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p53 regulates Mevalonate Metabolic pathway

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To my family

and to Andrea

... "If you can't be the sun, be a star;
It isn't the size that you win or you fail
Be the best of whatever you are".
Martin Luther King

... "Se non puoi essere il sole, sii una stella;
Non è la grandezza che ti fa vincere o fallire,
Sii sempre il meglio di ciò che sei".
Martin Luther King

TABLE OF CONTENTS

LIST OF PUBLICATIONS

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LIST OF ABBREVIATIONS

Akt/PKB: Protein Kinase B
AMPK: AMP-activated protein Kinase
ApoE: Apolipoprotein E
ARF: Alternate Reading Frame
ATM: Ataxia Teleangiactasia Mutated
ATP: Adenine Triphosphate
ATR: Serine/Threonine-protein kinase
BBB: Blood Brain Barrier
CaMKKβ: Calmodulin-dependent protein kinase-kinase β
CDKN1A: Cyclin Dependent Kinase inhibitor 1A (p21)
ChIP: Chromatin Immunoprecipitation
Chk2: Checkpoint homolog kinase 2
DBD: DNA Binding Domain
EtOH: Etanol
FDFT1: Farnesyl diphosphate Farnesyl Transferase1
FDPS: Farnesyl Diphosphate Synthetase
FPP: Farnesyl pyrophosphate
FTI: Farnesyl Transferase Inhibitor
GBM: Glioblastoma Multiforme
GLUT: Glucose Transporter
GOF: Gain of Function
HEK293: Human Embryonal Kidney 293
HMC-CoA: 3'-hydroxy-3'-methylglutaryl-CoA
HMGCR: 3'-hydroxy-3'-methylglutaryl-CoA Reductase
IGF: Insulin Like Growth Factor
Insig: Insulin-induced gene
JNK: Janus Kinase
LDLR: Low Density Lipoprotein Receptor
LKB1: Liver Kinase B
LRP1: LDLR Related Protein 1
LXR: Liver X Receptor
Mdm2: Mouse-double minute 2
mTOR: mammalian Target of Rapamicin
mutR273H-p53: plasmid containing mutation R273H in p53 sequence
MVA: Mevalonate
NES: Nuclear Export Signal
NHA: Normal Human Astrocytes
NLS: Nuclear Localization Sequence
p53RE: p53 Responsive Element
pCMV-p53: wild type p53 containing plasmid
Pgp: P-glicoprotein
PMG: Phosphoglicerate Mutase
PPP: Pentose Phosphate Pathway
PT-α: Pifithrin-α
qRT-PCR: quantitative RealTime-Polymerase Chain Reaction
RabGGTA: Rab-Geranyl-geranyl Transferase 1α
ROS: Reactive Oxygen Species
SCAP: SREBP-cleavage activated protein
shRNA: short hairpin RNA
SIN: SXL-interactor (SXL: Sex-Lethal)
SRE: Sterol Responsive Element
SREBP: Sterol Responsive Element Binding Protein
SSD: Sterol Sensing Domain
TCA: Tricarboxylicyclic acid

TIGAR: TP53-Induced Glycolysis and Apoptosis Regulator

TSS: Transcription Start Site

ABSTRACT

The mevalonate pathway is an important metabolic pathway implicated in multiple aspects of tumorigenesis. In this study, I provided evidences about genetic and pharmacologic perturbation of p53, which directly influenced expression of mevalonate pathway enzymes, including 3'-Hydroxy-3'-Methylglutaryl - CoenzymeA Reductase, Mevalonate Kinase, Farnesyl Diphosphate Synthase, Farnesyl Diphosphate Farnesyl Transferase 1.

Three different cell lines have been considered, U343 MG (U343) and U251 MG (U251) glioma cells, both classified as IV grade glioblastoma cell lines, with two different malignancy grade, and Normal Human Astrocytes (NHA), their normal counterpart.

In particular, NHA and U343 cells have wild type p53 (wtp53) while U251 bearing mutation (R273H)p53. This mutation affects p53 DNA binding site, preventing transcriptional function of the protein.

Different basal expression level of the mevalonate pathway's genes have found among the different cell lines considered and I hypothesized that this could be ascribable to p53 mutation status and function

Indeed, I observed that functional and active p53 recognized specific p53 Responsive Elements (p53REs) present in MVA enzymes gene-sequences. p53 bound to these regions correlated with increased transcription levels of mentioned genes and such effect has abolished in cells bearing mut(R273H)p53 or by site-directed mutagenesis of p53REs.

These new findings expose another facet of p53 functions, unrelated to tumor suppression, and render it a novel regulator of mevalonate pathway providing insight into the role of this pathway in cancer progression.

1. BACKGROUND

1.1 Mevalonate pathway in cancer cells metabolism

Mevalonate (MVA) pathway initiates with the enzymatic conversion of acetoacetyl-CoA into 3'-hydroxy-3'-methylglutaryl-CoA (HMG-CoA) catalyzed by the HMG-CoA synthase, the HMG-CoA Reductase (HMGCR), the rate-limiting enzyme of this metabolic pathway, conducts next step. Following a series of twenty-seven biochemical reactions, enzymes lead to cholesterol synthesis (Bloch K., 1965). Moreover, the cholesterol biosynthesis pathway generates a series of intermediates required to isoprenylation of small GTPase, such as Ras and Rho. This post-translational modification mainly affects proteins containing CAAX motif at their C-terminus, isoprenylation is important for protein function and promotes activation and translocation of modified proteins to plasma membrane (Konstantinopoulos PA et al. 2007).

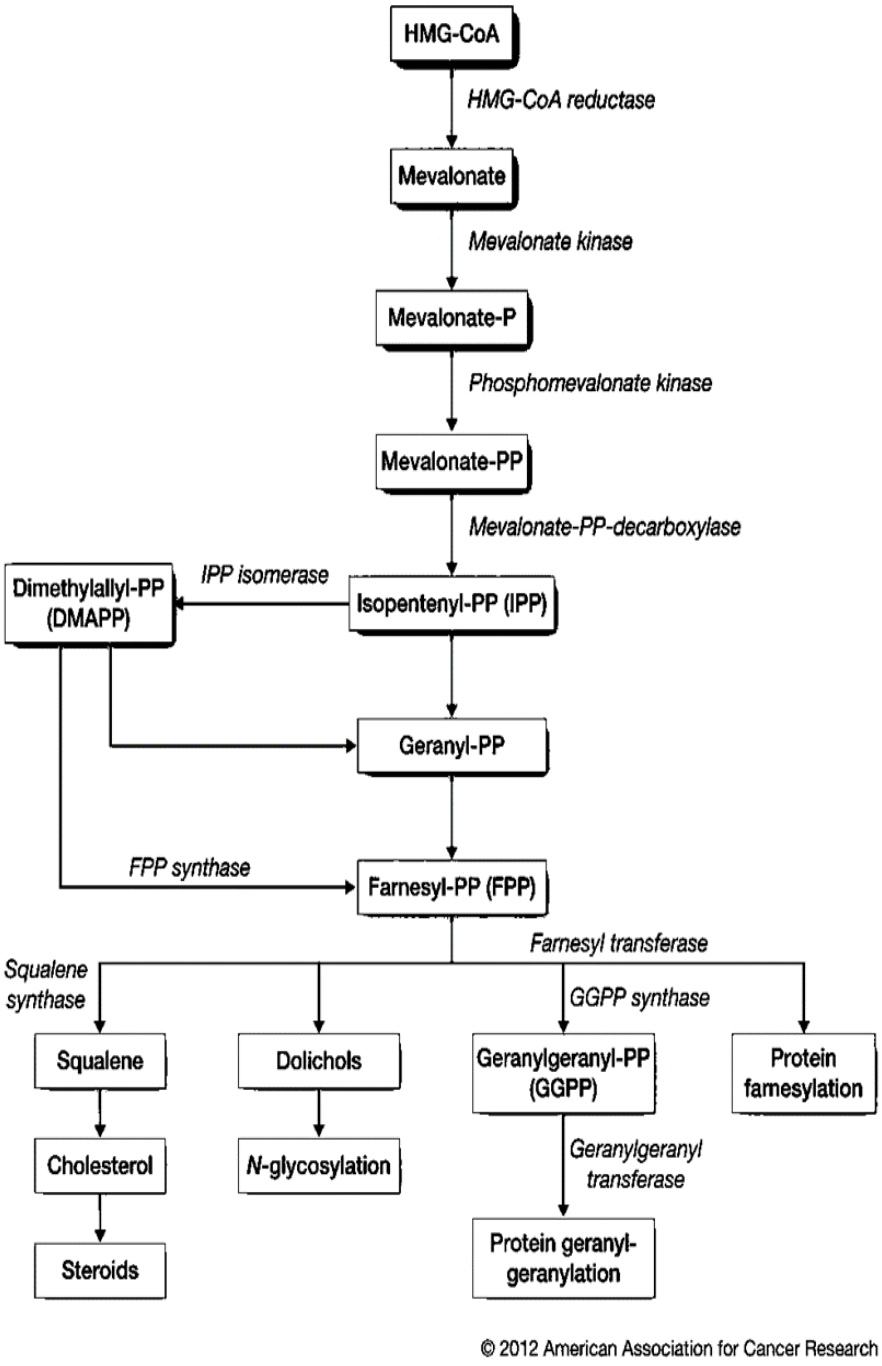


Figure 1. Schematic representation of Mevalonate Pathway enzymes and reactions

Generally, final product of MVA pathway, cholesterol, is necessary for membranes assembly, for steroid hormones' synthesis and bile acids formation. Endogenous synthesis maintained the steady levels by three main mechanisms: 1) Regulation of HMGCR levels and activity, 2) Control of excess intracellular cholesterol rate by regulation of Acyl-CoA cholesterol acyltransferase (also called Sterol O-acyltransferase), 3) Regulation of plasma cholesterol levels by LDL receptor (LDLR) endocytic activity, able to capture circulating Low-density Lipoproteins (LDL) and High-density Lipoproteins (HDL).

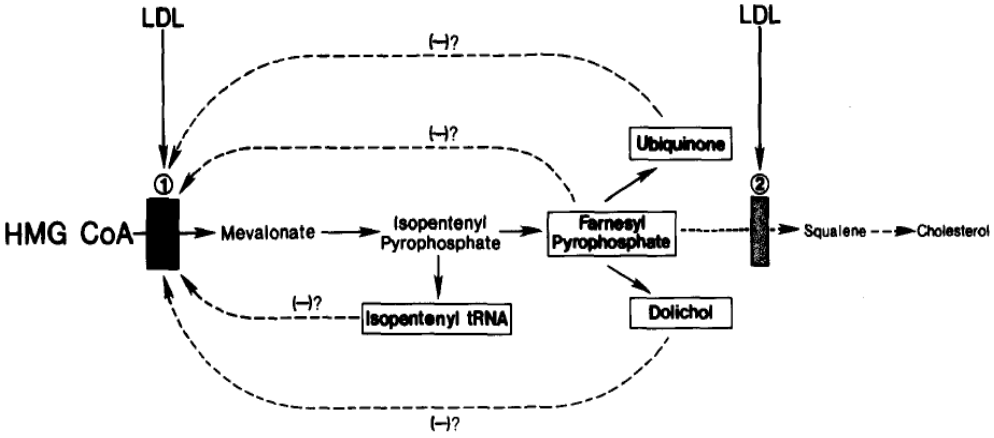
The primary mechanism in cholesterol synthesis controlling is HMGCR regulation, this happens through several mechanisms: feedback inhibition, rate of enzyme degradation, control of gene expression and post-translational phosphorylation/de-phosphorylation balance and cholesterol manages by itself these mechanisms.

When cholesterol levels increase, it binds the sterol-sensing domain (SSD) on HMGCR regulation domain, acting as a feedback inhibitor of pre-existing enzymes. It is able to induce rapid degradation of enzymes through the cholesterol-induced poly-ubiquitination of HMGCR and consequent proteasome degradation. When cholesterol and its intermediate mevalonate are in excess, they reduce the amount of mRNA of HMGCR and its gene expression (Nakanishi M. et al 1988).

Like cholesterol, non-sterol compounds, derived from mevalonate downstream reactions are essential for cell growth and participate with cholesterol in a type of multivalent feedback regulation of HMGCR (Brown MS and Goldstein JL, 1980) (Figure 2).

Post-translational modification such as phosphorylation and dephosphorylation could regulate HMGCR enzyme. Phosphorylations induced by AMP-activated protein kinase (AMPK) decrease HMGCR activity. AMPK is activated itself by two phosphorylation made by Liver Kinase B (LKB1), a serine-threonine kinase that directly phosphorylates and activates AMPK (Shackelford DB and Shaw

RJ, 2009) and Calmodulin-dependent protein kinase-kinase β (CaMKKβ), that phosphorylate AMPK in response to intracellular Ca^{++} increase.



J Lipid Res 21(5):505-17

Figure 2. Model for multivalent feedback regulation of HMG CoA reductase. N° 1 and n° 2 boxes indicate sites of action of LDL-derived cholesterol, able to reduce HMGCR activity (1) and squalene synthase (2). The dashed lines designate other HMGCR regulators non-sterol compunds.

Such hormones participate to control of AMPK/HMGCR regulation.

Insulin stimulates the removal of phosphates and, thereby, activates HMGR activity, while glucagon and epinephrine negatively affect cholesterol biosynthesis (Ness GC and Chambers CM, 2000)

The well-known transcriptional regulator of lipid pathways' enzymes is SREBP, the Sterol Responsive Elements Binding Protein. Transcriptional regulator SREBP includes a family of nuclear factors that bind to the sterol regulatory element (SRE) present in promoter regions of enzymes in charge of lipid synthesis, such as LDLR and HMGCR (Briggs MR et al, 1993; Vallett SM et al 1996). There are two distinct SREBP genes, SREBP-1 and SREBP-2. In

addition, the SREBP-1 gene codes for two different isoforms because of alternative splicing: SREBP-1a and SREBP-1c/ADD1.

SREBP-1a regulates all SREBP-responsive genes in both the cholesterol and fatty acid biosynthetic pathways. SREBP-1c controls the expression of genes involved in fatty acid synthesis and is involved in the differentiation of adipocytes and its homologous in rat is also called Adipocyte Determination and Differentiation factor 1 (ADD1). SREBP-1c is also an essential transcription factor downstream of the actions of insulin at the level of carbohydrate and lipid metabolism.

SREBP-2 is the predominant form in liver tissue and it exhibits preference at controlling the expression of genes involved in cholesterol homeostasis, including all of the genes encoding the sterol biosynthetic enzymes. In addition, SREBP-2 controls expression of the LDLR gene.

High sterols activate expression of the SREBP-1 gene but do not exert this effect on the SREBP-2 gene. The sterol-mediated activation of the SREBP-1 gene occurs via the action of the liver X receptors (LXRs). The LXRs are members of the steroid/thyroid hormone superfamily of cytosolic ligand binding receptors that migrate to the nucleus, upon ligand binding, and regulate gene expression binding specific target sequences.

All three isoforms of SREBPs have to be proteolytically activated and their cleavage is regulated by sterol intracellular levels. A protein called SREBP cleavage-activating protein (SCAP) binds the C-terminus of SREBP and is crucial for its activation.

Insig protein, the Insulin-induced gene, also participates in this process. When cells have sufficient sterol content, SREBP and SCAP are retained in the endoplasmic reticulum via the SCAP-Insig interaction. Both SCAP and Insig create a network able to regulate SREBP transcriptional activity organizing a kind of “sterol-sensor” in the cell.

Insig participates also in HMGCR sterol-induced degradation. When sterols are scarce in cells Insig-SCAP-SREBP inhibiting-interaction doesn't work and there is no-binding between Insig and SCAP, in this way SCAP drives SREBP to Golgi where it will activated by cleavage (http://themedicalbiochemistrypage.org; Shimano H, 2001).

All these evidence demonstrated that HMGCR, the rate-limiting enzymes of MVA pathway, has a very fine regulation.

During tumorigenesis, there are significant changes in energy metabolism, the Warburg effect is the well-known metabolic adaptation of cancer cells and it is a consequence of cell's increased energetic requirements and metabolic pathways activation (Warburg O, 1956). Recent data reported elevated mevalonate synthesis in transformed malignant cells (Ginestier C et al 2012; Duncan RE, 2004; Clendening JW et al, 2010). The possible mechanisms that lead to increased lipid synthesis could be: 1) the abundance of precursor derived from hyper-activation of glycolysis (such as acetyl-CoA) (Clendening JW et al, 2008), 2) the HMGCR loss of feedback control or 3) its increase in function and expression (Larsson O, 1996; Mo H and Elson CE, 2004).

Clendening et al. demonstrated, in 2010, that HMGCR ectopic expression contributes to cancer progression in hepatic and breast cancer cooperating with Ras oncogene (Clendening JW et al, 2010).

Furthermore, exogenous administration of mevalonate, in xenograft-bearing mice, was also shown to promote tumor growth (Duncan RE, 2004).

Other mevalonate enzymes are involved in cancer progression and resistance to chemotherapy that is the case of Farnesyl Diphosphate Synthetase (FDPS), able to catalyze the formation of the farnesyl pyrophosphate (FPP).

Isoprenylated proteins are involved in the pathogenesis and progression of some cancer (McTaggart SJ, 2006), specifically, in U87 glioma cell lines, FDPS is overexpressed and significantly attenuates apoptosis induced by paclitaxel, a mitotic inhibitor drug (Woo IS et al, 2010). Previous reports showed that

inhibition of FDPS in colorectal cancer play an important pro-apoptotic role (Notarnicola M et al, 2004) and use of specific inhibitors of this enzyme induce cell death in human cancer cells. (Senaratne SG et al, 2000; Riebeling C et al, 2002).

New therapeutic strategies aimed to use inhibitors of MVA enzymes to interfere with cancer progression and are supported by growing clinical and experimental evidences (Silvente-Poirot S and Poirot M, 2012).

Statins, a well-known drugs' family, reduce endogenous synthesis of cholesterol because they are specific inhibitors of HMGCR enzyme. This group of drugs includes: lovastatin, simvastatin, atorvastatin, fluvastatin and pravastatin and are used by millions of people to improve hypercholesterolemia and reduce cardiovascular risks.

Recent reports also investigated statins as candidate drug in cancer treatment protocols and many retrospective studies evidentiated a weak effect in reducing cell cycle activation and tumor growth in melanoma, colon, lung, breast, thyroid in vitro and in vivo (Wang Y et al, 2013; Zanfardino M et al,2013; Agarwal B et al, 2002; Yu X et al, 2013; Seeger H et al, 2003; Laezza C et al, 2008).

Statins inhibit Ras prenylation and reduce cholesterol biosynthesis, promote cell cycle arrest and proteasome activity (Bjarnadottir O et al, 2013; Singh PP et al, 2013; Corcos L et al, 2013). More, combined administration of statins with conventional and cytotoxic chemotherapy showed increase of apoptotic rate and differentiation state (Drucker L et al, 2004) and recent studies evidentiated that depletion of MVA intermediates, such as geranylgeranyl and farnesyl, by the use of statins, allows a reduction of the multidrug resistance (MDR) mechanism in cancer (Mehta NG and Mehta M, 2010).

Other molecules able to reduce MVA pathway enzymes activity had tested in recent years in cancer cells. Riganti and collegues showed a correlation between the rate of the cholesterol synthesis and the MDR using Zoledronic acid, a potent

aminobiphosphonate targeting the downstream enzyme of MVA pathway, the FDPS (Riganti C et al, 2013).

They showed that Zoledronic acid interrupted Ras- and RhoA-dependent downstream signaling pathways indicating that MVA pathway activity can directly regulate Pgp, P-glicoprotein, expression (Riganti C et al, 2013).

More, in recent clinical trials, different research groups, tested the Farnesyltransferase inhibitors (FTIs) as anti-cancer agents, with the purpose of farnesyl-transferase inhibition to disrupt oncogenic Ras farnesylation (Garcia AM et al, 1993; Sebti SM and Hamilton AD, 2000; Holstein SA and Hohl RJ, 2012).

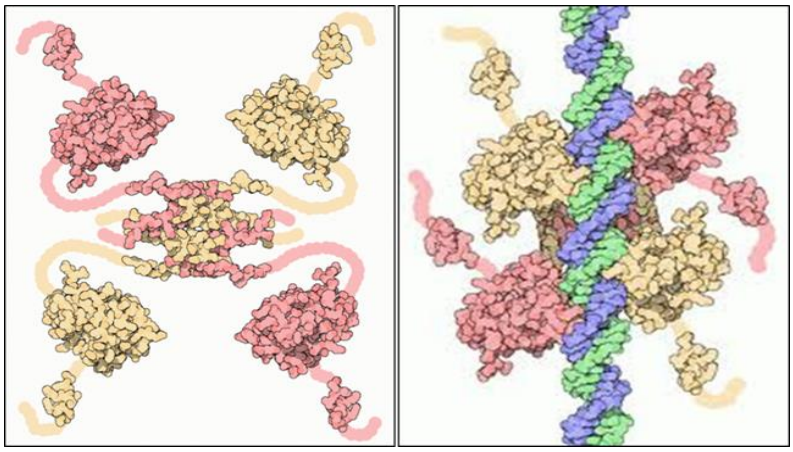
These reports highlighted the importance of metabolic perturbation and adaptation in cancer biology. In particular, MVA pathway seems to contribute to tumor progression and support a strong proliferation rate of transforming cells; at the same time, these evidences disclosed new understandings about cancer metabolism and propose new intriguing target in cancer therapy.

1.2 p53

The p53 is the most well studied tumor suppressor protein in cell biology and often it is referred to as the "*Guardian of the Genome*".

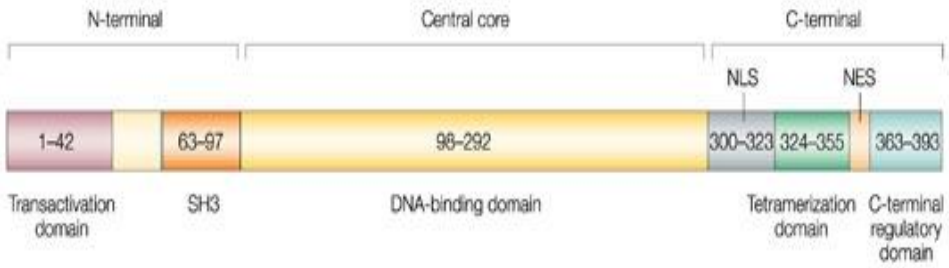
The list of known functions of p53 includes a pivotal role in apoptosis induction, DNA repair and cell senescence, regulatory roles in cell cycle arrest and angiogenesis. p53 binds to many regulatory sites in the genome and allow production of proteins that induce cell cycle arrest until the damage is repaired, or, if the challenge is too severe, p53 initiates the process of programmed cell death, or apoptosis, which directs the cell to commit suicide and permanently removing the damage.

Functional p53 arranged by four subunits (Figure 3), each subunit could be divided in three main domains (Figure 4): 1) the N-terminal domain containing the *Transactivation domain* and the *Src homology 3-like (SH3) portion*, 2) the central core with the *DNA binding domain* and 3) the C-terminal domain, containing the *Tetramerization* and *Regulatory domains*.



http://www.rcsb.org/pdb/101/motm.do?momID=31

Figure 3. **p53's tetramer structure.** p53 is active only as tetramer structure. Four subunits of p53 bind through their transactivation domains and compone the tetramer-active p53. In this conformation, p53 is able to bind and interact with specific DNA regions and activate transcription of target genes or recruit other transcription factors or co-factors and initiates genes' transcription.



Nature Reviews Cancer 4, 793-805 (2004)

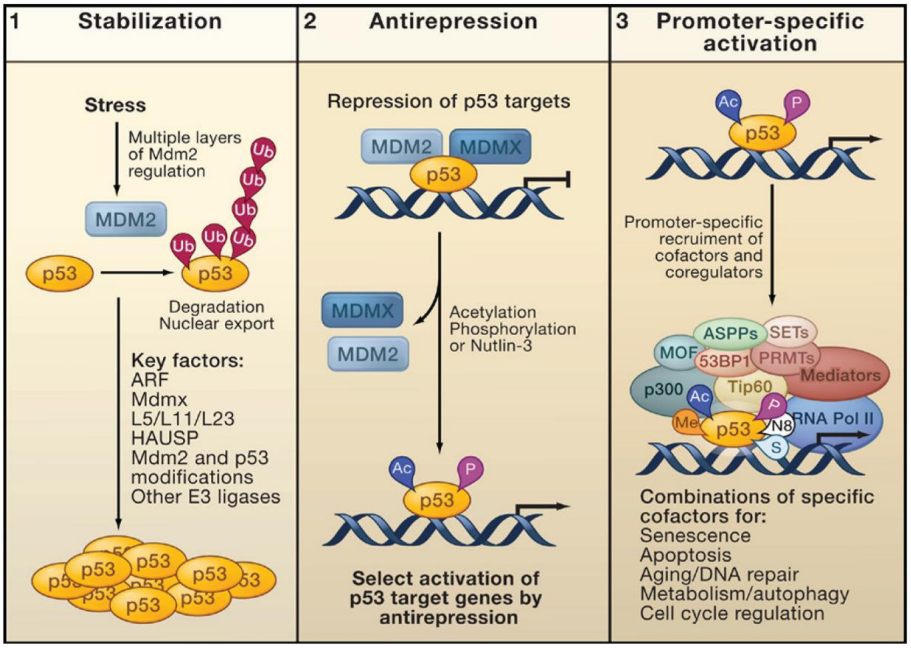
Figure 4. **p53's domains**: 1) The N-terminal domain containing the transactivation domain, it is required for transactivation activity and interacts with other transcription factors and with Mdm2 [1] and with acetiltransferases; the SH3 domain is a proline-rich domain required for interaction of p53 with SIN3[2], which protects p53 from degradation. 2) The central core is made up primarily of the DNA-binding domain that allow p53 to bind specific DNA responsive elements. 3) The C-end contains nuclear localization and export signals (NLS and NES, respectively), a regulatory domain and the tetramerization domain.

p53 tumor suppressor is normally found at extremely low levels because of its rapid degradation after synthesis. Stabilization of p53 is a common response to many different and diverse forms of stress, including DNA damage, oncogene activation, metabolic changes, hypoxia and alteration in pH or temperature, in this condition p53 levels rise and initiate protective measures (Ashcroft M and Vousden KH, 1999). The level of p53 mRNA and the rate of p53 protein synthesis remain low during G phase and increase markedly, reaching a peak near the G1/S transition just prior to initiation of DNA replication (Gudas JM et al, 1994).

1. MDM2, *Mouse double minute 2 homolog*, is an E3 ubiquitin-protein ligase it interacts with the N-terminal transactivation domain of p53 to inhibit p53 transcriptional activity.
2. SIN3/HDAC corepressor complex functions in transcriptional regulation of several genes and is therefore implicated in the regulation of key biological processes. Knockdown studies have confirmed the role of SIN3 in cellular proliferation, differentiation, apoptosis and cell cycle regulation, emphasizing Sin3 as an essential regulator of critical cellular events in normal and pathological processes (Kadamb R et al, 2013)

Regulation of p53 protein occurs by binding of proteins that promote its degradation via the ubiquitin/proteasome pathway thanks a "vital negative" feedback.

p53 activation is a three steps process. First, p53 protein stabilized and level increased via the inhibition of its interaction with Mdm2, its pivotal negative regulator, and the other regulators. Over translation of p53 mRNA is a complementary that will also ensure p53 accumulation.



Cell 137(4):609-22 (2009)

Figure 5. **Model of p53 activation**. 1) p53 stabilization: with reduction of Mdm2 binding; 2) Antirepression: this step requires acetylation of p53 to bind a specific subset of p53 target promoters. 3) Promoter activation: To complete activation of transcription machinery, p53 recruits co-factors and also induces histone modification of surrounding chromatin.

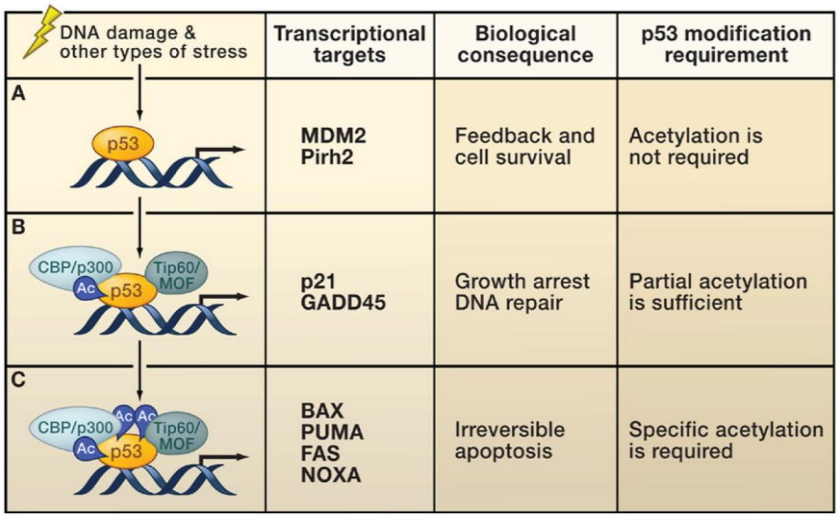


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Figure 6. **p53 acetylation and target genes regulation**. After stress-induced p53 activation, different subsets of target genes have different requirements for p53 posttranslational modifications.

Second, p53 is "anti-repressed", this step is mediated by post-translational modification of the protein mediated by acetylation and phosphorylation, and, finally, a series of modulators (kinases and acetylases again) activate p53 transcriptional activity. During third phase, promoter specific activation of p53 target genes allows to cofactor recruitments for target-genes transcription (Kruse JP and Gu W, 2009) (Figure 5 and Figure 6).

Mdm2 is one of the most important components of the p53 degradation pathway and is the product of a gene regulated by p53 itself in normal cells (Kubbutat MH and Vousden KH, 1998).

Expression of Mdm2 enhances by activated p53 and its principal role is to interact directly with p53 protein to inhibit its activity. Mdm2 binds to the N-terminus of p53 within the trans-activation domain, competing with components of the basal transcriptional machinery recruited by p53 as transcription factor. The binding of Mdm2 alone could inhibit normal function of this region of p53 (Figure 5), reducing the ability of p53 to activate genes' expression and,

moreover, induce poly-ubiquitination of p53 through its E3 ubiquitin-protein ligase activity (Momand J et al 1992; Oliner JD et al, 1993). Other ubiquitin ligases, such E6AP, and interaction with Janus Kinase (JNK) participates to p53 ubiquitin-dependent down – regulation (Talis AL et al, 1998; Fuchs SY et al 1998).

Stabilization of p53 is a common response to different and diverse forms of cellular stresses and arrest cell cycle at checkpoints until damage resolution.

The ability of Mdm2 to associate and target p53 outside nucleus to degradation highly depends on p53 phosphorylation status. Many kinases phosphorylate p53 on N-terminus residues with the aim to interfere in Mdm2/p53 binding. Ataxia telangiectasia mutated (ATM) and Serine/threonine-protein kinase (ATR) phosphorylate p53 on Serine 15 (Appella E and Anderson CW, 2001) while Chk2 phosphorylates it on Serine 20 (Hirao A et al, 2000).

p53, affected by these post-translational modifications, obtains stability and activates its transcriptional activity, in particular, ATM is able to phosphorylate both p53 and Mdm2. Phosphorylation of p53 causes its stability and activation, meanwhile ATM phosphorylation of Mdm2 on Serine 395 reduces nucleo-cytoplasm shuttling of p53 by its inhibitor and allows p53 nucleus accumulation (Maya R et al, 2001).

Among survival pathway, Akt targeting of Mdm2 was demonstrated to be required for its nuclear import, and allows the access to the cellular compartment in which it affects p53 (Mayo LD and Donner DB, 2001).

The p53-Mdm2 relationship is crucial not only for cellular surviving functions but also because it is integrated in a complex signalling network.

Important component that affect auto-regulatory feedback by p53 includes the tumor suppressive protein $p14^{ARF}$. Binding Mdm2 proteins, $p14^{ARF}$ interferes with E3 ubiquitin ligase function of Mdm2 and disrupt the negative feedback inhibition of p53 (Alarcon-Vargas D and Ronai Z, 2002).

Other regulatory mechanisms for p53 (such as phosphorylation, ubiquitination, methylation, sumoylation, neddylation, and acetylation) modify C-terminal region of p53 affecting the ability of p53 to bind DNA.

During malignant progression, cancer cells often accumulates mutant p53 isoforms. Mutations could affect different protein regions, determining loss of regulation and activity of p53. Mutated p53 participates to cancer progression in different ways:

1) mut p53 sequesters wt p53 and arranges a tetramer made up by wt p53/mut p53 subunits. Mutation determines the *loss of function* of p53 and abrogates the tumor suppressive function of p53. This effect is also known as "*dominant-negative effect*";

2) mut p53 may exhibit also a "*gain of function"*, it may acquire an "original" activity, not present in wtp53, which can contributes at various levels to tumorigenesis (Oren M and Rotter V, 2010).

Dittmer and colleagues introduced the concept of Gain of function (GOF) in 1993 (Dittmer D, 1993), they described the ability of mut p53 isoforms to transform and increase growth rate in p53 null-cells in human and mouse cells. Tumors with mut p53 present a great genomic instability and, in addition, mut p53 can interfere with DNA damage repair (Offer H et al, 1999) and influence cancer progression with the increase of DNA mutations accumulations.

Many factors influences the role of p53 mutation during cancer progression, including the stage during which p53 mutation and accumulation occur. In some types of cancer, p53 mutation is frequently a late events and correlate with a poor prognosis and with an enhancement in tumor aggressiveness (Vogelstein B and Kinzler KW, 1993).

Mutations on TP53 gene affected all exons despite the "hot-spot" mutation region consists of exon 4 to 9, this includes the central core of the protein containing the DNA binding domain (DBD) assigned to recognition of specific DNA sequences on promoters of target genes (Olivier M et al, 2010). Frequent

mutated residues are R175, G245, R248, R249, R273, and R282 and the most common are missense mutations (Cho Y et al, 1994).

Furthermore, mut p53 could protect cells against apoptosis induced by growth factor deprivation conferring to a selective advantage in proliferation (Peled A et al, 1996).

Even wtp53 has been involved in cellular protection against chemotherapy-induced apoptosis and emerging results presented new activities in survival promotion by wtp53 in cancer cells (Kim E et al. 2009).

As *guardian of the genome*, p53 is involved in cell damages control and in maintaining genomic integrity, wtp53 deals with control of cell cycle progression, DNA repair, centrosome duplication; it recognizes DNA lesions and activates repair mechanisms, as well as the transcriptional activation of DNA repair factors and of cell cycle regulators. The multitude of factors influencing p53 conformation, its subcellular distribution, and its DNA binding and transcriptional activity contribute to the dynamic balance between survival-promoting and death-inducing activities of wtp53.

Furthermore, TP53 mutations are rare in certain human cancer entities, or is a tardive occurring mutation during cancer progression as a paper by Kim E and colleagues described in 2009.

In particular, they highlighted as glioma cells with wtp53 exhibited a higher resistance to cytotoxic treatments used in clinical practice compared to glioma cells with transcriptionally inactive mutp53, moreover, inhibition of wtp53 exerts augmented apoptosis rate after chemotherapeutics treatments (Batista LF et al. 2009).

Surprisingly, induction of apoptosis by exogenously introduced wtp53 is more efficient in glioma cells expressing mutp53 than in glioma cells expressing wtp53 (Cerrato JA et al. 2001).

Functional duality seems to be an essential feature of the p53 functions, during development and in tissues homeostasis p53 undergoes to a fine-tuned balance

between survival and death induction and loss of death-inducing wtp53 functions, without loss of p53 activities, could provide a survival advantage in cell sub-population within tumor tissue (Kim E et al. 2009).

However, the diversity of cellular processes influenced by p53 is becoming more evident and the traditional view of p53 as simply a tumour suppressor is challenging, in fact, new emerging evidences ascribe to p53 an important role as well in cellular metabolic regulation (Maddocks OD and Vousden KH. 2011).

1.3 p53 and metabolism

Cancer cells are able to initiate replication without any extracellular stimulation, maintaining their own metabolic autonomy (de Berardinis RJ, 2008), in addition to the Warburg effect, tumors show alterations in many other aspects of metabolism, including altered metabolism of amino acids and lipid.

Ability of p53 in metabolic genes regulation is a new emerging topic in cancer cells biology knowledge in fact, several groups focused their attention about this subject showing that p53 is not only a "simple" oncosuppressor but it is able to deeply influence cell life in energetic management.

1.3.1 p53 and glucidic metabolism

"Metabolic obstacles" usually affect cancer cells during their development. First, they increase requirements of energy and reducing agents (ATP, Gluthatione and NADPH), second they need a huge amount of energetic substrates to sustain a rapid proliferation rate and finally they have to survive to metabolic stress, maintain an appropriate redox balance, face low oxygen rate and, at the same time, have to prevent cell death (Puzio-Kuter AM, 2011).

In cancer cells, glycolysis is the preferential way to obtain ATP in hypoxic condition or when cells rapidly need energy (Pfeiffer T, 2001). The product Pyruvate could be used for amino acids and fatty acids synthesis and utilize to maintain mitochondrial membrane potential supporting the energetic request of anabolic metabolism and cell growth.

A transforming cell regulates levels of genes involved in metabolic fate and increased activity of p53, during a chronic stress status, might allow synthesis of specific genes' sets.

For example, p53 is able to influence expression of glucose transporters (GLUT1 and GLUT4) through a direct transcriptional repression (Schwartzenberg-Bar-Yoseph F et al, 2004), moreover, p53 increases the

ubiquitin-dependent degradation of the glycolytic enzyme Phosphoglicerate mutase (PGM) and reduces the glycolytic rate (Kondoh H et al, 2005).

It is also able to reduce glycolytic rate by increased expression of TP53-induced glycolysis and apoptosis regulator (TIGAR) (Bensaad K et al, 2006) which lowers intracellular concentration of fructose 2,6 bisphosphate. All these metabolic changes address cells to hyperactivate the pentose phosphate pathway (PPP), the alternative way for glucose-6 phosphate use and a hallmark of proliferating cells because it is essential for the production of lipids and nucleotides required for cells to divide.

Furthermore, a current model suggested by Vousden assays that this process helps cell to survive to a moderate cellular damage in normal cells or in cells under mild level of stress (Vousden KH, 2009), because, at the same time, p53 directly interacts with and inhibits activity of glucose-6-phosphate dehydrogenase, the first rate limiting step enzyme of the PPP (Jiang P et al, 2011).

As the PPP is the major source of NADPH, which is needed for the scavenging of ROS by reduced glutathione (GSH), induction of this pathway by TIGAR can result in decrease of ROS levels and lowered cellular sensitivity to ROS-associated apoptosis (Bensaad K and Vousden KH, 2007).

p53 is involved also in ROS managing and it has, in this occasion also, both pro-oxidant and anti-oxidant functions. (Chen W et al, 2009; Faraonio R et al, 2006)

1.3.2 p53 and protein metabolism

Like glucose deprivation, even reduction of amino acids availability could activate p53 in a pro-survival manner.

Many tumors required glutamine, a non-essential amino acid, used for high-rate proteins and nucleotides synthesis, and ATP generation to sustain a rapid growth of cancer cells (Dang CV, 2010; Wise DR et al, 2008). At the same time, glutamine, converted first in glutamate and later in α-ketoglutarate, participated

among tricarboxyciclic acid (TCA) cycle in glutaminolysis, producing ATP in cells. Cells deficient of p53 were less viable under glutamine deprivation compared with those harboring functional p53 (Reid MA et al, 2013). Another amino acid, serine, also leads to p53-induced cell survival when its levels are depleted (Maddocks OD et al, 2013), low levels of nutrients are detected by kinases and phosphatases, which modulate downstream effector proteins such as transcription factors to reprogram cellular functions and promote survival (Reid MA and Kong M, 2013).

1.3.3 p53 and lipid metabolism

Cancer cells show an elevated rate of lipids synthesis. Lipids also participates in cancer growth and proliferation and contribute to tumor cells surviving, extensively, alteration in lipid metabolism are evident during tumorigenesis and cancer cells increase lipogenic rate compared to normal cells, that predominantly reach and metabolize diet lipids (Medes G et al, 1953; Kuhajda FP et al, 1994).

Nutrient deprivation contributes to p53 activation, in this circumstance p53 downregulates IGF1/Akt/mTOR pathway that signals for cell growth and proliferation in response to high levels of glucose and amino acids, induces glycolysis and protein synthesis through mTOR activation (DeBerardinis RJ et al, 2008).

There is a well-regulated network between *p53/IGF1/Akt/mTOR axis /nutrient balance* that allow cells to respond to cellular stress, under reduced energy balance, the Akt/mTOR axis and AMPK fail to be activated and p53 is induced. AMPK, the AMP-activated protein kinase, drives the catabolism of cellular fatty acids and participates in p53 activation (Puzio-Kuter AM, 2011)

Buzzai and collegues, showed as treatment of $p53^{+/+}$ cells with metformin (an anti-diabetic drug able to induce AMPK), enhances fatty acid β-oxidation in a p53 dependent manner. In fact, $p53^{-/-}$ cells, stimulated with metformin as well,

showed an increased apoptosis rate and a markedly susceptibility to apoptosis in case of in vitro nutrient deprivation. Taken together these evidences highlighted, again, the different behavior of cells in metabolic adaptation according to p53 status.

Even some very interesting evidences, the link between p53 and lipid metabolism is still under investigation.

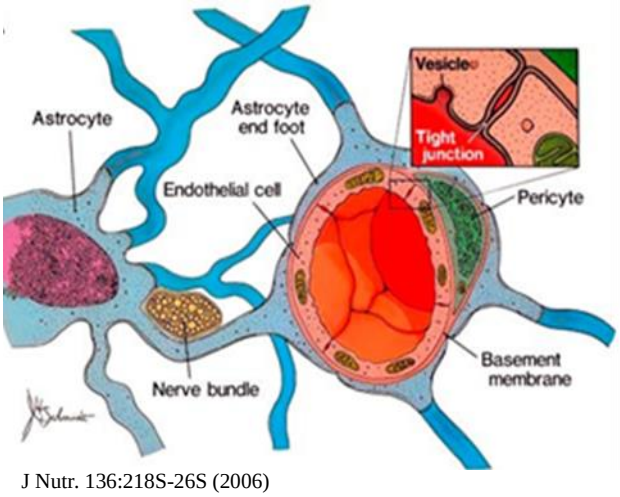
Recent study by Freed-Pastor et al., suggested an interaction between mutated p53 and SREBP2, a transcription factor controlling mevalonate pathway genes expression.

They focused their attention on breast-cancer cells and demonstrated that mutated p53 is able to induce levels of mevalonate genes, increasing tumorigenic feature of these cells and changing cell morphology. Using a specific small-hairpin RNA (shRNA) molecules targeting mutated p53, they demonstrated an attenuation of malignant phenotype of breast-cancer cells (Freed-Pastor WA et al, 2012).

Other findings, by Ido Goldstein at al., elucidated a new aspect of p53 on genes transcription regulation in different features of lipid metabolism. They observed a transcription induction of three genes: Pltp (Phospholipid transfer protein) and Cel (Carboxyl ester lipase), implicated in lipoprotein metabolism, and Abca12 (ATP-Binding cassette transporter 12), involved in lipids transfer across membranes. Promoters of these genes are activated directly by p53 and this activity suggests and reveals new roles for p53 in regulation of lipid homeostasis (Goldstein I et al, 2012).

1.4 Brain metabolic behavior

Brain has particular metabolic requirements, all vertebrates have a blood brain barrier (BBB) that allows metabolism inside the brain to operate differently from metabolism in other parts of the body (Figure 7).



J Nutr. 136:218S-26S (2006)

Figure 7. Schematic representation of BBB

Brain tissue consumes a large amount of energy (between 20 and 25% of basal metabolism of vertebrates) in proportion to its volume and most of energy consumption sustains the maintenance of membrane potential of neurons (Nieuwenhuys R et al, 1998; Mink JW et al, 1981)

The brain obtains the most of its energy from oxygen-dependent metabolism of glucose derived from the blood (Nieuwenhuys R et al, 1998) but alternative energetic sources are ketones, lactate, acetate and some amino-acids (Boumezbeur F et al, 2010; Deelchand DK et al, 2009).

Glucose is the elective molecule for brain metabolic reactions. In the brain, glucose is metabolized through glycolytic pathway, enter in the tricarboxylic acids cycle (TCA) and then into oxidative phosphorylation to produce ATP, it is used also as precursor for production of metabolic intermediates for biosynthetic reactions and to synthetize neurotransmitters.

More, glucose can participates in an alternative pathway: the pentose phosphate pathway (PPP). Glucose oxidation through PPP provides 5-carbon monosaccharides for nucleic acid synthesis and NADPH for reductive biosynthetic processes and for maintenance of the redox balance in the cell.

Brain glucose is also stored as glycogen but this stock is sufficient to sustain brain only for few minutes. Most of glycogen storage has localized in astrocytes, the metabolic supporter-cells for neurons (Vilchez D et al, 2007).

During special condition of prolonged deficiency of glucose, the brain utilizes protein as alternative source of energy and active neurons preferentially employ aminoacids comparing to resting neurons. The essential aminoacids, from the diet, cannot be synthetized by the brain and are necessary to make neurotransmitters that allow cells to develop networks and communicate each other. Phenylalanine, leucine, tyrosine, isoleucine, valine, tryptophan, methionine and histidine must to be supplied to brain metabolism and are precursor of dopamine, L-DOPA. These essential aminoacids enter the brain as rapidly as glucose and are transported into the brain by the leucine preferring or the L-type transporter-proteins through a competitive active transport. Although the BBB determines the availability and the brain content of essential aminoacids, astrocytes and neurons participate in maintaining the extracellular concentrations and regulate aminoacids concentration in extracellular fluid, that is important to maintain low concentration of neurotransmitter such a glutamate, aspartate, and glycine regulating brain's stimuli. (Hawkins RA et al, 2006).

Differently, lipids are not a major energy source for the brain.

Astrocytes are able to oxidize fatty acids and ketone bodies, while neurons and oligodendrocytes can only use ketone bodies. Ketone bodies are produced from Acetyl-CoA precursor and are synthesized by the liver after depletion of carbohydrates stocks or after a long glucose starvation period.

Moreover, Acetyl-CoA produced by the brain derived from pyruvate, but it can also originate from fatty acids, oxidized inside mitochondria, from ketone bodies, lactate and acetate.

Among brain's lipids the most abundant is cholesterol, in fact, its metabolism is very different from that in the rest of body's tissues and it is strictly regulated for optimal brain function. Recent evidences also showed that alteration of

cholesterol metabolism can lead to severe neurological disorders (Björkhem I et al, 2001; Wiegand V et al, 2003; Puglielli L et al 2004).

1.4.1 Brain cholesterol metabolism

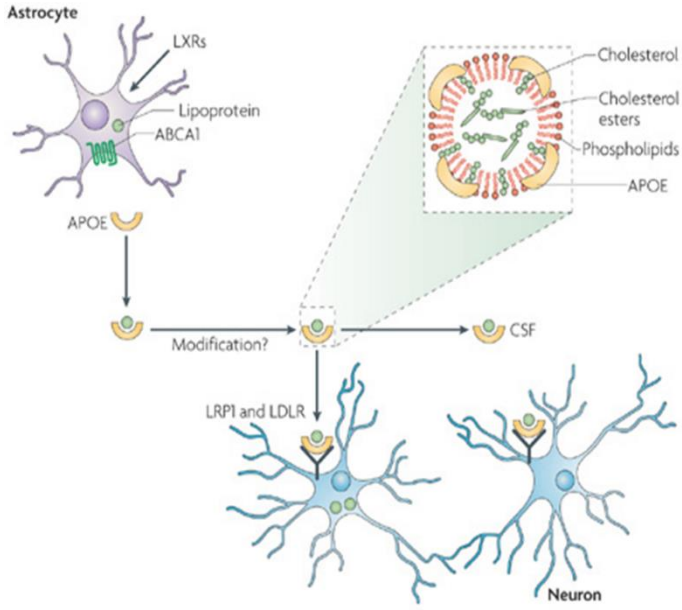
Cholesterol in the body can derived from the diet uptake and from "de novo" synthesis. It is delivered to other tissue by blood circulation. It is generally assumed that cholesterol carried by plasma lipoprotein not enter the brain because of the Blood-Brain Barrier (BBB), in fact, the brain obtains all its cholesterol from in situ synthesis (Dietschy JM and Turley SD, 2001) and cannot retrieve cholesterol from the circulation (Björkhem I and Meaney S, 2004).

Nevertheless, brain is the most cholesterol-rich organ of the body, it contains about the 25% of the whole body cholesterol content, even its weight represent only the 2% of the body mass.

Brain's cholesterol lies in three different compartment: 1) the larger pool, about 70-80%, is present in myelin membranes; 2) about the 10% resides in neurons and 3) the remaining 10% is contained in glial cells.

The huge amount of synthetized cholesterol is accompanied by a very slower turnover compared to other body's tissues, such as liver, for example, and its concentration is kept stable (Spady DK and Dietschy JM, 1983). Among brain cellular compartments, the astrocytes are committed to cholesterol synthesis and they supply this metabolite to neurons. In fact, neurons are not able to produce cholesterol by themselves after birth and required astrocytes complete differentiation and metabolic participation to a normal function. Neurons required cholesterol for membranes integrity and to repair damage after brain injuries, to form synapses and for vesicles formation, for maintaining a proper signal transduction and in neurons communication mechanisms (Göritz C et al, 2002).

Astrocytes are intimately associated with neurons as well as with endothelial cells forming the BBB, they provide not only metabolic but also structural and trophic support to neurons, release growth factors, control extra-cellular K^+ efflux and regulate brain immune response (C.Ehnholm. edition). Astrocytes secrete cholesterol in the form of ApoE-containing HDL-like lipoproteins (Gong JS et al, 2002), these particles are loaded with lipids through the ABCA1 plasma membrane transporter and needs additional ApoE molecules before binding to the neuronal ApoE receptors, the LDL receptor (LDLR) and LDLR-related protein 1 (LRP1) or being transported to the cerebrospinal fluid (CSF) to reach neurons. (Figure 8) (Bu G, 2009)



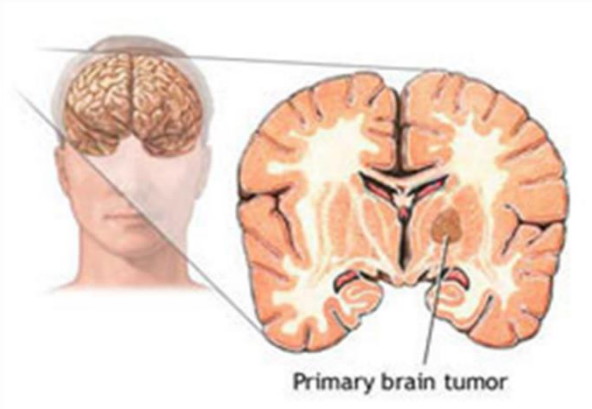
Nat Rev Neurosci 10(5):333-44 (2009)

Figure 8. Delivery of ApoE-containing HDL-like lipoparticles.
Cholesterol and other lipids transported to neurons have important roles in synapse formation and repair. ApoE – lipoprotein particles are composed by: cholesterol, cholesterol esters and phospholipids. These particles are assembled and released by astrocytes and reach neurons through the cerebrospinal fluid (CSF)

Within the brain, in a specific population of neurons, cholesterol can be modified by a cytochrome P450 isoform, the CYP46A1, and it is transformed in the 24(S)-hydroxycholesterol. This compound has a very lipophilic character but despite this, it could traverse the BBB (Björkhem I, 2007).

1.4.2 Glioma cancer cells and metabolic dysregulation

Glioma is a primary brain tumor that originates from the supportive cells of the brain, called glial cells (Figure 9). Glial cells are the most common cellular component of the brain, in fact, there are five to ten times more glial cells than neurons. Unlike neurons, glial cells have the ability to divide and multiply frequently and if this process occurs too rapidly and without control, a glioma forms.



http://neurosurgery.ucla.edu/body.cfm?id=159

Figure 9. **Glioma representation**

There are three principle types of glial cells: astrocytes, oligodendrocytes and ependymal cells, the different glial cells originate different kind of glioma. The 2000 World Health Organization system divides them in astrocytoma, oligodendroglioma and apendymoma respectively (Kleihues P and Cavenee WK, 2000)

Glioma cells present a strong infiltrative behavior and the majority of them are resistant to standard chemotherapy and radiotherapy treatments, and as tumor became more malignant, cells appear less differentiated. (Louis DN et al, 2001) Clinically, gliomas are divided into four grades and the most aggressive of these is the fourth grade glioma, called also glioblastoma multiforme (GBM). The latter has a really aggressive and poor prognosis, most patients' life expectancy is less than one year and none has a long term-survival (Holland EC, 2000).

New strategies are developing in order to improve patients' outcome and to interfere with cancer development, some pathways were targeted, such as EGFR (Mellinghoff, I.K et al, 2005), PI3K/Akt (Chakravarti A et al 2004), and VEGF (Ferrara N, 2005).

Recent and accumulating evidences show that cancer cells reprogramme their metabolism to supply high-energy requirements of proliferating cells, for this reason, targeting metabolism has become a new and promising strategy to interfere with tumor growth. GBM presents elevated glycolysis rate, enhanced glutaminolysis and high rate in lipid synthesis (Ru P et al, 2013).

In glioma tissues, aerobic glycolysis is one of the major characteristic and, although under aerobic conditions, this is the preferential pathway to produce ATP and, furthermore, glycolysis is up regulated by PI3K/Akt pathway (Warburg effect).

Several enzymes of glycolytic pathway are involved and de-regulated in GBM.

1) Hexokinase (HK), able to catalyze the formation of Glucose-6-phosphate (G6P). GBM showed enhanced levels of HK2 isoform, which is present at high levels in GBM samples and is associated with tumor proliferation and radio-resistance during treatment (Wolf A et al, 2011).

2) PKM2, an isoform of the Piruvate Kinase (PK), is mainly expressed in proliferating cells (Mazurek S, 2011) and through its nuclear translocation, it is involved in EGFR signaling pathway and correlates with increase of β-catenin levels in GBM tumors (Yang W et al, 2011).

3) IDH, the isocitrate dehydrogenase, catalyzes formation of α-ketoglutarate through the oxidative decarboxylation of isocitrate, and generates NADH and NADPH (Reitman ZJ and Yan H, 2010). Mutation of IDH often co-occurs together with mutation of p53 (Watanabe T et al, 2009), mutated enzyme acquired new activity and is able to produce a particular product called 2-hydroxyglutarate (2-HG), presents in a very low level in normal cells but is has elevated level in GBM patients sample (Andronesi OC et al, 2012).

Glutamine metabolism has been reported to be up-regulated in cancer (DeBerardinis RJ et al, 2007) and even if its contribution is much less known than glucose metabolism involvement in cancer metabolic redesign, recent investigations highlighted the roles of strong transforming oncogenes, such as c-Myc and RhoGTPase in glutamine pathway regulation in cancer cell metabolism (Dang CV et al, 2009; Rathore MG et al 2012).

Boost in lipid synthesis has been largely found in tumor cells (Santos CR and Schulze A, 2012) and GBM also present higher levels of unsaturated fatty acids compare to normal brain (Gopal K et al, 1963).

Lipid droplets accumulation, detected by Nile red staining, had recognized in glioma samples according to malignancy grade, in a paper by Benedetti and colleagues (Benedetti E et al, 2010) and they also suggested involvement of peroxisomes in malignant progression of GBM. In particular, data by Benedetti et al., showed that peroxisomes number increased as a function of GBM malignant grade together with β-oxydation enzymes. Peroxisomes, participate to acetyl-CoA cellular production by a β-oxydation mechanism that, unlike mitochondria, is not coupled to ATP production for cell's needs, moreover, peroxisome also influenced lipid synthesis directly on mevalonate pathway, because key enzymes of the pathway (Mevalonate Kinase, Mevalonate Diphosphate Decarboxylase and HMGCR) are located on cellular peroxisomes itself.

Other evidences highlighted upregulation of SREBP and downstream genes in GBMs and point out a regulatory role played by EGFR/PI3K/Akt (Guo D et al, 2011).

2. AIM OF THE STUDY

For my thesis work, I investigated regulation of Mevalonate pathway (MVA) enzymes in two different Glioblastoma multiforme (GBM) cell lines, to elucidate involvement of this pathway in cancer metabolic alterations.

The study focused on U343 MG and U251 MG glioblastoma multiforme cell lines, both classified as IV grade glioma, and their normal counterpart, the Normal Human Astrocytes (NHA) cells, in order to elucidate involvement of MVA pathway in cancer metabolism.

During the first phase of the study, I monitored the mRNA expression of HMGCR, MVK, FDPS, FDFT1, RabGGTA and LDLR in considered cell lines by quantitative RealTime-Polymerase Chain Reaction (qRT-PCR) and I evaluated the protein level of these enzymes by Immunoblotting assay.

Because results revealed an up-regulation of these enzymes in NHA and U343 cell lines, harboring wt-p53, compared to cell lines with mutant p53, the U251 cells, I investigated whether p53 activation participates in transcriptional induction of these enzymes.

To confirm this hypothesis, in the second part of the study, I evaluated the binding of p53 on promoter sequences of the considered genes by Chromatine Immuno-Precipitation assays (ChIP). Results, confirmed by luciferase-reporter assays, showed that p53 directly recognizes specific p53-responsive elements (p53-REs) in HMGCR, MVK, FDPS and FDFT1 sequences.

This study unrevealed a new aspect of p53 features, unrelated to tumor suppression, as metabolic regulator of mevalonate pathway activity.

3. MATERIALS AND METHODS

3.1 Cell cultures

Normal Human Astrocytes (NHA) are normal human cells derived from human brain tissue and were cultured in recommended medium AGM™ BulletKit™ (Lonza), these cells were used as normal cell control system. High-grade glioblastoma multiforme (IV grade glioblastoma, according to WHO classification) U343 and U251 cell lines were maintained in EMEM supplemented with 10% FBS. Human Embryonic Kidney 293 cells (HEK293) were expanded in DMEM supplemented with 10% FBS.

All cell lines were cultured at 37°C in 5% CO_2 controlled atmosphere.

3.2 Reagents

Etoposide (Eto), a p53 inducing drug (Karpinich NO et al 2002), and pifithrin-alpha (PT-α), an inhibitor of p53 transcriptional activity (Sohn D et al, 2009), were purchased from Sigma-Aldrich. To notice p53 and MVA enzymes induction, NHA cells were incubated with Eto 25 µM for 24 hours, I evaluated mRNA expression levels and investigated increase in p53 binding ability on MVA enzymes promoters in ChIP experiments.

PT-α was used in [^{14}C] -Acetate experiment at concentration of 30 µM for 24 hours, it is used to interfere with p53 in cholesterol synthesis regulation.

Nutlin-3a was purchased by Sigma-Aldrich, this drug activate p53 through Mdm2 binding (Mouraret N et al 2013), U343 cells were incubated for 24 hours with at a concentration of 10 µM for 24 hours.

Lovastatin (lova), a gift from dr. AW Alberts of the Merk, Sharp and Dohme institute, was used in ^{14}C acetate experiments to inhibit HMGCR activity, cells were incubated with 10 µM lova for 24 hours treatment.

3.3 Transfection procedure

Cells, at 80% confluence, were transfected using Lipofectamine®2000 (Life technology). Lipofectamine is cationic-lipid reagent formulated for the transfection of DNA into eukaryotic cells. Lipofectamine was diluted with culture medium EMEM, without FBS, and added to diluted DNA in a final volume of 100 µL, DNA-lipofectamine mixture was incubated for 5 min at room temperature and then added to cell culture. Ratio between DNA and Lipofectamine was 2,5 / 1. I transferred a total amount of 5 µg of plasmids for each well, while for co-transfection the total amount of transfected DNA was of 6 µM. After 6 hours, incubation medium was replaced with fresh and FBS added one. Cells were collected 36 hours later and analyzed for western blot, qRT-PCR and for luciferase assays.

3.4 quantitative Real Time – PCR (qRT-PCR)

Total RNA was isolated from 5 x 10^6 cells at least, using the NucleoSpin RNA II kit (Macherey-Nagel) which supplies all buffers for mRNA extraction. Cells were lysed with Lysis buffer, which immediately inactivated RNases and then vortexed vigorously. A first column, holding a violet ring-filter, was used to filtrate lysates by centrifugation for 1 min at 11,000xg. Filtrated lysates, added with 350 µL of 70% EtOH, were vortexed and loaded in a new column with light blue ring-filters to allow RNA binding to membrane. After tubes centrifugation, the Membrane Desalting Buffer (MDB) was added to filters to facilitate the following DNase digestion. 95 µL of DNA digestion mixture was loaded on filters and effected for 15 min at room temperature. Silica membranes were washed three times and finally dried. RNAs were eluted with 20-40 µL of RNase-free H_2O.

RNAs were quantified using UV absorption, the absorbance is measured at 260 and 280 nm, to calculate concentration and purity of samples and the instrument used was Nanoview spectrophotometer (GE Healthcare).

cDNA was synthesized with SuperScript II (Invitrogen).

For each RNA sample (1 µg of total RNA) were added 1 µL of dNTP Mix (10 mM each), 300 ng of random primers and sterile RNase-free water up to 12 µL. Reaction tubes were incubated in a termalcycler at 65°C for 5 min, later were added 4 µL of 5X First-Strand Buffer, 2 µL of 0.1 M DTT and 40 units of RNaseOUT™ and then incubated again at 25°C for 2 min. Finally 200U of SuperScript II were added to samples and they were heated at 25°C for 10 min and then at 42°C for 50 min. Enzymes used for retrotranscription procedure were inactivated at 70°C for 15 min.

With quantitative Real-Time and using specific primer sets (Table 1), I investigated the presence of MVA enzymes. SsoFast Eva Green supermix contains Sso7d polymerase and EvaGreen dye, a fluorescent nucleic acid dye, with 7,5 µL of this reagent, 1,5 µL of primers mix and with 4,75 µL of DEPC-H_2O (Sigma - Aldrich) I tested 2 µL of cDNA.

Amplification protocol is the following: 1) Enzyme activation: 95°C x 30 sec for 1 cycle; 2) Denaturation and Annealing/Extention phase, repeated for 40 cycles, respectively at 95°C x 5 sec and 60°C x 5 sec; 3) End-cycle at 70°C x 5 sec.

Cycle Threshold (Ct) obtained by Bio-Rad CFX96^{TM} instrument, were elaborated with the $2^{-\Delta\Delta Ct}$ method and provided indications about quantity of mRNA present in samples (Livak KJ and Schmittgen TD, 2001).

3.5 SDS-PAGE and Immunoblot analysis

Control and treated cells were washed twice with Phosphate Buffered Saline (PBS) (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM at pH 7.4) and resuspended in lysis buffer (Hepes 50 mM, NaCl 150 mM, EDTA 50 mM, NaF 100 mM, Na_3VO_4 2 mM, 10 % glycerol, $Na_4P_2O_7$ 10 mM, 10% triton pH 7.5). Lysated were passed through a 23-gauge needle, ten times before centrifugation at 12000×g at 4° C. Aliquots of the cellular lysates,

corresponding to 50 µg of proteins, were added with Laemli buffer (4% SDS, 10% 2-mercaptoehtanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris HCl) and boiled for 2 min at 95°C. Sample were loaded in gel's wells and electrophoresed at 40 mA on SDS-polyacrylamide gel.

Finally, proteins were transferred to nitrocellulose membranes, with a constant voltage of 100 V.

Filters were blocked with a 5% Non fat dry milk in TBS (50 mM Tris and 150 mM NaCl at pH 7.6) and incubated with specific antibodies.

Antibodies anti-p53 (DO-1), anti-p21waf, anti-HMGCR, anti-MVK, anti-FDFT1, anti-RabGGTA and anti-LDLR were from Santa Cruz Biotechnology while anti-FDPS was from Abcam and were used at a concentration of 1:1000.

3.6 Chromatin Immunoprecipitation Assays (ChIP)

Chromatin Immunoprecipitation (ChIP) experiments were carried out with MAGnify™ Chromatin Immunoprecipitation System (Invitrogen), using a minimum of $6x10^6$ cells. The kit supplied all buffers. NHA, NHA incubated with Eto (25µM for 24 h), U343, U251 and U251 transfected with pCMV-p53 cells were collected and washed with PBS and diluted at a concentration of $6x10^6$/mL.

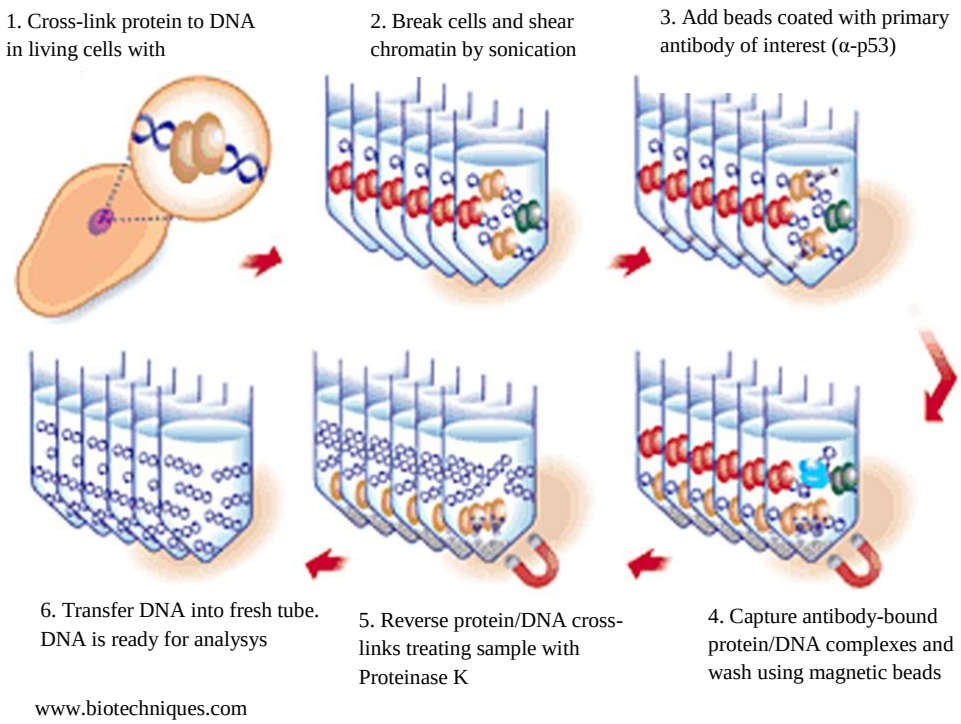


Figure 21. Schematic representation of ChIP procedure using magnetic beads

To ensure that protein linking chromatin were fixed on DNA, I cross-linked the cells with 13.3 µL of 37% formaldehyde. 15 minutes later, I added 57 µL of 1.25 M glycine to inactivate reaction. Cells were pelleted and washed with cold PBS and lysed in lysis buffer with protease inhibitors. I sheared chromatin by sonication in 200-500-bp fragments, employing BRANSON digital sonifier 250. An aliquot of sheared chromatin (~300 µL) was collected and processed as DNA input control. Remaining chromatin solution was incubated overnight with antibodies-coated magnetic Dynabeads®, respectively with α-p53 (Invitrogen) and with IgG non-specific antibodies, used as negative control for immunoprecipitation procedure.

Chromatin-Antibody-Dynabeads® complexes were washed to remove any unbound product. With Reverse Crosslinking Buffer added with Proteinase K, I reverse the formaldehyde crosslinking of the chromatin and this phase affected aliquots of input DNA. Immunoprecipitated samples and input control tubes were incubated at 55°C for 15 minutes in a water bath. The last step of ChIP procedure consider purification of the un-crosslinked DNA using the DNA Purification Magnetic Beads and buffers provided in the kit. Samples incubated with DNA Purification Beads were finally incubated at 55°C for 20 minutes in a water bath to divide DNA from the beads.

Collected immunoprecipitated (IP) DNA subdued to qRT-PCR analysis. Obtained results were compared with their own input and adjusted for their respective non-IP.

3.7 Plasmid constructs

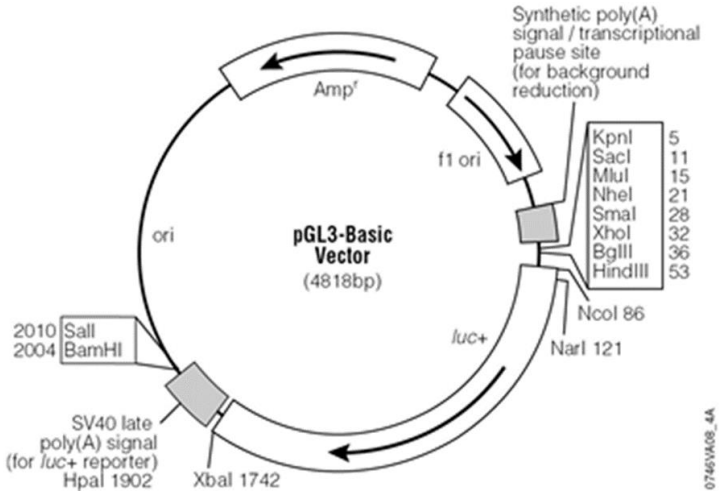
Human wild type p53 expression plasmid (pCMV-p53) was kindly provided by Professor Faraonio (Faraonio R et al, 2006), while mut(R273H)p53 plasmid was purchased by Addgene. Human genomic DNA from healthy donor were amplified using specific primer sets (Table 3). Cloning protocol considered the use of AccuPrime™ Pfx DNA Polymerase (Invitrogen) for high-fidelity amplification of DNA fragments for downstream applications such as cloning and mutagenesis. High fidelity is provided by the proofreading 3′→5′ exonuclease activity.

For PCR amplification procedure, I used 5 µL of 10X AccuPrime™ Pfx Reaction mix, 1.5 µL of primer mix (10 µM each), 50 ng of template DNA, 1unit of AccuPrime™ Pfx DNA Polymerase and autoclaved distilled water up to 50 µL.

Template were first denatured at 95°C for 2 min, then, with a three step cycling I performed 35 cycle as following: Denaturation step at 95°C for 15 sec, Annealing step at 60°C for 30 sec and Extending step at 70°C for 1 min long for

each template kb. Finally, I tested fragments' weight on 1 % agarose and purified them for digestion.

Cloning primers possess a 5' tail recognized by restriction endonucleases KpnI and HindIII (New England BioLabs® inc.) and same enzymes were used to digest pGL3 plasmids and open it in correspondence of the multi-restriction site region (Figure 21).



www.promega.com

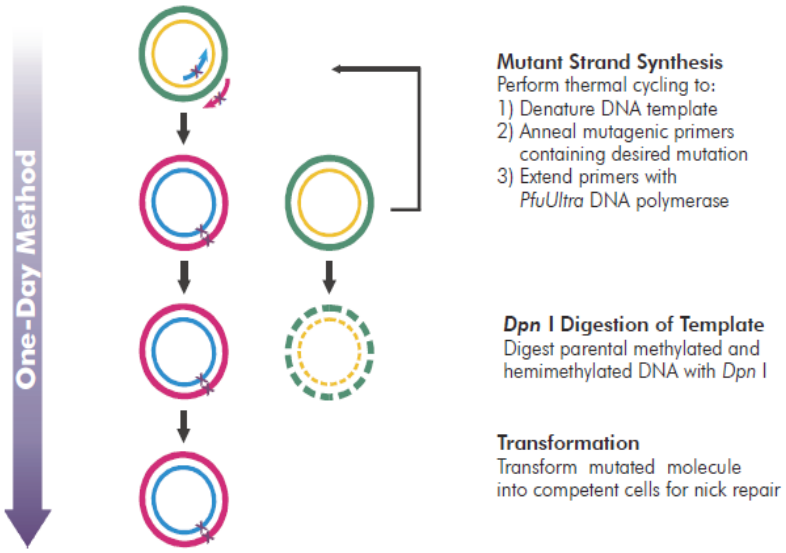
Figure 21. pGL3-Basic Vector schematic representation

Fragments were digested at first with KpnI enzyme for 1 hour (1µg of DNA, 5 µL of 10xNEB Buffer, 10 U (= 1 µL) of KpnI restriction endonuclease in a total of 50 µL) and later digested again with HindIII endonuclease (1µg of DNA, 5 µL of 10xNEB Buffer, 10 U (= 1 µL) of HindIII restriction endonuclease in a total of 50 µL).

Accomplished procedure the incubation with T4 DNA ligase (2 µL of 10xT4 DNA ligase Buffer, 50 ng of pGL3 digested vector, 50 ng of digested insert DNA and nuclease-free water up to 20 µL, 400 U of T4 DNA Ligase should be added at last).

Deleted constructs were obtained with the same procedure, but using specific deletion primer sets (Table 5).

Promoter constructs carrying mutations in the p53 responsive elements were achieved by site-directed mutagenesis employing the QuikChange II- XL Site-Directed Mutagenesis kit (Agilent technology). This kit, containing a particular Taq polymerase, the *PfuUltra* HF DNA polymerase, is optimized for large constructs amplifications and allows site-specific mutation in double-stranded plasmid using specific mutagenic primers (Figure 22) (Table 6).



http://www.bio-connect.nl/

Figure 22. Overview of the QuikChange II XL site-directed mutagenesis method

Experimental protocol was the following: 5 µL of 10xReaction Buffer, 10 ng of double strand DNA template, 125 ng of each mutagenic primers, 1 µL of dNTPs purchased by the kit, 3 µL of Quik solution and distilled water up to 50 µL. Reaction was drived for 1 cycle at 95°C for 1 min, for 18 cycles repeating 95°C

for 50 sec, 60°C for 50 sec, 68°C for 1 min/kb of plasmid length, for 1 cycle at 68°C for 7 min.

At the end of cycles, 1 µL of *DpnI* endonuclease was used to digested the parental DNA template and selected, in this way, for mutation-containing synthesized DNA.

3.8 Luciferase Reported Assays

U343, U251 and transfected U251 cells were cultured in 48-wells plates in triplicates, at an estimated cellular concentration of $5x10^4$ cells/well. 24 hours later cells were transfected using Lipofectamine (already described in 3.6 Transfection procedure) with 1 µg/well of pGL3 basic vector containing the promoter constructs of considered enzymes.

Transfected U251 cells were co-transfected with the above-mentioned concentration of promoter construct plasmids plus 400 ng/well of pCMV-p53.

To normalize transfection efficiency, cells were co-transfected with β-Galactosidase plasmid (500 ng), which contains a functional lacZ cloned downstream of SV40 promoter and enhancer (Promega).

Control cells were transfected with 400 ng/well of a pGL4 Luciferase Reporter Vector plasmid, containing luciferase gene that I considered as control-mock.

Luciferase assays were performed with Luciferase System Assay by Promega.

48 hours later transfection, cells were harvested, washed twice with PBS and then lysed to carry out the reporter assay with Reporter Lysis Buffer purchased by the kit, it is a mild lysis agent requiring a freeze-thaw cycle to achieve complete cell lysis. 20 µL of cell lysate was added with 100 µL of Luciferase Assay Reagent and read with Plate-Reading Luminometer (SpectraMax L Luminescence Microplate Reader). Another aliquot of 20 µL of cellular lysate was used to quantify β-Gal concentration using 100 µL of substrate solution. Luciferase's activity in cell lysates was normalized for protein concentration and to control β-Gal signal.

3.9 [^{14}C]-Acetate uptake

Actively growing, subconfluent cells were serum starved for 24 hours prior to incubation with lovastatin (10 µM for 24 hours) and PT-α (30 µM for 24 hours) and prior to radiolabelling with [^{14}C]-Acetate (American Radiolabelled Chemicals). After drugs' administration, cells were incubated with 7 µCi/mL of [^{14}C]-acetate. Cells were labelled for 14 hours, then washed and harvested with cold PBS, pelleted at 1300 rpm for 2 minutes, in a Beckman Spinchron DLX, and resuspended in 75% EtOH. Cellular extracts were normalized to protein content, dissolved in 0.5-1 mL of chloroform, and dried. Cellular lipids content were resolved by Thin Layer Chromatography (TLC), aliquots of 10 µL were loaded on TLC-Silica gel-60G (Millipore) in presence of cholesterol standard (NEN products - DuPont) and developed in hexane/diethylether/acetic acid (70:30:1) used as solvent system (Perillo B et al, 1995).

3.10 Patients

This study has the approval of the Hospital Ethics Committee and all patients signed an informed consent before participating in the study. Samples were collected between 2008 and 2013 from patients underwent surgical resection for a diagnosed primary brain glioma, and came from Department of Neurosurgery, "S.Salvatore" Hospital, L'Aquila, Italy and from Department of Neurological Sciences, O.U. Neurosurgery, "G.Rummo" Medical Hospital, Benevento, Italy. All patients were diagnosed according to the World Health Organization (WHO) classification in grade IV glioma (Glioblastoma Muliforme: GBM), grade III glioma (Anaplastic Astrocytoma: AA), grade II glioma (Low grade astrocytoma: LGA). Ages ranged between 26 and 73 years with a mean age of 52 years old. All patients underwent a complete clinical evaluation at the hospital admission in order to evaluate clinical conditions and Karnofsky Performance Status (Mor V et al, 1984).

Patients underwent a complete neuro-radiological study (TAC scan, MRI with and without gadolinium, Technetium^{99}MIBI brain APECT, and in case of tumor seated near motor and/or speech areas, functional MRI was performed), before surgical procedure. Surgical procedure started from the edematous brain surrounding the tumor. Complete post-operative neuro-radiological investigation was performed in all patients, including TAC scan with and without contrast within 48 hours from surgery and MRI without gadolinium performed immediately before an at the end of radiotherapy and three months later in association with Technetium 99 MIBI brain SPECT (Benedetti E et al 2010)

3.11 p53 mutations detection

Genomic DNA was extracted from patients' tissues using the Wizard genomic DNA Purification kit (Promega).

Tissues were homogenized in Nuclei Lysis Solution and incubated at 65°C for 30 minutes, 3µl of RNase Solution were added to lysates and samples were incubated for 20 minutes at 37°C. To induce proteins precipitation were used 200 µl of Protein Precipitation solution and tubes were centrifuged at 15,000xg for 4 minutes. Supernatants were collected in fresh and sterile tubes containing 600 µl of isopropanol and mixing gently I obtained DNA precipitation. I centrifuged tubes at 15,000xg for 1 minute and washed pellet twice with 70% EtOH and air-dryed. Finally, DNA was rehydrated in 50 µL of DNA Rehydration Solution overnight at 4°C. DNA concentration was quantified by Nanoview spectrophotometer (GE Healthcare). I investigated for the presence of p53 mutations in exons 5, 6, 7 and 8 representing a hot spot mutation region of the molecules. Using specific primers sets (Table 7) a Polymerase Chain Reaction (PCR) was carried out with high fidelity *Taq* polymerase, the Accuprime^{TM} *Pfx* DNA Polymerase (Invitrogen), the same used for plasmids cloning. Amplification mix contained: 20 ng of genomic DNA, 5 µL of 10x

Accuprime™ *Pfx* Reaction mix, 0.4 µL (1U) of Accuprime™ *Pfx* DNA Polymerase, 1.5 µL of primer mix (10 µM) and distilled autoclaved water up to 50 µL. Amplification conditions were: 1) Denaturing step at 95°C for 2 min, followed by 2) 34 cycles with Denaturation steps at 95°C for 20 sec, Annealing at 60°C for 30 sec and Extending phase at 72°C for 90 sec. Amplified exons were purified with Wizard SV Gel and PCR Clean-up system (Promega) using SV Minicolumn purchased by the kit and then sequenced with the BigDye Terminator v.3.1 kit (PE, Applied Biosystem).

Before DNA samples were amplified with BigDye terminator buffers to label sequences with fluorescent nucleotides. The cycle sequencing reaction was carried out adding 50 ng of PCR-purified samples, 8 µL of Terminator Ready Reaction Mix, 3.5 µL of Diluent, 3.2 pmol of primers (forward or reverse primer in Table 7) and DEPC water up to 20 µL. Labelling protocol was the following: denaturation of templates at 95°C for 5 min, 35 cycles at 95° for 15 sec, 58°C for 5 sec and 60°C for 4 min, finally a rapid thermal ramp to 4°C. Products were purified with Ethanol/EDTA precipitation method, and were run on the ABI 3500 Genetic Analyser (Applied Biosystem), obtained sequences were compared to the GenBank sequence file AF307851 corresponding to p53 nucleotide sequence.

3.12 Densitometric and statistical analysis

Densitometric measurement were carried out on triplicate experiments ([^{14}C]-acetate) and all values represented as mean ± SD.

For experiments represented in Figures: 12, 15, 16, 17 and 19 data were analyzed with a one-way Anova statistical analysis. For results of experiments in Figures: 13 and 14 a two-tailed *t*-Test was performed, while for experiment in Figure 20 I analyzed with one-tail *t*-Test.

All data were represented as mean of three different experiments ± SD. Data were considered significative when p-value was ≤ 0.05 ($p \leq 0.05$ = *; $p \leq 0.01$ = **; $p \leq 0.001$ = ***)

4. RESULTS

4.1 p53 induces expression of the Mevalonate metabolic pathway enzymes in human Glioblastoma cells

Elevated and deregulated activity of the mevalonate pathway has been demonstrated in several different tumors (El-Sohemy A and Archer MC, 2000; Bennis F et al, 1993; Harwood HJ Jr et al, 1991; Kawata S et al, 1990).

In order to evaluate the activity of this pathway in glioblastoma multiforme (GBM) cell lines, I designed specific primer sets to detect 3'-hydroxy-3'-metylglutaryl-CoenzimeA Reductase (HMGCR), Mevalonate kinase (MVK), Farnesyl Diphosphate Synthase (FDPS), Farnesyl Diphosphate Farnesyl Transferase 1 (FDFT1), Rab Geranylgeranyl Transferase 1α (RabGGTA) and Low-density Lipoprotein Receptor (LDLR) enzymes' expression by quantitative RealTime-PCR (qRT-PCR) (Table 1).

CDKN1A, a well-known and well-characterized p53 target gene, was considered as a control gene in all experiments (Table 1). Moreover, I compared results obtained between the two different GBM cell lines with a control cell line, the Normal Human Astrocytes (NHA) (Figure 10).

Lysates from cultured cells, analyzed by Western blot assay, showed an increase in enzymes' concentration correlated to p53 protein expression (Figure 11).

Results obtained by qRT-PCR (Figure 12) showed a significant up-regulation of MVA enzymes in cells bearing wtp53 (NHA and U343 cell lines) compared to cells with mut(R273H)p53 (U251 cells).

In order to verify the existence of a link between p53 and induction of MVA enzymes expression, NHA cells were incubated with etoposide (Eto) at a concentration of 25μM for 24 hours.

Etoposide is an anti-cancer drug able to induce DNA double strand breaks (DSBs) determining p53 activation, specifically, it is a topoisomerase II

inhibitor and it is widely used as apoptosis' inducer (Dai C et al, 2011). In this case, genes expression levels increased after treatment in NHA cells (Figure 12).

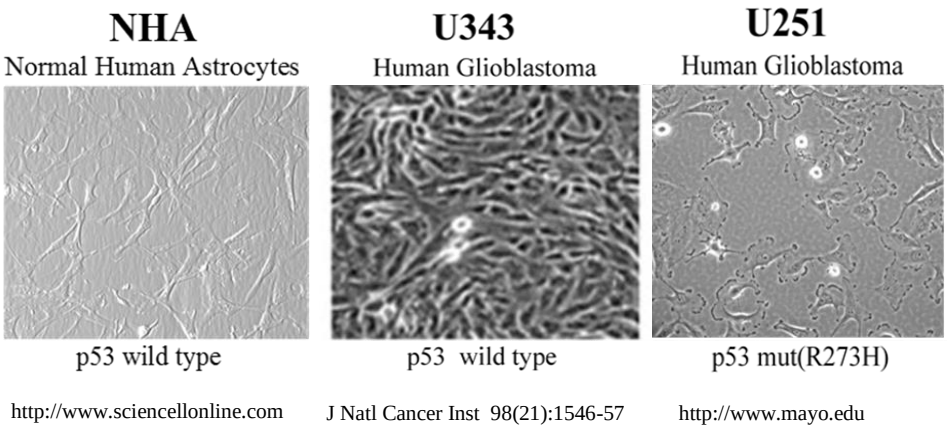


Figure 10. **Cells considered in this study: NHA, U343 and U251 cell lines**

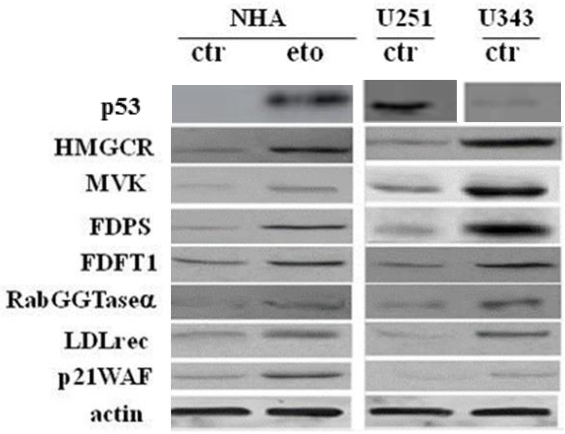


Figure 11. Western blot analyses of protein levels of MVA pathway enzymes. Protein expression levels of MVA enzymes in untreated and Eto-treated NHA cells and in U343 and U251 glioblastoma cell lines. A representative actin was used for all samples.

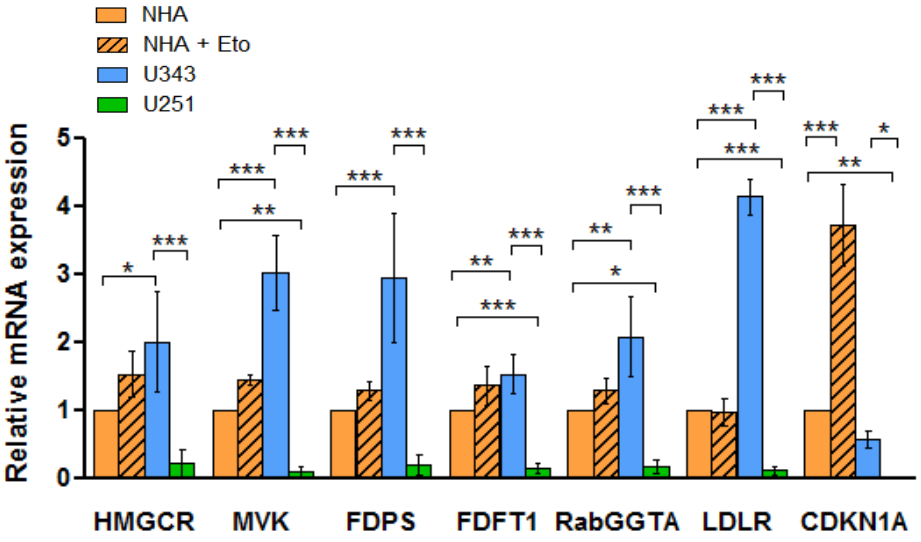


Figure 12. Basal mRNA expression of mevalonate pathway genes. qRT-PCR analyses of NHA cells untreated and treated with etoposide (25µM for 24 h), U343 and U251 cells. All the data represented mean +/- Standard Deviation (SD) of 3 independent experiments. Statistical analysis was conducted with one way ANOVA and indicated a significant difference in gene expression levels ($p \leq 0.001$ = ***; $p \leq 0.01$=**; $p \leq 0.05$=*).

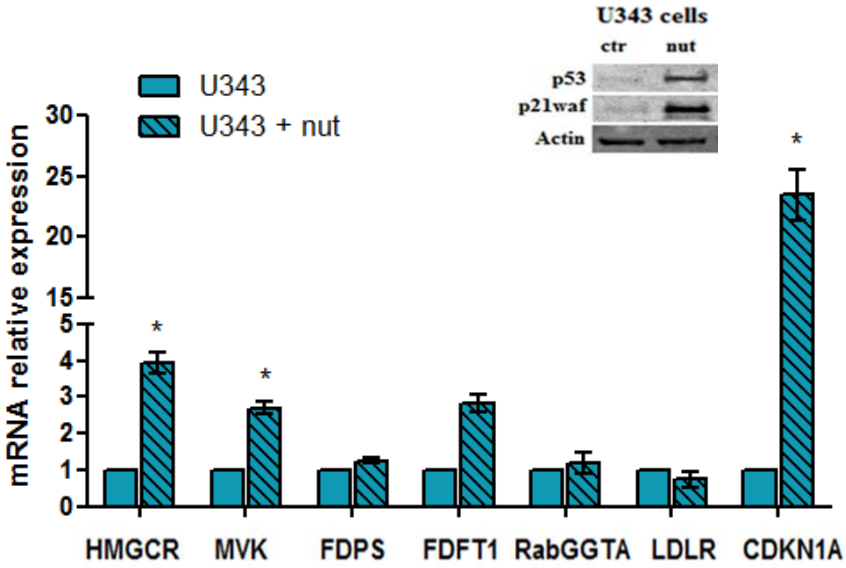


Figure 13. mRNA expression in U343 cells. U343 cells were incubated with Nutlin-3a (Nut). Nut is a specific activator of p53, it is able to interfere with Mdm2 binding of p53 and increases its transriptional activity

More, U343 cells were incubated with Nutlin-3a.

Nutlin-3a is an inhibitor of the ubiquitin ligase Mdm2, it disrupts Mdm2-p53 interaction and stabilizes and activates p53 (Poyurovsky MV et al, 2010). In U343 cells system, Nut-3a increased MVA genes' expression.

Furthermore, in order to better characterize a p53 involvement in regulation of this pathway, I examined the expression of MVA enzymes following ectopic expression of p53 in U251 (mut(R273H)p53) (Figure14). I transfected U251 cells with a plasmid containing the wild type p53 sequence, the pCMV-p53.

Cells, at a confluence of 80%, were transfected with 5μM of pCMVp53 using Lipofectamine 2000. Results obtained by this experiment showed that, all enzymes increased their mRNA expression levels after restoration of wtp53 in cells (Figure 14), with a significant difference for FDPS, LDLR and CDKN1A. Western blot assay showed a rise in p53 protein concentration and it was used as control for a good transfection procedure.

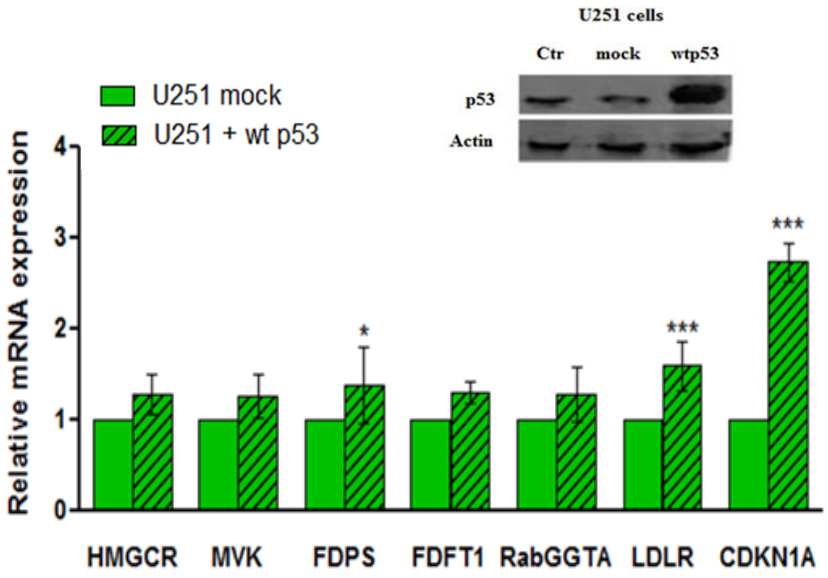


Figure 14. Expression levels of MVA genes in transfected U251. U251 cells were transfected with 5µM of empty vector control (mock) or with 5 µM of vector containing wtp53 (CMVp53). Cells transfected with wtp53 presented an increase of MVA genes expression compared to cells transfected with mock. Western blot assay confirmed p53 increase after transfection.

To corroborate these findings I examined other cellular control systems.

I transfected U343 cells with mut(R273H)p53 vector. This plasmid vector contains the same U251-p53 mutation. I hypotized that this construct could mimics the effect of a p53 allele mutation in U343 cells reducing MVA genes' expression levels. Results obtained confirmed hypothesis that mutated allele hinders wtp53 activity determining its *loss of function* in MVA enzymes transcription (Figure 15). CDKN1A gene expression resulted elevated with this treatment, probably due to compensative mechanisms linked to cellular stress (Macleod KF et al, 1995).

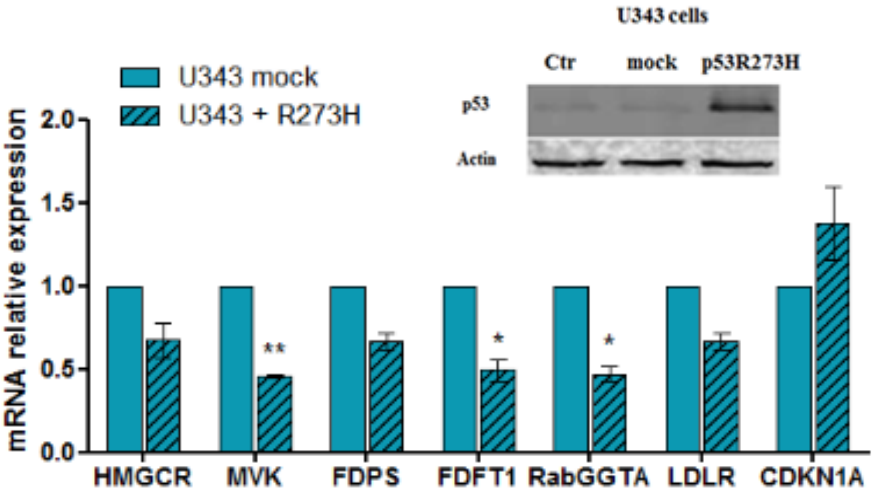


Figure 15. Expression levels of MVA genes in U343 transfected with mut(R273H) p53. U343 cells were transfected with 5µM of empty vector control (mock) or with 5 µM of vector containing mut(R273H)p53. Cells transfected with mutp53 presented a decrease of MVA genes expression, this confirm the hypothesis that mutated p53 is able to form inactive tetramers binding wtp53 subunits. Western blot assay confirmed p53 increase after transfection due to the accumulation of resident p53 plus transfected mutp53.

Human Embryonal Kidney 293 cells (HEK293) are null-p53 cells and were transfected with 5µM pCMVp53 or co-transfected with both pCMVp53 and mut(R273H)p53 vectors (3µM of each construct), to monitor MVA enzymes expression levels.

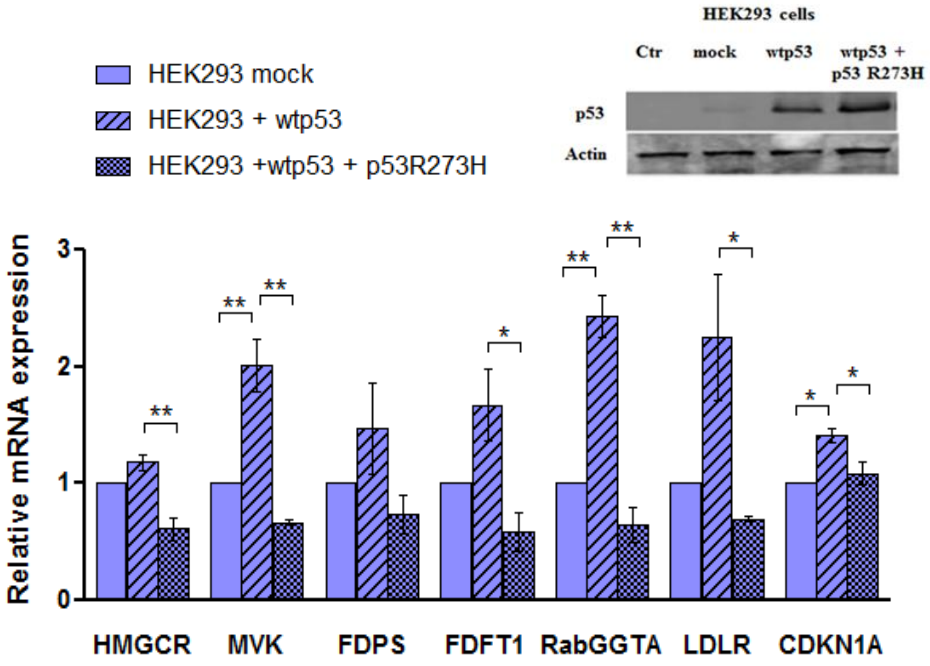


Figure 16. Expression levels of MVA genes in HEK293 cells transfected with wtp53 and with wtp53 + mut(R273H)p53. U343 cells were transfected with 5µM of empty vector control (mock) or with 5 µM of vector containing wt p53 or with a combination of wtp53 and mut(R273H)p53. Cells transfected with wtp53 presented an increase of MVA enzymes expression. In cells transfected with both plasmids expression of enzymes was reduced comparing result to cells transfected with wtp53 alone. Western blot assay confirmed p53 increase after transfection due to the accumulation of resident p53 plus transfected mutp53.

Even in this further control-cell system, hypothesis of p53 involvement in MVA enzymes regulation was confirmed by increase of mRNA expression levels after wtp53 transfection and attenuation of expression in case of co-transfection with wt53 and mut(R273H)p53 (Figure 16). These first findings suggested a direct p53 involvement in MVA enzymes' expression in transformed cells.

4.2 p53 directly binds specific p53 Responsive Elements in MVA enzymes gene-sequences.

First results suggested a relationship between p53 transcriptional activation and MVA enzymes expression in glioma.

To evaluate the link between MVA pathway's genes and p53 transcriptional activity in tumorigenesis I performed Chromatin-Immunoprecipitation assays.

p53 induces transcription of its target-genes by a direct binding of PuPuPuC(AT)(TA)GPyPyPy sequence presented in p53-Responsive Element (p53RE) of DNA regulatory elements (promoters and enhancers) (Hoh J et al, 2002).

I analyzed MVA genes' sequences using MatInspector software, by Genomatix, a tool projected to recognize transcription factors' binding sites (TFBS) in nucleotide sequences using a large library of weight matrices and assigning a probability value to the binding. (Cartharius K et al, 2005). By MatInspector I identified putative p53-Responsive Elements (p53REs) in regions including 3 kb upstream and 2 kb downstream relative to transcription start sites (TSS) of MVA enzymes sequences, while for FDFT1, MatInspector software, identified three sites located in intron in gene coding sequence (respectively positions +19178, +19412, +19577 relative to TSS) (Table 3).

To carry out ChIP assays, I designed specific primer sets to detect p53REs (Table 3, Table 4) to test the antibody anti-p53 immunoprecipitated chromatin samples belonging from U343, U251, pCMVp53 transfected U251, NHA and NHA treated with etoposide cells (Figure 17).

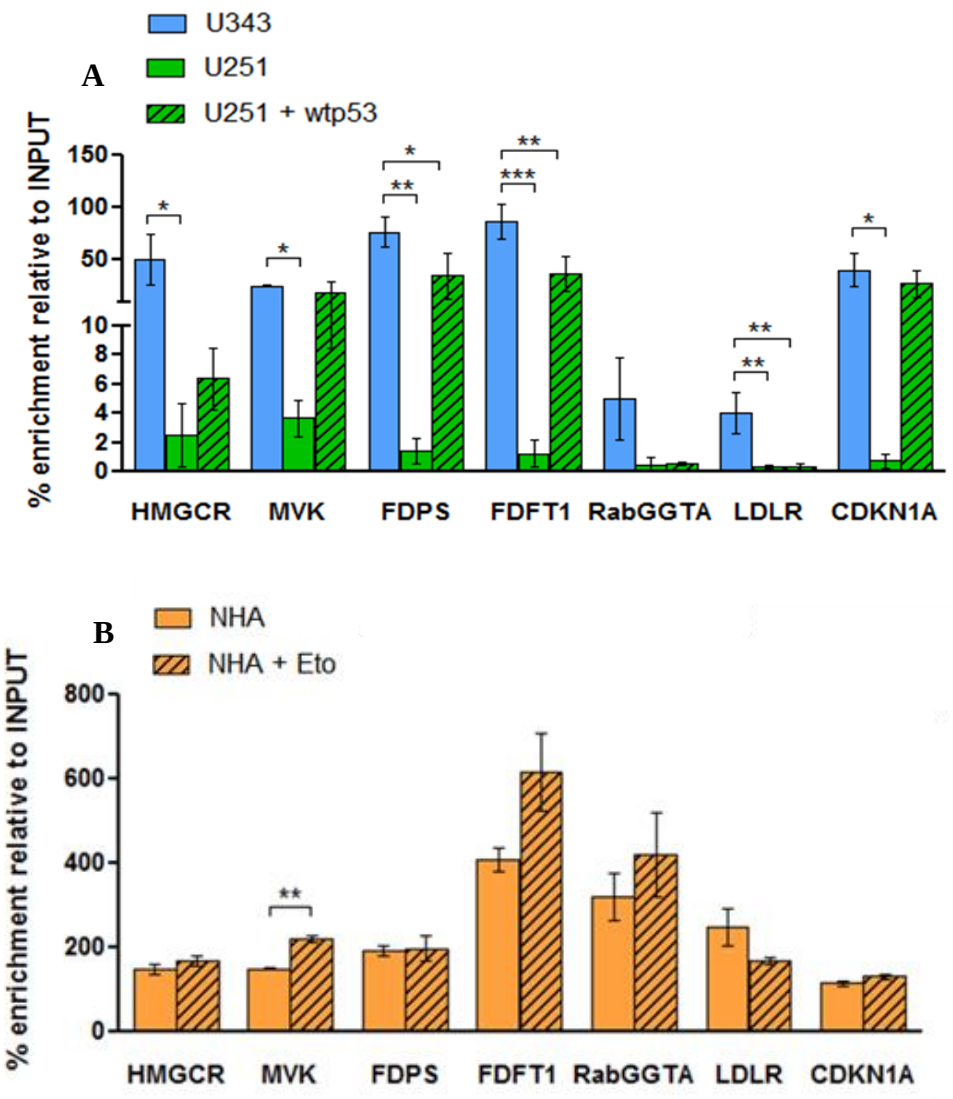


Figure 17. p53 binding with Mevalonate genes promoter regions. (A) Chromatine samples of U343, U251 and wtp53-transfected U251 cells were immunoprecipitated along with p53REs using the polyclonal anti-p53 antibodies. qRT-PCR analysis of immunoprecipitated chromatine were performed using specific primers sets (Table 2), results were normalized to 1% input. (B) qRT-PCR analysis of α-p53 immunoprecipitated chromatine of NHA cells, untreated and treated with etoposide at 25 μM for 24 h. All the data represented mean ± Standard Deviation (SD) at least two independent experiments, results were analyzed with a one-way ANOVA (A) and two-tailed Student *t*-test (B). Significant difference in immunoprecipitated chromatin was expressed as $p \leq 0.001$ = ***; $p \leq 0.01$=**; $p \leq 0.05$=*

Among all genes, HMGCR, MVK, FDPS and FDFT1 responsive elements regions were enriched in the anti-p53 immunoprecipitated chromatin of U343 and wtp53-transfected U251 and in normal control cells, NHA untreated and treated with etoposide at 25 μM for 24 hours.

Chromatin enrichment for the examined genes was compared to input and CDKN1A, a *bona fide* p53 target gene, was considered as control.

Furthermore, I observed:

i) U251 cells' immunoprecipitated chromatin presented no binding ability by p53 in considered putative REs

ii) p53 bond on REs of RabGGTA and LDLR enzymes was present in untreated and treated NHA cells.

Because physical association of a transcription factor with a genomic sequence in proximity to a gene does not necessarily imply a direct gene transcriptional increase, I cloned p53RE regions of HMGCR, MVK, FDPS and FDFT1 and transferred them in a pGL3 plasmid (Promega), where the luciferase (Luc) gene was under their promoter-activity control. I also cloned promoter regions deleted of p53REs, using sets of specific primers (Table 4) and, finally, I induced point mutation of the recognized p53 responsive sequence of the mentioned genes with specific mutation primers (Table 4) (Figure 18).

I transfected promoters-containing plasmids in U343 cells, transfected with control empty vector and with mut(R273H)p53, and in U251 cells, in mock and pCMVp53 transfected cells, to carry out Luciferase Reporter Genes assays and legitimate these regions as p53-responsive and activated elements (Figure 19).

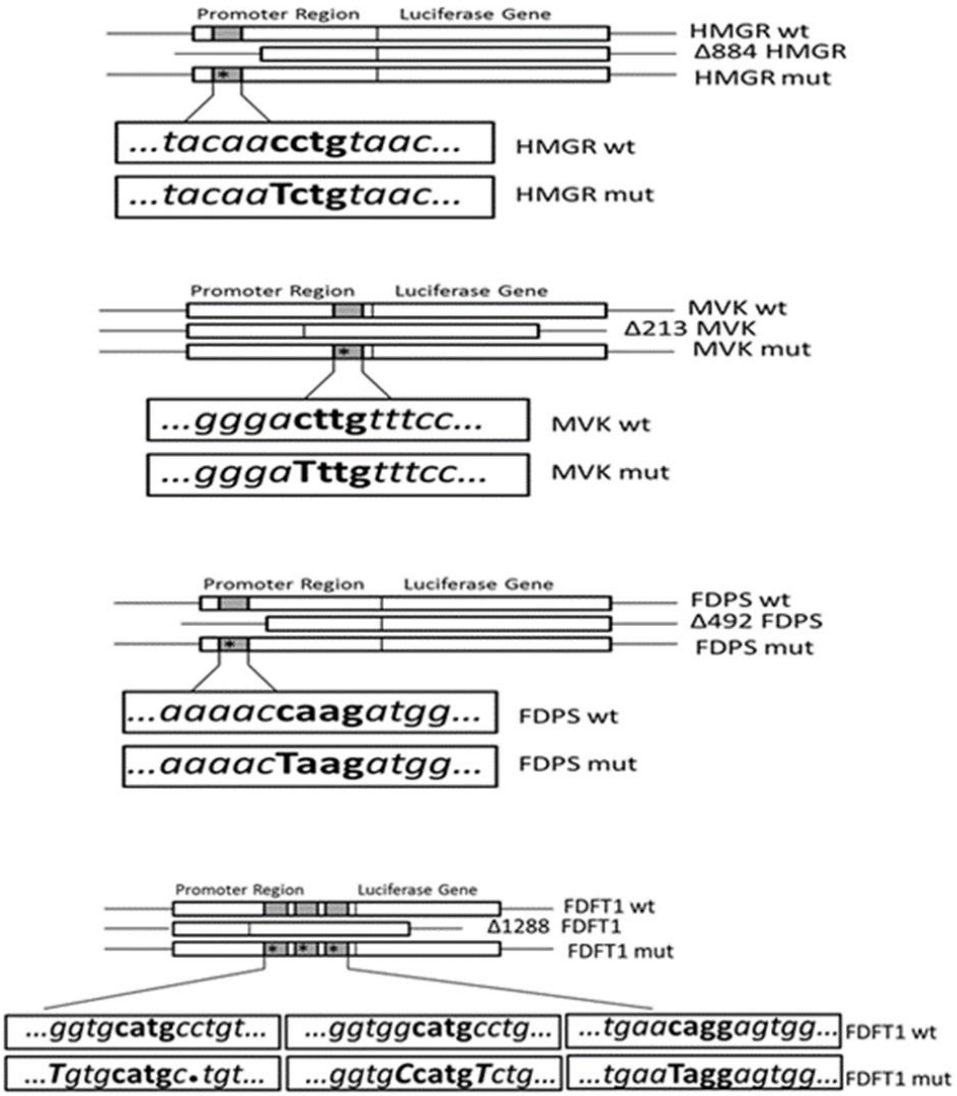


Figure 18. MVA enzymes' promoter regions were cloned in pGL3 luciferase reporter vector. pGL3 provides a system for the quantitative analysis of factor that regulate gene expression and it presents a coding region for firefly luciferase (Photinus pyralis) gene, preceded by a multi-restriction site region, in which is possible insert sequence of interest. p53REs positive to ChIP assays were cloned and integrated in a pGL3 luciferase reporter vector to evaluate the link between these regions promoter potential and p53 transcriptional activity. Regions cloned from HMGCR, MVK, FDPS, FDFT1 genes were also deleted and mutated in p53RE and subsequently tested for transcription of luciferase reporter gene.

U343 cells transfected with promoters-containing plasmids, presented an elevate rate of Luciferase production compared to cells transfected with mock empty vector, this suggested that in U343 cell system p53 is able to induce expression of HMGCR, MVK, FDPS and FDFT1 enzymes through the binding of these genes promoter or enhancer (this is the case of FDFT1) regions (Figure 19A). Co-transfection of U343 with promoters-containing plasmids and mut(R273H)p53 showed a reduction of Luciferase signal (Figure 19B).

A different scenario was observed in U251 cells, where the presence of R273H mutation in p53 DNA-binding site prevented p53 binding of responsive regions in MVA enzymes genes. In this case, expression of luciferase activity is comparable to control cells transfected with empty vector (mock) (Figure 19C) and there was any luciferase activity induction.

Instead, U251 cells transfected with pCMVp53 plasmid and co-transfected with pGL3-promoter-bearing constructs showed a luciferase expression pattern similar to U343 transfected cells (Figure 19D).

Cells transfected with deleted (Δp53RE) and mutated (mutp53RE) constructs of p53 responsive regions (Figure 18) presented a significant decreased luciferase signal compared to cells treated with wt-promoter constructs (Figura19).

All these evidences confirmed results obtained by ChIP assays, by Luciferase reporter assays, I demonstrated that p53 binds on promoters of HMGCR, MVK, FDPS and FDFT1 enzymes in correspondence of well-defined p53RE and, furthermore, binding and transcriptional activation of target genes were significantly reduced in cells bearing mut(R273H)p53.

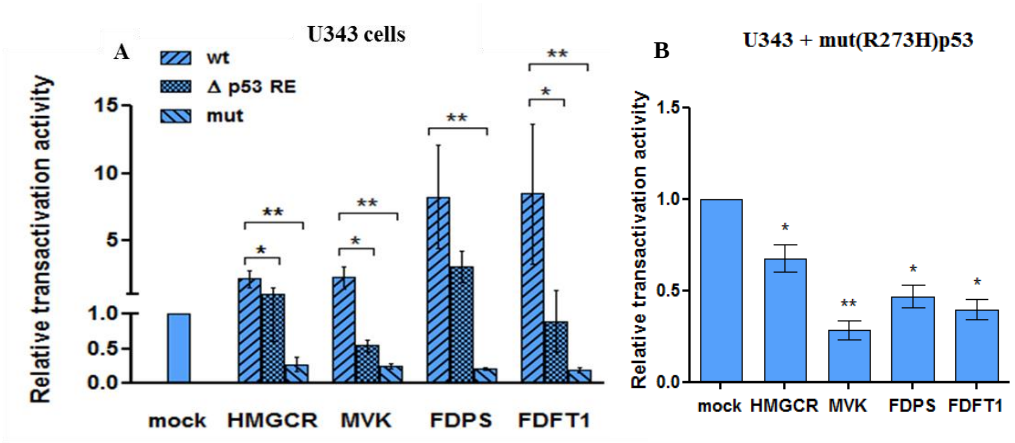
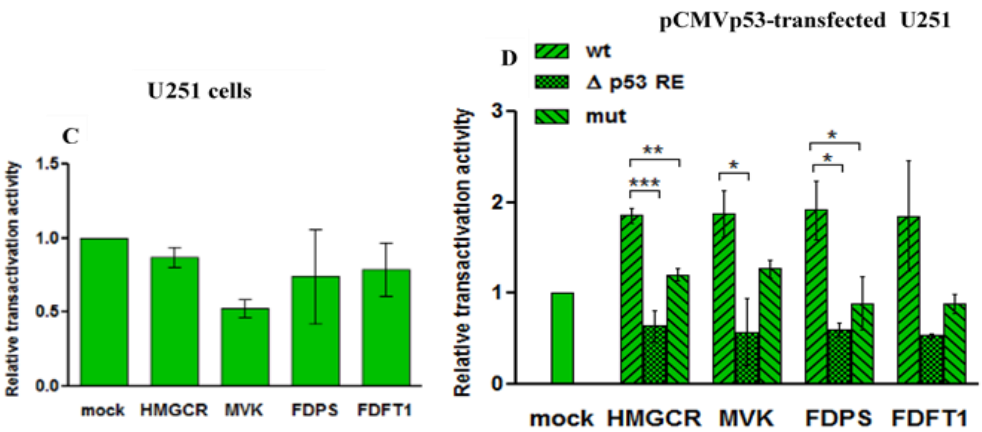


Figure 19. Luciferase Reporter Assays.

A) U343 cells were transfected with the three different pGL3 plasmids containing **i**. the cloned wild type (wt) p53RE from HMGCR, MVK, FDPS and FDFT1 gene sequences, **ii**. the deleted-cloned sequences of the mentioned genes (Δp53RE) and **iii**. the mutated constructs (mut). B) U343 were co-transfected with mut(R273H)p53 and promoter-containing plasmids showing decrease of luciferase reporter signal.

C) U251 cells were transfected with wt promoter-constructs of MVA enzymes and D) pCMVp53-transfected U251 cells were co-transfected with wt, Δp53RE and with the mutated constructs of p53 responding regions of HMGCR, MVK, FDPS and FDFT1 genes.

For all cellular system, luciferase synthesis of transfected cells with the MVA constructs is compared to luciferase synthesis of cells transfected with pGL3 empty vector (mock).



4.3 p53 modulates cholesterol biosynthesis in glioblastoma cells

Generally, cells obtain cholesterol by receptor-mediated endocytosis through LDLR or through endogenous synthesis where HMGCR is the key regulatory enzyme for MVA pathway. HMGCR has a complex feedback regulation (Goldstein JL and Brown MS, 1990) through transcriptional and post-translational mechanisms and by sterol and non-sterol pathway's products. (Nakanishi M et al, 1988).

Because p53 regulated the expression of some enzymes belonging to MVA pathway I measured synthesis of sterol products in NHA, U343 and U251 cell lines (Figure 20) using [^{14}C]-acetate, which was incorporated in neo-synthetized endogenous cholesterol.

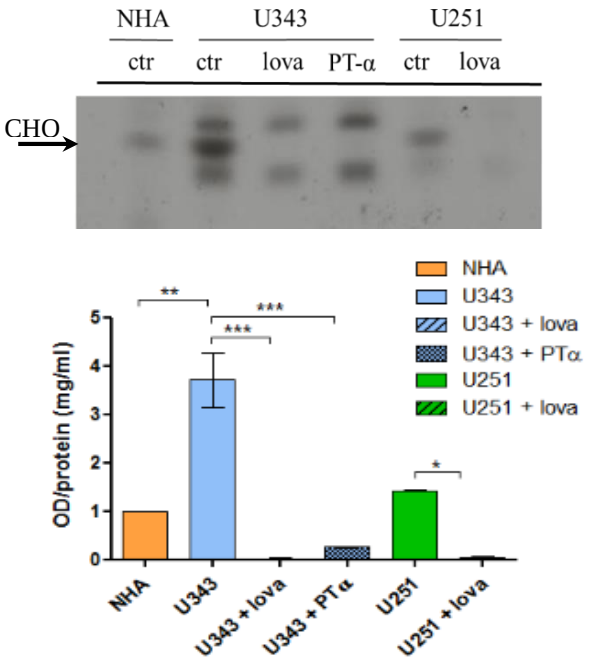


Figure 20.
[^{14}C] - acetate incorporation. Chromatogram of non-saponifiable lipids from cells radiolabeled during the 14-h period following treatment. U343 and U251 cells were incubated with lovastatin (lova) (10 µM) and cyclic pifithrin-α (PT-α) (30 µM). The chromatogram and values shown here are representative of two indipendent experiments. Cholesterol (CHO) bands are evidentiated with an arrow. Statistical analysis is performed with one tail T-test ($p \leq 0.001$ = ***; $p \leq 0.01$ = **; $p \leq 0.05$ = *)

Activation of MVA pathway in U343 cell line demonstrated an increase of *de novo* cholesterol synthesis compared to U251 and NHA cell lines. Furthermore, treatment of U343 and U251 cells with lovastatin (lova) (10 μM for 24h), a specific inhibitor of HMGCR enzyme, showed a considerable reduction of cholesterol biosynthesis demonstrated by a decrease of [^{14}C]-acetate incorporation.

U343 cells were also incubated with Pifithrin-α (PT-α) (30 μM for 24h), an inhibitor of p53 activity, and, even in this case, there was a reduction in endogenous cholesterol synthesis, suggesting a correlation between p53 and MVA pathway.

Indeed, U251, at basal conditions, showed a rate of endogenous cholesterol higher than NHA control cells.

Finally, I obtained preliminary results concerning MVA pathway enzymes' expression in human biopsies from brain tumor patients.

A collection of 38 samples was gathered from patients from University of L'Aquila (provided by professor Annamaria Cimini) and from the "G.Rummo" Medical Hospital, in Benevento (supplied by doctor Giuseppe Catapano).

Total RNA and genomic DNA were extracted from tissues and analyzed. A qRT-PCR was performed to evaluate mRNA of HMGCR, MVK, FDPS, FDFT1 and CDKN1A expression levels, while genomic DNA was sequentiated to look for mutation in exons 5, 6, 7 and 8 of p53 (Table 5), corresponding to p53's hot spot mutation region.

Majority of patients exhibited high levels of MVA enzymes compared to NHA, considered as normal control for this experiment. (Figure 21).

				Mevalonate genes expression level (fold induction)					
Sample ID	***WHO class***	***WHO Grade***	***p53 status***	***HMGCR***	***MVK***	***FDPS***	***FDFT1***	***CDKN1A***	
1	GBM	IV	WT	1,4	4,9	25,6	15,4	1,9	
2	GBM	IV	WT	2,4	6,8	8,2	8,4	3	
3	GBM	IV	WT	11.9	4.4	25,8	10,2	6,9	
4	GBM	IV	WT	> 50	11,9	> 50	29,4	> 50	
5	GBM	IV	WT	4,4	1,2	5	3,5	11,6	
6	GBM	IV	WT	> 50	26,3	> 50	> 50	> 50	
7	GBM	IV	WT	> 50	5,7	36,3	2,4	> 50	
8	GBM	IV	WT	7,5	> 50	> 50	> 50	> 50	
9	GBM	IV	WT	4,9	0,9	0,4	0,2	1,7	
10	GBM	IV	WT	6,8	2,5	3,4	4,8	0,03	
11	GBM	IV	WT	4,9	1,8	2,5	2,7	0,07	
12	GBM	IV	WT	6,8	3,9	21,5	16,9	1,1	
13	GBM	IV	WT	17,6	5,8	6,9	8,8	0,4	
14	GBM	IV	WT	0,6	0,06	0,1	0,2	0,01	
15	GBM	IV	WT	1,4	0,5	0,3	0,4	0,05	
16	GBM	IV	WT	3,11	13,2	2,4	2,5	0,1	
17	GBM	IV	WT	22,7	3,2	6,1	6,8	0,3	
18	GBM	IV	WT	0,3	0,3	0,4	0,3	0,01	
19	GBM	IV	WT	0,4	0,3	0,2	0,2	0,05	
20	GBM	IV	WT	0,7	0,41	0,94	0,65	0,08	
21	GBM	IV	WT	1,05	1,6	1,5	1,54	0,7	
22	GBM	IV	WT	2,03	3,2	1,5	3,2	3,1	
23	GBM	IV	WT	0,4	8,9	5,5	3,1	7,6	
24	GBM	IV	WT	2,6	1,2	1,4	1,7	0,05	
25	GBM	IV	WT	0,4	3,9	2,5	2,02	0,7	
26	GBM	IV	WT	5,3	6,7	16,8	10,3	1,4	
27	GBM	IV	WT	0,2	0,11	0,14	0,09	0,02	
28	GBM	IV	WT	2,7	4,7	7,5	12,3	0,9	
29	GBM	IV	WT	0,3	1,1	0,9	0,7	0,2	
30	GBM	IV	WT	0,56	1,05	0,23	1,47	0,36	
31	GBM	IV	WT	1,2	0,66	2,7	0,9	> 50	
32	AA	III	WT	13,2	15,4	19,2	31,4	44,4	
33	AA	III	WT	3,2	22,3	46,3	> 50	4,6	
34	AA	III	WT	0,8	0,4	1,5	0,9	0,2	
35	AA	III	WT	1	0,5	0,9	0,9	0,03	
36	LGA	II	WT	> 50	> 50	> 50	> 50	> 50	
37	LGA	II	WT	> 50	20,8	35,4	41,6	11,4	
38	LGA	II	WT	25,1	15	> 50	11,1	> 50	
NHA cells	Normal Human Astrocytes		WT	1	1	1	1	1	
U343 cells	GBM	IV	WT	2,01	3,03	2,99	1,54	0,6	
U251 cells	**GBM**	**IV**	**M**	**0,2**	**0,1**	**0,2**	**0,1**	**0,01**	**ex 8; 273 (CGT>CaT)**

Table 8. Patients' preliminary observations. Table showed patients' mRNA levels of HMGCR, MVK, FDPS and FDFT1 enzymes and CDKN1A expression levels. Patients are grouped according hystological classification of tumor including: GBM (Gliobastoma multiforme, IV grade), AA (Anaplastic Astrocytoma, III grade) and LGA (Low Grade Astrocytoma, II grade).

5. DISCUSSION

Malignant gliomas are highly dependent on the mevalonate pathway for cholesterol and isoprenoyds synthesis compared with normal cells counterpart (Prasanna P et al, 1996).

MVA pathway has a highly balanced metabolism in non-transformed cells, recent findings showed that loss of regulatory mechanisms may contribute to dysregulation of this pathway and could promote tumorigenesis (Clendening JW et al, 2010).

In this study I analyzed the expression profiles of some enzymes relative to this pathway in two different cell lines of glioblastoma multiforme, U343 cells expressing a wild type p53 and U251 cells containing a mutant p53 (R273H) alleles lacking of transcriptional activity (Olivier M et al, 2002).

I observed that 3'-Hdroxy-3'-Methylglutaryl-Coenzyme A Reductase (HMGCR), Mevalonate Kinase (MVK), Farnesyl Diphosphate Synthase (FDPS), Farnesyl Diphosphate Farnesyl Transferase (FDFT1), Rab Geranylgeranyl Transferase α (RabGGTA) and LDL receptor (LDLR) are upregulated in U343 cells and in normal human astrocytes compared with U251 cells bearing a mutant p53 (Figure 12).

I presented evidences that increased mRNA expression of MVA enzymes was supported by elevation of protein levels of mentioned genes as well (Figure 11).

These findings indicated the ability of p53 to bind a well-defined p53 responsive element (p53RE) in the promoter region of HMGCR, MVK, FDPS and FDFT1, and induce their transcription (Figure 17 and Figure 19).

Interestingly, p53 binds to the promoter regions of the RabGGTA and LDLR in normal human astrocytes but not in a glioblastoma cell line, U343 cells.

In the brain, the astrocytes are identified as cholesterol-producer cells and supply cholesterol to neurons via LDL receptors (Pfrieger FW and Ungerer N, 2011).

These results suggested the hypothesis that the transformation of these cells from normal to cancerous is accompanied by a dysregulation of MVA pathway enzymes and by a loss of p53 control of LDLR and RabGGTA, the latter has already been described to be a p53 target gene in a human lymphoblastoid cell lines (Jen KY and Cheung VG 2005).

How p53 discriminates among the several binding sites is unclear, although recent observations suggest that it might be due to aberrant DNA methylation and associated to chromatin changes (Botcheva K et al, 2011).

In this contest, we observed that low levels of p53 are sufficient in human glioblastoma cells to enhance mRNA levels of HMGCR and of the others enzymes by causing an increase of the metabolic flux through the mevalonate pathway, this contributes to cell survival according to the pro-survival roles of wild-type p53 (Kim E et al, 2009)

Recent work, by Freed-Pastor et al. (Freed-Pastor WA et al, 2012), showed that mutant p53 (mut (R280K)p53) is recruited to genes that encode MVA pathway enzymes by SREBPs to upregulate their expression in human breast cancer cells and contributing to the maintenance of the malignant phenotype; furthermore, they speculated that this activity of mutant p53 may be a "remnant of an unrecognized wild-type p53 function".

Here, I revealed that the p53 bound to specific regions into the promoters of the gene relative to mevalonate pathway activating their transcription. The upregulation of MVA pathway in U343 cell line caused a greater elevation in the *de novo* synthesis of cholesterol compared with U251 cells and NHA, in fact, in U251 cells we observed a 40% decrease in cell cholesterol. Furthermore, PT-α treatment of U343 cells inhibited the [^{14}C]-acetate incorporation in cholesterol and suggested a correlation between p53 and cholesterol pathway regulation (Figure 20).

Cholesterol is an essential component of cellular membranes; cells either obtain cholesterol through receptor-mediated endocytosis of low-density lipoprotein

(LDL) from plasma or through endogenous synthesis using acetate and the HMGCR, is a key regulatory enzyme for endogenous production of cholesterol. HMGCR is subjected to complex feedback regulation at the transcriptional, posttranscriptional and posttranslational levels by both sterol and non-sterol products, derived from the cholesterol biosynthetic pathway (Goldstein JL and Brown MS, 1990; Nakanishi M at al, 1988).

Coordinate control of LDLR and HMGCR by sterols is mediated through a specific regulatory element in the promoter regions of both genes known as the sterol regulatory element (SRE) (Osborne TF, 1995). Growth and survival of tumor cells are highly dependent on attenuation of sterol-mediated reductase gene regulation, a phenomenon referred to as "sterol-resistant" or "sterol-independent" regulation of reductase (Hentosh P et al, 2001). The sterol-resistant regulation may be a consequence of quantitative or qualitative alterations in transcription factors that modify SREBP-mediated transcriptional activity (Brown MS and Goldstein JL, 1997). Furthermore, SREBPs alone are inefficient transcriptional activators and require additional regulatory transcription factors for high activation levels (Goldstein JL and Brown MS, 1990).

The results showed here suggest that in U343 cells p53 could to be the major regulator of the MVA pathway allowing the elevation of cholesterol metabolism essential for cellular survival and then drive tumorigenesis, this hypothesis is currently under investigation.

Furthermore, I observed that human glioblastoma cells bearing mutant p53, U251 cells, are unable to upregulate the sterol biosynthesis genes but, at the same time, showed a rate of endogenous cholesterol synthesis higher than the normal human astrocytes allowing the elevated rate of cell proliferation. I consider that the reduction of cholesterol enzymes' expression and, as a consequence, a reduced amount of intracellular cholesterol, make them

particularly sensitive to inhibition of this pathway (Yongjun Y et al, 2013) and the understanding of this mechanism required further studies.

Indeed, the lovastatin treatment reduced considerably the amount of free cholesterol in both glioblastoma cell lines (U343 and U251) (Figure 20).

In this study, moreover, to investigate whether the regulation of the mevalonate pathway by p53 is generalizable to human patients, we evaluated the expression of HMGCR, MVK, FDPS, FDFT1 and CDKN1A gens and compared mRNA levels to p53 status. I screened for mutations affecting the "hot spot" mutation region of p53, including exon 5, 6, 7 and 8 of 38 human brain tumors. I observed, that in the most of samples, expression levels of MVA enzymes correlated with the mRNA levels of the CDKN1A, and they are overexpressed compared with normal human astrocytes (Table 8). Unfortunately, I did not observed mutations in analyzed exons of the p53 genes belonging to patients (even if this not excluded existing of other mutations in not examined exons) (Table 8).

TP53 mutations are rare in certain human cancer entities. In this study, I analyzed primary GBMs samples, which are the most frequently occurring and most aggressive form of GBMs. TP53 mutations are often found in secondary GBM (65%), but are considerably less frequent in primary GBM, comprising less than 30% of the cases and are a tardive events during glioblastoma progression (Ohgaki, H. et al. 2004; Ohgaki H and Kleihues P, 2005). However, it will be interesting to screen mutation in the entire gene of p53 for the samples where the expression levels of analyzed genes are low, because mutations affecting the transactivation domain of p53 are able to inactivate important function of the protein (von Eckardstein K et al, 1997).

In addition, the most of brain tumors exhibit a high chemo-resistance as glioma cells with wild type p53 to cytotoxic treatments used in clinical practice compared to glioma cells with transcriptionally inactive mutant p53 (Woo IS et al, 2010; Hirose Y et al, 2001). It thus appears that p53 activities associated with DNA repair contribute to the overall survival potential and drug resistance.

Supporting the notion, there are evidences that chemo-resistance, mainly mediated by p53 in glioma, relies at least in part on the ability of p53 to activate DNA repair machinery (Haar CP et al, 2012). Other p53 linked mechanisms are investigated during last years to understand better the relationship between p53 and chemoresistance.

A gene whose product directly contributes to drug resistance of gliomas is MGMT (O6-methylguanine-DNA methyltransferase), an enzyme, which removes methyl and chloroethyl groups from the O6-position of guanine (Hermisson M et al, 2006) which is directly regulated by wild type p53 (Blough MD et al. 2007).

Furthermore, the effects of p53 on drug resistance and the survival potential seen in different types of cancer cells could be also attributed to the activation of cholesterol pathway that, in addition to support cells survival, may modulate resistance to a variety of anticancer drugs, within a functional phenotype termed multidrug-resistance (MDR). The plasma membranes of MDR+ tumor cells are particularly rich in cholesterol, which facilitates the activity of Pglycoprotein (Pgp), an integral membrane transporter extruding chemotherapy drugs. Another hallmark of MDR+ tumor cells is the increased isoprenylation and activity of G-proteins which are also dependent on the rate of the MVA pathway activity (Riganti C et al, 2013).

In summary, obtained results demonstrate that p53 is an essential transcriptional factor for the regulation of MVA pathway.

Finally, further studies will be necessary to determine the mechanism by which p53 regulates enzymes relative to MVA pathway, and, more a better understanding of how p53 coordinates metabolic adaptation will facilitate the identification of novel therapeutic targets and will also illuminate the wider role of p53 in human biology.

6. CONCLUSIONS

These results elucidated a new role of p53 transcription factor in regulation of MVA pathway's enzymes.

Hyperactivation of p53, because of genotoxic stress or following oncogenic activation, deregulates MVA and participates in cancer progression. Cellular metabolic reorganization, during tumorigenesis, affects several pathways with the aim of fulfill energetic requirements of an active proliferating cell. Recent evidences explained as dysregulation of mevalonate pathway promotes transformation, in fact, many tumor cells presented elevated expression levels of MVA enzymes, associated with high synthesis of cholesterol and isoprenoyds intermediates.

However, it is not known whether the alterations of MVA pathway make a causal contribution to cancer etiology or whether its dysregulation occurs as a consequence of transformation. Given the importance of metabolic reprogramming in tumor development, it is not surprising that many oncogenes and tumor suppressor genes have been shown to participate in this pathway's control.

Mutation status of p53 influences directly MVA pathway activity as well.

Using U343 and U251, two high-grade glioblastoma multiforme cell lines, that differ for p53 mutation status, and NHA cells, used as normal control, I demonstrated that p53 recognizes specific regions of MVA enzymes promoters and in particular, it is able to induce transcription of HMGCR, MVK, FDPS and FDFT1 genes in early-transformed cells holding wild type p53 already (U343). When a p53 inactivation mutation occurs, this transcription factor could not bind responsive elements in promoters of its target genes and there are no mRNAs expression (as happens in U251, bearing mut(R273H)p53).

In this scenario, p53 prove to be involved in mechanisms committed to MVA enzymes regulation. Many studies demonstrated that other actors are engaged

in, such as SREBP, and further studies will be necessary to understand how p53 integrates its tasks in a so complex signaling network and how p53 coordinates metabolic adaptation during cancer progression.

These findings could facilitate the identification of novel therapeutic targets and will also unravel the wider role of p53 in human biology, not only as oncosuppressor.

7. TABLES

qRT-PCR primers			
Gene symbol	Forward Primer	Reverse Primer	Product size
HMGCR	taccatgtcaggggtacgtc	ccagtcctaatgaaaccttagaag	335 bp
MVK	gctcaagttcccagagatcg	atggtgctggttcatgtcaa	151 bp
FDPS	agcaggatttcgttcagcac	tcccggaatgctactaccac	169 bp
FDFT1	ggtcccgctgttacacaact	aaaactctgccatcccaatg	194 bp
RABGGTA	gacccctgctgtatgagaa	cacctcggcatactccatct	144 bp
LDLR	gaatttggccagacacaggt	caccgtacccagctgatttt	159 bp
CDKN1A	atgaaattcacccctttcc	ccctaggctgtgctcacttc	174 bp
β2M	cctgaattgctatgtgtctggg	aatgcggcatcttcaaacctc	267 bp

Table 1. qRT-PCR primer sets. All primers were optimized at Tm 60°C. β2microglobulin (β2M) was the housekeeping gene

ChIP primers			
Gene Symbol	Forward Primers	Reverse Primers	Product size
HMGCR	ttcccaattaggcagtttgc	gccgctagaagtctcaagga	151 bp
MVK	caccgctcaggtttcaattt	aagccgacacgggttttc	120 bp
FDPS	tgcatttccagttggtttagg	tgtcgacacagcatgtggta	197 bp
FDFT1	agggctagtggatcatgagg	gagtgcaatggcacgatct	201 bp
	ggcttatagatgaaccattgcag	tcggcaatcactgtttggta	170 bp
	acagtgattgccgacatttg	aaaccagaggggctgaaaat	201 bp
RABGGTA	atagtgcagaggccaagagc	gccttatgcagtaggtgcaa	165 bp
LDLR	cagcttattcctgggggaac	gtctctttggggactcatgg	119 bp
CDKN1A	ggtctgctactgtgtcctcc	tgcagaggatggattgttca	230 bp

Table 2. ChIP primer sets. All primers were optimized at Tm 60°C.

Cloned Promoter Region - Description					
Gene symbol	Chromosome location	Gene ID	Cloned Promoter Region (relative to TSS)	p53RE position relative to TSS	Matrix score (MatInspector)
HMGCR	5q13	3156	from - 2529 to +23	- 1736	0,8
MVK	12q24	4598	from - 937 to +124	- 68	0,92
FDPS	1q22	2224	from -1777 to +329	- 1255	0,78
FDFT1	8p23	2222	from +17932 to +20190	+ 19178	0,78
				+ 19412	0,79
				+ 19577	0,79
RabGGTA	14q11	5875	from -147 to + 1311	+ 894	0,92
LDLR	19p13	3949	from - 1450 to - 4	- 801	0,79
				-790	0,94

Table 3. Cloned promoter regions - Description. Description of p53 Responsive Elements containing regions with position relative to Transcription Statr Site (TSS) and MatInspector's putative site score.

Cloned Promoter Region - Primers			
Gene symbol	Forward Primers	Reverse Primers	Product size
HMGCR	TCGGGTACCagcc cagcaagactaagagc	CTCAAGCTTgacgag ccttcgaccaataa	2500 bp
MVK	TCGGGTACCagaa acctgagccacttcca	CTCAAGCTTctcccc aactcccacaac	1061 bp
FDPS	TCGGGTACCgctgg gctaattttggatttt	CTCAAGCTTcttcaaa acggacacaacag	2004 bp
FDFT1	TCGGGTACCgcttc gtccaagaaggacac	CTCAAGCTTgcacaa atcagcagttccaa	2258 bp
RabGGTA	TCGGGTACCatttca cacgcacgctacag	CTCAAGCTTcctaca gtcaacctcagctcac	1569 bp
LDLR	TCGGGTACCccag cccgctaattatttca	CTCAAGCTTacctca ctgcaagaggagga	1507 bp

Deleted Cloned Promoter Region - Primers				
Gene Symbol	Cloned Deleted Promoter Region (relative to TSS)	Forward Primers	Reverse Primers	Product size
ΔHMGCR	from -1593 to + 23	TCGGGTACCtggg tagttcaccegttttc	CTCAAGCTTgacg agccttcgaccaataa	1616 bp
ΔMVK	from – 937 to - 89	TCGGGTACCagaa acctgagccacttcca	CTCAAGCTTgtca ggtcccgtcaagca	848 bp
ΔFDPS	from -1186 to + 329	TCGGGTACCacct ctggtggtcttcaacg	CTCAAGCTTcttca aaacggacacaacag	1512 bp
ΔFDFT1	from + 17932 to + 18902	TCGGGTACCgctt cgtccaagaaggacac	CTCAAGCTTgag atgcttcaacataaagctg	970 bp

Mutated Cloned Promoter Region – Primers		
Gene symbol	Forward Primer	Reverse Primer
HMGCR *	gggcatgtgtacaa**T**ctgtaacttct gagatatttgg	ccaaatatctcagaagttacag**A**ttg tacacatgcc
MVK *	cgggacactcccaggga**T**ttgtttc ccattgg	ccaatgggaaacaa**A**tccctggga gtgtcccg
FDPS *	cctaccaaaac**T**aagatggcaaca agagtgacc	ggtcactcttgttgccatctt**A**gttttg gtagg
FDFT1 *	gacctctgaa**T**aggagtgggacaa ggttagtc	gactaaccttgtcccactcct**A**ttca gaggtc

Table 4. Cloned Constructs – Primer sets. Specific primer sets were used to cloned p53REs containing sequences and to induce mutations in cloned constructs. Primers used to clone p53REs regions hold a 5'- tail recognised by restriction enzymes, respectively: -GGTACC is target of KpnI endonuclease and -AAGCTT is recognized by HindIII enzyme. Primers used to induce deletions used same principles, while primers cointaining mutations were used to induce mutations in plsmids containing constructs using the QuikChange II- XL Site-Directed Mutagenesis kit

p53 – exon sequencing primers		
Exon	Forward Primer	Reverse Primer
ex. 5	ctgttcacttgtgccctgac	gcaaccagccctgtcgtct
ex. 6	agacgacagggctggttgc	ctcccagagaccccagttg
ex. 7	cctcatcttgggcctgtg	tgtgcagggtggcaagtggc
ex. 8	ctgcctcttgcttctctttt	tctcctccaccgcttcttgt

Table 5. p53 – exon sequencing primers. Primer sets used to sequentiated exons 5, 6, 7 and 8 representing p53 "hot spot mutations region".

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