The role of adenosine monophosphate-activated protein kinase (AMPK) in reactive oxygen species (ROS) signalling and cancer cell chemoresistance

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Abstract

The AMP-activated protein kinase (AMPK) is known to play a complex role in tumourigenesis. However, its role in advanced tumours, particularly in contexts such as chemoresistance, is poorly understood. Chemotherapeutic treatment can present tumour cells with unique metabolic challenges, including mitochondrial damage and reactive oxygen species (ROS) induction, suggesting a potential role for AMPK in mediating adaptive responses to these stressors. In this work, we investigate the role of ROS in AMPK signalling, as well as the roles of ROS signalling and particularly AMPK activity in chemoresistant cancers. We find that ROS are able to activate AMPK and induce a program of antioxidant response. When this program is lost due to AMPK ablation, elevated ROS levels induce a program of HIF-1α activation and glycolysis. In the context of chemoresistant p53-null cells, we find that treatment with chemotherapeutics induces both mitochondrial ROS and AMPK activity. AMPK loss does not alter susceptibility to chemotherapy in cells with wild-type p53, but rather abolishes chemoresistance in p53-null cells, a phenotype which is observed in both mouse embryonic fibroblasts and various cancer cell lines. AMPK activity in chemoresistant cells is found to induce a program of lysosomal biogenesis and autophagy, which can allow for recycling of cellular components damaged by drug treatment. This lysosomal program, controlled by the lysosomal transcription factors TFEB and TFE3, is necessary but not sufficient for the chemoresistance of p53-depleted cells. Future work will examine these phenotypes *in vivo*, and further characterize the roles of TFEB and TFE3 in this response.

Résumé

La protéine kinase activée par l'AMP (AMPK) est connue pour jouer un rôle complexe dans la tumorigénèse. Cependant, son rôle dans les tumeurs avancées, en particulier dans des contextes tels que la chimiorésistance, est incomplètement compris. Le traitement chimiothérapeutique peut présenter aux cellules tumorales des défis métaboliques uniques, y compris des dommages mitochondriaux et l'induction d'espèces réactives de l'oxygène (ROS), suggérant un rôle pour l'AMPK dans la gestion des réponses à ces facteurs de stress. Dans ce travail, nous étudions le rôle des ROS dans la signalisation d'AMPK, ainsi que les rôles de la signalisation de ROS et en particulier l'activité de AMPK dans les cancers chimiorésistants. Nous constatons que les ROS sont capables d'activer l'AMPK et d'induire un programme de réponse antioxydante. Lorsque ce programme est perdu en raison de l'ablation de l'AMPK, des niveaux élevés de ROS induisent un programme d'activation de HIF-1α et de glycolyse. Dans le contexte des cellules chimiorésistantes manquant la protéine p53, nous constatons que le traitement par chimiothérapie induit à la fois le production de ROS mitochondriale et l'activation de AMPK. La perte d'AMPK ne modifie pas la sensibilité à la chimiothérapie dans les cellules ayant p53, mais abolit la chimiorésistance dans les cellules sans p53, un phénotype qui est observé à la fois dans les fibroblastes embryonnaires de souris et dans diverses lignées cellulaires cancéreuses. L'activité de AMPK dans les cellules chimiorésistantes induit un programme de biogenèse lysosomale et d'autophagie, qui peut permettre le recyclage des composants cellulaires endommagés par le traitement avec chimiothérapie. Ce programme lysosomal, contrôlé par les facteurs de transcription lysosomale TFEB et TFE3, est nécessaire mais pas suffisant pour la chimiorésistance des cellules appauvries en p53. Les travaux futurs examineront ces

phénotypes *in vivo* et caractériseront davantage les rôles du TFEB et du TFE3 dans cette réponse.

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List of Abbreviations

AA, antimycin A

ABC, ATP binding cassette

ACC, acetyl CoA carboxylase

ADP, adenosine diphosphate

AMP, adenosine monophosphate

AMPK, AMP-activated protein kinase

Asc, ascorbate

ATG, autophagy-related protein

ATM, ataxia-telangiectasia mutated kinase

ATP, adenosine triphosphate

ATR, ataxia telangiectasia and Rad3-related protein

CAMKK2, calcium/calmodulin dependent protein kinase kinase 2

CLEAR, coordinated lysosomal expression and regulation

CTR, copper transporter

DCFDA, 2',7'-dichlorodihydrofluorescein diacetate

DKO, double knockout

DMEM, Dulbecco's modified Eagle medium

DNA, deoxyribonucleic acid

DNA-PK, DNA-dependent protein kinase

ECAR, extracellular acidification rate

ECM, extracellular matrix

ETC, electron transport chain

FACS, fluorescence activated cell sorting

FBS, fetal bovine serum

Glc, glucose

GLUT, glucose transporter

GYS, glycogen synthase

HIF, hypoxia inducible factor

KD, knockdown

KEAP1, Kelch-like ECH-associated protein

KO, knockout

LC3B, light chain 3B

LCMS, liquid chromatography-mass spectrometry

LDH, lactate dehydrogenase

LKB1, liver kinase B1

MDR, multidrug resistance

MEF, mouse embryonic fibroblast

MFI, mean fluorescence intensity

mTOR, mammalian target of rapamycin

mTORC1, mammalian target of rapamycin complex 1

NAC, N-acetyl cysteine

NADPH, nicotinamide adenine dinucleotide phosphate

NEAA, non-essential amino acid

NRF, nuclear factor erythroid 2-related factor 2

OCR, oxygen consumption rate

PARP, poly (ADP-ribose) polymerase

PBS, phosphate-buffered saline

PDK1, pyruvate dehydrogenase kinase 1

PGC-1α, Peroxisome proliferator-activated receptor γ coactivator 1 α

PPAR γ, Peroxisome proliferator-activated receptor γ

PPP, pentose phosphate pathway

PTEN, phosphatase and tensin homolog

qPCR, quantitative polymerase chain reaction

RISP, Rieske iron-sulfur protein

RNA, ribonucleic acid

SASP, senescence-associated secretory phenotype

SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

shRNA, short hairpin RNA

SOD, superoxide dismutase

TIGAR, TP53-inducible glycolysis and apoptosis regulator

ULK, Unc-51 like autophagy activating kinase

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Preface

This thesis incorporates two projects, investigating ROS signalling and AMPK, and the role of AMPK in chemoresistance, respectively. The first project is based on a paper published in Cell Reports (Rabinovitch et al. 2017). This work was completed in collaboration with several co-authors, most notably Brandon Faubert and Bozena Samborska. In this work, I made the PGC-1α ectopic expression and control cell lines, and produced figures 1 D-E, 2 F-G, 6C-F, and 7E,J-K, in addition to contributing to writing, editing, and data analysis.

In the second project, principal contributors were Takla Griss and Joelle Bekhazi, who did crucial preliminary work, Dr Russell Jones, who made the AMPK and p53 knockout cell lines, and Leeanna El-Houjeiri and Dominic Roy, who assisted in making the TFEB/TFE3/p53 knockdown cell lines. Amanda Swain and Maxim Artyomov ran and analyzed RNA-Seq. All other work in this section was my own.

1. Literature Review

1.1. Cancer

Cancer is a disease of uncontrolled growth and impaired death of cells within a tissue. Approximately half of Canadians will develop some form of cancer in their lifetime, and one quarter of Canadians are predicted to die of this disease (Canadian Cancer Statistics Advisory Committee, 2019). The main causes of mortality in cancer patient are chemoresistance (the ability of the tumour cells to survive drug treatment intended to eliminate the tumour) and metastasis (the spread of tumour cells to tissues outside the tissue of origin). Rather than being considered one unified disease, cancer can be better understood as a collection of diseases sharing a central disease process (Hiatt and Rimer 1999). Tumours may appear in a wide variety of tissues in the body, with varying prognoses and appropriate treatments. Risk factors include the presence of particular germline mutations (particularly in genes involved in DNA repair pathways), exposure to mutagens, and age. The specific mutations and mutagens that contribute to development of a tumour in a particular tissue vary. However, all of these factors contribute to the accumulation of mutations in precancerous lesions, thereby bringing the lesion closer to the transformed, malignant state.

Cancer-promoting mutations include those affecting both oncogenes and tumour suppressors. Oncogenes are genes which, when activated by mutation, are able to promote cancer progression. Many oncogenes are growth signalling factors or receptors, which if constitutively activated allow growth independent of external signals. This group of oncogenes includes such well characterized examples as EGFR and HER2 (Moasser 2007; Zandi et al. 2007). Tumour suppressors, on the other hand, are genes that promote oncogenesis when inhibited

by mutation. Tumour suppressors often function in cell cycle control or DNA damage repair, such as Rb, or, most famously, p53 (Levine, Momand, and Finlay 1991; Vélez-Cruz and Johnson 2017).

1.1.1. The tumour suppressor p53

The tumour suppressor p53 is mutated in up to 50% of cancers. However, p53 mutations are particularly common amongst certain types of cancer, with up to 90% of ovarian cancers hosting such mutations (Kandoth et al. 2013). There is also variation in the specific mutations observed, with the most common types including missense mutations (particularly within the DNA-binding domain) and nonsense or truncating mutations (Petitjean et al. 2008; Shirole et al. 2016). Missense mutations can be further classified as contact or structural mutants – that is, affecting either the protein's ability to properly bind DNA and initiate transcription, or affecting the overall structure of the protein, which can lead to an inappropriate shape for DNA binding, or to large-scale unfolding (Bullock, Henckel, and Fersht 2000). These unfolding events may in turn produce aggregates of unfolded mutant and even wild-type protein (Bullock and Fersht 2001; Bullock et al. 2000). Loss of heterozygosity of p53, however, more commonly occurs through segmental deletions (Liu et al. 2016).

The strong tendency of tumours to acquire mutations in p53 highlights this protein's importance in normal cellular function. In healthy cells, p53 plays a variety of roles, many of which relate to preservation of the integrity of the genome. In particular, p53 is used to detect DNA damage, and can initiate a repair response. However, if sufficient damage occurs, p53 will instead initiate an apoptotic response, which prevents a cell with highly damaged DNA from

replicating and transmitting potentially harmful mutations to daughter cells. In this way, p53 is an important safeguard against the development of tumours, which rely on unchecked replication and high mutation rates.

p53 has many sites available for post-translational modifications such as ubiquitination, acetylation, and phosphorylation. Ubiquitination of p53 by MDM2 promotes degradation of the p53 protein when the cell is not experiencing stress that requires p53 activity (Haupt et al. 1997; Kubbutat, Jones, and Vousden 1997). Acetylation of p53 at several C-terminal lysine residues can inhibit MDM2 binding, thereby promoting p53 activity (Tang et al. 2008). Phosphorylation of p53 can occur at several different sites, including serine 15, which promotes activation, serine 20, phosphorylation of which promotes acetylation, and serine 319, which promotes MDM2 binding (Ashcroft, Kubbutat, and Vousden 1999; Dornan and Hupp 2001; Katayama et al. 2004).

The various post-translational modifications that p53 can undergo allow it to integrate many cellular signals when determining cell fate (Joerger and Fersht 2008; Kruse and Gu 2008). Information about various types of DNA damage is relayed to p53, including double strand breaks, which can cause ATM or DNA-PK to phosphorylates p53, and single strand breaks, which can cause ATR to phosphorylate p53 (Canman et al. 1998; Lakin, Hann, and Jackson 1999; Lees-Miller et al. 1992). It is worth noting that in addition to DNA damage, oncogenic activation is relayed to p53 through ARF (Mellert et al. 2007). The integration of this information, accumulated through post-translational modifications, allows p53 to recruit the necessary effectors for either cell cycle arrest and damage repair, or apoptosis (Kumari, Kohli, and Das 2014). In the former case, p53 increases the transcription of p21, which is able to bind

cyclins and thereby inhibit progression through the cell cycle, creating a window in which DNA repair can occur before replication (El-Deiry et al. 1993; Reinhardt and Schumacher 2014; Wade Harper et al. 1993). However, sustained p21 expression can lead to senescence, in which cells become stably non-replicative and undergo phenotypic changes (Chang et al. 1999) (see below). When p53 is instead orchestrating apoptosis, it promotes the transcription of a wide variety of genes, including BH3 domain pro-apoptotic proteins such as Bad, Bax, Puma, and Noxa, as well as components of the extrinsic apoptosis pathway, such as the death receptor Fas (Chen 2016). The BH3 domain proteins upregulated by p53 promote mitochondrial outer membrane permeabilization, which allows release of proteins that promote apoptosome formation and cell death (Chen 2016). In either of these scenarios, p53 plays a crucial role in regulating the cellular response to genotoxic stress.

p53 is also known to play other roles in the cell, particularly in metabolism. Glucose deprivation is known to activate p53, through AMPK-controlled phosphorylation at serine 15 (Jones et al. 2005). Activation of p53 can have diverse effects on metabolism, including promotion of oxidative phosphorylation through transcription of cytochrome c oxidase 2 (SCO2), increased aspartate uptake through expression of SLC1A3, and altered responses to nutrient starvation (Maddocks et al. 2013; Matoba et al. 2006; Tajan et al. 2018). Another metabolic target of p53 is TP53- inducible glycolysis and apoptosis regulator (TIGAR). TIGAR (TP53- inducible glycolysis and apoptosis regulator) is upregulated by p53 under relatively low levels of cellular stress. TIGAR acts to convert fructose2,6-bisphosphate to fructose 6-phosphate, in reverse of the typical activity of glycolysis (Bensaad et al. 2006). High TIGAR activity acts to decrease the rate of glycolysis in the cell, and can push glucose to enter the

pentose phosphate pathway (PPP) instead. This increased PPP flux can in turn increase reducing equivalents available within the cell, a function that is in line with the ability of p53 to upregulate expression of several antioxidant genes (Hussain et al. 2004).

1.1.2. Treatment of cancer

Treatment of cancer can take several possible modalities. Common treatment avenues include surgery, radiation, and chemotherapies. Many cancer patients will experience multiple treatment modalities depending on the specific characteristics of their illness. Surgical interventions are the gold standard for cancer treatment and are frequently used for solid tumours, particularly in their earlier stages. The goal of surgical interventions is generally to remove the tumour, as well as a tumour-free margin of tissue surrounding it in order to reduce the likelihood of recurrence. Surgery may also be used in a palliative context, in order to reduce the bulk of a tumour that is compressing another structure or causing pain. However, many tumours cannot be surgically excised, whether due to an intractable location, the type of tumour, or the presence of metastases (the spread of tumour cells to sites distant from the initial tumour) at diagnosis. In these cases, other treatment modalities are used instead of or in addition to excision.

Tumours can also be treated with ionizing radiation, which acts to damage tumour DNA either directly or indirectly through formation of free radicals. Generally, several beams of radiation are directed at the tumour from different angles, in order to direct a high dose of radiation at the tumour while leaving the surrounding healthy tissue relatively undamaged.

Chemotherapy is the use of drugs to preferentially or selectively kill tumour cells. There are a wide variety of chemotherapeutics available, with varying levels of appropriateness for different tumour types. Broadly, chemotherapeutics may be considered to either targeted or cytotoxic. Targeted drugs act to interfere with the activity of a particular protein known to be overexpressed, mutated, or otherwise altered in a cancer. For example, drugs such as imatinib are used to inhibit activity of the BCR-ABL fusion present in chronic myeloid leukemia, enabling growth of this cancer to impaired (Iqbal and Iqbal 2014). Targeted drugs may also be used to pursue a strategy of synthetic lethality, wherein pharmacological inhibition of one protein or pathway synergizes with mutations already present in the cancer cell to lead to cell death. For example, in BRCA-mutant cancers, PARP inhibitors increase accumulation of single strand breaks, which cause replication forks to stall and convert these sites into double strand breaks. BRCA-mutant cells, which have impaired homologous recombination, then must repair these breaks with a lower-fidelity method, promoting cell death (Ashworth 2008). Cytotoxic drugs, on the other hand, are designed to take advantage of the high growth rates of cancer cells. These drugs tend to inhibit processes required for cellular replication and thereby kill cells that move through the cell cycle quickly, such as cancer cells, although healthy fast-cycling cells types such as hair follicle or digestive tract cells can also be killed, thereby contributing to some side effects of these drugs (Bodó et al. 2007; Keefe et al. 2000).

Two or even all three of these treatment modalities may be combined in the treatment of an individual patient, and there are many ways in which these treatments may reinforce one another. For example, chemotherapy may be used before surgery, to diminish the size of the tumour and ease its removal, or afterwards, to prevent recurrence. Since many

chemotherapeutics are unable to pass the blood-brain barrier, some chemotherapy regimens are used in combination with radiotherapy to the brain, to ensure that tumour cells cannot use the brain as a refuge site during treatment.

There are several classes of cytotoxic chemotherapeutics used in cancer treatment. These include antimetabolites, alkylating agents, topoisomerase inhibitors, anthracyclines, and mitotic inhibitors. Antimetabolites include drugs such as 5-fluorouracil and gemcitabine. These drugs resemble pyrimidines or purines, and consequently can bind enzymes involved in DNA synthesis. However, they cannot be successfully incorporated into DNA, leading to an inability of the treated cell to undergo DNA replication, which in turn leads to cytostasis. Because tumour cells undergo quick rounds of proliferation, they spend more time engaged in DNA replication, and so are more susceptible to these drugs than healthy cells.

1.1.2.1 Cisplatin

Alkylating agents were among the earliest chemotherapeutics to be identified, and act by directly alkylating DNA. When multiple alkylating events occur within the cell, this can lead to inter- or intra-strand crosslinks, which impair DNA replication and transcription, as well as interfering with the proper DNA sequence (Dasari and Tchounwou 2014). While this effect may occur in healthy cells in addition to within the tumour, healthy cells tend to have a robust DNA repair response able to counteract these drugs' effects. Tumour cells, on the other hand, tend to undergo rapid replication with little DNA repair, meaning that any errors introduced by alkylating agents can persist, leading to further damage and eventually cell death. It is worth noting that in addition to classical alkylating agents, other drugs known as alkylating-like agents

are also used in chemotherapy. This group includes the platinum group drugs, which do not alkylate DNA but nevertheless form crosslinks through binding to the drug.

Cisplatin is a member of the alkylating-like platinum group drugs, and therefore acts by creating crosslinks within the DNA by binding to guanine. Cisplatin can damage both nuclear DNA and mitochondrial DNA, which in addition to interfering with nuclear DNA replication, causes additional damage by impairing proper electron transport chain function and promoting reactive oxygen species (ROS) production in the mitochondria (Marullo et al. 2013; Yang et al. 2006). Cisplatin was initially noted for its ability to prevent cell division in bacteria (Rosenberg, van Camp, and Krigas 1965), and was first approved for use in human cancers in 1978. Since then it has become a common chemotherapeutic, used for cancers such as ovarian, lung, and breast (Florea and Büsselberg 2011).

1.1.2.2. Etoposide

Topoisomerase inhibitors are drugs that block the activity of the enzymes topoisomerase I or II. Topoisomerases are enzymes that cut DNA strands to allow resolution of tension in the double helix caused by over- or under-winding. Topoisomerase I cuts, unwinds, and re-ligates a single strand of DNA, and is targeted by drugs such as camptothecin. Topoisomerase II performs this procedure on both strands of a given double helix, and is targeted by two main types of drugs: inhibitors, which target the enzyme's ATPase domain, and poisons, which bind to the enzyme-DNA complex. Inhibition of either of these enzymes leads to single or double strand breaks, respectively, as well as other DNA abnormalities (Hande 2008).

Etoposide is a topoisomerase II poison, meaning that, as described above, it binds to the topoisomerase II-DNA complex and prevents DNA re-ligation, leading to double strand breaks. In addition to damaging nuclear DNA, etoposide can also promote mitochondrial dysfunction and ROS production, leading to further cellular damage, as well as inducing a mitochondrial recycling program (Shin et al. 2016; Yadav et al. 2015). Etoposide was initially derived from podophyllotoxin, and has been used as a chemotherapeutic since 1983. It is currently used in the treatment of a diverse set of cancers, including some leukemias and lymphomas, ovarian cancer, and lung cancer (Hande 1998).

1.1.2.3. Doxorubicin

Anthracycline antibiotics comprise several related drugs, inducing doxorubicin, daunorubicin, and epirubicin. The class of drugs is united by their discovery as a product of the bacteria *Streptomyces peucetius*, or their development as analogues of the naturally-occurring molecules. These drugs are known to act as topoisomerase II poisons (see above). However, other mechanisms of tumour cell killing have also been observed, including DNA intercalation and adduct formation (Cutts et al. 2005; Swift et al. 2006), as well as induction of ROS via inhibition of complexes I and II (Gammella et al. 2014; Mukhopadhyay et al. 2007). It is worth noting that intercalation into the DNA is also known to occur in the mitochondria, further contributing to ROS production in cells treated with these drugs (Serrano et al. 1999). These drugs' interference with DNA structure can prevent processes such as DNA replication that are crucial to tumour cell proliferation, while ROS production can lead to cell death through excessive oxidative damage.

Doxorubicin was one of the original anthracyclines to be discovered, in a mutated sample of *Streptomyces peucetius* after the discovery that daunorubicin was effective against murine tumours. Since 1974 doxorubicin has been used in the treatment of various cancers, including breast, ovarian, and lung tumours (Hortobagyi 1997). Doxorubicin treatment carries a risk of cardiotoxicity, which is thought to be caused by this drug's ability to impair mitochondrial function (Brookins Danz et al. 2009). This can be exacerbated by the ability of doxorubicin to accumulate in the mitochondria through direct binding of cardiolipin in the membrane (Aryal and Rao 2016; Parker, King, and Howard 2001).

1.1.2.4. Paclitaxel

Mitotic inhibitors are a class of drugs that prevent the cancer cell from undergoing its customary high rate of cell division by inhibiting microtubule function. Since microtubules are required for the separation of chromosomes during anaphase, they are indispensable for replication. There are two classes of mitotic inhibitors commonly used in the treatment of cancer: vinca alkaloids and taxanes. The vinca alkaloids, which are derived from the plant *Catharanthus roseus,* include drugs such as vincristine and vinblastine. They act by inhibiting tubulin polymerization (Jordan, Thrower, and Wilson 1991). Taxanes, by contrast, bind to microtubules and prevent their depolymerization (Ahn et al. 2004; Jordan et al. 1993; Schiff, Fant, and Horwitz 1979). This group of drugs is derived from plants of the genus *Taxus* and includes paclitaxel and docetaxel. Although their mechanisms are opposed, both groups of drugs disrupt normal microtubule function and thereby prevent cell division.

Paclitaxel, often known by its brand name Taxol, is a taxane. As described above, this means that this drug inhibits microtubule depolymerization, thereby interfering with mitosis. Paclitaxel was the first taxane to be discovered, in 1971, and was approved for use in cancer treatment in 1993. Paclitaxel is used in the treatment of ovarian, breast, lung, and prostate tumours, among others (Weaver 2014). While the primary mechanism of action of paclitaxel is via mitotic inhibition, it has also been found to induce mitochondrial dysfunction, particularly depolarization and permeabilization, which can also contribute to cell death (Galley et al. 2017; Kidd et al. 2002).

1.1.3. *Therapeutic resistance in cancer*

Any drug used in the treatment of cancer can be susceptible to the development of resistance by tumour cells. This resistance, also known as chemoresistance, may occur in one of two ways: tumours may harbour populations of cells that are inherently resistant, which are selected for upon treatment, or cancer cells may be able to induce survival programs when they are exposed to drugs. Evidence for both of these modes of chemoresistance have been found in several cancer types (Abolhoda et al. 1999; Meena et al. 2013). In the case of resistant populations, much work has suggested an overlap between these populations and cancer stem cell populations (Auffinger et al. 2014; Shien et al. 2013), with possible mechanisms of resistance including increased ROS scavenging (Diehn et al. 2009) and increased DNA repair (Bao et al. 2006).

Survival programs used by cancer cells under treatment with chemotherapeutic agents are varied. Components of the response may include upregulation of pro-survival signalling,

export of the drug, reduction of drug uptake, metabolism of the drug to a less harmful form, or sequestration of the drug. Export of drugs commonly occurs via expression of the multi-drug resistance protein, known as MDR, p-glycoprotein, or ABCB1. This protein is a transmembrane transporter able to export a wide range of substrates from the cell in an ATP-dependent manner, including doxorubicin, etoposide, and vinblastine (Zheng 2017). Several other members of the ABC transporter family can also be used by cancer cells to increase drug efflux (Korita et al. 2014; Wakamatsu et al. 2007).

Reduction of drug uptake can also promote resistance to treatment. A primary mechanism by which tumour cells reduce drug uptake is by reducing the expression of the proteins that can carry drugs into the cell. Members of the organic anion-transporting polypeptide or organic cation transporter families can be expressed at lower levels to reduce uptake of drugs ranging from paclitaxel to anthracyclines (Buxhofer-Ausch et al. 2013; Koepsell, Lips, and Volk 2007), while the expression of copper transporter 1 (CTR1) has been found to decrease in response to treatment with platinum derivatives, as this protein can transport this drug type in addition to its normal substrate (Kalayda, Wagner, and Jaehde 2012). In addition to this mechanism, tumours may also alter drug uptake through changes to the extracellular matrix (ECM) that impair drug arrival at the tumour site. These changes may include production of leaky blood vessels through disorganized angiogenesis, which disrupts drug transit to the tumour through the circulation, and increased expression of certain ECM proteins such as collagen that impair drug diffusion through the ECM (Senthebane et al. 2017).

Alterations in drug metabolism may also be used as a chemoresistance strategy in cancer cells. The exact nature of this strategy depends on the nature of the drug in question, as

some drugs are administered in an active form, while others require some amount of cellular processing to become active. Accordingly, cancer cells may upregulate or downregulate expression of relevant genes in order to reduce the amount of the active form of the drug within the cell (Marin et al. 2014). It is worth noting that glutathione S-transferase, in addition to its role in managing the redox status of the cell, can also function to transfer reduced glutathione groups onto drugs such as the platinum derivatives, an alteration which increases their water solubility and potential diffusion out of the cells (Marin et al. 2014; Meijer et al. 1992).

Drugs including chemotherapeutics can be sequestered in the lysosome. Many chemotherapeutic drugs, such as doxorubicin and vincristine, are hydrophilic and weakly basic. These properties allow the drug to travel trough cellular membranes passively, which is advantageous in that it precludes straightforward reduction of drug uptake. However, if a weakly basic drug diffuses into the highly acidic lysosome, it can be protonated, an alteration that renders the drug hydrophilic and unable to cross the lysosomal membrane back into the cytosol, resulting in accumulation of the drug. This phenomenon has been demonstrated in several different drugs, from various classes (Gotink et al. 2015; Herlevsen et al. 2007).

1.2. AMPK

1.2.1 Structure

AMPK is a protein kinase complex found in all eukaryotic cells that functions as a crucial cellular energy sensor. AMPK is a heterotrimeric protein complex consisting of an alpha, beta, and gamma subunit (Figure 1). Each subunit has multiple isoforms (two each for alpha and beta,

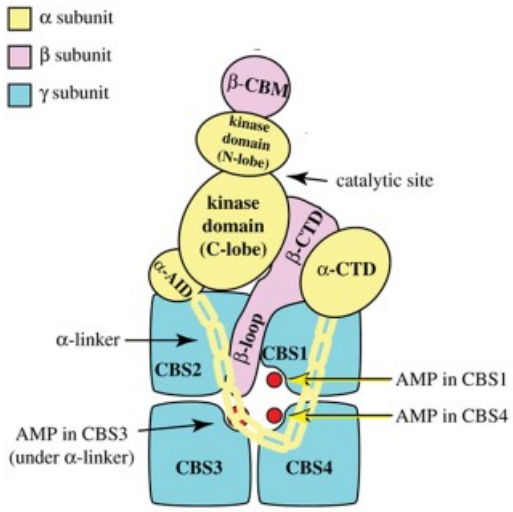


Figure 1. *Structure of AMPK*. Schematic diagram of the structure of the AMPK heterotrimer, with the different subunits colour coded (α, yellow; β, lilac; γ blue). The catalytic site in the α subunit and the CBS domains in the γ subunit are highlighted. (Vara-Ciruelos, Russell, and Hardie 2019)

and three for gamma), allowing for 12 combinations in total, although different subunits occur in different proportions from tissue to tissue (Garcia and Shaw 2017). Each of the subunits of AMPK has a specific function. The alpha subunit acts as the catalytic subunit, while the beta subunit performs a scaffolding role. The gamma subunit contains four CBS domains, which are able to bind adenylate molecules (e.g. AMP, ADP, and ATP). Of these four sites, CBS2 remains empty, CBS1 always binds ATP, and CBS 4 always binds AMP (Vara-Ciruelos, Russell, et al. 2019). At CBS3, however, there is no permanently bound adenylate molecule, and so it is at this site that the energetic status of the cell is assessed.

When the cell is not metabolically stressed, ATP is abundant. Under these conditions, binding of ATP to CBS3 results in blunting of AMPKα catalytic activity. However, when the cell becomes metabolically stressed, ATP levels are depleted and ADP levels rise. This cellular pool of ADP can then be processed by adenylate kinase to form AMP as well as regenerated ATP. While there is evidence that under some conditions ADP can bind to CBS3 of AMPK and promote its activation, AMP is the main signal in this pathway (Gowans et al. 2013). Since AMP is normally present in cells at very low concentrations, even a small increase in the number of molecules present has a dramatic effect on concentration and therefore AMPK binding, rendering this protein highly sensitive to energetic stress. When AMP binds this site, this promotes conformational changes that increase allosteric activation of AMPK, as well as promoting activating phosphorylation at threonine 172 by upstream kinases such as LKB1, and inhibiting dephosphorylation by regulatory phosphatases such as PP2C alpha (Davies et al. 1995; Hardie, Schaffer, and Brunet 2016). This activation of AMPK promotes its phosphorylation of downstream target proteins, which contributes to increases in catabolism and reductions in anabolism, in order to conserve ATP until better conditions arise.

1.2.2 Regulation of AMPK

In addition to regulation by the adenylate ratio of the cell, AMPK is also regulated by other kinases, specifically LKB1 and CamKK2 (Figure 2). LKB1, another serine-threonine kinase, is a known tumour suppressor in multiple cancers (Marignani 2005). Germline mutation of LKB1 promotes Peutz-Jeghers Syndrome, an autosomal dominant cancer predisposition characterized by the development of benign gastrointestinal polyps and an increased risk of

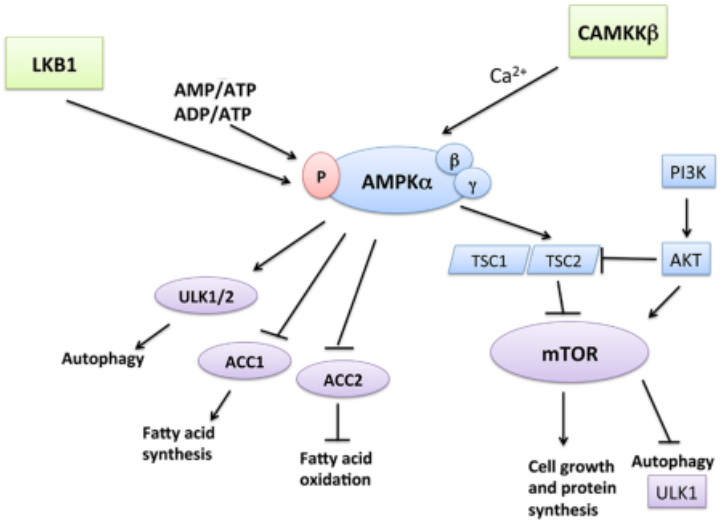


Figure 2. *AMPK signalling pathways*. AMPK is activated when the AMP/ATP ratio in the cell is increased by metabolic stresses such as hypoglycemia, leading to the activation of LKB1. CaMKKβ can also activate AMPK in response to increased intracellular calcium. AMPK acts to promote catabolic and inhibit anabolic pathways. For example, AMPK phosphorylation promotes autophagy through activation of ULK, and inhibits fatty acid synthesis through inhibition of ACC. AMPK also inhibits activity of mTOR through phosphorylation of TSC2. (Kim and He 2013)

developing cancer later in life. (Hemminki et al. 1998). LKB1 loss is also known to occur in up to 34% of non-small cell lung cancers (Ji et al. 2007). LKB1 phosphorylates AMPK at threonine 172 on the alpha subunit, and requires its two accessory subunits, STRAD and MO25 for proper function (Zeqiraj et al. 2009). Evidence suggests that the activity of LKB1 is constant regardless of energetic conditions, and that it is instead the changes caused by AMP binding to AMPK that

control phosphorylation and activation (Sakamoto et al. 2004). It is worth noting that LKB1 is also able to phosphorylate several other AMPK-related kinases (ARKs), which have roles in the control of cell polarity and survival (Sun et al. 2013).

Calcium/calmodulin-dependent protein kinase kinase 2 (CAMKK2) can also act as a kinase for AMPK, phosphorylating and promoting activity of AMPK under conditions of high intracellular calcium (Fogarty et al. 2016). When levels of calcium in the cell increase, a portion of this calcium binds the calcium-sensing protein calmodulin. Calmodulin is then able to transduce this signal by binding to other calcium-dependent proteins, including CAMKK2. CAMKK2 is not expressed in all tissues, and its interaction with AMPK is best characterized in the hypothalamus, where this pathway is involved in regulation of the expression of neuropeptide Y and thereby influences appetite (Anderson et al. 2008).

It is also worth noting that recent evidence has highlighted the importance of localization in AMPK function. In situations of low cellular energy, recruitment of axin, LKB1, and AMPK to regulator-vacuolar ATPase complexes at the lysosomal membrane can promote activation of AMPK (Zhang et al. 2013, 2014). One signal that can play a role in this process is fructose 1,6 bisphosphate (FBP), a glycolytic intermediate which is normally processed by aldolase. When glycolytic flux is low, FBP levels are reduced and unoccupied aldolase can promote formation of this lysosomal complex and thereby encourage AMPK activation (Zhang et al. 2017). This signalling through aldolase occurs via inhibition of TRPV channels, which can then prime the vacuolar ATPase to recruit other complex members (Li et al. 2019). AMPK localization, whether to the lysosome or perhaps to the mitochondria (Liang et al. 2015), is a

developing area of research and is likely to reveal layers of additional complexity in the metabolic regulation of the cell.

1.2.3. Role of AMPK in metabolism

The need for cells to meet the metabolic demands of growth and proliferation places pressure on the cell to properly coordinate cellular metabolism in response to external cues and environmental stresses. Resources must be expended in anabolic processes only when they are present in sufficient quantities that their use will not jeopardize the survival of the cell. AMPK acts to preserve cellular homeostasis by promoting catabolism and inhibiting anabolism, thereby conserving energy under metabolically stressful conditions. This is accomplished through several pathways, including those related to nutrient transport, fatty acid oxidation, mTOR suppression, autophagy, and mitochondrial biogenesis. Each of these pathways is crucial to the overall goal of preserving cellular ATP supply.

AMPK is able to promote nutrient uptake via several mechanisms, including increasing translocation of the glucose transporter GLUT4 to the cellular membrane, promoting glucose uptake (Kurth-Kraczek et al. 1999), increasing activity of the glucose transporter GLUT1 (Barnes et al. 2002), and increasing fatty acid uptake via CD36 (Habets et al. 2009). Each of these allow the cell to increase flux through glycolytic and oxidative phosphorylation pathways, which in turn allows increased ATP production. AMPK also inhibits the diversion of glucose from glycolysis to glycogen production through phosphorylation of the glycogen synthase enzymes GYS1 and GYS2 (Bultot et al. 2012). Similarly, AMPK phosphorylation of ACC1 and ACC2 inhibits fatty acid synthesis and promotes fatty acid oxidation, allowing further flux of this fuel into the

oxidative phosphorylation pathway (Fullerton et al. 2013), while also conserving NADPH pools (Jeon, Chandel, and Hay 2012).

In contrast to the catabolic processes regulated by AMPK, suppression of mTOR activity by AMPK acts to reduce anabolic activity in the cell. AMPK activates TSC2 via phosphorylation (Inoki, Zhu, and Guan 2003) and inhibits Raptor via promotion of 14-3-3 binding (Gwinn et al. 2008), both of which serve to reduce mTOR activity. mTOR, in turn, serves as another crucial energetic monitoring system within the cell, and can be incorporated into two complexes. mTORC2 promotes anabolism through Akt activation, but also plays a role in actin organization and promotion of cellular migration (Oh and Jacinto 2011). mTORC1 integrates signals including amino acid availability (Chantranupong et al. 2016; Wolfson et al. 2016) and growth factor signalling (Menon et al. 2014) to promote anabolic processes when conditions are appropriate. These processes include mRNA translation and biosynthesis of proteins, lipids, and nucleotides (Ben-Sahra and Manning 2017; Porstmann et al. 2008; Sonenberg and Hinnebusch 2009). mTORC1 is also known to inhibit autophagy via inhibitory phosphorylation of ULK1 and ATG13 (Kim et al. 2011; Puente, Hendrickson, and Jiang 2016). AMPK input on mTOR therefore inhibits these activities, conserving the ATP they would otherwise consume.

Another crucial downstream target of AMPK is PGC-1α. PGC-1α is a transcriptional co-activator, and is known to regulate the activity of several transcription factors, most prominently PPARγ, NRF1, and NRF2 (Puigserver et al. 1998; Wu et al. 1999). Consequently, PGC-1α functions primarily to promote mitochondrial biogenesis, as well as to increase transcription of antioxidant genes. AMPK upregulates the activity of PGC-1α by phosphorylating two sites, T177 and S538, which causes PGC-1α to promote increased transcription of its

targets, including itself (Jäger et al. 2007). AMPK is also thought to promote PGC-1α activity through its effects on NAD+ metabolism and sirtuin 1 activity (Cantó et al. 2009). It is worth noting that upon constitutive AMPK activity, PGC-1α-dependent mitochondrial biogenesis is dramatically increased, leading to a ROS-induced upregulation of glycolysis that outstrips the effects of PGC-1α on antioxidant function (Yan et al. 2014).

AMPK is also able to promote autophagy, the process whereby macromolecules or organelles are digested by the cell, allowing recycling of damaged cellular components or scavenging of nutrients in conditions of scarcity (Hale et al. 2013). Autophagy is a complex process involving the recruitment of a cascade of proteins to the autophagosome membrane, and eventually resulting in the fusion of the autophagosome with a lysosome to allow digestion (Hale et al. 2013). The autophagic process is initiated by the ULK protein complex, which is in turn regulated by phosphorylation by metabolic regulators. Specifically, while mTOR acts to inhibit ULK during energetically favourable conditions, AMPK acts to promote ULK activity and therefore autophagy (Egan et al. 2011; Kim et al. 2011). This dual regulation allows for fine-tuning of the autophagic response to the exact metabolic environment of the cell (Egan et al. 2011; Kim et al. 2011).

An additional pathway through which AMPK affects both mitochondrial homeostasis and autophagy is through interaction with TFEB and TFE3, which are members of the MITF transcription factor family. This group of transcription factors, which also includes MITF and TFEC, is known to homo- or hetero-dimerize with other MITF family members (Hemesath et al. 1994). This group of transcription factors is well known for their role in lysosomal homeostasis. MITF, TFEB, and TFE3 have been shown to upregulate transcription of lysosomal genes and

thereby promote lysosomal biogenesis (Martina et al. 2014; Palmieri et al. 2011; Ploper et al. 2015). These target genes are also referred to as the Coordinated Lysosomal Expression and Regulation (CLEAR) network (Palmieri et al. 2011). The CLEAR network comprises a wide variety of lysosomal genes, including components of the lysosomal membrane, the vacuolar ATPase essential for maintaining lysosomal pH, and numerous enzymes involved in digesting lysosomal contents (Palmieri et al. 2011). Regulation of TFEB transcriptional activity is largely through mTOR, which phosphorylates TFEB, upon which TFEB is bound by 14-3-3 proteins that sequester it in the cytosol and prevent transcription (Roczniak-Ferguson et al. 2012). However, AMPK has been shown to promote TFEB transcriptional activity (Young et al. 2016), potentially via PGC-1α (Tsunemi et al. 2012). Through these interactions, AMPK activity promotes lysosomal biogenesis, which in turn increases the cell's capacity for digestion of autophagosome contents, an effect consistent with the role of AMPK in promoting autophagy in particular and catabolism in general.

1.2.4. Role of AMPK in cancer metabolism

Metabolic reprogramming is known to be a hallmark of cancer (Hanahan and Weinberg 2011). The observation that cancer cells produce large amounts of lactate even when sufficient oxygen is present to allow for oxidative phosphorylation was made in the 1920s by Otto Warburg (Potter, Newport, and Morten 2016; Warburg, Wind, and Negelein 1923). While glycolysis produces ATP less efficiently than oxidative phosphorylation, many cancer cells can upregulate glucose uptake to compensate (Carvalho et al. 2011; Potter et al. 2016). This preference for glycolysis is thought to serve several possible functions for the cancer cell,

including more rapid production of ATP, as well as greater flux from glycolytic intermediates into various biosynthetic pathways including synthesis of nucleotides from glucose-6-phosphate and serine from 3-phosphoglycerate. This upregulation of biosynthetic pathways can better support cellular replication (Liberti and Locasale 2016). To further promote biosynthesis of macromolecules, many tumours have elevated mTOR activity, an observation that has been used to develop mTOR-targeting drugs for use against tumours (Fruman and Rommel 2014).

As a crucial metabolic regulator, one might expect AMPK to play a role in the metabolic alterations that occur in cancers, but its role is complex. While loss of AMPK is rare in most cancer types (Zadra, Batista, and Loda 2015), mutations in Stk11, the gene encoding LKB1, are common in non-small cell lung cancer and occur to some extent in other cancers, including pancreatic and HPV-associated cervical tumours (Su et al. 1999; Wingo et al. 2009). LKB1 loss in tumours can lead to changes in basal AMPK activity and the ability of cells to respond to energetic stress (Shackelford et al. 2013). Loss of AMPK activity is known to promote the Warburg effect via increased HIF-1α signalling (Faubert et al. 2013). Loss of AMPK activity can also allow for increased mTOR activity in tumours (Carretero et al. 2007). Perhaps unsurprisingly then, loss of AMPK has been shown to accelerate tumourigenesis in some cancer models (Faubert et al. 2013; Vara-Ciruelos, Dandapani, et al. 2019). Several epidemiological studies have suggested that pharmacological AMPK activation may reduce the incidence of cancer, although this remains controversial (Azoulay et al. 2011; DeCensi et al. 2010). However, several types of tumour appear to require AMPK for successful growth, particularly in the context of anchorage-independent growth, a metabolically and oxidatively stressful process that is important in the metastatic cascade (Eichner et al. 2019; Jeon et al. 2012; Saito et al.

2015). These data suggest that AMPK can play either a pro- or anti-tumourigenic role, depending on the specific context of the tumour and its metabolic environment (Faubert et al. 2015).

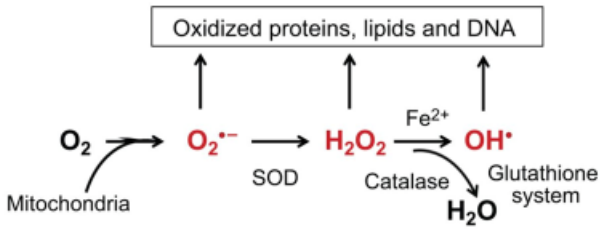
1.3. Reactive oxygen species (ROS)

Reactive oxygen species (ROS) are highly chemically reactive molecules containing oxygen. While oxygen-consuming processes throughout the cell may produce ROS, the main source of these molecules is the mitochondria. Normally in the process of oxidative metabolism, electrons are passed through the electron transport chain (ETC) and used to power ATP production at complex V. However, some leakage is inevitable, and this allows electrons to escape and react with oxygen molecules to form ROS. This leakage resulting in ROS production occurs in approximately 1-3% of electrons passing through the ETC (Valko et al. 2007). As mentioned above, the remainder of ROS production occurs elsewhere in the cell, most predominantly at the cell membrane through the activity of NADPH oxidases. NAPDH oxidases function to produce superoxide, which can then be employed in processes such as defense against pathogens (Graham et al. 2007).

Because of the large role of mitochondrial metabolism in ROS formation, the amount of ROS produced by the cell is heavily influenced by the rate of oxidative metabolism. As more electrons pass through the ETC, there is more opportunity for leakage and ROS formation. Furthermore, metabolically stressful conditions such as hypoxia can also increase ROS production (Bell et al. 2007). Consequently, any factors that affect the metabolic conditions in the cell can also affect the production of ROS.

1.3.1 Types of ROS and their interconversions

Three main types of ROS are particularly relevant to the cell (Figure 3). These are the superoxide anion, hydrogen peroxide, and the hydroxyl radical. The initial reaction between a free electron and a molecule of oxygen produces the superoxide anion. The superoxide anion is highly reactive with proteins that contain an iron-sulfur cluster, and so is at high risk of causing damage in the mitochondria, where such proteins are abundant (Stehling and Lill 2013). However, the cell expresses several isoforms of the superoxide dismutase protein (SOD1-3), which can convert the superoxide anion into hydrogen peroxide. Hydrogen peroxide is the primary vector of ROS signalling in the cell (see below for details), but this is not its only possible fate in the cell. Over time, two molecules of hydrogen peroxide can react to form water and molecular oxygen. Several enzymes within the cell, such as peroxiredoxins and catalase, can accelerate this reaction. Hydrogen peroxide can also react with ferrous ions to form the hydroxyl radical. The hydroxyl radical is able to oxidize and thereby damage or inactivate a variety of biological molecules, including proteins, lipids, and nucleotides. There are no enzymes in the cell capable of detoxifying the hydroxyl radical (Schieber and Chandel 2014). ROS damage to cellular components can have several detrimental effects on the cell. Of particular note are cessation of normal protein functions due to direct damage via oxidation, genomic instability due to damage to DNA, and senescence (Nelson et al. 2018; Rubattu et al. 2015). Cellular damage particularly to DNA by ROS, can induce cells to undergo senescence, in which cells cease replication and undergo changes in chromatin structure, metabolism, and the secretome (McHugh and Gil 2018). The secretory program that senescent cells undergo, also



known as the senescence-associated secretory phenotype (SASP), including many cytokines

Figure 3. *Interconversion of reactive oxygen species*. Mitochondrial O_2 metabolism is a significant source of reactive oxygen species (ROS) production. Types of ROS include superoxide ($O2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical ($OH^{\cdot}$), any of which can damage cellular components. $O2^{\cdot-}$ can be reduced to H_2O_2 by superoxide dismutase (SOD). H_2O_2 can be further reduced to water (H_2O) by catalase, or can spontaneously oxidize iron (Fe^{2+}) to form the highly reactive $OH^{\cdot}$. (Bigarella, Liang, and Ghaffari 2014)

that can act to promote senescence in other nearby cells as well (Kuilman and Peeper 2009; Nelson et al. 2018). Senescence can be induced through the action of p53, and is thought to play a role in limiting the growth of damaged or potentially tumourigenic cells, but has also been found to play a significant role in the progression of aging (Chang et al. 1999, 2015; Kang et al. 2011; Sun, Youle, and Finkel 2016).

Because of the potential for damage associated with high cellular levels of ROS, there are several mechanisms in place to control ROS levels. The expression of the antioxidants mentioned above, as well as others such as those involved in the glutathione system, are controlled by PGC-1α and Nrf2. PGC-1α is a transcriptional coactivator with a crucial role in mitochondrial biogenesis, as well as promoting the expression of many antioxidant genes

through its ability to co-activate Nrf2 (Wu et al. 1999) (see above for details). Nrf2 is a transcription factor that promotes the transcription of antioxidant genes, as well as detoxifying genes (Hayes and Dinkova-Kostova 2014; Itoh et al. 1997). It is regulated by KEAP1, which sequesters it in the cytoplasm and facilitates its ubiquitination by Cullin3. However, under conditions of oxidative stress, cysteine residues in KEAP1 can be oxidized, impairing its ability to bind Nrf2 and allowing Nrf2 to travel into the nucleus and aid in transcription of its targets (Cullinan et al. 2004; Kobayashi et al. 2004; Zhang et al. 2004).

1.3.2. ROS in signaling

While the ability of ROS to damage cellular components is important, so too is their ability to participate in signal transduction. Hydrogen peroxide, in particular, plays a role in several signalling pathways. In brief, hydrogen peroxide oxidizes cysteine residues on relevant proteins, converting the cysteine to its sulfene form, which leads to changes in protein conformation and therefore function. This change is not permanent, and can be reversed by activity of thioredoxins and glutaredoxins (Lee et al. 1998; Winterbourn and Hampton 2008). It is also important to note that this reaction can occur at much lower concentrations than are required for significant cellular damage, and may occur in a localized manner (Schieber and Chandel 2014; Woo et al. 2010).

While many proteins may be susceptible to this type of redox signalling, one of the best studied examples occurs in the PI3K/Akt pathway. Protein phosphatases such as PTEN or PP2A can be oxidized and inactivated in this manner, preventing dephosphorylation of PIP3 or Akt itself, respectively (Lee et al. 1998; Shimura et al. 2016). This allows Akt to promote mTOR

activity, glucose metabolism, proliferation, and numerous other pathways (Manning and Toker 2017). This ROS-induced activation is known to occur in several types of cancer (Schieber and Chandel 2014), a fact consistent with the well-documented tendency of tumours to have higher levels of ROS than comparable untransformed tissues (Yang et al. 2018).

1.4. Goals of this thesis

The goals of this thesis are to examine the role of AMPK in different cellular pathways, focusing on ROS signalling and on cancer cell chemoresistance. Both of these stressors affect cellular metabolism, and thus stimulate responses involving the AMPK pathway. More specifically, AMPK activity is known to be activated under conditions of mitochondrial dysfunction (Liang et al. 2015; Zhao et al. 2016), and there are several AMPK targets that feed back onto mitochondrial or antioxidant function (Egan et al. 2011; Jäger et al. 2007; Toyama et al. 2016), suggesting a role for AMPK in the cellular response to ROS production. With regards to cancer cell chemoresistance, AMPK is known to be activated by DNA damage (Bungard et al. 2010), and to activate p53 through phosphorylation (Jones et al. 2005). Furthermore, many chemotherapeutics are known to damage mitochondria and disrupt metabolism (Galley et al. 2017; Gammella et al. 2014; Marullo et al. 2013; Shin et al. 2016). While AMPK activation is a feature of chemotherapeutic treatment, the role of AMPK in the cellular response to DNA damaging agents is unclear. In the cases of both ROS signalling and chemotherapeutic treatment, an understanding of the role of AMPK and the downstream pathways it promotes will contribute to a more full picture of the functions of this metabolic regulator and the networks it controls within the cell. The data in section 3.1 describes results published in the

journal *Cell Reports* (Rabinovitch et al. 2017), while the data in section 3.2 are currently unpublished.

2. Materials and Methods

2.1. Cell lines and culture

Primary mouse embryonic fibroblasts (MEFs) conditional for *Prkaa1* and *Prkaa2* or ppargc1a were generated by timed mating as previously described (Jones et al. 2005). MEFs were immortalized via expression of SV40 Large T Antigen.

293T cells were obtained from the American Tissue Culture Collection (ATCC). Knockdown of *Uqcrfs1* (RISP) was performed with lentiviral shRNA vectors from the TRC shRNA collection (Sigma-Aldrich, St. Louis MO, ID: TRCN0000070108-10). Knockdown of p53 as performed as previously described (Xue et al. 2007). Ectopic expression of PGC-1α was achieved with GFP-tagged *Ppargc1a* lentivirus particles from OriGene (MR210710L2V). Transduction of cell lines was conducted as previously described (Jones et al. 2005). Retrovirus-infected cells were cultured in 2 ug/ml puromycin or sorted 7 days post-infection by flow cytometry (for hCD8-expressing cells). SOD2-mCat from the AAV-CMV-SOD-2A-Catalase-WMRE vector (a gift from Connie Cepko, Addgene plasmid #67635) was subcloned into pcDNA3 using EcoRI and NotI. For transient ectopic gene expression, 293Ts and MEFs were transfected using Lipofectamine 2000 or Lipofectamine 3000 (Thermo Fisher Scientific), respectively.

H1299 non-small cell lung carcinoma cells were obtained from the ATCC and were transduced with a control vector or a vector expressing shRNAs against *Prkaa1* and *Prkaa2* (Faubert et al. 2013). 634T lung cancer cells were a gift from Reuben Shaw (Eichner et al. 2019).

All MEFs and 634T cells used throughout the work were cultured in Dulbecco's modified Eagle's medium (DMEM) (Wisent) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 2 mM L-glutamine (Gibco), 100 IU penicillin (Wisent), and 100 mg/ml streptomycin (Wisent).

H1299s were cultured in the same medium as MEFs, with the addition of non-essential amino acids (NEAA).

2.2. Drugs

Doxorubicin, N-acetyl cysteine (NAC), and ascorbate were obtained from Sigma. A76 was obtained from Abcam. Trolox was obtained from Millipore. Cisplatin, etoposide, and paclitaxel were obtained from Tocris. Drugs were dissolved in water (doxorubicin, cisplatin, ascorbate, NAC) or DMSO (A76, Trolox, etoposide, paclitaxel) according to solubility.

2.3. ROS measurement

Cellular and mitochondrial ROS were assessed by incubating cells with 2',7'–dichlorofluorescin diacetate (DCFDA) or MitoSOX red, respectively, followed by analysis by flow cytometry. ROS levels were quantified as the mean florescence intensity (MFI). using BD FACSCalibur, BD LSRFortessa, BD FACSCanto (BD Biosciences, San Diego, CA), or Gallios (Beckman Coulter, Fullerton, CA) flow cytometers were used and data were analyzed with FlowJo software (Tree Star, Ashland, OR).

2.4. Senescence assays

Primary mouse embryonic fibroblasts wild-type or knocked out for *Prkaa1* and *Prkaa2* were obtained from Benoit Viollet (Laderoute et al. 2006). These cells were plated at a uniform density, cultured in normoxia (20%) or hypoxia (3%), and counted and re-plated every three days for 30 days, as previously described (Jones et al. 2005).

2.5. Metabolic assays

Metabolite concentrations in the media were analyzed with a BioProfile Analyzer (NOVA Biomedical, Waltham, MA). Cells were seeded in 6-well plates and media was collected after 48 hours for analysis. Metabolite concentrations were expressed relative to cell number. Rates of glycolysis and oxidative phosphorylation were measured using an XFe96 Extracellular Flux Analyzer (Agilent Technologies). Basal ECAR and OCR were measured, followed by additional measurements after injection of each of the following drugs: 30 µM oligomycin (Sigma), 20 µM FCCP (Sigma), a combination of 20 µM rotenone (Sigma) and 30 µM antimycin A (Sigma), and finally 200 µM monensin (Sigma), in order to obtain OCR and ECAR at baseline, OCR and ECAR under uncoupled conditions, maximal respiration, non-mitochondrial oxygen consumption, and maximal ECAR, respectively (Mookerjee, Nicholls, and Brand 2016). Data were then analyzed with Wave software (Agilent Technologies). Metabolite extracts from MEFs were harvested using ice cold 50% methanol, then bead beaten and analyzed via liquid chromatography coupled to mass spectrometry (LC-MS) as has been previously described (Ma et al. 2017). Metabolite concentrations were determined per million cells.

2.6. Apoptosis assays

Cells were cultured with or without drug treatment for 48 hours then collected and stained with eFluor 780 fixable viability dye (eBiosciences). This stain was diluted 1:2000 in FACS buffer (PBS with 2% FBS and 0.02% NaN3). FACS was performed on stained cells using BD LSRFortessa, BD FACSCanto (BD Biosciences, San Diego, CA), or Gallios (Beckman Coulter,

Fullerton, CA) flow cytometers, and analyzed with FlowJo software (Tree Star, Ashland, OR). Viability data were reported as percentage alive (gated based on control samples).

2.7. Lysates and Western blotting

Cell lysis was performed with modified AMPK lysis buffer (Faubert et al. 2013) or CHAPS buffer (10mM Tris-HCl, 1mM MgCl2, 1mM EGTA, 0.5mM CHAPS, 10% glycerol, 5mM NaF), which were supplemented as follows: protease and phosphatase tablets (Roche), DTT (1μg/ml), and benzamidine (1 μg/ml). Protein content was quantified by Bradford assay, then lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and incubated with primary antibodies overnight, followed by incubation with secondary antibody for one hour. Primary antibodies to AMPK (phosphoT172-specific and total), Acetyl-CoA-carboxylase (phosphoS79-specific and total), ULK (phosphoS555-specific and total), LCB3, p53, aldolase, LDHA, PDK1, and actin, as well as HRP-conjugated anti-rabbit and anti-mouse secondary antibodies were obtained from Cell Signaling Technology (Danvers, MA). Anti-HIF-1α antibodies were from Cayman Chemical (Baton Rouge, LO). Anti-RISP antibodies were obtained from Abcam (Cambridge, UK).

2.8. RNA and qPCR

Total mRNA was isolated from cells using Trizol (Invitrogen), or Qiazol (Qiagen) and the RNeasy Mini Kit (Qiagen). Following this cDNA was synthesized from 100ng of total RNA using the Superscript variable input, linear output (VILO) cDNA synthesis kit (Invitrogen) or the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR was performed

using SYBR Green qPCR SuperMix (Invitrogen) or SensiFAST SYBR Lo-ROX (Bioline) and an Mx3005 qPCR machine (Agilent Technologies). Primers against *Ant, Catalase, Cytochrome c, Gpx1, Tbp, Pgc1a, Pgc1b, Sod1, Sod2, Ucp2, Ucp3, Ldha, Aldolase, Hif1α, Pdk1, Ppargc1a, Tfeb, Gpnmb, Hexa, Hexb, Gaa, Gba, Rragc, Atp6v1g1, Atp6v0d1, Vps35, Zfyve26, Gnptg, Mcoln1, Lamp1, Tmem55b,* and *Ctns* were used. All primers used have been described previously (Cherqui et al. 2002; Faubert et al. 2013; Kawamura, Sun-Wada, and Wada 2015; Medina et al. 2015; Park et al. 2011; St-Pierre et al. 2006; Wang et al. 2012; Yagi et al. 2010) (see also Table 1). All samples were normalized to *β-actin* mRNA levels.

2.9. RNA-Seq

Wild-type, AMPK-deficient, p53-deficient, and combination AMPK- and p53-deficient MEFs were treated with vehicle, doxorubicin (0.1 μM), cisplatin (5 μM), etoposide (0.5 μM), or paclitaxel (10 μM) for 48 hours, then harvested for RNA following the protocol described in section 2.8. Following this, library construction and sequencing were performed as previously described (Izreig et al. 2016). Single-end fastqs were aligned to the mouse mm9 genome using TopHat, then gene expression was quantified and normalized using ht-seq and DESeq2. Finally, GSEA was performed using the GAGE R Bioconductor package (Luo et al. 2009).

2.10. Statistical analysis

Statistics were determined using paired Student's t test, ANOVA (one-way and two-way), or log-rank (Mantel-Cox) test using Prism software (GraphPad). Data are presented as the

mean ± SEM unless otherwise indicated. Statistical significance is represented in figures as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

3. Results

3.1. AMPK maintains cellular metabolic homeostasis through regulation of mitochondrial reactive oxygen species

3.1.1. Non-canonical activation of AMPK by ROS

"Previous work has implicated ROS in the activation of AMPK under certain conditions (Emerling et al. 2009; Park et al. 2006; Shafique et al. 2013), although this has remained controversial (Shao et al. 2014; Zmijewski et al. 2010). To study the effect of physiological cellular ROS on AMPK activation, we cultured mouse embryonic fibroblasts (MEFs) with Trolox, a vitamin E analog and antioxidant that reduces cellular ROS levels (Figure 4A). MEFs cultured with Trolox displayed reduced basal AMPK activation, as determined by reduced phosphorylation of the AMPKα catalytic subunit at Thr-172 (Figure 4B). AMPK is activated in cells in response to metabolic stresses such as glucose limitation (Jones et al. 2005; Salt et al. 1998), which is classically attributed to an imbalance in the AMP-to ATP ratio (Hardie 2011). Culturing MEFs with Trolox was sufficient to reduce the normal induction of AMPK phosphorylation by glucose withdrawal (Figure 4C). Interestingly, glucose withdrawal increased the AMP:ATP ratio (Figure 4D-E) in both control and Trolox-treated cells despite reduced AMPK activation in the presence of Trolox (Figure 4C). Phosphorylation of acetyl-coenzyme A (CoA) carboxylase (ACC), a direct downstream target of AMPK, at Ser-79 was still induced following glucose withdrawal in Trolox-treated cells despite reduced levels of AMPKα phosphorylation (Figure 4C). In contrast, AMPK-dependent phosphorylation of the autophagy-inducing kinase ULK1 (on Ser-555) was inhibited by Trolox treatment (Figure 4C)." (Rabinovitch et al. 2017)

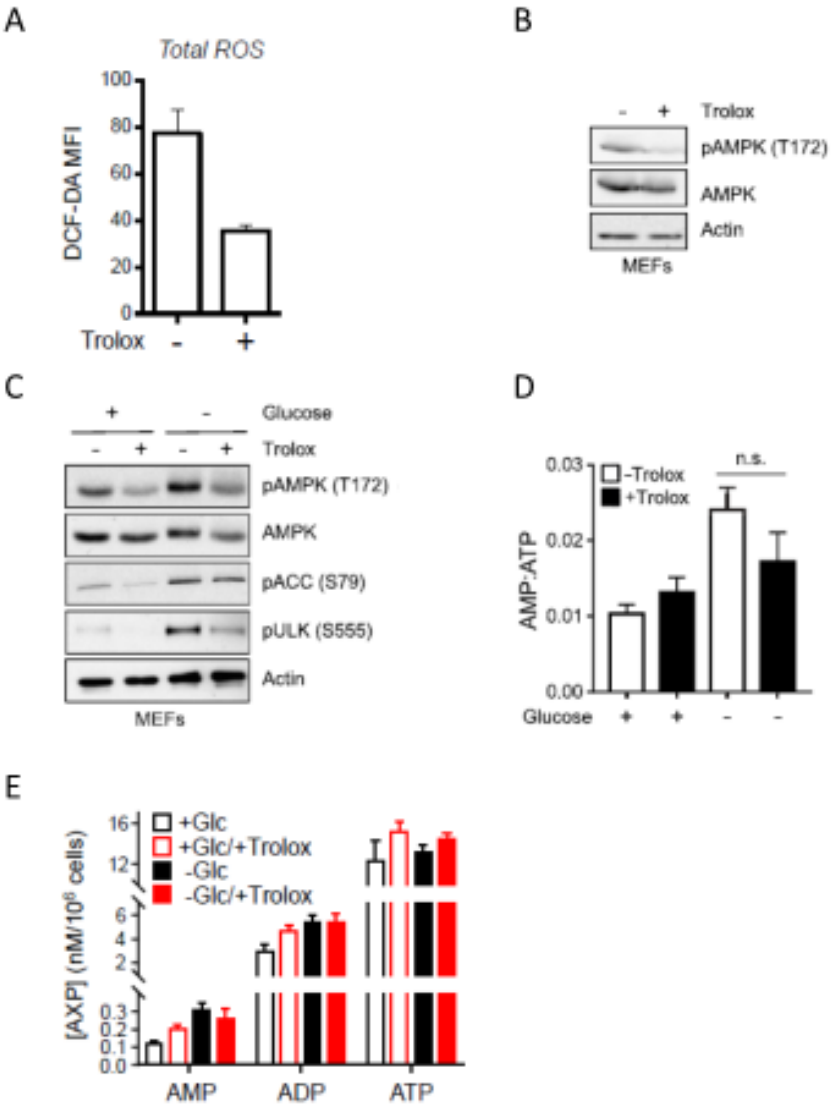


Figure 4. *Non-canonical activation of AMPK by ROS*. **A)** "Total cellular ROS levels in MEFs following treatment with Trolox. MEFs were incubated with Trolox (1 mM) for 1h, and total cellular ROS levels were measured by DCFDA staining and flow cytometry. Shown is the mean fluorescence intensity (MFI) for DCFDA staining for triplicate samples. **B)** Immunoblot for AMPKα (total and phospho-T172) and actin in MEFs treated with vehicle (-) or Trolox (+, 1 mM)

for 1 hr. **C)** Immunoblot for AMPKα activation (phospho-T172) and phosphorylation of the downstream AMPK effectors ACC (phospho-S79) and ULK1 (phospho-S555) in MEFs cultured with (+) or without (-) glucose and with or without Trolox (1 mM). **D)** AMP-to-ATP ratio in MEFs cultured with (+) or without (-) glucose for 1 hr in combination with (black) or without (white) 1 mM Trolox (mean ± SEM, n = 5). **E)** Concentration of adenlyates (AMP, ADP, and ATP) in MEFs. Cells were cultured with (empty bars) or without (filled bars) glucose (25 mM) for 1 h, in combination with (red) or without (black) Trolox (1 mM). Adenylate levels (nM per million cells) were determined by LCMS, and data expressed as mean ± SEM (n=5). These data were used to calculate the AMP to ATP ratios in Figure 1D. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$" (Rabinovitch et al. 2017)

3.1.2. Increased mitochondrial ROS production causes non-canonical AMPK activation

"To assess the role of mitochondrially derived ROS on AMPK activation, we used RNAi to silence the Rieske iron-sulfur protein (RISP), a component of complex III of the ETC that regulates ROS production from the mitochondria (Tormos et al. 2011). Silencing RISP decreased mitochondrial respiration in cells (Figure 5A), leading to a compensatory increase in lactate production in MEFs regardless of AMPK expression (Figure 5B). Notably, silencing RISP increased mitochondrial ROS production both at baseline and following complex III inhibition (Figure 5C). RISP knockdown increased phosphorylation of both AMPK, as shown previously (Moiseeva et al. 2009), and the downstream effectors ULK1 and ACC in unstressed cells (Figure 5D). Moreover, silencing RISP promoted increased AMPKα activation in cells following treatment with the complex III inhibitor Antimycin A (AA; Figure 5D) or glucose withdrawal (Figure 5E). This difference in AMPK activation could not be attributed to differential effects on OXPHOS because RISP short hairpin RNA (shRNA) lowered oxygen consumption in both control and AMPKα-deficient MEFs (Figure 5A). In addition, there was no significant difference in the AMP-to-ATP ratio between control and RISP shRNA-expressing cells (Figure 5F-G). AMPK-dependent phosphorylation of ULK1 was strongly dependent on the production of mitochondrial ROS because Trolox treatment reduced ULK1 phosphorylation in RISP shRNA-expressing cells (Figure 5E)." (Rabinovitch et al. 2017)

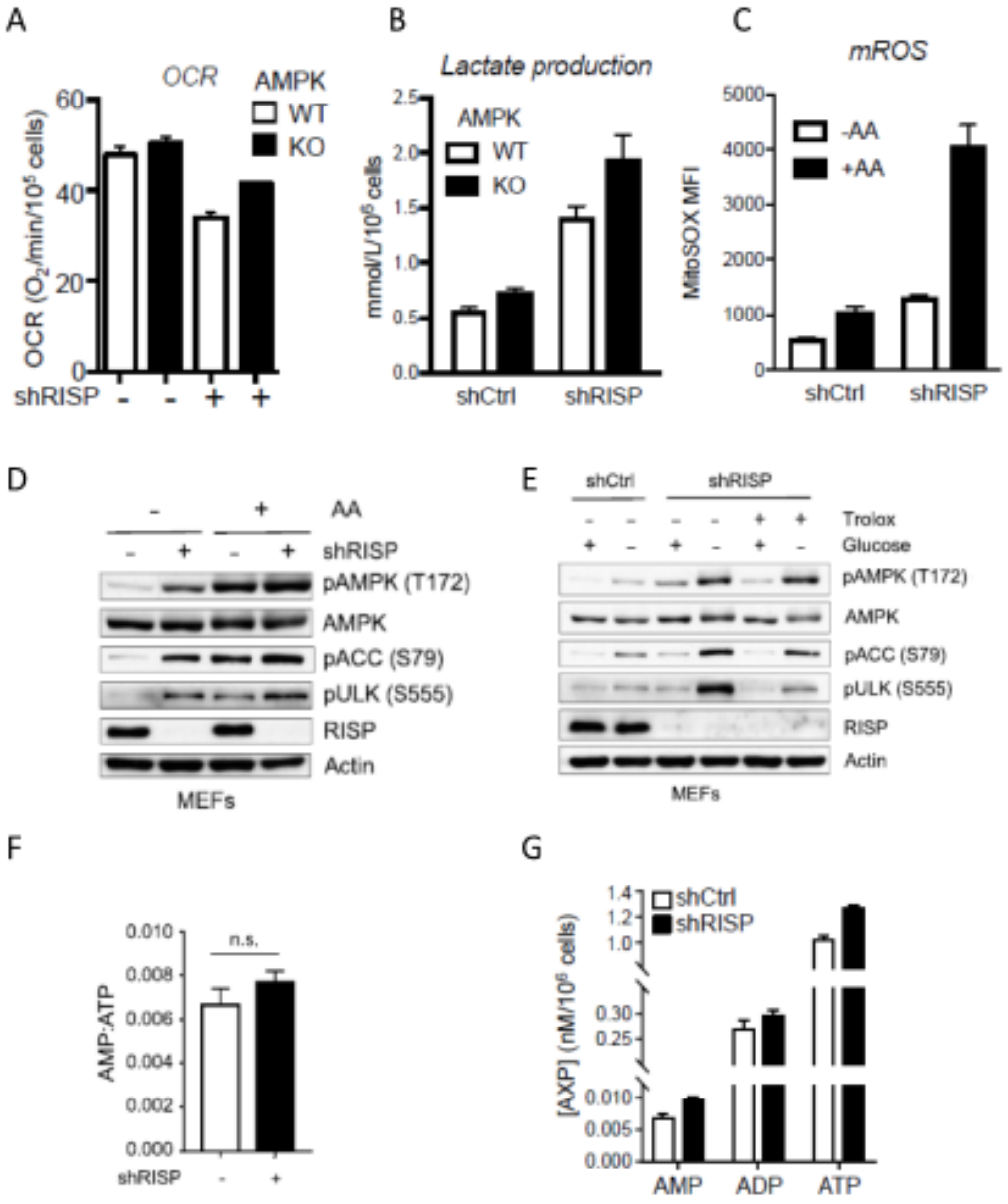


Figure 5. *Increased mitochondrial ROS production causes non-canonical AMPK activation.* **A)** "Oxygen consumption rate (OCR) of wild type (WT, white) or AMPKα-deficient (KO, black) MEFs expressing control (-) or RISP-specific (+) shRNAs as determined using a Seahorse XF96 extracellular flux analyzer. Data represent the mean $\pm$ SEM for biological replicates (n = 5). **B)**

Lactate production by control (WT) and AMPKα-deficient (AMPK KO) MEFs expressing control (shCtrl) or RISP-targeted (shRISP) shRNA. Cells were grown under standard culture conditions for 48 h prior to collection of media and metabolite measurements. Data are normalized to cell number and presented as mean ± SEM for triplicate cultures. **C)** Mitochondrial ROS levels in MEFs expressing control (shCtrl) or RISP-targeting (shRISP) shRNAs. Cells were treated with or without 1μM antimycin A for 2 h, and mitochondrial ROS 9 determined by MitoSOX staining. Data are presented as the MFI for MitoSOX staining (± SEM) for triplicate samples. **D)** Immunoblot for AMPKα (total and phospho-T172), ACC (phospho-S79), ULK1 (phospho-S555), RISP, and actin in MEFs expressing control (-) or RISP-targeted (+) shRNA. Cells were cultured with or without Antimycin A (AA; 1 mM for 2 hr). **E)** Immunoblot for AMPKα (total and phospho-T172), ACC (phospho-S79), and ULK1 (phospho-S555) in shCtrl- or shRNA against RISP (shRISP)-expressing MEFs following 1 hr culture with (+) or without (-) glucose (25 mM) and/or Trolox (1 mM) as indicated. **F)** AMP-to-ATP ratio in MEFs expressing control (-) or RISP-targeting (+) shRNAs and cultured under standard growth conditions (mean ± SEM, n = 5). **G)** Concentration of adenlyates (AMP, ADP, and ATP) in MEFs transduced with control (shCtrl) or RISP-targeting (shRISP) shRNAs. Cells were grown under standard conditions, and adenylate concentrations (nM per million cells) were measured as in Figure 1E. Data are represented as the mean ± SEM (n=5). These data were used to calculate the AMP to ATP ratios in Figure 2F. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$" (Rabinovitch et al. 2017)

3.1.3. Scavenging of mitochondrial ROS reduces AMPK activation

"We next transfected cells with a construct expressing Sod2 and mitochondrion-targeted Catalase (Sod2-mCat) to reduce mitochondrial ROS in cells (Xiong et al. 2015) (Figure 6A-B). Phosphorylation of AMPKα and ULK1 following glucose withdrawal (Figure 6C) or AA treatment (Figure 6D) was reduced in cells expressing Sod2-mCat compared with control cells. Collectively, these data indicate that mitochondrial ROS is a physiological activator of AMPK that affects distinct effectors downstream of AMPK signaling." (Rabinovitch et al. 2017)

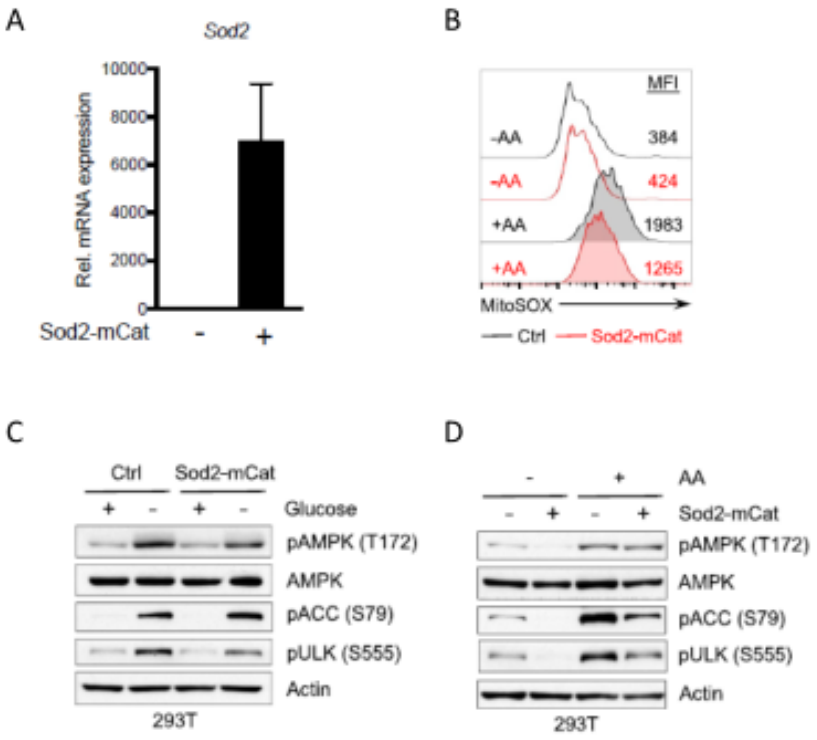


Figure 6. *Scavenging of mitochondrial ROS reduces AMPK activation*. **A)** "Relative expression of Sod2 transcript in 293T cells transiently transfected with control (white) or Sod2-mCat-expressing (black) vectors. Transcript levels were determined by qPCR, expressed relative to actin mRNA levels for triplicate samples, and normalized relative to expression in control cells. **B)** Histogram of mitochondrial ROS (mROS) levels in 293T cells expressing control (Ctrl, black) or Sod2-mCat (red) vectors, as measured by the mean fluorescence intensity (MFI) of MitoSOX staining. Cells were cultured without (-AA, open histograms) or with (+AA, shaded histograms) 0.5 mM AA for 2 hr. **C)** Immunoblot for AMPKα (total and phospho-T172), ACC (phospho-S79), and ULK1 (phospho-S555) in Ctrl or Sod2-mCat-expressing 293T cells following culture with (+) or without (-) glucose for 1 hr. **D)** Immunoblot for AMPKα (total and phospho-T172), ACC

(phospho-S79), and ULK1 (phospho-S555) in Ctrl or Sod2-mCat-expressing 293T cells following culture with (+) or without (-) AA (0.1 mM) for 2 hr. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$" (Rabinovitch et al. 2017)

3.1.4. AMPK-deficient cells display elevated mitochondrial ROS and undergo premature senescence

"Previous work by several groups has demonstrated changes in ROS in LKB1-deficient cells (Faubert et al. 2014; Li et al. 2015). Using paired isogenic MEF cell lines with (wild-type [WT]) or lacking (knockout [KO]) AMPKα catalytic activity, we found that cells lacking AMPKα expression displayed approximately 50% higher basal levels of mitochondrial ROS, as determined by MitoSOX staining (Figure 7A). In addition, MEFs lacking AMPKα featured higher levels of mitochondrial ROS following AA treatment or glucose withdrawal compared with control cells (Figures 7B-C). Mitochondrial ROS levels were elevated rapidly in AMPKα-deficient MEFs following glucose deprivation and remained elevated over 24 hr of nutrient deprivation (Figure 7D). One of the hallmarks of oxidative stress is the induction of cellular senescence, a state of irreversible growth arrest (Campisi and D'Adda Di Fagagna 2007). Consistent with increased levels of mitochondrial ROS in AMPKα-deficient cells, low-passage primary AMPKα-deficient MEFs rapidly underwent senescence (< 2–3 passages) compared with control cells, which typically underwent senescence after 7–8 passages (Figure 7E). This effect could not be rescued by culturing AMPKα-deficient cells under reduced O2 tension because AMPKα-deficient cells grown in 3% O2 still underwent senescence after 5–6 passages, similar to control cells grown under ambient (20%) O2 conditions (Figure 7F)." (Rabinovitch et al. 2017)

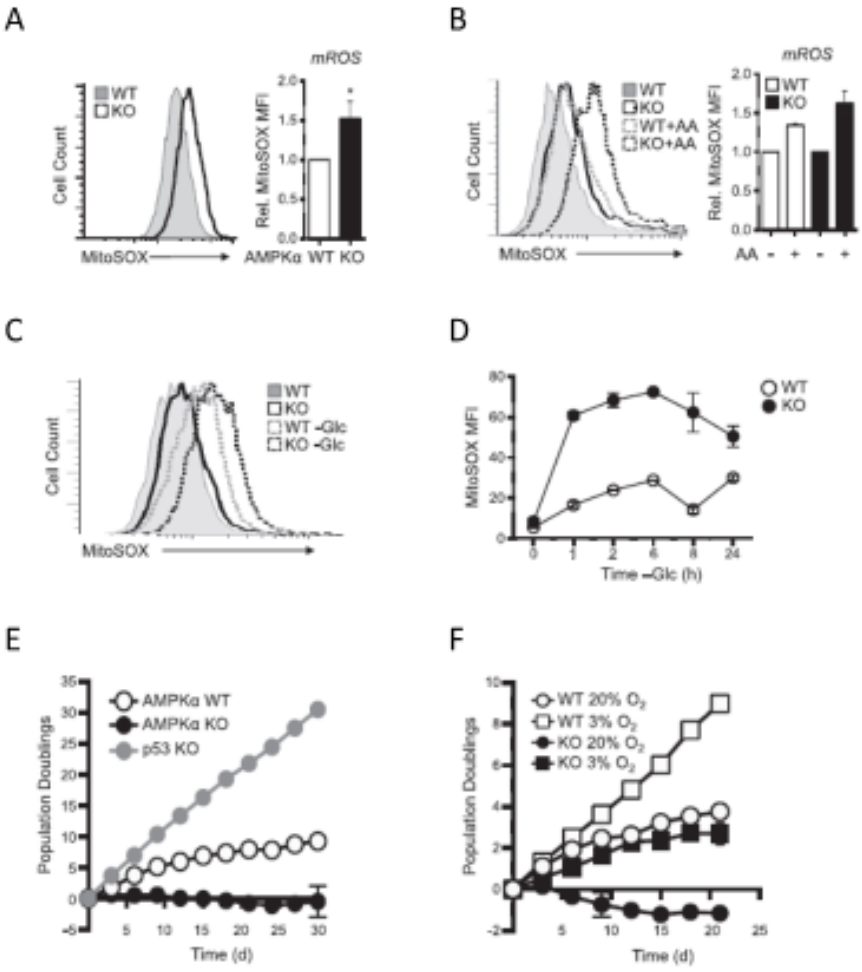


Figure 7. "*AMPK-deficient cells display elevated mitochondrial ROS and undergo premature senescence*. **A)** Mitochondrial ROS levels in wild-type (WT) or AMPKα-deficient (KO) MEFs grown under standard conditions. Left: representative histogram for MitoSOX staining. Right: relative MFI for MitoSOX staining in AMPKα WT and KO MEFs. Data represent the mean ± SEM relative to WT cells (n = 5). **B and C)** Mitochondrial ROS levels in WT or AMPKα-deficient (KO) MEFs treated with AA or glucose deprivation (-Glc) for 1 hr. Mitochondrial ROS levels were measured via MitoSOX staining as in (A). Representative flow cytometry plots for MEFs treated

with AA (B) or glucose withdrawal (C) are shown. Relative MitoSOX (MFI) following AA treatment is quantified in (B). **D)** Time course of mitochondrial ROS levels in WT and AMPKα KO MEFs following glucose deprivation. Cells were treated as in (C), and the MFI of MitoSOX staining in cells of the indicated genotypes were measured over time (mean ± SEM, n = 5). **E and F)** Serial passage growth curves of AMPKα-deficient primary fibroblasts. Primary MEFs WT for AMPK (open circle), lacking AMPKα (closed circle), or lacking p53 (gray circle) were cultured using a 3T3 passage protocol. Shown are the population doublings (PDLs) for cells under standard growth conditions (E) or growth under ambient (20%, circles) or 3% (squares) O2 conditions (F). Data represent the mean ± SEM (n = 3), measured at each passage. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$" (Rabinovitch et al. 2017)

3.1.5. AMPK activation induces PGC-1α expression and antioxidant response

"We next examined whether direct AMPK activation could influence mitochondrial ROS levels. Treatment of MEFs with the AMPK activator A-769662 (A-76) resulted in lower mitochondrial ROS levels in unstressed cells and blocked the increase in mitochondrial ROS production induced by glucose withdrawal (Figure 8A-B). To elucidate potential mechanisms by which AMPK activation influences mitochondrial ROS, we examined the expression of known antioxidant genes in control (WT) or AMPKα-deficient (KO) MEFs following A-76 treatment. A-76 treatment promoted an AMPK-dependent increase in several antioxidant genes, including *Catalase*, *Sod1*, *Sod2*, and *Ucp2* (Figure 8C). The transcriptional co-activator PGC-1α is a key regulator of mitochondrial biogenesis and antioxidant gene expression in response to oxidative stress (St-Pierre et al. 2006). Given that PGC-1α is a downstream effector of AMPK (Audet-Walsh et al. 2016; Jäger et al. 2007), we examined the involvement of PGC-1α in the AMPK-dependent control of mitochondrial ROS. At baseline, AMPKα-deficient cells exhibited lower levels of PGC-1α mRNA than WT cells (Figure 8D). Treating cells with A-76 increased PGC-1α but not PGC-1b mRNA levels in an AMPK-dependent manner (Figure 8E-F)." (Rabinovitch et al. 2017)

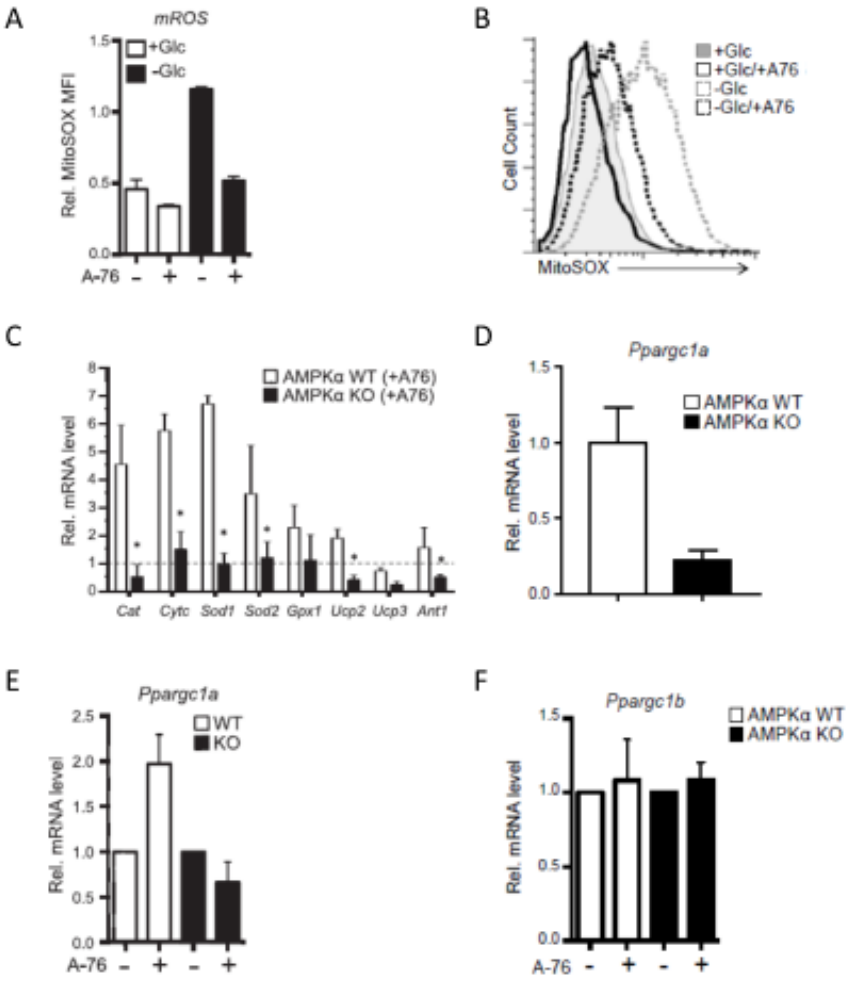


Figure 8. *AMPK activation induces PGC-1α expression and antioxidant response*. **A)** "Mitochondrial ROS levels in MEFs treated with the AMPK activator A-769662 (A-76). MEFs were pre-treated overnight with or without A-76 (25 mM), followed by culture in medium containing (+Glc) or lacking (-Glc) glucose. Relative MitoSOX staining intensities (MFI ± SD, n = 3) are shown. **B)** Mitochondrial ROS levels in MEFs treated with the AMPK activator A-769662. MEFs were pre-treated overnight with or without A-769662 (A-76, 25µM), followed by culture in medium containing (+Glc) or lacking (-Glc) glucose (25 mM) for 1h. Mitochondrial ROS levels

were quantified by MitoSOX staining and flow cytometry. Presented are representative flow cytometry plots for MitoSOX staining intensity. **C)** Relative expression of antioxidant genes in AMPKα WT (white) and null (KO, black) MEFs. Cells were cultured for 16 hr with or without A-76 (25 mM), and mRNA transcript abundance was determined by qPCR. Data are expressed relative to actin mRNA levels for triplicate samples and normalized relative to untreated Ctrl (WT) cells. **D)** *Ppargc1a* mRNA transcript expression in wild type (WT) or AMPKα-deficient (KO) MEFs. Transcript expression was determined by qPCR, expressed relative to actin mRNA levels for triplicate samples, and normalized relative to expression levels in control cells. **E)** Relative expression of *Ppargc1a* mRNA transcripts in WT (white) and AMPKα-deficient (KO, black) MEFs treated as in (C). Data are normalized to untreated Ctrl cells of their respective genotype. **F)** Relative expression of *Ppargc1b* mRNA transcript in wild type (WT, white) and AMPKα-deficient (KO, black) MEFs cultured with (+) or without (-) 25 μM A-76 for 16h. Data were normalized to untreated levels for both wild-type and AMPKα-deficient cells. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$" (Rabinovitch et al. 2017)

3.1.6. The AMPK-induced antioxidant response is PGC-1α-dependent

"Similar to AMPKα-deficient cells, MEFs harboring a conditional mutation for *Ppargc1a* (PGC-1α KO) displayed elevated mitochondrial ROS levels under basal growth conditions (Figure 9A). We next stimulated control or PGC-1α-deficient MEFs with A-76 and found that the expression of several AMPK-dependent antioxidant genes, notably *Catalase*, *Sod2*, and *Ucp2*, was also dependent on PGC-1α expression (Figure 9B). Ectopic expression of PGC-1α restored antioxidant gene expression in AMPKα-deficient MEFs (Figure 9C-D). Ectopic expression of PGC-1α in AMPKα-deficient MEFS also blunted the production of mitochondrial ROS induced by glucose deprivation (Figure 9E-F), implicating PGC-1α as a downstream effector of AMPK in the control of mitochondrial ROS homeostasis." (Rabinovitch et al. 2017)

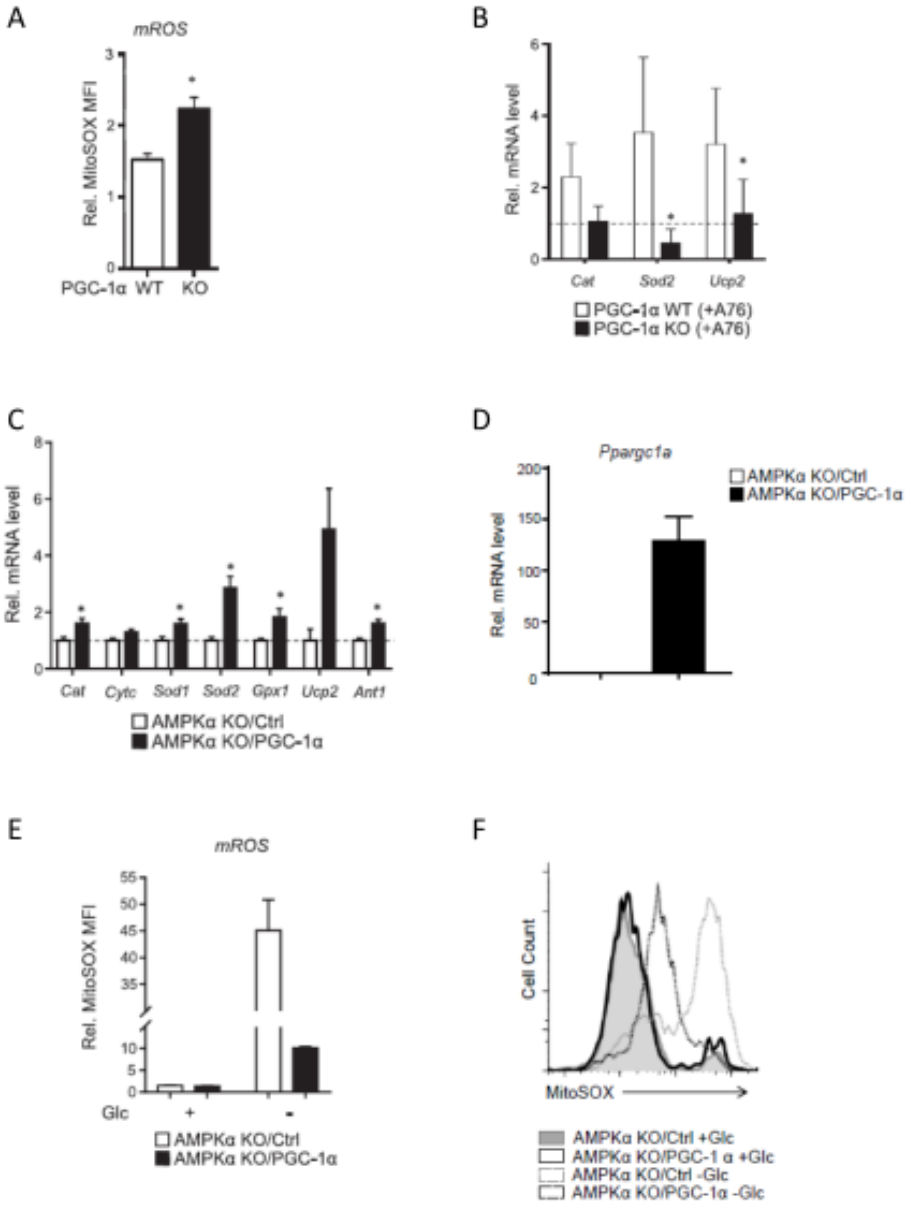


Figure 9. *The AMPK-induced antioxidant response is PGC-1α-dependent*. **A)** "Mitochondrial ROS levels in Ctrl (WT) and PGC-1α-deficient (KO) MEFs as determined by MitoSOX staining. Data represent the mean ± SEM (n = 3). **B)** Relative expression of *Catalase* (*Cat*), *Sod2*, and *Ucp2* mRNA transcripts in Ctrl (WT) and PGC-1α-deficient (KO) MEFs treated with A-76 (25 mM) for 16 hr. Data are expressed relative to *actin* mRNA levels and normalized relative to untreated

Ctrl (WT) cells (n = 3). **C)** Relative expression of antioxidant genes in AMPKα-null MEFs expressing Ctrl (white) or PGC-1α-expressing (PGC-1α, black) vectors, as determined by qPCR. Data are expressed relative to *Actin* mRNA levels and normalized relative to levels in AMPKα-null cells (n = 3). **D)** Relative expression of *Ppargc1a* mRNA transcript in AMPKα-deficient MEFs transduced with control (white) or PGC-1α-expressing (black) vectors. Transcript expression was determined by qPCR, expressed relative to *Actin* mRNA levels for triplicate samples, and normalized relative to expression levels in AMPKα-deficient control cells. **E)** Mitochondrial ROS levels in AMPKα-null MEFs expressing Ctrl (white) or PGC-1α-expressing (PGC-1α, black) vectors following culture with (+) or without (-) glucose (25 mM) for 24 hr. Data were normalized relative to MitoSOX MFI for untreated AMPKα-null MEFs (mean ± SEM, n = 3). **F)** Histograms of mitochondrial ROS levels in AMPKα-deficient MEFs expressing control or PGC-1α-expressing vectors. Cells were cultured with or without 25 mM glucose for 24 hr, and mitochondrial ROS levels were determined by MitoSOX staining. Histograms display representative flow cytometry plots for MitoSOX staining for cells treated as indicated. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$" (Rabinovitch et al. 2017)

3.1.7. AMPK-PGC-1α control of mitochondrial ROS regulates Warburg metabolism

" AMPK is required to maintain cellular metabolic homeostasis because both non-transformed and tumor cells display changes in cellular metabolism characteristic of the Warburg effect when LKB1 or AMPK signaling is disrupted (Faubert et al. 2013, 2014; Kishton et al. 2016). We previously demonstrated that this metabolic shift is due to elevated HIF-1α protein expression in cells lacking AMPK (Faubert et al. 2013). Given the links between mitochondrial ROS production and HIF-1α stabilization (Brunelle et al. 2005; Chandel et al. 2000), we tested whether increased mitochondrial ROS production because of defective AMPK-PGC-1α signaling could be a mechanistic driver of the Warburg effect. Consistent with this, treatment with Trolox or the antioxidants ascorbate (Asc) or N-acetyl cysteine (NAC), which lower cellular ROS levels (Figures 10A-B), reduced HIF-1α protein levels in AMPKα-deficient MEFs (Figures 10C-D). Trolox treatment also reduced the expression of several glycolytic genes transcriptionally induced by HIF-1α in AMPKα-deficient MEFs, including Aldolase, Ldha, and Pdk1 (Figure 10E). PGC-1α-deficient MEFs, which show elevated levels of mitochondrial ROS (Figure 9A), also displayed characteristics of the Warburg effect, including increased HIF-1α protein expression (Figure 10F), increased glucose consumption and lactate production (Figure 10G), and increased glycolysis (Figure 10H) compared with control cells. Similar to AMPKα-deficient MEFs (Figure 10C), Trolox treatment ablated the elevated HIF-1α protein expression observed in PGC-1α-deficient cells (Figure 10I). Ectopic expression of PGC-1α in AMPKα-deficient cells lowered HIF-1α protein levels as well as protein levels for HIF-1α target genes (*Aldolase, Ldha*, and *Pdk1*), similar to that observed following Trolox treatment (Figure 10J). Finally, we assessed the contribution of mitochondrial ROS to the glycolytic phenotype of

AMPKα-deficient cells. Two of the PGC-1α-dependent antioxidant genes induced by AMPK activation are Catalase and Sod2 (Figure 8C). Ectopic expression of Sod2 and mitochondrion-targeted Catalase was sufficient to reduce the elevated extracellular acidification rate (ECAR) of AMPKα-deficient MEFs (Figure 10K). Together, these data indicate that mitochondrial ROS are a physiological trigger for glycolysis that is regulated by AMPK-PGC-1α signaling." (Rabinovitch et al. 2017)

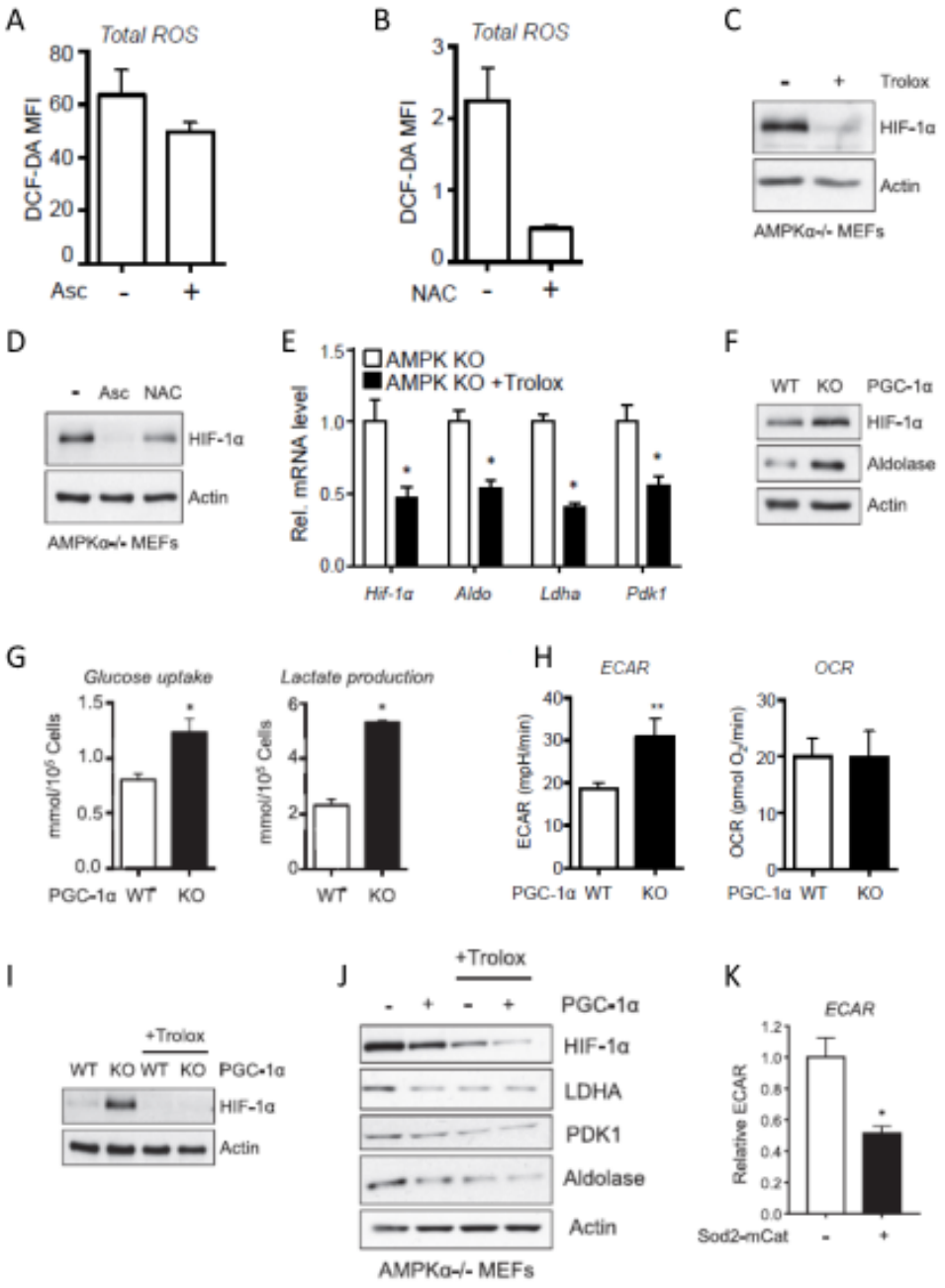


Figure 10. "*AMPK-PGC-1α control of mitochondrial ROS regulates Warburg metabolism*. **A-B)** Effect of antioxidant treatment on cellular ROS levels. MEFs were incubated with ascorbate (A) or N-acetyl-cysteine (NAC, B) for 1h, and total cellular ROS levels were measured by DCFDA staining and flow cytometry. Shown are the MFI of DCFDA staining for cells (n = 3) treated with

ascorbate (A) or NAC (B). **C-D)** Immunoblot for HIF-1α protein levels in AMPKα-deficient MEFs following culture for 1 hr with vehicle (-), Trolox (1 mM, C), ascorbate (Asc, 250 mM, D), or N-acetyl cysteine (NAC, 1 mM, D). **E)** Relative mRNA expression of glycolytic genes in AMPKα-deficient MEFs cultured with (black) or without (white) Trolox (1 mM) for 1h. Transcripts are expressed relative to actin mRNA levels, and normalized relative to expression levels in AMPKα-deficient control cells. **F)** Immunoblot for HIF-1α, aldolase, and actin protein levels in Ctrl (WT) or PGC-1α-deficient (KO) MEFs. **G)** Glucose uptake and lactate production by Ctrl (WT) and PGC-1α-deficient (KO) MEFs. Metabolite levels in culture medium were determined relative to cell number (mean ± SEM, n = 3). **H)** Bioenergetic profile of PGC-1α-deficient MEFs. ECAR (left) and OCR (right) for wild type (WT) and PGC-1α-deficient (KO) MEFs grown under standard culture conditions. Data represent the mean $\pm$ SEM for biological replicates (n = 5). **I)** Immunoblot for HIF-1α and actin protein levels in Ctrl (WT) and PGC-1α-deficient (KO) MEFs following 1 hr treatment with Trolox (1 mM). **J)** Immunoblot for HIF-1α, Ldha, Pdk1, and Aldolase protein levels in AMPKα-deficient MEFs expressing Ctrl (-) or PGC-1α-expressing (+) vectors. Cells were cultured with or without Trolox (1 mM) for 1 hr. **K)** ECAR of AMPKα-deficient MEFs expressing Ctrl (white) or Sod2-mCat-expressing (black) vectors. Data were normalized to cell number and expressed relative to untreated AMPKα-deficient cells (mean ± SEM, n = 5). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$" (Rabinovitch et al. 2017)

3.2. AMPK and its control of a TFEB/TFE3-induced lysosomal program are essential for chemoresistance in p53-null cells

3.2.1. Chemotherapeutic treatment induces ROS production and AMPK activation

My published work (Rabinovitch et al. 2017) described in section 3.1 indicates that ROS can function as an activator of AMPK. Furthermore, it has been shown that some chemotherapeutics can induce ROS production (Galley et al. 2017; Gammella et al. 2014; Marullo et al. 2013; Shin et al. 2016). Consequently, we decided to investigate AMPK and ROS activity in the context of treatment with chemotherapeutic agents. Mouse embryonic fibroblasts (MEFs) expressing wild-type AMPKα (WT) or deficient for AMPKα (AKO) were treated with cisplatin and etoposide, and levels of mitochondrial ROS were measured using a flow cytometric MitoSOX assay. Both WT and AKO MEFs demonstrated a similar increase in mitochondrial ROS production as drug dosage increased (Figure 11A-B). Following this, we found that chemotherapeutic treatment increased phosphorylation of AMPK as well as its targets ACC and ULK in WT MEFs, an effect which was absent in AKO MEFs (Figure 11C). This indicates that there is an increase in AMPK signalling upon treatment with chemotherapeutics such as cisplatin and etoposide, which may be due to ROS signalling or to other factors. It is worth noting that chemotherapeutic disruption of mitochondrial function and general perturbation of cellular functions could also produce changes in the adenylate ratio, so it is likely that canonical pathways of AMPK signalling are also involved.

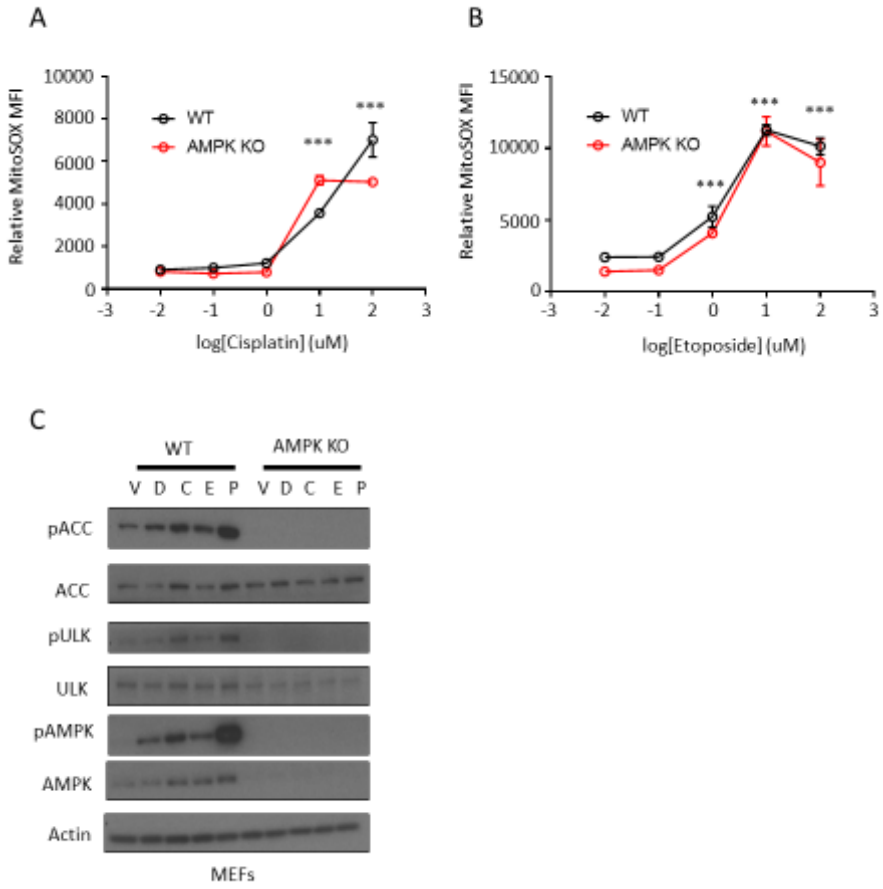


Figure 11. *Chemotherapeutic treatment induces ROS production and AMPK activation*. **A)** Relative MitoSOX mean fluorescence intensity (MFI) of WT (black) and AMPK KO (red) MEFs at increasing doses of cisplatin. Data represent the mean +/- SEM (N=3). **B)** Relative MitoSOX MFI of WT (black) and AMPK KO (red) MEFs at increasing doses of etoposide. Data represent the mean +/- SEM (N=3). **C)** Western blot of WT and AMPK KO MEFs treated with vehicle (V), doxorubicin (D, 1 µM), cisplatin, (C, 5 µM), etoposide (E, 1 µM), or paclitaxel (P, 10 µM) for 6 hr. Lysates were blotted for total and phospho-ACC (S79), total and phospho-ULK (S555), total and phospho-AMPK (T172), and actin was used as the loading control. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

3.2.2. AMPK knockout chemosensitizes p53-depleted but not WT MEFs

In order to further examine the role in AMPK signalling in chemotherapeutic treatment, we treated both WT and AKO MEFs with doxorubicin, cisplatin, etoposide, and paclitaxel and assessed cell viability with a flow cytometric assay. In all four cases, both cell types were susceptible to treatment and lost viability as the drug dose increased (Figure 12). Next, since p53 is known to regulate apoptosis (Chen 2016), we assessed viability of WT and AKO MEFs in comparison to that of MEFs with p53 knockdown (p53KD) and MEFs with both AMPK knocked out and p53 knocked down (DKO). As expected from the well-documented role of p53 in regulating apoptosis (Chen 2016), the p53KD MEFs were less susceptible to cell death than the WT and AKO MEFs, remaining highly viable even at high drug doses (Figure 12). However, the DKO MEFs did not retain this high level of chemoresistance, and instead demonstrated a susceptibility to chemotherapeutics similar to that of the WT and AKO MEFs (Figure 12). These effects appeared across all chemotherapeutics tested (Figure 12). This indicates that AMPK is required to maintain the viability of p53-depleted MEFs in response to chemotherapy-induced cell death.

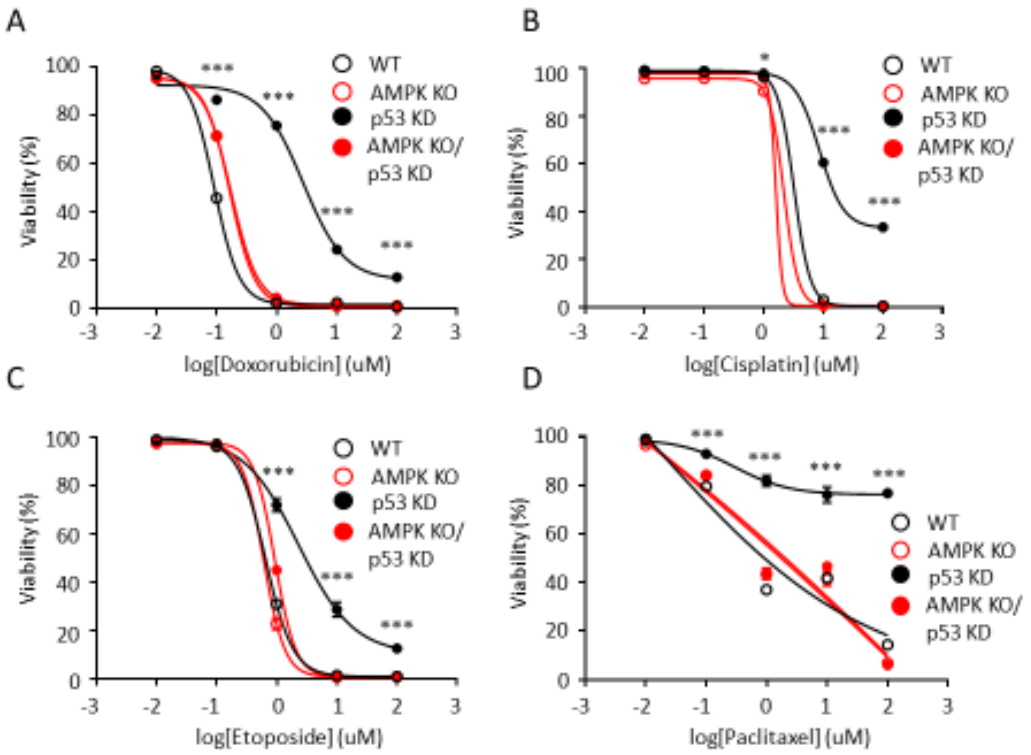


Figure 12. *AMPK knockout chemosensitizes p53-depleted but not WT MEFs*. **A-D)** Viability was measured with a flow cytometric assay for APC 780 viability dye for the following MEFs: WT (open black), AMPK KO (open red), p53 KD (filled black), and both AMPK KO and p53 KD (filled red). This experiment was performed using dose ranges of doxorubicin (A), cisplatin (B), etoposide (C), and paclitaxel (D). All drug treatments were for 48 hr. Data represent the mean +/- SEM (N=3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

3.2.3. AMPK knockdown chemosensitizes p53-null cancer cell lines

In order to determine the broader applications of this phenomenon, we examined viability in response to chemotherapeutic drug treatment in cancer cell lines. In a p53-null B cell lymphoma, knockdown of AMPKα 1 resulted in diminished viability upon etoposide treatment (Figure 13 A). Additionally, in H1299 non-small cell lung carcinoma cells, which are deficient in p53, AMPK knockdown resulted in lower viability upon etoposide treatment (Figure 13 B). Furthermore, we examined viability in the 634T lung cancer cell line, which was derived from mice with an oncogenic activation of KRAS and knockout of p53, with either knockout, addback, or kinase-dead addback of AMPK. This model also recapitulated the phenotype of AMPK loss reducing chemoresistance, with kinase-dead AMPK addback resembling AMPK knockout (Figure 13 C).

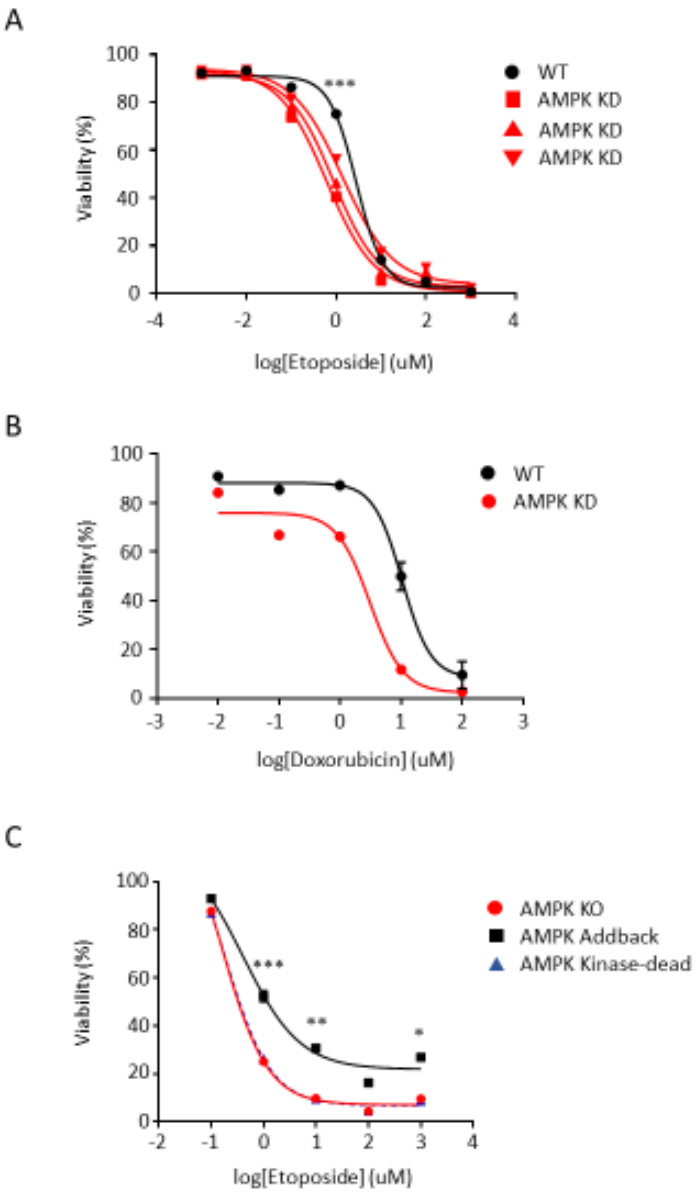


Figure 13. *AMPK knockdown chemosensitizes p53-null cancer cell lines*. **A)** Viability was measured with a flow cytometric assay for APC780 viability dye for the non-small cell lung cancer (NSCLC) cell line H1299 WT (black) or KD (red) for AMPK, at increasing doses of doxorubicin. Data represent the mean +/- SEM (N=3). **B)** Viability was measured with a flow cytometric assay for APC780 viability dye for p53-null B cell lymphomas WT (black) or KD (three

clones, red shapes) for AMPK, at increasing doses of etoposide. Data represent the mean +/- SEM (N=3). **C)** Viability was measured with a flow cytometric assay for APC780 viability dye for the NSCLC cell line 634T with AMPK KO (red), AMPK addback (black), and addback of kinase-dead AMPK (blue with dashed line), at increasing doses of etoposide. Data represent the mean +/- SEM (N=3). Drug treatments in all panels were for 48 hr. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

3.2.4. p53-depleted MEFs display a program of elevated autophagy and PGC-1α expression that is lost when AMPK is deleted

We next considered which AMPK targets might be responsible for its effects on survival. As shown previously (Fig 11C), chemotherapeutic treatment induced AMPK phosphorylation of ULK1, a kinase crucial for autophagy regulation. Consequently, we examined autophagy in MEFs deficient in AMPK and/or p53. We found that LC3B conversion from LC3B-I to LC3B-II, a marker of autophagic signalling, was increased with higher doses of etoposide treatment in WT cells, but was present at very high levels even at baseline in p53-null cells (Figure 14A). Both AMPK-deficient and doubly-deficient MEFs had lower LC3B levels than their corresponding AMPK-competent cell lines, which is as expected given the importance of AMPK in pro-autophagy signalling. Further highlighting the importance of AMPK for autophagy induction in this system was that treatment with the ROS scavenger Trolox could diminish LC3B levels in p53-deficient cells at baseline (Figure 14B). However, Trolox treatment was insufficient to reduce autophagy in p53-null cells co-treated with etoposide (data not shown).

One use of autophagy is the recycling of mitochondria, also known as mitophagy. AMPK is known to increase activity of PGC-1α, a transcriptional coactivator involved in mitochondrial biogenesis. Given that mitochondria are known to be damaged by chemotherapeutics, initiation of mitophagy and mitochondrial biogenesis may act as a compensatory mechanism, degrading damaged mitochondria and producing functional ones. We examined this idea by measuring expression of PGC-1α in MEFs deficient for AMPK and/or p53. We found that PGC-1α is expressed at constitutively high levels in p53-null cells (Figure 14C), reminiscent of the high levels of autophagy found in these cells. At a baseline, however, doubly-deficient cells

expressed PGC-1α to a lesser extent (Figure 14C). We also examined expression of PGC-1α-controlled antioxidant genes, including catalase and SOD2. The expression of these genes remained approximately constant regardless of genotype or treatment (Figure 14D-E), suggesting that it is not the antioxidant-promoting function of PGC-1α that is most crucial for this phenotype. These data suggest that in WT cells, chemotherapeutic treatment induces a program of mitochondrial recycling, but that this program is insufficient to avoid apoptosis. In p53-null cells there is a constitutively high activity of this program, which in concert with the loss of apoptosis programming induced by p53 loss, contributes to improved survival upon stress. When AMPK is lost in the p53 null context, however, this constitutive activity is lost as well and survival is impaired.

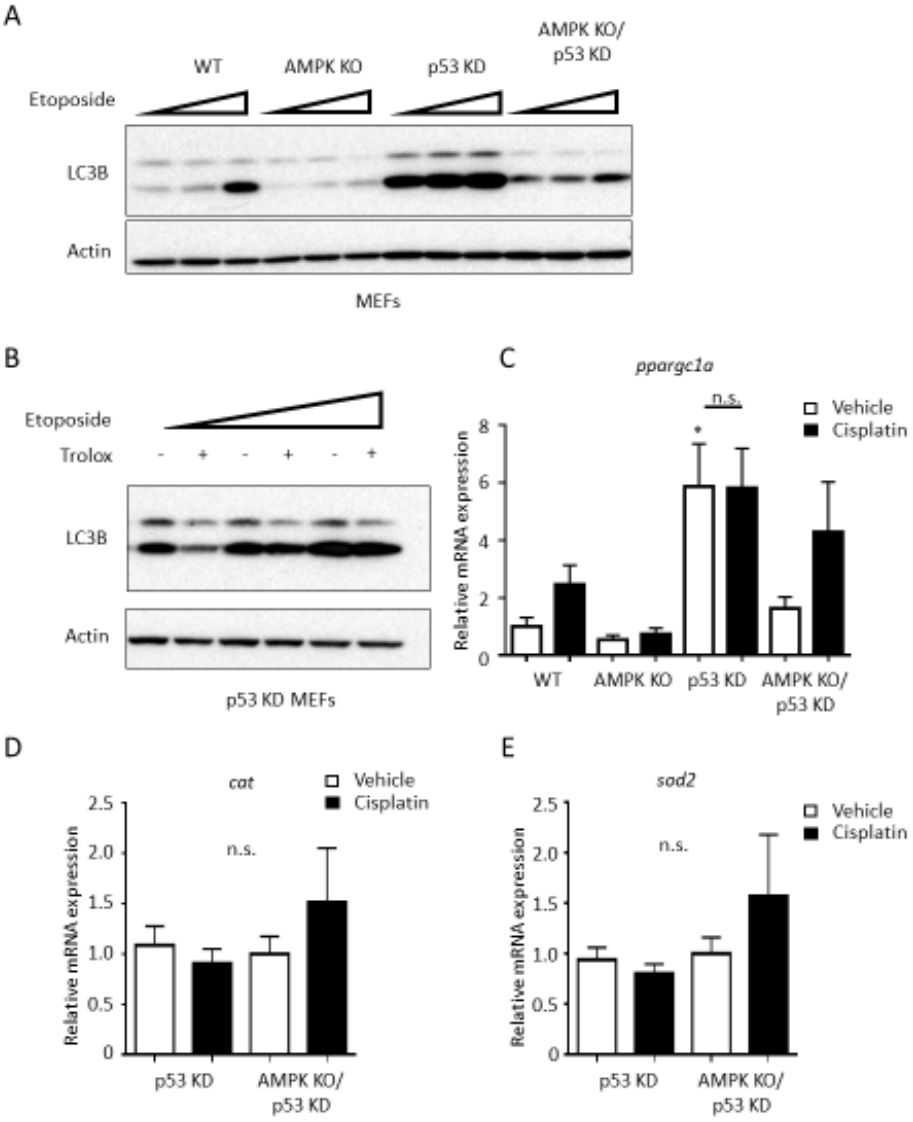


Figure 14. *p53-depleted MEFs display a program of elevated autophagy and PGC1a expression that is lost in AMPK- and p53-depleted MEFs*. **A)** Western blot of WT, AMPK KO, p53 KD, and AMPK KO p53 KD MEFs treated with etoposide (0, 0.1, 1 µM) for 48 hr. Lysates were blotted for LC3B, with actin as the loading control. **B)** Western blot of p53 KD MEFs treated with etoposide (0, 0.1, 1 µM for 48 hr), with or without additional Trolox treatment (1mM, 48 hr). Lysates were

blotted for LC3B, with actin as the loading control. **C)** RT-qPCR of WT, AMPK KO, p53 KD, and AMPK KO p53 KD MEFs treated with or without cisplatin (1 µM, 48 hr) for *Ppargc1a*. Data represent means +/- SEM, with all data normalized to *Actin* mRNA. **D-E)** RT-qPCR was performed for *Cat* (D), and *Sod2* (E) on p53 KD and AMPK KO p53 KD MEFs after treatment with cisplatin (1 µM, 48 hr). Data represent means +/- SEM, with all data normalized to *Actin* mRNA. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

3.2.5. AMPK loss and chemotherapeutic treatment promote alterations in lysosomal gene expression

In order to better understand what changes AMPK loss produced in p53-null cells upon chemotherapeutic treatment, we performed RNA-Seq analysis of p53-deficient and AMPKα/p53 deficient cells treated with doxorubicin. Gene set enrichment analysis of these data revealed that the most significantly downregulated pathway in the doubly-deficient cells as compared to the p53-null cells was lysosomal genes (data not shown), which is consistent with the importance of autophagy for survival upon stress in the p53-null context.

3.2.6. p53-depleted but not AMPK- and p53-depleted MEFs induce a program of lysosomal biogenesis that is required for chemoresistance

We next examined TFEB target genes by probing the Coordinated Lysosomal Expression and Regulation (CLEAR) network. The majority of CLEAR network gene assessed were expressed at a higher level in p53-deficient than in doubly-deficient drug-treated MEFs (Figure 15A). These data indicate that induction of a lysosomal program by TFEB contributes to the upregulation of autophagy used by p53-null cells to maintain survival upon chemotherapeutic stress.

In order to further examine the role of TFEB and TFE3 in chemoresistance, we used MEFs with or without p53 knockdown and added knockdown of TFEB and TFE3, then investigated the viability of these cell lines in response to etoposide treatment. We found that depletion of TFEB and TFE3 in the p53-null context led to a decrease in viability upon etoposide treatment, although this lowered viability did not reach the level of chemosensitivity of wild-type MEFs (Figure 15B). This partial loss of chemoresistance suggests that TFEB and/or TFE3

play an important role downstream of AMPK, but do not account for the entire effect of AMPK in the chemoresistance phenotype.

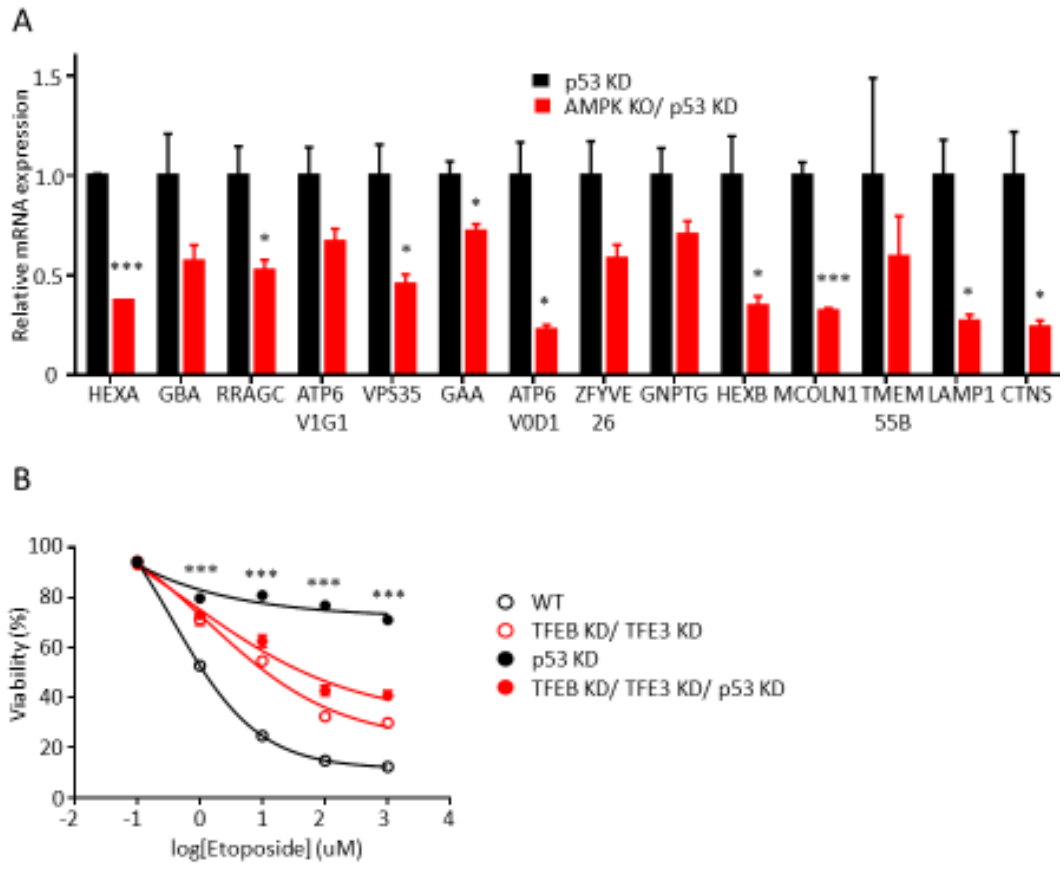


Figure 15. *p53-depleted but not AMPK- and p53-depleted MEFs induce a program of lysosomal biogenesis that is required for chemoresistance*. **A)** p53 KD and combined AMPK KO p53 KD MEFs were treated with cisplatin (1 µM) and RT-qPCR was performed for the following genes: *Hexa, Gba, Rragc, Atp6v1g1, Vps35, Gaa, Atp6v0d1, Zfyve26, Gnptg, Hexb, Mcoln1, Tmem55b, Lamp1*, and *Ctns*. Data represent normalized means +/- SEM, with all data normalized to respective *Actin* mRNA. **B)** Viability was measured with a flow cytometric assay for APC 780 viability dye for the following MEFs: WT (open black), TFEB/ TFE3 KD (open red), p53 KD (filled black), and both TFEB/ TFE3 KD and p53 KD (filled red). This experiment was performed using a

dose range of etoposide (48 hr). Data represent the mean +/- SEM (N=3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

4. Discussion

4.1. Summary of results

In the present work we have found that AMPK plays a complex and important role in both ROS signalling and in the chemoresistance of p53-depleted cancer cells. "Firstly, we report a role for mitochondrial ROS in promoting non-canonical AMPK activation and shaping AMPK-dependent metabolic reprogramming. Our data demonstrate that mitochondrial ROS are required for AMPK activation by various metabolic stressors and promote the activation of AMPK-dependent downstream effectors that influence mitochondrial homeostasis, such as ULK1 and PGC-1α. AMPK activation triggers a PGC-1α-dependent antioxidant response. Consequently, cells lacking either AMPKα or PGC-1α display increased mitochondrial ROS levels. Our data indicate that this AMPK-dependent circuit is essential for cellular metabolic homeostasis because loss of AMPKPGC-1α signaling leads to ROS-dependent activation of HIF-1α and stimulation of the Warburg effect. These data highlight a physiological role for mitochondrial ROS in coupling mitochondrial fitness to AMPK-dependent programs that help maintain cellular metabolic balance." (Rabinovitch et al. 2017)

We also report a role for AMPK in chemoresistance, specifically in the context of p53 deficiency. Treatment with a wide range of chemotherapeutics induces ROS production and AMPK activation. While loss of AMPK does not affect chemosensitivity of wild-type cells, cells lacking p53 become highly chemosensitive when AMPK is knocked out. This effect is present both in MEFs and in a variety of cancer cell lines, suggesting that this phenotype may be of relevance to cancer in vivo. Our data suggest the importance of autophagy, mitochondrial biogenesis, and particularly lysosomal biogenesis as processes downstream of AMPK in this

phenotype. The role of lysosomal biogenesis in chemoresistance is further reinforced by our finding that knockdown of TFEB and TFE3 chemosensitizes p53-depleted cells, although this does not fully recapitulate the effect of AMPK knockout, suggesting the importance of other targets such as ULK and PGC-1α as well.

4.2. Limitations and future work

"AMPK has emerged as an important regulator of mitochondrial function, coupling stress signals to processes that regulate mitochondrial homeostasis (Egan et al. 2011; Jäger et al. 2007; Kim et al. 2011; Toyama et al. 2016). Interestingly, AMPK-dependent phosphorylation of ACC, which mediates the effects of AMPK on lipid metabolism, was largely unaffected by blocking mitochondrial ROS production. Rather, AMPK-dependent pathways associated with mitochondrial quality control, such as ULK1 phosphorylation and upregulation of PGC-1α, were highly dependent on mitochondrial ROS. We speculate that mitochondrial ROS may affect a pool of cellular AMPK associated with mitochondria, effectively promoting the bifurcation of AMPK signaling to favor pathways that affect mitochondrial function. In such a scenario, mitochondrially localized AMPK could sense local regions of mitochondrial dysfunction, coupling increased ROS production by the ETC in these regions to a coordinated program of mitochondrial fission and ULK1-mediated mitophagy. Consistent with this, artificially targeting AMPK to mitochondria is sufficient to induce mitophagy and cell survival (Liang et al. 2015). We speculate that non-canonical methods of AMPK activation may function to tether AMPK activity to specific subcellular locations to exert distinct biological effects at these locations. Recent work by (Zhang et al. 2017) demonstrated that non-canonical activation of AMPK via

recruitment to the lysosome was induced by loss of fructose-1,6-bisphosphate (FBP) binding to aldolase. Mitochondrial ROS may contribute to this process through HIF-1α-dependent control of aldolase expression, thereby allowing greater sensitivity of AMPK to changing FBP levels." (Rabinovitch et al. 2017)

In addition to localization effects, the role of AMPK in ROS signalling may be regulated by direct oxidation. Hydrogen peroxide is known to directly oxidize numerous other protein targets, including enzymes involved in the antioxidant response (Rabilloud et al. 2002; Schieber and Chandel 2014; Shimura et al. 2016). AMPK alpha has been found to possess two cysteine sites that can be oxidized under high ROS conditions (Zmijewski et al. 2010). However, it remains controversial whether oxidation at one or both of these sites is sufficient to induce AMPK activity (Hinchy et al. 2018). Further work will be required to determine the exact mechanisms required for AMPK activation by ROS.

"One consequence of chronically increased mitochondrial ROS is the stabilization of HIF-1α, which acts as a survival mechanism by re-directing energy production away from the mitochondria to glycolysis. This increase in glycolysis can also support additional nicotinamide adenine dinucleotide phosphate (NADPH) production to fuel the glutathione antioxidant system to deal with elevated ROS levels. AMPK has previously been shown to affect cellular ROS by maintaining cellular NADPH levels (Jeon et al. 2012). In cells with normal mitochondrial function, disruption of LKB1-AMPK signaling (Faubert et al. 2013; Kishton et al. 2016; Shackelford and Shaw 2009) or PGC-1α (as shown here) promotes HIF-1α-dependent reprogramming of glucose metabolism characteristic of the Warburg effect. Reducing mitochondrial ROS production abolished the elevated HIF-1α expression characteristic of

AMPKα-deficient cells, implicating elevated ROS production as the driver of this metabolic phenotype. Interestingly, both low and high levels of AMPK signaling are associated with increased ROS production and Warburg metabolism. These data position AMPK-PGC-1α signaling as a regulator of ROS-dependent metabolic balance under both low- and high-energy conditions." (Rabinovitch et al. 2017)

Our finding that AMPK loss induces a phenotype of elevated ROS production and senescence may be relevant to the study of aging. It is well established that high ROS levels contribute to senescence, via DNA damage and activation of p53 (Chang et al. 1999, 2015; Kang et al. 2011). Senescence, in turn, is a central process in aging. Senescence of cells within tissues and especially of stem and progenitor cells can lead to inability to replace damaged cells and overall organ dysfunction (McHugh and Gil 2018). Reduced mitophagy in senescent cells can also contribute to metabolic impairment and further tissue damage (Sun et al. 2016). In addition to these cell-autonomous effects, the inflammatory components of the SASP can induce senescence in other nearby cells and promote the generalized inflammation common in aging (Kuilman and Peeper 2009). Much evidence suggests a role of AMPK in delay of aging, often through regulation of mTOR function and autophagy (Salminen and Kaarniranta 2012). However, the present work suggests that the role of AMPK in ROS regulation may also be an important factor in its control of aging.

ROS-dependent regulation of AMPK may also play a part in the complex role of AMPK in cancer. Many cancer cells exhibit elevated ROS levels, which may enhance tumor cell growth through increased signaling but also damage cellular components (Nelson et al. 2018; Rubattu et al. 2015; Yang et al. 2018). AMPK-dependent stimulation of antioxidant genes may benefit

cancer cells when ROS levels are sufficient to promote cell death; for example, during radiotherapy. However, mitochondrial ROS can also be pro-oncogenic by promoting genomic instability (Sallmyr, Fan, and Rassool 2008). In this context, AMPK-dependent regulation of ROS homeostasis could exert tumor suppressor functions. This corresponds to observations that AMPK may be pro- or anti-tumorigenic, depending on context (Faubert et al. 2015; Vara-Ciruelos, Russell, et al. 2019).

Our finding of TFEB and/or TFE3 as important downstream targets of AMPK in the chemoresistance response is consistent with recent work finding that the transcription factors are crucial mediators of AMPK in both immunity and tumourigenesis (Eichner et al. 2019; El-Houjeiri et al. 2019). However, this discovery leads to the further question of what aspect of TFEB/TFE3 function is needed for cellular survival under these conditions. These transcription factors play a crucial role in promoting lysosomal biogenesis, but the lysosome has several functions that could play a role in chemoresistance. For example, increased production of lysosomes in the cell could improve chemoresistance directly, by sequestering weakly basic chemotherapeutic drugs (Gotink et al. 2015; Herlevsen et al. 2007). The importance of the lysosome as a localization site for metabolic regulators to integrate signals may also play a role, as an increase in such sites could allow greater sensitivity and localization of cellular responses to the stress of chemotherapeutic treatment. The role of the lysosome in autophagy may also play an important role. The high levels of autophagy and PGC-1α expression present in chemoresistant cells and lost upon AMPK depletion suggest a program of mitochondrial recycling may be at play. In this model, chemotherapeutic treatment leads to mitochondrial damage, which then leads to metabolic disarray and apoptosis. However, if AMPK is present,

the cell may instead be able to recycle damaged mitochondria through mitophagy, and replace them through biogenesis promoted by PGC-1α. Further work will be needed to distinguish between these possibilities, particularly through determining the roles of autophagy and mitochondrial biogenesis through additional knockout models.

While we have demonstrated the importance of AMPK in chemoresistance across several different models *in vitro*, it will be important to acquire a better understanding of this effect *in vivo*. It is well known that effects found *in vitro* cannot necessarily be recapitulated *in vivo*, and this phenomenon has occurred in works on cancer metabolism (Muir, Danai, and Vander Heiden 2018). While cell lines *in vitro* share many qualities with *in vivo* tumours, they cannot fully represent the tumour microenvironment, which plays an important role in tumour metabolism (Muir et al. 2018). Furthermore, much as *in vitro* work cannot capture the full complexity of nutrient and oxygen supply to the tumour, similar problems apply to the difficulties of modeling the assortment of microenvironmental factors that can affect chemotherapeutic uptake and processing (Marin et al. 2014; Zhang et al. 2012). Consequently, in further work on this topic, it will be necessary to implant tumours lacking p53 and/or AMPK in mice and measure their sensitivity to chemotherapeutic treatment.

All of the drugs used in this work revealed a reliance on AMPK for chemoresistance in p53-depleted cells. Because of this, it is important to note that these drugs act through a wide range of mechanisms and represent different classes of chemotherapeutic. Specifically, doxorubicin is an anthracycline, cisplatin is an alkylating-like agent, etoposide is a topoisomerase II poison, and paclitaxel is a mitotic inhibitor. This diversity of drug mechanisms that are nonetheless capable of producing a similar phenotype indicates that the AMPK

response to chemotherapeutic treatment is not a reaction to some specific drug mechanism, but rather to some aspect of the general cellular derangement induced by cytotoxic drug treatment.

The exact mechanisms of AMPK activation in response to chemotherapeutic treatment are not yet clear. Likely factors include both canonical and non-canonical activators. Many chemotherapeutics impair the mitochondria, whether through disrupting mitochondrial DNA, directly binding components of the ETC, or depolarizing mitochondria through the mitochondrial permeability transition pore (Galley et al. 2017; Gammella et al. 2014; Marullo et al. 2013; Shin et al. 2016). This disruption can lead to the production of multiple signals that can induce AMPK activity, including lowered ATP production and increased ROS production. However, AMPK is also known to be activated by several genotoxic stressors (Bungard et al. 2010). Induction of AMPK upon DNA damage may be due to phosphorylation by ATM (Luo et al. 2013; Sanli et al. 2014), but some work has suggested other kinases such as CamKK2 may be responsible (Vara-Ciruelos et al. 2018). It is worth noting that AMPK is known to promote p53 activity (Jones et al. 2005), and may also contribute to genomic stability through other pathways (Chen et al. 2020; Wu et al. 2013). It will be useful in future work on this topic to examine the burden of mitochondrial DNA damage as well as mitochondrial turnover in cells expressing and lacking AMPK and p53, as well as to use ROS scavengers and strategies such as inhibitors and knockouts to determine the exact upstream regulators controlling the response of AMPK to chemotherapeutics.

This pro-survival role of AMPK in cancer cells under chemotherapeutic treatment is worth considering in the context of the ambiguous role of AMPK in cancer. AMPK has been

identified as playing both pro-tumourigenic and anti-tumourigenic roles in various tumour types and stages (Vara-Ciruelos, Russell, et al. 2019). However, we and others speculate that in general, AMPK most frequently plays a pro-tumourigenic role under conditions of particular metabolic difficulty (Eichner et al. 2019; Faubert et al. 2015). When under adequate metabolic circumstances, the tumour is able to prioritize anabolic growth, a circumstance under which AMPK may play an anti-tumourigenic role. However, when metabolic conditions are suboptimal, such as during anchorage-independent growth or in low nutrient environments, AMPK can promote survival and tumour progression (Jeon et al. 2012; Saito et al. 2015). Our findings that AMPK activity promotes survival under chemotherapeutic treatment, another metabolically stressful paradigm, provides further support for this model.

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