LPIN1: A NEW P53 REGULATED GENE INDUCED BY DNA DAMAGE AND GLUCOSE WITHDRAWAL AND ITS INVOLVEMENT IN FATTY ACID OXIDATION

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy, Medical Biophysics
University of Toronto

Your file Votre référence
ISBN: 978-0-494-44769-7
Our file Notre référence
ISBN: 978-0-494-44769-7

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Doctor of Philosophy, 2008
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Abstract

The upregulation and activation of the p53 tumour suppressor gene in response to a variety of stress signals leads to cell cycle arrest and DNA repair, senescence or apoptosis. In addition to these functions, emerging evidence indicates a role for p53 in regulating metabolism. p53 was recently shown to be phosphorylated by AMPK in response to glucose withdrawal. p53 has also been shown to regulate expression of genes involved in mitochondrial respiration as well as glycolysis.

In this study, we provide further evidence for a role of p53 in metabolism. We show that p53 protein is phosphorylated and accumulates in myoblasts cultured under low glucose conditions. Surprisingly, phosphorylation of p53 at Ser-15 in this context occurs in an ATM-dependent but AMPK-independent manner. Furthermore, we identify a new p53-regulated gene, Lpin1, which encodes lipin1. Lpin1 is essential for fat metabolism and mutation in this gene is responsible for the lypodystrophy phenotype in *fld* mice. Lpin1 was also demonstrated to regulate fatty acid oxidation in transgenic mice. We show that Lpin1 is induced in response to DNA damage and glucose deprivation in a p53-dependent manner in cell lines as well as tissues. In addition, we show that Lpin1regulates fatty acid oxidation under normal and reduced glucose conditions.

Lpin1 provides another link between p53 and metabolism. The fact that this connection exists under DNA damage as well as a nutritionally relevant stress suggests at least two things. First, DNA damage mediated regulation of metabolism may be an important biological process and may have significance in cancer. Second, the upregulation of p53 and Lpin 1 under glucose withdrawal in a muscle cell line potentially expands the role of p53 from a tumour suppressor to a metabolic regulator. Thus, p53 may be important in disease and non-disease contexts.

Acknowledgements

I would like to thank my supervisor Dr. Sam Benchimol for talking a chance with me, his limitless patience, unparalleled mentorship, and for always putting me first before the work. He always listened intently to what I had to say even when at times I knew I wasn't saying much.

I would also like to thank my committee members Rob Bristow and Dwayne Barber for being there when I needed help, for supporting me through my re-class and not giving up on me. I am deeply grateful.

Members of the Benchimol past and present have been like a second family to me. I thank Stephen Chung for his endless help and his humor; Jenny for her giving and our discussions; Keith for his critical input and companionship; Elizabeth for always willing to help; and Weili Ma for her kindness, eagerness to help and always keeping me straight. Weili... I will miss you dearly.

What would my PhD years be with out friendship? Thanks to my friends for being effective counselors during the hard times and plain old buddies during the good. Many beautiful memories were forged during the past years. Thanks to Ryan, Fernando, Joe, Mariano, Khaldoun, Luciano, Anne, Sheila and Shane and many others that have enriched my life. When I am on my death bed it's the smiles and laughs that will be remembered and not Lpin1.

Special thanks to my family: my brother for supporting me through school and my father for his tenacity to secure a future for the family, even when it meant risking his

life. Finally, I'd like to thank my mother. It is not easy to describe the courage it takes to break free from traditions, to set sights on foreign lands, and without an education or money, take three kids and dare to go for something better. We were refugees a decade ago and now we are Canadians. She did it. My PhD is a testament to her courage. Thank you mom. This was for you.

To my Mother, Inaam, who defied fate and traditions and crossed oceans to secure a better future for her children.

To my father who risked his life for us.

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List of Abbreviations:

7-AAD-7-Amino-actinomycin D

ACC-acetyl CoA carboxylase

ARF-alternate reading frame protein

AMPK- 5'AMP-activated protein kinase

ATM-Ataxia Telangiectasia protein

ATP-adenosine triphosphate

BER-Base excision repair

BSA-bovine serum albumin

C.C.-Compound C

CDK-cyclin dependent kinase

C-14-carbon 14

DAG-diacylglycerol

DGAT-diacylglycerol acetyl trasferase

DMEM-Dulbecco's modified Eagles medium

DMSO-di-methyl sulfoxide

FACS-Fluorescence activated cell sorting

FAO-Fatty acid oxidation

H2AX-Histone 2AX

HCL-hydrochloric acid

H_2SO_4-sulfuric acid

HR-homologous recombination

IL2-inteleukin 2

Lpin1-Lipin 1

kDA-kilodalton

Mdm2-mouse double minute 2 oncogene

MEM-modified eagle's medium

MMR-mismatch repair

NE growth-Nuclear/endoplasmic reticulum growth

NLS-nuclear localization signal

NO-nitric oxide

NHEJ- nonhomologous end joining repair

PA-phosphatidic acid

PBS- phosphate buffered saline

PCR-polymerase chain reaction

PGC-α-PPAR gamma co-activator

PI-propidium iodide

PIN1- peptidyl-prolyl cis/trans isomerase

PPP-pentose phosphate pathway

PPAR- peroxisome proliferator-activated receptors

Pu-purine

Py-pyrimidine

RB-retinoblastoma

ROS- reactive oxygen species

RPMI- Roswell Park Memorial Institute

TBP-tata box binding protein

TIGAR-TP53 induced glycolysis and apoptosis regulator

TOR-Target of rapamycin

TLS-translesion synthesis

CHAPTER 1: INTRODUCTION

1.1 p53 and tumour suppression

The role of p53 in tumour suppression stems from observations in human cancers, animal models and cell culture systems. Mutational analysis of the p53 gene revealed mutations in about 50% of all human cancers ranging from greater than 50% in lung and colon to 12% in hematological malignancies such as leukemia and lymphoma [1, 3]. In addition, the identification of p53 germline mutations in patients with Li-Fraumeni syndrome, an inherited familial predisposition towards early-onset of cancer, provided further evidence of p53 as a tumour suppressor [4]. Li-Fraumeni patients are highly susceptible to cancer with 50% of individuals developing cancer by the age of 30. In these patients, a mutant p53 allele is inherited in the germline with a subsequent loss of the remaining wild-type allele during tumour formation. These data have argued in favor of a critical role for p53 in tumour suppression and stressed the need for a greater understanding of p53 biology with the hope that this knowledge will prove clinically useful.

The significance of the data on p53 in humans was underscored by model studies of p53 in genetically-altered animals. For example, p53 knockout mice demonstrated the dispensability of p53 in development, but showed high rates of tumour incidence [5]. p53 -/- mice were born normal with no obvious developmental defects but developed tumors by 3-6 months of age with a high incidence of lymphoma and soft tissue sarcomas. Furthermore, these mice demonstrated very clear differences in the stress response of certain tissues when compared to wild type mice. For example, p53 -/- primary murine embryonic fibroblasts (MEFs) were deficient in G1 arrest compared to wild type counterparts in response to DNA damage (e.g. γ-irradiation) and thymocytes

from p53 -/- mice failed to undergo apoptosis in response to γ-irradiation as compared to p53 +/+ thymocytes [6].

Over-expression studies using wild type p53 have consistently shown either a growth arrest or an apoptotic response in a number of established human and mouse cell lines [7-9]. Mutational analysis of p53, and data gained from Li-Fraumeni patients combined with p53 knockout mice and cell culture systems, have thus provided clear and compelling evidence for p53 as a tumour suppressor gene.

1.2 The mechanisms of p53 mediated suppression:

How p53 mediates tumor suppression has been the main focus in the p53 field. Three main questions characterize the approach to understanding p53: 1) What are the signals upstream of p53 important for p53 mediated tumor suppression?; 2) How is p53 stabilized and activated once these signals are initiated?; and, 3) What are the p53 outcomes that are essential for tumor prevention?

1.2.1 Upstream of p53: What are the relevant signals?

p53 is known to respond to a number of stresses that include DNA damage, oncogenic activation, hypoxia, nutrient deprivation as well as stresses such as heat shock or NO [10]. A key issue is which of these signals is relevant for p53-dependent tumour suppression. Among the many candidates, p53's ability to sense immediate DNA damage through DNA damage sensors and its ability to respond to oncogenic stress through p14Arf have been extensively studied [11, 12].

DNA damage signaling to p53 is thought to be crucial to its tumour suppression ability. Important lines of evidence include the many ways in which DNA damage is communicated to p53 [12, 13] and the predisposition to cancer in mice and humans with mutations occurring in these pathways [14]. p53 is activated by UV through ATR (1) and gamma radiation through Atm [15]. The latter is among the most understood activators of p53 and Atm is a key communicator of this damage to p53.

Atm is 370 kDa protein belonging to the large PI3 kinase ser/thr protein family that includes, ATR and DNA-PK. Mutations in Atm give rise to a rare human disease, ataxia telangiectasia, in which patients have a neurodegenerative disorder, are radiosensitive and are susceptible to tumors [16]. Atm normally exists as a dimer and upon DNA damage is rapidly autophosphorylated on ser 1981 leading to activation [17]. One of the first targets phosphorylated is the well established double strand break marker, H2AX [18]. H2AX, is one the five types of histones, H2A, H2B, H3, H4 and H1. H2AX is a variant of H2AX, that is phosphorylated rapidly upon DNA damage into the active form H2AX gamma. H2AX gamma provides an important localization signal for DNA repair machinery that organizes in large foci around damage sites. Knock out mice for H2AX are deficient in double strand break repair [19].

Once activated, Atm phosphorylates a number of targets that promote a G1, intra S and a G2/M phase checkpoints aimed at arresting the cell and allowing repair to occur [20]. The Atm mediated G1/S cell cycle checkpoint is achieved through direct p53 ser 15 phosphorylation and ser 20 phosphorylation through Chk2. This is thought to promote p53 stabilization and activation. p53 in turn up regulates p21 blocking the cyclin E and cyclin D-dependent CDK2 complexes, essential for G1/S progression [20].

In addition to DNA damage, oncogenic stress has long been recognized as an activator of p53 and this represents an important block to cellular transformation and cancer progression [21]. Oncogenes like c-myc and Ras activate p53 inducing senescence or apoptosis. Interestingly, all oncogenic signaling have so far been linked to p53 through Arf. Recently, the importance of the oncogenic pathway relative to DNA damage has come under new light. Recent work published provided evidence that tumour suppression ability of p53 is dependent on the oncogenic pathway but not its ability to respond to acute DNA damage [22]. It was shown that if p53 is activated prior to irradiation and left on until DNA damage signaling subsided, tumour incidence is not affected. If on the other hand, p53 is turned on after DNA damage signaling has subsided, tumour incidence is reduced. Furthermore, this reduction is inhibited in the absence of Arf indicating that the tumour suppressor ability of p53 depends on oncogenic signaling.

1.2.2 p53 protein structure:

p53 is a 393 amino acid protein that can be divided into four major functional domains based on structure function analysis [23]. At the N-terminus is a transcriptional activation domain (amino acids 1-42) that is responsible for transactivation properties of p53. The N-terminal domain is believed to recruit basal transcriptional proteins including the TATA box binding protein (TBP) and TBP associated factors. Amino acids 102-292 contain the central DNA binding domain and four highly conserved regions (II-V) in which 80-90% of p53 mutations occur. The C-terminal portion of the protein contains two distinct domains: an oligomerization domain (amino acids 323-365) which mediates tetramerization of p53 and a regulatory domain (amino acids 360-393) which is believed

to mediate positive and negative regulation of p53 through post-translational modifications such as acetylation and phosphorylation.

1.2.3 How is p53 stabilized and activated?

Regulation of p53 stability and activity can be grouped into core regulation involving the p53-Mdm2 network and secondary regulation involving a variety of posttranslational modifications on p53 affecting function. Mdm2 is a critical negative regulator of p53 stabilization and activation while post-translational modifications make the process of p53 activation efficient.

The evidence for the important role Mdm2 plays in regulating p53 was convincingly shown when Mdm2 knockouts were rescued from embryonic lethality by crossing to p53 -/- mice [24]. In support of this, transient and stable knockdown of Mdm2 expression in cell lines and amplification of Mdm2 observed in many tumors have led to this conclusion [25]. A widely accepted model of Mdm2 regulation of p53 involves Mdm2 binding and subsequent mono-ubiquitination of the p53 protein in the nucleus. This leads to shuttling p53 into the cytoplasm where it is poly-ubiquitinated leading to proteosomal degradation. In response to stress, this mechanism is disrupted in multiple ways to inhibit degradation leading to stabilization. For example, sequestering Mdm2 in the nucleolus by Arf, Pml and Nucleophosmin, have been shown promote p53 stabilization and activation [20]. Another mechanism involves Atm phosphorylation of Mdm2 at S395 promoting dissociation from p53 [26]. Hausp, a recently discovered de-ubiquitinating enzyme, actively counters Mdm2 activity by removing ubiquitin moieties on p53 [27]. Positive regulation of p53-Mdm2 interaction has also been described. Under growth conditions, Akt

phosphorylates Mdm2 on ser 166 promoting nuclear export of p53 and subsequent degradation [28].

The existence of multiple layers of regulation suggests the importance of tight regulation of the p53-Mdm2 network. How these pathways come together to influence p53 mediated suppression, is an important question will undoubtedly be focus of research for years to come.

Apart from Mdm2 regulation of p53, it has long been recognized that p53 is post-translationally modified and that these modifications promote stabilization and activation of p53. Phosphorylation and acetylation of p53 occurs extensively with 14 phosphorylation sites and at least 5 acetylation sites identified so far [20]. Two of the most studied phosphorylation sites are Ser 15 and Ser 20, phosphorylated by Atm and Chk2, respectively. They are consistently phosphorylated in response to many genotoxic stresses and thus raise interest as to their role in p53 activation and stability. Furthermore, they are located in the transactivation domain and are therefore potentially important regulators of two crucial aspects of p53 -transcription and stability, the latter due to being the site of Mdm2 binding. Ser 15 phosphorylation has been reported to inhibit Mdm2 binding, increase stability and activation, and might be a priming event for other modifications such Ser 20 and Thr 18 phosphorylation [15, 29]. Although it is generally thought that these modifications are important, it is not clear exactly how they affect p53 function. This is because controversial evidence shows mutations of Ser 15 and Ser 20 alone or in combination do not affect p53 stability and activation [30]. Obviously, much work needs to be done to reconcile these opposing views.

Acetylation of p53 represents another major form of post-translational modification with at least 7 lysine residues known to be acetylated in the C-terminus [20].

Acetylation is thought to induce unfolding of the C-terminus from the DNA binding domain promoting activation. Furthermore, since these same sites are also ubiquitinated, acetylation may interfere with Mdm2-mediated ubiquitination and degradation of p53. Again, there is much controversy as to how important these types of modifications are to p53 function. A number of studies have shown lysine residues to be acetylated by PCAF and p300 promoting DNA binding and activation [31, 32]. Furthermore, the discovery that the enzyme Sir2 de-acetylates p53, inactivating it, supports the idea that this form of regulation is important to p53 function [33]. On the other hand, transgenic mice in which 7 lysine residues were mutated to arginine were phenotypically normal [34]. This means that p53 retained normal transcription abilities required for tumour suppression. As with phosphorylation, acetylation probably plays a role in overall p53 function. However, the complexity and redundancy of these modifications have proved a major challenge in understanding their role on different aspects of p53 function.

Another important activator of p53 recently described is Pin1 [35]. Pin1 is a prolyl isomerase that binds p53 promoting stability and transactivation. This binding requires Thr 81, Ser 33 and Ser 315 phosphorylation and this dependency provides further evidence that posttranslational modifications are important for p53 function.

In spite of the complexity of p53 regulation and activation, a general model has emerged in which different types of post-translational modification come together to produce a fully functional p53 protein [20]. In this model, S15 is the earliest modified residue promoting dissociation of p53 from Mdm2 and priming thr18 phosphorylation by Chk2 [13]. This promotes Ser 20 and Ser 9 phosphorylations by Atm [29]. Together, Ser 15, Thr 18 and Ser 20 help promote p53 interaction with P/CAF, CBP, and p300. Acetylation of the C-terminus blocks ubiquitination, stabilizing p53 and leads to

unfolding of the C-terminal domain from the DNA binding domain promoting stability [36]. Finally, phosphorylation of Ser33, Thr81, and Ser20 helps PIN 1 (prolyl isomerase) bind leading to a structural change for final activation [35].

1.2.4 What are the p53-dependent outcomes?

p53-dependent outcomes are largely dependent on its ability to act as a transcription factor. p53 is a sequence-specific tetrameric transcription factor that binds DNA to activate transcription of genes. The consensus DNA binding site of p53 is defined by two repeats of a 10 bp motif consisting of PuPuPuC(A/T)(T/A)GPyPyPy-3' separated by 0-13bp [37]. Transcription of a number of genes including p21 has been shown to depend on the presence of this motif in the promoter or intronic regions of the target gene [38]. p53 has also been implicated as a transcriptional repressor and this is associated with hypoxia. The contribution of p53-dependent transcriptional repression to p53 tumour suppressor function remains unclear.

Transcriptional regulation by p53 is believed to be an essential component of its tumour suppressor activity. Several lines of research provide evidence in support of this hypothesis. Mutational analysis of tumour-derived p53 alleles shows that 90% of mutations occur in the conserved DNA binding domain of p53 [3]. Many of these mutations are missense mutations and the encoded p53 proteins are unable to bind DNA *in vitro* and, when reintroduced into p53 null cell lines, fail to transactivate known target genes such as p21 [39]. Furthermore, transgenic mice expressing a mutant p53 allele with changes at Leu25 and Trp26 have a phenotype that is similar to p53 knockout mice [40]. This p53 mutant retains the ability to bind DNA *in vitro* but is incapable of activating

gene transcription. Like p53 knockout mice, p53 Leu25 and Trp26 transgenic mice develop tumors at an early age. Moreover, these mice display a tumour spectrum similar to that of p53 -/- mice. In addition, MEFs derived from Leu25/Trp26 mice do not undergo cell cycle arrest in response to DNA damage and thymocytes from these transgenic mice do not undergo apoptosis compared with wild type cells. These observations indicate that the transactivation function of p53 is important for its tumour suppressor function.

A number of p53 responses have been discovered that result directly from the ability of p53 to act as a transcription factor. p53-dependent cell cycle arrest, apoptosis, DNA repair and senescence are the most studied. Recently, metabolism has been added to this list. Our understanding of how each of these outputs contributes to p53-mediated tumour suppression is still incomplete but significant progress has been made.

1.2.5 Cell cycle arrest

p53-mediated G1 arrest is mediated to a large extent through the cyclin dependent kinase inhibitor, p21 [38]. p21 inhibits CDK2 and CDK4 which are required for cell cycle progression from G1 to S phase. The extent to which p21 is required for p53-dependent cell cycle arrest was revealed from studies on p21 -/- mice. Experiments on MEFs from these mice showed that p21 accounts for most of the p53 mediated G1 arrest in response to DNA damage. Given this discovery, it was surprising that these mice did not develop tumors. In support of this fact, p21 is not commonly mutated in cancers.

In addition to p21, other genes have been implicated in p53-mediated G1 arrest. They include, BTG2 and GADD45 but their roles in cell cycle arrest and tumour suppression is less well understood [12].

p53 also plays a role in the G2/M checkpoint which prevents damaged chromosomes from being segregated into daughter cells. A number of p53 regulated genes have been implicated in G2/M checkpoint. p21 itself is thought help promote the checkpoint by binding the cdc2-cyclin B complex [41]. 14-3-3σ is another G2/M checkpoint regulator shown to be regulated by p53. It blocks entry into mitosis by inhibiting two key proteins required for this process- Wee1 kinase and Cdc25C [42].

1.2.6 Apoptosis

Apoptosis, also known as programmed cell death, is characterized by DNA fragmentation, chromatin condensation, cell shrinkage, and externalization of membrane proteins such as phosphatidylserine which serves as a signal for engulfment by phagocytes. It is now widely recognized that apoptosis is critical in a variety of physiological processes that include normal embryonic development and proper immune function. Deregulation of apoptosis is thought to be involved in autoimmune and neurodegenerative diseases, and many types of cancers.

There are two general mechanisms by which apoptosis is initiated: the extrinsic and intrinsic pathway [43]. Both are initiated by specific signals that lead to activation of a family of cysteine proteases, the caspases, an irreversible step that begins the programmed killing of the cell. The extrinsic pathway is intitiated from outsided the cell by ligand activation of the TNF superfamily of death domain containing receptors and

include TNF-α/TNFR and the FasL/FasR pathways [44]. Both lead to recruitment of adaptor proteins and caspase 8 on the cytoplasmic side of the death receptors followed by formation of a disc complex. This is followed by autocatalysis and activation of caspase 8 causing the activation of downstream caspases.

The intrinsic pathway is initiated from within the cell [43, 45]. At the heart of this pathway is the integrity of the mitochondria which if compromised results in release of cytochrome c, normally residing in the inter-mitochondrial membrane space. Cytochrome c release triggers the formation of the apoptosome complex that includes Cyt c, Caspase 9 and Apaf 1 [46]. This leads to Caspase 9 activation which then cleaves downstream executionary caspases. Since release of Cytochromes c is a critical step and apoptosis becomes irreversible with its release, it's not surprising that multiple layers of control exist over this step. Control is carried out by the interplay of a large number of Bcl-2 domain containing proteins [47]. They are divided into 3 groups: BH3 only (Noxa, Puma), anti-apoptotic Bcl-2 (Bcl-2 and Bcl-Xl), and proapoptotic BCL-2 family members (Bax). Under basal conditions, anti-apoptotic proteins, Bcl-2 and Bcl-x long are bound to the pro-apoptotic members Bax and Bak inactivating them. Under stressful conditions that promote expression of BH3-only proteins, these interactions are effectively terminated releasing bax and bak to attach to the mitochondria, forming pore channels and thus causing loss of membrane integrity and release of cytochrome c.

p53 has now been shown to regulate both extrinsic and intrinsic pathways. The discovery that p53 regulates the extrinsic pathway added an exciting prospect that this might be important to its tumour suppressing ability. p53 regulates a number of genes involved in the death receptor pathway. Death receptor 4 and 5 are both upregulated by p53 promoting their activation in response to trail ligand as well as chemotherapeutic

drugs [48]. Fas, a potent death receptor ligand, was also shown to be upregulated in response to ionizing radiation in a p53-dependent manner [49]. This regulation was tissue-specific and in many cases p53 independent. Perp, a member of the PMP-22/gas family of proteins, was shown to mildly contribute to p53-dependent apoptosis in thymocytes and neurons in response to gamma radiation but not upon oncogenic activation [50].

All described target genes regulating the extrinsic pathway have failed to recapitulate the full p53 response shown in physiological systems such as thymocytes, neurons or intestinal cells. This combined with the observation that blocking the intrinsic pathway fully blocks p53-dependent apoptosis, has provided strong evidence that p53 primarily relies on the intrinsic pathway for apoptosis.

p53 has now been shown to influence this process through transcription dependent and independent mechanisms. A number of genes have now been identified that directly influence release of Cytochrome c and they include Bax and Puma. Bax was the first bcl2-domain containing protein shown to be important for p53-dependent apoptosis.[51]. Loss of Bax was also shown to inhibit tumour formation in a brain tumour model [52]. The excitement that bax was the critical mediator of p53-dependent apoptosis was weakened when it was found to be dispensable for p53 mediated apoptosis in thymocytes and intestinal cells in response to gamma radiation [53]. Aside from Bax, other genes found to influence Cytochrome c release include Noxa and Pidd (p53 Inducible Death Domain). Pidd is relatively recent addition to the list. Pidd is directly induced by gamma irradiation in mefs and other cell lines in a p53-dependent manner and contributes to p53-dependent apoptosis [54]

Puma, a recently discovered p53 target gene, seems to be crucial for p53-dependent apoptosis since Puma -/- thymocytes, neurons and MEFs show complete

impairment of p53-dependent apoptosis in response to gamma radiation [55, 56]. Puma on its own is a very potent apoptotic inducer killing cells within hours of induction. The discovery of Puma also resolved a long standing controversy on the relative importance of transcription dependent and independent function of p53 for apoptosis. Evidence from a number of papers had already indicated that some p53 localizes to the mitochondria in response to DNA damage and that this could be the mechanism for p53-dependent apoptosis [57]. The discovery of Puma provided a mechanism in which transcription-dependent and independent aspects of p53 come together to induce apoptosis efficiently. This model involves p53 acting as a pro-apoptotic, BH-3 protein normally bound to Bcl-Xl. Puma, after being transcribed by p53, binds to Bcl-Xl releasing p53 to activate Bax and Bak [57]. The model is relatively new and many questions still abound. For example, how prevalent is this model? Does it apply to p53-dependent apoptosis only in certain types of cells?

1.2.7 DNA repair

p53 has been hypothesized to promote genomic integrity by regulating DNA repair pathways through transcription dependent and independent mechanisms [12]. Evidence has implicated p53 in MMR, BER, TLS, HEJR and NHEJ although the picture is complicated. Its role as a positive or negative regulator of DNA repair depends on context. For example, p53 inhibits BER pathway in response to nitric oxide but promotes repair in response to gamma radiation [58]. p53 also promotes BER in G1 but inhibits it in G2/M phase of the cell cycle [59]. MMR and TLS, which are used to repair base mismatches and bypass stalled replication forks have been shown to be positively

regulated by p53 [60]. In spite of much work on the subject, it is still not clear if p53 plays a direct role in DNA damage repair and what significance this has on tumour suppression.

1.2.8 Senescence

Senescence is a state of permanent arrest characterized by enlarged and flattened morphology, increase in β-galactosidase activity, and sustained metabolic activity. Although senescence was first described as a response to telomere shortening, it is also induced by a number of other stresses including oncogenic activation, ROS generation and DNA damage [12]. Telomere induced senescence is p53-dependent and is brought about by the p53-p21-RB pathway while premature senescence is brought about by both the p53-p21-Rb pathways and p16-RB pathways [61]. Although senescence was discovered in cell culture models, emerging evidence indicates that senescence occurs physiologically in vivo. Senescence as a mechanism of tumour suppression has now been shown in both mice and human tumors [62].

1.2.9 p53 and antioxidants

A fundamental change in how p53 is viewed occurred with the discovery that p53 regulates a number of potent antioxidant genes [63]. Firstly, p53 was thought to exist between inactive and active states depending on the presence or absence of stress. This study revealed that even in the absence of exogenous stress, there is a certain amount of functional p53 responsible for upregulating a number of antioxidant genes called sestrins. Secondly, this regulation is essential for preventing oxidative DNA damage, a major cause of mutations. The study suggested that the ability of p53 to transactivate antioxidant

genes provided a first line of defense against tumour development. By adding an antioxidant drug to the diet of p53-/- mice, tumour formation was significant delayed. This indicates that the ability of p53 to regulate antioxidant genes is an important function of its tumour suppression ability.

1.3 p53 and Metabolism

For the last two decades, p53 was thought to limit cell growth and division by solely promoting cell cycle, apoptosis, and senescence. The role of p53 on cellular metabolism, if any, was a secondary effect produced by other cellular p53 responses. Recent work suggests that p53 can directly affect metabolic pathways by upregulating metabolic genes. These genes are implicated in metabolic pathways that include glycolysis and oxidative phosphorylation. In addition, p53 can communicate with the central metabolic regulators AMPK and mTor. This has established a strong basis to propose a significant role for p53 in metabolism.

1.3.1 p53 and glycolysis

p53 can regulate glycolysis by directly up regulating two glycolytic genes: Pgm (phosphoglycerate mutase) [64]and Tigar (p53 inducible glycolysis and apoptosis regulator) [65]. Pgm upregulates glycolysis, and in contrast, Tigar inhibits glycolysis.

Pgm is a widely used marker of differentiation of skeletal and heart muscle and controls the eight step in glycolysis, converting 3-phosphoglycerate to 2-phophoglycerate. The evidence supporting p53-dependent regulation of Pgm is based on work by Riuz-Lozano et. al. showing that PGM is upregulated during differentiation of muscle cells

and this induction is inhibited by blocking p53 [64]. Furthermore, p53 is shown to bind to a consensus site in the promoter of Pgm establishing it as a direct target. It is not clear, however, if this relationship has functional significance on glycolysis or metabolism in general.

Tigar is a fructose-1,6-biphosphatase, a molecule that positively regulates PFK-1, an enzyme that catalyzes the commitment step in glycolysis [65]. Reduction of F-1,6-bisphosphate inhibits PFK-1 effectively inhibiting glycolysis. The net result is the diversion of G-6-P, the product of step 1, into the pentose phosphate pathway (PPP) which serves at least two important functions: the recycling of NADPH required for many processes including reduction of glutathione, the form required to oxidize ROS species, and supplying ribose-5-phosphate intermediates used in DNA repair and synthesis. p53-dependent up-regulation of Tigar inhibits glycolysis, increases the pentose phosphate pathway and ultimately interferes with p53-dependent apoptosis. The authors argue that p53-dependent apoptosis is compromised due to the PPP-dependent reduction in ROS species. In this context, it seems that p53 is playing a protective effect through PPP complimenting the antioxidant role of p53 that occurs through another class of antioxidant genes, sestrins [63]. The observations also raise other important questions that remain unaddressed: what is the significance of the down regulation of glycolysis on the cells? Is p53 regulating energy levels and ultimately survival?

1.3.2 p53 and oxidative phosphorylation

Evidence for p53 regulation of oxidative metabolism was strengthened from a recent discovery that p53 directly regulates Sco2 (synthesis of cytochrome c oxidase), a

critical component of the electron transport chain [66]. Liver preparations from p53 -/- mice showed lower Sco2 levels than wild type. Furthermore, p53 or Sco2 knockdown reduced O_2 consumption while increasing lactate production, indicating a shift from oxidative phosphorylation (anaerobic glycolysis) to aerobic glycolysis. This, the authors point out, shows how p53 might contribute to the Warburg effect, a condition in which cancer cells rely more on glycolysis for ATP production than on mitochondrial respiration [67]. Thus, in a hypoxic tumour environment, p53-deficient cells would be unable to down regulate glycolysis through Tigar induction and would be unable to promote oxidative phosphorylation through Sco2 induction. As a result, the p53-deficient cells would exhibit a selective growth advantage provided through enhanced glycolysis. Aside evidence for a possible role for p53 in the Warburg effect, the Matoba et. al. study was unique in two respects. Firstly, it provided further evidence that p53 is functional even in unstressed cells. Secondly, for the first time, p53 was shown to directly regulate respiration. In addition to shedding light on the Warburg effect, p53's regulation of Sco2 raises the possibility that p53 regulates metabolism in a non-disease context.

1.3.3 p53 communicates with key metabolic regulators, AMPK and mTor

p53 is known to communicate with AMPK and mTor , potentially regulating metabolism in a significant way. AMPK integrates energy demands with a wide variety of processes including fatty acid oxidation and synthesis, glycolysis, and mitosis [68]. mTor responds to amino acids, energy levels and growth factors, integrating nutrient status with protein translation, autophagy, and cell growth and division [69].

ATP, the energy currency in cells, is vital to most essential processes in cells. Therefore, it is not surprising that cells have evolved mechanisms to maintain proper ATP levels. One key mechanism of control evolved is the AMPK pathway. AMPK senses levels of ADP/ATP and responds by promoting energy producing pathways and shutting down energy consuming ones.

AMPK is a serine/threonine kinase made of three subunits: a catalytic alpha subunit, and non-catalytic beta and gamma subunits [70]. Three known mechanisms of activation exist for AMPK. LKB1, a recently discovered tumour suppressor, directly phosphorylates the alpha subunit in response to rising ADP levels [71]. Secondly, ADP binds to and allosterically activates AMPK. Thirdly, ADP makes AMPK a poor phosphatase substrate, thereby keeping the kinase active for longer. Once activated, AMPK phosphorylates a variety of substrates including, acetyl CoA carboxylase (ACC) and TSC complex [70].

A key mechanism in which AMPK regulates fatty acid metabolism is through ACC. ACC is a key enzyme responsible for the conversion of acetyl CoA to malanoyl CoA, a rate limiting step in the fatty acid synthesis pathway [1]. Malonyl CoA is the basic carbon base used to make all fatty acids and is a potent inhibitor of carnitine palmatoyl transferase (CPT1), which transfers fatty acids into the mitochondria for oxidation. Thus, the conversion of acetyl CoA to malanoyl CoA becomes an important step in coordinating fatty acid synthesis with fatty acid oxidation. When nutrients such as glucose are high, AMPK is off and unphosphorylated ACC is free to produce malonyl CoA increasing fatty acid synthesis. High malanoyl CoA inhibits CPT-1 shutting down fatty acid oxidation. When nutrient levels are low, the reverse happens. The end result of this process is

shutting ATP consuming processes (fatty acid synthesis) and turning on ATP generating activities (fatty acid oxidation).

Recently, AMPK was shown to phosphorylate p53 directly on Ser 15 leading to a G1 cell cycle check point [72]. Under low glucose levels, the G1 block enhanced survival presumably because G1 arrest allowed cells to conserve ATP. Cells lacking p53 continued through S phase without proper energy levels and died. A different study also showed that glucose deprivation led to phosphorylation of ser 15 in HCT-116 colon cancer cells [73]. However, this was transient and did not lead to p53 stabilization or a p53-dependent response. Interestingly, another study by the same group showed that communication between p53 and AMPK is in fact bidirectional [74]. p53 in response to DNA damage binds to the promoter of AMPK beta 1 subunit and up regulates it in a number cancer cell lines, although this up-regulation was absent in gamma irradiated wild type tissues.

mTor is another key regulator in metabolism linked to p53. mTor is a highly conserved PI3-like kinase that integrates growth factor signaling and nutrient availability to cell growth and proliferation [69]. Growth factor signaling feeds directly to mTor through the PI3 kinase/Akt pathway. mTor also senses amino acids through a yet unknown mechanism, and also senses ADP/ATP levels through AMPK. When activated, mTor turns on protein translation by phosphorylating p70SK and 4EBP while suppression of mTor leads to inhibition of protein translation and activation of autophagy. Hyper-activation of the mTor pathway by activated Akt, loss of Pten or the mTor inhibitor TSC1/2 has been shown to promote cancer [69]. p53 regulates mTor indirectly by regulating Pten and TSC2 [74]. Pten, a phosphatase that represses PI3 kinase signaling, is a

direct target of p53 [75, 76]. p53 also represses mTor by upregulating TSC2 promoting its activation [74].

These studies provide strong evidence in support of the hypothesis that p53 plays an important role in metabolism and raise some pressing questions. What is the appropriate stress that leads p53 to regulate metabolism? Many of these observations were done using DNA damage as a stress signal and suggest the possibility of DNA damage dependent regulation of metabolism. Since cells and tissue are under constant stress from internal ROS and external agents, metabolism would then be constantly regulated by DNA damage induced pathways. The other possibility is that there are metabolism-related signals that activate p53 and glucose may be a critical one. This thesis argues for glucose being an important stress signal for p53 and presents a new p53 regulated gene, Lpin1, which may be significant in regulating metabolism.

1.4 Lipin1

Lpin1 is a phosphatidic acid phosphatase that was discovered to be mutated in lypodystrophic mice [77]. Lpin1 has since been shown to be responsible for the phenotype which is characterized by a block in adipogenesis and related metabolism disorders such as diabetes [78, 79]. Lpin1 is now understood to be essential for adipocyte differentiation, and regulates fatty acid oxidation and synthesis depending on the context, see Figure 1.2 [80]. The importance of Lpin1 in metabolism suggests that p53-dependent regulation of Lpin1 could be of significant importance.

1.4.1 Lypodystrophy and the fld phenotype:

In 2001, Peterfy et. al. identified two strains of Fld mice mutated in a gene they termed Lpin1 [79]. A homology search revealed that Lpin1 was one of three family members in mammals (Lpin 1, 2 and 3) and is evolutionarily conserved in mammals as well as single celled organisms, indicating an important role for this gene family. The search also revealed three highly conserved homology domain (HD) sequences, HD1 and HD2, which are at the N and C-terminus respectively (see Figure 1.1). Their function is not known. A nuclear localization motif (NLS) is conserved in mammals but not lower organisms. Significance of the NLS domain was revealed from the nature of the fld mutations. Although both strains show no differences in the lypodystrophy phenotype, Lpin1 DNA was rearranged leading to loss in mRNA and protein expression in one strain while the other displayed a point mutation in the nuclear localization signal. Over expression of the Lpin1 point mutant revealed cytoplasmic localization while reversal of the mutation to wild type localized Lpin1 to the nucleus, suggesting localization is important to Lpin1 function [79].

Recent work has revealed important new functions about Lpin1 that help explain its role in the fld phenotype. These functions include Lpin1 being a phosphatidic acid phosphatase, and a regulator of both fatty acid synthesis and fatty acid oxidation.

1.4.2 Lpin1 is a phosphatidic acid phosphatase and is essential for adipocyte differentiation:

In 2006, Han et al, published that Lpin1 is a phosphatidic acid phosphatase (PAP), belonging to a family of lipid phosphatases [77]. There are two groups of PAPs: PAP1 and PAP2 [81]. PAP1 enzymes are Mg^{+2} dependent, specific for phosphatidic acid (PA) and

include Lpin1, 2 and 3 so far. PAP2 enzymes are Mg^{+2} independent and have a broader range of targets that include lysophosphatidate (LPA), sphingosine-1-phosphate (S1P), phosphatidate, and ceramide-1-phosphate (CIP). PAP2 enzymes play diverse and complicated roles in signaling cascades involving cells division, cytoskeletal rearrangement, and membrane movement.

Lpin1 and its ancestral yeast homologue, Pah1, unlike PAP2 enzymes, are specific for phosphatidic acid, catalyzing its breakdown into diacylglycerol and inorganic phosphate [81]. This means PAP1 controls the concentration of PA and DAG and can influence both DAG and PA pathways. DAG formation is the quintessential step in fat triglyceride synthesis, while PA, is essential to forming membrane phospholipids. Han et. al. showed that in yeast Pah1 maintains proper PA levels and this is required for proper nuclear/ER (NEM) membrane growth [77]. Pah1 mutants resulted in unusually large NEM and 90% loss in TAG levels due to lower DAG levels. Consistent with Pah1 function in TAG synthesis in yeast, Lpin1 also increases TAG synthesis in mammals and this function is essential for adipogenesis. There are at least two lines of evidence supporting this. In a tissue culture model, primary mefs derived from wt mice differentiate into adipocytes under stimulatory conditions while fld derived mefs do not [82]. In addition, over expression of Lpin1 in the fld derived mefs rescues this defect. Consistent with this, Lpin1 A overexpression was shown to induce the lipogenic program that involves an increase in PPAR gamma, an essential transcription factor for adipocyte differentiation, while Lpin1 B upregulated lipogenic genes DGAT and AP2 [82].

Further understanding of the role of Lpin 1 has come from transgenic mice over expressing Lpin1 in muscle and fat under tissue specific promoters [83]. These studies revealed a number of important findings that were consistent with earlier work. Lpin1

over expression under adipocyte specific promoter in an fld (Lpin1 null) background restored fat mass demonstrating that the fld phenotype is due to loss of Lpin1. Furthermore, Lpin1 was shown to be an obesity gene as well because when fed a high fat diet these mice were prone to obesity compared to wt mice counterparts.

Consistent with physiological observations, biochemical analysis on mice expressing Lpin1 under muscle specific promoter showed reduced fatty acid synthesis and increased fatty acid oxidation in muscle. Consistent with this finding, Lpin1 over expression in muscle resulted in a decrease in fatty acid oxidation and an increase in fatty acid synthesis genes [83].

Although the function Lpin1 as an enzyme is consistent with its role in adipogenesis, evidence suggests Lipin1 has other functions. Firstly, Lpin1 seems required for induction of lipogenic transcription factors such as PPAR gamma long before TAG accumulation could be measured. It is possible, DAG, given its role in signaling, results a cascade culminating in the induction PPAR gamma and other important factors. It is also possible that Lpin1 plays a transcriptionally related role as well. This is consistent with its nuclear localization signal, previously published data on Pah1 and Ned1 homologues [77, 84], as well as recently published data showing that Lpin1 is a co-transcription factor [80]. The recently discovered role for Lpin1 as a transcription factor is particularly puzzling given its role in this context is to increase fatty acid oxidation and decrease fatty acid synthesis in striking contrast to the role of Lpin1 predicted from the mouse transgenic studies.

1.4.3 Lpin1 as a co-transcription factor and promoter of fatty acid oxidation:

In addition to being a phosphatidic acid phosphatase and a promoter of fatty acid synthesis, Lpin1 was shown to also function as a co-transcriptional activator serving to increase fatty acid oxidation [80]. This study showed that Lpin1 mRNA increased in response to fasting and dexamethasone treatment in livers of mice. This induction fully depends on Pgc-α, a well know inducer of fatty acid oxidation. Secondly, Pgc-α and Lipin co-transactivate PPAR alpha, another important promoter of fatty acid oxidation. Thirdly, Pgc-1/PPAR alpha together with Lpin1 lead to regulation of liver energy metabolism. A dysfunctional Pgc-1/PPAR circuit leads to liver steatosis in which fat accumulates due to decreased fatty acid oxidation. Furthermore, Lipn1 overexpression by viral transduction, resulted in decreased fatty acid synthesis, increased fatty acid oxidation and a reduction of fat secretion.

Thus, Lpin1 is an enzyme and a co-transcription factor. Lpin1 promotes fatty acid synthesis and adipogenesis or increases fatty acid oxidation depending on the context. The first has only been shown to occur in muscle and fat and the latter in liver. How Lpin1 carries out two functions is probably the most puzzling question about Lpin1 biology. One possible clue is the relationship between Lpin1 function and localization. Firstly, the NLS domain of Lpin1 indicates that Lpin1 probably moves between the nucleus and cytoplasm and that cellular localization might be important for function. Secondly, Lpin1 is a phosphatidic acid phosphatase and this function is thought to be a cytoplasmic function while its role as a co-transcription factor, is a nuclear function. One possible mechanism for this dual role of Lpin1 is that when Lpin1 is a promoter of fatty acid oxidation when nuclear and a promoter of fatty acid synthesis when cytoplasmic.

1.4.4 Lpin1 orthologues: adding complexity to an already complex picture

Studies in S. *cerevisiae* and S. *pombe have* revealed pleiotropic roles for Lpin1 orthologues, some which are consistent with mouse studies. The Pah1/Smp2 orthologue in S. cerevisiae functions as a phosphotidic acid phosphatase catalyzing PA to DAG, consistent with the role of Lpin1 in mice [77]. Thus Pah1 controls both PA and DAG levels. An active Pah1 increases DAG and thus triglyceride synthesis. On the other hand a dysfunctional Pah1/Smp2 leads to overgrowth of the nuclear/ER membrane. This is believed to be due to an increase of PA, a positive regulator of the nuclear/ER (NE) membrane growth. Thus, by catalyzing PA to DAG, Pah1 maintains proper levels of PA concentration and thus NE growth. Furthermore, Pah1 is phosphorylated by CDK1, essential to G2/M transition, potentially linking cell division to NE growth.

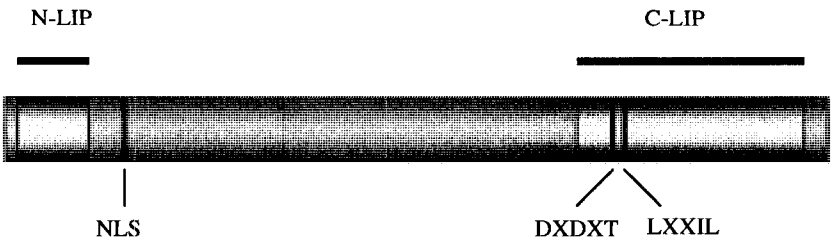
Studies in S. *pombe* reveal a different picture for the Lpin1 orthologue Ned1 [84]. Ned1 mutants, lead to pleiotropic effects. Ned1 over expression causes the formation of a microtubule bundle in the nucleus. More interestingly, Ned1 mutants resulted in chromosome missegregation, a deformed nuclear membrane, and were sensitive to microtubule destabilizing drugs. In addition Ned1 was found to interact with a number of proteins, including a nuclear pore complex protein, nucleoporin, chromosome segregation protein, Pim1, and nuclear transport protein, Dis1 [84].

It is difficult from the above to form a coherent view of how Ned1/Pah1 and Lpin1 function. One consistent observation is that all three are phosphatidate acid phosphatases, and this includes the other Lpin1 family members Lpin 2 and Lpin 3 based on mapping analysis. An important question is how the enzyme and co-transcription abilities translate into chromosomal, nuclear and microtubule integrity observed in yeast. And just as importantly, if these observations persist in mammals.

1.4.5 An emergent p53 model

Recent discoveries have argued for a modified model of how p53 promotes tumour suppression (Figure 1.3). An important shift in understanding p53 occurred with the discovery that p53 is constitutively active under low stress. In this state p53 upregulates a number of antioxidant genes that scavenge free radicals. This is the first role of p53 as a tumour suppressor: prevention of cancer by eliminating potent carcinogens. Associated with this is its role in G1 arrest and DNA repair that helps the cell repair DNA damage and reduce the possibility of genomic instability. The second line of defense is elimination of cells that have undergone transformation. Transformation may be communicated to p53 in many ways. This includes increased DNA damage signaling because of genomic instability and/or the oncogenic pathway through the Arf pathway. Other pathways may include hypoxia, glucose deprivation and telomere shortening. Once activated, p53 contains the transformed cell by means of senescence and/or apoptosis. In addition, p53 may employ other mechanisms. The emerging role for p53 in metabolism, for example, might be a way to limit growth of cancer cells when elimination is not an option. In this context, the ability of p53 to regulate processes important for cancer growth such as glycolysis or fatty acid metabolism, may slow down growth and be an effective means of tumour suppression.

A.



B.

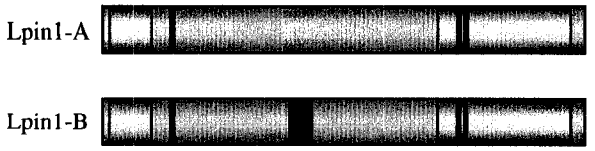


Figure 1.1: Lpin1 domain structure. A. Lpin1 contains two stretches of amino acids, N-LIP and C-LIP, conserved in mammals and single celled organisms such as yeast. Lpin1 also contains a functional nuclear localization domain (NLS),a phosphatidate acid phosphatase motif (DXDXT) and a transcriptional co-activator domain (LXXIL). B. Lpin1 exists in two different isoforms, Lpin1-A and Lpin1-B, resulting from alternative mRNA splicing. Lpin1-B contains an extra 33 amino acid sequence with a yet unknown function.

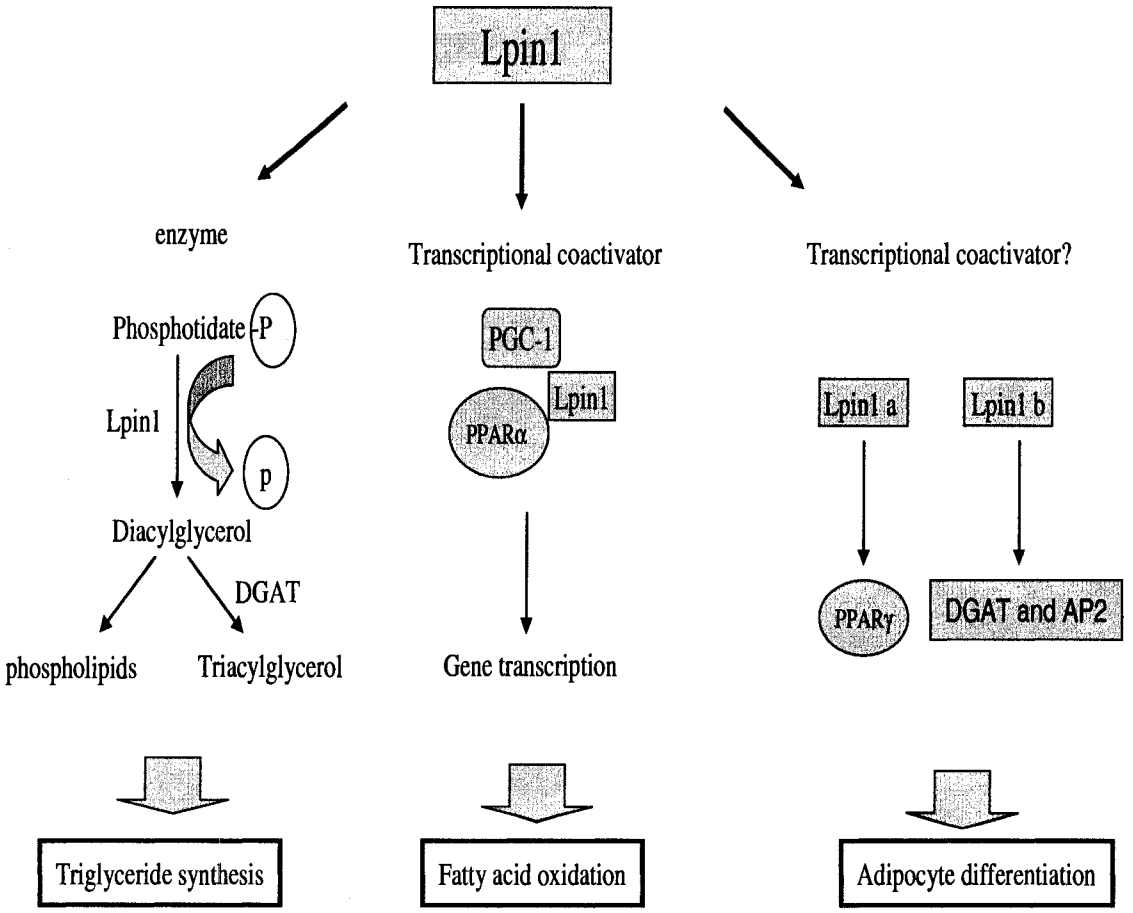


Figure 1.2: Lpin1 function. Lpin1 has been show to be an enzyme as well as a transcriptional coactivator. As an enzyme, Lpin1 is a phosphotidic acid phosphatase, serving to remove the phosphate group from phosphotidate to form diacylglycerol. In this context Lpin1 plays an important role in triglyceride synthesis and adipocyte biology. As a transcriptional coactivator, lpin1 binds PGC-1 alpha and regulates the expression of genes involved in fatty acid oxidation. Lpin1a has also been shown to be necessary for PPARγ expression during adipocyte differentiation while Lpin1b has been shown to upregulate genes important in lipid accumulation (DGAT and AP2). It is not known if this function is also mediated by transcriptional coactivation properties of lpin1.

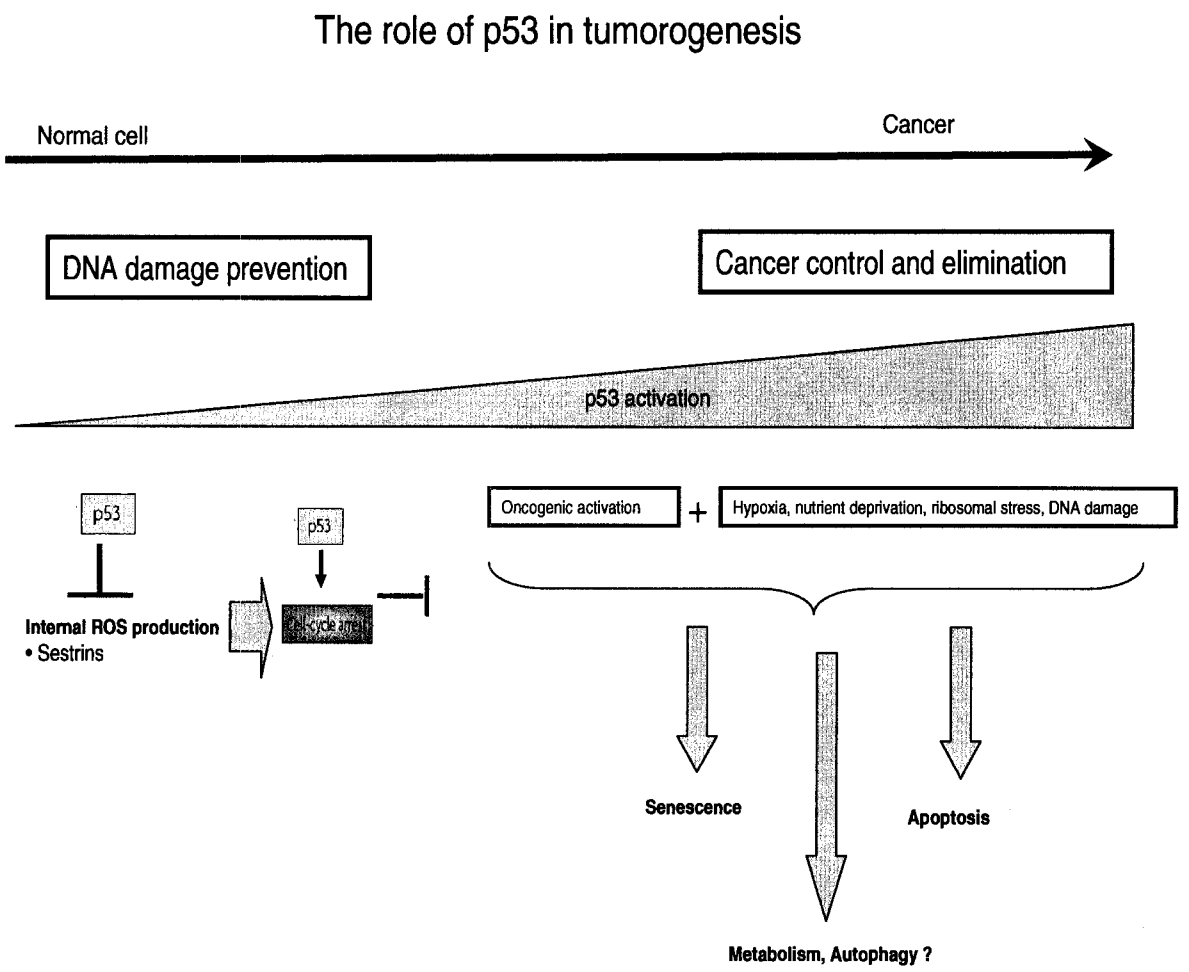


Figure 1.3: A summary of the role of p53

1.5 Purpose and Rationale of thesis

p53 is a potent tumour suppressor. Its ability to regulate a large number of genes as a transcription factor is crucial to its function. The identification and understanding of genes regulated by p53 is therefore important to understanding how p53 mediates tumour suppression. This thesis identifies and investigates the role of a new p53 regulated gene, Lpin1. Our investigations involved two approaches, firstly examining the role of Lpin1 under the classical p53 activator, γ-radiation, and secondly, examining the function of Lpin1 under a metabolically relevant signal, glucose deprivation. Under γ-radiation, our objectives were three fold: 1) Is Lpin1 a p53 regulated gene? 2) Is Lpin1 involved in p53-dependent apoptosis? 3) Does lipin1 exhibit tissue dependent expression pattern in response to DNA damage? Our second approach had three objectives: 1) Is Lpin1 induced by glucose deprivation in a p53-dependent manner? 2) What is the mechanism of p53 activation by glucose deprivation? and finally, 3) What is the role of Lpin1 under glucose deprivation?

The first approach revealed a number of findings. Lpin1 is upregulated in a p53-dependent manner in response to γ-radiation in cell lines as well as tissues. This up-regulation is not important for p53-dependent apoptosis in response to γ-radiation. Induction of Lpin1 occurs in a tissue-dependent manner with strong induction in hematopoietic systems but not in mefs. When overexpressed, Lpin1 does not affect cell viability or proliferation in 3T3 fibroblasts and DP16.1 cells.

The second approach revealed that Lpin1 is upregulated in response to glucose withdrawal in a p53-dependent manner. p53 is upregulated and activated in response

glucose withdrawal in an Atm dependent manner that is DNA damage-independent . In this context, Lpin1 is an important regulator of fatty acid oxidation suppressing it under high glucose but promoting it under glucose withdrawal.

CHAPTER 2: MATERIALS AND METHODS

Materials and Methods:

T and B cell proliferation assays: For T cell proliferation assays, mice were killed and the neck, armpit and mesenteric lymph nodes were extracted, passed through a 70 μm nylon cell strainer, washed in PBS, and seeded in RPMI with 10 % FBS. Activation of T-cells was achieved by incubating cells for 1 hr in 10 ug/ml CD3e (BD biosciences 557306) and 100U/ml, IL-2 (BD pharmingen # 550069). Cells were then washed in PBS and cultured in RPMI with 10 % FBS with IL-2. For B cell proliferation assays, the spleen was removed, passed through a mesh, and cultured in RPMI with 10 % FBS. Cells were then stimulated with LPS (SIGMA # L-3012). For all experiments, cells were seeded in 24 well plates at $1x10^5$ cells/well and then shifted to RPMI adjusted for varying amounts of glucose, with 10% dialyzed serum containing IL2 for T cells and LPS for B-cells.

Antibody manufacture: Lpin1 rabbit IgG were developed by Biosynthesis Inc. using the Lpin1 peptide NH2-SKTDSPSRKKDKRSRHLGADG-OH.

Mice and genotyping: Fld mice, strain BALB/cByJ-Lpin1 fld/+, were purchased from Jackson laboratory. p53 +/- mice were a gift from Dr. Tak Mak's laboratory. Fld +/- mice were mated and genotyped 2 weeks after birth using the following primers: WT primers: Lpin1- forward: 5'-CCCTTGAGCACGTTCACA-3', Lpin1-reverse 5'-CTGATCGTTGTCAGTCTCT-3' ;

Mutant primers: Lpin1 forward: 5'-GGTTGTGGGGACCCTGGA-3', reverse: 5'GCCTGCTGCAGATGCGTT-3'. p53 +/+ and -/- mice were genotyped using the following primers: Wild type, forward: 5'-GTGTTTCATTAGTTCCCCACCTTGAC-3', reverse: 5'-ATGGGAGGCTGCCAGTCCTAACCC-3'. Mutant primers: forward 5'-GTGGGAGGGACAAAAGTTCGAGGCC-3'; reverse: 5'-TTTACGGAGCCCTGGCGCTCGATGT-3'.

Cloning:

Lpin1 was cloned from a mouse liver cDNA library using the following primers: forward primer: 5'-CCTGCTCGTGAATCCTCTTG-3', reverse primer: 5'-TTTAATGCTCGGTCGCGTC-3' using standard PCR protocol.

shRNA vectors. Lpin1 shRNA retroviral vectors #1 (V2SHS-68550: 5'-CGCAGAACTCTTCCTAATGATA-3') and #2 (V2MM-36936: 5'-AAGAAATGCCACAATCAAA-3') were purchased from Open Biosystems. The p53 shRNA retroviral vector [85] was obtained from Dr. Scott Lowe (Cold Spring Harbor Laboratories, New York). DNA transfections into C2C12 cells were done using the FuGENE 6 reagent (Roche)

Antibodies: p53 (FL393, Santa Cruz, CA, SC-6243), phospho-Atm (ser 1981, Calbiochem, DR1002), p53 Ser-15 (Cell Signaling, #3662), p53 Thr-18 (Santa Cruz, SC-16716-r), ACC (cell signaling #3662), phospho-ACC (Ser 79, cell signaling, #3661S), p21 (Santa Cruz, sc397), gamma-H2AX (Hycult Biotechnology, HP5001)

Inhibitor studies: Atm inhibitor (Calbiochem #118500) was dissolved in DMSO and added directly to cells for a working concentration of 10 uM. AMPK inhibitor (compound C, Calbiochem, 171260) was dissolved in DMSO and used at a working concentration of 40 uM.

HCl acid histone extraction - Cells were washed twice in ice-cold PBS and scraped off the plate in Triton extraction buffer (TEB: PBS containing 0.5 % Triton X 100, v/v, 0.02% sodium azide at cell density of $1x10^7$/ml. Cells were then lysed for 10 minutes with gentle agitation, centrifuged at 2000 rpm for 10 min at 4°C and the supernatant discarded. The pellet was then resuspended in 0.2N HCl at $4x10^7$ cells/ml and extracted overnight at 4°C with gentle agitation. The next day, cells were centrifuged at 2000 rpm for 10 minutes at 4°C and the supernatant aliquoted and stored at -20°C. For westerns, samples were diluted with 2x SDS loading buffer and run on a 15 % ployacrylamide gel, transfered and blotted with H2AX-γ antibody.

***FBS dialysis*:** FBS was dialyzed in snakeskin pleated dialysis tubing with a 3.5 kDa cut off value (Pierce, cat. # 68035). Dialysis was carried out on 150 ml of FBS at a time. FBS was placed in dialysis tubing and dialyzed in 1x PBS for 24 hours at 4°C. PBS was changed 5 times during the course. FBS was then filtered in 0.2 μ filter before use.

Tissue culture: The DP16.1/p53ts cell line were maintained at 37 °C in α-minimal essential medium supplemented with 10% fetal calf serum (Hyclone). AML3, AML4 and K562 cells were maintained in α-minimal essential medium (α-MEM) supplemented with

10% fetal bovine serum. Ba/F3 and Ba/F3 DD cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 0.1 ng/ml interleukin-3 (IL-3), and 0.0004% ß-mercaptoethanol. C2C12 cells were maintained in DMEM high glucose with 20% FBS. For glucose deprivation experiment, DMEM (-glucose, -pyruvate) was used.

Retroviral infections- For retroviral infections, 293TV cells were transfected using calcium phosphate with PCL-ECO and various retroviral constructs. Viral supernatants were collected 48 h later and incubated with DP16.1 and 3T3 fibroblasts along with 8 μg/ml Polybrene for 16 h. Cells were evaluated 24 to 48 h postinfection.

Northern blot analysis. Total RNA was isolated from cells and tissues using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For each sample, 10 μg of RNA was run on a denaturing agarose gel and transferred to a positively charged nylon membrane. The blots were hybridized with ^{32}P-radiolabeled c-myc, p21, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes, followed by standard washes. Phosphorimaging analysis was carried out, and RNA transcript levels were quantitated using ImageQuant software (Molecular Dynamics)

Apoptosis and death. Apoptosis was assessed using Annexin V-phycoerythrin and 7-amino-actinomycin D (7-AAD) staining according to the manufacturer's instructions (BD PharMingen). The proportion of apoptotic cells (Annexin V positive, 7-AAD negative) was determined using a FACScan flow cytometer and CellQuest software (Becton Dickinson). For C2C12 cell death assays, cells were washed once and stained with propidium iodide (5 μg/ml) and quantified using flow cytometery.

Fatty acid oxidation assay. Palmitate oxidation was measured by the production of $^{14}CO_2$ from [1-^{14}C]palmitic acid and was adapted from a protocol described earlier [86]. Briefly, palmitate was conjugated with essential fatty acid-free BSA to generate a 10X stock solution of 25% BSA (w/v) and 6 mM palmitate in DMEM (-glucose) and mixing overnight at 37°C. [1-^{14}C]palmitic acid (Amersham) was then added to the cold palmitate/BSA mixture by mixing for 1 h at 37°C to give a working concentration of 0.2 mCi/ml. C2C12 ($5x10^4$) cells were plated in 6 cm dishes and grown overnight. The next day, cells were subjected to glucose withdrawal and 24 h later, cells were washed once with PBS (-Ca^{++}, -Mg^{++}) and then incubated for 1 h with 2 ml of conjugated [1-^{14}C] palmitic acid solution. Each dish was sealed with parafilm, which had a piece of Whatman paper taped facing the inside cover of the dish. After a 2-h incubation, the Whatman paper was wetted with 100 µl of phenylethylamine-methanol (1:1) to trap the CO_2 produced during the incubation period. Next, 200 µl of H_2SO_4 (4 M) was added to the cells and incubated for an additional 1 h at 37°C. Finally, the Whatman paper was carefully removed from the dish covers and transferred to scintillation vials for measurement of radioactivity.

CHAPTER 3: IDENTIFICATION AND CHARACTERIZATION OF LPIN1 INDUCTION AND FUNCTION IN RESPONSE TO DNA DAMAGE

Chapter 3 Summary:

Lpin1 is upregulated in a p53-dependent manner in cell lines as well as primary tissues. This induction is not important for p53-dependent apoptosis as loss of Lpin1 does not compromise p53-dependent apoptosis in thymocytes and splenocytes. Lpin1 is upregulated in a tissue-dependent manner with strong induction in haematopoietic and lymphoid systems but not in MEFs in response to γ-irradiation. Over expression of Lpin1 in DP16.1 and 3T3 fibroblasts did not affect growth.

Chapter 3.1 Introduction:

p53 is a well known transcription factor that regulates a large number of target genes [23]. In response to stress, these targets initiate well known p53 responses that include cell cycle arrest, DNA repair, apoptosis and senescence. In spite of extensive research undertaken to understand p53 and its role in biological responses, many questions remain unanswered. For example, it is not clear if all important p53 regulated genes have been identified. Identifying critical p53 regulated genes might help us understand how individual processes such as apoptosis are influenced by p53 or help identify new processes that p53 might be involved in.

Earlier, our lab has addressed this issue by carrying out a cDNA microarray screen with the aim of identifying p53-regulated genes in response to p53 overexpression. We have utilized the p53-null Friend virus induced erythroleukemic cell line (DP16.1) transfected with a temperature-sensitive p53 mutant (DP16.1/p53ts). The mutated p53ts protein ectopically expressed in the DP16.1 line has a valine in place of alanine at position 135 and behaves as a mutant at 37°C but as a wild type at 32°C [87]. These cells grow normally at 37°C, but upon temperature shift, they transiently arrest in G1, rapidly lose viability and undergo apoptosis while the parental DP16.1 cells continue to grow normally at 32°C. The microarray screen produced a number of potential candidates from which Lpin1 was selected for further study.

To investigate the potential for Lpin1 as bona-fide p53 regulated gene, we examined the dependency of Lpin1 up-regulation on p53 in response to γ-irradiation in a variety of systems in mouse and human systems. Furthermore, the role of Lpin1 in

apoptosis and growth arrest was examined by looking at attenuation of apoptosis in the absence of Lpin1 and suppression of growth with Lpin1 overexpression.

3.2 Chapter 3 Results:

3.2.1 Lpin1 is a p53-regulated gene

Lpin1 was identified through miccroarray analysis of cDNA expression in a screen for p53 regulated genes using DP16.1/p53ts cells. These cells express a temperature-sensitive p53 allele that is activated when the cells are cultured at 32°C. To confirm that Lpin1 is a p53-regulated gene, we studied the kinetics of Lpin1 induction in DP16.1/p53ts cells in response to p53 activation at 32°C by Northern blot analysis (Figure 3.1a). Lpin1 mRNA increased within 3 hr of p53 activation and remained elevated at 6 hr. The amount of Lpin1 mRNA was unchanged in DP16.1 cells at 32°C , indicating that Lpin1 mRNA induction in DP16.1/p53ts cells is dependent on p53 and not the result of a change in temperature. This pattern of expression is similar to that of the p53-responsive gene, $p21^{WAF1}$. We also observed induction of Lpin1 mRNA in response to γ-irradiation in the wild-type p53-expressing, mouse myeloid cell line, Ba/F3 , but not in Ba/F3-DD cells, an isogenic derivative expressing the C-terminal fragment of p53 that has been shown to act as a potent trans-dominant repressor of wild-type p53 through its ability to prevent the formation of functional p53 tetramers (Figure 3.1b). Lpin1 mRNA levels were also induced in the spleen, thymus and bone marrow of γ-irradiated p53+/+ mice but not p53-/- mice (Figure 3.1c). We did not detect any increase in the level of Lpin1 mRNA in response to γ-irradiation in mouse fibroblasts.

3.2.2 Lpin1 is not important for gamma radiation induced p53-dependent apoptosis

To determine if the induction of Lpin1 in response to γ-irradiation is important for p53-dependent apoptosis, we measured apoptosis in splenocytes and thymocytes from

irradiated wild type, p53 -/-, and fld mice. Fld mice contain a mutation in the Lpin1 gene and are Lpin 1 deficient. The mice were exposed to whole body irradiation (5 Gy), and the spleen and thymus were harvested and analyzed at 0, 5, 12 and 20 hours post radiation. Cells were stained with Annexin V as a marker for apoptosis and the proportion of apoptotic cells that were Annexin V-positive and 7-AAD-negative was determined by flow cytometry (Figure 3.2). p53-dependent apoptosis was unaffected in the irradiated Lpin1-deficient thymocytes and splenocytes. We conclude that Lpin1 induction is not involved in p53-dependent apoptosis in hematopoietic cells.

3.2.3 Lpin1 induction is tissue dependent

Thus far Lpin1 induction was observed only in lymphoid and hematopoietic systems. We wanted to see if Lpin1 was also induced in other systems. We therefore looked at Lpin1 induction in wild type and p53 -/- mefs, HCT-116, primary human fibroblasts and human leukemia cell lines in response to gamma radiation and cisplatin. Figure 3 a, b, c and d show lack of Lpin1 induction in both human and mouse primary fibroblasts in response to γ radiation. Cisplatin treatment of AML-3 and 4 showed no consistent pattern of Lpin1 induction (Figure 3.3c). Thus based on Figures 1 and 3, we conclude Lpin1is induced in a cell line and tissue-dependent manner.

3.2.4 Lpin1 over-expression in fibroblasts and DP16.1 has no effect on growth rate.

To assess whether Lpin1 promotes or suppresses growth of cells, Lpin1 was cloned in to a retroviral vector containing an IRES allowing expression of the gene of interest and GFP simultaneously. DP16.1 and 3T3 fibroblasts were infected with pbabe vector and pBabe Lpin1 and GFP expression was followed over time as a measure of

viability. Tob, a known antiproliferative gene, was also cloned and expressed together with Lpin1 in DP16.1 for positive control. Figure 3.4 a shows that while Tob has clear negative effect on growth of DP16.1, there was no measurable effect due to Lpin1 overexpression. The same pattern was also observed in 3T3 fibroblasts (Figure 3.4 b).

3.2.5 Tissue expression pattern of Lpin1

Lpin1 is highly expressed in testis and liver and to a lower extent in kidney and heart and is barely detectable in lung, brain and skeletal muscle (see Figure 3.5). The heart and liver show two detectable transcripts of Lpin1 consistent with the two transcripts detected by our northern blot analysis (Figure 3.5).

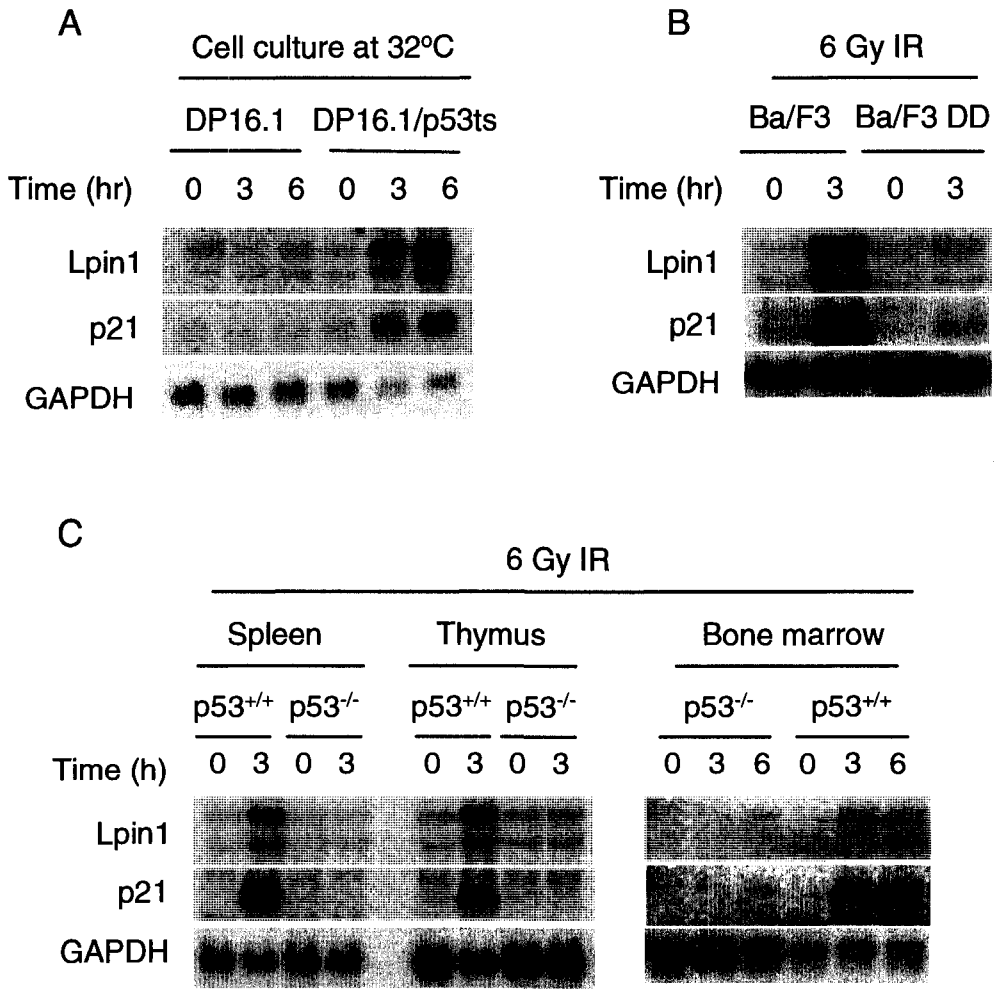


Figure 3.1 p53 activates Lpin1 expression

(A) Northern blot analysis of RNA isolated from parental DP16.1 cells and DP16.1/p53ts cells after incubation at 32°C for the times indicated. RNA (15 μg) was run in each lane and hybridized to *Lpin1*, *p21*^{WAF1} and *GAPDH* cDNA probes. RNA was visualized by autoradiography.

(B) Northern blot analysis of RNA isolated from mouse Ba/F3 and Ba/F3 DD cells after γ-irradiation with a dose of 6 Gy at the times indicated. The blot was hybridized with the indicated cDNA probes.

(C) Northern blot analysis of RNA isolated from the spleen, thymus and bone marrow of wild-type (*p53*$^{+/+}$) and *p53*$^{-/-}$ mice at the times indicated after whole body γ-irradiation with a dose of 6 Gy. The blot was hybridized with the indicated cDNA probes.

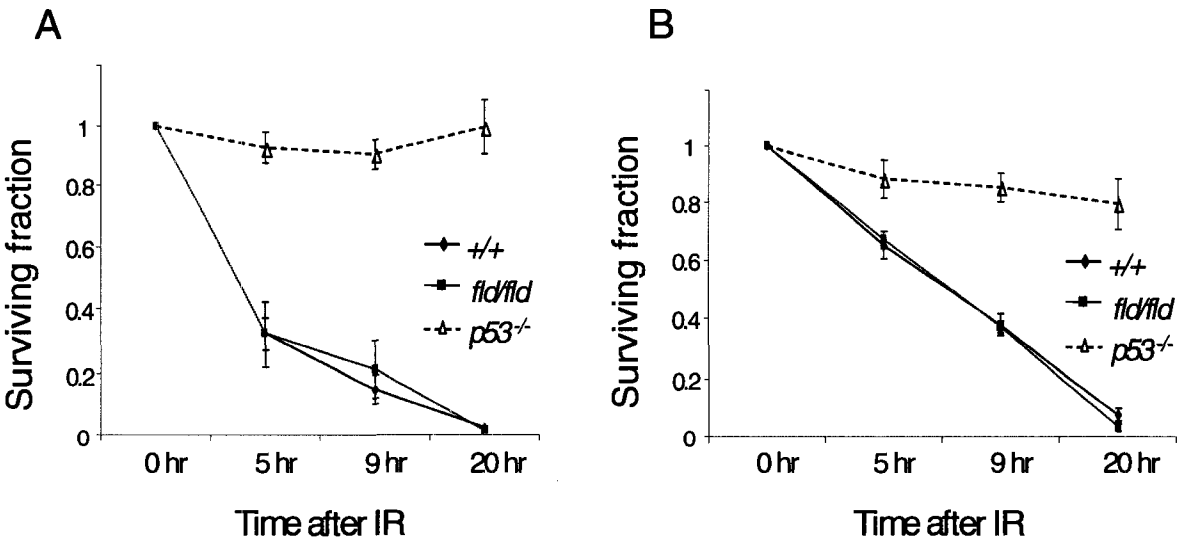


Figure 3.2 Lpin1 is not required for p53-dependent apoptosis:
Survival (mean ± SD) of thymocytes **(A)** and splenocytes **(B)** isolated from $p53^{+/+}$, $p53^{-/-}$ and *fld/fld* mice after whole body γ-irradiation with a dose of 5 Gy. Cells were cultured for the indicated times. Surviving fraction represents the proportion of cells that were 7-AAD negative and Annexin V negative compared with unirradiated cells.

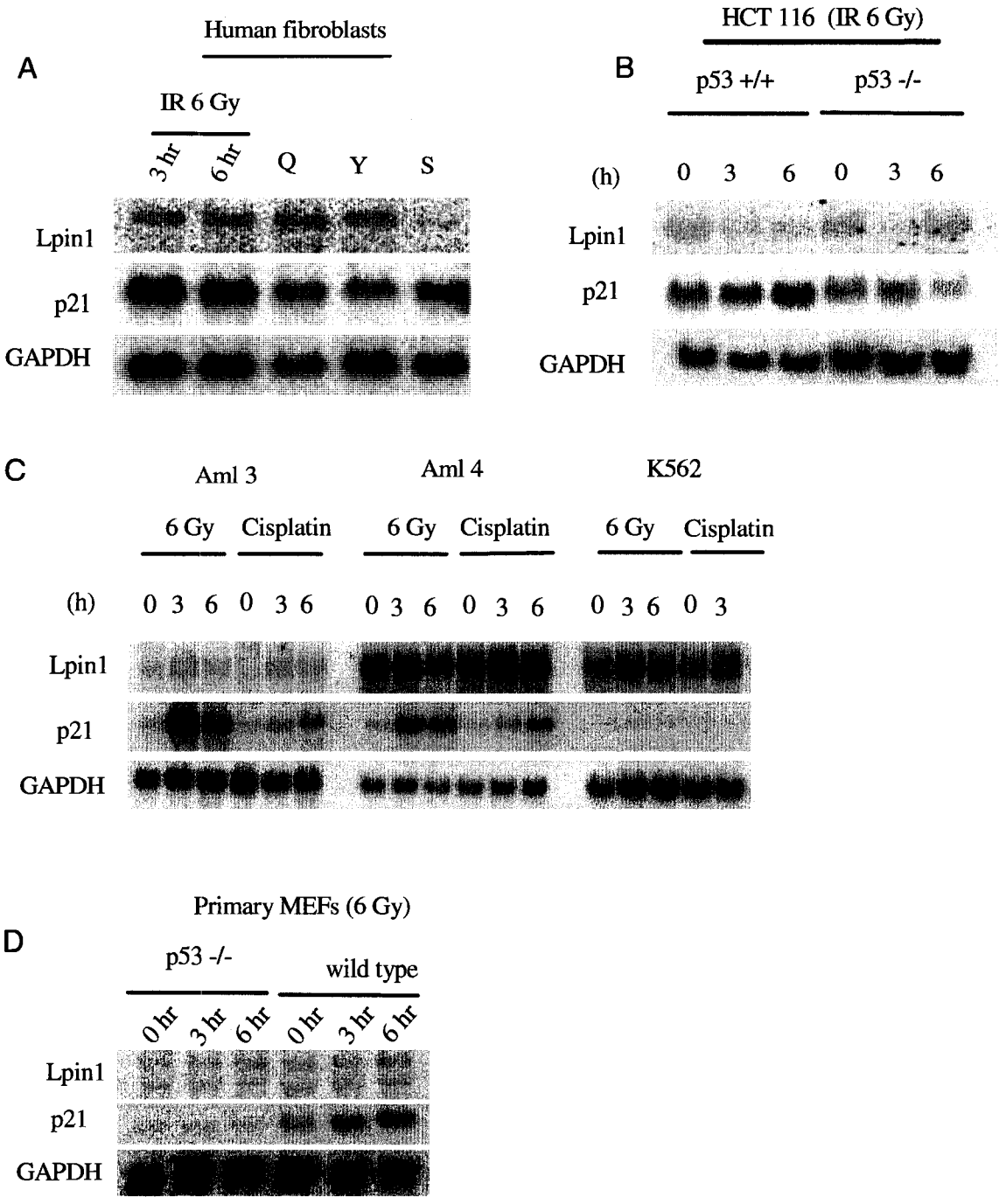


Figure 3.3: Lpin 1 mRNA expression in human and mouse cell lines using flull length human Lpin 1 cDNA **(A)** Lpin 1 mRNA levels in response to gamma radiation (lanes 1 and 2); Lpin1 mRNA levels are similar in quiescent human fibroblasts (Q) to young (Y) and is repressed in senescent cells (S). **(B)** Lpin1 expression in the colon cancer cell line HCT 116 with and without p53 in response to gamma radiation. **(C)** Lpin1 expression in the human leukemic cell lines p53 wild type Aml3 and Aml4 and p53 -/- K562. **(D)** Lpin1 expression in wild type and p53 -/- Mefs.

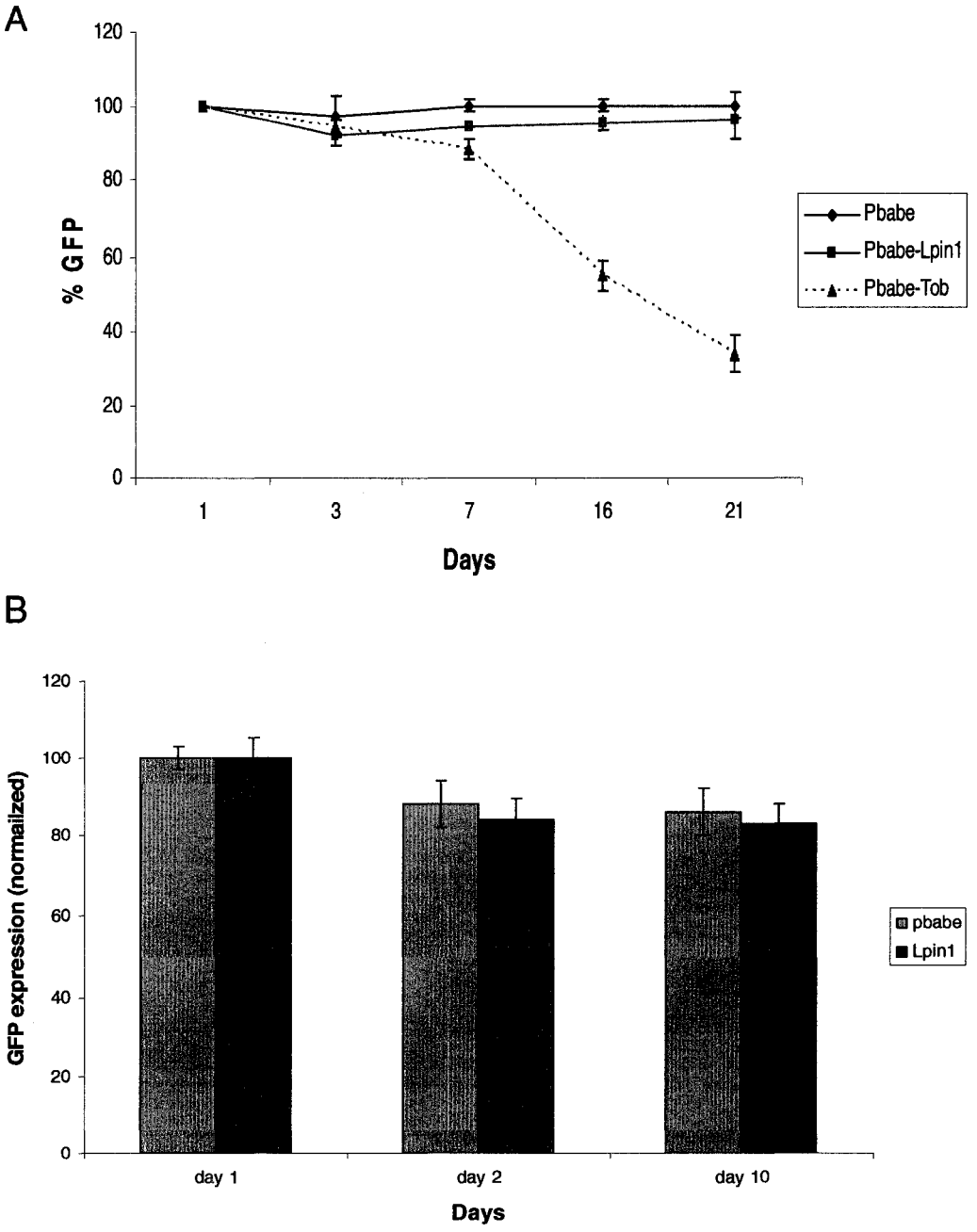


Figure 3.4: Effect of Lpin1 on growth.
(A) Pbabe vector, Lpin1 and Tob infected DP16.1 cells were analysed for GFP expression over a period of 21 days; Tob is known to have a negative effect on growth and was used as a positive control **(B)** pbabe and Lpin1 infected 3T3 fibroblasts analysed for GFP expression for a period of 21 days.

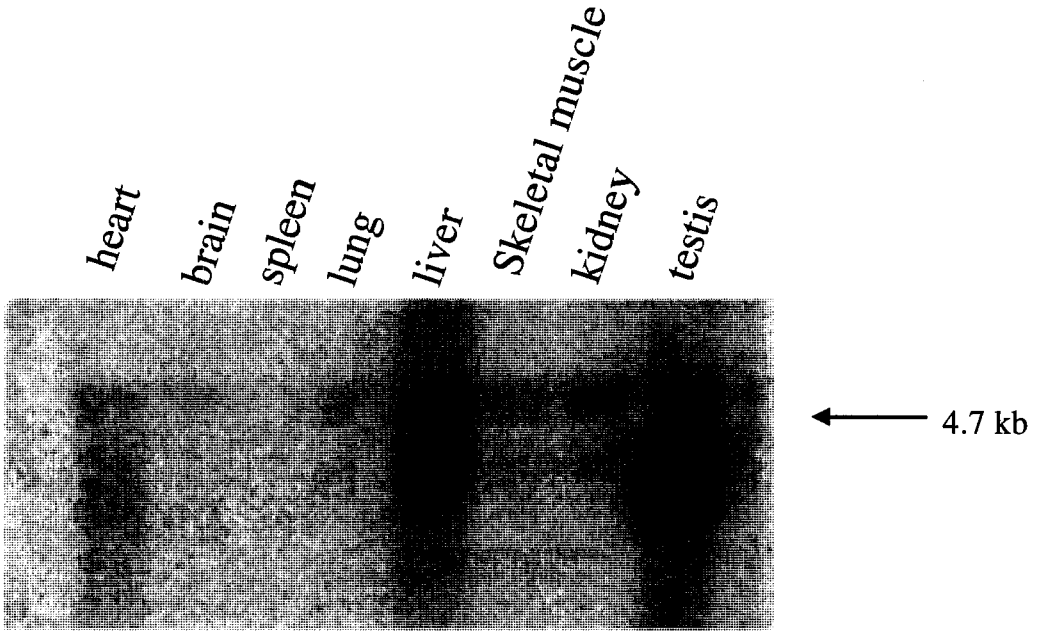


Figure 3.5: Tissue pattern expression of mouse Lpin1mRNA on pre-run blot of enriched poly A+ RNA.

CHAPTER 4: MECHANISM AND SIGNIFICANCE OF p53 AND LPIN1 UP-REGULATION IN RESPONSE TO GLUCOSE WITHDRAWAL

Chapter 4 Summary:

This chapter demonstrates a glucose withdrawal pathway that leads to p53 up-regulation and phosphorylation at Ser-15 and Thr-18. Ser-15 phosphorylation of p53 in this context occurs in an Atm dependent but AMPK independent manner. Atm activation occurs in a DNA damage-independent manner. This glucose withdrawal pathway also leads to Lpin1 up-regulation in a p53-dependent manner. We also show that p53 and Lpin1 involvement in glucose regulation of fatty acid oxidation.

4.1 Introduction:

The up-regulation and activation of the p53 tumour suppressor protein in response to a variety of stress signals leads to cell cycle arrest, senescence or apoptosis [88]. The level of p53 protein and its transcriptional activity have been shown to be dependent on the severity of various stress signals. Low levels of p53 protein induce the expression of highly responsive, antioxidant genes including Sestrin 1 and Sestrin 2 that serve to decrease intracellular ROS levels that might otherwise contribute to genomic instability [63]. p53 can also promote the expression of cell cycle arrest genes that modulate the cell cycle and provide time for DNA repair. Higher levels of p53 protein promote the expression of pro-oxidant genes and pro-apoptotic genes that lead to cell death [88]. Hence, p53 has both protective and death-promoting functions and both functions are likely to be involved in suppressing tumour development. In addition to these functions, emerging evidence indicates a role for p53 in regulating metabolism. Glucose deprivation has been shown to lead to ser-15 phosphorylation in two separate studies [72, 73], and AMPK-dependent activation of p53 was shown to lead to a p53-dependent cell cycle arrest that helped cells survive in the absence of glucose [72]. The relationship between p53 and AMPK appears to be bi-directional since p53 is not only a substrate for AMPK but also promotes the expression of an AMPK subunit in response to DNA damage [74]. p53 has also been shown to regulate the expression of Sco2, a gene that encodes a component of the electron transport chain and is important in the generation of ATP by oxidative phosphorylation [66]. In the absence of p53, Sco2 levels decreased, shifting ATP generation from the oxidative phosphorylation pathway to glycolysis, a phenomenon widely observed in cancer cells and known as the Warburg effect. p53 also regulates the

expression of Tigar, a protein that reduces glycolysis and protects cells from ROS-associated apoptosis likely through enhancement of the pentose phosphate pathway [89].

Although, the above studies point to a strong connection between p53 and metabolism, our knowledge of the mechanism by which nutrient deprivation activates p53 remains incomplete. For example, it is not clear if glucose deprivation leads to p53 protein stablization and phosphorylation, if this is AMPK dependent, and if this occurs in a nutritionally relevant system. In addition, it is not known if p53 can be activated under physiologically relevant low glucose concentrations. Although Jones et. al., reported that AMPK activity triggered by glucose deprivation promotes p53 phosphorylation at Ser-15, it remains possible that other mechanisms exist to regulate p53 activity in response to low glucose.

In addition to the lack of data on the connection between glucose withdrawal and p53, it is not known if glucose withdrawal upregulates Lpin1 and if this is p53-dependent. Lpin1 has been shown to be an important regulator of fatty acid oxidation [79, 82] and is upregulated by fasting [90]. However, the exact signal responsible for Lpin1 upregulation in fasting is still not known.

This chapter examines the role of p53 and Lpin1 in response to glucose withdrawal in a nutritionally relevant system, C2C12 (an undifferentiated muscle derived cell line). We first examine the mechanism of p53 upregulation and activation and then ask what roles do p53 and Lpin1 play in glucose withdrawal induced cell cycle arrest, cell death and fatty acid oxidation.

4.2 Chapter 4 Results:

4.2.1 Glucose deprivation upregulates Lpin1 in a p53-dependent manner

Lpin1 has been shown to be an important regulator of metabolism promoting and inhibiting fatty acid oxidation depending on tissue context. Importantly, Lpin1 mRNA levels were shown to be elevated in muscle in a fasting induced cachexia model. Glucose deprivation has also been shown to activate p53 in Mefs. To investigate the relationship between p53 and Lpin 1 expression in response to nutrient deprivation in a physiologically relevant context, we used C2C12 myoblasts cultured under normal, glucose-rich medium and under conditions of low glucose. To assess whether p53 was upregulated by glucose deprivation, C2C12 myoblasts were grown in high glucose DMEM (25 mM) containing dialyzed FBS (20%) and then shifted to 0 mM glucose DMEM containing dialyzed FBS (20%) for different periods of time; p53 levels were measured by western blotting (Figure 4.1a). p53 protein levels increased as early as 6 hours after glucose withdrawal and reached their highest level at 18 hours. p53 phosphorylation at Ser18 (equivalent to human p53 Ser15) and Thr 21 (equivalent to human p53 Thr18) provides a widely accepted marker for p53 activation. To investigate p53 activation in response to glucose withdrawal, we performed a Western blot using phospho-specific antibodies to p53 (Figure 4.1b). Phosphorylation of Ser18 and Thr21 was evident 18 hr and 24 hr after glucose withdrawal. These data indicate that glucose withdrawal promotes the accumulation of p53 protein and the phosphorylation of p53. We next investigated whether Lpin1 was induced under similar conditions in C2C12

myoblasts by Northern blot analysis. Figure 4.1c shows Lpin1 mRNA induction at 24 hours after glucose withdrawal.

To determine whether Lpin1 induction upon glucose withdrawal is dependent on p53, we generated C2C12 cells stably expressing p53 shRNA or empty shRNA vector. The cells expressing p53 shRNAi showed effective knockdown of endogenous p53 under gamma radiation compared with empty vector control cells (Figure 4.1 d). C2C12 cells stably expressing p53 shRNA showed a reduction in the induction of Lpin1 mRNA levels upon glucose withdrawal (Figure 4.1 e). We conclude that Lpin1 induction in response to glucose deprivation is dependent on p53.

4.2.2 Low glucose levels are sufficient to upregulate p53 and Lpin1

Glucose levels in the blood stream are tightly regulated and do not vary much even under extreme conditions such as fasting. Hence, culturing cells in the complete absence of glucose is not physiologically relevant. We investigated whether p53 and Lpin1 levels are modulated under low glucose conditions.. Figure 2a shows that total p53 levels increased slightly at 5 mM and further at 1 mM glucose after a 48 hour period. The increase in p53 protein at 1 mM was also associated with ser 18 and thr 21 phosphorylation (Figure 4.2 b). Northern blot analysis was used to measure Lpin1 mRNA levels in C2C12 cells and C2C12 cells expressing p53 shRNA under various concentrations of glucose (25 mM , 5 mM, 1 mM, 0.1 mM). We observed a 3-fold increase in Lpin1 mRNA levels at 5 mM glucose, a 4-fold increase at 1 mM glucose, and a 3.4-fold increase at 0.1 mM relative to C2C12 cells grown in 25 mM glucose (Figure 4.2c). The induction of Lpin1 was abolished or reduced in C2C12 cells expressing p53

shRNA. We conclude that Lpin1 mRNA is induced in response to low glucose levels in a p53-dependent manner.

4.2.3 Glucose deprivation leads to p53 Ser-15 phosphorylation in an AMPK-independent but Atm-dependent manner

To understand how p53 is activated in response to glucose withdrawal we investigated the involvement of Atm, a classical activator of p53 in response to DNA damage and AMPK, a sensor of glucose availability. Atm is known to communicate double strand break damage to p53 by phosphorylating Ser-15 directly while AMPK has been reported to phosphorylate Ser-15 in response to glucose deprivation in primary MEFs and other non-muscle related cell lines [72, 73]. To test whether AMPK or Atm is required for p53 activation in response to glucose deprivation, we treated glucose-deprived C2C12 cells with the AMPK-specific inhibitor, compound C, or with the Atm-specific inhibitor Ku-55933. The level of phosphorylated ACC served as an indicator of AMPK activity. Reducing the glucose concentration in the culture medium to 1 mM resulted in p53 phosphorylation on Ser-15 and ACC phosphorylation (Figure 4.3 a). AMPK inhibition prevented the phosphorylation of ACC but had no effect on p53 Ser-15 phosphorylation (Figure 4.3a). Atm inhibition on the other hand led to a significant decrease in p53 Ser-15 phosphorylation in C2C12 cells deprived of glucose and in C2C12 cells treated with adriamycin. We conclude that p53 Ser-15 phosphorylation upon glucose deprivation in C2C12 cells is dependent on Atm and not on AMPK.

Since the inhibition of Atm lead to lower Ser-15 levels, we sought to confirm Atm activation under low glucose by examining phosphorylation of Atm at amino acid 1981, a hallmark of Atm activation. Figure 4.3 c shows Atm is phosphorylated as early as 2 hours in response to low glucose. This confirms that glucose withdrawal leads to Atm activation.

As seen in figure 4.3c, Atm phosphorylation occurs quite rapidly, 2 hours after glucose withdrawal. This raised the possibility that AMPK, also known to respond rapidly to changes in ATP levels, could be responsible for early Atm activation. We thus treated cells for 1 hour with the AMPK inhibitor compound C, and then shifted the cells to low glucose while maintaining inhibitor concentrations. Figure 4.3d, shows that while AMPK was completely inhibited as indicated by loss in ACC phosphorylation, Atm phosphorylation was unaffected. Thus, we conclude that phosphorylation of Atm at 1981 is AMPK independent.

4.2.4 Glucose withdrawal leads to Atm phosphorylation in a DNA damage independent manner

The activation of Atm in response to low glucose was unexpected and raised the possibility that cell growth under conditions of low glucose generates double-stranded breaks in DNA possibly as a result of increased levels of reactive oxygen species. Glucose deprivation leads to reduced glycolysis and reduced formation of NADPH through the pentose phosphate pathway. As a result, the NADPH-dependent reduction of glutathione disulphide to glutathione by glutathione reductase will be impeded. This would potentially lead to an increase in ROS species and thus DNA damage. Atm

phosphorylation, however, was also reported to occur in a DNA damage-independent manner in response to chromatin modifying agents [17]. To determine if glucose deprivation activates Atm through the formation of DNA strand breaks, we measured H2AX phosphorylation at Ser 139 since H2AX phosphorylation represents an early marker of DNA strand breaks. Figure 4.3 e shows glucose deprivation did not lead to any appreciable detection of H2AX phosphorylation while H2AX phosphorylation could be detected when C2C12 cells were treated with the DNA damaging agent, adriamycin. These data suggest that Atm phosphorylation in response to glucose deprivation occurs independently of DNA double strand breaks.

4.2.5 Loss of Lpin1 is dispensable for glucose induced cell cycle arrest and glucose withdrawal induced cell death

C2C12 cells maintained at low concentrations of glucose proliferate much more slowly than cells maintained at 25 mM glucose and these cells die after prolonged incubation in medium containing 1 mM glucose. To determine whether p53 and/or Lpin1 are required for reduced cell proliferation and for cell death after glucose deprivation, we developed two independent double-stranded oligonucleotides that target Lpin1 – Lpin 1 shRNAi-1 and Lpin 1 shRNAi-2, and showed that each was capable of efficiently downregulating Lpin1 protein levels in transfected C2C12 cells (Figure 4.4 a). We performed flow cytometric analysis on C2C12 cells 48 hr after culture in 1 mM glucose. p53 or lipin shRNAi and control cells all had similar cell cycle profiles (Figure 4.4 b). We then measured the death of C2C12 cells over a 72 hour period and found no differences in cells when p53 or Lpin1 was repressed (Figure 4.4 c). We conclude Lpin1

and p53 are not involved in inhibiting cell proliferation nor promoting glucose induced cell death after glucose deprivation.

4.2.6 Lpin1 represses fatty acid oxidation under normal conditions but promotes fatty acid oxidation under glucose withdrawal

Lpin1 has been shown to inhibit fatty acid oxidation in muscle and to promote fatty acid oxidation in liver tissue [80, 91]. In order to assess the involvement of p53 and Lpin1 in regulating fatty acid oxidation in C2C12 myoblasts, we generated C2C12 cells that overexpress Lpin1 (Figure 4.5a) and compared these with cells expressing empty vector. C2C12 over-expressing Lpin1 or containing the empty pBabe vector were cultured under normal (25 mM glucose), 3 mM or 1 mM glucose for 24 hr prior to assay for fatty acid oxidation (Figure 4.5b). Lpin1 overexpressing cells demonstrated an increase in FAO in a similar fashion to pBabe controls. Thus Lpin1 overexpression does not affect FAO. This might be because two Lpin1 isoforms exist and this one has no impact on fatty acid oxidation. The other isoform was not tested.

The two Lpin1 shRNAi developed for Lpin1 in our studies target both isoforms of Lpin1. Therefore, examining fatty acid oxidation under normal and 1 mM glucose conditions would be more revealing. To determine whether Lpin1 regulates fatty acid oxidation in C2C12 myoblasts, we measured palmitate oxidation under normal (25 mM) and 1 mM glucose conditions in the presence and absence of Lpin1 shRNA and p53 shRNA (Figure 4.5c). C2C12 cells expressing Lpin1 shRNA exhibited significantly higher fatty acid oxidation levels than control cells (untransfected and transfected with

empty vector) indicating that under normal cell culture conditions (25 mM glucose), Lpin1 represses fatty acid oxidation. This is consistent with a previous study demonstrating that enhanced Lpin1 transgene expression in skeletal muscle repressed fatty acid oxidation [83]. Interestingly, when the glucose level was reduced to 1 mM for 24 hr, control C2C12 cells exhibited an elevated level of fatty acid oxidation while the cells expressing Lpin1 shRNA showed a reduced rate of fatty acid oxidation. In the absence of Lipin1, cells may be unable to promote fatty acid oxidation under low glucose conditions. This could reflect a function of Lpin1 in promoting fatty acid oxidation in low glucose. It is also possible that in the absence of Lipin1, cells have an elevated rate of fatty acid oxidation in high glucose and consequently are unable to increase further the rate of palmitate oxidation when the glucose concentration is reduced. The rate of palmitate oxidation in cells cultured in high glucose was unchanged in cells expressing p53 shRNA. The increase in palmitate oxidation observed in control cells (untransfected and transfected with empty vector) upon glucose limitation was significantly reduced in p53 shRNA-expressing cells. This reveals an involvement of p53 in promoting fatty acid oxidation under reduced glucose conditions and is consistent with the idea that Lpin1 induction in response to low glucose is dependent on p53.

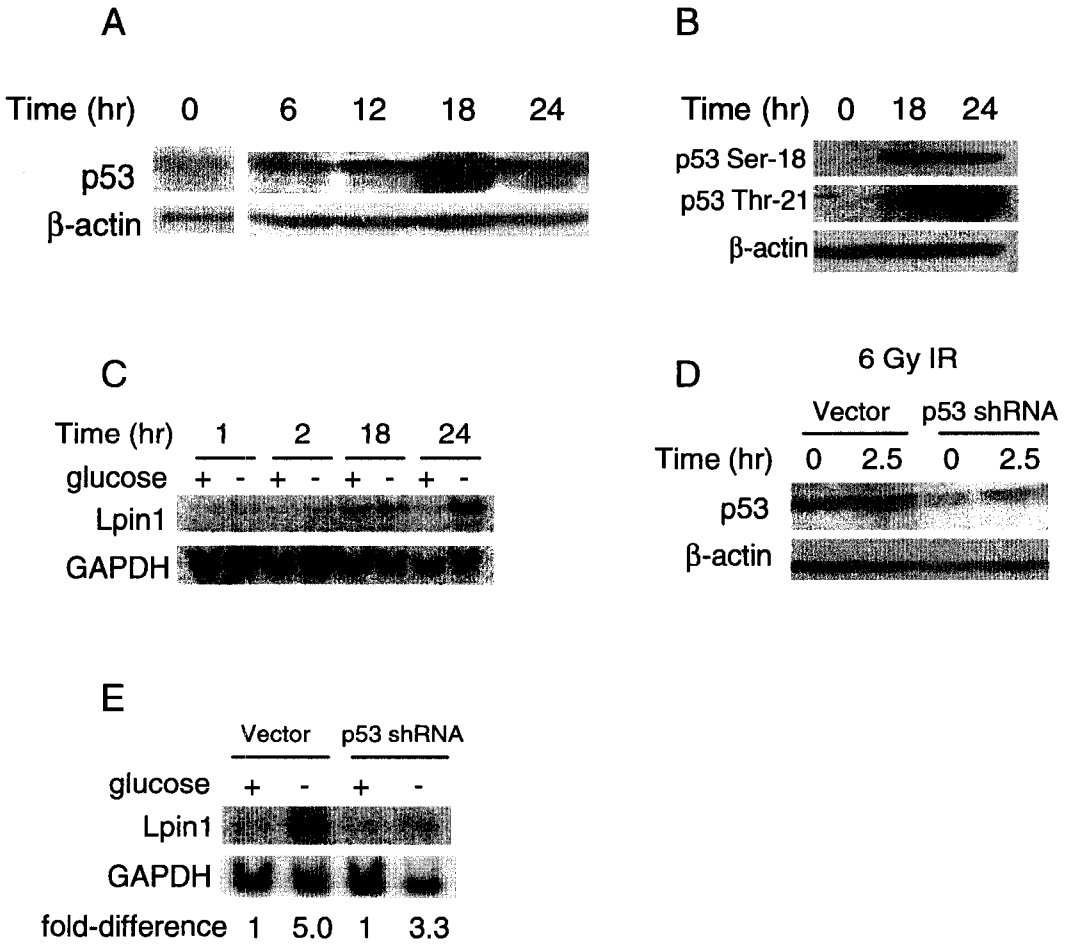


Figure 4.1 Glucose deprivation upregulates Lpin1 expression in a p53-dependent manner
(A) Western blot analysis of p53 protein expression in C2C12 cells after glucose withdrawal for the indicated times. The blot was reprobed with an antibody against β-actin as a loading control.
(B) Western blot analysis of p53 phosphorylation on Ser-18 and Thr-21 in C2C12 cells after glucose withdrawal for the indicated times.
(C) Northern blot analysis of Lpin1 expression in C2C12 cells using RNA isolated at different times after glucose withdrawal. The blot was reprobed with GAPDH cDNA as a loading control.
(D) C2C12 cells stably expressing p53 shRNA or empty vector were γ-irradiated (6 Gy). Cell extracts were prepared 2.5 hr after treatment and separated by gel electrophoresis. Knockdown of endogenous p53 was evaluated by Western blot analysis. β-actin served as a loading control.
(E) Northern blot analysis of Lpin1 expression in C2C12 cells stably expressing p53 shRNA or empty vector after glucose withdrawal for 24 hr.

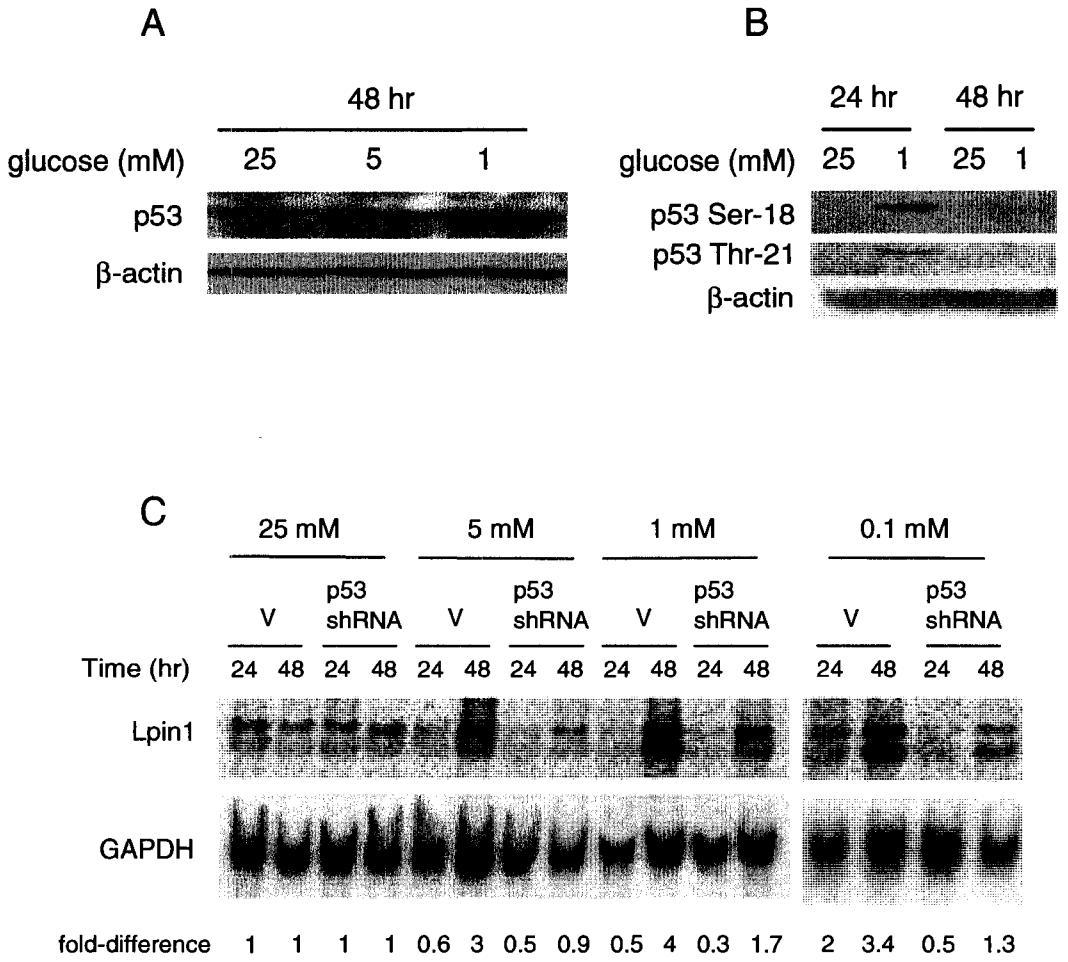


Figure 4.2 Lpin1 is induced by p53 under low glucose conditions
(A) Western blot analysis of p53 protein expression in C2C12 cells cultured under different concentrations of glucose.
(B) Western blot analysis of p53 phosphorylation on Ser-18 and Thr-21 in C2C12 cells cultured in 25 mM or 1 mM glucose for 24 and 48 hr.
(C) Northern blot analysis of Lpin1 expression in C2C12 cells stably expressing p53 shRNA or empty vector under various concentrations of glucose for the indicated amount of time. For the 48 hr time point, the medium was changed after 24 hr. Signal intensities were quantified by phosphorimage analyses and the relative abundance of Lpin1 mRNA was determined after normalizing to the level of GAPDH mRNA in each sample. The fold-difference in Lpin1 mRNA is expressed relative to the amount of Lpin1 in cells cultured in 25 mM glucose.

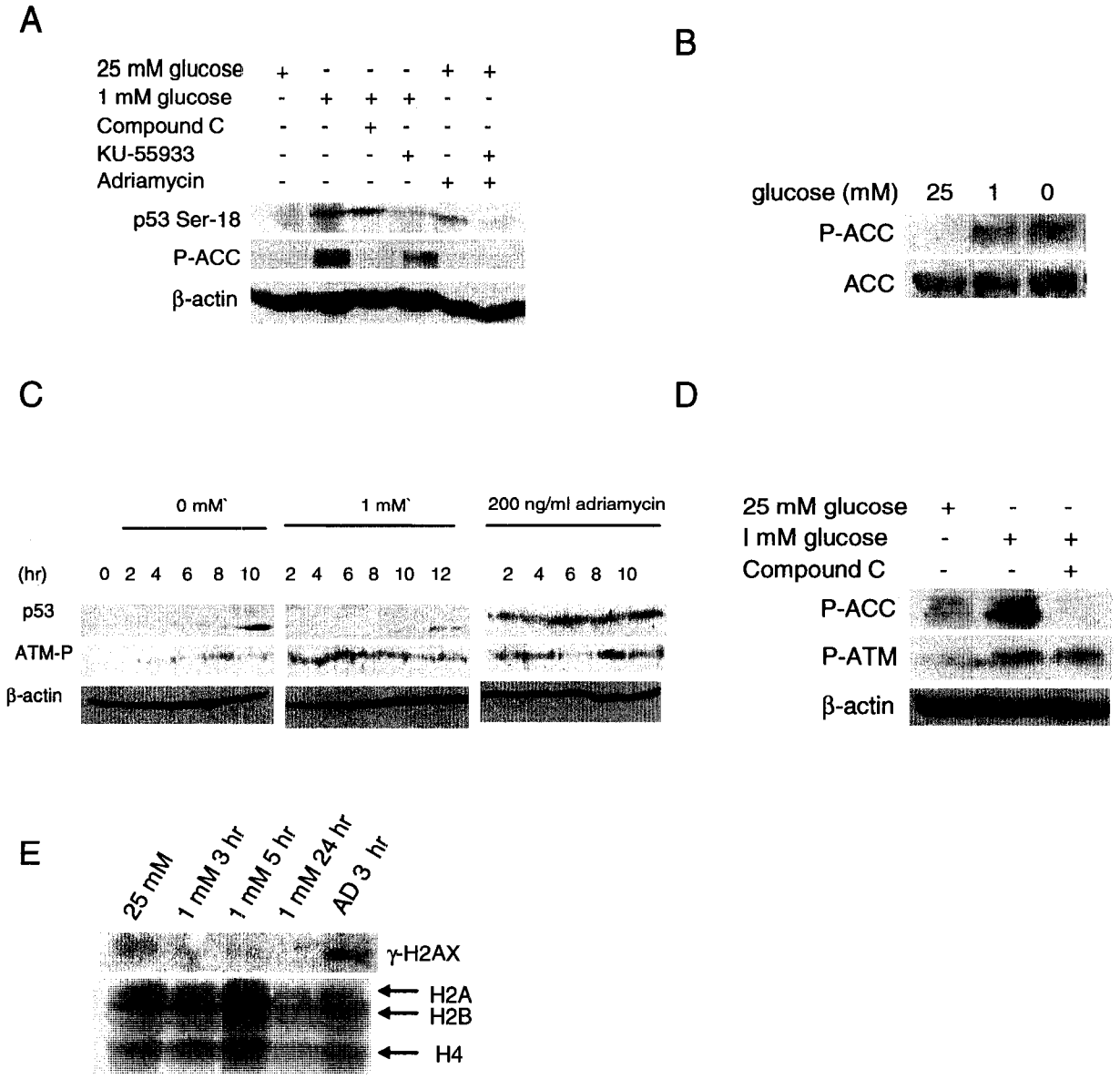


Figure 4.3. Low glucose-induced p53 phosphorylation on Ser-18 is dependent on ATM and not on AMPK

(A) C2C12 cells were cultured for 24 hr in the presence of 25 or 1 mM glucose either in the absence or the presence of Compound C or KU-55933. The two inhibitors were added during the final 1.5 hr of the culture period. Cells grown under normal conditions (25 mM glucose) were treated with Adriamycin (200 ng/ml) for 24 hr either in the absence or presence of KU-55963, added during the final 1.5 hr of the culture period. Cell lysates were analyzed for phospho-p53 (Ser-18) and phospho-ACC (ser-79) by Western blotting. β-actin served as a loading control.

(B) Activation of AMPK in C2C12 cells in response to low glucose. C2C12 cells were cultured for 24 hr in the presence of 25, 1 or 0 mM glucose. Cell lysates were analyzed for phospho-ACC (ser-79) and total ACC by Western blotting.

(C) Western blot for p53 and ATM phosphorylation under 0 and 1 mM

glucose with adriamycin (AD) as positve control and
(D) Activation of ATM in C2C12 cells in response to low glucose. C2C12 cells were cultured for 2 hr in the presence of 25 or 1 mM glucose either in the absence or presence of Compound C (40 μM). Cell lysates were analyzed for phospho-ACC (ser-79) and phospho-ATM (Ser-1981) by Western blotting
(E) Western blot analyses for phosphorylated H2AX (γ-H2AX) on HCl acid extracted histones from C2C12 cells cultured in 25 mM glucose or 1 mM glucose for different periods of time. Cells in 25 mM glucose were also treated with Adriamycin (AD, 200 ng/ml) for 3 hr prior to histone extraction. The Coomassie blue stained gel is presented in the bottom panel and shows the histone enrichment; histone identification is based on gel mobility.

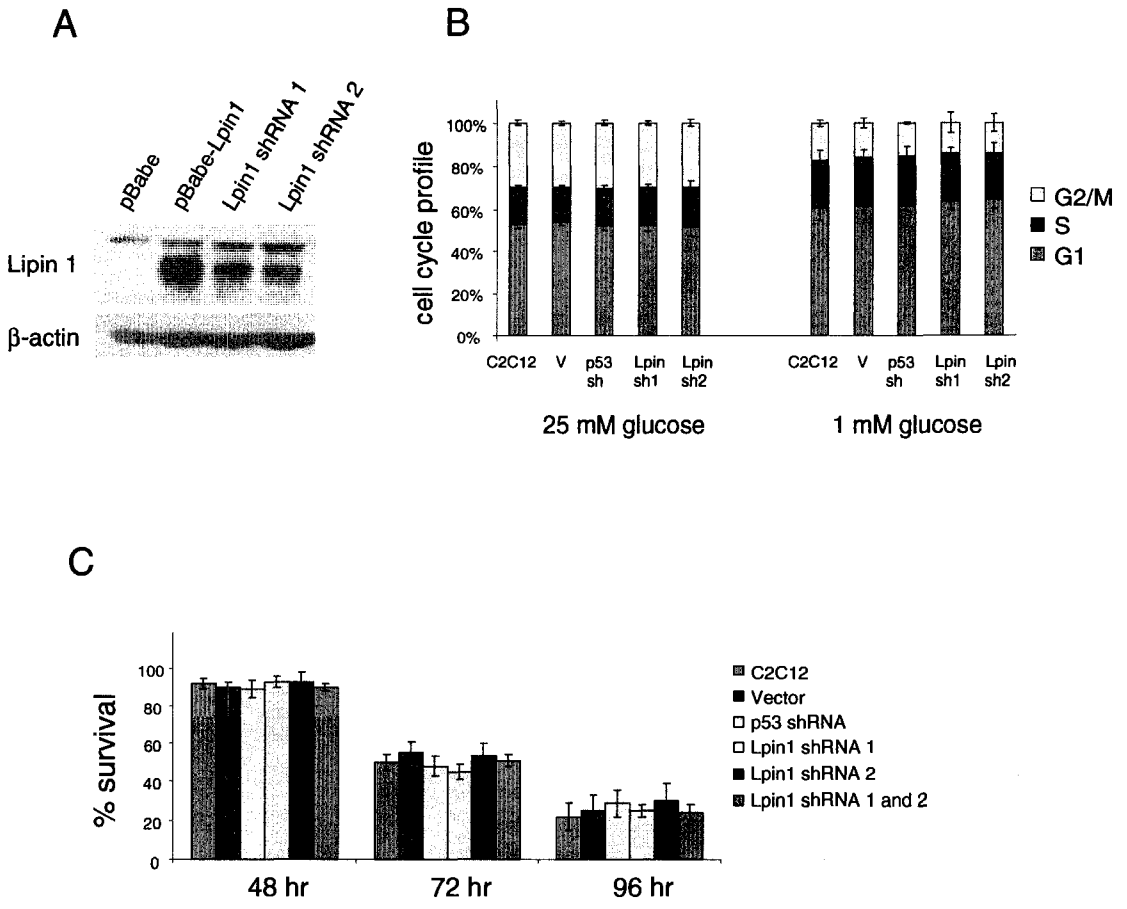


Figure 4.4. Low glucose-induced changes in cell cycle progression and cell death are independent of p53 and Lpin1

(A) shRNA-mediated downregulation of lipin1 in C2C12 cells. Cells stably expressing pBabe-Lpin1 or pBabe were transfected with Lpin1 shRNA and extracts were prepared 48 hr afterwards. Samples were analyzed by Western immunoblotting with the indicated antibodies. Lpin1 shRNA-1 and shRNA-2 are two independent shRNA sequences that target different regions in the Lpin1 mRNA transcript.

(B) C2C12 cells stably expressing p53 shRNA, Lpin1 shRNA-1, Lpin1 shRNA-2, or empty vector (V) were cultured in normal medium (25 mM glucose) or in medium containing 1 mM glucose for 48 hr. Medium was replaced after 24 hr. Cell cycle profiles were determined by flow cytometry after staining with propidium iodide.

(C) C2C12 cells were cultured in 1 mM glucose for the times indicated prior to measurement of cell viability by propidium iodide staining. Medium was replaced every 24 hr.

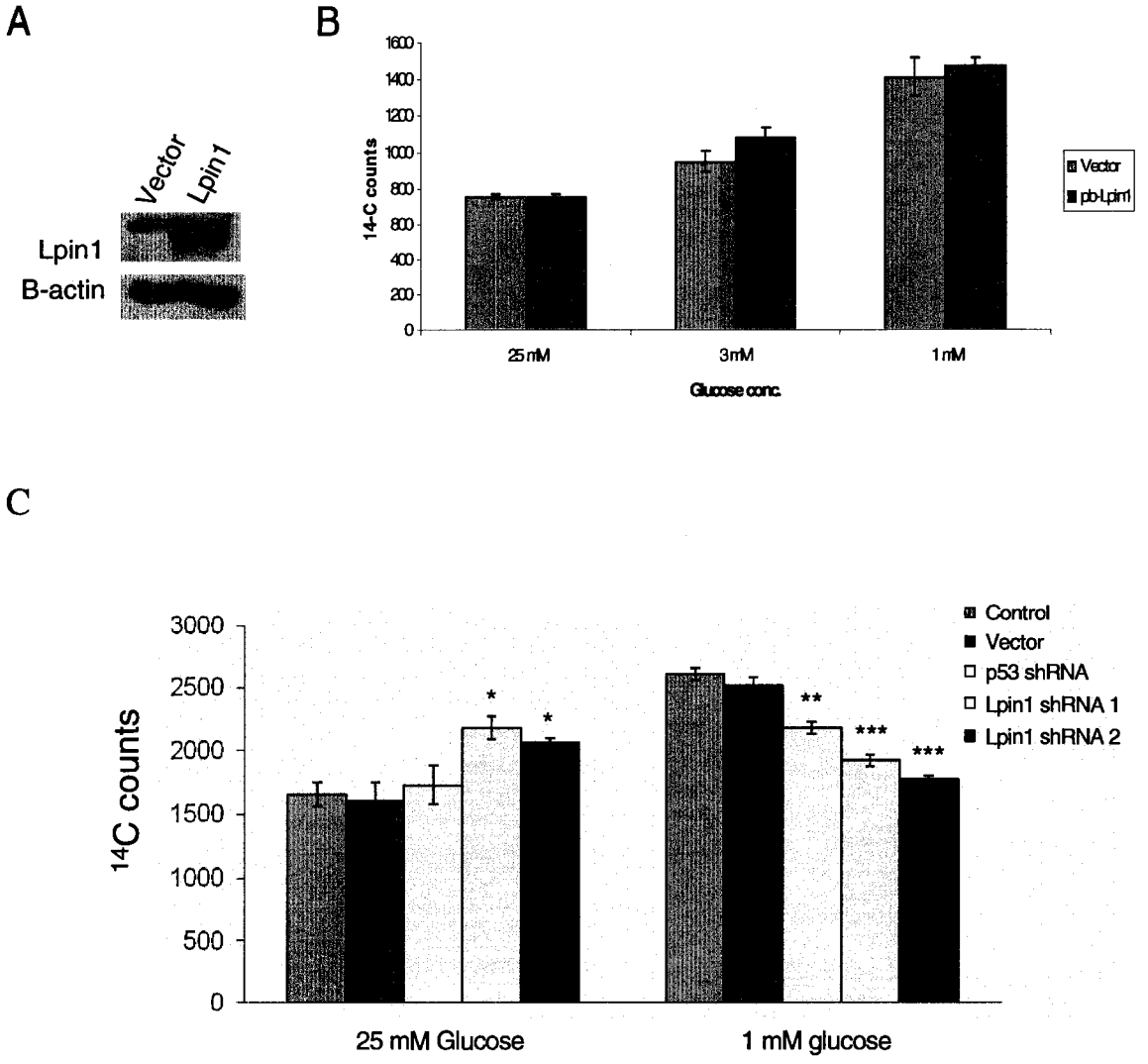


Figure 4.5 Regulation of fatty acid oxidation by Lpin1 in response to glucose deprivation
(A) western blot shows Lpin1 over-expression
(B) C2C12 subjected to 1 and 3 mM glucose and assayed for fatty acid oxidation
(C) Lipin 1 regulates palmitic acid oxidation in response to low glucose
Untransfected C2C12 cells (Control) or C2C12 cells stably expressing p53 shRNA, Lpin1 shRNA-1, Lpin1 shRNA-2 or empty vector (Vector) were cultured in normal medium (25 mM glucose) or in medium containing 1 mM glucose for 24 hr before measuring fatty acid oxidation. Values shown represent the mean ± SEM. *p = 0.018 versus vector control at 25 mM glucose. **p = 0.047 versus vector control at 1 mM glucose. ***p < 0.003 versus vector control at 1 mM glucose. For the differences between Lpin1 shRNA expressing cells at 1 mM versus 25 mM glucose, p = 0.05 (Lpin1 shRNA 1) and p = 0.03 (Lpin1 shRNA 2).

CHAPTER 5: DISCUSSION AND FUTURE DIRECTIONS

5.1 Discussion:

A number of previous studies have linked glucose deprivation to p53. Earlier work by Feng et. al. demonstrated transient Ser-15 phosphorylation in HCT1-116 after complete glucose withdrawal with no change in stability of p53 [73]. Evidence of p53 Ser-15 phosphorylation by AMPK activation and glucose withdrawal was also provided by Jones et. al. [72]. While both studies present important evidence linking glucose withdrawal to p53, several important questions remain unanswered. In the Jones et. al. study, it was not clear if glucose deprivation leads to stabilization and phosphorylation of endogenous p53. The glucose withdrawal induced Ser-15 phosphorylation of p53 was in response to over-expressed p53 and makes a conclusion about endogenous p53 difficult. Our study in C2C12 cells with endogenous p53 eliminates this complication. A similar argument can be made about p53 phosphorylation demonstrated by over-expression of an active form of AMPK in the same study-it may or may not be the case in a natural setting. Inhibiting AMPK with Compound C in the absence of glucose allows for a more valid interpretation. Furthermore, both studies are limited to systems with limited nutritional relevance: MEFs, H1299 and HCT-116 cells. Demonstrating that p53 responds to glucose in the C2C12 cell line suggests p53 may be involved in muscle metabolism.

One of the main shortcomings of studies on glucose withdrawal is that they are performed in glucose levels (0.5 mM or 0 mM) that would be very difficult to relate to physiological conditions given glucose is tightly buffered in the blood stream. Showing that p53 is stabilized by higher levels of glucose withdrawal (1mM and 5mM) indicates that p53 responds to glucose withdrawal conditions much higher that previously thought.

This might be significant in intra and interstitial tissue where glucose levels could be much lower than the bloodstream.

The pathway from glucose withdrawal to p53 has also been difficult to pin down. Over-expression of AMPK leading to Ser-15 phosphorylation or a dominant negative AMPK to block p53 phosphorylation has been given as evidence that AMPK mediates Ser-15 phosphorylation in response to glucose deprivation [72]. However, this again was performed with overexpressed p53. Blocking AMPK in the absence of glucose with endogenous p53 is a more direct test of this hypothesis. The lack of any significant effect on Ser-15 phosphorylation even after complete AMPK inhibition suggests that AMPK is not the mediator of p53 activation. On the other hand, inhibition of Atm significantly inhibited Ser-15 phosphorylation in the absence of glucose. This is supported by our observation that Atm is phosphorylated in response to glucose deprivation at an early stage (2 hours).

Atm phosphorylation in response to glucose deprivation has not been reported to our knowledge and therefore suggested the occurrence of double strand breaks as the mechanism of Atm activation. The lack of H2AX phosphorylation under the same condition of Atm activation suggests a DNA damage independent mechanism. Recent work has provided evidence that, Atm can be activated by DNA damage-dependent and independent mehanisms [17]. The latter involves chromatin modifying drugs that interestingly lead to Atm phosphorylation but not to H2AX-γ phosphorylation. What could be activating Atm in response to glucose deprivation? It is tempting to speculate that glucose deprivation could be mimicking the drug by changing chromatin structure. Alternatively, there may be a glucose-dependent but AMPK-independent signaling pathway that leads to Atm activation.

A number of p53 regulated genes have now been identified that link p53 to metabolism. This list includes Pten, Tigar, Sco2 and here we add Lpin1 [65, 66, 76]. Our data show that Lpin1 is upregulated in response to DNA damage, as well glucose deprivation in a p53-dependent manner. Previously, Lpin mRNA was shown to be upregulated in different cachexia models in mice suggesting Lpin1 is induced by stress [90]. Here, we identify two specific stress signals, DNA damage and glucose withdrawal that upregulate Lpin 1. It is interesting to speculate if the stresses identified here may be related to the stresses involved in chachexia in which Lpin1 is also induced and whether p53 is involved as well.

An important question is what is the significance of p53-dependent regulation of Lpin1? We hypothesized that Lpin1 may play a role in traditional p53-dependent responses such as cell cycle arrest and/or apoptosis in response to DNA damage, or may be a mediator of p53-dependent regulation of fatty acid oxidation, a pathway demonstrated to be regulated by Lpin1 [80, 83]. Our data indicate that Lpin1 does not play a role in p53-dependent cell death (thymocytes and splenocytes using gamma radiation) or glucose-induced cell death (C2C12). Nor is it likely to play a role in p53-dependent cell cycle arrest given it is not induced in MEFS in response to gamma radiation. In addition, in C2C12 cells, Lpin1 knockdowns have the same cell cycle profile compared to controls in response to glucose withdrawal.

Lpin1 was identified by Peterfy et. al. in 2001 as the cause of the fld phenotype, a disease characterized by a severe deficiency in adipocyte development. Later, two separate studies revealed that Lpin1 is an important player in fat metabolism mediating fatty acid oxidation in liver [80] but inhibiting it in muscle [83]. Consistent with work done in muscle showing that Lpin1 over expression inhibits fatty acid oxidation, Lpin1

knockdown in C2C12 showed a reduction in fatty acid oxidation under 25 mM glucose. However, when glucose was withdrawn, fatty acid oxidation failed to increase to the same level as controls. In this context, Lpin1 may be required to increase fatty acid oxidation, an observation similar to the observed role that Lpin1 played in liver tissue. How Lpin1 carries out the two opposing roles in different tissues is one of the most interesting aspects about Lpin1 biology. The mechanism that allows Lpin1 to function uniquely in different tissues may be the same that glucose deprivation is triggering. Thus, the glucose deprivation model in C2C12 cells may be an important tool available to decipher the pathways involved in Lpin1 dependent regulation of fatty acid metabolism.

4.2 Future directions:

This thesis has established a clear link between glucose deprivation, p53 activation, Lpin1 up-regulation and fatty acid oxidation. There are at least four pressing questions/issues that arise out of this work: 1) Is the glucose withdrawal induction of p53 and Lpin1 reproducible in primary myoblasts and myotubes? 2) What is the mechanism of p53 activation by glucose deprivation? 3) What is the significance of fatty acid oxidation regulation by Lpin1 in response to glucose deprivation? 4) What is the significance Lpin1 induction under DNA damage?

The C2C12 muscle cell line is a spontaneously immortalized cell line capable of differentiating into myotubes. It was originally established in 1974 and has likely acquired changes that are not representative of normal physiology [92]. Does glucose induced activation of p53 and Lpin1 occur in primary as well as immortalized systems? To address this, primary muscle myoblasts can be derived and established from wild type and p53 -/- mice, subjected to glucose withdrawal and examined for p53 stabilization, p53 phosphorylation and Lpin1 induction. The p53 -/- cell lines would address whether Lpin1 up-regulation is dependent on p53 and whether fatty acid regulation is p53- dependent. In order to confirm role of Lpin 1 in FAO regulation, fld-/- myoblasts can be established and examined for deficiencies in fatty acid oxidation in response to glucose withdrawal.

Once the glucose-p53-Lpin1 pathway is confirmed in primary myoblasts another important question can be asked: Is this pathway active in myotubes? Adult muscle tissue in mammals consists mostly of differentiated contracting myotubes with myoblast stem cell populations representing a small percentage of total muscle mass. Our work suggests

that the p53-Lpin1 pathway could exist in myotubes and thus play an important role in muscle metabolism. To confirm this, both C2C12 and primary myoblasts obtained from wild type, p53-/- and fld mice could be differentiated into myotubes and examined for changes in p53 expression, Lpin1 expression, and FAO in response to glucose withdrawal. Furthermore, apoptosis could be measured as well as other parameters not feasible in myoblasts. For example, myotubes increase in size due to accumulation of muscle-specific proteins during differentiation. Nutrient withdrawal leads to a decrease in myotube size [90]. Generally, glucose is the primary energy source with fatty acids and proteins representing secondary and tertiary sources, respectively [1]. Given that Lpin1 is important for the switch from glucose to fatty acid oxidation, an interesting hypothesis can be proposed: glucose withdrawal in myotubes leads to p53 and Lpin1 up-regulation and this response determines the selection of the energy substrate. According to this model, in the absence of p53 or Lpin1, myotubes would switch from glucose to protein usage for survival bypassing fatty acids. Two potential biological outputs that could be affected are survival and myotubes size. p53 -/- as well as Lpin1 -/- myotubes would have compromised survival and/or smaller size due to a deficiency fatty acid oxidation.

Our studies have shed some light on the mechanism of p53 activation as a result of glucose withdrawal. Contrary to evidence in the literature, we could not find evidence that that p53 is phosphorylated at Ser-15 by AMPK. Rather, we showed that Atm is phosphorylated shortly after glucose withdrawal and Atm activation is responsible for at least some of Ser-15 phosphorylation observed. Glucose withdrawal has not previously been shown to lead to Atm activation. Our findings raise the important question of how this occurs especially since we could not obtain evidence for double strand breaks. Two approaches may be used to address this question. The first involves looking at what

mechanism/s other than DNA damage activate Atm and then examining if any of those mechanisms can be activated by glucose withdrawal. The second approach is to ask if the effect of glucose withdrawal on glycolytic offshoot pathways can directly contribute to Atm activation. Atm activation can occur as a result of both DNA damage dependent and independent mechanisms [17]. We ruled out the classical Atm activation mechanism by double strand breaks because Atm activation was not accompanied by H2AX phosphorylation. Atm activation has been shown to be activated by chromatin modifying agents that do not cause DNA damage and include hypotonic conditions, Chloroquine and Trichostatin [17]. These agents cause relaxation of chromatin that through a yet undiscovered mechanism lead to Atm activation. This raises the intriguing possibility that glucose withdrawal is causing chromatin modifications in a similar manner to these drugs. Is glucose withdrawal affecting pathways that ultimately lead to chromatin modification? Two known mechanisms that alter chromatin structure are acetylation of histones by Hats (histone acetyl transferases) and deacetylation by Hdacs (histone deacetyl transferases) [93]. The hypothesis that chromatin modification leads to Atm activation could be tested by first looking at changes in histone acetylations early in response to glucose withdrawal and then devising ways to block these changes and examining the impact on Atm and p53 phosphorylation.

An important pathway that stems from glycolysis is the pentose phosphate pathway also known as PPP [94]. Two important functions of the PPP is to supply nucleotide intermediates for DNA synthesis and maintain a supply of NADPH, which is required to generate reduced glutathione and is a potent antioxidant [95]. When glucose is withdrawn, glycolysis shuts down and so does the PPP. It is possible that inhibition of the PPP as a result of glucose withdrawal could be causing Atm activation. Inhibiting PPP

using available inhibitors and examining Atm and p53 activation would address this question.

This thesis argues that at least one role for p53 in response to glucose withdrawal is to regulate fatty acid oxidation by upregulating Lpin1. Lpin1 has been shown by a number of studies to be a very important regulator of lipid metabolism with dualistic functions of promoting fatty acid oxidation in liver and inhibiting it in muscle. It is not clear how Lpin1 carries out these functions. Our studies show that the dual role of Lpin1 is reproducible in the C2C12 system under high and low glucose conditions. This model therefore could be an important tool to understand how Lpin1 functions under normal and stressful conditions. Lpin1 has two intrinsic biochemical activities: transcriptional co-activator function that in associated with FAO and a phosphatidic acid activity that is associated with triglyceride synthesis [80, 82]. The first activity is dependent on nuclear localization, mediated by a nuclear localization domain, and the latter is dependent on cytoplasmic localization. This suggests that localization is key to function which immunohistochemical studies can address. In a simple model, Lpin1 would be localized to the cytoplasm under high glucose. Here, Lpin1 would be unavailable to interact with key transcription factors necessary to regulate FAO. Under low glucose conditions, Lpin1 would then localize to the nucleus where co-transcription activities necessary for increase in FAO can be carried out. Nuclear localization of Lpin1 is associated with co-transactivator function and has been shown to bind PPAR-α in the liver. This interaction is the suggested mechanism of how Lpin1 promotes fatty acid oxidation.

So far, our investigation into the significance of Lpin1 induction by p53 has been restricted to cell lines. The next logical step is to investigate the potential significance of p53-lpin induction in a physiological setting, i.e., does a low glucose level in blood of

mammals activate p53 and Lpin1 in muscle tissue? One way to test this hypothesis is to use fasting as a stress signal to induce lower glucose levels. Lpin1 has been shown to be upregulated by fasting in muscle tissue but it is not clear if this is p53-dependent and if low glucose is the trigger [90]. To address these questions, wild type and p53 -/- mice would be fasted and muscle tissue isolated and examined for p53 and Lpin1 up-regulation by western and northern blots. Fld mice that lack Lpin1 expression would serve as control for Lpin1 detection in these experiments. This will determine if p53 and Lpin1 are upregulated by fasting and whether Lpin1 up-regulation is p53-dependent. To determine if lower glucose levels is the cause of p53 and Lpin1 up-regulation, water with glucose could be fed to mice under the same fasting conditions. This should raise glucose levels in the blood stream to normal levels. If low glucose is the stress signal, p53 and Lpin1 should not go up in glucose fed fasting mice.

This thesis demonstrates a link between p53 and Lpin to glucose withdrawal as well as DNA damage. γ-irradiation of thymocytes, splenocytes and bone marrow demonstrated that Lpin1 was upregulated in a p53-dependent manner. What is the significance of this induction? One hypothesis is that p53 may regulate fatty acid oxidation in response to DNA damage and this may be important to p53-dependent tumour suppression. To address this, proliferating T and B cells from wild type and p53 -/- and fld (Lpin -/-) mice could be exposed to a low dose of DNA damage agent that does not cause death. The first step is to find as DNA damage stress signal that is strong enough to activate p53 and Lpin1 but not cause apoptosis. H_2O_2 is one possibility given how quickly cells repair damage caused by it. p53, Lpin1 and fatty acid oxidation could then be measured. This would establish if DNA damage regulates fatty acid oxidation and whether this occurs through p53 and Lpin1.

If DNA damage regulates FAO in a p53-dependent manner, an important question is raised: is this an important mechanism for p53 mediated tumour suppression? It has long been known that a common feature of most cancers is the increase in glycolytic capacity known as the Warburg effect [67]. It is also known that FAO increase results in the inhibition of glycolysis [96]. This raises the interesting possibility that p53 mediated regulation of fatty acid oxidation through Lpin1 is a means of inhibiting glycolysis and thus tumour suppression. To address whether Lpin1 is involved in tumorigenesis, the Eu-myc transgenic mouse model could be employed. These mice express c-myc in the B cell lineage and develop B-cell lymphomas in a few months [97]. To assess whether Lpin1 might be involved in tumorigenesis, Eu-myc mice would be crossed to Lpin1 +/- mice to generate Eu-myc, Lpin1 +/- mice. Eu-myc Lpin1 +/- mice can then be mated to each other to produce Eu-myc Lpin1 -/- mice. The length and aggressiveness of tumors formed in these mice can then be compared to Eu-myc Lpin1 +/- or +/+.

The work put forth by this thesis and the above suggested experiments are aimed at understanding the significance of Lpin1 induction by p53. The implications of this relationship could be wide-ranging. It is possible that Lpin1 mediated regulation by p53 could be important in fasting/starvation and exercise and may be a new mechanism of p53-mediated tumor suppression. p53 may help organisms adapt to fasting/starvation by up-regulating Lpin1 and increasing fatty acid oxidation in muscle. The increase in fatty acid oxidation would allow the muscle to shift to an alternate energy supply and help preserve glucose for essential organs such as the brain. The same mechanism could also be useful during exercise where muscle demands of energy increase tremendously. p53 upregulation of Lpin1 and subsequent increase of ATP by fatty acid oxidation my help supply an extra energy source. Finally, p53 upregulation of Lpin1 may be mechanism of

suppressing a ubiquitous phenomenon in cancer-the increase in the rate of glycolysis. An increase in fatty acid oxidation is known to decrease glycolysis and vice versa- a relationship referred to as the Randle cycle[2]. This may be an attractive pathway to target for cancer treatment. It is my hope that this thesis leads to the exploration of these ideas and eventually to human benefit.

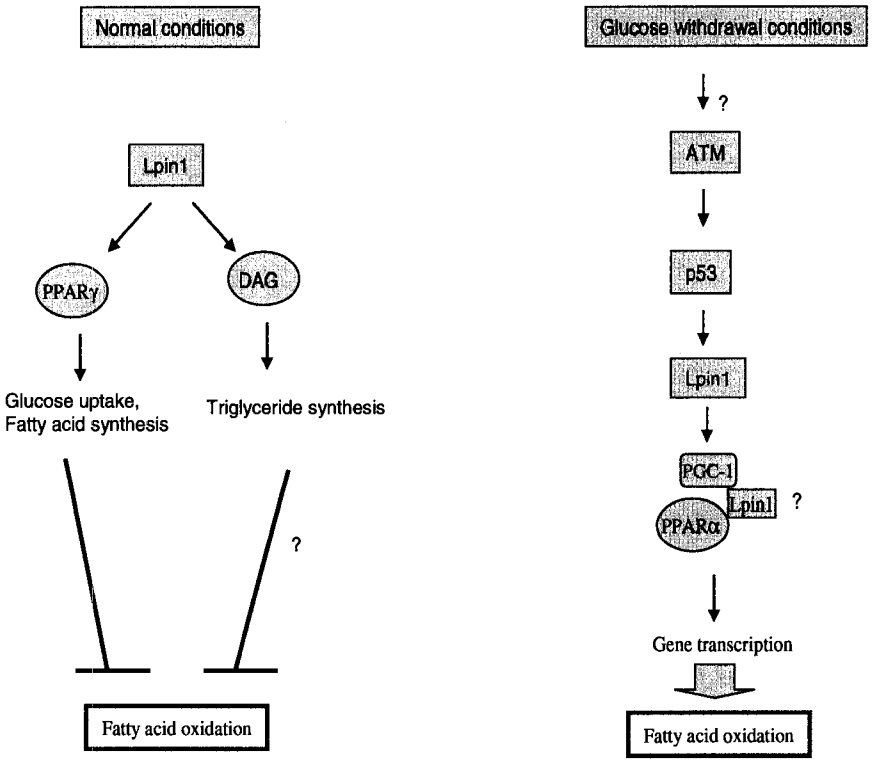


Figure 5.1 Proposed model of how Lpin1 functions in C2C12. Under normal conditions Lpin1 interaction with PPARγ promotes glucose uptake, fatty acid synthesis and triglyceride synthesis. Intermediates of glycolysis and fatty acid synthesis have been shown to inhibit fatty acid oxidation[1, 2]. Under glucose limiting conditions, Lpin1 interacts with different partners, PPARα and PGC-1, leading to upregulation of genes that increase fatty acid oxidation.

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APPENDIX

Summary of Figure 6.1:

Lpin1 polyclonal rabbit antibodies were produced against the peptide NH2-SKTDSPSRKKDKRSRHLGADG-OH.(Lpin1 a.a. 377 -397). To verify that rabbit sera recognized Lpin1 peptide, a GST-peptide fusion was constructed and run on western blot together with GST only and GST-PIRH2 serving as negative controls. Later, the antibody sera was tested against over-expressed full length Lpin1 and verified using two Lpin1 shRNAi constructs. (see chapter 4).

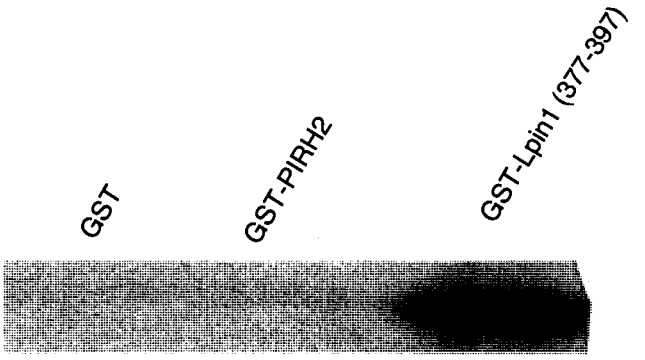

Figure 6.1: Verification of lpin1 antibodies. Western with GST alone, GST-PIRH2 fusion and GST-lpin1peptide were run on a western and rabbit sera was used as a primary.

Summary of Figure 6.2:

p53 and Lpin1 are not necessary for glucose deprivation induced cell death

In chapter one, we showed that Lpin1 is not necessary for p53-dependent death under DNA damage conditions. We therefore wanted to see if the same was true for glucose induced cell death. Since the involvement of p53 in glucose withdrawal cell death is not well studied, we also asked if p53 was involved. To address both questions, we utilized two different models: proliferating T-cells and splenocytes extracted from wild type, p53 -/- mice and fld mice. T cells and splenocytes were chosen because Lpin1 was robustly induced in these tissues earlier (thymus and spleen) by DNA damage in a p53-dependent manner (see chapter 3).

T-cells stimulated with IL-2 and CD3 and splenocytes stimulated with LPS from wild type and p53 -/- mice were grown in 5 mM glucose and then subjected to glucose starvation. Figure 6.2 a) and b) show no difference in viability between wild type and p53-/- splenocytes and T-cells after 24 hours as measured by 7-AAD. Splenocytes extracted WT and fld mice and stimulated with LPS were also subjected to dose dependent decreases of glucose. Figure 6.2 c) shows that cells from fld background have similar sensitivity to glucose deprivation compared with wild type cells. Thus neither p53 nor Lpin1 seems to be necessary for glucose deprivation induced death in lymphoid systems.

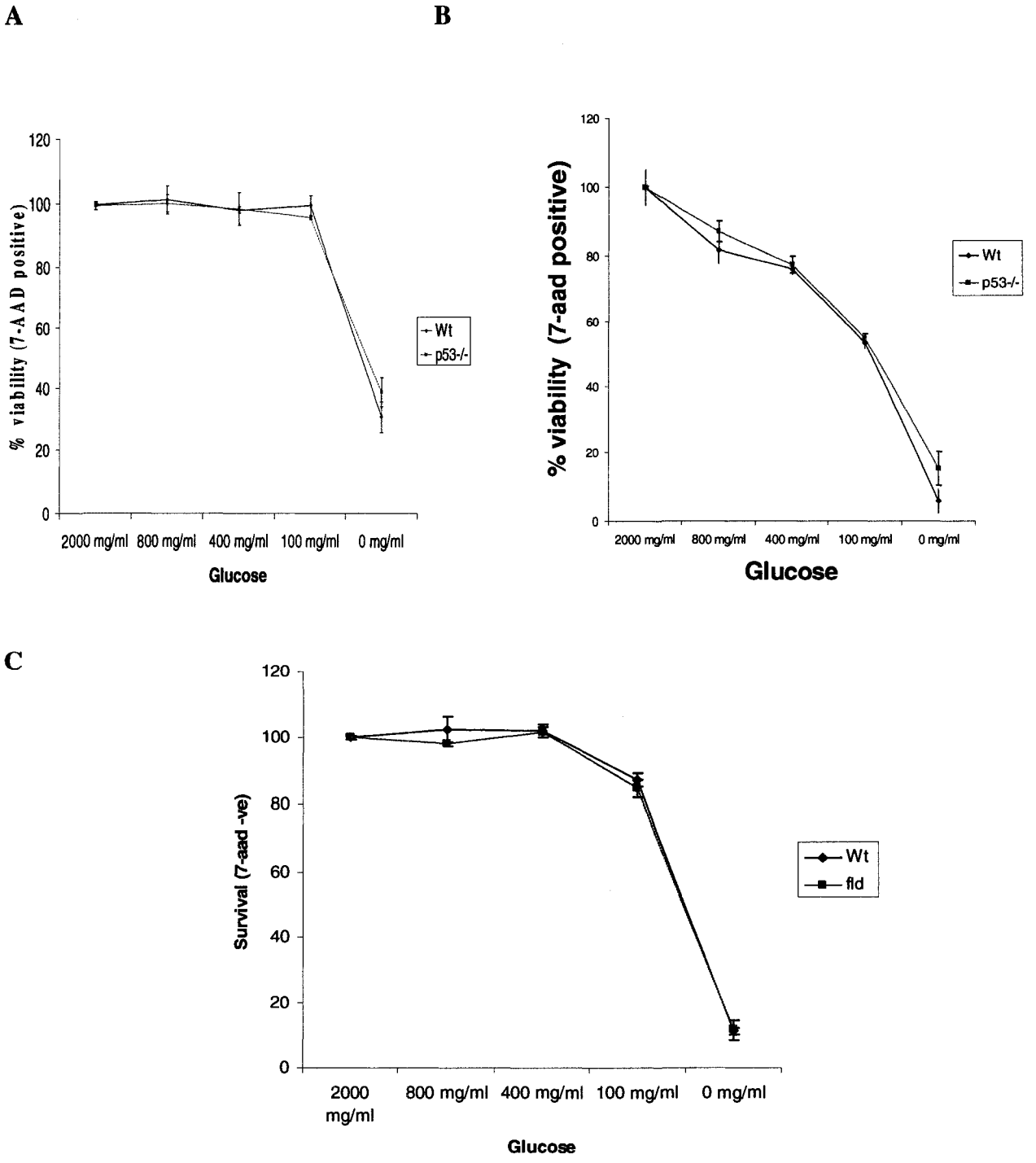

Figure 6.2: Glucose withdrawal induced death in the presence or absence of p53 and lpin1 in various models a) Viability of T-cells from p53 wild type and p53 -/- mice subjected to glucose withdrawal b) Viability of LPS stimulated splenocytes from p53 and wild type mice subjected to glucose withdrawal c) Viability of of wt and fld derived splenocytes subjected to glucose deprivation