the Macherey-Nagel EC 150/2 NUCLEODUR 100-5 HILIC column. The mobile phase consisted methanol-water pumped at a flow rate of 0.9 ml/min at 25°C with a chromatographic duration of approximately 15 minutes. The flush, or rinsing injection phase, consisted of acetronitrile/$H_2O$ (50/50). MfCl was detected by ultraviolet absorbance at 234 nm. The calibration curve range was between 0.05 and 5mg/L.

**Analysis of blood glucose in mice**

Mice were bled retro-orbitally and blood was collected in heparin-coated tubes. Immediately after bleeding, blood was analyzed for glucose levels using an Ascensia BRIO blood glucose meter with corresponding Ascencia Easyfill test strips.

**Diabetic-induction in mice**

NOD-SCID mice were injected with a single dose of streptozotocin (STZ) at concentrations of 150 mg/kg for males and 200 mg/kg for females. Mouse blood sugars were tested one week later and had reached diabetic ranges of 200-300 $mg/dL^{-1}$ in females and 300-400 $mg/dL^{-1}$ in males. A total of 10 days following STZ injection, mice were injected intraperitoneally with 200 mg/kg metformin. Groups of three mice were then bled retro-orbitally at 0.25, 0.50, 1.0, 2.0, 4.0 or 24.0 hours following metformin injection for analysis of blood glucose concentrations.

**Tumor xenografts**

Nude: Xenograft tumors were generated by injecting 2 x $10^6$ MOLM14 cells (in 100 ml of PBS) subcutaneously on both flanks of NU/NU Nude mice (Charles River) (n=5 mice [10 tumors] per group). Mice were given daily intraperitoneal injections with 200 □l of 300 mg/kg/day metformin or vehicle (PBS). Tumor dimensions were measured with a caliper on days indicated and volume calculated using the formula: $v=\pi/6 \times A \times B^2$, where A is the larger diameter and B is the smaller diameter. At the end of the experiment, tumors were dissected, weighed, photographed and fixed with alcohol formalin acetic acid fixative for 48 hours. Tumors were embedded in paraffin and sectioned for immunohistochemistry.

NOD-SCID: Xenograft tumors were generated by injecting either 4 x $10^6$ HL60 cells (in 100 ml of PBS) or 2 x $10^6$ MOLM14 cells (in 100 ml of PBS) subcutaneously on both flanks of NOD-SCID mice (n = 3 mice [6 tumors] per group). Mice were given daily intraperitoneal injections with 200 □L 250 mg/kg/day. Tumor dimensions were measured with a caliper on days indicated and volume calculated using the formula: $v=\pi/6 \times A \times B^2$, where A is the larger diameter and B is the smaller diameter.

**Immunohistochemistry**

Apoptosis in xenografts was determined by immunohistochemistry for active caspase-3 (R&D Systems). For quantification, positively stained cells in 6 consecutive fields at x 20 magnification were counted from the edge towards the center of each section. Photographs for quantification were taken with a Leica DM4000B microscope.

**siRNA transfections**

siRNA transfection of AMPKα1/2 and REDD1 was performed using the NEON Transfection System (Sigma-Aldrich). siRNA for AMPKα1 and α2 was purchased from Invitrogen and previously published (Tomic et al. 2011). siRNA for REDD1 was purchased from Applied Biosystem (ID #s29168) and previously published (Ben Sahra et al. 2011). In summary, 1 million cells were transfected with siControl or siRNA for either AMPKα1/2 or REDD1 at a final concentration of 700 nM and then resuspended in 2.0 mL of MEMα + 10% FBS. Cells were incubated for 24 hours to obtain complete inhibition of desired protein. Cells were then resuspended in fresh MEMα + 10% FBS and treated with vehicle or 10 mM metformin for an additional 48 hours.

**CFU colony assays**

Patient samples were obtained from The University of Pennsylvania School of Medicine's Stem Cell Core Facility. All samples were obtained after informed consent and provided to researchers as previously collected, anonymous samples. Frozen cells were thawed and washed twice in PBS. Viable cells were resuspended at 100,000 cells/mL in PBS and 400 uL were added to 3.6mL Human Methylcellulose Enriched Media from R&D Systems. 1mL of the methylcellulose and cell combination were replated in NUNC suspension dishes for a total of 10,000 cells per dish. Treatments were done in triplicate with metformin dissolved in PBS (or PBS alone for control) added to the plated dish. Dishes were incubated at 37°C with 5% $CO_2$ for 14 days, after which colonies were counted. The cell line CFU colony assays were performed using the same protocol listed for patient samples except only 1000 cells per 1 mL of methylcellulose were plated. The methylcellulose used was 80% methylcellulose (MethoCult H4320) and 20% Iscove's Modified Dulbecco's Medium (IMDM) from Invitrogen. Additionally, the colonies were counted after 5-7 days.

**Cell cycle by flow cytometry**

Cell cycle analysis by flow cytometry was performed using staining for Ki67 (FITC mouse anti-human Ki67 Set from BD Pharmingen) and PI (propidium iodide from Sigma-Aldrich). In summary, 500,000 cells were collected, washed with PBS, centrifuged and resuspended in 1 mL PBS. Cells were fixed with 2 mL 100% ethanol and stored overnight at -20°C. The next day, cells were centrifuged, washed in PBS+BSA (1%), centrifuged again and resuspended in 1 mL 0.25% Triton X-100 for permeabilization. Cells were left for 10 minutes at 4°C. Following this incubation, cells were washed, centrifuged and then treated with 18 uL of Ki67 in 100uL PBS+BSA (1%). Cells were left for 30 minutes at room temperature and in the dark. Cells were then washed, centrifuged and resuspended with 300 uL PBS-RNase (1ug/mL) and 3 uL PI. Cells were incubated for 2 hours at room temperature and in the dark. Cells were then analyzed using a FACS Calibur flow cytometer with CELL Quest-Pro software.

**Electron transport chain complex I activity.**

Five million cells were used to measure enzymatic activities. Rotenone-sensitive NADH ubiquinone oxidoreductase activity (respiratory complex I activity) was determined as described previously (Gutierrez Cortes et al. 2012). Normalization was done by citrate synthase (CS) activity, which is a mitochondrial matrix enzyme, as described previously (Rocher et al. 2008).

**Measurement of oxygen consumption in cultured cells**

Cell lines were incubated for 24 hours in conditions of untreated, metformin and antimycin A. Following incubation, four million cells were resuspended in 155 uL of fresh media and analyzed in a water-jacketed, air-tight chamber with a Strathkelvin Clark oxygen electrode. Measurements were taken at 37□C with constant stirring. The specific oxygen consumptions described in Results Figure 9 represent the following measurements: Basal is the basal rate; ATP turnover is the basal oligomycin A-insensitive rate (i.e. oligomycin sensitive); $H^+$ leak is oligomycin A *minus* insensitive rate; maximal is the uncoupler rate; spare respiratory capacity is the uncoupler-stimulated minus basal rate.

**ATP analysis**

ATP was measured using the Promega Cell Titer Glo kit and protocol. In summary, following treatment, 50,000 cells were resuspended in 80 uL and distributed in a 96 well plate. Cells were then treated in replicates of five with control (PBS), oligomycin A, FCCP or sodium iodoacetate both either or in combination with oligomycin A or FCCP. Following a 1-hour incubation, 100 uL of cell titer glo reaction mix were added to each well for a final volume of 200 uL. Plates were then analyzed for luminescence with a Perkin Elmer Luminoscan. By comparing the different conditions, global ATP and percentages of both glycolytic and mitochondrial ATP were determined.

**Lactate production**

Lactate production was determined using the Abnova Lactate Assay Kit and protocol. In summary, supernatants were collected following a 24 hour treatment and frozen at -80□C. For testing, samples were thawed, mixed, centrifuged and diluted in the assay buffer. 50 uL of sample were added to each well followed by 50 uL of reaction mix, which contained 47 uL lactate assay buffer, 2uL probe and 2 uL enzyme mix. Samples were mixed. The plate was incubated for 30 minutes at room temperature and the absorbance was read at 570nM. The final lactic acid concentration in nmol/well was determined by comparing absorbance values to a standard curve prepared with the sample conditions.

**Metabolic flux analysis**

Macro-kinetics analysis of MOLM14 and U937 cells in MEMα (5.6mM) + 10% FBS after 24 hours incubation with untreated, 10 mM metformin or 10 uM antimycin A. The concentration of glucose, glutamine and lactate in the medium were taken by the Nova Biomedical Bioprofile Flex Analyzer. The oxygen consumption data was taken from the oxygen consumption assay previously described above. The uptake rates ($q_s$) were calculated using the following equation, $S_{(24)} = S_{(0)} - q_s/m*X_{(0)}*EXP(m*24)$; where $S_{(24)}$ and $S_{(0)}$ are the substrate concentrations at 24h and 0h respectively, m is the growth rate in $h^{-1}$, and $X_{(0)}$ is the mass of cell at 0h calculated from cell density measurement (0.5E10 cells per mL) multiplied by the cell dry weight conversion (1 cell = 400 $pg_{(CDW)}$). Consumption rate ($p_s$) were calculated using the equation, $S_{(24)} = S_{(0)} + p_s/m*X_{(0)}*EXP(m*24)$. Calculations were done using a growth rate of 0.045 $h^{-1}$ (calculated from proliferation measurement) or assuming that cells treated with metformin or antimycin had no cell growth. In this last case, the growth components were removed from the equations leading to the following formula, $S_{(24)} = S_{(0)} - q_s*X_{(0)}*24$.

**Liquid chromatography coupled to electrospray-LTQ-Orbitrap mass spectrometry with data processing and multivariate statistical analysis.**

Method 1 – Intracellular metabolites were analyzed as described by (Bolten et al. 2007). Briefly, analysis was performed by high performance anion exchange chromatography (Dionex ICS 2000 system, Sunnyvale, USA) coupled to a triple quadruple QTrap 4000 (Applied Biosystems, Foster City, USA) mass spectrometer. All samples were analyzed in the negative mode giving [M-H]- ions, which were monitored in the multiple reaction monitoring (MRM) mode. The injection volume was 15 µL. To get highly accurate quantification, the Isotope-Dilution Mass Spectrometry (IDMS) method was applied (Wu et al. 2005).

Method 2 – A total of 10 µL of samples was injected and separated on a 2.1 × 150-mm Hypersil gold C18 1.9-µm column (Thermo Electron) equipped with an on-line prefilter (Interchim). The flow rate was 0.5 mL/min with mobile phases A (100% water) and B (100% acetonitrile), both containing 0.1% formic acid. The gradient consisted of an isocratic step of 2 minutes at 100% phase A, followed by a linear gradient from 0% to 100% of phase B for the next 13 minutes, before returning to 100% A for 4 minutes. Mass spectrometric detection was performed using an LTQ-Orbitrap hybrid mass spectrometer (Thermo Electron) fitted with an electrospray source operated in the positive and negative ion modes. The detection was achieved from 50 to 1000 u at the maximum resolving power of 30000 (expressed as full width at half maximum for an ion at 400 U). The mass spectrometer was operated with capillary voltage at 4 kV and capillary temperature at 275°C. The sheath gas pressure and the auxiliary gas pressure were set, respectively, at 45 and 10 arbitrary units with nitrogen gas. Samples were randomly analyzed together by ultra-high performance liquid chromatography coupled

to mass spectrometry (UHPLC/MS) and a quality control sample was injected every 10 RBC samples to check the performance of the analytical system in terms of retention times, accurate mass measurements and signal intensities.

Collision-induced dissociation spectra were acquired using the data-dependent scanning function for identification purpose. The scan event cycle composed a full scan mass spectrum at a resolution power of 30000 and one or 2 data-dependent (MS2 and MS3) events acquired with a resolution set to 7500. Microscan count was set to unity and a repeat count for dynamic exclusion was set to 3. MSn acquisition parameters were an isolation width of 1 u, normalized collision energy ranging from 15% to 45%, and an activation time of 30 ms. Our data processing for method 2 included 2 main steps: (1) Automatic peak detection. Raw data were analyzed using the XCMS software Version 1.14.1 running under R Version 2.8.1,19 (http://www.bioconductor.org/packages/bioc/html/xcms.html). The UHPLC/MS data were peak-detected and noise-reduced in both the LC and MS domains to exclude peaks related to noise. The resulting datasets consisted of a single matrix containing features with m/z-retention time pair as an identifier and their intensity values for each sample. (2) Annotation of the datasets. The datasets resulting from the XCMS process were then annotated using tools developed in-house. The resulting data from the XCMS process were first mean-centered and scaled either to unit variance or to Pareto variance, then introduced into SIMCA-P11 (Umetrics) for multivariate analyses by unsupervised principal component analysis (PCA) or by supervised projection to latent structure-discriminant analysis (PLS-DA). The PLS-DA models were validated using the cross-validation function of SIMCA-P11 and by permutation tests (k = 100). In the 2 analyses, the first principal component t[1] accounts for as much of the variability in the data as possible, and the second t[2] and third t[3] components account for as much of the remaining variability.

**Metabolic gene expression**

Cells treated for 24 hours were centrifuged and stored as frozen cell pellets at -80°C. RNA was extracted from frozen cell pellets using the procedure described in the Qiagen RNeasy Mini Kit. RNA concentrations were then calculated using the NanoDrop ND-1000 spectrophotometer and computer program. Following concentration analysis, we used the PTC-100 Programmable Thermal Controller to produce cDNA with the iScript cDNA Synthesis Kit (Bio-Rad) following the PTC iScript program guide. Final gene expression was determined using a 7500 Real Time PCR System (AB Applied Biosystems).

**Thermodynamic analysis**

To determine how far from thermodynamic equilibrium the metabolic reactions in our cells operate, we used an equation previously described (Klimacek et al. 2010): $\Delta G = \Delta G K_{eq} - \Delta G_{MAR}$. Herein, $\Delta G K_{eq}$ and $\Delta G_{MAR}$ are free energies of a reaction ($\Delta G$) at equilibrium ($K_{eq}$) and at a particular mass-action ratio (MAR), respectively. Values of free energies were calculated by applying the equation $\Delta G = -RT\ln K$, where $R$ and $T$ are gas constant and absolute temperature (310.15 K), respectively, while $K$ is $K_{eq}$ or a MAR. $\Delta G K_{eq}$ in case of the PRPP biosynthesis was calculated from the theoretical standard free energy of formation of the compounds and MAR was determined using the quantified metabolite pool sizes.

**Total reactive oxygen species production**

Following a 24-hour incubation, 500,000 cells per sample were collected in FACS tubes. As a positive control, a sample from each cell line was incubated with 500 uM $H_2O_2$ for 15 minutes at 37°C. Cells were then washed with PBS and incubated with 5 uM 2,7-dichloro-fluorescein (DCF) in 200 uL PBS for 15 minutes at 37°C. Cells were then washed with PBS supplemented with 0.5% FBS. Finally, cells were incubated with 1 uL AnnexinV APC in 200 uL PBS for 15 minutes to control for cell death. Samples were analyzed using a FACS Calibur flow cytometer and CELL Quest-Pro software.

**Mitochondrial reactive oxygen species production**

To measure mitochondrial ROS production, we injected our cells with a hydroethidium (HE) probe that freely enters cells and reacts with superoxides ($O^{2-}$) to form ethidium. Ethidium will then interact with DNA to emit fluorescence. In preparation, cells were washed twice in PBS and resuspended in MEM-$\alpha$ medium with 10% serum at a final concentration of 500,000 cells per mL. Cells were then incubated with the HE probe at a final concentration of 100 nM. Afterwards, cells were individually treated with 10 mM metformin, 200 nM rotenone or 2 uM cytarabine and incubated at 37°C for 24 hours. After incubation, cells were washed twice with PBS, resuspended in a final volume of 10,000 cells per tube and stored on ice to block further enzymatic activity. Cells were then analyzed by flow cytometry on a FACScan cytometer (Becton Dickinson) on the FL3 channel (excitation: 488 nm, emission: 580 nm) and analyzed with CELL Quest-Pro software.

**Quantification of γH2AX staining on the S-phase fraction by arrayscan analysis (DNA Damage)**

Staining for γH2AX was conducted as described by the manufacturer (Euromedex/Upstate Biotechnology; mouse monoclonal anti-phospho-histone γH2AX antibody; 1:500 dilution). DNA content for blast cells was processed using a coulter DNA Prep reagent kit (Beckman Coulter, Fullerton, CA, USA). In summary, cells were treated with control, 10 mM metformin, 200 nM rotenone or 2 uM AraC for 24 hours. Cells were then subjected to an immunofluorescence assay using anti-Proliferating Cell Nuclear Antigen (PCNA; detection of S-phase cells) and anti-γH2AX (the phosphorylated form of histone H2AX that accumulates in cells containing damaged DNA) antibodies and finally stained with DAPI. Quantification was carried out with a Cellomics Array Scan device (ThermoScientific). For each analysis, 590 PCNA-positive cells were analyzed for the number of γH2AX foci/spot in at least 3 independent experiments. PCNA-positive cells containing more than 1 foci/spot were included in the analysis.

**Acridine orange autophagy assay**

100,000 cells were collected and centrifuged in FACS tubes at 1200 rpm for 5 minutes. Cells were then resuspended in 1 mL PBS, stained with 7 uL of 100 ug/mL acridine orange (Sigma-Aldrich) and vortexed for homogenization. Cells were incubated in the dark for 17 minutes at 37°C. After incubation, cells were centrifuged at 1200 rpm for 5 minutes, washed with 1 mL PBS, recentrifuged at 1200 rpm for 5 minutes and resuspended in a final volume of 300 uL PBS. Samples were analyzed using a FACS Calibur flow cytometer and CELL Quest-Pro software.

**Statistical analysis**

Unpaired *t*-test was used to calculate final *P* values. Significance is represented by stars in which * is $p<0.05$ ** is $p<0.01$ and *** is $p<0.005$

# – Results –

# IV Results

## 1 Anti-tumoral activities of metformin in human acute myeloid leukemia

We first determined the *in vitro* effect of metformin on cell proliferation and apoptosis of three AML cell lines and six primary AML patient specimens (Table 8 in "Materials and methods" page 111 lists the characteristics of cell lines and patient samples). Metformin induced a strong inhibitory effect on cell proliferation in all leukemic cell lines (Figure 1A) and significantly reduced leukemia colony-forming units (CFU-L) of three AML cell lines and six primary AML patient samples in a concentration-dependent manner (Figure 1B-C). Interestingly, metformin induced a cell cycle block in G0/G1 (U937) or S-G2/M (HL60 and MOLM14) phase in all three AML cell lines (Figure 2). However, we observed striking differences in metformin-induced cell death. HL60 and MOLM14 cells exhibited significant apoptosis-dependent cell death in response to metformin while U937 cells were resistant to this treatment (Figure 3A-B). These results were determined with two different readouts, which include AnnexinV-/7AAD-based flow cytometry and western blot analysis of caspase-3 and PARP cleavage following metformin treatment.

To determine if these results apply *in vivo*, we first performed pharmacokinetic studies of metformin and found that an intraperitoneal injection of 200 mg/kg of metformin in NOD-SCID mice induced a maximum plasma concentration of approximately 1.0 mM metformin that could be detected at decreasing levels up to four hours following the injection (Figure 4A). However, we did not observe any changes in the glucose level of these normoglycemic mice following metformin treatment, although we did observe that male mice had a slightly higher glucose level than female mice (Figure 4B). To do determine if we could affect the glucose level of NOD-SCID mice with metformin, we injected our mice with streptozotocin (STZ) at concentrations of 150 mg/kg for males and 200 mg/kg for females, which chemically induced diabetes within 1 week post-injection. Male mice were more responsive to STZ as they exhibited more significant increases in blood glucose levels than female mice. We then injected our mice intraperitoneally with 200 mg/kg of metformin and observed a slight decrease in the blood glucose level for approximately two hours following the injection (Figure 4C-D).

We then established a subcutaneous xenograft model for AML in female nu/nu nude mice using MOLM14 cells and treated these mice with daily intraperitoneal injections of metformin (300 mg/kg/day). Metformin strongly reduced the tumor growth and weight in this model (Figure 5A-C). Additionally, analysis of treated tumors demonstrated that metformin induced apoptosis *in vivo* with an increase in active caspase-3 staining (Figure 5D-E). Thus, metformin is capable of inducing cell death in AML cells *in vivo*.

## 2 The effect of metformin on AMPK and related pathways

We next studied the effects of metformin on cell signaling to better understand the mechanism of sensitivity in AML. As Green and colleagues (2010) showed that metformin activates AMPK in various AML cell lines, we analyzed expression of AMPK and phospho-T172-AMPK in HL60, MOLM14 and U937 cells following metformin treatment. We noted no changes in phospho-T172-AMPK after 24 hours metformin in all leukemic cells (Figure 6A). However, metformin transiently increased phosphorylation of AMPK in all AML cell lines as early as 1 hour after incubation with 1 and 10 mM metformin with a maximum activation at 1 and 3 hours for U937 and MOLM14 cells, respectively (Figure 6B). To determine whether the anti-proliferative and apoptotic effects of metformin are secondary to this transient activation of AMPK, we blocked this pathway using siRNA of the two catalytic subunits, AMPK$\alpha$1 and $\alpha$2. While we strongly diminished expression of AMPK in all three AML cell lines (Figure 6C), we did not prevent either inhibition of cell proliferation or induction of apoptosis in response to metformin (Figure 6D-E). Thus, AMPK activation by metformin is not necessary for metformin-induced cell death in AML cells.

Despite the AMPK-independent nature of metformin's anti-tumoral effects, we investigated the effect of metformin on mTORC1 activity by analyzing the activation/phosphorylation of two well-known mTORC1-downstream targets: (1) ribosomal protein S6 (S6), which mTORC1 activates *via* activation of ribosomal protein S6 kinase 1 (P70S6K1), a protein which will phosphorylate and activate S6 leading to increased translation of ribosomal proteins and (2) eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1), which mTORC1 phosphorylates, resulting in its dissociation from eukaryotic translation initiation factor 4E (eIF4E), promoting assembly of the eIF4F complex and increased translation. Indeed, 24-hour treatment with 10 mM metformin inhibited phosphorylation of S6 and 4EBP1 in HL60, MOLM14 and U937 cells (Figure 6). The effect on pS6 was extremely potent in all three cell lines. The effect was not as strong in p4EBP1, but it is clear there is a decrease in 4EBP1 activity as measured by the level of total 4EBP1, which was collapsed into the gamma subunit, a signature of decreased activity. Surprisingly, mTORC1 inhibition was also completely independent of AMPK, as siRNA of AMPK$\alpha$1/$\alpha$2 was not able to abrogate this effect. Several alternative AMPK-independent mechanisms of mTORC1 repression have been considered in the literature {cf introduction; (Kalender et al. 2010; Ben Sahra et al. 2011)}. In particular, as increased expression of REDD1 has been identified as a mechanism in which metformin inhibits mTORC1 activity, we measured protein expression of REDD1 and its role in the anti-tumoral effects of metformin in HL60, MOLM14 and U937 cells (see "Future directions" pages 155-157).

Additionally, as mTORC1 is known to control downstream translation of both pro- and anti-apoptotic factors, including Mcl-1, we performed western blot analysis of multiple apoptotic proteins in HL60, MOLM14 and U937 cells treated with or without 10 mM metformin for 24 hours (Figure 8).

Interestingly, we found that metformin significantly decreased the level of anti-apoptotic factor, Mcl-1, in all AML cell lines, without any significant changes in other anti-apoptotic proteins, Bcl-2 or Bcl-xL, or pro-apoptotic proteins, Bak, Bax, BIM or AIF (Figure 8). This inhibition of Mcl-1 was particularly strong in HL60 cells, which are also the most sensitive to metformin-induced apoptosis. Surprisingly, we also observed that metformin decreased the expression of pro-apoptotic factor, PUMA, in HL60 and MOLM14 cells, for which we have no current explanation. Regardless, decreased Mcl-1 is responsible for the induction of apoptosis in response to metformin by inducing cytochrome c release and subsequent cleavage of caspase-3. If confirmed, the fact that cytotoxically-insensitive U937 cells also exhibit decreased Mcl-1 suggests that the block in apoptosis occurs downstream of this inhibition. Thus, we next analyzed the metabolic response to metformin in order to attempt to elucidate the mechanism of survival in U937 cells.

## 3 The role of the mitochondrial ETCI and energetic status in the response to metformin

As metformin is known to inhibit electron transport chain complex I (ETCI), we examined the correlation between cell survival and respiratory characteristics of AML cell lines grown under the same conditions (MEMα containing 5.6 mM glucose, 2.5 mM glutamine+glutamate and 1 mM pyruvate). First, we assessed ETCI activity following metformin treatment and observed a significant decrease at a similar level to rotenone, a specific ETCI inhibitor (Figure 9A). Accordingly, determination of oxygen consumption confirmed that metformin elicited a concentration-dependent decrease in the respiratory rate in HL60, MOLM14 and U937 cells (Figure 9B). Metformin inhibited oxygen consumption by 90% in leukemic cells after 24 hours, an effect comparable to that observed with specific ETCIII inhibitor, antimycin A. Furthermore, all measured rates of oxygen consumption (oligomycin-sensitive, oligomycin-insensitive, uncoupler-stimulated [maximal] respiration and spare respiratory capacity of cells) decreased upon treatment with either 10 mM metformin or 10 uM antimycin A (Figure 9C). Hence, our data is consistent with previously published *in vitro* studies demonstrating a similar concentration-dependent inhibition of oxygen consumption over a range of 1 to 15 mM metformin after 24 hours in several cell types (El-Mir et al. 2000; Owen et al. 2000).

We next asked which metabolic alterations are involved in metformin-induced cell death. The decrease in mitochondrial ATP production that occurs as a result of inhibition of mitochondrial oxidative phosphorylation in most proliferating cells is compensated by an increase in ATP production through glycolysis, the so-called Pasteur effect. Therefore, we first measured glycolytic ATP following metformin-induced inhibition of oxygen consumption and found that glycolytic ATP production is increased to a larger extent in HL60 (+186%) and MOLM14 (+292%) compared to U937 (+37%) after 24 hours treatment with 10 mM metformin (Figure 10A). As other surrogates of

the Pasteur effect, we also measured lactate production and release to the extracellular medium (Figure 10B). HL60 and MOLM14 cells exhibited a large (+60% and +142%, respectively) increase in lactate production when treated with either 10 mM metformin or 10 uM antimycin A, while U937 cells only exhibited a limited (+21%) increase. This confirmed that sensitive HL60 and MOLM14 cells show a more significant induction of the Pasteur effect in response to metformin than less sensitive U937 cells. To understand why U937 cells did not undergo apoptosis and did not exhibit a pronounced Pasteur effect, we analyzed different oxidizable substrates in the culture medium (glutamine, glucose) after treating HL60, MOLM14 and U937 cells with metformin or antimycin A. A significant increase was observed in glutamine consumption upon treatment in all cell types (Figure 10D). Consistent with the increase in glycolytic ATP and lactate production, glucose consumption was markedly increased in metformin- and antimycin-treated HL60 and MOLM14 cells (Figure 10C). Interestingly, both metformin and antimycin A only mildly increased glucose utilization in U937 cells (+23% *versus* +138% and +113% in HL60 and MOLM14, respectively; Figure 10C). These results again demonstrate that only metformin-sensitive HL60 and MOLM14 cells have a pronounced induction of the Pasteur effect following metformin treatment.

Interestingly, basal glucose consumption was two-fold higher in untreated U937 cells compared to MOLM14 cells, which correlated with higher basal lactate production in untreated U937 cells (Figure 11A). This difference in basal glucose metabolism between MOLM14 and U937 cells was also evident in their respective glycolytic and oxidative phosphorylation contributions to ATP synthesis (26%/74% for MOLM14 *versus* 73%/27% for U937 of ATP produced by glycolysis/oxidative phosphorylation; Figure 11B). These results demonstrated that the weak Pasteur effect observed in U937 cells in response to metformin is correlated to a decreased dependency on mitochondrial energetic metabolism in the basal state.

To ascertain whether mitochondrial changes and inhibition of ETC complex I account for the observed Pasteur effect and cell survival of metformin-treated AML cells, we examined ATP production at different time points (1-3-6 hours compared to 24 hours metformin). Our data indicated that the global ATP content of metformin-treated MOLM14 and U937 cells did not change during the experiment (Figure 12A). In addition, we found that metformin-induced glycolytic ATP production occurred as early as 1 hour with a maximum at 3 hours of metformin treatment in MOLM14 and U937 (Figure 12B). However, the induction of the Pasteur effect was much stronger in MOLM14 than U937 cells, confirming previous data (Figure 10B). Of note, the increase in lactate production in the culture medium in response to metformin was significantly delayed when compared to the increase of glycolytic ATP production and occurred only after 24 hours treatment, and markedly in MOLM14 cells (Figure 12C compared to Figure 12B). The discrepancy between the kinetics of these two readouts of the Pasteur effect is likely due to the time required to induce expression of plasma membrane monocarboxylic acid transporters implicated in lactate release.

Because basal glucose uptake and glycolytic rate seem to be critical parameters of the Pasteur effect induction and of the mitochondrial energetic status, we asked how this balance is regulated. Thus, we analyzed the activation of the PI3K/AKT pathway, a key metabolic regulator of aerobic glycolysis in cancer cells. Interestingly, while metformin did not affect the expression of PTEN, metformin treatment increased and sustained phosphorylation of AKT at Ser473 (and not Thr308) only in metformin-sensitive HL60 and MOLM14 cells (Figure 10E). In contrast, we detected strong basal AKT phosphorylation in untreated, PTEN-null U937 cells and did not observe further increase upon metformin treatment (Figure 10E). This is consistent with the notion that U937 cells have a strong glycolytic basal status, as illustrated by higher basal levels of lactate secretion and glucose uptake, lower levels of oxygen consumption and lower dependence on mitochondrial ATP. In summary, these data suggest that U937 cells are resistant to metformin-induced cell death because they exhibit a constitutive glycolytic energetic phenotype in the basal state and thus derive a significantly smaller fraction of ATP from mitochondrial oxidative phosphorylation, thereby requiring a smaller compensatory response to metformin-driven inhibition of ETCI.

## 4 Metabolic response to metformin

To better characterize the metabolic adaptations of AML cells in response to metformin, we analyzed the metabolomic signature of metformin using quantitative LC-MS/MS mass spectrometry (Figure 13A, 14, 15A) as well as protein and gene expression analysis of key metabolic enzymes (Figure 13B-C, 15B). Genome-scale metabolic network of *Homo sapiens* (Duarte et al. 2007) was downloaded from BiGG database (Schellenberger et al. 2010) to perform integrated analysis of the data using the Gene to Protein to Reaction association for each leukemic cell line treated with 10 mM metformin for 24 hours.

The results demonstrated a significant decrease in intermediates of the upper segment of glycolysis (G1P, G6P, F6P, FBP, Man6P) and the oxidative segment of the pentose phosphate pathway (6-PG, Sed7P) and a strong accumulation of purines (PRPP, IMP, AMP) in both MOLM14 and U937 cells after 24 hours metformin treatment (Figure 13A). We also observed a strong increase in acetylcarnitine and decrease in carnitine, suggesting that metformin promotes high mitochondrial fatty acid shuttling for oxidation, as expected (Figure 13A). Interestingly, we observed a significant difference between these two cell types in TCA metabolites (α-KG, fumarate, malate) and glycolytic intermediates (Gly-3P, 2/3PG, PEP), which were decreased in U937 and increased in MOLM14 after metformin treatment. Finally, comparing U937 to MOLM14 cells, we found a significant reduction in basal metabolites in the lower segment of glycolysis (Gly3P, 1,3-DiPG, 2/3-PG, PEP), pentose phosphate pathway (6-PG, Rib5P+Ribu5P+Xylu5P) and amino acid biosynthesis (methionine, glutamine), but an increase of purines (orotate, IMP, GMP, total NAD+/NADH pool), glutathione,

carnitine and acetylcarnitine (Figure 15A), suggesting that the control of flux through key metabolic pathways is significantly different between MOLM14 and U937.

Metformin globally increased gene expression of enzymes involved in anaplerotic reactions (*Pck2*, *Idh2*, *Got1* and *Gls1*) in MOLM14 and U937 cells (Figure 13B) and downregulated *Pfkfb4, AldoC, Eno2, Glut1, Slc7A5, Tkt* and *Acly* as an adaptive mechanism only in metformin-treated MOLM14 cells (Figure 13B). Interestingly, we also observed that metformin decreased phosphorylation of key rate-limiting enzyme of mitochondrial pyruvate oxidation, pyruvate dehydrogenase (PDH), only in metformin-sensitive leukemic cells (Figure 13C). However, we observed no significant changes in HK-II, GAPDH, PK-M2, LDHA, IDH1, IDH2, CPT1 or ACC (Figure 13C). Of note, the calculated free energy changes for three key reactions (GPI, PFK, ENO) in the glycolytic pathway were similar between treated and untreated MOLM14 and U937 cells (Figure 16). However, the PRPP biosynthetic equilibrium was significantly displaced by metformin in MOLM14 and the free energy change of the PFK reaction was significantly more negative in U937 under basal growth conditions, suggesting FBP formation by PFK is strongly favored in U937 cells (Figure 16).

## 5 Inhibition of glycolysis or AKT activation induces cell death upon metformin treatment of resistant U937 cells

According to the U937-specific phenotype, we tested whether the strong basal AKT activation and glycolysis protects U937 cells from metformin-induced apoptosis. First, we inhibited glycolytic metabolism with deoxyglucose (DXG) to determine if this would preferentially sensitize U937 cells to metformin-induced apoptosis following the shift from non-glucose oxidizing substrates (glutamine, fatty acids) to glucose oxidation through mitochondria. We observed marked sensitivity to DXG alone in MOLM14 without any synergistic effect with metformin. In U937 cells, by contrast, DXG had no effect on apoptosis alone even though DXG significantly decreased global and glycolytic ATP production. However, in combination with metformin, DXG induced marked apoptosis in U937 cells and induced a significant Pasteur effect (i.e. glycolytic ATP production) (Figure 17A-B; Figure 18A). These results suggest that U937 cells depend heavily on glycolysis after metformin treatment. We confirmed these results by starving U937 of glucose by culturing the cells at high cell density in media with 5.6 mM glucose. In these conditions, U937 cells are highly sensitive to metformin, which can be abrogated by growing high cell density cells in the presence of 25.6 mM glucose (Figure 17C). Culturing U937 at high cell density also significantly decreased AKT phosphorylation, suggesting the key role of glucose uptake and the AKT pathway in metformin sensitivity (Figure 17D).

To test the hypothesis that high AKT activation can drive glycolysis and metformin insensitivity in U937 cells, we studied the effects of AKT inhibition on cell metabolism and survival. We observed that a 4-hour pretreatment with an AKT inhibitor completely reduced AKT phosphorylation in both leukemic cells (Figure 18E). Subsequent treatment with metformin induced a strong Pasteur effect (increased lactate and glycolytic ATP production; Figure 18F-G) and apoptosis, the latter of which was markedly higher in U937 cells (Figure 18H). Overall, these results support our hypothesis that high AKT phosphorylation leads to high basal glycolysis and insensitivity to metformin-induced cell death in U937 cells.

## 6 The manipulation of other major metabolic pathways can induce cell death upon metformin treatment in AML cells

Based on the combined apoptotic effect of metformin with glycolytic or AKT inhibitors, we decided to test metformin in combination with additional metabolic drugs. We hypothesized that if metformin induces a metabolic shift that confers some resistance to its apoptotic effects, than inhibiting other metabolic pathways could be extremely synergistic. We thus analyzed the apoptotic effect of combining metformin and positive controls, rotenone and antimycin A, with 8 other drugs that could impact metabolism in both MOLM14 and U937 cells. An overview of each drug and its target can be found in Figure 19A. For the purpose of comparing metformin treatments to drug combinations, metformin induced, on average, a 1.6-1.8-fold increase in apoptosis in MOLM14 cells and did not induce any apoptosis in U937 cells throughout all experiments. In both cell lines, our positive controls of rotenone and antimycin A had higher impacts on apoptosis.

We first analyzed the combination of metformin with inhibitors of the pentose phosphate pathway (PPP). We analyzed the combinatorial effect of 100 uM 6-Aminonicotinamide (6-ANA), an inhibitor of 6PGD of the oxidative branch of the PPP, and 100 uM oxythiamine (OXY), a competitive inhibitor of transketolase of the non-oxidative branch of the PPP (Figure 19B-C). Alone, 6-ANA induced a slight, 1.3-fold, induction of apoptosis in MOLM14 while it had no effect on U937 cells. However, in combination, 6-ANA and metformin induced synergistic effects on apoptosis, with 3.5- and 1.4-fold increases in apoptosis. Oxythiamine, however, did not induce apoptosis alone and had no effect in combination with metformin, rotenone or antimycin A in MOLM14 or U937 cells. These results may suggest that metformin-treated cells have an increased dependence on the oxidative branch of the PPP as MOLM14 and U937 cells were only significantly sensitive to 6-ANA in combination with metformin and its positive controls.

We next tested the combination of metformin with inhibitors of fatty acid metabolism, including 20 uM orlistat (ORLI) to inhibit FAS and 50 uM etomoxir (ETO) to inhibit CPT1 and block fatty acid oxidation (Figure 19D-E). Alone, ORLI induced a much higher level of basal apoptosis of

4.8-fold and 3.6-fold in MOLM14 and U937, respectively. Etomoxir, on the other hand did not induce apoptosis as a single-agent. This may suggest a basal cell dependency on FAS. The combination of ORLI and metformin was additive in MOLM14 cells, while the combination was synergistic in U937 cells with respective 8.3-fold and 6.6-fold increases in apoptosis. The combination of ETO and metformin was also additive and synergistic in MOLM14 and U937 cells, respectively, but had a less significant effect on apoptosis with only 2.0- and 1.3-fold increases. For both ORLI and ETO, the combination with either rotenone or antimycin had similar impacts as metformin.

We then attempted to combine PPARα agonist, 0.5 mM bezafibrate (BEZA), with mitochondrial inhibitors (Figure 19F). As PPARα exhibits control over fatty acid uptake, synthesis and esterification, we hypothesized that PPARα could work in combination similar to orlistat. Alone BEZA induced only a slight 1.2-fold increase in apoptosis in both MOLM14 and U937 cells. However, BEZA had a small synergistic effect in combination with metformin, and its positive controls, in both MOLM14 and U937 cells. BEZA increased the apoptosis level to 2.3-fold in MOLM14 and to 1.5-fold in U937 cells.

We also tested the effect of 5 mM dichloroacetate (DCA), an inhibitor of PDK1. PDK1 inhibition releases its negative feedback on PDH, the reactivation of which increases pyruvate entry into the TCA cycle. We hypothesized that DCA would increase glucose oxidation and mitochondrial ATP production, which could sensitize cells to mitochondrial inhibition by metformin. Alone, DCA had no apoptotic effect on U937 or MOLM14 and only a very slight combinatorial effect in both cell lines with metformin, rotenone and antimycin A (Figure 19G).

10 uM 6-diazo-5-oxo-L-norleucine (DON), a glutamine antagonist that blocks glutaminolysis through competitive inhibition, induced a 2.0- and 2.2-fold increase in apoptosis in MOLM14 and U937 cells, respectively (Figure 19H). However, in combination with metformin, rotenone or antimycin A, the level of apoptosis did not increase beyond that induced by DON alone in MOLM14 or U937 cells. While we expected a synergistic effect, it is possible that these cells are very dependent on glutamine as a carbon source to fuel the TCA cycle. Thus, pre-inhibition of glutaminolysis could decrease mitochondrial function and reduce the effect of mitochondrial inhibition.

Finally, we tested the effect of 10 mM buthionine sulfoxide (BSO), which inhibits *de novo* GSH synthesis and thereby decreases antioxidant capacities (Figure 19I). Thus, if metformin's mechanism of action involves ROS production and/or the antioxidant levels in our cell types counteract metformin effects, it is possible we would see a synergistic effect with BSO. Alone, BSO induced a slight 1.2-fold increase in apoptosis in MOLM14 cells, while there was no effect in U937 cells. Additionally, BSO was only additive with metformin, rotenone and antimycin A in MOLM14 cells, while it had no combinatorial effect in U937 cells (Figure 19I).

# – Figures –

## V Figures

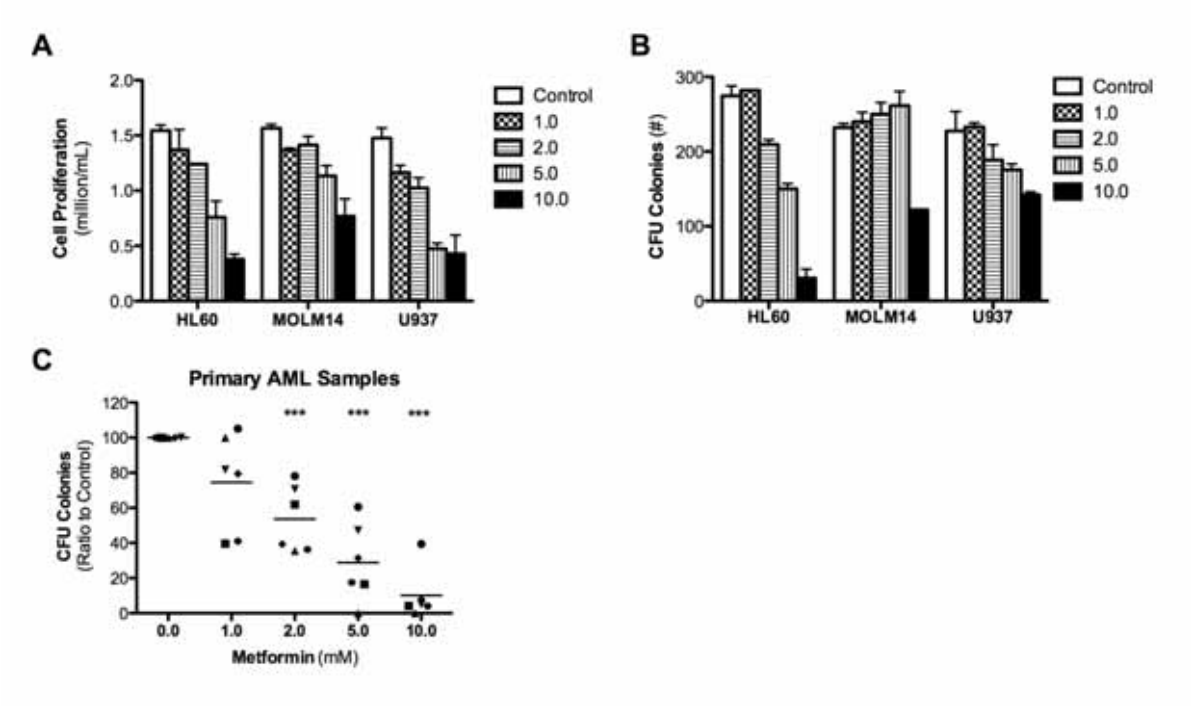



**Figure 1. Metformin inhibits cell proliferation in AML cells.** (A) HL60, MOLM14 and U937 cells after 48 hrs with 0.0, 1.0, 2.0, 5.0, or 10.0 mM metformin. Proliferation is calculated by ratio of final to initial cell count (with trypan blue exclusion) over the 48 hrs of incubation. (B) Average CFU growth from HL60, MOLM14 and U937 cells in methylcellulose enriched media after 5-7 days incubation with 0.0, 1.0, 2.0, 5.0 or 10.0 mM metformin. (C) CFU growth calculated as an average from 6 patient samples in methylcellulose enriched media after 14 days incubation with 0.0, 1.0, 2.0, 5.0 or 10.0 mM metformin, statistics by unpaired t-test with p-value as *** for $p<0.005$.

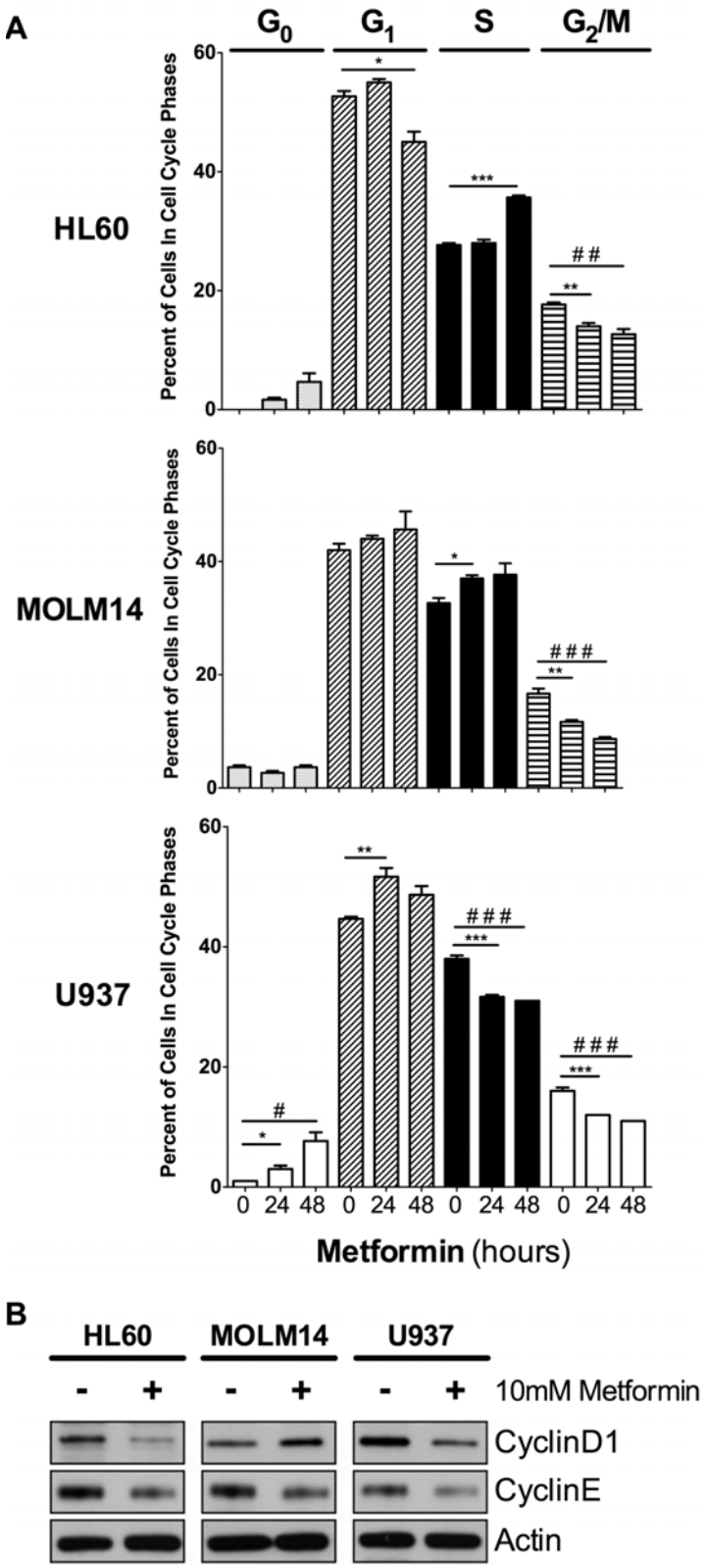



**Figure 2. Metformin blocks cell cycle progression in AML cell lines.** (A) Flow cytometry analysis of cell cycle using PI and Ki67 staining of HL60, MOLM14 and U937 cells following 24 and 48 hrs treatment with control or 10 mM metformin. (B) Western blot analysis of the cells in Figure 2A for cyclin D1 and cyclin E expression, normalized to β-actin. Statistics were performed with an unpaired t-test with p-values represented as *** for $p<0.005$, ** for $p<0.01$, and * for $p<0.05$ (# substituted for * if two instances of significance for a control).

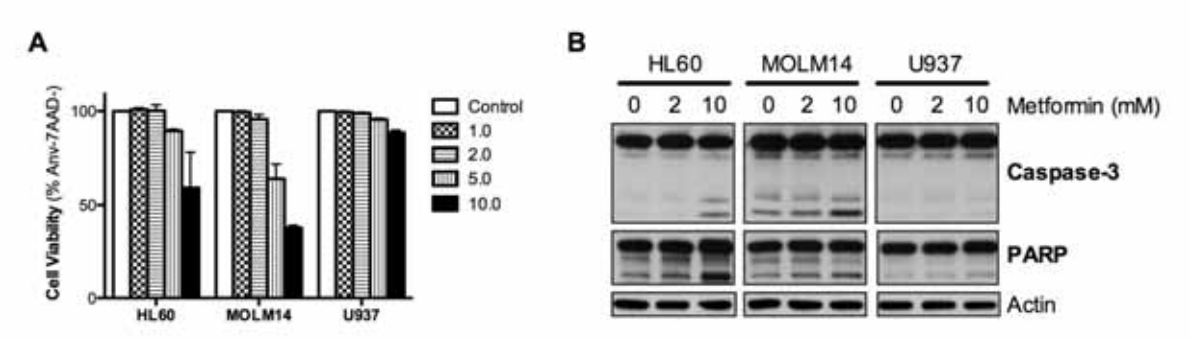



**Figure 3. Metformin has varying effects on apoptotic-dependent cell death in AML cell lines.**
(A-B) Viable cells as the percentage of Annexin-V negative and 7AAD negative cells carried out on the same cells listed for Figure 1A and the cleavage of caspase-3 and PARP, respectively, and normalized to β-actin.

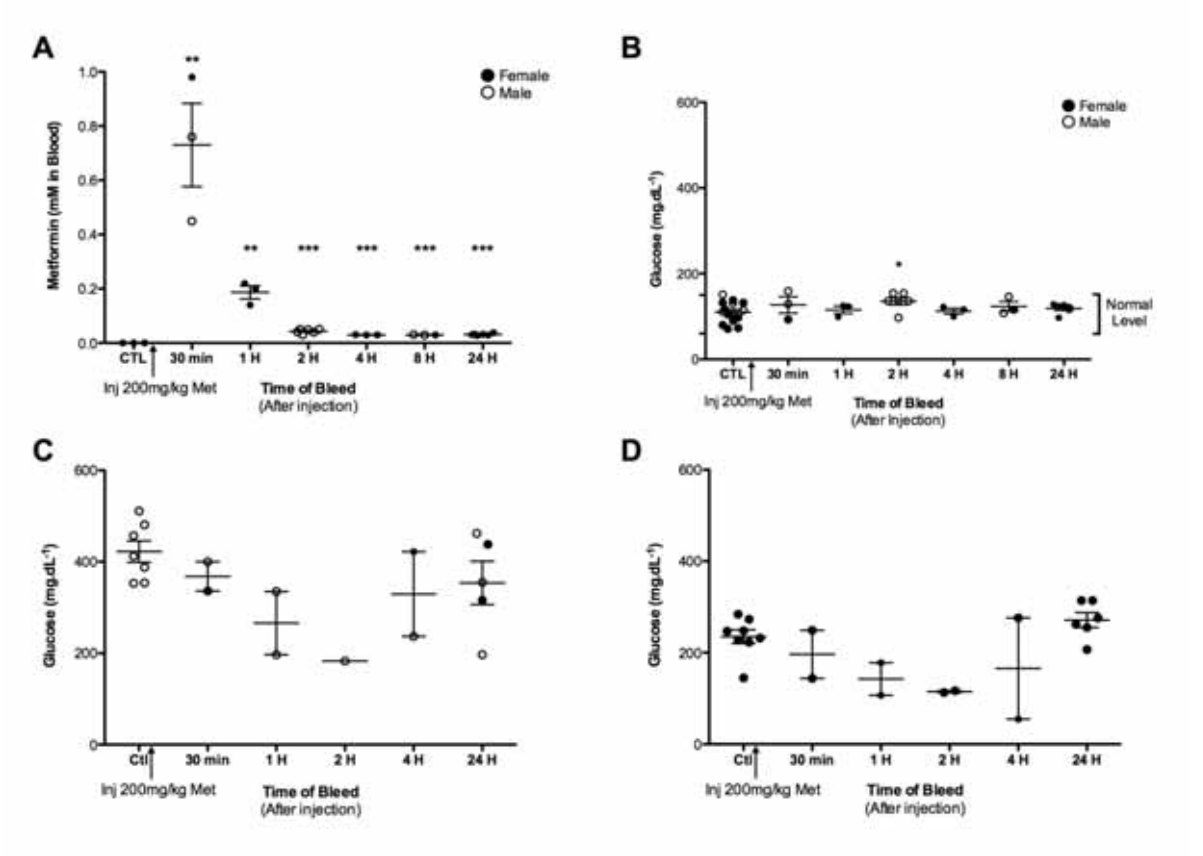



**Figure 4. Metformin's blood serum concentration and effect on blood glucose in mice.** (A) Average plasma concentration of metformin (mM) and (B) blood glucose (mg/dL$^{-1}$) of NOD-SCID mice at 0, 0.5, 1, 2, 4, 8 and 24 hrs following an intraperitoneal injection of metformin at 200 mg/kg. Average blood glucose (md/dL$^{-1}$) in (C) male and (D) female NOD-SCID mice pre-induced with streptozotocin-induced diabetes, intraperitoneally with metformin at 200 mg/kg and then bled 0.25, 5.0, 1, 2, 4 and 24 hrs after injection for blood analysis. Statistics were performed with an unpaired t-test with p-values represented as * for $p<0.05$. ** for $p<0.01$ and *** for $p<0.005$.

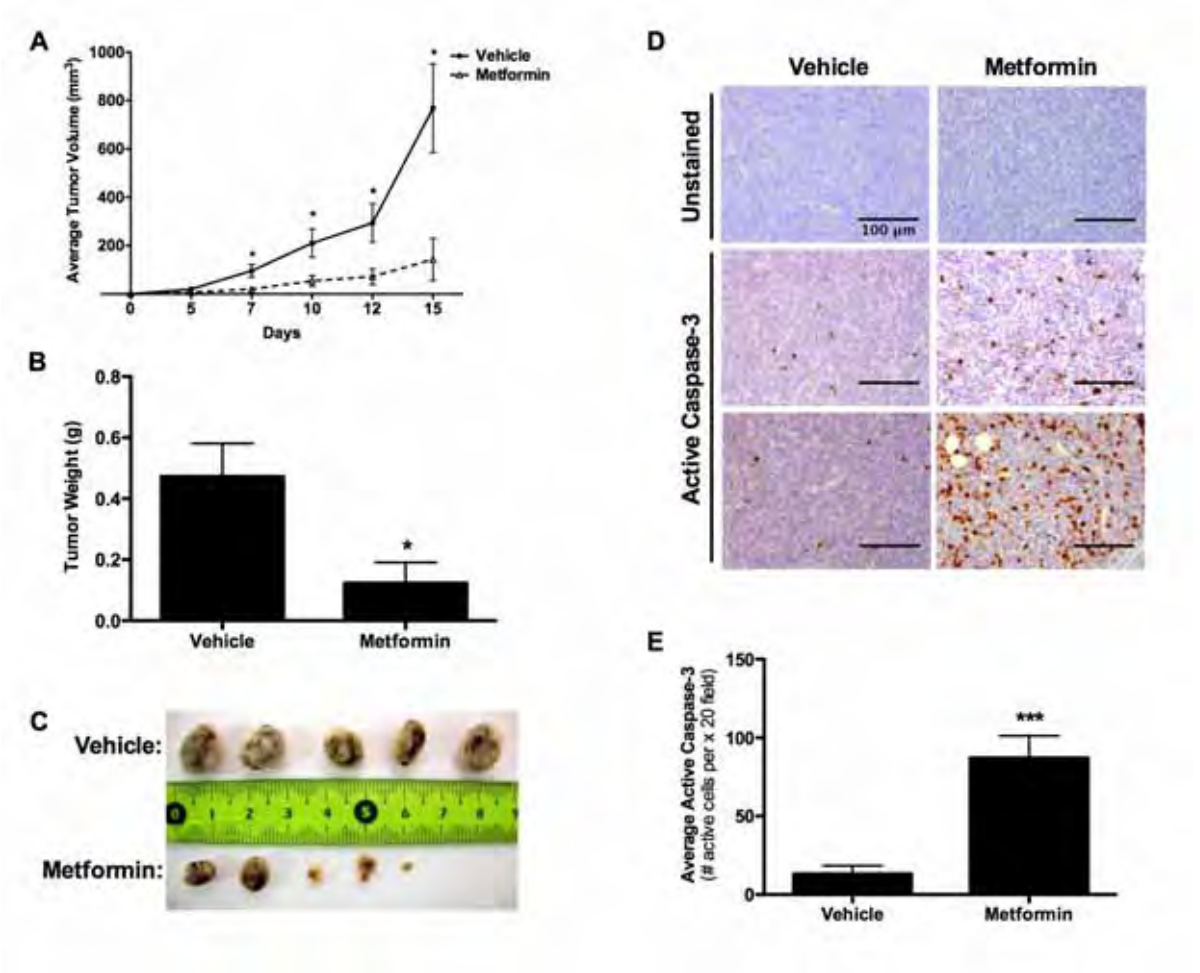



**Figure 5. Metformin inhibits tumor growth of AML cells *in vivo*** (A) Mean tumor size ($mm^3$) and (B) tumor weight (grams) was assessed in nude mice xenografted with MOLM14 cells and treated with either vehicle or 300 mg/kg/day metformin (n = 10 tumors in each group). (C) Representative photographs of xenografted tumors at day 17. (D) Tumor sections of mice injected with either vehicle or metformin were stained for active caspase-3. Representative photos of 3 metformin and 3 vehicle tumors are shown. Statistics were performed with an unpaired t-test with p-values represented as * for $p<0.05$.

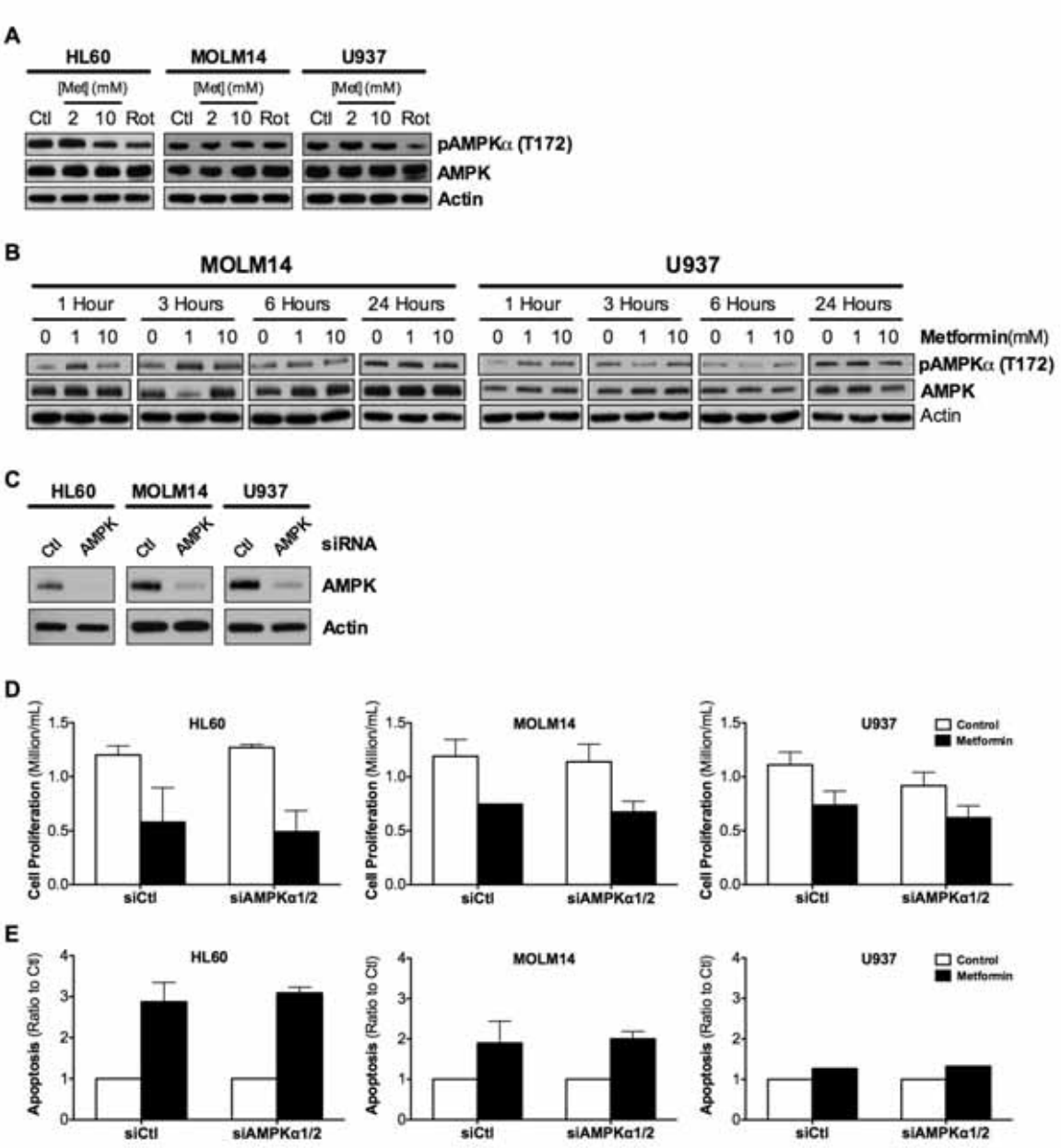



**Figure 6. AMPKα1/2 does not effect the proliferation inhibition or apoptosis induced by metformin.** (A) Western blots in HL60, MOLM14 and U937 cells after 24 hrs with untreated, 2 mM metformin, 10 mM metformin or 200 nM rotenone (Rot). (B) Western blots in MOLM14 and U937 cells after 1, 3, 6 and 24 hrs with untreated, 1 mM metformin and 10 mM metformin. (C) Western blots in HL60, MOLM14 and U937 cells 24 hrs after control siRNA or AMPKα1/2 siRNA. Following 24 hrs inhibition, these cells were treated for 48 hrs with control or 10 mM metformin and then analyzed for (D) cell proliferation, calculated by ratio of final to initial cell count (with trypan blue exclusion) and (E) apoptosis, measured as AnnexinV-positive cells and presented as ratio to control.

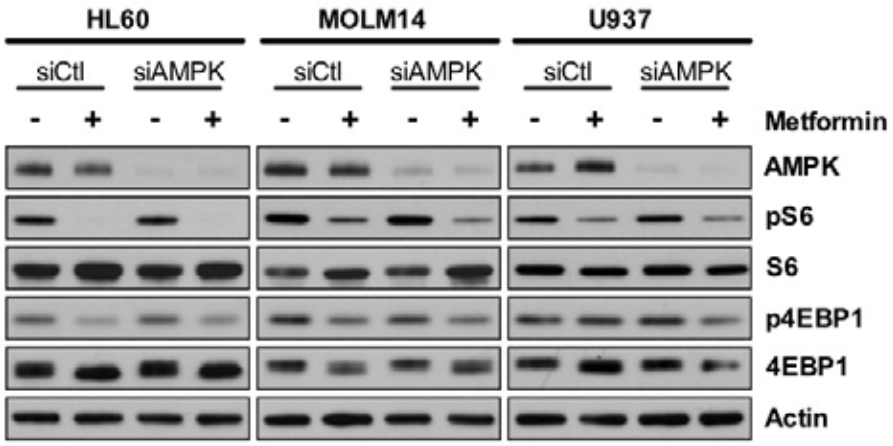



**Figure 7. Metformin decreases mTORC1 activity independent of AMPK in all AML cell lines.** Western blots in HL60, MOLM14 and U937 cells after an initial 24 hr treatment with either siRNA control or siRNA AMPKα1/2 followed by a 24 hr treatment with either control or 10 mM metformin. Western blots were analyzed with primary antibodies for AMPK, pS6, S6, p4EBP1 and 4EBP1, all normalized to β-actin.

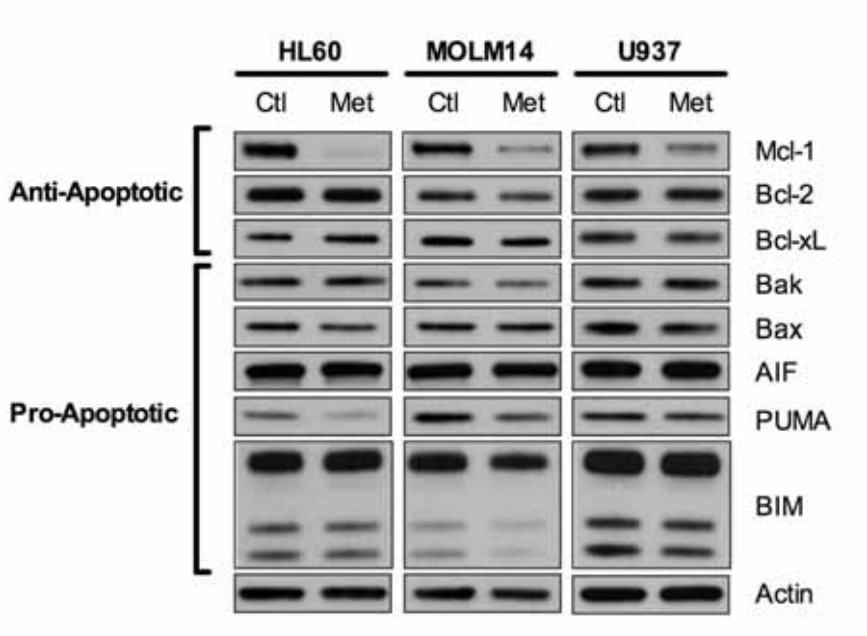



**Figure 8. Metformin significantly decreases Mcl-1 expression in AML cells.** Western blots of anti-apoptotic proteins, Mcl-1, Bcl-2 and Bcl-xL, as well as pro-apoptotic proteins, Bak, Bax, AIF, PUMA and BIM, in HL60, MOLM14 and U937 cells after 24 hrs with control or 10 mM metformin.

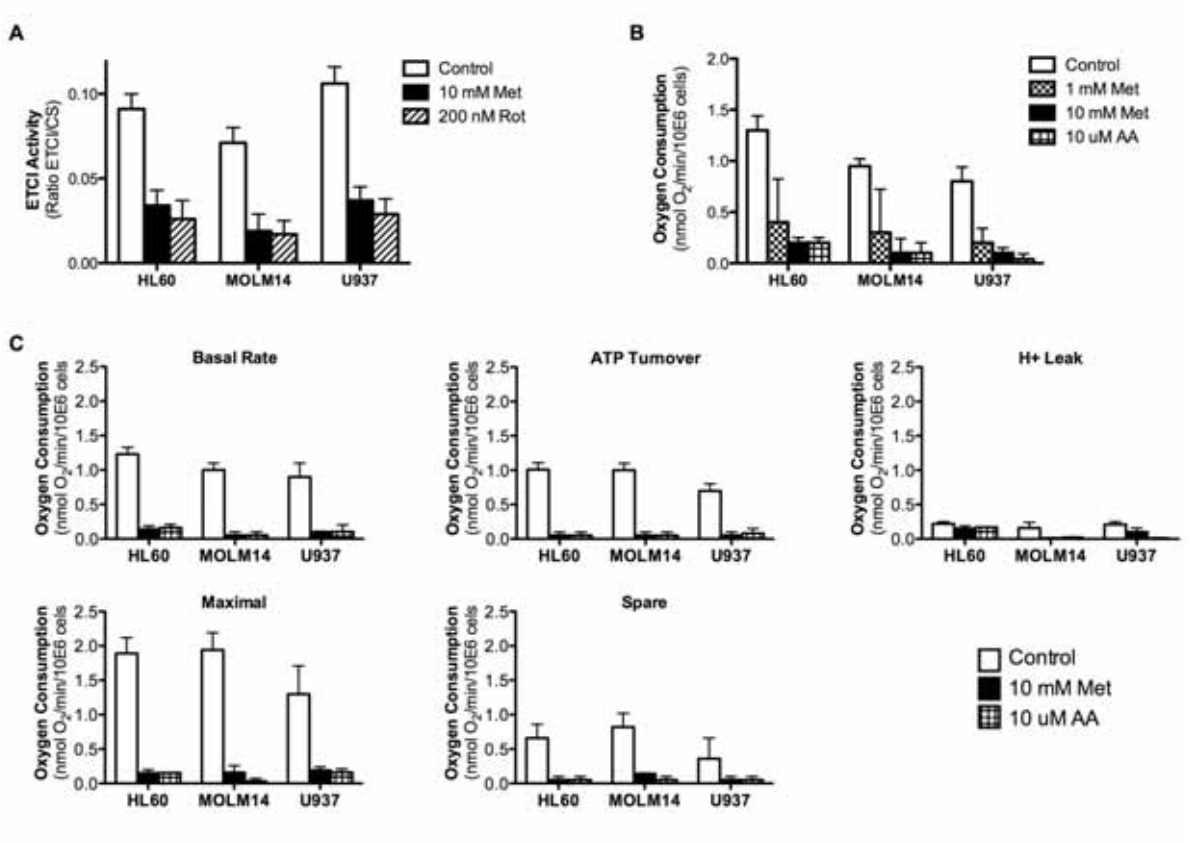



**Figure 9. Metformin inhibits ETCI and oxygen consumption in all AML cell lines.** (A) Electron transport chain complex I (ETCI) activity measured as a ratio of ETCI activity to citrate synthase activity in HL60, MOLM14 and U937 cells after 24 hrs with control, 10 mM metformin or 200 nM rotenone (Rot) (B) The basal oxygen consumption rate, measured as nmol/min/million cells, in HL60, MOLM14 and U937 cells after 24 hrs with control, 1.0 mM metformin, 10.0 mM metformin or 10 uM antimycin A (AA). (C) The graphs represent specific oxygen consumption rates, measured as nmol/min/million cells, in HL60, MOLM14 and U937 cells in after 24 hrs with control, 10 mM metformin or 10 uM antimycin A (AA). The specific oxygen consumptions described represent the following equations: Basal is the basal rate; ATP turnover is the basal minus oligomycin rate; H+ Leak is oligomycin minus insensitive rate; Maximal is the uncoupler rate; spare is the uncoupler minus basal rate.

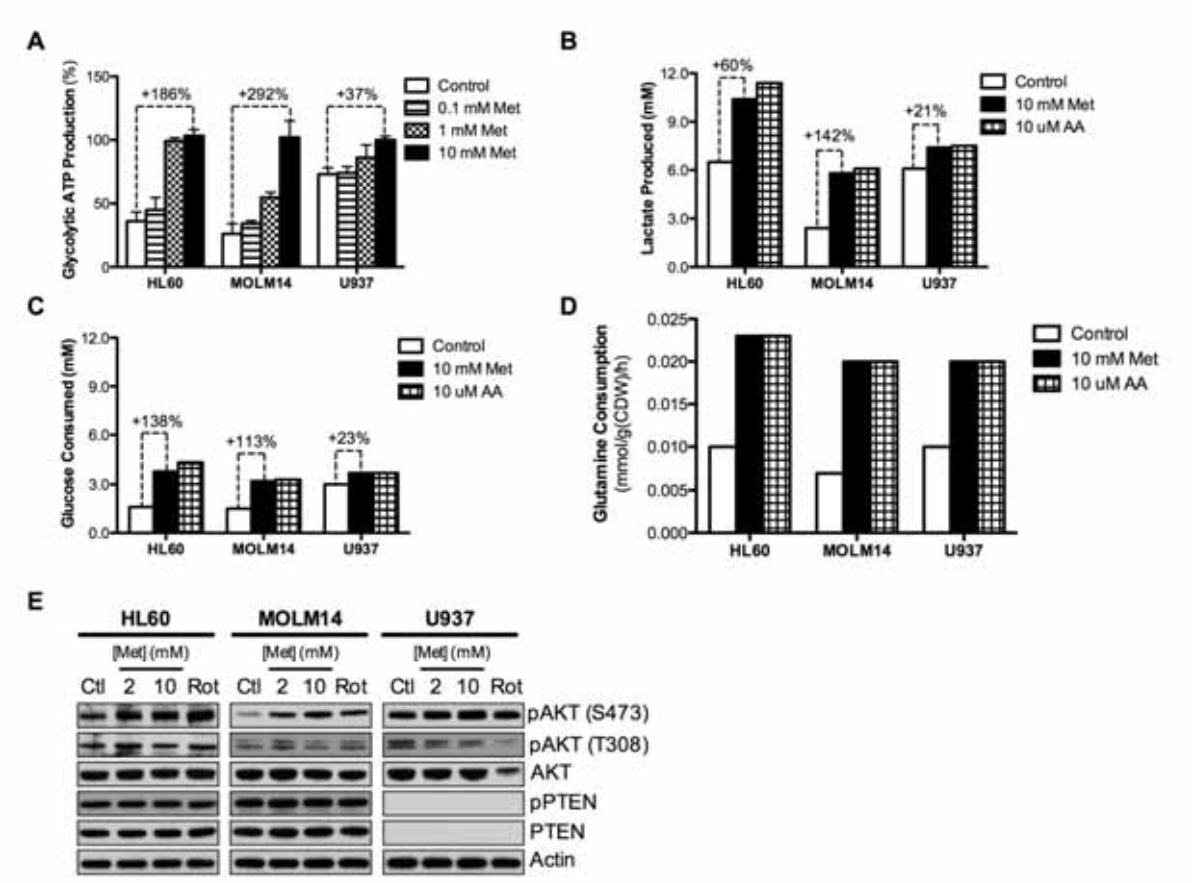



**Figure 10. Metformin induces a stronger Pasteur effect in MOLM14 and HL60 cells due to lower glucose consumption for ATP production.** (A) Glycolytic ATP production measured as a percentage of total ATP production in HL60, MOLM14 and U937 cells after 24 hrs with 0.0, 0.1, 1.0 or 10.0 mM metformin. Comparison of (B) lactate produced (mM) and (C) glucose consumed (mM) in HL60, MOLM14 and U937 cells after 24 hrs with control, 10 mM metformin and 10 uM antimycin A. (D) Glutamine consumption (mmol/g(CDW)/h) in HL60, MOLM14 and U937 cells after 24 hrs with control, 10 mM metformin or 10 uM antimycin A. (E) Western blots in HL60, MOLM14 and U937 cells after 24 hrs with control, 2 mM metformin, 10 mM metformin or 200 nM rotenone. Western blots were analyzed using primary antibodies for pAKT (S473), pAKT (T308), AKT, pPTEN, and PTEN, all normalized to β-actin.

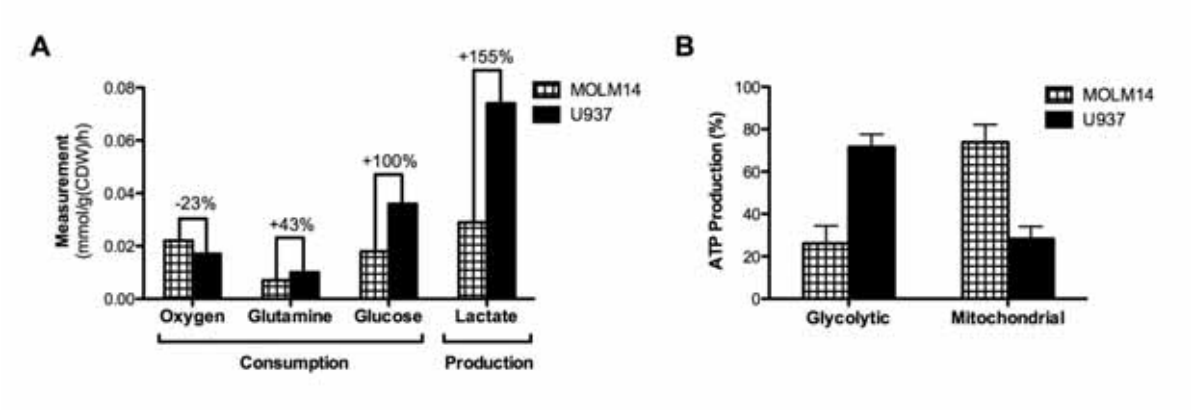



**Figure 11. Basal metabolic activity in MOLM14 *versus* U937.** (A) Basal oxygen, glutamine and glucose consumption and lactate production measured as mmol/g(CDW)/h in MOLM14 and U937. (B) The basal percentage of glycolytic or mitochondrial ATP production in MOLM14 and U937 cells.

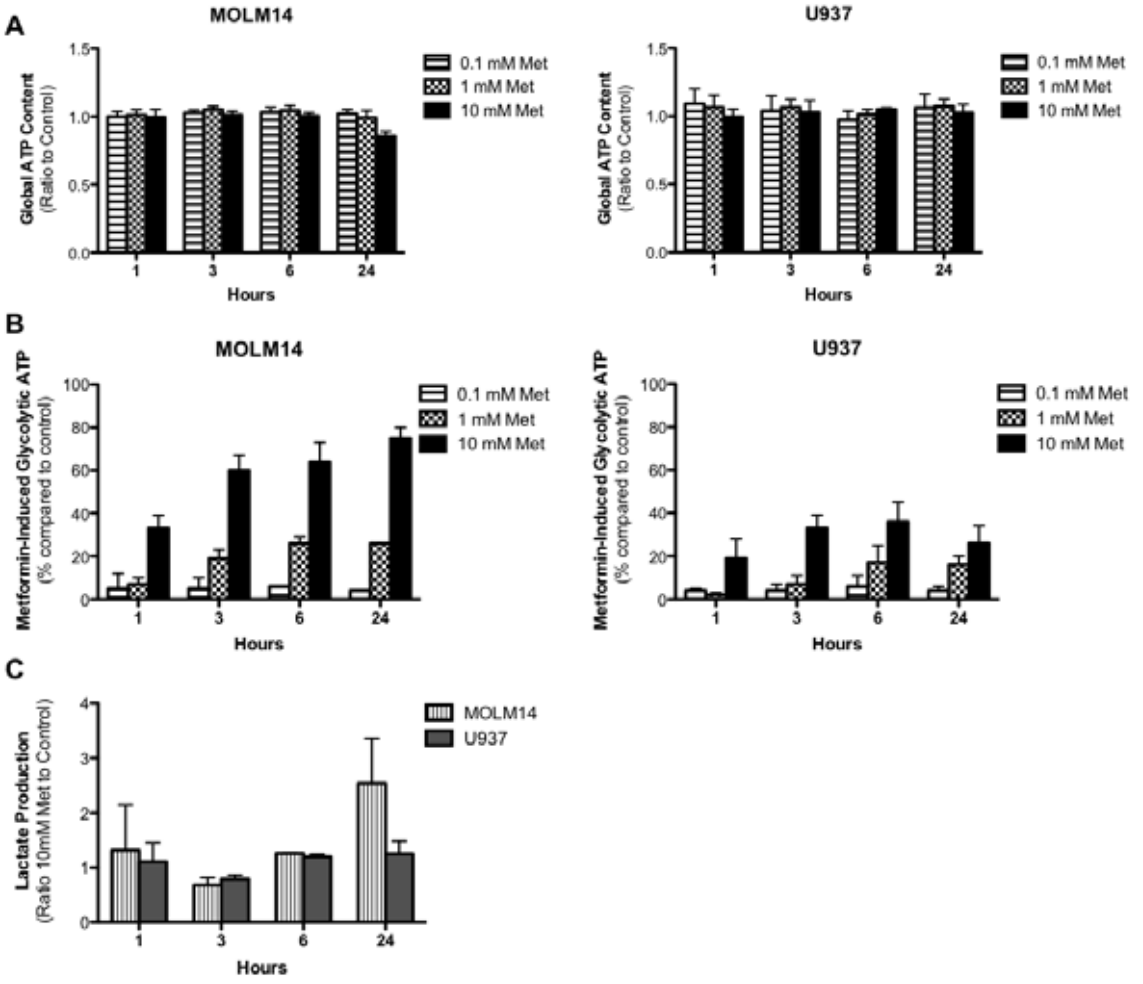



**Figure 12. Metformin kinetics of ATP and lactate production in MOLM14 and U937 cells.** (A) Global ATP content presented as ratio to control in MOLM14 and U937 cells after 1, 3, 6 and 24 hrs with 0.0, 0.1, 1.0 and 10.0 mM metformin. (B) Pasteur effect induction measured as a percentage of glycolytic ATP induced by metformin in MOLM14 and U937 cells after 1, 3, 6 and 24 hrs with control or 10 mM metformin. (C) Lactate production, represented as ratio to control, of MOLM14 and U937 cells after 1, 3, 6, and 24 hrs with control or 10 mM metformin.

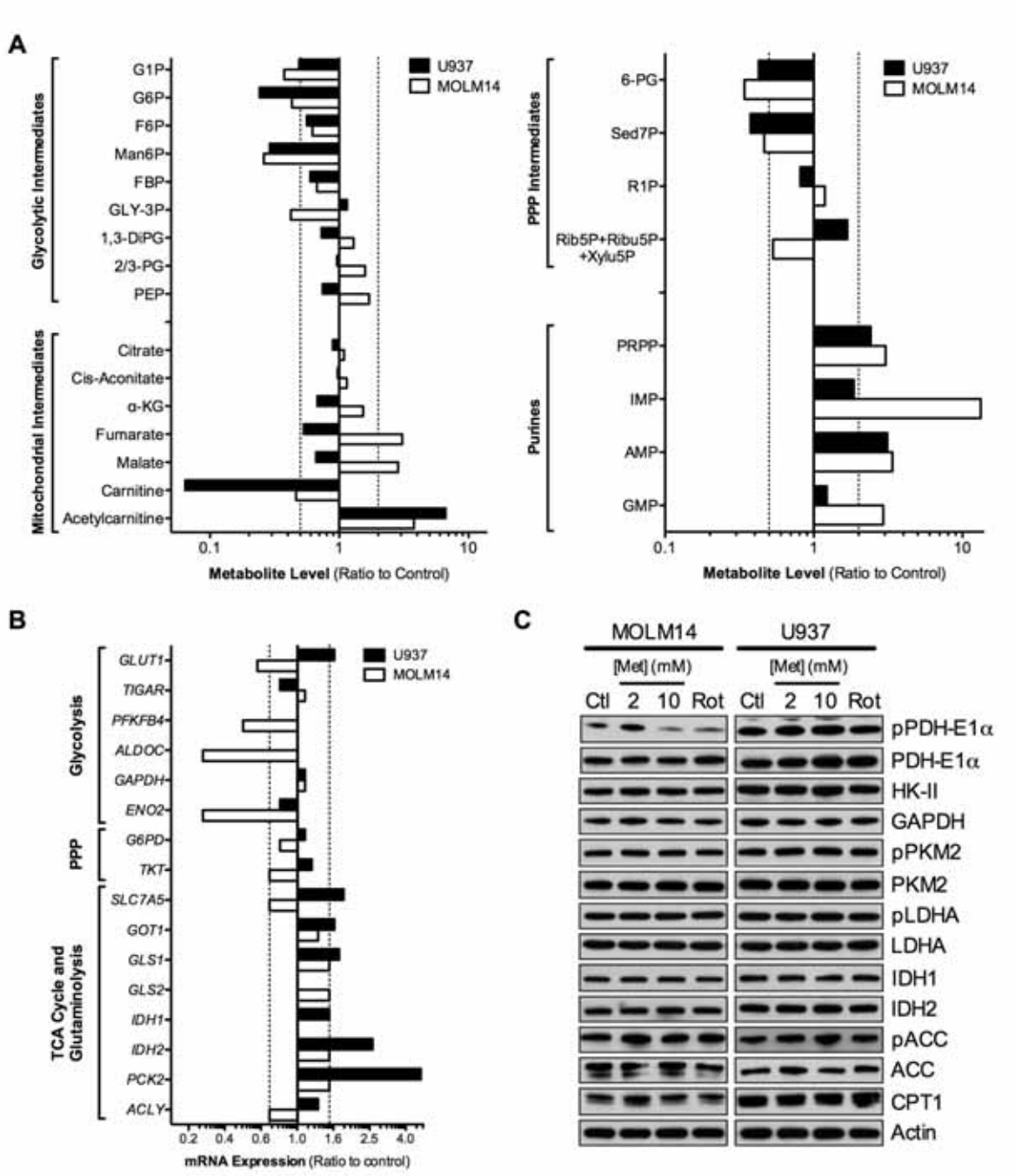



**Figure 13. Metformin induces a metabolic adaptation in all leukemic cells, but insensitive cells have additional adaptive mechanisms to sustain cell survival.** (A) Quantified intracellular concentrations of metabolites and (B) gene expression, calculated as ratio of 10 mM metformin to control, for MOLM14 and U937 cells after 24 hrs. (C) Western blots in MOLM14 and U937 cells treated for 24 hrs with untreated, 2 mM metformin, 10 mM metformin and 200 nM rotenone (ROT).

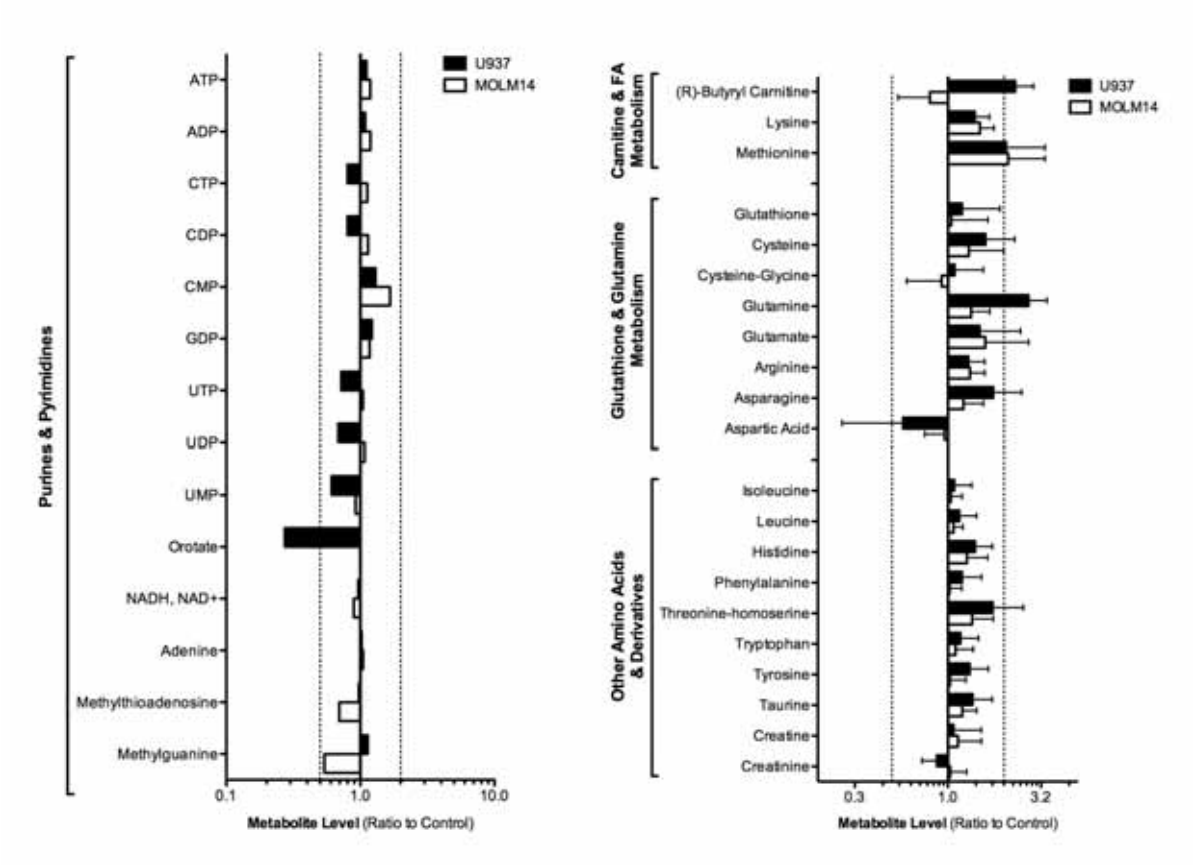



**Figure 14. Additional metabolite data in U937 and MOLM14 cells that did not exhibit significant change in response to metformin.** Quantified intracellular concentrations of metabolites, calculated as ratio of 10 mM metformin to control, for MOLM14 and U937 cells after 24 hrs.

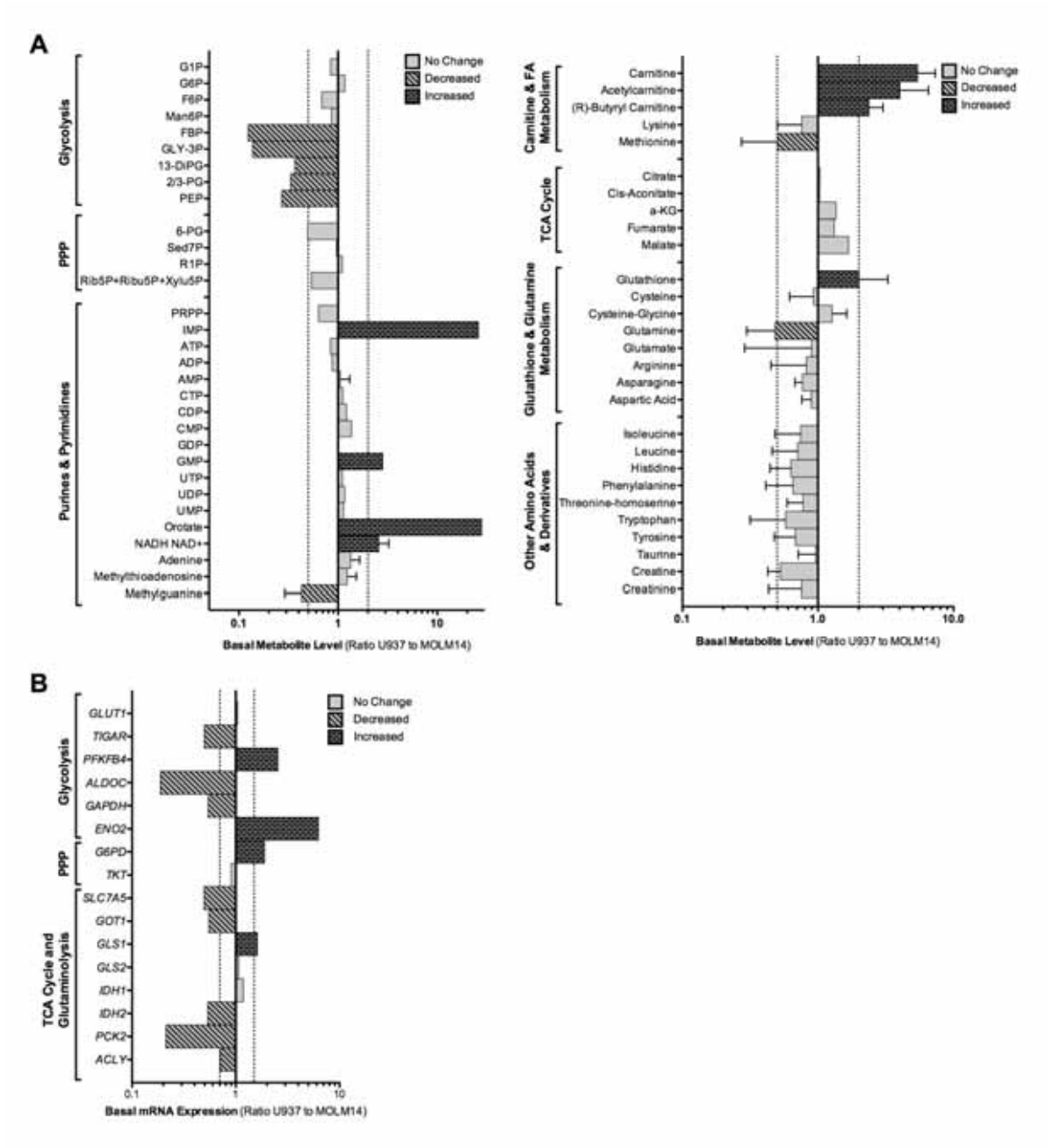



**Figure 15. Basal metabolite levels in U937 and MOLM14 have significant differences that correlate to the cytotoxic response to metformin.** Comparison of (A) metabolite levels and (B) gene expression as a ratio of U937 to MOLM14.

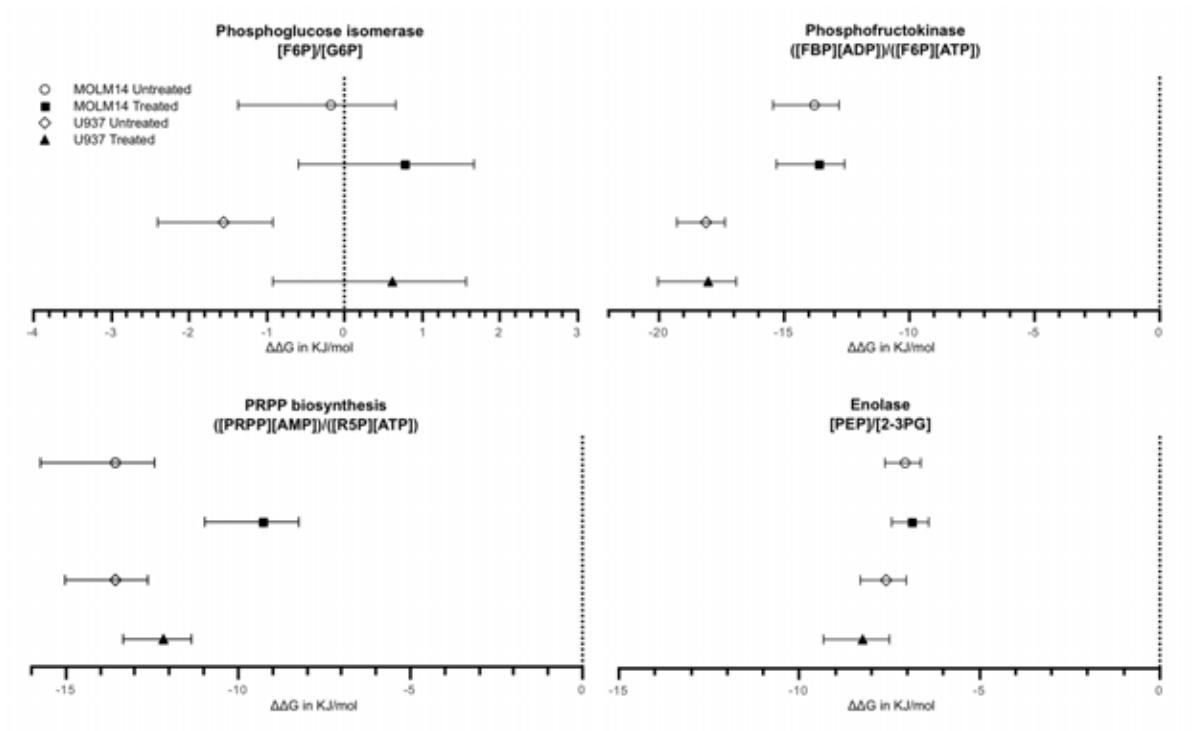



**Figure 16. Thermodynamic status of U937 and MOLM14.** (A) Thermodynamic states, measured as ΔΔG in KJ/mol, of phosphoglucose isomerase (PGI), phosphofructokinase (PFK), enolase (ENO) and PRPP biosynthesis in MOLM14 and U937 cells after 24 hrs with control or 10 mM metformin.

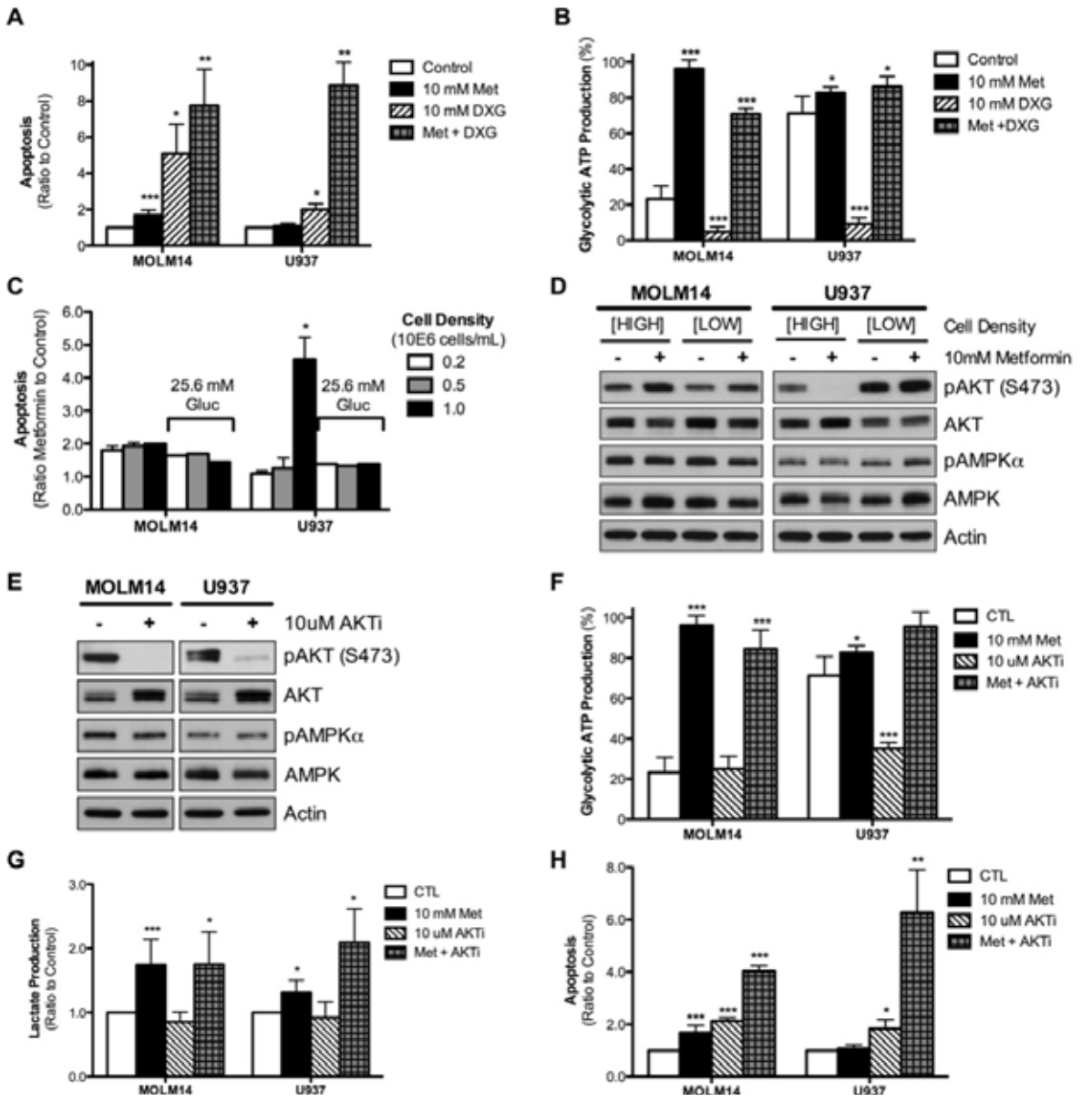



**Figure 17. The pro-apoptotic effect of metformin can be increased or induced in AML cells by specific metabolic manipulation.** After a 4 hr pre-incubation with or without 10 mM 2-deoxyglucose (DXG), U937 and MOLM14 cells were incubated for 24 hrs with control, 10 mM metformin, 10 mM DXG, or the combination and analyzed for (A) apoptosis, measured as AnnexinV-positive cells and presented as ratio to control and (B) glycolytic ATP production, presented as a percentage of total ATP production. (C) Apoptosis, measured as AnnexinV-positive cells and presented as ratio of 10mM metformin-treated to untreated, in MOLM14 and U937 cells at starting cell densities of 0.2, 0.5, or 1.0 million cells/mL either in MEMα (5.6mM) or MEMα (25.6mM). (D) Western blots in MOLM14 and U937 cells after 24 hrs with control or 10 mM metformin with either high or low starting cell densities. (E) Western blots in MOLM14 and U937 cells after 4 hrs pretreatment with untreated or 10 uM AKTi to confirm AKT inhibition. Following the 4 hr pre-incubation with either control or 10 uM AKTi, U937 and MOLM14 cells were then incubated for 24 hrs with control, 10 mM metformin, 10 uM AKTi, or the combination of met and AKTi and analyzed for (F) lactate production, presented as a ratio to control, (G) glycolytic ATP production, presented as a percentage of total ATP production, and (H) apoptosis, measured as AnnexinV-positive cells and presented as ratio to control. Statistics were performed with an unpaired t-test with p-values of *** for $p<0.005$, ** for $p<0.01$, and * for $p<0.05$.

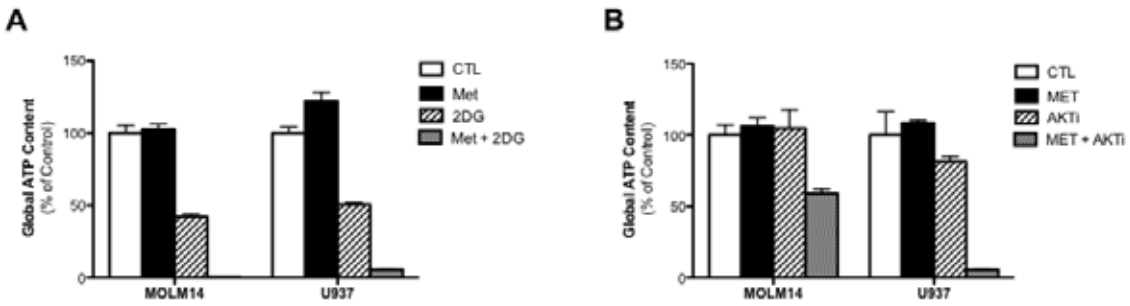



**Figure 18. Global ATP production following 2-deoxyglucose and AKTi treatments.** Global ATP production, measured as percent of control, for (A) MOLM14 and U937 cells incubated for 24 hrs with control, 10 mM metformin, 10 mM 2-deoxyglucose (DXG) or the combination of metformin and DXG and (B) MOLM14 and U937 cells incubated for 24 hrs with control, 10 mM metformin, 10 uM AKTi or the combination of metformin and AKTi. Complementary data for these cells can be found in Figure 17A-B and 17E-H, respectively.

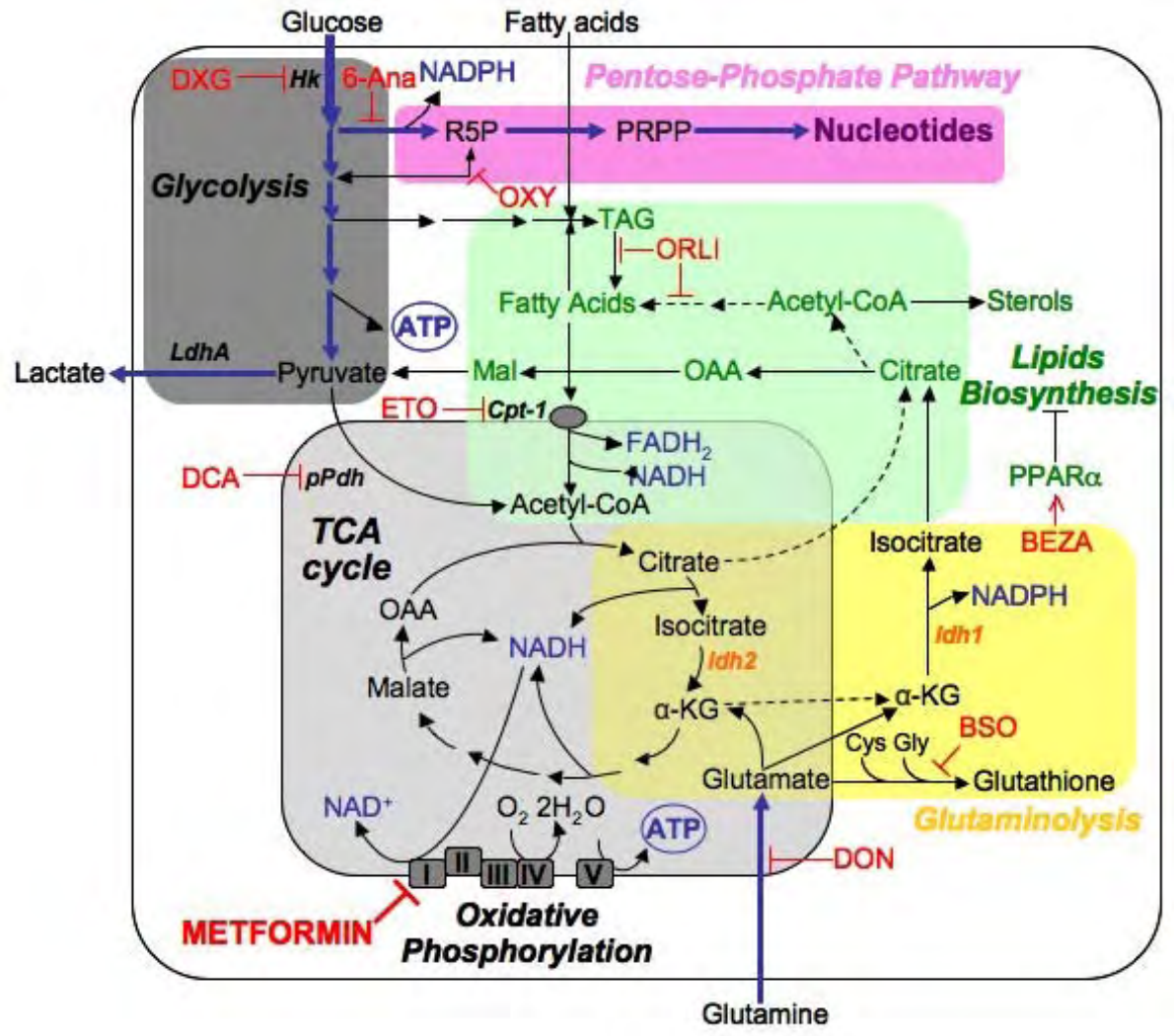



**Figure 19. The apoptotic effect of the combination of metformin with other metabolic drugs.**
(A) Overview of metabolic drugs tested in combination with metformin. These drugs include 2-deoxyglucose (DXG), 6-aminonicotinamide (6-ANA), oxythiamine (OXY), orlistat (ORLI), etomoxir (ETO), dichloroacetate (DCA), 6-diazo-5-oxo-L-norleucine (DON), buthionine sulfoxide (BSO), bezafibrate (BEZA).

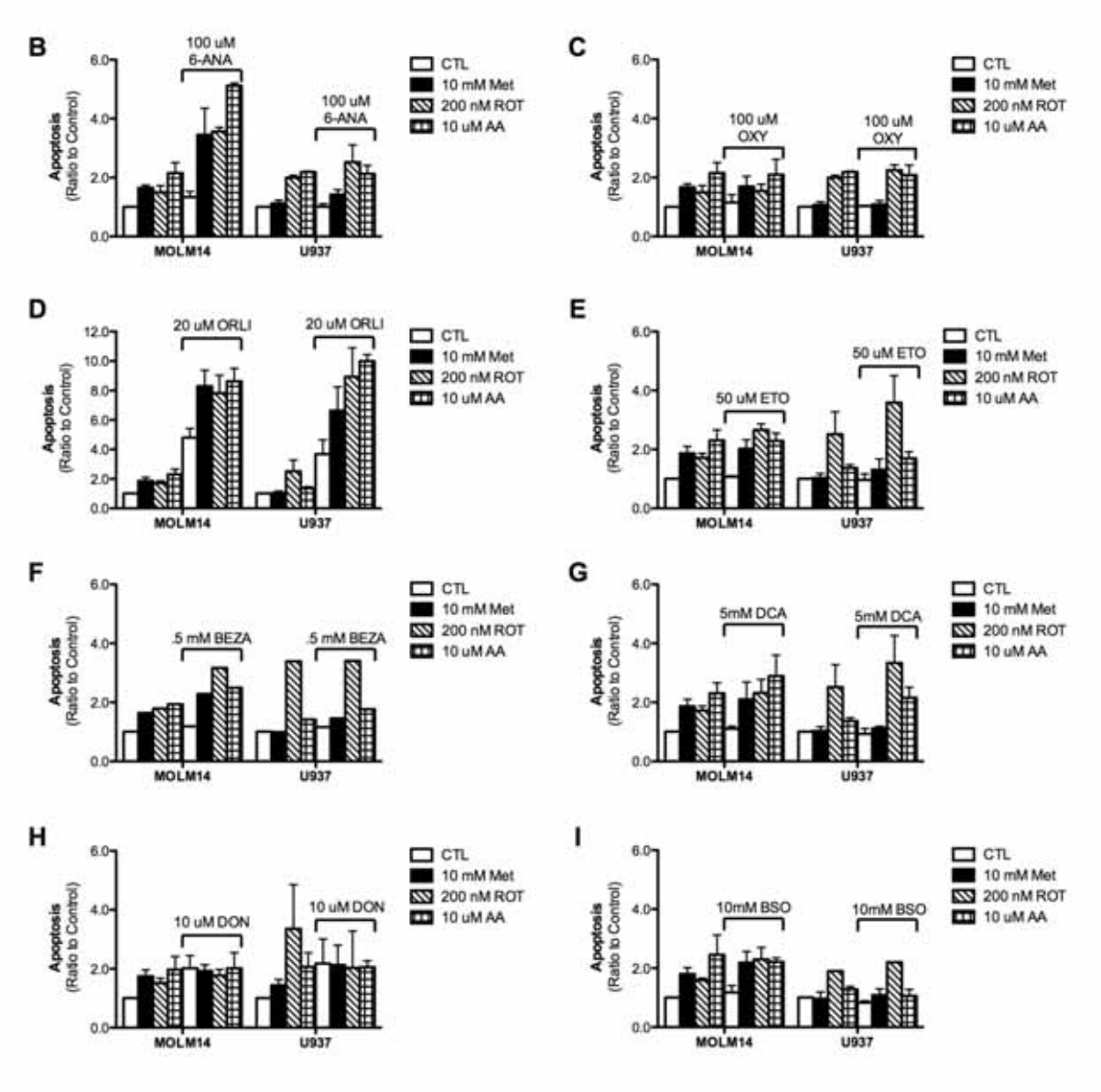



**Figure 19. The apoptotic effect of the combination of metformin with other metabolic drugs.** Following the 4 hr pre-incubation with either control or (B) 100 uM 6-aminonicotinamide (6-ANA), (C) 100 uM oxythiamine (OXY), (D) 20 uM orlistat (ORLI), (E) 50 uM etomoxir (ETO), (F) 5 mM dichloroacetate (DCA), (G) 10 uM 6-diazo-5-oxo-L-norleucine (DON), (H) 10 mM buthionine sulfoxide (BSO), (I) 0.5 mM bezafibrate (BEZA), U937 and MOLM14 cells were then incubated for 24 hrs with control, 10 mM metformin, 200 nM rotenone or 10 uM antimycin A and then analyzed for apoptosis, measured as AnnexinV-positive cells and presented as ratio to control.

# – Discussion and Conclusions –

# VI Discussion and conclusions

## 1 Anti-tumoral activities of metformin in human leukemic cells

While metformin inhibited cell cycle progression, cell proliferation and CFU-L activities in all leukemic cells, metformin induced apoptosis only in certain cell types. Furthermore, daily treatment of xenograft nude mice with metformin induced apoptosis and decreased tumor growth *in vivo*. While we observed a transient activation of AMPK at the Thr172 site in all leukemic cell lines in response to metformin, we determined with siRNA of AMPK$\alpha$1/2 that the anti-proliferative and pro-apoptotic effects of metformin in AML are not mediated by activation of AMPK, which is consistent with recent studies (Ben Sahra et al. 2008; Kalender et al. 2010; Ben Sahra et al. 2011; Tomic et al. 2011). However, we did observe an AMPK-independent decrease in mTORC1 activity, which correlates to the decrease in cell proliferation and the block in cell cycle in all AML cells. We also observed a decrease in protein expression of anti-apoptotic protein, Mcl-1, in all AML cells. This is especially interesting, because previous reports have demonstrated that decreases in mTORC1 activity can induce a decrease in Mcl-1 through translational inhibition (Mills et al. 2008; Zang et al. 2012). Additionally, inhibition of PI3K, and potentially a decrease in the AKT-mTOR axis, leads to Mcl-1 suppression and subsequent apoptosis (Thomas 2013, in revision). Interestingly, while mTORC1 inhibition clearly correlates to anti-proliferative activities of metformin, the decrease in Mcl-1 could integrate both anti-proliferative and pro-apoptotic activities of metformin in one mechanism of action. Additionally, the fact that the decrease in Mcl-1 also occurs in cytotoxically-insensitive U937 cells may suggest that the block of apoptosis in U937 cells may occur downstream of Mcl-1 inhibition. Currently, we are in the process of investigating potential mechanisms by which metformin could inhibit mTORC1, such as activation of REDD1, previously established in prostate cancer cell lines (Ben Sahra et al. 2011), the inhibition of Rag GTPase activity in response to metformin or phenformin (Kalender et al. 2010), and the effect of cyclic AMP, which can be diminished by the inhibition of adenylate cyclase and PKA through the metformin-induced accumulation of AMP and related nucleotides (Miller et al. 2013) and could potentially affect mTORC1.

## 2 Effects of metformin on mitochondrial function and intermediate metabolic pathways in human leukemic cells

To understand metformin's apoptotic mechanism, and more specifically the cytotoxic insensitivity in U937 cells, we analyzed mitochondrial function and global metabolism in three different (sensitive, HL60 and MOLM14 *vs*. insensitive, U937) leukemic cell lines. We demonstrated

that metformin, similar to rotenone or antimycin A, inhibited both electron transport chain complex I (ETCI) and oxygen consumption and shifted energetic ATP production from oxidative phosphorylation to glycolysis, but without affecting global ATP content in all leukemic cell types. Furthermore, metformin induced a common metabolic adaptation to its early inhibitory effects on mitochondrial energetics with increased glucose uptake and consumption as well as a redirection of carbon flux towards lactate production, the pentose phosphate pathway (PPP) and purine and pyrimidine biosynthesis. However, despite increased flux through the PPP, we observed an increase in purine and pyrimidine precursor metabolites (PRPP, AMP, IMP, GMP), suggesting a block in DNA synthesis. Not only does this correlate to the decrease in cell cycle, but it may also occur as a result of mTORC1 inhibition. Ben-Sahra et al. (2013) recently published a novel downstream metabolic pathway of mTORC1, *via* downstream targeting of S6 kinase 1, which directly phosphorylates S1859 on CAD (carbamoyl-phosphate synthetase 2, aspartate transcarbamoylase, dihydroorotase). CAD is a tri-functional enzyme that catalyzes and post-translationally regulates the first three steps of *de novo* pyrimidine synthesis and thereby regulates DNA synthesis and potentially the cell cycle (Ben-Sahra et al. 2013). Furthermore, metformin increased mitochondrial and anaplerotic reactions (eg. fatty acid shuttling and oxidation, glutamine consumption and glutaminolysis) in all leukemic cells. However, between the two cells lines, metformin had the opposite effect on TCA cycle intermediates (α-ketoglutarate, fumarate, malate) and PDH phosphorylation, confirming the importance of mitochondria on both basal energetic status and metformin sensitivity. Accordingly, metformin-insensitive U937 cells exhibited lower basal OCR and are less oxidative and more glycolytic under basal conditions, thereby requiring a smaller metabolic response (e.g. Pasteur effect) following ETC1 inhibition by metformin.

The basal metabolome also demonstrated significantly reduced carbon metabolites in the lower segment of glycolysis (Gly-3P to PEP) of U937 compared to MOLM14. This is consistent with our thermodynamic analysis showing FBP formation by PFK1 is strongly favored in U937 and rapidly consumed in the lower segment of glycolysis as intermediates are withdrawn for biosynthetic reactions. In addition to being converted to lactate, glycolytic intermediates in U937 might be diverted to increase flux through the PPP, to operate the glycerol-3-phosphate shuttle (dihydroxyacetone phosphate) and/or to support glycerol and serine metabolism for biosynthetic purposes, including amino acids and triglycerides. Increased flow through the PPP increases production of purines and NADPH to maintain high GSH levels, which provides high antioxidant capacity and links energetic reprogramming and redox balance in cancer cells (Anastasiou et al. 2011; Hamanaka and Chandel 2011). Rerouting glucose flux from glycolysis to the PPP does not limit substrate availability in U937 because they sustain survival by alternative, non-glycolytic pathways and substrates (eg. glutamine or fatty acids) as supported by the marked basal accumulation of carnitine and acetylcarnitine. Accordingly, and as recently discovered in other leukemic cells (Hitosugi et al. 2009), we observed significant phosphorylation of mitochondrial PDH in both U937 and MOLM14, which suggests

mitochondrial acetyl-CoA is derived from glutaminolysis (MOLM14) or glutaminolysis and fatty acid oxidation (U937, $PTEN^{NULL}$ AKT constitutively activated cells) rather than pyruvate oxidation. Additionally, accumulation of fumarate and malate in MOLM14 following metformin also suggests these cells might preferentially use glutamine-derived reductive carboxylation of α-KG to produce acetyl-CoA for four carbon intermediates for the TCA cycle, fatty acids, lipids, sterols and lactic acid, as very recently observed in cancer cells upon mitochondrial mutation, acute pharmacological ETC inhibition or hypoxia (Wise et al. 2011; Metallo et al. 2012; Mullen et al. 2012). This is also consistent with the high metformin sensitivity observed in c-Myc-driven glutamine-addicted cells, such as HL60 (Javeshghani et al. 2012).

## 3 The metabolic and anti-tumoral effects of metformin can be augmented by other metabolic inhibitors

In accord with our metabolic signature (eg. metformin increases flux through the PPP), we observed a significant synergistic effect of metformin in combination with 6-ANA, an inhibitor of the oxidative branch of the PPP (glucose-6-phosphate dehydrogenase inhibitor) and especially in MOLM14 cells. This glucose diversion away from glycolysis could occur as a mechanism to increase antioxidant production or to respond to the decrease in cell cycle by promoting nucleotide biosynthesis. One mechanism through which this could occur is activation of NOXA, a pro-apoptotic, BH3-only protein, that can be activated by DNA damage, hypoxia and proteasome inhibition to increase glucose consumption and PPP flux (Lowman et al. 2010). Thus, it is possible that inhibition of the oxidative branch of the PPP with 6-ANA significantly eliminates a metformin response tool, leading to cell death. Additionally, we did not observe a significant induction of apoptosis from oxythiamine, an inhibitor of the non-oxidative branch of the PPP (transketolase inhibitor), suggesting the absence of re-entry of pentose into the glycolytic flux *via* glyceraldehyde-3-P and fructose-6-P, otherwise known as the Embden-Meyerhof pathway.

Metformin was also extremely effective in combination with orlistat, which is an inhibitor of fatty acid synthesis (inhibitor of the thioesterase domain of fatty acid synthase) and fatty acid/triglyceride storage (lipase inhibitor) but not etomoxir, which is an inhibitor of fatty acid oxidation. Unlike 6-ANA, orlistat also induced a significant level of apoptosis alone in MOLM14 and U937 cells, indicating a dependency of leukemic cells on lipogenesis to support their proliferation in the basal status (Samudio et al. 2010). The fact that etomoxir did not significantly impact apoptosis alone or with metformin was especially interesting considering we observe an increase in FAO, as evidenced by decreased carnitine and increased acetylcarnitine, in both MOLM14 and U937 cells in response to metformin. This may indicate that etomoxir is not fully effective in our cells and requires further investigation to determine the true FAO status of our cells.

Finally, and of prime importance, we confirmed that metformin-insensitive U937 cells have high basal pAKT due to PTEN deletion that renders cells constitutively active in the PI3K/AKT pathway. This activation plays a pivotal role in metformin resistance, especially during early stages of treatment. Increased pAKT might also be induced in sensitive cells following metformin treatment as an adaptive mechanism to early AMPK activation and mitochondrial inhibition. As a key regulator of glucose metabolism, AKT increases translocation of glucose transporters, GLUT1 and GLUT4, to the plasma membrane to increase glucose uptake and aerobic glycolysis (Kohn et al. 1996; Barthel et al. 1999; Wieman et al. 2007). This confers resistance to the Pasteur effect elicited by metformin in U937 cells, but renders them susceptible to death following glucose withdrawal or glycolytic inhibitors (Buzzai et al. 2005; Ben Sahra et al. 2010). AKT also favors hexokinase-II activity and its association with the outer mitochondrial membrane, thereby increasing ATP affinity and providing direct access to mitochondrial ATP (Mathupala et al. 2009). Additionally, hexokinase-II binds specifically to the mitochondrial voltage-dependent anion channel (VDAC1), preventing opening of the mitochondrial permeabilization transition pore, cytochrome c release and subsequent apoptosis (Majewski et al. 2004a; Arzoine et al. 2009). As we demonstrated that metformin decreases the level of anti-apoptotic protein, Mcl-1, in all AML cells, a blockage of VDAC1 by AKT-induced HK-II binding is a reasonable mechanism of apoptotic resistance in U937 cells. While we have performed preliminary co-immunoprecipitation of HK-II and VDAC1, we have not yet perfected this experiment and therefore cannot conclude on this point. However, metformin-mediated apoptosis was induced in U937 and augmented in MOLM14 by metabolic manipulations with AKT and glycolytic inhibitors and glucose starvation. These results prove that the basal AKT status of leukemic cells is crucial in determining the ability to elicit a Pasteur effect and to regulate apoptosis in response to metformin.

Overall, performing studies with metabolic drugs, such as metformin, DXG, 6-ANA and orlistat, allows us to begin to truly understand leukemic cell metabolism and to better target this cancer feature in AML. First, metabolic drugs help us to better characterize cancer cell metabolism both in the basal state, but also after inhibition of metabolic pathways, such as inhibition of ETCI with metformin in human AML. Whereas the therapeutic use of one metabolic drug, such as metformin, may induce only a small anti-tumoral effect, the downstream changes in metabolism may provide an Achilles' heel that, when targeted in combination with metformin, could have an extremely synergistic effect against leukemic cells, including stem cells. It is for this reason that we must continue analyzing both anti-tumoral and downstream metabolic impacts of metabolic drugs and the energetic status of leukemic cells in order to better understand how to best target and treat leukemia.

# 4 Conclusions

From this metformin study, and as represented in Figure 20, we propose that metformin induces an early (within 3 hours) effect on mitochondrial energetics leading to long-term (6-24 hours) metabolic adaptations (common- and sensitive- cell specific) followed by an extremely crucial inhibition of mTORC1 and subsequent decreases in cell proliferation, cell cycle progression and Mcl-1 protein levels and, finally, an induction of the caspase-3-dependent mitochondrial apoptotic pathway (24-48 hours) in leukemic cells. However, the late specific metabolic adaptation (strong induction of the Pasteur effect) and apoptosis do not occur in U937 cells, which already exhibit high basal glucose consumption, glycolysis and AKT activation and are thus metabolically poised to withstand inhibition of ETCI by metformin. Specifically, these cells may have high HK-II phosphorylation and binding to VDAC1, due to constitutive activation of AKT, which blocks the release of cytochrome c from the mitochondria and prevents downstream apoptosis. Taken together, our study allows for a more general conclusion: metabolic fluxes of leukemic cells for energy production and generation of biosynthetic precursors are important determinants in metformin sensitivity. Our findings concerning the integration and regulation of energetic and metabolic crosstalks between mitochondria and cytosol, and other recent discoveries of cancer-related changes in metabolic pathways (Frezza et al. 2011; Possemato et al. 2011; Metallo et al. 2012; Mullen et al. 2012; Zhang and Du 2012), demonstrate that metabolic and energetic flexibility are a common feature of tumor cell metabolism. A deeper understanding of this intrinsic capacity and its targeting are crucial steps to establish new therapeutic strategies in oncology.

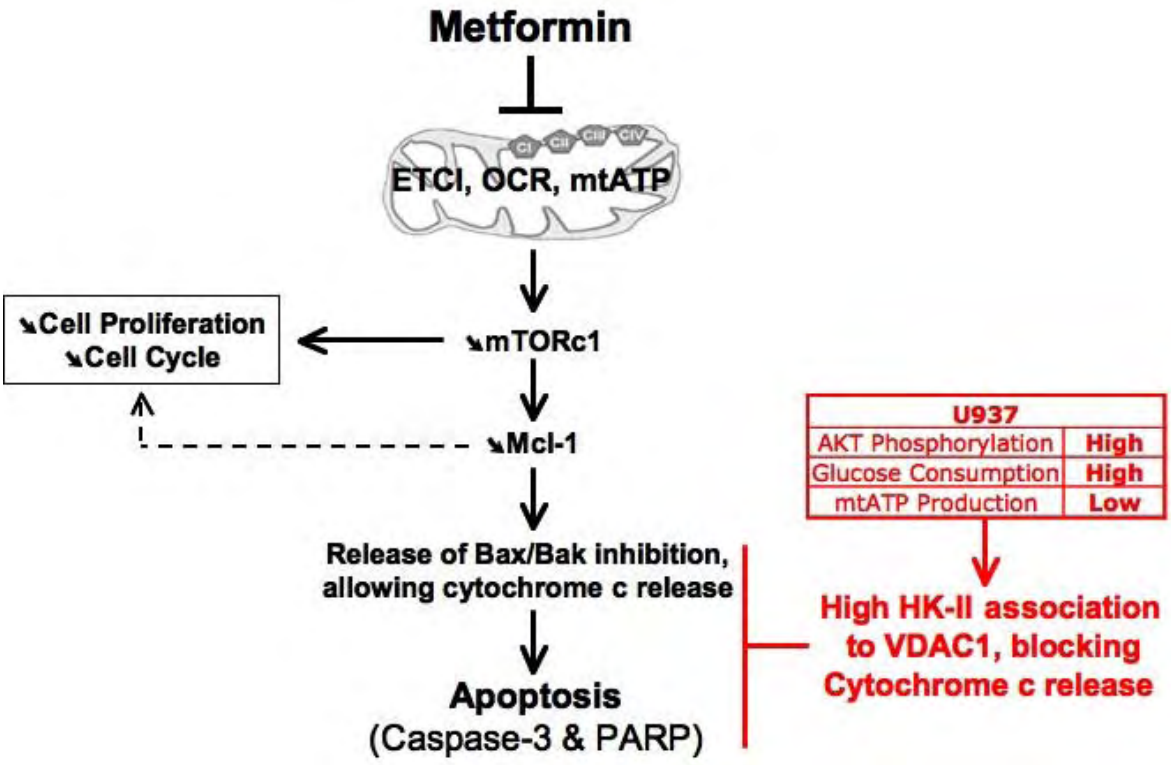



**Figure 20. Working model of the multi-faceted mechanism of action of metformin in leukemic cells.** *In vitro*, metformin induces early energetic changes through the inhibition of electron transport chain complex I (ETCI), mitochondrial oxygen consumption (OCR) and mitochondrial ATP production (mtATP). The resulting metabolic response leads to an inhibition of mTORC1, leading to decreases in cell proliferation the cell cycle and Mcl-1 expression. Decreased Mcl-1 activity releases the inhibition of pro-apoptotic factors, Bax and Bak, which can then signal cytochrome c release and downstream activation of caspase-3 and PARP to induce mitochondrial-dependent apoptosis. Cells that are insensitive to metformin-induced apoptosis, like U937, have high basal AKT phosphorylation and glucose consumption as well as low mitochondrial ATP production, which leads to high hexokinase-II (HK-II) association to VDAC1, thereby blocking cytochrome c release and downstream apoptosis.

# – Future Directions –

# VII Future directions

## 1 Going deeper into metformin's mechanism of action *in vitro*

### 1.1 Role of mTORC1 inhibition in the anti-tumoral effects of metformin

While we have established many aspects of the molecular mechanism of action of metformin in AML, there are several questions that remain unanswered. First, it is clear that metformin inhibits mTORC1 in all AML cells regardless of their energetic and PI3K/AKT status. However, while literature has previously proven the link between mTORC1 inhibition and decreases in cell proliferation, cell cycle and Mcl-1, which can also lead to apoptosis, we have not yet proven that mTORC1 inhibition is the cause of these anti-tumoral effects in AML. However, we have established a number of correlations. For example, in response to metformin, we have observed increases in purine and pyrimidine precursor metabolites, which correlates to the cell cycle arrest as these metabolites are precursors for DNA synthesis and are evidently not being consumed by this process. Additionally, recent papers have formed a correlation between a block in *de novo* pyrimidine biosynthesis and inhibition of mTORC1 (Ben-Sahra et al. 2013; Robitaille et al. 2013). The inhibition of mTORC1 and the subsequent decrease in phosphorylated S6 kinase 1, which we observe in response to metformin, decreases its phosphorylation of S1859 on CAD, the enzyme that catalyzes the first three steps of *de novo* pyrimidine synthesis, to post-translationally decrease *de novo* pyrimidine synthesis.

To directly correlate mTORC1 inhibition to anti-tumoral effects, we could manipulate this inhibition either directly or through upstream and downstream targets. While it would be difficult to perform siRNA of mTORC1 to potentially abrogate these effects, as this may induce too much cell death, we could attempt to over-express mTORC1 activity to rescue these effects. We could also inhibit TSC1/2 with siRNA to prevent its ability to inhibit mTORC1. However, we will need to consider that there are TSC1/2-independent mechanisms to inhibit mTORC1, such as Rag GTPase (Kalender et al. 2010), which could limit the conclusiveness of this experiment. Additionally, it is possible that overexpression of AKT with myrAKT transfections in either HL60 or MOLM14 could inhibit mitochondrial-dependent apoptosis, as observed in U937 cells.

To further understand mTORC1's role in anti-tumoral effects of metformin we must also identify the mechanism by which mTORC1 is inhibited in AML cells, as our data has previously demonstrated that the effect is AMPK-independent. Several alternative AMPK-independent mechanisms of mTORC1 repression have been considered in the literature (Kalender et al. 2010; Ben Sahra et al. 2011; Miller et al. 2013). In particular, as increased expression of REDD1 has been identified as a mechanism in which metformin inhibits mTORC1 activity (Ben Sahra et al. 2011) we

analyzed REDD1 protein expression in HL60, MOLM14 and U937 cells. In all three cell lines, we observed a significant increase in REDD1 after 24 hours treatment with 10 mM metformin (Figure 21A). Despite this strong correlation with decreased mTORC1 activity, siRNA of REDD1 did not provide an obvious conclusion on the involvement of REDD1 in the anti-tumoral effects of metformin (Figure 21B-D). In a 24-hour western blot, we found that siREDD1, as compared to siRNA control, only significantly blocked REDD1 activation in HL60 cells (Figure 21B). Thus, only HL60 can provide any potential conclusions on the role of REDD1 in metformin's mechanism of action. Overall, siREDD1 did not significantly rescue the inhibition of S6 phosphorylation, Mcl-1 protein levels or cell proliferation in response to metformin in HL60 cells (Figure 21B-D), suggesting that HL60 cells may depend on another mechanism than REDD1 for inhibition of mTORC1.

U937 cells, on the other hand, exhibited a higher than normal activation of REDD1 in transfected cells, as compared to non-transfected cells, suggesting there might be an element of stress that induces REDD1 through transfection (Figure 21B). Additionally, while metformin inhibited pS6, Mcl-1 and cell proliferation in siCtl cells, this effect did not correlate to activation of REDD1, as we did not observe a strong metformin-induced increase in REDD1 in siCtl cells. This may suggest that REDD1 is not responsible for metformin activities in U937. Additionally, U937 cells pretreated with siREDD1 and without metformin exhibited a significant decrease in pS6, Mcl-1 and cell proliferation compared to the U937 siCtl untreated cells. This is particularly interesting because we did not observe the same inhibitory effects with siAMPK, which suggests it is specific to siREDD1 rather than overall translation. We propose that this is correlated to the fact that we see increased REDD1 expression in siCtl cells, compared to previously non-transfected cells, suggesting that inhibiting REDD1 with siRNA may remove the mechanism of stress-response to the transfection process.

Before we can conclude on the role of REDD1 in metformin's anti-tumoral effects, there are multiple technical issues that need to be addressed. First, the transfection process appears to be very taxing for AML cells, especially U937, and can actually change the response of cells to metformin in the siCtl as compared to non-transfected cells. Additionally, it appears that siREDD1 does not inhibit the basal level of REDD1 in MOLM14 or HL60 and only inhibits the activation of REDD1 following metformin treatment in HL60 cells. We might also speculate on the dependency or activation of REDD2 compensating for the loss of REDD1 in our transfected cells, which could account for the difference. Currently, we are also investigating the role of other potential mechanisms by which metformin could inhibit mTORC1 in AML cells, such as the effect of cyclic AMP, which can be diminished by increases in AMP leading to downstream changes in metabolism (Miller et al. 2013) that could potentially affect mTORC1. We will also investigate the inhibition of Rag-GTPase activity as a mechanism to inhibit mTORC1, which has been previously observed in response to metformin or phenformin (Kalender et al. 2010). In line with this mechanism, we will determine the role of glutaminolysis on mTORC1 activity as a recent work demonstrated that the inhibition of glutaminolysis prevented GTP loading of RagB and lysosomal translocation, leading to mTORC1

inhibition (Duran et al. 2012). This mechanism is particularly interesting as c-Myc cells are addicted to glutamine and are particularly sensitive to metformin, which could suggest an inhibition of glutaminolysis in the anti-tumoral effects of metformin (Javeshghani et al. 2012). However, we observe increased glutamine consumption and glutaminolytic gene expression in response to metformin in all AML cells, which contradicts this hypothesis. Thus, this mechanism requires further investigation before a final conclusion can be made.

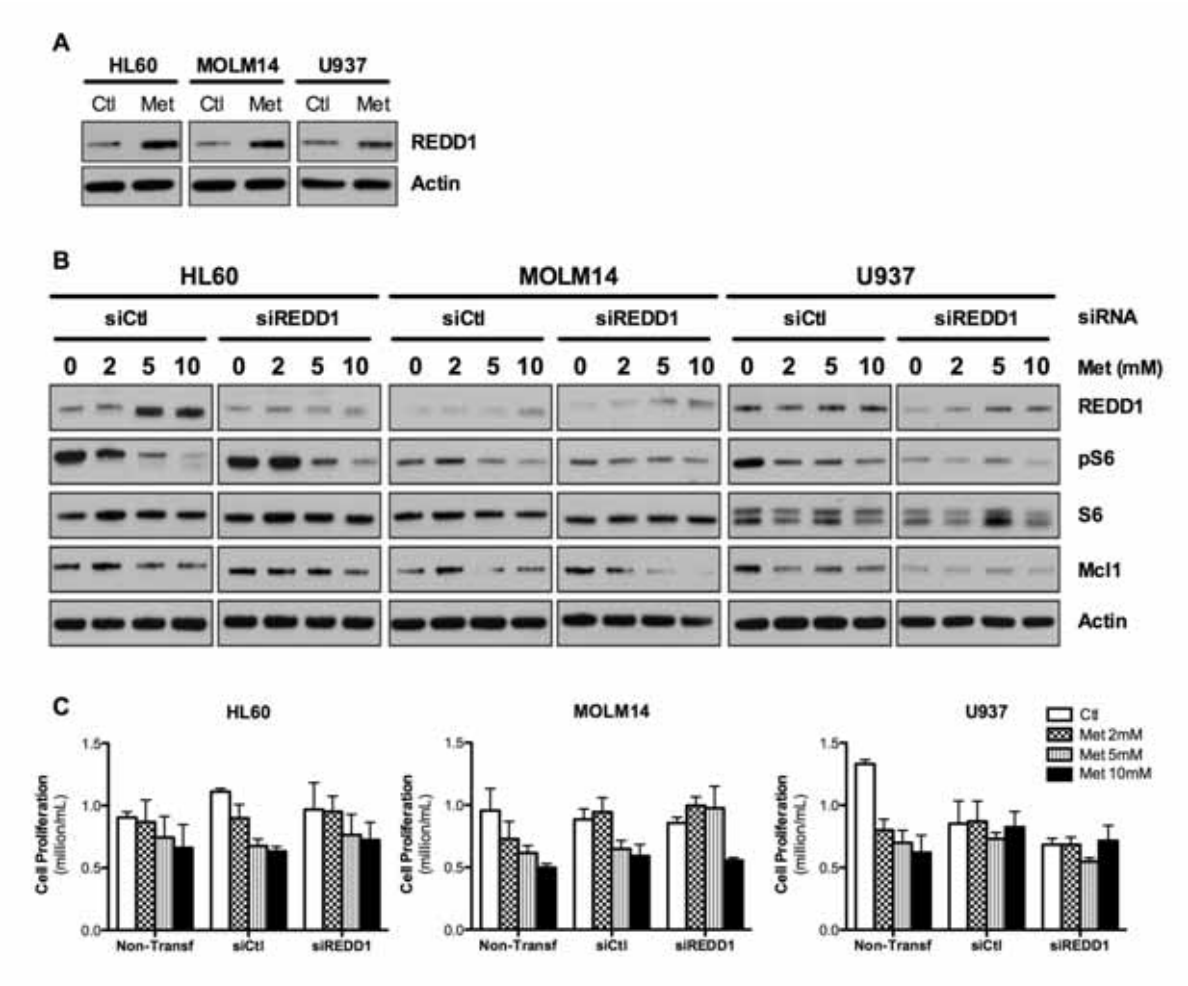



**Figure 21. Metformin increases REDD1 expression in AML cells, but siRNA to elucidate the role of REDD1 in metformin's anti-tumoral effects is not conclusive.** (A) Western blot of REDD1 in HL60, MOLM14 and U937 cells after a 24 hr treatment with either control or 10 mM metformin. (B) Western blot analysis in HL60, MOLM14 and U937 cells after an initial 24 hr treatment with either siRNA control or siRNA REDD1 followed by a 24 hr treatment with either control or 10 mM metformin. Western blots were analyzed with primary antibodies for REDD1, pS6, S6, p4EBP1, 4EBP1 and Mcl-1, all normalized to β-actin. Following another 24 hrs control or 10 mM metformin treatment of the cells in (B), these cells were analyzed for (C) cell proliferation, calculated by ratio of final to initial cell count (with trypan blue exclusion).

## 1.2 The role of reactive oxygen species, mitochondrial bioenergetics and the mitochondrial permeability transition pore in response to metformin

Regardless of whether or not REDD1 is directly involved in the inhibition of mTORC1 and downstream proliferation or apoptosis, determining the mechanism by which REDD1 is activated in our cells could help us understand the complete mechanism of action of metformin. In multiple cell types, REDD1 can be activated downstream of endoplasmic reticulum stress, reactive oxygen species, energy depletion, hypoxia/HIF and DNA damage (Ellisen et al. 2002; Brugarolas et al. 2004; Sofer et al. 2005; Jin et al. 2009; Regazzetti et al. 2010; Kimball and Jefferson 2012). To address activation of REDD1 in our cells, we have begun by performing analysis of the endoplasmic reticulum stress response, which can lead to unfolded proteins and the unfolded protein response (UPR). However, we analyzed XBP-1 splicing (activated during UPR) and found no differences, treated or not with metformin, between the AML cell lines. In each case, no XBP-1 splicing was detectable (data not shown). This demonstrates that, in these cells, neither UPR induction nor variation occurred in response to metformin, suggesting that UPR is not involved in the inhibition of cell proliferation.

Additionally, we have considered that activation of REDD1 and/or downstream effects could be due to ETC1 inhibition by metformin, which could produce changes in ROS, energy status, hypoxia and DNA damage. We have already demonstrated that metformin's inhibition of ETCI induces a decrease in mitochondrial ATP production. While global ATP production does not change, due to a responsive increase in glycolytic ATP production, it is possible that the compartmentalized decrease of mitochondrial ATP production could elicit downstream changes in REDD1. Thus, we will induce a Rho0 condition in our cells, in which mitochondria will be depleted with ethidium bromide, to determine the overall role of ETCI inhibition and attempt to rescue the anti-tumoral effects of metformin. This will be particularly interesting for confirming mitochondrial-dependent apoptosis.

We have also considered the possibility that inhibition of ETCI could lead to an activation of HIF-1α through a number of mechanisms. First, we considered that a decrease in the TCA cycle and subsequent accumulation of fumarate and succinate could block the degradation of HIF-1α by inhibition of prolyl hydroxylases. While our metabolite data does indeed suggest an increase in fumarate in MOLM14 cells in response to metformin, we had the opposite result in U937 cells. Thus, this does not provide a clear response. Additionally, HIF-1α can be activated downstream of ataxia-telangiectasia mutated (ATM) activation, which occurs as a result of hypoxia and in coordination with increases in ROS and decreases in oxidative phosphorylation. ATM is a serine/threonine protein kinase that is recruited and activated by DNA double-strand breaks and often mutated in cancer cells. It phosphorylates several key proteins that initiate activation of the DNA damage checkpoint, leading to cell cycle arrest, DNA repair or apoptosis. Several of these targets, including p53, CHK1/2 and γH2AX, are tumor suppressors. Very recently, two studies have proposed new functions of ATM on

the regulation of mitochondrial oxidative phosphorylation (Zakikhani et al. 2012; Ambrose and Gatti 2013). Additionally, ATM activation can also lead to downstream activation of REDD1 and a decrease in mTORC1, which we observe in response to metformin in all three AML cell lines tested (Cam et al. 2010; Kim et al. 2010).

To analyze these potential mechanisms, we measured the levels of ROS in our cells at various time points following metformin treatment. We did observe a slight increase of total ROS (DCF assay) at 3 hours in HL60 cells (data not shown) and a significant increase of mitochondrial ROS (DHE assay) at 24 hours in MOLM14 and U937 cells (Figure 22A). In fact, the induction of mitochondrial ROS by metformin was as strong as our positive control, rotenone, which is a more potent complex I inhibitor. This is particularly interesting, because metformin's impact on ROS has been highly debated in the literature. Many groups studying non-cancerous cells of patients with type 2 diabetes have demonstrated that metformin decreased mitochondrial ROS, potentially through decreased oxygen consumption and/or increased PPP-related NADPH and antioxidant formation (Leverve et al. 2003; Fujisawa et al. 2009; Piwkowska et al. 2010). Our data is also contrary to published data that has suggested that metformin, unlike rotenone, did not increase ROS production because it blocked the reverse (succinate) electron flow in rat liver and skeletal muscle cells (Batandier et al. 2006; Kane et al. 2010). However, our data is in line with previous literature demonstrating that metformin increases ROS in several cancer cells in parallel with anti-tumoral activity (Anedda et al. 2008). Additionally, our data suggests that metformin is inducing ROS at the same level as rotenone, a drug which has been previously observed to induce mitochondrial-dependent apoptosis and sensitize leukemic cells, including HL60, to anti-cancer agents (Li et al. 2003; Pelicano et al. 2003). These effects were inhibited by antioxidants, demonstrating a causative link between ROS and anti-tumoral effects (Li et al. 2003). Similar to other anti-tumoral mechanisms of metformin, it is likely that the effect on ROS could be cell- and cancer-dependent, which could help explain the controversy in the literature. In the future, it will be necessary to simultaneously analyze mitochondrial and total ROS levels at other time points to determine if the ROS induction in MOLM14 or U937 cells occurs later than 3 hours and can be implicated in cellular signaling changes leading to the anti-tumoral effects of metformin.

We have also analyzed the level of γH2AX staining, as a readout for DNA damage and ATM induction, in U937 and MOLM14 cells following a 24-hour treatment with metformin, rotenone or chemotherapy agent, AraC, as a positive control (Figure 22B). We found that metformin induced a slight increase in γH2AX staining, which was comparable to the effect of rotenone, but low compared to the level induced by AraC. However, the fact that there is a slight increase could suggest that ATM, and potentially REDD1, could be activated in our cells through a DNA damage-dependent mechanism (Cam et al. 2010; Kim et al. 2010). In the future, we will analyze γH2AX staining following a longer incubation with metformin as the effect may be delayed. Additionally, we will

evaluate both basal and metformin-induced expression of ATM, p-ATM, CHK1/2, p-CHK1/2, HIF-1α and CAIX, the latter of which will indicate hypoxia levels in our cells. One issue to remember is that mTORC1 inhibition can then have negative feedback on HIF-1α through translation so a kinetics analysis will be required.

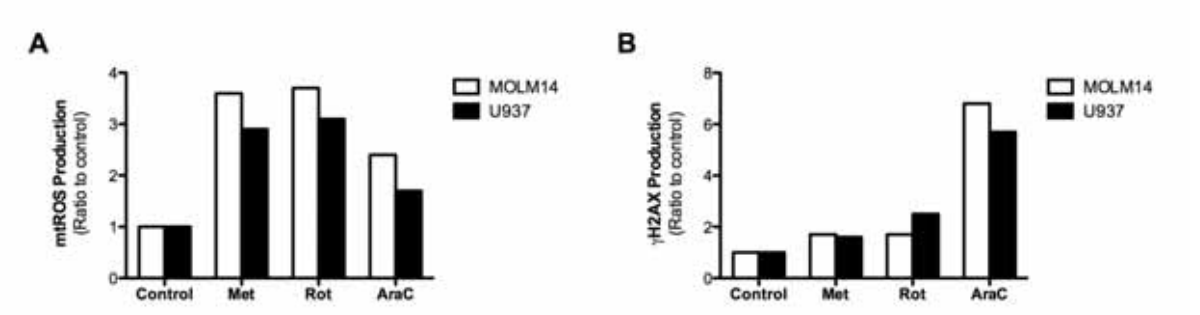



**Figure 22. Metformin induces mitochondrial ROS and DNA damage in U937 and MOLM14.**
(A) Mitochondrial ROS production and (B) γH2AX staining, as a readout for DNA damage, both represented as a ratio to control in U937 and MOLM14 cells following a 24 hr incubation with control, 10 mM metformin (Met), 200 nM rotenone (ROT) and 2 uM cytarabine (AraC).

It will also be essential to further elucidate metformin's mechanism of action in apoptosis. We have identified a decrease in anti-apoptotic protein, Mcl-1, as a potential molecular target by which metformin offsets the balance between pro- and anti-apoptotic proteins in the pro direction. Interestingly, it is possible that this decrease comes in response to decreases in mTORC1 activity and subsequent decreases in specific translation, as previously described (Mills et al. 2008; Zang et al. 2012). However, the fact that the decrease in Mcl-1 also occurs in cytotoxically-insensitive U937 cells suggests a number of possible conclusions that need to be addressed. First, it is possible that the decrease in Mcl-1 is not responsible for downstream apoptosis or that other anti-apoptotic factors could override this inhibition in U937 cells, which express high AKT activation leading to high survival capacity. For example, as the decrease in Mcl-1 leads to increased association of Bak with VDAC and subsequent apoptosis, it is possible that low Bak activity could be responsible for insensitivity to metformin-induced Mcl-1 suppression (Thomas 2013 In revision) although we did not observe any difference in Bak expression in our three cell lines. Another mechanism of apoptosis induction that we will investigate involves the pro-apoptotic, BH3-only protein, NOXA, which can be induced by DNA damage, hypoxia and proteasome inhibition to induce apoptosis in human cells (Lowman et al. 2010). NOXA is particularly interesting in hematopoietic cells as it is constitutively expressed in proliferating cells and required for apoptosis in response to glucose stress or the loss of Cdk5. NOXA helps cells respond to stress by increasing glucose consumption and flux through the PPP to reduce ROS and DNA damage, which is a metabolic shift that we have observed in our 3 AML cell lines. When this response is not enough to compensate the apoptotic stimuli or metformin's glucose consumption leads to glucose stress, NOXA could induce apoptosis in response to metformin

(Lowman et al. 2010). Thus, NOXA activity is a potential apoptotic mechanism of action of metformin, but requires further investigation.

Additionally, and in AML specifically, high Bcl-2 activity is linked to drug resistance and poor prognosis (Moon et al. 2010; Cluzeau et al. 2012). A very recent publication demonstrated that leukemic stem cells (LSCs) exhibit relatively low levels of ROS, which is correlated to significantly high levels of Bcl-2. Additionally, Bcl-2 inhibition in these cells not only eradicated quiescent LSCs, but also reduced oxidative phosphorylation, suggesting a link between LSC dependence on Bcl-2 and its promotion of oxidative phosphorylation (Lagadinou et al. 2013). Additional groups have demonstrated that MM, AML and ALL patients exhibit a strong correlation between chemotherapeutic response and mitochondrial "priming," which refers to the level of anti-apoptotic members of the Bcl-2 family and their ability to prevent a mitochondrial outer membrane permeabilization (MOMP) event in response to the addition of pro-apoptotic peptides (Ni Chonghaile et al. 2011; Vo et al. 2012). They demonstrated that patients with highly primed cancers, which exhibited a strong MOMP in response to pro-apoptotic peptides, respond better to chemotherapy than cancers and normal tissues that are poorly primed. Thus, the level of anti-apoptotic Bcl-2 family proteins could explain not only chemoresistance, but provides an explanation for the fact that normal cells do not respond to cytotoxic chemotherapy agents, which target all nucleated human cells (Ni Chonghaile et al. 2011; Vo et al. 2012). Additionally, this data increases the potential of anti-apoptotic proteins as a therapeutic target in cancer treatments. Thus, we will test the mitochondrial priming of our AML cell lines to determine if this basal condition could determine the response to metformin and thus provide an additional target to test in combination with metformin.

Based on our work, however, our current hypothesis is that U937 cells could have a block in apoptosis downstream of the decrease in Mcl-1, such as activity at the mitochondria permeability transition pore (MPTP complex including several core proteins eg. VDAC-1, ANT, cyclophilin D, and their interaction with pro- and anti-apoptotic proteins), which is the site of cytochrome c release and subsequent caspase-dependent apoptotic induction. Hyperactivation of AKT, which we observe in cytotoxically-insensitive U937 cells, activates and phosphorylates HK-II, driving its association with VDAC (Robey and Hay 2005; Robey and Hay 2006). When HK-II is bound to VDAC, it competes with members of the anti-apoptotic protein family, such as Bax and Bak, thereby preventing cytochrome c release, activation of downstream caspases and subsequent apoptosis (Danial et al. 2003; Pastorino and Hoek 2008; Pedersen 2008; Tagscherer et al. 2008). Additionally, it appears that overexpression of HK together with GLUT1 can phosphorylate and inhibit GSK3 through increased glycolysis (Zhao et al. 2007). Inhibition of GSK3 is key because this protein has been reported to directly phosphorylate VDAC and inhibit it's binding to HK (Pastorino et al. 2005), but also because GSK3 can phosphorylate and destabilize anti-apoptotic protein, Mcl-1 (Maurer et al. 2006). Interestingly, we have also shown that U937 has high phosphorylation, and subsequent inhibition, of GSK3β (Ser9), which could also be linked to this anti-apoptotic behavior (Figure 23). The fact that

HL60 cells do not exhibit high GSK3β (Ser9) phosphorylation could also suggest that these cells are extremely sensitive to metformin and the decrease in Mcl-1 due to pre-destabilization of Mcl-1 by GSK3β activity. To better analyze this potential block at the VDAC-1 pore, we will perform co-immunoprecipitation studies of hexokinase-II and VDAC-1 to determine if the two are highly bound in U937 cells, as compared to MOLM14 or HL60 cells. In addition, we will perform mitochondrial separation assays to localize hexokinase-II, VDAC-1, cytochrome C as well as other anti- and pro-apoptotic factors in either the cytosol or mitochondria. Other aspects of the MPTP, such as Ant or cyclophilin, should be also investigated. Together, these experiments should help us elucidate the mechanism by which U937 blocks downstream apoptosis and provide a more specific mechanism in which HL60 and MOLM14 activate this pathway.

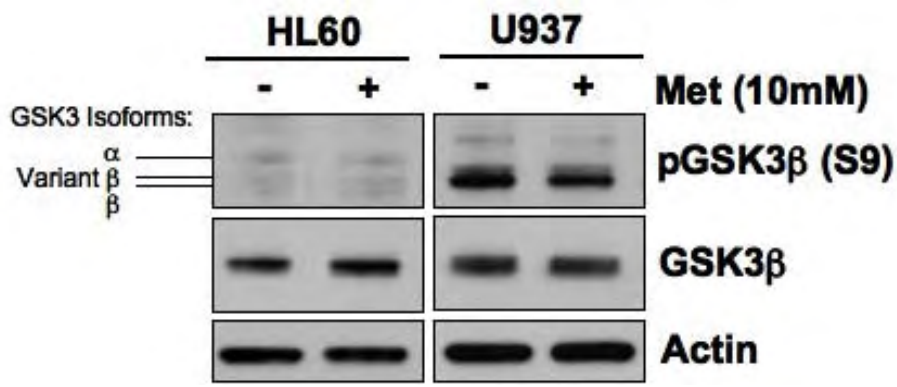



**Figure 23. GSK3-β activity in HL60 and U937 cells.** HL60 and U937 cells were treated for 24 hrs with control or 10 mM metformin. Western blots were then performed to analyze p-GSK3-β (Ser9) and total GSK3-β, as normalized to β-actin.

### 1.3 Analysis of metabolic and lipid fluxes in response to metformin

From our metabolic signature and inhibitors studies performed during this thesis, several points appear crucial – PPP, FA synthesis and recycling, the citrate cycle and the serine/glycine biosynthetic pathway. In light of our data on the synergistic combination of metformin and 6-aminonicotinamide *in vitro*, we will perform more in-depth analysis of the flux from glucose oxidation to the pentose phosphate pathway. These types of experiments will be especially interesting in the context of metformin, as we have previously demonstrated that metformin induces increased flux through the PPP to increase antioxidants to combat ROS and increase nucleotide biosynthesis.

We will perform similar analysis, as well as *in vivo* experimentation, of the combination of metformin and orlistat in MOLM14 and U937 cells, which was also synergistic *in vitro*. However, to determine the appropriate dose of orlistat and to determine which cells could be most responsive to this combination, it will be necessary to first determine the relative dependence of each cell line on either fatty acid synthesis or oxidation, both in the basal state and after treatment with metformin. We first performed a dose-response analysis of the cytotoxic response to 24 hours of orlistat treatment in

HL60, MOLM14 and U937 cells, as we expect that the comparative dose required for cytotoxicity should indicate the dependence on fatty acid synthesis (Figure 24). Interestingly, sensitivity to orlistat correlates with sensitivity to metformin, with the highest sensitivity in HL60 and MOLM14, as compared to U937 cells (Figure 24), and may also correlate to dependence on fatty acid synthesis as well. Next, we will perform lipidomic analysis to determine both basal and metformin-induced lipid metabolite levels in our cells. We will also analyze the accumulation of lipid droplets to further understand fatty acid and lipid storage, using Nile Red or BODIPY staining followed by analysis with confocal microscopy or flow cytometry. Finally, and specifically for fatty acid oxidation, we can perform a precise flow cytometry assay (abcam kit, Catalog #ab118183) to measure protein expression of very long chain specific acyl-CoA dehydrogenase, medium-chain and long-chain 3-hydroxyl-CoA dehydrogenases.

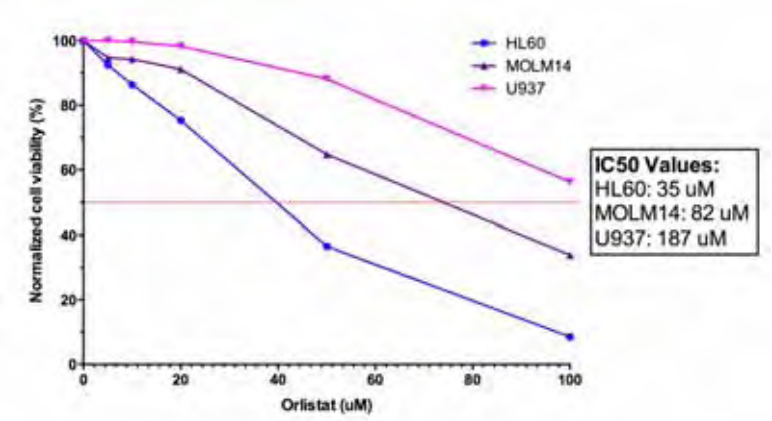



**Figure 24. Orlistat dose-response curve in HL60, MOLM14 and U937 cells.** Normalized cell viability, represented as a percentage, in HL60, MOLM14 and U937 cells treated for 24 hrs with control or 5, 10, 20, 50 and 100 uM orlistat.

Recent data has also suggested a role of mTORC1 in lipid metabolism, as mTORC1 activates SREBP1/2, which is involved in activation of lipogenic enzymes, ACC, FAS and SCD (Peng et al. 2002; Brown et al. 2007; Mauvoisin et al. 2007). Additionally, mTORC1 appears to exercise translational control over PPARα activity and its role in fatty acid uptake, synthesis, esterification and storage in adipocytes (Carnevalli et al. 2010). Interestingly, blocking mTORC1 with rapamycin in human BJAB B-lymphoma cell lines and murine CTLL-2 T lymphocytes increased β-oxidation and catabolism of free fatty acids due to increased very long acetyl CoA dehydrogenase (VLACD) and carnitine acyl transferase (CAT) expression (Peng et al. 2002). This appears to be dependent on the S6K1 activity downstream of mTOR as mice deficient in S6K1 show increased β-oxidation and lipolysis compared to normal mice (Um et al. 2004). Additionally, knockdown of 4EBP1/2 genes leads to decreased lipolysis (Le Bacquer et al. 2007). However, while many publications have focused on metformin-induced AMPK activation and subsequent inhibition of ACC as the key regulator of FAO, this does not seem to be the case in our cells. First, we only have transient and slight activation

of AMPK. Additionally, we do not see increased phosphorylation and inhibition of ACC in response to metformin over time. Based on our strong inhibition of pS6 in metformin-treated AML cells, it is possible that this is the cause of the increased β-oxidation leading to increased acetylcarnitines and carnitines in metformin-treated AML cells. We will therefore more deeply analyze the role of mTORC1 in lipid metabolism in AML cells.

Furthermore, several groups (DeBerardinis 2011; Locasale and Cantley 2011; Possemato et al. 2011; Zhang et al. 2012) have shown that the serine/glycine biosynthetic pathways are upregulated in cancer cells as a major consequence of the global metabolic rewiring with PKM2 activity or an increase of c-Myc-driven glutaminolysis (Wise and Thompson 2010). Chaneton et al (2012) showed that serine can bind to and activate human PKM2 and that PKM2 activity in cells is reduced in response to serine deprivation. This reduction in PKM2 activity shifts cells to a fuel-efficient mode in which more glucose-derived carbon is channeled into serine biosynthesis to support cell proliferation (Chaneton et al. 2012). Recent work from Craig Thompson's lab has also shown that PKM2 drives a redirection of the glycolytic flux to serine biosynthesis through the mTORC1 pathway to sustain cell growth (Ye et al. 2012). Furthermore, serine biosynthesis with one carbon catabolism and the glycine cleavage system represents a novel pathway for ATP generation. Finally, Vazquez et al. (2013) have proposed that overexpression of the mitochondrial folate and glycine-serine pathway is a new determinant of methotrexate selectivity in tumors. They propose that patients whose tumors show this phenotype will be sensitive to folate antagonists targeting thymidylate or purine biosynthesis (Vazquez et al. 2013). Thus, we will analyze the effect of metformin on serine metabolism and the potential therapeutic combination of metformin with inhibition of serine metabolism.

Finally, and to respond to a majority of present questions, we will also perform $C^{13}$ glucose/glutamine NMR or LC-MS to complete the metabolomic studies on carbon tracing in our cells both in the basal state and following metformin treatment. These experiments will help us understand a number of questions that still remain following the broad metabolic analyses we have already performed. In particular, glucose and glutamine are major metabolic substrates for cancer cells, providing a carbon source for generating ATP. Glutamine also provides precursors for the synthesis of nucleic acids, proteins and lipids by replenishment of the tricarboxylic acid (TCA) cycle intermediates via anaplerosis. Oncogenic levels of c-Myc induce a transcriptional program that increases glutamine consumption and reliance on glutamine as a bioenergetic substrate. $C^{13}$ experiments will allow us to analyze the major carbon source of our cells before and after metformin to elucidate the mechanism of action. Additionally, one of the most intriguing results we should be able to understand with $C^{13}$ experiments is why we observe decreases in TCA metabolites in U937 and increases in MOLM14 in response to metformin. Studies from Michael Pollak's lab have demonstrated that the carbon source (glucose *versus* glutamine) and c-Myc (which is consistent with glutamine addiction of c-Myc-transformed cells) influence metformin sensitivity and suggest that the metformin-induced increase in glycolysis attenuates the anti-proliferative effects of the compound

(Javeshghani et al. 2012). Furthermore, inhibitors of ETCI (metformin) and ETCII (KU *via* ATM) induce significant changes in TCA metabolites (Zakikhani et al. 2012). We also need to determine where TCA intermediates are coming from, such as glucose, glutamine, pyruvate, fatty acids, ketone bodies and proline, and what is the benefit of cells compensating in these manners. Finally, we would like to understand the unique basal cell metabolism of U937 cells, which have decreased metabolites, thereby increased flux, through the lower segment of glycolysis, PPP and amino acid biosynthesis.

### 1.4 Metformin may decrease autophagy in human AML cells

In the literature, a decrease in mTORC1 normally corresponds to an increase in autophagy (Shi et al. 2012). In fact, multiple papers have suggested an autophagic mechanism of action of metformin in cancer cells through the AMPK-dependent inhibition of mTORC1 (Bellodi et al. 2009; Harhaji-Trajkovic et al. 2009; Tomic et al. 2011). However, this appears to be cell-dependent, because metformin may induce a decrease in autophagy in our AML cells, as demonstrated by decreases in acridine orange mean fluorescence intensity, a readout for autophagic activity, and through western blots of LC3, a protein cleaved from LC3-I to LC3-II in the process of autophagy (Figure 25). Considering that the induction of autophagy has been proposed as a mechanism for leukemic cell survival and resistance to tyrosine-kinase inhibitors, like imatinib, metformin could offer a promising therapeutic agent to target resistant cells (Calabretta and Salomoni 2011; Crowley et al. 2013; Elzinga et al. 2013; Zhu et al. 2013).

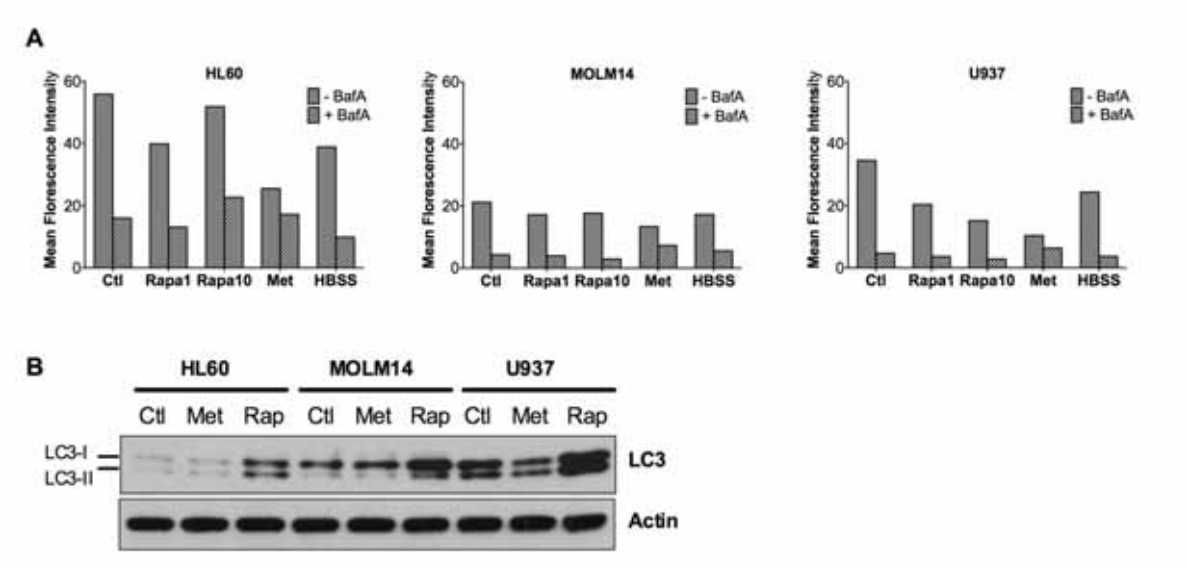



**Figure 25. Metformin may inhibit autophagy in AML cells.** (A) Mean-florescence intensity of acridine orange staining in HL60, MOLM14 and U937 cells treated for 24 hrs with 1uM or 10 uM rapamycin (RAPA), 24 hrs with 10 mM metformin (Met) or a final 4 hr starvation period in HBSS all with or without 2 hrs final incubation with 10 uM bafilomycin A (BafA) as a negative control. (B) Western blot analysis of LC3 conversion from LC3-I to LC3-II in HL60, MOLM14 and U937 treated for 24 hrs with control, 10 mM metformin (Met) or 10 uM rapamycin (Rap) and normalized to β-actin.

Our acridine orange data, which includes bafilomycin A as a negative control that blocks autophagy, provides a number of interesting conclusions. In the basal status of our cells, it appears that HL60 may exhibit a very high basal level of autophagy based on the high level of mean fluorescence intensity of acridine orange (Figure 25A). Regardless, in all cell types tested, metformin induced a decrease in acridine orange fluorescence intensity, suggesting a decrease in autophagy. Our LC3 western blot was not as conclusive for HL60 and MOLM14 as there appears to be no significant difference between control and metformin, although rapamycin is an effective positive control of mTORC1 inhibition and autophagy induction (Figure 25B). However, metformin does appear to decrease LC3 cleavage in U937 cells treated with metformin (Figure 25B). To better understand these results, it will be necessary to repeat these western blots with a negative control of bafilomycin A to confirm the result we observed in metformin- and rapamycin-treated cells. We will continue to analyze the role of autophagy in the response to metformin, but also at the level of its intrinsic importance for basal cell metabolism in AML cells.

One of the major complications with these studies, however, is determining the best methods and conditions to analyze autophagy. In AML cells, a major problem we have is finding positive controls to test against both our control and metformin-treated cells. It is possible that this could be due to a high level of autophagy and/or lysosomal processes in the control, as it has been shown that leukemic cells may depend on autophagy in the basal state and in resistance to drugs, like CML resistance to imatinib (Bellodi et al. 2009; Calabretta and Salomoni 2011; Crowley et al. 2013; Elzinga et al. 2013; Zhu et al. 2013). To attempt to resolve this problem, we will test our cells in different conditions. While we use MEMα media for our cells, as the 5.6 mM glucose level matches that observed in blood, this may not be the best condition for measuring autophagy as low glucose levels may induce basal autophagy. Thus, testing the appropriate media and serum conditions will be necessary for clear and reproducible results. Another issue is that multiple analyses can provide confusing and non-conclusive results. An example is our acridine orange *versus* LC3 western blot data. LC3 data does not suggest a high level of basal autophagy in HL60 or MOLM14 cells, while acridine orange data suggests the opposite. In the case of LC3 cleavage, an increased autophagic flux could be responsible for this quick LC3 turnover and explain why it is not observed in our western blot. Thus, going forward we will analyze autophagy through new experiments, such electron microscope analysis of LC3 droplets. By performing several different experiments, we hope to resolve some of the experimentation issues and find the best methods to analyze autophagy in AML.

# 2 Going deeper into metformin's mechanism of action *in vivo*

## 2.1 Impact of mouse background on the metformin response *in vivo*

While still pursuing metformin's mechanism of action *in vitro*, it is also crucial to deeper analyze metformin's anti-leukemic capabilities *in vivo*. We have already established that metformin can inhibit subcutaneous tumor growth in a mitochondrial-dependent apoptotic mechanism. However, to truly establish metformin as an anti-leukemic agent we need to test this drug in the proper setting of AML with intravenous cell injections. Currently, our lab has subcutaneous and intravenous xenograft models established in NOD-Scid and more highly immunodeficient (NOD/LtSz-scid IL2Rγchain null) NSG models (Sanchez et al. 2009; Sarry et al. 2011). Thus, we first decided to test our subcutaneous xenograft model in NOD-Scid mice by injecting MOLM14 or HL60 cells and treating daily with intraperitoneal injections of metformin at 250 mg/kg/day. Unlike nude mice, we did not observe any significant difference in tumor size between control and metformin-treated groups in either female or male NOD-Scid mice injected with HL60 cell or MOLM14 cells (Figure 26A-B).

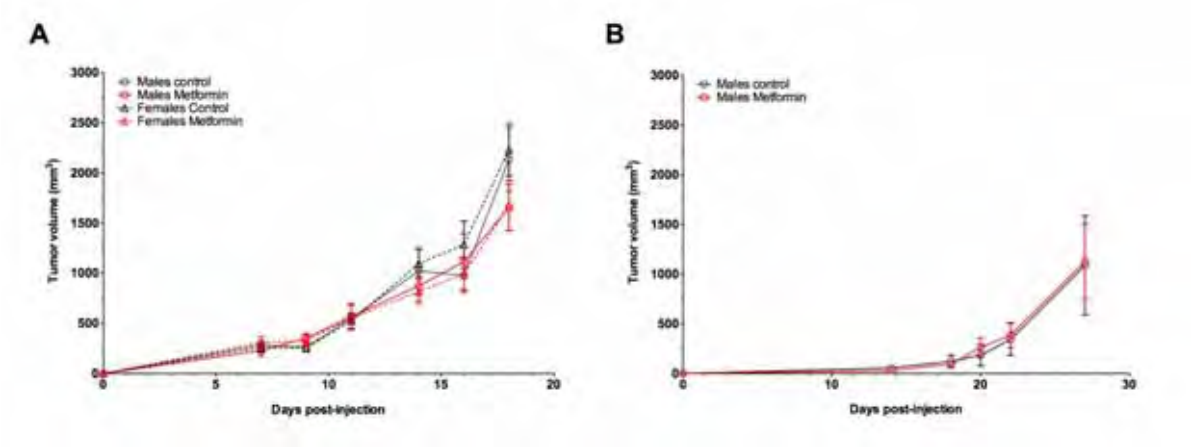



**Figure 26. Metformin does not inhibit tumor growth of AML cells in a NOD-SCID mouse xenograft model.** Average tumor size ($mm^3$) of (A) female and male NOD-SCID mice xenografted subcutaneously with HL60 cells (n = 6 tumors per group) and (B) male NOD-SCID mice xenografted subcutaneously with MOLM14 cells (n = 6 tumors per group).

A potential explanation for the results observed in NOD-Scid mice is that daily intraperitoneal injections are too stressful for NOD-Scid mice compared to nude mice. Thus it may be necessary to determine another mode of treatment, such as metformin in drinking water, for these mouse backgrounds. Additionally, it is possible that the non-obese diabetic (NOD) background of both NOD-Scid and NSG mice may play a role in inhibiting metformin anti-tumoral effects. Thus, we can consider the possibility that NOD-Scid mice may not be sensitive to metformin alone, but could be sensitive in combination with other drugs. For example, Hirsch et al. (2009) previously demonstrated that in a breast nude xenograft model, metformin alone had no effect but decreased almost all tumor

growth in combination with chemotherapy agent, daunorubicin. Based on our data suggesting the strong synergistic effect between metformin and AKT inhibitors, especially in constitutively activated AKT cell types, we plan to treat mice with the combination of the two in both a subcutaneous and intravenous xenograft model before potentially testing this combination with an additional chemotherapy agent.

While it is a priority to induce metformin-sensitivity in NOD-Scid or NSG mice, it would also be interesting to attempt to establish an intraveneous xenograft mouse model in nude mice to attempt to repeat the anti-tumoral effects of metformin observed in the subcutaneous model. Although nude mice are primarily used in subcutaneous tumor xenograft models, several cancer cell types, including primary Hodgkin's disease (Zamecnik and Long 1977), colon carcinoma, prostate adenocarcinoma, renal adenocarcinoma (Kozlowski et al. 1984), human glioma and human medulloblastoma cells (Mandybur et al. 1984) have induced metastatic development in nude mice. The main issue is that, unlike NOD-Scid and NSG mice, nude mice have natural-killer cells, which can decrease tumor metastasis (Klein et al. 1979). However, the fact that there is data presenting tumor metastasis in nude mice suggests that it would at least be worth attempting to inject AML cells intravenously. Additionally, as our lab has established that AML cell lines engraft extremely quickly and can lead to death in NOD-Scid and NSG mice, unlike primary AML cells, it is possible that AML cell lines would be efficient enough to engraft nude mice.

One of the issues we face in all *in vivo* endeavors is the high dose of metformin used preclinically *in vitro* and its relationship to reachable doses for *in vivo* experiments and clinical studies. Many *in vitro* studies test metformin at doses between 1 and 50 mM (165-6600 mg/l), which is well above the obtainable therapeutic plasma levels (0.465-2.5mg/l or 2.8-15uM) in humans (Stambolic et al. 2009; Dowling et al. 2012). Thus, it is possible that the metformin effects seen *in vitro* would not be obtainable *in vivo* or in humans due to the dose. Additionally, it also raises the question of whether higher doses in mice or humans would have off-target and/or harmful effects. Nonetheless, recent studies have indicated that metformin can reduce cancer cell proliferation *in vitro* as low as 10uM, which is in the therapeutic range (Liu et al. 2009). Metformin has also been shown to inhibit tumor growth in mouse models at doses equivalent to those used in humans without causing toxicity (Anisimov et al. 2005; Ben Sahra et al. 2008). This may, however, be dependent on tumor location in the body, which is of particular interest for studying AML cells both in the blood, but also in bone marrow and other organs. It must be determined if metformin will reach these niches at appropriate concentrations.

In parallel, the mircroenvironment of cancer cells may play a role in how those cells will respond to metformin. *In vitro*, cancer cells are often grown in non-physiological conditions. Growth media often contains excessive amounts of nutrients, like glucose, serum and growth factors. This may account for the high doses of metformin required to see *in vitro* effects as well as the differences seen *in vitro* versus *in vivo*. Take, for instance, the fact that p53 and LKB1 presence *in vitro* aids the

effects of metformin, while the two are often knocked down in the most effective metformin-responses *in vivo*. We must understand the relationship between *in vitro* and *in vivo* experiments if we want to have the most effective models to prepare clinical trials.

## 2.2 Impact of the nutritional and liver energetic status of mice on the response to metformin *in vivo*

Metformin may also be more effective *in vivo* when combined with alterations of the nutritional and energetic status of mice. For example, Algire et al. (2011) previously demonstrated that metformin effectively inhibits tumor growth and reduces insulin receptor activation in tumors of mice with high-fat induced hyperinsulinemia, regardless of LKB1 expression. However, in the absence of hyperinsulinemia, metformin only inhibited tumor growth in mice transfected with short hairpin RNA against LKB1, which was not attributable to either host insulin levels or tumor activation of AMPK (Algire et al. 2011). Thus, in diabetic-like conditions, LKB1, which is normally considered a tumor suppressor, is unimportant whereas in normal mice LKB1 actually benefited tumor cells by activating energy-conservation mechanisms. Going further with the objective of understanding the physiological effects of a high-fat diet, a second recent study analyzed the effect of a high-fat diet either ad libitum, eating frequently throughout the day and night, or with time-restricted feeding for 8 hours per day (Hatori et al. 2012). This group demonstrated that time-restricted feeding reset the metabolic cycle and effectively reversed the obesity, hyperinsulinemia, hepatic steatosis, inflammation and negative motor coordination induced by the high-fat diet. Additionally, time-restricted feeding improved CREB, mTOR and AMPK pathway function, oscillations of the circadian clock and overall lipid metabolism, nutrient utilization and energy expenditure (Hatori et al. 2012).

Overall, these nutritional and energetic conditions may be crucial for *in vivo* studies, but are also important issues concerning patients, as we question whether or not metformin has its highest efficacy only in diabetic-like conditions. In light of these issues, we plan to analyze the impact of high-fat diet, LKB1 status and the overall metabolic status of our mice on the effect of metformin. We will also establish a xenograft mouse model in diabetic mice to determine if metformin is truly more effective in diabetic-like conditions.

## 2.3 Impact of metformin on the chemotherapeutic response *in vivo*

One of the issues we face in treating AML is the residual, chemoresistant stem cells that induce relapse in patients. As such, it is important to find new therapeutic targets to work in combination with chemotherapy to eliminate the residual disease. Thus, we have performed several *in vitro* experiments combining metformin with chemotherapy agent, cytarabine, which suggest the two could have a synergistic effect *in vivo* (Figure 27). Interestingly, we first found that AraC alone increases both oxygen consumption (Figure 27A) and lactate production (Figure 27B) in HL60, MOLM14 and U937 cells. This suggests that this particular chemotherapy agent increases metabolic pathways, but especially oxidative phosphorylation. We hypothesized that this could increase the dependence on mitochondrial ATP production, which could sensitize cells to ETCI inhibition by metformin. Thus, it was not surprising to find that the combination of metformin and AraC increased the anti-proliferative and apoptotic effects compared to either drug alone in the three AML cell lines tested (Figure 27C-D). Going forward, this will be very interesting to test *in vivo*, especially for AML samples that do not respond well to chemotherapy in this model. Additionally, it is possible that we could observe a response to metformin in NOD-Scid or NSG mice in this setting compared to metformin alone.

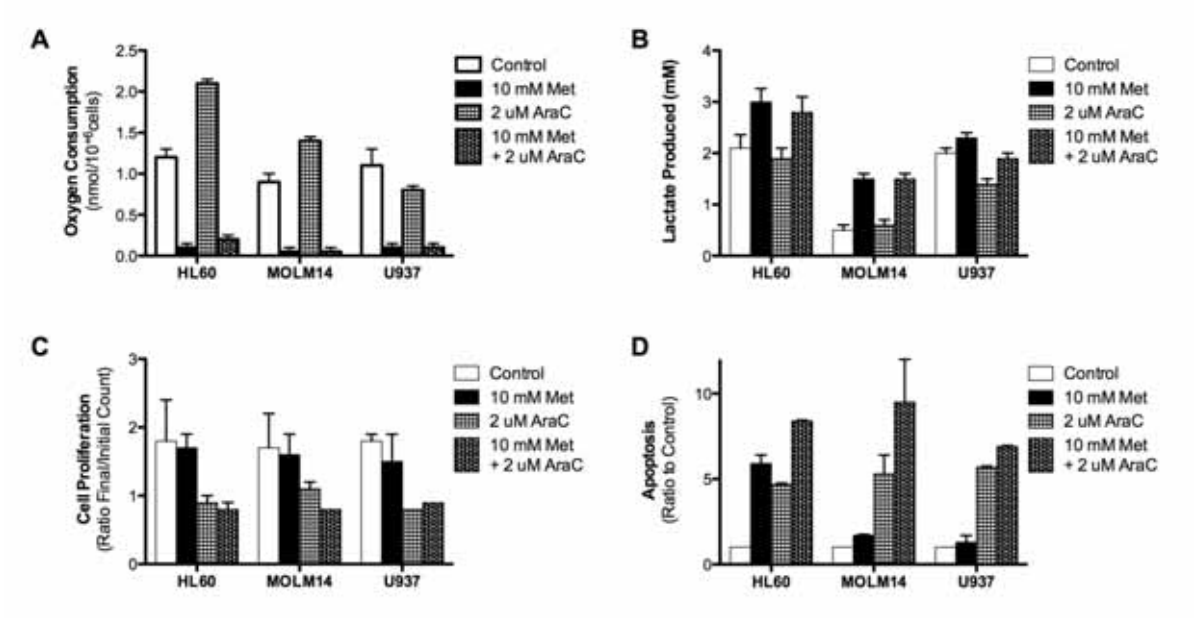



**Figure 27. Metformin augments the anti-tumoral effect of AraC *in vitro*.** HL60, MOLM14 and U937 cells were treated for 24 hrs with control, 10 mM metformin, 2 uM AraC or the combination of metformin and AraC and then analyzed for **(**A**)** the basal oxygen consumption rate, measured as nmol/min/million cells, (B) lactate produced (mM), (C) proliferation, calculated by ratio of final to initial cell count (with trypan blue exclusion) and (D) apoptosis, presented as the ratio to control of AnnexinV-positive cells.

## 2.4 Biomarkers for the metformin response *in vivo*

While further *in vivo* studies with both AML cell lines and primary samples will be crucial to identify biomarkers of both the biological and cytotoxic responses to metformin, we have already identified several potential candidates through our *in vitro* studies. First, it is quite interesting that glycolytic cells are insensitive to metformin compared to oxidative cells, which could allow us to identify the primary mode of ATP production in patient samples to determine their expected response to metformin. One mechanism by which this can be tested is an easily facilitated ATP test with Cell Titer Glo that would allow us to immediately test fresh leukemic cells for their relative dependence. Additionally, we could take advantage of FDG-based CT-PET scans to analyze the relative glycolytic dependence of cells within the patient. We will be able to first test this second potential biomarker *in vivo* by injecting AML cell lines into mice and performing CT-PET scans to determine if there is a correlation between *in vivo* metabolism and sensitivity to metformin.

In terms of specific biomolecular markers, we have also identified AKT, VDAC1 and PFK. Basal levels of AKT have been identified in our AML cells as readouts for the response to metformin and could easily be tested in patient samples to determine the potential response to the drug. Additionally, while cells constitutively activated in AKT may not be sensitive to metformin alone, this readout would suggest combining metformin with an AKTi, which could be an extremely successful combined therapy. VDAC1 is the voltage-dependent mitochondrial pore responsible for the activation of mitochondrial-dependent apoptotic pathways. We have found that VDAC1 is expressed at higher levels in oxidative HL60 and MOLM14 cells compared to glycolytic U937 and KG1 cells (Figure 28). Interestingly, this correlates to the apoptotic sensitivity to metformin, as we have found that KG1 cells are also resistant to metformin-induced apoptosis but not cell proliferation.

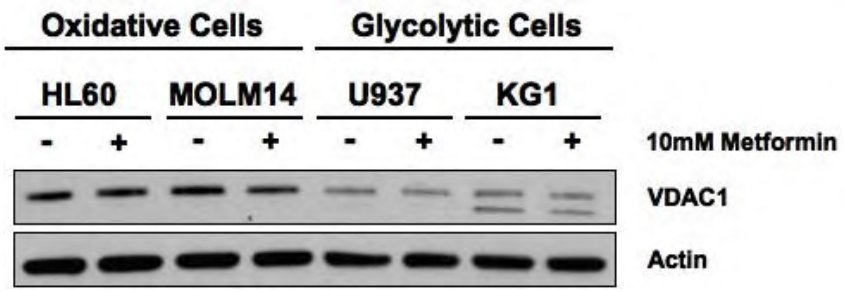
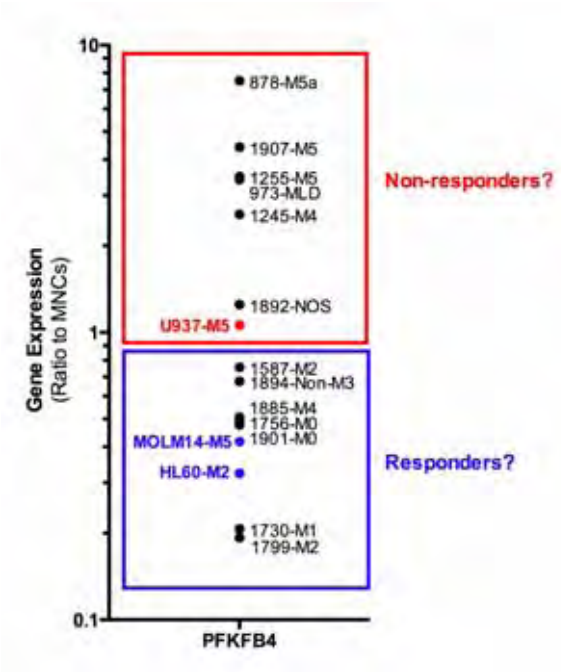



**Figure 28. VDAC1 expression correlates to metabolism in AML cell lines.** HL60, MOLM14, U937 and KG1 cells were treated for 24 hrs with control or 10 mM metformin. Western blots were then performed to analyze VDAC1, as normalized to β-actin.

Finally, we have identified PFKFB4, which is a member of the PFKFB family, as a potential marker of sensitivity to metformin. Selective activity of members of the PFKFB family controls the flux through the pentose phosphate pathway and glycolysis. PFKFB enzymes with high PFK2 activity,

such as PFKFB3, will preferentially catalyze the ATP-dependent phosphorylation of F6P to F-2,6-BP, the later of which promotes activity of PFK1 and high glycolytic flux (Okar and Lange 1999; Okar et al. 2001). However, PFKFB enzymes with high FBPase activity, like PFKFB4, will actually promote the conversion of F-2,6-BP back to F6P, which decreases positive regulation of PFK1 and subsequently increases flux through the PPP (Ros et al. 2012). This flux is crucial because it increases antioxidant levels to combat ROS and apoptosis while it can also promote nucleotide biosynthesis to recover from DNA damage. Of note, this activity can also be promoted by high levels of HIF-1α and p53 (Minchenko et al. 2002; Minchenko et al. 2003; DeBerardinis et al. 2008; Bensaad et al. 2009). In terms of metformin, high *PFKFB4* gene expression, as observed in cytotoxically-insensitive U937 cells compared to sensitive HL60 and MOLM14 cells, may promote high PPP flux to withstand metabolic effects downstream of metformin's inhibition of ETCI, such as ROS or DNA damage (Figure 29). Additionally, we have been able to analyze the *PFKFB4* gene expression levels of 13 patient samples and found that they do exhibit stratified levels of *PFKFB4*, which is linked to the FAB subtype of our samples. M0/1/2/3 patient samples tend to have lower levels of *PFKFB4*, correlating to a higher level of glycolytic flux, rather than PPP flux. M4/5 samples, on the other hand, have higher *PFKFB4* activity, correlating to higher PPP flux potentially due to increased oxidative phosphorylation and the need to combat ROS production. In the future, we will collect fresh patient samples and immediately test for both *PFKFB4* gene expression levels as well as the *in vitro* response to metformin to determine if there is a correlation between the two.




| ID | Sex | FAB | Cytogenetics | FLT3 |
|---|---|---|---|---|
| 878 | | M5a | | D835 |
| 1907 | M | M5 | t(11q23) | |
| 1255 | F | M5 | | WT |
| 973 | | MLD | | ITD |
| 1245 | M | M4 | | ITD |
| 1892 | M | NOS | | WT |
| | | | | |
| 1587 | M | M2 | t(8:21) | WT |
| 1894 | F | Non-M3 | der(9) t(1:9) | WT |
| 1885 | F | M4 | | WT |
| 1756 | M | M0 | | ITD |
| 1901 | M | M0 | | ITD |
| 1730 | M | M1 | | ITD |
| 1799 | F | M2 | t(8:21) | WT |

**Figure 29. PFKFB4 gene expression may correlate to the apoptotic response to metformin.** PFKFB4 gene expression, expressed as a ratio to MNCs, in HL60, MOLM14 and U937 AML cell lines as well as 13 primary samples, which are further characterized in the accompanying table.

# – References –

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# – Appendix –

# IX Appendix

1 Publications:

**S Scotland**, E Saland, N Skuli, F de Toni, H Boutzen, E Micklow, I Sénégas, R Peyraud, L Peyriga, F Théodoro, E Dumon, Y Martineau, G Danet-Desnoyers, F Bono, C Rocher, T Levade, S Manenti, C Junot, J-C Portais, N Alet, C Récher, M A Selak, M Carroll, J-E Sarry, Mitochondrial energetic and AKT status mediate metabolic effects and apoptosis of metformin in human leukemic cells, Leukemia accepted article preview 9 April 2013; doi: 10.1038/leu.2013.107.

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ORIGINAL ARTICLE

# Mitochondrial energetic and AKT status mediate metabolic effects and apoptosis of metformin in human leukemic cells

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Previous reports demonstrate that metformin, an anti-diabetic drug, can decrease the risk of cancer and inhibit cancer cell growth. However, its mechanism in cancer cells is still unknown. Metformin significantly blocks cell cycle and inhibits cell proliferation and colony formation of leukemic cells. However, the apoptotic response to metformin varies. Furthermore, daily treatment with metformin induces apoptosis and reduces tumor growth *in vivo*. While metformin induces early and transient activation of AMPK, inhibition of AMPK$\alpha$1/2 does not abrogate anti-proliferative or pro-apoptotic effects of metformin. Metformin decreases electron transport chain complex I activity, oxygen consumption and mitochondrial ATP synthesis, while stimulating glycolysis for ATP and lactate production, pentose phosphate pathway for purine biosynthesis, fatty acid metabolism, as well as anaplerotic and mitochondrial gene expression. Importantly, leukemic cells with high basal AKT phosphorylation, glucose consumption or glycolysis exhibit a markedly reduced induction of the Pasteur effect in response to metformin and are resistant to metformin-induced apoptosis. Accordingly, glucose starvation or treatment with deoxyglucose or an AKT inhibitor induces sensitivity to metformin. Overall, metformin elicits reprogramming of intermediary metabolism leading to inhibition of cell proliferation in all leukemic cells and apoptosis only in leukemic cells responding to metformin with AKT phosphorylation and a strong Pasteur effect.

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**Keywords:** metabolism; mitochondria; apoptosis; therapeutics; metformin

## INTRODUCTION

An expanding body of evidence indicates that numerous metabolic pathways are altered in cancer cells.[1,2] The Warburg effect, or aerobic glycolysis, is a common characteristic of cancer cells. However, metabolic reprogramming of transformed cells extends far beyond glucose metabolism.[3,4] For example, numerous perturbations in the Krebs cycle, fatty acid and lipid metabolism and NADPH/ROS pathways have been identified in cancer cells, possibly as adaptations to their microenvironment, oncogenic stress or other factors. Understanding metabolic differences between normal and tumor cells offers promising possibilities for therapeutic strategies.

Acute myeloid leukemia (AML) is the most common adult acute leukemia and is characterized by clonal expansion of immature myeloblasts, initiating from rare leukemic stem or progenitor cells. Previous works have suggested that energetic metabolism has a role in cellular differentiation and chemoresistance in AML *in vitro*.[5–7] Leukemic cells, like most cancer cells, may be addicted to glucose for generation of ATP and key biosynthetic intermediates, but recent data show that these cells also rely on fatty acid metabolism to grow and evade apoptosis.[8] Furthermore, several groups[9–12] found that 20–25% of AML patients harbor mutations in isocitrate dehydrogenase (*IDH1* or *IDH2)*, inducing a neomorphic enzymatic activity and production of oncometabolite, 2-hydroxyglutarate.[13,14] These findings have changed our vision about the importance of cell metabolism in leukemia.

Metformin is a biguanide molecule used for treatment of diabetes mellitus. Several epidemiological studies have shown that although patients with type 2 diabetes have a higher risk for cancer, treatment with metformin is linked to a reduction in the risk of developing various types of cancers.[15–17] Both *in vitro* and *in vivo*, metformin inhibits growth of pancreatic, colon, prostate, ovarian and breast cancer cells.[18,19] Additionally, studies have shown that patients with breast cancer treated with a combination of chemotherapy and metformin have higher pathological response rates compared with those treated with chemotherapy alone.[18,19] To date, two major effects of metformin have been described: inhibition of mitochondrial electron transport chain complex I (ETCI) and LKB1-dependent and independent activation of AMPK, a key energy sensor in cells (Supplementary Figure 1). Metformin can also induce a decrease in protein synthesis through a direct and AMPK-dependent activation of tuberous sclerosis complex 1/2 (TSC 1/2), which inhibits mammalian target of rapamycin.[20–22] By targeting complex I, metformin mediates changes in AMP/ATP ratios, calcium levels, mitochondrial matrix pH and transmembrane potential, magnesium ions, inorganic phosphate, cyclophilin D and adenine nucleotides concentrations that commonly correlate

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with increased oxidative stress.[23–25] These effects lead to a local and whole-body metabolic shift away from energy-consuming biochemical pathways to an energy-producing status, which profoundly reprograms cell metabolism. In brief, metformin activates catabolism and mitochondrial biogenesis while inhibiting protein synthesis and anabolic pathways. Unlike other indirect AMPK agonists (for example, AICAR), metformin inhibits ETCI, thereby preventing fatty acid oxidation and restricting glucose oxidation by mitochondria. The therapeutic potential of metformin might stem from this dual mechanism of action, which increases catabolism and limits substrate availability and oxidation. Importantly, both the effect of and sensitivity to metformin vary from cell to cell, providing a rationale for a therapeutic index for use of metformin in cancer therapy.

Herein, we investigate the metabolic, signaling and cytotoxic responses of diverse human leukemic cells to metformin. Metformin induces an early metabolic shift (for example, decreased oxygen consumption and mitochondrial ATP production while increasing glucose uptake and glycolytic ATP synthesis) and transient AMPK phosphorylation in all leukemic cells. Metformin inhibits cell proliferation in an AMPK-independent manner. The inhibition of mitochondrial respiratory capacities triggers a delayed and profound adaptation in the carbon flux balance and central metabolism in all leukemic cells, while inducing apoptosis in only some leukemic cells. Sensitivity to metformin-induced cell death is mediated by the metabolic capability to elicit the Pasteur effect, as well as by the basal level of AKT phosphorylation and glucose consumption. Collectively, these data demonstrate that metformin is a promising therapeutic drug and a useful tool for determining the contribution of each major metabolic pathway to leukemic cell survival.

## MATERIALS AND METHODS

### Cell proliferation and apoptosis

Cell proliferation was calculated by measuring the cell density using a Nexcelom cellometer with trypan blue dye. The cytotoxic response was determined by measuring apoptosis by flow cytometry after staining with AnnexinV-APC (Invitrogen, Saint Aubin, France) and 7AAD (BD Biosciences-Pharmingen, Le Pont de Claix, France). Samples were washed with cold HBSS (Invitrogen) and resuspended in 100 ul $1\times$ annexin-V-binding buffer (BD Biosciences). Cells were stained, on ice and in the dark, with Annexin V-APC for 15 min followed by 7AAD for another 15 min. We added 200 ul of $1\times$ Annexin-V-Binding buffer. Samples were analyzed using a FACSCalibur flow cytometer and BD CELLQuest-Pro software (BD Biosciences-Pharmingen).

### Tumor xenografts

Xenograft tumors were generated by injecting $2\times10^6$ MOLM14 cells (in 100 ml of PBS) subcutaneously on both flanks of NU/NU Nude mice (Charles River, Wilmington, MA, USA) ($n=5$, mice (10 tumors) per group). Mice were given daily intraperitoneal injections with 200 μl of 300 mg/kg/day metformin or vehicle (PBS). Tumor dimensions were measured with a caliper on days indicated and volume calculated using the formula: $v=\pi/6\times A\times B\times B$, where A is the larger diameter and B is the smaller diameter. At the end of the experiment, tumors were dissected, weighed, photographed and fixed with alcohol formalin acetic acid fixative for 48 h. Tumors were embedded in paraffin and sectioned for immunohistochemistry.

### Immunohistochemistry

Apoptosis in xenografts was determined by immunohistochemistry for Active Caspase-3 (R&D Systems, Lille, France). For quantification, positively stained cells in six consecutive fields at $\times20$ magnification were counted from the edge towards the center of each section. Photographs for quantification were taken with a Leica DM4000B microscope (Leica, Saint Jorioz, France).

### Small interfering RNA transfections

Small interfering RNA transfection of AMPKα1/2 was performed using the NEON Transfection System (Sigma-Aldrich, Lyon, France). Small interfering RNA for AMPKα1 and α2 was purchased from Invitrogen and previously published.[26] In summary, 1 million cells were transfected with siControl or siAMPKα1/2 at a final concentration of 700 nM and then resuspended in 2.0 ml of MEMα + 10% FBS. Cells were incubated for 24 h to obtain complete inhibition of AMPKα1/2. Cells were then resuspended in fresh MEMα + 10% FBS and treated with vehicle or 10 mM metformin for an additional 48 h.

### Measurement of metabolic parameters

We measured ETCI activity, oxygen consumption, adenosine triphosphate (ATP), lactate production and flux levels of glucose, lactate and glutamine. An extended description of the material and methods is provided in Supplementary Information Materials and Methods.

### Liquid chromatography coupled to electrospray-LTQ-Orbitrap mass spectrometry with data processing and multivariate statistical analysis

Intracellular metabolites were analyzed with high performance anion exchange chromatography coupled to a triple quadrupole QTrap 4000 mass spectrometer (Method 1) and with ultra-high performance liquid chromatography coupled to mass spectrometry (UHPLC/MS), performed using an LTQ-Orbitrap hybrid mass spectrometer (Method 2). Raw data were analyzed using the XCMS software Version 1.14.1 running under R Version 2.8.1,19 (http://www.bioconductor.org/packages/bioc/html/xcms.html). The data sets resulting from the XCMS process were then annotated using tools developed in-house. An extended description of the Material and Methods is provided in Supplementary Information Materials and Methods.

### Statistical analysis

Unpaired *t*-test was used to calculate final *P*-values. Significance is represented by stars in which $*P<0.05$, $**P<0.01$ and $***P<0.005$.

## RESULTS

### Metformin, independent of AMPK, inhibits cell growth in human leukemic cells

We first determined the *in vitro* effect of metformin on cell proliferation and apoptosis of three AML cell lines and six primary AML patient specimens (Supplementary Table 1 shows the characteristics of cell lines and patient samples). Metformin induced a strong inhibitory effect on cell proliferation in all leukemic cell lines (Figure 1a) and significantly reduced leukemia colony-forming units of three AML cell lines and six primary AML patient samples in a concentration-dependent manner (Figures 1b and c). Interestingly, metformin induced a cell cycle block in G0/G1 (U937) or S-G2/M (HL60 and MOLM14) phase in all three cell lines (Supplementary Figure S2A and B). However, we observed striking differences in metformin-induced cell death. HL60 and MOLM14 cells exhibited significant apoptosis-dependent cell death in response to metformin while U937 cells were resistant to this treatment (Figures 1d and e).

To determine if these results apply *in vivo*, we established a xenograft model for AML in female nude mice using MOLM14 cells and treated these mice with daily intraperitoneal injections of metformin (300 mg/kg/day). Metformin strongly reduced the tumor growth and weight in this model (Figures 2a and c). Additionally, analysis of treated tumors demonstrated that metformin induced apoptosis *in vivo* with an increase in active caspase-3 staining (Figures 2d and e). Thus, metformin is capable of inducing cell death in AML cells *in vivo*.

We next studied the biochemical effects of metformin on AML cells to understand the mechanism of sensitivity. As Green *et al.* (21) showed that metformin activates AMPK in various AML cell lines, we analyzed expression of AMPK and phospho-T172-AMPK in HL60, MOLM14 and U937 cells following metformin treatment. We noted no changes in phospho-T172-AMPK after 24 h metformin in all leukemic cells (Figure 3a). However, metformin transiently increased phosphorylation of AMPK in all AML cell lines as early as 1 h after incubation with 1 and 10 mM metformin with a


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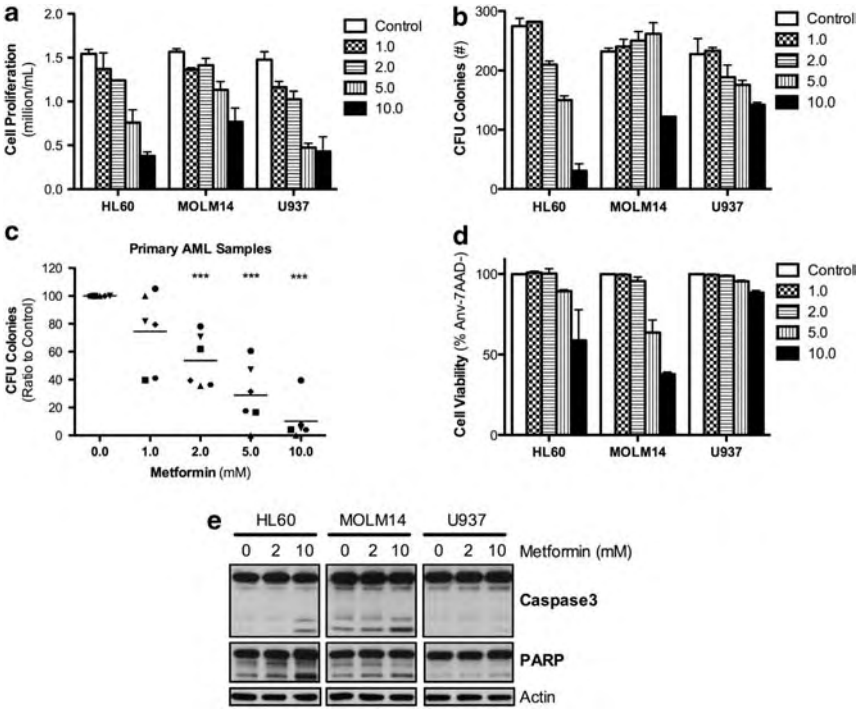


**Figure 1.** Metformin inhibits cell proliferation in all human leukemic cell lines but has varying effects on apoptotic-dependent cell death. (**a**) HL60, MOLM14 and U937 cells after 48 h with 0.0, 1.0, 2.0, 5.0 or 10.0 mM metformin. Proliferation is calculated by ratio of final to initial cell count (with trypan blue exclusion) over the 48 h of incubation. (**b**) Average number of colony-forming units after 5-7 days incubation of HL60, MOLM14 and U937 cells in methylcellulose enriched media with 0.0, 1.0, 2.0, 5.0 or 10.0 mM metformin. (**c**) Average number of colony-forming units after 14 days incubation of six AML patient samples in methylcellulose enriched media with 0.0, 1.0, 2.0, 5.0 or 10.0 mM metformin, statistics by unpaired *t*-test with *P*-value as $^{***}P<0.005$. (**d**, **e**) Viable cells as the percentage of Annexin-V negative and 7AAD negative cells carried out on the same cells listed for Figure 1a and the cleavage of capase-3 and PARP, respectively.

maximum activation at 1 and 6 h for U937 and MOLM14 cells, respectively (Supplementary Figure S3). To determine whether the anti-proliferative and apoptotic effects of metformin are secondary to this transient activation of AMPK, we blocked this pathway using small interfering RNA of the two catalytic subunits AMPK$\alpha$1 and $\alpha$2. While we strongly diminished expression of AMPK in all three AML cell lines (Figure 3b), we did not prevent either inhibition of cell proliferation or induction of apoptosis in response to metformin (Figures 3c and d). Thus, AMPK activation by metformin is not necessary for metformin-induced cell death in AML cells.

### Metformin inhibits ETCI in all leukemic cells but resistance to metformin-induced cell death correlates with a weak induction of the Pasteur effect

As metformin is known to inhibit ETCI, we examined the correlation between cell survival and respiratory characteristics of AML cell lines grown under the same conditions (MEM$\alpha$ containing 5.6 mM glucose, 2.5 mM glutamine + glutamate and 1 mM pyruvate). First, we assessed ETCI activity following metformin and observed a significant decrease at a similar level to rotenone, a specific ETCI inhibitor (Figure 4a). Accordingly, determination of oxygen consumption showed that metformin elicited a concentration-dependent decrease in the respiratory rate in HL60, MOLM14 and U937 cells (Figure 4b). Metformin inhibited oxygen consumption by 90% in leukemic cells after 24 h, an effect comparable to that observed with specific ETCIII inhibitor, antimycin A. Furthermore, all measured rates of oxygen consumption (oligomycin-sensitive, oligomycin-insensitive, uncoupler-stimulated (maximal) respiration and spare respiratory capacity of cells) decreased upon treatment with either 10 mM metformin or 10 $\mu$M antimycin A (Supplementary Figure S4A). Hence, our data is consistent with previously published *in vitro* studies showing a similar concentration-dependent inhibition of oxygen consumption over a range of 1 to 15 mM metformin after 24 h in several cell types.[23,27]

We next asked which metabolic alterations are involved in metformin-induced cell death. As observed in proliferating cells undergoing inhibition of mitochondrial OXPHOS, the decrease in mitochondrial ATP production was compensated by an increase in ATP production through glycolysis, the so-called Pasteur effect. Therefore, we first measured glycolytic ATP following metformin-induced inhibition of oxygen consumption and found that glycolytic ATP production is increased to a larger extent in HL60 (+186%) and MOLM14 (+292%) compared with U937 (+37%) after 24 h treatment with 10 mM metformin (Figure 4c). As other surrogates of the Pasteur effect, we also measured lactate production and release to the extracellular medium (Figure 4d). HL60 and MOLM14 cells showed a large (+60% and +142%, respectively) increase in lactate production when treated with either 10 mM metformin or 10 $\mu$M antimycin A, while U937 cells only exhibited a limited (+21%) increase. This demonstrated that sensitive HL60 and MOLM14 cells show a more significant induction of the Pasteur effect in response to metformin than less sensitive U937 cells. To understand why U937 cells did not undergo apoptosis and did not exhibit a pronounced Pasteur effect, we analyzed different oxidizable substrates in the culture medium (glutamine, glucose) after treating HL60, MOLM14 and U937 cells with metformin or antimycin A. A significant increase was observed in glutamine consumption upon treatment in all cell types (Supplementary Figure S4B). Consistent with the increase in glycolytic ATP and lactate production, glucose consumption was markedly increased in metformin- and antimycin-treated

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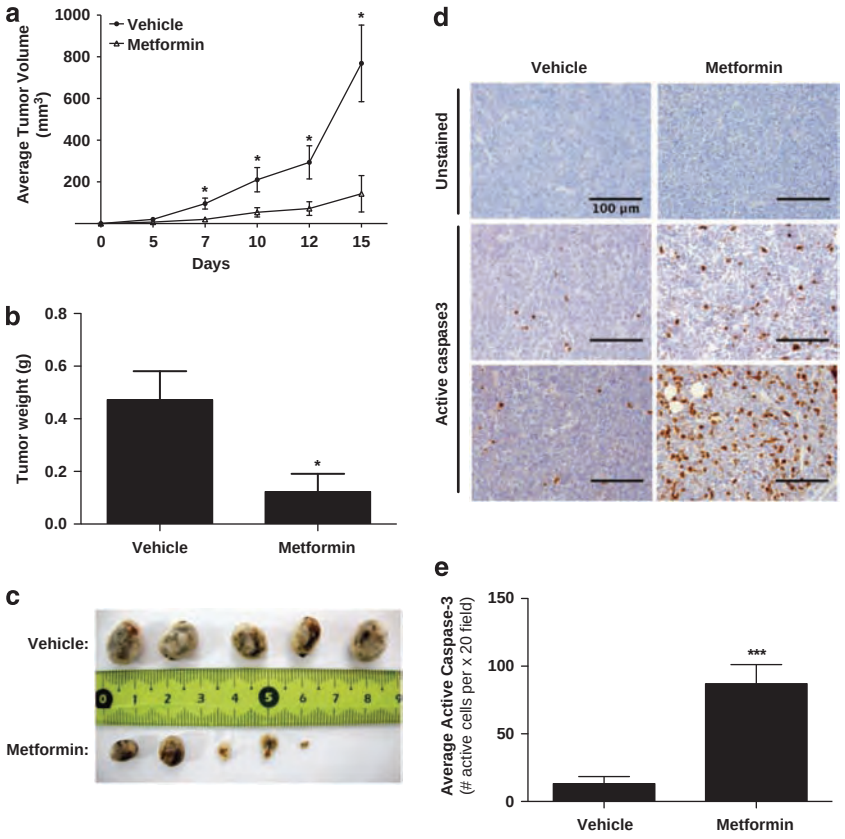


**Figure 2.** Metformin inhibits tumor growth of AML cells *in vivo*. (**a**) Mean tumor size ($mm^3$) and (**b**) tumor weight (g) was assessed in nude mice xenografted with MOLM14 cells and treated with either vehicle or 300 mg/kg/day metformin ($n = 10$, tumors in each group). (**c**) Representative photographs of xenografted tumors at day 17. (**d**) Tumor sections of mice injected with either vehicle or metformin were stained for active caspase-3. Representative photos of three metformin and three vehicle tumors are shown. (**e**) Quantification of the average number of active caspase-3 cells in 6 consecutive fields at x 20 magnification in tumors treated with either vehicle or metformin. Statistics were performed with an unpaired *t*-test with *P*-values represented as $*P < 0.05$ and $**P < 0.005$.

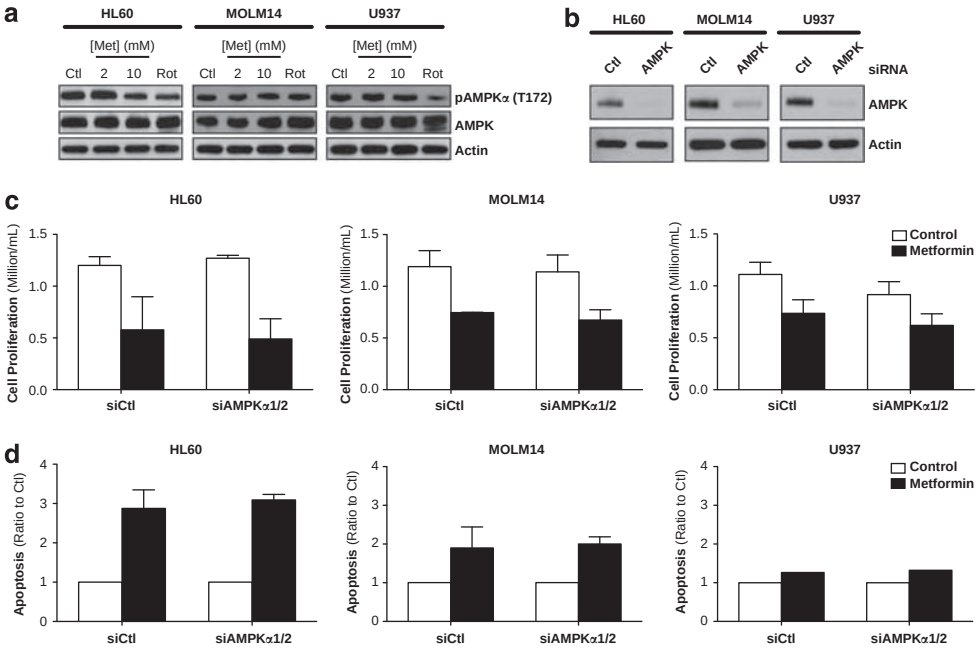


**Figure 3.** AMPKα1/2 does not effect the proliferation inhibition or apoptosis induced by metformin. (**a**) Western blots in HL60, MOLM14 and U937 cells after 24 h with untreated, 2 mM metformin, 10 mM metformin or 200 nM rotenone (Rot). Western blots were analyzed using primary antibodies for pAMPKα (T172) and AMPKα, all normalized to β-actin. (**b**) Western blots in HL60, MOLM14 and U937 cells 24 h after control small interfering RNA or AMPKα1/2 small interfering RNA. Following 24 h inhibition, these cells were treated for 48 h with control or 10 mM metformin and then analyzed for (**c**) cell proliferation, calculated by ratio of final to initial cell count (with trypan blue exclusion) and (**d**) apoptosis, measured as 7AAD-positive and AnnexinV-positive cells and presented as ratio to control.


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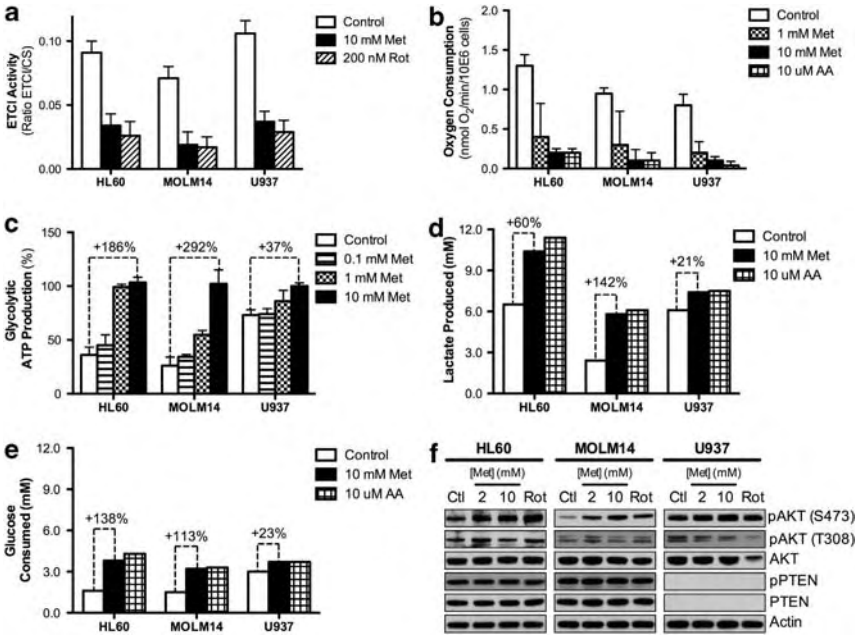


**Figure 4.** Inhibition of oxygen consumption by metformin induces a stronger Pasteur Effect in HL60 and MOLM14 cells due to lower basal glucose consumption for ATP production. (**a**) ETCI activity measured as a ratio of ETCI activity to citrate synthase activity in HL60, MOLM14 and U937 cells after 24 h with control, 10 mM metformin or 200 nM rotenone (Rot). (**b**) The basal oxygen consumption rate, measured as nmol/min/million cells, in HL60, MOLM14 and U937 cells after 24 h with control, 1.0 mM metformin, 10.0 mM metformin or 10 μM antimycin A (AA). (**c**) Glycolytic ATP production measured as a percentage of total ATP production in HL60, MOLM14 and U937 cells after 24 h with 0.0, 0.1, 1.0 or 10.0 mM metformin. Comparison of (**d**) lactate produced (mM) and (**e**) glucose consumed (mM) in HL60, MOLM14 and U937 cells after 24 h with control, 10 mM metformin and 10 μM antimycin A. (**f**) Western blots in HL60, MOLM14 and U937 cells after 24 h with control, 2 mM metformin, 10 mM metformin or 200 nM rotenone. Western blots were analyzed using primary antibodies for pAKT (S473), pAKT (T308), AKT, pPTEN, and PTEN, all normalized to β-actin.

HL60 and MOLM14 cells (Figure 4e). Interestingly, both metformin and antimycin A only mildly increased glucose utilization in U937 cells (+23% versus +138% and +113% in HL60 and MOLM14, respectively; Figure 4e). These results again demonstrate that metformin-sensitive HL60 and MOLM14 cells have a pronounced induction of the Pasteur effect following metformin treatment.

Interestingly, basal glucose consumption was two-fold higher in untreated U937 cells compared to MOLM14 cells, which correlated with higher basal lactate production in untreated U937 cells (Supplementary Figure S5A). This difference in basal glucose metabolism between MOLM14 and U937 cells was also evident in their respective glycolytic and oxidative phosphorylation contributions to ATP synthesis (26%/74% for MOLM14 versus 73%/27% for U937 of ATP produced by glycolysis/OXPHOS; Supplementary Figure S5B). These results demonstrated that the weak Pasteur effect observed in U937 cells in response to metformin is correlated to a decreased dependency on mitochondrial energetic metabolism in the basal state.

To ascertain whether mitochondrial changes and inhibition of ETC complex I account for the observed Pasteur effect and cell survival of metformin-treated AML cells, we examined ATP production at different time points (1–3–6 h compared with 24 h metformin). Our data indicated that the global ATP content of metformin-treated MOLM14 and U937 cells did not change during the experiment (Supplementary Figure S6A). In addition, we found that metformin-induced glycolytic ATP production occurred as early as 1 h with a maximum at 3 h of metformin treatment in MOLM14 and U937 (Supplementary Figure S6B). However, the induction of the Pasteur effect was much stronger in MOLM14 than U937 cells as above-mentioned. Of note, the increase in lactate production in the culture medium in response to metformin was significantly delayed when compared with the increase of glycolytic ATP production and occurred only after 24 h treatment, and markedly in MOLM14 cells (Supplementary Figure S6C compared with Supplementary Figure S6B). The discrepancy between the kinetics of these two readouts of the Pasteur effect is likely due to the time required to induce expression of plasma membrane monocarboxylic acid transporters implicated in lactate release.

Because basal glucose uptake and glycolytic rate seem to be critical parameters of the Pasteur effect induction, we asked how this balance is regulated. Thus, we analyzed the activation of the PI3K/AKT pathway, a key metabolic regulator of aerobic glycolysis in cancer cells. Interestingly, while metformin did not affect the expression of PTEN, metformin treatment increased and sustained phosphorylation of AKT at Ser473 (and not Thr308) only in metformin-sensitive HL60 and MOLM14 cells (Figure 4f). In contrast, we detected strong basal AKT phosphorylation in untreated, PTEN-null U937 cells and did not see further increase upon metformin treatment (Figure 4f). This is consistent with the notion that U937 cells have a strong glycolytic basal status, as illustrated by higher basal levels of lactate secretion and glucose uptake, lower levels of oxygen consumption, and lower dependence on mitochondrial ATP. In summary, these data suggest that U937 cells are resistant to metformin-induced cell death because they constitutively exhibit a strong glycolytic phenotype in the basal state and thus derive a significantly smaller fraction of ATP from mitochondrial OXPHOS, thereby requiring a smaller compensatory response to metformin-driven inhibition of ETCI.

### Metformin-induced mitochondrial changes lead to profound metabolic adaptations in leukemic cells

To better characterize the metabolic adaptations of AML cells in response to metformin we analyzed the metabolomic signature of metformin using quantitative LC-MS/MS mass spectrometry (Figure 5a and supplementary Figure S7A), as well as protein and gene expression analysis of key metabolic enzymes (Figure 5b

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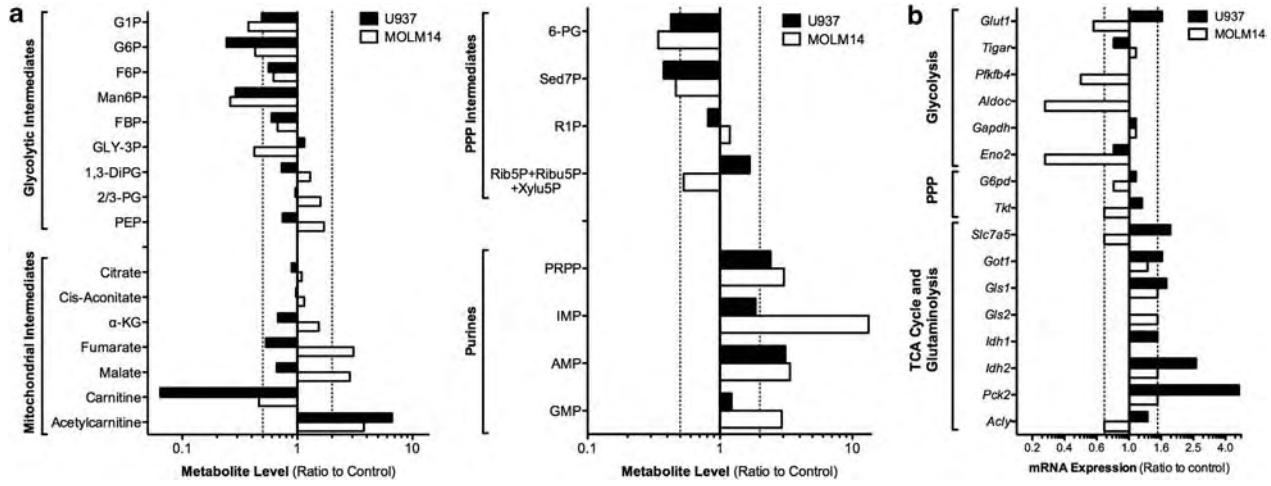


**Figure 5.** Metformin induces a metabolic adaptation in all leukemic cells, but insensitive cells have additional adaptive mechanisms to sustain cell survival. (**a**) Quantified intracellular concentrations of metabolites and (**b**) gene expression, calculated as ratio of 10 mM metformin to control, for MOLM14 and U937 cells after 24 h. For metabolite abbreviations, glucose-1-phosphate (G1P); glucose-6-phosphate (G6P); fructose-6-phosphate (F6P); mannose-6-phosphate (Man6P); fructose-1,6-bisphosphate (FBP); glyceraldehyde-3-phosphate (GLY-3P); 1,3-phosphodiglycerate (1,3-DiPG); 2/3-phosphoglycerate (2/3-PG); phosphoenolpyruvate (PEP); alpha-ketoglutarate (α-KG), 6-phosphogluconate (6-PG); sedoheptulose-7-phosphate (Sed7P); ribose-1-phosphate (R1P), ribose-5-phosphate (Rib5P); ribulose-5-phosphate (Ribu5P); xylulose-5-phosphate (Xylu5P); phosphoribosyl pryphosphate (PRPP); inosine monophosphate (IMP); adenosine monophosphate (AMP); guanine monophosphate (GMP). For gene expression abbreviations, glucose transporter 1 (*Glut1*); TP53 (Tumor Protein 53)-induced glycolysis and apoptosis regulator (*Tigar*); 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4 (*Pfkfb4*); aldolase c (*Aldoc*); glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*); enolase 2 (*Eno2*); glucose-6-phosphate dehydrogenase (*G6pd*); transketolase (*Tkt*), solute carrier family 7 (amino acid transporter light chain, L system); member 5 (*Slc7a5*), glutamate oxaloacetate transaminase 1 (*Got1*); glutaminase 1 (*Gls1*); glutaminase 2 (*Gls2*); isocitrate dehydrogenase 1 (*Idh1*); isocitrate dehydrogenase 2 (*Idh2*); phosphoenolpyruvate carboxykinase 2 (*Pck2*); ATP citrate lyase (*Acly*).

and Supplementary Figure 7B). Genome-scale metabolic network of *Homo sapiens*[28] was downloaded from BiGG database[29] to perform integrated analysis of the data using the Gene to Protein to Reaction association for each leukemic cell line treated with 10 mM metformin for 24 h. The results showed a significant decrease in intermediates of the upper segment of glycolysis (G1P, G6P, F6P, FBP, Man6P) and the oxidative segment of the pentose phosphate pathway (6-PG, Sed7P) and a strong accumulation of purines (PRPP, IMP, AMP) in both MOLM14 and U937 cells after 24 h metformin (Figure 5a). We also found a strong increase in acetylcarnitine and decrease in carnitine, suggesting that metformin promotes high mitochondrial fatty acid shuttling (Figure 5a). Interestingly, we observed a significant difference between these two cell types, decreased in U937 and increased in MOLM14, in TCA metabolites (α-KG, fumarate, malate) and glycolytic intermediates (Gly-3P, 2/3PG, PEP) after metformin. Finally, comparing U937 to MOLM14 cells, we found a significant reduction in basal metabolites in the lower segment of glycolysis (Gly3P, 1,3-DiPG, 2/3-PG, PEP), pentose phosphate pathway (6-PG, Rib5P + Ribu5P + Xylu5P) and amino acid biosynthesis (methionine, glutamine), but an increase of purines (orotate, IMP, GMP, total NAD + /NADH pool), glutathione, carnitine and acetylcarnitine (Supplementary Figure 8), suggesting that control of flux through key metabolic pathways is significantly different between MOLM14 and U937.

Metformin globally increased gene expression of enzymes involved in anaplerotic reactions (*Pck2*, *Idh2*, *Got1* and *Gls1*) in MOLM14 and U937 cells (Figure 5b) and downregulated *Pfkfb4, AldoC, Eno2, Glut1, Slc7A5, Tkt* and *Acly* only in metformin-treated MOLM14 cells (Figure 5b). Interestingly, we also observed that metformin decreased phosphorylation of key rate-limiting enzyme of mitochondrial pyruvate oxidation, pyruvate dehydrogenase only in metformin-sensitive leukemic cells (Supplementary Figure S7B). However, we observed no significant changes for HK-II, GAPDH, PK-M2, LDHA, IDH1, IDH2, CPT1 or ACC (Supplementary Figure S7B). Of note, the calculated free energy changes for three key reactions (GPI, PFK, ENO) in the glycolytic pathway were similar between treated and untreated MOLM14 and U937 cells (Supplementary Figure S9). However, the PRPP biosynthetic equilibrium was significantly displaced by metformin in MOLM14 and the free energy change of the PFK reaction was significantly more negative in U937 under basal growth conditions, suggesting FBP formation by PFK is strongly favored in U937 cells.

### Inhibition of glycolysis or AKT induces cell death upon metformin treatment of resistant U937 cells

Finally, we tested whether the strong basal AKT activation and glycolysis protects U937 cells from metformin-induced apoptosis. First, we inhibited glycolytic metabolism with deoxyglucose (DXG) to determine if this would preferentially sensitize U937 cells to metformin-induced apoptosis following the shift from non-glucose oxidizing substrates (glutamine, fatty acids) to glucose oxidation through mitochondria. We observed marked sensitivity to DXG alone in MOLM14 without any synergistic effect with metformin. In U937 cells, by contrast, DXG had no effect on apoptosis alone even though DXG significantly decreased global and glycolytic ATP production. However, in combination with metformin, DXG induced marked apoptosis in U937 cells and significantly induced the Pasteur effect (that is, glycolytic ATP production) (Figures 6a and b; Supplementary Figure S10A). These results suggest that U937 cells depend heavily on glycolysis after metformin treatment. We confirmed these results by starving U937 of glucose by culturing the cells at high cell density in media with 5.6 mM glucose. In these conditions, U937 cells are highly sensitive to metformin, which can be abrogated by growing high cell density cells in the presence of 25.6 mM glucose (Figure 6c). Culturing U937 at high cell density also significantly decreased AKT phosphorylation, suggesting the key role of glucose uptake and the AKT pathway in metformin sensitivity (Figure 6d).

To test the hypothesis that high AKT activation can drive glycolysis and consequently metformin insensitivity in U937 cells, we studied the effects of AKT inhibition on cell metabolism and


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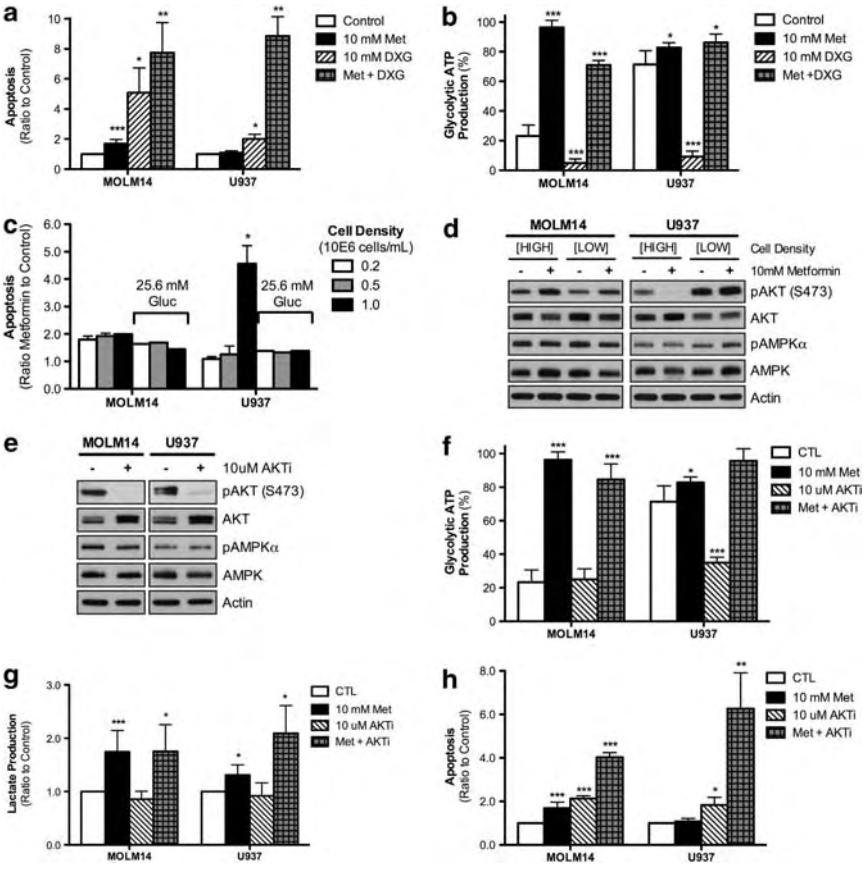


**Figure 6.** The pro-apoptotic effect of metformin can be increased or induced in AML cells by specific metabolic manipulation. After a 4 h preincubation with or without 10 mM DXG, U937 and MOLM14 cells were then incubated for 24 h with control, 10 mM metformin, 10 mM DXG, or the combination of metformin and DXG and then analyzed for (**a**) apoptosis, measured as 7AAD-positive and AnnexinV-positive cells and presented as ratio to control and (**b**) glycolytic ATP production, presented as a percentage of total ATP production. (**c**) Apoptosis, measured as 7AAD-positive and AnnexinV-positive cells and presented as ratio of 10 mM metformin-treated to untreated, in MOLM14 and U937 cells at starting cell densities of 0.2, 0.5 or 1.0 million cells/mL either in MEMα (5.6 mM) + 10% FBS or MEMα (25.6 mM) + 10% FBS. (**d**) Western blots in MOLM14 and U937 cells after 24 h with control or 10 mM metformin with either high or low starting cell densities. Western blots were analyzed using primary antibodies for pAKT (S473), AKT, pAMPKα (T172) and AMPKα, all normalized to β-actin. (**e**) Western blot data in MOLM14 and U937 cells after 4 h pretreatment with untreated or 10 μM AKTi to confirm AKT inhibition. Western blots were analyzed using primary antibodies for pAKT (S473), AKT, pAMPKα (T172) and AMPKα, all normalized to β-actin. Following the 4 h preincubation with either control or 10 μM AKTi, U937 and MOLM14 cells were then incubated for 24 h with control, 10 mM metformin, 10 μM AKTi, or the combination of metformin and AKTi and then analyzed for (**f**) glycolytic ATP production, presented as a percentage of total ATP production, (**g**) lactate production, presented as a ratio to control, and (**h**) apoptosis, measured as 7AAD-positive and AnnexinV-positive cells and presented as ratio to control. Statistics were performed with an unpaired *t*-test with *P*-values represented as $^{***}P<0.005$, $^{**}P<0.01$ and $^{*}P<0.05$.

survival. We observed that 4 h-pretreatment with an AKT inhibitor completely reduced AKT phosphorylation in both leukemic cells (Figure 6e) and induced a strong Pasteur effect (increased glycolytic ATP and lactate production Figures 6f and g) and apoptosis in response to metformin that was markedly higher in U937 (Figure 5h). Overall, these results support our hypothesis that high pAKT leads to high basal glycolysis and insensitivity to metformin-induced cell death in U937 cells.

## DISCUSSION

While metformin inhibited cell cycle progression, cell proliferation and leukemia colony-forming activities in all leukemic cells, metformin induced apoptosis only in certain cell types. Furthermore, daily treatment of xenograft mice with metformin induced apoptosis and decreased tumor growth *in vivo*. To understand the pro-apoptotic effect of metformin, we analyzed mitochondrial function and signaling as well as global metabolism in three different (sensitive, HL60 and MOLM14 versus insensitive, U937) leukemic cell lines. We demonstrated that metformin, similar to rotenone or antimycin A, inhibited both ETCI and oxygen consumption and shifted energetic ATP production from oxidative to glycolytic. Of note, we observed significant and transient AMPK phosphorylation at the Thr172 site in all leukemic cell lines in response to metformin, with a time course similar to inhibition of oxygen consumption and mitochondrial ATP production, but without any changes in global ATP content, as previously described in cells with defective AMPK signaling or effectors pathways.[30,31] However, the anti-proliferative and pro-apoptotic effects of metformin are not mediated by activation of AMPK, which is also consistent with recent studies.[20,26,32,33]

Next, we interrogated the role of metabolism on metformin-induced cell death. In all leukemic cells, metformin induced a common metabolic adaptation to its early inhibitory effects on mitochondrial energetics with increased glucose uptake and consumption, as well as redirection of carbon flux towards lactate, the pentose phosphate pathway and purine biosynthesis. Furthermore, metformin increased mitochondrial and anaplerotic reactions (for example, fatty acid shuttling and oxidation, glutamine consumption and glutaminolysis) in all leukemic cells.

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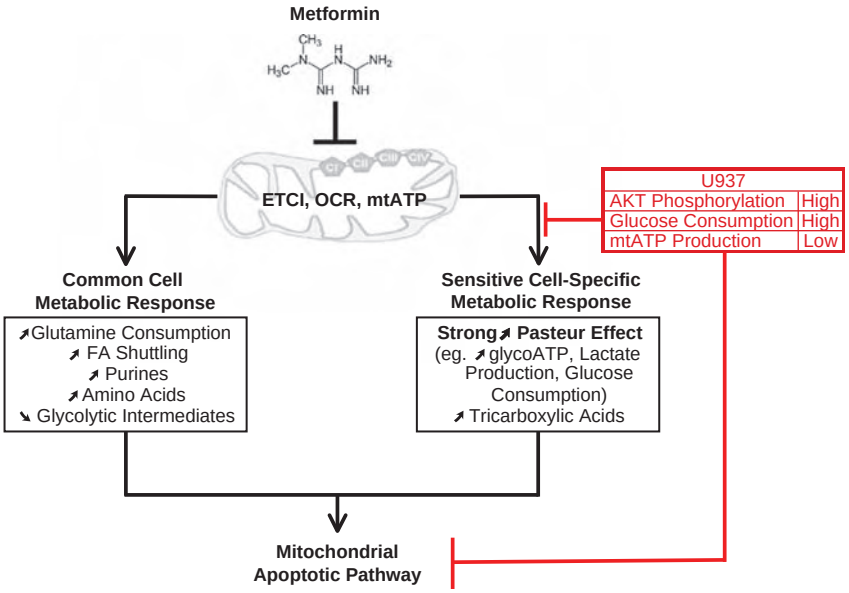


**Figure 7.** Working model of the multifacted mechanism of action of metformin in leukemic cells. *In vitro* metformin induces early energetic changes through the inhibition of ETCI, mitochondrial oxygen consumption (OCR) and mitochondrial ATP production (mtATP). The resulting metabolic response in all cells is an increase in glutamine consumption, fatty acid (FA) shuttling (acetylcarnitine), purines (PRPP, IMP, AMP) and amino acids (methionine), as well as a decrease in glycolytic intermediates. For cells that are sensitive to metformin-induced apoptosis, including HL60 and MOLM14, the specific metabolic response includes a strong increase in the Pasteur effect (for example, increased glycolytic ATP production, glucose consumption and lactate production), as well as an increase in tricarboxylic acids (fumarate and malate) which leads to activation of the mitochondrial apoptotic pathway. Cells that are insensitive to metformin-induced apoptosis, such as U937 cells, block this sensitive cell-specific metabolic response and apoptosis due to high AKT phosphorylation, high glucose consumption and low mitochondrial ATP production in the basal state.

However, between the two cell lines, metformin had the opposite effect on TCA cycle intermediates (α-ketoglutarate, fumarate, malate) and pyruvate dehydrogenase phosphorylation, confirming the importance of mitochondria on both basal energetic status and metformin sensitivity. Accordingly, metformin-insensitive U937 cells exhibited lower basal OCR and are less oxidative and more glycolytic under basal conditions, thereby requiring a smaller metabolic response (for example, Pasteur effect) following ETC1 inhibition by metformin.

The basal metabolome also showed significantly reduced carbon metabolites in the lower segment of glycolysis (Gly-3P to PEP) of U937 compared with MOLM14. This is consistent with our thermodynamic analysis showing FBP formation by PFK is strongly favored in U937 and rapidly consumed in the lower segment of glycolysis as intermediates are withdrawn for biosynthetic reactions. In addition to being converted to lactate, glycolytic intermediates in U937 might be diverted to increase flux through the PPP, to operate the glycerol-3-phosphate shuttle (dihydroxyacetone phosphate) and/or to support glycerol metabolism for biosynthetic purposes, including amino acids and triglycerides. Increased flow through the PPP increases production of purines and NADPH to maintain high GSH levels, which provides a high antioxidant capacity and links energetic reprogramming and redox balance in cancer cells.[34,35] Rerouting glucose flux from glycolysis to PPP does not limit substrate availability in U937 because they sustain survival by alternative, non-glycolytic pathways and substrates (for example, glutamine or fatty acids) as supported by the marked basal accumulation of carnitine and acetylcarnitine. Accordingly, and as recently discovered in other leukemic cells,[36] we observed significant phosphorylation of mitochondrial pyruvate dehydrogenase in both U937 and MOLM14, which suggests mitochondrial acetyl-CoA is derived from glutaminolysis (MOLM14) or glutaminolysis and FA oxidation (U937) rather than pyruvate oxidation. Additionally, accumulation of fumarate and malate in MOLM14 following metformin also suggests these cells might preferentially use glutamine-derived reductive carboxylation of α-KG to produce acetyl-CoA for four carbon intermediates for the TCA cycle, fatty acids, lipids, sterols and lactic acid, as very recently observed in cancer cells upon mitochondrial mutation, acute pharmacological ETC inhibition or hypoxia.[37–39] This is also consistent with high sensitivity to metformin of cMyc-driven glutamine addicted cells (such as HL60).[40]

Finally, and of prime importance, we showed that metformin-insensitive U937 cells have high basal pAKT that has a pivotal role in metformin resistance, especially during early stages of treatment. Increased pAKT might also occur in sensitive cells following metformin treatment as an adaptive mechanism to early AMPK- and mitochondrial-induced metabolic responses. As a key regulator of glucose metabolism, AKT increases translocation of glucose transporters, GLUT1 and GLUT4, to the plasma membrane to increase glucose uptake and aerobic glycolysis.[41–43] AKT also favors hexokinase-II activity and its association with the outer mitochondrial membrane, thereby increasing ATP affinity and providing direct access to mitochondrial ATP.[44] Additionally, hexokinase-II binds specifically to the mitochondrial voltage-dependent anion channel, preventing opening of the permeabilization membrane pore, Cytochrome *c* release and subsequent apoptosis.[45–48] In U937 cells, constitutive activation of the PI3K/AKT pathway by PTEN deletion leads to AKT-dependent stimulation of glucose uptake and metabolism. This confers resistance to the apoptotic and Pasteur effects of metformin in U937 cells, but renders them susceptible to death following glucose withdrawal or glycolytic inhibitors.[49,50] Supporting this conclusion, metformin-mediated apoptosis was induced in U937 and augmented in MOLM14 by metabolic manipulations with AKT and glycolytic inhibitors and glucose starvation. These results show that the basal AKT status of leukemic cells is crucial in determining the ability to elicit a Pasteur effect and to regulate apoptosis in response to metformin.


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In conclusion, and represented in Supplementary Figure 1 and Figure 7, metformin induces an early (within 3 h) effect on mitochondrial energetics leading to long-term (6–24 h) metabolic adaptations (common- and sensitive-cell specific) followed by inhibition of cell cycle progression and cell proliferation and induction of the caspase-3 dependent mitochondrial apoptotic pathway (24–48 h) in leukemic cells. However, the late specific metabolic adaptation (strong induction of the Pasteur effect) and apoptosis do not occur in U937 cells, which already exhibit high basal glucose consumption, glycolysis and AKT activation and are thus metabolically poised to withstand inhibition of ETCI by metformin. Taken together, our study allows for a more general conclusion: metabolic fluxes of leukemic cells for energy production and generation of biosynthetic precursors are important determinants in metformin sensitivity. Our findings concerning the integration and regulation of energetic and metabolic cross-talks between mitochondria and cytosol, and other recent discoveries of cancer-related changes in metabolic pathways,[38,39,51–53] demonstrate that metabolic and energetic flexibility are a common feature of tumor cell metabolism. A deeper understanding of this intrinsic capacity and its targeting are crucial steps to establish new therapeutic strategies in oncology.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## Abstract

Normoglycemic agent, metformin, decreases the risk of cancer in type 2 diabetics and inhibits cell growth in various cancers. Metformin activates AMPK and inhibits electron transport chain complex I (ETCI), but its mechanism of action in cancer cells is unknown. Thus, we investigated metformin's activity in human acute myeloid leukemia (AML) cells. Metformin significantly blocks cell cycle progression and inhibits cell proliferation and colony formation. However, the apoptotic response to metformin varies among AML cell types. Furthermore, daily treatment with metformin induces apoptosis and reduces tumor growth *in vivo*. Metformin induces an AMPK-independent inhibition of mTORC1, which could be responsible for metformin's anti-tumoral activities. Additionally, metformin decreases ETCI activity, oxygen consumption and mitochondrial ATP synthesis, while stimulating glycolysis for ATP and lactate production (so-called Pasteur Effect). AML cells with high basal AKT phosphorylation or glycolysis exhibit a markedly reduced induction of the Pasteur effect in response to metformin and are resistant to metformin-induced apoptosis. Accordingly, glucose starvation or treatment with deoxyglucose or an AKT inhibitor induces sensitivity to metformin. Thus, we propose that activation of AKT, which can phosphorylate hexokinase-II to induce its translocation to the mitochondrial outer membrane pore, VDAC1, blocks cytochrome c release and mitochondrial-induced apoptosis. Overall, this work has allowed us to decipher the role of certain signaling and metabolic pathways in the anti-tumoral response to metformin in AML.

## Résumé

La metformine, utilisée pour le traitement du diabète de type 2, a été décrite comme pouvant réduire le risque de cancer. Cependant, les mécanismes responsables de cette activité n'ont pas encore été élucidés. Nous avons ici décidé d'étudier les effets de la metformine dans les cellules de leucémie aiguë myéloïde (LAM). La metformine bloque la progression dans le cycle cellulaire, inhibe la prolifération et la formation de colonies *in vitro*. *In vivo*, un traitement quotidien à la metformine induit l'apoptose et réduit la progression tumorale. Toutefois, cette induction d'apoptose est variable en fonction des cellules leucémiques. La metformine induit une inhibition de mTORC1 indépendante de l'AMPK, qui peut être responsable des effets anti-tumoraux observés. Par ailleurs, la metformine réduit fortement l'activité du complexe I de la chaîne respiratoire, la consommation d'oxygène et la production d'ATP par la mitochondrie alors qu'elle stimule la glycolyse pour la production d'ATP et de lactate (effet Pasteur). Les cellules leucémiques avec une forte glycolyse ou activation d'AKT montrent une réduction significative de l'induction de l'effet Pasteur et de l'apoptose en réponse à la metformine. Ainsi, la déprivation en glucose ou un inhibiteur de la glycolyse ou d'AKT sensibilisent ces cellules à la metformine. Nous proposons que le mécanisme d'apoptose est bloqué par la phosphorylation et la translocation au pore mitochondrial VDAC1 de l'hexokinase-II en aval d'AKT. En conclusion, ces travaux ont permis de mieux caractériser la signalisation et le métabolisme impliqués dans la réponse anti-tumorale induite par la metformine dans des lignées cellulaires de LAM.