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Characterization of an APEX2-p53 Fusion Protein for the Identification of New p53 Interactants

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Abbreviation List

53BP1: p53-binding protein 1

AIF: apoptosis-inducing factor

AP-1: activator protein 1

APAF1: apoptotic protease activating factor 1

APEX2: ascorbate peroxidase 2

Apo3L/Apo2L: apoptosis-inducing ligand

ASPP: apoptosis stimulating proteins of p53

ATM: protein kinase ataxia-telangiectasia mutated

ATP: adenosine triphosphate

ATR: ataxia telangiectasia and rad3-related protein

BAD: BCL-2 associated agonist of cell death

BAG: BCL-2 associated athanogene

BAK: BCL-2 homologous antagonist/killer

BAX: BCL-2 associated X-protein

BCL-2: B-cell lymphoma 2

BCL-XL: B-cell lymphoma-extra large

BH3: BCL-2 homology domain 3

BID: BH3 interacting-domain death agonist

BioID: biotin identification

BirA: biotin-protein ligase from E. coli

c-MYC: c-myelocytomatosis

CAD: caspase-activated DNase

CARM1: coactivator associated arginine methyltransferase 1

CDKN1A: cyclin dependent kinase inhibitor 1A

cDNA: complementary DNA

CHK1/CHK2: checkpoint kinase 1/2

CPT: camptothecin

CRM1: chromosomal region maintenance 1

DAPI: 4′,6-diamidino-2-phenylindole

dATP: 2-deoxy adenosine triphosphate

DISC: death-inducing signaling complex

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

DOX: doxycycline

DR3/DR4/DR5: death receptor 3/4/5

E1B-55: adenovirus E1B protein (55 kDa)

FADD: FAS associated death domain

GADD45: growth arrest and DNA damage inducible 45

GRP75: glucose-regulated protein 75

HAT: histone acetyltransferase

HPV: human papillomavirus

HRP: horseradish peroxidase

HSP60/70: heat shock protein 60/70

HTRA2/Omi: high-temperature requirement protein A2

IAP: inhibitor of apoptosis proteins

ICAD: inhibitor of CAD

IMM: inner mitochondrial membrane

L3MBTL1: L3MBTL histone methyl-lysine binding protein 1

LFS: Li-Fraumeni Syndrome

MAX: myc-associated factor X

MCL-1: myeloid leukemia cell differentiation protein

MDM2/MDM4: murine double minute 2/4

miRNA: microRNA

MOM: mitochondrial outer membrane

MOMP: mitochondrial outer membrane permeabilization

MOZ: monocytic leukemia zinc finger protein

mRNA: messenger RNA

MS: mass spectrometry

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

NES: nuclear export sequence/signal

NF1: neurofibromin 1

NFBD1: nuclear factor with BRCT domains protein 1

NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells

NLS: nuclear localization sequence/signal

NMR: nuclear magnetic resonance

OD: oligomerization domain

OMM: outer mitochondrial membrane

p53AIP1: p53-regulated apoptosis-inducing protein 1

PARP: poly ADP-ribose polymerase

PAX(2, 5, or 8): paired box 2, 5, or 8

PCNA: proliferating cell nuclear antigen

PIDD: p53-induced protein with a death domain

PPIs: protein-protein interactions

PRD: proline-rich domain

P_{tet}: tetracycline-responsive promoter

PTMs: post-translational modifications

PUMA: p53 upregulated modulator of apoptosis

RAS protein: rat sarcoma

RIP1: tumor necrosis factor receptor type 1-associated DEATH domain

RNA: ribonucleic acid

ROS: reactive oxygen species

RPA: replication protein A

RSF1: remodeling and spacing factor 1

RSV: resveratrol

rtTA: reverse tTA

RUNX: runt-related transcription factor

SETD8: SET domain containing (lysine methyltransferase) 8

SH3: SRC Homology 3 domain

SILAC: stable isotope labeling by amino acids in cell culture

Smac/DIABLO: second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI

SMYD2: SET And MYND Domain Containing 2

SSB: single-strand break

SV40: simian vacuolating virus 40

TAD: transactivation domain

TAF: TBP-associated factors

TBP: TATA-Box Binding Protein

TFIID/TFIIH: transcription factor II D/H

TIP60: histone acetyltransferase KAT5

TNF: tumor necrosis factor

TNFR1: tumor necrosis factor receptor 1

TP53: tumor protein p53

TRADD: tumor necrosis factor receptor type 1-associated DEATH domain

tTA: tetracycline-controlled transcriptional activator

USF: upstream stimulatory factor 1

UTR: untranslated region

WRAP53: WD40-encoding RNA antisense to p53

YY1: Yin Yang 1

Abstract

The tumor suppressor p53 is nicknamed the guardian of the genome and is involved in many processes that maintain cell integrity. Under stress, p53 is activated to exert its crucial functions. The gene encoding for p53, *TP53*, is mutated in 50% of human cancers. The protein p53 executes its role of tumor suppressor through the induction of cell cycle arrest or cell death. It does this by firstly acting as a transcription factor, regulating the expression of a variety of genes encoding for proteins which promote cell cycle arrest or apoptosis. A second pro-apoptotic activity of p53 implies its mitochondrial localization in stressed cells. At the mitochondrial outer membrane, it has direct interactions with various members of the BCL-2 protein family, triggering mitochondrial outer membrane permeabilization. This phenomenon is seen as the point of no return in apoptotic cell death, as it provokes the release of mitochondrial apoptosis effectors.

Recently, our lab showed that the phosphorylation of serine 392 is important for the mitochondrial localization of p53 in cells exposed to genotoxic stress. The role of S392 phosphorylation is yet unknown, but two hypotheses exist: (1) S392 phosphorylation is induced by stress signals and promotes interaction between p53 and a cytosolic interaction partner that helps in localizing p53 to the mitochondria; (2) In absence of stress, the cytosolic fraction of p53 is sequestered by one or multiple proteins. Upon stress, S392 is phosphorylated and disrupts the inhibitory complex, allowing p53 to reach the mitochondria.

The identification of these unknown cytosolic interaction partners of p53 is the ultimate goal of this project. This will allow us to explain the role of S392 phosphorylation in p53 mitochondrial localization and apoptosis induction. For this, a proximity-dependent labeling method is employed. APEX2 (ascorbate peroxidase 2), has been fused to the N-terminus of p53. The construct has been stably transfected in the H1299 ($p53^{-/-}$) cell line and the expression of APEX2-p53 is inducible by doxycycline via the Tet-On system. Here, we will characterize the fusion protein: the induction of its expression, its subcellular localization, its transcriptional activity, and its ability to trigger apoptotic cell death.

While induction of expression and mitochondrial localization of the APEX2-p53 fusion protein are successful, we failed to see any transcriptional activity. Moreover, APEX2-p53 appears unable to trigger cell cycle arrest or apoptosis. Characterization of our cell lines using immunofluorescence microscopy showed that a small proportion of cells correctly express the APEX2-p53 fusion protein. We conclude that currently, the cell lines generated are not suitable for future experiments and we propose different approaches to solve the issues encountered.

Résumé

Le suppresseur de tumeurs p53 est surnommé le gardien du génome et est impliqué dans de nombreux processus de la cellule qui contribuent à maintenir son intégrité. En cas de stress, p53 est activé pour exercer ses fonctions cruciales. Le gène codant pour p53, TP53, est muté dans 50% des cancers humains. La protéine p53 exécute principalement son rôle de suppresseur de tumeur par l'induction de l'arrêt du cycle cellulaire ou de la mort cellulaire par apoptose. Pour ce faire, elle agit d'abord comme un facteur de transcription, en régulant l'expression d'une variété de gènes codant pour des protéines favorisant l'arrêt du cycle cellulaire ou l'apoptose. Une deuxième activité pro-apoptotique de p53 implique sa localisation mitochondriale dans des cellules stressées. Suite à des interactions protéiques avec certains membres de la famille de protéines BCL-2, p53 déclenche la perméabilisation de la membrane externe de la mitochondrie. Ce phénomène est considéré comme le point de non-retour dans la mort cellulaire apoptotique, car elle provoque la libération des effecteurs mitochondriaux de l'apoptose.

Récemment, notre laboratoire a montré que la phosphorylation de la sérine 392 est importante pour la localisation mitochondriale de p53 dans des cellules exposées à un stress génotoxique. La fonction précise de la phosphorylation de la sérine 392 est encore inconnu, mais deux hypothèses existent : (1) la phosphorylation de S392 est induite par des signaux de stress et favorise l'interaction entre p53 et un partenaire protéique cytosolique qui amènera p53 à la mitochondrie ; (2) en l'absence de stress, p53 est séquestrée dans le cytosol par une ou plusieurs protéines. Lors d'un stress, S392 est phosphorylée, perturbant cette séquestration, et p53 est alors capable d'atteindre les mitochondries.

L'identification de ces partenaires d'interaction cytosolique inconnus de p53 est le but ultime de cette étude. Cela permettra d'expliquer le rôle de la phosphorylation de S392 dans la localisation mitochondriale de p53 et l'induction de l'apoptose. Pour cela, une technique de marquage de proximité par l'enzyme APEX2 (ascorbate peroxydase 2) sera utilisée. Cette enzyme a été fusionnée à l'extrémité N-terminale de p53. La construction a été transfectée de manière stable dans la lignée cellulaire H1299 ($p53^{-/-}$), l'expression de APEX2-p53 étant inductible par la doxycycline via le système Tet-On. Ici, nous allons caractériser la protéine de fusion : l'induction de son expression, sa localisation subcellulaire, son activité transcriptionnelle et sa capacité à déclencher la mort cellulaire apoptotique.

Les données montrent que l'induction de l'expression et la localisation mitochondriale de notre protéine de fusion sont correctes. Cependant, nous n'observons aucune activité transcriptionnelle de la protéine. De plus, APEX2-p53 n'induit pas l'arrêt du cycle cellulaire ni l'apoptose. La caractérisation de nos lignées cellulaires par immunofluorescence a montré que seule une proportion faible de cellules exprime APEX2-p53. Nous concluons que les lignées cellulaires générées ne sont actuellement pas appropriées pour de futures études.

I. INTRODUCTION

1. Cancer

1.1. General information

Cancer is a term used to describe a large group of diseases, whose common feature is the rapid and abnormal growth of cells, forming a tumor. Some cells can break away from the initial site and form new tumors in other body parts by travelling through the lymph or blood systems. This process is called metastasis formation. (1,2)

Cancer can affect any part of the body and destroys millions of people's lives every year. In 2020 alone, 10 million people died of cancer worldwide, which corresponds to nearly one in six deaths. The most commonly diagnosed cancers are breast cancer (2.26 million new cases in 2020), lung cancer (2.21 million), colorectal cancer (1.93 million), and prostate cancer (1.41 million). The deadliest forms of cancer are found in the lung, colon and rectum, liver, stomach, and breast, as they show the highest number of deaths. (1)

The causes of cancer are numerous. Some are intrinsic, like random DNA replication errors leading to mutations. But there are also non-intrinsic risk factors that can lead to cancer, which can either be exogenous or endogenous. Among the exogenous, modifiable risk factors, you can find radiation, tumor-causing viruses, or an unhealthy lifestyle (smoking, drinking alcohol, imbalanced diet, lack of exercise). For example, 33% and 12% of cancer cases were linked to tobacco exposure and alcohol consumption, respectively. Among the endogenous factors are aging, dysfunction of the immune system (chronic inflammation has been linked to cancer), hormone levels, and metabolism, but a big part is also played by genetic factors like heredity and epigenetics. Hormonal imbalance is especially dangerous for women, as estrogen and progesterone can promote tumor growth. An unusually high level of these hormones can increase the risk of getting breast, ovary and uterine cancer. (1,3–5)

1.2. Oncogenes and tumor suppressors

Cancer is a result of several factors, including a deregulation of the cell cycle, which leads to abnormal and rapid cell growth promoting tumor formation. The cell cycle is a highly regulated process. Genes coding for some important regulators of the cell cycle are called proto-oncogenes. The overexpression or mutation of these genes can turn them into oncogenes. Oncogenes have the potential to promote cancer formation by accelerating cell growth and proliferation. (6)

RAS (rat sarcoma) and *c-MYC* (c-myelocytomatosis) are examples of proto-oncogenes. The RAS protein stimulates proliferation in its activated state, which is highly regulated via phosphorylations and dephosphorylations. If mutations arise, RAS can be trapped in the activated state and lead to uncontrolled proliferation. *c-MYC* is a gene encoding for a transcription factor, and the products of some of its transcriptional targets induce cell cycle progression. If the number of copies of the *c-MYC* gene increases by gene amplification, the expression of its targets also increases. (6,7)

While oncogenes abnormally drive cellular proliferation, tumor suppressor genes do the opposite. As their name suggests, the proteins encoded by this group of genes have a role in suppressing tumor formation by inhibiting cell cycle progression and therefore proliferation. They can also have a role in promoting cell death. (6,7)

TP53 is a major tumor suppressor gene, which is mutated in over 50% of human cancers. It encodes for the protein p53, which is also nicknamed as "guardian of the genome". In response to DNA damage and other stresses like hypoxia, ionizing radiation or oxidative stress, it can promote cell cycle arrest (to allow DNA repair) or even apoptosis to prevent cells with unrepairable DNA damage from proliferating. (7)

2. The tumor suppressor p53

The protein p53 was discovered in 1979, originally as a protein binding the large oncogenic T-antigen in cells infected by the SV40 virus (simian vacuolating virus 40). Studies performed on several p53 cDNA clones suggested that *TP53*, the gene coding for the p53 protein, might be an oncogene itself. This hypothesis was supported by the fact that levels of p53 protein were high in cancer cells. However, researchers realized that each of the cDNA clones previously studied contained mutations, and that the wild-type form of p53 is actually a tumor suppressor. (8,9)

Ever since, p53 is one of the main cancer research targets. It is involved in DNA repair, cell cycle regulation, senescence, apoptosis, and more. (10) As stated above, somatic mutations in the *TP53* gene are present in around half of human cancers. Germline *TP53* mutations have been connected to the LFS (Li-Fraumeni syndrome), a disease where the affected individual often suffers from multiple cancers, starting already at a young age. (9) $p53^{-/-}$ knockout mice undergo normal embryonic development, but they are susceptible to a variety of tumors (mainly sarcomas and lymphomas), starting when they are 3-6 months old. (11)

The p53 protein has multiple functions. It firstly acts as a tetrameric transcription factor, regulating the expression of genes associated with DNA repair like *RPA* (Replication protein A). Other target genes are associated with cell cycle arrest like *GADD45* (growth arrest and DNA damage inducible 45) and *CDKN1A* (cyclin dependent kinase inhibitor 1A), but p53 also regulates the expression of genes involved in apoptosis, including members of the BCL-2 (B-cell lymphoma 2) family as for example *NOXA* and *PUMA* (p53 upregulated modulator of apoptosis) [Figure 1]. (10)

The protein p53 also can, when cells are exposed to a genotoxic stress, localize to the mitochondrial outer membrane (MOM) to induce its permeabilization, the "point of no return" in apoptotic cell death. This mechanism is transcription-independent and is based on interactions of p53 with BCL-2 family members. (12)

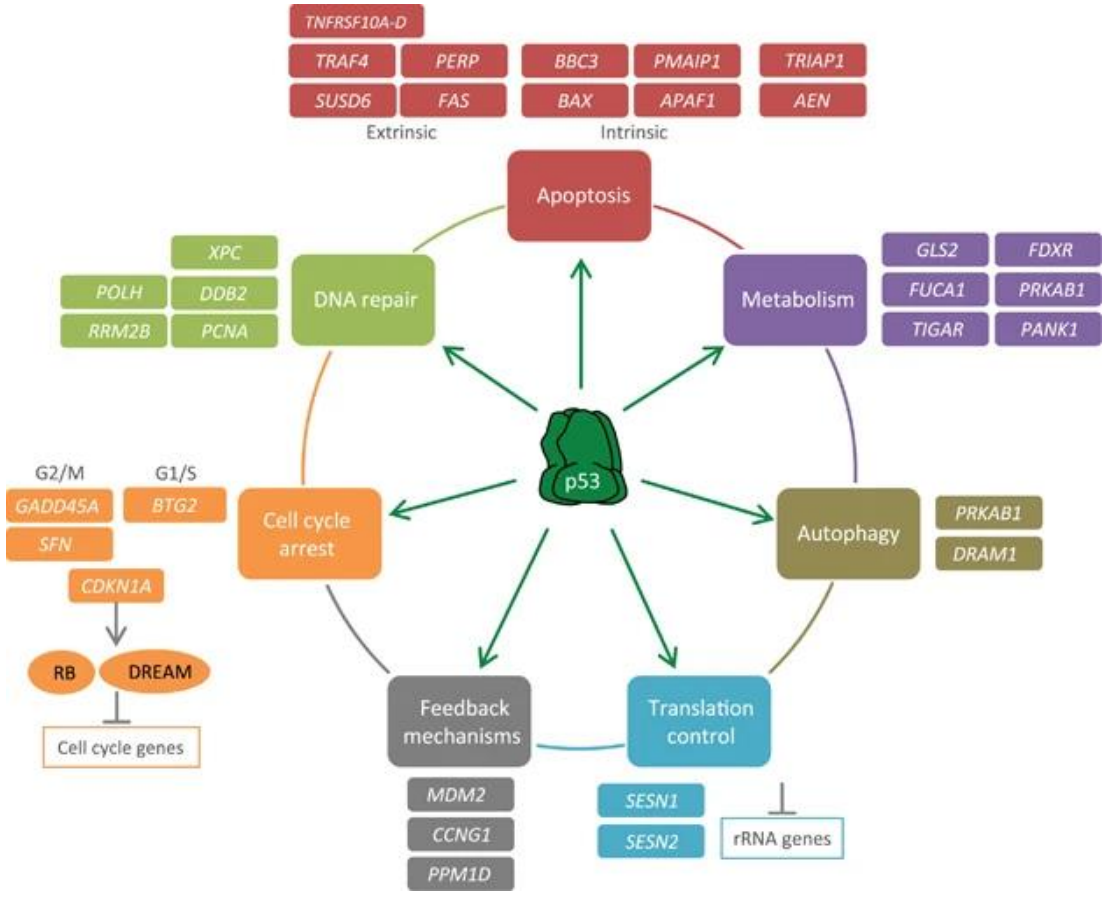

Figure 1: *The transcription factor p53 directly activates target genes that mediate various functions. Proteins encoded by p53 target genes function in multiple processes that include, but are not limited to, cell cycle arrest, DNA repair, apoptosis, metabolism, autophagy, translation control and feedback mechanisms. Citation from* (13).

2.1. The *TP53* gene

The *TP53* locus is found on chromosome 17p13.1 and has a length of 20 kb, where half of the sequence is taken up by the first intron. The gene contains 11 exons in total (the first one being non-coding) and five highly conserved regions (among vertebrates) can be found in exons 2, 5, 6, 7, and 8 [Figure 2]. There are 15 polymorphisms in total in the *TP53* gene, and allele frequencies vary with ethnic origin. A well-known polymorphism concerns codon 72, that can encode for either an arginine or a proline. The Arg allele is the most common in Northern populations, but when going further south, a proline residue is more prevalently found. There is also a rarer polymorphism at codon 47. Most of the time, this residue encodes for a proline, but in less than 5% of African Americans, the residue is a serine. (10,14)

The *TP53* gene has eight different mRNA transcripts and one natural antisense transcript named *WRAP53* (WD40-encoding RNA antisense to p53). There are also 2 start codons (ATG1 and ATG40), which create even more diversity and possible isoforms. *TP53* has two homologous genes, *TP73* and *TP63*, which are located on chromosomes 1p36 and 3p28, respectively. They share the main structure, meaning they all have an acidic N-terminal region, a large central DNA-binding domain and a C-terminal domain containing an oligomerization domain. All of them are sequence-specific transcription factors, but they vary in function and their location of expression. In fact, expression of *TP63* and *TP73* is specific to some tissues, while expression of *TP53* is ubiquitous. (10,14–16)

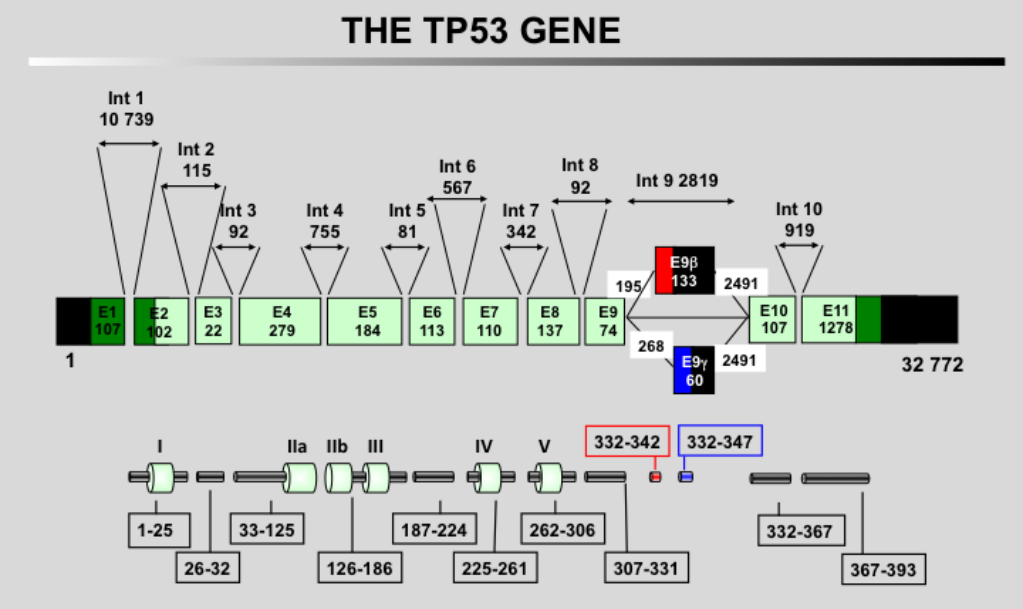

Figure 2: *Structural organization for the human TP53 gene. Exons 1 to 11 are shown in green with exon 1 which is noncoding. Exons 2 to 11 are translated into a full-length TP53 protein (P1, 393 residues). Exons 9 beta (red) and gamma (blue) are used via an alternative splicing leading to different transcripts. Modified citation from (14).*

2.2. Structure of the p53 protein

In animals, the *TP53* gene has been well conserved during evolution, and as previously described, it (and therefore also the p53 protein), has five conserved regions. In the protein, these regions, which are crucial for p53 functions, are found within the amino acid residues 13 – 23, 117 – 142,171 – 181, 234 – 250, and 270 – 286. (17)

Human p53 has a length of 393 amino acids and can be divided into three regions. The acidic N-terminal region (amino acid residues 1 – 94) consists of two transactivation domains (TADs) and a proline-rich domain (PRD), the latter being important for p53 regulation. The central core contains a sequence-specific DNA-binding domain (residues 102 – 292). The importance of the integrity of this domain is shown by the fact that 95% of mutations associated with cancer are found within it. The C-terminal region contains two domains: an oligomerization domain (residues 323 – 356) and a basic regulatory domain (residues 360 – 393) [Figure 3a]. (10,17,18)

2.2.1. N-terminal region

The N-terminal region of p53 is acidic and is mainly responsible for the transactivational function of p53. It contains two TADs: one major, TAD I (residues 1 – 40), and a smaller, TAD II (residues 43 – 63). These two TADs recruit multiple components of the basal transcriptional machinery, such as for example the TATA-binding protein (TBP) and TBP-associated factors (TAFs) like TFIID. It also interacts with RPA, a single-stranded DNA-binding protein, as well as the p62 subunit of TFIIH. (7,17)

Regulation of p53 activity is another important function of the N-terminal region. Multiple proteins like the adenovirus E1B-55 kDa (adenovirus E1B) protein, the human proteins murine double minute 2 and 4 (MDM2 and MDM4) as well as the hepatitis B virus X protein can bind to the first part of the N-terminal region of p53 to inhibit its transactivation function. Amino acids 22 and 23 are essential for MDM2 to bind. *In vivo*, these residues, along with residue 19, are important for transcriptional activation by p53. *In vitro*, they are involved in binding TAFII60 and TAFII40. These functions highlight the fact that MDM2 is a powerful inhibitor of p53 by binding its TAD. However, MDM2 has a second way of regulating p53. In fact, MDM2 is an E3 ubiquitin-protein ligase and will ubiquitinate p53 to induce its degradation by the proteasome. At the same time, the transcription of *MDM2* (gene encoding for the MDM2 protein), is activated by p53, resulting in a negative feedback loop. Mutants of the p53 protein found in cancerous cells are often more stable than wild-type p53, which is a result of their incapacity to induce *MDM2* expression. (17,19)

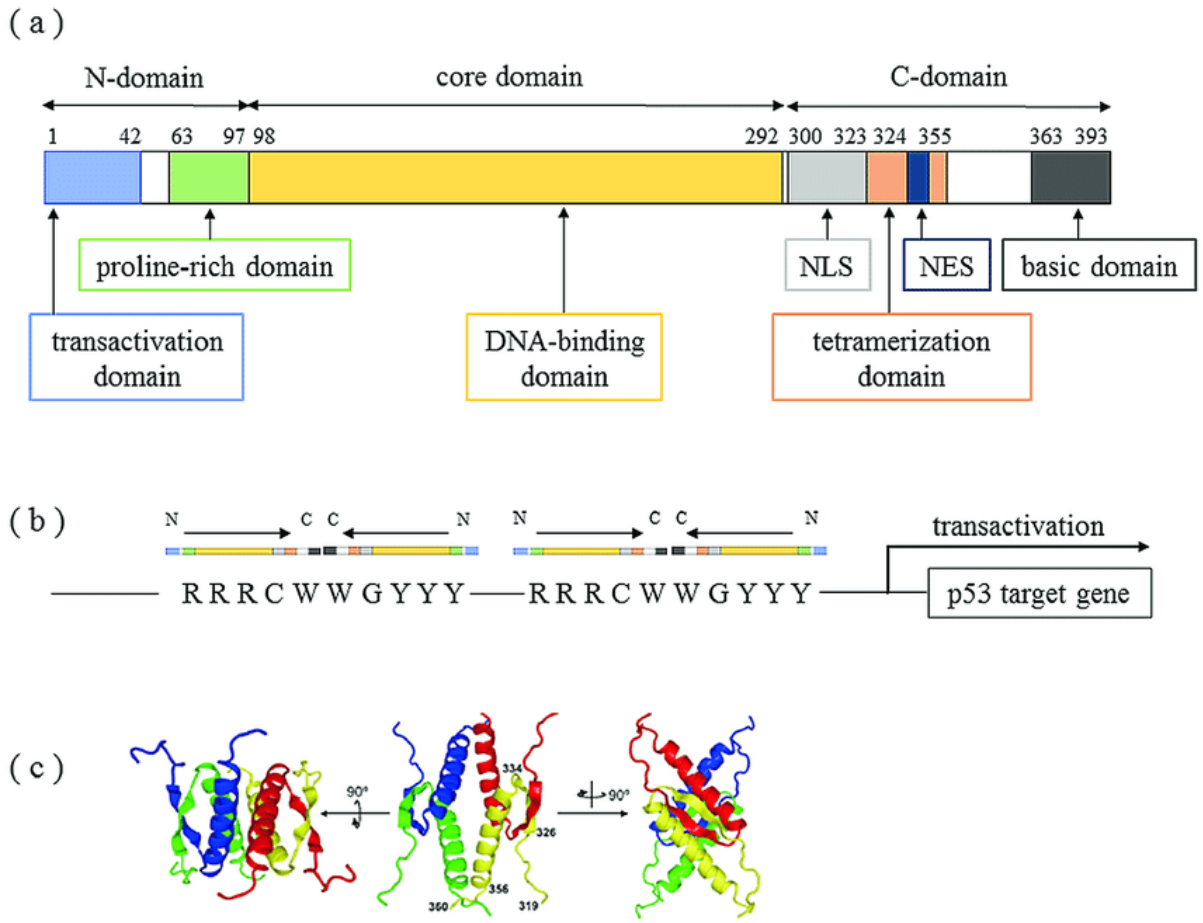

Figure 3: *p53 domains and transactivation function. (a) Domain structure of p53. Human p53 is composed of 393 amino acid residues and has a transactivation domain, a proline-rich domain, a DNA-binding domain, a nuclear localization signal, a tetramerization domain, a nuclear export signal, and a basic domain. (b) Transcriptional activation mechanisms of target genes by the p53 protein. Various stresses activate p53, which then recognizes the sequence 2xRRRCWWGYYY (R: purine base, Y: pyrimidine base, W: adenine or thymine) and combines with the sequence to form a tetramer. (c) NMR (nuclear magnetic resonance) structure of tetramerization domains. The tetramerization domain contains a [β strand-turn-α helix] structure and forms a tetramer from two antiparallel β sheets and four helix bundles. Citation modified from (19).*

After the transactivation domains lies a proline-rich domain (residues 63 – 97) said to be important for p53 regulation. In fact, this domain contains five repeats of the PXXP amino acid motif (P = proline, X = any other amino acid). These motifs are able to bind Src homology 3 (SH3) domains, which can be found in proteins involved in signal transduction. In some experimental systems, this domain is important for p53-mediated apoptosis and tumor growth suppression. (17,18) A marked decrease of p53 pro-apoptotic activity is caused by a deletion of this region. (7)

The previously mentioned polymorphism at codon 72 can either encode for a proline (p53 Pro^{72}), or an arginine (p53 Arg^{72}). The p53 protein will, if transcribed from the arginine allele, have one PXXP motif removed and only have four left. A study reported that individuals homozygous for the p53 Arg variant are more susceptible to tumorigenesis by the human papillomavirus (HPV, mostly HPV 16 and 18) than heterozygotes. (17,18,20) Another consequence of this polymorphism is the difference in localization to the mitochondria, and in induction of apoptosis. In a previous study, it has been shown that the p53 Arg^{72} variant has greater mitochondrial localization, as well as a higher capacity to trigger cell death. (21)

2.2.2. Central region

The central region (residues 102 – 292) holds four (II – V) conserved regions and consists of a large, sequence-specific DNA-binding domain. X-ray crystallography analysis was able to give profound information about the structure of this domain. It consists of two antiparallel β-sheets, consisting of four and five β-strands. These β-sheets form a scaffold, which supports a group of three flexible loops and α-helices. These helices and loops are able to directly contact DNA. While the first loop L1 binds to the major groove of the DNA, the second and third loops (L2 and L3) bind to the minor groove of the DNA. L2 and L3 are connected and stabilized by a zinc atom which is tetrahedrally coordinated on three cysteines residues and one histidine residue. (17)

The sequence that this domain binds to, found on the promoter region of p53 target genes, is called the p53-responsive element. It consists of two tandem repeats with an RRRCWWGYYY sequence (R = purine, W = adenine/thymine, Y = pyrimidine) and a spacer of up to 13 bp between the two repeats [Figure 3b]. Upon activation by various stress signals, p53 will (as a transcription factor) induce cell cycle arrest or apoptosis, depending on the severity of the stress. (7,10,19)

Most of the mutations found in p53 from cancer cells are encountered in the three loops contacting the DNA, which overlap with the highly conserved regions. Two classes of mutations can be distinguished: Class I mutations affect residues that directly contact DNA, while class II mutations hit residues whose mutation affects p53 structure stability. The two most frequent mutations of p53 are class I mutations involving residues Arg^{248} and Arg^{273}. To summarize, it is obvious that the integrity of the p53 DNA-binding domain is crucial for its tumor suppression activity. (17,18)

2.2.3. C-terminal region

After the central region is a linker connecting it to the C-terminal region, which ranges from residues 300 – 393. This domain contains an oligomerization domain (residues 323 – 356), since p53 exerts its function of transcription factor as a homo-tetramer. NMR (nuclear magnetic resonance) as well as X-ray crystallography data have given structural insights on the highly symmetrical p53 tetramer. In fact, the tetramer can be seen as a pair of dimers, since two monomers need to interact and form a dimer before the tetramer is put in place. Each monomer has a C-terminal region consisting of a turn, followed by a β-strand, a second turn and lastly an α-helix. When two monomers form a dimer, the β-strand and the α-helix are antiparallel. By interacting through their α-helices, two dimers will form a tetramer [Figure 3c]. (7,17)

Since p53 is a transcription factor, it needs to be able to locate to the nucleus. To achieve this, it has three nuclear localization signals (NLS). NLS1 (residues 316 – 325) seems to be the main NLS, since its mutagenesis results in a completely cytosolic p53 protein without any nuclear localization. NLS2 (residues 369 – 375) and NLS3 (residues 379 – 384) seem to be secondary, as a p53 protein containing mutations in their sequences is to some extent still able to localize to the nucleus. The NLS of p53 are recognized by the importin α/β complex, and a dysfunctional importin α leads to a solely cytoplasmic p53 protein. (7,17)

Within amino acid residues 339 – 352, p53 holds a nuclear export signal (NES). It is recognized by the CRM1 (chromosomal region maintenance 1) protein, responsible for its export into the cytosol. (7)

The last domain of p53 (residues 363 – 393) constitutes a basic regulatory domain. It contains multiple lysine residues (able to undergo post-translational modifications) and seems to be important in negative regulation of p53 transcriptional activity, as well as regulation of p53 stability. (17,18)

Another particularity of the C-terminal domain of p53 is that it is able to interact with DNA. These interactions with DNA are independent of the sequence, but non-linear (including damaged) segments of DNA are preferred. (22)

2.3. p53 protein activities

The p53 protein has numerous functions and is involved in many aspects of cellular homeostasis. These functions will form an indispensable network of regulation. Inside this network, p53 can either be seen as a direct actor, a conductor of an orchestra (by supervising and controlling the correct execution of different programs), or as a judge (by deciding the fate of the cell depending on its situation). In fact, p53 is involved in numerous processes like signal transduction (as an adaptor/regulatory protein), genome reparation, regulation of the cell cycle, and cell death (p53 can directly induce apoptosis at the mitochondria), communication between cells as well as coordination of different metabolic processes. p53 is crucial to animals because it puts the survival of the organism over that of a cell. This depends on the severity of the stress. In response to low stress levels, p53 can activate processes to repair and thereby protect the cell. However, in the event of a severe stress, p53 may irreversibly stop the cell cycle or induce apoptotic cell death to favor the survival of the organism. (22)

Despite these important roles, the presence of p53 is not a requirement for the development of the organism or cellular differentiation. This is highlighted by the fact that the phenotype of p53-knockout mice and wild-type mice are almost identical. (22) However, these knockout mice show early tumorigenesis and generally die of cancer at an early age. (11)

2.3.1. Transcriptional p53 activities

The transactivation function of p53 is probably the most known and studied of all, as well as the most significant one among the many p53 activities. p53 can activate or repress genes by binding to specific p53 response elements, of which today more than 200 are known. (23) Repression of genes is mostly happening indirectly, without p53 binding to DNA, through interaction with other proteins. (22,23)

As previously described, the p53 response element consists of two adjacent tandem repeats, with a length of 10 nucleotides and a spacer of 0 – 13 nucleotides. The sequence of the response element is RRRCWWGYYY (R = purine, W = adenine/thymine, Y = pyrimidine), and these sequences can either be found in the direct upstream region of a gene, but it is also possible to find them at large distances or even in introns. The degeneracy influences binding of p53, since the interaction affinity between DNA and the p53 tetramer decreases with the increasing number of mismatches in the response element. (22,23) Another factor influencing interaction affinity between p53 and its response element is the length of the spacer. A spacer length of more than two bp drastically decreases the cooperativity of p53-DNA association. (24)

All regions participate in p53 transcriptional activity. The tumor suppressor p53 can bind specifically to the p53 response element via its central region, which contains a large DNA-binding domain. The C-terminal domain is responsible for p53 tetramer formation, as well as non-specific DNA binding. Finally, the N-terminal domain will interact with parts of the transcription machinery as well as transcriptional co-activators and co-repressors. (22) Some examples of p53-binding cofactors are histone modifying proteins like p300 HAT (histone acetyltransferase) or CARM1 (coactivator associated arginine methyltransferase 1, as well as chromatin remodeling complexes like SWI/SNF. (25)

Target genes of p53 belong to many different families. Some of the first genes whose expression regulated by p53 that have been identified encode for GADD45 and $p21^{WAF1}$. These proteins are involved in cell cycle control, and there are many other genes implicated in cell cycle regulation whose expression p53 can regulate. Other target gene groups have functions in DNA repair, angiogenesis, antioxidation, metabolism and induction of cell death, the latter being the most represented one. Some pro-apoptotic proteins whose expression is upregulated by p53 are for example p53AIP1 (p53-regulated apoptosis-inducing protein 1), NOXA, PUMA, BAX (BCL-2 associated X-protein), or BAK (BCL-2 homologous antagonist/killer), the last four being part of the BCL-2 protein family. (7,22) It also increases synthesis of death receptors (FAS, DR5/KILLER) and the APAF1 (apoptotic protease activating factor 1) protein, which is important for caspase-9 activation. (26,27)

2.3.2. Mitochondrial p53 activities

The mitochondria are organelles with an outer and an inner membrane. Between these two membranes is the intermembrane space. Mitochondria are important organelles in every eukaryotic cell, being crucial for energy production/ATP synthesis. They achieve this through oxidative phosphorylation, which is executed by five enzyme complexes that are embedded in the inner membrane. Other metabolic functions assigned to the mitochondria are involved in stress response, signaling, cell cycle control as well as the initiation and the progression of apoptosis. Alterations in the mitochondrial membrane potential, permeabilization of the outer membrane or excessive ROS (reactive oxygen species) accumulation are some mitochondrial apoptosis triggers. To successfully reach the execution phase of apoptosis, the mitochondria will release multiple proapoptotic factors into the cytosol such as cytochrome *c*, APAF1, AIF (apoptosis-inducing factor), or endonuclease G. (26)

p53, in addition to being a nuclear transcription factor, will act at the mitochondria whether it is through transcription-dependent or -independent ways. It can for example switch the metabolism from glycolysis to aerobic respiration by regulating gene expression of proteins involved in both metabolic processes. Mitochondrial DNA repair is also a function of p53. (26)

The protein p53 is also a direct inducer of apoptosis at the mitochondria. In presence of a genotoxic stress, p53 will localize to the mitochondria and directly interact with BCL-2 family members to induce mitochondrial outer membrane permeabilization (MOMP). This is known as the "point of no return" of apoptosis, and it is provoked by BAX and BAK oligomerization, which will form pores in the outer mitochondrial membrane. These pores will in turn provoke the release of proapoptotic factors such as cytochrome *c*, which will interact with APAF1 to form the apoptosome complex and activate caspase-9.

There are currently three coexisting hypotheses regarding p53 mechanism of action at the outer mitochondrial membrane (OMM):

First hypothesis [Figure 4, a]: BCL-XL (B-cell lymphoma-extra large) is an anti-apoptotic protein, trapping BAX and preventing its oligomerization. p53 interacts with BCL-XL, which will release BAX. BAX is now able to oligomerize and form pores.

Second hypothesis [Figure 4, b]: MCL-1 (myeloid leukemia cell differentiation protein) is an anti-apoptotic BCL-2 family member that traps BAK at the OMM. p53 would in this case interact with BAK, displacing MCL-1. BAK, assisted by p53, will then undergo a conformational change and oligomerize at the OMM.

Third hypothesis [Figure 4, c]: p53 is here linked to BCL-XL in the absence of a stress. When its expression is triggered by p53, PUMA can release p53 from BCL-XL inhibition. p53 can then activate BAX oligomerization. (9,28)

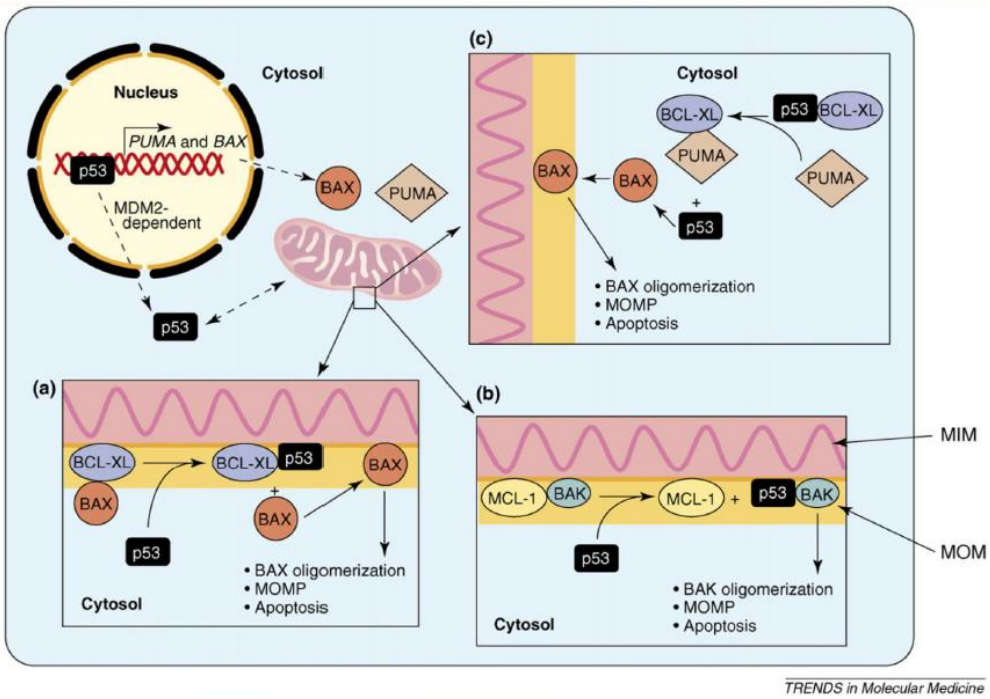

Figure 4: *Transcription-independent pathways of p53-induced MOMP. (a) p53 interacts with BCL-XL to release BAX. Oligomerization of BAX can then take place and induce MOMP. (b) p53 interacts with BAK, which displaces MCL-1. BAK changes conformation and oligomerizes, inducing MOMP. (c) Nuclear p53 induces expression of PUMA, which will release mitochondrial p53 from BCL-XL. BAX oligomerization is then induced by p53. Figure from (28)*

2.4. Regulation of p53

Since p53 is a protein regulating many cellular processes including cell death induction, it needs an extremely tight control of its activity. There are various levels to this regulation. The transcription of the *TP53* gene involves the activity of multiple transcription factors. However, the major mechanisms of regulation of p53 are based on the modulation of its activity and stability once synthesized. (7)

As long as the cell is not under any form of stress, p53 levels are extremely low. This is caused by MDM2 ubiquitination (along with MDM4) which will result in p53 degradation by the 26S proteasome complex. (22) However, in presence of a stress such as DNA damage, kinases like ATM (protein kinase ataxia-telangiectasia mutated), ATR (ataxia telangiectasia and rad3-related protein), CHK1 and CHK2 (checkpoint kinase 1 and 2) will phosphorylate p53. These phosphorylations inhibit the binding of MDM2 to p53, thus increasing p53 stability. (7,22,29) p53 degradation by MDM2 is also inhibited by the activation of $p14^{ARF}$ through oncogenic stress. In this case, $p14^{ARF}$ forms complexes with MDM2 to inhibit its binding to p53, resulting in an increase of p53 levels [Figure 5]. (30)

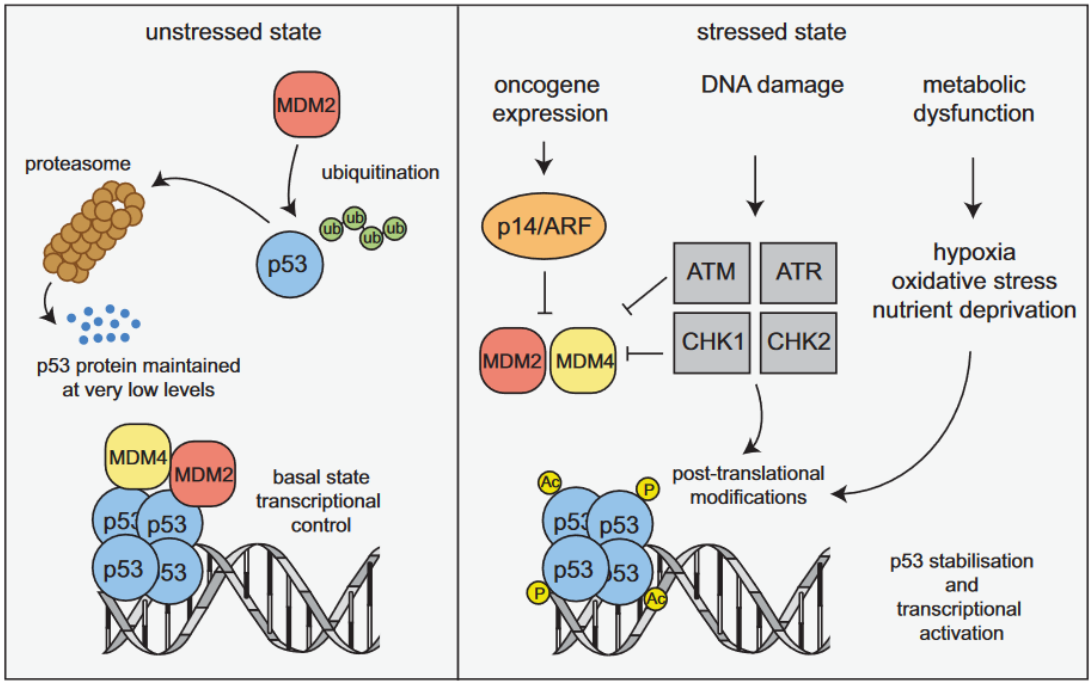

Figure 5: *Activation of p53. Unstressed cells contain very low levels of p53 through constant degradation mediated by MDM2 and MDM4. In stressed cells, p53 is activated by post-translational modifications, binds to the DNA and recruits elements of the transcriptional machinery to induce expression of its target genes (Ub, ubiquitin; P, phosphorylation; Ac, acetylation). Oncogenic stress also induces $p14^{ARF}$ expression, which inhibits binding of MDM2/MDM4 to p53. Thus, p53 levels as well as its stability increase. Figure from (9).*

2.4.1. Regulation of p53 expression

The transcription of the *TP53* gene is regulated in many different ways. *TP53* does not have a TATA box in its promoter region, and it is under the influence of numerous transcription factors. Some of the activating transcription factors are MYC/MAX (myelocytomatosis/myc-associated factor X) heterodimers, USF (upstream stimulatory factor 1), AP-1 (activator protein 1) and NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells). YY1 (Yin Yang 1) and NF1 (neurofibromin 1) are two other transcription factors upregulating *TP53* gene expression, but they bind to the same site and are therefore in competition. The *TP53* promoter also contains a PAX (paired box) binding site, and overexpression of PAX2, PAX5 or PAX8 were shown to inhibit *TP53* expression. Mutations in the binding sites of PAX, AP-1 or NFκB completely suppressed any activity of the promoter. There are many other transcription factors for the *TP53* gene, but another interesting one is p53 itself. The p53 protein binds to the promoter of its gene and activates its expression, creating a positive feedback loop. (10,31)

Another mechanism of negative regulation on the *TP53* promoter is epigenetic silencing, which can be achieved by DNA methylation. But there are also processes that hinder p53 synthesis after transcription. In this category, multiple miRNAs were found that target the 3'-UTR of p53 mRNA. Lastly, a newly found anti-sense transcript of *TP53*, named *WRAP53*, is also involved in transcriptional regulation of p53. The sense and antisense transcripts interact head-to-head in vitro, and this interaction is essential for the correct transcription of *TP53*. This fact is highlighted by the drastic reduction of p53 mRNA levels when knocking down the *WRAP53* transcript or blocking the interaction of sense and anti-sense transcripts. (31)

2.4.2. Regulation of activity

Activation of p53 can happen under various stresses like hypoxia, DNA damage, oncogene activation or ribosomal stress. Since p53 will then decide the fate of the cell by promoting either cell cycle arrest, DNA repair mechanisms, changes in metabolism or even cell death, the activity of the guardian of the genome needs to be highly regulated. There are numerous ways to this regulation. It involves chromatin structure remodeling and interactions of p53 with cofactors and other proteins. (32) Post-translational modifications also play a key role in regulating many aspects of p53 like conformation, stability, binding partners and intracellular localization. (33)

2.4.2.1. Chromatin structure

Remodeling of the chromatin structure is an indirect way of regulation of p53 activity. It is still a matter of debate whether chromatin structure has a major influence on p53 binding to DNA, but there is at least one p53 target gene (the gene coding for the adaptor protein 14-3-3-σ) that was unresponsive after DNA methylation. By comparing cancer cells to normal cells, they found a diminution of CpG islands and hypomethylated DNA in cancer cells (containing wild-type p53) in comparison to normal cells. This phenomenon may be due to chromatin landscape changes in cancer cells which could limit p53 binding site accessibility. RSF1 (remodeling and spacing factor 1), a chromatin remodeler, was found to be necessary for p53-DNA binding. This has been highlighted by the fact that a decrease in RSF1 correlates with a lower expression of genes responsible for cell cycle arrest and apoptosis. In addition, it has been shown that in open chromatin regions as well as regions in which binding of p53 promoted chromatin remodeling, basal gene expression levels were increased. (32)

2.4.2.2. Protein-protein interaction

As previously mentioned, MDM2 and MDM4 are some of p53 most well-known interaction partners. MDM2 is a ubiquitin E3 ligase whose expression is promoted by p53 itself. It will ubiquitinate p53 and make it a target for the 26S proteasome complex, where it is degraded. A closely related protein, known as MDM4 or MDMX, is involved in this process as well. It binds the N-terminal region of p53 and suppresses its transcriptional activity. Oligomerization of MDM2 and MDM4 leads to MDM2 stability. These mechanisms are all highly regulated, showing again the depth and complexity of the p53 regulatory network. (22,29)

There are many proteins which interact with p53. The protein 14-3-3-σ for example is an upregulator of p53 activity, and its loss has been observed in cancer development. The 14-3-3-σ protein will, via its C-terminal domain, interact with p53 and increase its stability by blocking MDM2 binding. It has also been shown that 14-3-3-σ promotes tetramerization of p53 as well as its transactivation function. (7,32)

ASPP1 (apoptosis-stimulating protein of p53 1) and ASPP2 are, as their name suggests, proteins which interact with p53 and increase its ability to induce cell death by apoptosis. They achieve this by inhibiting BCL-2, an anti-apoptotic protein, as well as by increasing recruitment of p53 to promoters of genes encoding for pro-apoptotic proteins, for example the promoter of *BAX*. Interestingly, expression of genes involved in cell cycle arrest are less affected by the interaction of ASPP1 (or ASPP2) with p53. (7,32)

Another positive regulator of p53 is 53BP1 (p53-binding protein 1), which binds to the DNA-binding domain to enhance p53 transcriptional activity. The RUNX (runt-related transcription factor) protein has the same effect on p53, in addition to promoting its pro-apoptotic activity. An example of an inhibitory p53 protein partner is NFBD1 (nuclear factor with BRCT domains protein 1). This protein binds to the N-terminal part of p53, inhibiting ATM phosphorylation and thus apoptosis, to protect cells with repairable DNA damage. (7,32)

2.4.2.3. Post-translational modifications

The regulation of p53 via post-translational modifications (PTMs) is the most effective and widespread type of the many layers of regulation. There are many types of PTMs that target p53, among them are phosphorylation, acetylation, methylation, ubiquitination, sumoylation, neddylation, hydroxylation and others [Figure 6]. (33)

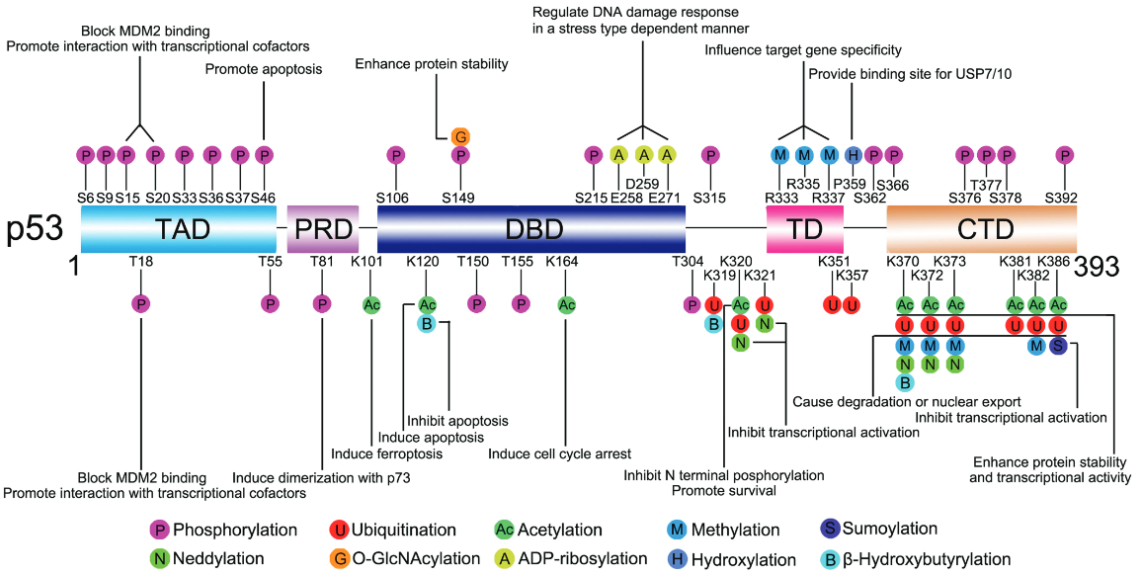

Figure 6: *Overview of p53 PTMs. The major sites for p53 modifications (phosphorylation, ubiquitination, sumoylation, neddylation, acetylation, methylation, O-GlcNAcylation, ADP-ribosylation, hydroxylation, and β-hydroxybutyrylation) are plotted. Different colors are used to differentiate distinct modification types. Representative functions of some modifications are indicated. Citation from* (33).

Some of the characteristics of these PTMs are that each type of modification can affect multiple amino acid residues. They have multiple functions which are dependent on the site, type and context in the cell. This is highlighted by the fact that different modifications can execute the same role, and the same modification can change its function if it is on different sites. A third feature is that they are reversible, and they are also able to perform crosstalk, which means that modifications can influence one another. PTMs of p53 regulate its subcellular localization, stability, transcriptional activity, capacity to trigger apoptosis and its interaction with multiple protein partners. (33)

On the p53 protein sequence are numerous serine (S) and threonine (T) residues that are phosphorylation sites. They are mainly found in the N-terminal region of p53, but they span across the whole protein. In unstressed cells, residues T55, S376 and S378 are constantly phosphorylated. The nuclear export protein CRM1 targets p53 phosphorylated on T55, leading to cytoplasmic localization of p53 and therefore promoting its degradation. Under stress conditions in form of DNA damage, this phosphorylation is removed, increasing p53 stability and promoting cell cycle arrest. The dephosphorylation of S376 also happens under stress. It then allows binding of 14-3-3-σ, which results in a higher p53-DNA binding affinity. (33,34)

Most phosphorylations occur following a stress. For instance, the kinases ATM, ATR, and CHK1/2 phosphorylate the residues S15 and S20, hindering MDM2 binding and therefore increasing p53 stability. This also allows induction of the expression of genes involved in cell cycle arrest and apoptosis. Strong DNA damage provokes phosphorylation of S46, which promotes transcription-dependent p53-mediated apoptosis. (7,22,33,35)

Some phosphorylations influence p53 interactions with different partners. For example, phosphorylation of T81 promotes dimerization of p53 with p73. p53 with a phosphorylated S392 residue displays an increased mitochondrial localization and an enhanced capacity to trigger transcription-independent apoptosis. (33,36)

Acetylations are also common p53 PTMs and affect lysine residues. Six C-terminal lysines (K370, K372, K373, K381, K382, and K386) will, upon acetylation, increase p53 binding to target genes and therefore favor processes like cell cycle arrest, senescence, or apoptosis. The same lysines are targeted by the MDM2 E3 ubiquitin ligase activity. Therefore, acetylation of these residues decreases p53 ubiquitination and augments p53 stability. (7,33)

The residue K120 is another well studied acetylation site. TIP60 (histone acetyltransferase KAT5) for example may modulate the conformation of the DNA-binding domain when performing acetylation on K120, causing pro-apoptotic genes to be preferably expressed. However, if the enzyme MOZ (monocytic leukemia zinc finger protein) acetylates K120, p53 induces cell cycle arrest and not apoptosis. Acetylation of C-terminal lysines like K317 or K320 can suppress apoptosis or promote it, respectively. (18,32,33)

Methylation is also possible on some lysines of p53. Methylated K372 traps p53 in the nucleus and increases its stability. A methylation on K370 however inhibits transcriptional activity of p53. Arginine residues are also able to be methylated. The specificity of p53 target gene binding is influenced by R333, R335 and R337 methylation. (18,26,33) The dimethylation of K382 (or K370) induces 53BP1 recruitment. This recruitment stabilizes p53 and promotes repair of damaged DNA. SMYD2 (SET And MYND domain containing 2) and SETD8 (SET domain containing lysine methyltransferase 8) monomethylate K370 or K382, respectively. This provokes localization of p53 to the *CDKN1A* (cyclin dependent kinase inhibitor 1A) promoter, which encodes for the $p21^{WAF1}$ protein, promoting cell cycle arrest [Figure 7].

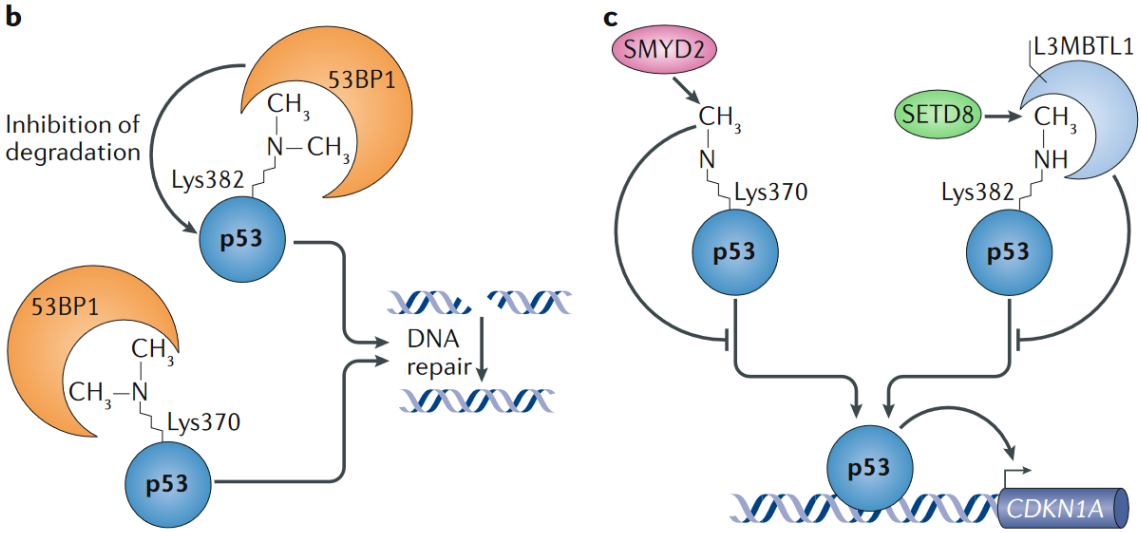

Figure 7: *b) Dimethylation of Lys370 or Lys382 recruits 53BP1(p53-binding protein 1), which promotes DNA damage repair. Specifically, 53BP1 binding to dimethylated Lys382 inhibits p53 degradation. c) Monomethylation of Lys370 by SMYD2 or of Lys382 by SETD8 inhibits p53 localization to the promoter of CDKN1A (encoding for $p21^{WAF1}$). The effect of Lys382 monomethylation is mediated by binding of the transcription repressor L3MBTL1. Citation modified from (32).*

Ubiquitination, which involves the covalent attachment of an 8.5 kDa protein called ubiquitin, is performed by three enzymes (an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin-ligating enzyme). There can either be mono- or polyubiquitinations, most often responsible for targeting substrates to the proteasome, where they are degraded. But there are also other roles to this PTM, like regulation of localization, activity, or interactions with protein partners. As mentioned before, MDM2 is the most important E3 ubiquitin ligase of p53 and a major negative regulator. It targets the six lysines at the C-terminus previously mentioned (K370, K372, K373, K381, K382, and K386) and ubiquitinates them. While polyubiquitination (high MDM2 activity) provokes degradation, monoubiquitination (low MDM2 activity) induces nuclear export and mitochondrial localization of p53. Other E3 ubiquitin ligases can influence the transcriptional program that p53 will execute, or even promote carcinogenesis by causing p53 degradation. (33,37)

Neddylation and sumoylation are two PTMs that involve similar mechanisms as ubiquitination and target lysines as well. While they do not seem to affect localization or stability of the p53 protein, they can inhibit its transcriptional activity. (33)

2.5. Mutations in *TP53* and their role in disease

While wild-type p53 is called the guardian angel of the genome, mutated and therefore non-functional p53 can be compared to a fallen angel. This metaphorical comparison reflects the importance of functional p53 in preventing disease, as well as the devastating consequences that a mutated p53 can have on health. The deregulated activity of p53, including both loss of function and hyperactivity, can provoke numerous diseases. Next to cancer, these diseases can be cardiovascular, neurodegenerative, infectious, or metabolic. Deregulated p53 activity can also speed up the aging process. (22)

Mutations of p53 can be divided into two categories: germline and somatic mutations. (38)

2.5.1. Germline mutations

Germline mutations in the *TP53* gene can cause the Li-Fraumeni syndrome (LFS). The LFS is a heterogenous, autosomal dominant disorder that provokes early-onset tumor formation. Unexpectedly, the tumors associated with this disease are different from the ones provoked by somatic mutations and include rarer tumors like sarcomas, brain tumors, leukemias, adrenocortical carcinomas, or pre-menopausal breast cancer. Pre-menopausal breast cancer is also caused by the LFS, which may be responsible for the higher risk of cancer in women. The lifetime risk of getting cancer for an individual affected by the LFS is over 90%, making it a highly penetrant disorder. (38,39)

The most frequently found p53 germline mutations are in the DNA-binding domain. Another variety of the LFS is due to mutations in the *CHEK2* gene, which codes for checkpoint kinase 2 (CHK2). Following DNA damage, this kinase phosphorylates p53 to increase its stability and therefore activity. A mutant CHK2 protein favors tumor formation by compromising at least in part the DNA damage response. (38)

2.5.2. Somatic mutations

Somatic mutations of p53 are present in more than 50% of human cancers, with varying frequency depending on the cancer type. The vast majority of somatic mutations in *TP53* are found in the DNA-binding domain, and 87.9% of these mutations are missense. Outside of the DNA-binding domain however, only 40% of mutations are missense, while nonsense and frameshift mutations represent the majority. (38,39)

2.5.3. Influence of mutations in disease

Overall, 95% of *TP53* mutations are found in the DNA-binding domain. Among these numerous mutations are seven which are particularly frequent in cancer: codons 175, 220, 245, 248, 249, 273, and 282 [Figure 8]. These so-called hotspot mutations are regularly observed in patients. These mutations can be divided into two types. They either are residues that directly establish contacts with the DNA promoter of target genes (class I), or they are important for the structure of the DNA-binding surface of p53 (class II). (39)

Mutant p53 acts in a dominant-negative way toward wild-type p53. As previously described, p53 acts as a tetramer to regulate gene expression. Therefore, if we have one mutated and one wild-type allele, the chance to have a wild-type homotetramer is only 1/16. While most mutations provoke a loss of tumor suppressor functions, they can also cause a gain of function, where mutant p53 becomes an oncogenic protein. (7,10)

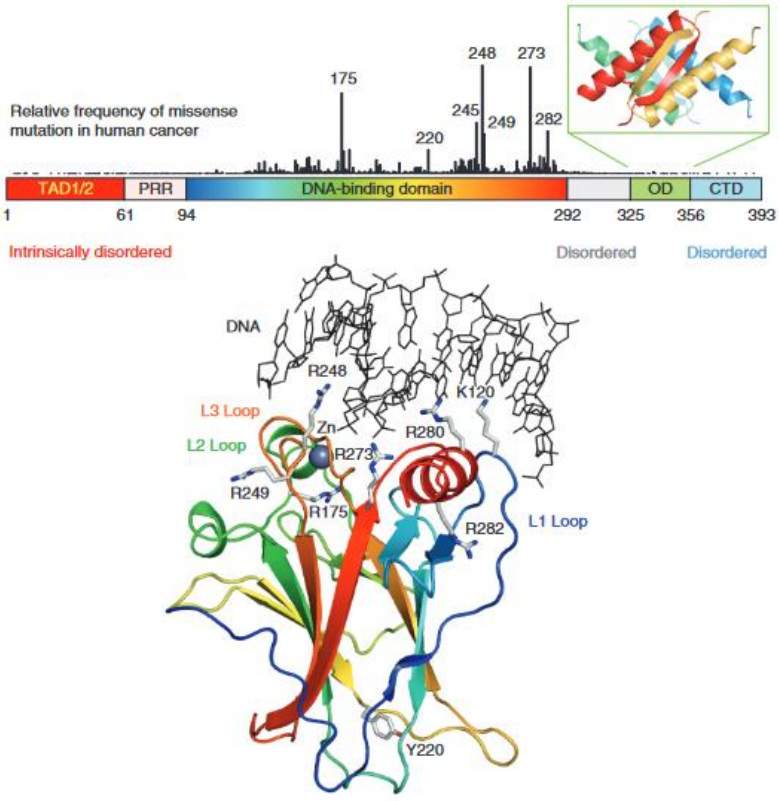

Figure 8: *Domain structure of p53 and mutations in cancer. p53 contains a natively unfolded amino-terminal transactivation domain (TAD), which can be further subdivided into the subdomains TAD1 and TAD2, followed by a proline-rich domain (PRD). The structured DNA-binding and tetramerization domains (OD) are connected through a flexible linker region. The regulatory domain at the extreme carboxyl terminus (CTD) is also intrinsically disordered. The vertical bars indicate the relative missense-mutation frequency in human cancer for each residue, showing that most cancer mutations are located in the DNA-binding domain. The structure of the DNA-binding domain is shown as a ribbon representation and colored with a rainbow gradient from the amino terminus (blue) to the carboxyl terminus (red). Sites of cancer hotspot mutations and essential DNA contacts are shown as stick models. Citation modified from (40).*

3. Apoptosis

Apoptosis, also known as type I programmed cell death, is a normal process during development. In the adult organism, it helps maintaining healthy and stable cell populations in tissues. Apoptosis can also occur as a response to cell damaging stimuli, acting as a defense mechanism. There is a wide variety of death-inducing stimuli, and they can be both physiological as well as pathological. However, not all cells are affected the same way by a certain stimulus. The deregulation of apoptosis is a characteristic of multiple pathologies such as neurodegenerative diseases, autoimmune disorders, or cancer. (41,42)

Apoptosis is a form of programmed cell death displaying distinct morphological and biochemical features, allowing it to be easily distinguishable from other types of cell death. A first feature is observable in early apoptosis: the cell shrinks, and chromatin is irreversibly condensed in the nucleus, a process known as pyknosis. Afterwards, a process called "budding" occurs, involving plasma membrane blebbing and karyorrhexis (nucleus fragmentation). Finally, the cell is disintegrated into apoptotic bodies which are subsequently phagocytosed by macrophages, parenchymal or neoplastic cells. During this process, no intracellular components are released in the extracellular medium and the integrity of organelles is maintained. As a result, apoptosis is a process that does not provoke an inflammatory reaction. (41,42)

Apoptosis is functionally irreversible and is a process involving a highly complex and energy-dependent cascade of molecular events. There are two main apoptotic pathways, depending on the apoptotic stimulus: the extrinsic (or death receptor) pathway, and the intrinsic (or mitochondrial) pathway [Figure 9]. These two pathways are linked, as effectors of one pathway can influence the other, and they both have the same outcome, which is the activation of the execution phase of the apoptotic program. In this final step of apoptosis, a family of cysteine proteases known as caspases are activated, resulting in protein cleavage and DNA fragmentation, as well as degradation of cellular components like the cytoskeleton or the nuclear lamina. The formed apoptotic bodies present a signal at the plasma membrane (phosphatidylserine exposure) that promote their phagocytosis. (41,42)

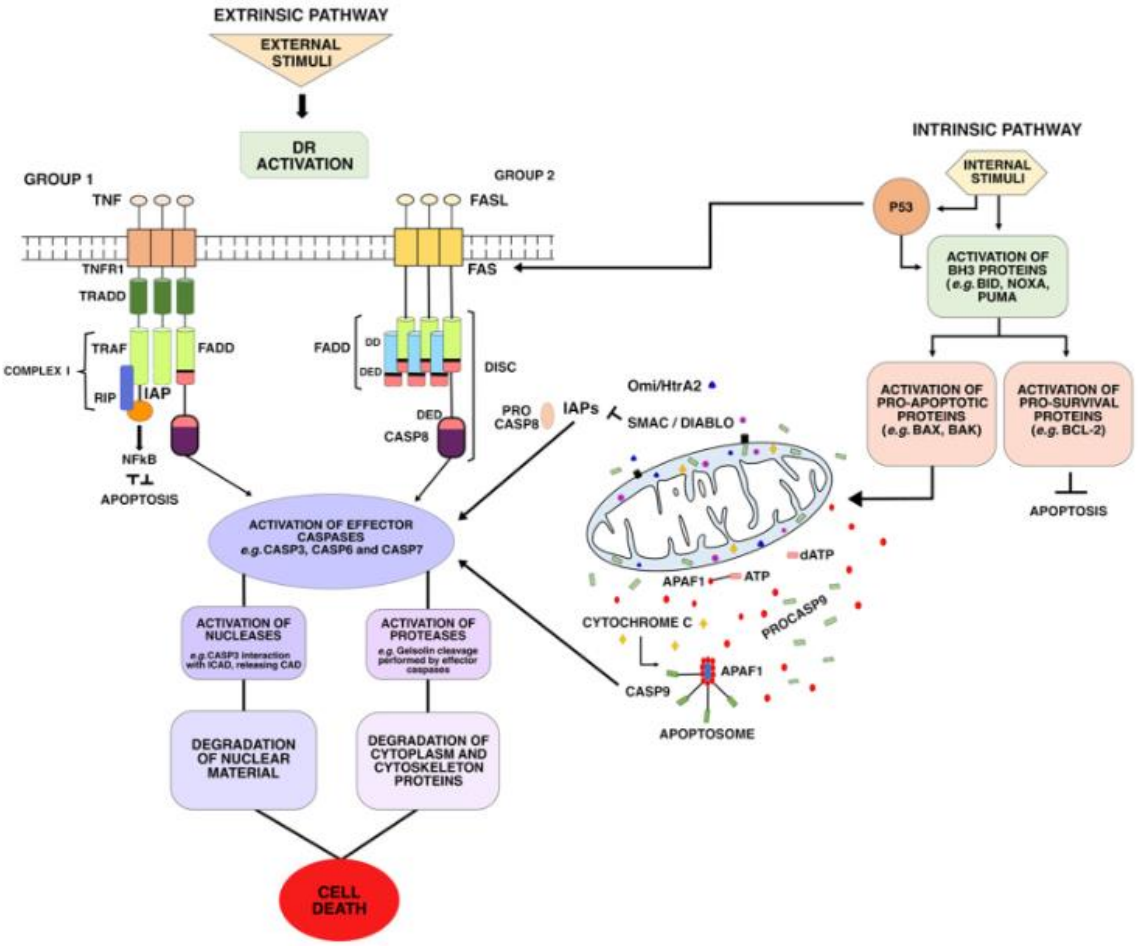

Figure 9: *General scheme of the process of apoptotic cell death. The extrinsic pathway is characterized by the activation of the death receptors (like FAS or TNFR1) by an external stimulus (binding of ligands like TNF or FASL). Upon death receptor activation, FADD binds and recruits procaspase-8, resulting in its activation into caspase-8. The intrinsic pathway implies the activation of BH3-only proteins. Activation of pro-apoptotic proteins (BAX, BAK) provokes MOMP. Cytochrome c release induces activation of procaspase-9 into caspase-9 following formation of the apoptosome. Caspase-8 and caspase-9 both give rise to the execution phase of apoptosis by activating executioner caspases. Citation modified from (42).*

3.1. Extrinsic pathway

The extrinsic or death receptor pathway of apoptosis involves, as its name suggests, the binding of specific ligands to death receptors (DR) on the plasma membrane. These death receptors are transmembrane proteins and are members of the tumor necrosis factor (TNF) superfamily of receptors. Characteristics of this family are a cysteine-rich extracellular domain as well as an about 80 amino-acid-long cytoplasmic domain called the death domain. The latter has an important role in the transduction of the death signal to the signaling pathways inside the cell. Among the most studied ligand/death receptor pairs are FASL/FASR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/ DR4 and Apo2L/DR5. (41,42)

The successive events leading to apoptosis are best characterized for the FASL/FASR and the TNF-α/TNFR1 models. The first step involves binding of the ligand, provoking trimerization of the receptors. Cytosolic adapter proteins are then recruited via the previously mentioned death domains. This adapter is FADD (FAS-associated death domain protein) for the FASL/FASR pair, and TRADD (Tumor necrosis factor receptor type 1-associated death domain protein) for the TNF-α/TNFR1 model. Binding of TRADD provokes the recruitment of FADD and RIP1 (Receptor-interacting serine-threonine kinase 1). FADD will then, in both models, recruit procaspase-8. The receptor, the adaptor(s), and procaspase-8 form the DISC (death-inducing signaling complex), and its formation results in the so-called proximity-induced auto-activation of procaspase-8 into caspase-8. This activation triggers eventually the execution phase of apoptosis. (41,42)

3.2. Intrinsic pathway

The intrinsic, or mitochondrial pathway of apoptosis is initiated by a stimulus that does not require the action of transmembrane receptors. A great variety of stimuli generates intracellular signals, that can either be positive or negative. Some examples of negative signals include the absence of growth factors, cytokines, or hormones which may result in a failed suppression of cell death. The cell will, in this case, undergo apoptosis. Positive signals are for example radiation, toxins, free radicals, viral infections, hypoxia, and hyperthermia. (41,42)

These stimuli will provoke mitochondrial outer membrane permeabilization (MOMP). As mentioned before, MOMP is considered the “point of no return” of apoptosis and involves the release of pro-apoptotic proteins. These pro-apoptotic factors that were previously trapped inside the mitochondrial intermembrane space get released into the cytosol in two different groups. (41,42)

The first group of pro-apoptotic proteins contains cytochrome *c*, Smac/DIABLO (second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI) and HTRA2/Omi (high-temperature requirement protein A2), a serine protease. These proteins will activate the caspase-dependent mitochondrial pathway. Cytochrome *c* will bind to APAF1 and procaspase-9 in the presence of ATP or dATP, forming the structure known as the “apoptosome”. Via this mechanism, procaspase-9 forms clusters, leading to its auto-activation into caspase-9. The other members of the group, Smac/DIABLO and HtrA2/Omi suppress the action of IAP (inhibitors of apoptosis proteins), thereby further promoting apoptosis. (41,42)

In the second group of pro-apoptotic proteins are AIF (apoptosis-inducing factor) and the endonuclease G. Their release is a late event happening during apoptosis. AIF will translocate to the nucleus, where it causes DNA fragmentation. It also induces condensation of peripheral nuclear chromatin. Endonuclease G is also found to translocate to the nucleus in order to cleave nuclear chromatin into oligonucleosomal DNA fragments. AIF and endonuclease G are independent of caspase activity. (41,42)

Members of the BCL-2 family of proteins, which are at least partially regulated by p53, control MOMP. There are pro-apoptotic members like BAK, BAX, BID (BH3 interacting-domain death agonist), BAD (BCL-2 associated agonist of cell death), PUMA and NOXA and anti-apoptotic members like BCL-2, BCL-XL, and BAG (BCL-2 associated athanogene). This protein family is important in the decision-making process between cell death through apoptosis or cell survival. The regulation by p53 can happen at two different levels. Firstly, through its activity as a transcription factor, p53 can control the expression of some BCL-2 family members. This is the case of four important pro-apoptotic BCL-2 family members: BAX, BAK, PUMA, and NOXA. (41,42) But p53 can also regulate some BCL-2 family members through direct interaction, which happens for BAX, BAK, and possibly other members. As previously described, a fraction of p53 can, following exposure to a genotoxic stress, localize to the mitochondria and interact with various BCL-2 family proteins to induce MOMP. (28)

While the extrinsic and intrinsic pathways are different in their activating stimuli, there is some crosstalk happening between them. One example of this crosstalk is the cleavage of BID by caspase-8. (41) The active form of BID, tBID, further promotes MOMP. (43)

3.3. Apoptosis execution phase

The last phase of apoptosis, which is the execution phase, follows both the intrinsic and extrinsic pathways. The main actors of this phase are caspase-3, caspase-6, and caspase-7, the executioner caspases. These proteases will firstly activate apoptosis effectors like the main apoptotic nuclease CAD (caspase-activated DNase). In healthy cells, CAD is found in the cytosol and nucleus but stays inactive as long as it is bound to the inhibitor of CAD, ICAD. Upon cleavage of ICAD by caspase-3, CAD is activated and causes oligonucleosomal DNA fragmentation as well as a more advanced and pronounced chromatin condensation. (41,42)

The second function of the executioner caspases is destroying numerous proteins of the cell, for example those of the nuclear lamina. They will also inactivate proteins that are involved in DNA repair, like PARP (poly ADP-ribose polymerase). The presence of cleaved PARP

(c-PARP) is a marker of apoptosis. Many biochemical and morphological changes that are seen during the apoptotic process are linked to the action of the executioner caspases. (41,42)

The executioner caspases are one of two groups of caspases that act during apoptosis. The other group are the initiator caspases: caspase-2, -8, -9, and -10. Their common feature is a large (> 90 amino acid residues) N-terminal pro-domain, and their activation requires large protein complexes (the apoptosome, the DISC and the PIDDosome, PIDD standing for p53-induced protein with a death domain) to which they will be recruited when needed. Caspases are indeed continuously synthesized but exist in an inactive form. After their recruitment, they will first undergo a process of proximity-induced auto-activation and then activate the executioner caspases, which are the main substrates of the initiator caspases. Caspase-3 is the most important caspase during the execution phase, and it is a substrate of all initiator caspases. (19,44)

Caspase-3 has multiple functions in the execution phase of apoptosis: Firstly, it degrades ICAD, the inhibitor of the endonuclease CAD, resulting in DNA fragmentation. Cytoskeletal reorganization and formation of apoptotic bodies is another role of caspase-3. Lastly, caspase-3 helps with recognition of the apoptotic bodies by phagocytes. This recognition is facilitated by translocation of phosphatidylserines to the outer leaflet of the plasma membrane. While the exact mechanisms are still unknown, research implies the involvement of caspase-8, caspase-3, and FAS in this process. Phosphatidylserine externalization is important to ensure that apoptosis is a non-inflammatory event. (41,42)

4. Proximity-dependent labeling: APEX2

Protein-protein interactions (PPIs) are important in every cellular process. To understand these processes, it is crucial to map the protein interaction network, the "interactome". Classical methods to achieve this are often limited by the necessity of non-physiological conditions or the ability to only map high-affinity and non-transient PPIs. (45)

More recently, different proximity-dependent labeling methods have been developed to overcome these limitations. By using these methods, interactions can be mapped in more physiologically relevant conditions and even transient/low affinity PPIs can be detected. (45)

These mapping methods use enzymes producing reactive molecules that will covalently link to adjacent proteins. Labeled proteins can then be extracted, purified, and identified by using, for example, mass spectrometry (MS). In practice, these labeling enzymes are either fused to a protein of interest or fused to targeting sequences that send the enzyme to the wanted subcellular structure or compartment. (45)

By using a variety of different labeling enzymes, numerous protein interactions can be detected in living cells, either over a longer period of time or to generate a snapshot. Examples of labeling enzymes are BioID, a biotinylating enzyme based on the *Escherichia coli* biotin ligase BirA, horseradish peroxidase (HRP), or modified ascorbate peroxidases (APEX and APEX2). (45)

4.1. APEX2, a second-generation enzyme

APEX2 (ascorbate peroxidase 2) is a second-generation enzyme derived from the wild-type soybean ascorbate peroxidase. This 27 kDa enzyme is monomeric and does not contain any disulfide bonds, however it requires a heme cofactor to function correctly. (46)

In the presence of hydrogen peroxide, APEX2 catalyzes the oxidation of biotin-phenol to generate highly reactive and short-lived (< 1 ms) biotin phenoxyl radicals. These radicals bind covalently to proteins in a radius of 10-20 nm (the biotinylation radius) on four different amino acid residues: cysteine, histidine, tryptophan, and tyrosine. (46)

However, the shape of the biotinylation contour map is not fixed and depends on variable factors of the local environment like the concentration in glutathione or the protein density. This creates the possibility of far proteins being biotinylated, creating false positives. A solution to this problem is using the SILAC (Stable Isotope Labeling by Amino acids in Cell culture) approach, which is a necessity in order to attain high spatial specificity. By combining APEX2 with SILAC, unwanted background noise can be successfully eliminated. (46)

4.2. Stable Isotope Labeling by Amino acids in Cell culture (SILAC)

The SILAC approach [Figure 10] is based on using two different cell culture media in which the samples are grown: The first one has no particularity, but the second one is supplemented with non-radioactive, isotopically labeled amino acids (also called heavy amino acids). While there are no differences on cell morphology, growth and other characteristics between heavy cell media and normal (light) media, true interaction partners can be distinguished from the background noise via the H/L ratio. (47)

There are multiple advantages to using stable isotopes when labeling proteins in mammalian cells. For instance, labeling efficiency is the same for all samples because the incorporation of the heavy isotopes is 100%. This incorporation efficiency of 100% is also useful as a control by comparing changes in several peptides of the same protein, as the extent of change should be the same for each peptide. Moreover, this method allows detection of changes in small proteins and proteins that do not contain any cysteine (thiol-reactive dyes are used to map proteomic changes). (47,48)

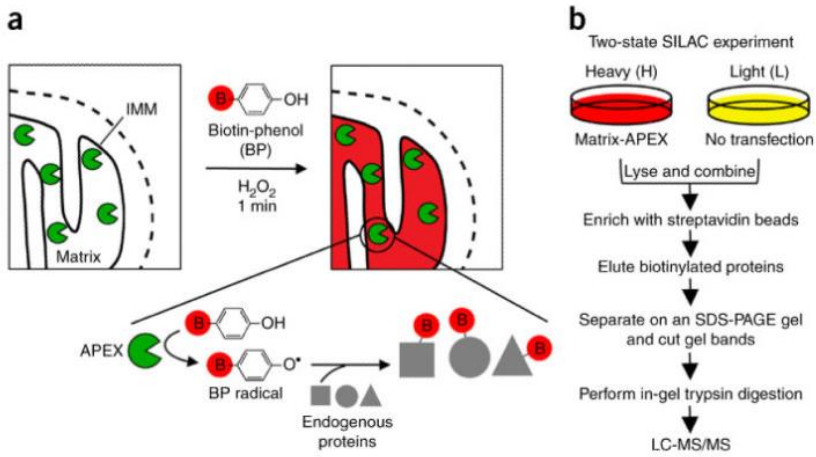

Figure 10: *Live-cell proteomics using APEX. (a) Scheme showing APEX-catalyzed biotinylation. The mitochondrial matrix is enclosed by the IMM. This example shows APEX (green Pac-Man) targeted to the mitochondrial matrix. Live cells are incubated with biotin-phenol probe (red B = biotin) for 30 min and then treated for 1 min with 1 mM H2O2 to initiate biotinylation. APEX and APEX2 catalyze the one-electron oxidation of biotin-phenol into a biotin-phenoxyl radical, which covalently tags proximal endogenous proteins. The biotin-phenoxyl radical does not cross the IMM. (b) Two-state SILAC experiment used to map the proteome of the mitochondrial matrix. The experimental sample was cultured in heavy (H) arginine and lysine amino acids. The negative control sample, with APEX omitted, was cultured in light (L) arginine and lysine amino acids. Each dish of cells was separately treated with biotin-phenol and H2O2 and then lysed, and streptavidin enrichment was performed on the combined lysates. Subsequent processing steps are shown. For each detected peptide, the H/L intensity ratio reflects the extent to which that peptide was biotinylated by APEX. Nonspecific binders have H/L ratios close to 1. Citation modified from (46).*

4.3. Comparison of APEX2 to BioID

As previously mentioned, there are various techniques to label proteins in a proximity-dependent manner. BioID, for example, is an enzyme which is based on the *E. coli* enzyme BirA. It generates an activated form of a biotin adenylate ester (biotin-AMP) that is capable of covalently binding to any lysine residues of neighboring proteins. However, this method has two weaknesses. The first weakness is that to achieve a sufficient amount of biotinylation, the reaction needs to go on for 18 to 24 hours. This could have consequences like cytotoxicity, changes in protein abundance, subcellular localization, or others. The second weakness is the long half-life of biotin-AMP (several minutes), which greatly increases the labeling radius of BioID in comparison to APEX2, where the biotin-phenoxyl radicals have a half-life of approximately 1 millisecond. APEX2 has therefore a much higher spatial selectivity. (46,49,50)

II. OBJECTIVES

C. Castrogiovanni's thesis (36) features the importance of S392 phosphorylation for the mitochondrial localization of p53 in response to a stress. Analyses on a non-phosphorylatable p53 S392A mutant showed that the absence of S392 phosphorylation resulted in a reduced sensitivity to the DNA-damaging agent camptothecin, a topoisomerase I inhibitor. The mutant also portrayed less mitochondrial localization compared to wild-type p53, and cells expressing the mutant displayed less apoptosis than cells expressing wild-type p53 or p53 S392E, a phosphomimetic mutant. It had, however, no influence on p53 activity as a transcription factor.

To explain these data, C. Castrogiovanni proposed two hypotheses [Figure 11]:

The first hypothesis is that p53 needs to interact with a cytosolic, yet unknown, protein partner in order to localize to the mitochondria in stressed cells and trigger MOM permeabilization. This interaction would be facilitated by phosphorylation of S392.

The second hypothesis states that in the absence of a stress, cytosolic p53 could be sequestered through interaction with one or more proteins, preventing mitochondrial localization and MOMP. The increased phosphorylation of serine 392 in cells exposed to a genotoxic stress would prevent formation of this inhibitory protein complex, allowing cytosolic p53 to reach the MOM to trigger apoptosis.

Thus, the goal of the project is to identify protein partners of p53 that are important for its mitochondrial localization, and whose interaction is affected by the phosphorylation status of S392. To identify these proteins, a proximity-dependent labeling method will be used. A genetic construct containing an N-terminal fusion of APEX2 to p53, inducible by doxycycline (Tet-On system), has been stably transfected in H1299 ($p53^{-/-}$) cells, generating three cell lines: C2, C5, and C7.

The objective of this master's thesis is to characterize the three cell lines, in order to ensure that they are suitable for further experiments. This characterization involves three specific goals: 1) Analysis of p53 expression when fused to APEX2; 2) Assessment of mitochondrial localization of the fusion protein in cells exposed to an apoptotic stress; and 3) Determination of the capacity of APEX2-p53 to trigger apoptosis and function as a transcription factor.

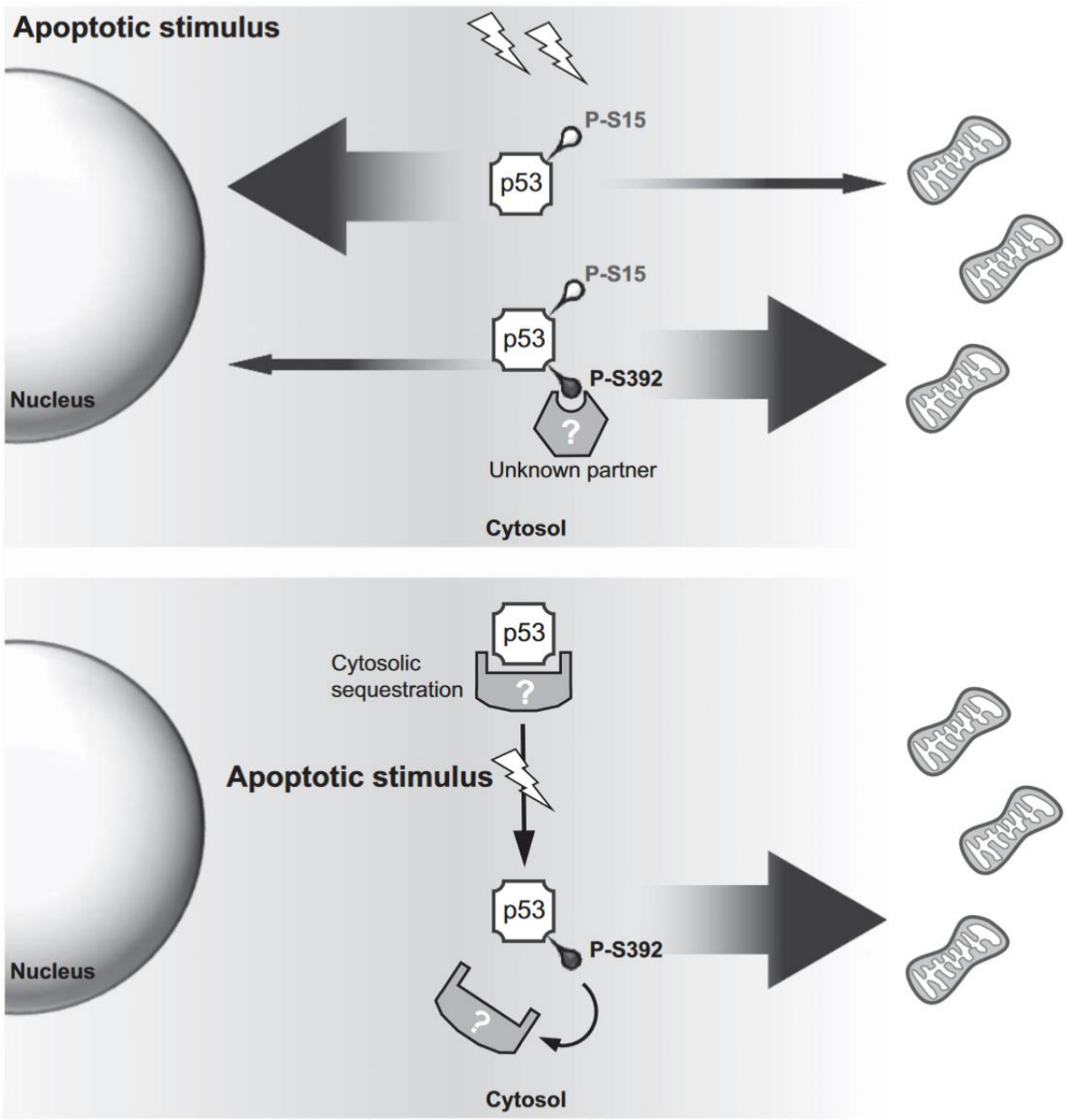

Figure 11: *Hypotheses on the involvement of S392 phosphorylation in the regulation of p53 trafficking to mitochondria. In the top diagram, we postulate that p53 needs to interact with a cytosolic, yet unknown, protein partner in order to localize to the mitochondria and trigger OMM permeabilization; and that the interaction is facilitated by phosphorylation of S392. In the bottom diagram, we propose that in the absence of a stress, mitochondrial localization of p53 could be largely prevented through interaction with one or more proteins able to interact and sequester the tumor suppressor. Moreover, formation of this inhibitory complex is prevented by the increased phosphorylation of serine 392 in cells exposed to a genotoxic stress, allowing p53 to reach the OMM to trigger apoptosis. Modified from (36).*

III. RESULTS

1. Characterization of the cell lines

Three different H1299 cell lines have been generated via stable transfection of the genetic construction shown on figure 12. The H1299 cell line is a p53 null non-small cell lung cancer cell line. It has a homozygous partial deletion of *TP53* and no p53 protein expression. (51) Because of the lack of endogenous p53, this cell line is suitable for the transfection of our genetic construction. In this plasmid, the sequence coding for APEX2 is fused to that of wild-type p53. The sequences are connected by a flexible linker of 10 amino acids. The tumor suppressor p53 has a length of 393 amino acid residues, which is why we fused APEX2 to the N-terminus of p53, so as not to interfere with S392 phosphorylation. This PTM is important for mitochondrial localization of p53 and transcription-independent apoptosis. (36)

In the following experiments, we assessed different characteristics of our fusion protein APEX2-p53: its inducible expression by treatment with doxycycline, its mitochondrial localization, its transcriptional activity, and lastly its ability to induce apoptosis or cell cycle arrest.

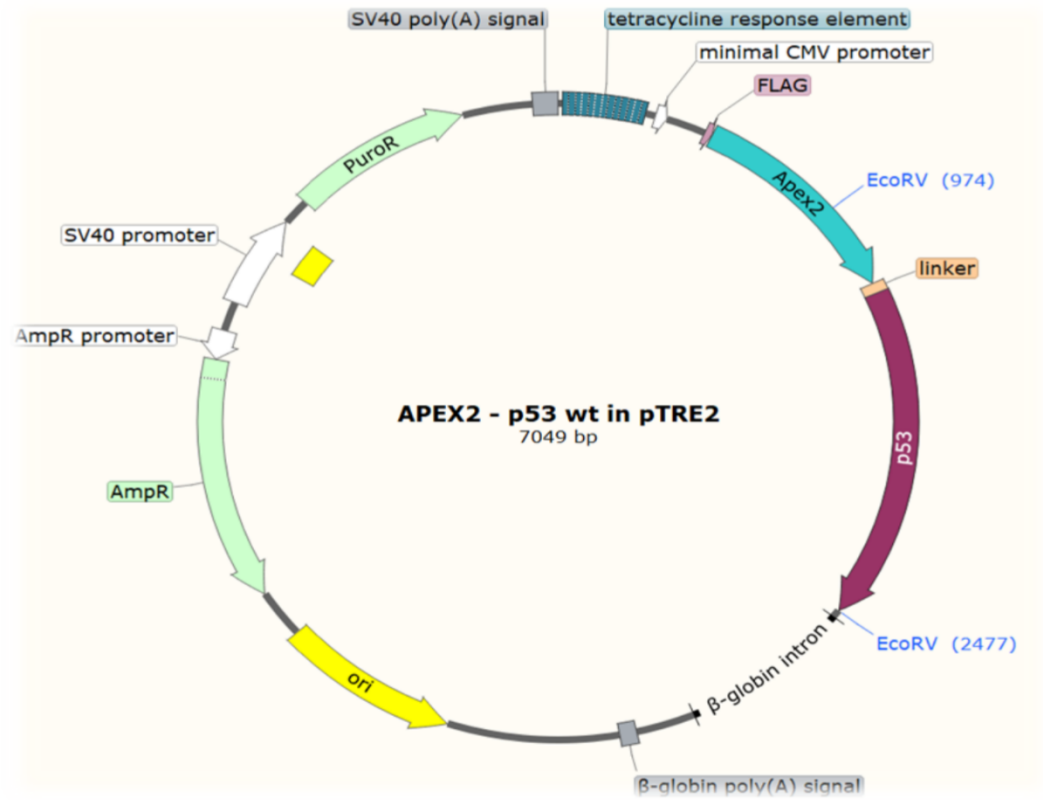

Figure 12: *Graphical representation of the APEX2-p53 wt expression vector. This demonstration is obtained by using the Snapgene program. Light blue: APEX2 sequence; Dark purple: wt p53 sequence; Dark blue: tetracycline response element (TRE); Light purple: flag; orange: flexible linker; Green: resistance elements (PuroR and AmpR); White: promoters; Yellow: replication origin. Citation modified from* (52).

1.1. Induction of APEX2-p53 expression

The regulation of the expression of APEX2-p53 uses a tetracycline (or derivatives) inducible system (Tet-ON system). This is required since a constitutive expression of p53 wt is known to be lethal in most cellular models (cell death by apoptosis). (53) The expression of APEX2-p53 is thus inducible via the addition of doxycycline (a tetracycline derivative) to the culture medium. The transcriptional regulation requires multiple components: rtTA, P_{tet}, and doxycycline. rtTA is a modified version of tTA, a hybrid transcription factor. tTA is a fusion of a prokaryotic repressor TetR, and a eukaryotic transactivation domain. While tTA binds to DNA in absence of doxycycline, rtTA does the opposite: it binds to the P_{tet} promoter only in presence of doxycycline [Figure 13]. P_{tet} is a synthetic promoter and is also known as the TRE (tetracycline responsive element). It responds to both tTA and rtTA. (54)

To verify the inducible expression of APEX2-p53 in our H1299 cell lines, we treated our cells with 2 µg/ml of doxycycline (DOX). We also performed treatment with 5 µM camptothecin (CPT, a topoisomerase I inhibitor that induces DNA damage), as well as a treatment with both DOX and CPT, in order to check if the presence of an additional stress changes p53 levels. A control without any treatment was also performed. Cells were at 70-80% confluency when treatments were added to the flasks. Treatments were stopped after 40 hours. Afterwards, total protein extracts were obtained using chemical lysis and quantified using the Bradford protein assay. (55) After SDS-PAGE, immunodetection was performed using a monoclonal anti-p53 antibody. Detection of β-actin was used as a loading control.

On figure 14, we observe for the cell lines C2, C5, and C7, that in absence of DOX, independent of the presence of CPT, very little to no expression of APEX2-p53 is detected. When cells were treated with doxycycline, a strong expression of APEX2-p53 was observed in C5 and C7 cells with a major band at ~80 kDa, which is the expected molecular weight of the APEX2-p53 fusion protein. In the C2 cell line, no band at 80 kDa was detected, however, a band at around 55 kDa was observed in doxycycline-treated samples. This corresponds to the molecular weight of p53. Loading controls were as expected in all samples. These results show that the APEX2-p53 fusion protein is correctly induced in two out of three cell lines in the presence of doxycycline.

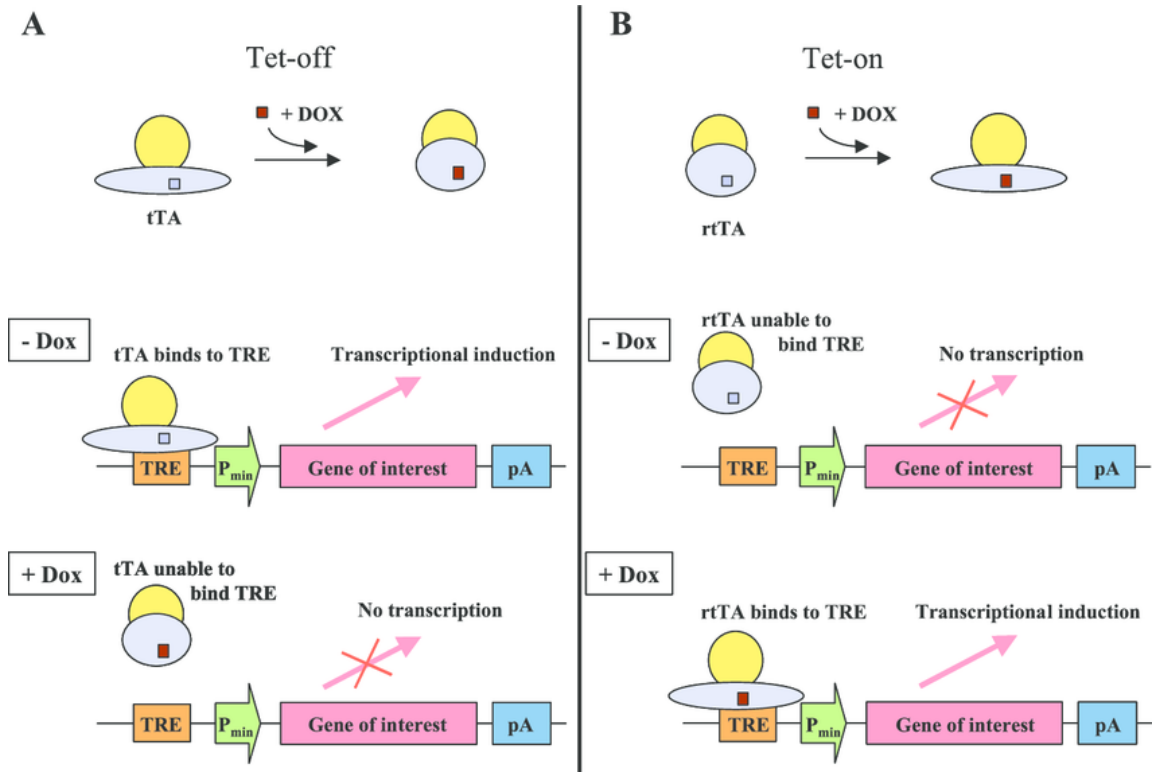

Figure 13: *The Tet system. Tet-On and Tet-Off expression systems can be used to conditionally activate gene expression. A: in the Tet-off system (tTA), expression is activated in the absence of doxycycline (DOX, shown as red box). Upon addition of DOX, transcription of the gene of interest is extinguished. B: in contrast, in the Tet-On system (rtTA), addition of DOX triggers the expression of the gene of interest. tTA, tetracycline-dependent transactivator; rtTA, reverse tetracycline-dependent transactivator; DOX, doxycycline; TRE, Tet-responsive element. Citation modified from* (56).

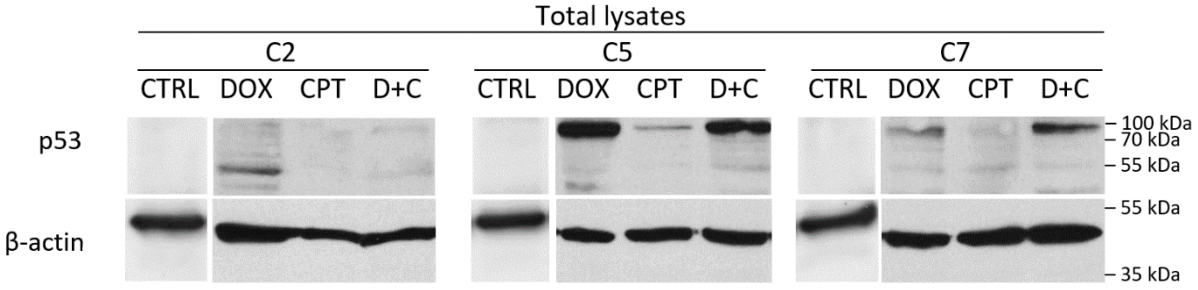

Figure 14: *Immunodetection of APEX2-p53 in total cell lysates. Cells were treated with 2 µg/ml of doxycycline and 5 µM of camptothecin for 40 hours. CTRL: no treatment; DOX: cells treated with doxycycline; CPT: cells treated with camptothecin; D+C: cells treated with both doxycycline and camptothecin. Samples are from three different cell lines (C2, C5, and C7) as indicated. Expression of APEX2-p53 was detected using an anti-p53 antibody. Loading control was immunodetection of β-actin.*

1.2. Subcellular localization of APEX2-p53

Next, we wanted to assess the mitochondrial localization of the fusion protein. As previously described, S392 phosphorylation seems to be important for p53 mitochondrial localization. A non-phosphorylatable p53 S392A mutant displays a deficient mitochondrial localization and hence less apoptosis. (36) Apoptosis can be induced by p53 at the mitochondria, via its interaction with different members of the BCL-2 protein family. (28) We therefore wanted to see if the N-terminal fusion of APEX2 hinders or not the localization of p53 to the mitochondria.

The four conditions previously described were used: no treatment, doxycycline (DOX, to induce expression), camptothecin (CPT, a DNA-damaging agent to induce stress), and both treatments combined. Cells were treated at 70-80% confluency for 40 hours with 2 µg/ml DOX and 5 µM CPT. Mitochondrial and cytosolic fractions were obtained by using mechanical lysis of cells and differential centrifugation. This extraction consists of performing multiple low-speed centrifugations (500xg) to remove intact cells, nuclei as well as large cellular debris, after which high-speed centrifugations (9000xg) are used to isolate a mitochondrial pellet.

After SDS-PAGE electrophoresis, detection of APEX2-p53 was carried out by performing immunoblotting with an anti-p53 antibody. PCNA (30 kDa) served as a loading control for the cytosolic fraction and as a purity control for the mitochondrial fraction. The loading control of the mitochondrial fraction, which simultaneously acted as a purity control for the cytosolic fraction, was GRP75 (75 kDa). (36)

In the cytosolic fractions, in the absence of doxycycline, no or only very little APEX2-p53 was detected in all three cell lines [Figure 15]. In the samples treated with doxycycline, a robust expression of APEX2-p53 was observed in the C5 and C7 cell lines with an apparent molecular weight of around 80-90 kDa. In the C2 cell line, multiple bands at a lower-than-expected molecular weight were observed, suggesting a degradation, at least partial, of the APEX2-p53 fusion protein. This is also observed in the cell line C5, although to a lesser extent. We see bands at around 80 kDa in the mitochondrial fractions of all three cell lines, although very weak in the C2 cell line. These results indicate that mitochondrial localization of APEX2-p53 takes place, and that the fusion with APEX2 does not prevent p53 mitochondrial localization.

Purity assessment of mitochondrial fractions was successful [Figure 16], as there is GRP75 but no PCNA in those fractions. Cytosolic fractions contain PCNA as expected, but their purity cannot be validated, as they also contained GRP75. For the C7 cell line, only mitochondrial fractions were tested.

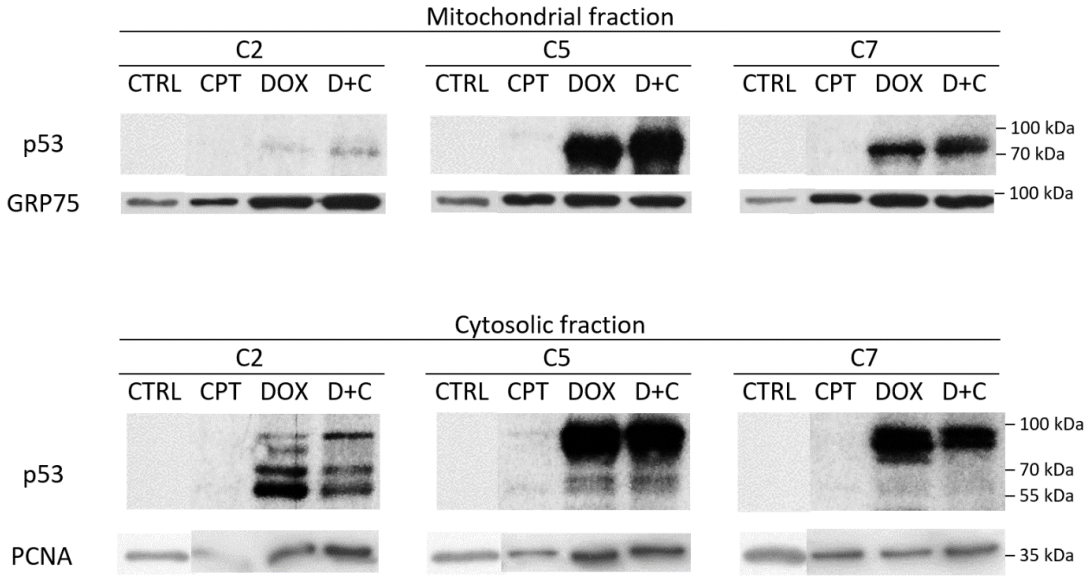

Figure 15: *Immunodetection of APEX2-p53 in cytosolic and mitochondrial fractions. Cells were treated with 2 µg/ml of doxycycline and 5 µM of camptothecin for 40 hours. CTRL: no treatment; DOX: cells treated with doxycycline; CPT: cells treated with camptothecin; D+C: cells treated with both doxycycline and camptothecin. Samples are from three different cell lines (C2, C5, and C7) as indicated. On the left, the antibody that was used is shown. Loading control was immunodetection of PCNA for the cytosolic fraction and GRP75 for the mitochondrial fraction.*

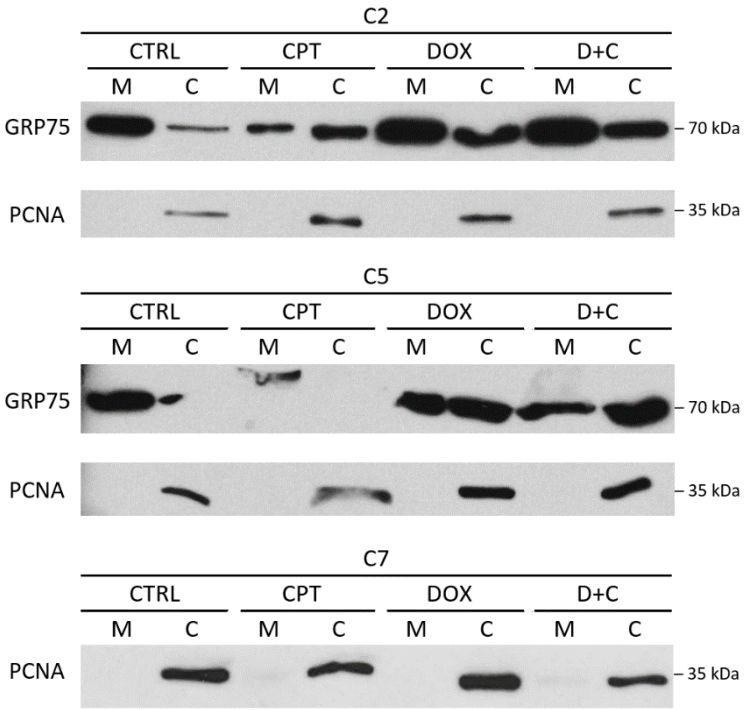

Figure 16: *Purity control of cytosolic and mitochondrial fractions. Cells were treated with 2 µg/ml of doxycycline and 5 µM of camptothecin for 40 hours. M: mitochondrial fraction; C: cytosolic fraction; CTRL: no treatment; DOX: cells treated with doxycycline; CPT: cells treated with camptothecin; D+C: cells treated with both doxycycline and camptothecin. Samples are from three different cell lines (C2, C5, and C7) as indicated. Detection of PCNA and GRP75 was performed to assess the quality of the cytosolic and mitochondrial fractions.*

1.3. Transcriptional activity of APEX2-p53

The tumor suppressor p53 exerts its many functions mainly through its activity as a transcription factor, by regulating the expression of a variety of genes. One of the target genes of p53 is $p21^{WAF1}$, a protein which is able to stop the cell cycle. (7) The protein p53 has a strong affinity for the promoter of the $p21^{WAF1}$ encoding gene, *CDK1NA*, resulting in high expression upon p53 activation. (57) To detect presence of $p21^{WAF1}$, immunoblotting was used.

Protein samples are total lysates of cells treated with doxycycline (DOX), camptothecin (CPT), or a combination of the two molecules DOX+CPT (D+C on figures) as previously described. Untreated protein samples have not been tested and will need to be tested in the future as a control. After SDS-PAGE, immunoblotting was performed using an anti-$p21^{WAF1}$ antibody. β-actin detection was used as a loading control.

No proteins were detected in any of the samples [Figure 17]. Loading controls were successful. These results are not as expected, since previous works (52) showed an expression of $p21^{WAF1}$ as well as of MDM2 after induction of APEX2-p53 expression with doxycycline.

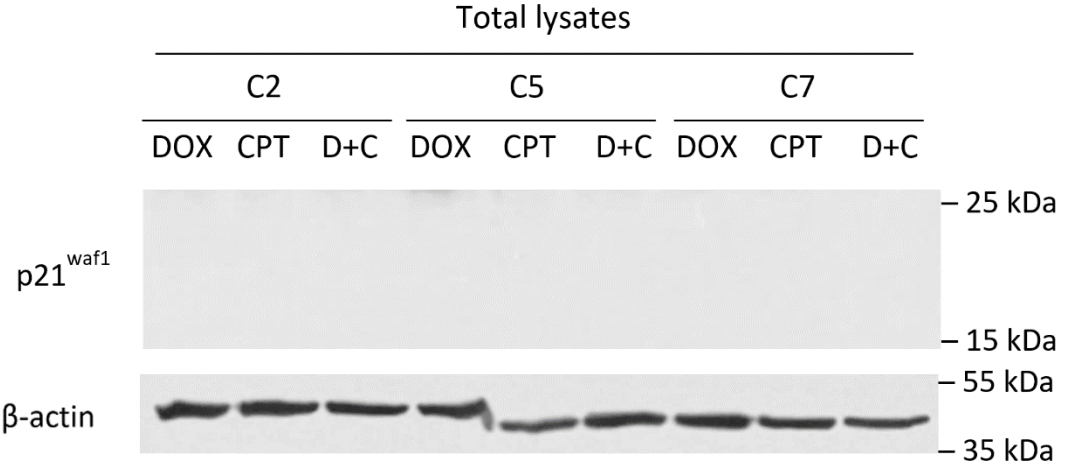

Figure 17: *Immunodetection of $p21^{WAF1}$ in total cell lysates. Cells were treated with 2 µg/ml of doxycycline and 5 µM of camptothecin for 40 hours. DOX: cells treated with doxycycline; CPT: cells treated with camptothecin; D+C: cells treated with both doxycycline and camptothecin. Samples are from three different cell lines (C2, C5, and C7) as indicated. The loading control was the immunodetection of β-actin.*

1.4. Induction of apoptosis and cell growth inhibition by APEX2-p53

In the presence of a stress, p53 localizes to the mitochondria to induce apoptosis. (58) It can also localize to the nucleus, tetramerize and regulate gene expression to induce cell cycle arrest or type I programmed cell death (apoptosis). (7) We wanted to check if our fusion protein APEX2-p53 can still exert its crucial function of apoptosis or cell cycle regulator. To do this, we performed multiple experiments. Firstly, we detected the presence of cleaved PARP (a marker of apoptosis) by immunoblot. Afterwards, we performed different assays to measure global cell growth inhibition by p53 such as colony formation assays (59) and MTT assays (60).

1.4.1. Induction of PARP cleavage

Poly (ADP-ribose) polymerase (PARP) is an enzyme found in the nucleus. Its main function is to detect single-strand breaks (SSBs). (61) Cleavage of PARP (producing a main fragment of 85 kDa, known as c-PARP) happens in the execution phase of apoptosis and is caspase-dependent. (41) Therefore, detecting the presence of cleaved PARP in cells will give an indication whether they are undergoing apoptosis or not.

Protein samples are total lysates with the four different treatments (control, DOX, CPT, and D+C), as previously described. SDS-PAGE was performed, and an anti-c-PARP antibody was used for the immunoblot. This antibody only recognizes the cleaved PARP form and not the full-length form. (62) Loading control was executed via β-actin detection.

We did not detect c-PARP in untreated or doxycycline-treated cells [Figure 18]. However, we detected strong levels of c-PARP in camptothecin-treated samples, independently of doxycycline presence. Loading controls were positive in each sample.

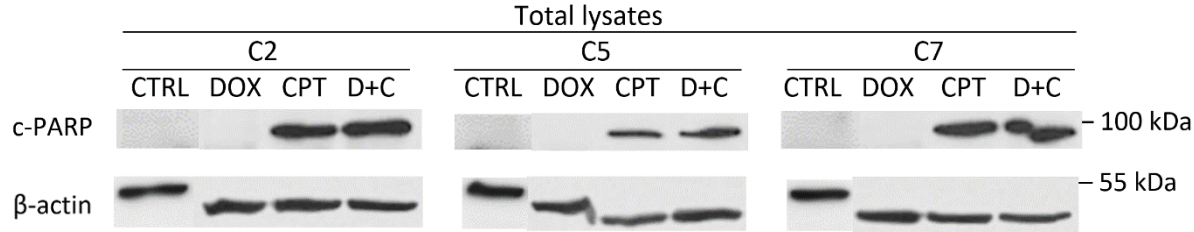

Figure 18: *Immunodetection of cleaved PARP. Cells were treated with 2 µg/ml of doxycycline and 5 µM of camptothecin for 40 hours. CTRL: no treatment; DOX: cells treated with doxycycline; CPT: cells treated with camptothecin; D+C: cells treated with both doxycycline and camptothecin. Samples are from three different cell lines (C2, C5, and C7) as indicated. Left indications show the antibody that was used. Loading control was performed using immunodetection of β-actin.*

1.4.2. Colony formation assays

The colony formation assay or clonogenic assay is an *in vitro* cell survival assay. (59) Here, our three cell lines were either untreated or treated with 2 µg/ml of doxycycline, to induce expression of APEX2-p53.

Colony formation assays were performed in six-well plates once with a concentration of 5000 cells/well and twice with a concentration of 500 cells/well. Medium and treatment was refreshed every two days for one week. Cells were fixed with ethanol and stained with crystal violet. No difference between doxycycline-treated and untreated cells was observed [Figure 19], suggesting that the tumor suppressor activity of p53 is somehow impaired as a consequence of the N-terminal fusion with APEX2.

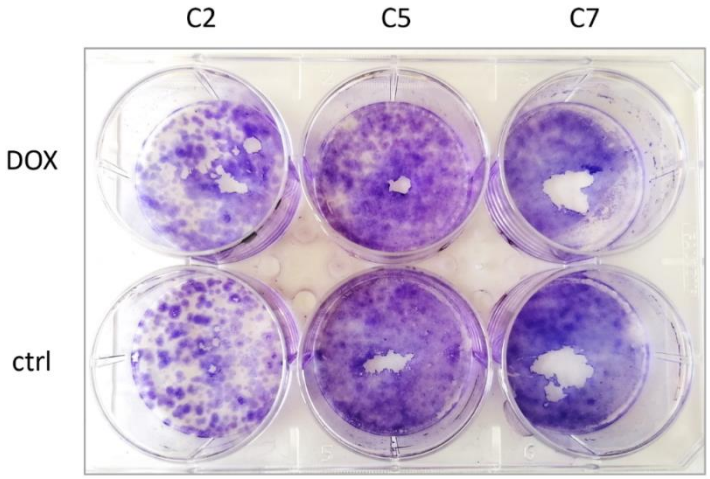

Figure 19: *Colony formation assay. Cells were seeded at low density (2500 cells/ml). Cells were treated or not with 2 µg/ml doxycycline for 1 week. After fixation and staining, no difference between DOX and control cells was observed.*

1.4.3. MTT assay

The MTT assay is a colometric cell viability assay. In this experiment, the yellow dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is added to cells. Living (metabolically active) cells reduce the yellow dye into an insoluble blue-violet product, which is dissolved in DMSO. The absorbance at 570 nm is then measured, and cell viability is expressed in percentage, relative to the control (untreated) condition. (60)

The three cell lines (C2, C5, and C7) were seeded in a 96-well plate. Increasing concentrations of camptothecin (0 µM, 2,5 µM, 5 µM, and 10 µM), in the presence or not of doxycycline (DOX and NO DOX, respectively) were used to create a total of eight different experimental conditions. Each condition was performed in sextuplicates and treatments were stopped after

three days to perform the colometric assay. Firstly, cell viability (%) was compared between NO DOX/DOX subgroups using one-way analysis of variance (ANOVA). No statistically significant difference was shown between the samples without doxycycline and the samples treated with doxycycline in each subgroup (Figure 20 top left to bottom left, Table S1). Next, a linear regression model to predict cell viability showed that neither the cell line nor the presence of doxycycline had a statistically significant effect on cell viability. However, a statistically significant effect of camptothecin (CPT) concentration on cell viability was shown (Table S2). Further analysis demonstrated that each increase of CPT concentration showed a statistically significant decrease in cell viability (Figure 20 bottom right, Table S3). The statistical analysis was conducted using R (version 4.2.2) software. The script can be found in the supplementary data.

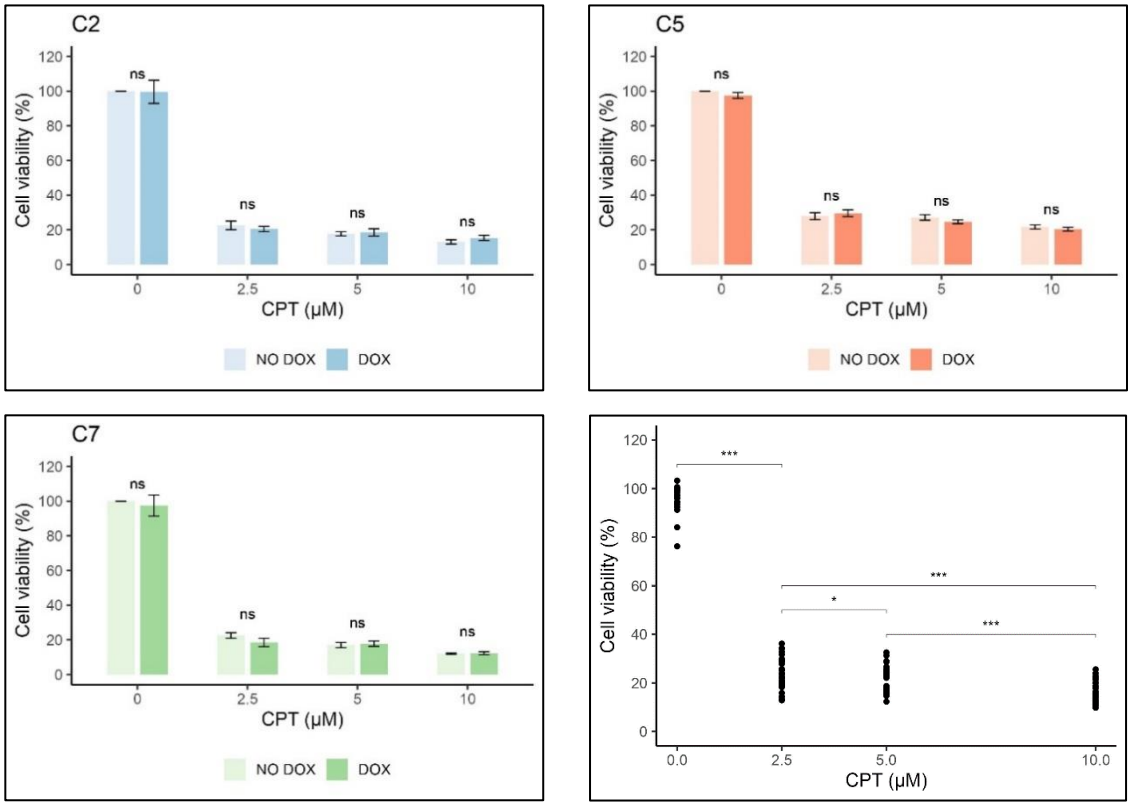

Figure 20: *Statistical analysis of MTT assays. The three H1299 cell lines were seeded in a 96-well plate at a concentration of 2000 cells/well. Two days later, cells were treated for 3 days with or without doxycycline (2 µg/ml) in presence of different concentrations of CPT (0 – 10 µM). Each condition was repeated 6 times.* ***Top left, top right, and bottom left:*** *Cell viability (%) with or without doxycycline, in function of cell line (C2, C5 or C7) and CPT concentration. Histogram represents Mean ± Standard Error (N=6). Comparison of DOX vs NO DOX using one way ANOVA: all p-values > 0,05 (ns = not significant).* ***Bottom right:*** *Cell viability (%) in function of CPT concentration. Points represent N=36 observations for each CPT concentration (DOX and NO DOX together, C2-C5-C7 together).*
****: p <0,001, **: p<0,01, *: p<0,05*

2. Immunofluorescence characterization of APEX2-p53 expression

We next wanted to assess APEX2-p53 expression using immunofluorescence microscopy. This technique allows visualization of proteins or intracellular structures of interest inside tissues or cells by combining specific antibodies that are tagged with different fluorophores. Afterwards, their presence/localization is detected using a fluorescence microscope. (63)

Cells were seeded in 24-well plates at 50% confluence. Four conditions were used: untreated (control), DOX (2 µg/ml doxycycline), CPT (10 µM camptothecin), and D+C (2 µg/ml doxycycline and 10 µM CPT). Treatments were stopped after 26 hours. DAPI (64) was used as a nuclear marker. To label APEX2-p53, a primary mouse anti-p53 antibody was used. The secondary antibody was the fluorescent goat anti-mouse Alexa546 antibody.

To assess if induction of expression by doxycycline was successful, we used an inducible (Tet-On system) H1299 p53 wt cell line previously developed in the laboratory. As a second control, the PA-1 cell line (ovarian cancer cell line (65)), expressing endogenously wt p53, was used.

In all three cell lines (C2, C5, and C7), only few cells emitting a red fluorescent signal (corresponding to APEX2-p53 expression) were observed in the doxycycline-treated cells or the cells treated with a combination of doxycycline and camptothecin. Untreated cells, as well as camptothecin-treated cells did not display any red signal. However, in the H1299 p53 wt Tet-On cell line, a red fluorescent signal corresponding to wt p53 was detected in the majority of the cells when cells were treated with doxycycline, with or without camptothecin. No signal was observed in the negative control (untreated cells) and camptothecin-treated cells [Figures 9-12]. The second control, which was achieved by using the PA-1 cell line, shows strong signal of p53 in camptothecin-treated cells, with or without doxycycline. Moreover, doxycycline does not seem to have any influence on signal distribution, as expected [Figure 13]. We are not able to make any comparisons on signal intensity (which would potentially be proportional to APEX2-p53 expression) between the different samples due to problems encountered with the fluorescence microscope ("flickering" of the intensities when taking images). Detection of chromatin condensation (66), which is an indicator of apoptosis, was not assessed. We also need more repetitions to be able to draw more accurate conclusions.

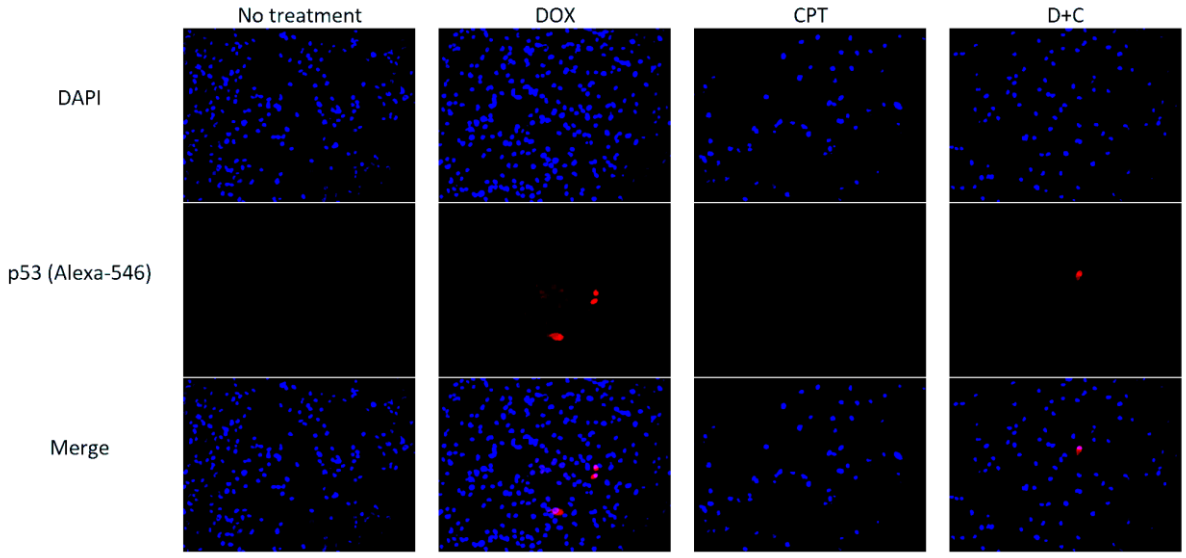

Figure 21: *Immunofluorescence assay (cell line C2). Cells were seeded at medium density and treated for 26 hours with 2 µg/ml of doxycycline and 5 µM of camptothecin. DOX: cells treated with doxycycline; CPT: cells treated with camptothecin; D+C: cells treated with both doxycycline and camptothecin. Nuclei were labeled with DAPI. The primary antibody for APEX2-p53 detection was a monoclonal mouse anti-p53 antibody and the secondary antibody was a goat anti-mouse Alexa Fluor™ 546 antibody. Exposure time: DAPI: 5 ms; p53: 100 ms. Pictures have been modified for better contrast and visibility.*

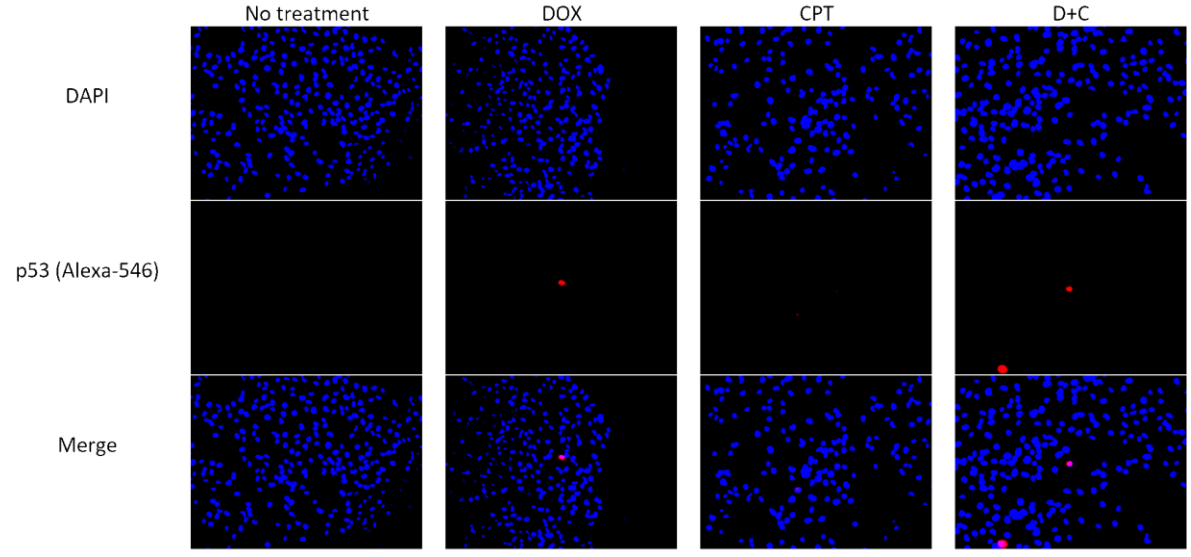

Figure 22: *Immunofluorescence assay (cell line C5). Cells were seeded at medium density and treated for 26 hours with 2 µg/ml of doxycycline and 5 µM of camptothecin. DOX: cells treated with doxycycline; CPT: cells treated with camptothecin; D+C: cells treated with both doxycycline and camptothecin. Nuclei were labeled with DAPI. The primary antibody for APEX2-p53 detection was a monoclonal mouse anti-p53 antibody and the secondary antibody was a goat anti-mouse Alexa Fluor™ 546 antibody. Exposure time: DAPI: 5 ms; p53: 100 ms. Pictures have been modified for better contrast and visibility.*

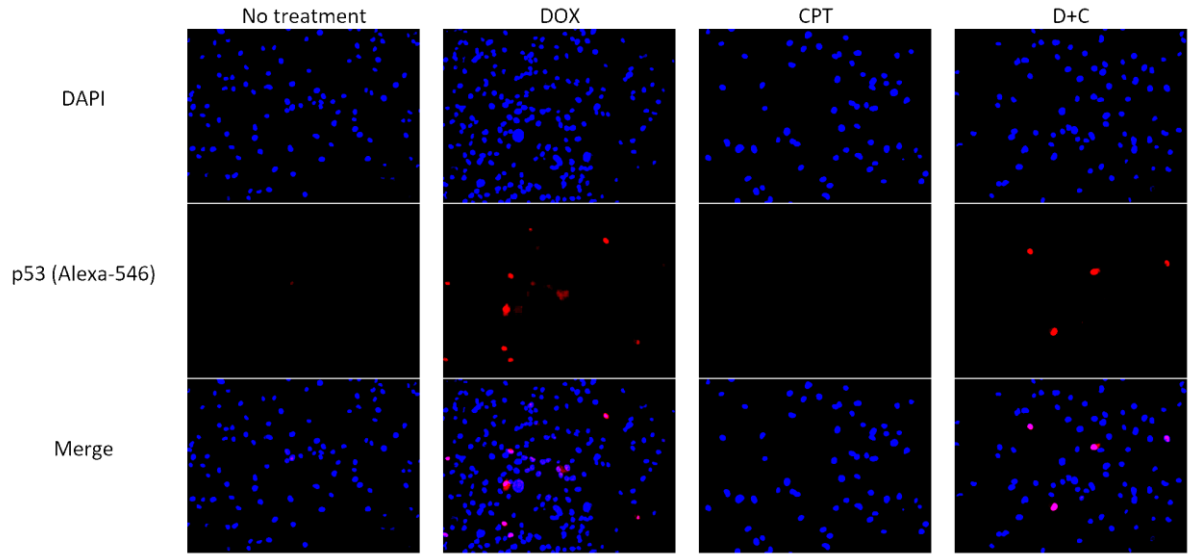

Figure 23: *Immunofluorescence assay (cell line C7). Cells were seeded at medium density and treated for 26 hours with 2 µg/ml of doxycycline and 5 µM of camptothecin. DOX: cells treated with doxycycline; CPT: cells treated with camptothecin; D+C: cells treated with both doxycycline and camptothecin. Nuclei were labeled with DAPI. The primary antibody for APEX2-p53 detection was a monoclonal mouse anti-p53 antibody and the secondary antibody was a goat anti-mouse Alexa Fluor™ 546 antibody. Exposure time: DAPI: 5 ms; p53: 100 ms. Pictures have been modified for better contrast and visibility.*

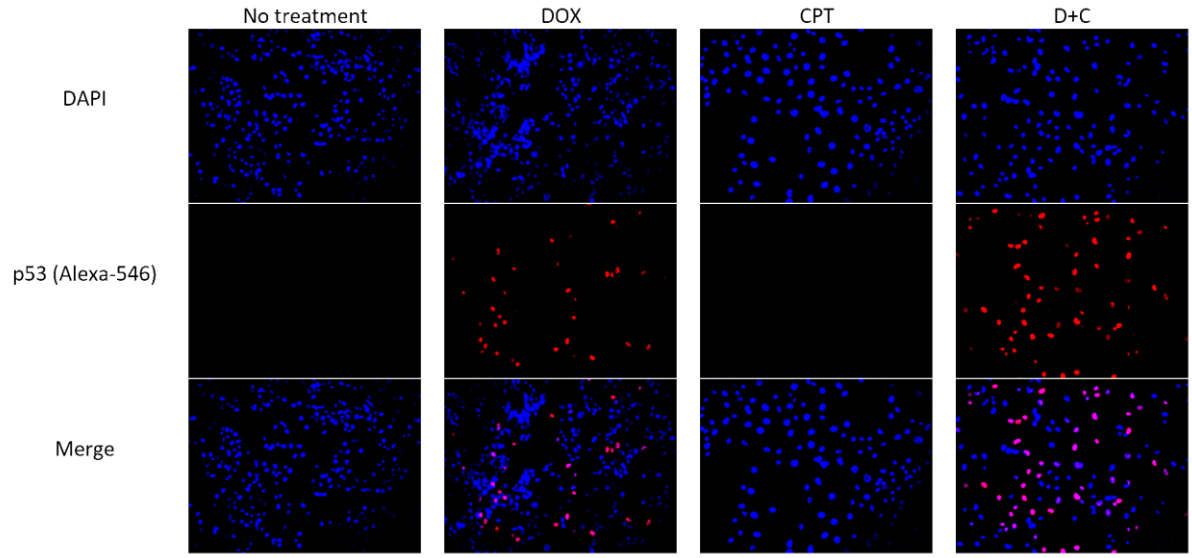

Figure 24: *Immunofluorescence assay (cell line H1299 Tet-On p53wt). Cells were seeded at medium density and treated for 26 hours with 2 µg/ml of doxycycline and 5 µM of camptothecin. DOX: cells treated with doxycycline; CPT: cells treated with camptothecin; D+C: cells treated with both doxycycline and camptothecin. Nuclei were labeled with DAPI. The primary antibody for APEX2-p53 detection was a monoclonal anti-p53 antibody and the secondary antibody was a goat anti-mouse Alexa Fluor™ 546 antibody. Exposure time: DAPI: 5 ms; p53: 100 ms. Pictures have been modified for better contrast and visibility.*

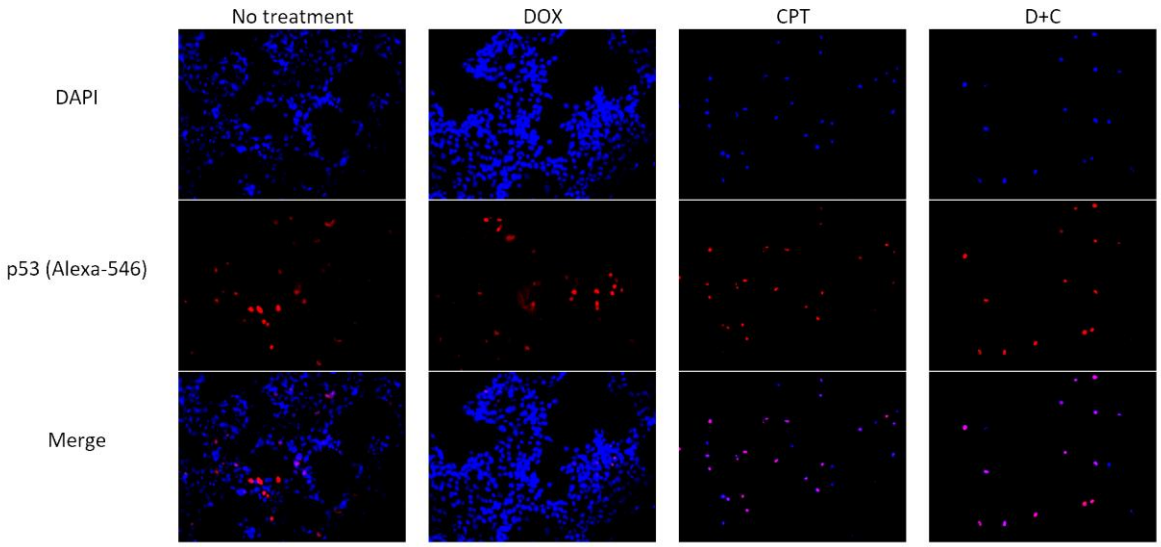

Figure 25: *Immunofluorescence assay (cell line PA-1 p53wt). Cells were seeded at medium density and treated for 26 hours with 2 µg/ml of doxycycline and 5 µM of camptothecin. DOX: cells treated with doxycycline; CPT: cells treated with camptothecin; D+C: cells treated with both doxycycline and camptothecin. Nuclei were labeled with DAPI. The primary antibody for APEX2-p53 detection was a monoclonal anti-p53 antibody and the secondary antibody was a goat anti-mouse Alexa Fluor™ 546 antibody. Exposure time: DAPI: 10 ms; p53: 100 ms. Pictures have been modified for better contrast and visibility.*

IV. DISCUSSION

1. Discussion

The tumor suppressor p53 is a major regulator of cell growth and death. Under stress signals, which reach p53 by the means of post-translational modifications, p53 is activated and induces cell cycle arrest, senescence, or even cell death through apoptosis. While p53 mainly functions as a transcription factor, our team focuses on its activity as a direct apoptosis inducer at the mitochondria. It achieves this by localizing to the mitochondria in cells exposed to a detrimental stimulus and interacting with various members of the BCL-2 protein family including the pro-apoptotic BAK protein to trigger MOMP. (58)

The mechanisms of p53 mitochondrial localization remain largely unknown. Recently, Castrogiovanni et al. (34) showed that the phosphorylation of S392 is crucial to this phenomenon. The two resulting hypotheses imply the involvement of one or multiple cytosolic interaction partners of p53. The first hypothesis suggests that upon stress, p53 interacts with a protein partner in order to localize to the mitochondria and trigger MOM permeabilization, and that the interaction is promoted by S392 phosphorylation. The second hypothesis states that in the absence of a stress, one or several proteins could sequester cytosolic p53, thus preventing mitochondrial localization and MOMP. The increased phosphorylation of serine 392 in cells exposed to a stress would prevent formation of this inhibitory protein complex, allowing cytosolic p53 to reach the MOM to trigger apoptosis.

Identifying these protein partners is the ultimate goal of this project, and we want to reach this goal by performing proximity-dependent labeling using APEX2. Previously, Zhen et al. (67) and Dong et al. (68) showed that the fusion of APEX2 to a protein of interest is a promising tool to identify new protein interactions. Therefore, the H1299 p53 null cell line was stably transfected with a genetic construction containing p53 with an N-terminal fusion of APEX2, generating three different cell lines, denominated C2, C5, and C7. The purpose of our work is to characterize these cell lines. More specifically, this means: 1) Checking the induction of expression of our fusion protein; 2) Assessing the subcellular localization of the APEX2-p53 fusion protein; 3) Determining its transcriptional activity and ability to function as a tumor suppressor.

Using immunoblotting, induction of expression of the APEX2-p53 fusion protein by doxycycline (Tet-On system) was found to be successful in cell lines C5 and C7, however it was very weak in cell line C2. This experiment was repeated two times with comparable results. In the absence of doxycycline treatment, no or very weak bands were observed, meaning that

the Tet-On expression system in our cell lines is not very leaky which, unfortunately, is a reoccurring problem in numerous studies. (69,70) In the C2 cell line, the detected band presented a molecular weight of approximately 55 kDa, suggesting that in these cells, the APEX2-p53 fusion protein is somehow cleaved or degraded. Of note, this molecular weight corresponds to that of p53 in SDS-PAGE. Further evaluation could include an immunoblot using an anti-APEX2 antibody. Moreover, in the three cell lines, additional experiments regarding the expression of APEX2-p53 could be performed by changing the concentration of doxycycline employed, or the duration of the treatment.

Differential centrifugation and immunoblotting of mitochondrial and cytosolic fractions suggest that mitochondrial localization of APEX2-p53 takes place in cells treated with doxycycline as well as cells treated with both doxycycline and camptothecin. This is the case for two out of three cell lines. In the C2 cell line, only a faint band is detected in the mitochondrial fraction. This is in accordance with the abnormal molecular weight of the major band detected in the total lysates of this cell line, as well as the extensive degradation of the APEX2-p53 fusion protein observed in the cytosolic fractions. We do not detect any of these lighter molecular weight bands in the mitochondrial fractions. A possible reason for this is a degradation of the fusion protein via the proteasome before reaching the mitochondria. This experiment was repeated two more times and each repetition showed similar results.

Purity controls of mitochondrial and cytosolic fractions were performed. To achieve this, PCNA was used to assess purity of mitochondrial fractions, while GRP75 served as a purity control for the cytosolic fractions. PCNA (proliferating cell nuclear antigen) is a cofactor for DNA polymerase and acts as a scaffold during replication to avoid detachment of the enzyme during DNA synthesis. It executes this function in the nucleus, but it can also be found in the cytosol. (71) GRP75 (glucose-regulated protein 75) is a chaperone of the HSP70 family regulating mitochondrial import and intracellular trafficking. (72) It is therefore mainly found in the mitochondria.

While the mitochondrial fractions do not contain any (or contain very little) PCNA, we detect GRP75 in mitochondrial as well as cytosolic fractions. However, this does not necessarily mean that these fractions are of a poor quality. For instance, Ran et al. (73) previously showed, that depending on the cell line, GRP75 (also known as Mortalin) can be found in the cytosol in high quantities [Figure 26]. Therefore, the integrity of cytosolic fractions will need to be reassessed using a different mitochondrial marker, like HSP60. (74)

Further experiments that need to be performed is checking the phosphorylation status of S392 in all samples, since this post-translational modification is crucial for mitochondrial localization. (36)

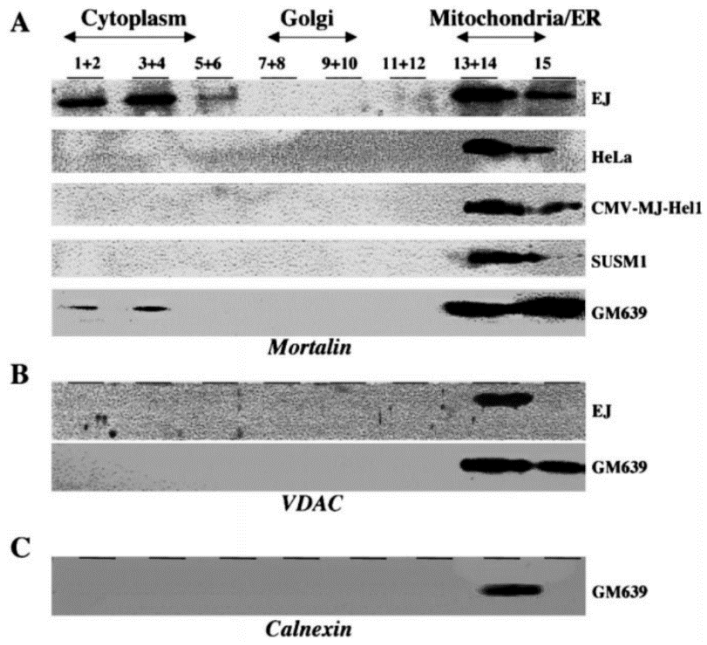

Figure 26: *Western blot analysis of sucrose/deuterium oxide gradient fractions. Fractions obtained from indicated cells by a shallow discontinuous sucrose gradient in buffered deuterium oxide were subjected to Western blotting stained with anti-mortalin or anti-VDAC antibody. Mortalin was detected in fractions 13 to 15 in all the cell lines. These fractions were positive for the mitochondrial and ER markers (panels B and C). EJ and GM639 cells showed sedimentation of mortalin in fractions as early as 1 to 4 (panel A). These fractions were negative for the mitochondrial (panel B) as well as ER markers (panel C). Fraction 15 of EJ cells (mortalin positive and VDAC negative) revealed ER resident pool of mortalin and fractions 1– 4 represented nonmitochondrial and nonER pool of mortalin. Citation from* (73).

In order to assess the transcriptional activity of our APEX2-p53 fusion protein, immunoblotting on total cell lysates was performed using an anti-$p21^{WAF1}$ antibody. The experiment was carried out a total of four times, but no $p21^{WAF1}$ was detected in any of our samples, even when using high antibody concentration. This is unexpected, as in previous works (52), $p21^{WAF1}$ as well as MDM2 were detected in samples that were treated with doxycycline, thus in presence of APEX2-p53 expression. The anti-$p21^{WAF1}$ antibody used here is known as very reliable and efficient in our team. A first hypothesis to explain our failure to detect $p21^{WAF1}$ implies that somehow, for instance due to poor storage conditions, the antibody is degraded. Another hypothesis involves that the APEX2-p53 fusion protein is a poor transcription factor. For example, the N-terminal fusion of APEX2 could interfere with the function of the TAD1 of p53 (residues 1 – 40). Samples could be tested with a newly bought $p21^{WAF1}$ antibody to confirm the results, and MDM2 presence could also be detected via immunoblotting. As of now, a positive control using a p53wt cell line is missing and therefore it is not certain that the antibody is at fault. Luciferase assays could also be used to assess the transcriptional activity of APEX2-p53.

To evaluate p53 ability to induce apoptosis or cell cycle arrest, multiple experiments were performed. Firstly, immunoblotting with an anti-c-PARP antibody was carried out, however no cleaved PARP was detected in doxycycline-treated samples (without camptothecin as an additional stress inducer). The bands corresponding to c-PARP that are present in samples with camptothecin treatment likely result from an apoptotic process that is activated by other pathways than via those involving p53 activation, apoptosis being a highly regulated form of programmed cell death that can be provoked by many different pathways. For example, Park et al. (75) showed that fucoidan-treated HCT116 p53 K.O. cells showed similar apoptosis levels than wt cells in presence of DNA damage.

As this experiment was carried out only once, more repetitions are needed. Furthermore, a positive control (cell lines expressing wild-type p53) could be tested.

Cell viability was assessed via colony formation and MTT assays, nevertheless the results were similar to those obtained from the c-PARP immunoblot. In both experiments, treatment with doxycycline showed no visible (colony formation assays) or statistically significant (MTT assays) changes in cell viability.

Previous studies (colony formation assays) from Castrogiovanni et al. (36) showed that induction (Tet-On system) of wt p53 expression in H1299 cells reduced the viability of cells drastically [Figure 27]. However, additionally to 2 µg/ml of doxycycline, 1 µM of camptothecin was used as treatment in this previous study. Repetition of these experiments with our cell lines would require the same conditions.

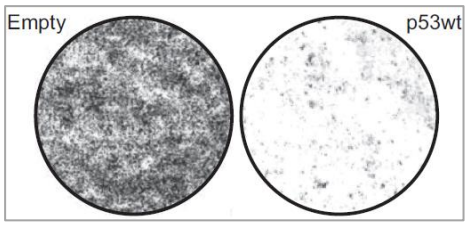

Figure 27: *Colony formation assay of H1299 Tet-On clones expressing p53 wt or transfected with the empty vector were treated simultaneously with 2 µg/ml of doxycycline and 1 µM of CPT for 3 days. Modified from* (36).

Lastly, immunofluorescence microscopy was performed on our three cell lines as well as two control cell lines. Results show that in our cell lines expressing APEX2-p53 (C2, C5, and C7), only a small fraction of the cells is labeled by the anti-p53 antibody. As a positive control, we used a H1299 cell line stably expressing wt p53 (Tet-On system). The PA-1 cell line, which endogenously expresses wild-type p53, served as a second control. In these two control cell lines, a drastic increase of fluorescent red signal corresponding to wt p53 was observed following the adequate treatment. Using the PA-1 cell line, we could determine that the labeling reaction worked well. Under stress condition, every cell expressed p53. As expected, the

presence of doxycycline did not make any difference as in these cells, the expression of p53 is not induced by doxycycline.

These results indicate that in the three APEX2-p53 cell lines, only a small proportion of cells correctly express the fusion protein. This low expression could possibly be due to a lack of subcloning directly after the initial transfection, leading to cell populations quite heterogenous regarding APEX2-p53 inducibility by doxycycline, as well as expression. (76) By performing subcloning, we can choose a single cell colony displaying an inducible and strong expression of APEX2-p53, and from this cell we can create a more homogenous and stable cell line. Another possibility is repeating transfection of the plasmids in another cell model such as the SAOS-2 ($p53^{-/-}$) cell line. (77)

Subcloning (or retransfection) could clarify our results obtained regarding cleaved PARP detection and cell viability assays. In fact, cleavage of PARP could potentially be present in DOX samples, however in very small, undetectable quantities. This could be due to the fact that each cell does not express APEX2-p53. Regarding colony formation and MTT assays, we have no visible/statistically significant change in cell viability when comparing doxycycline-treated cells to untreated cells. It is therefore possible that our protein is functional but only in a small fraction of our cells.

It is also possible that the failure to detect $p21^{WAF1}$ following induction of p53 expression by doxycycline is linked to the small proportion of cells responding correctly to the treatment.

A second hypothesis to explain the scarce signal in C5 and C7 cell lines in our immunofluorescence microscopy experiments is that the antibody does not correctly bind to the protein. In fact, for these two cell lines we see strong bands of APEX2-p53 in our immunoblots, where the proteins are completely denatured before antibody labeling. However, in our immunofluorescence experiments, we detected very weak signal for APEX2-p53. The antibody that we are using (78) binds to the N-terminus of p53 (the epitope maps between amino acid residues 11-25), so the antibody could potentially not be able to bind to the fusion protein if not denatured, as APEX2 is fused to the N-terminus.

Immunofluorescence microscopy experiments using an anti-APEX2 antibody could be performed. This could bring further valuable information regarding the expression of the APEX2-p53 fusion protein.

Induction of apoptosis can also be detected using fluorescence microscopy. The DAPI stain has a blue fluorescence and binds to AT regions in DNA, thus staining the nucleus. A marker of apoptosis is chromatin condensation, which translates to the presence of bright blue spots inside the nucleus [Figure 28]. (64,66) This was not assessed due to difficulties encountered with the microscope ("flickering" of signal intensity while taking pictures, resulting in images of varying intensity while using the same exposure parameters). Therefore, we cannot draw any conclusions and need to repeat these experiments.

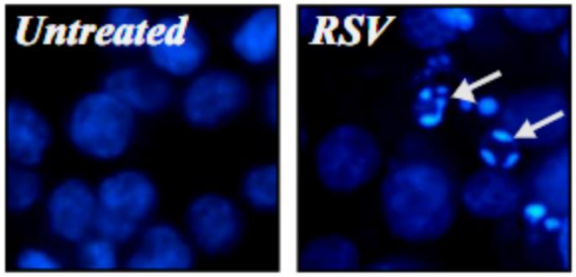

Figure 28: *DAPI staining reveals that resveratrol (RSV) induces chromatin condensation. HCT-116 p53 wt cells were treated or not with RSV. Photos are representative fluorescence microscopy images of DAPI stained cells. The white arrows indicate nuclei showing condensed chromatin. Figure and citation modified from* (79).

In conclusion, our results show that induction of expression as well as mitochondrial localization of the fusion protein take place, but its transcriptional and mitochondrial activities (and thus ability to induce cell cycle arrest or apoptosis) remain unclear. We found that only a small fraction of cells in each cell line correctly expresses APEX2-p53.

2. Future perspectives

As of now, the cell lines generated for this study are not suitable for further experiments. In the future, we will assess the expression of the fusion protein by performing immunofluorescence experiments using an anti-APEX2 antibody.

If the data confirm that the fusion protein is only expressed in a small proportion of cells, we will need to perform subcloning on the C5 and C7 cell lines which will allow to isolate clonal populations where the majority of cells express APEX2-p53. Alternatively, H1299 cells (51), or an alternative model like the SAOS-2 ($p53^{-/-}$) cell line (77) could be retransfected with the plasmid to generate new cell lines. Only by obtaining stable cell lines where all cells correctly express the APEX2-p53 fusion protein, the tumor suppressor activity of p53 fused to APEX2 can be correctly assessed. For instance, luciferase assays with p53 target promoters to assess transcriptional activity, as well as cell viability assays to check its ability to trigger cell cycle arrest or cell death by apoptosis.

We also need to determine if the phosphorylation of S392, which is crucial for mitochondrial localization and activity, takes place for our fusion protein. (36)

After this, we can begin to set up the proximity-dependent labeling reaction. Optimal parameters like the incubation time and the concentrations of biotin phenol and H_2O_2 need to be determined. (49)

Furthermore, the activity of APEX2, in particular its ability to produce biotin phenoxyl radicals from biotin phenol, needs to be verified. For this, immunofluorescence staining of biotinylated proteins, or a streptavidin blot analysis of cell lysates can be used. (49)

Lastly, multiple parameters concerning the streptavidin pull-down assay need to be optimized. The correct washing duration and conditions, as well as the quantity of streptavidin beads used need to be determined, in order to minimize background noise. (49)

MATERIAL AND METHODS

1. Cell culture

The cells used during this thesis are an H1299 cell line (human lung cancer cell line not expressing any endogenous p53). This cell line was transfected with the pTRE2 APEX2-p53 wt expression vector.

To avoid contaminations, the following manipulations are carried out in a sterile environment, which is obtained by using a laminar flow hood and gloves. All solutions and material coming in contact with the cells are sterilized. Any non-sterile material used in the laminar flow hood is thoroughly disinfected before use.

1.1. Culture medium preparation

Material:

- 500 ml of DMEM high glucose (4°C) *[Gibco™ 42430-025]*
- 50 ml (10%) of decomplemented fetal bovine serum (-20°C) *[BioWhittaker™ Lonza DE 14-802F]*
- 5 ml (1%) of penicillin/streptomycin antibiotics (-20°C) (Penicillin 10,000 units/ml and Streptomycin 10,000 µg/ml) *[Gibco™ 15140122]*
- 50 µl (1 µg/ml) puromycin *[Invivogen™ QLL-40-04]*
- 400 mg (800 µg/ml) of geneticin *[Gibco™ 11811-031]*
- 1 falcon™ tube (15 ml)
- 1 sterile syringe filter, pore size 0.22 µm
- 1 sterile syringe (10 ml)

Operating mode:

- Put 400 mg of geneticin in a 15 ml falcon tube.
- In a laminar flow hood, add 7-8 ml of high glucose DMEM medium to the falcon tube.
- Homogenize until the powder is fully dissolved.
- In a laminar flow hood, decant the content of the falcon into the syringe, equipped with the 0.22 µm filter.
- Filter directly into the high glucose DMEM medium bottle.
- Add 50 ml of FBS, 5 ml of the penicillin/streptomycin solution and 50 µl of puromycin.
- Store for 1 month at 4°C.

1.2. Thawing of cells

Thawing should be performed as quickly as possible to remove the cytotoxic DMSO that is used to cryogenize cells.

Material:

- Cryotube containing cells (stocked in liquid nitrogen)
- Supplemented DMEM culture medium (previously heated to 37°C)
- 1 falcon™ tube (15 ml) per cryotube
- 1 cell culture flask (75 cm^2) per cryotube
- 1 berlin containing water (42°C)

Operating mode:

- Take the cryotube out of the liquid nitrogen.
- Place it quickly in the berlin containing water which was previously heated to 42°C.
- In a laminar flow hood, put the content of the cryotube into a 15 ml falcon™ tube containing 9 ml of culture medium.
- Centrifuge at 20°C and 500xg for 10 minutes.
- Remove the supernatant containing the toxic DMSO.
- Resuspend the pellet containing the cells with 13 ml of culture medium.
- Transfer to a 75 cm^2 flask and place the flask in an incubator at 37°C, 5% CO_2.

1.3. Cryogenization of cells

Material:

- Sterile PBS *[Gibco™ 18912014]*
- Trypsin-EDTA (4°C) (10x trypsin, diluted 10x in sterile PBS)
- Sterile cell culture medium (4°C)
- Sterile serum + DMSO (10%) solution
- Cryotube
- 1 falcon™ (15 ml)
- Cell culture flask (75 cm^2) at 80% confluency

Operating mode:

- Preheat PBS, trypsin, and culture medium to 37°C.
- In a laminar flow hood, remove the culture medium from the flask.
- Rinse with 8 ml of sterile PBS.
- Add 3 ml of trypsin and let the cells detach for 10 minutes.
- Add 7 ml of culture medium and put the cell suspension in a 15 ml falcon™ tube.
- Centrifuge for 10 minutes at room temperature and 500xg.
- Discard the supernatant.
- Resuspend the cell pellet with 1 ml of serum + DMSO (10%).
- Transfer 1 ml to a cryotube.
- Place the cryotubes in isopropanol (room temperature) for 24 hours.
- Place the cryotubes in liquid nitrogen for storage.

1.4. Change of culture medium

Material:

- Sterile PBS (4°C)
- Sterile cell culture medium (4°C)

Operating mode:

- Preheat the PBS and the culture medium to 37°C.
- Remove the culture medium of the cell containing recipient.
- Wash with 7-8 ml of PBS.
- Add 13 ml of fresh culture medium.

1.5. Cell passage

Material:

- Sterile PBS
- Sterile Trypsin-EDTA
- Sterile cell culture medium
- 75 cm^2 flask containing confluent cells

Operating mode:

- Preheat the PBS, trypsin, and cell culture medium to 37°C.
- Remove the culture medium of the flask.
- Wash 1-2 times with 6-7 ml of PBS.
- Add 3 ml of trypsin and let the cells detach for 10-15 minutes in the incubator.
- Add 7 ml of cell culture medium to neutralize the trypsin.
- Homogenize gently by up & down.
- Transfer the desired volume cell suspension to a new annotated 75 cm² flask.
- Adjust to a volume of 13 ml with cell culture medium.

2. Treatments of cells

The following treatments can be performed independently or simultaneously.

2.1. Doxycycline treatment for p53 expression induction

Material:

- Sterile PBS (4°C)
- Sterile cell culture medium containing 2 µg/ml of doxycycline *[Sigma-Aldrich™ D9891*] (added the day of the manipulation)
- Recipient containing cells

Operating mode:

- Preheat the PBS and culture medium to 37°C.
- In a laminar flow hood, remove the culture medium of the cell containing recipient.
- Wash with 7-8 ml of PBS.
- Put 13 ml (for a 75 cm² flask, volume may vary depending on the recipient) of doxycycline supplemented cell culture medium in the recipient.
- Place the flask in the incubator (37°C, 5% CO2) for the desired incubation time.
- Perform the desired experience.

2.2. CPT treatment for stress induction

Material:

- Sterile PBS (4°C)
- Sterile cell culture medium containing 5 µM of camptothecin *[Sigma-Aldrich™ C9911]* (added the day of the manipulation)
- Recipient containing cells

Operating mode:

- Preheat the PBS and culture medium to 37°C.
- In a laminar flow hood, remove the culture medium of the cell containing recipient.
- Wash with 7-8 ml of PBS.
- Put 13 ml (for a 75 cm² flask, volume may vary depending on the recipient) of camptothecin supplemented cell culture medium in the recipient.
- Place the flask in the incubator (37°C, 5% CO2) for the desired incubation time.
- Perform the desired experience.

3. Cell viability tests

3.1. Colony formation assay

3.1.1. Initiation of the assay

Material:

- Sterile PBS
- Sterile trypsin-EDTA
- Sterile cell culture medium
- 75 cm² flask at ~50% confluency
- Cell counting device *[LUNA-II™ Automated Cell Counter BioCat L40001-LG]*
- Eppendorf™ tubes (1.5 ml)
- 6-well cell culture plates

Operating mode:

- Preheat PBS, trypsin, and cell culture medium to 37°C.
- In a laminar flow hood, remove the old cell culture medium.
- Wash with 7-8 ml of PBS.
- Add 3 ml of trypsin and let the cells detach for 10-15 minutes in the incubator.

- Add 7 ml of cell culture medium to neutralize the trypsin.
- Homogenize gently and thoroughly before putting one drop of cell suspension into and Eppendorf™ tube.
- Homogenize again before counting the cells on a cell counting device.
- Put the necessary volume of cell suspension in each well to get the desired number of cells and perform a treatment if necessary. The final volume of a well should be ~2 ml.
- Readjust the 75 cm² flask volume to 13 ml or perform a cell passage depending on your needs.

Note: Cell culture medium (with or without treatment) needs to be renewed every 2-3 days.

3.1.2. Fixation of cells

Note: This can be done in a non-sterile environment. All solutions should be introduced very carefully into the wells so as not to destroy the cells.

Material:

- Non-sterile PBS
- Ethanol (70%)
- Water (milliQ)
- Crystal violet solution (hexamethyl pararosaniline chloride, 1x)
- 6-well cell culture plates

Operating mode:

- After the desired incubation time, remove the cell culture medium and wash twice with PBS.
- Add 70% ethanol and let the cells fixate for 15 minutes.
- Rinse the cells twice with milliQ water.
- Add crystal violet solution and let it stain for 15-20 minutes.
- Rinse thoroughly with milliQ water.
- Let dry.

3.2. MTT assay

3.2.1. Initiation

Material:

- Sterile PBS
- Sterile trypsin-EDTA
- Sterile cell culture medium
- 75 cm² flask at ~50% confluency
- Cell counting device
- Eppendorf™ tubes (1.5 ml)
- 96-well cell culture plates

Operating mode:

- Preheat PBS, trypsin, and cell culture medium to 37°C.
- In a laminar flow hood, remove the old cell culture medium.
- Wash with 7-8 ml of PBS.
- Add 3 ml of trypsin and let the cells detach for 10-15 minutes in the incubator.
- Add 7 ml of cell culture medium to neutralize the trypsin.
- Homogenize gently and thoroughly before putting one drop of cell suspension into and Eppendorf™ tube.
- Homogenize again before counting the cells on a cell counting device.
- Put the necessary volume of cell suspension in each well to get the desired number of cells. The final volume of a well should be 200 µl.
- Readjust the 75 cm² flask volume to 13 ml or perform a cell passage depending on your needs.
- After two days of cell growth, perform the treatments of your choice.

3.2.2. Assessing cell viability

Note: This can be done in a non-sterile environment.

Material:

- MTT *[Invitrogen™ M6494]*
- DMSO
- UV-vis photospectrometer
- 96-well plates

Operating mode:

- After the desired treatment time, remove the cell culture medium and put 200 µl of cell culture medium containing MTT (0.5 mg/ml) in each well.
- Let incubate for 3 hours.
- Centrifuge for 15 minutes at 1000xg and 20°C.
- Remove the medium and redissolve the precipitate with 200µl DMSO per well.
- Incubate for 40 minutes on a benchtop shaker.
- Measure by using a UV-vis photospectrometer the absorbances at 655 nm (background noise) and 570 nm (MTT signal).
- Calculate relative cell viability.

4. Protein extractions and protein assay

4.1. Total protein extraction

Note: This manipulation can be done in a non-sterile environment

Material:

- Non-sterile PBS (4°C) (to be put on ice for this manipulation)
- 1 rake per condition
- RIPA (radioimmunoprecipitation assay) buffer:
    - 50 mM Tris-HCl (pH 7.4) *[MP Biomedicals™ 819623]*
    - 150 mM NaCl *[Acros Organics™ 207790010]*
    - 0.1% SDS *[Sigma-Aldrich™ L-5750]*
    - 1% Sodium deoxycholate *[Sigma-Aldrich™ 30970]*
    - 1% Triton X-100 *[Acros Organics™ 215682500]*
- Lysis buffer (prepared on the day of the manipulation):
    - 10 ml RIPA buffer
    - 10 µl Aprotinin *[Sigma-Aldrich™ A-6279]*
    - 100 µl PMSF (1 M) *[Sigma-Aldrich™ 78830]*
    - 100 µl Leupeptin (10 mM) *[Sigma-Aldrich™ L2884]*
- 75 cm^2 flask at 90% confluency
- Falcon™ tubes (15 or 50 ml, depending on the volume)
- Eppendorf™ tubes (1.5 ml)

Operating mode:

- Scrape the cells off the flask using one sterile rake per condition.
- Collect the cell suspension in a falcon™ tube.
- Centrifuge for 10 minutes at 500xg and 4°C.
- Discard the supernatant and resuspend the cell pellet in 10 ml of ice-cold PBS.
- Centrifuge for 10 minutes at 500xg and 4°C.

Note: You can pause the manipulation after this step by discarding the supernatant and storing the cell pellet at -20°C. Otherwise, perform as noted below.

- Discard the supernatant and resuspend the cell pellet in 200 µl of lysis buffer.
- Transfer the solution into an Eppendorf™ tube (1.5 ml).
- Incubate on ice for 15 minutes.
- Sonicate for 30 seconds.
- Centrifuge for 15 minutes at 15,000xg and 4°C.
- Collect the supernatant in an Eppendorf™ tube.
- Store the obtained total lysates at -20°C.

4.2. Mitochondrial protein extraction

This manipulation is based on differential centrifugation. Following this protocol, mitochondrial as well as cytosolic protein fraction can be isolated. The purity of these fractions can be assessed by western blots using fraction specific antibodies. Note that the mitochondrial fraction contains proteins of organelles which have a similar density, like lysosomes and peroxisomes.

Material:

- Non-sterile PBS (4°C) *[Gibco™ 18912014]*
- 1 syringe (5 ml) per condition
- 1 needle per condition
- Trypan blue
- Counting blade and coverslip
- 1 rake per condition
- 1 falcon™ tube (50 ml) per condition
- 3 Eppendorf™ tubes (2 ml) per condition
- 5 Eppendorf™ tubes (1.5 ml) per condition

- Buffer 1:
    - 10 mM Hepes-NaOH (pH 7.4) *[Sigma-Aldrich™ H3375]*
    - 1 mM EDTA (pH 7.4) *[Sigma-Aldrich™ E5134]*
    - 0.2 mM EGTA (pH 7.4) *[Sigma-Aldrich™ E4378]*
    - 300 mM D-Mannitol *[Sigma-Aldrich™ 63559]*

Note: Add the protease inhibitor (10 µl of Aprotinin per 10 ml of buffer) on the day of the manipulation.

- Buffer 2:
    - 10 mM Hepes-KOH (pH 7.4) *[Sigma-Aldrich™ H3375]*
    - 1 mM KH_2PO_4-KOH (pH 7.4) *[Sigma-Aldrich™ P5655]*
    - 0.2 mM EGTA (pH 7.4) *[Sigma-Aldrich™ E4378]*
    - 300 mM D-Mannitol *[Sigma-Aldrich™ 63559]*

Note: KOH is used to bring the buffer to the correct pH to ensure the viability of mitochondria.

⇨ **All operations are carried out at 4°C (on ice).**

Operating mode:

- Scrape the cells off the flask using one sterile rake per condition.
- Collect the cell suspension in a falcon™ tube (50 ml).
- Centrifuge for 10 minutes at 500xg and 4°C.
- 2 washes with ice-cold PBS:
    - Discard the supernatant and resuspend the cell pellet in 10 ml of ice-cold PBS.
    - Centrifuge for 10 minutes at 500xg and 4°C.
    - Discard the supernatant and resuspend the cell pellet in 10 ml of ice-cold PBS.
    - Centrifuge for 10 minutes at 500xg and 4°C.
- Discard the supernatant and suspend the cell pellet in 1 ml of buffer 1. Transfer to an Eppendorf™ tube (2 ml).
- Perform 40 up & down using a syringe and needle to mechanically lyze the cells.
- Check if enough cells are lyzed (≥ 50%) by examination under microscope of 20 µl of cell suspension + 20 µl of blue trypan.
- Place the homogenized cells in Eppendorf™ tubes (2 ml) and store on ice.
- Centrifuge for 10 minutes at 500xg and 4°C.

- Transfer the supernatant into a new Eppendorf™ tube (2 ml) by using a pipette (the pellet can be discarded at the end of the manipulation as it consists of intact cells and nuclear debris).
- Centrifuge for 5 minutes at 500xg and 4°C.
- Transfer the supernatant into a new Eppendorf™ tube (1.5 ml) by using a pipette (the pellet can be discarded at the end of the manipulation as it consists of intact cells and nuclear debris).
- Centrifuge for 10 minutes at 500xg and 4°C.
- Transfer the supernatant into a new Eppendorf™ tube (1.5 ml) by using a pipette (the pellet can be discarded at the end of the manipulation as it consists of intact cells and nuclear debris).
- Centrifuge for 10 minutes at 9000xg and 4°C.
- Collect the supernatant (cytosolic fraction) in a new Eppendorf™ tube (1.5 ml).
- The pellet (mitochondrial fraction) are kept on ice, remove all traces of supernatant.
- Resuspend the pellet in 600 µl of buffer 1.
- Centrifuge for 6 minutes at 500xg and 4°C.
- Collect the supernatant (mitochondrial fraction) in a labeled Eppendorf™ tube (1.5 ml).
- Centrifuge cytosolic as well as and mitochondrial fractions for 10 minutes at 9000xg and 4°C.

Cytosolic fraction:

- Collect the supernatant (avoid the pellet) in a new labeled Eppendorf™ tube (1.5 ml).
- Store at -20°C.

Mitochondrial fraction:

- Remove as much supernatant as possible using a pipette.
- Resuspend the pellet (mitochondrial fraction) in 100 µl of buffer 2.
- Store at -20°C.

4.3. Protein assay

Material:

- Bio-Rad DC Protein Assay Kit: Reagents A, B and S *[Bio-Rad™ 500-0114, 500-0113 and 500-0115]*
- Reaction solution: 20 µl of reagent S per 1 ml of reagent A (25 µl of mix / well)
- 96-well plate(s)
- BSA diluted in RIPA buffer (2 mg/ml, -20 ° C) *[Promega™ R396D]*
- UV-vis spectrophotometer

Operating mode:

- Using a 96-well plate, put 2 µl of each sample in triplicates.
- Triplicate a BSA calibration line: 0, 1, 2, 5 and 10 µl.
- Add 25 µl of reaction solution to each well.
- Add 200 µl of reagent B to each well.
- Incubate at room temperature for 20 minutes.
- Measure the protein absorbance at 655 nm.
- Calculate the protein concentration of each sample using the calibration line.

5. Western blot

5.1. SDS-PAGE

Material:

- Plastic and glass equipment for SDS-PAGE
- Protein samples
- RIPA buffer
- Syringe needle
- Heating block
- 0.5 M Tris-HCI buffer pH 6.8 *[MP Biomedicals™ 819623]*
- 1.5 M Tris-HCl buffer pH 8.8 *[MP Biomedicals™ 819623]*
- 10% SDS solution in milliQ water *[Sigma-Aldrich™ L-5750]*
- 5x electrophoresis buffer. For 500 ml:
    - 250 mM Tris (15,14 g) *[MP Biomedicals™ 819623]*
    - 750 mM Glycine (28.15 g) *[MP Biomedicals™ 808822]*
    - 0.5% SDS (2.5 g) *[Sigma-Aldrich™ L-5750]*

    - Adjust the volume to 500 ml using milliQ water.
- Laemmli 5x Denaturation Buffer. For 5 ml:
    - 50% Ultra-pure glycerol (2.5 ml)
    - 112 mM 0.5M Tris-HCl pH 6.8 (2.25 ml) *[MP Biomedicals™ 819623]*
    - 5% SDS (250 mg) *[Sigma-Aldrich™ L-5750]*
    - 250 mM DL-1,4-Dithiothreitol (DTT, 192.8 mg) *[Fluka Analytical™ 43819]*
    - Less than 0.125% of Bromophenol Blue (spatula tip) *[Sigma-Aldrich™ B8026]*
    - Adjust with 125 µl of milliQ water to a final volume of 5 ml.
    - Store at -20 ° C.
- 30% Acrylamide/Bis Solution 29:1 (CAUTION: Neurotoxic product. Use gloves and handle with precaution) *[Bio-Rad™ 1610156]*
- Water (milliQ)
- 10% Ammonium persulfate (APS) *[MP Biomedicals™ 802811]*
- TEMED (Tetramethylethylenediamine) *[Acros Organics™ 138450500]*
- Isopropanol *[Janssen Chimica™ 18.413.80]*
- Eppendorf™ tubes (1.5 ml)

5.1.1. Sample preparation

Operating mode:

- Take the desired amount of sample (to have 10 µg for example) and adjust the volume to 20 µl with milliQ water or RIPA buffer (if necessary) in an Eppendorf™ tube.
- Add 5 µl of 5x Laemmli buffer and pierce the cap with a syringe needle.
- If necessary, perform a quick spin to collect the sample to the bottom of the tube.
- Place the samples for 10 minutes on a heating block preheated to 95°C.
- Perform a quick spin to collect the sample to the bottom of the tube.

5.1.2. Pouring the gels

- Separation gel (for 2 gels):
    - 2.5 ml of 1.5 M Tris-HCl buffer pH 8.8 *[MP Biomedicals™ 819623]*
    - 6.6 ml of 30% Acrylamide/Bis Solution 29:1 *[Bio-Rad™ 1610156]*
    - 200 µl of 10% SDS *[Sigma-Aldrich™ L-5750]*
    - 8 ml of milliQ water
    - 150 µl of 10% APS *[MP Biomedicals™ 802811]*
    - 15 µl of TEMED *[Acros Organics™ 138450500]*

- Concentration gel (for 2 gels):
    - 2.5 ml of 0.5 M Tris-HCl buffer pH 6.8 *[MP Biomedicals™ 819623]*
    - 1.6 ml of 30% Acrylamide/Bis Solution 29:1 *[Bio-Rad™ 1610156]*
    - 100 µl of 10% SDS *[Sigma-Aldrich™ L-5750]*
    - 5.7 ml of milliQ water
    - 75 µl of 10% APS *[MP Biomedicals™ 802811]*
    - 7.5 µl of TEMED *[Acros Organics™ 138450500]*

Operating mode:

- Prepare the separation gel (add APS and TEMED last, these two components trigger polymerization).
- Pour the separation gel into the intended equipment by leaving 2.5 cm of empty space before the superior limit of the glass plate. This is for pouring the concentration gel.
- While the gel is still liquid, pour a thin layer of isopropanol on its surface to remove any bubbles and create a straight surface.
- After polymerization, remove the isopropanol using for example blotting paper.
- Prepare the concentration gel.
- Pour the gel on top of the separation gel and place the comb in it.
- Leave to polymerize.

5.1.3. Electrophoresis

Operating mode:

- Place the gels carefully in the intended tank.
- Dilute the electrophoresis buffer 5x with milliQ water (200 ml buffer + 800 ml water)
- Fill the tank with buffer and remove any foam, especially in the inner chamber.
- Remove the combs and refill with some buffer if necessary.
- Load 5 µl of weight marker (PageRuler™ Prestained Protein Ladder) in the first well.
- Load the samples into the wells (25 µl per well).
- Migrate the samples at 160 V for approximately 1 hour.

5.2. Wet electroblotting transfer

Material:

- Transfer equipment (tanks, sandwich cassettes, etc.)
- Nitrocellulose membranes
- PBS-Tween 20 (0.2%) *[Sigma-Aldrich™ P1379]*
- Blotting paper
- Transfer buffer (for 5 liters):
    - 15 g of Tris *[MP Biomedicals™ 819623]*
    - 72 g of Glycine *[MP Biomedicals™ 808822]*
    - 5 ml of SDS 10% *[Sigma-Aldrich™ L-5750]*
    - 1 liter of methanol
    - 4 liters of milliQ water

Operating mode:

For 1 gel:

- Cut out 4 blotting papers (7.5 * 8.5 cm).
- Cut out 1 transfer membrane (7.5 * 8.5 cm).
- Setup in the sandwich cassette, starting on the black side:
    - Sponge soaked in transfer buffer
    - 2 blotting papers soaked in transfer buffer
    - SDS-PAGE gel (concentration gel was previously cut off) → Needs to stay wet!
    - Transfer membrane soaked in transfer buffer
    - 2 blotting papers soaked in transfer buffer
    - Sponge soaked in transfer buffer
- Close the cassette and place it in a tank filled with transfer buffer.
- Place an “ice cube” block in the tank to cool the system down.
- Transfer for 1h30 at 300 mA.
- Collect the membrane, roll it up (gently, membrane must stay wet at all times!) and place it in a falcon™ tube (50 ml) filled with PBS-Tween 20 (0.2%).
- Store at 4°C for approximately 1-2 weeks.

5.3. Immunodetection

Material:

- PBS-Tween20 (0.2%) *[Sigma-Aldrich™ P1379]*
- Milk powder *[Régilait™ skim milk powder]*
- BSA *[MP Biomedicals™ 160069]*
- Cling film
- Plastic brochure sleeve
- Autoradiography cassette
- Chemiluminescent substrate *[Perkin Elmer™ NEL104001EA]*
- Developer solution *[Ilford™ Multigrade Developer 1757855]*
- Fixer solution *[Agfa HealthCare™ G354]*
- Water bath (milliQ)
- Primary antibodies:
    - Anti-p53: mouse monoclonal IgG *[Santa Cruz Biotechnology™ sc-126]* Dilution: 1/1000
    - Anti-$p21^{WAF1}$: mouse monoclonal IgG *[Santa Cruz Biotechnology™ sc-6246]* Dilution: 1/1000-1/100
    - Anti-β actin: mouse monoclonal IgG (Dilution 1/3000-1/2000)
    - Anti-GRP75: mouse monoclonal IgG *[Santa Cruz Biotechnology™ sc-133137]* Dilution: 1/1000
    - Anti-PCNA: mouse monoclonal IgG *[Santa Cruz Biotechnology™ sc-56]* Dilution: 1/1000
    - Anti-cPARP: rabbit monoclonal IgG *[Cell Signaling Technology™ 5625]* Dilution: 1/1000 → use PBS-Tween20 (0.2%) BSA (3%) instead of PBS-Tween20 (0.2%) milk (5%).
- Secondary antibodies:
    - Goat anti-mouse-IgG coupled to horseradish peroxidase *[Agilent™ P0447]* Dilution: 1/3000-1/2000
    - Goat anti-rabbit-IgG coupled to horseradish peroxidase *[Agilent™ P0160]* Dilution: 1/2000

Note: All incubation and washing steps are to be done under agitation. The membranes should always be wet.

Operating mode:

- Incubate the nitrocellulose membranes with 10 ml of PBS-Tween 20 (0.2%) milk (5%) for 30 minutes. Throw away.
- Incubate the membranes for 1 hour at room temperature with the primary antibody diluted in 10 ml of PBS-Tween 20 (0.2%) milk (5%).
- Perform 3 washes of 15-20 minutes using 10 ml of PBS-Tween 20 (0.2%).
- Incubate the membranes for 1 hour at room temperature with the secondary antibody diluted in 10 ml of PBS-Tween 20 (0.2%) milk (5%).
- Perform 3 washes of 15-20 minutes.
- Prepare the chemiluminescence reagent: Mix equal volumes (4 ml) of both reagents.
- Attach some cling film to the bench and add 1 big drop of 4 ml of the chemiluminescence reagent per membrane.
- Place the nitrocellulose membrane, proteins facing downwards, on the reagent. Incubate for 1 minute.
- Collect the membrane and gently remove the excess reagent. Place the membrane in a plastic brochure sleeve.
- Place the plastic sleeve inside the autoradiography cassette.
- In the dark room:
    - Put photographic film inside the cassette, on the membranes emitting chemiluminescence. Incubate the desired time.
    - Recover the film and place in the developer solution until bands start to appear.
    - Rinse thoroughly in a water bath.
    - Place the film in the fixer solution.
    - Rinse thoroughly with water and let dry.

6. Immunofluorescence

6.1. Cell seeding & treatment

Material:

- 24-well cell culturing plate
- Sterile PBS
- Sterile cell culture medium containing 2 µg/ml of doxycycline, camptothecin, or both (added the day of the manipulation)
- Round microscope cover slips
- Cell flasks at 90-100%

Operating mode:

- Remove cell medium from the flasks, wash with PBS, and incubate with 3 ml of trypsin for 10-15 minutes.
- Meanwhile, put a sterile glass cover slide into each well and fill the wells with untreated or treated medium.
- Add the volume of cells necessary to have the desired confluence in the wells. The volume of a well is 1 ml.
- Put the cells in the incubator for the desired treatment time.

6.2. Fixation & antibody incubation

Material:

- Non-sterile PBS (4°C)
- PBS-Formaldehyde 4% (-20°C) *[Sigma-Aldrich™ F8775]*
- PBS-Triton X-100 0.2% (4°C) *[Acros Organics™ 10591461]*
- PBS-Tween20 0.05% BSA 3% (4°C) *[Sigma-Aldrich™ P1379, MP Biomedicals™ 160069]*
- PBS-Tween20 0.05% (4°C) *[Sigma-Aldrich™ P1379]*
- Primary antibody: Anti-p53: mouse monoclonal IgG *[Santa Cruz Biotechnology™ sc-126]* Dilution: 1/200
- Secondary antibody: Anti-mouse: goat IgG Alexa 546 *[Thermo Fisher Scientific™ A-11003]* Dilution: 1/100
- VECTASHIELD Antifade Mounting Medium with DAPI *[Vector Laboratories™ H-1200-10]*
- Microscope slides
-

Operating mode:

- Remove cell medium from each well and rinse with PBS.
- Fix the cells for 20 minutes with PBS-Formaldehyde 4% at room temperature.
- Perform three washes with PBS.
- Permeabilize by incubating with PBS-Triton X-100 0.2% for 15 minutes at room temperature and perform three washes with PBS.
- Incubate in the dark with PBS-Tween20 0.05% BSA 3% for 10 minutes at 37°C.
- Incubate over-night with PBS-Tween20 0.05% BSA 3% supplemented with primary antibody at 4°C.
- Perform three washes with PBS-Tween20 0.05%.
- Incubate in the dark with PBS-Tween20 0.05% BSA 3% supplemented with secondary antibody for one hour at room temperature.
- After two washes with PBS-Tween20 0.05%, the cover slips are mounted on the microscope slides using 5 µl of VECTASHIELD Mounting Medium with DAPI. They can then be observed using the fluorescence microscope.

7. Statistical analysis

All the experimental conditions were performed six times, considered independent. Results were expressed as mean and standard deviation (± SD). Cell viability (%) was compared between NO DOX / DOX subgroups using one-way analysis of variance (ANOVA). Multiple linear regression model was built in order to analyze the impact of CPT concentration, cell line (C2, C5, C7), and presence of DOX on cell viability. Results were presented using estimated coefficients and their standard error (± SE) and p-values. Results were considered statistically significant at 95% level ($p<0,05$). Statistical analysis was conducted using R (version 4.2.2) software.

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Supplementary Data

Table S1: *Comparison between DOX and NO DOX in each subgroup. Each experimental condition was performed in sextuplicates, considered independent. Results are portrayed as mean ± SD (Standard Deviation). One-way ANOVA analysis was carried out using R (version 4.2.2.) software. No statistically significant difference between DOX and NO DOX was shown. Results were considered statistically significant at 95% level (p<0,05).*

		NO DOX	DOX	Comparison
Cell line	CPT (µM)	Mean ± SD	Mean ± SD	p-value ANOVA
C2	0	100 ± 0,0	99,6 ± 16,4	0,95
	2,5	22,6 ± 6,2	20,5 ± 3,7	0,50
	5	17,8 ± 3,0	18,5 ± 5,2	0,77
	10	13,0 ± 3,0	15,3 ± 3,6	0,26
C5	0	100 ± 0,0	97,5 ± 4,2	0,18
	2,5	27,9 ± 4,9	29,6 ± 4,8	0,57
	5	27,1 ± 3,9	24,7 ± 2,7	0,23
	10	21,7 ± 2,9	20,4 ± 2,7	0,44
C7	0	100 ± 0,0	97,4 ± 14,8	0,68
	2,5	22,5 ± 3,9	18,5 ± 5,9	0,19
	5	17,0 ± 3,7	17,8 ± 3,7	0,72
	10	12,1 ± 0,87	12,3 ± 2,1	0,80
All	0	100 ± 0,0	98,2 ± 12,2	0,68
	2,5	24,3 ± 5,4	22,9 ± 6,8	0,48
	5	20,6 ± 5,8	20,3 ± 4,9	0,86
	10	15,6 ± 5,0	16,0 ± 4,3	0,79

Table S2: *Linear regression to predict cell viability (N=144). Multiple linear regression model was built in order to analyze the impact of CPT concentration, cell line (C2, C5, C7), and presence of DOX on cell viability. Results were presented using estimated coefficients and their standard error (± SE) and p-values. Results were considered statistically significant at 95% level (p<0,05).*

	Coef. ± SE	p-value
Intercept	68,9 ± 4,7	-
CPT (µM)	-6,9 ± 0,55	<0,001
DOX (1=yes)	-0,80 ± 4,0	0,84
Cell line (ref=C2)		
C5 vs C2	5,2 ± 5,0	0,30
C7 vs C2	-1,2 ± 5,0	0,81
-> Cell viability decreases when CPT increases (p<0,001)		
-> No effect of DOX on Cell viability (p=0,84)		
-> No effect of cell line on cell viability		

Table S3: *Effect of CPT concentration on cell viability. Multiple linear regression model was built in order to analyze the impact of CPT concentration with different concentrations taken as reference value. Results were presented using estimated coefficients and their standard error (± SE) and p-values. Results were considered statistically significant at 95% level (p<0,05).*

	Coef. ± SE	p-value
Intercept	99,1 ± 1,0	-
CPT (Ref=0 µM)		
2,5 µM vs 0 µM	-75,5 ± 1,5	**<0,001**
5 µM vs 0 µM	-78,6 ± 1,5	**<0,001**
10 µM vs 0 µM	-83,3 ± 1,5	**<0,001**
	Coef. ± SE	p-value
Intercept	23,6 ± 1,0	-
CPT (Ref=2,5 µM)		
0 µM vs 2,5 µM	75,5 ± 1,5	**<0,001**
5 µM vs 2,5 µM	-3,1 ± 1,5	**0,037**
10 µM vs 2,5 µM	-7,8 ± 1,5	**<0,001**
	Coef. ± SE	p-value
Intercept	23,6 ± 1,0	-
CPT (Ref=5 µM)		
0 µM vs 5 µM	75,5 ± 1,5	**<0,001**
2,5 µM vs 5 µM	-3,1 ± 1,5	**0,037**
10 µM vs 5 µM	-7,8 ± 1,5	**<0,001**

Supplementary data: *Script used in R software (version 4.2.2.)*

```
library(plyr)

library(ggplot2)

library(readxl)


# Lecture des donnees ----

MTT_assay_DataStat <- read_excel("C:/Users/Desktop/MTT
assay_DataStat.xlsx")

View(MTT_assay_DataStat)

MTT_assay_DataStat$Echantillon<-factor(MTT_assay_DataStat$Echantillon)

MTT_assay_DataStat$Dox<-factor(MTT_assay_DataStat$Dox)

MTT_assay_DataStat$CPT_c<-factor(MTT_assay_DataStat$CPT)
```

```
# Calcul des distributions par echantillon/Dox/CPT ----
Groups<-ddply(MTT_assay_DataStat,c("Echantillon","Dox","CPT_c"),summarise,
      N=sum(!is.na(ViabiliteCellulaire)),
      mean = mean(ViabiliteCellulaire),
      sd   = sd(ViabiliteCellulaire),
      se   = sd / sqrt(N),
      med = quantile(ViabiliteCellulaire,prob=0.50),
      p25 = quantile(ViabiliteCellulaire,prob=0.25),
      p75 = quantile(ViabiliteCellulaire,prob=0.75),
      min= min(ViabiliteCellulaire),
      max=max(ViabiliteCellulaire))
Groups

# Calcul des distributions par Dox/CPT (tous les echantillons ensemble) ---
-
Groups_All<-ddply(MTT_assay_DataStat,c("Dox","CPT_c"),summarise,
              N=sum(!is.na(ViabiliteCellulaire)),
              mean = mean(ViabiliteCellulaire),
              sd   = sd(ViabiliteCellulaire),
              se   = sd / sqrt(N),
              med = quantile(ViabiliteCellulaire,prob=0.50),
              p25 = quantile(ViabiliteCellulaire,prob=0.25),
              p75 = quantile(ViabiliteCellulaire,prob=0.75),
              min= min(ViabiliteCellulaire),
              max=max(ViabiliteCellulaire))
Groups_All

# Representations graphiques par Echantillon ----
Groups$upper = Groups$mean + Groups$se
Groups$lower = Groups$mean - Groups$se

C2<-subset(Groups,Echantillon=="C2")
p<-ggplot(data=C2,aes(x=CPT_c,y=mean,fill=Dox))+
  geom_bar(stat="identity",width=0.5, position=position_dodge(width=0.6)) +
  scale_fill_brewer(palette="Blues",name="",labels=c("NO DOX","DOX")) +
  geom_errorbar(aes(ymin=C2$lower, ymax=C2$upper),
width=0.2,size=0.4,position=position_dodge(width=0.6)) +
```

```
  labs(x="CPT (??M)",y="Cell viability (%)")+
  scale_y_continuous(limits=c(0,120),breaks=seq(0,120,20))+
  ggtitle("C2") +
  theme_classic(base_size = 14)+theme(legend.position="bottom")
p
ggsave("C:/Users/Desktop/Fig_C2.jpg",
       width = 14, height = 10, units = "cm")


C5<-subset(Groups,Echantillon=="C5")
p2<-ggplot(data=C5,aes(x=CPT_c,y=mean,fill=Dox))+
  geom_bar(stat="identity",width=0.5, position=position_dodge(width=0.6)) +
  scale_fill_brewer(palette="Reds",name="",labels=c("NO DOX","DOX")) +
  geom_errorbar(aes(ymin=lower, ymax=upper),
width=0.2,size=0.4,position=position_dodge(width=0.6)) +
  labs(x="CPT (??M)",y="Cell viability (%)")+
  scale_y_continuous(limits=c(0,120),breaks=seq(0,120,20))+
  ggtitle("C5") +
  theme_classic(base_size = 14)+theme(legend.position="bottom")
p2
ggsave("C:/Users/Desktop/Fig_C5.jpg",
       width = 14, height = 10, units = "cm")


C7<-subset(Groups,Echantillon=="C7")
p2<-ggplot(data=C7,aes(x=CPT_c,y=mean,fill=Dox))+
  geom_bar(stat="identity",width=0.5, position=position_dodge(width=0.6)) +
  scale_fill_brewer(palette="Greens",name="",labels=c("NO DOX","DOX")) +
  geom_errorbar(aes(ymin=lower, ymax=upper),
width=0.2,size=0.4,position=position_dodge(width=0.6)) +
  labs(x="CPT (??M)",y="Cell viability (%)")+
  scale_y_continuous(limits=c(0,120),breaks=seq(0,120,20))+
  ggtitle("C7") +
  theme_classic(base_size = 14)+theme(legend.position="bottom")
p2
ggsave("C:/Users/Desktop/Fig_C7.jpg",
       width = 14, height = 10, units = "cm")


# Comparaisons des tous les sousgroupes - ANOVA ----
```

```
MTT_assay_DataC2_0<-subset(MTT_assay_DataStat,Echantillon=="C2"&CPT=="0")

fit <- aov(MTT_assay_DataC2_0$ViabiliteCellulaire ~ MTT_assay_DataC2_0$Dox)

summary(fit)


MTT_assay_DataC2_25<-
subset(MTT_assay_DataStat,Echantillon=="C2"&CPT=="2.5")

fit <- aov(MTT_assay_DataC2_25$ViabiliteCellulaire ~
MTT_assay_DataC2_25$Dox)

summary(fit)


MTT_assay_DataC2_5<-subset(MTT_assay_DataStat,Echantillon=="C2"&CPT=="5")

fit <- aov(MTT_assay_DataC2_5$ViabiliteCellulaire ~ MTT_assay_DataC2_5$Dox)

summary(fit)


MTT_assay_DataC2_10<-subset(MTT_assay_DataStat,Echantillon=="C2"&CPT=="10")

fit <- aov(MTT_assay_DataC2_10$ViabiliteCellulaire ~
MTT_assay_DataC2_10$Dox)

summary(fit)


MTT_assay_DataC5_0<-subset(MTT_assay_DataStat,Echantillon=="C5"&CPT=="0")

fit <- aov(MTT_assay_DataC5_0$ViabiliteCellulaire ~ MTT_assay_DataC5_0$Dox)

summary(fit)


MTT_assay_DataC5_25<-
subset(MTT_assay_DataStat,Echantillon=="C5"&CPT=="2.5")

fit <- aov(MTT_assay_DataC5_25$ViabiliteCellulaire ~
MTT_assay_DataC5_25$Dox)

summary(fit)


MTT_assay_DataC5_5<-subset(MTT_assay_DataStat,Echantillon=="C5"&CPT=="5")

fit <- aov(MTT_assay_DataC5_5$ViabiliteCellulaire ~ MTT_assay_DataC5_5$Dox)

summary(fit)


MTT_assay_DataC5_10<-subset(MTT_assay_DataStat,Echantillon=="C5"&CPT=="10")

fit <- aov(MTT_assay_DataC5_10$ViabiliteCellulaire ~
MTT_assay_DataC5_10$Dox)

summary(fit)


MTT_assay_DataC7_0<-subset(MTT_assay_DataStat,Echantillon=="C7"&CPT=="0")
```

```
fit <- aov(MTT_assay_DataC7_0$ViabiliteCellulaire ~ MTT_assay_DataC7_0$Dox)

summary(fit)


MTT_assay_DataC7_25<-
subset(MTT_assay_DataStat,Echantillon=="C7"&CPT=="2.5")

fit <- aov(MTT_assay_DataC7_25$ViabiliteCellulaire ~
MTT_assay_DataC7_25$Dox)

summary(fit)


MTT_assay_DataC7_5<-subset(MTT_assay_DataStat,Echantillon=="C7"&CPT=="5")

fit <- aov(MTT_assay_DataC7_5$ViabiliteCellulaire ~ MTT_assay_DataC7_5$Dox)

summary(fit)


MTT_assay_DataC7_10<-subset(MTT_assay_DataStat,Echantillon=="C7"&CPT=="10")

fit <- aov(MTT_assay_DataC7_10$ViabiliteCellulaire ~
MTT_assay_DataC7_10$Dox)

summary(fit)


MTT_assay_DataAll_0<-subset(MTT_assay_DataStat,CPT=="0")

fit <- aov(MTT_assay_DataC7_0$ViabiliteCellulaire ~ MTT_assay_DataC7_0$Dox)

summary(fit)


MTT_assay_DataAll_25<-subset(MTT_assay_DataStat,CPT=="2.5")

fit <- aov(MTT_assay_DataAll_25$ViabiliteCellulaire ~
MTT_assay_DataAll_25$Dox)

summary(fit)


MTT_assay_DataAll_5<-subset(MTT_assay_DataStat,CPT=="5")

fit <- aov(MTT_assay_DataAll_5$ViabiliteCellulaire ~
MTT_assay_DataAll_5$Dox)

summary(fit)


MTT_assay_DataAll_10<-subset(MTT_assay_DataStat,CPT=="10")

fit <- aov(MTT_assay_DataAll_10$ViabiliteCellulaire ~
MTT_assay_DataAll_10$Dox)

summary(fit)


# Linear regression ----
```

```
linreg<-
lm(MTT_assay_DataStat$ViabiliteCellulaire~MTT_assay_DataStat$CPT+MTT_assay_
DataStat$Dox+MTT_assay_DataStat$Echantillon, data = MTT_assay_DataStat)

summary(linreg)

linreg<-
lm(MTT_assay_DataStat$ViabiliteCellulaire~MTT_assay_DataStat$CPT_c, data =
MTT_assay_DataStat)

summary(linreg)

MTT_assay_DataStat_c <- within(MTT_assay_DataStat, CPT_c <- relevel(CPT_c,
ref = 2.5))

linreg<-
lm(MTT_assay_DataStat_c$ViabiliteCellulaire~MTT_assay_DataStat_c$CPT_c,
data = MTT_assay_DataStat_c)

summary(linreg)

MTT_assay_DataStat_c <- within(MTT_assay_DataStat, CPT_c <- relevel(CPT_c,
ref = 5))

linreg<-
lm(MTT_assay_DataStat_c$ViabiliteCellulaire~MTT_assay_DataStat_c$CPT_c,
data = MTT_assay_DataStat_c)

summary(linreg)


# graphe lien CPT vs viabilite cellulaire ----

p<-ggplot(data=MTT_assay_DataStat,aes(x=CPT,y=ViabiliteCellulaire))+

  geom_point() +

  labs(x="CPT (??M)",y="Cell viability (%)")+

  scale_y_continuous(limits=c(0,120),breaks=seq(0,120,20))+

  scale_x_continuous(limits=c(0,10),breaks=c(0,2.5,5,10))+

  theme_classic(base_size = 14)+

  geom_bracket(xmin = c(0),xmax = c(2.5),y.position = c(110),label =
c("***"),tip.length = 0.01,color="black",size=0.2)+

  geom_bracket(xmin = c(2.5),xmax = c(5),y.position = c(50),label =
c("*"),tip.length = 0.01,color="black",size=0.2)+

  geom_bracket(xmin = c(5),xmax = c(10),y.position = c(40),label =
c("***"),tip.length = 0.01,color="black",size=0.2)+

  geom_bracket(xmin = c(2.5),xmax = c(10),y.position = c(60),label =
c("***"),tip.length = 0.01,color="black",size=0.2)

p

ggsave("C:/Users/Desktop/Fig_CPT_2.jpg",

       width = 14, height = 10, units = "cm")


# graphe lien CPT vs viabilite cellulaire : idem avec p-valeurs ----

p<-ggplot(data=MTT_assay_DataStat,aes(x=CPT,y=ViabiliteCellulaire))+
```

```
  geom_point() +
  labs(x="CPT (??M)",y="Cell viability (%)")+
  scale_y_continuous(limits=c(0,120),breaks=seq(0,120,20))+
  scale_x_continuous(limits=c(0,10),breaks=c(0,2.5,5,10))+
  theme_classic(base_size = 14)
p
ggsave("C:/Users/Desktop/Fig_CPT.jpg",
       width = 14, height = 10, units = "cm")

# Calcul des distributions par echantillon/CPT et graphes ----
Groups_cpt<-ddply(MTT_assay_DataStat,c("Echantillon","CPT_c"),summarise,
              N=sum(!is.na(ViabiliteCellulaire)),
              mean = mean(ViabiliteCellulaire),
              sd   = sd(ViabiliteCellulaire),
              se   = sd / sqrt(N),
              med = quantile(ViabiliteCellulaire,prob=0.50),
              p25 = quantile(ViabiliteCellulaire,prob=0.25),
              p75 = quantile(ViabiliteCellulaire,prob=0.75),
              min= min(ViabiliteCellulaire),
              max=max(ViabiliteCellulaire))
Groups_cpt
Groups_cpt$upper = Groups_cpt$mean + Groups_cpt$se
Groups_cpt$lower = Groups_cpt$mean - Groups_cpt$se
p<-ggplot(data=Groups_cpt,aes(x=CPT_c,y=mean,fill=Echantillon))+
  geom_bar(stat="identity",width=0.5, position=position_dodge(width=0.6)) +
  scale_fill_brewer(name="",labels=c("C2","C5","C7")) +
  scale_fill_manual(name="",values =
c("lightskyblue2","coral","darkseagreen2"))+
  geom_errorbar(aes(ymin=Groups_cpt$lower, ymax=Groups_cpt$upper),
width=0.2,size=0.4,position=position_dodge(width=0.6)) +
  labs(x="CPT (??M)",y="Cell viability (%)")+
  scale_y_continuous(limits=c(0,120),breaks=seq(0,120,20))+
  theme_classic(base_size = 14)+theme(legend.position="bottom")
p
ggsave("C:/Users/Desktop/Fig_Cpt_sample.jpg",
       width = 14, height = 10, units = "cm")
```

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