

Functional Analysis of the Oncogene Carnitine Palmitoyltransferase 1C and Its Regulation under Hypoxia

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1 Abstract

Cancer cells are able to survive and proliferate under various challenging environmental conditions such as hypoxia. They gain the ability to re-organize their metabolic machinery to fulfill their energy needs, termed as metabolic transformation. The most well-known phenomenon is the Warburg effect, where cancer cells predominantly generate energy through glycolysis even in the presence of oxygen. In fact, altered metabolism is well accepted as an emerging hallmark of cancer cells and recent studies highlight the significance of one specific metabolic pathway, the fatty acid metabolism.

Fatty acids are carboxylic acids which usually derive from triglycerides or phospholipids. They are an essential fuel for the body, as a significant amount of energy will be generated when they get metabolized. The main mechanism to burn fatty acids is the fatty acid oxidation (FAO) which takes place mainly in the mitochondria. It recently has been discovered that FAO is required for certain types of cancer cells to survive and proliferate. Thus, this signaling pathway is of increasing importance. FAO requires a process termed the carnitine shuttle to translocate long chain fatty acids into the mitochondrial matrix. A group of enzymes which catalyzes the rate limiting step of this process is called the carnitine palmitoyltransferase 1 (CPT1). There are three CPT1s in the mammalian genome and CPT1C is the last discovered and most enigmatic member. While the function of the other two members of the family (CPT1A and CPT1B) has been well characterized, emerging evidence reveals the unique role of CPT1C.

Despite the fact that under physiological conditions CPT1C is mainly expressed in brain, we found that many cancer cell lines as well as human cancer tissues show an up-regulated expression of CPT1C. In a previous publication from our laboratory CPT1C has been identified to have some protective effects under metabolic stresses such as glucose deprivation and hypoxia. Since we identified CPT1C as a novel p53 target gene we next focused on investigating the role of CPT1C in carcinogenesis. Depletion of CPT1C in the neurofibromatosis type 1 murine tumor model leads to a significant increase in survival and to a reduced tumor rate.

To better understand the physiological function of CPT1C, we next clarified its subcellular localization. Compared to the other CPT family members CPT1C is localized to the ER and not to the mitochondria. In addition, we were able to identify the domain responsible for the translocation to the ER.

Since CPT1C is upregulated upon hypoxic stress and has been found to be a bona fide p53 target gene, we further investigated CPT1C's role in the crosstalk of HIF and p53 and we found that both, p53 and HIF1 α were required for the proper activation of CPT1C.

The results presented in this thesis show new evidence that CPT1C might play a key role in carcinogenesis by impacting the crosstalk between p53 and HIF1 and therefore modulating the metabolic transformation. Understanding the role of CPT1C as a key target in the hypoxic adaptation may provide an interesting approach for new cancer therapies.

2 Zusammenfassung

Krebszellen sind in der Lage, auch unter erschwerten Bedingungen - wie zum Beispiel unter Sauerstoffmangel – zu überleben. Sie entwickeln die Fähigkeit, ihren Stoffwechsel sowie ihre Signalwege der Umgebung anzupassen. Dieser Prozess wird metabolische Transformation genannt. Das bekannteste Phänomen ist der sogenannte Warburg-Effekt, wobei die Krebszellen ihren Energieverbrauch optimieren, indem sie auf Zuckerverbrennung umstellen und so auch in sauerstoffarmem Milieu besser überleben können. Diese metabolische Transformation hat zur Folge, dass Krebszellen, welche eine erhöhte Resistenz gegenüber Hypoxie aufweisen, sich aggressiver verhalten und auf konventionelle Therapiemethoden bedeutend schlechter ansprechen. In der aktuellen Literatur gibt es immer mehr Hinweise, dass bei der metabolischen Transformation nicht nur der Zuckerstoffwechsel, sondern auch der Fettstoffwechsel eine wichtige Rolle spielt. Fettsäuren sind Karbonsäuren, welche gewöhnlich von Triglyceriden oder Phospholipiden abgeleitet sind. Sie tragen in den Zellen wesentlich zur Energiegewinnung bei, vor allem durch die sogenannte Fettsäureoxidation (FAO), welche hauptsächlich in den Mitochondrien stattfindet. Durch den Carnitin-Shuttle werden langkettige Fettsäuren in die mitochondriale Matrix transportiert. Dieser Prozess wird durch die Carnitinpalmitoyltransferase 1 (CPT1A und CPT1B) katalysiert.

In einer Reihe von Experimenten konnten wir zeigen, dass das Gene Carnitine Palmitoyltransferase 1C (CPT1C) vom Tumor Suppressor p53 reguliert wird. Zudem wird es in den meisten Krebszellen über-exprimiert und schützt diese gegen Hypoxie-induzierten Zelltod. In einem Mäusetumormodell konnten wir zeigen, dass eine Reduktion von CPT1C-Expression zu einem signifikant erhöhten Überleben und zu einer deutlichen Verringerung der spontanen Tumorentstehung führt. Diese Resultate unterstützen unsere Hypothese, dass CPT1C eine entscheidende Rolle spielt in der Krebsentstehung, wahrscheinlich über eine metabolische Transformation der Tumorzellen.

Um den molekularen Mechanismus von CPT1C und seine potentielle Rolle bei der metabolischen Transformation besser zu verstehen, haben wir zunächst die in der Literatur kontrovers diskutierte Frage der intrazellulären Lokalisation geklärt. Wir konnten zeigen, dass CPT1C zum endoplasmatischen Retikulum transloziert und konnten die dafür verantwortlichen Gensequenzen identifiziert. Da CPT1C im ER und nicht wie initial beschrieben in den Mitochondrien lokalisiert ist, konnten wir in der hochauflösenden Respiration (OROBOROS) kein signifikanter Unterschied zwischen wt und CPT1C ko Zellen feststellen.

Zudem konnten wir zeigen, dass sowohl p53 als auch HIF1 α für die Regulation von CPT1C unter Hypoxie notwendig sind. Die Ergebnisse dieser Dissertation liefern weitere Hinweise, dass CPT1C eine wichtige Rolle spielt in der Karzinogenese, wahrscheinlich durch die subtile Regulation des Zusammenspieles von p53 und HIF sowie der hierdurch beeinträchtigten metabolischen Transformation.

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4 List of Abbreviations

ACC	Acetyl-CoA Carboxylase
Acetyl-CoA	Acetyl Co-Enzyme A
AD-HSP	Adult-onset Hereditary Spastic Paraplegias
AMP	Adenosine Monophosphate
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid
AMPAR	AMPA Receptor
AMPK	AMP activated Kinase
Arc	Arcuate Nucleus
ATM	Ataxia Telangiectasia Mutated
ATP	Adenosine Triphosphate
ATR	Ataxia Telangiectasia and Rad3 Related
CACT	Carnitine-Acylcarnitine Translocase
CDK	Cyclin Dependent Kinase
CNS	Central Nervous System
COX	Cytochrome c Oxidase
CPT	Carnitine Palmitoyltransferase
DMH	Dorsomedial Hypothalamus
ER	Endoplasmic Reticulum
FA	Fatty Acid
FADH ₂	Flavin Adenine Dinucleotide, Reduced
FAO	Fatty Acid Oxidation
FAS	Fatty Acid Synthesis
GSH	Reduced Glutathione
HIF	Hypoxia Inducible Factor
HK	Hexokinase
IR	Ionizing Radiation
LCFA	Long Chain Fatty Acid
MEF	Mouse Embryonic Fibroblast
mTOR	Mammalian Target of Rapamycin

NADH	Nicotinamide Adenine Dinucleotide, Reduced
NMD	N-Methyl-D-Aspartate
OMM	Outer Mitochondrial Membrane
ORF	Open Reading Frame
OXPPOS	Oxidative Phosphorylation
P53-RE	P53-Responsive Elements
PPP	Pentose Phosphate Pathway
PTEN	Phosphatase and Tensin Homolog
PUMA	P53 Upregulated Modulator of Apoptosis
PVN	Paraventricular Nucleus
RER	Rough Endoplasmic Reticulum
ROS	Reactive Oxygen Species
SER	Smooth Endoplasmic Reticulum
TCA	Tricarboxylic Acid
TIGAR	Tp53-Inducible Glycolysis and Apoptosis Regulator
VMC	Ventromedial Hypothalamus

5 General Introduction

5.1 Carnitine palmitoyltransferase 1C

5.1.1 The carnitine palmitoyltransferases

Fatty acid oxidation (FAO) is the major process to utilize fatty acids for energy homeostasis. During this process, fatty acids are broken down to generate acetyl co-enzyme A (acetyl-CoA) which could enter the tricarboxylic acid (TCA) cycle as fuels, and NADH (nicotinamide adenine dinucleotide, reduced) and FADH₂ (flavin adenine dinucleotide, reduced), which could serve as co-enzymes in the electron transport chain. The majority of fatty acid oxidation takes place in the mitochondrial matrix, while very long chain fatty acids can undergo oxidation in peroxisomes.

The major fraction of fatty acids in target tissues is the long-chain fatty acids (LCFA). In order to get access to the oxidation machinery, LCFA need to enter the mitochondrial matrix. While short or medium-chain fatty acids can enter simply by diffusion, LCFA need a dedicated system termed the carnitine palmitoyltransferases (CPT)^[1]. Before oxidation, fatty acids need to be activated by fatty acyl CoA synthase, forming corresponding acyl-CoA esters. The transport of LCFA into mitochondria could be divided into three steps: firstly, the acyl group of LCFA is transferred to the hydroxyl group of carnitine which results in the formation of acyl-carnitine. This step is catalyzed by carnitine palmitoyltransferase 1 (CPT1) on the outer mitochondrial membrane (OMM) and considered the rate limiting step of FAO. Secondly, acyl-carnitine is translocated through inner mitochondrial membrane by carnitine-acylcarnitine translocase (CACT). Finally, acyl-carnitine is converted back to acyl-CoA and carnitine, which is catalyzed by the inner mitochondrial membrane bound carnitine palmitoyltransferase 2 (CPT2). The whole process is known as “carnitine shuttle” (**Figure 5.1**).

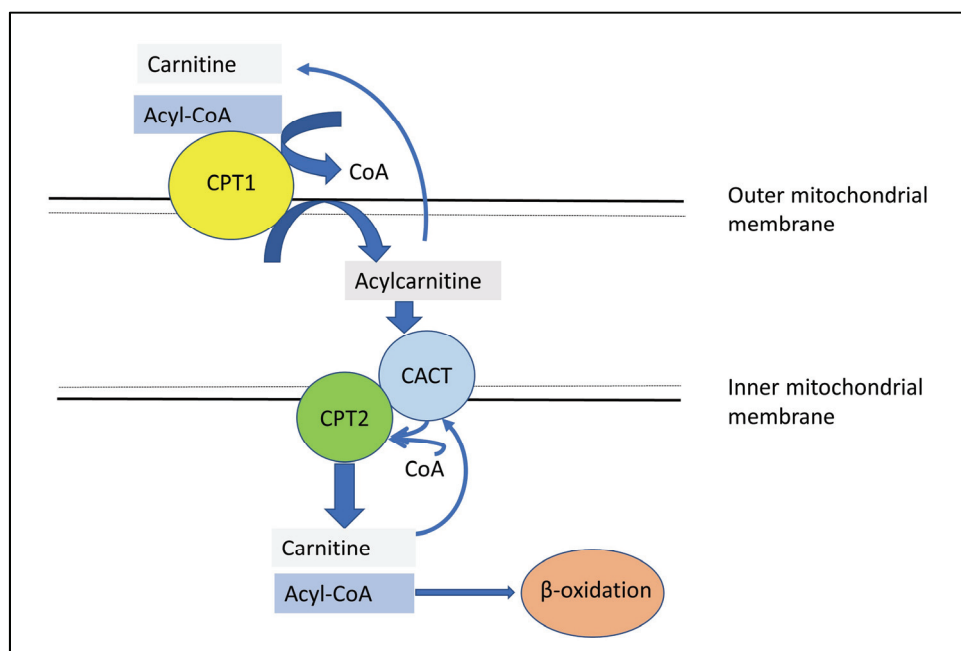


Figure 5.1 The carnitine palmitoyltransferases and carnitine shuttle. Acyl-CoA esters derived from long chain fatty acids enter the mitochondrial matrix with the help of CPTs. CPT, carnitine palmitoyltransferase; CoA, coenzyme A; CACT, carnitine-acylcarnitine translocase. Adapted from Lehninger et al^[2].

In this system, two groups of CPTs are involved: CPT1 and CPT2. While one sole CPT2 exists across all different tissues in the body, three different CPT1s (CPT1A, CPT1B and CPT1C) are expressed in a tissue specific manner. CPT1A is ubiquitously expressed in many tissues (liver, kidney, lung, spleen, intestine, pancreas, ovary, and brain) but predominantly exerts its function in the liver. CPT1B is expressed in tissues undergoing massive fatty acid oxidation like muscle and brown adipose tissue^[3]. CPT1C, the last identified member of CPT1 family, is expressed in the brain and testis. Since CPT1C has distinct function and localization when compared to the other two CPT1s, “mitochondrial CPT1s” will be used hereby referring to CPT1A and CPT1B.

The translocation of LCFA into mitochondria is delicately regulated through both the expression and activity of mitochondrial CPT1s. CPT1 gene expressions are regulated by dietary and hormonal signals as well as diverse physiologic or pathologic stimuli such as fasting, fat feeding, induction of diabetes or treatment with peroxisomal/mitochondrial proliferating agents^[1]. On the other hand, the activity controls of mitochondrial CPT1s are mainly regulated through the binding of malonyl-CoA, a conformational inhibitor of mitochondrial CPT1s^[4]. Malonyl-CoA is a metabolic intermediate in the process of fatty acid synthesis. It is formed from acetyl-CoA carboxylation with the help of acetyl-CoA carboxylase (ACC) and used as 2-carbon unit donors for fatty acid elongation catalyzed by fatty acid synthase (FAS). As fatty acid synthesis and oxidation are both concurrent and opposing reactions coexist in many tissues, a substrate in one reaction inhibits the other reaction, without introducing additional regulatory substrates, tunes fatty acid metabolism in a simple but efficient manner. Essentially, CPT1A and CPT1B has different sensitivity towards the inhibitory effect of malonyl-CoA binding. CPT1B proved to be far more sensitive to malonyl-CoA and to have a much higher K_m for carnitine than CPT1A^[5, 6]. In central nerve system, malonyl-CoA plays a key role in energy sensing. It is proved that increasing hypothalamus malonyl-CoA could reduce food intake and cause weight loss^[7]. Since all isoforms of CPT1 exist in brain but brain doesn't rely on fatty acids as fuel for energy burning, regulating free malonyl-CoA thus affecting energy sensing could be a possible hint.

5.1.2 Discovery of CPT1C

Carnitine palmitoyltransferase 1C was identified in 2002 based on the homology with the other two known isoforms of the CPT1 family^[8]. The primary amino acid sequence of CPT1C has a high degree of identity to CPT1A and CPT1B (52.4% and 50.6% identity, respectively)^[9]. Other than human and mouse, CPT1C gene was also discovered in other mammalian species including rat, cow, pig, and rabbit^[8]. In non-mammalian vertebrates, CPT1C is generally not present while one report suggests chicken CPT1B gene is pro-orthologous of the mammalian CPT1C^[10].

The primary sequence of CPT1C contains all important motifs for carnitine acyltransferase activity. However, in vitro expressed proteins showed no detectable catalytic activity with several different acyl-CoA esters that are substrates of other CPT1s in the original paper

identifying CPT1C^[8]. Additional research confirmed that isolated mitochondria from CPT1C overexpression mammalian cells failed to catalyze acyl transfer reaction with acyl-CoA thioesters of various chain lengths and saturations^[9]. While one more recent research using isolated microsomal fractions of transfected PC-12 and HEK293T cells has detected very low acyl transferase activity of CPT1C. For example, efficiencies for palmitoyl-CoA and carnitine were 320 and 25 times lower than that of CPT1A, respectively^[11]. The primary sequence responsible for malonyl-CoA binding is also conserved in CPT1C and experiments confirmed CPT1C can bind to malonyl-CoA^[8] with a similar dissociation constant ($K_d \approx 0.3 \mu\text{M}$) to that of CPT1A^[8, 9]. It is worth noting that this K_d is within the dynamic range of hypothalamic malonyl-CoA in fasted and refed states which enables this binding to be happening in physiological state^[12].

The structure of CPT1C protein shares the common features of CPT1s^[13], consisting an N-terminal regulatory domain and a C-terminal catalytic domain with two transmembrane helices in between (**Figure 5.2**). A close comparison of the N-terminal regulatory domain between CPT1C and CPT1A by NMR spectroscopy has been performed^[14]. Based on membrane topology, the N domain and the C domain of CPT1A both face towards the cytosol and CPT1C is predicted to adopt a similar conformation. The N-terminal domain of CPT1A has two distinct structural states representing different activity status: the inhibitory state $N\alpha$ and non-inhibitory state $N\beta$, respectively^[15]. $N\alpha$ is the default state, while the transformation of these two states are dependent on factors representing metabolic state, e.g. malonyl-CoA concentration, OMM enzyme location (membrane curvature) and OMM fluidity and composition^[15]. The N domain of CPT1C (isotope-labeled peptides encoding Met1-Phe50) were prepared and tested with different conditions promoting $N\alpha$ or $N\beta$ states as verified in CPT1A. Results showed that the inhibitory $N\alpha$ state is structurally similar between CPT1C and CPT1A, but the non-inhibitory $N\beta$ state of CPT1C is destabilized relative to CPT1A^[14]. The $\beta 1$ - $\beta 2$ arrangement (residues 9–24) which is important for the amphiphilic, bent $N\beta$ structure is absent in CPT1C and two amino acids substitutions in CPT1C compared to CPT1A (Ala9Gly and Pro16Ser) are considered responsible. In CPT1A, these two mutations together would reduce the IC_{50} with a factor up to 51^[15] and this will put the catalytic activity of CPT1A close to the activity observed in CPT1C.

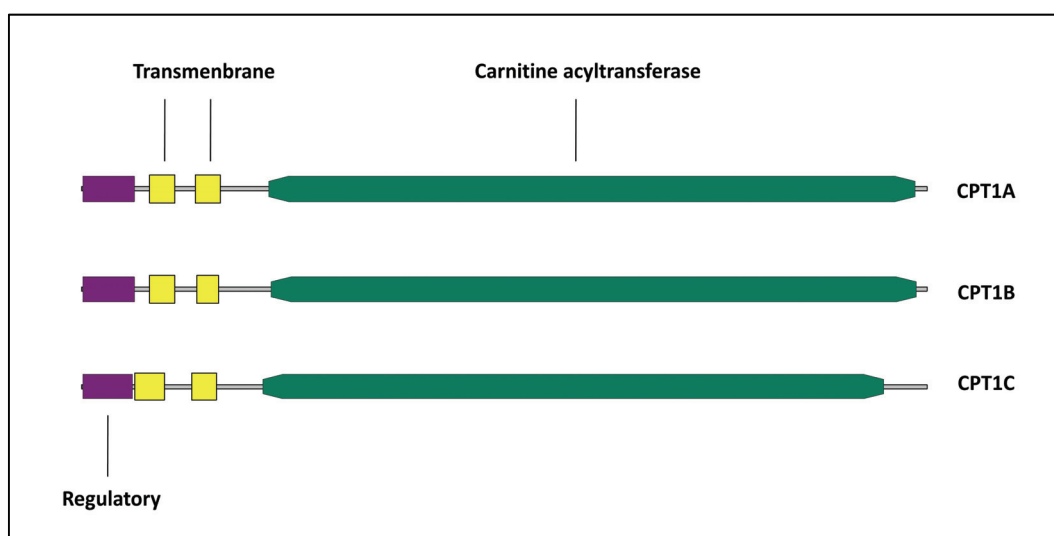


Figure 5.2 The protein structures of three CPT1 family members in human. CPT1A, CPT1B and CPT1C share some common features in structure, an N-terminal regulatory domain and a C-terminal catalytic domain with two transmembrane helices in between.

More recently, the first CPT1C associated disease has been reported^[16]. A family of adult-onset hereditary spastic paraplegias (AD-HSP) has been identified bearing a c.109C>T mutation in exon 3 of CPT1C, which results in a revolutionarily conserved arginine substituted to a cysteine (p. Arg37Cys). The mutation is clearly related to the disease as 5 out of 5 family members with HSP have this mutation, but is overall of low incidence as the same mutation was not found in 163 other unrelated pure HSP patients of unknown genetic cause^[16]. The NMR spectra showed this mutation had caused a shift of many backbone hydrogen-nitrogen resonances, and induced a destabilization of the helical structure near Cys 37 in the non-inhibitory N β state. When the same mutant CPT1C was overexpressed in COS-7 cells, the average number and size of lipid droplets (LDs) per cell were dramatically reduced^[16]. A similar phenotype was found in CPT1C $-/-$ cortical neurons^[17, 18], indicating the involvement of CPT1C in LD biogenesis. Interestingly, the second most common cause of AD-HSP (accounting for approximately 10% of all cases)^[19, 20], atlastin-1 (SPG3A), also has a similar reduction in LD size when mutated or expressed in a dominant negative form^[21]. Most importantly, CPT1C can physically interact and co-localize with atlastin-1 in vitro, although the Arg37Cys mutation did not affect their binding. Atlastin-1 drives membrane fusion in a GTP-dependent manner and is required for the ER network formation^[22], but a recent study showed that membrane fusion activity of atlastin-1 may not be required to cause HSP^[23]. How the modification of this specific amino acid associates with the onset of HSP and lipid metabolism requires further elucidation.

Unlike the embryonic lethality of CPT1A and CPT1B knockout mouse^[24, 25], at least four independently generated CPT1C knockout/null mouse models confirmed that mice depleted of CPT1C are viable and have a normal life span^[9, 17, 26, 27]. The emerging evidence of the distinctive CPT1C function of CPT1C makes it the most unique member in the CPT1 family.

5.1.3 Tissue specific expression and subcellular localization of CPT1C

Upon discovery, the expression pattern of CPT1C became of great interest. Like the mitochondrial CPT1s, CPT1C is also expressed in a tissue specific manner. CPT1C has been detected to be highly expressed in mammalian brain and testis, in addition, human CPT1C is also highly represented in tumor-derived ESTs (expressed sequence tags)^[8]. Within the central nerve system, high levels of CPT1C mRNA was found in discrete areas including the hippocampus, the paraventricular, arcuate, and superchiasmatic nuclei that are responsible for feeding and energy homeostasis^[8, 28]. With regard to protein expression, CPT1C was detected throughout the CNS with particular enrichment in the hypothalamus, amygdala and hippocampus^[29]. In hypothalamus, CPT1C is especially highly expressed in regions critical for the regulation of energy balance, notably the paraventricular nucleus (PVN), ventromedial hypothalamus (VMH), and the arcuate nucleus (Arc). Interestingly, CPT1C protein was found predominantly in neuronal cell bodies but not the endothelial or glial cells. Also in hippocampus, CPT1C was found both in pyramidal cell bodies and dendrites^[29]. More recently, Carrasco et al showed in isolated neurons, the expression of CPT1C protein was found not only across neuronal bodies and dendrites but also in the spines^[27] indicating the possibility

of CPT1C existing in the peripheral nerve system. Since it is a transfection driven overexpression experiment, the results should be interpreted with caution.

As mentioned before, mitochondrial CPT1s are localized on the outer mitochondrial membrane and this subcellular localization is tightly related to their function. Similar study on subcellular localization of CPT1C provides us novel information on its function. In the first CPT1C paper in 2002 Price et al described that CPT1C protein was present in both the mitochondrial and microsomal fractions isolated from mouse or rat brains in 2002^[8]. Five years later, Dai et al verified the co-localization of CPT1C and mitochondria-specific marker in transfected GT1-7 hypothalamic cells utilizing immunofluorescent microscopy. And they detected by western blot that CPT1C was present within the mitochondrial fraction, thus concluded CPT1C as an integral membrane protein of the outer mitochondrial membrane, like the other CPT1s^[29]. Surprisingly, one year later, Sierra et al performed similar transfection driven co-localization experiments in several cell lines (SH-SY5Y cells, PC-12 cells, and HEK293T cells) and found CPT1C co-localized with the endoplasmic reticulum (ER) marker, but not with mitochondrial or peroxisomal markers. Regarding to endogenous proteins, they fractionated mouse cellular extracts from different tissues and found CPT1C was only present in brain, predominantly in the microsomal fraction. Furthermore, with the expression of chimeric proteins, i.e. swapping the N terminal sequences of CPT1C and CPT1A, they proved CPT1C doesn't have the mitochondrial localization signal that exists in CPT1A^[30], instead it contains a potential microsomal targeting signal in its N terminal sequence (1 – 153 aa) that is responsible for ER localization^[11]. Ever after, more evidence supporting the ER localization emerged^[27, 31] and widely accepted. However, the detailed ER localizing sequence of CPT1C has not yet been identified and how this unique localization affects the function of CPT1C is still not well understood.

5.1.4 CPT1C and energy homeostasis

To better understand the unique physiological role of CPT1C, genetically modified knockout (KO) mice were generated. Analysis of KO mice phenotypes gives us more insights into the molecular function of CPT1C.

Since CPT1C has all primary sequences for an acyl-transferase, efforts never stopped trying to identify the enzymatic activity and substrates of CPT1C. Although it has been proven that CPT1C has minimal acyl-transferase activity with known CPT1 substrates *in vitro*^[11], considering mitochondrial CPT1s' co-existing in the same brain regions, it is probable that even the brain needs to burn fatty acid for energy, CPT1C's contribution to fatty acid oxidation will be masked by the activity of other CPT1s. A metabolomic profiling on brains of CPT1C WT and KO mice was performed by Lee et al^[32] and they found lack of CPT1C does not cause major deficiencies in fatty acid oxidation. Although certain metabolites, such as free carnitine, 3-dehydrocarnitine, glutaroyl-carnitine, and betaine did show significant changes, the direct involvement of CPT1C in fatty acid oxidation could not be proven. Interestingly, in this study endogenous endocannabinoids were found to be decreased in KO mice as well. Since the endocannabinoids were related to the regulation of food intake and energy expenditure (reviewed by DiPatrizio and Piomelli^[33]), it implies that CPT1C could also be involved in those processes.

The phenotype of CPT1C KO mice is complex. They are viable and have a normal life span. It is reported from two independently generated KO mouse lines^[9, 17] that CPT1C KO mice have significantly less body weight and food intake than WT littermates but show an equivalent body length, indicating a normal growth^[9]. But when fed with a high fat diet (HFD, 45% calories from fat), KO mice gained much more weight than WT mice, interestingly, they still ate less than WT mice. At the end of the HFD feeding, KO mice showed a significant increase of plasma phospholipids content and mild insulin resistance^[9, 17]. On the other hand, when CPT1C was overexpressed in the murine ventral hypothalamus by stereotactic injection of adenoviral expression vector^[34], mice were protected from weight gain consuming high fat diet^[29]. Similar results were obtained from a CPT1C overexpressing mouse model as well^[35]. All evidence confirms CPT1C is related to this unusual weight gain.

How could CPT1C KO mice gain more weight while they actually eat less? To answer this, an in vivo whole-body fatty acid oxidation study was performed in CPT1C WT and KO mice. Fasted mice were injected with ¹⁴C-labeled fatty acids^[36] then the ¹⁴CO₂ they expired was trapped in NaOH and quantified. Results from this experiment showed that in fasted status, KO mice had a much lower fatty acid oxidation, suggesting the overall low energy expenditure in these animals^[9]. In the liver of HFD fed KO mice, total CPT1 activity and fatty acid oxidation were decreased, and triacylglycerol content was increased. Also, lack of CPT1C increased the gluconeogenesis process and decreased muscle glucose uptake indicating an impaired glucose tolerance^[17]. Depletion of CPT1C increased mRNA expression of other CPT1s in the hypothalamus and resulted in a considerably increased CPT1 activity in this region. This effect was not found in other CPT1C expressing areas like hippocampus and cortex and the significance of this effect remains unknown.

5.1.5 CPT1C in ceramide metabolism

A study in 2011 first provided a correlation between CPT1C and ceramide metabolism^[37]. Gao et al overexpressed CPT1C into the arcuate nucleus of rats using stereotaxic surgery and found that long-chain acylcarnitines were not changed, which is consistent with other reports. However, they found following fasting, the rats with CPT1C overexpression ate more food than null rats with a concurrent increase of the Arc orexigenic neuropeptide Y (NPY) and its transcription factor, brain-specific homeobox factor (Bsx)^[38]. Both NPY and Bsx are subjected to the regulation by leptin, an important physiological regulator of food intake and body weight. Leptin reduces feeding and body weight partly through down-regulating NPY and Bsx levels^[39]. Further experiments showed overexpression of CPT1C antagonized the anorectic effects of leptin and cerulenin (a FAS inhibitor), because leptin ultimately induces malonyl-CoA in the hypothalamic arcuate nucleus. Finally, CPT1C expression was connected with changes in ceramide metabolism. Under fasting condition, overexpressing CPT1C in the Arc increased ceramide level, and depleting CPT1C in the Arc decreased ceramide level. The ceramide level was also seen responsive to Arc leptin and malonyl-CoA levels as they work in the upstream of CPT1C in this pathway. With the introduction of myriocin, a ceramide biosynthesis inhibitor which inhibits serine palmitoyltransferase (SPT), the key rate-limiting enzyme of ceramide de novo biosynthesis^[40, 41], they proved the increased feeding after fasting with Arc overexpression of MCD (lowering malonyl-CoA) or CPT1C can be stopped by the blockage of the de novo ceramide metabolism.

Further research on CPT1C and ceramide was performed by Carrasco et al^[27]. They found in cultured primary neurons, overexpression of CPT1C resulted in elevated saturated ceramides (C16:0, C18:0, and C20:0). Although ceramide in the ER comes mainly from de novo synthesis, this process was not promoted in CPT1C-expressing cells, quite to the opposite, they found that the de novo synthesis of ceramide was increased in CPT1C KO cells when compared to WT cells, indicating the presence of compensation mechanism. Interestingly, one CPT1C KO phenotype they reported, reduction of mature spines of cultured hippocampal neurons, can be rescued by addition of soluble C6:0 ceramide (which can be converted to long chain ceramides inside the cell^[42]), suggesting this CPT1C KO phenotype was caused by ceramide deficiency.

In addition, it has been described that hypothalamic CPT1C and ceramides mediated the effect of ghrelin^[43]. Ghrelin is the hormone produced in the stomach which exerts its orexigenic effect through the growth hormone secretagogue receptor 1a (GHSR, or ghrelin receptor) in the hypothalamus^[44, 45]. Ghrelin was shown to inhibit fatty acid synthesis by AMP-activated protein kinase (AMPK) and resulted in a decreased hypothalamic malonyl-CoA and increased CPT1 activity^[46, 47]. Ramirez et al found that the administration of ghrelin induced a short-term increase of ceramides in the hypothalamus, but this effect was absent in CPT1C KO mice. As a result, ghrelin failed to induce food intake in these KO mice despite the evidence that many components of the canonical ghrelin signaling pathway (pAMPK, pACC, UCP2, FoxO1 and pCREB) were activated. When hypothalamic ceramide synthesis was blocked with myriocin, mice showed the same insensitivity to ghrelin. It was proposed that ghrelin must activate two parallel pathways, the mitochondrial pathway (by activation of CPT1A and fatty acid oxidation) and the ER pathway (by activation of CPT1C and ceramide synthesis), for its orexigenic effect to be effective^[43].

To sum up, CPT1C expression is required for neuronal ceramide metabolism in an unknown process which is not the de novo synthesis. When CPT1C is depleted, the effects of leptin and ghrelin are blunted due to the loss of corresponding ceramide level changes.

5.1.6 CPT1C and neuron function

Since CPT1C has the unique expression pattern that concentrates in neurons in the CNS, it is plausible that the function of CPT1C lies in the context of brain. The phenotypes of a CPT1C overexpression mice model were reported in 2011^[35]. CPT1C expression was controlled by neural specific Nestin-Cre and 3-fold more proteins within the CNS was achieved. These CPT1C overexpressing mice exhibited significantly smaller body size and lower body weights. Furthermore, they had a normal brain size during the first few weeks of life, but resulted in a marked reduction in brain size upon adulthood. This is consistent with the report showing expression of CPT1C were low from birth to postnatal day 10, then increased gradually and peaked on postnatal day 21^[18]. Another report regarding the hippocampal expression of CPT1C protein found that CPT1C had the same expression pattern as many proteins that are important in neuron development (synapsin I, PSD95, GluA1, GluA2 and GluN2A), which increased after birth and reached their maximum values after weaning^[48]. Taken together, CPT1C is required for proper brain development, especially in the period between birth and adulthood. It is not surprising when CPT1C KO mice were reported to show significantly delayed learning ability in the Morris water maze (MWM) test, a test that is usually used to

measure hippocampus-dependent spatial learning and memory in mice^[49]. During the test, escape latency was found significantly higher in CPT1C KO mice while swimming speed, visuospatial memory and cognitive flexibility were not altered, indicating a pure spatial learning impairment^[27].

With respect to the study of molecular mechanism, a high-resolution proteomic screening performed in 2012 draw our attention to the AMPA-type glutamate receptors^[50]. Glutamate is the main excitatory neurotransmitter in the CNS, and the ionotropic receptors of glutamate fall into three families named after their preferred agonists: NMDA (N-methyl-D-aspartate), AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), and Kainate receptors. The AMPA receptors (AMPA) are account for 90% of the fast-excitatory neurotransmission in the CNS and are shown to promote formation and maturation of synapses during the early phase of synaptogenesis^[51, 52]. The investigators separated affinity purified rat brain membrane fractions with the Blue-Native gel following by nanoflow liquid chromatography tandem mass spectrometry (nano-LC-MS/MS) and identified CPT1C as a native interacting partner of AMPAR complex, to be specific, the subunits GluA1 and GluA2^[50]. Further experiments performed within an in vitro overexpressing system in tsA201cells confirmed co-immunoprecipitation of CPT1C with both GluA1 and GluA2^[31]. However, co-expressing CPT1C and GluA1 could increase glutamate-evoked whole cell current density but this is not seen in CPT1C with GluA2 co-expression. It turned out to be the effect of increased surface expression of GluA1 mediated by CPT1C, probably by enhancing trafficking of GluA1-containing AMPAR. Since CPT1C has the potential to catalyze palmitoylation process and this type of posttranslational modification has shown to affect AMPAR trafficking^[53-55], the authors also tried to explore if CPT1C participates in the palmitoylation of AMPAR subunits. Intriguingly, overexpressing CPT1C didn't change the palmitoylation state of GluA1. But one mutant form of GluA1 that cannot be palmitoylated, C585S, resembled the phenotype of CPT1C overexpression (increased whole cell current and surface expression of GluA1) and CPT1C cannot further increase surface expression in these mutant cells, indicating cysteine 585 is important for this phenotype but the mechanism remains unknown.

In cultured hippocampal neurons, whole-cell voltage clamp experiments revealed reduced AMPAR-mediated miniature excitatory postsynaptic current (mEPSC) amplitude due to loss of CPT1C, indicating a reduction in synaptic transmission^[48]. This phenomenon was found to be related to the decreased quantity of AMPARs in synaptic puncta and CPT1C was identified to positively regulate the protein expression of GluA1 and GluA2. This regulation most likely happened at post transcription level as the mRNA of GluA1 or GluA2 were not changed and the protein degradation of AMPARs were also unchanged. Further analysis demonstrated CPT1C affected the protein synthesis of GluA1 under both basal and stimulated conditions^[48]. And this is in accordance with previous findings that CPT1C co-localizes with AMPARs only at the ER level but not at the Golgi apparatus or at the plasma membrane^[31].

5.1.7 CPT1C promotes tumor progression

The research findings from our laboratory have for the first time linked CPT1C to tumor progression. It started with the finding that CPT1C was overexpressed in many tumor cell lines and human malignancies^[26, 56].

As previously mentioned in the first CPT1C paper, human CPT1C was found abundant in tumor-derived ESTs^[8]. Since then more evidence of deregulated CPT1C expression in tumors was emerging. Furuta et al reported an aberrant methylation of CpG islands in CPT1C promoter region existing in 12/13 of melanoma cell lines but not in two cultured normal melanocytes^[57]. Generally, CpG islands methylation will result in transcriptional silencing^[58, 59], but in this experiment, the methylation didn't depress the expression of CPT1C. One possible explanation could be a misannotation as the authors selected the promoter region based on transcription start site. In a similar research performed by Maeda et al in 5-fluorouracil (5-FU) and cisplatin (CDDP) resistant gastric cancer cells, CPT1C was demethylated in 5-FU-resistant cells and increased in expression, indicating the relationship between CPT1C and drug resistant^[60]. There are some reports showing CPT1C negatively correlates with cancer malignancies. For instance, Cirillo et al found that in a cohort of 80 patients, high grade glioblastoma is associated with downregulated CPT1C mRNA^[61]. Kim et al showed in a study including 135 primary bladder cancer (BCA) samples, CPT1C was significantly downregulated in tumor tissues compared to normal bladder tissues of patients with non-muscle invasive as well as muscle invasive BCA^[62]. However, none of above mentioned studies provided mechanical analysis on CPT1C's role in tumor.

In 2011, Zaugg et al found CPT1C mRNA was highly expressed in 108 out of 168 mouse primary tumors that had a low mTOR (mammalian target of rapamycin) index (average of the mean centered expression values of the mTOR pathway genes *Pik3ca*, *Frap1*, *Pik3r3*, *Pik3r1*, and *ErbB3*) and rapamycin sensitivity. Furthermore, in paired normal and tumor tissues from 19 non-small-cell lung carcinoma (NSCLC) patients, 13 sample pairs showed an increased CPT1C mRNA expression in tumor tissues. In breast cancer cell line MCF7, overexpressing CPT1C can significantly increase fatty acid oxidation and ATP production, more importantly, also increases cell survival and growth under nutrient deficient conditions like hypoxia and limited glucose. On the other hand, when CPT1C was knocked down by siRNA (small interfering RNA), the protective effect under hypoxia was gone and the growth of xenograft tumors was severely delayed. More convincing data was retrieved from a neurofibromatosis type 1 (*Nf1*) +/-: *p53* +/- tumor model by Sanchez-Macedo et al^[63]. These mice bear mutations in both tumor suppressor genes *Nf1* and *p53*^[64] thus have a high tendency to spontaneously develop various malignancies^[65], for example, soft tissue sarcomas of the limbs and abdomen as well as lymphomas. The tumors emerged at around 3–6 months of age with a penetrance of over 70%, resulting in an extremely short life span of these animals. CPT1C protein was found to be overexpressed in sarcomas compared to normal tissue. When these mice were crossed to a CPT1C *gt/gt* (a mouse strain with a gene trap introduced insertional null mutant of CPT1C) background, tumor progression was dramatically delayed and survival rate of the animals increased significantly. The incidence of sarcomas, metastases as well as splenic hyperplasia was decreased in *Nf1* +/-: *p53* +/-: *Cpt1c* *gt/gt* mice, showing a direct link between CPT1C and tumor progression.

5.1.8 Regulation of CPT1C

There are less reports on the regulation of CPT1C. Our group identified CPT1C as a direct downstream target gene of tumor suppressor *p53* in mouse genome. CPT1C was highlighted as responsive target in a cDNA microarray screening when *p53* was activated in transformed

mouse erythroleukemia cells (DP16.1/p53ts) and later two p53 response elements (p53 RE) were found within the promoter region of mouse CPT1C by bioinformatic approaches as well as a ChIP-sequencing assay^[63, 66]. Sanchez-Macedo et al validated with luciferase reporter assay and ChIP analysis (chromatin immunoprecipitation) that one of these two p53 REs was functional. For in vivo evidence, p53 +/- and p53 -/- mice were subjected in utero to 5 Gy irradiation and mRNA changes were detected by in situ hybridization 8 hours later. CPT1C upregulation was found in p53 +/- embryos but not in p53 -/- embryos. Moreover, hypoxia could induce expression of CPT1C in p53 +/- mouse embryo fibroblasts but not in p53 -/- MEFs, and under this condition, p53 was recruited to the promoter region of CPT1C, indicating a direct transcription factor binding and activation process^[63].

Intriguingly, in human genome, no p53 REs have yet been discovered for CPT1C, but evidence showed p53 may also regulate CPT1C in human. CPT1C was seen to be upregulated upon multiple treatments (irradiation, UV, etoposide, 5-FU) that activate p53 in human acute myeloid leukemia cell line AML4 and human colorectal cancer cell line HCT116 in a p53 dependent manner. In addition, CPT1C is the only CPT family member that responds to p53 activation^[63]. Since p53 is the key tumor suppressor gene and more than 50% tumors bear mutations in p53^[67], a p53 status checking in CPT1C overexpressing human lung tumors was performed. More interestingly, only 2 out of 13 tumor samples possess wildtype p53, the rest of them have either amino acid substitution, frameshift mutation, nonsense mutation or no detectable p53^[26]. This indicates that either mutant p53 or other unknown mechanisms could also regulate CPT1C in tumor tissues.

One possible regulator of CPT1C is AMPK. As tumor cells grow rapidly into surroundings, they are facing the challenge of insufficient nutrients. In many cases, they could alter their own metabolism to adapt to the environmental changes^[68, 69]. This process, also known as metabolic transformation, is mediated by AMPK and mTOR^[70-72]. As CPT1C contributes to FAO and ATP production in tumor cells and inversely correlates with mTOR activation, it seems apparent to link CPT1C to metabolic transformation. While no evidence showing that the mTOR pathway can regulate CPT1C, AMPK on the other hand seems more involved. It has been described that CPT1C RNAi xenograft tumors didn't show growth inhibition to the AMPK agonist metformin. Metformin can activate the AMPK pathway and inhibits tumor growth by reducing free glucose within the tumor microenvironment. Further experiments confirmed that metformin increases CPT1C mRNA level in both human MCF7 cells and wildtype MEFs, but not in AMPK-deficient MEFs. Moreover, ectopic expression of CPT1C prevented AMPK-deficient cells from metabolic stressor 2-deoxyglucose (2-DG) induced apoptosis^[63]. All evidence leads to the fact that CPT1C is a downstream target in the AMPK signaling pathway that promotes cell survival in response to metabolic stress.

Observation from conditional double knockout of p53 and AMPK MEF cells provides insights between p53 and AMPK in the regulation of CPT1C. During metabolic stress, AMPK regulates a variety of downstream targets including p53. Many publications focused on illustrating the correlation between AMPK and p53^[73-76]. Most prominently, AMPK activation causes a p53 dependent cell cycle arrest, with the presence of p53 phosphorylation at serine 15, although it is not clear whether serine 15 is a direct AMPK target. Furthermore, in hepatocellular carcinoma (HCC) cells, AMPK enhances the acetylation and stability of p53 through

phosphorylating and inactivating a p53 deacetylase, SIRT1^[73]. In addition, p53 upregulates the regulatory subunit AMPK β 1 under multiple stresses^[77]. Or in an indirect manner, target genes of p53, sestrin 1 and sestrin 2, can activate AMPK thus concurrently inhibit the mTOR pathway^[78]. Regarding CPT1C, AMPK is activated by metformin in MEF cells, where p53 is phosphorylated at serine 18 (equivalent to serine 15 in human). CPT1C is also upregulated under this condition. With either AMPK or p53 deficiency, metformin fails to induce CPT1C. Notably metformin cannot induce p53 phosphorylation in AMPK deficient cells^[63], indicating that AMPK works as an upstream regulator of p53 and both are required for proper regulation of CPT1C.

The regulation of CPT1C also has been described to happen on the translational level. Human CPT1C mRNA has an upstream open reading frame (uORF) within its 5' untranslated region (5'-UTR) with potential regulatory function^[79]. Since the existence of uORF usually leads to inhibited translation of the main ORF because ribosomes scan the mRNA and bind the first initiation codon for translation^[80], Lohse et al examined the effect of this uORF of CPT1C. Wildtype and mutated (from ATG to ACG) uORF were cloned into luciferase vectors and the translation of luciferase was shown to be inhibited with the wildtype uORF. In many cases, uORF could regulate the translation of main ORF as a reaction to cellular environment^[81-83]. Interestingly, glucose deprivation could disrupt the depressing effect of this uORF, which is in accordance with previous finding that glucose deprivation could induce endogenous CPT1C^[26]. Further study revealed the suppression of translation is relieved with either palmitate-BSA treatment or AMPK subunit α 1 knockdown, and these two mechanisms seem to work independently. Although CPT1C has minimal acyl-transferase activity, palmitoyl-CoA was found to be a substrate of CPT1C, so it looks plausible that palmitate could induce CPT1C's expression. In addition, these results provide a new perspective how CPT1C could be possibly regulated by AMPK.

5.2 P53

P53 is one of the most important genes that has been defined as tumor suppressor (gene symbol, human: tumor protein p53, TP53; mouse: transformation related protein 53, Trp53). It is described that most human cancers bear p53 malfunctions caused by all sorts of mutations in either the p53 gene itself or other members in the same pathway^[67]. At the beginning of its finding in 1979, p53 was regarded as an oncogene, this notion was challenged by further analysis within the following ten years, then p53 was identified as a tumor suppressor gene^[84]. Although this gene has been studied for over 30 years, the emerging discoveries on p53 still amaze researchers how versatile this gene can be. P53 has been shown to modulate multiple processes like cell cycle arrest, stress induced apoptosis, cell senescence, autophagy as well as cellular metabolism (**Figure 5.3**). More intriguingly, under certain circumstances, p53 has been proven to support cell survival, indicating the many facets of p53 function.

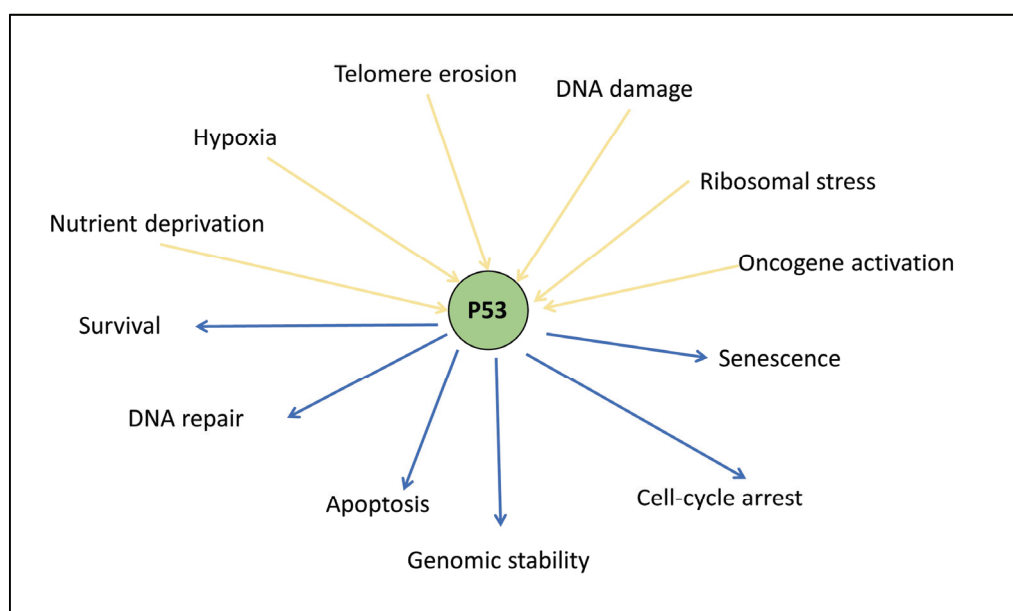


Figure 5.3 The many functions of p53. P53 could be activated by multiple stresses and mediate diverse downstream effects. Adapted from Vousden and Lane^[85].

5.2.1 P53 and tumor suppression

Tumor suppression is the most prominent function of p53 upon its discovery. Unless cells encounter certain stress or damage, the p53 pathway normally remains inactive. This is achieved by controlling the protein stability of p53. Under normal conditions, p53 protein has a very short half-life (from 5 to 30 minutes)^[86, 87] and the principal regulator of p53 stability is MDM2 (mouse double minute 2, HDM2 in human). MDM2 is a E3 ubiquitin ligase which mediates ubiquitination of lysine residue usually near the C-terminus of p53, resulting in a rapid degradation of p53 protein through the ubiquitin dependent proteasome pathway^[88]. MDM2 alone catalyzes the mono-ubiquitination of p53^[89], which by itself preferably promotes nuclear export of p53^[90, 91], but together with the assistance of p300/CREB binding protein (CBP), p53 will undergo poly-ubiquitination and degradation^[92, 93]. Another key repressor of p53 is MDM4 (also known as MDMX). MDM4 is a MDM2 homologue but does not ubiquitinate p53 to promote its degradation. Instead, MDM4 binds to both p53 and MDM2 and facilitates

MDM2 activity towards p53^[94]. There are several other E3 ligases have been reported as p53 specific regulator like E6-AP (E6-Associated Protein)^[95, 96], Arf-BP1 (Arf Binding Protein 1)^[97], COP1 (Constitutively Photomorphogenic 1)^[98], Pirh2^[99], synoviolin^[100] and Cullin4B^[101] which increase the complexity of this regulation.

The activation of this pathway starts with the stabilization of the p53 protein. P53 can be activated through a wide range of cellular stresses like DNA damage, replication stress, oncogene activation and hypoxia. The activation process relies on the post translational modifications on the p53 protein. For instance, when cells are challenged by DNA damage, p53 protein can be phosphorylated at serine 15 and serine 20 by many protein kinases (ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia related; and DNA-PK. Please refer to reviews^[102, 103]); in the meanwhile, MDM2 will also get phosphorylated at serine 395 and tyrosine 394 by the kinases ATM and c-Abl^[104, 105], resulting in a reduced binding affinity between p53 and MDM2. Therefore, p53 protein can be stabilized and subject to further post translational modifications that activates p53. It is worth noting that p53 can positively regulate its key repressor, MDM2, forming a fine-tuned negative feedback loop^[106, 107].

The next step in p53 activation is DNA binding. The classical working model of p53 is a transcription factor, although many functions of p53 are transcription-independent. The role as a transcription factor defines p53's structural properties (**Figure 5.4**). Human p53 protein can be divided into five major domains: N-terminal transactivation domain (also for MDM2 binding), the proline rich domain (contains a second transactivation domain), the DNA binding domain in the middle, the tetramerization domain (also contains nuclear export signal), and the C-terminal basic domain (contains 3 nuclear localization signals). Most mutations in cancer cells are found within the DNA binding domain^[108], compromising the binding specificity of p53. The consensus sequence that p53 recognizes and binds to is known as the p53 responsive element (p53 RE). In most cases, a functional p53 RE contains two copies of the inverted pentameric sequence RRRC(A/T)(T/A)GYYY (R, purines; Y, pyrimidines) , separated by a 0 to 13 base-pair spacer fragment. Binding of p53 can result in either a transcriptional activation or repression^[109, 110]. It recently has been discovered that p53 mediated repression through differences in its p53 REs, in other words, the p53 RE contains the information whether a target gene can be activated or repressed^[111].

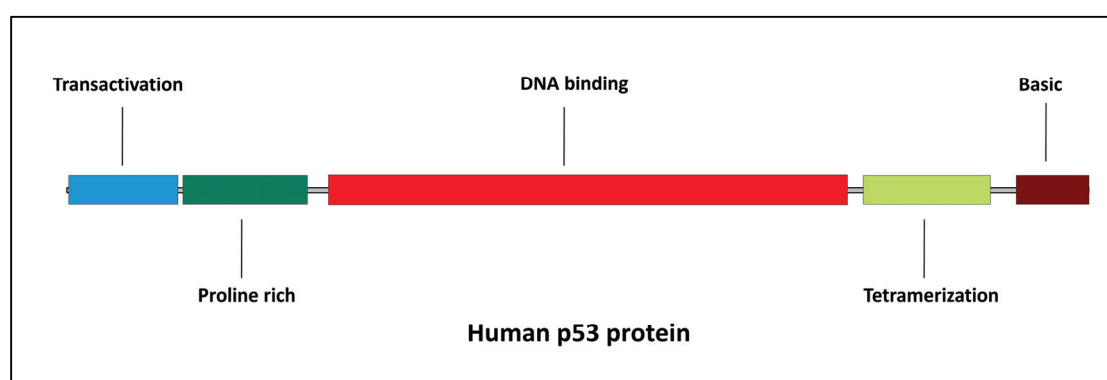


Figure 5.4 The structural model of human p53 protein. There are five major functional domains: transactivation domain, proline rich domain, DNA binding domain, tetramerization domain, and the basic domain.

Finally, after binding specifically to its responsive element, p53 regulates the transcription of its target genes with the help of many co-factors. The tumor suppression function of p53 relies mainly on the activities of its downstream target genes.

In response to numerous different stress signals, p53 induces a cell cycle arrest. The most prominent and well-defined way is through the direct expression stimulation of its target gene, p21^{WAF1/CIP1} (also known as CDKN1A), an inhibitor of cyclin-dependent kinases (CDKs). CDKs can associate with different cyclins to regulate cell cycle transitions. P21 expression leads to a proliferation halt during both the G1/S and the G2/M phases. P53 can also stimulate G1/S arrest by interfering with the CDK-activating kinase (CAK) complex (CDK7/CyclinH1/Mat1), which is needed for CDK2/Cyclin A activation^[112] and G2/M arrest by inducing expression of GADD45 and 14-3-3- σ , which inhibits CDK1/Cyclin B1 interaction^[113, 114] or Cdc25C phosphorylation^[115, 116], respectively.

Moreover, the induction of apoptosis constitutes a crucial part of p53's tumor suppressing ability which keeps the body away from defective or potentially carcinogenic cells. Apoptosis is the programmed cell death process and there are mainly two pathways to activate it, the extrinsic and intrinsic pathways. The extrinsic pathway involves transmembrane receptor-mediated interactions to receive death signal from outside of the cell and initiates apoptosis. Usually members from the tumor necrosis factor (TNF) receptor superfamily mediate the signal transduction^[117]. The internal pathway on the other hand does not require apoptotic signal from outside the cell but relies on intracellular signals, leading to compromised integrity of inner mitochondrial membrane and release of pro-apoptotic proteins into the cytosol^[118]. P53 induces apoptosis in both pathways. For example, in response to DNA damage, p53 transcriptionally activates a target gene called Fas (also known as apoptosis antigen 1, APO-1 or CD95)^[119], which is a key component of the death-inducing signaling complex (DISC) that triggers the extrinsic apoptotic pathway after binding with the Fas ligand^[120]. For the internal pathway, p53 activates the transcription of many pro-apoptotic BCL-2 (B-cell lymphoma 2) family members including Bax^[121], Puma (p53 upregulated modulator of apoptosis)^[122] and Bid^[123], therefore increases the protein ratio of proapoptotic/antiapoptotic BCL-2 members and triggers the downstream apoptosis-promoting effects like cytochrome c release and caspase activation (please refer to review^[124]). In addition, p53 activates the downstream effector of the apoptotic machinery like Apaf-1^[125-127] and caspase-6^[128] which can potentially lower the apoptotic threshold and enhance apoptosis.

P53 also triggers cell senescence to prevent abnormal cells from uncontrolled growth. Cell senescence is the state that cells irreversibly cease to divide and proliferate, accompanied by changes in morphology, metabolism and differentiated functions^[129]. The common causes of this phenomenon are telomere shortening, oncogenic/mitogenic stimuli and DNA damage. P53 can be activated by the DNA Damage Repair (DDR) pathway and establish cell senescence through a not well understood mechanism. It is implied that target genes of p53, p21^[130-132] and E2F7 (E2F transcription factor 7)^[133], are potential candidates that responsible for the cell cycle halt. Evidence also suggests that the mTOR pathway, reactive oxygen species (ROS) mediated damage and mitochondrial aging are also involved (please refer to reviews^[134, 135]). There are two other p53 homologues, p63 and p73, which have been identified. They share some similar structural features^[136] and play either a redundant or distinct role depending on

the context (please refer to reviews^[137-139]). In summary, p53 provides potent strategies to stop the cell cycle of abnormal cells and/or eliminate them for the sake of suppressing tumor development.

5.2.2 P53 in cellular metabolism

The effect of p53 in cellular metabolism is a relatively new addition to the many functions of p53. The most well established metabolic pathway which involves p53 is the glucose metabolism.

Glucose is the primary energy source in almost every cell type. Especially in CNS, it is the sole substrate for energy metabolism. It is well described that cancer cells usually undergo aerobic glycolysis even with the presence of oxygen (“Warburg effect”). Evidence shows that p53 regulates glycolysis in multiple ways. P53 limits the glucose transport into the cells through either transcriptionally represses the expression of glucose transporters (GLUT1 and GLUT4)^[140], phosphoglycerate mutase (PGM)^[141] or in an indirect manner, inhibits the NF-κB (nuclear factor-kappaB) pathway, which is accounted for elevated rate of aerobic glycolysis and upregulation of GLUT3^[142, 143]. Moreover, the p53 target gene, TP53-induced glycolysis and apoptosis regulator (TIGAR) negatively regulates glycolysis by lowering the substrate (fructose-2,6-bisphosphate) for phosphofructokinase 1 (PFK1)^[144], an intermediate step for glycolysis, and shifts the cellular metabolism towards the pentose phosphate pathway (PPP). P53 also enhances the expression of hexokinase II (HK2)^[145], an enzyme catalyzing the first step in glycolysis. While HK2 and TIGAR are both activated, glycolytic intermediates will be further supplied from glycolysis towards the PPP (Figure 5.5).

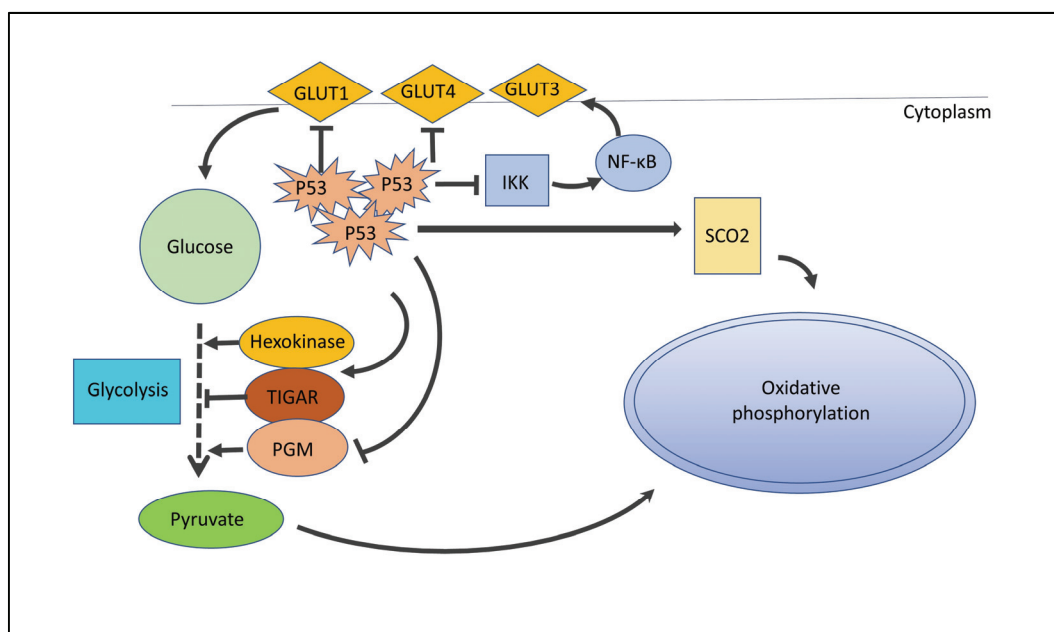


Figure 5.5 P53 in cellular metabolism. P53 reduces glucose influx and glycolytic pathway while increases oxidative phosphorylation. GLUT, glucose transporter; IKK, IκB kinase; NF-κB, nuclear factor-κB; SCO2, synthesis of cytochrome c oxidase 2; PGM, phosphoglycerate mutase. Adapted from Vousden and Ryan^[146].

In addition, p53 also regulates other metabolic pathways like oxidative phosphorylation (OXPHOS), glutamine metabolism and fatty acid oxidation (FAO). P53 promotes OXPHOS by

inducing the expression of cytochrome c oxidase 2 (SCO2)^[147], which is an essential component for the catalytic core of cytochrome c oxidase (COX, also known as Complex IV). Loss of p53 diverts cells from aerobic respiration to glycolysis because of the dysfunctional COX, leading to a lower dependence on oxygen (more like cancer cells). Another p53 target gene, glutaminase 2 (GLS2)^[148, 149], a key enzyme converting glutamine to glutamate, can also contribute to the OXPHOS by providing glutamate as an intermediate for tricarboxylic acid (TCA) cycle thus producing ATP and antioxidant activities^[150]. In addition, when cells are facing glucose starvation, p53 activates the FAO pathway as an alternative energy source. This is partly achieved by the activation of guanidinoacetate methyltransferase (GAMT)^[151], which is shown to be related to FAO in response to glucose starvation.

P53 signaling is activated under numerous metabolic stresses and the overall outcome of metabolic regulation by p53 is regarded to be tumor suppressing. But with the rapid changes of nutrient availability in tissue, p53 usually does not initiate the process to simply eliminate cells facing metabolic difficulties. To the opposite, it sometimes regulates cell fate towards survival under this condition.

5.2.3 P53 and cell survival

Although p53 is well recognized as a tumor suppressor, emerging evidence shows under certain circumstances (especially metabolic stress), p53 promotes cell survival.

For example, the before mentioned p53 target gene TIGAR, who serves as fructose-2,6-bisphosphatase, has been proven to be required for intestinal tumorigenesis in a mouse intestinal adenoma model^[152]. Significant decrease in both total tumor burden and average tumor size as well as increase in survival were observed in TIGAR KO mice compared to WT mice. Moreover, immunohistochemistry of WT tumors showed TIGAR expression was elevated in the adenoma compared with surrounding normal tissue. And this upregulation resulted in more proliferation and less ROS (reactive oxygen species) damage in tumor tissue. TIGAR catalyzes the dephosphorylation of fructose-2,6-bisphosphate therefore lowering the substrates for glycolysis. Consequently, cellular metabolism can be diverted into the pentose phosphate pathway (PPP) to generate NADPH and reduced glutathione^[144, 153]. It has been suggested in an earlier study that TIGAR protects glioma cells from glucose and oxygen starvation-induced cell death^[154]. The effect was probably caused by improving glucose catabolism yield through increased respiration and providing more defense against ROS^[155]. It is also suggested that TIGAR has the ability to inhibit both apoptosis and autophagy^[156] thus increase survival and high level of TIGAR expression in cytogenetically normal acute myeloid leukemia associated with poor prognosis^[157]. Taken together, this evidence reveals that TIGAR plays a critical role in cell survival and tumor development.

Another example is AMPK, the major cellular energy sensor. When treated with glucose deprivation, AMPK triggers a reversible G1 cell-cycle arrest that requires the phosphorylation of p53 on serine 15. MEFs with WT p53 can undergo cell cycle arrest and remain alive during the starvation, while MEFs with p53 KO genotype enter cell cycle and lose their viability by apoptosis^[75]. This effect could be highly cell type specific as in human osteosarcoma-derived U2OS cells, glucose deprivation induced AMPK activation and a p53-dependent apoptosis.

Notably, under this situation, p53 was phosphorylated at serine 46, but not at serine 15 or 20, suggesting a distinct mechanism^[74].

There are more reports on the pro-survival role of p53. Maddocks et al showed upon serine starvation, p53 can induce p21 mediated cell cycle arrest and redirect serine metabolism to glutathione synthesis thus protect cells from oxidative stress. Cells without p53 cannot respond to environment changes and suffer from an impaired anti-oxidant capacity, resulting in reduced viability and proliferation^[158]. Jiang et al reported when MEFs were challenged with glucose deprivation, p53 can promote cell survival through upregulating OXPHOS. Multiple p53 target genes were activated during this process including Acad11 (Acyl-CoA Dehydrogenase Family Member 11) and Hmgcll1 (3-hydroxymethyl-3-methylglutaryl-CoA lyase like 1), contributing to cell survival^[66].

From these studies, we can conclude that p53 plays a pro-survival role under certain conditions such as metabolic stress. While this might more likely be part of its physiological function, possibilities exist that tumors can overtake these survival signaling pathways and benefit from it coping better with metabolic stress.

5.3 Hypoxia Inducible Factor 1 (HIF1)

Hypoxia Inducible Factor 1 (HIF1) is the key regulator of hypoxic response that is involved in many crucial processes including angiogenesis, glucose metabolism and cell survival. Similar to p53, it exerts its function as a transcription factor that recognizes a specific binding sequence, called the hypoxia response element (HRE), and through which it regulates transcription of its target genes.

5.3.1 Hypoxic response mediated by HIF1 α

HIF1 works as a heterodimer consisting of two subunits, a constitutively expressed HIF1 β subunit (also known as aryl hydrocarbon nuclear translocator, ARNT) and a tightly regulated HIF1 α subunit^[159, 160]. HIF2 α ^[161-164] and HIF3 α ^[165] subunits have also been identified by sequence homology. Like HIF1 α , they can associate with HIF1 β subunit and serve as transcription factors. HIF2 α shares most target genes with HIF1 α but it does have non-redundant function^[166, 167], while the role of HIF3 α has been less understood. Since HIF2 α and HIF3 α are not ubiquitously expressed, they will not be further discussed in this dissertation.

The structure of HIF1 α and HIF1 β share some common features (**Figure 5.6**). Both of them contain a N terminal bHLH (basic helix-loop-helix) domain which is usually seen in transcription factors and is necessary for dimerization. In addition, they have a second domain that is known for dimerization, the PAS domain (PER-ARNT-SIM), which works together with bHLH domain promoting proper DNA binding and dimerization. In addition, HIF1 α has two transactivation domains (TAD) for transcriptional activation and an oxygen dependent degradation domain (ODD, also known as PSTD) for its stability control.

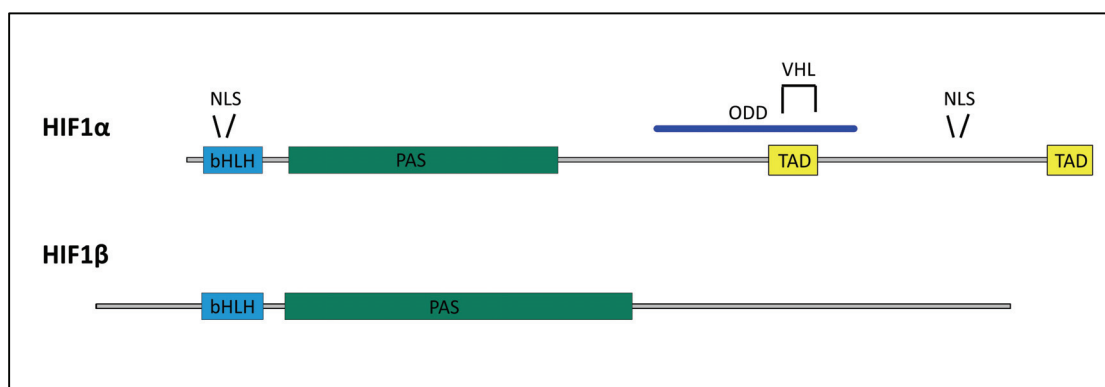


Figure 5.6 Structure of HIF1 α and HIF1 β proteins. Domain structures are indicated: bHLH, basic helix-loop-helix; NLS, nuclear localization signal; PAS, PER-ARNT-SIM domain; TAD, transactivation domain; ODD, oxygen dependent degradation domain. Interaction site with VHL is also indicated. Adapted from Semenza^[168].

The activity of HIF1 mainly relies on the protein availability of HIF1 α . Under normoxia, HIF1 α protein is negatively regulated and remained at a very low level, like in the case of p53. In fact, the half-life of HIF1 α protein is very short (about 5 minutes)^[169]. It is controlled through the ubiquitination and proteasome dependent degradation mediated by the tumor suppressor von Hippel-Lindau protein (VHL)^[170-172]. And this process is regulated through hydroxylation of proline residues (Pro 402 and 564) located within ODD by a set of enzymes that contain the prolyl hydroxylase domain (PHD)^[173, 174]. VHL protein interacts with the ODD and forms an E3 ubiquitin ligase complex thus leading HIF1 α protein to degradation^[175]. In addition, VHL

protein and HIF1 α both bind to the factor-inhibiting HIF 1 (FIH1). FIH1 is an asparaginyl hydroxylase and works on HIF1 α protein (Asn 803), resulting in a reduced binding of the coactivators CBP (CREB-binding protein) and p300^[176-178] and impaired transcriptional capability of HIF1 α .

It is reported that HIF1 α starts to get stabilized in cells under conditions containing approximately 6% of oxygen^[179]. Under low oxygen conditions, the prolyl hydroxylation is inhibited, and HIF1 α protein is released from interaction with VHL protein and gets stabilized. The accumulation of HIF1 α promotes its translocation into the nucleus, where it forms a heterodimer with HIF-1 β and bind to HRE. With both subunits bound within the HRE region, HIF1 activates the transcription of its target genes and mediates various responses to hypoxic stress^[180].

For example, HIF1 α has been shown to regulate many genes that are involved in the process of angiogenesis, inducing the formation of blood vessels. The best-known gene is the vascular endothelial cell growth factor (VEGF)^[181]. VEGF and its receptor are required for the initial assembly of the vasculature therefore is a key regulator of angiogenesis, not only under physiological but also under pathological conditions (please refer to review^[182]). Moreover, HIF1 α upregulates glucose uptake by inducing the expression of glucose transporters 1 and 3 (GLUT1, GLUT3)^[183] and glycolytic pathway by activating transcription of almost all glycolytic enzymes (HKI, HKII, PFK-L, ALD-A, ALD-C, PGK1, ENO-alpha, PYK-M2, LDH-A, PFKFB-3, please refer to review^[184]), thus contributing to more energy production. The products of glycolysis such as lactate and pyruvate can induce HIF1 α activation even under normoxia, forming a positive feedback between HIF1 α and glycolysis which could be potentially misused by cancer cells^[185]. Furthermore, HIF1 α induces some growth factors like insulin-like growth factor-2 (IGF2)^[186] and transforming growth factor- α (TGF- α)^[187] which eventually promote cell proliferation and survival.

As a result, HIF1 α activation helps cells to better adapt to oxygen changes in the environment and because of this, overexpression of HIF1 α has been found in various cancers.

5.3.2 The interplay between HIF1 α and p53

As p53 can be activated by numerous stress factors, hypoxia is one of them. This effect seems to be very cell type and condition dependent as many contradictory results have been reported. While in some reports p53 was responsive to hypoxia^[188, 189], some reports investigating hypoxia cannot induce p53 at all^[190] or can only induce p53 together with other stress like DNA damage^[191] or acidosis^[192]. There is increasing evidence that p53 only can be activated under very stringent oxygen condition ($\leq 0.02\%$) and under prolonged incubation time. Even then, the transcription activity of p53 is distinct from genomic stress induced p53 as many p53 target genes will not be activated^[193, 194].

Great interest has been drawn to a study published in 1998 showing wild type p53 can be stabilized by HIF1 α ^[195]. An et al found in several cell lines, hypoxia mimicking reagents induced p53 protein accumulation and this process was dependent on concomitant induction of HIF1 α . Furthermore, they found that HIF1 α and p53 proteins have a direct physical interaction which has been confirmed by another study^[196]. Shortly, this notion was challenged by the finding

that hypoxic conditions inducing HIF1 α cannot induce p53^[197]. Later, it has been demonstrated that dephosphorylated HIF1 α , not the phosphorylated form, binds to p53 and abrogates the inhibitory effect of MDM2 thus activating p53^[198]. Soon after, a striking report showed HIF1 α directly binds to MDM2 both in vitro and in vivo, and p53 fails to directly interact with HIF1 α ^[199], implying the previously identified interaction between HIF1 α and p53 was mediated through MDM2. Besides the above mentioned positive regulation of p53 by HIF1 α , evidence was also found that HIF1 α can negatively regulate p53 through downregulation of the homeodomain-interacting protein kinase-2 (HIPK2)^[200] which is required for p53 phosphorylation and activation. In addition, HIF1 α protects from CEP1 (worm homologue of p53) dependent apoptosis in *C. elegans* by transcriptional upregulation of the tyrosinase family member TYR2 and this effect is conserved in human melanoma cells^[201].

It has been reported that p53 when activated by severe hypoxia promoted HIF1 α protein degradation^[202] through MDM2 mediated ubiquitination and 26S proteasomal degradation^[203]. Therefore, in p53 KO cells^[203] or mice^[204] exposed to chronic hypoxia, usually an increase of the HIF1 α level was monitored. Functionally, it has been demonstrated that p53 and HIF1 α compete with each other for the binding to p300, a coactivator that is required for both transcription factors. As a result, p53 can inhibit HIF1 α activity without changing its protein level^[205].

Apart from the above discussed direct mutual interactions, both HIF1 α and p53 mediate the adaptation to cellular stresses and their influence overlaps in many identical pathways. Usually they play competing roles, but sometimes they work in a synergistic manner (**Figure 5.7**).

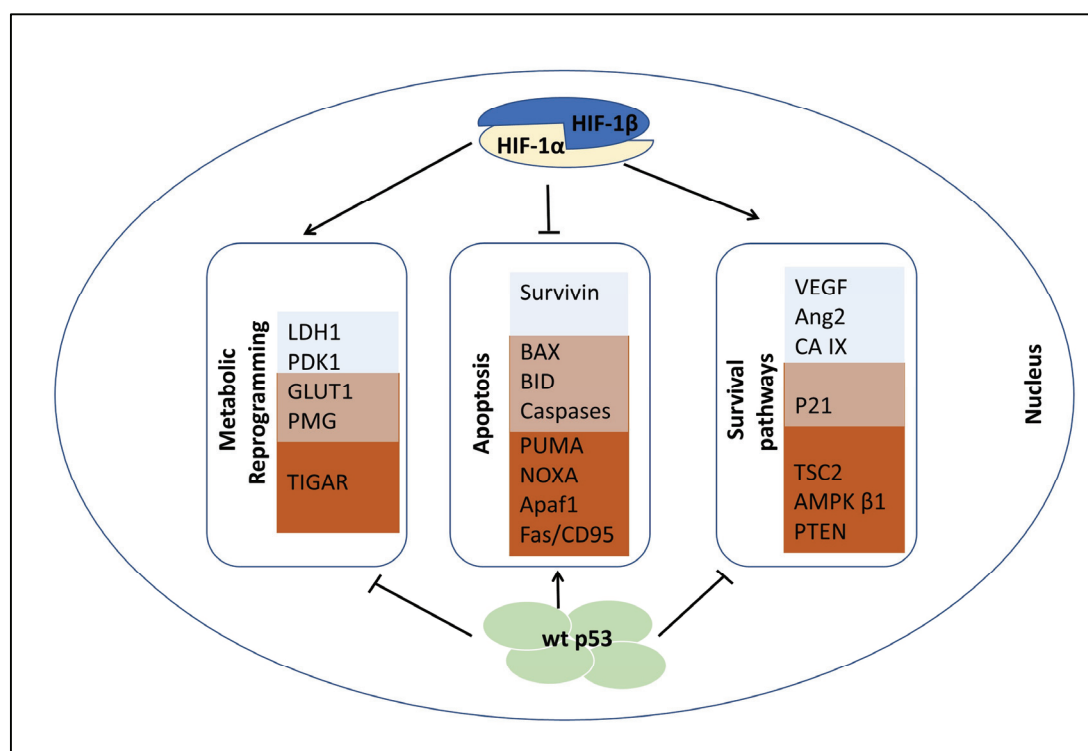


Figure 5.7 The interplay between HIF1 α and p53. HIF1 α and p53 could regulate overlapping pathways in adaptation to cellular stress. Please see text for details. Adapted from Obacz et al^[206].

For metabolic reprogramming under hypoxia, HIF1 α promotes glycolysis by activating lactate dehydrogenase (LDH1), pyruvate dehydrogenase kinase 1 (PDK1), phosphoglycerate mutase (PGM) and glucose transporter 1 (GLUT1)^[207]. P53 on the contrary represses the glycolytic pathway by downregulating the expression of GLUT1, PGM and upregulating TIGAR^[208]. In regard to apoptosis, HIF1 α induces survivin expression and represses BAX, BID and caspases^[207, 209], decreasing apoptosis in cells while p53 promotes apoptosis by inducing several target genes including PUMA, NOXA, Fas/CD95, Apaf1, BAX, BID and caspases^[210]. In addition, HIF1 α promotes cell survival by regulating downstream genes such as VEGF^[211], angiopoietin2 (Ang2)^[207], carbonic anhydrase IX (CA IX)^[212] and p21^[213]. On the p53 side, while hypoxia driven p21 (shared target gene of HIF1 α and p53) upregulation is shown to be more related to HIF1 α ^[194], p53 suppresses Akt (also known as protein kinase B, PKB) and mTOR pathways by activating its target genes Tuberous Sclerosis Complex 2 (TSC2), AMPK β 1 and phosphatase and tensin homolog (PTEN)^[208], thus decreasing cell proliferation and survival.

The interplay between HIF1 α and p53 is an interesting topic in understanding how cells react and adapt to cellular stress. Since HIF1 α and p53 both play important roles in cancer progression, insights from this crosstalk may be of therapeutic significance.

6 Aims of the Study

The aim of this PhD thesis divides into two major parts. The first part is to shed light on the possible physiological function of carnitine palmitoyltransferase 1C (CPT1C); the second is to investigate the regulation of CPT1C under one specific condition that all solid tumors are facing, namely hypoxia.

CPT1C is the last discovered and most enigmatic member of the CPT1 family. It possesses the potential of acyltransferase but this has not yet been proven to be important for its physiological role. It most likely localizes to the endoplasmic reticulum with unknown interacting partners or function. Notably, it is overexpressed in tumor cells, despite the normal extremely low protein level in tissues other than the CNS, indicating CPT1C grants tumor cells survival or proliferative advantages to their normal counterparts. Furthermore, we observed in our previous study that CPT1C is upregulated upon metabolic stress such as hypoxia, which draw our attention to elucidate the underlying mechanism of how CPT1C is regulated under such condition.

This dissertation is divided in two parts. In the first part (chapter 7), we conducted a series of experiments in order to explore the physiological function of CPT1C. We clarified the subcellular localization of CPT1C with a mouse monoclonal antibody generated in our laboratory and further mapped the sequences responsible for the ER-targeting of CPT1C. We also performed a proteomic screening to identify the interacting partners of CPT1C and verified one of the identified targets, namely dynamin 1, *in vitro*. Furthermore, an untargeted metabolomic screening revealed no major changes upon CPT1C depletion, which is consistent with previous studies. Performing high resolution respirometry, we found that lack of CPT1C does not seem to impair the oxidative phosphorylation function of mitochondria neither in extracts from whole mouse brain nor from the hypothalamus, where CPT1C is predominately expressed.

The second part of this dissertation (chapter 8) was directed towards investigating on how CPT1C is regulated under hypoxia, a common metabolic stress occurring in tumor tissue. We discovered that upregulation of CPT1C only occurred under severe hypoxia using mouse embryonic fibroblasts, and we confirmed that under this condition, CPT1C expression is also dependent on p53. However, the results we obtained from HIF1 α knockout cells demonstrated HIF1 α is also required in CPT1C regulation. Since under hypoxia, p53 and HIF1 α usually direct cell fate into opposite directions, we were interested to further investigate how p53 and HIF1 α work together in the regulation of CPT1C. Further experiments suggested that lack of HIF1 α probably affected the activation of p53, featured by a decreased phosphorylation of mouse p53 at position serine 18 and alleviated target gene transactivation. Notably, HIF1 α is not needed for DNA damage induced p53 activation and CPT1C upregulation, implying a specific cooperation between HIF1 α and p53 to regulate CPT1C under hypoxic conditions.

7 The Functional Analysis of CPT1C

7.1 Introduction

It has been highlighted by recent research that fatty acid oxidation (FAO) plays an essential role in the survival and proliferation in certain types of cancer^[214-216]. The majority reaction of FAO, the β oxidation, takes place in the mitochondria and the rate limiting step of this process is regulated by members of the carnitine palmitoyltransferase 1 (CPT1) family.

More than 14 years ago, the latest member of the family, CPT1C was discovered in mammalian genome and since then much effort has been invested in studying the function of it. Although it was expected to be an acyl transferase because of high homologue to other CPT1 members (CPT1A and CPT1B), it instead exhibits distinct features including predominant expression in the central nerve system (CNS)^[8], minimal acyl transferase activity in vitro^[11] and unique subcellular localization to the endoplasmic reticulum (ER)^[11]. Since the CNS does not rely on FAO for energy production, the relevance between CPT1C and FAO is debatable.

The understanding of CPT1C's subcellular localization was once misled by the low abundance of CPT1C protein^[29]. More emerging evidence revealed CPT1C is localized mainly in the ER. Unlike the mitochondrial locating sequence CPT1A^[30], to date no sequence has been identified to be responsible for the ER locating of CPT1C, while it has been suggested that the N terminal region is required for this specific localization^[11].

Current evidence also implied that CPT1C participates in the control of food intake and energy expenditure^[9], probably through its interaction with malonyl-CoA, the key regulatory component of energy sensing system in the hypothalamus^[7]. Some interacting partners of CPT1C have been identified and relevant functional changes have been investigated. For example, neuron function is altered due to lack of CPT1C because of its interaction with the neurotransmitter glutamate receptor subunits, GluA1 and GluA2^[31, 48]. Cellular lipid droplets formation is deregulated due to CPT1C's interaction with atlastin 1^[16]. Moreover, ceramide metabolism in neurons has also been described to be deficient because of CPT1C depletion^[27, 43]. More interacting partners and metabolic pathways are needed to be identified and analyzed for the influence of CPT1C. Interestingly, overexpression of CPT1C in vitro increases FAO rate and ATP production^[26], which raises the possibility that even localized mainly to the ER, CPT1C can regulate mitochondrial respiration. The underlying molecular mechanism remains unknown.

Here we performed a series of experiments in order to explore the physiological functions of CPT1C. We identified the ER-localization sequence that is required for the proper subcellular localization of CPT1C. In addition, we performed proteomic and metabolomic screenings that identified new interacting protein partners and new metabolites that potentially associate with CPT1C. Finally, the high resolution respirometry performed with mouse whole-brain/hypothalamus extracts revealed no mitochondrial function defects were observed in CPT1C knockout animals.

7.2 Material and methods

Cell lines. HEK 293 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 IU penicillin and 100 µg/ml streptomycin in a 37 °C 5% CO₂ atmosphere.

Primers. Primers were synthesized by Microsynth (Switzerland) with desalted purification. The following primers were used to generate different fragments of mouse CPT1C. Forward primers: mCPT1c_531: GCC AAC ATG GAG GGA GCC AAG ACC TTG T; mCPT1c_1: GCC ACC ATG GCT GAG GCA CAC CAG; mCPT1c_49: GCC ACC ATG GAC TTT CTT GCT GGT GTG GTC; mCPT1c_187: GCC ACC ATG CTA GAG TCT GT A CGC CCT GTG. Reverse primers: mCPT1c_553: GCT CTT GCC GAA GTG GGA GAA; mCPT1c_798: CAA GTT GGT GGA TGT; mCPT1c_189: AGA CTC TAG GTA CTT GCG CAC; mCPT1c_58: GGT TGC AGG GAC CAC ACC AG.

Plasmid construction. CPT1C fragments were amplified by Phusion High-Fidelity DNA Polymerase (New England Biolabs) and cloned into pEF6/V5-His TOPO vector (Life technologies) according to the manufacturer's instructions.

Cell transfection. Cell transfection was carried out with X-tremeGENE HP DNA Transfection Reagent (Roche) according to the manufacturer's instructions. The amount of DNA and transfection reagent were determined by pilot experiments.

Protein isolation and fractionation. Cells were collected 48 hours after transfection, briefly washed with PBS and re-suspended in fractionation buffer (10 mM HEPES, pH 7.8, 250 mM sucrose, 1 mM EGTA, 25 mM KCl, supplemented with benzamidine and PMSF). Cell disruption was achieved by passing through a 25G syringe needle 10 times. Homogenates were centrifuged at 1,000 X g for 10 minutes at 4°C to remove cell debris. The supernatants were centrifuged at 10,000 X g for 15 minutes at 4°C to obtain the mitochondrial pellets. Microsomal pellets containing ER fractions were precipitated by further centrifugation at 100,000 X g for 60 minutes at 4°C. The remaining supernatants were soluble cytosolic proteins. Microsomal and mitochondrial pellets were re-suspended in lysis buffer containing 1% DDM (n-dodecyl-D-maltoside) and quantified for western blotting.

Antibodies. Mouse CPT1C antibody (clone 1E11) was generated in our laboratory. PDI, PHB2 antibodies were purchased from Cell Signaling Technology. Pan-actin antibody was purchased from Abcam. His-tag, V5-tag, Dynamin 1 antibodies and normal mouse/rabbit IgG were purchased from Santa Cruz Biotechnology.

Mouse strains and animal care. C57BL/6 and CPT1C gt/gt mice were maintained within the Central Animal Facilities, University of Bern. Generation of the CPT1C gt/gt mouse was described in previous study^[26].

Immunoprecipitation. Immunoprecipitation was performed using standard protocol with the Protein G-Agarose beads from Roche. Briefly, samples were lysed in IP buffer (50 mM Tris-HCl, pH 7.5; 150 mM sodium chloride; 1% Nonidet P40 supplemented with benzamidine and PMSF) and precleaned with beads overnight at 4°C. Antibodies were incubated with beads for 1 hour at room temperature on a roller. Samples were added to the washed antibody-beads overnight at 4°C to form the protein-antibody-beads complexes. These complexes were

washed 3 times and denatured by heating the suspension to 100°C for 3 min. The supernatant parts were used in western blotting.

Mass spectrometry. Mice (CPT1C wildtype and knockout) were sacrificed and dissected, whole brains were collected and frozen immediately into liquid nitrogen. Tissues were lysed and fractionated using the above-mentioned method and immunoprecipitation was performed with anti-mouse CPT1C antibody (1E11) to enrich the protein fractions containing CPT1C. For LC-MS/MS, IP samples were denatured and separated by 1D SDS-PAGE. The mass spectrometry was performed by Dr. Jianhua Feng at the Functional Genomics Center Zurich. LC-MS/MS experiment and analysis were carried out with standard protocol^[217]. For the gel-free ESI-QTOF MS/MS, samples were analyzed in Mass Spectrometry and Proteomics core facility at the Department of Clinical Research, University of Bern.

Untargeted metabolomic profiling. Untargeted metabolic profiling of the samples was performed by ultra-performance liquid chromatography coupled to a quadrupole time-of-flight mass spectrometer (UPLC-QTOFMS). Mice (CPT1C wildtype and knockout) were sacrificed and dissected, whole brains were collected and frozen immediately into liquid nitrogen. The followed experiments were performed by Dr. Cédric Bovet in the Department of Clinical Chemistry, University Hospital Bern. Briefly, frozen brain tissue samples (-140°C) were homogenized and analyzed in triplicate in randomized order on a QTOF mass spectrometer (Synapt G2-S HDMS, Waters) coupled to an UPLC Acquity system (Waters). The raw mass spectrometric data were processed with the Progenesis QI software (version 1.1). After ion pattern deconvolution, possible ion features (defined by m/z and retention time pairs) were exported to EZInfo. Principle component analysis (PCA) was performed followed by orthogonal partial least square discriminant analysis (OPLS-DA) of the Pareto-transformed ion features in the two groups (i.e., KO and wild type mouse brains). The ion features of each technical replicate were separately analyzed. For each technical replicate, ion features were selected as potential metabolite candidates if their loading vectors $p(\text{corr})$ were < -0.5 or > 0.5 , the OPLS-regression loadings were < -0.05 or > 0.05 and the q-values were ≤ 0.05 (adjusted p-value). The list of possible metabolite candidates was refined by considering only those meeting the selection requirements in the technical triplicate. Structure for the possible chemical formula were searched against the Metlin Metabolite Database containing 242,766 metabolites (state on 04.04.2014).

High resolution respirometry. Whole brain/hypothalamus homogenates were extracted from CPT1C WT or KO mouse. Substrate-uncoupler-inhibitor titration (SUIT) protocols were performed with OROBOROS high-resolution respirometry (OROBOROS Instruments Corp)^[218]. Two SUIT protocols were used: PM+D+G+cc+S+F+Rot+Ama and PalM+D+G+S+F+Rot+Ama. PM, pyruvate (final concentration, 5 mM) and malate (2 mM); PalM, palmitoyl-carnitine (20 μM) and malate (2 mM); D, ADP (1-5 mM); G, glutamate (10 mM); cc, cytochrome c (10 μM); S, succinate (10 mM); F, FCCP (0.5-1 μM); Rot, rotenone (1 μM); Ama, antimycin A (2.5 μM).

7.3 Results

7.3.1 The determination of CPT1C domains responsible for ER localization

As above reviewed, the subcellular localization of CPT1s profoundly affect their function. Contradicting reports on CPT1C's subcellular localization hinder further functional investigation. Although it is suggested that the N terminal part (1-153 aa, amino acids) is important to translocate CPT1C to the ER, we would like to confirm and further narrow down the responsible sequence for this unique localization. We overexpressed HIS-V5 tagged functional domains of mouse CPT1C protein in HEK 293 cells and fractionated different cell compartments with differential centrifugation. Then, we examined if respective truncated protein can be detected in either mitochondrial, microsomal (containing ER fraction) or cytosolic fraction with Western blotting.

Firstly, full-length CPT1C protein was overexpressed and detected only in the ER fraction (**Figure 7.1**), which correlated with the results from endogenous CPT1C protein in our previous studies in mouse brain. The mitochondrial or ER specific protein, prohibitin 2 (PHB 2) and protein disulfide isomerase (PDI), were blotted respectively as markers. This result also indicated the system is reliable in discriminating proteins from different cellular fractions.

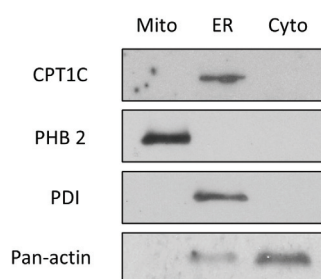


Figure 7.1 Expression of CPT1C full length protein in different cell fractions. Cell extracts were separated and blotted with indicated antibodies. PHB2, prohibitin 2; PDI, protein disulfide isomerase; Mito, mitochondrial fraction; ER, endoplasmic reticulum fraction; Cyto, cytosolic fraction.

Then we divided full length protein into two parts, the N terminal (1-553 aa) and the C terminal part (531-789 aa). While the N terminal part showed a clear ER localization, the C terminal part lost its ability to translocate to ER but was detected in both the mitochondrial and the cytosolic fractions (summarized in **Figure 7.2**). Next, within the N terminal region, we made fragments containing sequence from the beginning until end of the two transmembrane (TM) domains (1-187 aa) and the remaining part (189-553 aa). As expected, only fragment containing two TM domains was found in the ER (This part of experiments was performed by Ana Propadalo as her Master thesis project).

Then, shorter regions were tested to further discriminate which TM domain is dispensable for the ER-localization. Strikingly, either one of the two TM domains (1-95 aa or 93-553 aa) was able to guide the truncated protein to the ER. Furthermore, sequence before the two TM domains (1-58 aa) alone was also sufficient to guide protein to the ER. After calculating the overlapping regions, two ER localization signals were identified (1-58 aa and 93-187 aa).










Amino acids	Structure	Localization
1-798 (FL)		ER
1-553		ER
531-798		Mito/Cyto
1-187		ER
189-553		Cyto
1-95		ER
93-553		ER
1-58		ER
48-187		ER

Figure 7.2 Localization of different fragments of CPT1C. The subcellular localization for each fragment was summarized. Mito, mitochondrial fraction; ER, endoplasmic reticulum fraction; Cyto, cytosolic fraction. Domain structure was indicated as in introduction: purple, N terminal regulatory domain; yellow, transmembrane domain; green, acyltransferase domain. FL, full length.

Our experiments suggested that multiple ER localization signals exist in the N terminal region of mouse CPT1C protein and either of them is sufficient for the proper subcellular localization.

7.3.2 The identification of interacting partners of CPT1C

In order to get a comprehensive understanding on the interacting network of CPT1C, three proteomic screenings were performed. Since CPT1C is mainly expressed in brain and we have confirmed CPT1C's presence in ER fraction, protein samples of ER fractions were obtained from CPT1C WT and KO mouse brains. In the first two screenings, protein samples were further purified by immunoprecipitation (IP) with anti-mouse CPT1C antibody to enrich potential interacting proteins. For the first screening, samples after IP were applied to 1-D SDS-PAGE coupled with LC-MS/MS for analysis (1-D, one dimensional; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; LC, liquid chromatography; MS, mass spectrometry). Similar samples were subjected to a second screening performed with the gel-free ESI-QTOF MS/MS (ESI, electrospray ionization; QTOF, quadrupole time-of-flight). In the third screening, ER protein samples were not purified by IP but subjected to 1-D blue native gel electrophoresis. Gel region containing CPT1C (pre-defined by western blot) was cut out and analyzed by ESI-QTOF MS/MS. Results from the first two screenings were analyzed together and the third screening was used for further validation.

In the first screening, 94 proteins were identified to be present only in the CPT1C WT samples, while from the second screening, 17 unique proteins were found. Proteins on both target lists were highlighted and the top five (based on WT/KO peptides difference, excluding CPT1C protein itself) were annotated (**Table 7.1**). Surprisingly, the top three proteins were all localized to the inner mitochondrial membrane and related to ATP production (the presence of both ADP/ATP translocase 1 and 2 may be because of grouping ambiguity). CPT1C has been shown to promote ATP production in vitro but since CPT1C is mainly localized to ER, how CPT1C can physically interact with mitochondrial proteins under physiological conditions requires further validation.

Accession Number	Gene	Protein	MW	Localization	Function
P48962	Slc25a4	ADP/ATP translocase 1	33 kDa	Mitochondrial inner membrane	Catalyzes the exchange of cytoplasmic ADP with mitochondrial ATP across the mitochondrial inner membrane.
P51881	Slc25a5	ADP/ATP translocase 2	33 kDa	Mitochondrial inner membrane	Catalyzes the exchange of cytoplasmic ADP with mitochondrial ATP across the mitochondrial inner membrane. As part of the mitotic spindle-associated MMXD complex it may play a role in chromosome segregation.
D3Z6F5	Atp5a1	ATP synthase subunit alpha	60 kDa	Mitochondrial inner membrane	Produces ATP from ADP in the presence of a proton gradient across the membrane.
P39053	Dnm1	Dynamin-1	97 kDa	Cytoplasm/Cytoskeleton	Microtubule-associated force-producing protein involved in producing microtubule bundles and able to bind and hydrolyze GTP. Most probably involved in vesicular trafficking processes. Involved in receptor-mediated endocytosis
P68040	Rack1	Guanine nucleotide-binding protein subunit beta-2-like 1 (RACK1)	35 kDa	Cell membrane/Peripheral membrane protein/Cytoplasm	Component of the 40S ribosomal subunit involved in translational repression. Binds to and stabilizes activated protein kinase C (PKC). Inhibits cell growth by prolonging the G0/G1 phase of the cell cycle. Promotes apoptosis by increasing oligomerization of BAX and disrupting the interaction of BAX with the anti-apoptotic factor BCL2L.

Table 7.1 Potential CPT1C interacting candidates detected from proteomic screenings. These proteins were highlighted in both two proteomic screenings and rated based on WT/KO peptides difference.

Other than mitochondrial proteins, our interest grew towards the highlighted interacting candidate, dynamin 1. Dynamin 1 has also been identified in the third proteomic study we

performed with native protein complexes containing CPT1C, indicating it is a native interacting partner of CPT1C. It belongs to a GTPase family that often described to be involved in endocytic membrane fission. More interestingly, dynamin 1 has a similar expression pattern like CPT1C: highly expressed in neurons and is generally absent in non-neuronal tissues. To verify this interaction, co-IP was performed with mouse CPT1C antibody using microsomal fractions from CPT1C WT and KO mice brains. Results confirmed the physical interaction between CPT1C and dynamin 1 was specific, as CPT1C antibody can pull down dynamin 1 protein together with CPT1C but a control normal IgG failed to precipitate any of them (**Figure 7.3**). In CPT1C KO samples, although dynamin 1 expression was not affected, this interaction was totally abrogated, which further proved the specificity of this interaction. Other potential interacting proteins are currently under validation.

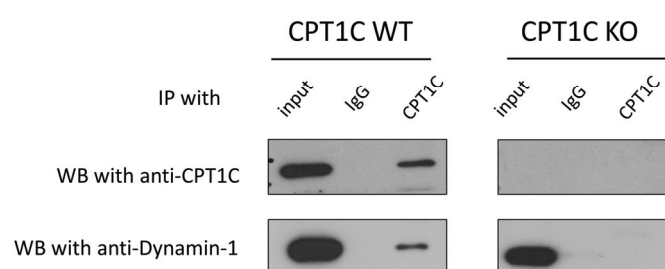


Figure 7.3 Physical interaction between CPT1C and dynamin 1 protein. Co-IP with CPT1C antibody and control normal IgG were performed with CPT1C WT and KO samples from ER fraction of whole mouse brains. IP samples were then detected by CPT1C and dynamin 1 antibodies in western blot.

7.3.3 Untargeted metabolomic screening reveals unique metabolites associated with CPT1C

As previously mentioned, one targeted metabolomic screening has been performed in CPT1C KO mouse by Lee et al and few significant findings were identified^[32]. Since our CPT1C KO mouse is different in origin, we would like to confirm and only focus on significant changes in metabolism caused by CPT1C. Therefore, the untargeted metabolomic profiling method was selected (please refer to review^[219]). It does not aim on certain metabolic pathways, instead it highlights only the significantly different metabolites between samples and further identifies them, which provides a comprehensive scope on cellular metabolism, enabling the finding of unknown metabolic pathways.

The untargeted metabolomic profiling was performed with whole brain homogenates from CPT1C WT and KO mice. Samples were subjected to UPLC-QTOFMS (ultra-performance liquid chromatography coupled to a quadrupole time-of-flight mass spectrometer) at the Department of Clinical Chemistry, University Hospital Bern and analyzed with EZinfo. Metabolites that have an adjusted p-value ≤ 0.05 (between CPT1C WT and KO groups) were identified as significant and possible chemical formulas were searched with the Elemental Composition software available in MassLynx.

In this screening, 2697 compound ions were recognized and 4 metabolite candidates were highlighted for their significant differences (**Table 7.2**). Two metabolites ($C_{24}H_{30}O_6$ and $C_9H_{21}N$) were found to be upregulated in CPT1C KO group while two ($C_{23}H_{43}NO_4$ and $C_{21}H_{39}NO_4$) were down regulated.

ID	m/z	Ions	Possible formula	Mass error (ppm)	Isotope similarity (%)	Max fold change	Highest mean
1	415.2117	[M+H] ⁺	$C_{24}H_{30}O_6$	0.5	98	6.2	CPT1C KO
2	144.1755	[M+H] ⁺	$C_9H_{21}N$	5.5	99.8	1.9	CPT1C KO
3	398.327	[M+H] ⁺	$C_{23}H_{43}NO_4$	1.2	99.3	1.5	CPT1C WT
4	370.2954	[M+H] ⁺	$C_{21}H_{39}NO_4$	0.7	98.4	1.4	CPT1C WT

Table 7.2 Possible metabolite candidates that were significantly changed due to CPT1C depletion. Untargeted metabolomic profiling was performed with whole brain samples from CPT1C WT and KO mice. Only significantly changed metabolites were further analyzed and identified (N=5).

While the underlying biological processes in which these metabolites are involved requires further validation, we proved CPT1C contributes to certain metabolic changes, which could possibly shed light on the physiological function of CPT1C.

7.3.4 Lack of CPT1C does not interfere with the oxidative phosphorylation function of mitochondria

We have previously reported that CPT1C may be related to mitochondrial function. For example, overexpression of CPT1C in vitro induces FAO and ATP production, and depletion of CPT1C induces abnormal morphologic changes in the mitochondria and increases lipid droplets in mouse embryonic stem cells^[26]. Since in the proteomic screening, we identified potential CPT1C interacting partners localized to mitochondria, it is plausible that CPT1C can interact with mitochondria. Furthermore, it recently has been proven that ER and mitochondria form a network structure called mitochondria-ER associated membranes (MAMs) and different processes can be regulated in this fashion (Please refer to review^[220]). Strikingly, CPT1C was identified as one of the MAM proteins in a proteomic analysis^[221]. Based on these findings, we examined the oxidative phosphorylation function of mitochondria upon CPT1C expression.

We obtained whole brain homogenates from CPT1C WT and KO mice and analyzed their abilities for oxidative phosphorylation with high resolution respirometry (**Figure 7.4**). Two different substrate-uncoupler-inhibitor titration (SUIT) protocols were applied to the samples. The first protocol (PM+D+G+cc+S+F+Rot+Ama) was aimed to detect OXPHOS from glucose metabolism while the second protocol (PalM+D+G+S+F+Rot+Ama) test FAO with OXPHOS as well. No difference was found in either the function of mitochondrial complex I (CI) or complex II (CII) linked respiration, or total OXPHOS or electron transfer system (ETS) capacity. Since CPT1C is specifically concentrated in the hypothalamus of the brain, we performed additional experiments with isolated hypothalamus homogenates, similar results were observed (data not shown).

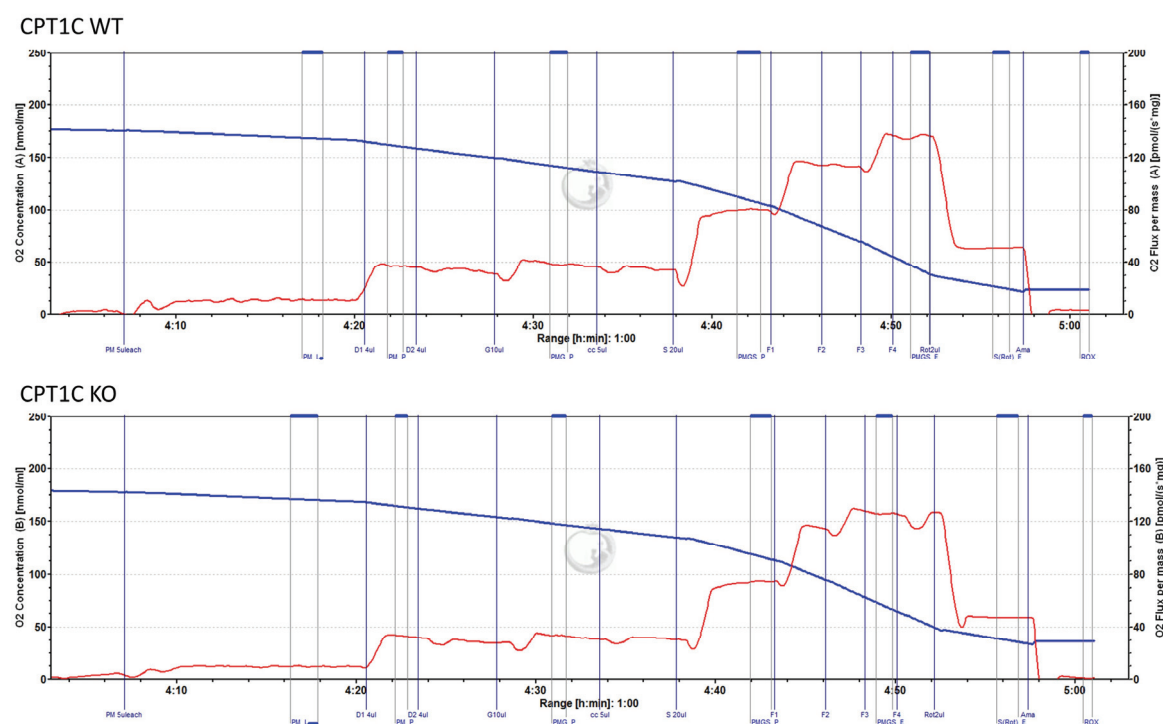


Figure 7.4 High resolution respirometry with homogenates from CPT1C WT and KO mouse brains. Oxygen concentration [nmol/ml] (blue line) and oxygen flux per mass [pmol/s*mg] (red line) were

shown. PM+D+G+cc+S+F+Rot+Ama protocol was applied to samples. PM, pyruvate and malate (CI substrate); D, ADP; G, glutamate (CI substrate); cc, cytochrome c; S, succinate (CII substrate); F, FCCP (uncoupler); Rot, rotenone (CI inhibitor); Ama, antimycin A (CIII inhibitor).

These data showed that lack of CPT1C doesn't affect mitochondrial OXPHOS in mouse brains, suggesting CPT1C's influence on FAO or ATP production may not be resulting directly from regulating mitochondrial respiration. The possible mechanism requires further validation.

7.4 Discussion

Here we reported the existence of multiple ER-locating sequences within the N terminal domain and two transmembrane domains in CPT1C. It remains poorly understood why and how CPT1C is localized to the endoplasmic reticulum but not the mitochondria, like the other two CPT1s. ER is a continuous membrane system that capable of many biological functions. Majorly ER could be categorized into two types: the rough ER (RER) and the smooth ER (SER), depending on the presence of ribosomes. While RER is dedicated to protein synthesis, processing and transport, SER is more closely linked with lipid metabolism. By far no information regarding whether CPT1C is associated with RER or SER has been reported, otherwise more specific functions could be investigated.

Many ER resident proteins contain retrieve signals that can be recognized by specific receptors at their C terminus to ensure their transport back and retention to the ER. The typical signal sequence for soluble protein is “KDEL” (Lys-Asp-Glu-Leu) and for membrane bound protein is “KKXX” (X represents for any residue)^[222-224]. Implied from sequence similarity with other CPT1 proteins, CPT1C most likely serves as a transmembrane protein, and we found a ER retention signal “KKSS” near its C terminus which is conserved from mouse (734-737 aa, 798 aa in total) to human (736-739 aa, 803 aa in total). We were surprised to see the C terminus is not necessary for the ER localization. It is also a surprising finding that multiple ER targeting sequences localized in the N terminal region, especially before the TM domains, the 1-58 aa fragment. Since this fragment contains 8 amino acids belonging to the first TM domain, to clarify if this is relevant to the ER localization, a new fragment without any residues from the TM domain is in urgent need. Besides, protein with deletion in either one of the TM domains could be an ideal subject for studying the potential physiological function of CPT1C, since with one TM domain the protein can be transported to ER but the topology will be changed as the N terminus and the C terminus cannot be facing the same side of the membrane.

Results from two proteomic screenings provided us new insights into the possible functions of CPT1C. The presence of several proteins from inner mitochondrial membrane with high WT/KO peptides difference implies it could be more than mere sample cross contamination. Most likely, the physical interaction between CPT1C and potential mitochondrial proteins are achieved through the formation of MAM, as all proteins on our list, as well as CPT1C, were identified as MAM proteins in a previous proteomic study (ADP/ATP translocase 1/2, ATP synthase subunit alpha, Guanine nucleotide-binding protein subunit beta-2-like 1 and Dynamin 1)^[221]. Furthermore, the mitochondrial defects found in CPT1C KO ES cells^[26] implied this physical interaction may result in functional changes. Although we cannot detect any defects in OXPHOS in CPT1C KO samples, validations with more interaction candidates and possible functional consequences are needed for clarification.

Dynamin 1 is a promising new interacting partner of CPT1C. It has been identified in all three proteomic studies we performed. There are three dynamin isoforms in mammalian genome which are expressed in a tissue specific manner. Dynamin 1 is selectively expressed in the CNS at high level^[225]; dynamin 2 is ubiquitously expressed^[226] and dynamin 3 has a similar expression pattern like dynamin 1, but at much lower level^[227-229]. The function of dynamin 1 is believed to be involved in endocytosis, vesicle budding, organelle fission and fusion^[230]. Specifically in CNS, it is responsible for recycling of neuron transmitting vesicles, and its

depletion will impair synaptic vesicle endocytosis during strong exogenous stimulation^[227]. Since dynamin 1 and 3 share the specific CNS expression pattern as well as several interacting partners^[228], it is of great interest to verify if dynamin 3 also interacts with CPT1C. It is also worth mentioning that the other potential interacting partner we found in our proteomic screening, RACK1 (Receptor of activated protein C kinase 1), has physical interaction with dynamin 1^[231]. Besides, the previously identified CPT1C interacting protein atlastin 1 belongs to the dynamin like protein family as well^[16, 232]. Furthermore, CPT1C was previously found in a protein complex with the molecular weight of approximately 0.4 MDa^[50] and larger than 0.6 MDa in our native electrophoresis. Multiple lines of evidence imply that CPT1C can interact with several proteins and serve as a component of a large protein complex.

Our findings suggest that CPT1C possesses more than one ER localization signals within its N terminal region. It can bind to unique interacting proteins partners and participate in metabolic reactions that are distinct from other CPT1 members, providing new insights in understanding the physiological role of CPT1C.

8 The Regulation of CPT1C under Hypoxia Involves p53 and HIF1 α

8.1 Introduction

Hypoxia, the status of restricted oxygen availability, can occur both under physiological conditions or during tumor progression. Oxygen tensions in different tissues vary significantly as a result of limited oxygen transport capacity in blood vessels and distance from the lung. While the oxygen concentration goes as high as 19.7% in the trachea, typically a much lower concentration is monitored in muscle (3.8%)^[233]. Although compromised oxygen content may restrict functions of tissues or cells, physiological hypoxia is required for certain processes especially during embryonic development. On the other hand, solid tumor cells also frequently face hypoxia because of the rapid rate of growth and lack of normal microvessels^[234].

The key regulator that mediates the necessary response to adapt to the hypoxic condition is the hypoxia-inducible factor (HIF) complex. HIF works as a heterodimer that is composed of a constitutively expressed HIF1 β subunit and a tightly regulated HIF α subunit (HIF1 α , HIF2 α , HIF3 α). Either HIF α subunit can form dimer with the HIF1 β subunit and serves as a transcription factor that binds to specific DNA sequence known as the hypoxia response element (HRE) and transcriptionally regulates expression of its target genes. HIF1 α is the ubiquitously expressed isoform which has been shown to be closely related to hypoxic adaptation in most cell types.

Under hypoxia, other critical transcription factors like p53 can also be activated, but only a few hypoxic p53 target genes have been identified^[235, 236]. The condition for hypoxic p53 protein stabilization is much stricter than that for HIF1 α . It is described that p53 can be stabilized only by severe hypoxia to anoxia (0.02% oxygen and less^[189, 202]) while HIF1 α can be induced with an oxygen concentration as high as 6%^[179]. It has been highlighted that p53 and HIF1 α have intensive interplay not only on the level of direct interaction between each other, but also on the overlapped target pathways. Since p53 and HIF1 α bind to the same domain of the essential coactivator p300^[237-239] for their transcription activity, under most conditions they play competing roles.

Carnitine palmitoyltransferase 1C (CPT1C) is a recently identified oncogene and downstream target gene of p53^[63]. It has been reported that CPT1C responds to metabolic stress like glucose deprivation and hypoxia and cells under stress benefit from the elevated expression of CPT1C by increased survival and proliferation^[26]. Little is known about the regulation of CPT1C under hypoxia. Since CPT1C is a p53 target gene and has potential HREs near promoter region, a feature shared in currently found hypoxic p53 target genes, we would like to investigate if p53 and/or HIF1 α participates in the expression regulation of CPT1C under hypoxia.

Here we reported CPT1C can be induced under severe (0.2% oxygen) but not mild (1.5% oxygen) hypoxic condition in a p53 dependent manner. While HIF1 α is required for the proper

activation of CPT1C under hypoxia, probably through affecting the p53 pathway, stabilization of HIF1 α alone is not sufficient to regulate CPT1C expression. Our results also suggested hypoxic activation of p53 requires different factors compared to genotoxic conditions.

8.2 Material and methods

Cell lines. Wild type, HIF1 α -/- (kindly provided by PD Dr. Deborah Stroka) and p53 -/- mouse embryonic fibroblasts were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 IU penicillin and 100 μ g/ml streptomycin in a 37 °C 5% CO₂ atmosphere.

RNA isolation and reverse transcription. Total RNA was isolated from fresh cultured cells with the High Pure RNA Isolation Kit (Roche) according to the manufacturer's instructions. RNA was then quantified by NanoDrop 1000 Spectrophotometer (Thermo Scientific) and same amount of RNA was used for every sample in the reverse transcription reaction with the SuperScript III Reverse Transcriptase (Thermo Fisher Scientific). cDNA products were diluted and same amount of sample was used in QPCR.

Quantitative PCR. Quantitative PCR was performed on Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific) with FastStart Universal SYBR Green Master (Rox) from Roche. All gene expressions were normalized to the geometric mean of three reference genes: PPIA (peptidylprolyl isomerase A), HPRT1 (hypoxanthine phosphoribosyltransferase 1) and RPL13a (ribosomal protein l13a). The bar graph shows mean \pm SD.

Primer list. Primers were synthesized by Microsynth (Switzerland) with desalted purification. Primer sequence used in mouse sample QPCR: BNIP3 forward: GGC GTC TGA CAA CTT CCA CT; BNIP3 reverse: AAC ACC CAA GGA CCA TGC TA. CA9 forward: GCT GTC CCA TTT GGA AGA AA; CA9 reverse: GGA AGG AAG CCT CAA TCG TT. CPT1C forward: TTC CTC TGG AGG TGG ATT TG; CPT1C reverse: GCT ACA TCC AAC AGG GCA TT. Fas/CD95 forward: ATG CAC ACT CTG CGA TGA AG; Fas/CD95 reverse: CAG TGT TCA CAG CCA GGA GA. HPRT1 forward: GCT GGT GAA AAG GAC CTC T; HPRT1 reverse: CAC AGG ACT AGA ACA CCT GC. PPIA forward: GCT GGA CCA AAC ACA AAC G; PPIA reverse: ATG CCT TCT TTC ACC TTC CC. RPL13a forward: ATG ACA AGA AAA AGC GGA TG; RPL13a reverse: CTT TTC TGC CTG TTT CCG TA.

Protein lysates and immunoblotting. Cells were collected, briefly rinsed in PBS and lysed in buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM sodium chloride, 1% Nonidet P40, supplemented with benzamidine and PMSF. Cell lysates were then centrifuged at 10,000X g for 10 min at 4°C to remove cell debris. The supernatant was analyzed by western blotting. Antibody sources are indicated as follow: HIF1 α (Abcam), HIF2 α (Abcam), p53 (Santa Cruz Biotechnology), phosphorylated p53 serine 18 (Cell Signaling Technology), pan-actin (Novus Biologicals).

8.3 Results

8.3.1 CPT1C is upregulated by severe hypoxia in a p53 dependent manner

In order to explore possible specific effects of p53 and HIF1 α , isogenic mouse embryonic fibroblast (MEF) cell lines with either wildtype (WT), p53 -/- (p53 KO) or HIF1 α -/- (HIF1 α KO) genotypes were employed. These cells have an extra advantage for this study as they lack the hypoxic reaction mediated by HIF2 α because of the inability of HIF2 α protein accumulation and nuclear translocation^[240], providing a clearer scope on the effect of HIF1 α . To validate if CPT1C responds to hypoxia and whether p53 is required, we treated WT and p53 KO MEF cells with oxygen concentration representing either mild (1.5%) or severe (0.2%) hypoxia within 24 hours. Since we can only reliably detect endogenous CPT1C in brain tissues, we performed QPCR to analyze gene expression on mRNA level.

Under mild hypoxia, CPT1C expression was not induced regardless of the p53 status (**Figure 8.1 A**). The expression of a HIF1 α target gene, BNIP3 (BCL2/adenovirus E1B 19 kDa protein-interacting protein 3) verified the presence of HIF1 α mediated transactivation under this condition, while the p53 target gene Fas/CD95 showed that p53 mediated transactivation was absent. Western blotting revealed HIF1 α protein was stabilized but no changes in p53 total protein or phosphorylation of serine 18 was monitored (**Figure 8.1 B**).

Next, we treated the same cells with severe hypoxia (0.2% oxygen). CPT1C was found to be upregulated under this condition from 12 to 24 hours in WT cells, but not in p53 KO cells (**Figure 8.1 C**). Fas/CD95 and BNIP3 were transcriptionally induced, indicating both the p53 and the HIF1 α pathways were activated. While Fas/CD95 showed a clear p53 dependence, BNIP3 expression was not affected by lack of p53. Further protein expression analysis showed while the total protein of p53 remained largely unchanged, the phosphorylation of serine 18 increased with time under this hypoxic condition, also implying p53 was activated under 0.2% of oxygen (**Figure 8.1 D**).

These findings indicated that the hypoxic activation of CPT1C is tightly correlated to the oxygen content. CPT1C can only be induced under severe hypoxia, when both p53 and HIF1 α pathways were activated. P53 depletion abrogated the hypoxic upregulation of CPT1C, confirming our previous findings that CPT1C is a p53 target gene.

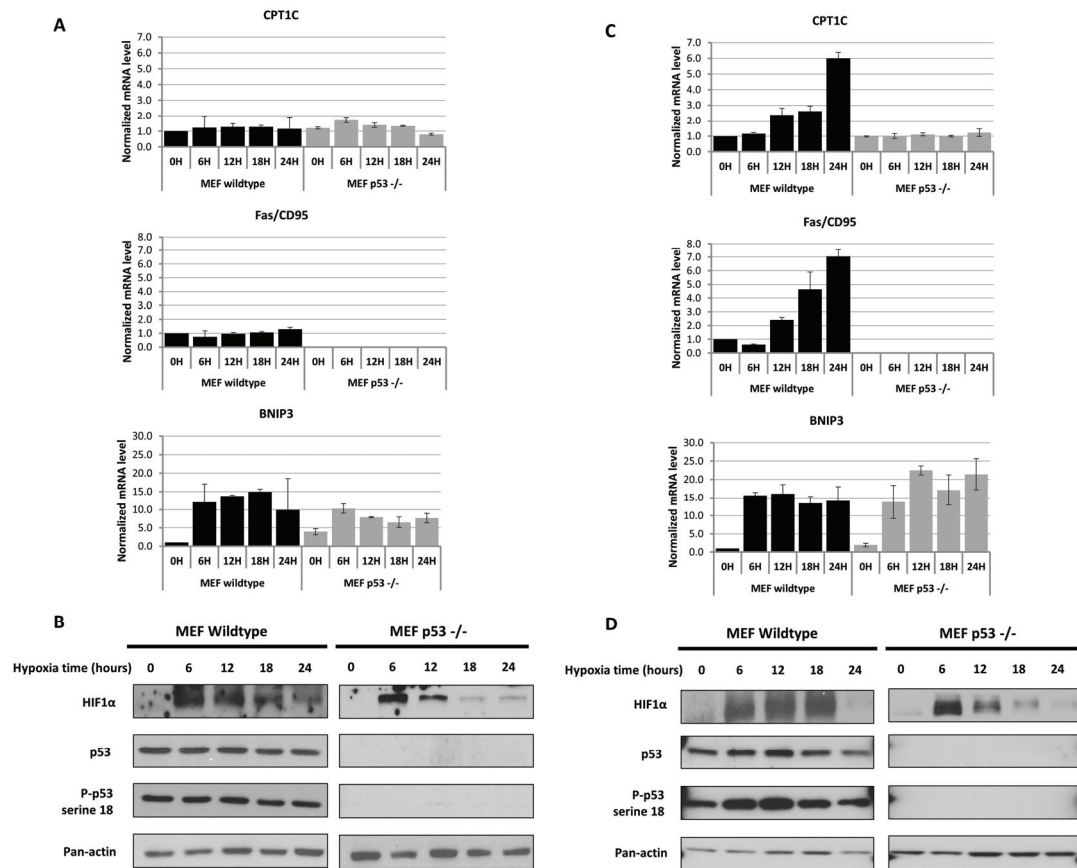


Figure 8.1 mRNA and protein expression of MEFs under hypoxia. (A and B) P53 WT and KO MEFs were incubated under 1.5% of oxygen for the indicated time. mRNA and protein expression were detected by QPCR and western blot, respectively. (C and D) P53 WT and KO MEFs were incubated under 0.2% of oxygen for the indicated time. mRNA and protein expression were detected by QPCR and western blot, respectively.

8.3.2 HIF1 α is required for hypoxia mediated upregulation of CPT1C

As described in the introduction, p53 and HIF1 α mediate cellular adaptation to hypoxia and their crosstalk significantly affects hypoxic response. Since we have proven hypoxic upregulation of CPT1C is also p53 dependent, we would like to examine if HIF1 α is involved.

We applied the same oxygen settings to isogenic HIF1 α WT and KO MEF cells and examined mRNA and protein expression (**Figure 8.2**). Interestingly, the hypoxic induction of CPT1C was dramatically impaired in HIF1 α deficient cells (**Figure 8.2 C**). As expected, the HIF1 α target BNIP3 displayed a HIF1 α dependence, but expression of the p53 target gene, Fas/CD95 was also repressed in HIF1 α KO cells. Although we cannot detect any changes in total protein level of p53, the p53 phosphorylation of serine 18 showed a substantial decrease in HIF1 α depleted cells (**Figure 8.2 D**).

These results implied that lack of HIF1 α attenuated the hypoxic response mediated by p53, featuring by decreased transactivation of CPT1C and Fas/CD95, as well as phosphorylation of p53 serine 18. It also suggested proper hypoxic response of CPT1C requires both p53 and HIF1 α , hence the two transcription factors work in a synergistical fashion.

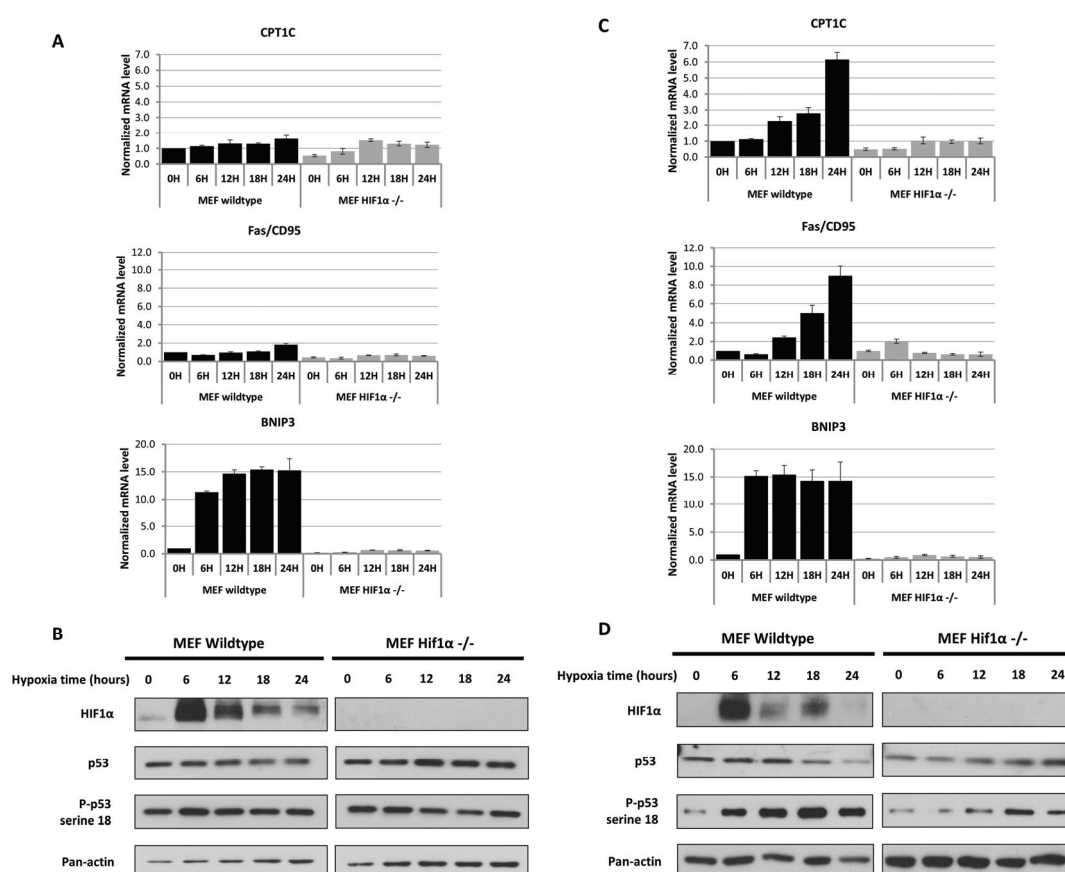


Figure 8.2 mRNA and protein expression of MEFs under hypoxia. (A and B) HIF1 α WT and KO MEFs were incubated under 1.5% of oxygen for the indicated time. mRNA and protein expression were detected by QPCR and western blot, respectively. (C and D) HIF1 α WT and KO MEFs were incubated under 0.2% of oxygen for the indicated time. mRNA and protein expression were detected by QPCR and western blot, respectively.

8.3.3 Activation of HIF1 α alone is not sufficient for CPT1C activation

It is described that the hypoxic threshold for HIF1 α or p53 activation is different. HIF1 α starts to be activated by oxygen content as low as 6%. In the case of p53, it is well accepted that p53 will be stabilized by oxygen concentration ranging from 0.2% to 0.02%, until anoxia. Since we can only detect CPT1C induction under severe hypoxia (0.2% oxygen) when both pathways are activated, we would like to further test conditions where only a single pathway is activated.

In previous studies we proved p53 single activation (IR, UV or etoposide) is able to induce CPT1C expression^[63]. For HIF1 α , although the evidence that CPT1C did not response to mild hypoxia (1.5% oxygen), a condition under which HIF1 α is clearly activated, implied the incapacity of HIF1 α alone for CPT1C induction. We tested two hypoxia mimicking reagents, cobalt chloride (CoCl₂) and DMOG (dimethyloxallylglycine). WT MEF cells were treated with either DMSO (dimethyl sulfoxide), 200 μ M CoCl₂ or 1 mM DMOG for 24 hours and mRNA expressions were detected. Both reagents activated mRNA expression of VEGF (vascular endothelial growth factor), a HIF1 target gene, while none of them had a significant effect on the expression of CPT1C (**Figure 8.3**).

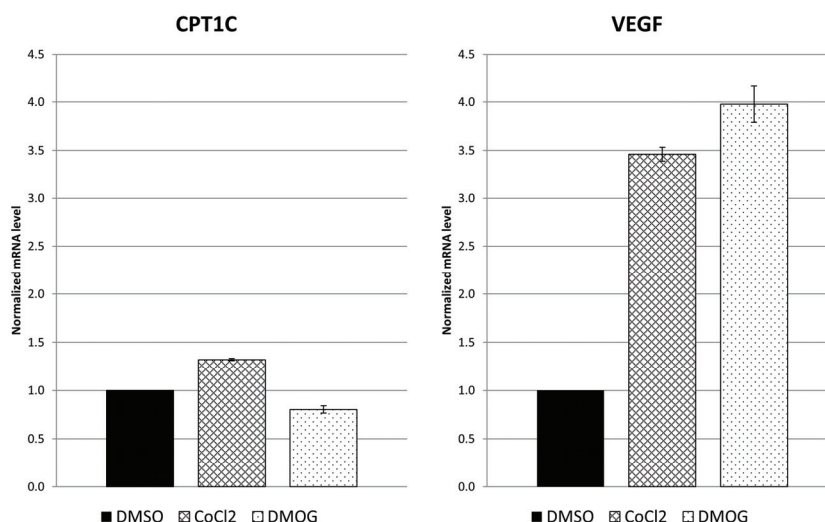


Figure 8.3 mRNA expression of MEF following treatment with hypoxia mimicking reagents. Cells were treated with either 200 μ M CoCl₂ or 1 mM DMOG for 24 hours.

Taken together, we concluded that activation of HIF1 α alone is not sufficient for hypoxic CPT1C expression. These results also implied that HIF1 α may not be able to regulate CPT1C expression directly, possibly in an indirect manner, through modulating the activation of p53.

8.3.4 DNA damage induced CPT1C does not require HIF1 α

Since deficiency of CPT1C induction was only detected in HIF1 α KO MEFs, and they exhibited a slightly reduced endogenous CPT1C expression (about 60% to 80% compared to WT cells). Possibilities exist that the effect monitored was due to the inability of these cells to induce CPT1C. To exclude this scenario, HIF1 α WT and KO MEFs were challenged with ionizing radiation (IR) and DNA damaging reagent, etoposide (10 μ M), both have been found capable of inducing p53 dependent CPT1C activation. The induction of CPT1C after both treatment is similar to that of WT cells, even has a stronger response to IR (**Figure 8.4**). The other p53 target gene, Fas/CD95 also showed a similar tendency, confirming the capability of HIF1 α KO cells to activate the p53 pathway and to express CPT1C.

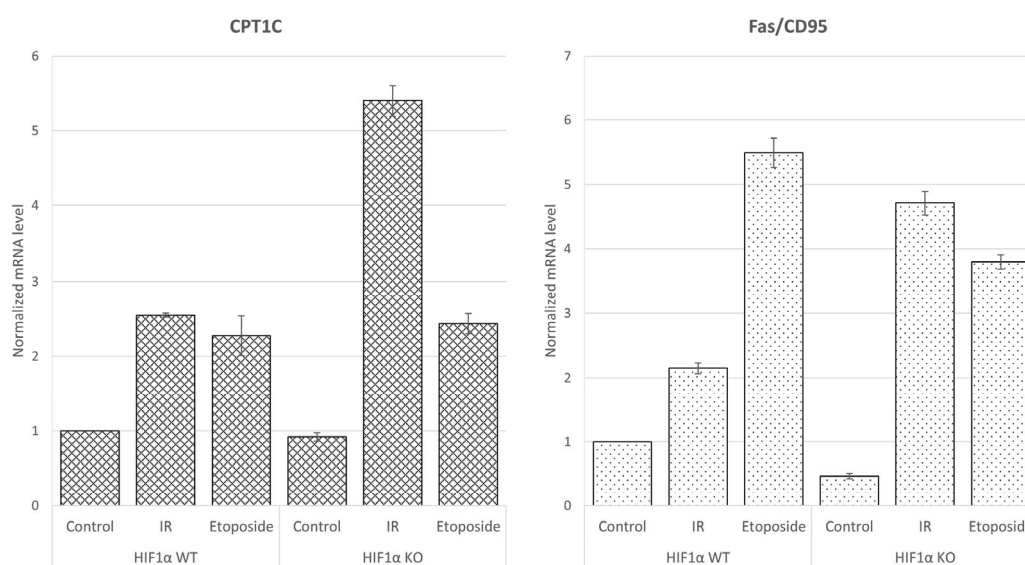


Figure 8.4 mRNA expression of HIF1 α WT and KO MEFs following treatment with DNA damaging conditions. For IR, cells were treated with 10 Gy of IR and collected 6 hours afterwards. For etoposide, cells were treated with 10 μ M and collected 24 hours after treatment.

Additional experiments were performed by treating WT MEFs with DNA damaging reagents together with either chemically induced hypoxia or mild hypoxia (1.5%), in order to confirm that HIF1 α and p53 double activation provides a more potent induction on CPT1C than p53 single activation. No difference was detected in these experiments (data not shown), indicating that the induction capacity of CPT1C does not depend on HIF1 α . These findings suggested HIF1 α is only required under hypoxic regulated CPT1C activation but not in DNA damage induced CPT1C.

8.4 Discussion

In our previous work, CPT1C was revealed to be related to carcinogenesis. It has been described that CPT1C overexpression increases cell proliferation as well as decreases apoptosis in response to metabolic stress^[26], which provides insights on why the brain specific protein is overexpressed in many cancer cell lines and tumors. But the finding that murine CPT1C serves as a direct p53 responsive target gene has made its role complex. It is well accepted that p53 is a critical tumor suppressor gene monitoring genome integrity and stability, therefore its direct activation on the potential oncogene CPT1C seems erratic. The most convective explanation is, this regulation (p53-CPT1C) is part of the physiological pro-survival function of p53 (please refer to review^[241]) and is hijacked by cancer cells with an unknown mechanism. But it remains elusive that since p53 is frequently mutated in cancer cells, whether it can still regulate CPT1C. Our previous study analyzed 13 tumor samples that have an overexpressed CPT1C compared to surrounding normal tissues and found intriguingly that only 2 tumor samples possess wildtype p53^[26]. Possibly p53 is not the main CPT1C regulator in those tumor cells. Besides, we failed to identify the p53-RE in the genomic region of human CPT1C. Although evidence showed in human cell lines CPT1C activated following DNA damaging reagents is p53 dependent^[63], whether human CPT1C is a direct target of p53 requires further elucidation.

Our results demonstrated that mouse CPT1C can be upregulated by severe hypoxia in a p53 dependent manner. Transcriptional activity of p53 under hypoxia differs from that under genotoxic condition. In most cases, hypoxia-induced p53 is not able to induce most common p53 target genes^[242]. It is often described that hypoxia-induced p53 promotes apoptosis by transcriptional repression, but not activation^[193]. Among a small set of identified hypoxic p53 activating genes e.g. Fas/CD95^[235] or BNIP3L (BNIP3-like)^[236], potential HREs were found within their promoter region. CPT1C also shares the same feature. As HRE is a relatively short sequence (RCGTG) that is very common across the genome^[243], analysis is needed to validate the functional binding of HIF1 α . Since mild hypoxia or hypoxia-mimicking reagents failed to induce CPT1C expression, current evidence does not support the hypothesis that CPT1C is a direct HIF1 target gene.

Hypoxia can increase, decrease or impose no effect on p53 protein accumulation^[244]. In our experiments, p53 transactivation is monitored but accompanied without obvious changes in total p53 protein level. Similar regulations have been reported by other studies^[245]. As possible mechanism has been suggested that hypoxia induces phosphorylation of p53 on serine 15 (equivalent to mouse p53 serine 18) by ATR, while reoxygenation induces the same phosphorylation site by ATM^[189, 246, 247]. Phosphorylation on p53 serine 15 does not increase its stability^[248] but is required for interaction with coactivator CBP^[249] and histone acetylation^[250], both promoting p53 transcription function. In our experiments, phosphorylation of mouse p53 serine 18 correlates with its transcriptional activity, which is severely impaired in HIF1 α KO cells following hypoxia. One possible mechanism by which HIF1 α is involved is through the activity of ATR. HIF1 α has been shown to be related to the hypoxia induced γ H2AX, another ATR phosphorylation target under hypoxia^[251]. The underlying mechanism is unclear. Since hypoxia generally does not induce detectable DNA damage, replication fork stalling could be one possibility. It has been also suggested that HIF1 α

regulates the expression of an ATR positive regulator, ATR interacting protein (ATRIP), thus affects ATR activation^[252].

Hypoxia induced p53 dependent apoptosis has been often described^[253], and the presence of both HIF1 α and p53 seems to be essential^[244]. Here we demonstrated a novel role of HIF1 α mediating the regulation of pro-survival target gene of p53, CPT1C, specifically in response to severe hypoxia. Our results are indicative of the crosstalk between two key cellular response pathways upon the pro-surviving gene CPT1C that might have an important role in carcinogenesis.

9 General Discussion and Outlook

With the rapid growth of cancer cells, the surrounding microenvironment becomes depleted of nutrients like glucose and oxygen. Cancer cells often deal with these metabolic difficulties by reprogramming their metabolic pathways to better adapt to the environmental changes. CPT1C is believed to be part of this process known as metabolic transformation. While the primary sequence of CPT1C implies its ability as an acyltransferase, the key enzyme needed for FAO like other members of the CPT1 family, its unique properties make it the most distinct member in the CPT1 family.

It remains unclear whether the potential acyltransferase activity is critical for its physiological function. The enzymatic activity of CPT1C has only been monitored by in vitro experiment and no changes in the FAO pathway were seen in a metabolomic screening^[32]. Chances are low that CPT1C is indeed a conventional acyltransferase. It is shown in a NMR spectroscopy study that CPT1C has similar N terminal regulatory domain as CPT1A, but the same point mutations found in CPT1C will reduce the enzymatic activity of CPT1A by more than 50 folds^[14]. Since all three CPT1s are expressed in brain, especially the ubiquitously expressed CPT1A, the contribution to FAO by CPT1C will be most possibly masked by the other two CPT1s.

Another important fact is that brain does not rely FAO for energy generation. As glucose is the sole energy source in brain, the reason why CPT1C predominately expresses in brain is not resolved yet. Due to CPT1C's ability of malonyl-CoA binding, it is possible that CPT1C serves as a regulator of malonyl-CoA. Studies showed CPT1C works as an upstream regulator of ceramide metabolism and as a downstream effector of ghrelin and leptin, with regards to food intake control. It is not clear by which mechanism CPT1C participates in neuronal ceramide metabolism (not the de novo synthesis) and whether the enzymatic activity is required for this process. But with the blockage in ceramide metabolism, the effect of malonyl-CoA and CPT1C are both abolished. It is demonstrated that CPT1C more likely works as a downstream effector of malonyl-CoA in order to control ceramide metabolism.

However, if CPT1C can utilize substrates other than long chain acyl CoA and carnitine, the situation will be different. Palmitoylation modification of protein is a minor post translational modification that affects the properties and localization of proteins (please refer to reviews^[254, 255]). It takes place in the cytoplasmic face of endoplasmic reticulum, Golgi apparatus and the plasma membrane^[256]. The chemical reaction is catalyzed similar to that of CPT1s, transferring the acyl group (in most cases palmitate) to the cysteine residues. If CPT1C is involved in the palmitoylation or de-palmitoylation, the existence of CPT1C in the ER can be easily interpreted.

There are several lines of evidence supporting this hypothesis. Firstly, the physical interaction with dynamin 1. Dynamin 1 is a GTPase specifically expressed in neurons. The most prominent function of dynamin 1 is to regulate the recycling of the synaptic vesicles by endocytosis. It mediates the membrane fission with energy from GTP hydrolysis. Interestingly, it has been reported that the palmitoyl protein thioesterase-1 (PPT1), which catalyzes protein de-palmitoylation, also participates in synaptic vesicle recycling. PPT1 depletion results in prolonged membrane association of palmitoylated synaptic vesicle (SV) proteins and

deficiency at nerve terminals^[257]. Secondly, the interaction between CPT1C and AMPAR subunits GluA1. CPT1C interacts with GluA1 only at ER but increases the surface expression of GluA1. Further analysis shows CPT1C does not change the protein level of GluA1. More interestingly, although palmitoylation state of GluA1 remains unchanged, one mutant form of GluA1 that cannot be palmitoylated, C585S, resembled the phenotype of CPT1C overexpression, suggesting CPT1C is still possibly linked to protein palmitoylation. Thirdly, the ER localization and possible interaction with mitochondria. Since palmitoylation takes place in the cytoplasmic side of ER, CPT1C will not face the ER lumen, which increases the mobility for potential interaction with other organelles.

Whether CPT1C possesses the ability for protein palmitoylation requires further validation, the pro-survival function of CPT1C has been confirmed. CPT1C increases cell proliferation and decreases apoptosis under metabolic stresses like glucose deprivation and hypoxia^[63]. The specific mechanism remains unknown, but elevated FAO and ATP production were monitored. How p53 and HIF1 α regulate the same gene and guide cell fate into survival as glucose shortage as well as hypoxic conditions are commonly met in the body. CPT1C is predominantly expressed in the brain, the vital organ in the body. To be more specific, in the neurons whose regeneration ability is restricted, it's crucial for these gene regulators to ensure the surviving of these cells.

The urgent scientific question is how CPT1C is regulated in cancer. Results from MEF cells demonstrate the concurrent requirement for both p53 and HIF1 α under hypoxia, while cancer cells that have an upregulated expression of CPT1C in most cases bear dysfunctional p53. Although upregulation of HIF1 α is a common event in cancer, our results do not support the notion that HIF1 α directly regulates CPT1C. Validation is needed to be carried out in cancer cells to identify new regulators. It has been predicted that mouse CPT1C has an alternative transcription start site that results in much shorter mRNA transcripts (XM_011250917.1 and XM_011250918.1). And in human glioma cell lines, Wakamiya et al detected a shorter CPT1C protein band (\approx 60 kDa)^[258], suggesting these potential transcript variants may be related to cancer cells. Since no p53 RE has been identified in human CPT1C genome, it remains possible that human CPT1C is not a direct p53 downstream gene.

Our findings in the functional analysis of CPT1C provide insights on the new physiological role of CPT1C. And understanding the crosstalk between p53 and HIF1 α signaling on CPT1C under limited oxygen content may provide interesting potential targets for the development of new cancer therapies.

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11 Appendix

Depletion of the Novel p53-target Gene Carnitine Palmitoyltransferase 1C Delays Tumor Growth in the Neurofibromatosis Type I Tumor Model

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11.1 Abstract

Despite the prominent pro-apoptotic role of p53, this protein has also been shown to promote cell survival in response to metabolic stress. However, the specific mechanism by which p53 protects cells from metabolic stress-induced death is unknown. Earlier we reported that carnitine palmitoyltransferase 1C (CPT1C), a brain-specific member of a family of mitochondria-associated enzymes that play a central role in fatty acid metabolism promotes cell survival and tumor growth. Unlike other members of the carnitine palmitoyltransferase family, the subcellular localization of CPT1C and its cellular function remains elusive. Here, we report that CPT1C is a novel p53 target gene with a bona fide p53 responsive element within the first intron. CPT1C is up-regulated in vitro and in vivo in a p53 dependent manner. Interestingly, expression of CPT1C is induced by metabolic stress factors such as hypoxia and glucose deprivation in a p53 and AMPK-dependent manner. Furthermore, in a murine tumor model, depletion of Cpt1c leads to delayed tumor development and a striking increase in survival. Taken together, our results indicate that p53 protects cells from metabolic stress via induction of CPT1C and that CPT1C may play a crucial role in carcinogenesis. CPT1C may therefore represent an exciting new therapeutic target for the treatment of hypoxic and otherwise treatment-resistant tumors.

Keywords:

CPT1C, p53, AMPK, Nf1, tumor growth, metabolic stress.

Abbreviations:

CPT1, carnitine palmitoyltransferase 1; CPT1C, carnitine palmitoyltransferase 1C, AMPK, AMP-activated kinase; ER, endoplasmic reticulum; Nf1, neurofibromatosis type 1; p53-RE, p53-responsive element; MEFs, mouse embryonic fibroblasts; ChIP, chromatin immunoprecipitation; FAS, fatty acid synthesis; FAO, fatty acid oxidation; ACC, Acetyl-CoA carboxylase; Irrad, ionizing radiation; UV, ultraviolet; 5-FU, 5-fluorouracil; WT, wild type; KO, knockout; gt, gene trap.

11.2 Introduction

Hypoxia is an important chronic stress on tumor cell growth and has been shown to correlate with poor disease-free and reduced overall survival in a variety of carcinomas and sarcomas.¹ To enhance survival in an altered environment such as hypoxia cancer cells undergo a so-called metabolic transformation.²⁻⁴ The best-known aspect of metabolic transformation is the Warburg effect, whereby cancer cells up-regulate glycolysis to limit their energy consumption. However, there is increasing evidence that not only glucose metabolism, but also fatty acid oxidation is involved in metabolic transformation. Although glucose seems to be the major energy source for tumor growth and survival, there is increasing evidence that alternative energy sources such as fatty acid metabolism are altered in cancer cells, even under hypoxic conditions. Indeed, fatty acid synthase has been found to be up-regulated in many human cancers,⁵ and inhibitors of the fatty acid synthase show antitumor activity.⁶

As recently published, we identified carnitine palmitoyltransferase 1C (*CPT1C*) as a potential novel p53 target gene.⁷ By their restriction of fatty acid import into mitochondria,⁴ the carnitine palmitoyltransferase 1 (CPT1) family of enzymes represent key regulatory factors of fatty acid oxidation. There are three tissue-specific isoforms of CPT1: CPT1A, which is found in liver, CPT1B in muscle, and CPT1C in brain and testes.⁸ Loss-of-function of CPT1C was generated in mouse embryonic stem cells (*Cpt1c^{gt/gt}* ES cells). Importantly, *Cpt1c^{gt/gt}* ES cells readily succumbed to cell death under hypoxic conditions, whereas control cells were resistant. ES cells deficient for *Cpt1c* showed a spontaneous induction in cell death through the mitochondrial apoptosis pathway. Using transient knock-down models for *Cpt1c*, we reported that *Cpt1c* promotes tumor growth in response to metabolic stress.⁷ These results suggest that cells can use a novel mechanism involving CPT1C to protect against metabolic stress.

Cpt1c-deficient mice show a complex metabolic phenotype characterized by decreased food intake and lower body weight when fed a normal diet. However, the mice show a higher tendency to obesity on a high fat diet when compared to wild-type mice.^{9,10} Conversely, using a transgenic mouse model, *Cpt1c* gain-of-function results in postnatal microcephaly and when fed a high-fat diet, these mice are protected from weight gain.¹¹ These data suggest a role for CPT1C in feeding behavior or metabolic sensing in the brain.

Metabolic stress stimulates the activation of intracellular sensors which mediate cellular adaptation in order to evade apoptosis. The tumor suppressor gene p53 is a well-studied transcriptional factor that is activated and stabilized by many cellular insults such as DNA damage, hypoxia, starvation and oncogenic activation.

The AMP activated kinase (AMPK) is a cellular energy sensor activated by conditions of metabolic stress characterized by an increase in the intracellular AMP/ATP ratio.¹² AMPK is now known to be activated by multiple factors, including AMP and ADP, as well as many nucleotide independent factors acting through upstream kinases.¹³ Starvation or low energy levels initiate activation of AMPK, which results in the induction of p53.¹⁴ Depending on the intensity and duration of the stress, p53 activation either leads to cell cycle arrest, ROS clearance or survival signals or induces apoptosis and cell death signals. There is increasing

evidence that p53, which plays a key role in determining apoptotic cell fate, is involved in metabolic reprogramming, one of the key alterations in tumorigenesis.

Recent studies showed that CPT1C is localized both in the endoplasmic reticulum (ER) and mitochondria, but predominantly in ER.¹⁵ However, the exact subcellular localization of CPT1C and its cellular function remains unclear. Here we show that CPT1C is upregulated in vitro and in vivo in a p53-dependent manner. We also demonstrate that CPT1C expression is induced by metabolic stress in an AMPK- and p53-dependent manner. Furthermore, we show that CPT1C can protect cells from cell death induced by hypoxia. Interestingly, CPT1C-depletion increases survival and suppresses tumor development in the *Nf1^{+/-};p53^{+/-}* tumor model. Analysis of these tumors confirms an activated AMPK/p53 signaling pathway. In addition, depletion of CPT1C leads to decreased proliferation. Our findings have implications for the cell survival effects of p53 under conditions of metabolic stress and might play a key role in carcinogenesis. Understanding the roles of CPT1C as a key downstream target in the AMPK/p53 pathway via regulation of metabolism may provide interesting potential targets for the development of new cancer therapies.

11.3 Results

11.3.1 CPT1C is a p53 target gene

We previously reported on a cDNA microarray screen designed to identify novel p53 transcription targets and identified CPT1C as a potential novel p53 target.⁷ This screen employed Friend virus-transformed mouse erythroleukemia cells that lack endogenous p53 and express a temperature-sensitive form of p53 (DP16.1/p53ts cells). At the permissive temperature of 37°C, mutated p53 protein is inactive and cells continue to proliferate. At the restrictive temperature of 32°C, mutated p53 becomes active and cells are induced to undergo apoptosis. To identify genes differentially regulated upon p53 activation, we compared microarray mRNA expression patterns of DP16.1/p53ts cells cultured at 37°C or 32°C. Additionally, we recently published that upon p53 activation in DP16.1/p53ts cells, mRNA for EST AA050178.1, which represents a partial cDNA for Cpt1c, was increased 1.9-fold and 2.8-fold after 3 and 6 h at 32°C, respectively.⁷ There were no significant changes in CPT1C mRNA in the parental DP16.1 cells (-1 and 1-fold change at 3 and 6 h after temperature shift, respectively. data not shown). We next examined whether CPT1C regulation was truly an effect of p53 activation. Real time RT-PCR in a variety of cancer cell lines revealed that CPT1C is upregulated in multiple cell lines in a p53-dependent manner in response to several different stress stimuli such as ionizing radiation (6 or 12 Gray), ultraviolet (UV) radiation, etoposide and 5-fluorouracil (5-FU) (Figures 1a and b). Moreover, in U87 cells, CPT1C was the only CPT family member regulated by p53 (Figure 1c). Similar results were obtained using A549 and other human cancer cell lines (data not shown). Due to the fact that the current available commercial antibodies were unable to detect endogenous level of human CPT1C in the tested cells, we could not confirm these results at the protein level.

11.3.2 p53 directly activates CPT1C transcription

We analysed the promoter of the murine CPT1C gene¹⁶ and identified two putative p53-responsive elements (p53-RE)¹⁷ in the first intron that were 330 bp apart: p53-RE-A, +174-219; p53-RE-B, +504-533 (Figure 2a). To investigate whether p53 could bind directly to either of these sites, we performed ChIP analyses on DP16.1/p53ts cells grown at 37°C or 32°C for 8 h. Using immunoprecipitation with anti-p53 antibody and PCR with primers specific for the two potential p53-binding sites, we observed a specific amplification of p53-RE-A at 32°C under which p53 was activated (Figure 2b). It should be pointed out that the non-specific bands were also observed for p53-RE-B at all conditions. The proximity of p53-RE-A and p53RE-B (330 bp) and the use of sonication which allows analysis of 700 bp fragments make it difficult to clearly separate p53 binding. To further determine the specificity of these two binding sites, we cloned the p53-RE-A and p53-RE-B sequences into separate luciferase reporter vectors to test transcriptional activity. These constructs were co-transfected into *p53*^{-/-} mouse embryonic fibroblasts (MEFs) with either WT p53 or p53 bearing a mutation in its DNA binding domain. Only the luciferase vector containing p53-RE-A and not p53-RE-B showed increased luciferase activity in the presence of WT p53 (Figure 2c). This p53-dependent luciferase activity was blocked by a point mutation at position 42 (G->T) of p53-RE-A (p53-RE-A*) (Figure 2c). Co-transfection of p53-RE-A with the DNA binding domain p53 mutant showed no increase in luciferase activity (data not shown), demonstrating that luciferase activation associated with p53-RE-A is dependent on the DNA-binding activity of p53. Taken together, these data suggest

that the p53-consensus motif p53-RE-A is both sufficient and necessary to drive the p53-dependent transcription of CPT1C.

11.3.3 p53 upregulates CPT1C in vivo

To determine whether CPT1C could be upregulated in response to p53 activation in vivo, we performed in situ hybridization to detect CPT1C mRNA in irradiated mouse embryos. At day 12.5 post coitum, embryos of C57BL/6 *p53*^{+/-} and *p53*^{-/-} mice were subjected in utero to 5 Gy irradiation. At 8 h post-irradiation, various tissues were excised and fixed for detection of CPT1C mRNA by in situ hybridization. Consistent with previous reports, the highest base levels of CPT1C mRNA were detected in neuronal tissues of non-irradiated embryos (Figure 3, midbrain). Irradiated *p53*^{+/-} embryos showed a strong upregulation of CPT1C mRNA in most tissues examined, including the midbrain (Figure 3c) and heart (data not shown). This CPT1C upregulation was not detected in irradiated *p53*^{-/-} embryonic midbrain (Figure 3d). These data indicate that CPT1C expression can be transcriptionally activated by p53 in vivo in response to DNA-damaging stimuli.

11.3.4 CPT1C expression is induced by hypoxia and glucose deprivation in a p53-dependent manner

Key targets of p53 under hypoxic conditions are of special interest, since p53 is mutated in over 50% of all solid tumors. Rapidly growing cancer is often associated with hypoxia. Therefore, we investigated whether CPT1C is upregulated in response to hypoxia. We exposed transformed wild-type and mutant p53 MEFs to 0.2% O₂ over 7 h and measured the expression levels of all CPT family members by real time PCR. As shown in Figure 4a, CPT1C is the only family member upregulated in response to hypoxia in a p53-dependent manner.

We reported earlier that CPT1C depletion confers sensitivity to metabolic stress including hypoxia and glucose withdrawal.⁷ To investigate whether p53 directly binds to the verified p53-RE of *Cpt1c* upon hypoxia or glucose withdrawal, we performed ChIP analyses on p53 wild-type MEFs treated with either glucose-free DMEM (Figure 4b) or low oxygen (Figure 4c) using anti-p53 antibodies. Using immunoprecipitation with anti-p53 antibody and PCR with primers specific for the p53-binding sites of *Cpt1c*, we observed a strong amplification of the *Cpt1c* p53-RE (p53-RE-A) under hypoxia and glucose withdrawal conditions (Figure 4b and c). Primers specific for the p53-RE in the *Bax* gene were used as a positive control for p53 activation.

11.3.5 CPT1C is induced by energetic stress in an AMPK- and p53-dependent manner

AMPK activation leads to increased catabolic metabolism, which can activate a p53-dependent cell cycle checkpoint.¹⁴ Our observation that glucose withdrawal induces CPT1C expression and regulates cell proliferation suggests that CPT1C may be a target of AMPK, the major sensor of cellular energy levels.¹² We treated control or AMPK α -deficient MEFs (*AMPK α 1*^{-/-}, *α 2*^{fl/fl} MEFs expressing Cre recombinase) with 1mM Metformin and found that increased endogenous CPT1C levels parallel increased phosphorylation of AMPK α and its downstream targets (ACC and p53) (Figure 5a). These results indicate that CPT1C is up-regulated by Metformin in an AMPK-dependent manner. To verify whether up-regulation is dependent on p53, we examined by Western blotting the endogenous level of CPT1C in 3T3 MEFs wild-type and p53-deficient cells. Western blotting analysis revealed that CPT1C protein

levels in $p53^{-/-}$ cells were much lower than that in $p53^{+/+}$ cells (Figure 5b, left). The CPT1C level was significantly elevated after Metformin treatment, where AMPK was activated in $p53^{+/+}$ cells, but not in $p53^{-/-}$ cells. (Figure 5b, right). These data strongly support the observation that CPT1C is up-regulated in a p53 and AMPK-dependent manner. We previously showed that CPT1C-depletion in mouse ES cells leads to activation of the intrinsic mitochondrial apoptosis.⁷ Moreover, AMPK-deficient cells display increased sensitivity to apoptosis induced by metabolic stress.¹⁸ Interestingly, the ectopic expression of CPT1C protects AMPK-deficient cells from apoptosis induced by the metabolic stressor 2-deoxyglucose (Figures 5c and d). These data suggest that CPT1C is a key downstream mediator of AMPK signaling, important for mediating cell survival in response to metabolic stress.

11.3.6 CPT1C depletion increases survival and suppresses tumor development in the $Nf1^{+/-};p53^{+/-}$ tumor model

Based on our results that show upregulation of *Cpt1c* after activation of the tumor suppressor p53, we hypothesize that CPT1C plays a crucial role in carcinogenesis by protecting tumor cells from hypoxic and metabolic stresses. To verify this hypothesis, we used the neurofibromatosis type I tumor model.^{19, 20} $Nf1^{+/-};p53^{+/-}$ mice, which are prone to develop soft tissue sarcomas, were crossed with $Cpt1c^{gt/gt}$ mice to generate $Nf1^{+/-};p53^{+/-};Cpt1c^{gt/gt}$ mice in a C57BL/6 background. Survival as well as tumor incidence was compared to C57BL/6, $Cpt1c^{gt/gt}$ and $Nf1^{+/-};p53^{+/-}$ mice (Figure 6a). Similar to previous reports^{20, 21}, we observed that $Nf1^{+/-};p53^{+/-}$ mice developed soft tissue sarcomas of the limbs and abdomen as well as lymphomas at around 3 to 6 months of age and with a penetrance of over 70%. CPT1C depletion in this murine tumor model highly increases the median survival time from 5 to 15 months ($p < 0.0001$). Sarcomas developed in 59.86% of $Nf1^{+/-};p53^{+/-}$ mice, and metastases in 29.41%. In contrast, depletion of *Cpt1c* in $Nf1^{+/-};p53^{+/-}$ mice significantly decreased the incidence of sarcomas and metastases to 13.89% and 19.44%, respectively (Figure 6b). Similarly, splenic hyperplasia was also significantly less in $Nf1^{+/-};p53^{+/-};Cpt1c^{gt/gt}$ mice (25%) compared to that in $Nf1^{+/-};p53^{+/-}$ mice (41.18%). In our cohort, there was no obvious difference in the onset of brain tumors. The reason why our $Nf1^{+/-};p53^{+/-}$ mice developed fewer brain tumors than previously reported is probably due to the different C57BL/6 background.¹⁹

11.3.7 Histological analysis reveals less proliferation in tumors from $Nf1^{+/-};p53^{+/-};Cpt1c^{gt/gt}$ mice

It has been shown that hypoxia in solid tumors is associated with rapid disease progression and poor outcome. Since CPT1C protects tumor cells from apoptotic cell death, we hypothesize that CPT1C depletion in our murine tumor model reduces markers of tumor aggressiveness such as proliferation and apoptosis. In order to analyze the proliferative and apoptotic rate in $Nf1^{+/-};p53^{+/-}$ and $Nf1^{+/-};p53^{+/-};Cpt1c^{gt/gt}$ tumors, we performed immunohistochemistry with Ki67 and cleaved Caspase-3 (CC3) in paraffin-embedded tumor samples. As shown in Figure 6c, positive staining for Ki67 was significantly reduced in $Nf1^{+/-};p53^{+/-};Cpt1c^{gt/gt}$ tumor samples compared to $Nf1^{+/-};p53^{+/-}$. There is also an apparent tendency towards CC3 positive staining in $Nf1^{+/-};p53^{+/-};Cpt1c^{gt/gt}$ tumors when compared to $Nf1^{+/-};p53^{+/-}$, though no significant difference was observed between those two groups. Taken together, *Cpt1c* depletion significantly decreases the incidence of sarcomas and metastases in the murine neurofibromatosis type I tumor model $Nf1^{+/-};p53^{+/-}$ which might be caused by

the cumulative effects of altered metabolism, an increase in sensitivity to hypoxia, and down-regulation of proliferation.

11.3.8 Cpt1c is overexpressed in *Nf1^{+/-}:p53^{+/-}* sarcomas

Previous studies^{20, 22} performed with the murine *Nf1^{+/-}:p53^{+/-}* tumor model revealed that the majority of sarcomas are malignant peripheral nerve sheath tumors (MPNSTs) and malignant triton tumors (MTTs) that arise within peripheral nerves. We next investigated whether Cpt1c is overexpressed in the tumors extracted from *Nf1^{+/-}:p53^{+/-}* mice. We performed real time PCR and Western blotting to analyze the expression level of CPT1C in normal muscle tissue and tumor samples from *Nf1^{+/-}:p53^{+/-}* mice that developed soft tissue sarcomas (Figure 7). Even though CPT1C is mainly expressed in brain^{10, 15, 23, 24}, we found high expression levels of Cpt1c in sarcomas extracted from *Nf1^{+/-}:p53^{+/-}* mice. In contrast, no Cpt1c expression was found in normal muscle tissues by performing real time PCR and immunoblotting experiments. Motivated by our in vitro results that Cpt1c is regulated by p53 and AMPK, we next analyzed whether increased Cpt1c expression in tumors correlates with p53 and AMPK activation. p53 and p21 protein levels, and AMPK phosphorylation at T172 were highly upregulated in sarcomas which showed elevated expression of Cpt1c (Figure 7b). It is worth noting that we observed p53 expression in all *Nf1^{+/-}:p53^{+/-}* sarcomas tested by Western blotting (Figure 7b) and by PCR (data not shown) indicating that the *Nf1^{+/-}:p53^{+/-}* tumors did not undergo loss of heterozygosity of p53. These results imply that Cpt1c may give a p53-dependent growth advantage in tumors.

11.4 Discussion

Here we have shown that CPT1C is a bona fide p53 target and plays a crucial role in sensitizing tumor cells to hypoxia and glucose withdrawal. In a murine tumor model, depletion of Cpt1c significantly reduces tumor development and increases survival of tumor bearing mice. These results suggest that CPT1C may act as an oncogene to promote cell survival in response to metabolic stress.

p53 is a tumor suppressor activated in response to a variety of cellular stresses.^{25, 26} Up to now, activation of p53 by hypoxia^{27, 28} has been commonly considered a death-inducing strategy of the cell because of its pro-apoptotic role in cancer cells. Alternatively, under acute cellular stress p53 is known to signal DNA repair, cell-cycle arrest, or senescence to maintain the viability of the cell.²⁹ Intriguingly, there is increasing evidence that p53 can promote cell survival by activating pathways of metabolic adaptation that seem to be crucial for successful cancer progression.³⁰ Matoba et al.³¹ reported that p53 directly stimulates oxidative phosphorylation by activating the synthesis of cytochrome c oxidase 2. Interestingly, disrupting the synthesis of cytochrome c oxidase 2 in cancer cells with wild-type p53 leads to glycolytic metabolism in p53-deficient tumor cells. In addition, Bensaad et al.³² demonstrated that expression of TIGAR (TP53-induced glycolysis and apoptosis regulator) attenuates glycolysis. The ability of p53 to suppress glycolysis and to promote oxidative phosphorylation might help to prevent the unrestrained glycolytic flux that is associated with malignant cell growth, which represents another manifestation of the tumor suppressive activity of p53.³³

While alterations in glucose metabolism seem to represent a major source for metabolic transformation in cancer cells, there is increasing evidence that fatty acid metabolism plays a crucial role. Fatty acid synthesis (FAS) is an energy-depleting process required for cell growth and proliferation, while fatty acid oxidation (FAO) is an oxygen-dependent catabolic process that occurs in the lumen of mitochondria. Cytokines cause cells to activate FAS and concurrently reduce FAO.³⁴ In conditions of ATP depletion, FAS is turned off in favour of FAO by AMPK-dependent inactivation of acetyl-CoA carboxylase (ACC).³⁵ In light of energy and oxygen use implications, it is likely that the hypoxic response tightly regulates the balance between FAS and FAO. Thus far, the mechanism by which cells regulate a potential switch between FAS and FAO under hypoxic conditions has not been suggested. To date, no correlation has been found between CPT family members, which are key regulators of fatty acid oxidation, and metabolic adaptation in tumor cells.

The CPT1 family (see review Bonnefont et al.⁸) consists of three members encoded by separate genes that appear to be expressed in a tissue-specific manner: CPT1A (liver isoform), CPT1B (muscle isoform), and CPT1C (brain isoform). CPT1A and CPT1B function to translocate free fatty acids to the lumen of mitochondria, where they can be degraded by beta-oxidation as source of energy. While CPT1C appears to represent a more distant family member by homology,²³ database searches suggest that CPT1C arose from a relatively recent gene duplication event.¹⁰ Indeed, unlike other CPT1 family members, two separate biochemical studies have failed to show palmitoyltransferase activity for CPT1C.^{10, 23, 36} CPT1C has been recently demonstrated to be expressed in pyramidal neurons of hippocampus and is located in the endoplasmic reticulum. Sierra et al.¹⁵ demonstrated that CPT1C possessed carnitine palmitoyltransferase activity, while Carrasco et al.²⁴ indicated that CPT1C may regulate

ceramide levels in neurons. Nevertheless, it has been reported that CPT1C conserves the affinity for Malonyl-CoA, which inhibits all the CPT1 family members.³⁷ However, it is still not clear whether the binding of Malonyl CoA to CPT1C causes a decrease in food intake and weight loss. Current experiments in our laboratory are directed at elucidating the exact subcellular localization and molecular mechanism of CPT1C to better understand the functions of this protein.

The present study demonstrates that transcription of the mouse CPT1C gene emanates from a putative p53-RE in the first intron that is sufficient and necessary to drive the p53-dependent transcription of CPT1C. We were able to detect an increase of the levels of CPT1C mRNA in human cancer cells cultured under DNA damage conditions known to activate p53 (Figure 1). Interestingly, we found that CPT1C but not p21 was induced by Staurosporin and UV240 stimuli in MCF7 cells (data not shown). This observation indicates that CPT1C is also regulated by certain unknown mechanisms apart from the p53 pathway. Unfortunately, it was not possible to confirm the increased CPT1C expression at the protein level due to lack of a good quality antibody to detect endogenous levels of human CPT1C protein.

AMPK activation stimulates a number of biological pathways in order to conserve cellular energy. AMPK achieves this by two main mechanisms: (1) by limiting cellular energy usage through the inhibition of anabolic pathways such as mTOR-dependent mRNA translation or ACC-mediated FAS, (2) or by activating pathways of catabolic metabolism to generate ATP. Recent results suggest that AMPK-dependent inhibition of anabolic growth plays a key role mediating cell survival under nutrient limitation. Abrogating lipid synthesis by inhibiting ACC activity rescues AMPK-deficient cells from glucose deprivation.³⁸ The data presented here suggest that AMPK- and p53-dependent activation of lipid catabolism via Cpt1c plays an important role in this process as well. We show here that CPT1C protein levels accumulate in cells under energy stress conditions and its expression is dependent on AMPK α and p53 (Figure 5). Moreover, ectopic expression of Cpt1c is sufficient to rescue AMPK α -deficient cells from apoptosis induced by glycolytic inhibition. Together our data indicate that Cpt1c, the newest member of the CPT family, is a downstream target of the AMPK/p53 pathway, and provides a direct link between AMPK, p53 signaling and metabolic adaptation in tumor cells.

The current literature together with our results suggests that CPT1C may have a unique function in the tumor milieu. Current targeting strategies against cancer mainly focus on specifically blocking molecular signals, which promote cell proliferation, hinder cell death, modulate the immune response or enhance neoangiogenesis. However, most of these signaling pathways are either redundant or essential in healthy tissue. A further strategy is to target the altered metabolism of cancer cells. The metabolic transformation that occurs in cancer cells and in response to hypoxia seems to represent an intrinsic part of carcinogenesis and might be altered by modulating CPT1C. Evidence that hypoxia-resistant tumors are highly aggressive and have a worse prognosis underscores that overcoming hypoxia is a major hurdle for viability in the tumor microenvironment.¹

11.5 Materials and methods

cDNA microarray screen. The screen was conducted as previously described^{7, 39}

Cell lines. DP16.1 and DP16.1/p53ts cell lines were maintained in α -modified Eagle's medium (α -MEM) containing 10% foetal calf serum (FCS). $p53^{+/+}$ and $p53^{-/-}$ MEFs were derived from 14 day old embryos, transformed with E1A/ras, and cultured in a 5% CO₂ atmosphere in Dulbecco's MEM containing 10% FCS. XL823, a gene trap ES cell line targeting CPT1C (BayGenomics, San Francisco, CA), was maintained on 1% gelatin-coated dishes in DMEM supplemented with leukemia inhibitory factor, 15% FCS, L-glutamine, and β -mercaptoethanol. $AMPK\alpha1^{-/-}$, $\alpha2^{fl/fl}/Cre^{+/+}$ MEF cells were maintained in DMEM supplemented with 10% FCS, 100 IU penicillin, 50 μ g/ml streptomycin, and transfected with Flag-tagged CPT1C or vector control using Lipofectamine 2000 (Invitrogen, New York, NY) as described previously.⁷

Prediction of promoter and p53-binding sites. Mouse genomic DNA sequence was obtained from National Center for Biotechnology Information Entrez Gene (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene>). Promoter sequence was predicted using WWW Promoter Scan program (<http://www-bimas.cit.nih.gov/molbio/proscan/>). Potential p53-responsive elements were sought using TFBIND (<http://tfbind.hgc.jp/>).

ChIP analysis. Chromatin immunoprecipitation was carried out as previously described.⁴⁰ Cells were cross-linked in formaldehyde and sonicated with 6 x 10s pulses at 50 Watt, 50% max power (Vibra Cell TM, Sonics and Material Inc, Newtown, CT). Extracts were subjected to ChIP assays using the Acetyl-Histone H3 ChIP Assay Kit (Upstate Biotechnology, New York, NY) and anti-mouse p53 antibody (FL-393; Santa Cruz Biotechnology, Dallas, TX). PCR amplification was performed using primers specific for the two regions in CPT1C intron 1 that contained consensus p53-binding sequences. The primers used were as follows: p53-RE-A, forward primer (GTACTAGTACCAGGTACAGGAGGGGC) and reverse primer (GAAGCACCTACTGCGCATGCCC); p53-RE-B, forward primer (GCCTGGCAATTGGAAATGAACAG) and reverse primer (AGTTGGAGAGGGCTTTGGGACC).

Luciferase Assay. The two potential p53-binding sites in CPT1C intron 1 were individually PCR-amplified from murine E14K ES cells and cloned into a pGL3-promoter vector (Promega, Madison, WI). These constructs were co-transfected with WT p53 or a DNA-binding mutant of p53 into $p53^{-/-}$ MEFs using Lipofectamine 2000 (Invitrogen). Luciferase activity was measured in the presence or absence of p53 and normalized to β -galactosidase activity. A luciferase construct containing the p21 promoter region, and a p53 construct with a mutation in the DNA binding site, were used as positive and negative controls, respectively.

Real time PCR. Cells were treated with various stress stimuli (sham treatment, 12 Gy of irradiation, UV 240 μ J/cm², 1 μ M staurosporine for 8 h, 10 μ M etoposide for 8 h, and 50 μ g/ml 5-fluorouracil for 8 h). Total RNA was extracted using the Qiagen Mini Kit (Sigma, Saint Louis, MO). RNA was reverse-transcribed using Superscript (Invitrogen). Specific primers for mouse GAPDH, CPT1A, CPT1B, CPT1C, CPT2 and p21 were generated using either Oligo 5 or PrimerBank. Primer sequences are available upon request. Real time PCR was performed using an SDS 7900 (Becton Dickinson, Franklin Lakes, NJ) with SYBR Green fluorescence (Applied

Biosystems, Bedford, MA). The samples were normalized to the stably expressed reference gene GAPDH. From tumor samples: RNA was isolated from frozen tumor samples using the Trizol method (Invitrogen) and the PureLink RNA Mini kit (Ambion, Austin, TX). The RNA was quantified with NanoDrop (Thermo Scientific, Asheville, NC). 2 µg of total RNA were reverse transcribed using the SuperScript III CellsDirect cDNA Synthesis kit (Invitrogen). Real Time PCR was performed using a LightCycler 480 System and SYBR Green I Master mix (Roche, Indianapolis, IN). Raw Ct values were normalized against control housekeeping genes (GAPDH, beta actin and HPRT) and analyzed using the $\Delta\Delta Ct$ method.

In situ hybridization. In situ hybridization was performed as previously described.^{41, 42} Briefly, E12.5 embryos of C57BL/6 *p53^{+/-}* and *p53^{-/-}* mice were sham-irradiated or subjected in utero to 5Gy X-ray irradiation. At 8 h post-irradiation, recovered embryos were dissected, fixed in 4% paraformaldehyde (PFA), processed and embedded in paraffin. Tissue sections (4-6 mm) were cut, deparaffinized, acetylated and exposed to ³³P-UTP-labelled riboprobes. The CPT1C cDNA template (from which the riboprobes were made) was a 700 bp fragment cloned into pBluescript SK (Invitrogen). The p21 cDNA template was a full-length fragment. Sense and antisense probes were synthesized from linearized templates using T3 or T7 RNA polymerase, labelled with [³³P]-UTP (Amersham, Arlington Heights, IL), and processed as previously described.

Mouse models and animal care: Cis *Nf1^{+/-}:p53^{+/-}* mice^{19, 20} were kindly provided by K. Cichowski,²² Dana-Farber/Harvard Cancer Center, USA, and mice depleted in *Cpt1c* were generated using a gene trap approach as described (BayGenomics).⁷ Both mouse models were inbred in the C57BL/6 background. All mice were maintained within the Biologisches Zentrallabor barrier facility, University Hospital Zürich and all the experiments were approved by the Zurich Kantonales Veterinäramt (license number 161/2007, 15/2011).

We crossed *Cpt1c^{gt/gt}* mice into the *Nf1^{+/-}:p53^{+/-}* background. The mice were monitored three times per week. As soon as the animals showed signs of tumors and distress, they were euthanized and the tumors were isolated.

Statistical analysis and survival studies: The survival of *Nf1^{+/-}:p53^{+/-}:Cpt1c^{gt/gt}* mice was compared with *Nf1^{+/-}:p53^{+/-}* mice, *Cpt1c^{gt/gt}* and wild-type control (C57BL/6). The number of mice per group is indicated in the legend. The survival of the mice was plotted on a Kaplan-Meier curve for individual genotypes against the animal age in months. The survival probabilities were calculated using a public survival calculator (<http://www.hutcheon.net/Kaplan-Meier.htm>) and GraphPad Prism 5. The results were analyzed with the log-rank ($p < 0.0001$) test using GraphPad Prism 5.

Histological analysis of tumors: Tumors were fixed in 4% formalin for one week at 4 °C. Then, they were embedded in paraffin, sectioned, and stained with haematoxylin and eosin. Immunohistochemistry was performed to test for apoptosis and proliferation using a rabbit polyclonal anti-cleaved Caspase-3 (Cell Signaling Technology, Beverly, MA) and a rabbit monoclonal anti-Ki67 (clone SP6, Thermo Scientific). The samples were analyzed with Adobe Photoshop and ImageJ and the histological scores were obtained by calculating the ratio between the number of positive-stained cells and the total cell number/field.

Protein lysates and immunoblotting: Frozen tumor samples were thawed, washed in PBS and minced in extraction buffer containing 50 mM NaHCO₃ pH 8.3, 0.25 M NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X100 and protease inhibitors. The tissues were then disrupted by Polytron homogenization and incubated on ice for 30 min. The homogenates were then centrifuged at 10000 x g for 30 min at 4°C to remove the cell debris. The supernatant was analyzed by Western blotting. 50 µg of protein lysates were subjected to SDS-PAGE and immunoblotted with antibodies indicated. Antibody sources are as follow: mouse monoclonal anti mouse CPT1C antibody (clone 1E11, generated in our Laboratory); mouse monoclonal anti-p53, rabbit polyclonal anti-Pan-actin, anti-AMPKα, anti-phospho-AMPKα (Thr172), and anti-phospho-ACC (Ser79) (Cell Signaling Technology); mouse monoclonal anti-p21 (Santa Cruz Biotechnology).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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11.6 Figures

Figure 1. Induction of endogenous CPT1C by stress stimuli in a p53-dependent manner

a and b: AML (p53 WT), K562 (p53 mutant) and p53 WT and mutant HCT116 cells were subjected to different stress stimuli known to activate p53 and RT-PCR was performed using SYBR Green to detect CPT1C mRNA. p21 served as a positive control for p53 activation. All values shown were normalized to GAPDH expression. Results shown are one trial representative of at least 3 experiments. c: The U87 cell line was treated with the indicated DNA-damaging stimuli and real time RT-PCR was performed to detect upregulation of expression of the indicated CPT family members. 6 Gy, 6 Gray of irradiation; UV240, 240 μ J/cm² of ultraviolet; 5-FU, 50 μ g/ml of Fluorouracil; Etoposide, 10 μ M of Etoposide. p21 was used as positive control. All values shown were normalized to GAPDH and the response level was calculated relative to the untreated control. Results shown are one trial representative of at least 3 experiments.

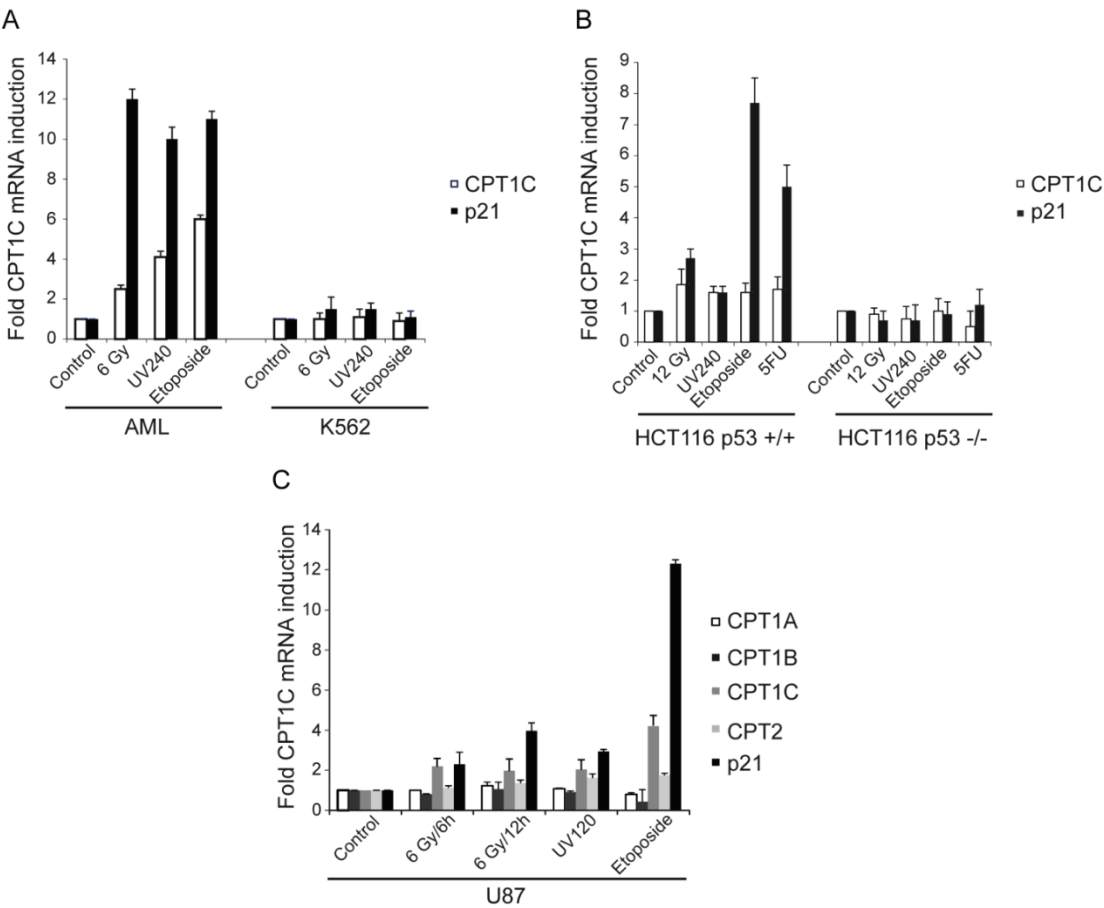


Figure 2. CPT1C is a p53 target gene

a: p53 binding sites. Computational analysis revealed two putative p53-responsive elements, p53-RE-A and p53-RE-B, located in intron 1 in the CPT1C promoter region as indicated.

b: p53 binding to p53-RE-A. ChIP analysis was performed on DP16.1/p53ts cells cultured at 37°C or 32°C. The p53-RE of p21 was used as positive control; unprecipitated genomic DNA was the loading control. N.A: no p53 antibody. Results shown are one result representative of 3 trials.

c: p53-RE-A binds to p53 and activates transcription. The indicated luciferase reporter constructs were transfected into E1A/ras-transformed *p53*^{-/-} MEFS, with or without co-transfection of WT p53. Relative luciferase activity was taken as the relative transcriptional activity. pGL3-SV40, vehicle control; p53-RE-A*, mutated p53-RE-A (G→T at position 42); p21 was used as positive control for p53 transcription.

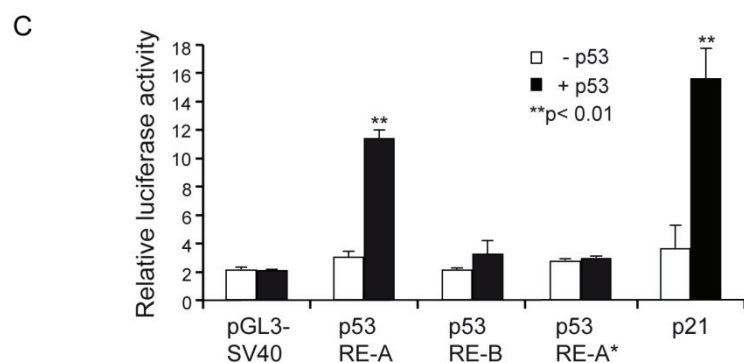
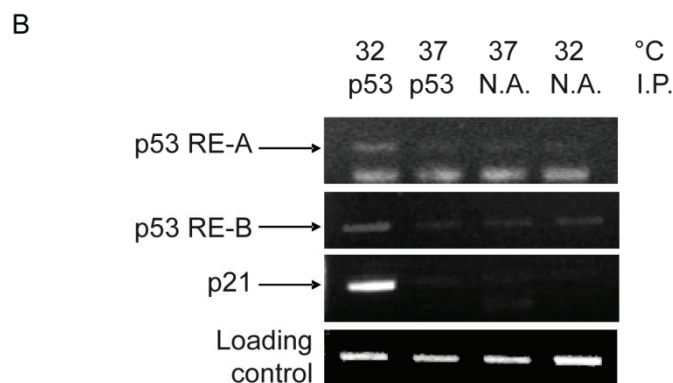
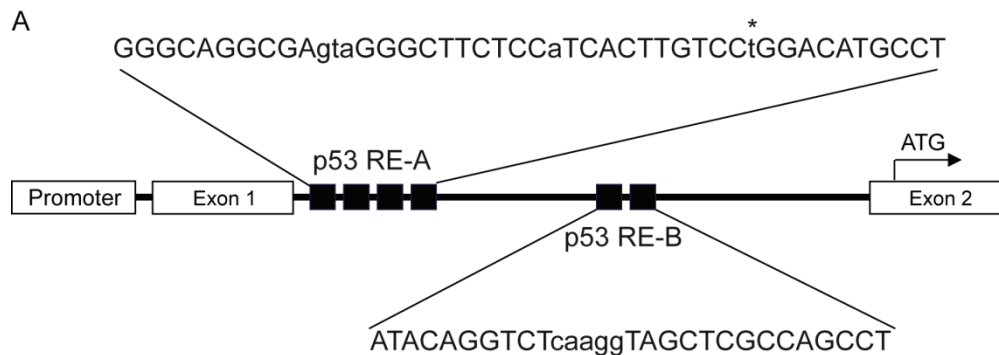


Figure 3. p53 upregulates CPT1C in vivo

E12.5 C57BL/6 embryos from $p53^{+/-}$ and $p53^{-/-}$ mice were subjected to 5 Gy ionizing radiation (Irrad) in utero. Embryos were harvested and prepared for in situ hybridization at 8 h post-irradiation. Incubation of midbrain sections with a CPT1C riboprobe showed that CPT1C mRNA was upregulated in irradiated $p53^{+/-}$ cells (c) but not in irradiated $p53^{-/-}$ cells (d) compared to sham-irradiated controls (a and b).

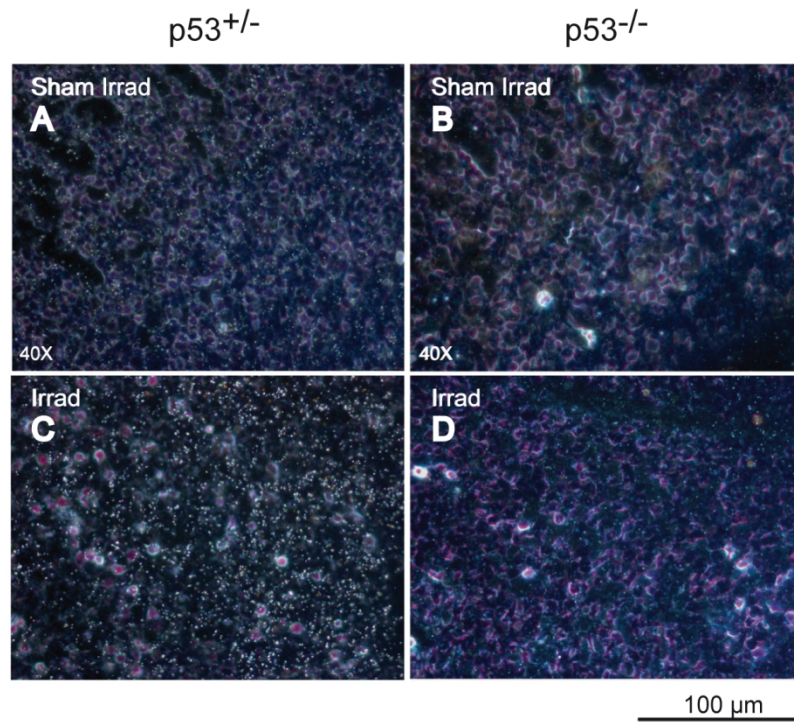


Figure 4. Cpt1c is induced by hypoxia and glucose deprivation in a p53-dependent manner.

a: Mouse embryo fibroblasts from $p53^{+/+}$ and $p53^{-/-}$ mouse embryos at day 13.5 were treated with either normoxia (20% O_2) or hypoxia (0.2% O_2) for 7 hours, then the cells were harvested for total RNA isolation. The CPT1A, B, C and CPT2 mRNA levels were measured using real time PCR. RNA levels were normalized to GAPDH. VEGF was used as positive control.

Using ChIP analysis and PCR with primers specific for the p53-binding sites of Cpt1c, amplification of p53-RE was tested in p53 WT MEFs treated with either glucose deprivation (b) or hypoxia (c). Bax was used as positive control.

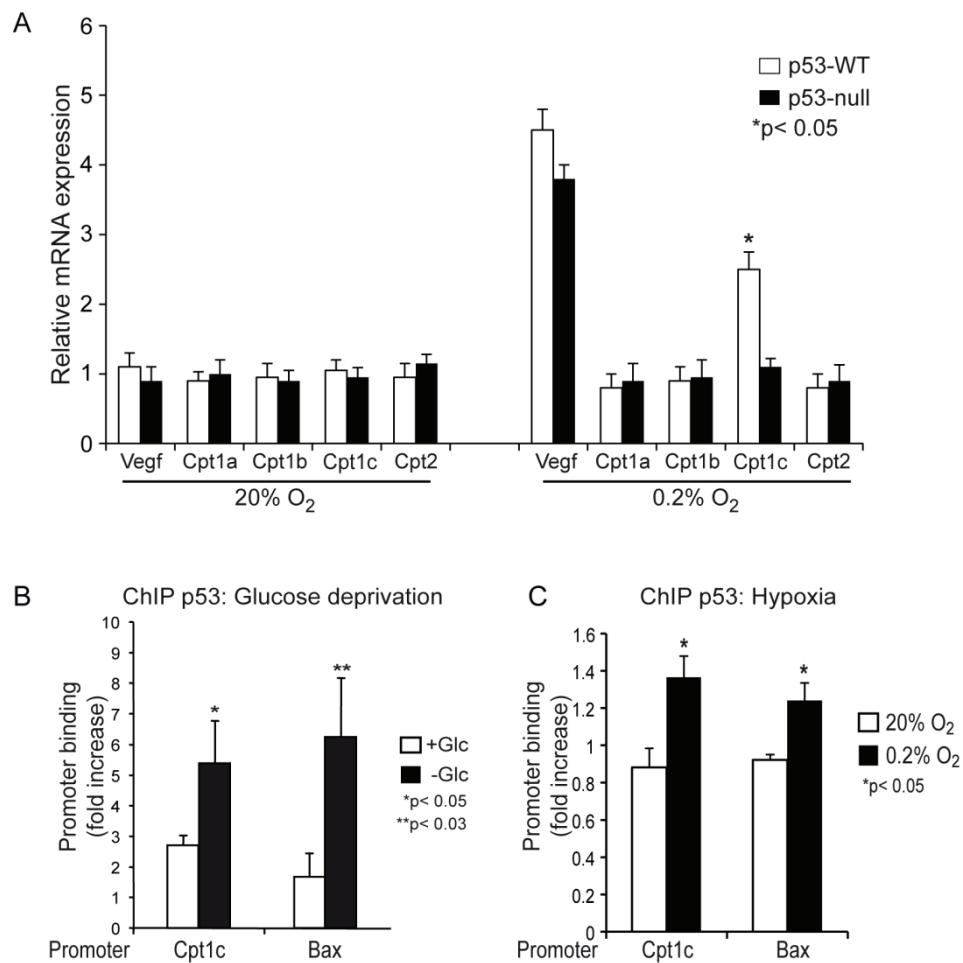


Figure 5. Cpt1c is induced by energetic stress in an AMPK and p53 dependent manner

a: AMPK $\alpha 1^{-/-}$, $\alpha 2^{fl/fl}$ MEFs +/- Cre cells were treated for 16 hours in 1mM Metformin. Cells were lysed with CHAPS buffer, and immunoblotted with antibodies indicated.

b: $p53^{+/+}$ and $p53^{-/-}$ 3T3 MEF cells were treated for 16 hours in 1mM Metformin. Cells were lysed with CHAPS buffer and immunoblotted with antibodies indicated.

c: Cell lines stably expressing CPT1C protein were generated in control (Cre -) and AMPK α -deficient (Cre +) MEFs. Clones of each cell type were immunoblotted with antibodies indicated.

d: A PI-exclusion apoptosis assay was performed with AMPK $\alpha 1^{-/-}$, $\alpha 2^{fl/fl}$ MEFs (Cre -, open bar; Cre +, closed bar) expressing either Flag-Cpt1c (+) or control vector (-). MEFs were treated with 2-deoxyglucose. Data expressed in Mean \pm SEM.

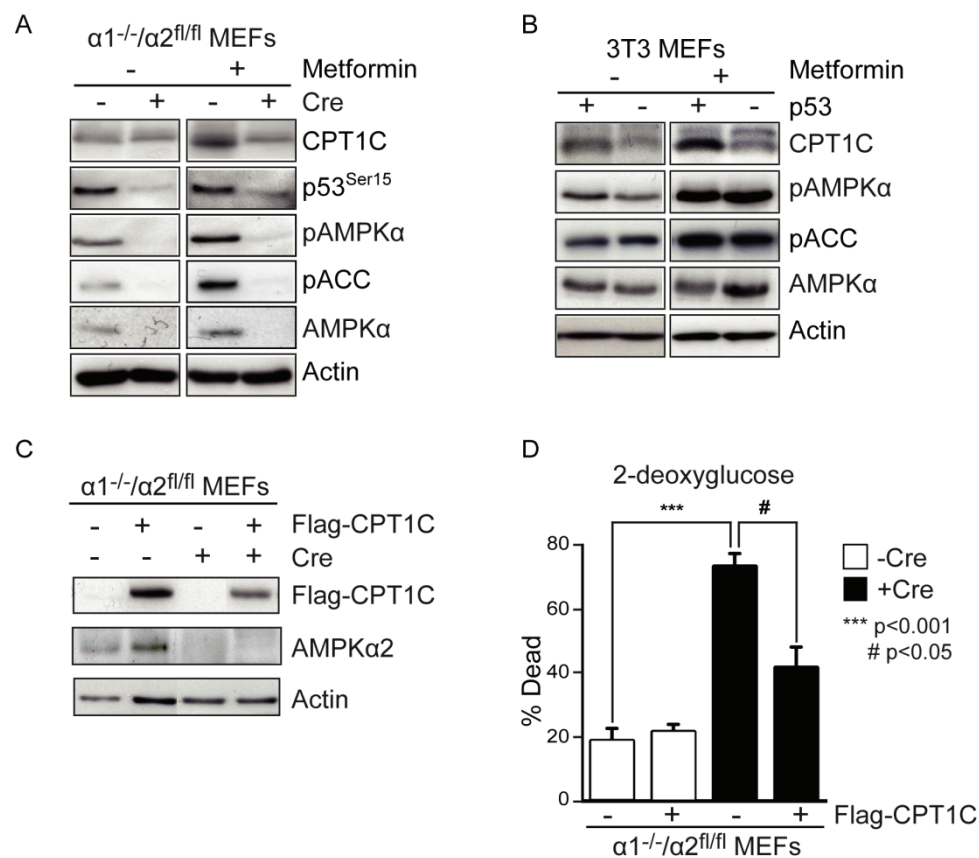


Figure 6. Cpt1c depletion in the murine neurofibromatosis type I tumor model significantly increases their survival rate and suppresses tumor transformation and metastasis

a: Kaplan Meier survival curve. The survival of the *Nf1*^{+/-}:*p53*^{+/-}:*Cpt1c*^{gt/gt} mice (green, n=53) was compared with the *Nf1*^{+/-}:*p53*^{+/-} mice (blue, n=53), the *Cpt1c*^{gt/gt} mice (red, n=54) and a wild-type control (C57BL/6 strain, black, n=33). The survival of the mice was plotted on a Kaplan-Meier curve for individual genotypes against the animal age in months.

b: *Nf1*^{+/-}:*p53*^{+/-}:*Cpt1c*^{gt/gt} mice showed less metastasis cases and fewer cases of spleen hyperplasia and sarcomas. There is no variation in the brain tumor cases when compared with *Nf1*^{+/-}:*p53*^{+/-} mice.

c: Histological analysis of tumor phenotypes in *Nf1*^{+/-}:*p53*^{+/-} and *Nf1*^{+/-}:*p53*^{+/-}:*Cpt1c*^{gt/gt} mice. *Nf1*^{+/-}:*p53*^{+/-} (n=6) and *Nf1*^{+/-}:*p53*^{+/-}:*Cpt1c*^{gt/gt} (n=4) sarcomas were analyzed using immunohistochemistry staining as indicated. Paraffin sections were stained with Ki67 (brown) and cleaved Caspase-3 (CC3, red) to analyze the proliferation and the apoptotic rate respectively. Data expressed is Mean ± SD.

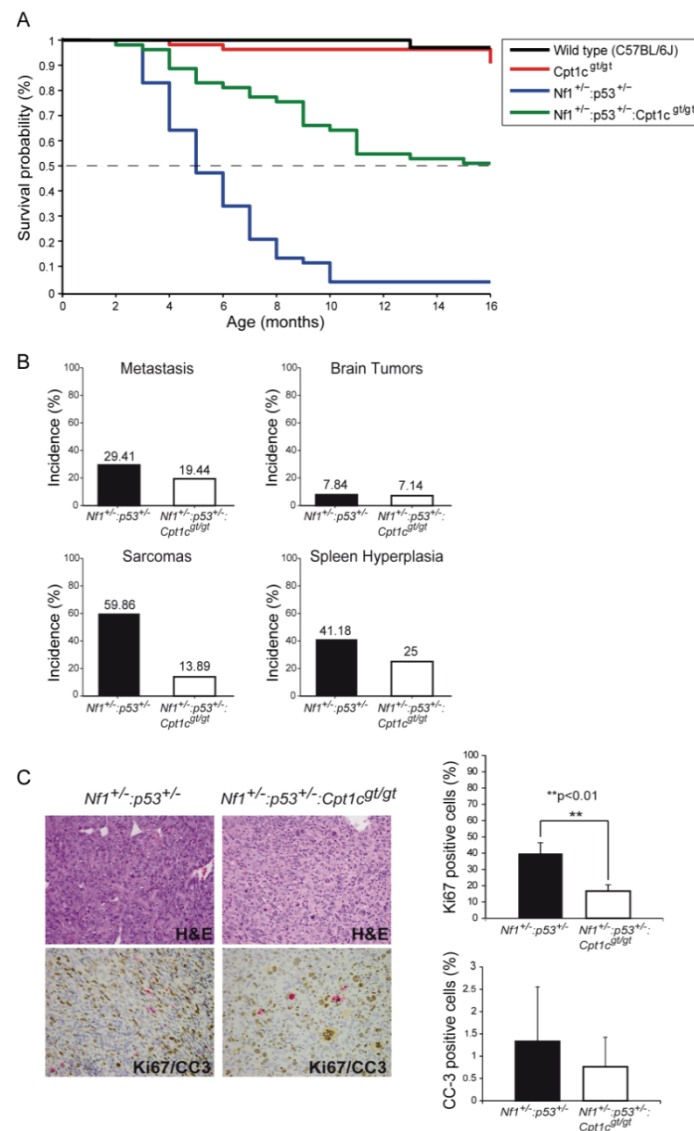
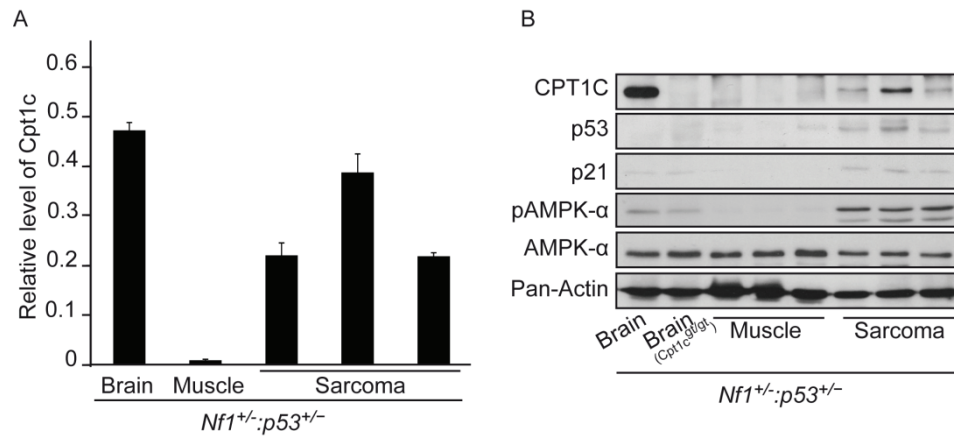


Figure 7. CPT1C is upregulated in tumor tissues

Sarcomas and normal muscle tissues were isolated from *Nf1*^{+/-}:*p53*^{+/-} mice. RT-PCR analysis (a) and Western blot (b) showed that Cpt1c is upregulated in sarcomas. Data represents at least 3 independent experiments. The brain tissues from *Nf1*^{+/-}:*p53*^{+/-} and *Nf1*^{+/-}:*p53*^{+/-}:*Cpt1c*^{gt/gt} mice were used as Cpt1c positive and negative controls as indicated.



11.7 References

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Publication

- ❖ Sanchez-Macedo N; Feng J; Faubert B; Chang N; Elia A; Rushing EJ; Tsuchihara K; Bungard D; Berger SL; Jones RG; Mak TW and Zaugg K. Depletion of the novel p53-target gene carnitine palmitoyltransferase 1C delays tumor growth in the neurofibromatosis type I tumor model. Cell Death Differ. 2013 Apr; 20 (4): 659-68.

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