

Characterization of a p53 tumor suppressor mutant deficient for mitochondrial localization

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Academic year: 2020-2021

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I am forever indebted to Mr. Dumont for his unwavering and unconditional support, encouragements and patience through this process. I can never pay you back for all the help you have provided me, the experience you have helped me gain by working in your lab and the precious time you spent making sure my thesis is always on track. I hope you find some kind of satisfaction in this modest paper. Thank you so much!

I am very fortunate and grateful to Mrs. Gofflot and to Mr. Rezsóhazy for providing readership and ideas; paramount to the realization of this thesis. Thank you for your support and helpful suggestions, I will be forever thankful to you.

I was very lucky to have such amazing PhD students around me with a big heart, always attending to help. Thank you, Marine, Laure, André, Maryse, Damien from the bottom of my heart.

Thank you, Jade, Carole, Johana, Handy from all my heart for being there for me and helping me whenever I lose track. I was blessed by the work with you.

Not least of all, I owe so much to my whole family for their undying support, their unwavering belief that I can achieve so much. Unfortunately, I cannot thank everyone by name because it would take a lifetime but, I just want you all to know that you count so much. Had it not been for all your prayers and benedictions; were it not for your sincere love and help, I would never have completed this thesis. So, thank you all!

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Abbreviation list

IARC: International Agency for Research on Cancer

TSGs: tumor suppressor genes

POC: proto-oncogenes

TP53: tumor protein p53

NLS: nuclear localization signal

NES: nuclear export signal

TADs: transcription activation domains

PRD: proline-rich domain

DBD: DNA-binding domain

TD: tetramerization domain

TBP: TATA box binding protein

TAF: TBP-associated factors

TFIID: Transcription factor IID

TFIIH: Transcription factor IIH

MDM2: murine double minute 2

MDMX: murine double minute X

E1B-55: Adenovirus E1B protein (55 kDa)

Bcl-2: B-cell lymphoma 2

p21^{waf1}: wild-type activating fragment-1/cyclin-dependent kinase inhibitory protein-1

CDK: Cyclin-Dependent Kinase

SV40: simian virus 40

53BP1: p53-binding protein 1

53BP2: p53-binding protein 2

ATM: protein kinase ataxia-telangiectasia mutated

ATR: Ataxia telangiectasia and Rad3-related protein

OD: oligomerization domain

XPD: Xeroderma Pigmentosum group D protein

ChIP: Chromatin immunoprecipitation

SCO2: Synthesis of Cytochrome c Oxidase 2

NF- κ B: Nuclear Factor-kappa B

Sp1: Specificity protein 1

c-jun: Jun proto-oncogene, AP-1 transcription factor subunit

PTMs: post-translational modifications

RSF1: Remodeling and spacing factor 1

PCAF: P300/CBP-associated factor

HIPK2: Homeodomain-interacting protein kinase 2

PUMA: p53 upregulated modulator of apoptosis

DDR: DNA damage response

CHK1/2: Checkpoint kinase ½

CHK2: Checkpoint kinase 2

LFS: Li-Fraumeni Syndrome

DISC: death inducing signaling complex

BID: BH3 interacting-domain death agonist

MOM: mitochondrial outer membrane

BAK: BCL2-antagonist/killer

BAX: BCL-2-associated X

BID: BH3 interacting-domain death agonist

tBID: truncated BID

Apaf-1: Apoptotic peptidase activating factor 1

TNF: tumor necrosis factor

FasL/FasR: Fas ligand/ Fas receptor

TNF- α /TNFR1: Tumor necrosis factor-alpha/Tumor necrosis factor receptor 1

Apo2L/DR4: apoptosis-inducing ligand/death receptor 4

FADD: Fas-associated protein with death domain

TRADD: Tumor necrosis factor receptor type 1 associated death domain

RIP1: Receptor-interacting serine-threonine kinase 1

MOMP: Mitochondrial outer membrane permeabilization

SMAC/Diablo: Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low p-I

HtrA2/Omi: High-temperature requirement protein A2

IAPs: inhibitors of apoptosis proteins

AIF: Apoptosis Inducing Factor

CAD: Caspase-activated DNase

ICAD: Inhibitor of Caspase-activated DNase

DFF40: DNA fragmentation factor

APEX2: engineered ascorbate peroxidase 2

MS: mass spectrometry

SILAC: Stable isotope labeling by amino acids in cell culture

HEK: Human embryonic kidney

IMS: the intermembrane space

IMM: the inner mitochondrial membrane

OMM: outer mitochondrial membrane

iTRAQ: Isobaric tag for relative and absolute quantitation

TMT: Tandem Mass Tags

BioID: proximity-dependent biotin identification

BirA: Bifunctional ligase/repressor

HRP: Horseradish peroxidase

ELISA: enzyme-linked immunosorbent assay

ER: endoplasmic reticulum

PCR: polymerase chain reaction

Tet-ON system: tetracycline (Tc)-regulated gene expression system

KLD: Kinase-Ligase-DpnI

Tc: Tetracycline

DOX: Doxycycline

TetR: Tet repressor proteins

tetO: tet operator

TRE: Tetracycline response element

RIPA buffer: Radioimmunoprecipitation assay buffer

CPT: camptothecin

PCNA: proliferating cell nuclear antigen

GRP75: chaperone glucose-regulated protein 75

ANOVA: analysis of variance

HC: heterochromatin regions

Abstract

The p53 protein is the guardian angel of our genome. It participates in several processes by maintaining integrity and correctness because of its diverse activities. The deviation from optimum triggers p53 to react and perform its functions. A mutation of *TP53*, the gene encoding for p53, is present in 50% of human cancers. Indeed, the tumor suppressor p53 is a potent cell growth inhibitor and cell death inducer. First, p53 can act as a transcription factor to regulate the expression of many genes whose products promote cell cycle arrest or apoptosis. Second, p53 has a pro-apoptotic mitochondrial activity. In stressed cells, a fraction of p53 can localize to mitochondria and directly interact with different BCL-2 family members to trigger mitochondrial outer membrane permeabilization and the subsequent release of the mitochondrial effectors of apoptosis.

Many works indicate that post-translational modifications regulate the activity of p53. Previous studies by C. Castrogiovanni show that serine 392 phosphorylation is crucial for p53 mitochondrial localization in stressed cells. By using CRISPR-Cas9, S392 was replaced by an alanine (non phosphorylable amino acid) in HCT-116 colon carcinoma cells. This substitution markedly impaired the p53 mitochondrial localization and its ability to trigger apoptosis, underlying the role of S392 phosphorylation. To explain the role of S392 phosphorylation, two hypotheses were proposed: (1) the phosphorylation of S392, induced by a stress, allows p53 to interact with a cytosolic protein that promotes its mitochondrial localization; (2) in unstressed cells, cytosolic p53 could be sequestered by one or several proteins. S392 phosphorylation, after a stress, leads to disruption of the inhibitory complex, thus allowing p53 to reach the mitochondria.

The ultimate goal here is thus to determine the cytosolic interactions of wt p53 and S392A, which can be helpful to explain the relation between S392 phosphorylation and p53 mitochondrial localization. A proximity-dependent labeling method using APEX2, a modified ascorbate peroxidase, will be employed. The APEX2-p53 fusion has been performed already. We also stably transfected the construction in the H1299 (p53^{-/-}) cell line. Further step will include the characterization of the activity of the fusion protein (p53 transcriptional activity, subcellular localization and ability to trigger apoptosis).

I. Introduction

1. Cancer

Cancer is a main cause of death in the world. In 2020, the statistics of the International Agency for Research on Cancer (IARC) displayed 10 million deaths. The deadliest cancers are lung cancer (1.80 million deaths) because of its poor prognosis, colorectal cancer (935 000 deaths), stomach cancer (830 000 deaths), liver cancer (769 000 deaths) and breast cancer (685 000 deaths). Nowadays, it is estimated that one in 5 men and one in 6 women will develop cancer during their life. Moreover, one in 8 men and one in 11 women will die from the disease. In men, the most commonly diagnosed cancer is lung cancer (14.5% of total cases). In women, the most commonly diagnosed cancer is breast cancer (24.2%). [1]

Normal cell growth is a precisely regulated process in which cell proliferation only engages when requested. However, gene alterations may alter the various pathways that regulate it. These alterations can target proto-oncogenes (POC) or tumor suppressor genes (TSGs). Besides, the mutations of other genes can contribute to the development of cancer such as genes involved in DNA repair.[2]

POCs are normal cellular genes. The products of POCs regulate cell growth and proliferation. Gain-of-function mutations can convert POCs to oncogenes, which promotes cancer. Genome alterations can also lead to a markedly increased expression of POCs, converting them into oncogenes [3]. The products of TSGs negatively regulate cell growth and/or promote cell death. p53 and Rb are two examples of tumor suppressor proteins. These two tumor suppressors are involved in many processes including DNA repair, transcriptional regulation, induction of cell cycle arrest... [4].

Tumors can be divided into two main groups: benign or malignant. Benign tumors are a mass of cells that cannot invade the neighboring tissues or metastasize (spread to other places of the body). Malignant tumors are the opposite of the first group. These tumors can spread to different areas of the body and generate new tumors. This ability to metastasize is life-threatening. Tumor cells have many properties that differentiate them from normal cells, such as: (1) self-sufficiency in growth signals, (2) anchorage-independent growth, (3) unlimited replicative potential, (4) increased resistance to cell death (5) and altered sensitivity to anti-growth signals [2].

The protein p53, as indicated before, is a major tumor suppressor. It's the guardian of the genome. It maintains genome integrity and prevents the proliferation of cells with damaged DNA. A mutation of the *TP53* gene is observed in half of human cancers. It is known to be activated in response to many things such as DNA damage, oncogene expression or ribosome dysfunction. Once activated, p53, as transcription factor, can induce the expression of an important number of genes mainly involved in cell cycle regulation and cell death promotion. [5]

2. p53 tumor suppressor

The tumor suppressor p53 is encoded by the gene *TP53*. If somebody receives one mutated copy of *TP53* from its parents, he is at a great risk to develop cancer during his life. This case is rare and known as Li-Fraumeni Syndrome (LFS). However, it does not need to be always hereditary. It could come from mutations in somatic cells. *TP53* mutations also commonly exist in many cancer types. As a transcription factor, the p53 protein binds specific sequences on DNA to induce the expression of various genes involved in cell cycle arrest, cell death, and DNA repair. For instance, once activated, p53 induces the expression of p21^{waf1} that binds to cyclin-CDK2 complexes to prevent cell cycle progression. [6]. p53 also induces the expression of pro-apoptotic proteins such as the BH3-only proteins PUMA, and NOXA, and other BCL-2 family members, [7].

2.1. *TP53* gene

The *TP53* gene is located on chromosome 17p13.1. It spans 20 Kb, with a non-coding exon 1 and a very long first intron of 10 Kb. The coding sequence contains regions within exons 2, 5, 6, 7, and 8 that are highly conserved in vertebrates. There are 15 polymorphisms in *TP53* identified in human populations, and two of them affects the coding sequence (codons 47 and 72). The p53 polymorphism at codon 72 is very common, it can encode for either an arginine (Arg) or a proline (Pro) amino acid. The Arg allele is the most common in northern populations, and the frequency of the Pro allele increases from north to south and is predominant near the equator. In our genome, we have two genes related to *TP53* and identified on chromosome 1p36 and 3p28. These two genes give proteins of high homology with the p53 protein in terms of the overall structure. [8]

2.2. p53 protein structure

The p53 protein contains five highly conserved regions within its amino acid sequence (amino acids 13-23, 117-142, 171-181, 234-250, and 270-286). These regions are essential for p53 functions. [9]

The p53 protein consists of 393 amino acids and can be divided in three main regions (figure 1a). The N-terminal region contains two transactivation domains (TADs) as well as a proline-rich

domain (PRD). The central part of the protein (amino acids 102 – 292) is occupied by a large DNA-binding domain (DBD). The C-terminal region contains different important elements for p53 regulation and overall function. [9]

2.2.1. N-terminal region

The N-terminal region contains two tandem TADs [10]. These TADs allow the p53 protein to recruit the basal transcriptional machinery such as TATA box binding protein (TBP), TBP-associated factors (TAF) elements of Transcription factor IID (TFIID), and the p62 subunit of the transcription/repair factor IIH (TFIIH). The N-terminal region can also interact with the single-stranded DNA-binding protein RP-A as well as chromatin modifiers such as the histone acetyltransferase p300/CBP. [9]

Some molecules such as murine double minute 2 (MDM2) and Adenovirus E1B protein (E1B-55 kDa) can bind the N-terminal region of p53 and inhibit its transcriptional functions. MDM2 can negatively regulate p53 in two ways. Firstly, MDM2 binds the TAD1 of p53 and frustrates its functions. Secondly, MDM2 regulates p53 stability. Indeed, MDM2 is a ubiquitin E3 ligase that targets p53 for degradation by the proteasome. The p53 protein itself regulates the expression of MDM2, creating a negative feedback loop that controls p53 activity. Moreover, most tumor-derived p53 mutants are incapable of inducing the expression of MDM2. [9]

The N-terminal region also includes a PRD (amino acid 63 – 97) that is known to interact with various protein partners. This domain is important for the ability of p53 to trigger apoptosis and prevent tumor cell growth. The deletion of the proline-rich domain does not affect the transactivation of diverse p53-target genes such as those encoding for p21^{waf1}, MDM2 or the BCL-2 family member BAX. The replacement of a proline by an arginine at codon 72, a common p53 polymorphism, suppresses one of the five PXXP motifs of the proline-rich domain [9]. This replacement has effects. For example, the Arg allele increases the mitochondrial localization of p53 in stressed cells and apoptosis induction in presence of stress. The Pro allele shows a lower apoptotic potential and an increased growth arrest in the G1 phase of the cell cycle [11].

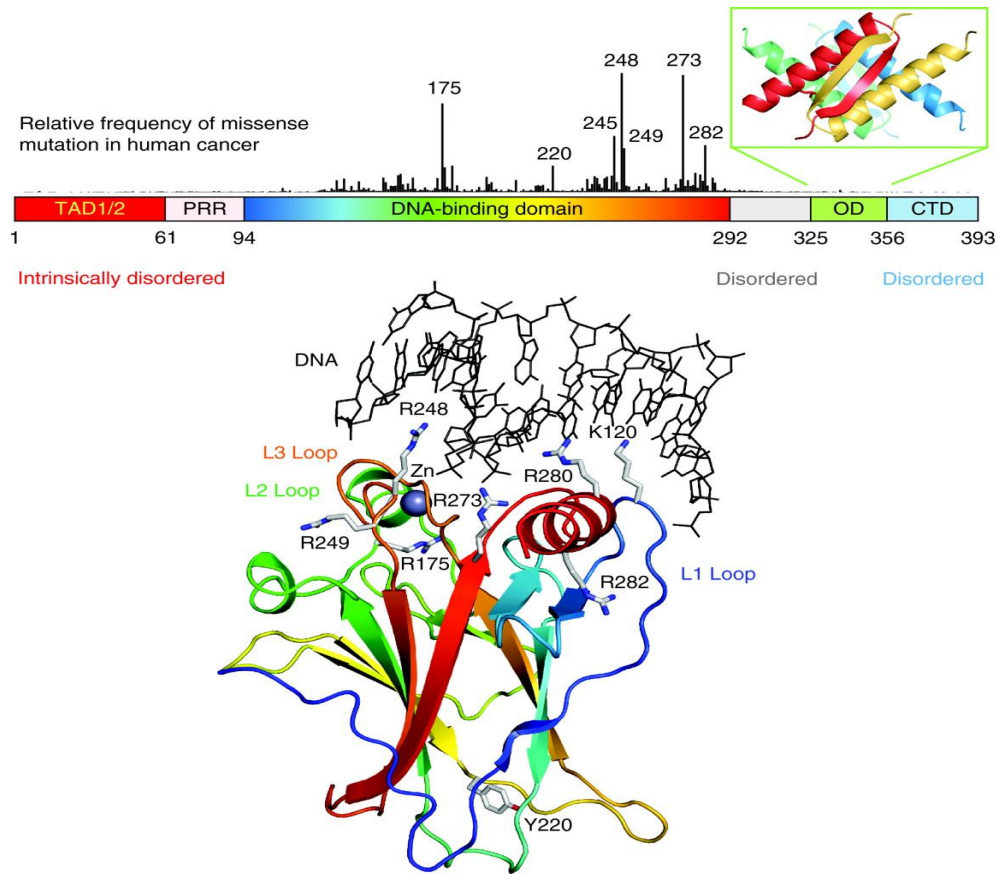


Figure 2: Structure of the p53 protein. Above: p53 contains an amino-terminal transactivation domain (TAD1/2) in red, followed by a proline-rich domain in pink. Like the TAD region, the regulatory domain at the extreme carboxyl terminus (CTD) is intrinsically disordered. The vertical bars indicate the relative missense mutation frequency in human cancer for each residue based on the TP53 Mutation Database of the International Agency for Research on Cancer. Most cancer mutations are located in the DNA-binding domain (central region of the protein). Below: the structure of the DNA-binding domain is 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. Modified citation from [23].

2.2.2. Central region

The central region of p53 protein is bounded approximately by amino acids 102 to 292. This part contains four of the five conserved regions (II – V). About 80 - 90% of tumor mutations are detected within this central region (figure 2). It consists of a large sequence-specific DBD. It is noteworthy that the central part can also interact with the SV40 large T antigen and other cellular proteins such as p53-binding protein 1 (53BP1) and p53-binding protein 2 (53BP2).

The crystal structure of the core DBD displays many pieces of information (figure 2). The central part is a large beta-sandwich that acts as a scaffold for three loop-based elements. The sandwich is two antiparallel beta-sheets carrying four and five beta-strands. The loops of the scaffold can bind to the DNA. The first loop L1 binds to DNA in the major groove. Loop L2 and L3 bind to the DNA minor groove. There is a zinc atom that connects the L2 with the L3 (amino acids Cys 176, His 179, Cys 238, and Cys 242). This zinc atom stabilizes the structure of these loops.

The three loops of the DNA-binding domain are extremely conserved. The majority of mutations take place in these three loops. These mutations can be divided into two classes. Class I hit residues in direct contact with the DNA like Arg248 and Arg273, which are the most frequently mutated in p53 (figure 2). Class II does not hit residues that are in direct interaction with the DNA but those how preserve p53 structure stabilization. [9]

2.2.3. C-terminal region

The C-terminal region (residues 300 – 393) contains a linker that connects the central and C-terminal regions. It also contains a tetramerization domain (TD) (amino acids 323-356) that permits the oligomerization of p53 proteins to form a tetramer [9]. This domain is not often mutated in cancer but it is essential for DNA binding and hence p53 transcriptional activity [12].

X-ray crystallography analysis shows that the C-terminal of each monomer is formed of a turn (Asp324 – Gly325), a β -strand (Glu326 – Arg333), a second turn (Gly334) and an α -helix (Arg335

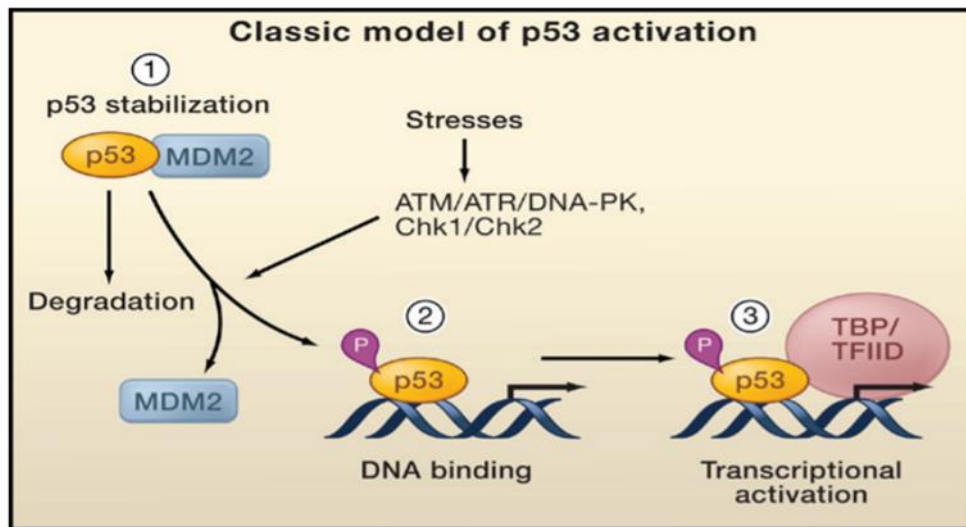


Figure 3: Classical model for p53 activation. p53 activation involves three steps: (1) the stress-induced stabilization of p53 mediated by phosphorylation, (2) DNA binding, and (3) recruitment of the general transcriptional machinery. During normal homeostasis, p53 is degraded after MDM2-mediated ubiquitination. Stress induces p53 phosphorylation by ATM, ATR and other kinases which stabilizes p53 and promotes DNA binding. DNA bound p53 then recruits the transcriptional machinery to activate transcription of target genes. Citation from [24].

– Gly356) (figure 1c). Two monomers give a dimer where the β -strand and the α -helix of the C-terminus are antiparallel. Two dimers interact to give a tetramer by their α -helix. In the outside of this tetramer, we can see the linkage of β -strands on the opposite faces (figure 1c). [9]

The C-terminal region also contains three nuclear localization signals (NLSs) (figure 1a). The mutation of NLS1 (amino acids 316 – 325) prevents p53 nuclear localization. Moreover, when the NLS2 (amino acids 369 – 375) and NLS3 (amino acids 379 – 384) are altered, p53 displays both a cytoplasmic and nuclear localization. [9]

In addition, there is a remarkable region (amino acids 363 – 393) close to the oligomerization domain (OD) (323 – 356). It contains different sites for post-translation modifications, including several lysines that can be ubiquitinated by MDM2. This region impacts on p53 stability and ability to regulate gene expression. It is also believed to realize non-specific interactions with DNA. Various proteins can bind to the C-terminal region such as the hepatitis B virus X protein or TFIIF subunits. [9]

2.3. p53 protein activities

The p53 protein plays a central role by controlling many processes in the cell. p53 is called the guardian protein or the guardian angel of the genome. The p53 protein is not required for normal differentiation and development of the organism. This explains why the development of p53-knockout mice is almost the same as the one of wild-type mice. So, p53 is required in cases of damages to cell components, stresses and many deviations from the optimum. At first, the functional abilities of p53 come from its capacity to act as a transcriptional factor that can induce or repress the expression of various genes (figure 3). Moreover, the p53 protein realizes interactions with many protein partners and this is also important for its functions as a tumor suppressor. For instance, when cells are exposed to an apoptotic stimulus, a fraction of p53 localizes to mitochondria where it interacts directly with different members of the BCL-2 family to trigger mitochondrial outer membrane permeabilization (MOMP), a key event in the course of the apoptotic process. [13]

2.3.1. Transcriptional activities of p53

The transcriptional activity of p53 is well known and characterized (figure3). The binding of p53 to its specific DNA response elements can result in gene induction or gene repression. The p53 protein can also cause gene repression without binding to DNA: it can prevent other transcriptional factors from acting through protein-protein interactions [13].

As a transcription factor, the active p53 tetramer binds to two decameric half-sites separated by a variable number of nucleotides (spacer) [14]. The half-site consensus is RRRC(A/T) (T/A)GYYY (R is a purine and Y a pyrimidine) (Figure 1b). In each 10bp half-site, we find two 5bp quarter sites; these are palindromic sequences that can adopt a head-to-head orientation as well as head-to-tail or tail-to-tail arrangements [10]. The spacer between the two half-sites can contain a different number of nucleotides but short spacers are preferred. The p53-binding elements can be located in the immediate or far upstream regions of genes or in intronic regions [14].

p53 binding to its response elements involves its large central DBD. In addition, the C-terminal region can also be involved in interactions with DNA: this region is capable of non-specific sequence-independent DNA binding. In addition, a model suggested that different post-translational modifications of the C-terminal region influence the ability of p53 to bind DNA with its DBD in a sequence-specific manner. Subsequent studies rejected this model: the absence of an effect was clear by using a ChIP method, which displays that those modifications of the C-terminal region do not change the affinity of p53 for its response element.

After p53 interaction with its response element, the N-terminal region promotes the transcriptional activities. The N-terminal region contains many residues that can interact with components of the transcription machinery as well as chromatin modifier. [13]

2.3.2. Mitochondrial activities of p53

The mitochondria have essential roles, for example, the production of energy, the initiation and progression of apoptosis, the participation in intracellular signaling pathways, etc. The p53

protein is tightly connected to mitochondria. p53 can regulate a switch between glycolysis and aerobic respiration. On the one hand, it can control aerobic respiration by regulating the expression of SCO2. SCO2 is important for the assembly of cytochrome *c* oxidase. On the other hand, p53 can control the expression of glycolytic enzymes to regulate glycolysis. p53 is also involved in the maintenance of mitochondrial DNA by stimulating the activity of DNA polymerase- γ . [15]

The mitochondria play a central role in apoptosis (this point will be discussed later in this document). p53 itself is a major regulator of apoptosis. In cells exposed to a detrimental stimulus, p53 can induce the expression of different pro-apoptotic proteins (transcription-dependent p53 mediated apoptosis). In addition, a fraction of p53 can localize at the mitochondrial outer membrane to trigger its permeabilization and the release of the mitochondrial effectors of apoptosis. This involves interactions of p53 with different BCL-2 family members (transcription-independent p53 mediated apoptosis) [15]. Notably, p53 was shown to interact with BAK and BAX, two pro-apoptotic BCL-2 family members. These interactions lead to a conformation change of BAX and BAK, promoting their oligomerization at the mitochondrial outer membrane (MOM) and the formation of pores. Regarding BAK, it was well established that the interaction requires p53 DBD. [48]

2.4. Regulation and functions

2.4.1. Regulation of p53 expression

The regulation of *TP53* transcription has been less studied than p53 protein activities. The *TP53* gene does not carry a conventional TATA box. It is under the control of numerous ubiquitous transcriptional factors such as NF- κ B, Sp1, and c-jun.

The mRNA that encodes for full length p53 has two translation initiation sites. The main translation initiation site exists in exon 2 (ATG 1) and the alternative site exists at codon 40 (ATG 40) in exon 4. In addition, the gene encoding for p53 has several alternative splicing sites. In the end, this complexity can potentially lead to the production of at least twelve different p53 isoforms. [8]

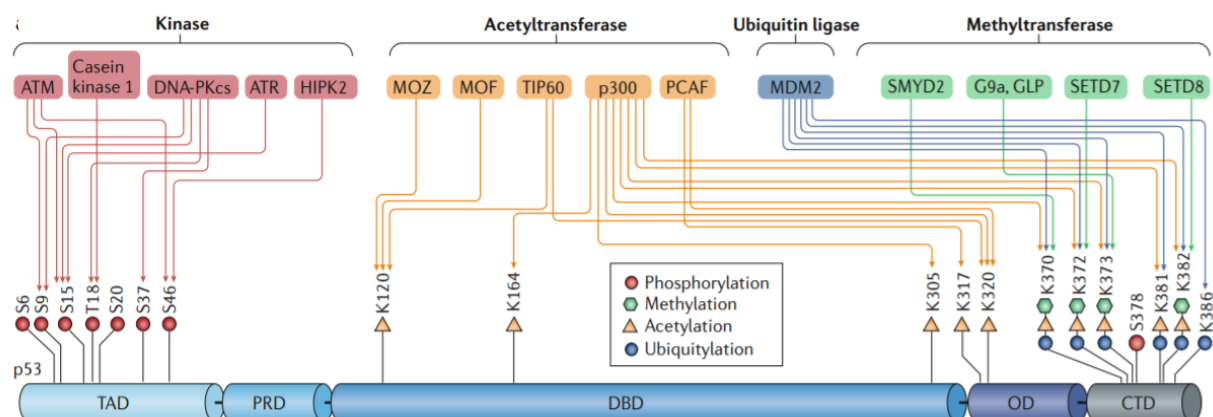


Figure 4: Main p53 post-translational modifications and the enzymes responsible for catalyzing them. Experimentally validated modifications known to affect p53 function are shown. Phosphorylation sites are prevalent in the TAD, whereas acetylation sites occur in the DNA binding domain (DBD), oligomerization domain (OD) and carboxy-terminal domain (CTD). Methylation sites are enriched in the CTD. The proline-rich domain (PRD) contains few well-studied modifications. Modified citation from [10].

2.4.2. Regulation of p53 activity

The p53 protein is activated by cellular stresses such as DNA damage, hypoxia, oncogene activation, and ribosomal stress. In return, p53 engages to arrest the cell cycle and promote DNA repair or to trigger cell death pathways. How the selection is made between cell survival or cell death in response to DNA damage as cellular stress is still a matter of intense debate. Different mechanisms have been proposed. These mechanisms include the modulation of p53 DNA binding by chromatin remodeling and p53 regulation by post-translational modifications (PTMs) (figure 4). [10]

2.4.2.1. Chromatin remodeling

In response to DNA damage or other stimuli, p53 binds to specific DNA sequences. The chromatin state of the target genes can modulate p53 ability to induce transcription. A comparison of p53 target promoters in normal and cancer cells shows a high presence of hypomethylated CpG islands in normal but not in cancer cells. These chromatin modifications in cancer cells can affect the accessibility of binding sites to p53 or other factors. The chromatin remodeler RSF1 was also showed to be important for p53 DNA binding in response to DNA damage. [10]

2.4.2.2. Post-translational modifications

PTMs have an important role for the regulation of p53 function. They regulate p53 binding to DNA, target-gene selection, stability, and others. More than 300 PTMs have been identified by mass spectrometry. Some of these PTMs have been well studied and shown to be essential for controlling p53 levels and activity. For example, the ubiquitination of p53 on C-terminal lysines by MDM2 leads to its proteasome-dependent degradation, while the phosphorylation of N-terminal serine and threonine residues (TAD1 domain) impairs the p53-MDM2 interaction and stabilizes p53. [10]

In human cells, a lysine to arginine (KR) substitution of six residues in the C-terminal region (lysines 370, 372, 373, 381, 382, and 386) generates a p53 protein that remains a potent

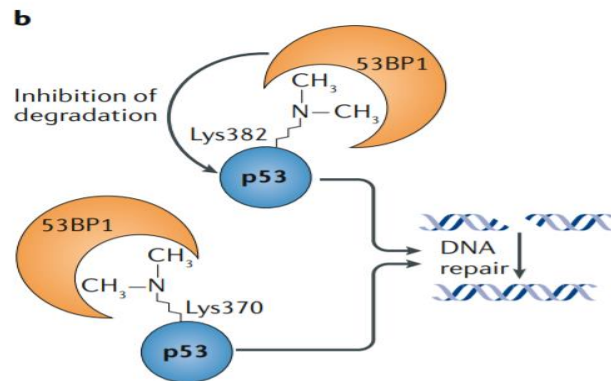


Figure 5: Dimethylation of Lys370 or Lys382 recruits p53-binding protein 1 (53BP1), which promotes DNA damage repair. 53BP1 binding to dimethylated Lys382 inhibits p53 degradation. Modified citation from [10].

transcription factor. In addition, p53 becomes insensitive to MDM2-mediated degradation since MDM2 fails to ubiquitinate the tumor suppressor. [49]

Acetylation of lysines in the p53 C-terminal region is also common (figure 4). The consequences of these PTMs are varied. For example, the combined acetylation of Lys370, Lys372, and Lys373 (C-terminal region) or the acetylation of Lys120 (DBD) promotes apoptosis. However, the acetylation of Lys317 by PCAF suppresses apoptosis and favors growth arrest induction [10].

p53 also contains various phosphorylation sites and many of them are located in the N-terminal region (figure 4). The phosphorylation of serine 46 (TAD2) by HIPK2 promotes the expression of p53 target genes involved in apoptosis, such as the gene encoding for the BCL-2 family member PUMA. This phosphorylation clearly favors apoptosis induction [10]. Many phosphorylations occur in the presence of DNA damage. These phosphorylations are mediated by the major DDR kinases ATM and ATR [10]. The tumor suppressor p53 is a major target of ATM and ATR activity. Both kinases can phosphorylate the tumor suppressor directly or indirectly through the activation of CHK1/2. At least 8 serine residues of p53 are targeted by this group of kinases. The phosphorylation events promoted by these kinases trigger cell cycle arrest, facilitating DNA repair, or cell death [17].

Other PTMs have important roles in p53 regulation such as methylation (figure 4). The methylation of lysine residues can enhance or decrease p53 transcriptional activity. These effects depend on the residue targeted and the extent of methylation (monomethylation, dimethylation, or trimethylation). For example, the monomethylation of Lys382 or the dimethylation of Lys373 by G9a (KMT1C) and GLP (KMT1D) represses p53 activity. In the case of DNA damage, the monomethylation and the dimethylation of Lys372 promote p53 stability, target genes induction and apoptosis. The dimethylation of Lys370 and Lys382 residues promotes p53 binding to 53BP1(p53-binding protein 1) and DNA repair (figure 5). However, the monomethylation of Lys370 or Lys382 inhibits p53 localization to target genes promoters. [10]

PTMs appear to also regulate p53 mitochondrial activity. For instance, it has been proposed that p53 ubiquitination favors its mitochondrial localization. Sykes and his colleagues showed that p53

acetylation on lysine 120 increases its ability to release BAK from MCL-1, an antiapoptotic BCL-2 family member. In addition, a recent study indicates that serine 392 phosphorylation is important for p53 mitochondrial localization. Failure to phosphorylate S392 results in a markedly reduced p53 mitochondrial localization, and consequently decreased apoptosis. [33, 50, 51, 45]

2.5. *TP53* role in disease

While the wild type p53 protein is the guardian angel of the genome, mutant p53 was compared to a fallen angel. p53 is known for its important role in different physiological processes thus preventing the development of pathologies. Altered p53 activities lead to malignant diseases and promote several others such cardiovascular, neurodegenerative, and metabolic diseases [13]. We can separate p53 mutations into two categories: germline p53 mutations and somatic p53 mutations [18].

2.5.1. Germline p53 mutations

The Li-Fraumeni Syndrome (LFS) is related to germline p53 mutations. This syndrome is an autosomal dominant hereditary disorder that gives rise to early-onset cancers. LFS patients show tumors such as sarcomas, brain and pre-menopausal breast tumors, adrenocortical carcinomas and leukemias. The LFS has a high penetrance: during the lifetime, the risk of developing cancer is over 90%. Most *TP53* germline mutations are missense mutations in the DNA binding domain (codons 175, 248, 249, 273, and 281). There is another variety of LFS that is caused by mutation in *CHEK2*, the gene encoding for CHK2. This is a serine/threonine protein kinase that activates the p53 protein in response to DNA damage. [18]

2.5.2. Somatic p53 mutations

Somatic p53 mutations exist in 50% of human cancers. According to tumors types: 10% of p53 mutations are found in leukemia, 20% in breast cancer, 30% in gastric cancers, and 60% in ovarian and colorectal cancer [18]. The examination of the whole coding sequence shows that 86% of mutations take place between codons 125 and 300, thus targeting the DBD of p53. Most alterations of the DBD are missense mutations (87.9%). However, missense mutations outside the DBD represent only 40% of the recorded alterations, the majority being here nonsense or frameshift

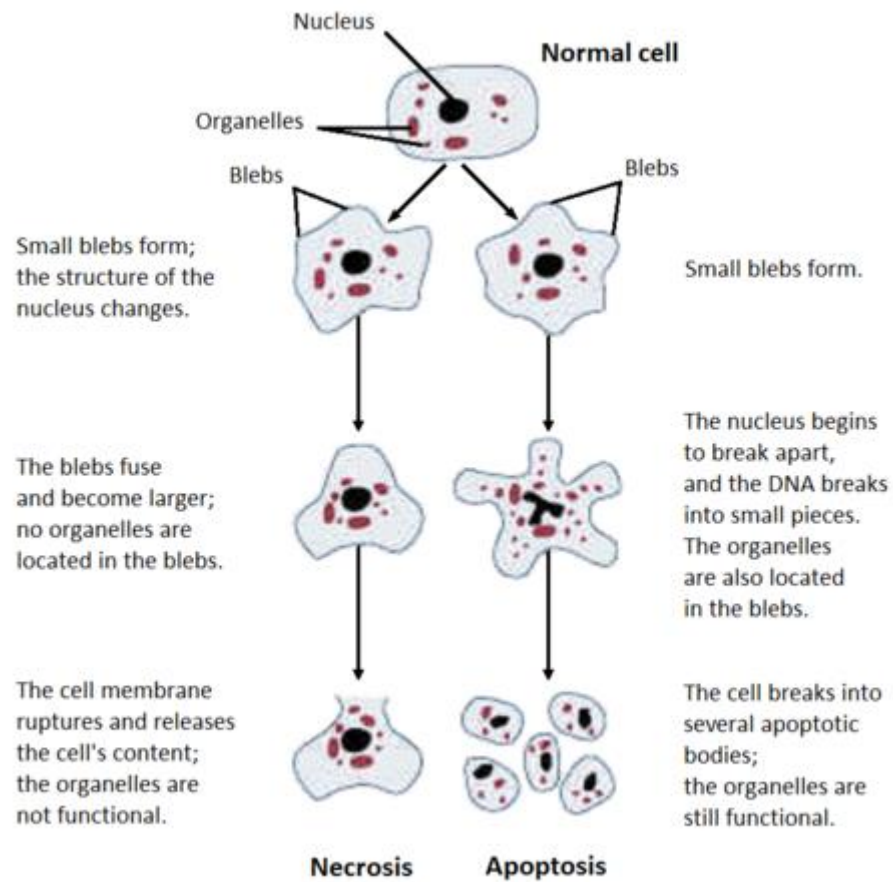


Figure 6: Apoptosis morphology vs necrosis morphology. Modified citation from [25].

mutations. [19]

3. Apoptosis: type I programmed cell death

Apoptosis or type I programmed cell death is distinguished by morphological characteristics and energy-dependent biochemical mechanisms. Apoptosis works as a homeostatic mechanism to maintain cell populations in tissues and as a defense mechanism when cells are damaged, and it can be triggered by many factors, physiological or pathological. This procedure is vital for many processes such as normal cell turnover, proper development, functioning of the immune system, hormone-dependent atrophy, embryonic development and chemical-induced cell death. Moreover, a deregulation of the apoptotic process can be observed in different pathologies such as neurodegenerative diseases, ischemic damages, autoimmune disorders and cancers. [20]

The apoptotic cell, as said above, displays typical morphological features. This allows to distinguish apoptosis from other cell death processes (figure 6). During early apoptosis, at the beginning of the process, cell shrinkage and pyknosis can be observed. The cell presents a smaller size, a dense cytoplasm and closely packed organelles. Pyknosis refers to chromatin condensation in the nucleus. Next, a dramatic plasma membrane blebbing happens followed by karyorrhexis and detachment of cell fragments under a process called "budding". There is no release of intracellular components in the extracellular medium. Moreover, under the plasma membrane, organelles integrity is maintained. At the end of the process, the cell disintegrates into apoptotic bodies that are phagocytosed by macrophages, parenchymal cells or neoplastic cells. Of note, apoptosis does not lead to an inflammatory reaction. [20]

Apoptosis is an evolutionarily conserved and functionally irreversible process. This process is highly complex and involves an energy-dependent cascade of molecular events. Depending on the apoptotic stimulus, two main apoptotic pathways are distinguished: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. These two pathways are linked: effectors of one pathway can influence the other one. The two pathways culminate ultimately in the triggering of the execution phase of apoptosis. This execution phase is common to both pathways. It starts with the activation of the caspases, a family of proteases, and results in DNA fragmentation, cleavage of various proteins, degradation of different cell components (cytoskeletal, nucleus), formation of apoptotic bodies and presentation at the membrane of ligands for phagocytic cells.[20]

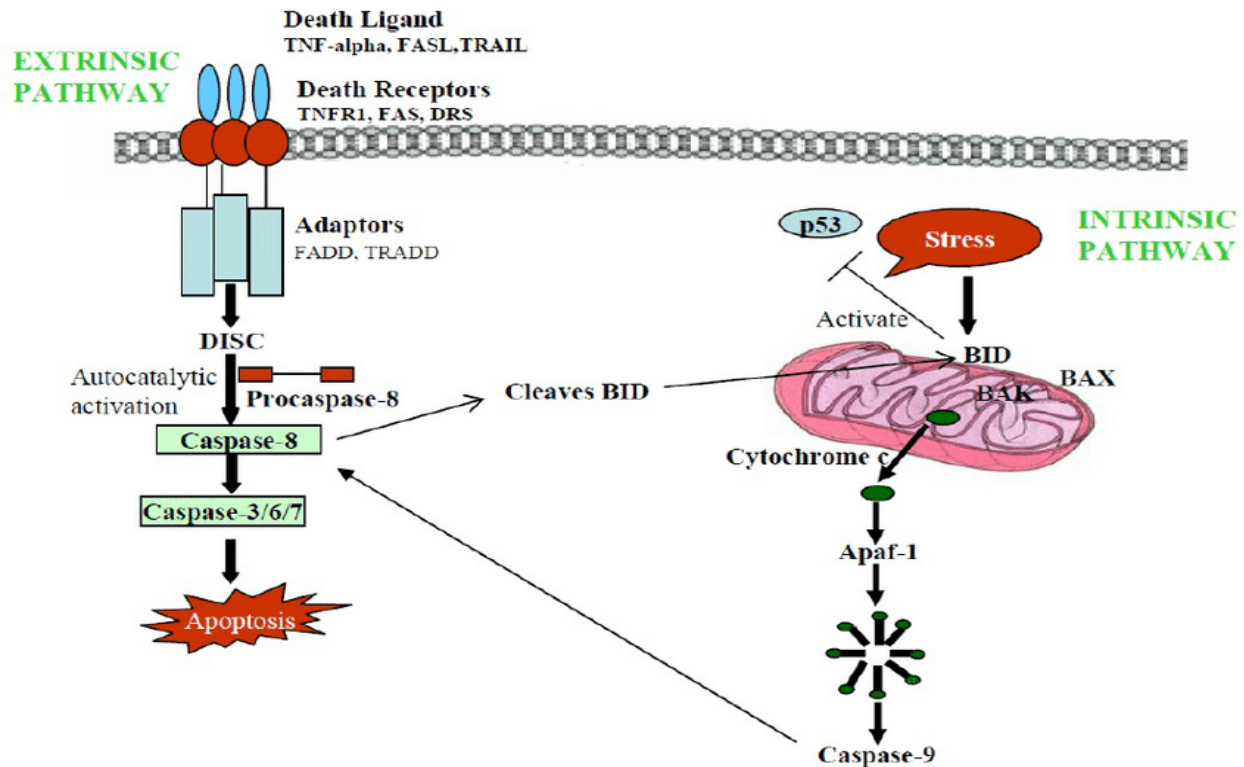


figure 7: Extrinsic and Intrinsic pathways of apoptosis. In the extrinsic pathway, the death ligands bind to transmembrane death receptors present at the cell surface. The binding of the ligand to its receptors on the target cell triggers receptors clustering. This allows the recruitment of adaptor proteins on the cytoplasmic side of the receptors, and subsequently of procaspases-8 and -10. Formation of an active DISC (death inducing signaling complex) brings procaspases molecules close to one another, which facilitates their autocatalytic activation and release into the cytosol where they activate the caspase cascade. Active caspase-8 also mediates the cleavage of the proapoptotic protein BID (into tBID, truncated BID) which promotes mitochondrial outer membrane permeabilization (MOMP). In the intrinsic pathway, a stress causes the activation of the cytoplasmic protein BAX and its localization at the mitochondrial outer membrane (MOM). BAK can also be activated. Both proteins are able to oligomerize in the MOM to generate pores that will allow the release into the cytosol of the mitochondrial effectors of apoptosis such as cytochrome c. BAX and BAK oligomerization can be induced through interactions with several proteins, including the active form of BID, tBID. Cytochrome c, once in the cytosol, binds to Apaf-1 to form the apoptosome complex. Formation of this complex leads to the activation of procaspase-9. Activated caspase-9 further initiates the caspase cascade leading to apoptosis. The tumor suppressor p53 protein is a sensor of cellular stress and plays a vital role in initiating apoptosis. As transcription factor, it induces the expression of different pro-apoptotic proteins, including BAX and BAK. Citation from [26].

3.1. Extrinsic pathway

The extrinsic pathway of apoptosis starts with the interaction of specific ligands with receptors present on the plasma membrane of the target cell. These transmembrane receptors are called the death receptors. They are members of the TNF superfamily of receptors. Each death receptor consists of a cysteine-rich extracellular domain and a cytosolic portion that contains a death domain. This death domain is crucial to carry in the signal from the cell surface to the intracellular signaling pathways. To illustrate, here are the main ligands and their corresponding death receptors: FasL/FasR, TNF- α /TNFR1, Apo2L/DR4, and Apo2L/DR5. [20]

The chain of events during the activation of the extrinsic pathway are well defined for the FasL/FasR and TNF- α /TNFR1 models. Following binding of their ligand, receptors cluster at the membrane. Next, a cytosolic adapter protein binds the intracellular death domain of the receptors. For instance, the binding of the Fas ligand to the Fas (also named CD95) receptor results in the binding of the adapter protein FADD (Fas-associated protein with death domain) (figure 7). The binding of TNF- α to TNFR1 results in the binding of the adapter protein TRADD (Tumor necrosis factor receptor type 1 associated death domain) to the intracellular death domain of the receptor. The binding of TRADD will allow the subsequent recruitment of FADD and RIP1 (Receptor-interacting serine-threonine kinase 1). FADD, in both cases, will then recruit procaspase-8. Right now, we can say that the DISC (death inducing signaling complex) is formed. Its formation results in the auto-catalytic activation of procaspase-8. At this point, apoptosis is triggered. [20]

3.2. Intrinsic pathway

The intrinsic pathway of that apoptosis does not require transmembrane receptors because the initial stimulus is intracellular. Stimuli that initiate the intrinsic pathway of apoptosis are diverse. It can be the lack of certain hormones, growth factors or cytokines. It can also be radiations exposure, hypoxia, viral infections, free radicals, etc. [20]

These stimuli are all capable of triggering MOM permeabilization, an event often considered as “a point of no return” in the course of apoptosis. MOM permeabilization leads to the release of mitochondrial effectors of apoptosis into the cytosol (figure 8). A first group of proteins includes cytochrome c, Smac/DIABLO and the serine protease HtrA2/Omi. [20]

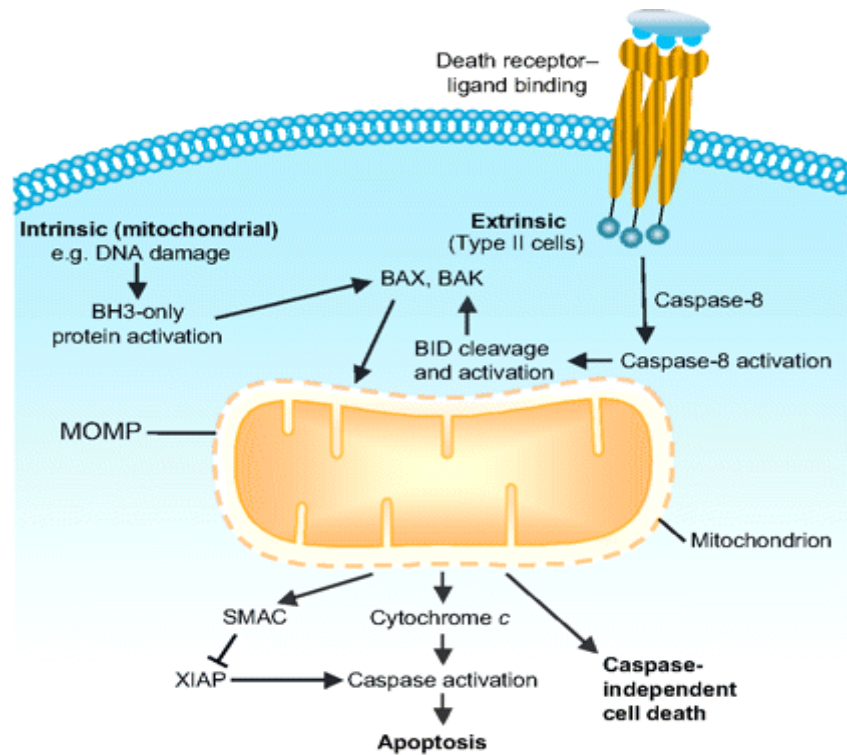


Figure 8: Mitochondrial regulation of apoptosis. Left: in the intrinsic pathway of apoptosis, pro-apoptotic stimuli such as DNA damage activate BH3-only proteins that, in turn, activate BAX and BAK. Active BAX and BAK cause mitochondrial outer membrane permeabilization (MOMP), leading to release of proteins from the intermembrane space, including cytochrome *c* and SMAC/Diablo (as well as Omi/Htra2). This triggers the activation of caspases and thereby causes apoptosis. MOMP can also lead to caspase-independent cell death. Right: in the extrinsic pathway, activation of cell surface death receptors leads to activation of the initiator caspase-8. In type II cells, mitochondria are required for apoptosis. Following formation of the DISC, caspase-8 is activated and cleaves the BH3-only protein BID (to form tBID, truncated BID). tBID interaction with BAX or BAK leads to their activation and consequently MOMP. Citation from [27].

Cytochrome c, once released, binds and activates Apaf-1, in the presence of ATP. Several activated Apaf-1 then aggregate to form a large complex called the apoptosome (this complex is equivalent to the DISC in the extrinsic pathway of apoptosis). Formation of the apoptosome allows the recruitment of procaspase-9 and its auto-processing and activation. Smac/DIABLO and HtrA2/Omi promote apoptosis by inhibiting the inhibitors of apoptosis proteins (IAPs) (figure 8). [20]

The second group of pro-apoptotic proteins includes Apoptosis Inducing Factor (AIF), endonuclease G, and Caspase-activated DNase (CAD). Once in the nucleus, AIF promotes DNA condensation. The nuclease CAD is already present in the cytosol and nucleus of “healthy” cells. However, it is kept inactive by a protein-protein interaction with the inhibitor of Caspase-activated DNase (ICAD). The cleavage of ICAD by caspase-3, releases CAD (figure 9). Endonuclease G, once released from the mitochondria, translocates to the nucleus and also cleaves chromatin. [20]

The apoptotic mitochondrial events are regulated by members of the BCL-2 family of proteins. These proteins are regulated, at least partly, by the tumor suppressor p53. The BCL-2 family includes both pro-apoptotic (BAX, BAK, BID, BAD, BIM, BIK, Bak, PUMA, NOXA...) and anti-apoptotic proteins (BCL-2, BCL-XL, BCL-W, DIVA...). [20]

The main cross-talk between the intrinsic and extrinsic pathways of apoptosis is the cleavage of BID into tBID by caspase-8 (figure 7) [20]. The active tBID protein is a potent promoter of MOM permeabilization [21].

The expression of different members of the BCL-2 family is under the control of the transcription factor p53. This is for instance the case of BAK, BAX, PUMA and NOXA, four important mediators of p53-mediated apoptosis. *In vitro*, it has been shown that the overexpression of PUMA leads to BAX conformational change and its localization to the mitochondria. Once at the mitochondrial outer membrane, BAX oligomerizes and generates pores that will allow the release of mitochondria apoptosis effectors, such as cytochrome c, into the cytosol. BAK, following its activation, is also able to oligomerize at the mitochondrial outer membrane to permeabilize it. NOXA is another mediator of p53-induced apoptosis. This pro-apoptotic protein can inhibit different anti-apoptotic BCL-2 family members. [20]

DNA fragmentation

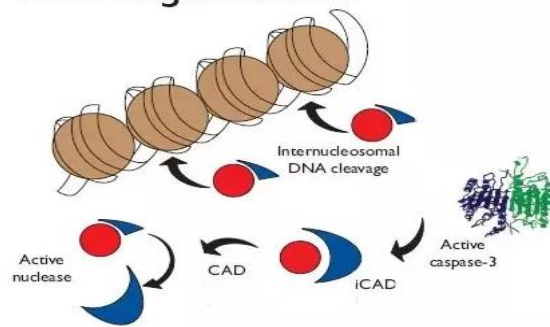


Figure 9: DNA fragmentation by CAD. ICAD, the inhibitor of CAD, is cleaved by active caspases-3. Caspases are indirectly involved in DNA fragmentation by activating cellular nucleases. CAD (caspase activated DNase) also called DFF40 (DNA fragmentation factor) exists in an inactive form by complexing with ICAD (inhibitor of CAD). During apoptosis caspase 3 cleaves ICAD, releasing and activating CAD which causes DNA degradation and chromatin condensation. Modified citation from [28].

In addition to regulating the expression of different BCL-2 family members, p53 can also directly interact with some of them. This the case of BAX and BAK. For instance, in cells exposed to an apoptotic stimulus such as DNA damaging agent, a fraction of p53 localizes to the mitochondrial outer membrane where it interacts with BAK. This interaction triggers BAK oligomerization and MOMP. [20]

3.3. Execution phase of apoptosis

The execution phase is the final step of apoptosis. The actors involved are shared between the two pathways of apoptosis described previously. The executioner caspases (caspase-3, caspase-6, and caspase-7) play a major role during this phase. First, they are needed to activate some effectors of apoptosis such as the BCL-2 family member BID as well as CAD, the main nuclease of apoptosis. Secondly, as proteases, they will cleave various cell proteins such as, for instance, the nuclear laminas. The activity of the executioner caspases is responsible for many morphological and biochemical changes seen during apoptosis. [20]

The caspases involved in the apoptotic process can be divided in two groups, the initiator and the executioner caspases. In “healthy” cells, caspases exist in an inactive form after their synthesis. The initiator caspases (caspases-8, -9, -10) are characterized by a large N-terminal pro-domain (>90 amino acids). Their activation involves their recruitment into large complex such as the DISC or the apoptosome, as discussed earlier. One recruited in this complex, they undergo a process of auto-activation. The main substrates of the initiator caspases are the executioner caspases (caspases -3, -6 and -7). The most important caspase for the execution phase is caspase-3. All the initiator caspases can activate caspase-3. [22]

Caspase-3 can activate the endonuclease CAD by degrading its inhibitor ICAD, leading to DNA digestion (figure 9). Caspase-3 is also responsible for cytoskeleton reorganization during apoptosis and apoptotic body formation. Moreover, during apoptosis, an externalization of phosphatidylserines can be observed. The mechanism of phosphatidylserine externalization (their presence in the outer leaflet of the plasma membrane) is not well understood. However, using oxidative stress, the involvement of Fas, caspase-8, and caspase-3 was shown. The presence of phosphatidylserines in the outer leaflet of the apoptotic cell membrane is important. It facilitates recognition of the apoptotic bodies by phagocytic cells (figure 6). [20]

4. Proximity-dependent labeling using APEX2

APEX2 is being used for proteomic mapping within living cells. It is helpful to skip some limitations of mass spectrometry and identify protein components of some organelles. Biologists are using mass spectrometry to study and identify the proteome of cellular compartments. Mass spectrometry needs pure samples for the analysis. However, the purification methods are imperfect leading to an important rate of false-positive and thus rendering difficult data interpretation. Proximity dependent labeling methods were developed to improve this.

APEX2 (engineered ascorbate peroxidase 2) is a second-generation enzyme. Wild-type soybean ascorbate peroxidase was mutated four times to obtain APEX2. The molecular weight of APEX2 is 27 kDa. It is a monomer, free from disulfide bonds, and it requires a heme cofactor for its activity.

APEX2, in the presence of hydrogen peroxide (H_2O_2), is able to catalyze the oxidation of biotin phenol to form biotin-phenoxy radicals. These radicals are very reactive, short-lived ($<1ms$), and conjugate covalently with surrounding molecules. Precisely, they can bind to amino acids like tyrosine, tryptophan, cysteine, and histidine.

The biotinylation radius is not a fixed value. The local environment (glutathione concentration, endogenous protein density) influences the biotinylation contour map in different ways. Therefore, far proteins could be exposed to the biotinylation. The ratiometric SILAC approach (figure 10) is a solution to this problem. It is crucial for achieving a high spatial specificity. Binding APEX proteomic method and ratiometric SILAC approach is so helpful to reduce unwanted background.

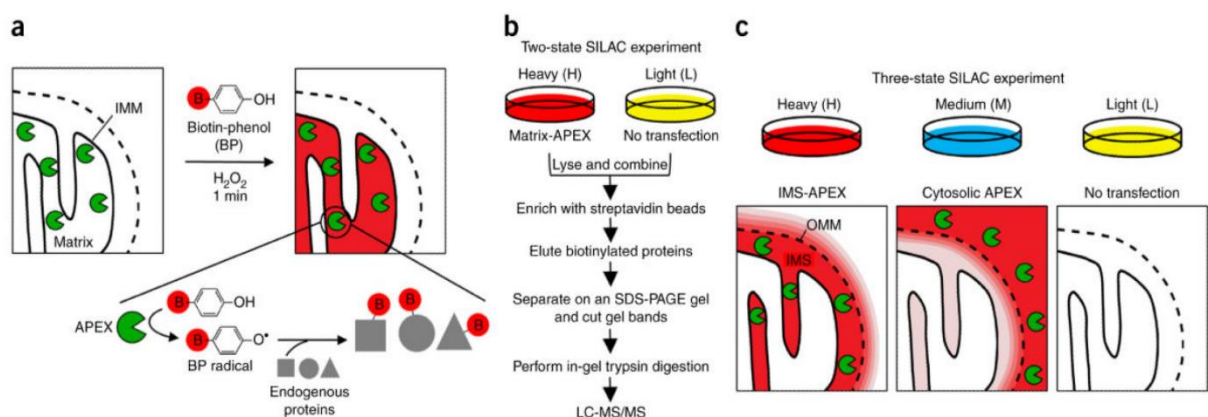


Figure 10: Live-cell proteomics using APEX. **(a)** Scheme showing APEX-catalyzed biotinylation. The mitochondrial matrix is enclosed by the IMM. This example shows APEX (green Pac-Man) targeted to the mitochondrial matrix. Live cells are incubated with biotin-phenol probe (red B circles = biotin) for 30 min and then treated for 1 min with 1 mM H_2O_2 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 H_2O_2 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. **(c)** Three-state SILAC experiment used to map the proteome of the IMS. The IMS lies in between the IMM and the OMM, which contains porins that allow the exchange of molecules < 5 kDa. Consequently, biotin-phenoxyl radicals generated by IMS-localized APEX can escape into the cytosol, leading to background (biotinylation contour map outside the OMM shown in drawing). To subtract this background, a three-state rather than two-state SILAC experiment. The heavy sample expresses APEX targeted to the IMS. The light sample is a nonbiotinylated negative control, just as in b. The medium (M) sample expresses APEX outside (in this case, the cytosol, via fusion to a nuclear export sequence peptide). Streptavidin enrichment is performed on the mixture of H, M and L lysates, as shown in b. Modified citation from [29].

The first demonstration of the APEX proteomic method used the first generation of APEX enzyme to get a proteomic map of the mitochondrial matrix in HEK 293T cells. They used two-state SILAC to distinguish between biotinylated proteins and non-specific streptavidin binders. They found a proteome of 495 proteins with > 94% of mitochondrial specificity, > 97% of mitochondrial matrix specificity, and 85% of coverage of known mitochondrial matrix proteins. The second demonstration was about the proteome of the intermembrane space (IMS). The space exists between the inner mitochondrial membrane (IMM) and outer mitochondrial membrane (OMM). The last one contains pores that can allow biotin-phenoxyl radicals to pass through and label cytosolic molecules by mistake. Therefore, to increase the spatial specificity in this region, they decided to use three-state SILAC instead of a two-state. They found 127 proteins, with a mitochondrial specificity of > 94% and an IMS specificity of > 99%. [29][30]

4.1. SILAC (stable isotope labeling by amino acids in cell culture) approach

The two-state SILAC protocol is suitable for proteome identification in membrane-enclosed cellular compartments that could trap biotin-phenoxyl radicals within (figure 10a, b). The protocol here carries out two states which are the heavy- and light-state samples. The experimental samples are cultured in two separated culture mediums with heavy and light amino acids. The ratio H/L allows for the distinction of true-labeled proteins and non-true proteins.

For the ratiometric three-state SILAC protocol (figure 10c). The heavy- and the light state samples remain the same as before. However, the medium state sample is required. If we are talking about mapping the IMS, APEX or APEX2 should be in touch with NES (nuclear export sequence). This construction allows the enzyme to be in the cytosol and to label the cytosolic proteins. The ratio H/M reflects the degree to which that peptide is preferentially biotinylated by IMS-APEX versus cytosolic APEX.[29]

4.2. Comparison with BioID (Proximity-dependent biotin identification)

BioID is another biotinylation technique based on a variant of an *Escherichia coli* biotin ligase called BirA. BirA generates an activated biotin adenylate ester (biotin-AMP) that links covalently with the side chain of lysines of surrounding proteins. This technique has two weaknesses compared with the APEX2 approach. First, it takes 18 to 24 hours to achieve the appreciable level of biotinylation. This long period could lead to cell toxicity or affect the protein composition of the region under the study. Second, biotin-AMP has a half-life of minutes in water. This makes the labeling radius much greater than that of biotin-phenoxy radicals (half-life <1ms), and we cannot use SILAC or iTRAQ for spatial specificity as in the APEX2 approach. So, the APEX2 approach gives high spatial specificity and short labeling windows. [29][31]

HRP is one of the most vital enzymes obtained from plants. It is a heme-containing enzyme involves in many physiological roles such as the indole-3-acetic acid metabolism, the cross-linking of biological polymers, and lignification. Many practical and commercial applications use this enzyme. Biology is one of the most fields that uses HRP in many techniques such as ELISA, western blot ... etc. [32]

II. Objectives

The thesis of C. Castrogiovanni [33] highlights that PTMs have effects on p53 mitochondrial activity. More specifically, it revealed the importance of S392 for the mitochondrial localization of p53 in stressed cells. It was observed that the phosphorylation of S392 leads to the mitochondrial localization of p53. Mutation of this residue (S392A) impaired p53 mitochondrial localization, which has an impact on apoptosis. It was also observed that S392 phosphorylation has little impact on p53 transcriptional activity. To explain these results, C. Castrogiovanni proposed two hypotheses:

First hypothesis: during a stress, the phosphorylation of p53 S392 promotes p53 binding to an unknown cytosolic protein partner. This unknown partner will then guide p53 to mitochondria, making the tumor suppressor able to trigger MOMP.

Second hypothesis: in normal conditions (in the absence of a stress), cytosolic p53 is sequestered and inhibited by one or more protein partners. This prevents p53 from reaching the mitochondria and inducing apoptosis. Phosphorylation of S392 following a stress disturbs the inhibitory complex, promoting p53 mitochondrial localization.

The ultimate goal of our project is to identify cytosolic p53 protein partners whom interaction with p53 is affected by S392 phosphorylation. To identify these proteins, we will use a proximity-dependent labeling method. The required genetic construction is already available in the lab. It consists of a fusion between p53 and APEX2, a modified plant peroxidase. APEX2 was fused to the N-terminal of p53, serine 392 being located in the C-terminal region of p53.

Our specific objectives during this master thesis are the following: (1) the stable transfection of two constructions (APEX2-p53 wt and APEX2-p53 S392A) in H1299 (p53^{-/-}) cells; (2) the characterization of p53 activity when fused to APEX2 (transcriptional activity, ability to localize to mitochondria and to induce apoptosis).

III. Results

1. Coupling APEX2 to p53

To use the proximity-dependent labeling approach in order to identify protein partners that regulates the mitochondrial localization of p53, an APEX2-p53 fusion protein must be created and then validated by *in vitro* experiments aimed at assessing p53 activity.

1.1. APEX2-p53 wt construction

The APEX2-p53 wt genetic construction was previously generated in our laboratory. The NEBuilder® HIFI DNA Assembly Reaction (Gibson assembly method) was employed to obtain the fusion protein (figure 1). The construct was verified by sequencing.

The APEX2 protein is merged to the N-terminal of wt p53 protein to prevent any effect on S392 phosphorylation status, this residue being on the C-terminus of p53. Furthermore, sequences of APEX2 and wt p53 are linked together by a flexible linker of ten amino acids. The expression of the construction in under the control of a Tet-responsive promoter (figure 1).

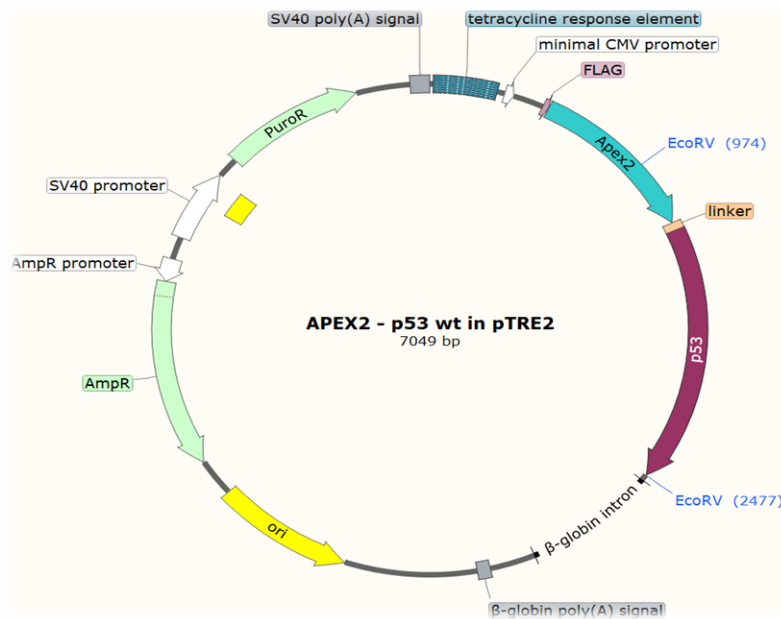


Figure 1: Graphical representation of the APEX2-p53 wt expression vector. This demonstration is obtained by using the Snapgene program. Light blue: APEX2 sequence; Dark purple: wt p53 sequence; Dark blue: tetracycline response element (TRE); Light purple: flag; orange: linker; Green: resistance elements (PuroR and AmpR); White: promoters; Yellow: replication origin.

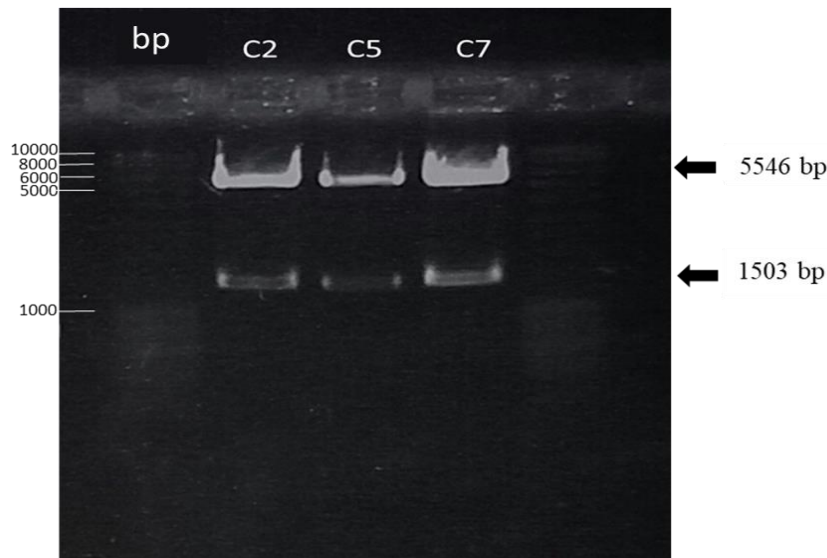


Figure 2: Enzymatic restriction of the pTRE2-p53 wt by EcoRV. bp: base pair number; C2: restricted plasmids from the C2 sample; C5: restricted plasmids from the C5 sample; C7: restricted plasmids from the C7 sample. Arrows show two fragments of 1503 bp and 5546 bp.

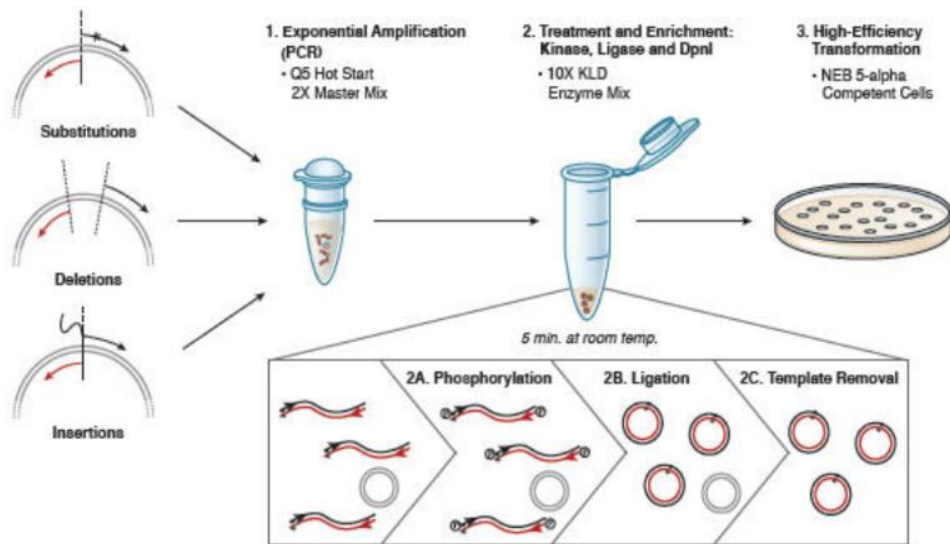


Figure 3: Q5 Site-Directed Mutagenesis Kit Overview. This kit is designed for the rapid and efficient incorporation of insertions, deletions, and substitutions into double-stranded DNA plasmids. The first step is the exponential amplification using standard primers and a master mix formulation of Q5 Hot Start High-Fidelity DNA Polymerase. The second step involves incubation with a unique enzyme mix containing a kinase, a ligase, and DpnI. These enzymes promote the rapid circularization of the PCR product and the removal of the template DNA. The last step is a transformation into high-efficiency chemically competent cells (provided). Citation from [34].

1.2. Verification of the APEX2-p53 wt construction by restriction digest

This verification is just an extra step to be sure that our genetic construction is correct. To do this, we used a restriction digest. EcoRV was the chosen enzyme. It cuts at two sites: the first one is in the sequence of APEX2 and the second is at the end of the p53 sequence. This digestion is supposed to give two fragments of respectively 1503 bp and 5546 bp. (figure 1)

We had three samples to verify (vectors purified from bacterial cultures). After restriction and agarose gel electrophoresis, we observed as expected the presence of two DNA fragments in each lane (figure 2). The small fragment (1503 bp) includes sequences belonging to APEX2 and wt p53. The big fragment (5546 bp) corresponds to the rest of the pTRE2 vector. These data suggest that our genetic constructions from three samples are correct and ready to be used for next experiments. As said before, the construction was also verified by sequencing.

1.3. APEX2-p53 S392A construction

The only difference between APEX2-p53 S392A and APEX2-p53 wt is the substitution of S392 by an alanine. So, we used the pTRE2 APEX2-p53 wt vector as a template DNA to introduce the mutation. We employed the Q-5 Site-Directed Mutagenesis Protocol to replace AGT (Ser) with GCA (Ala).

The Q5® Site-Directed Mutagenesis Kit allows a fast site-specific mutagenesis (less than 2 hours) (figure 3). Here, primers are non-overlapping to ensure robust exponential amplification, and generate a high efficiency of introduction of the desired mutation. Besides, the error rate in this process is low thanks to the Q5 Hot Start High-Fidelity DNA polymerase. After performing the PCR, we incubated the amplified material to the KLD enzyme mix for DNA circularization and template removal. For the transformation, we used NEB high-efficiency competent cells. [34]

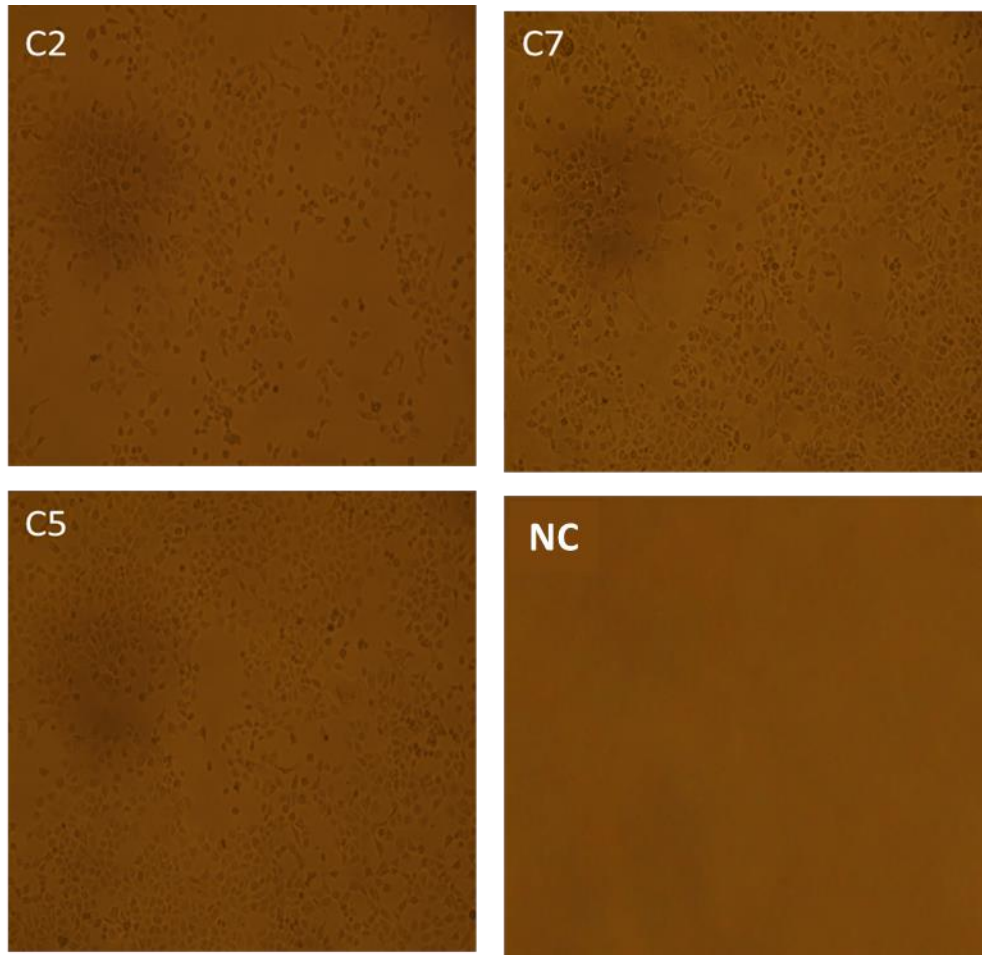


Figure 4: Stable transfection of the H1299 cell line with pTRE2-APEX2 p53 wt. Phase contrast microscopy images showing cultures of H1299 cells transfected or not (negative control) with the vector pTRE2-APEX2 p53 wt. C2: H1299 cells contain plasmids of the C2 sample; C5: H1299 cells contain plasmids of the C5 sample; C7: H1299 cells contain plasmids of the C7 sample; NC: negative control (untransfected cells).

2. Stable transfection of H1299 cells

In this study, we used the H1299 cell line as a model. It is a p53 null human non-small cell lung cancer cell line. These cells display a homozygous partial deletion of *TP53* and a lack of p53 protein expression. Using these cells as a model to host our genetic constructions is therefore a good choice because of the lack of an intrinsic p53 protein. [36]

We needed to transfect H1299 cells with pTRE2 APEX2-p53 wt. We performed the transfection using the X-tremeGENE HP DNA Transfection Reagent. This reagent includes different molecules that form a complex with DNA and transport it into cells. It is used for many cell lines because of its high performance (high transfection efficiency and low toxicity). [37]

We prepared three flasks containing H1299 cells. Each flask was destined for one of the three plasmid samples (C2, C5, and C7). After obtaining the optimal cell confluency (70% to 90%), we added the transfection complex (DNA, transfection reagent, and culture medium without serum) and incubated the cells for 18 hours. Following transfection, cells were selected under puromycin. The selection step took twenty days. We used non-transfected cells as a negative control, and we performed the same treatment on them. In figure 4, we can observe that cells transfected with pTRE2-p53 wt vector grown in the presence of puromycin. In contrast, no growth is observed in the negative control (non-transfected cells). These data suggest that the stable transfection was successful.

3. Characterization of wt p53 activity

In the construction described before, the APEX2 enzyme is fused to the N-terminal of p53 wt. In addition, the two elements of the fusion are separated by a flexible linker of 10 amino acids. Serine 392 is located in the C-terminal end of the p53 protein (393 amino acids). Considering the distance, we expect that the fusion will not affect S392 phosphorylation, a PTM previously shown to be important for p53 mitochondrial localization. [38]

In this section, we verified four things. First, we checked the expression of APEX2-p53 wt in H1299 cells. Second, we determined whether the fusion protein can still translocate to mitochondria. Third, we analyzed whether p53 was still able to induce some of its target genes. Finally, we checked the induction of apoptosis by p53.

3.1. Expression of APEX2-p53 wt in H1299 cells

The first step is to verify the expression of APEX2-p53 wt in H1299 cells. The APEX2-p53 wt construct is under the control of a Tet-ON system that modulates gene expression, gives control over quantitative and temporal manners. Tc or DOX is a key player in Tet-on systems as well as TetR. TetR proteins bind with a high affinity to the tetO DNA sequence and inhibit the expression. 7 repetitions of the tetO sequence form a TRE (figure 1). The binding of Tet or DOX to TetR results in conformation changes that prevent TetR to set on tetO. Consequently, the expression become permissible.[39]

We verified the expression of APEX2-p53 wt within H1299 cells in the absence (negative control) and presence of doxycycline (DOX). Besides, all treatments were performed after having a cell confluency of 70 to 80 % in each flask. As said before, DOX in the culture medium is required to trigger APEX2-p53 wt expression. The concentration of DOX used here is 2 µg/ml. The treatments took 16 hours (overnight). Total proteins were then extracted from all samples by using RIPA buffer. We did the same with non-transfected H1299 cells (wt H1299) for controlling the

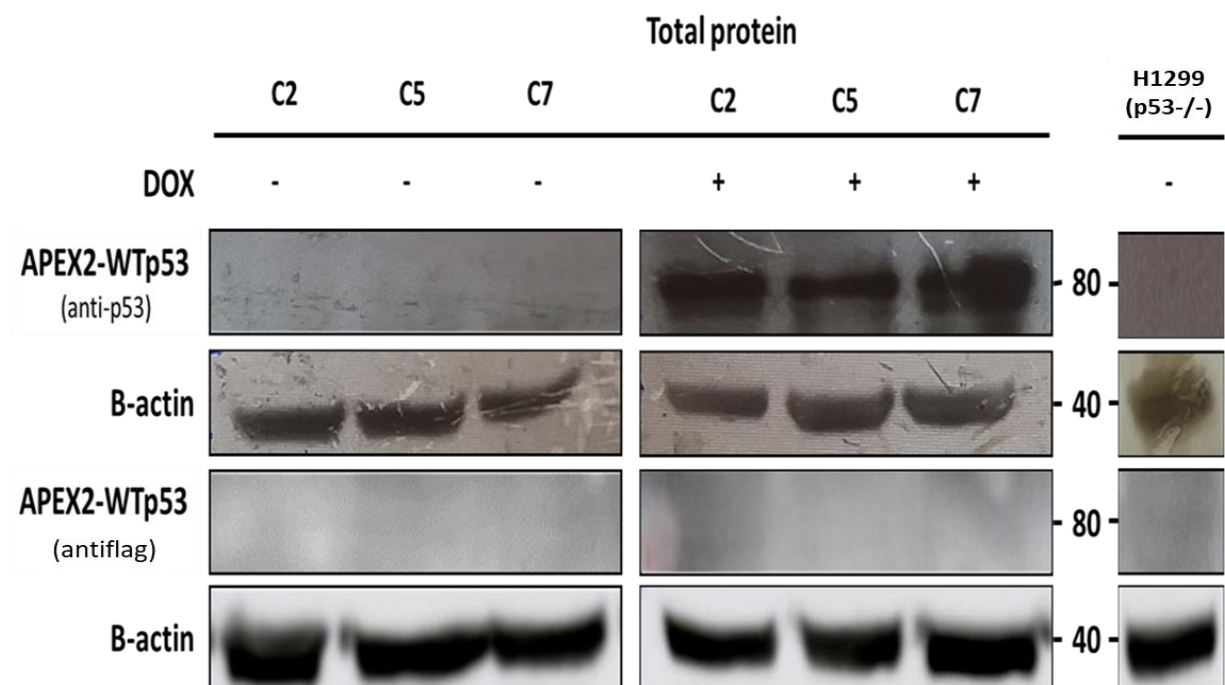


Figure 5: Immunodetection of APEX2-p53 wt proteins using the anti-p53 and anti-flag antibodies. DOX-: no treatment with doxycycline; DOX+: treatment with doxycycline. Total protein samples are above: C2, C5, and C7 for transfected H1299 cells; H1299 (p53 -/-) non-transfected cells. A detection of β -actin proteins was performed as loading control.

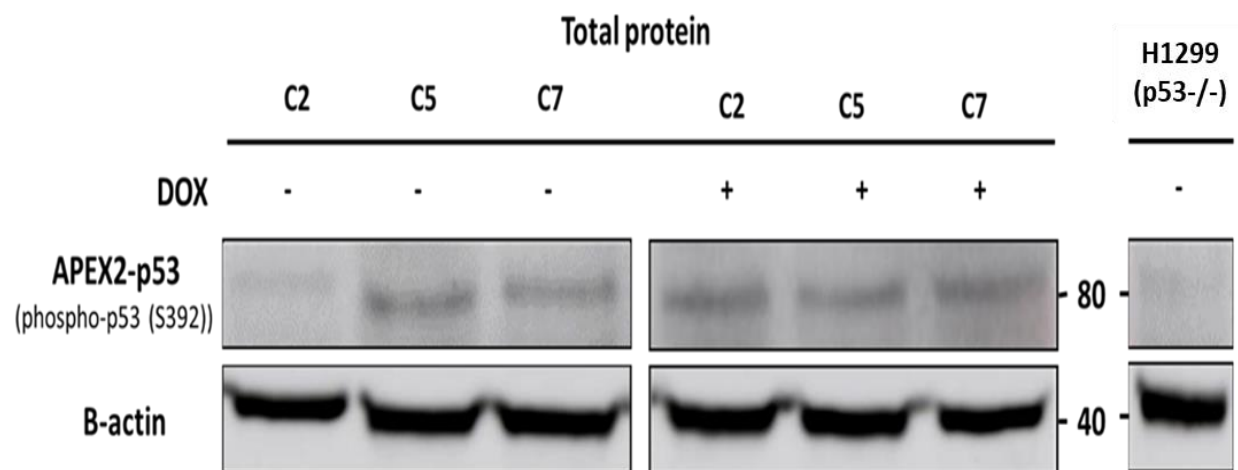


Figure 6: Immunodetection of APEX2-p53 wt proteins a using phospho-specific antibody that recognize the p53 form phosphorylated on S392. DOX-: no treatment with doxycycline; DOX+: treatment with doxycycline. Total protein samples are above: C2, C5, and C7 for transfected H1299 cells; H1299 for non-transfected cells. A detection of β -actin was performed as loading control.

specificity of the antibodies. After SDS-page electrophoresis, we performed an immunodetection for APEX2-p53 wt proteins (80 kDa) using anti-p53 and anti-flag antibodies. Furthermore, we did a loading control by detecting β -actin proteins (40 kDa).

In the absence of DOX, we saw no sign of proteins that may correspond to APEX2-p53 wt (80 kDa). In the presence of DOX, we detected a strong presence of APEX2-p53 wt proteins with the p53 antibody. There were no proteins detected with the anti-flag antibody. The western blot for β -actin showed an equal presence of proteins in each well. Thus, these data indicate that the presence of DOX molecules allows the expression of APEX2-p53 wt within all three samples. (Figure 5)

3.2. Mitochondrial and cytosolic localization of wt p53

As described before, a fraction of p53 localizes to mitochondria in stressed cells and promotes MOMP through interaction with different BCL-2 family members. In addition, phosphorylation of S392 seems to be crucial for mitochondrial localization. The mutation of S392 with alanine decreases the mitochondrial localization of p53 resulting in low apoptosis. We expect that the fusion between APEX2 and p53 has no effect on the phosphorylation of S392. However, it could still influence the mitochondrial localization of p53 somehow. That is what we monitored next. [33]

We extracted total proteins from stably transfected H1299 cells (treated or not with DOX overnight). A SDS-page electrophoresis was then performed. For immunodetection, we used a phospho-specific antibody that recognizes the p53 form phosphorylated on S392. We saw a noticeable level of phosphorylated p53 proteins at S392 residue in both conditions (with or without DOX). On the other hand, there was no sign of phosphorylated p53 proteins in wt H1299 p53 -/- cells. (Figure 6)

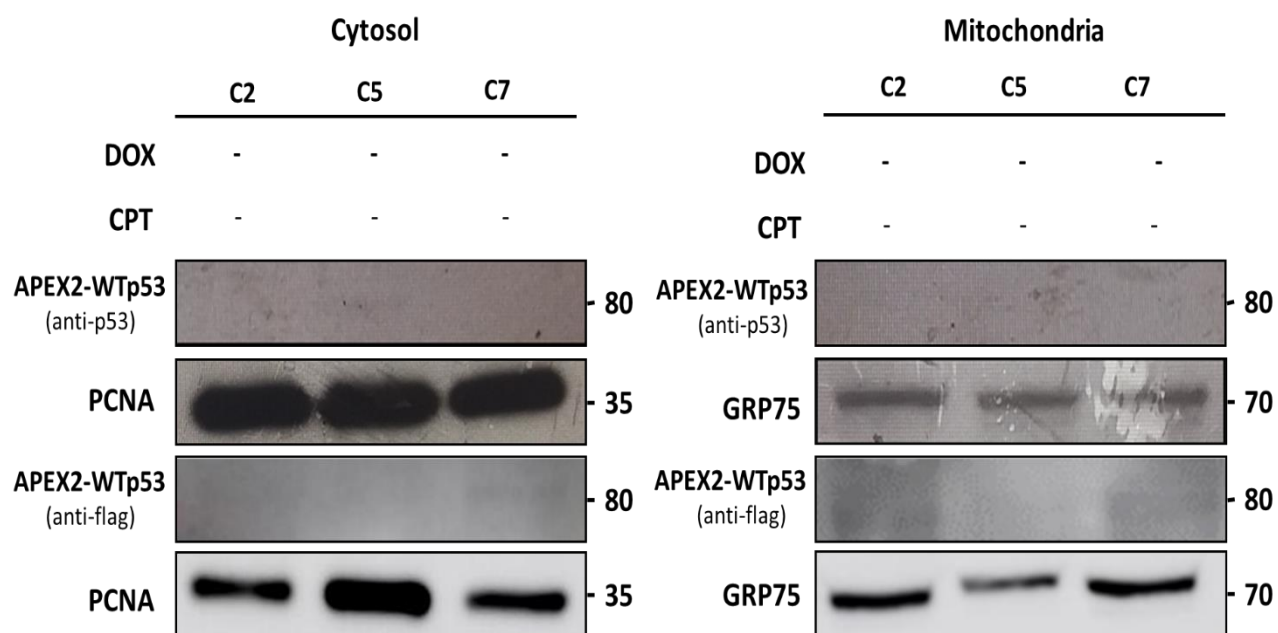


Figure 7: Immunodetection of APEX2-p53 wt proteins in cytosolic and mitochondrial fractions. DOX-: no treatment with doxycycline; CPT-: no treatment with camptothecin. Protein samples of each fraction are indicated (C2, C5, and C7). As loading controls, we performed immunodetection of PCNA (cytosolic and nuclear localization) and GRP75 (mitochondrial protein).

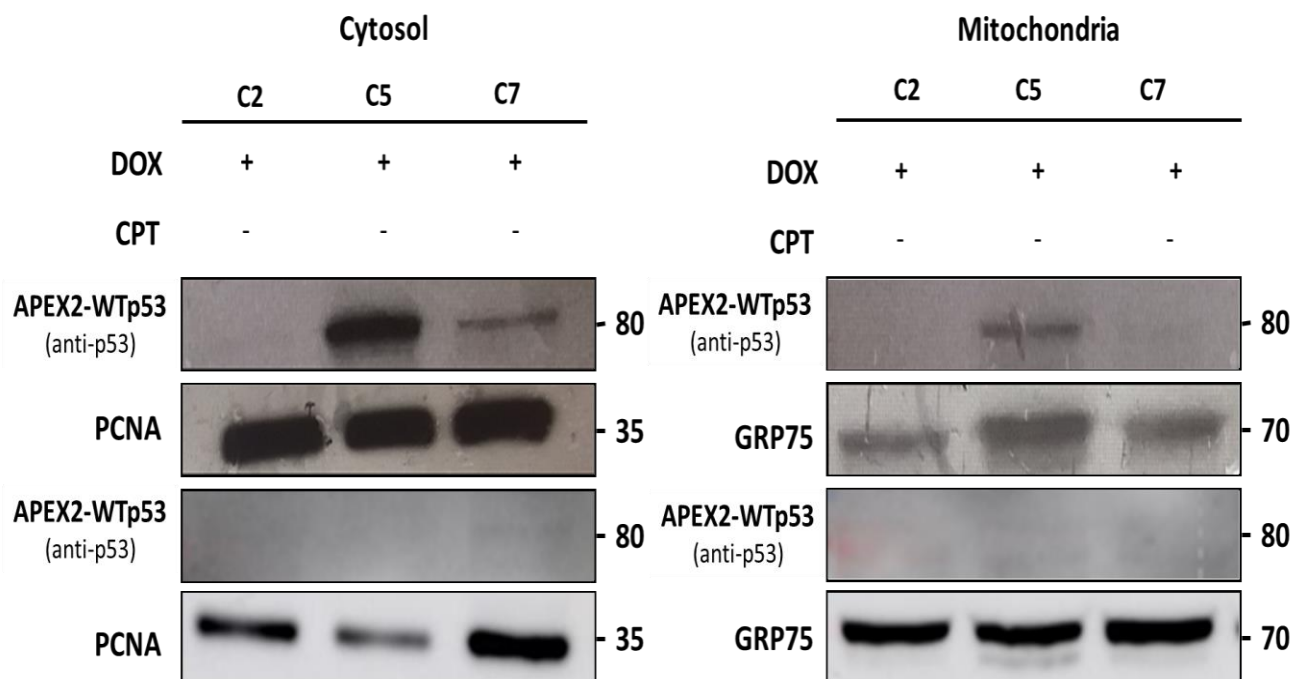


Figure 8: Immunodetection of APEX2-p53 wt proteins in cytosolic and mitochondrial fractions. DOX+: treatment with doxycycline; CPT-: no treatment with camptothecin. Protein samples of each fraction are indicated (C2, C5, and C7). As loading controls, we performed immunodetection of PCNA and GRP75, for respectively the cytosolic and the mitochondrial fraction.

We checked the mitochondrial localization of APEX2-p53 wt in four conditions: no treatment, treatment with DOX, treatment with camptothecin (CPT), and treatment with DOX + CPT. We used CPT to induce DNA damage. CPT is a topoisomerase inhibitor. We treated cells at 70% to 80% of confluency. We treated the necessary flasks with 2 µg/ml of DOX and with 1µM of CPT. Next, mitochondrial fractions were extracted from samples by using differential centrifugation. We used a low-speed centrifugation to remove intact cells, cell debris, and nuclei from whole-cell extracts followed by a high-speed centrifugation to pellet the mitochondria and separate them from other organelles. [40]

After purification of the cytosolic and mitochondrial fractions, we performed SDS-page electrophoresis. Western blot then allowed to detect APEX2-p53 wt proteins using both anti-p53 and anti-flag antibodies. As loading controls, PCNA proteins (35 kDa) were determined in the cytosolic fraction, and GRP75 proteins (70kDa) were detected in the mitochondrial fraction.

Without treatment (Figure 7), the APEX2-p53 wt protein (80 kDa) was not detected within both fractions of C2, C5, and C7 samples. Loading controls (PCNA for cytosolic Fraction; GRP75 for mitochondrial fraction) showed a similar amount of protein in the different wells.

After the treatment with DOX for 16 hours (figure 8), we detected APEX2-p53 wt proteins (80kDa) in both fractions using anti-p53 antibodies. The cytosolic localization of APEX2-p53 wt was remarkable in the C5 and C7 samples. Furthermore, the mitochondrial localization of APEX2-p53 wt was rich in the C5 sample but not in C7. On the other hand, there was no detection of APEX2-p53 wt proteins using the anti-flag antibody. Moreover, APEX2-p53 wt was not detected in the C2 sample.

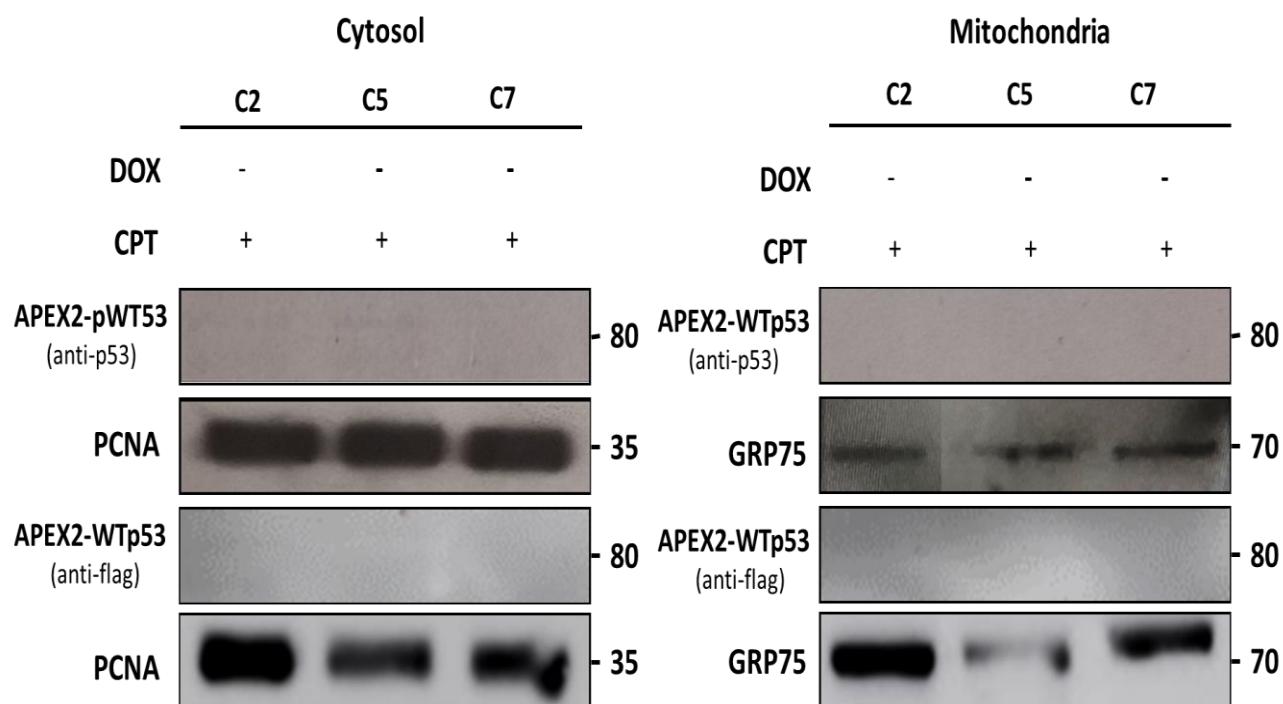


Figure 9: Immunodetection of APEX2-p53 wt proteins in cytosolic and mitochondrial fractions. DOX-: no treatment with doxycycline; CPT+: treatment with camptothecin. Protein samples of each fraction are indicated (C2, C5, and C7). As loading controls, we performed immunodetection of PCNA and GRP75, for respectively the cytosolic and the mitochondrial fraction.

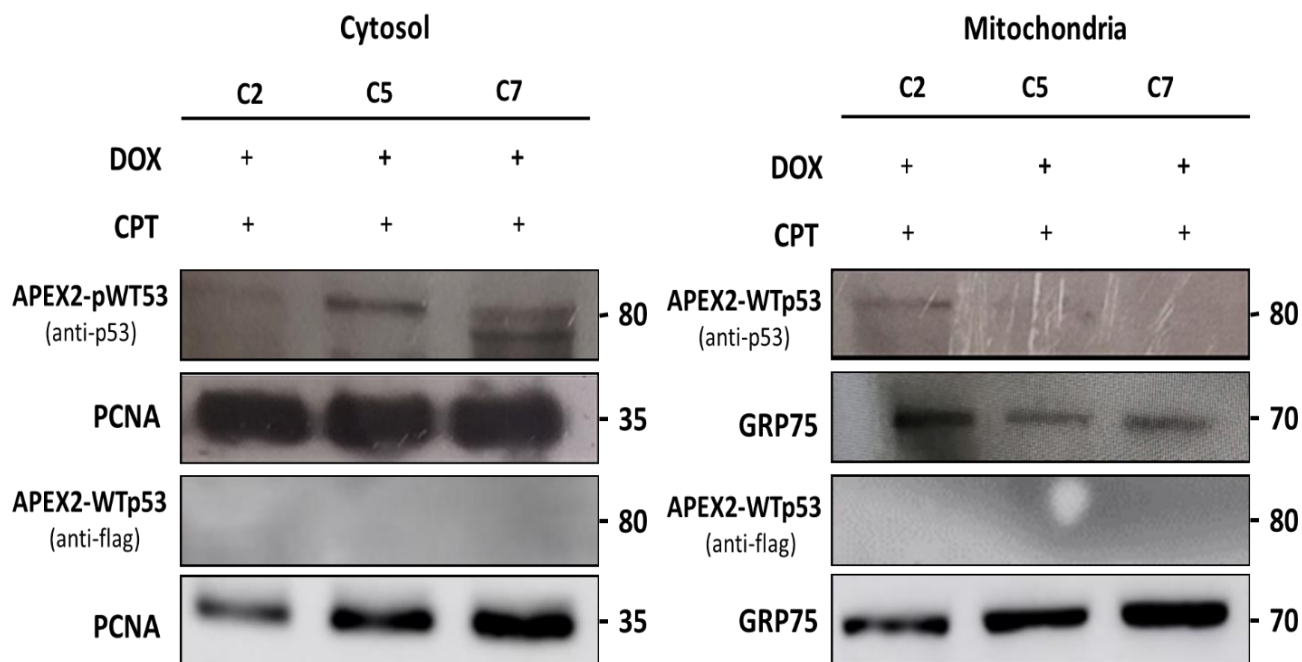


Figure 10: Immunodetection of APEX2-p53 wt proteins in cytosolic and mitochondrial fractions. DOX+: treatment with doxycycline; CPT+: treatment with camptothecin. Protein samples of each fraction are indicated (C2, C5, and C7). As loading controls, we performed immunodetection of PCNA (cytosolic and nuclear localization) and GRP75 (mitochondrial protein).

After the treatment with CPT alone for 15 hours (figure 9), we as expected did not detected APEX2-p53 wt protein (80 kDa) in the cytosolic and mitochondrial fractions. For joint treatments (DOX for 6 hours followed by CPT for 15 hours) (figure 10), the localizations of APEX2-p53 wt proteins seemed to be different between samples. The anti-p53 antibody was able to detect our protein of interest, which was not the case of the anti-flag antibody. The cytosolic localization of APEX2-p53 wt protein was clear in the C5 and C7 samples. Besides, a mitochondrial localization was present in both C2 and C5 samples although weaker in the second. Thus, these data suggest that APEX2-p53 wt can translocate to the mitochondria.

3.3. Transcriptional activity of wt p53

The p53 protein is a transcriptional factor. It can promote or repress the expression of a variety of genes. We verified the transcriptional activity of wt p53 proteins to see if it is undamaged and normal. We checked the expression of MDM2 and p21^{waf1} proteins because these genes are mainly regulated by p53 [40, 42, 43].

The expression of MDM2 and p21^{waf1} proteins was verified under both the absence and presence of APEX2-p53 wt proteins. After treatment or not with DOX for 16 hours, total proteins were extracted from cell suspensions using RIPA buffer. Besides, we did the same for non-transfected H1299 cells (p53 -/-). All protein samples were subjected to a SDS-page electrophoresis. Next, we performed western blot to detect MDM2 (56 kDa) and p21^{waf1} (21 kDa) proteins. Furthermore, we used β -actin (40 kDa) as loading control.

In total protein fractions without DOX (C2, C5, and C7), we could not detect MDM2. On the other hand, we noticed a weak presence of p21^{waf1}. In total protein fractions treated with DOX (C2, C5, and C7), we observed an expression of both MDM2 and p21^{waf1} proteins. Thus, these data suggest that APEX2-p53 wt is able to regulate at least some of its target genes. (Figure 11)

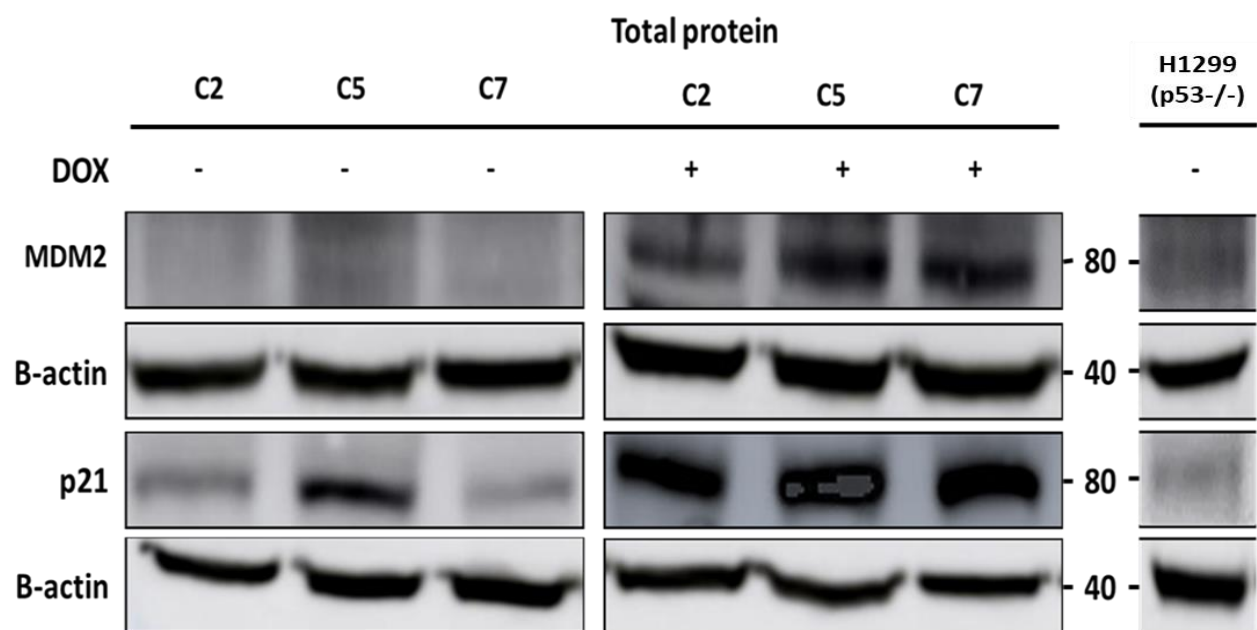


Figure 11: Immunodetection of MDM2 and p21^{waf1} proteins in total protein fraction. DOX -: no treatment with doxycycline; DOX+: treatment with doxycycline. Total protein samples are indicated: C2, C5, and C7 for transfected H1299 cells; H1299 (p53-/-) are non-transfected cells. A detection of β -actin was performed as loading control.

3.4. Induction of apoptosis APEX2-p53 wt

Previously, we verified the mitochondrial localization of APEX2-p53 wt proteins, which was noticeable in C5 sample cells (figure 8). For this reason, we selected C5 cells to highlight the induction of apoptosis. The mitochondrial localization of p53 participates at triggering the intrinsic pathway of apoptosis. The tumor suppressor p53 proteins interacts with different BCL-2 family members to promote outer mitochondrial membrane permeabilization and release of apoptosis effectors with lethal impacts (section 3.2 of the introduction). It is possible to observe a mitochondrial localization of the APEX2-p53 fusion protein without an induction of apoptosis. For this reason, we wanted to check the capacity of the fusion protein to induce cell death.

A flow cytometry method was chosen method to reveal the viability of C5 sample cells in four conditions: no treatment, treatment with DOX (2 µg/ml) overnight; treatment with CPT (10µM) for 48 hours; and treatment with both DOX and CPT. We used LDS-751 and propidium iodide as markers to detect and quantify cell death in each condition. LDS-751 is a cell permeant nucleic acid stain. It can interact with DNA molecules of both healthy and apoptotic cells. Moreover, propidium iodide can penetrate cells because of their loss of membrane integrity and interact with DNA molecules. We generated three replicates in each condition to analyze and compare results through a one-way ANOVA test.

In cell populations treated with CPT alone, we observed 7% of apoptotic cells (figure 12, graph b). In this first condition, the standard deviation of the data was not that important (SD=0.77). Moreover, the ANOVA test demonstrated that the difference between the control and the treatment with CPT was significant (p-value<0.05). When there was only induction of APEX2-p53 wt, the percentage of apoptotic cells was low (~ 1.5%) and similar to the control (~ 1.3%). The presence of APEX2-p53 wt alone appeared not sufficient to induce apoptosis. Therefore, the occurrence of apoptotic cells was weak in graph c (figure 12). The ANOVA test displayed a non-significant difference between treatment with DOX and the control (no treatment).

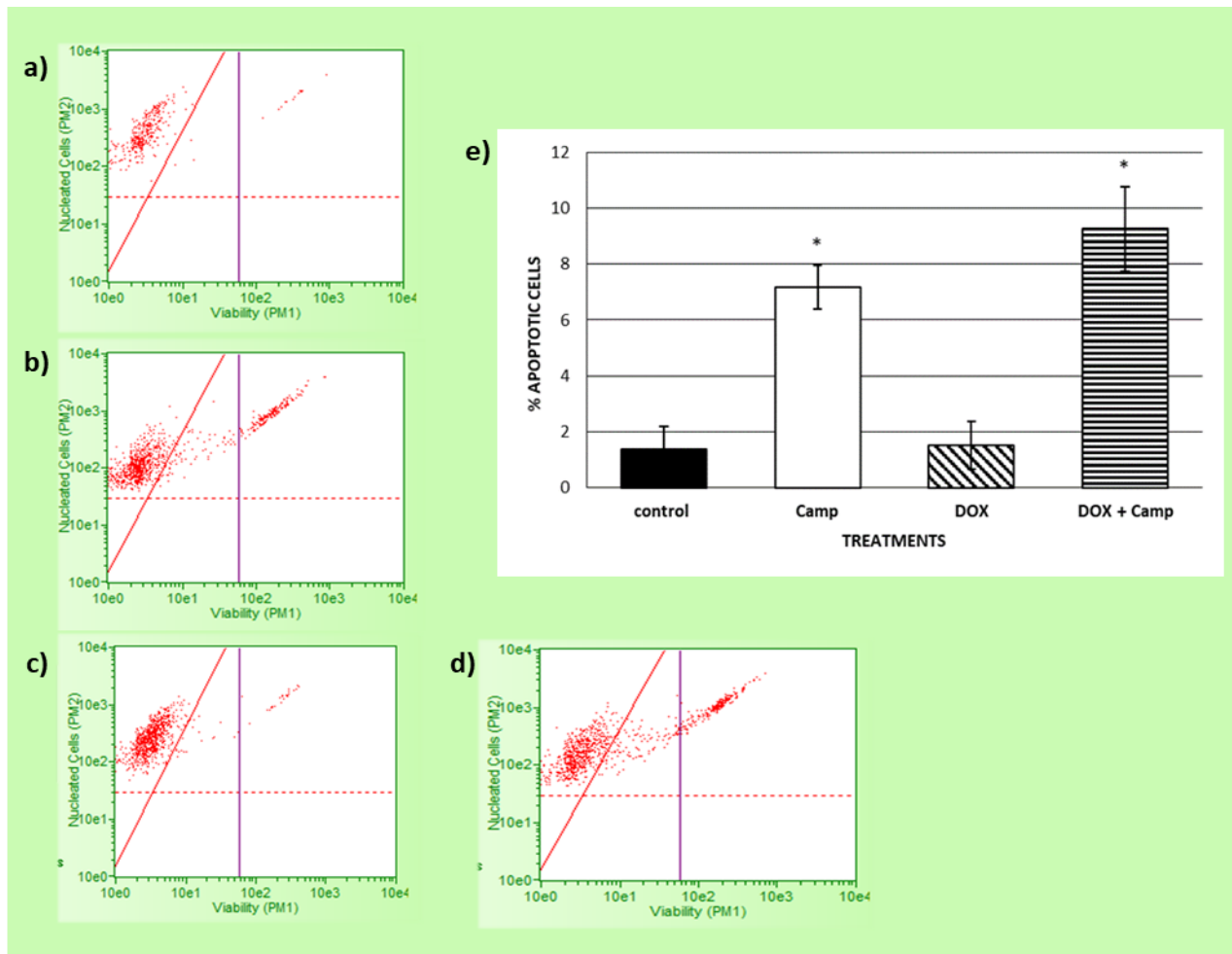


Figure 12: flow cytometry analysis of cell viability. a) example of cell viability data without treatment; b) example of cell viability data after treatment with CPT for 48 hours; c) example of cell viability data after treatment with DOX for 16 hours; d) example of cell viability data after treatment with DOX (16 hours) followed by CPT (48 hours). The red line is a viability marker; The purple line is an apoptosis marker. PM1: propidium iodide staining (viability). PM2: LDS-751 staining to detect apoptotic cells. e) graph describing the flow cytometry data obtained: each column is a mean of the data in each condition; error bars refer to the standard deviation. The asterisks show the significant differences in comparison with control cells (p-value < 0.05).

The induction of APEX2-p53 wt followed by a stress with CPT was the condition presenting the highest percentage of apoptotic cells (figure 12, graph d). The percentage of apoptotic cells (almost 9.2%) is higher and significantly different from the control. We did an ANOVA test to analyze the difference between the CPT and DOX + CPT conditions. The difference although noticeable was not significant (p-value > 0.05).

IV. Discussion

1. Conclusion

p53 tumor repressor is so vital to maintain the integrity of the genome. The p53 pathway is activated in response to stress that could affect and disrupt the fidelity of DNA replication and cell division. Stress signals are transmitted to p53 by post-transcriptional modifications resulting in its stabilization and activation, which leads to cell cycle arrest, cell senescence or apoptosis. The activation of p53 means turning on a whole network of genes in order to respond to stress situation [44]. Here, we are focusing on p53 pro-apoptotic mitochondrial activity. The mitochondrial localization is important for p53 to interact with BCL-2 family members and promote MOMP.

Phosphorylation of S392 is crucial for p53 mitochondrial activity. This PTM occurs after stress induction. The mutation of S392 reduces the mitochondrial localization of p53 and lower the capacity of p53 to induce apoptosis. In this study, we are trying to understand the relation between S392 phosphorylation and p53 mitochondrial translocation. Two hypotheses were established: First, during a stress, the phosphorylation of S392 promotes p53 binding to an unknown cytosolic protein partner. This unknown partner will then guide p53 to mitochondria, making the tumor suppressor able to trigger MOMP. Second, p53 is sequestered and inhibited by one or more cytosolic protein partners. The phosphorylation of S392 that follows the stress disturbs the inhibitory complex, promoting p53 mitochondrial localization [33]. The ultimate goal of this work is the identification of cytosolic p53 protein partners whom interaction with p53 is affected by S392 phosphorylation. The specific goals during this work are: 1) the stable transfection of two constructions (APEX2-p53 wt and APEX2-p53 S392A) in H1299 cells (p53^{-/-}); 2) the characterization of p53 activities when fused to APEX2 (transcriptional activity, ability to localize to mitochondria and induce apoptosis).

We performed a chemical transfection of H1299 cells with the APEX2-p53 wt construction. The selection was performed using puromycin (the vector carrying a puromycin resistance gene). After a selection assay of several weeks, the cells that could grow up in a culture medium supplemented with puromycin, showing that they were stably transfected.

We observed by western blot presence of APEX2-p53 wt proteins in the total protein fraction of samples (figure 5). The expression of APEX2-p53 wt was visible after a treatment with DOX, demonstrating that the Tet-On inducible system is functioning as expected. Of the note, H1299 cell line that we are using has been modified previous by stable transfection of the “regulatory plasmid” (expression of the tetracycline-controlled transactivator rtTA).

The phosphorylation of S392 was observed in all transfected cell samples (figure 6). In the absence of DOX molecules, we noticed a low expression of APEX2-p53 wt. This is expected from Tet-ON systems, and we call it a background expression. Because of the imperfection of Tet-On systems, low background expressions may occur even in the absence of effectors (Dox molecule). Many studies try to minimize these background expressions. [46]

The mitochondrial translocation of APEX2-p53 wt proteins was not the same between samples. After inducing the expression of APEX2-p53 wt with DOX, we observed a noticeable mitochondrial localization in the C5 sample. The cytosolic localization of APEX2-p53 wt was strong in C5 sample cells than in others. It could be hypothesized that the more we have APEX2-p53 wt proteins in the cytosol, the greater the mitochondrial localization will be. The APEX2-p53 wt protein was not detected in the cytosol of C2 sample cells (fig 8, 10), although the APEX2-p53 wt construction is indeed expressed within these cells (figure 5). The cytosol is a common localization of p53 proteins. However, p53 also localizes to other cellular compartments such as the nucleus because of its NLSs (12). We noticed weak mitochondrial localizations in both C5 and C7 samples under both DOX and CPT treatments (figure 10). Although a strong mitochondrial localization of APEX2-p53 wt in the presence of DNA damage (CPT) was expected.

We noticed transcriptional activities after the induction of APEX2-p53 wt. The expression of MDM2 and p21^{Waf1} was strong and important (figure 11). We know that MDM2 proteins inhibit p53 tumor suppressor functions through direct interaction, blockade of the MDM2-p53 protein-protein interaction would liberate p53 to regain its functions again. We must also know about the reciprocal influences between p53 and MDM2 [41]. p53 induces the expression of MDM2, which in return promotes the degradation of p53 and quenches the p53 cellular activity [42]. The expression of the cell cycle-dependent kinase (cdk) inhibitor p21^{Waf1} is also induced by p53. Moreover, p21^{Waf1} plays a role in cell cycle arrest and DNA damage. [43]

We checked the induction of apoptosis in C5 sample cells because they present a clear mitochondrial localization of APEX2-p53 wt proteins (figure 12). We observed apoptotic cells after a stress with CPT (figure 12b). CPT is known to trigger an apoptosis that is essentially p53 and mitochondria-dependent. However, in the absence of p53 proteins, other mechanisms can supplement [47]. In the presence of both CPT (DNA damage induction) and DOX (APEX2-53 expression), the apoptosis rate was similar to CPT alone (figure 12d, e). There was not significant difference between the two treatments The expression of APEX2-p53 wt with no stress showed a low level of apoptosis as well as the control (non-significative differences).

2. Perspectives

We used site directed mutagenesis to create the S392A substitution within 2466 bp and 2468 bp of the p-TRE2 vector. This construction (APEX2-p53 S392A) should be verified by enzymatic restriction and sequencing. Primers are required to sequence the entire construction. Samples will be sent for sequencing as a mixture of 0.5 µg from plasmids and 30 pmol from primers.

We attempted to analyze the ability of APEX2-p53 wt to induce apoptosis during stress but the data obtained were not optimal. For this reason, additional experiments must be performed, taking in consideration the time and concentration of treatments.

The labeling reaction is important to develop before the verification of APEX2 activity. We should determine the optimal parameters for non-toxic labeling reaction. Main parameters are concentrations and time of incubations with biotin phenol (BP) and H₂O₂.

After determining the parameters for the labeling reaction, we must verify the activity of APEX2. In other words, we should check the ability of APEX2 (engineered ascorbate peroxidase 2) to transform biotin phenol to biotinphenoxy radicals. We can use immunofluorescence staining of biotinylated proteins or streptavidin blot analysis of whole-cell lysates.

Another important thing to do is the optimization of streptavidin pull-down of cytosolic biotinylated proteins. we must focus on two things here: the number of beads and series of washes to remove unwanted proteins.

V. Materials & methods

1. Cell culture

All cell culture manipulations are carried out in a sterile medium in order to avoid any contaminations. Cell culture is done in a laminar flow hood with the use of gloves and sterile equipment. Any other non-sterile material is first disinfected.

1.1. Cell lines

The line used during this thesis is the H1299 cell line (cells from lung cancer with the particularity of not expressing p53).

1.2. Preparation of the culture medium

Material:

- 500 ml of DMEM high glucose (4 ° C) (Dulbecco's Modified Eagle Medium, Invitrogen cat. No. 42430)
- 10% (50 ml) of decompemented FBS (fetal bovine serum) (-20 ° C) (BioWhittaker™ Lonza cat. n ° DE 14-802F)
- 1% (5ml) of Streptomycin / Penicillin antibiotics (-20 ° C) (Penicillin 10,000 g / ml and Streptomycin 10,000 ug / ml, GIBCO® Invitrogen™ cat. n ° 15140)

Operating mode:

- In a laminar flow hood, supplement the high glucose DMEM medium with 50ml of FBS and 5ml of antibiotics.
- Store at 4 ° C for 1 month.

1.3. Thawing H1299 cells

Because the cells have been cryogenized in DMSO that is toxic, the thawing should take place as quickly as possible in order to remove this DMSO.

Material:

- Cryotube containing cells (liquid nitrogen).
- Supplemented DMEM culture medium, previously heated to 37 ° C.

- 1 faclon™ of 15 ml / cryotube to thaw.
- 1 flask 75cm / cryotube to thaw.
- 1 berlin containing water at 42 ° C.

Operating mode:

- Take the cryotube out of the liquid nitrogen.
- Place the cryotube in the berlin containing the water at 42 ° C.
- Take the content of the cryotube and put it in a 15ml faclon™ "containing 9ml of the culture medium.
- Centrifuge the faclon™ "at 20 ° C, 10 min at 500xg.
- Remove the supernatant.
- Resuspend the cell pellet with 13 ml of culture medium and transfer to a flask.
- Place the flask in the incubator at 37 ° C, 5% CO2.
- Renew the culture medium the day after unfreezing.

1.4. Cryogenization

Materials:

- Sterile PBS (phosphate-buffered saline, GIBCO® Life Technologies™ cat. N ° 18912-014).
- Trypsin-EDTA (stored at 4 ° C) (10x trypsin, 10x diluted in sterile PBS Bio Whittaker).
- Sterile cell culture medium (stored at 4 ° C).
- Sterile serum + DMSO (10%) solution.
- Cryotube.
- 1 faclon™ of 15 ml.

Operating mode:

- Preheat PBS, trypsin and culture medium to 37 ° C.
- Flask of 75 cm² at 80% confluence.
- Aspirate the culture medium.
- Rinse with 8ml of PBS.
- Unhook the cells with 2ml of trypsin.

- Add 8ml of culture medium to 2ml of trypsin, take up the cell suspension and place it in a faclon™ of 15 ml.
- Centrifuge at room temperature, 10min at 500xg.
- Discard the supernatant.
- Resuspend the pellet with 1ml of serum + DMSO (10%) and transfer 1ml to a cryotube.
- Place the cryotubes in a container containing isopropanol at room temperature.
- After 24 hours, place the cryotubes in liquid nitrogen.

1.5. Change of culture medium

Equipment:

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

Operating mode:

For a 75 cm² flask:

- Preheat the PBS and the culture medium to 37 ° C.
- Aspirate the culture medium.
- Wash with 8 ml of PBS.
- Add 13 ml of culture medium.

1.6. Passage of cells

Material:

- Sterile PBS.
- Sterile Trypsin-EDTA.
- Sterile cell culture medium.

Operating mode:

For a 75 cm² flask (Cellstar® Culture Flasks, Greiner Bio-One cat. N ° 658 175):

- Preheat PBS, trypsin and culture medium to 37 ° C.

- Aspirate the culture medium using a vacuum pump.
- Wash with 8 ml of PBS.
- Peel off the cells with 2 ml of trypsin.
- Neutralize the trypsin by adding 8 ml of culture medium.
- Homogenize by up & down (do it gently).
- Transfer the volume of cell suspension to a new annotated flask.
- Adjust to a volume of 13 ml with the culture medium for a 75 cm² flask.

2. Treatments

2.1. Treatment with CPT for stress induction

Material:

- Sterile PBS (stored at 4 ° C).
- Sterile cell culture medium (stored at 4 ° C) to supplement on the day of manipulation with 1 µM of CPT (Sigma® cat. N ° C9911).
- Flask 75 cm² at 80% confluence.

Operating mode:

- Heat the PBS and the culture medium to 37 ° C.
- Flask 75 cm² at 80% confluence.
- Remove the culture medium.
- Rinse with 8 ml of PBS.
- Put 12 ml of medium containing 1 µM of CPT.
- Place the flask in the 37 ° C incubator and wait for the desired incubation time.
- Perform the desired experience.

2.2. Treatment with DOX to induce expression

Material:

- Sterile PBS (stored at 4 ° C).
- Sterile cell culture medium (stored at 4 ° C) to supplement on the day of manipulation with 2 µg/ml of DOX (Sigma® cat. N ° D9891-1G).
- Flask 75 cm² at 80% confluence.

Operating mode:

- Heat the PBS and the culture medium to 37 ° C.
- Flask 75 cm² at 80% confluence.
- Remove the culture medium.
- Rinse with 8 ml of PBS.
- Put 12 ml of medium containing 2 µg/ml of DOX.
- Place the flask in the 37 ° C incubator and wait for the desired incubation time.
- Perform the desired experience.

3. Cell viability test by flow cryometry

3.1. LDS-751 marking / Propidium iodide

Principle:

This test allows cells to be counted and their viability to be determined. The flow cytometer distinguishes viable and non-viable cells based on their difference in permeability to different markers. LDS-751 is a cell permeable DNA intercalator while propidium iodide, a second intercalating agent, enters cells only when there is a loss of membrane integrity. The dead cells therefore contain the dye LDS-751 and propidium iodide.

Equipment:

- Guava ViaCount reagent (Millipore® cat. N ° B76075).
- Flow Cytometry Device, Guava EasyCyte™ 6. Millipore®).
- Material used for stress induction (see 2).

- faclon™ 15 ml / flask.

Operating mode:

- 1 flask of 75 cm² at 80% confluence / treatment.
- Treat the cells (see 2).
- After the desired treatment time, empty the flasks, keeping the medium in an annotated faclon™ of 15 ml.
- Wash the flasks with 8 ml of PBS.
- Peel off cells with 2 ml of trypsin. **Warning:** In order to avoid a high basal cell death rate, avoid up & down and / or mechanical shocks to unhook the cells, they must come off naturally under the action of trypsin.
- Inhibit trypsin with the flask medium recovered previously.
- Gently homogenize and transfer to the annotated 15 ml falcon™ and place them on ice cream.
- In an Eppendorf tube, mix 20 µl of the cell medium with 80 µl of reagent Guava ViaCount
- Incubate for 5 min at room temperature. **Warning:** In order to protect the markers, proceed in a place far from natural light.
- Analyze the samples with the flow cytometer (ViaCount module of the Guava program CytoSofiT™ system).

4. Total protein extraction

Equipment:

- Non-sterile PBS at 4 ° C (Phosphate-Buffered Saline, GIBCO® Life Technologies™, cat. N° 18912-014)
- 1 rake / condition
 - RIPA buffer (radioimmunoprecipitation assay buffer):
 - 50 mM Tris-HCl pH = 7.4 (MP Biomedicals cat. N ° 819623).
 - 150 mM NaCl (Sodium chloride 99.5%, AcrosOrganics® cat. N ° 207790010).
 - 0.1% SDS.
 - 1% Sodium deoxycholate (Sigma® cat. N ° 30970).
 - 1% Triton X-100 (Sigma® cat. N° T9284).

- Lysis buffer to be prepared on the day of the extraction:
 - 10 ml RIPA + 1 tablet of protease inhibitors at 4 ° C (cOmplete Mini EDTA-free, Roche cat. n° 11 836 170 001)

Note: Phosphatase inhibitors are added if we want to study the presence of phosphorylation:
Sigma cocktail 2: 40ul / 10ml and Sigma cocktail 3: 40ul / 10ml.

Operating mode:

- When the cells are at 90% confluence, unhook / scrape the cell layer in the culture medium using a sterile rake (1 / condition).
- Collect the cell suspension in a faclon™ of 15 ml.
- Centrifuge at 4 ° C, 10 min at 500xg.
- Discard the supernatant, resuspend the cells in 10 ml of ice-cold PBS.
- Centrifuge at 4 ° C, 10 min at 500xg.
- Discard the supernatant, resuspend the cells in 200 µl lysis buffer and transfer the solution in an Eppendorf tube of 1.5 ml.
- Incubate on ice for 15 minutes.
- Sonicate for 30 sec.
- Centrifuge at 4 ° C, 15 min at 15,000xg.
- Collect the supernatant in an "annotated" Eppendorf tube.

5. Cell fractions

5.1. Mitochondrial extraction

Principle:

Based on differential centrifugations, the mitochondria as well as the cytoplasm are isolated. Note that the mitochondrial fraction isolated in this way could also contains organelles of similar densities, mainly lysosomes and peroxisomes.

Equipment:

- Non-sterile PBS at 4 ° C (Phosphate-Buffered Saline, GIBCO® Life Technologies™ cat. No. 18912-014).
- Dounce-Type B homogenizer.
- Trypan Blue (Trypan Blue Solution 0.4%, Sigma - Aldrich cat. N ° T8154).
- Counting blade and coverslip (Marienfeld Fuchs-Rosenthal, depth 0.200mm, surface 0.0625mm).
- 1 rake / condition.
- 1 falcon™ 50 ml / condition.
- 2 Eppendorf tubes of 2 ml / condition.
- 6 Eppendorf tubes of 1.5 ml / condition.

Buffer N ° 1:

- 10 mM Hepes-NaOH pH 7.4 (Sigma-Aldrich cat. N ° H3375).
- 1 mM EDTA pH 7.4 (Sigma - Aldrich cat. N ° E5134).
- 0.2 mM EGTA PH 7.4 (Sigma - Aldrich cat. N ° E4378).
- 300 mM D-Mannitol (Biochemika cat. N ° 63559).

Add the protease inhibitor on the day of manipulation: 1 tablet of E-64 10mg for 10 ml of buffer N° 1 (Roche - cat. N ° 10 874 523 001).

Note: Phosphatase inhibitors are added if we want to detect the phosphorylation of a protein by western blot: Sigma cocktail 2: 40 µl / 10ml and Sigma cocktail 3: 40 µl / 10ml.

Buffer N ° 2:

- 10 mM Hepes-KOH pH 7.4 (Sigma-Aldrich cat. N ° H3375).
- 1 mM KH PO-KOH pH 7.4 (Merck Chemicals cat. N° 1048729025).
- 0.2 mM EGTA pH 7.4 (Sigma-Aldrich cat. N ° E4378).
- 300 mM D-Mannitol (Biochemika cat. N ° 63559).

Note: The buffer must be brought to pH with KOH for the viability of mitochondria (ex: oligomerization of BAK). In other cases (ex: extractions mitochondria for SDS-Page gel) the pH can be adjusted with NaOH.

Note: All operations are carried out at 4 ° C

→ On ice

→ Centrifuges at 4 ° C

Operating mode:

- Supplement 20 ml of buffer No. 1 with 2 tablets of inhibitors (2.6 ml TI / conditions)
- From the culture flask at 80% confluence, remove the cells mechanically using a scraper.
- Place the cell suspension in a falcon™ of 50 ml.
- Centrifuge at 4 ° C, during 6 min at 500xg.
- 2 washes with PBS (PBS should be cold):
 - Discard the supernatant and suspend the pellet with 10 ml of cold PBS.
 - Centrifuge at 4 ° C, 6 min at 500xg.
 - Discard the supernatant and suspend the pellet with 10 ml of cold PBS.
 - Centrifuge at 4 ° C, 6 min at 500xg.
- Discard the supernatant and suspend the pellet in 2 ml of buffer N °1.
- Place the suspension in the homogenizer. Perform “back and forth” for a lysis cellular greater than or equivalent to 50% (40 times are enough for H1299 cells).
- Check the percentage of lysis by removing 20 µl of suspension + 20 µl of blue trypan and examine under a microscope.
- Place the homogenized in Eppendorf tube of 2 ml (s), store on ice.
- Centrifuge at 40C. 10 min at 500xg.
- Collect the supernatant in a new Eppendorf tube of 2 ml (the pellet consisting of intact cells and nuclear debris). Transfer the supernatant by pouring directly the contents of the Eppendorf tube in a new one, without using a pipette.
- Centrifuge at 4 ° C, 5 min at 500xg.
- Recover the supernatant in a new 1.5 ml Eppendorf tube (the pellet being constituted intact cells and nuclear debris). Transfer the supernatant by pouring directly the contents of the Eppendorf tube a new one, without using a pipette.
- Centrifugate at 4 ° C 10 min at 500xg.

- Collect the supernatant in a new Eppendorf tube (the pellet consisting of cells intact and nuclear debris). Transfer the supernatant by directly pouring the contents of the Eppendorf tube into a new one, without using a pipette.
- Centrifuge at 4 ° C. 6 min at 500xg.
- Recover the supernatant in a new Eppendorf tube (the pellet consisting of cells intact and nuclear debris). Transfer the supernatant by directly pouring the contents of the Eppendorf tube in a new one, without using a pipette.
- Centrifuge at 4 ° C, during 10 min at 9000xg.
- Recover the supernatant in new Eppendorf tube, it being composed of the cytoplasmic fraction.
- The pellets containing the mitochondria are kept on ice but remove all traces of supernatant + quick-spin to remove traces of buffer.
- Resuspend the pellets in 600 µl of buffer N ° 1.
- Centrifuge at 4 ° C, during 6 min at 500xg.
- Recover the supernatant containing mitochondria, in an "annotated Eppendorf tube".
- Centrifuge at 4 ° C, during 10 min at 9000xg with the cytoplasmic fraction recovered earlier.

Mitochondrial fraction:

- Remove as much supernatant as possible with a pipette.
- Perform a "Quick-Spin" to pellet the mitochondria and facilitate elimination of remaining traces of supernatant.
- Resuspend the mitochondrial pellet with 10-200 µl of Buffer N° 2.
- Store at -20 ° C.

Cytoplasmic fraction:

- recover the supernatant and avoid the pellet.
- Store at -20 ° C.

6. Protein assay

Equipment:

- Bio-Rad DC Protein Assay Kit: Reagents A, B and S (Bio-Rad DCT Protein Assay, Bio-Rad cat N ° 500-01 13.500-0114 and 500-0115)
- Reaction solution: 20 µl of reagents per 1 ml of reagent A (25 µl of mix / well)
- 96-well plate (s)
- BSA 2 mg / ml diluted in RIPA (see V.0) + 1 tablet of protease inhibitors for 10 ml (Roche-cat. N ° 10 874 523 001) (-20 ° C)
- Spectrophotometer (Microplate Reader Model 680 XR. Bio-Rad)

Operating mode:

- In the 96-well plate, put 2 µl of each sample well in duplicate or triplicate.
- On the same plot, also triplicate the BSA calibration line: 0 µl. 1 µl. 2 µl. 5 µl. 10 µl
- Add 25 µl of reaction solution per well.
- Add 200 µl of Reagent B per well.
- Incubate for 20 minutes at room temperature.
- Measure the absorbance at 650 nm.
- Calculate the protein concentration of the sample based on the calibration line.

7. Western blot

7.1. SDS-Page gel electrophoresis

Equipment:

- Equipment for SDS-Page electrophoresis.
- 0.5 M Tris-HCl buffer pH 6.8 (MP Biomedicals-Cat N ° 819623).
- 1.5 M Tris-HCl buffer pH 8.8 (MP Biomedicals-Cat N ° 819623).
- 10% SDS solution in milliQ water (Sodium dodecyl Sulfate - Sigma-Aldrich - Cat. N° 13771).
- 5x electrophoresis buffer. For 500 ml:
 - 250 mM Tris-15,14 g (MP Biomedicals-Cat. N° 819623).
 - 750 mM Glycine -28.15 g (MP Biomedicals-Cat. N° 04808822)

- 0.5% SDS = 2.5 g (Sodium Dodecyl Sulfate -Sigma-Aldrich-Cat N° L3771).
- Adjust the volume to 500 ml with 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-Cat N° 819623).
 - 5% SDS = 250 mg (Sodium Dodecyl Sulfate -Sigma-Aldrich-Cat. N° L3771).
 - 250 mM DL-1,4-Dithiothreitol (DTT) = 192.8 mg (Acros organics - Cat. N° 327190100).
 - Less than 0.125% of Bromophenol Blue = spatula tip (Sigma-Aldrich-Cat. N° B8026).
 - Adjust with 125 µl of milliQ water.
 - Store at -20 ° C.

Gel preparation:

Gels	Products	Volumes
Separation gel (preparation for 2 gels)	1.5M Tris-HCl buffer pH 8.8	2.5 ml
	30% Acrylamide / Bis Solution 29: 1 (BioRad-Cat N° 161-10156) CAUTION: Neurotoxic product. So, wear gloves and handle with precaution.	6.6 ml
	10% SDS	200 ml
	H2O milliQ	8 ml
	APS 10% (Ammonium persulfate - Sigma-Aldrich - Cat N° A3678)	150 µl
	TEMED (N, N, N', N-Tetramethyl-ethylene Organics Cat N° 138450500)	15 µl
Concentration gel (preparation for 2 gels)	0.5M Tris-HCl buffer pH 6.8	2.5 ml
	30% Acrylamide / Bis Solution 29: 1 (BioRad-Cat N° 161-10156) CAUTION: Neurotoxic product. So, wear gloves and handle with precaution.	1.6 ml
	10% SDS	100 µl
	10% SDS	5.7 ml

	APS 10% (Ammonium persulfate - Sigma-Aldrich - Cat N° A3678)	75 µl
	TEMED (N, N, N', N-Tetramethyl-ethylene Organics Cat N° 138450500)	7.5 µl

Operating mode:

- Prepare the separation gel (IAPS and TEMED triggering the polymerization of the gel).
- Pour the separation gel, leave 2.5cm of empty space before reaching the limit superior of the glass plate. (Space for pouring concentration gel).
- Pour a thin layer of isopropanol on the surface of the gel in order to remove air and have a straight surface.
- Leave to polymerize and remove isopropanol.
- Prepare the concentration gel.
- Pour the gel and place the comb in it

Preparation of samples:

- Take the desired amount of sample, adjust the volume if necessary, to 20 µl with milliQ water.
- Add 5 µl of 5X Laemmli buffer.
- Pierce the cap of the Eppendorf tube with a needle.
- Perform a rapid centrifugation to pellet the sample at the bottom of the Eppendorf tube.
- Place the tubes for 10min on the heating block at 95 ° C.
- Perform a rapid centrifugation to pellet the sample at the bottom of the Eppendorf tube.

Performing electrophoresis:

- Place the gels in the tanks provided for this purpose
- Prepare the electrophoresis buffer by diluting it 5x with milliQ water.
- Fill the tanks.
- Remove the combs from the gels, replace with buffer if necessary.
- Load the samples into the wells (25pLwells). Load 5 µl of Weight Marker (Page Ruler™ Prestained Protein Ladder Fermentas Cat. No. SM0671)
- Migrate the samples at 200 V for 45min

7.2. Transfer in liquid condition

Material:

- Tanks and equipment provided for this purpose.
- Transfer membranes (GE Healthcare-Amersham HybondM ECLTM Cat N° RPN2020D).
- 0.2% PBS-Tween208 (Sigma-Aldrich-Cat N ° P 7949-500ML).
- Blotting paper.
- Transfer buffer (for 5 L).
 - 15g Tris (MP Biomedicals- Cat N ° 819623).
 - 72g Glycine (MP Biomedicals-Cat N ° 04808822).
 - SDS 10% = 5 ml (Sodium dodecyl Sulfate-Sigma-Aldrich-Cat N° 13771).
 - 4 l of milliQ water.
 - 1 l of methanol.

Operating mode:

For 1 gel:

- Cut out 4 blotting paper (7.5x6cm).
- Cut out a transfer membrane (7.5x8.5cm).
- In the planned device.
- Place a sponge soaked in transfer buffer.
- Place 2 blotting papers soaked in transfer buffer.
- Place the SDS-Page gel on which the concentration gel is removed.
- Place the transfer buffer soaked in transfer buffer.
- Place 2 blotting papers soaked in transfer buffer.
- Place a sponge soaked in transfer buffer.
- Close the device and place it in the tank filled with transfer buffer.
- transfer membrane must be on the positive terminal side.
- Place an “ice cube” block in the tank.
- Perform the transfer: 1h30 at 300mA.
- Collect the transfer membrane, gently roll it up and place it in a 50mL falcon filled with PBS Tween20 (0,2%)

7.3. Immunodetection

Materials:

- PBS-Tween208 (Sigma-Aldrich-Cat N° P7949-500ML)
- Milk (Regular & Skimmed milk 0% Fat)
- BSA (MP Biomedicals - Cat N° 160069)
- Plastic shirts.
- X-ray cassette.
- Chemiluminescence reagent (Perkin Elmer Cat N° NEL 104001EA)
- Developer (Iford Multigrade Developer Cat N° 1757855)
- MQ water bath
- Fixer (Agfa HealthCare-Cat. N° G354)
- Primary antibodies:
 - Anti-p53: mouse monoclonal IgG. (Santa Cruz Biotechnology-p53 FL-393-Cat N° sc-126). Dilution used: (1/400)
 - Anti-flag: mouse monoclonal IgG. Dilution 1/1000.
 - Anti-MDM2: Mouse mAb (IF2). (Sigma-Alidrich-Cat N° OP46). Dilution: 1/1000.
 - Anti-p21^{waf1}: mouse monoclonal IgG. (Santa Cruz Biotechnology. Cat N°sc-6246). Dilution 1/1000.
 - phospho-p53 (S392): mouse monoclonal IgG. Dilution 1/1000.
 - Anti-β actin: mouse monoclonal IgG. Dilution 1/2000.
 - Anti-GRP75: mouse monoclonal IgG. (Santa Cruz Biotechnology. Cat N°sc-133137). Dilution 1/1000.
 - Anti-PCNA: mouse monoclonal IgG (Santa Cruz Biotechnology. Cat N°sc-56). Dilution 1/1000.
- Secondary antibodies:
 - Mouse Anti-IgG: Polyclonal Goat Ac (Cat. No. P0447). Dilution used: 1/2000
 - Goat Anti-IgG: Rabbit polyclonal Acid coupled to horseradish peroxidase. (Dako Cat N° PO160). Dilution used: 1/2000

Operating mode:

- Incubate the western blot membranes with 10mL of PBS-Tween 0.2% -5% milk for 30 min; throw away
- Add the Primary Ab diluted in 10 ml of PBS-Tween20% 0.2-5% milk (PBS-Tween200 0.2% - 3% BSA for Anti-BAK antibody).
- Incubate for 1 hour at room temperature (3 hours for Ab Anti-BAK) or 12 hours at 4 ° C.
- Perform 4 washes of 5 min with 10ml of PBS-Tween20 0.2%
- Incubate the membranes with the secondary antibody diluted in 10 ml of PBS-Tween20 0.2%-5% milk for 1 hour at room temperature.
- Perform 4 washes of 5 min with 10ml of PBS-Tween20 0.2%
- Prepare the chemiluminescence reagent: mix in equal volumes the two reagents.
- Attach a plastic sheet to the bench. Add 4 ml of the reagent chemiluminescence on its surface.
- Place the western blot membrane on the reagent (proteins facing the reagent). Incubate 1min.
- Recover the membrane, remove the excess reagent against a blotting paper. Place the membrane in a plastic jacket.
- Place everything inside the autoradiography cassette.

In dark room:

- Place photographic film in the cassette. Incubate desired time according to the antibody.
- Collect the film, place the film in the developer.
- Rinse in a water bath.
- Place the film in the fixer.
- Rinse with water and dry.

7.4. “Stripping” of the western blot membranes

Equipment:

- Stripping solution:
 - 2% SDS - 2g / 100mL (Sigma-Aldrich – Cat N° L3771).
 - 100mM β -Mercaptoethanol - 704HL / 100mL (Sigma-Aldrich – Cat N° M6250).
 - 50mM Tris-HCl pH 6.8-5mL / 100mL (MP Biomedicals – Cat N° 819623).
 - Adjust to 100mL with milliQ water.
 - Store at 4 ° C

Operating mode:

- Preheat the “stripping” solution to 50 ° C in a water bath.
- Place the western blot membrane in the “stripping” solution, with agitation for 30 min.
- Recover the stripping solution (can be used 5 times again).
- Rinse 5 times 10min and under.
- Store the membrane at 4 ° C in 0.2% PBS-Tween20®.
- Shake the membrane with 0.2% PBS-Tween 20®.

Caution: β -Mercaptoethanol is toxic. So, work under a hood, use gloves and proceed with extreme caution.

8. Site-Directed Mutagenesis

8.1. Exponential Amplification (PCR)

Equipment:

- 12.5 μ l Q5 Hot Start High-Fidelity 2X Master Mix. (NEW ENGLAND BioLabs inc. Cat N°M0494A).
- 1.25 μ l (10 μ M) Forward Primer: AGGGCCTGACgcaGACTGAGATA.
- 1.25 μ l (10 μ M) Reverse Primer: TCTGTCTTGAACATGAGTTTTTTATGG.
- 1 μ l template DNA (1-25 ng/ μ l).
- 9.0 μ l nuclease-free water.
- PCR EppendorfTM tubes.
- PCR machine.

Operating mode:

- In a PCR tube, mix all products together starting from above (greater volume).
- Using a PCR machine, enter the parameters of cycling conditions:

STEP	TEMP	TIME
Initial Denaturation	98 °C	30 seconds
25 Cycles	98 °C	10 sec
	64 °C*	10-30 sec
	72 °C	20-30 seconds/kb

Final Extension	72 °C	2 minutes
Hold	4-10C	∞

*The recommended annealing temperature of 64 °C is specific for the mutagenic primers listed above when amplified with Q5 DNA Polymerase.

8.2. Kinase, Ligase & DpnI (KLD) Treatment

Equipment:

- 1 µl PCR Product.
- 5 µl 2X KLD Reaction Buffer. (NEW ENGLAND BioLabs inc. Cat N°B0554A)
- 1 µl 10X KLD Enzyme Mix. (NEW ENGLAND BioLabs inc. Cat N°M0554A)
- 3 µl Nuclease-free water.
- PCR Eppendorf™ tubes.

Operating mode:

- Mix well all elements by pipetting gently up and down
- incubate at room temperature for 5 minutes.

8.3. Transformation

High proficiency E. coli bacteria were used to amplify our DNA samples (NEW ENGLAND BioLabs inc. C2987 competent E. Coli).

Material:

- A bin of ice.
- A water bath at 42 ° C.
- A water bath at 37 ° C.
- An incubator at 37 ° C.
- LB / SOC liquid culture medium. (NEW ENGLAND BioLabs inc. Cat N° B9020S).
- Petri dishes of LB medium with the antibiotics provided for the selection of transformed bacteria

Operating mode:

- Add 5 µl of the KLD reaction mixture directly from step II to 50 µl of chemically-competent cells.
- Incubate on ice for 30 minutes.
- Heat shock at 42 °C for 30 seconds.
- Incubate on ice for 5 minutes.
- Add 950 µl of SOC, gently shake at 37 °C for 1 hour.
- Spread 40-100 µl onto appropriate selection plate and incubate overnight at 37 °C (if necessary, make a 10-100-fold dilution of recovered cells before plating to avoid a lawn of colonies).

8.4. bacterial culture medium

Equipment:

- 2 IL berlins.
- 2 graduated cylinders.
- LB Broth Base.
- LB Agar.
- 2 bottles of 500 ml.
- Magnetic agitator.
- Magnetic bar.

Operating mode:

- Preparation of LB liquid cultures
 - In a berlin, weigh 15g of LB Broth Base.
 - Add 300 ml of milliQ water.
 - Dissolve the powder in water using the magnetic stirrer.
 - Transfer the solution to a graduated cylinder and adjust the volume to 500 ml.
 - Transfer the solution to a 500 ml bottle and autoclave.
 - Let it cool.
 - Near the Bunsen burner, add 50ug / mL of antibiotic and mix.
- Preparation of the LB to be poured into the Petri dishes:

- In a beaker, weigh 15g of LB Broth Base and 10g of LB Agar.
- Add 300 ml of milliQ water.
- Dissolve the powder in water using the magnetic stirrer.
- Transfer the solution to a graduated cylinder and adjust the volume to 500 ml.
- Transfer the solution to a 500 ml bottle and autoclave.
- Let it cool until you can hold it between your hands.
- Near the Bunsen burner, add 50 µg / mL of antibiotic and mix.
- Near the Bunsen burner, prepare the Petri dishes.
- Pass the neck of the bottle over the flame and pour the LB medium.
- Let them cool.
- Put on parafilm.
- Store at 4 ° C.

8.5. MiniPrep Plasmid DNA Extraction

Equipment:

- Use of the kit: Macherey-Nagel NucleoSpin Plasmid Cat. N° 740 588.250.

Operating mode:

- Culture of the selected clones:
 - Take a pipette tip and pick the desired colony
 - Add the tips in a falcon™ with 6 mL of LB + the appropriate antibiotic
 - Incubate 16h at 37 ° C with agitation.
- Purification of plasmids:
 - Centrifuge the falcon™ containing the bacterial culture at 3220×g for 10 min.
 - Discard the supernatant.
 - Resuspend the bacterial pellet with 250 µl of A1 buffer.
 - Add 250 µl of buffer A2 and do 6 to 8 up / down to lyse the cells.
 - Incubate 5 min at room temperature.
 - Add 300 µl of buffer A3 and do 6 to 8 up / down to neutralize cell lysis.
 - Centrifuge at 11,000×g for 5 min.
 - Place the "Nucleospin" columns on the 2ml collection tube

- Transfer 750 µl of the supernatant to the columns.
- Centrifuge at 11000×g for 1 min.
- Discard the liquid in the collection tube.
- Add 600 µl of buffer A4 to rinse the column.
- Centrifuge at 11000×g for 1 min.
- Discard the liquid in the collection tube.
- Centrifuge at 11000×g for 2 min to remove remaining residues.
- Place the columns on 1.5 ml Eppendorf™.
- Add 50 µl of milliQ water to the columns.
- Wait 5 min.
- Centrifuge at 11000×g for 1 min to retrieve our DNA.
- Store at -20 C.

8.6. MaxiPrep Plasmid DNA Extraction

Equipment:

- Use of the kit: QIAfilter Plasmid purification (QIAGEN Cat. N° 12163)

Operating mode:

- Culture of the selected clones:
 - Take a pipette tip and prick the desired colony.
 - Add the tips in a "falcon" with 6 mL of LB + the appropriate antibiotic.
 - Incubate 16h at 37 ° C with stirring.
- Purification of plasmids:
 - Centrifuge the falcon™ containing the bacterial culture at 3220×g for 10 min at 4°C.
 - Discard the supernatant.
 - Resuspend the bacterial pellet with 10 ml of P1 buffer
 - Add 10 ml of buffer P2 and mix gently by inverting the tube (4-6x)
 - Incubate 5 min at room temperature.
 - Prepare the QIAfilter Holder while waiting for the 5 min incubation, fix the cap and place the column on a suitable tube

- Add 10 ml of P3 buffer to the lysate and make 6 to 8 tube inversions to neutralize the cell lysis.
- Transfer the lysate to the QIAfilter holder column and incubate at room temperature for 10 min. This step allows the precipitate to rise to the surface and prevent clogging of the filter when pressure is applied with the piston.
- Prepare the QIAGEN-tip 500 by adding 10 ml of QBT buffer and allow it to flow through. Then discard the filtrate.
- Once the QIAGEN-tip 500 is ready, remove the cap at the end of the QIAfilter holder and gently insert the plunger.
- Transfer the contents of the QIA filter holder into the QIAGEN-tip 500 by applying pressure on the piston.
- Let flow by gravity.
- Wash with 2x 30 ml with QC buffer the QIAGEN-tip 500.
- Elute the DNA with 15 ml of QF buffer and collect the eluate in a 50 ml falcon™.
- Precipitate the DNA by adding 10.5 ml (0.7 volumes) of isopropanol at room temperature.
- Distribute everything in different Eppendorf™ and centrifuge at 15000×g at 4 ° C for 20 min.
- Add 500 µl of 70% ethanol per tube and centrifuge at 15000g at 4 ° C for 10 min.
- Gently remove the supernatant.
- Allow the pellet to air dry then resuspend in 500 µl of water.

8.7. Restriction of plasmids

Material:

- Appropriate restriction enzyme.
- Enzyme specific buffer (10%).
- milliQ water.
- 1 Eppendorf™ tube.
- Water bath at 37 ° C.
- Plasmid DNA.

Operating mode:

For a final volume of 20 µl:

- Calculate the volume of DNA to be taken so to have a quantity of 1 µg/µl,
- Calculate the volume of enzyme to be sampled knowing that for a 1 µg of DNA, 5U is needed of enzyme (usually a volume of 0.5 µl of enzyme is obtained).
- 2 µl of 10x buffer specific for the selected restriction enzyme.
- Adjust the volume with milliQ water to reach 20 µl.
- Incubate for 1 hour at 37 ° C in a water bath.

8.8. Agarose gel

Materials:

- TBE buffer (Tris Borate EDTA).
- Agarose (MP Biomedicals Cat. N° 0210026725).

Operating mode:

- Preparation of the 1% Agarose solution:
 - Weigh 1 g of agarose and dissolve it in 100 ml of 1xTBE buffer
 - Heat in the microwave, monitor and stir every 30 seconds. Agarose is ready when the solution has become completely clear.
 - Pour the heated agarose into the gel mold and place the desired comb, let cool until the gel gets harder.
- Preparation of TBE 5x:
 - 54 g of Tris (MP Biomedicals Cat N° 819623).
 - 27.5 g boric acid (MP Biomedicals Cat N° 02191411)
 - Dissolve in 900mL of milliQ water.
 - Add 20 ml of 0.5 M EDTA.
 - Store at room temperature

8.9. Verification of the digestion profile on agarose gel

Equipment:

- 1% agarose gel previously heated in the microwave.
- 20 µl of DNA restriction.
- 2 µl of charge blue.
- Molecular Weight Marker Sul (Eurognetec Smartladder Cat. N° WM-1700-10).
- Electrophoresis tank.
- Electrophoresis cuvette mounted with the comb.
- Ethidium bromide bath.

Operating mode:

- Preparation for electrophoresis tank:
 - Fill the tank with TBE IX buffer
 - Assemble the cuvette.
 - Pour the warm agarose into the cuvette.
 - Add the comb.
 - Leave to polymerize.
- Preparation of samples:
 - Add charge blue to the 20 µl of restricted DNA and homogenize.
 - When the agarose gel is polymerized, remove the comb gently.
 - Place the gel in the electrophoresis tank and immerse it in TBE buffer.
 - In the first well, add 5µl of the molecular weight marker.
 - Fill the other wells with the samples (maximum 25 µl of samples per well).
 - Turn on the generator and program the electrophoresis: 128 volts, for 1 hour.
 - Observe the gel after 45min (pay attention: samples can come out of the gel).
 - Incubate 10 to 20 min in the ethidium bromide bath.
 - Expose the gel to UV (put at 70% exposure if you want to recover the DNA)

9. Cell transfection

Cell preparation for transfection:

Plate cells approx. 24 hours before transfection making sure cells are at optimal concentration (70–90 % confluency).

Steps prior to transfection:

Allow X-tremeGENE 9 DNA Transfection Reagent (Sigma-Aldrich – Cat N° XTGHP-RO), DNA and diluent (Opti:MEM® I Reduced Serum Medium or serum-free medium) to warm to +15° C to +25° C, and vortex gently.

- Place diluent in a sterile tube.
- Add **plasmid DNA**. Pipet gently to mix.
- Add **X-tremeGENE HP DNA Transfection Reagent** to the diluted DNA.
- Incubate for 15 min at +15° C to +25° C.
- Add **transfection complex** to the cells in a dropwise manner.
- Gently shake or swirl the wells or flasks to ensure even distribution over the entire plate.
- Incubate cells for 18 – 72 hours before measuring protein expression.

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