AMPK Inhibition Enhances Chemotherapy-Induced Apoptosis in p53-Deficient Cells

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DEDICATION

This document is dedicated to my parents.

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LIST OF ABBREVIATIONS

7-AAD, 7-amino actinomycin

ABC, ATP binding cassette

ACC, acetyl CoA carboxylase

Adr, adriamycin

AICAR, AMP-mimetic amino-imidazole carboxamide ribonucleotide

AMP, adenosine mono-phosphate

AMPK, adenosine mono-phosphate kinase

Apaf-1, apoptotic protease activating factor-1

ARF, alternate reading frame

ATP, adenosine tri-phosphate

Bcl, B-cell lymphoma

BH, Bcl-2 homology domain

CamKKß, calmoldulin-dependent protein kinase kinase ß

Cis, cisplatin

CPT, camptothecin

DMEM, dulbecco's modified eagle medium

DNA, deoxy ribonucleic acid

Doxo, doxorubicin

EndoG, endonuclease G

ETC, electron transport chain

Etop, etoposide

FACS, fluorescence-activated cell sorter

FBS, fetal bovine serum

FH, fumarate hydratase

GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Glc, glucose

HEK, Human embryonic kidney

HtrA2, high-temperature requirement protein A2

IC_{50}, 50 percent inhibitory concentration

IDH, isocitrate dehydrogenase

IR, ionizing radiation

LC3B, long chain 3

LKB1, liver kinase-B 1

MDM2, murine double minute

MDR, multidrug resistance

MEF, mouse embryonic fibroblast

MNNG, N-methyl-N'-nitro-N-nitrosoguanidine, nitrogen mustard

MRP, multidrug resistant associated protein

NSCLC, Non-Small-Cell Lung Cancer

OXPHOS, oxidative phosphorylation

p21, cyclin-dependent kinase inhibitor 1A

p53, transformation related protein 53

PBS, phosphate buffered saline

PI, propidium iodide

PI3K, phosphatidylinositol 3'-kinase

PGC1, peroxisome proliferator-activated receptor γ coactivator 1

P-gp, P-glycoprotein

PTEN, phosphatase and tensin homolog

qPCR, quantitative real time polymerase chain reaction

RING, really interesting new gene

RNA, ribonucleic acid

SD, standard deviation

SDH, succinate dehydrogenase

SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Ser, serine

shRNA, short hairpin RNA

Smac/Diablo, second mitochondrial activator-of-caspases/direct IAP-binding protein

TAK1, TGF-ß-activated kinase-1

tBID, truncated BID

TCA, tricarboxylic acid

Thr, threonine

TORC1, target of rapamycin complex 1

ULK1, unc-51-like kinase 1

UV, ultraviolet

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ABSTRACT

Despite efforts in developing targeted therapies, cytotoxic chemotherapy remains the standard treatment method of most primary tumours. However, the emergence of tumour chemoresistance contributes to the majority of cancer-related deaths. Understanding the mechanisms that underscore chemoresistance is critical for the development of therapies that target tumour relapse and drug resistance. A key regulator of cellular homeostasis that responds under conditions of low energy is the AMP-activated protein kinase (AMPK). AMPK is a modulator of cell viability in response to metabolic stress. AMPK activation promotes activation of the tumour suppressor p53 following cytotoxic stress. However, the involvement of AMPK in the cellular response to chemotherapeutic agents is not well characterized. In this work we investigated the influence of AMPK signalling on the apoptotic response of p53-deficient cells to different classes of cytotoxic drugs, including topoisomerase inhibitors (etoposide), DNA intercalating agents (doxorubicin), and alkylating agents (cisplatin). Interestingly, we observed that p53-deficient cells, which are normally resistant to DNA damage-induced apoptosis, become sensitive to chemotherapeutic agents when AMPK signalling is blocked. We tested the importance of AMPK for chemoresistance in two different settings: p53-deficient mouse embryonic fibroblasts (MEF) and p53-deficient tumour cells (H1299). We observed that p53-deficient MEFs and H1299 cells become sensitive to DNA damaging agents upon AMPK loss. This effect was not observed with AMPK knockdown alone. Moreover, AMPK appears to promote metabolic adaptation in chemoresistant and chemosensitive cells. We subsequently investigated the influence of AMPK on adaptive mechanisms in p53-deficient MEFs by characterizing their bioenergetic

profiles under stress conditions. Taken together, our data highlight a role for AMPK in stress response and survival of chemoresistant cells. Furthermore, we show that blocking AMPK signalling is sufficient to overcome chemoresistance of p53-deficient MEF and tumour cells (H1299s). These results suggest that AMPK inhibition has the potential to overcome cancer chemoresistance when combined with chemotherapeutic agents.

ABRÉGÉ

Malgré les efforts déployés dans le développement de thérapies ciblées, la chimiothérapie cytotoxique reste la méthode de traitement standard de la plupart des tumeurs primaires. Cependant, l'émergence de la chimiorésistance de tumeur contribue à la majorité des décès liés au cancer. Comprendre les mécanismes qui mettent en évidence la chimiorésistance est essentiel pour le développement de thérapies qui ciblent la rechute de la tumeur et la résistance aux médicaments. Un régulateur clé de l'homéostasie cellulaire qui réagit dans des conditions de faible énergie est la protéine kinase activée par l'AMP (AMPK). AMPK est un modulateur de la viabilité des cellules en réponse à un stress métabolique. L'activation de l'AMPK favorise l'activation du suppresseur de tumeur p53 suite à un stress cytotoxique. Toutefois, l'implication de l'AMPK dans la réponse cellulaire aux agents chimiothérapeutiques n'est pas bien caractérisée. Dans ce travail, nous avons étudié l'influence de la signalisation de l'AMPK de sur la réponse apoptotique des cellules déficientes en p53, à différentes classes de médicaments cytotoxiques, y compris les inhibiteurs de la topoisomérase (étoposide), les agents intercalants de l'ADN (doxorubicine), et des agents alkylants (cisplatin). Chose intéressante, nous avons observé que des cellules déficientes en p53, qui sont normalement résistants aux dommages à l'ADN qu'induit l'apoptose, deviennent sensibles à des agents chimiothérapeutiques lorsque la signalisation d'AMPK est bloquée. Nous avons testé l'importance de l'AMPK de chimiorésistance dans deux milieux différents: des fibroblastes embryonnaires de souris déficientes en p53 (MEF) et des cellules tumorales déficientes en p53 (H1299). Nous avons observé que les MEF déficientes en p53 et les cellules H1299 deviennent sensibles à des agents endommageant à l'ADN lors de la perte de l'AMPK. Cet effet n'a pas été observé avec l'AMPK

knockdown seul. En outre, l'AMPK semble favoriser l'adaptation métabolique dans les cellules de chimiorésistance et chimiosensibles. Nous avons ensuite étudié l'influence de l'AMPK sur les mécanismes d'adaptation dans des MEF déficientes en p53 en caractérisant leurs profils bioénergétiques dans des conditions de stress. Dans l'ensemble, nos données mettent en évidence un rôle pour l'AMPK en réponse et la survie des cellules chimiorésistantes stress. En outre, nous montrons que le blocage de la signalisation AMPK est suffisante pour surmonter la chimiorésistance des cellules tumorales et MEF déficientes en p53 (H1299s). Ces résultats suggèrent que l'inhibition de l'AMPK a le potentiel de surmonter la chimiorésistance du cancer en combinaison avec des agents chimiothérapeutiques.

LITERATURE REVIEW

1. INTRODUCTION

In order to grow and survive, all cells must manage their energetic resources. This is especially true for cancer cells, which often display changes in cellular metabolism to fuel cell growth and proliferation. Metabolic adaptation also allows tumour cells to survive periods of metabolic stress induced by energetic stresses. A key regulator in cellular homeostasis in mammalian cells is the AMP-activated protein kinase (AMPK). AMPK is a highly conserved serine/threonine kinase complex activated under conditions of low energy (ATP). This protein kinase intersects with a number of tumour suppressors including LKB1 [1, 2], TSC1/TSC2 [3], and p53 [4]. Our laboratory recently discovered that the loss of AMPK cooperates with Myc oncogene to promote tumourigenesis [5]. Silencing AMPK reprograms cancer cell metabolism to promote aerobic glycolysis (i.e. the Warburg effect) and biosynthetic programs that favour tumour growth [6]. Nonetheless, silencing AMPK also sensitizes cancer cells to cell death induced by energetic stress [6-8]. The mechanisms by which AMPK regulates tumour progression is still unclear. Moreover, the concept of inhibiting AMPK activity to modulate or enhance cancer therapy has not been extensively explored.

1.1 Cancer

Cancer describes a group of diseases that share the common feature of abnormal division with the potential to invade other tissues and metastasize within the body. Cancer is the leading cause of death worldwide, primarily associated with aging that accounts for 30% of deaths in the developed world [11]. Furthermore, over 10 million people will be diagnosed

with this disease this year and the mortality rate for those affected is significantly elevated [11]. According to the Canadian Cancer Statistics of 2014, 2 out of 5 Canadians (46% of men, 45% of women) are expected to develop cancer during their lifetimes, where 1 out of 4 (28% of men and 24% of women) are expected to die from cancer [12]. These numbers are projected to increase in the upcoming years due to a largely aging population.

Tumour development proceeds through a process in which a series of genetic changes, each presenting a growth or survival advantage, leads to the progressive alteration of normal human cells into cancer cells [9, 10]. Cancer can originate from any part of the body, and in late stages can metastasize to invade normal tissue. This invasion of cancer cells into normal tissue affects the function of normal cellular machinery, and can overcome the entire organism, ultimately leading to death [12, 13]. Most cancers are detected during late stages of development, and treatment is necessary to remove or diminish the spreading of cancerous cells throughout the body. Currently, the conventional treatment options for cancer are surgery, cytotoxic chemotherapy, and radiotherapy. However, increasing knowledge about tumour biology and genetic mutations associated specific tumour types have allowed for more selective treatments, often referred to as “targeted therapy” [14].

The most effective treatment for most cancers still remains the use of high doses of toxic chemotherapeutic agents. Chemotherapy describes the use of chemicals that target DNA transcription and replication of any rapidly dividing cells in a largely non-specific fashion. Normal cells can usually recover from any chemical-induced damage while cancer cells cannot [15]. In order to achieve effective concentrations of chemotherapeutic drugs within the body, patients need to go through extensive rounds of chemotherapy with

many adverse side effects due to the effort on other healthy tissues and organs [16]. This is a significant problem that highlights the need for developing therapeutic treatments that are less toxic and more efficient in eliminating cancerous cells over somatic cells, to improve the quality of life of patients and provide a permanent and more successful recovery.

1.1.1 Cancer and metabolism

Over the last several decades, the molecular mechanisms of cancer progression have been better elucidated identified through advances in molecular biology. Key biological control points are affected by several defined oncogene or tumour suppression mutations observed in tumours. Key biological pathways deregulated in cancer include cell cycle entry, DNA damage checkpoints, and apoptosis [17]. Proliferation presents a significant challenge to rapidly dividing cancer cells. A cell must produce enough energy and acquire or synthesize biomolecules at a necessary rate to meet the demands of proliferation. In order to meet the increased requirements of proliferation, tumour cells often exhibit changes in pathways of nutrient uptake and energy metabolism [18].

It has been shown that mutations observed in cancer can also control tumour cell metabolism as part of their mechanism of action [19, 20]. For example, loss of PTEN or p53 tumour suppressor activity or activation of the c-Myc or phosphatidylinositol 3'-kinase (PI3K) oncogene pathways have been shown to affect cellular metabolism [21-24]. Mutations in genes encoding metabolic enzymes from the tricarboxylic acid (TCA) cycle, such as fumarate hydratase (FH), succinate dehydrogenase (SDH), and isocitrate dehydrogenase (IDH), have also been implicated in cancer [25, 26]. These observations highlight potential links between tumour suppression and

metabolism, raising the possibility that mutations affecting metabolic pathways may have an impact on cancer.

1.1.2 Metabolic reprogramming in cancer

Metabolic changes in cancer have stimulated the broader concept that a metabolic transformation accompanies tumourigenesis. One of the primary metabolic changes associated with cellular transformation in cancer cells is increased glycolytic activity. Glucose is catabolized in cells by two independent biochemical pathways: glycolysis, the non-oxidative arm of glucose catabolism, and the TCA cycle/oxidative phosphorylation (OXPHOS) system in mitochondria. Oxidative phosphorylation is a mitochondrial process where ADP is phosphorylated to ATP as a direct consequence of oxidizing NADH and $FADH_2$. Non-transformed cells primarily oxidize glucose-derived pyruvate by OXPHOS to generate ATP. However, cancer cells convert the majority of their glucose-derived pyruvate to lactate (>85%) regardless of oxygen consumption. This observation was first reported by German biochemist Otto Warburg [27], and is now known as the "Warburg Effect". The "Warburg Effect" is used to illustrate a metabolic phenotype in which tumour cells engage in high rates of lactate production and glucose uptake, regardless of the presence of oxygen [28, 29].

1.2 Cancer Therapeutics

There are several different types of cancer treatments, which may be used alone or in combination. In addition to conventional cancer treatments such as surgery, cytotoxic chemotherapy, and radiotherapy, our knowledge on tumour biology and specific tumour types have allowed for more selective

treatments [14]. Chemotherapy still remains the most effective treatment in controlling metastasis and preventing tumour recurrence. However, chemotherapeutic drug resistance is the single largest obstacle. Resistance to chemotherapy is fatal once developed, and usually occurs following initial treatment. Unfortunately, resistant cancers are not only resistant to the agents they were exposed to, but often to a wide range of chemicals related and unrelated drugs [30].

1.2.1 Genotoxic agents in cancer therapy

Genotoxic agents either bind or indirectly damage DNA affecting enzymes involved in replication, ultimately inducing apoptosis [31]. Therefore, proliferating cancer cells are more sensitive than the less frequently dividing normal cell. This class of drugs is subdivided into several groups: enzyme inhibitor agents (etoposide), intercalating agents (doxorubicin), and alkylating agents (cisplatin) [32, 33] (Figure 1). These drugs and their mechanisms of action are described below.

1.2.1.1 Etoposide

In 1983, FDA approval was granted for the anticancer agent epipodophyllotoxin-derivative etoposide. The antineoplastic activity of etoposide is used in the treatment of acute myeloid leukaemia (AML), Hodgkin's lymphoma, lung, gastric, breast, and ovarian cancers [37-39]. Etoposide primarily targets topoisomerase II, an enzyme important in preventing DNA tangles and supercoils, and functions in cellular processes such as DNA recombination, replication, and transcription [40]. In the absence of topoisomerase II, cells die of mitotic failure due to cells being incapable of segregating daughter chromosomes; thus topoisomerase II is

important in chromosomal segregation [41]. Inhibition of topoisomerase II causes double-stranded breaks in DNA, which are re-ligated by the enzyme itself. This occurs by producing two staggered cuts in the sugar phosphate backbone, one on each strand, creating cleavage complexes, which are transient covalent cleavage intermediates [42, 43]. The mechanism of action of etoposide is binding to the topoisomerase II enzyme and stabilizing the cleavage complexes. This results in inhibition of the enzyme's ability to re-ligate cleaved DNA and preventing DNA replication [44]. Etoposide induces an accumulation of double-strand breaks in treated cells by blocking the relegation of cleaved DNA, which overwhelms cellular repair capacity. This triggers programmed cell death, affecting cells in G2 and S phase of the cell cycle [45]. Cytotoxic resistance to etoposide treatment occurs by decreased intracellular accumulation of topoisomerase II inhibitors, due largely to the presence of elevated P-glycoprotein (either by gene amplification or over expression of the gene that codes for P-gp, *mdr1* [46-48]).

1.2.1.2 Doxorubicin

Developed in the 1970's, doxorubicin is an anthracycline antibiotic used to treat many human cancers, including non-Hodgkin's lymphoma and multiple myeloma, as well as lung, ovarian, gastric, thyroid, and breast cancers [49]. The cytotoxic mode of action of doxorubicin is intercalation into DNA, which interferes with polymerase activity during replication and transcription, or by poisoning of topoisomerase II; generation of free radicals, leading to DNA and membrane damage. Its genotoxic working mechanism includes oxidative damage of DNA, the formation of reactive oxygen species, disturbances in nitric oxide pathway that leads to protein nitration, lipid peroxidation, and apoptosis [50-56]. Doxorubicin is limited in it's use due to

severe cardiotoxic effects [57]. The alterations in myocardial energy metabolism play an important role in the cardiac toxicity induced by doxorubicin, mainly due to the mitochondrial malfunction [58]. While doxorubicin is an important antineoplastic agent, along with problems in cardiotoxicity, resistance is also a limiting problem [59]. The resistance mechanism involves MDR1, P-gp, and MRP1 as well as other transporters. This occurs by acting as an ATP-dependent drug efflux pump [60].

1.2.1.3 Cisplatin

In 1965 was the discovery of the biological action of platinum complexes [61]. Cisplatin (cis-diammine-dicholoro-platinum(II)) was grown from bacteria into one of the largest-selling cancer chemotherapeutic drugs, having a major impact in cancer therapy. This platinum complex is the most active chemotherapeutic agent in testicular, ovarian, bladder, and head and neck carcinoma [62, 63]. It's genotoxic working mechanism is by targeting DNA to form adducts, where intra- or inter-strand crosslinks occur [64]. In order for adduct formation to arise, a series of spontaneous aquation reactions must activate the neutral cisplatin by sequential replacement of the chlorine groups with water molecules, which are more chemically reactive and positively charged [65]. Intra-strand crosslinks are preferentially formed and interact with nucleophilic N7-sites of guanine-rich DNA, favourably forming 1,2-intra-stand ApG and GpG crosslinks [66]. DNA damage caused by cross-linking atoms is claimed to facilitate cytotoxicity by inhibition of DNA synthesis, suppression of RNA transcription, effects on cell cycle, and ultimately lead to cellular apoptosis [67].

Although cisplatin is a potent anticancer agent, resistance is a major limitation of cisplatin-based chemotherapy. Resistance occurs when

endogenous nucleophiles, specifically glutathione (GSH), methionine, and metallothionein, found in the cytoplasm, become more abundant after cisplatin treatment. This inactivates and decreases the level of anticancer agent available for interacting with DNA [67, 68]. Other mechanisms that contribute to drug resistance include decreased intracellular drug uptake, increased drug efflux, increased drug deactivation, increased DNA adduct repair, and inhibition of apoptosis [69, 70].

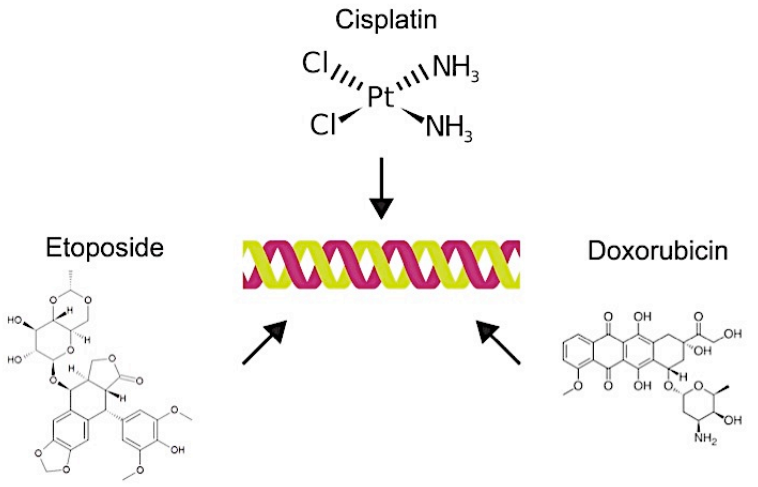

Figure 1: Cellular response to genotoxic agents that initiate double-strand breaks.

1.3 Multidrug Resistance in Cancer Therapy

1.3.1 General Aspects

Chemotherapy still remains the most effective treatment in controlling metastasis and preventing tumour recurrence. However, chemotherapeutic drug resistance is the single largest obstacle to long-term remission. Drug resistance is defined as the capability of a resistant cell or an organism to survive concentrations of drug that would typically kill or prevent

the proliferation of tumour cells. Resistance to chemotherapy is fatal once developed, and usually occurs following initial treatment. Unfortunately, resistant cancers are not only resistant to the agents they were exposed to, but often to a wide range of drugs related to the original chemotherapy [30].

Treatment with genotoxic agents can select for cells resistant to drugs that are structurally and functionally different, leading to multidrug resistance (MDR) of tumour cells and subsequent relapse [71]. Currently, 90% of drug failures in metastatic cancers are due to chemoresistance [72]. Exploration of the biochemical source of MDR led to the discovery of a group of membrane proteins, 170-kDa P-glycoproteins (P-gp) [73], that are overexpressed in MDR cells [74, 75]. In 1994, it was found that P-gp identifies drug molecules through the interaction of transmembrane domains and that both nucleotide-binding sites participate in ATP binding and/ or hydrolysis, within the cell lipid-bilayer. This results in the stimulation of drug transport, conceivably by signal transduction to the transmembrane domains [76].

Multidrug resistance arises either by impaired delivery of cytotoxic drugs to tumour cells or by arising in the tumour cells itself due to genetic modifications that affect drug sensitivity [77, 78]. Many different types of MDR biochemical mechanisms have been characterized in tumour cells. Classical drug resistance results from ATP-dependent efflux pumps with broad drug specificity. These pumps belong to a large superfamily of ATP-binding cassette (ABC) genes of transporters known as traffic ATPases [79]. Alterations in efflux has been attributed to increased activity of ABC transporter proteins P-gp and multidrug resistance proteins (MRP) [72, 80]. Other characteristics of MDR include decreased drug accumulation, activation of detoxifying systems, and blocked apoptotic pathways [78].

1.3.2 Mechanisms of resistance: Drug metabolism

Increased drug efflux is the classical form of multidrug resistance. In this case, drug resistance occurs due to increased drug efflux, which lowers the intracellular concentration of the drug, thus reducing its efficacy. P-gp and MRP actively transport drugs out of cells and specifically target natural hydrophobic drugs. Typical classes of drugs impacted by this mechanism are the vinca alkaloids (vinblastine and vincristine), the anthracyclines (doxorubicin and daunorubicin), the RNA transcription inhibitor (actinomycin-D), and the microtubule-stabilizing drug (paclitaxel) [81].

Resistance can also occur from reduced drug accumulation. This occurs when water-soluble drugs "piggyback" on transporters that are used to transport nutrients into the cell, or by agents that enter by endocytosis. Drugs that are affected by this are the antifolate methotrexate, nucleotide analogues 5-fluorouracil and 8-azaguanine, and cisplatin [82, 83].

MDR can also arise from activation of regulated detoxifying systems that include DNA repair mechanisms and cytochrome P450 mixed-function oxidases, through the transporter P-gp and cytochrome P450 3A [78]. This type of MDR can be induced after exposure non-specifically to any drug. It has been recently shown that cytochrome P450 is regulated by orphan nuclear receptors, such steroid and xenobiotic receptor (SXR), which mediates this response to environmental stress [84].

1.3.3 Mechanisms of resistance: Cellular stress responses

Cellular stress response is a defence mechanism that allows for organisms to survive in response of environmental changes disturbing cellular homeostasis. Proteins responsible in stress response have been conserved

through evolution and various homologues in different pathways are induced by energetic stress in a number of organisms. Moreover, around 18% of conserved human proteins are responsible in the stress response [85]. Stressful stimuli can induce changes in protein conformation and damage in the DNA. Cells activate various mechanisms in order to diminish damaging changes to its macromolecules. There are five major mechanisms involved in cellular stress response, including DNA repair, heat shock proteins and chaperones, antioxidant defence, apoptosis, and cell cycle checkpoints. In present day oncology, the primary goal of chemotherapy is the death of cells from apoptosis [86]. The strategies used to attack tumourous cells or get rid of cancerous cells include, DNA damage to malignant cells, suppression of anti-apoptotic genes, or induction of pro-apoptotic genes [67, 87, 88]. One of the ways that tumour cells can gain resistance to chemotherapy is through inhibition of apoptotic pathways [86]. For example, cancers with mutant or non-functional p53 are resistant to apoptosis and treatment with chemotherapeutic agents [89].

1.4 Apoptosis

Programmed cell death or apoptosis refers to the initiation of cellular suicide mechanisms to eliminate cells from the organism. This is an important system for cellular homeostasis, as apoptosis is important for the removal of infected, damaged, or senescent cells [90, 91]. Beginning in embryogenesis, the apoptotic pathway is initiated causing the removal of excess cells and the remodelling of the organism and its tissues [92]. It is a distinct from other cell death processes, as cell integrity is maintained until apoptotic bodies are formed and eliminated by phagocytic cells, and the inflammatory response is not triggered. Programmed cell death is influenced

positively and negatively by various genes that are either mutated and/or dysfunctional in human tumours [93], including the tumour suppressor gene p53 and members of the bcl-2 gene family [93, 94]. Exogenous agents, including anticancer treatments, can activate apoptosis [95, 96].

Defects in programmed cell death can lead to the development of specific diseases, including Type 1 and Type 2 diabetes [97] and autoimmune disorders [98]. When apoptosis is blocked, removal of normal or mutated cells cannot occur efficiently and unchecked proliferation can result in cancer and tumour formation [99].

In 1972, Kerr and others first described apoptosis and characterised the morphology of apoptotic cells [100]. What differentiate apoptotic cells from necrotic cells are specific features including: shrinking of the cytoplasm, nuclear condensation, DNA fragmentation, membrane blebbing, and the formation of apoptotic bodies. Intrinsic and extrinsic signalling pathways can trigger the apoptotic response. Extrinsic factors initiate death receptors present on the cell membrane, through the death receptors Fas and TNFR, DNA damage, UV or gamma irradiation, inhibition of protein synthesis, and viral infection. The activation of the intrinsic pathway then follows, resulting in the loss of mitochondrial membrane potential, ultimately leading to proteolysis of zymogen caspases [101-105]. Many genes regulate programmed cell death, including the tumour suppressor p53, caspases, the bcl-2 family, and Fas ligands [106-109].

1.4.1 Mitochondrial functions in apoptosis

The mitochondrion is often considered the “powerhouse” of the cell, as it is the major source of energy production in the form of ATP. This organelle is formed by lipid bilayers that constitute the outer mitochondrial

membrane and the highly folded inner mitochondrial membrane, which forms the cristae. The machinery needed for oxidative phosphorylation (OXPHOS) is contained within these two membranes (also known as the mitochondrial intermembrane space). The electron-transport chain (ETC) performs a series of oxidation-reduction reactions by pumping protons into the intermembrane space from the matrix of the mitochondria. This creates an electrochemical proton gradient across the inner membrane of the mitochondria that drives the synthesis of ATP by the F_0F_1-ATPase. The mitochondria not only functions as the energy producer of the cell, but also plays an important role in apoptosis. After mitochondrial permeabilization, toxic proteins located within the intermembrane space trigger the destruction of the cell if released into the cytosol.

1.4.2 Proapoptotic intermembrane proteins

The permeabilization of the outer mitochondria membrane releases cytochrome c, an essential component of the ETC. Release of cytochrome c leads to activation of the Apaf-1- and caspase-9-dependent apoptosome, a holoenzyme complex capable of activating downstream caspases [110]. Caspases are dormant proenymes in normal healthy cells and are activated through proteolysis [91]. This permeabilization can also release apoptosis-inducing factor (AIF) [111], the second mitochondrial activator of caspases/direct IAP (caspase inhibiting protein) binding protein with low pI (Smac/DIABLO) [112, 113], endonuclease G (EndoG) [114, 115], high-temperature requirement protein A2 (HtrA2/Omi) [116], triggering caspase-dependent and independent cell death.

Smac/DIABLO is a protein released from the mitochondria that can antagonize IAPs, leading to apoptosis [112, 113]. Smac is released, during

apoptosis, from the mitochondria and suppresses IAPs inhibition, leading to the reactivation of caspases. The serine mitochondrial matrix protein, HtrA2/Omi, also can inactivate IAPs, promoting caspase activation [117-119].

Additionally, AIF released from the mitochondria, translocates to the nucleus, where chromatin condensation and DNA fragmentation are initiated in a caspases independent manner [111, 120]. It also contributes to the apoptotic mitochondrial membrane permeabilization regulation, which triggers cell death through ROS [111].

The DNA degrading enzyme, EndoG, is important for mitochondrial DNA repair and is released from the mitochondria during apoptosis in response to Bid insertion into the mitochondrial membrane [121].

1.4.3 The Bcl-2 family

Bcl-2 family members regulate the release of mitochondrial intermembrane proteins, and as such can regulate the intrinsic apoptotic pathway. Bcl-2 was originally identified as a proto-oncogene isolated from B-lymphoma, where over 20 members have been discovered. Bcl-2 proteins comprise four conserved Bcl-2 homology domains (BH1-BH4) that promote or suppress apoptosis. This family is classified into three groups (Figure 2). The first group contains the multidomain anti-apoptotic proteins Bcl-2, Bcl-x_L Bcl-w, Mcl-1, A1/Bfl-1, Boo/Diva, and NR13. This group acquires BH1-BH4 and they mostly contain C-terminal hydrophobic tails, which allows them to anchor to the intracellular membranes of the mitochondria, endoplasmic reticulum, and the nuclear envelope [122]. Their anti-apoptotic activity is elicited through the BH4 domain, since the removal of BH4 converts these proteins to pro-apoptotic proteins [123]. The second group contains the multidomain pro-apoptotic proteins Bax, Bak, and Bok/Mtd,

which consist of BH1-BH3 domains. The final group of proteins (Bid, Bad, Bik, Bim, Blk, Bmf, Hrk/DP5, Bnip3, Nix, Noxa, and Puma) consist of only the BH3 domain-only, and are pro-apoptotic.

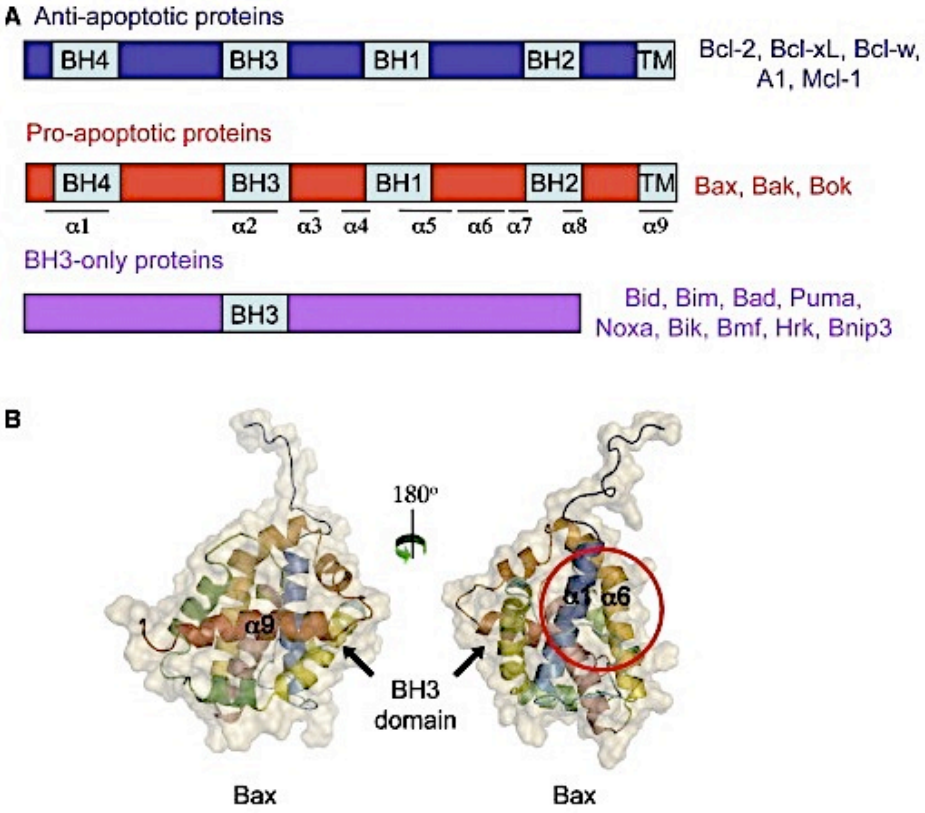

Figure 2: The Bcl-2 family

(A) Group 1 contains anti-apoptotic members. Group II and group III contain pro-apoptotic members. (B) Structure of Bax showing the α helix 9 (transmembrane domain) embedded in the hydrophobic groove (left structure). A 180° rotation (structure on the right) shows the α helices 1 and 6. The red circle represents the binding site for the Bim BH3 domain. Bcl-2 homology (BH) and transmembrane (TM) domains are depicted [124].

Bcl-2 family members are able to form homodimers and heterodimers. Studies have revealed that the structure of BH1, BH2 and BH3 domains form a hydrophobic pocket in the BH3 domain of another Bcl-2 protein [125]. It has been suggested that Bcl-2 antagonizes apoptosis by acting at the mitochondrial membrane through the binding of pro-apoptotic Bax-like and BH3-only proteins, neutralizing their activity. Current models propose that for the cell to undergo death, they become dependent on the ratio of pro-apoptotic to anti-apoptotic proteins at the mitochondrial membrane [126].

BH3 proteins connect proximal death signals to the core apoptotic pathway [127-131]. Activation of the death receptors Fas or TNFR1 leads to cleavage of BID, resulting in formation of p15 tBID [132, 133]. tBID is a caspase-activated pro-death molecule that triggers the oligomerization of BAK [134] and BAX [135], resulting in cytochrome c release from the mitochondria [136] (Figure 3). Cells lacking both Bax and Bak are completely resistant to tBID-induced cytochrome c release and apoptosis. Therefore, the pro-apoptotic member, BAX or BAK, are an essential gateway to mitochondrial dysfunction necessary for cell death in response to multiple apoptotic stimuli [136]. Of note, apoptosis triggered by energetic stress occurs through activation of the BH3-only proteins [137].

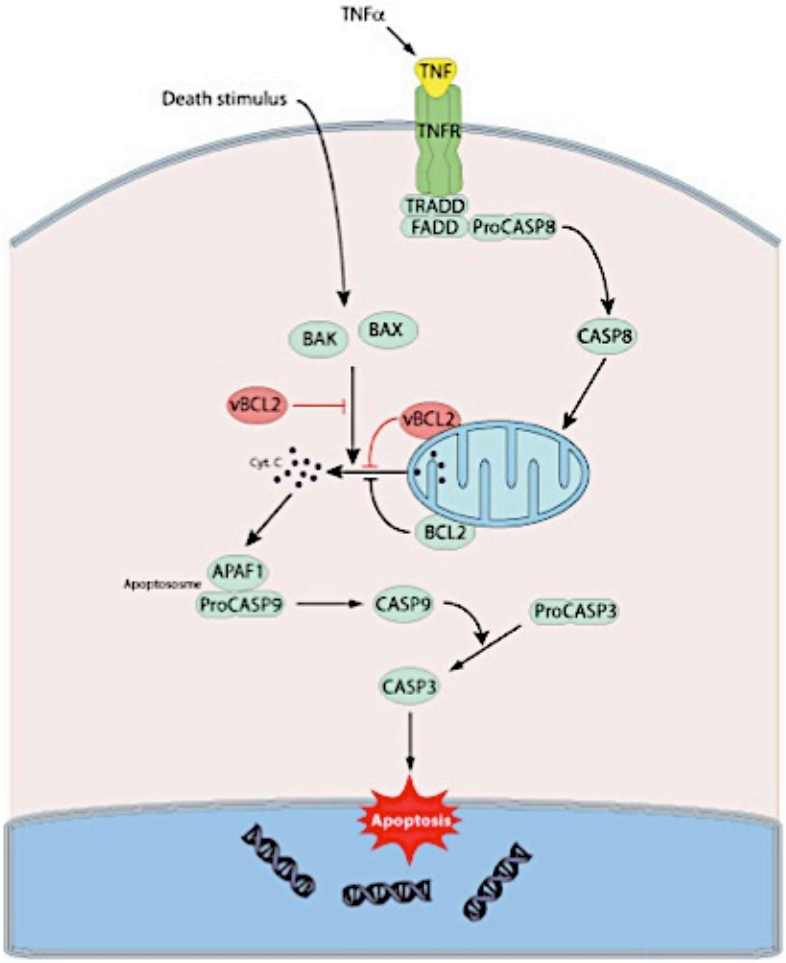

Figure 3: Pro-apoptotic pathway cascade.

A major checkpoint is the ratio of pro-apoptotic (BAX) to anti-apoptotic (BCL-2) members. Downstream are two major effecting programs: the caspase pathway and mitochondria dysfunction. Mitochondrial dysfunction includes a change in the mitochondrial membrane potential, production of reactive oxygen species (ROS), permeability transition (PT), and the release of the intermembrane space protein, cytochrome c (Cyt c). Released cytochrome c activates Apaf-1, which in turn activates a downstream caspase program. Activated caspases can also affect the function of mitochondria. Caspases could be activated through Apaf- 1/cytochrome c or directly by activation of cell surface death receptors. Caspases (e.g. caspase-3) are activated by cleavage events and the activated caspases cleave death substrates (e.g. PARP), which ultimately lead to cell death [138] (figure taken from SIB Swiss institute of bioinformatics).

1.4.4 Apoptosis and sensitivity to chemotherapeutic agents

It is widely accepted that apoptosis is the primary mechanism of cell death after DNA damage induced by anticancer agents. Cells that can elude apoptosis can gain resistance to genotoxic agents [86]. The role of mutated p53 in chemotherapeutic drug and radiation resistance and a highly significant association of mutated p53 with drug resistance.

1.5 The p53 Tumour Suppressor

The p53 tumour suppressor protein has been called the "guardian of the genome" [139] and the "cellular gatekeeper" [140], because of its important role in coordinating cellular responses to a wide range of cellular stress factors [141]. p53 is defined as a gene for which loss-of function mutation is oncogenic and is the most frequently mutated gene in all human cancers [142]. In about half of all human cancers, perturbations in pathways that regulate p53 signalling or mutations in the p53 gene occur [143, 144]. The loss or inhibition of p53 prevents apoptosis and cellular senescence. Where p53 function is compromised, additional key processes in tumour progression are altered, including cell migration and invasion [144]. p53 organizes whether cells undergo apoptosis, cell cycle arrest, senescence, DNA repair, cell metabolism, or autophagy, in response to different types and levels of cellular stress [141]. The most-understood cellular functions of p53 are those that inhibit the proliferation of cells during malignant transformation. p53 functions as a transcription factor that can activate and repress a variety of downstream target genes [145-147]. p53 is regulated by a variety of posttranslational modifications during both normal homeostasis and cellular stress responses [148].

There are three simple and rate-limiting steps in the classical models of p53 activation: p53 stabilization induced by ATM/ATR-mediated phosphorylation, sequence-specific DNA-binding, and target gene activation by interacting with transcriptional machinery [141] (Figure 4).

The signalling pathway of p53 can be activated by oncogene activation, telomere erosion, loss of stromal support, and nutrient and oxygen deprivation, or genotoxic agents by transactivating genes required to induce cell-cycle arrest and apoptosis [149, 150]. This tumour suppressor gene is affected by deletions, rearrangements or point mutations in at least 50% of human cancers [151-153]. Consequently, p53 protein characterizes a critical hurdle in tumour development.

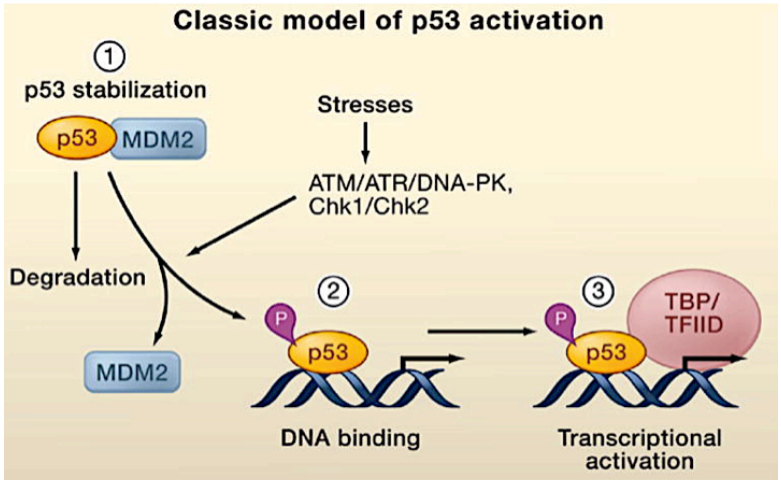

Figure 4: Classical model of p53 activation

The classic model of p53 activation consists of three sequential activating steps: (1) stress-induced stabilization mediated by phosphorylation (P), (2) DNA binding, and (3) recruitment of transcriptional machinery. p53 is degraded after Mdm2-mediated ubiquination (left), while stress signal-induced p53 phosphorylation by ATM, ATR, and other kinases stabilizes p53 and promotes DNA binding. DNA-bound p53 then recruits the transcriptional machinery to activate transcription of p53 target genes [141].

1.5.1 Pathways of p53 Activation

There have been three major pathways described in the activation of the p53 network. The first is induced by DNA damage and involves a wide range of kinases, including ATM/ATR/DNA-PK, which become activated and phosphorylate the checkpoint kinases Chk1/Chk2, in response to double-strand breaks [154-157]. Phosphorylation of Ser-15 and Ser-20, within the N-terminal p53 transactivation domain, is understood to stabilize p53 by inhibiting the interaction of p53 and Mdm2 (Figure 4). These kinases take part in a serine/threonine kinase signalling cascade responsible for post-translational phosphorylation, ultimately leading to the activation of p53 [154].

The second is triggered by the expression of the oncogenes Ras and Myc, which stimulate transcription of the alternative reading frame protein (ARF), which binds and interferes with the MDM2-p53 interaction, thus acting to stabilize and activate p53 [158, 159]. The mouse double minute protein 2 (MDM2) is the main endogenous E3-ligase with high specificity for p53 [160-162]. The control of p53 is mainly achieved through its ubiquitin-mediated proteosomal degradation [163].

Lastly, p53 can be activated as a result of chemotherapeutic agents, ultraviolet light, and protein-kinase inhibitors [164]. This functions through the involvement of kinases ATR and casein kinase II [164]. p53 is highly unstable in normal cells with a very short half-life, but their half-life increases significantly upon stimulation of any of these pathways, which leads to an accumulation of p53 and the transcription of p53-target gene p21 [154].

1.5.2 Cellular effects of p53 activation

Inhibition of cell-cycle progression of damaged cells and initiation of apoptosis by transcription-dependent and –independent mechanisms, are two of the major outcomes of p53 activation [165, 166]. p21 is the principal effector involved in p53-mediated cell cycle arrest [167]. p21 is an inhibitor of cyclin-dependent kinases at the G1-S and the G2-M phase transitions of the cell-cycle [168]. LKB1 cooperates with p53 to activate the expression of p21 [169]. The importance of cyclins for cell-cycle progression occurs when for example cyclin E-CDK2 phosphorylates Rb to disrupt the Rb-E2F association [168]. Fas ligand is another p53-regulated gene with similarities to the "death signal" receptors and can initiate the extrinsic apoptotic pathway [170].

During normal homeostasis, MDM2-mediated ubiquitination degrades p53. However, upon cellular stress, the activation of p53 induces apoptosis. p53-dependent apoptosis follows the mitochondrial pathway, but p53 can also induce cell death through death receptors (APO-1/Fas, Puma, Noxa, Bax, and others) [171, 172]. The primary contribution of p53 to apoptosis is mainly dependent on transcriptional activity. p53 has the ability to activate transcription of various proapoptotic genes, such as the BH-3 only proteins Bax, Noxa, and Puma [173]. Alternatively, p53 can also induce apoptosis by repressing antiapoptotic genes to promote caspase activation [174].

p53 activation is also important in the activation of the apoptosome, by inducing Apaf-1, where interaction with cytochrome c released into the cytoplasm, to initiate a protease cascade. This leads to the activation of caspase 9 and 3, which results in apoptosis [175].

1.5.3 p53 mutations in cancer

In response to a variety of stress signals, p53 induces transcription programs that contribute to tumour suppression [176]. The majority, over 98%, of p53 gene mutations in human cancers are somatically acquired mutations [152]. Cells lacking p53 have increased tumour susceptibility [177]. Under normal conditions, p53 levels are degraded by the activation of the E3 ubiquitin ligase MDM2 [160]. Nevertheless, cellular stresses, such as DNA damage or oncogene activation, alleviate the suppression, which allows for p53 stabilization [144]. Thus, tumour formation occurs in cells lacking p53 expression, due to the inability to engage in apoptosis or senescence after stressful insults. In addition to influencing apoptosis and cellular senescence, the loss of p53 effects cell motility that can contribute to tumour invasion and metastatic potential [144].

50% of human cancers contain mutations in the *p53* gene. The genetic changes in cancer cells are most commonly missense mutation in one allele, which produces a faulty protein followed by a decrease in homozygosity. Missense mutations, which alter the tertiary structure of p53, inhibit the DNA-binding and the transcriptional activation function of p53 [100, 178]. More rarely, deletion or chain-termination mutations occur in the *p53* gene, which lead to the null phenotype inclined to cancer [179]. The faulty protein produced by the missense mutation is suggested to contribute to "gain-of-function" phenotype [180]. A strong selection for subset of mutations localized primarily in the DNA-binding domain of the protein and a strong set of diverse environmental mutagens combine to generate mutations in the p53 gene in tumours [140] . The mutations are found in 4 evolutionally conserved regions of residues 117-142, 171-181, 234-258, and 270-286. Due to the high conservation in these regions of the p53 protein

among amphibians, mammals, birds, and fishes, it is believed that these regions are important for p53 function [178].

p53 mutations lead to oncogenesis through either loss-of-function mutations and gain-of-function mutations [153]. Excess mutant p53 proteins can form complexes with wild type p53, leading to inhibition of wild type activity in a dominant-negative fashion, effectively phenocopying a loss-of-function p53 mutation [153, 181, 182].

1.6 AMPK

1.6.1 AMPK: a cellular energy sensor

The adenosine monophosphate-activated protein kinase (AMPK) is a highly conserved regulator of energy homeostasis at a cellular level. AMPK is also a modulator of cell viability in response to metabolic stress [185]. AMPK is involved in many metabolic processes, such as gluconeogenesis, fatty acid oxidation, and glucose transport [186, 187]. AMPK is a highly conserved heterotrimeric serine/threonine kinase complex composed of a catalytic subunit (α) and two regulatory subunits (ß and γ) (Figure 5).

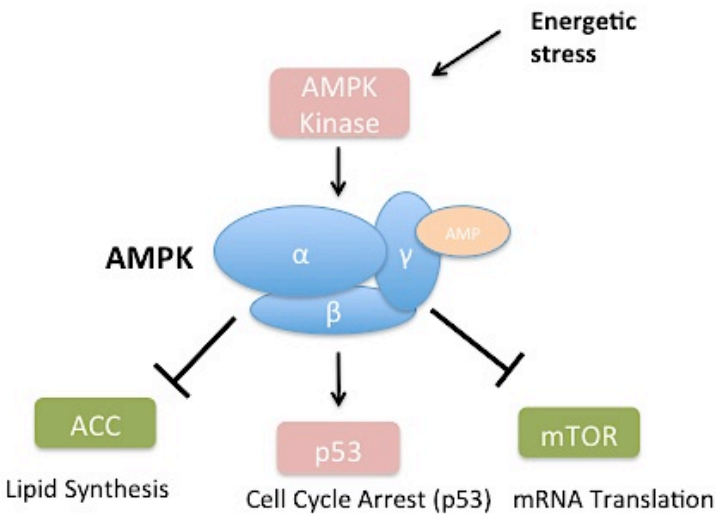

Figure 5: AMPK is a cellular energy sensor

AMPK is a heterotrimeric kinase complex consisting of α, β, and γ subunits. AMPK is activated in response to energetic stress through upstream AMPK kinases. Upstream AMPK kinases phosphorylation of the α subunit at Thr-172 is sustained under conditions of low ATP concentration when AMP binds to the γ subunit.

AMPK is activated by an increase in the cellular AMP/ATP ratio; this can be stimulated by conditions of excessive ATP consumption or inhibition of ATP production, such as what occurs under nutrient limitation or hypoxia [188, 189]. AMP binding to the γ subunit leads to phosphorylation of the AMPKα subunit at threonine 172 (Thr-172). This phosphorylation event promotes increased AMPK activity, which is monitored experimentally by detecting the AMPK-dependent phosphorylation of Acetyl CoA carboxylase (ACC) at serine 79 (Ser-79) [190]. The activity of AMPK is dependent on both its phosphorylation at Thr-172 by upstream kinases and its de-phosphorylation by phosphatases [191-194]. To date, three upstream AMPK kinases have been identified – tumour suppressor liver kinase-B 1 (LKB1), calmoldulin-dependent protein kinase kinase ß (CamKKß) [195-197], and

TGF-ß-activated kinase-1 (TAK1) [198, 199]. Each activates AMPK in response to distinct stimuli. AMPK intersects with a number of tumour suppressors including LKB1, TSC1/TSC2, and p53 [19].

1.6.2 Regulation of cell growth by AMPK

Activation of AMPK promotes net ATP conservation by activating pathways of catabolic metabolism and inhibiting anabolic processes that consume energy, before the cell reaches bioenergetic catastrophe that triggers cell death [189, 200, 201]. One way AMPK regulates energy homeostasis is by inhibiting lipid synthesis and stimulating lipid oxidation through phosphorylation of ACC1 [202] and inactivation of ACC2 through phosphorylation [203]. AMPK-dependent phosphorylation of ACC inhibits its enzymatic activity to suppress malonyl-CoA synthesis, thus decreasing inhibition of fatty acid uptake into mitochondria and increasing fatty acid oxidation [204]. It has recently been shown that AMPK-dependent inactivation of ACC promotes cellular conservation of NADPH pools, which are necessary to maintain cell viability and redox homeostasis [8]. Activation of AMPK also impacts cell growth through the control of mTOR-dependent protein translation. This occurs through the activation of the TSC1-TSC2 [1, 3, 205] complex or by suppressing the mTOR-binding protein Raptor [201], when ATP levels are low.

1.6.3 Metabolic adaptation regulated by AMPK

By responding to energetic stress, AMPK can mediate adaptation to metabolic perturbations. AMPK has been implicated in autophagy, a catabolic process for the maintenance of cellular energy and cell survival in starved cells [206]. AMPK-dependent phosphorylation of ULK kinases can

directly induce autophagy in stressed cells. This pathway results in the removal of damaged mitochondria through specific activation of "mitophagy" [207, 208]. The ULK kinase in turn can negatively regulate AMPK signaling through the phosphorylation of AMPK subunits [209]. ULK1 has been identified as a direct substrate of AMPK. It was found that there are six different AMPK sites (Ser-317, Ser-476, Ser-555, Thr-575, Ser-637, and Ser-777) [210]. The phosphorylation of these sites is AMPK-dependent and mirrors the phosphorylation of the known AMPK substrates ACCα and raptor. Under low-energy and nutrient-poor conditions, autophagy is initiated as a survival mechanism. This mechanism ensures availability of critical metabolic intermediates and to eliminate damaged organelles, such as mitochondria [211].

AMPK can also promote adaptation to bioenergetic stress through the regulation of transcriptional programs. AMPK can alter gene expression through a number of mechanisms including phosphorylation of transcriptional factors and transcriptional co-activators including: PGC1α [212], Foxo3a [213], CRTC2 [214], HIF-1α [215], and p53 [4, 216, 217]. Under poor nutrient conditions, it has been shown that AMPK-dependent phosphorylation of p53 induces a metabolic cell cycle checkpoint that inhibits proliferating cells [4, 188]. AMPK can also bind to chromatin and promote the phosphorylation of Histone H2B at Ser-36 [34], which is required to mediate p53-dependent transcriptional responses in glucose-starved cells.

1.6.4 AMPK in cancer progression and therapy

The loss of AMPK cooperates with Myc oncogene to promote tumouregenesis, and it has been shown that silencing AMPK reprograms cancer cell metabolism to stimulate aerobic glycolysis (i.e. the Warburg

Effect) [6]. This results in increased glucose uptake, glycolytic flux, and flow of carbon into tricarboxylic acid (TCA) cycle to fuel biosynthetic programs that favour tumour growth. Cells lacking AMPKα expression display lower cell viability in response to metabolic stress.

In addition to its effects on glucose metabolism, silencing AMPK promotes unchecked mTORC1 activity [1, 3]. Maintaining mTORC1 activity by the silencing of AMPK would support tumour cell growth. Therefore, AMPK has an important function in limiting tumour cell growth, as a "metabolic" tumour suppressor, by regulating Warburg metabolism and key components necessary to support unchecked proliferation [5]. Thus, AMPK functions as a key metabolic regulator during tumour initiation and progression, conferring a metabolic advantage in tumour growth.

Downstream of AMPK pro-survival pathways such as p53-dependent cell cycle arrest (p53) are promoted in response to energetic stress. AMPK activation inhibits the growth of cells by phosphorylating p53 at Ser-15 and the accumulation of the cyclin-dependent kinase inhibitors, p21 and p27 [216, 218]. This has raised the possibility that AMPK may serve primarily a pro-survival role in stressed cancer cells. Interestingly, agents that induce genotoxic stress also activate AMPK (Figure 6) [34-36]. This thesis explores the role of AMPK in cellular responses to genotoxic agents.

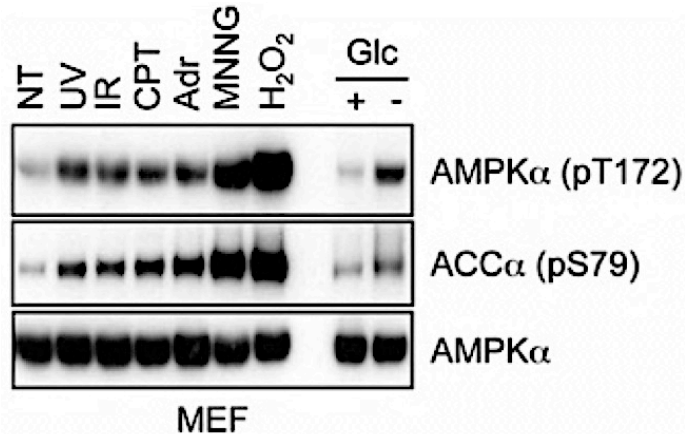

Figure 6: AMPK activation by genotoxic agents and radiation.

Immunoblot of AMPKα activation seen by phosphorylation of Thr-172 and phosphorylation of ACCα at Ser-79 in MEF cells after were treatment with various genotoxic agents. UV, ultraviolet radiation; IR, ionizing radiation; CPT, camptothecin; Adr, Adriamycin; MNNG, nitrogen mustard; Glc, glucose. UV, IR, CPT, and Adr were treated for 6 hrs; MNNG and H2O2 were treated for 30 min. *Work by Russell G. Jones and L. Berger Shelley [34].

MATERIALS AND METHODS

Cell Lines

AMPKα-deficient mouse embryonic fibroblasts (MEFs) (lacking both AMPKα1 and AMPKα2) were obtained from Dr. Benoit Viollet as previously described [219]. Bax and Bak-deficient MEF cells (lacking both Bax and Bak) were obtained from Dr. Craig B. Thompson. 293T cells, human colorectal cancer cell line HCT116, and human non-small lung cancer cell line H1299 were obtained from the American Tissue Culture Collection (ATCC; Manassas, VA).

Cell Culture, Transfection, and Retroviral Transduction

MEFs, HCT116, and HEK 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO Invitrogen; Langley, OK) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 2 mM L-glutamine (Invitrogen), 100 IU penicillin (Invitrogen), and 100 mg/ml streptomycin (Invitrogen). H1299 cells were cultured similarly in RPMI 1640 (Roswell Park Memorial Institute media) medium (Invitrogen), 100 mg/ml streptomycin (Invitrogen), and non-essential amino acids (NEAA; catalogue # 11140-050; Invitrogen). All cell lines were grown as monolayers in 10 cm petri dishes with 10 ml of growth medium and maintained in a humidified 5% CO_2, 37°C incubator. Cells were sub-cultured to maintain them at a logarithmic growth rate such that the dishes at approximately 80% confluency were split at a ratio of 1:8 (MEFs and HCT116) or 1:3 (H1299).

For subculturing, growth medium was aspirated and adherent cells were washed with 5 ml of phosphate buffered saline (PBS; Invitrogen). The cells were detached from the dish by using 1 ml of trypsin and incubating at

37°C for 1-5 min. The cells were then resuspended in the appropriate growth medium, pre-warmed to 37°C.

For cell treatments, cells were cultured in the presence of medium with or without genotoxic drugs etoposide (Sigma-Alderich; St-Louis, MO), doxorubicin (Sigma), or cisplatin (Sigma), or the AMPK agonists AICAR (Toronto Research and Chemicals), or metformin (Sigma) for 1, 6, 24, 48, or 72 hours.

Silencing of mouse AMPKα was achieved by retroviral transduction of cells with LMP shRNA vectors expressing GFP markers, as described in [34]. shRNA constructs targeting mouse p53 (p53.1224 miR30 hairpin) were previously reported by Dr. Scott Lowe [220]. Briefly, transduced cells were selected in 20 ug/ml puromycin and GFP double-positive cells enriched by cell sorting MoFlo sorting (Beckman Coulter, Mississauga, Canada). The shRNA sequences are listed in Table 1.

Transfection was conducted using Lipofectamine 2000 (Invitrogen). HEK293T cells (2×10^6 / 6 cm dish) were cultured in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen), 2 mM L-glutamine (Invitrogen), and 25mM choloroquine (Sigma). Cells were transfected by titering retrovirus generated by transfecting retroviral vector (5 μg) and vector DNA (10 μg). Virus was collected 48 and 72 hrs postransfection and cleared prior to use. MEFs (30×10^4 /12 well dish) were infected three times over 2 days using 0.5 ml of retroviral supernatant and 8 μg/ml polybrene. Cells were then expanded into 10 cm dishes. Cells were selected with 10 μg/ml puromycin for 2-7 days (time point when non-infected cells were all dead). Prior to use in proliferation assays, GFP+ cells were sorted 4 days postinfection and grown for 48 hrs. The level of infection was measured by flow cytometry by FACS

using GFP antibodies (eBioscience). Cell lysates were harvested 24h post-transfection.

Analysis of Cell Viability by flow cytometry

Cells were seeded at a density of 20, 000 cells/well in 96-well plates and allowed to adhere overnight. Cells were then washed in phosphate buffered saline (PBS; Invitrogen) and left untreated or treated for 24, 48, 72h with the indicated drugs as follows: etoposide (0.1, 0.3, 1, 3, 10, 30 μM), doxorubicin (0.03, 0.1, 0.3, 1, 3, 10 μg/ml), or cisplatin (3.125, 6.25, 12.5, 25, 50 μM) in growth medium. For analysis, cells were harvested by trypsinization, and resuspended in PBS containing 10 μg/ml propidium iodide (PI; Sigma-Alderich), 1:1000 7-Aminoactinomycin D (7-AAD; Sigma-Alderich), or 1:1500 Fixable Viability Dye eFluor® 780 (eBioscience, San Diego, CA), and incubated for 30 min at 4°C. Cells were then spun down for 5 min at 1350 rpm, resuspended in 200-300 μl of FACS buffer (10% FBS, 0.02% sodium azide in PBS), and analyzed on a Gallios cytometer (Beckman Coulter). Size of viable cells was determined by flow cytometry and quantified as the mean of fluorescence intensity for forward scatter (FCS). All FACS data were analyzed using FlowJo (Tree Star inc., Ashland, Oregan) software, version 10.0.

Assessment of Mitochondrial Load and Potential

Mitochondrial content was analyzed using an Operetta High Content Imaging System. Cells were seeded at 5, 000 cells/well in a 96-well μ-clear black bottom plate and allowed to adhere overnight. Cells were washed in PBS, and then left untreated or treated for 6 or 24 hrs with etoposide at 10 μM in growth medium. Following culture, cells were washed in PBS, then

incubated in phenol red-free medium (Invitrogen) supplemented 2 mM L-glutamine (Invitrogen), 100 IU penicillin (Invitrogen), and 100 mg/ml streptomycin (Invitrogen), containing mitochondrial stains: 150 nM of mitotracker red FM (Life technologies M-7512) and 100 nM of mitotracker green FM (Life technologies M-7515). Cells were incubated for 30 min at 37° C and read on the Operetta high content imaging system (Perkin Elmer, Waltham, MA). All Operetta data was analysed using Columbus software (Perkin Elmer).

Quantitative real-time PCR

After drug treatment, cells were washed with sterile PBS, and total mRNA was extracted with 1 ml TRIzol® reagent (Invitrogen; Carlsbad, CA, 15596-026) on ice. Cells were harvested with a cell lifter, kept at room temperature for 1-5 min, and 0.2 ml of chloroform was added. After vigorous shaking for 30 sec, mixture was centrifuged at 11, 000g for 15 min at 4°C, and the upper clear phase was collected. An equal amount, than the collected solution, of 70% ethanol was added to the precipitate RNA and mixed by vortexing. The samples were pipetted onto RNeasy columns and centrifuged at 8, 000g for 30 sec at 4°C. Centrifuging and discarding of the supernatant and on-column DNAse digestion was done following the QIAGEN protocol (#79254). After the RNeasy columns were placed in sterile microfuge tube, 0.05 ml of RNase-free water was added and incubated at room temperature for 1-5 min. After centrifuging at 8, 000g for 1 min, the RNA was quantified using a Thermo Scientific nanodrop and stored at -80°C.

cDNA synthesis was carried out using Omniscript kit (QIAGEN; Toronto, Canada, # 2051100). This kit uses a combination of oligo(dt) primers and random hexamers to prime cDNA synthesis. Quantitative PCR

was performed using SYBR® Green qPCR SuperMix (Invitrogen), and an Mx3005 qPCR machine (Agilent) using validated qPCR primers against p21, PGC1α, actin (QIAGEN). The mRNA levels were normalized to β-actin mRNA, and the relative mRNA levels were determined by using the $2^{-\Delta\Delta Ct}$ method. The primer sequences are listed in Table 2.

Thermal cyclic protocol: (1) 95°C for 2 min; (2) 95°C for 30 sec; (3) 60°C for 30 sec; (4) 68°C for 30 sec; (5) return to step (2) 39 times. Primers (Table 1) were used at a final concentration of 200 nM.

Protein Extraction and Western Blot Analysis

For immunoblot experiments, MEFs, HCT116, and H1299 cells were grown in 6 cm dishes and treated with or without drugs for 1, 6, or 24 hrs. Following the culture period, cells were lysed in 80 μl of modified CHAPS buffer (10 mM Tris-HCL, 1 mM $MgCl_2$, 1mM EGTA, 0.5 mM CHAPS, 10% glycerol, 5mM NaF) supplemented with the following additives: protease and phosphatase tablets (Roche; Indianapolis, IN), DTT (1 M), and benzamide (500 mM). Cell extracts were incubated on ice for 30 min, after which lysates were sonicated and cell debris removed by centrifugation (12, 000 rpm for 10 minutes at 4°C). Protein concentration of lysates was determined by BioRad Protein Assay Dye Reagent Concentrate (Bio-Rad). Bradford assay readings were determined using Softmax Pro software. 20 μg of protein was boiled for 5 min at 100°C and separated by 10% SDS-PAGE at 100V for 90-120 min.

Following SDS-PAGE, proteins were transferred to nitrocellulose membrane over night at 30 V in transfer buffer (10% of 10x Transfer buffer, 30% methanol, and 70% Milli-Q water). Non-specific binding sites were blocked by incubating membranes in blocking buffer (4% skim milk in 0.05% (v/v) Tween in 1x PBS (PBS-T)) for 45-60 min at room temperature with

gentle shaking. Membranes were incubated overnight at 4°C with shaking in the appropriate dilution of primary antibodies. Primary antibodies were diluted in 4% bovine serum albumin (BSA) in PBS-T and 0.1% sodium azide. Primary antibodies used are as follows: AMPKα (pThr172 and total), Acetyl-CoA Carboxylase (pSer79 and total), p53 (pSer15 and total), ULK (pSer555 and total), LC3B, Actin, and GAPDH. All primary antibodies, as well as Anti-HRP-conjugated anti-rabbit secondary antibodies were purchased from Cell Signalling Technologies (Danvers, MA). All primary antibodies were diluted 1:1000 and the secondary antibody was diluted 1:5000. Membranes were washed 2x 5 min in 4% skim milk and 3x 5 min in PBS-T. Membranes were then diluted in secondary antibody in 4% skim milk and PBS-T with appropriate secondary antibody. Westerns were developed using chemiluminescence reagent (ECL; Thermo, Amersham, Germany) for 3 min and exposed to Amersham Hyperfilm for sufficient time to obtain a clear signal (typically 15 sec to 10 min).

Seahorse XF96 Respirometry

Oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) of cells were measured using an XF96 Extracellular Flux Analyzer (Seahorse Bioscience, Massachusetts, USA). Cells were seeded at 7, 000 – 10, 000 cells/well in 96-well plates in 80 µl DMEM (Invitrogen) containing 10% FBS, 2mM L-glutamine (Invitrogen), 100 IU penicillin (Invitrogen), and 100 mg/ml streptomycin (Invitrogen) and allowed to adhere overnight. Cells were then left untreated or treated for 6 or 24 hrs with etoposide at 10 µM in the appropriate medium. Cells were then washed in nonbuffered DMEM containing 25 mM glucose, 5 µl of 10 N NaOH. Cells were incubated in a CO_2-free incubator at 37°C for 1h to allow for

temperature and pH equilibration prior to loading into the XF96 apparatus. XF assays consisted of sequential mix (2 min), pause (2 min), and measurement (5 min) cycles, allowing for determination of OCR/ECAR every 10 min. Cells were normalised to cell number by fixing the cells in 4% paraformaldehyde (PFA) for 20 min at 4°C, washed 2 x 5 min in PBS, and then incubated in crystal violet (0.05% crystal violet, 20% EtOH 2%, H_2O) for 30 min. Crystal violet was then removed and cells washed 3 x 5 min in PBS before solubilizing on rocker for 1 hr in 1% SDS/PBS. Crystal violet content was determined for all samples at 595 nM using Softmax Pro software.

Statistical Analysis

All experiments were repeated at least three times and each experiment was performed at least in biological duplicates. The data was expressed as means of ± SD (standard deviation). Statistical analysis was performed using Student's t-test using Prism software (GraphPad). The criterion for statistical significance was represented in figures as follows: *P<0.05; **P<0.01; ***P<0.001.

Table 1: shRNA sequences used for retrovirus infection

	Sequence for shRNA 5'→3'
AMPKα1	GAGGAGAGCTATTTGATTA
AMPKα2	GCCATAAAGTGGCAGTTAA
p53	CACTACAAGTACATGTGTA

Table 2: PCR Primers used for real-time PCR to determine gene expression levels

Gene	Forward Primer Sequence	Reverse Primer Sequence
Mouse Actin	ATGCTCCCCGGGCTGTAT	CATAGGAGTCCTTCTGACCCATTC
Human Actin	TCCCTGGAGAAGAGCTACG	GTAGTTTCGTGGATGCCACA
mPGC1α	AATCAGACCTGACACAACGC	GCATTCCTCAATTTCACCAA

RESULTS

Establishing a model system to test cellular responses to genotoxic agents

Due to a known link between AMP-activated protein kinase (AMPK) and p53 [4], we wanted to assess the interdependence of these two agents in response to DNA damage. Specifically we wanted to assess cell viability in response to different genotoxic agents. To test this, I developed an experimental cell culture model as follows: MEFs or cancer cells were cultured overnight, treated with different chemotherapeutic drugs the following day, and cell viability determined at different time points (24, 48, or 72 hours) following drug treatment using viability dyes (Figure 7A). As a proof of concept for this assay system, I used SV40-transformed $bax^{-/-}$ $bak^{-/-}$ MEF cells. MEFs lacking expression of both Bax and Bak are resistant to apoptosis induced by intrinsic death signals [136]. Bax Bak DKO and control cells were treated with three different chemotherapeutic drugs in a dose dependent manner (1/3 log step): etoposide over 24 hrs (Figure 7B), doxorubicin over 48 hrs (Figure 7C), and cisplatin over 24 hrs (Figure 7D). Cell viability was assessed using viability dies propidium iodide (PI) in response to etoposide and cisplatin treatment, and 7-amino actinomycin (7-AAD) in response to doxorubicin treatment. 7-AAD was used in response to doxorubicin treatment due its red colour, which can lead to interference in the FL2 channel of the Gallios flow cytometer. In all three conditions, Bax Bak DKO cells were resistant to apoptosis induced by these agents, whereas control cells were susceptible to the genotoxic agents in a dose dependent manner.

p53-deficient MEF cells are susceptible to death in the absence of AMPK signalling

I next wanted to test whether disrupting AMPK confers increased sensitivity to genotoxic agents. To do this I developed a MEF cell model system in which p53 activity was decreased in both wild type (WT) and AMPKα-deficient cells (lacking both $\alpha1^{-/-}$, $\alpha2^{-/-}$; AKO) using a p53-specific shRNA (shp53) (Figure 8A). WT or AMPKα-deficient MEFs were infected with shRNA vectors targeting p53 or firefly luciferase (as a control). To validate AMPKα-deficiency I treated cells with the AMPK agonist AICAR (1.25 mM, 1 hour), and assessed the activation and activity of AMPKα by immunoblot (Figure 8B). I observed an increase in activation and activity of AMPK in both control (shCtrl) and shp53 cells, seen by an increase in the phosphorylation of AMPKα at Thr172 and phosphorylation of ACCα at Ser79, respectively. In contrast, Thr172 and Ser79 phosphorylation was absent in AMPKα-deficient cells and AMPKα-deficient/p53 shRNA-expressing cells (AKO/shp53) in response to AICAR treatment.

Next, I validated the knockdown of p53 by treating the MEF cells with a topoisomerase II inhibitor, etoposide, at 10 μM for 6 hours to assess the activation of p53 by immunoblot (Figure 8C). Cells expressing wild type p53 displayed increased Ser15 phosphorylation in response to etoposide treatment, regardless of AMPKα status. The activation of p53 was lower in shp53 and AKO/shp53 cells, seen by reduced phosphorylation of p53 at Ser15, and reduced total levels of p53.

p53-dependent cell cycle arrest is primarily mediated by the activation of the cyclin-dependent kinase inhibitor p21 [169]. I next tested the relative mRNA levels of p21 by quantitative real time polymerase chain reaction (qPCR) in my cell lines in order to assess the activity of p53 (Figure 8D).

Transcript levels were determined relative to actin mRNA levels and normalized relative to the control (shCtrl) cells. Relative p21 mRNA levels were significantly reduced in shp53 and AKO/shp53 cells in comparison to the control cells (shCtrl, AKO).

p53-deficient cells are resistant to death induced by DNA damage [221]. I next assessed whether loss of AMPKα expression could impact the viability of cells with reduced p53 expression. To do this I measured the viability of my cell lines (from Figure 8) to increasing doses of etoposide (1/3 log step, μM) (Figure 9A), doxorubicin (1/3 log step, μg/ml) (Figure 9C), and cisplatin (1/2 log step, μM) (Figure 9E). As expected, MEF cells expressing p53-specific shRNA (shp53) maintained cell viability in response to all three genotoxic agents. However, AKO/shp53 cells displayed increased susceptibility to cell death induced by all three agents, at similar levels to that observed with control cells or AKO cells. Cell viability, in the absence of AMPK, was also shown at one specific dose of etoposide at 30 μM over 24 hours (Figure 9B), doxorubicin at 3 μg/ml over 48 hours (Figure 9D), and cisplatin at 10 μM over 48 hours (Figure 9F). These data suggest that shp53 cells lose resistance to chemotherapeutic drug treatment when AMPK is absent.

p53-null Cancer Cells are susceptible to death in the absence of AMPK

I next wanted to test if the phenotype described in Figure 9 was also true for p53-deficient cancer cell lines. We chose p53-null H1299 non-small cell lung carcinoma (NSCLC) cells as a model system. Takla Griss from the Jones laboratory generated H1299 cells expressing shRNAs targeting AMPKα1 and α2 [6]. We first validated AMPK knockdown in these cells

using metformin, an AMPK agonist. AMPKα activity and activation in H1299/shCtrl and H1299/shAMPKα was assessed by immunoblot following treatment with metformin (5 mM for 1 hour) (Figure 10A). Upon metformin treatment, the activation of AMPK in shAMPKα cells were significantly lower than in shCtrl cells, as determined by the phosphorylation of AMPKα at Thr172. AMPK activity was also lower, as determined by reduced phosphorylation of ACCα at Ser79. We next assessed the viability of H1299 cells using the apoptosis assay as in Figure 9. Viability of H1299/shCtrl and H1299/shAMPKα was determined in response to increasing doses of doxorubicin (1/3 log step, μg/ml) (Figure 10B) and cisplatin (1/2 log step, μM) (Figure 10D). shCtrl (p53-deficient) cells were resistant to both doxorubicin and cisplatin, while shAMPKα cells displayed increased sensitivity to drug treatment in a dose-dependent manner. I have also presented cell viability at one specific dose of doxorubicin at 10 μg/ml over 72 hours (Figure 10C) and cisplatin at 50 μM over 72 hours (Figure 10E), where we see significant levels of cell death when AMPK signal is blocked in these p53-deficient cancer cells.

p53-dependent apoptosis relies on Bax and Bak

In Figures 9 and 10 I have shown that p53-deficient cells gain sensitivity to apoptosis induced by genotoxic agents when AMPK is absent. I next tested the cell death response of Bax Bak DKO cells lacking AMPK expression (Figure 11). To test this I generated Bax Bak DKO cells with AMPK knockdown using the techniques described in Figure 8. I determined the level of AMPKα knockdown for three different cell clones (clones 1-3) by assessing the activation and activity of AMPK by immunoblot in response to 1.25 mM of AICAR for 1 hour (Figure 10A). The decreased level of activation of AMPKα, in shAMPKα clones 1, 2, and 3, was seen by the

reduced phosphorylation of AMPKα at Thr172. The decrease in activity was seen by the reduced phosphorylation of ACCα at Ser79. shAMPKα clones 1 and 2 were chosen for further validation based on their reduced level of AMPK activation (as seen in Figure 11A). Bax Bak DKO and Bax Bak DKO/AMPKα shRNA-expressing cells (clones 1 and 2) all displayed resistance to apoptosis in response to etoposide (1/2 log step, μM) (Figure 11B), doxorubicin (1/3 log step, μg/ml) (Figure 11C), and cisplatin (1/2 log step, μM) (Figure 11D) after 72 hours of treatment, while the control cells were susceptible to death in a dose-dependent manner. These data suggest that AMPKα loss is not sufficient to confer sensitivity to genotoxic agents in cells lacking Bax and Bak.

Loss of AMPK promotes a glycolytic signature in p53-deficient cells

One possibility for the increased susceptibility of p53-null cells to chemotherapy-induced apoptosis is the disruption of cellular bioenergetics. To assess this possibility, I conducted bioenergetic profiling of my cell lines using the Seahorse Bioscience XF96 Extracellular Flux Analyser, which enables the measurement of cancer cell metabolism in real time in a microplate. The Seahorse measures oxygen consumption rate (OCR), a measure of mitochondrial respiration, as well as extracellular acidification rate (ECAR), a measure of glycolysis [222]. Figure 12A represents an optimal OCR curve.

These measurements occur upon drug injection in real time. In a standard protocol, we inject 4 drugs in sequence to identify mitochondrial fitness. The first port injects the drug oligomycin, an ATP-synthase blocker (compound V inhibitor) of ETC. This allows for the measurement of

uncoupled respiration, which represents the proton leak. The second port injects the drug FCCP, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone ionopore that transports the H^+ through the membrane before it can be used for ATP synthesis resulting in maximal respiration. The third port injects a mixture of both the drug rotenone, a complex I inhibitor, and antimycin A, a complex III inhibitor by binding to the Q_i site of the cytochrome C reductase, inhibiting the oxidation of ubiquinol. This combination measures the non-mitochondrial respiration, by ultimately shutting down the ETC.

To examine the metabolic consequences of AMPK loss in p53-deficient MEF cells, I examined the effect of AMPK loss in response to DNA damage by etoposide treatment. I first measured the OCR and ECAR (Figure 12B-C) for shp53 or AKO/shp53 cells under basal growth conditions and in response to treatment. MEF cells expressing p53 shRNA displayed no significant difference in OCR (Figure 13A) but displayed almost a twofold increase in ECAR (Figure 13B) following 10 μM etoposide treatment over 24 hours.

I next was able to measure the contribution of ATP production versus proton leak to total mitochondrial respiration. shp53 cells displayed a slight decrease in response to etoposide treatment (Figure 13C). A similar trend was seen for maximal respiration of shp53 with etoposide treatment (Figure 13D). I then measured the spare respiratory capacity (SRC) in these cells, which had almost a twofold decrease in response to etoposide treatment (Figure 13E), whereas their mitochondrial efficacy has no significant change to drug treatment (Figure 13F).

In the absence of AMPKα in shp53 MEF cells, there was more than a twofold increase OCR at baseline. Although in response to etoposide treatment over 24 hrs, the OCR was non-detectable (ND; Figure 14A). The

ECAR of AKO/shp53 displayed a threefold increase at baseline and was non-detectable (ND) in response to etoposide treatment over 24 hrs (Figure 14B). This observation of increased basal ECAR, in cells lacking AMPK, suggests that downregulation of AMPK signalling enhances the Warburg effect in these cells [6]. In the absence of AMPK in shp53 cells, there was no significant difference in the total respiration at baseline, and was non-detectable (ND) in response to the genotoxic agent (Figure 14D). A similar trend was seen for the maximal respiration (Figure 14E) and in the percent of mitochondrial efficacy (Figure 14F). Due to the absence of AMPK, cells were not able to promote mitochondrial turnover and therefore upon cellular stress, the absence of AMPK ultimately led to cell death.

AMPK promotes mitochondrial biogenesis in response to genotoxic stress

The data presented suggest that maintenance of mitochondrial respiration in p53-null cells in response to etoposide treatment requires AMPK. Thus, I next measured the impact of AMPK expression on mitochondrial content (load) and membrane potential in shp53 and AKO/shp53 MEF cells using high content imaging. Mitochondrial content and membrane potential were determined using the imaging dyes Mitotracker green and Mitotracker red, respectively (see materials and methods). Following 24 hours of etoposide treatment (10 μM), mitochondrial load increased by more than seven-fold in p53 shRNA-expressing MEFs compared to untreated cells (Figure 15A). AKO/shp53 cells displayed a similar increase in etoposide-stimulated mitochondrial load (Figure 15A).

I next measured mitochondrial membrane potential in shp53 and AKO/shp53 MEF cells. MEFs expressing p53 shRNA displayed a four-fold

increase in membrane potential upon exposure to etoposide compared to untreated cells (Figure 15B). Likewise, mitochondrial membrane potential increased in AKO/shp53 cells in response to etoposide treatment relative to non-treated cells. At baseline, AKO/shp53, show a slight increase in mitochondrial potential to shp53, but upon etoposide treatment, the mitochondrial potential significantly increases about twenty-fold (Figure 15B). Finally, we determined the ratio of mitochondrial potential relative to total mitochondrial content in shp53 and AKO/shp53 cells following etoposide treatment (Figure 15C). We observed a decrease in mitochondrial load versus membrane potential only in p53 shRNA-expressing cells lacking AMPK.

Genotoxic stress induces AMPK-dependent ULK phosphorylation

One of the downstream targets of AMPK is the autophagy-initiating protein ULK1 [207, 208, 210]. Upon AMPK activation, AMPK directly phosphorylates ULK1 to trigger mitophagy. ULK1 is phosphorylated by AMPK on four sites: Ser-467, Ser-555, Thr-574, Ser-637 [207]. To assess whether ULK1 activation could be triggered by AMPK in response to genotoxic stress we measured Ser-555 phosphorylation of ULK1 after etoposide treatment by immunoblot (Figure 16A). We examined levels of Ser-555 ULK1 in shp53 and AKO/shp53 MEFs over 0, 2, 4, 8, and 24 hours of etoposide treatment (10μM). We observed phosphorylation of ULK1 at Ser-555 in an AMPK-dependent manner in shp53 cells; with maximal ULK1 phosphorylation occurring 24 hours post treatment. ULK1 phosphorylation followed the same trend as both AMPKα and ACCα phosphorylation.

However, ULK1 phosphorylation at Ser-555 was dramatically reduced in AKO/shp53 cells (Figure 16A).

To assess whether autophagy induction was affected by etoposide treatment in p53 shRNA-expressing cells, I measured the expression of the autophagy long chain 3 marker (LC3B) in shp53 and AKO/shp53 MEF cells following etoposide treatment. Cells were left untreated or treated with 10 μM etoposide for 6 hours, and cell lysates were processed by immunoblot (Figure 16B). I observed increased phosphorylation of AMPKα at Thr-172 and ULK1 at Ser-555 in shp53 but not AKO/shp53 cells as in Figure 16A. Similarly, LC3B expression was reduced in AKO/shp53 MEFs relative to shp53 MEFs.

We then wanted to assess AMPK-dependent autophagy in HCT116 cancer cells. Using control (shCtrl), p53 knockout clones 1 and 2 (p53KO cl.1 or 2), and p53 knockout/AMPKα-specific shRNA (p53KO/shAMPKα) HCT116 cells, we examined the phosphorylation of ULK1 at Ser-555 after treatment with the AMPK agonist, AICAR, at 2 mM over 1 hour by immunoblot (Figure 16C). In the control, p53KO cl. 1 and 2, we observed phosphorylation of ULK1 at Ser-555 in an AMPK-dependent manner, following that same trend as the phosphorylation of AMPKα at Thr-172. We also observed the activity of AMPK by the phosphorylation of ACCα at Ser-79. In the absence of AMPK, no phosphorylation was seen in ULK1 at Ser-555, AMPKα at Thr-172, or ACCα at Ser79.

One of the key regulators of mitochondrial biogenesis in mammalian cells is the peroxisome proliferator-activated receptor c coactivator 1α (PGC1 α) transcriptional coactivator [223]. Thus, I measured the relative levels of PGC1α mRNA level p53 shRNA-expressing MEFs by qPCR (Figure 16D). MEF cells expressing p53 shRNA displayed a significant increase in PGC1α

mRNA levels regardless of etoposide treatment (Figure 16D). This increase in PGC-1α mRNA expression was reduced in AKO/shp53 MEFs.

Figures

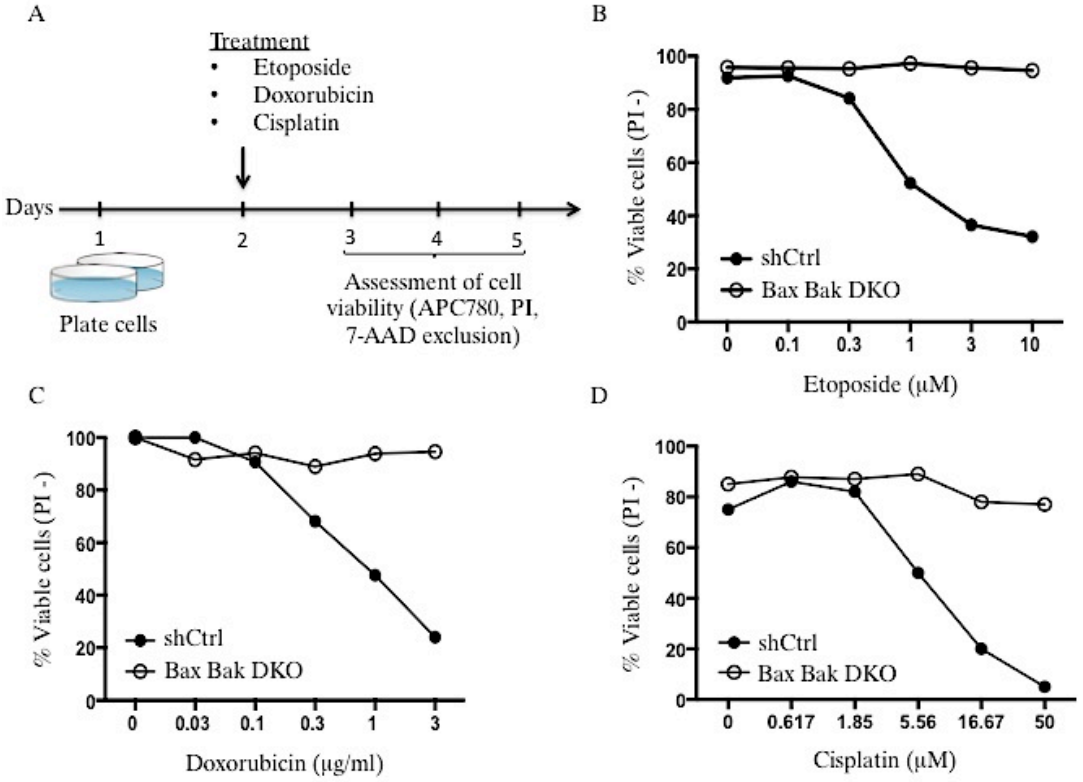

Figure 7: Validation of chemotherapy-induced apoptosis assay.

(A) Assay design for cell viability where we plated cells over night, treated with the genotoxic agent the following morning, and assessed the cell viability over 24, 48, or 72 hrs by viability dye exclusion. (B-D) Assay validation of a series of doses over a series of days. These are dose response curves of cell viability of control (shCtrl, closed circle), or Bax Bak-null cells (Bax Bak DKO, open circle) in response to increasing doses of (B) etoposide (1/3 log step) (24 hrs), (C) doxorubicin (1/3 log step) (48 hrs), or (D) cisplatin (1/3 log step) (24 hrs). Cell viability was assessed by flow cytometry, using (B and D) PI, or (C) 7-AAD dyes. These experiments are representative of four independent experiments in single measurements. Viability data for a minimum 10,000 cells per condition.

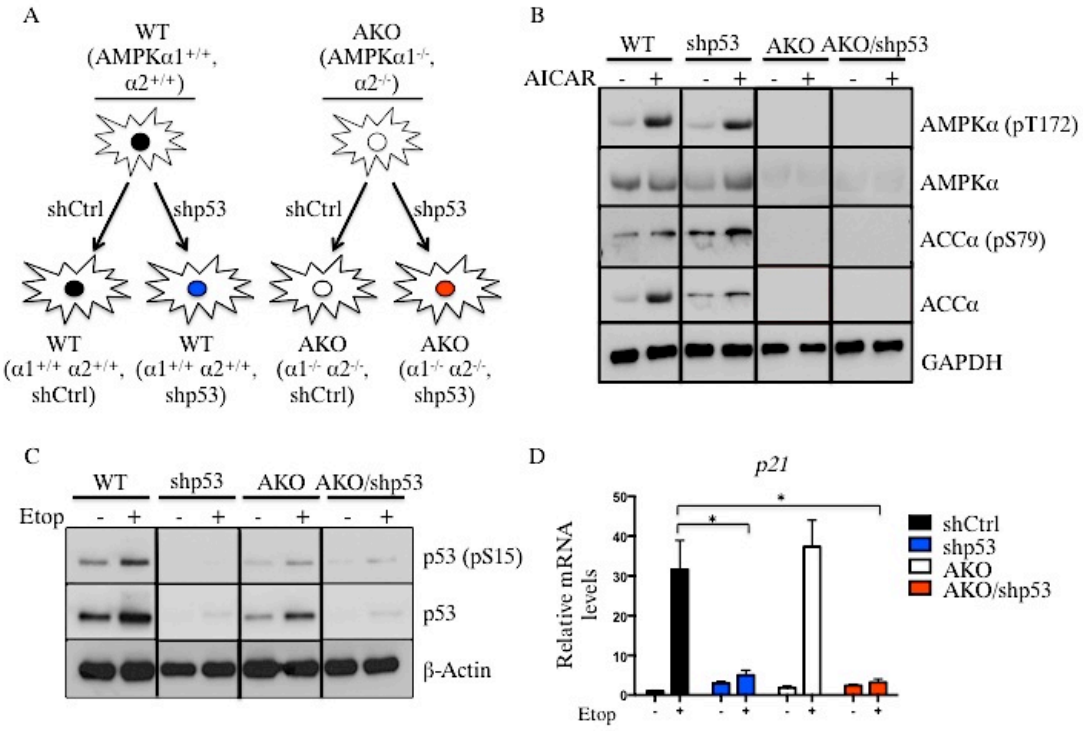

Figure 8: Generation of AMPK-deficient, p53 knockdown lines.

(A) Procedure for generating cells. (B and C) Immunoblot analysis for (B) ACCα Ser79 phosphorylation, total ACCα, AMPKα Thr172 phosphorylation, total AMPKα, (C) p53 Ser15 phosphorylation, and total p53 in whole-cell lysates from control (shCtrl), p53-specific shRNA (shp53), AMPKα-null (AKO), or AMPKα-null/ p53-specific shRNA (AKO/shp53) MEFs following treatment with (B) AICAR (1.25mM, 1 hr) and (C) etoposide (10 μM, 6 hrs). Actin levels are shown as controls. (D) Relative p21 mRNA expression in wild type (shCtrl, closed bar), p53-specific shRNA (shp53, blue bar), AMPKα -null (AKO, open bar), or AMPKα null/ p53-specific shRNA (AKO/shp53, red bar) MEF cells as determined by qPCR following treatment with etoposide (10 μM, 6 hrs). Transcript levels were determined relative to actin mRNA levels and normalized relative to control (shCtrl-) cells. Error bars represent the SD. *$p<0.05$ relative to control (shCtrl+).

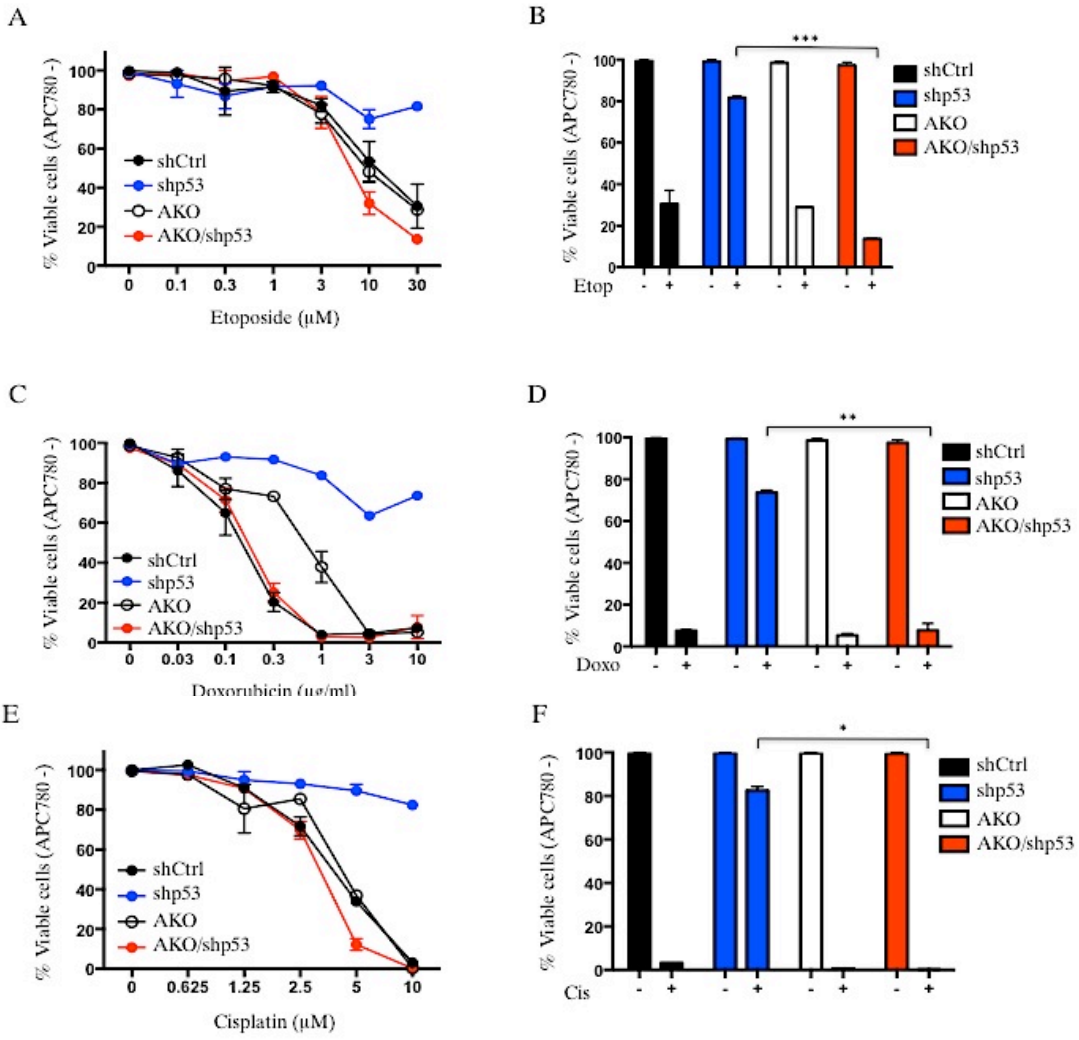

Figure 9: p53-deficient MEFs are susceptible to death in the absence of AMPK.

(A, C, and E) Dose response curves of cell viability of control (shCtrl, closed circle), p53-specific shRNA (shp53, blue circle), AMPKα-null (AKO, open circle), or AMPKα-null/ p53-specific shRNA (AKO/shp53, red circle) in response to increasing doses of (A) etoposide (1/3 log step) (24 hrs), (C) doxorubicin (1/3 log step) (48 hrs), or (E) cisplatin (1/2 log step) (48 hrs). Cell viability was seen by flow cytometry with the viability dye APC 780. (B, D, F) Cell viability in response to (B) etoposide (30 μM, 24 hrs), (D) doxorubicin (3 μg/ml, 48 hrs), or (F) cisplatin (10 μM, 48 hrs). These experiment were done in triplicate and repeated three times. ***p<0.001; **p<0.01; *p<0.05 relative to p53-specific shRNA (shp53+) cells.

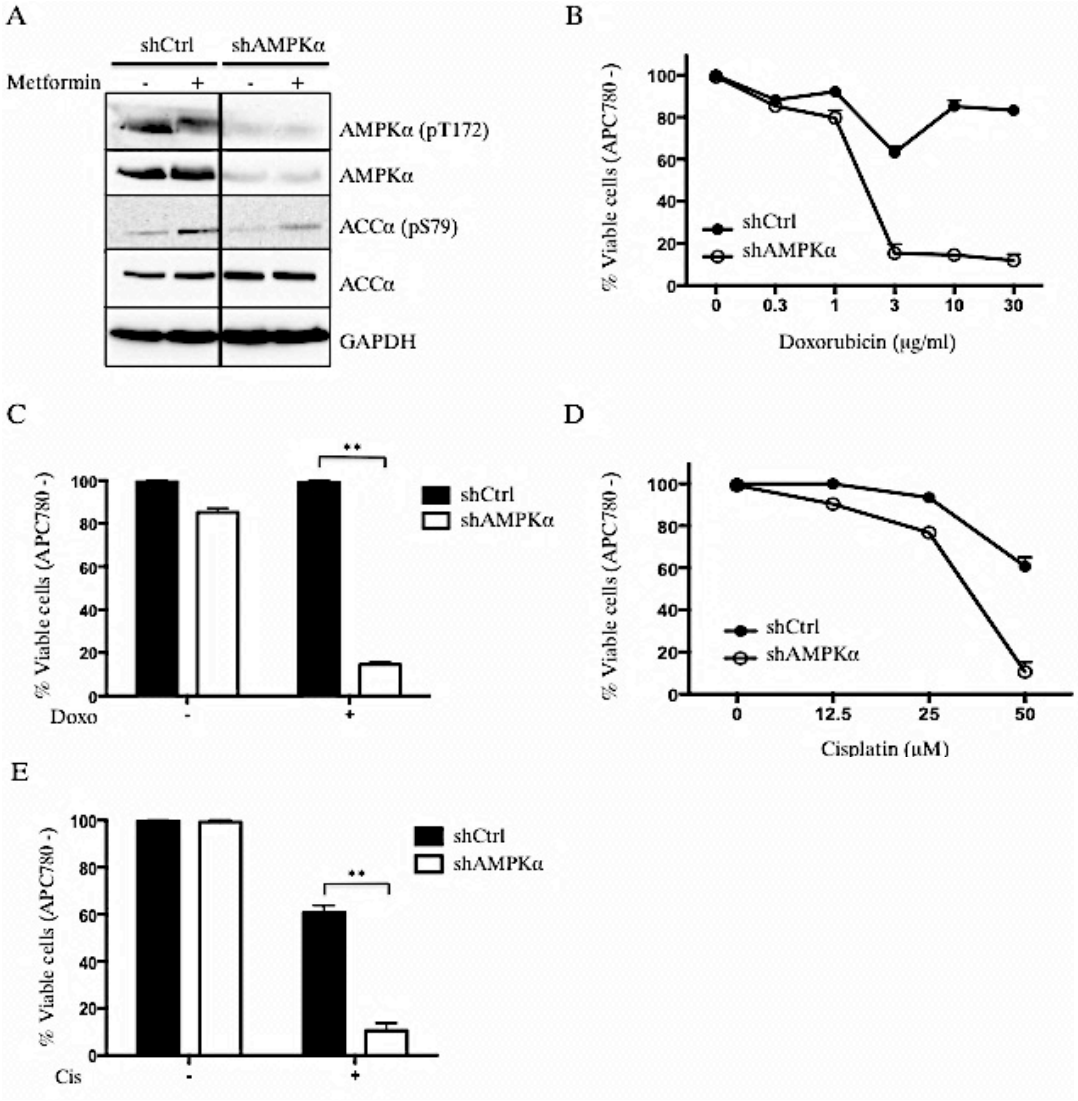

Figure 10: Cancer cells (p53-deficient) are susceptible to death in the absence of AMPK.

(A) Immunoblot analysis for ACCα Ser79 phosphorylation, total ACCα AMPKα Thr172 phosphorylation, or total AMPKα in H1299 control (shCtrl) or AMPKα-specific shRNA (shAMPKα) cells following metformin treatment (5 mM, 24 hrs). (B and D) Dose response curves for cell viability of control (shCtrl, closed circle) or AMPKα-specific shRNA (shAMPKα, open circle) cells in response to (B) doxorubicin (1/3 log step) (72 hrs), or (D) cisplatin (1/2 log step) (72 hrs). Cell viability was seen by flow cytometry with the viability dye APC 780. (C and E) Viability of H1299 cells in response to (C) doxorubicin (3 μg/ml, 48 hrs) or (E) cisplatin (10 μM, 48 hrs). **$p<0.01$ relative to control (shCtrl+) cells.

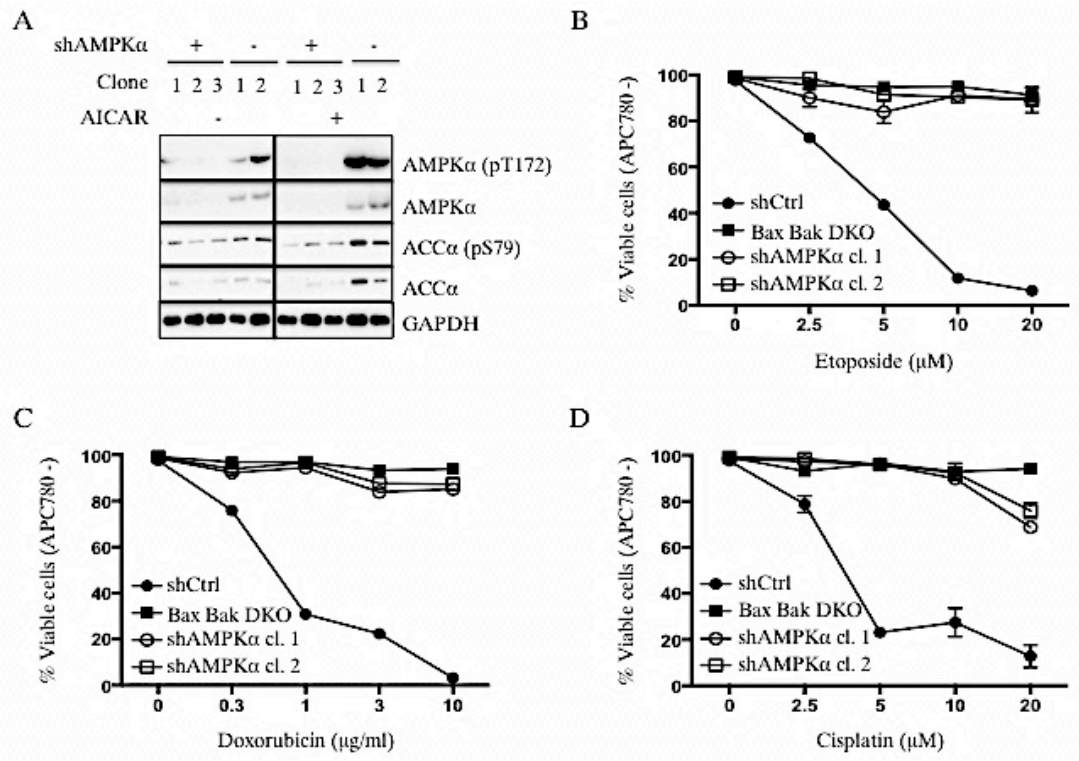

Figure 11: AMPK knockdown in Bax Bak DKO MEFs.

(A) Immunoblot analysis for ACCα Ser79 phosphorylation, AMPKα Thr172 phosphorylation, or total AMPKα in Bax Bak DKO control (shCtrl) or AMPKα-specific shRNA (shAMPKα) following AICAR treatment (1.25 mM, 1 hr). GAPDH levels are shown as a control. (B-D) Dose response curves of cell viability of control clone 1 and 2 (shCtrl, closed circle and closed square), AMPKα-specific shRNA clone 1, and 2 (shAMPKα, open circle and open square) in response to increasing doses of (B) etoposide (1/2 step log) (72 hrs), (C) doxorubicin (1/3 step log) (72 hrs), or (D) cisplatin (1/2 step log) (72 hrs). Cell viability was seen by flow cytometry with the viability dye APC 780. This experiment was done in duplicates and repeated three times. Viability data for a minimum 10,000 cells per condition. These are representative graphs of three independent experiments. Error bars represent the SD.

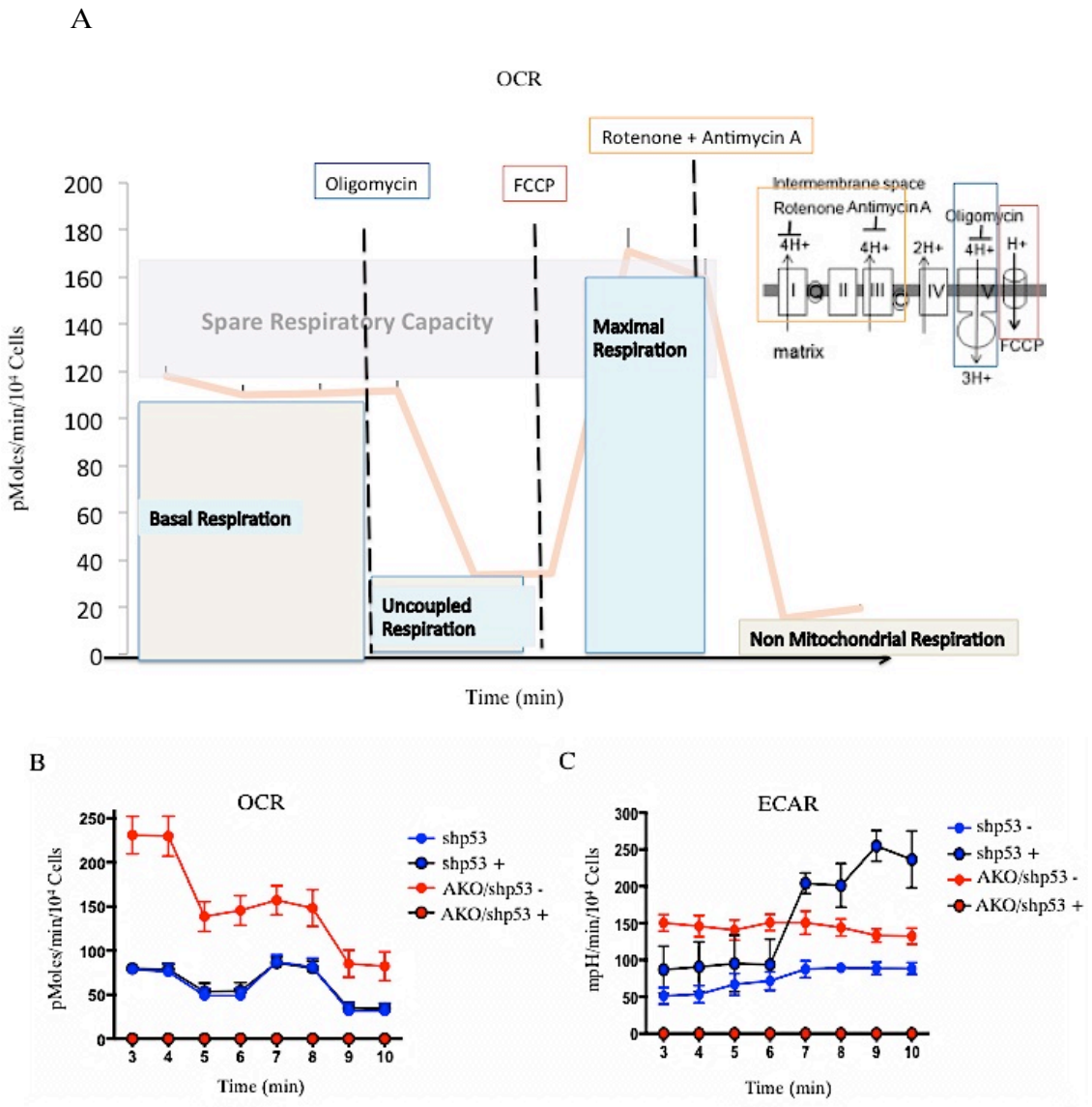

Figure 12: Bioenergetic profiling of p53-deficient MEFs in response to etoposide treatment

(A) Optimal oxygen consumption respiration (OCR) curve with oligomycin, FCCP, rotenone and antimycin A and the effect of each drug on electron transport chain. (B-C) Bioenergetic profile of etoposide-treated MEFs. p53 shRNA-expressing (shp53) and AMPKα-deficient/p53 shRNA-expressing (AKO/shp53) MEFs were exposed to etoposide (10 μM, 24 hours) prior to Seahorse analysis. Oligomycin (80 μM), FCCP (20 μM), and rotenone (32 μM) plus antimycin A (40 μM) were added in succession to the cultures. Profiles for (B) OCR and (C) ECAR are shown.

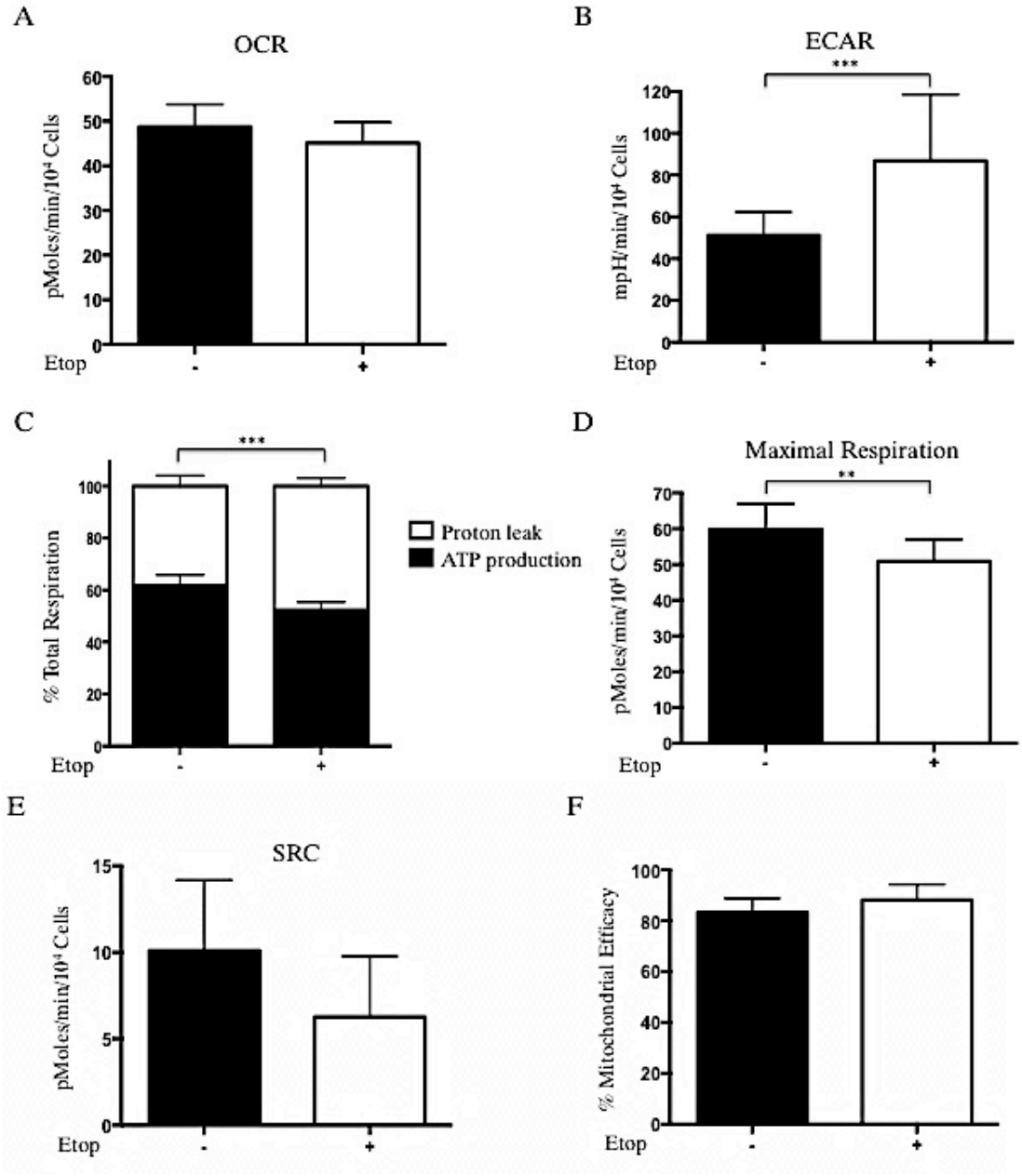

Figure 13: Metabolic analysis of p53-deficient MEF cells.

(A) Baseline OCR, in p53-expressing shRNA MEF cells, in response to etoposide treatment (10 μM, 24 hours). (B) Basal ECAR for cells as in (A). (C) Total respiration (proton leak/ATP production) for etoposide-treated MEFS. (D-F) Maximal mitochondrial respiration (D), spare respiratory capacity (SRC, E), and mitochondrial efficacy (F) for cells treated as in (A). in response to etoposide (10 μM, 24 hours). Cells were normalised by crystal violet analysis, and each data point represents mean ± SD (n=24). ***p<0.001; **p<0.01 relative to control (shp53 -) cells.

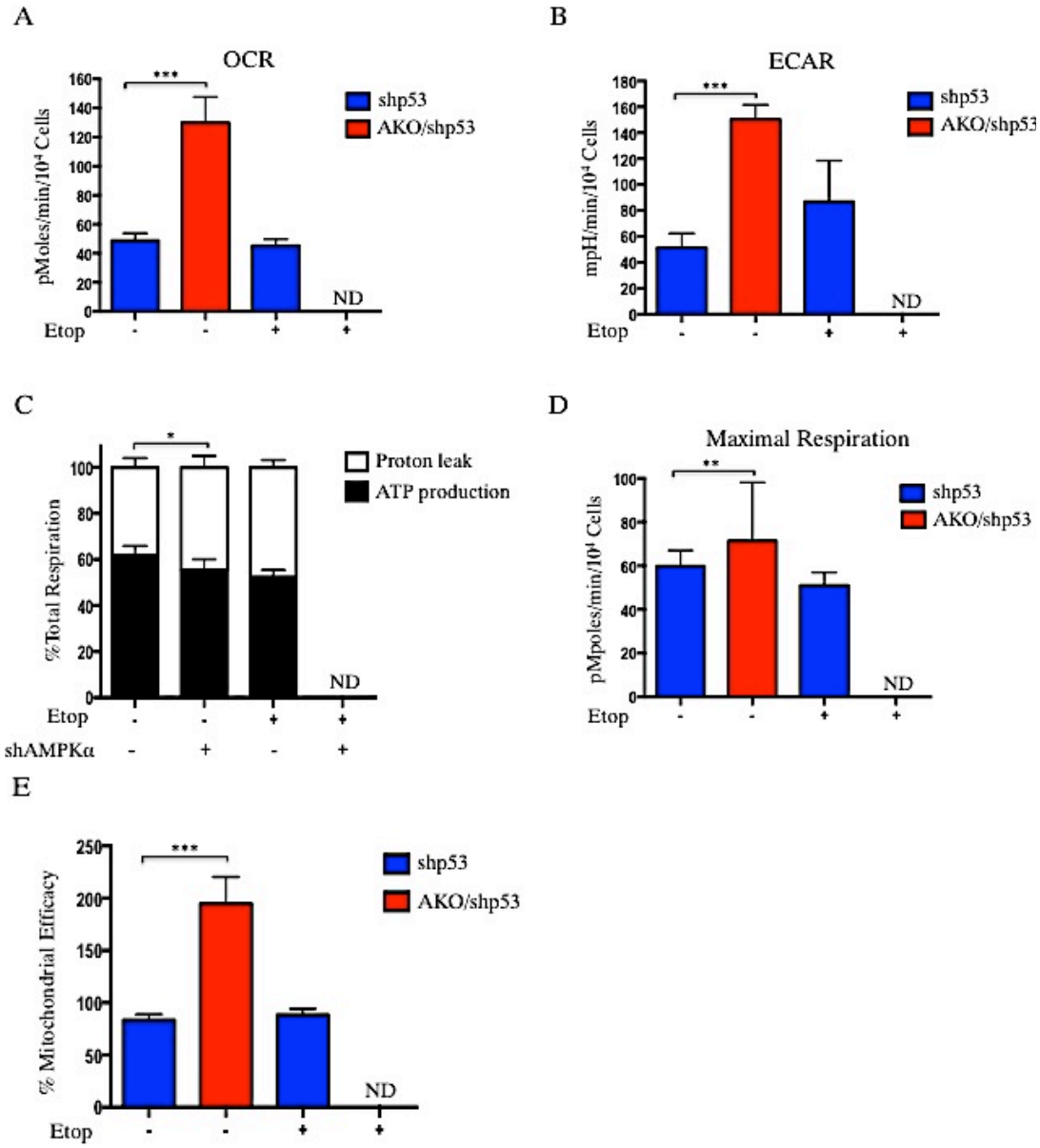

Figure 14: Metabolic analysis of AKO/shp53 MEFs.

(A) Baseline OCR of shp53 and AKO/shp53 MEFs following etoposide treatment (10 μM, 24 hours). (B) Basal ECAR was measured in parallel with respiration before the addition of oligomycin. (C) % Total respiration (proton leak/ATP production). (C) Maximal mitochondrial respiration. (D) Spare respiratory capacity (SRC) in response to etoposide (10 μM, 24 hours). (E) The percent of mitochondrial efficacy in response to etoposide treatment (10 μM, 24 hours). Cells were normalised by crystal violet analysis. Each data point represents mean ± SD (n=24). ***p<0.001; **p<0.01; *p<0.05 relative to control (shp53 -) cells.

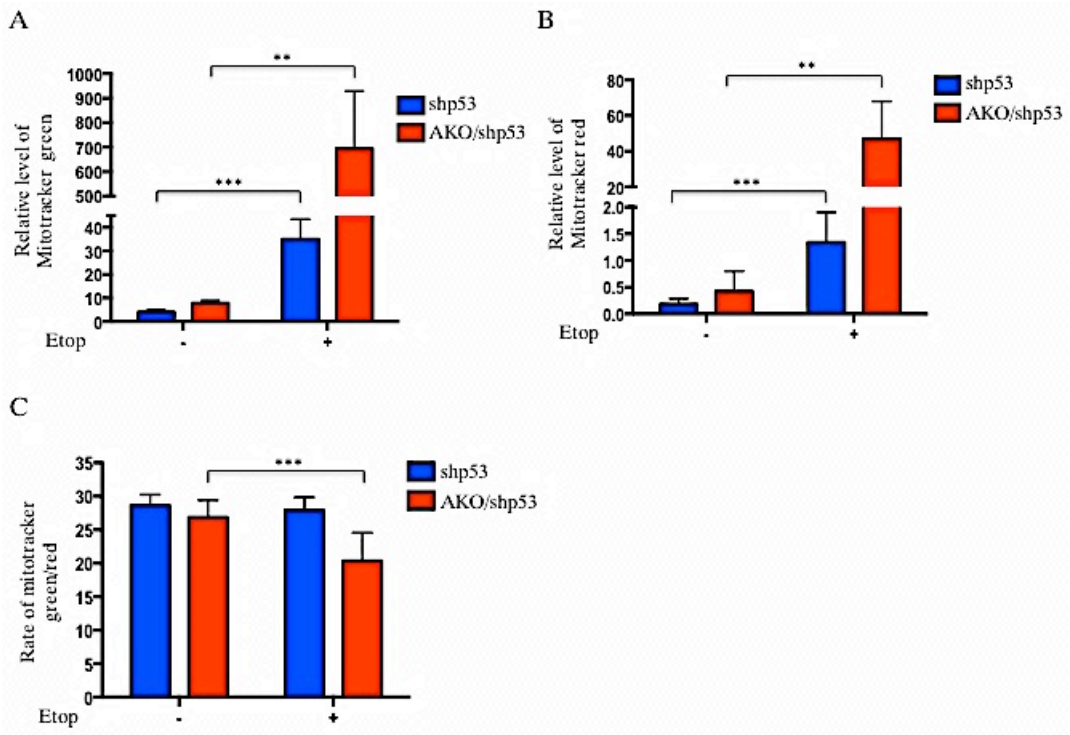

Figure 15: AMPK promotes mitochondrial biogenesis in p53-deficient cells in response to genotoxic agents

(A) p53-expressing shRNA (shp53) and AMPKα-deficient/p53-expressing shRNA (AKO/shp53) MEF cells were exposed to etoposide (10 μM, 24 hours). Total mitochondrial load was measured high content imaging using the Mitotracker green FM dye. (B) Mitochondrial membrane potential was measured using Mitotracker red FM dye. (C) Rate of mitochondrial membrane potential over mitochondrial load (Green/red). This experiment was done in triplicate. Mitotracker data for a minimum of 6,000 cells per condition. Error bars represent the SD. ***$p<0.001$; **$p<0.01$ relative to control (shCtrl- or AKO/shp53-).

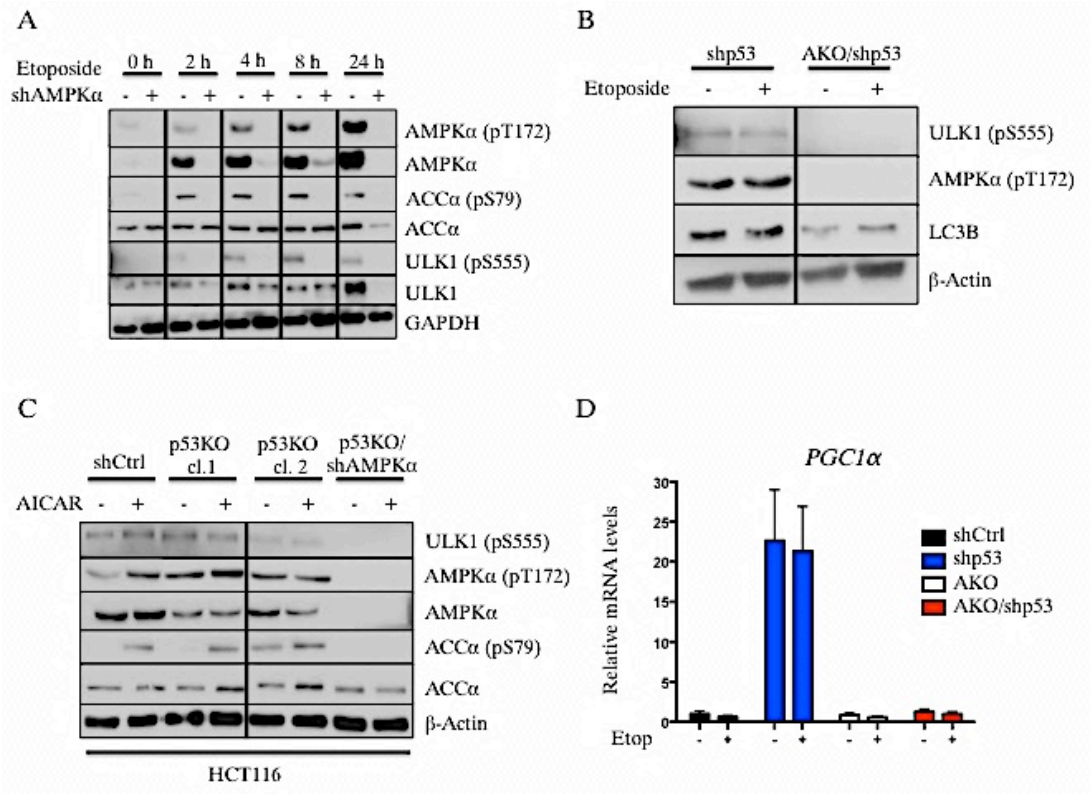

Figure 16: Loss of AMPK results in the loss of ULK1 and PGC1α expression in p53-deficient MEF cells.

(A) Immunoblot analysis for pT172 AMPKα, total AMPKα, pS79 ACCα Ser79, total ACCα, pS555 ULK1, and total ULK1 in p53-specific shRNA (shp53), or AMPKα-null/ p53-specific shRNA (AKO/shp53) expressing MEFs following etoposide treatment (10 μM for 0, 2, 4, 8, and 24 hrs). GAPDH levels are shown as a control. (B) Immunoblot analysis of pS555 ULK1, pT172 AMPKα, and LC3B levels in shp53 or AKO/shp53 MEFs following etoposide treatment (10 μM, 6 hrs). β-actin levels are shown as a control. (C) Immunoblot analysis for ULK1 Ser555 phosphorylation, AMPKα Thr172 phosphorylation, total AMPKα, ACCα Ser79 phosphorylation, or total ACCα in HCT116 from control (shCtrl), p53-knockout clones 1 and 2 (p53KO cl.1-2), or p53 knockout/AMPKα-specific shRNA (p53KO/shAMPKα) following treatment with AICAR (2 mM, 1 hr). β-actin levels are shown as a control. (D) Relative PGC1α mRNA expression in control (shCtrl, closed bar), p53-specific shRNA (shp53, blue bar), AMPKα-null (AKO, open bar), or AMPKα null/ p53-specific shRNA (AKO/shp53, red bar) MEF cells as determined by qPCR following treatment with etoposide (10 μM, 24 hrs). Transcript levels were determined relative to actin mRNA levels and normalized relative to control (shCtrl-) cells. Error bars represent the SD for triplicate samples.

DISCUSSION

Cancer is the leading cause of death in Canada and despite efforts in developing targeted therapies, cytotoxic chemotherapy remains a first-line therapy for most primary tumours. However, the emergence of tumour chemoresistance contributes to the majority of cancer-related deaths. One of the main factors that contributes to chemoresistance in human cancer is mutations in the p53 gene or perturbations in pathways that regulate p53 signalling [143]. Previous work from our laboratory has established a link between the energy sensor AMPK and p53, where AMPK activation can induce p53 phosphorylation at Ser-15; this phosphorylation is necessary to initiate AMPK-dependent cell-cycle arrest in response to metabolic stress [4]. Another link between AMPK and p53-regulated stress pathways is that AMPK is activated in response to DNA damaging agents [34], which in turn promotes cell survival in response to stress. Given these links, we wanted to assess the interdependence of AMPK and p53 in response to genotoxic agents. As p53-deficient MEF cells are resistant to cell death [221], we wanted to determine the impact of blocking AMPK signalling on the chemoresistance of p53-disrupted cells. Overall our results suggest that chemoresistant p53-null cells require AMPK to maintain cell viability in response to genotoxic agents. Mechanistically, p53-null cells lacking AMPK display defects in mitochondrial respiration, mitophagy, and mitochondrial biogenesis normally triggered by genotoxic stress. Our results suggest that cells lacking p53 rely on AMPK to maintain mitochondrial function in the face of cellular stress. Our data have revealed an “Achilles’ heel” for p53-null cells, and may represent a novel mechanism for targeting chemoresistant tumour cells.

The goal of my thesis was to investigate novel mechanisms that control cellular resistance to chemotherapy. Our first step was to validate a cell viability assay using SV40-transformed $bax^{-/-}$ $bak^{-/-}$ MEF cells, which are normally resistant to apoptosis induced by intrinsic death signals [136]. After treatment with three different genotoxic agents of different classes (etoposide, doxorubicin, and cisplatin), we observed that Bax and Bak-deficient cells are resistant to apoptosis, whereas the control cells were sensitive to the drug (Figure 7). We next developed a series of cell lines to test the interdependence of AMPK and p53 on cell survival by decreasing the activity of p53 in both wildtype (WT) and AMPKα-deficient cells using p53-specific shRNAs. These cells were validated by immunoblot in response to the AMPK agonist, AICAR (Figure 8B), and the topoisomerase II inhibitor, etoposide (Figure 8C), and the relative mRNA levels of p21 in order to assess the activity of p53 by quantitative real time polymerase chain reaction (Figure 8D). I then used these cells for cell viability assays using the chemotherapeutic agents etoposide, doxorubicin, and cisplatin. Interestingly, I found that blocking AMPK in p53-deficient MEF cells conferred sensitivity to genotoxic stress (Figure 9). To explore this question further, I tested the response of p53-null lung cancer cells (H1299 NSCLC cells) expressing AMPKα shRNA to various genotoxic agents. A similar trend was observed where control cells were resistant to chemotherapeutic drugs, while H1299 cells expressing AMPKα shRNA became susceptible to cell death (Figure 10). These results suggest that the loss of AMPK in p53-deficient cells can convert chemoresistant cells to chemosensitive cells.

My data demonstrates that both p53-deficient MEFs and cancer cells are susceptible to DNA damaging agents when AMPK is blocked. Given the role of p53 in the apoptotic pathway induced by DNA damage, one

possibility was that AMPK loss could bypass the normal apoptotic machinery in p53-null cells. Thus, we assessed the viability of cells lacking the intrinsic apoptotic pathway (MEFs lacking Bax and Bak) and AMPK activity (via expression of AMPKα shRNA) in response to etoposide, doxorubicin, and cisplatin. While there was some response of DKO/shAMPKα cells at high doses of cisplatin (Figure 11D), Bax Bak DKO cells with reduced AMPK activity were largely resistant to apoptosis induced by these agents (Figure 11B-E). One caveat of these experiments is that there may be some residual AMPK activity due to incomplete AMPKα knockdown that is sufficient to protect Bax Bak DKO cells from apoptosis. An alternative strategy would be to target AMPKα1 and α2 using a Crisper/Cas genome editing approach to disrupt AMPKα gene expression in Bax Bak DKO cells.

The susceptibility of p53-deficient cells to chemotherapy-induced apoptosis could be due to the disruption of mitochondrial bioenergetics. Given the central role of AMPK in regulating cellular adaptation to metabolic stress, I examined the metabolic consequence(s) of AMPK loss in p53-deficient cells. Remarkably, p53-deficient MEF cells displayed no significant difference in cellular oxygen consumption in response to etoposide treatment (OCR, Figure 13A) with a significant increase in ECAR levels (Figure 13B), suggesting increased aerobic glycolysis. In these cells, upon stress, there is a significant increase in uncoupled respiration, proton leak, suggesting that there are not enough protons to drive back through the ATP synthase to generate ATP. After the injection of FCCP, there is also a significant decrease in their maximal respiration, upon etoposide treatment, indicating that their mitochondria aren't able to increase their oxygen consumption upon stress. The spare respiratory capacity of shp53 cells decreased upon energetic stress, indicating that the cells are working at

maximal capacity and have no spare respiratory capacity. Finally, the mitochondrial efficacy has no significant different with or without treatment, suggesting that their mitochondria are working just over 80% of their maximal efficacy (Figure 13A-F).

Now I wanted to compare these results of shp53 cells to when AMPK is blocked. There is a significant increase in OCR at baseline and upon etoposide treatment the cells seem to undergo apoptosis. My data suggests that AMPK loss disrupts normal metabolic homeostasis in cells, which surprisingly has a positive effect on cell growth and proliferation. This downregulation in AMPK activity is sufficient to induce the Warburg Effect in MEF cells, seen by the significant increase in ECAR at baseline. These results suggest that the downregulation of AMPK activity eliminates a key metabolic checkpoint that normally antagonizes anabolic progrowth cellular metabolism. Thus, AMPK may act in MEF cells as a metabolic gatekeeper that functions to establish metabolic checkpoints that limit cell division, and its loss of function can enhance both tumourigenesis and tumour progression.

In the absence of AMPK we also see a significant increase in uncoupled respiration, meaning there is a decrease in the generation of ATP. Upon AMPK loss, there is a significant increase in maximal respiration at baseline, indicating that their mitochondria are used to increase their oxygen consumption. Although upon stress, the maximal respiration becomes non-detectable, possibly due to the early induction of apoptosis in AMPKα-deficient cells. Finally, the mitochondrial efficacy increases fourfold at baseline in AMPKα-deficient cells, but upon etoposide treatment, respiration in these cells is non-detectable (Figure 14 A-E). These results suggest that the mitochondria in AKO/shp53 cells are working 4 times as hard at baseline, whereas upon treatment the cells can no longer survive

without AMPK. These results may suggest that cells lacking AMPK activity were not able to promote mitochondrial biogenesis and therefore, upon stress, cells underwent apoptosis. A hallmark of cells defective for autophagy is a susceptibility to undergo apoptosis after cellular stress that normally would activate autophagy to induce cell survival [224].

Similar to AMPK activation, the cellular process of autophagy is induced under low-energy conditions and nutrient-deprivation as a survival mechanism to guarantee accessibility of important metabolic intermediates and the elimination of damaged organelles, such as mitochondria [211]. It has been shown that AMPK phosphorylation on downstream substrates (ULK1) is required for mitochondrial homeostasis and cell survival during nutrient deprivation [207]. Since I established that loss of AMPK activity in p53-deficient cells allows these cells to become chemosensitive to genotoxic agents, I then examined whether AMPK-deficiency in MEFs exhibited mitochondrial defects. In order to test whether AMPK-deficiency exerted effects on mitochondrial homeostasis, I used high content imaging and mitochondrial-selective dyes on shp53 and AKO/shp53 MEF cells. High content imaging and Mitotracker green, which is used for morphological and functional measurements of mitochondria, revealed that AKO/shp53 cells have increased levels of dye, indicating altered mitochondrial homeostasis as compared to shp53 cells, denoted by increase in the overall number of mitochondria (Figure 15A). This increase was enhanced upon etoposide treatment. To test whether these mitochondria were functionally impaired, I analyzed the mitochondrial membrane potential using the mitochondrial-selective Mitotracker red dye in these same cells (Figure 15B). The slight increase in membrane potential of AKO/shp53 cells, as compared to shp53 MEFs, revealed that their mitochondrial membrane potential was

compromised. When I measured the ratio of mitochondrial load versus potential, I observed a significant decrease in AKO/shp53 MEFs upon etoposide treatment, while there was no significant change in p53 shRNA-expressing cells with or without treatment (Figure 15C). AMPK is an important component in autophagy, where autophagy is a key mechanism by which damaged mitochondria are removed from the cell. Thus, my data may suggest that AMPK has an important role in mitochondrial homeostasis and cell survival in response to genotoxic drugs.

Autophagy is a cellular damaging process that functions to retain biological activities during cellular stress [225]. The regulation of autophagy by TORC1 is conserved in eukaryotes [226]. It has been shown that mammalian ULK1 is involved in autophagy regulation and lies downstream of TORC1 [227, 228]. mTORC1 phosphorylates ULK1 and represses the kinase activity of ULK1, thereby suppressing the initiation of autophagy [208, 229]. Not surprisingly, as an energy sensor, AMPK is also involved in autophagy [230] by inhibiting mTORC1 through phosphorylation of TSC2 [3] and Raptor [201, 230]. AMPK can induce autophagy by suppressing mTORC1 in response to cellular stress.

There is evidence that genotoxic agents trigger mitophagy [210] and my data suggests that AMPK is a regulator in this process. I observed that ULK1 is activated by etoposide treatment in a manner that depends on AMPK-mediated phosphorylation. ULK1 cannot be activated when AMPK-knockout MEFs are subjected to genotoxic agents, which suggests that AMPK has an essential role on ULK1 activation in response to chemotherapy (Figure 16A). Egan et al. [207] also showed that AMPK directly targets ULK1, by showing that AMPK lies upstream of ULK1 and that AMPK regulation of ULK1 is necessary for proper autophagy.

My data reveals a direct connection between energy sensing and core conserved autophagy proteins. In mammals, AMPK phosphorylation of ULK1 is necessary for ULK1 function in response to cellular stress. Due the suppression of mTOR activity and mTOR-dependent inhibition of ULK1 [228], AMPK can indirectly control ULK1. However, as mentioned, AMPK can directly target ULK1 and promote its activation. AMPK targets multiple ULK1 sites (Ser-317, Ser-467, Ser-555, Thr-575, Ser-637, and S777), matching the AMPK substrate motif, and promotes ULK1 function in autophagy [204, 207]. Given the conservation of AMPK sites in ULK1, I also wanted to examine autophagy through the autophagy marker light chain 3 B (LC3B). Similarly, no phosphorylation was observed in the absence of AMPK and an increase in LC3B phosphorylation was seen upon etoposide treatment (Figure 16B). This solidifies the idea that AMPK is required for initiation of autophagy. I next tested whether this same trend held true in cancer cells. I observed that a genotoxic agent, etoposide, activates ULK1 in an AMPK-dependent manner in HCT116 colon cancer cells (Figure 16C). In the absence of AMPK, ULK1 cannot be activated upon cellular stress. Thus, the same trend held true in both MEF cells and cancer cells, where ULK1 is targeted by AMPK in response to etoposide. Together these data establish that AMPK is a key component for the process of autophagy in response to genotoxic drugs. AMPK has opposing effects with mTORC1 on autophagy induction by coordinating phosphorylation of ULK1. Although, many questions still remain on how these energy-sensing kinases accomplish and organize their functions in response to various conditions that induce autophagy.

It has been suggested that AMPK-dependent phosphorylation of PGC1 α stimulates mitochondrial biogenesis [231, 232]. Since AMPK-

dependent mitochondrial biogenesis is mediated by the transcriptional coactivator peroxisome proliferator-activated receptor c coactivator 1α (PGC-1α), I measured the relative levels of PGC-1α mRNA in order to assess the dependence on AMPK (Figure 16D). Relative mRNA levels of PGC-1α were significantly increased in shp53 MEF cells regardless of genotoxic treatment. Therefore, if PGC-1α levels don't change with etoposide treatment, this may suggest that the PGC-1α program is always turned on. Interestingly, when you lose AMPK, this effect is no longer present, indicating that AMPK may lie upstream of PGC-1α and that p53 may be inhibiting/slowing down transcription until DNA damage is fixed.

My data indicates a role for AMPK in the stress response and survival of chemoresistant cells. Furthermore, inhibition of AMPK is sufficient to overcome chemoresistance of p53-deficient MEF and tumour cells. In Figure 17 I have included a model of how I think AMPK functions to protect p53-deficient cells from cellular damage during chemotherapeutic treatment. My data suggest that AMPK is important in promoting mitophagy and mitochondrial biogenesis programs in p53-deficient cells; without AMPK, p53-null cells cannot adapt to cellular stress and ultimately undergo cell death. One-way p53-deficient tumour cells may be adapting to cellular stress is by activating AMPK, which mediates a constant checking for mitochondrial fitness. Following from this, AMPK activation may confer resistance to chemotherapy in hypoxic tumours.

In a number of human cancers, AMPK activation is defective, due to inactivating mutations in one of its upstream kinases – LKB1. Therefore, ULK1 may play a key role downstream of the LKB1/AMPK pathway in tumour suppression or in metabolic disease treatment. This hypothesis is supported by my data demonstrating etoposide-dependent stimulation of

ULK1 phosphorylation and defects in autophagy in AMPK-deficient MEF cells. These results suggest that disruption of AMPK has the potential to prevent or treat cancer chemoresistance when given in combination with chemotherapeutic agents. ULK1-dependent effects on cell survival and mitochondrial homeostasis may add beneficial effects of AMPK activators in an organism's overall health and survival [185]. However, one must use caution as inactivation of AMPK may not only have deteriorative effects on cancer cell survival, but may also contribute to the development of metabolic disorders such obesity and type 2 diabetes [204].

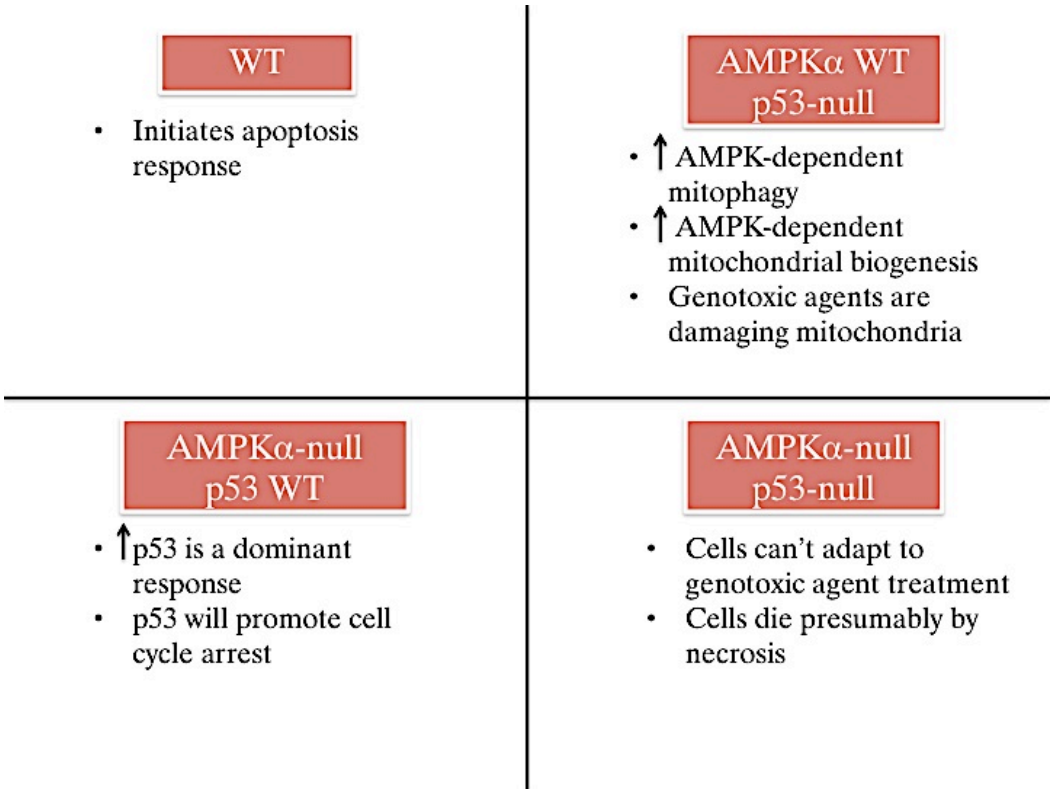

Figure 17: Model of the importance of AMPK to the survival of p53-deficient cells

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