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Demoulin, Benjamin

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Influence of MAGEA and effects of resveratrol on p53 biology

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A thesis submitted for the degree of Ph.D.

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Abstract

MAGEA1 and -A2 impair the stabilization and activation of p53 in presence of a genotoxic stress.

MAGEA (Melanoma AntiGEn-A) genes are members of the cancer testis antigens (CTAs). They are silenced in the vast majority of healthy human adult tissues. However, a re-expression is commonly observed in various types of cancer. Several studies demonstrated a correlation between MAGEA expression and advanced cancer stages, malignant transformation as well as increased resistance to chemotherapy. These observations emphasize that MAGEA proteins could have oncogenic properties and favor cancer development. However, the functions of MAGEA proteins in cancer cells remain largely unknown. In order to study the functions of the MAGEA commonly expressed in cancer, we used the MCF-7 breast cancer cell line to derive clones stably expressing them. Here, we present evidence that MAGEA1 and MAGEA2 (but not -A3, -A4, -A6 and -A12) diminish the sensitivity of breast cancer cells to the chemotherapeutic agent doxorubicin. We link this resistance to reduced DNA damage-induced apoptosis in presence of MAGEA expression. Furthermore, we demonstrate that the mechanism is p53-dependent. MAGEA1 and -A2 proteins directly interact with the DNA binding domain of the tumor suppressor and inhibit the stabilization and activation of p53 in cells exposed to a genotoxic stress. We link the decreased activation and lowered half-life of stress-induced p53 to an impaired phosphorylation of its N-terminal

region in presence of MAGEA1 or -A2. Our data suggest that preventing MAGEA proteins to bind and inhibit p53 could be an interesting strategy to combat cancers that harbor the wt version of the tumor suppressor.

Resveratrol induces DNA damage in colon cancer cells by poisoning topoisomerase II and activates the ATM kinase to trigger p53-dependent apoptosis.

Resveratrol (trans-3,4',5-trihydroxystilbene) is a natural polyphenol synthesized by various plants such as grape vine. Resveratrol (RSV) is a widely studied molecule, largely for its chemopreventive effect in different mouse cancer models. We propose a mechanism underlying the cytotoxic activity of RSV on colon cancer cells. Our data show that resveratrol induces apoptosis, as observed by the cleavage of PARP-1 and chromatin condensation. We show that the tumor suppressor p53 is activated in response to RSV and participates to the apoptotic process. Additionally, we show that HCT-116 p53 wt colon carcinoma cells are significantly more sensitive than HCT-116 p53 -/- cells to RSV. RSV induces DNA damage including double strand breaks, as evidenced by the presence of multiple γ-H2AX foci in 50% of cells after a 24h treatment with 25 µM RSV. The formation of DNA damage does not appear to rely on a pro-oxidant effect of the molecule, inhibition of topoisomerase I, or DNA intercalation. Rather, we show that DNA damage is the consequence of type II topoisomerase poisoning. Exposure of HCT-116 cells to RSV leads to activation of the Ataxia Telangiectasia Mutated (ATM) kinase, and ATM is required to activate p53.

Abbreviations

A

ADAM:
Disintegrin And Metalloproteinase
ADP: Adenosine diphosphate
AF1: activation function-1 domain
AF2: activation function-2 domain
Angptl4: angiopoeitin-like-4
Apo-1: see Fas
AIF: Apoptosis-inducing factor
Aka: also known as
Akt: protein kinase B
Ala: Alanine
AMP: Adenosine monophosphate
AMPK: AMP-activated kinase
Apaf-1: apoptotic peptidase activating factor-1
Apo-2: TNF-related apoptosis-inducing ligand receptor 1.
Apo-3: Death receptor 3
AR: Androgen receptor
Ara-C: cytarabine
Ara-CTP: tri-phospho-cytarabine
ARF-BP1: ARF binding-protein 1
ARF: Alternate Reading Frame protein
ATM: Ataxia telangiectasia mutated
ATP: Adenosine triphosphate
ATR: Ataxia telangiectasia and Rad3-related protein

B

B-Raf: B- Rapidly Accelerated Fibrosarcoma
BAD: BCL2-associated agonist of cell death
BAK: Bcl-2 homologous antagonist killer
BAX: BCL-2 associated X protein
BCL-2: B-cell CLL/Lymphoma 2
BCL-2A1: BCL2-related protein A1
BCL-XL: B-cell lymphoma-extra large
BCL-X_S: BCL2-like 1 protein
BCL-W: Bcl-2-like protein 2
BH: BCL-2 homology domains
BH3S: BH3-only sensitizers
BH3A: BH3-only activators
BIK: BCL2-interacting killer
BIM: BCL2-like 11
BIRC2: Baculoviral IAP repeat-containing protein 2
BIRC3: Baculoviral IAP repeat-containing protein 3
BMF: Bcl2 modifying factor
BOK: BCL2-related ovarian killer
BOO: Bcl2-like 10 protein
BORIS: Brother of the Regulator of the Imprinted Site
BP: base pair

C

C: cytosine

c-FLIP: Cellular FLICE-inhibitory protein

c-FLIP$_L$: long form of c-FLIP

c-FLIP$_R$: Raji form of c-FLIP

c-FLIP$_S$: short form of c-FLIP

c-IAP1: see BIRC3

c-AIP2: see BIRC2

CARD: caspase recruitment domain

CASH: see c-FLIP

Casp: caspase

CASPER: see c-FLIP

CAT: collective to amoeboid transition

CBF-1: C-promoter binding factor-1

CBP: CREB-binding protein

CCL-3: chemokine (C-C motif) ligand 3

CD-95: see Fas

CDK: Cycline dependent kinase

CEA: carcinoembryonic antigen

ced-3: Cell death-3

CEM cells: ATCC n° : CCL-119

ChIP: Chromatin immunoprecipitation

CHK1: check point kinase 1

CHK2: check point kinase 2

CLARP: see c-FLIP

COP1: constitutively photomorphogenic 1

COX-2: cytochrome c oxidase subunit II

CpG: cytosine-guanine dinucleotides

CRM-1: chromosome region maintenance 1

CTL: cytotoxic T lymphocytes

CSF-1: colony-stimulating factor-1

CTA: cancer testis antigens

CTC: circulating tumor cell

CTCF: CCCTC-binding factor

CXCL12: stromal cell derived factor

cyt C: Cytocrome C

D

DBD: DNA binding domain

dCTP: (deoxycytosine tri-P)

DD: death domain

DED: Death Effector domain

DIABLO: direct IAP binding protein with low pI

DISC: Death-Inducing-Signaling-Complex

DLL: (notch ligand)

DNA: Deoxyribonucleic acid

Dox: Doxorubicin

DR1: see TNFR1

DR2: see Fas

DR3: Death receptor 3

DR4: TNF-related apoptosis-inducing ligand receptor 1

DR5: TNF-related apoptosis-inducing ligand receptor 2

DR6: Death Receptor 6

DYRK2: dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2

E

E_2: oestradiol

E6AP: E6/E6-Associated Protein

ECM: extracellular matrix

EDAR: ectodysplasin A receptor

EGF: Epidermal growth factor

EMT: Epithelial to mesenchymal transition

EndoG: Endonuclease G

ER: Estrogen receptor

ERK1: Extracellular signal-regulated kinase 1

F

FADD: Fas-associated Death Domain

FAK: focal adhesion kinase

Fas: TNF receptor superfamily member 6

FasL: Fas ligand

FGF: fibroblast growth factor

FLICE: FADD-like IL-1β-converting enzyme

FOXO: Forkhead-O transcription factor

G

G: guanine

GADD45α: Growth arrest and DNA-damage-inducible protein

GRP75: mortalin

Grs: granzymes

GST: Glutathione S-transferase

H

HAT: Histone acetyl transferase

HAUSP: see USP7

HCT-116: colon carcinoma cell line

HDAC: Histone deacetylase

MDM2: Human double minute 2

HDM4: see MDMX

MDMX: Human double minute X

hFADD: human FADD

HGF: hepatocyte growth factor

HIPK2: homeodomain interacting protein kinase 2

HPV: human papillomavirus

HRK: harakiri

HSP: Heat Shock Proteins

HSP70: Heat shock protein 70

HSP74: mortalin

HTATIP: HIV-1 Tat interacting protein,60kDa

HtrA2: high-temperature requirement A2

I

IAP: Inhibitors of apoptosis proteins

ICAD: Inhibitor of caspase-activated DNase

IGF-1: insulin like growth factor-1

IKK: IκB kinase

IL-1α: interleukine-1 α

IL-8: interleukine-8

Ile: Isoleucine

ILP2: IAP-like protein 2

J

JMY: Junction-mediating and -regulatory protein

K

KAP1: see TRIM28

KILLER: TNF-related apoptosis-inducing ligand receptor 2

L

LARD: Death receptor 3

LKB1: Liver Kinase B1

LTc: cytotoxic T Lymphocytes

M

MAD: mothers against decapentaplegic

MAGE: Melanoma Antigen

MAPK: Mitogen-activated protein kinase

MAT: mesenchymal to amoeboid transition

MCF-7: Michigan Cancer Foundation-7 : breast cancer cell line

MCL-1: myeloid cell leukemia 1

MDM2: Mouse double minute-2

MDMX: Mouse double minute-X

MEFs: Mouse embryonic fibroblast

mFas: mouse Fas receptor

mg: milligram

MHD: Mage Homology Domain

miR: MicroRNA

ML-IAP: Melanoma-IAP

MLS: mitochondrial localization signal

MMP9: matrix metalloproteases-9

MMPs matrix metalloproteases

MOM: mitochondrial outer membrane

MOMP: Mitochondrial Outer Membrane Permeabilization

Mot-2: mortalin

MRIT: see c-FLIP

mRNA: messenger RNA

Myc: v-myc avian myelocytomatosis viral oncogene homolog

N

NAIP: Neuronal apoptosis-inhibitory protein

NAD: Nicotinamide adenine dinucleotide

NADPH: Nicotinamide adenine dinucleotide phosphate

NBs: Nuclear bodies

NDUFS3: NADH Dehydrogenase (Ubiquinone) Fe-S Protein 3

NEDD4: neural precursor cell expressed developmentally down-regulated protein 4

NES: nuclear export signal

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

NGFR: nerve growth factor receptor

NKs: Natural Killers

NICD: Notch intracellular domain

NLS: Nuclear localization signal

NM23-H1: nm23-human homolog1

NOTCH1: Notch homolog 1

NOXA: "damage" in Latin

NY-ESO-1: New York Esophageal Squamous Cell Carcinoma 1

O

OMI: See HtrA2

P

p300: E1A binding protein

p53AIP1: p53-regulated apoptosis-inducing protein 1

Parc: Parkin-like ubiquitin ligase

PARP: Poly ADP ribose polymerase

PCD: Programmed cell death

PCR: polymerase chain reaction

PDGF: platelet derived growth factor

PES: 2-Phenylethynesulfonamide

PGC-1α: PPAR-γ-coactivator-1α

PI-9: Protease inhibitor-9

PI3-Kinase: Phosphoinositide 3-kinase

PIDD: p53-induced protein with a death domain

Pin1: prolyl isomerase

PIRH2: p53-induced protein with a RING-H2 domain

PMEPA1: Prostate transmembrane protein-1

PML: Promyelocytic leukemia

PML-NB: PML-Nuclear bodies

PKA: Protein kinase A

PP2A: serine/threonine phosphatase

PRD: proline-rich domain

PRIMA-1: (2.2-bis (hydroxymethyl)-1-azabicyclo(2.2.2) octan-3-one

PRIMA-MET: Methyl version of PRIMA

Pro: Proline

PTEN: Phosphatase and tensin homolog

PUMA: p53 upregulated modulator of apoptosis

R

R: Adenine or guanine

RAIDD: RIP-associated ICH-1/CED-3 homologous protein with a death domain

Ras: resistance to audiogenic seizures

RE: response element

RING: Really Interesting New Gene

Rb: Retinoblastoma-associated protein

ROS: reactive oxygen species

RSV: Resveratrol

RT: reverse transcription

S

Saos-2: Sarcoma osteogenic-2

SET: Inhibitor of granzyme A-activated DNase

SET9: methyltransferase

SIM: SUMO interacting motif

SIP1: see ZEB2

Sir2: Silent information regulator 2

SIRT1: Sirtuin1

SKIP: SKI Interacting Protein

Slug: see SNAI2

SMAC: see Diablo

Smad: human MAD homolg

SMYD2: (methyltransferase)

SNAI1: snail family zinc finger 1

SNAI2: snail family zinc finger 2

Snail: see SNAI1

SP100: Speckled 100 KDa

Src: contraction of sarcoma

SSX-2: Synovial Sarcoma/X Breakpoint 2

SUMO: Small Ubiquitin-like Modifier

SV-40: Simian Virus 40

T

TAD1: transactivation domains

TAD2: transactivation domains

TAMs: tumor activated macrophages

tBid: truncated Bid

Tf: tissue factor

TGF-β: Transforming growth factor beta

TIF2: transcriptional mediators/intermediary factor 2

TIP60: see HTATIP

TMPRSS2: Transmembrane protease, serine 2

TNF: tumor necrosis factor

TNFR1: tumor necrosis factor receptor 1

TOPOI: topoisomerase I

TOPOII: topoisomerase II

TP53: tumor protein 53

TRADD: TNFRSF1A-associated via death domain

TRAF2: TNF receptor-associated factor 2

TRAILR1: TNF-related apoptosis-inducing ligand receptor 1

TRAIL2: TNF-related apoptosis-inducing ligand receptor 2

TRAMP: see TNFR1

Tregs: regulatory T cells

TREX-1: Three prime repair exonuclease 1

TRICK2: see TRAIL2

TRIM28: Tripartite motif-containing 28

TSP-1: Trombospondin-1

U

Ubc13: ubiquitin-Conjugating Enzyme 13

uPA: Urokinase-type plasminogen activator

USP7: Ubiquitin Specific Peptidase 7 (Herpes Virus-Associated)

V

Val: valine

VEGF-A: Vascular endothelial growth factor-A **VEGF**: vascular endothelial growth factor

VEGFR: VEGF receptors

W

W: adenine or thymine

WB: western blot

WD-40: 40 amino acids sequence terminating by Trp-Asp

WH: winged helix

WSL1: see DR3

WT: Wild type

WWP1: NEDD4-like E3 ubiquitin-protein ligase

X

X-CTAs: chromosome X-located CTAs

XIAP: X-linked inhibitor of apoptosis protein

Y

Y : thymine or cytosine

Z

ZEB2: zinc finger E-box binding homeobox 2

INTRODUCTION

Chapter 1: cancer

World wild cancer statistics published by the International Agency on Cancer estimates that 32.6 million people are currently living with cancer. In 2012, more than 14 million new cancer cases were diagnosed and 8.2 million people died from it[1]. Among men, lung, prostate, colorectum, stomach and liver cancers were the most represented cancer types while breast, colorectum, lung, cervix and stomach cancers were the most prevalent among women. In developed countries such as USA, cancer was the second cause of death in 2012 (22.9% of total death) behind heart diseases (23.7%) [Hoyert *et al*., 2012]. Data available for Belgium are generally older. In 2009, among 103,816 deaths, 27,973 were due to cancer. Digestive tract and respiratory organs tumors were the main represented types of cancer (50,6%) [2]. According to the World Health Organization, some cancers may be prevented by a healthier behavior. Indeed, high body mass index, low fruit and vegetable intake, lack of physical activity, tobacco and alcohol (ab)use are presumably responsible for 30% of cancers. In the next two decades, previsions expect an increase of 70% in cancer cases reaching 22 million annual cases[3].

[1] http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx
[2] http://statbel.fgov.be/fr/binaries/FR%20-%20Tableau%201.3_T_pdf_tcm326-210617.pdf
[3] http://www.who.int/mediacentre/factsheets/fs297/en/

Cancer is a general term that includes a group of diseases in which abnormal cells divide without control and invade other tissues and organs. Cancer cells can spread to other parts of the body through the blood and lymph systems[4]. Cancers are classified based on their origin. For example a carcinoma describes a type of cancer developing form epithelial cells. Carcinoma may further be divided into subclasses based on tissue architecture and cancer cell features (adenocarcinoma, squamous cell carcinoma, etc…). On the other hand, sarcomas arise from non-hematopoetic mesenchymal cells (cartilage, fat, muscle or bones). Finally, hematopoetic cells give rise to leukemias or lymphomas[5].

[4] National cancer institute website
[5] www.cancer.org

1.1. Hallmarks of cancer

In their famous review article, Hanahan and Weinberg described ten distinctive and complementary cellular characteristics that enable tumor growth and metastatic dissemination [Hanahan and Weinberg, 2011]. In this chapter, a short review of each of these will be described. On Figure 1, these hallmarks of cancer are enumerated and symbolized by an icon.

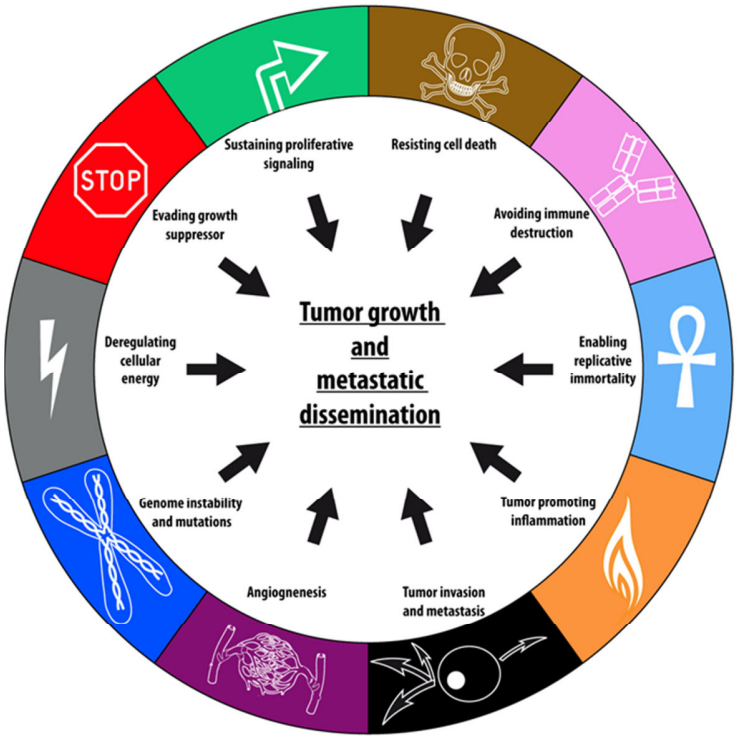

Figure 1 : Hallmarks of cancer. This chart summarizes ten hallmarks observed in cancer cells. All hallmarks are further described below. Modified from Hanahan and Weinberg, 2011.

1.1.1. Resisting Cell death

Programmed cell death (PCD) defines different pathways involved in the removal of unnecessary or damaged cells in our tissues. Observed in any animals yet analyzed, PCD shapes our body during development by sculpting (e.g. fingers), removing unwanted structures (e.g. tail of the frog), controlling the cell numbers and eliminating non-functional cells [Jacobson *et al.*, 1997].

Apoptosis, reviewed in the following chapter, plays a critical role in protecting our tissue from cancer development. [Kroemer *et al.*, 1995; Hanahan and Weinberg, 2011]. The number of cellular conditions leading to apoptosis is significant and pathways variable: oncogene signaling, DNA damage, hypoxia and reactive oxygen species (ROS) are all able to trigger apoptosis [Kroemer *et al.*, 1995]. Unfortunately, cancer cells often acquire resistance to apoptosis by corrupting key regulators. Combinations of mutations leading to impaired apoptosis are probably as high as the number of cancers itself but some events are defined as crucial. Among them, mutation of *TP53* occurs in more than 50% of all cancers. The tumor suppressor p53 is a major regulator of apoptosis since it regulates, as transcription factor, the expression of many genes such as BAK, BAX, NOXA, PUMA, DR5, Fas…

Autophagy, another form of PCD, seems to play a bivalent role. It may either confer a protection against the transformation of a normal cell or be beneficial for the cancer cell submitted to stresses. Autophagy degrades and recycles proteins and organelles to regenerate nucleotides, amino acids, fatty acids, sugars as well as energy, thus supporting the metabolism and allowing cell survival in starvation situation [Rabinowitz and White, 2010]. In normal cells, autophagy prevents tumor initiation by suppressing inflammation [White *et al*., 2010] and chronic tissue damages [Guo *et al.*, 2013]. On the other hand, autophagy sustains cancer cells metabolism by recycling nutrient and energy in tumors ineffectively vascularized. Moreover, after radiotherapy or chemotherapy treatments, autophagy-mediated cytoprotective effects have been observed. Indeed, it has been proposed that severely stressed cancer cells can enter a state of reversible dormancy thanks to autophagy [White and Dipaola, 2009]. If the environment becomes favorable again, the tumor cell quits dormancy and may recreate tumors.

1.1.2. Avoiding immune destruction

Ability to avoid immune-dependent destruction remains to be firmly established as one of the hallmarks of cancer but several studies

point out a relationship between cancer progression and a deficient immune system.

In 2009, an Australian team demonstrated that immunocompromised persons were more prone to develop cancer [Vajdic and van Leeuwen, 2009]. Reports on mice deficient for various components of the immune system further reinforced the link between immune deficiency and cancer [Teng *et al.*, 2008; Kim *et al.*, 2007]. Epidemiological studies also support a role of the immune system in cancer prevention: patients showing tumors massively infiltrated with cytotoxic T lymphocytes (CTLs) and Natural Killers (NKs) have better prognosis than patients with tumors less infiltrated. In the same context, transplants from a cancer-free donor to an immunocompromised recipient may develop tumors [Strauss and Thomas, 2010]. Authors raised the possibility that the donor functional immune environment maintained cancer cells in dormancy. Once transplanted, the immunosuppressed environment of the new host allows cell division.

Mechanistically speaking, immune evasion requires immune system impairment. For example, TGF-β is often produced by cancer cells in order to paralyze infiltrated CTLs or NKs. Also, tumor cells can actively recruit immunosuppressive cells such as regulatory T cells (Tregs) [Hanahan and Weinberg, 2011].

All the above elements seem to highlight immune evasion as a highly plausible hallmark of cancer.

1.1.3. Enabling replicative immortality

Immortality is the quality or state of something that will never die. In a cellular context, replicative immortality is the ability to divide indefinitely. Usually, the number of cell division is limited by two hurdles: senescence and cell death [Hanahan and Weinberg, 2011]. When a cell reaches a determined amount of division, it enters in an irreversible state of non-proliferation called replicative senescence. When a cell contains mutations allowing to bypass senescence, additional divisions will lead to the second barrier, cell death. Nevertheless, sometimes cells overcome both senescence and cell death. This transition has been defined as cell immortalization.

Each time a human cell replicates, each chromosomes ends, the telomeres, is shortened by 50-150 nucleotides [Smogorzewska *et al*., 2000]. When critically short telomeres are detected, a signal driving cell entry into replicative senescence is triggered. This process can be seen as a clocking device that allows replication induction until probabilities of oncogene activation or deleterious mutations are too high [Shay and Wright, 2000; Shay and Wright, 2002].

As a consequence, transition to immortality is only conceivable with the existence of a mechanism that counteracts telomere shortening. In cancer, multiple evidences demonstrate that protecting the ends of chromosome plays a central role in unlimited proliferation

[Smogorzewska *et al*., 2000]. It seems that more than 90 percent of cancers reactivate or overexpress the telomerase [Shay and Wright, 2002]. By extending telomeric DNA, the telomerase counters replication dependent chromosome erosion and allows resistance to senescence and cell death.

Without surprise, telomerase is an important field of research in cancer biology. Inhibiting the telomerase would force the cancer cell to enter into senescence. Nevertheless, side effects on cells naturally expressing the telomerase, such as hematopoetic progenitors, germline cells, stem cells and cells with a high renewal rate may represent a clear drawback of telomerase based therapies.

1.1.4. Inducing Angiogenesis

Angiogenesis is the ability to build a capillary network. A majority of tumors grow capillaries to receive oxygen and nutrients and in the same time get rid of carbon dioxide and metabolic wastes. Even if not perfect, cancer angiogenesis is based on the same inducers and inhibitors as embryonic angiogenesis. Among many factors that trigger or inhibit vessels formation, Vascular endothelial growth factor-A (VEGF-A) and Trombospondin-1 (TSP-1) are two good examples. VEGF-A is a ligand that binds to VEGF receptors (VEGFR-1, -2 or -3) and triggers new blood vessel formation. On the other hand, TSP-1 binds transmembrane

receptors at the surface of endothelial cells and represses vessel formation. In tumors, the balance between the two factors is deregulated, therefore vessel formation leads to incomplete or abnormal networks. Amid aberrations inventoried, precocious capillary sprouting, excessive capillary branching, distorted and enlarged vessels, erratic blood flow and even micro-hemorrhages are frequently observed [Nagy and Dvorak, 2010; Baluk *et al*., 2005]. Worthy of note, in the majority of cancers, the size of the blood vessel network is unrelated to cancer aggressiveness. For example, pancreatic ductal adenocarcinomas are almost free from capillaries but 5-year survival rates are lower than 15% [6] [Olive *et al*. 2009]. This could be surprising since blood stream is important for cancer cells colonization. However, the lymphatic system is also responsible for metastasic dissemination. Finally, it is interesting to note that some oncogenes, like Myc or Ras, enhance angiogenic factors production [Hanahan and Weinberg, 2011].

[6] http://www.cancer.org/cancer/pancreaticcancer/detailedguide/pancreatic-cancer-survival-rates

1.1.5. Metastasis formation

Metastasis formation is a multi-step process that involves the journey of a cancer cell from a primary tumor site to another localization. On the invasion site, cancer cells nest, grow and rebuild a secondary tumor, the metastasis. The metastatic process is clearly inefficient. It is estimated that a tumor of 1cm size, corresponding to one billion cells, can infiltrate the circulatory system with one million cells per day. The number of natural defenses and the high number of non-compatible invasion sites limit to less than 0.1% the number of cells actually developing a metastasis. Unfortunately for us, it still represents thousands of cells and 90% of all cancer deaths are associated to the presence of metastasis [Van Zijl *et al.*, 2001; Valastyan and Weinberg, 2011]. For a carcinoma, the metastatic process is divided into 7 steps:

Local invasion of the surrounding tissue. This first step is crucial and differs by the type of invasion induced. Cancer cells may move collectively, as a tissue, or by single cell progression via mesenchymal or amoeboid transition and invasion. During cancer progression, tumor cells modify their physiological and morphological characteristics to get around obstacles. Among those transformations, there is the epithelial to mesenchymal transition (EMT : “molecular and cellular program by which epithelial cells shed their differentiated characteristics, including

cell-cell adhesion, planar and apical-basal polarity, and a lack of motility, and acquire instead mesenchymal features, including motility, invasiveness and a heightened resistance to apoptosis." [Polyak and Weinberg, 2009]), the collective to amoeboid transition (CAT: this specifies the detachment of a specific carcinoma cell from the tumor by the use of amoeboid migration. Cells undergoing CAT show little connection with the extracellular matrix (ECM) and migrate through "holes" in the ECM without degrading it) and the mesenchymal to amoeboid transition (MAT : Mesenchymal cells produce matrix metalloproteases (MMPs) that help them to move through the ECM. If, for any reason, the extracellular proteolysis is blocked, MAT transition allows the cancer cell to continue its progression through amoeboid progression) [Van Zijl *et al.*, 2001].

Intravasation in the lumen of lymphatic or blood vessels. Although lymphatic dissemination is regularly observed, it seems that blood dissemination represents the main path of metastatic dissemination [Gupta and Massagué, 2006]. Blood vessels possess a basement membrane usually inhibiting cell movement. However, as mentioned earlier, cancer capillaries networks are tortuous and prone to leakiness. Moreover, weak interactions between endothelial cells facilitate the intravasation of cancer cells. Finally, intravasation of carcinoma cells may be assisted by cytokine such as TGF-β [Giampieri *et*

al., 2009] or by cells like tumor activated macrophages (TAMs) [Hernandes *et al.*, 2009].

Survival in the circulation. In the circulation, carcinomas cells are renamed "circulating tumor cells" (CTCs). The circulation entails many stresses that cells have to deal with to survive. Firstly, lack of anchorage to the ECM is supposed to trigger anoikis, an apoptotic process triggered by the loss of anchorage to the substratum. However, since the average diameter of a carcinoma cell is 30µm and since average capillaries possess a diameter of less than 8µm, carcinoma cells are rapidly blocked in some capillaries, re-enter the surrounding ECM and evade anoikis before it even starts [Valastyan and Weinberg, 2011].

In the bloodstream, CTCs also suffer from predation by circulating immune cells and from physical stresses (pressure, movement…). A rather unexpected mechanism of defense consists in the expression at their surface of the membrane bound tissue factor (Tf) which is the receptor for coagulation factors VIIa and X. As consequence, CTCs shield themselves in platelets aggregation allowing them to escape physical stresses or immune destruction [Van Zijl *et al.*, 2011; Valastyan and Weinberg, 2011].

Arrest at a distant organ site. It is common knowledge that cells originating from a particular cancer do not nest and create metastasis anywhere in the body. Some organs seem more appropriate than others. Already in 1889, Stephen Paget described an organ specific pattern of metastasis. For example, breast carcinoma rather invades bones, lungs and brain while colorectal cancers show a preference for the liver. This last example may be explained by the liver vascularization acting as a trap. CTCs, coming from the guts, are outgushed in the portal vein and rapidly arrive in liver capillaries with a diameter smaller than their size [Valastyan and Weinberg, 2011]. It remains to be determined whether CTCs are able to actively select the best environment for them, or if passive trapping in the capillaries is the sole mechanism for site selection.

Extravasation. Here, several hypothesis stand and may be applied in parallel by CTCs. First, Al Medhi *et al*. proposed a mechanism in which CTCs trapped in microcapillaries continue to grow and develop a microtumor [Al-Mehdi *et al.*, 2000]. Tumor growth may eventually break endothelial tight junctions, creating a hole in the capillaries allowing cells to invade the stroma. This hypothesis does not take into account anoikis, nevertheless, it is likely that specific CTCs are able to downregulate anoikis due to the spectrum of mutations they contain. In

addition, CTCs could undergo extravasation, the transfer of the cell from the lumen of the capillaries to the ECM of the colonized organ. Mechanistically, evidences suggest differences with intravasation, even if TAMs also help CTCs to cross the endothelial barrier [Qian and Pollard, 2010].

Micrometastasis formation. The new environment differs from the initial tumor site. Characteristics of the invaded organ, such as the type of stromal cells, ECM constituents or growth factors available may not be suitable for the newly arrived carcinoma cells. Some recent studies raised the hypothesis that cancer cells may prepare the invasion site before actually leaving the primary tumor. Systemic signals may be released in the blood to modify the normal behavior of fibroblasts in the future invaded organ. For example, on the invasion site, cells expressing MMP9 were shown to be recruited, remodel the ECM and release cytokines. This hypothesis is currently questioned and under intense debate [Psaila and Lyden, 2009]. Another hypothesis to CTCs adaptation to a new environment is simply its autonomous program. Cells express proteins and use pathways that dodge the harmful conditions imposed by the foreign environment.

Metastatic colonization. Once survival on the new site is achieved, proliferation is not guaranteed. Indeed, cells may enter a quiescent state and fail to replicate. In breast cancer, this has been linked to a lack of adhesion with the surrounding ECM. Moreover, if cells effectively begin to divide, the apoptotic rate may be so intense that they will never be able to develop a metastasis. Mechanisms responsible for apoptosis, in this case, are poorly understood but failure to induce neoangiogenesis may be a key to understand the high apoptotic rate [Chambers *et al.*, 2002].

1.1.6. Genome instability and mutation

A majority of hallmarks displayed by cancer cells are acquired following successive mutations. Among mutations inventoried, mutations in the components of the genomic maintenance machinery, in the surveillance system or in processes triggered in response to severe stresses (apoptosis, senescence…) are observed. In normal condition, tumor suppressors inhibit cell transformation. Unfortunately, when a mutation directly strikes a tumor suppressor gene impairing its function, these processes are altered. With a sufficient amount of mutations, cancer cells progress and develop metastasis.

There is an endless combination of mutations leading to cancer but cancer requires mutations of genes involved in the regulation of cell division and in the cell control/surveillance mechanisms. Moreover, mutations altering genome maintenance and repair are selectively conserved since they increase the probability of new mutations [Hanahan and Weinberg, 2011].

1.1.7. Tumor promoting inflammation

It has been observed that every tumor is at least slowly infiltrated by immune cells [Dvorak, 1986]. It has first been thought that it was a consequence of tumor formation and therefore a normal reaction of the body. However, since 2000, compelling evidences demonstrate that inflammation may enhance progression of cancer instead of preventing it. More precisely, inflammation can facilitate tumor progression by delivering growth factors (e.g. EGF), survival factors, proangiogenic factors (e.g. VEGF) and ECM modifying enzymes (e.g. MMPs) [Hanahan and Weinberg, 2011].

1.1.8. Deregulating the energy metabolism

In tumors, due to limited access to oxygen, an adjustment of the energy metabolism is compulsory. The tumor is a micro-environment in which glycolysis becomes favored. Interestingly, even in presence of a sufficient amount of oxygen, cancer cells rather use glycolysis. Otto Warburg was the first scientist to observe this effect thereafter named "Warburg effect" in his honor. As consequence, GLUT1, the glucose transporter, is overexpressed and the oncogene RAS increases the levels of HIF1α and HIF2α in order to boost glycolysis. It is difficult to explain why cancer cells favor an energy pathway 18 fold less efficient in term of

ATP generated by mole of glucose engaged. A hypothesis is that intermediates of glycolysis may be used in other pathways involved in cell division.

Hanahan and Weinberg question this hallmark as a real one or as side effect carried out by protein involved in other hallmarks [Hanahan and Weinberg, 2011].

1.1.9. Sustaining proliferative signaling

Normal cell division of is tightly controlled by growth-promoting signals. One of the main characteristics of cancer cells consists in freeing themselves from external signals in order to sustain chronic proliferation [Hanahan and Weinberg, 2011]. Mechanisms responsible for the autonomous cell division are various. Cancer cells may produce growth factors, resulting in an autocrine stimulation of cell division. They may also activate stromal cells to receive growth factors. For example, cancer cells secrete CSF-1 or TGF-β1 and in return receive EGF or stromal cell derived factor (CXCL12) from activated TAMs or cancer-associated fibroblasts, respectively. Another mechanism consists in overexpressing membrane receptors. The cell, thus, becomes hypersensitive to growth stimuli. Finally, growth factor independence may proceed from mutations leading to the constitutive activation of actors regulating cell division. Concerning activating mutations, several well-known

mechanisms have been described. In melanoma, B-Raf mutation affects the protein structure and activates the MAPK pathway. Mutations may also occur in negative feedback loops, leading to constant proliferation. A good example could be inhibitory mutations affecting PTEN phosphatase which normally counteracts PI3-Kinase effects [Datta *et al.*, 1997; Cantley and Neel, 1999].

1.1.10. Evading growth suppressors

Cell division is tightly supervised by powerful programs analyzing molecular check-points and taking decision based on information received. Those programs depend on tumor suppressor genes such as Rb and p53.

RB controls the cell cycle based on the integration of signals coming from the intra- and extra-cellular signaling. A cell deficient for Rb activity, will typically be compromised in its ability to regulate the so-called “restriction point” of the G1 phase of the cell cycle. This condition, therefore favors a non-controlled proliferation.

On the other hand, p53 nicknamed “guardian of the genome” is activated in response to various detrimental stimuli such as DNA damage or oncogene activation. In response, p53 triggers cell cycle arrest, senescence or even apoptosis.

Those two tumor suppressors, even if capital, are part of a larger network. Hence, mutation leading to the impairment of one tumor suppressor is not sufficient to generate a cancer cell. Indeed, many additional events are required [Hanahan and Weinberg, 2011].

1.2. Future direction

As explained by Hanahan and Weinberg, hallmarks of cancer are useful but incomplete tools to fully describe cancer. It only represents current knowledge about the disease and many layers of complexity will surely be added in the future. Therefore, until a complete understanding of the molecular processes leading to cancer development and progression, accent must be put on prevention to reduce cancer prevalence and death among the population.

Chapter 2: Apoptosis.

First observed in 1885 by Fleming, apoptosis is a programmed cell death (PCD) that has been defined by Kerr and his colleagues in 1972. The apoptotic phenomenon is characterized by a **nuclear and cytoplasmic condensation**, **DNA fragmentation**, **membrane blebbing** and formation of apoptotic **bodies**. Importantly, apoptosis does not lead to **inflammation.** This last characteristic allows preservation of intact surrounding tissues [Kerr *et al.*, 1972; Lawen, 2003; Fink and Cookson, 2005; Parrish *et al.,* 2013].

PCD describes types of cell death that are programmed in time and localization during the development of an organism. Programmed features of PCD lead to the hypothesis that pathways involved had to be genetically encoded and that cell death was part of the cell biology such as glycolysis or mitosis [Lockshin and Zakeri, 2001]. Concrete evidence came in 1966 with observations on the frog *Rana temporaria*. If RNA and protein synthesis is inhibited (by treatment with actinomycin D and cycloheximide), regression of the *Rana temporaria* tadpoles is impeded [Tata, 1966; lockshin, 1969]. Further evidences of genetically induced apoptosis came in 1977 when Sulston and Horvitz demonstrated that 13% of somatic cells of *C. elegans* embryo die predictably, shortly after appearing [Sulston and Horvitz, 1977]. In 1982, a gene determining the fate of *C. Elegans* cells is described, *ced-3*. Sequencing of *ced-3* gene led

to the identification of a mammalian isoform of ced-3, the “interleukin-1β-converting enzyme” later renamed caspase-1 [Yuan *et al*., 1993; Pop and Salvesen, 2009]. This breakthrough led to the description of the mammalian caspases family, proteases currently linked to apoptosis or inflammation [Lawen, 2003; Creagh *et al*., 2003].

2.1. Caspases

In humans, 12 caspases are identified. They are divided into two groups on the basis of their function.

The **first group of caspases** contains all caspases involved in apoptosis and is divided into two subgroups based on structure and timing of activation during cell death. The first subgroup is referred to as the **initiator caspases** (caspases-2, -8, -9 and -10). They possess a long pro-domain and are responsible for the activation of caspases belonging to the second subgroup. The second subgroup contains the **effector caspases** or “downstream caspases” (caspases-3, -6 and -7). They contain a small pro-domain and are responsible for the cleavage of cellular proteins [Fink and Cookson, 2005; Pop and Salvesen, 2009; Parrish *et al.*, 2013; McIlwain *et al.*, 2013].

The **second group** of caspases contains caspases related to **caspases-1** (Caspase-1, -4, -5, -12). These caspases play a role in the

inflammation process. For example, caspase-1 was shown to mature pro-IL-1β into mature IL-1β but also IL-18 and IL-33 [Chowdhury *et al*., 2008].

Finally, caspase-14 is not classified a member of these two groups since it has specific functions and seems implicated in the differentiation process of keratinocytes [Chowdhury *et al*., 2008; McIlwain *et al.*, 2013].

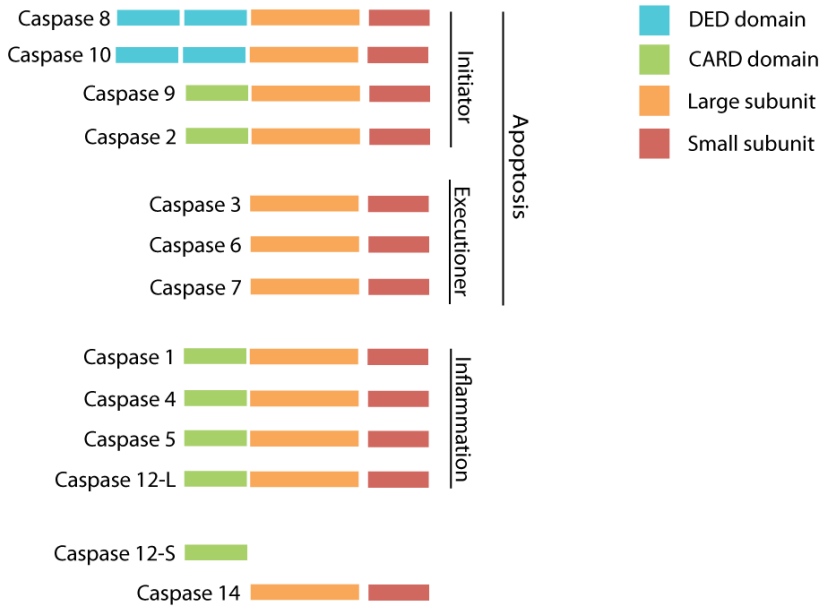

Figure 2 : Structures and association of human caspases. All caspases possess a similar structure composed of a large and a small subunit. Caspases are proteases either responsible for apoptosis with initiator and executioner caspases or responsible for inflammation. The caspase family includes 13 isoforms differing by their cellular functions. They possess or not DED or CARD domains depending on their cellular role. Modified from Taylor *et al.*, 2008.

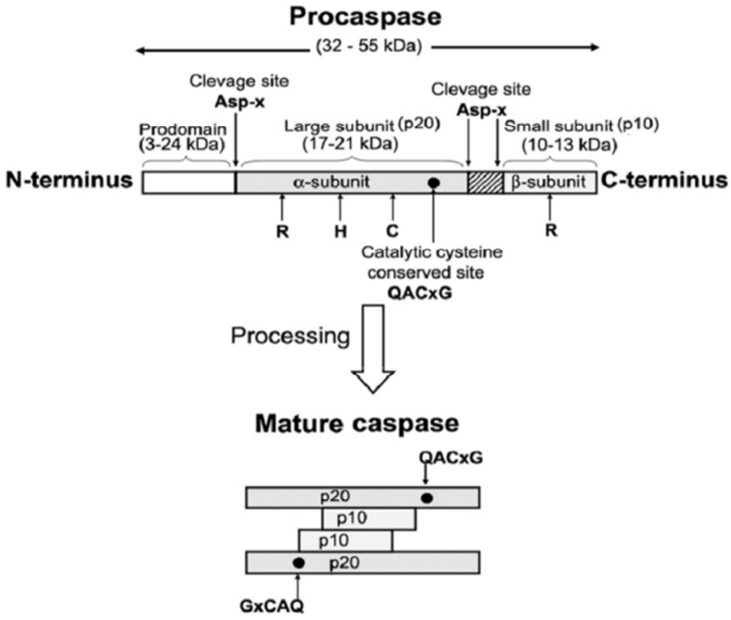

Figure 3 : General mechanism of maturation of pro-caspases into active caspases. Two procaspases in close proximity will be cleaved on two specific cleavage site (Asp-X). This induces a structure rearrangement that allows the binding of their respective small units creating and ultimately, formation of an active heterotetramer containing two large subunits and two small subunits. Modified from Chowdhury *et al.,* 2008.

2.1.1. Apoptotic caspases

2.1.1.1. Initiator caspases

Caspase-2 (Casp-2), **caspase-8** (Casp-8), c**aspase-9** (Casp-9) and **caspase-10** (Casp-10) are the initiator caspases. They may further be divided into two groups depending on upstream signals inducing their activation. Casp-8 and -10 are type I initiator caspases since their activation is “extrinsic” while the activation of casp-9 is “intrinsic” (type II initiator caspase). Those modes of activation will be discussed later in the chapter [Pop and Salvesen, 2009; McIlwain *et al.*, 2013].

Structurally, type II initiator caspases contain a CARD domain (CAspase Recruitment Domain) while type I caspases have two DED domains (Death Effector Domain) (Figure 2). CARD and DED domains interact with other proteins to bring two monomers in close proximity. Moreover, they all possess a catalytic domain divided in two subunits: a small one (± 10kDa) and a long one (± 20kDa) (Figure 2) [McIlwain *et al.*, 2013; Fuentes-prior *et al.*, 2004; Salvesen and Ashkenazi, 2011].

In the absence of an apoptotic stimulus, initiator caspases exist in an inactive form, the procaspase monomer. Initiator caspases are activated by dimerization of two procaspases under the model of “induced proximity”. This model hypothesizes that upstream signals triggers the dimerization of two procaspases monomers by inducing a

close proximity between them [Muzio *et al.*, 1997; Boatright and Salvesen, 2003; Chang *et al.,* 2003]. This proximity allows self-cleavage into two small subunits and two large subunits which together form a heterotetramer (Figure 3) [Fuentes-prior and Salvesen, 2004; Salvesen and Ashkenazi, 2011].

Caspase 2 is an initiator caspase (**Erreur ! Source du renvoi introuvable.**) that deserves a specific paragraph since it does not directly activates effector caspases. Caspase-2 is also found as an inactive monomer and like other initiator caspases, is activated through close proximity-induced dimerization. Pro-caspase-2 activation requires formation of the so-called PIDDosome. The PIDDosome is a protein complex composed by five “p53-induced protein with a death domain” (PIDD), seven “RIP-associated ICH-1/CED-3 homologous protein with a death domain” (RAIDD) and seven caspase-2 molecules (Figure 10) [Parrish *et al.*, 2013]. It has been proposed that active caspase-2 is able to cleave Bid into tBid thus leading to mitochondrial membrane permeabilization [Bouchier-Hayes *et al.*, 2009; Olsson *et al.*, 2009]. PIDD genes expression is induced following p53 activation, therefore caspase-2 is thought to be important for p53-dependent apoptosis. Finally, recent studies demonstrate that caspase-2 can be activated in absence of PIDD suggesting that other mechanisms may exist [Manzl *et al.*, 2009].

2.1.1.2. Effector caspases

Caspase-3 (Casp-3), **caspase-6** (Casp-6) and **caspase-7** (Casp-7), the effector caspases, are characterized by the lack of a DED or CARD domain. Only the catalytic domain remains [Fuentes-prior and Salvesen, 2004].

Like initiator caspases, effector caspases are inactive and exist in absence of stress as procaspases. Inactive effector caspases are in a dimeric form and require cleavage by initiator caspases to be operative. As shown in Figure 4, initiator caspases cleave effector procaspases on the L2 loop (and L2') that holds the two subunits of each monomer. This cleavage allows more flexibility from the L3 and L4 loops and helps to change the conformation from an inactive into an active dimer [Riedl and Shi, 2004].

Activated effector caspases are responsible for all morphological changes that occur during apoptosis. Casp-3 and -7 share almost the same substrate and inhibitor specificity. Still, they diverge by the N-terminal part of the protein thought to be responsible for subcellular localization [Fuentes-prior and Salvesen, 2004]. Substrates cleaved by Casp-6 are different which suggests a role slightly different from the other effector caspases.

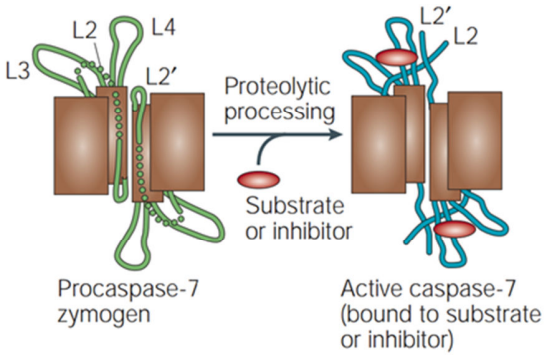

Figure 4 : Effector pro-caspase 7 activation. On the left of the panel, the active-site loops are presented in green before the cleavage and in blue after the proteolytic processing. Cleavages increase the general flexibily of the protein and allow the activation [Riedl and Shi, 2004].

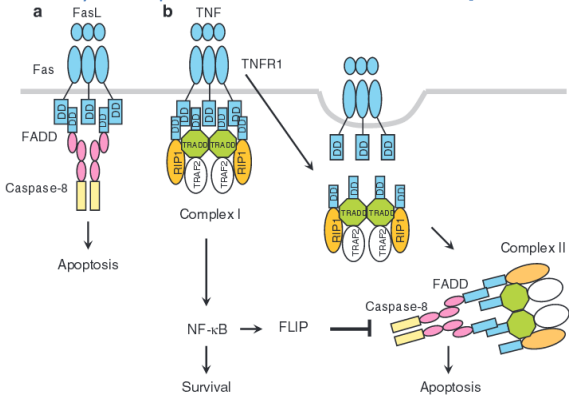

Figure 5 : FAS and TNF receptor activation. Death receptors are activated by external binding of a specific ligand. A) Activation of Fas induces the recruitment of procaspase-8 via FADD molecules and leads to caspase-8 activation. B) Activation of TNF receptors lead to two different outcomes. Either complex I is formed and induces NF-kB activation or formation and detachment of complex II induces cell death by caspase-8 recruitment and activation via FADD molecules. From Li and Yuan, 2008.

2.2. Pathways of apoptosis

Many stimuli induce caspases activation but three main pathways are defined: (1) the extrinsic pathway of apoptosis (or death receptor pathway), (2) the intrinsic pathway of apoptosis (or mitochondrial apoptosis pathway) and finally (3) the granzyme-dependent pathway. Those pathways differ by the adapters and initiators required but all aim at activating effector caspases. A recapitulative figure of the apoptotic pathways may be found at the end of this chapter (Figure 10).

2.2.1. Extrinsic apoptosis

The extrinsic apoptosis pathway or "death receptor pathway of apoptosis" is triggered by external signaling. At the surface of the cell stands a family of transmembrane death receptors. These receptors are members of the tumor necrosis factor (TNF) receptor superfamily of receptors. They contain multiple cysteine-rich repeats as well as a death domain (DD) and are able to transduce the external death signal to the inside of the cell following the binding of a specific ligand. Members of this family include **tumor necrosis factor receptor 1** (TNFR1 aka DR1, CD120a, p55 and p60), **Fas** (aka DR2, Apo-1 an CD-95), **Death receptor 3** (DR3 aka Apo-3, LARD, TRAMP and WSL1**), TNF-related apoptosis-inducing ligand receptor 1** (TRAILR1 aka DR4 or APO-2), **TNF-related**

apoptosis-inducing ligand receptor 2 (TRAIL2 aka DR5, KILLER and TRICK2), **Death Receptor** 6 (DR6), **ectodysplasin A receptor** (EDAR) and **nerve growth factor receptor** (NGFR) [Li and Yuan, 2008; Andera, 2009].

The classical model for **Fas-induced cell death** states that Fas receptors are activated by binding of the Fas ligand (FasL) mainly produce by activated cytotoxic T Lymphocytes (LTc) and by Natural killers (NK). Binding of FasL induces Fas receptor trimerization and triggers a conformational change in the cytosolic part of the receptor. It exposes the DD of Fas allowing the recruitment of FADD (FAS-Associated Death Domain) through DD-DD interaction (Figure 5). Indeed, FADD contains a DD as well as a DED similar to the DED of caspases. FADD acts as an intermediate between pro-caspase-8 and the Fas receptor to create a complex called the **Death-Inducing-Signaling-Complex (DISC**). DISC allows several pro-caspases 8 to be close enough to be activated [Carrington *et al.*, 2006]. Nevertheless, this model does not fit with recent observations made by NMR. Recently, Wang *et al*. created and analyzed by X-ray a hybrid crystal composed of mouse Fas receptor (mFas) and Human FADD (hFADD). This crystal seems to reflect rather well the human complex since analysis in solution of this chimera trough electron microscopy and mass spectrometry demonstrates that the size, the shape and stoechiometry appears to be conserved. According to Wang *et al.*, the complex is composed by five Fas receptors and five

FADD. This new model bears a close similarity to the PIDDosome responsible for the activation of caspase-2 [Wang *et al.*, 2010].

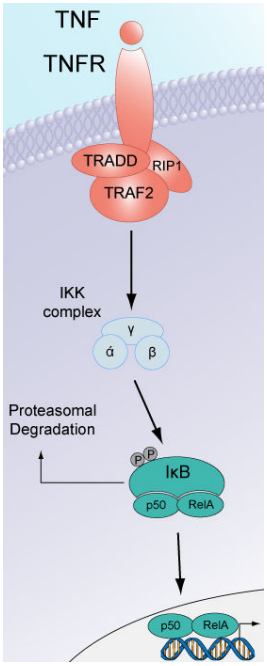

Figure 6: NF-kB activation by TNFR1. Triggering of TNFR induces activation of the IKK complex. IKK complex triggers the phosphorylation of IκB, the Nf-κB (p50 and RelA complex) inhibitor. Released from its inhibition, NF-kB enters the nucleus and induces genes related to inflammation or cell survival. Modified from Pelzer and Thome, 2011.

Activation of death receptors does not necessarily cause cell death. For example, the binding of **TNFα** on **TNFR1** may lead to two different outcomes. Shortly after binding, the activated TNFR1 oligomerizes and forms a complex with TRADD, RIP, TRAF2 and c-IAP1 (Figure 5 and Figure 6). This structure called “complex I” activates the Iκκ complex, induces phosphorylation of IκB, the NF-κB repressor. NF-κB

controls the expression of numerous genes including pro-inflammatory cytokines like IL-1α, IL-8, COX-2, CCL-3… Interestingly, NF-κB also promotes cell survival by expression of anti-apoptotic proteins like BCL-2, BCL-XL, cIAP, FLIP… (Figure 6) [7].

On the other hand, the DD domain of TRADD and RIP can recruit procaspase-8 to form "complex II". This leads to caspases-8 activation according to the "induced proximity model" and ultimately to apoptosis [Creagh *et al*., 2003; Micheau and Tschopp, 2003; Li and Yuan, 2008]

The final outcome between cell death or survival depends on the balance between complex I and II. For example, NF-κB is able to induce the expression of c-FLIP (Cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (aka FLAME-1, FLICE-1, CASPER, CASH, MRIT, CLARP and usurpin)). Three c-FLIP isoforms exist: a long, a short and a "Raji" one (respectively c-$FLIP_L$, c-$FLIP_S$ and c-$FLIP_R$). They act as repressors of apoptosis induced by receptors such as Fas, TNFR1… Briefly, all c-FLIP isoforms possess two DED domains and therefore compete for FADD binding. The mechanism is quiet simple, with c-$FLIP_S$ and c-$FLIP_R$ proximity between procaspases-8 is inadequate and procaspases-8 are unable to induce their auto-activation. c-$FLIP_L$ acts in a similar manner if at high concentration at the DISC. Nevertheless, some studies demonstrate that c-$FLIP_L$ may act as a pro-apoptotic molecule under the condition of strong receptor activation or in the presence of a

[7] http://www.bu.edu/nf-kb/gene-resources/target-genes/

high amount of the short isoforms (c-FLIP$_S$ and c-FLIP$_R$). If one of these conditions occur, c-FLIP$_L$ stimulates the activation of caspase-8 at the DISC. In addition to caspases-8, two other c-FLIP$_L$ targets have been listed to this day: p43-FLIP and p22-FLIP. Both cleaved forms are inducers of NF-κB activity by binding to the Ικκ complex [Lavrik and Krammer, 2012].

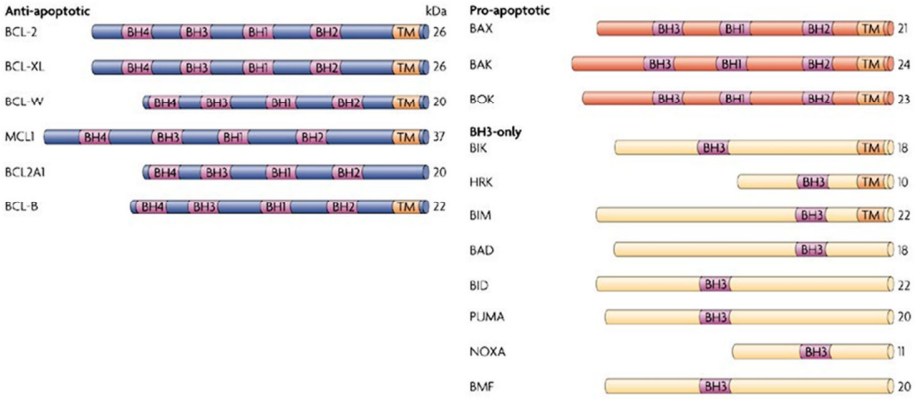

Figure 7: BCL-2 family members. BCL-2 proteins are composed by one to four BCL-2 homology (BH) domains. The number of BH domains determines the classification into subcategories: anti-apoptotic (four different BH domains), pro-apoptotic (three BH domains) and BH3-only with only one BH domain.

2.2.2. Intrinsic pathway of apoptosis

Also called “mitochondrial pathway”, this pathway of apoptosis is triggered by different stimuli including DNA-targeting drugs [Chipuck and Green, 2008; Li and Yuan, 2008]. Intrinsic apoptosis involves the permeabilization of the mitochondrial outer membrane (MOMP for Mitochondrial Outer Membrane Permeabilization) by the pro-apoptotic members of the B-cell CLL/Lymphoma 2 (BCL-2) family. MOMP allows the release, into the cytosol, of apoptotic effectors such as cytochrome C (cyt C), SMAC/DIABLO, Omi/htrA2, AIF and EndoG localized in the mitochondrial intermembrane space. These proteins are important for caspases activation and DNA fragmentation. The release in the cytosol of such effectors often seals cell fate. This part aims to describe the most important features of the intrinsic pathway of apoptosis.

2.2.2.1. BCL-2 family

Mitochondria are constantly protected by and under the threat of BCL-2 family members. This family can be subdivided into three different groups: the anti-apoptotic proteins (BCL-2, BCL-XL, BCL-W, BCL-2A1, MCL-1 and BOO), the multi-domain pro-apoptotic proteins (BAX, BAK, BOK) and the pro-apoptotic “BH3 only” proteins (NOXA, PUMA, BAD, BIK, HRK, BIM, BMF). These three groups of proteins differ

from each other by the number of BCL-2 homology domains (BH) and the presence or not of a C-terminal transmembrane domain (Figure 7).

Although additional subcellular localization are known, **Pro-survival BCL-2 family proteins** are usually cytosolic or integrated to the outer membrane of the mitochondria. These proteins directly bind and inhibit the pro-apoptotic members. All pro-survival proteins possess four different BH domains: BH1, BH2, BH3 and BH4. It has been shown that an overexpression of each of these proteins protects cultured cells against different apoptotic stimuli [Müller-Röver *et al*., 2000; Lin *et al*., 2001; Alam *et al*., 1997].

The pro-apoptotic multidomain proteins contain at least two BH domains but never more than three. Once activated, BAX and BAK oligomerize at the mitochondrial outer membrane (MOM) creating pores that allow the release of apoptotic effectors. It has been demonstrated that each of these proteins, when overexpressed in a cell line, triggers apoptosis. Finally, MEFs deficient for both BAX and BAK are unable to respond to a wide variety of apoptotic stimuli. These studies underline the essential role of BAX and BAK in the regulation of the apoptotic process [Lindsten *et al.*, 2000; Rathmell *et al.*, 2002].

The activity of **BH-3 only proteins** is regulated by different mechanisms including cleavage, sequestration, post-translational modification or gene expression. For example, in non-stressed cells, the BAD protein is phosphorylated on S112, S136 and S155 by Akt and PKA.

BAD phosphorylation on S112 and S136 induces 14-3-3 proteins binding and prevent the access of BAD to BCL-2 pro-survival proteins. In addition, phosphorylation of S155 inhibits the interaction of BAD with BCL-2 and BCL-XL [Dougherty and Morrison, 2004; Liu and Lin, 2005]. Under apoptotic stress, the serine/threonine phosphatase PP2A directly binds BAD and dephosphorylates S112. This dephosphorylation inhibits the interaction with 14-3-3 proteins and increases the access of other phosphatases. As mentioned above, BH3-only proteins can also be regulated through their gene expression. For example, in presence of DNA damages, activated p53 can induce PUMA expression and thus increase its intracellular levels leading to massive apoptosis [Nakano and Vousden, 2001].

2.2.2.1. Mechanisms of action

Different models explain how BCL-2 members interact at the mitochondrial surface and induce MOMP. Recently, a unified model has recently been proposed to explain all observation made so far.

The "unified" model

The unified model proposes a mechanism that explains all observations made experimentally. In this model, anti-apoptotic members of the family inhibit apoptosis by two "modes" and apoptosis relies on the cellular levels of BH3-only proteins [Llambi *et al*., 2011]. BCL-2 anti-apoptotic members bind BH3-only proteins. This inhibits multi-domain pro-apoptotic members activation by the same BH3-only proteins (MODE 1). On the other hand, anti-apoptotic members also bind active BAX or BAK and therefore inhibit MOMP formation (MODE 2). When BH3-only concentration is too high, all anti-apoptotic members are on mode 1 and active pro-apoptotic members are free to generate MOMP (Figure 8).

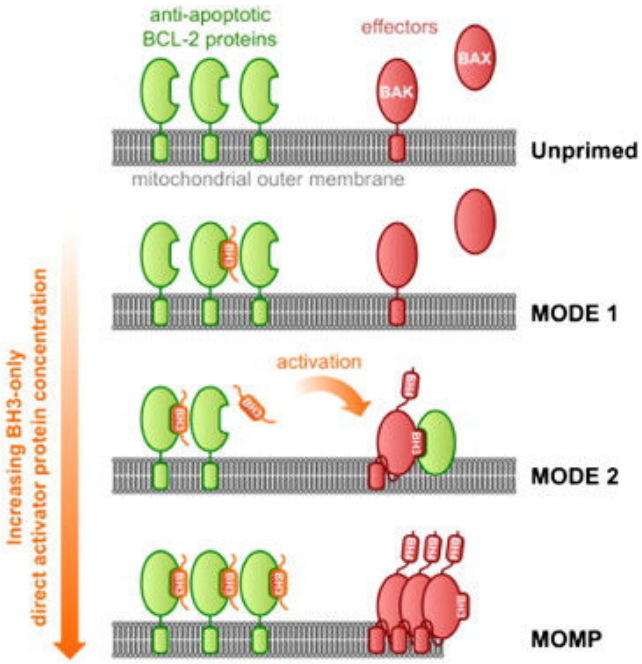

Figure 8 : The unified model. Anti-apoptotic members of the BCL-2 family inhibit BAX or BAK by two modes. First, anti-apoptotic BCL-2 bind and sequestrate BH3-only protein (MODE1). BAK or BAX are loosely bound to the membrane but not embedded. If the concentration of BH3-only protein increases, some BAX and BAK proteins may be activated and insert into the membrane potentially inducing MOMP. Therefore, a second mode of inhibition exists. Once inserted, in the membrane, anti-apoptotic BCL-2 family member can directly sequestrate BAX or BAK. Finally, if too many BH3-only proteins are in presence, all anti-apoptotic proteins are in MODE1 and unable to inhibit BH3-only activated BAX or BAK. From Llambi *et al.*, 2011.

2.2.2.3. Effects of Apoptotic effectors

When MOMP is achieved, different mitochondrial intermembrane space apoptotic effectors (cyt C, SMAC/DIABLO, Omi/HTrA2, EndoG and AIF) relocate into the cytosol or nucleus. Those molecules act in complementarity to induce apoptosis by several mechanisms reviewed in this section.

2.2.2.3.1. Cytochrome C

In case of mitochondrial membrane disruption, cyt C is thrown out of the mitochondria into the cytosol and interacts with the apoptotic peptidase activating factor-1 (Apaf-1). Apaf-1, a major component of the "apoptosome" complex, contains two WD-40 domains, a CARD domain and an ATPase domain[8]. In normal condition, the CARD domain of Apaf-1 is trapped between the two WD-40 domains leading to Apaf-1 self-inhibition. However, in the presence of cyt C, the CARD domain is exposed and Apaf-1 activated. ATP hydrolysis by the ATPase domain of Apaf-1 allows its oligomerization with six other Apaf-1-cyt C complexes, forming the apoptosome. The apoptosome recruits procaspase-9 through CARD domains interaction. The close proximity of several

[8] NCBI, gene, APAF1.

procaspases-9 triggers their auto-activation into caspase-9 [Ledgerwood and Morison, 2009; Hengartner, 2000; Riedl and Shi, 2004] (Figure 9).

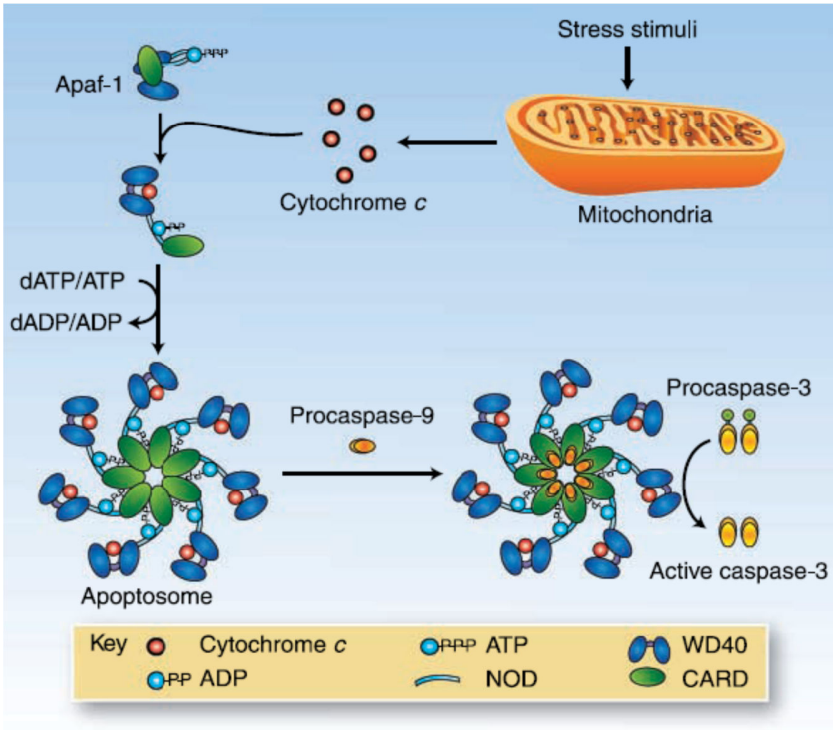

Figure 9 : Apoptosis induction by Apoptosome formation. Following Cytochrome C release from the mitochondria, CARD domain of Apaf-1 is displaced by Cyt C, activating Apaf-1. Seven activated Apaf-1 combine to form a platform that recruits Pro-caspase-9. Pro-caspases-9 are in sufficient proximity to induce self-activation. From Ledgerwood and Morison, 2009.

2.2.2.3.2. SMAC/DIABLO and Omi/HtrA2

The SMAC / DIABLO gene codes for an "inhibitor of apoptosis protein (IAP)-binding protein"[9]. Usually, IAPs inhibit caspase-3,-7 and -9 activity (but not caspase-6 and -8) by obstructing the catalytic site and/or by ubiquitinating caspases for proteasome-dependent degradation [Huang *et al*., 2000]. In mammals, two types of IAPs exist. Those with a RING domain such as XIAP, c-AIP1, c-AIP2, Melanoma-IAP (ML-IAP) and IAP-like protein 2 (ILP2), and those without a RING domain: Neuronal apoptosis-inhibitory protein (NAIP), Bruce and survivin [Riedl and Shi, 2004]. XIAP inhibits caspase-9 by sequestering the pro-caspase form but in the same time inhibits caspase-3 and -7 [Shiozaki *et al*., 2003]. On the other hand, casp-3 and -7 are also inhibited by c-IAP1, c-IAP2 and NAIP trough catalytic site occlusion and degradation induction [Maier *et al.*, 2002; Salvesen and Duckett, 2002]. SMAC/DIABLO inhibits procaspase-9 sequestration by XIAP. SMAC proteins hold a tetrapeptide IAP-binding motif composed of Ala-Val-Pro-Ile amino acids. This motif binds XIAP and competitively displaces procaspase-9 allowing its recruitment by the apoptosome. Worthy of note, SMAC/DIABLO cannot disrupt IAPs inhibition of effector caspases [Riedl and Shi, 2004]. Clear understanding of IAPs antagonists is lacunar and needs further investigations [Feltham *et al.*, 2012]

[9] NCBI, gene, Diablo

Omi/HtrA2 also target IAPs through a tetrapetide IAP-binding domain. For example, it has been shown to interact with XIAP. In addition, Omi/HtrA2 seems to be able to cleave IAPs thanks to a serine protease activity. Finally, reports suggest that once in the cytosol, Omi/HtrA2 is able to induce caspase-independent cell death [Suzuki *et al*. 2004].

2.2.2.3.3. AIF and EndoG

AIF and EndoG need caspases processing to be released from the intermembrane space of the mitochondria. Therefore, AIF and EndoG action is delayed by comparison to cyt C or SMAC/DIABLO [Arnoult *et al*., 2003].

Apoptosis-inducing factor (AIF) was described by G. Kroemer [Susin *et al*., 1997]. AIF is a protein able to induce chromatin condensation as well as DNA fragmentation into large fragments [Susin *et al*., 1999]. In parallele to DNA fragmentation, AIF induces mitochondrial membrane permeabilization generating a kind of positive feedback loop. This action of AIF is inhibited in presence of an overexpression of BCL-2 [Susin *et al*., 1999].

Endonuclease G (EndoG) is able to relocate to the nucleus where it processes DNA into small fragments. Moreover, EndoG cooperates

with exonucleases and DNase I to facilitate DNA processing [Widlak *et al.*, 2001].

2.2.3. Crosstalk between extrinsic and intrinsic pathways

Extrinsic apoptosis may be enhanced by crosstalk with the intrinsic pathway. Observations report that in specific cells, such as Jurkat cells or CEM cells, activation of pro-caspase-8 leads to insufficient amount of activated caspase-8. In those cells, called Type II, an amplification of the apoptotic signal is required. The small amount of extrinsically-activated caspases-8 triggers the cleavage of Bid, a BH3-only BCL-2 family member, releasing a truncated form of Bid (tBid). tBid translocates to the mitochondria where it interacts with pro- and anti-apoptotic members of the BCL-2 family to trigger MOMP (Figure 10). The activation of effector caspases by caspase-9 is the add-on needed to induce apoptosis [Schütze *et al*., 2008].

2.2.4. Granzyme

In lymphocytes, specific cellular compartments, called granules, sequestrate Granzymes (Grs) and perforin proteins. Grs are released by cytotoxic T lymphocytes and NK to induce cell death of cancer cells and

also virus infected cells. Perforins are pore-forming proteins that facilitate Grs transit to the cytosol [Bots and Medema, 2006; Ewen *et al*., 2012]. Grs, on the other hand, are serine proteases and in human consist in 5 isoforms: A, B, H, K and M. Up to day, Granzyme H and K are orphan enzymes since no target has been identified and the mechanism of apoptosis induction remain unclear [Grossman *et al*., 2003].

2.2.4.1. Granzyme A.

Once in the cell, Granzyme A (GrA) attacks the mitochondria by cleaving NDUFS3, a protein implicated in the complex I of the electron transport chain. NDUFS3 cleavage disrupts the electron transport chain, generates reactive oxygen species (ROS) and induces the loss of mitochondrial potential. Increased ROS in the cytosol promotes the translocation of the ER-associated complex SET (Inhibitor of granzyme A-activated DNase) into the nucleus. There, GrA induces SET degradation and releases NM23-H1 and TREX-1 known to induce nicks in the DNA. Moreover, GrA possesses other characteristics such as the ability to trigger lamins degradation leading to nuclear membrane disruption, PARP-1 degradation inhibiting DNA repair as well as degradation of transcription factors involved in the early repair response [Lieberman, 2010; Bots and Medema, 2006]. Importantly, caspases are not activated

by GrA and loss of mitochondrial membrane integrity is not observed. [Bots and Medema, 2006]

2.2.4.2. Granzyme B.

On the contrary to GrA, Granzyme B (GrB) activates caspases. GrB induces apoptosis following several pathways. For example, GrB directly targets the BH3-only protein Bid and processes it into tBid leading to MOMP and apoptosome formation. On the other hand, GrB directly binds and activates caspases-8, -3 and -7 [Rousalova and Krepela, 2010]. Finally, GrB directly processes PARP, ICAD, Lamin B … [Bots and Medema, 2006; Rousalova and Krepela, 2010]

2.2.3.3. Granzyme M.

Granzyme M (GrM) was originally thought to induce apoptosis in a caspase- and mitochondrial-independent manner. Cells treated with granzyme M show a rapid degradation of the plasma membrane [Kelly *et al.,* 2004]. However, contradictory informations were published recently suggesting the formation of ROS and the release of cyt C from mitochondria following GrM treatment [Hua *et al*., 2007]. Finally, GrM was reported to cleave PI-9, the main inhibitor of GrB. Therefore GrM may be a direct activator of GrB [Mahrus *et al*., 2004].

Cytoplasm
Nucleus
Perforin
FAS
FASL
TNF
TNFR
Pro-caspase 8
C-FLIP
Active Caspase-8
Complex II
Complex I
IKK complex
IκB
NF-κB
Inflammation and survival
Active Caspase-3-6-7
Protein degradation and cell death
Active Caspase-9
Apoptosome
Cyt C
Mitochondria
Bid
tBid
Bcl-2
Bax / Bak
ROS
Increased ROS
NDUFS3
Cleavage :
- Rock1
- α-tubulin
- Filamine
- ICAD
- PARP
- Lamin
- ...
Active Caspase-2
PIDDosome
PIDD
RAIDD
PUMA
Bax
DNA fragmentation
Granzyme A
Pro-caspase-8
DED
FADD

Figure 10 : Summary of apoptosis induction. All proteins and mechanisms discussed above are summarized in this figure allows a more general view and points all interconnections between pathways. TNFR activation may lead to two different outcomes. First, activation may induce complexI formation and phosphorylate NF-κB's inhibitor: the Iκκ complex. NF-κB enters the nucleus and induces the expression of inflammation-related gene. Otherwise, complex II may be induced and triggers caspasase-8 activation. Fas receptor activation only leads to caspase-8 activation but may be inhibited by C-FLIP. Grz introduction through the cellular membrane via perforin channel also triggers cell death. Grz B directly cleaves proteins such as Bid but also activates caspase-3 while Grz A, however, seems to cleave NDUFS3 and to increase cellular ROS levels. tBid, activated by Grz but also by the caspase-2, activates the mitochondrial pathway of apoptosis and releases the apoptotic effectors such as cyt C (apoptosome activation), SMAC/DIABLO (XIAP inhibitors), AIF and EndoG (DNA cleavage). Finally, p53 is able to induce genes that directly induce apoptosis such as PUMA, BAX or even RAIDD that participate to PIDDosome formation.

Chapter 3: p53

3.1. Introduction to p53

The tumour suppressor p53 is one of the most studied protein in cancer research since it is estimated that at least 50% of cancers bear a mutated form of p53 [Levine and Oren, 2009]. In the remaining 50%, wild type p53 activity is altered following different mechanisms that will be discussed in this chapter. Today identified as a *bona fide* tumor suppressor, p53 was previously considered as an oncogene. This conclusion came following works from different teams analysing cancers related to Simian Virus 40 (SV-40) infection. A protein of ~53kDa was co-precipitated along with the Large T antigen of SV-40 [Lane and crowford, 1979; Linzer *et al.*, 1979; Kress *et al.*, 1979; Melero *et al.,* 1979; Smith *et al.,* 1979; Deleo *et al*., 1979]. Moreover, this protein was present in huge amount in different types of cancers in absence of SV-40 infection. The protein also seemed absent from normal cells [Benchimol *et al*., 1982; Crawford *et al.*, 1982; Rotter, 1983]. With these observations, a hypothesis rapidly grew among the scientific community: this protein must be an oncoprotein favouring cell transformation. To demonstrate this theory, p53 was cloned from cancer cells and expressed in various cellular models. In each case, p53 promoted immortalization and

transformation [Eliyahu *et al.,* 1984; Parada *et al.,* 1984; Jenkins *et al*., 1984].

The oncogenic properties of p53 were challenged when a p53 cDNA clone was shown to be unable to promote cell transformation. Rapidly, comparison with DNA sequences of clones used in previous studies demonstrated that mutated versions of p53 were employed. Moreover, this wild type form of p53 was prone to protect cells from transformation.

In order to be defined as a tumor suppressor, a protein must meet different criteria. First, the coding gene must be frequently mutated or deleted in cancer. This is the case for p53 as mentioned above and originally demonstrated by Vogelstein and colleagues. A second hallmark of tumor suppressors is that they inhibit cell transformation once activated or expressed. Indeed tumor suppressors are most often cell cycle inhibitors or programmed cell death inducers, which is the case of p53. Moreover, mice carrying a deletion of a tumor suppressor show a higher propension to develop cancer. Finally, tumor suppressors are often associated to a hereditary cancer syndrome. The Li-Fraumeni syndrome is a human pathology where humans bear a germline mutation of p53 and transmit it to their offspring [Levine and Oren, 2009]. Such individuals are prone to develop different cancers and have a reduced life expectancy. With knowledge of the Li-Fraumeni

syndrome, works on mouse models and the known cellular functions of p53, the protein was recognized in 1989 as a tumor suppressor.

The gene *TP53* encodes for a transcription factor controlling several cellular pathways such as cell cycle [Jain *et al.,* 2012; Tanaka *et al.*, 2000], apoptosis [Mellert *et al.*, 2011; Concannon *et al.*, 2007], replicative senescence [Gannon *et al*., 2011; Vigneron and Vousden, 2010], energy metabolism [Maddocks and Vousden, 2011; Hu *et al.*, 2009], angiogenesis [Ravi *et al*., 2002; Yamakuchi *et al.*, 2009], immune response [Li *et al*., 2011], cell migration [Kim *et al.*, 2011; Hwang *et al.*, 2010], cell differentiation [Jain *et al.*, 2012; Aranha *et al.*, 2011], autophagy [Livesey *et al.*, 2012] and even EMT [Kim *et al.*, 2011]. The protein p53 is stabilized and activated following a wide range of cellular stresses such as DNA damage [Shieh *et al*., 1997] or oncogene activation [Attardi *et al.*, 1996; Hermeking and Eick, 1994]. In addition to its role as transcription factor, p53 is also able to relocate at the mitochondria and play a direct role in MOMP during apoptosis.

3.2. p53 structure

The p53 protein contains 393 amino acids and is divided into three main parts, the N-terminal region, the DNA binding domain and the C-terminal region (Figure 11).

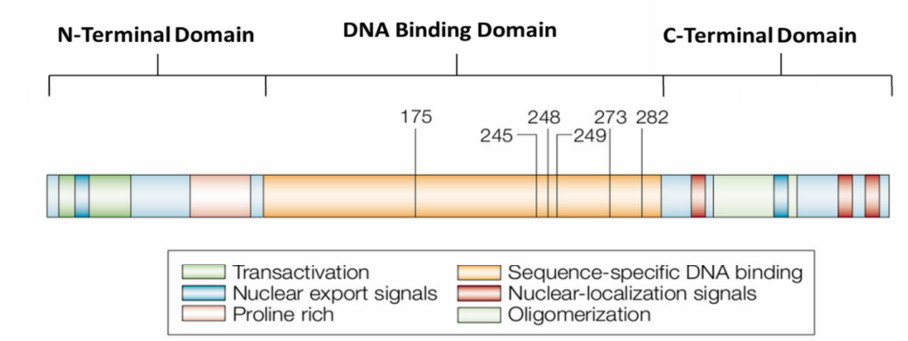

Figure 11 : p53 protein structure. p53 N-terminal region (N-terminal domain on the figure) contains two transactivation domains (TAD1 and TAD2) subject to various post translational modifications. Moreover, the N-terminal region possesses a proline-rich domain where a polymorphism influencing p53 activity was identified. The central part of p53 is the DNA-binding domain. This region, frequently mutated in cancer, is responsible for the selective binding of p53 to DNA. In this figure, six hotspots of mutation are presented (R175, G245, R248, R248, R273 and R282) and represent almost 30% of all p53 mutations in cancer. Finally, the C-terminal region (C-terminal domain on the figure) contains a domain of tetramerization, as well as a nuclear localization and a nucler export signal. The C-terminal region is critical for the regulation of p53 stability. It contains different lysines that can be ubiquitinated (K370, K372, K373, K381, K382 and K386). Modified from Vousden and Lu, 2002.

The N-terminal region of p53 encompasses the 94 first amino acids of the protein. The domain is further divided into two transactivation domains, TAD1 (AA 1 to 40) and TAD2 (AA 40 to 61), and a proline-rich domain (PRD: residues 64 to 92) [Joerger and Fersht,

2008]. TAD domains are naturally weakly folded and responsible for interactions with several other proteins such as MDM2 and p300/CBP. They are subject to post-translational modifications that modulate p53 stability, cellular localization or activity. There is 10 different phosphorylation sites located between Ser6 and Thr81. Each of them is thought to play a role in the modulation of p53 behavior. For example, Thr18 phosphorylation acts like a binary switch that disrupts the p53-MDM2 complex. MDM2 is the main p53 repressor and affinity analysis demonstrated that Thr18 phosphorylation decreases MDM2 binding by more than 20-fold [Lee and Gu, 2010; Jenkins *et al*., 2012]. Moreover, a nuclear export signal (NES) is located in the N-terminal domain of p53. According to Zhang, the NES includes residues 11 to 27 and displays two serine residues phosphorylated after DNA damage (Ser15 and Ser20). Following stress, phosphorylation of these serines inhibits MDM2 binding and in the same time inhibits NES functionality, allowing retention of p53 in the nucleus [Zhang and Xiong, 2001]. Finally, PRD function remains elusive. Some studies point its importance for proteins docking. For example, the prolyl isomerase Pin1 binds PRD and induces cis-trans prolyl isomerizations to inhibit MDM2 binding [Toledo and Wahl, 2007]. More recently, a team showed *in vivo* that mice lacking p53 PRD were unable to elicit response to DNA damages induced by γ-radiation and concluded that this must be an important region for signal integration [Campbell *et al*., 2012]. Finally, PRD possesses an interesting

polymorphism at residue 72 which can be either a proline or an arginine. Studies showed that this polymorphism could modify p53 structure and that the Pro72 allele was more represented in populations exposed to a higher levels of ultraviolet light. By looking at the functional differences between the two polymorphisms, Dumont *et al.* identified the Arg72 variant as more efficient to induce apoptosis than the Pro72 variant due to its enhanced mitochondrial localization [Dumont *et al*., 2003].

The DNA binding domain (DBD), located between amino acids 95 and 292 is responsible for specific interactions of p53 with DNA. Structurally, p53 DBD has an immunoglobulin-like β sandwich architecture. Two structural motifs bind the minor and major groove of the DNA. The L1 loop, the β-strand S2 and S2' as well as the C-terminal helix are responsible for binding to the major groove. Arg273 located in the S10 β-strand also contacts the major groove (Figure 12 and Figure 13). Binding to the minor groove relies on two large loops (L2 and L3) stabilized by a zinc ion. More specifically, the Zn ion interacts with Cys176, His179 from the H1 α-helix (inserted into the L2 loop), Cys238 and Cys242 from the L3 loop. Finally, Arg248 and Ser241 directly contact the minor groove while the highly mutated residue Arg273 directly contacts the DNA backbone (Figure 13) [Joerger and Fersht, 2008].

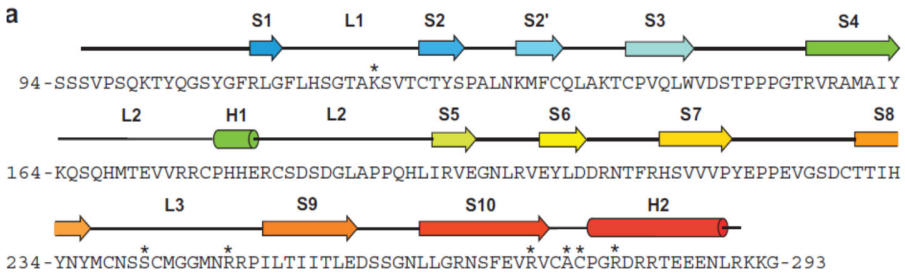

Figure 12 : Structural representation of p53 DNA binding Domain. p53 DBD contains three main loops: L1, L2 and L3. Alpha helices are represented by cylinder and beta strand sheats by arrow. Asteriks represents residues directly binding DNA. From Joerger and Fersht, 2008.

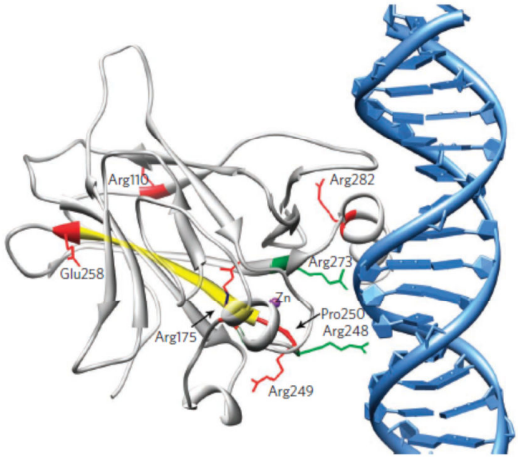

Figure 13 : Structural binding of p53 to DNA. Arg282 contained in the C-terminal helix directly contacts the major groove of the DNA. On the other hand, Serine 241 and Arginine 248 from L3 loop move into the minor groove and directly contact DNA. On this picture, it is possible to observe the Zn ion (purple dot) linking the H1 helix with the L3 loop (red and yellow arrow) [Xu *et al*., 2011].

p53 binds to specific response element (RE) located in the promoter of its target genes. The DNA binding sequence of RE is composed by two decameric half sites separated by a spacer. The sequence may vary but a decameric consensus has been defined: 5'-RRRCWWGYYY-3'. In other words, the sequence begins with three

purines (Adenine or guanine: R) followed by a cytosine (C), two adenines or thymines (W), a guanine (G) and three pyrimidines (thymine or cytosine: Y). Between each decameric half site stands the spacer. It consists in a variable sequence of 0 to 13 nucleotides. Analyses demonstrate that the shorter the spacer is, the stronger is p53 affinity for the RE. Therefore, a vast majority of p53 RE validated *in vitro* and *in vivo* possess a spacer shorter than 3 base pairs (BP) [Menendez *et al*., 2009].

The nature of the decameric sequence is extremely important. For example, replacing the C or the G in the CWWG sequence of the decameric motif drastically reduces p53 affinity for the RE. Furthermore quantitative analyses showed that the best CWWG sequence for enhancing p53 affinity was CATG [Veprintsev and Fersht, 2008]. This arrangement, found for example in the promoter of *CDKN1*, the gene coding for $p21^{Waf\text{-}1}$, [Riley *et al*., 2009], allows a stronger activation than the other combinations [Menandez *et al*., 2009; Veprintsev and Fersht, 2008]. The reason seems to be linked to the bending abilities of this sequence as compared to the other combinations [Menandez *et al*., 2009; Nagaich *et al*., 1997].

The majority of p53 mutations observed in cancer are located in the DBD. Those mutations abolish the sequence specific DNA binding of p53 [Brosh and Rotter, 2009]. By analyzing 25,902 cancer patients carrying a p53 mutation, six residues were found to be highly mutated.

These hot spots residues are: R248, R273, R175, G245, R249 and R282 and represent together 28% of p53 mutation in cancer [Wong *et al*., 1999; Freed-pastor and Prives, 2012]. Usually, mutations in p53 DBD are divided in two categories. Mutations of residues directly involved in DNA binding are called "DNA-contact mutation" (e.g. R248Q and R273H) and mutations that destabilize p53 structure are called "conformation mutant". Finally, conformation mutants are further divided into local distortion mutants (e.g. R249S or G245S) or global structure distortion mutants (e.g. R175H or R282W) [Brosh and Rotter, 2009].

The C-terminal region of p53 is 100 bp long and begins with the 293th amino acid. It includes three different nuclear localization signals (NLS), a NES and a tetramerization domain. This part of p53 is subject to massive post-translational modifications impacting its stability, subcellular localization and its activity.

The tetramerization domain (AA 325 to 356) is responsible for the assembly of p53 into a dimer of dimers [Joerger and Fersht, 2008]. In this tetrameric form, each p53 monomer binds a half site (RRRCW or WGYYY) of the double decameric sequence composing the RE. Nicholls *et al*. showed that p53 dimerization occurs directly after translation whereas the tetrameric form needs a high concentration of the dimer [Nicholls *et al.*, 2002; Joerger and Fersht, 2008] and some stabilizing post-translational modifications (such as phosphorylation of Ser392) to form [Sakaguchi *et al*., 1997].

The tetramerization domain of p53 contains a NES. This NES is efficient to relocate p53 in the cytosol via exportin 1. During tetramerization, NES of all four p53 monomers are masked in the center of the structure. This favors the nuclear retention of p53 [Stommel *et al*., 1999]. MDM2 also plays a role in p53 localisation. MDM2 is an E3 ubiquitin ligase binding the N-terminal region of p53. MDM2 is able to ubiquitinate p53 on specific lysines of its C-terminal region. Ubiquitination has been demonstrated to inhibit dimerization and therefore to unmask the NES. Therefore, ubiquitinated p53 undergoes nuclear export [Liang and Clarke, 2001].

There are also three NLS in the C-terminal part of p53: NLSI (313-322), NLSII (366-372) and NLSIII (376-381) [Shaulsky *et al.,* 1990]. NLSI seemed to be the most active NLS among all but more recent studies demonstrates that alone, NLSI is unable to induce p53 nuclear localization. It emerges that a motif containing Lys305 and Arg306 is essential, in association with NLSI, for the binding of p53 to importin α [Liang and Clarke, 2001].

3.3. Central regulation

The tumor suppressor p53 is a transcription factor involved in the transcription of many target genes. p53 reacts to several stresses such as hypoxia, DNA damage or oncogene activation by modulating cellular processes such as cell cycle, senescence or even cell death. It is therefore compulsory to ensure that such pathways are inactive in normal condition and rapidly induced following a stress. Under normal condition, p53 is constantly expressed and degraded. The physiological advantage lies in the quicker response that may be induced following a stress. Indeed, the expressed p53 just needs to be stabilized in order to become functional [Hock and Vousden, 2014].

Human double minute 2 (MDM2) is a RING (Really Interesting New Gene) domain E3 ubiquitin ligase that mainly ubiquitinates p53. MDM2 promotes p53 degradation by adding ubiquitin residues on six lysines located in the C-terminal region of p53: K370, K372, K373, K381, K382, and K386 [Lee and Gu, 2010]. Following polyubiquitination, p53 undergoes proteasome-dependent degradation. This mechanism represents the main pathway regulating p53 levels. MDM2 is the major ubiquitin ligase targeting p53, however, it is not the only one [Hoffman *et al*., 2014; Haupt *et al*., 1997; Honda *et al*., 1997]. PIRH2 [Leng *et al*., 2003], ARF-BP1 [Shmueli and Oren, 2005] and COP1 [Dornan *et al*., 2004] also ubiquitinate p53 *in vitro* and *in vivo*. Interestingly, small molecules

inhibiting the binding between MDM2 and p53 are efficient to induce a p53-dependent cellular response. This underlines the importance of MDM2 [Issaeva *et al.,* 2004].

Human double minute X (MDMX or MDM4) is closely related to MDM2. It also targets p53 and possesses a RING domain. Nevertheless, it seems that MDMX is unable to ubiquitinate p53 [Hock and Vousden, 2014]. More analyses showed that MDMX interacts with MDM2. It has been demonstrated *in vivo* that this heterocomplex was required for a proper regulation and polyubiquitination of p53 [Huang *et al*., 2011; Wang *et al*., 2011]. Loss of MDMX or MDM2, the mouse variants, results in embryonic lethality in null-mice. Interestingly, loss of *TP53* [Jones *et al*., 1995; Parant *et al*. 2001] rescues the embryonic lethality [Steinman *et al*., 2005]. In addition to point the real importance of MDM2, those studies also indicate that other ubiquitin ligases are not efficient, alone, in controlling p53 activity during embryonic development.

The main p53 repression is due to MDM2-dependent ubiquitination. However, both HMD2 and MDMX repress p53 function by other mechanisms. For example, both proteins bind p53 on its N-terminal region and inhibit its transcriptional activity. Indeed, they prevent access to co-activators such as CBP/p300. MDM2 was also shown to recruit HDACI [Ito *et al*., 2002] and MDM2 binding slightly modifies p53 conformation so that DNA binding is impaired [Cross *et al*., 2011]. Finally, MDM2 binds and ubiquitinates JMY. JMY ("Junction-

mediating and -regulatory protein"), is a transcription factor important for p53 target genes expression. It is recruited by p300 and favor gene transcription. In normal cells, MDM2 induces the degradation of JMY, impairing expression of it target genes. This adds a new layer of complexity in the regulation of p53 transcriptional activity regulation [Coutts *et al*., 2007].

More recently, MDM2 was shown to mono-ubiquitinate p53 with a different outcome. Indeed, instead of targeting p53 to the proteasome, monoubiquitination in presence of a low levels of MDM2 [Wang *et al*., 2011] induces nuclear export of the protein [Li *et al*., 2003]. In the cytosol, p53 monoubiquitinated forms may either be sequestrated by E3 ligases such as WWP1 [Laine and Ronai, 2007] or Ubc13 [Laine *et al*., 2006] or either relocated to the mitochondria.

Finally, MDM2 expression is regulated by p53. p53 is able to bind with high affinity to the MDM2 promoter and directly triggers its expression. Therefore, a negative feedback loop exists in order to precisely control p53 cellular levels and activity [Wu *et al*., 2004].

3.4. Regulation of p53 transcriptional activity

The classical activation pathways of p53 are reviewed by several papers [Riley *et al*., 2009; Prives and Hall, 1999; Vousden and Prives, 2009]. It consists in successive steps: p53 stabilization, sequence specific DNA binding and target genes expression.

3.4.1. p53 stabilization

Following stresses (such as DNA damage or oncogene activation) p53 is stabilized and activated. Its half-life increases from minutes to hours. The crucial step leading to p53 increased half-life resides in the suppression of the MDM2/MDMX-p53 interaction. Stresses are different by nature, therefore mechanisms targeting p53-MDM2 interaction may vary. For example, oncogene activation leads to the induction of ARF. ARF directly binds MDM2 on its acidic region (residues 237-268) [Manfredi, 2010] and inhibits its interaction with p53. On the other hand, DNA double strand breaks will rather activate the ATM kinase ("Ataxia telangiectasia mutated") while DNA damages induced by replication stresses or DNA crosslink will rather involve ATR ("Ataxia telangiectasia and Rad3-related protein") [Meek, 2009]. ATM and ATR are both kinases that activate p53 by different mechansims: 1) ATM and ATR phosphorylate and activate other kinases involved in the

stabilization of p53 such as CHK1 and CHK2 [Gatei *et al.*, 2003; Branzei and Foiani, 2008]; 2) ATM and probably ATR phosphorylate MDM2 and MDMX. MDM2 phosphorylation stimulates auto-ubiquitination and destruction while phosphorylation of MDMX stimulates MDM2-dependent ubiquitination and degradation of MDMX [Stommel and Wahl, 2004; Chen *et al.,* 2012; Chen *et al*., 2005]; 3) Finally, ATM and ATR directly phosphorylate p53 on Ser15 [Ollson *et al.,* 2007]. This phosphorylation is important for MDM2-p53 complex disruption [Momand *et al*., 2000]. Indeed, pSer15 was shown to be necessary for the subsequent phosphorylation of Thr18 and Ser20 [Saito *et al*., 2003]. Altogether, these mechanisms inhibit p53 ubiquitination and stabilize the tumor suppressor.

3.4.2. Sequence-specific DNA binding and target genes expression

In the classical model, the binding of p53 to specific DNA sequences is the second main step. This step is influenced by the nature and the intensity of the stress. In function of the intensity, different p53 post-translational modifications may occur which modulate its stability or improve its binding to the so-called low-affinity promoters. Among modifier proteins that further stabilize p53, there are acetyltransferases (p300, CBP, TIP60 [Ollson *et al*., 2007; Riley *et al*., 2009]),

methyltransferases (SET9 or SMYD2 [Ollson *et al.,* 2007]) and kinases (HIPK2, DYRK2, p38MAPK [Kruse and Gu, 2009; Ollson *et al*., 2007]). As discussed earlier, the RE of a promoter may be more or less suitable to p53 binding. For example, p53 possesses a high affinity for the promoter of the genes coding for $p21^{WAF-1}$, MDM2 or GADD45α. Therefore, after stabilization, p53 preferentially binds and induces expression of those genes [Riley *et al*., 2009]. In other circumstances, genes involved in senescence may be expressed. However, this requires modifications such as phosphorylation of Ser376 and loss of phosphorylation of Ser392 [Webley *et al.*, 2000]. Finally, if the stress reaches a critical threshold, apoptosis is induced. Here, important post-translationnal modifications include phosphorylation of Ser46 [Oda *et al.,* 2000; Li *et al*., 2005] and acetylation of Lys120 for the expression of p53AIP1, BAX and PUMA [Sykes *et al.,* 2006]. These additional post-translational modifications increase p53 affinity for the RE located in the promoter of pro-apoptotic genes. According to the authors, it is not clear if these post-translational modifications enhance p53 affinity for the DNA sequence or if it helps to recruit specific cofactors [Oda *et al.,* 2000; Sykes *et al.,* 2006]. Concerning K120, since this residue is directly located in the DBD, recruitment of cofactors would seem less plausible than enhancement of p53 affinity. However, the K120R p53 mutant is still located on the PUMA and BAX promoters [Sykes *et al.,* 2006] (Figure 12 and Figure 13). Further investigations are needed to confirm the mechanisms involved.

3.5. Recent advances on p53 function.

Recently, several papers challenged the relative importance of the three canonical cellular responses of p53 and highlighted the importance of p53 in metabolism regulation, stem cell maintenance, invasion and metastasis.

The tumor suppressor p53 classically promotes cell cycle arrest, senescence or apotosis. However, some teams recently suggested that these pathways of p53 were mainly built using radiation and doxorubicin, agents triggering acute DNA damages and the above mentonned p53-mediated responses. Therefore, our current knowledge about p53 would only be a part of all p53 abilities.

In 2012, Li *et al.* generated mice expressing $p53^{3KR}$. In this model, three normally acetylated lysines important for p53 binding to DNA are replaced by three arginines. MEFs generated from this model are unable to trigger cell cycle arrest, apoptosis and senescence in response to ionizing radiation. The reason lies in the unability of $p53^{3KR}$ to induce several genes among which *Puma*, *Cdkn1a* (gene coding for p21), *Serpine1* and *Pml* (two genes involved in senescence induction). However, mice $Trp53^{3KR/3KR}$ are less prone to develop tumors than $Trp53^{-/-}$ mice. By analyzing the remaining transcriptional activity under acute DNA damage, they showed that $p53^{3KR}$ cells were able to activate genes such as glutaminase 2 (*Gls2*), TpP53-induced glycolysis and apoptosis

regulator (*Tigar*) and gluthation peroxidase 1 (*Gpx1*). In addition, basal levels of $p53^{3KR}$ were able to regulate glucose uptake, restrain glycolysis and inhibit ROS accumulation [Li *et al.,* 2012]. These remaining activities were suggested to play a crucial role in tumor suppression.

Another clue of the importance of other activities of p53 came from Valente *et al*. They generated triple knock out mice for *Cdkn1a, Puma* and *Noxa.* These mice are deficient for p53 dependent apoptosis and cell cycle arrest following DNA damage. Furthermore, fibroblasts isolated from the triple KO are partially deficient for senescence induction in presence of etoposide. However, these mice were free from tumor until the age of 500 days while $Trp53^{-/-}$ mice developped tumors after 250 days. Therefore, in normal cellular condition, *Cdkn1a, Puma* and *Noxa* are not necessary to prevent tumorigenesis. This underlines, the importance of other p53 pathways in tumor prevention [Valente *et al*., 2013].

Finally, Brady *et al.* analyzed a $p53^{25,26}$ knock-in mouse model where p53 is mutated in the first TAD domain (L25Q, W26S) and therefore unable to trigger the expression of the most common p53 dependent genes. However, p53 retained a tumor suppressive ability. By analyzing differences in genes expressed by wild type p53 and $p53^{25,26}$ in oncogene-expressing MEFs, genes implicated in DNA repair, signaling and regulation of the cytoskeleton were identified. Further experiments

where those “hits” were downregulated showed an increase of tumor growth in allografts tumor assays [Brady *et al*., 2011].

The above studies suggest that other pathways than the canonical cell cycle arrest, apoptosis and senescence pathways are able to be induced by p53 and participate to the maintainance of cell integrity. A role of p53 has now been identified in various pathways such as glycolysis inhibition, autophagy induction, inhibition of the reprogramming of differientiated cells into pluripotent cells, inhibition of EMT and inhibition of the modification of the surrounding ECM (e.g. by expression of anti-angiogenesis molecules such as Tsp1) [Bieging *et al*., 2014]. It is however clear that cells harboring full p53 functions will rapidly undergo cell cycle arrest, senescence or apotosis in response to an insult such as DNA damage. These other mechanisms may also represent a back-up plan in case of problems with classical p53 pathways.

To conclude, earlier studies determined that p53 inhibits tumor development by triggering cell cycle arrest, senescence or apoptosis. In order to continue our road towards the most potent p53 based therapies, a full understanding of p53 many functions is crucial. All p53 dependent mechanisms controlling cell growth and proliferation must be identified and characterized to determine their potential for the development of therapies. In the future, therapies will focus on p53

dependent mechanisms that are the most relevant for the cell type and the tumor environment. [Bieging *et al*., 2014].

3.6. Non-transcriptional functions of p53

First evidences for a non-transcriptional function of p53 came from observations describing non-nuclear p53 accumulation during apoptosis [Speidel, 2009] as well as p53-dependent apoptosis in the absence of transcription or protein synthesis [Caelles *et al*., 1994; Gao and Tsuchida, 1999]. In 2000, Marchenko *et al*. crossed these observations with the fact that in eukaryotic cells, mitochondria are critical player in apoptosis. They found small amounts of p53 localized at the mitochondria following a stress such as DNA damage or hypoxia. Mitochondrial p53 was absent in normal non-stressed cells [Marchenko *et al.,* 2000]. Since this first observation, mitochondrial functions of p53 as well as the mechanisms allowing its mitochondrial localization have been investigated.

3.6.1. p53 mitochondrial translocation requires MDM2-mediated mono-ubiquitination

Mitochondrial relocalization of p53 is observed following different stresses such as DNA damage, hypoxia, irradiation and cytotoxic drugs [Speidel, 2009]. Moreover, targeting p53 to the mitochondria by fusing it to a mitochondrial localization signal (MLS) is sufficient to induce massive cell death [Dumont et al., 2003]. Rapidly, data suggested that the non-transcriptional apoptotic activity of p53 relies on its ability to trigger MOMP through interactions with BCL-2 family members [Speidel, 2009].

In unstressed cells, observations established that p53 shuttles between the nucleus and the cytosol. Furthermore, it accumulates in both locations when stabilized [Speidel, 2009]. Muyang Li et al. confirmed that a high MDM2 concentration leads to the poly-ubiquitination of p53 and its proteasome-dependent degradation. Nevertheless, a low levels of MDM2 induces mono-ubiquitination and p53 nuclear export [Li et al., 2003]. Mechanisms leading to p53 mitochondrial relocalization are not completely resolved. However, a first description of the importance of p53 ubiquitination was published in 2003. It has been shown that the p53 Arg72 polymorphism variant was more relocated to the mitochondria and more prone to induce apoptosis as compared to the Pro72 variant. Authors linked this

observation to a better binding of the Arg72 variant to MDM2. This enhanced binding leads to a greater ubiquitination and CRM-1 dependent nuclear export of the tumor suppressor. Moreover, the authors presented evidence of ubiquitinated forms of p53 at the mitochondria [Dumont et al., 2003]. Marchenko et al. who already demonstrated the presence of p53 at the mitochondria, later made the observation that mono-ubiquitination was a mitochondrial relocalization signal for p53. Moreover, by analysing a p53-BCL-2 protein complex, they found that all p53 proteins involved in the complex were ubiquitine free. Therefore they hypothesize that the protein HAUSP deubiquitinates p53 at the mitochondria and that non-ubiquitinated active forms of p53 are able to induce mitochondrial apoptosis [Marchenko et al., 2007].

3.6.2. Mitochondrial apoptosis: mechanisms of induction

At the mitochondria, p53 acts as a BH3-only protein and modulates BCL-2 members activity. Three mechanisms were reported [Fuster et al., 2007].

3.6.2.1. BAK-MCL-1 complex disruption

This mechanism relies on disruption of an inhibitory MCL-1-BAK complex located at the surface of the MOM. Following a stress, p53 is relocated to the mitochondria and directly interacts with BAK. p53-BAK complex formation correlates with BAK oligomerization, release of Cyt C and loss of interaction between BAK and MCL-1. Therefore, a model in which p53 displaces BAK from its inhibitory complex was suggested. In addition, p53 was shown to induce a conformation change of the BAK protein required for its oligomerization [Leu *et al*., 2004].The authors also describe that p53 does not interact with BAX or BCL-XL. Moreover, p53 is unable to trigger MOMP and Cyt C release in presence of BAK -/- mitochondria. Further studies revealed that Lys120 acetylated forms of p53 were enriched at the mitochondria and that this post-translational modification was important for the ability of p53 to disrupt the BAK-MCL-1 complex [Sykes *et al.*, 2009].

3.6.2.2. Cytosolic BAX activation

Chipuk *et al.* demonstrated that, following a stress, p53 accumulation in the cytosol was efficient to activate cytosolic BAX and trigger its oligomerization at the MOM. Here, however, no binding between BAX and p53 was detected by co-immunoprecipitation. Therefore, authors hypothesize a “hit and run” mechanism to explain BAX activation by p53 [Chipuk *et al*., 2003]. They proposed that cytosolic p53 binds the inhibitory BCL-XL protein. *De novo* synthetized PUMA, a transcriptional target of p53, displaces p53 from this inhibitory complex. This allows p53 to activate cytosolic BAX. However, authors also raised the possibility that the high p53 concentration in stressed cell may exceed cytosolic BCL-XL concentration [Chipuk *et al*., 2005].

3.6.2.3. Inhibition of BCL-2 and BCL-XL

Moll and co-workers showed the direct binding of p53 with BCL-XL but also BCL-2 and hypothesize that p53 binding neutralizes the inhibitory effect of these proteins on BAX and BAK. This hypothesis is supported by evidences establishing that following stress, concentration of p53-BCL-2 complex was rather enhanced than diminished. Moreover, this is correlated with the loss of BCL-2-BAX or BCL-XL-BAX complexes

present at the mitochondria. Therefore the idea is that p53 displaces pro-apoptotic BCL-2 members from their inhibitory complex [Mihara *et al.*, 2003]. However, Chipuk *et al*. described that in stressed cells, p53-BCL-XL complex concentration decreases. According to that, the hypothesis is made that the BCL-2-p53 complex is an inhibitory complex that sequestrates p53 and prevents it to exert its pro-apoptotic function by activating BAX and BAK proteins [Chipuk and Green, 2004].

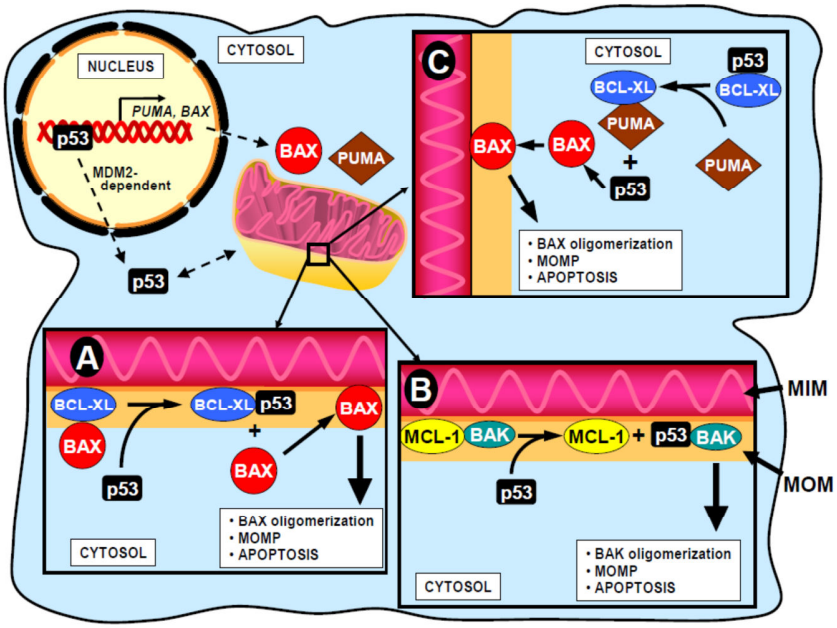

Figure 14 : Mitochondrial pro-apoptotic activities of p53. Following a stress, activated p53 enters the nucleus and induces the expression of genes implicated in apoptosis induction such as PUMA, NOXA or BAX. In parallel, p53 is relocated to the mitochondria and induces MOMP formation by three mechanisms. A) p53 displaces BAX from its inhibitory complex with BCL-XL and allows BAX to oligomerize and trigger MOMP. B) p53 displaces BAK from its complex with MCL-1. p53 activates BAK by inducing a change in BAK conformation allowing MOMP formation by BAK oligomerization. C) p53 activates cytosolic BAX following the "hit and run" model. Worthy of note, this model requires the implication of PUMA to displace p53 from its complex with BCL-XL. From Fuster *et al*., 2007.

3.7. p53 alteration in cancer and p53-based therapies.

Reasons for p53 impairment in cancer are numerous: mutations, viral infection, overexpression of upstream inhibitors, loss of activators or downstream effectors, nuclear exclusion... (Figure 15). They will be discussed below.

3.7.1. p53 mutation

As described earlier, p53 impairment may be the results of a mutation (missense, nonsense or insertion/deletion) of the *TP53* gene leading to the production of a non-functional form of p53. According to Brosh, mutations of p53 occur in approximately 50% of cancers but incidence varies among cancer types. For example, *TP53* mutation reaches only 10% in haematopoietic malignancies but reaches up to 70% in ovarian, colorectal and head and neck cancers [Brosh and Rotter, 2009]. It is demonstrated that restoring p53 function sensitizes established cancers to apoptosis or senescence and leads to tumor regression [Sharpless and Depinho, 2007]. Several molecules were recently shown to restore p53 function and inhibit the oncogenic properties of p53 mutants. For example, Y220C is a mutation commonly

found in cancer and is responsible for the destabilization of the core structure of p53. Recently, several teams used compounds (such as benzothiazoles) and demonstrated a regain of activity of p53. For example, Liu *et al*. demonstrated that PK7088 was able to bind p53 and increase the concentration of mutant $p53^{Y220C}$ with the correct conformation. Moreover, transcriptional analysis showed an increased level of p21 and NOXA whereas nutlin-3a had no effect [Liu *et al*., 2013].

Another example is PRIMA-1 (2,2-bis(hydroxymethyl)-1-azabicyclo(2.2.2) octan-3-one) and its more active derivative PRIMA-1^{MET}. So far, PRIMA-1^{MET} is the most advanced drug in clinical studies, already reaching phase II clinical trials [Hao and Cho, 2014]. PRIMA-1 and also its MET version were identified by library screening for their ability to restore active conformation to mutants of p53, allowing DNA binding and apoptosis induction [Bykov *et al*., 2002]. Analyses demonstrate that PRIMA-1^{MET} is active against several types of cancer in xenograft mouse models and has only little side effects [Zache *et al*., 2008; Zandi *et al*., 2011]. Interestingly, almost all mutants are sensitive to PRIMA-1^{MET} and only the Phe176 mutant was shown to be insensitive to the molecule [Duffy *et al*., 2014]. In addition, PRIMA-1 enhanced the action of drugs currently used in cancer chemotherapy such as doxorubicin [Magrini *et al*., 2008].

Finally, 8% of all mutations occurring in *TP53* give rise to a truncated form of p53 due to nonsense mutation. Recent work identified drugs

able to promote read-through non-sense mutation [Khoo *et al.,* 2014]. For example, Floquet *et al.* showed that aminoglycoside antibiotics, such as G418, are able to stabilize the mutant mRNA of p53 and to inhibit its degradation by non-sense mediated decay. Treatment with G418 leads to the production of a functional full-length p53 and a reduction of viability of cells bearing such mutations due to P21 and BAX expression [Floquet *et al.,* 2011].

3.7.2. Viral infection

Some cancers, such as cervix cancers, are linked to a viral infection with human papillomavirus (HPVs). HPVs family consists in more than 100 subtypes of small DNA viruses classified in high and low risk HPVs [Duensing and Münger, 2004]. High-risk HPVs express two oncoproteins, E6 and E7 targeting p53 and RB respectively. E6 enhances the proteasome-dependent degradation of p53, FADD and caspase-8 [Yuan *et al.,* 2012] while E7 binds and inhibits Rb protein [Thomas and Laimins, 1998]. In addition to inhibit p53 function, E6 protein was recently shown to trigger cancer cell migration. E6 downregulates miR-23b expression and increases the expression of its main target, the urokinase-type plasminogen activator (uPA) [Au Yeung *et al*., 2014]. uPA favors EMT, mesenchymal stem cell migration and transendothelial migration, all characteristics of metastatic dissemination [Jo *et al*., 2009; Krstic *et al*.,

2014]. Although, E6 and E7 alone are not able to transform cells and additional events are required [Duensing and Münger, 2004], it is clear that HPVs favour cell transformation by inhibiting critical cellular gatekeepers function and by enhancing cell motility.

Currently, there is no effective antiviral agent available on the market. Although some molecules, like bortezemib were recently shown to block the function of the E6 protein in HPV-positive HNSCC cells [Li and Johnson, 2013]. Therefore, specialists focus on vaccination with Gardasil (Merck) or Cervarix (GlaxoSmithKline) in order to prevent infections by HPV-16 or -18. In the future, high throughput screening approaches will aim to identify compounds inhibiting E6/E6-Associated Protein (E6AP) association responsible for the E6-mediated degradation of p53 [Yuan *et al*., 2012].

3.7.3. Upstream regulators

Upstream activators inefficiency may be responsible for a decreased p53 activity. For example, loss of ARF reinforces MDM2 inhibition and therefore compromises p53 stabilization in presence of diverse oncogenes [Sherr and Weber, 2000]. In the same manner, dysfunction of kinases involved in p53 stabilization such as ATM or CHK2 may alter p53 stabilization and activity [Johnstone *et al.,* 2002]. On the other hand, the overexpression of a direct repressor of p53 like MDM2 or MDMX may also lead to p53 enhanced degradation [Ramos *et al*., 2001; Tovar *et al*., 2006; Reifenberger *et al*., 1993]. Development of molecules inhibiting MDM2 and/or MDMX is currently performed. Nutlins are probably the most investigated family of molecules so far. Nutlins mimic p53 residues responsible for MDM2 binding (Phe19, Trp23 and Leu26 [Chène, 2003; Duffy *et al.,* 2014]). They block the docking pocket of MDM2 and inhibit its interaction with p53. In animal models, a low toxicity was observed. Moreover, an increase in p53 concentration and an enhanced apoptosis is observed [Duffy *et al.,* 2014]. However, so far, complete tumor regression in animal models remains elusive. Therefore the development of a new generation of molecules such as MI-219 has been undertaken with good results in cell models. Another molecule, RG7112 binds MDM2 similarly to Nutlin-3a but with a much higher affinity. Moreover,

in vivo experiments demonstrate a regression of tumors from various origins. RG7112 currently undergoes phase I of clinical trials.

Unfortunetly, these molecules are designed to target MDM2 only. It is well known that in addition to MDM2, MDMX can also be overexpressed in cancer cells, notably in melanomas. Therefore a coumpound targeting both molecules would be interesting [Khoo *et al.,* 2014]. Even if inhibitors of MDMX exist, for example SJ-172550 [Reed *et al*., 2010], efforts are now made to develop small molecules inhibiting the binding of both MDMX and MDM2 to p53.

3.7.4. Nuclear exclusion

In 1994, Ryan *et al.* described, for the first time, a mechanism in which p53 trafficking was impaired in presence of an overexpression of BCL-2 [Ryan *et al*., 1994]. A year later, Moll *et al.* also noticed that wt p53 could be sequestrated into the cytosol and hypothesized that sequestration may be a novel mechanism for p53 inactivation [Moll *et al*., 1995]. Years later, Nikolaev *et al.* demonstrated that Parc, a Parkin-like ubiquitin ligase, acts as a cytoplasmic anchor for p53 and inhibits its nuclear relocalization. Moreover, RNAi targeting of Parc significantly reduces cell resistance to DNA damages [Nikolaev *et al.*, 2003].

Chaperones of the HSP70 family can also be responsible for a reduced nuclear relocalization of p53. This is the case, for instance, of

the mitochondrial heat shock protein mortalin (aka Mot-2, GRP75 or HSP74) also located at the endoplasmic reticulum and in the cytosol. An overexpression of mortalin is observed in MCF-7, HCT-116 and Saos-2 cell lines as well as in leukaemia and liver metastasis [Wadhwa *et al*., 2006; Gestl and Böttger, 2012]. This is correlated with an enhanced p53 cytosolic localization. The complex between p53 and mortalin inhibits p53 entry into the nucleus and can also abolish its interaction with BCL-2 family members. Therefore, mitochondrial and transcriptional apoptosis are both inhibited. Targeting wt p53 sequestration may be an efficient approach to restore p53 function. Maureen Murphy and colleagues are currently developing molecules, notably 2-Phenylethynesulfonamide (PES) that interacts selectively with HSP70 and disrupts its association with partners such as p53. Data demonstrate that PES can suppress tumor development in a mouse model [Leu *et al*., 2011]. Besides, and in association with professor Murphy, our laboratory will soon test next generation compounds to establish their potential effects on inhibition of p53 sequestration as well as reactivation of mutant forms of p53.

3.7.5. Downstream inhibition

Finally, every inhibition or alteration of effectors of the p53-dependent response as well as overexpression of inhibitors of pathways located downstream of p53 may also compromise the activity of the tumor suppressor. A rather well documented example is the overexpression of the anti-apoptotic BCL-2 family members. When activated, p53 induces the expression of BH3-only proteins such as PUMA and NOXA and therefore favours MOMP. In the case of an overexpression of one or several anti-apoptotic BCL-2 members as observed in hematologic malignancies [Reed, 2008], the ratio between pro- and anti-apoptotic proteins is disturbed and apoptosis may be inhibited.

Several molecules called BH3-mimetics were developed to restore the mitochondrial apoptosis pathway. Gossypol is one of them and has been discovered for its antifertility properties. Apogossypol, an analogue, seems to better target BCL-2 and MCL-1 and therefore induce fewer side effects. Mechanistically, gossypol targets the hydrophobic groove of anti-apoptotic BCL-2 members that interacts with BH3-only proteins. Therefore, in its presence, p53-dependent expression of BH3-only proteins may activate BAX or BAK and trigger MOMP [Kang and Reynolds, 2009].

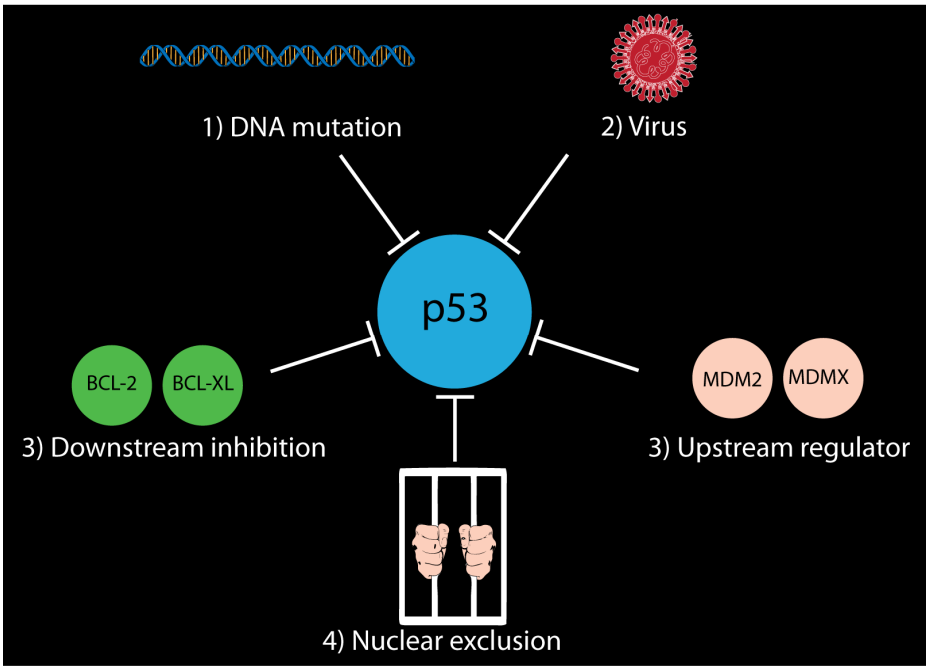

Figure 15 : Heterogeneity of p53 inactivation in human cancer. Mutation, viral infection, upstream inhibitor overexpression, nuclear exclusion and downstream inhibition are among the main modes of p53 inactivation in cancer. Therapies focus on restoring the wt activity to the mutant forms of p53, on inhibiting interactions that compromise wt p53 function as well as on prevention of viral infection.

Therapies based on p53 were developed on our current knowledge of p53. As discussed above, molecules inhibiting protein-protein interactions or refolding mutated forms of p53 are among the most advanced p53 based therapies. In addition, it seems more and more clear that the cell type and the alterations beared by the tumor will be important factor to determine the best suited therapy. With the recent demonstration of unknown or rather underestimated functions of p53, new therapies will possibly rise and bring new alternatives to treat

patients. Combination of old and new methods may represent the future of clinical therapies.

	MAGE-A1	MAGE-A2	MAGE-A3	MAGE-A4	MAGE-A6	MAGE-A12
COLON CARCINOMAS						
MZ-CO-2	++	++	-	-	-	+
SK-CO-11	-	++	+++	-	+	++
LiB 150	-	-	-	+	-	-
HSR 320	-	+++	+++	+	++	+++
LEUKEMIAS						
K562	-	++	+++	-	++	+++
MELANOMAS						
MI10221	+	+++	+++	+++	+++	+++
MZ2-MEL 3.0	+++	+++	+++	-	+++	+
LB265	-	++	-	-	-	+
LG7	-	++	-	-	-	-
LGII	++	++	++	-	-	+++
LB27	-	++	+++	-	++	+++
LUNG CANCERS						
LBI78 (NSCLC)	++	-	-	+++	-	-
LB 175 (NSCLC)	++	++	+++	+++	++	+++
LBII (SCLC)	++	+++	+++	-	-	+++
LBI2 (SCLC)	-	+++	+++	-	-	+++
SARCOMAS						
LB23	-	-	-	++	-	-
LB408	-	-	-	++	-	-
LB258	+	++	+	-	-	++
BREAST CARCINOMAS						
LB280	++	-	++	-	-	+
LB284	++	++	++	+	-	++
NORMAL TISSUES						
Stomach	-	-	-	-	-	-
Lung	-	-	-	-	-	-
Breast	-	-	-	-	-	-
Colon	-	-	-	-	-	-
Skin	-	-	-	-	-	-
Uterus	-	-	-	-	-	-
Testis	++	++	++	++	++	++
Thymocytes	-	-	-	-	-	-
EBV-lymphocytes	-	-	-	-	-	-
Foetal liver	-	-	-	-	-	-
Foetal brain	-	-	-	-	-	-
Placenta LB694	-	-	+	+++	-	-

Table 1 : MAGEA expression measured by RT-PCR in several cancer lines, tumor samples (underlined entries) and normal tissues. The levels of expression is presented by +++ (strongly expressed), ++ (moderately expressed) and + (lightly expressed). - indicate that no signal has been detected. As described in the text, MAGEA genes are not expressed in normal tissues to the exception of testis and placenta while cancers commonly expressed 6 of them. MAGEA5, -A8, -A9, -A10 and -A11 are almost not expressed in all samples analyzed (less than 2 copies per cell). Modified from De Plaen *et al.,* 1994.

Chapter 4: MAGEA

4.1. Background

In the early 90's cancer research focused its attention on the identification of antigens that would be cancer specific. With such breakthrough, the hope for a cancer vaccine could be reached and a cure found. In 1991, Van der Bruggen *et al*. published encouraging results. It was known that cytolytic T lymphocytes (CTL) could elicit a reaction towards melanoma tumor cells *in vitro*. Van Der Bruggen *et al*. selected CTL that were particularly effective against MZ2-MEL cells, a melanoma cell line derived from patient MZ2-E, and identified the antigen MZ2-E responsible for CTL activity . This antigen is encoded by a 2.4 kb DNA fragment containing an unknown gene that was named MAGE for melanoma antigen (known today as MAGEA1). That gene contains three different exons and an open reading frame located entirely in the third exon. During this identification phase, they also noticed by southern blot analysis that the probe hybridized to several additional bands suggesting the existence of closely related genes. Finally, two other genes were identified and named MAGE-2 and -3 (later renamed MAGEA2 and MAGEA3) [Van der Bruggen *et al*., 1991]. Three years later, the same team demonstrated that MAGEA1 was expressed in several cancers such as colon cancer, breast cancer, melanoma, lung cancer but never in

normal tissues at the exception of testis and placenta (Table 1). Moreover, other genes were identified. The family now contains eleven expressed genes that share the same structure as MAGEA1: three exons with the last one coding for the entire protein. The percentage of base pair homology among MAGEA genes varies from 64% to 84% and the overall protein structure is predicted to share similar features. Dissimilarities among MAGEA genes are mainly located in the promoter and in the first non-coding exon suggesting that members of the family may be differentially activated depending on stimuli and pathways activated [De Plaen *et al.,* 1994].

4.2. Cancer testis antigens

MAGEA genes are part of the the larger group of cancer testis antigens. Indeed, shortly after the identification of MAGEA1 by Van Der Bruggen *et al.*, other teams identified different genes that could express tumor specific antigens. In 1997, the highly immunogenic tumor antigen New York Esophageal Squamous Cell Carcinoma 1 (NY-ESO-1) and, in 1998, the Synovial Sarcoma/X Breakpoint 2 (SSX-2) were discovered [Sahin *et al*. 1997, SEREX technology]. Later, a variety of genes with cancer and testis restricted pattern of expression were identified using various techniques such as comparison of genes expression between tumor and normal tissues. Today, these genes only expressed in cancer

and in normal testis are called the cancer testis antigens (CTAs). They include at least seventy subfamilies and more than 140 members all referenced online[10] [Fratta *et al*., 2011].

CTAs are further divided into CTAs encoded on the X chromosome (X-CTAs) and CTAs encoded elsewhere in the genome (non-X CTAs) [Simpson *et al*. 2005]. The X-CTAs represent more than 50 percent of all CTAs and constitute 10 percent of all genes located on the X chromosome. Usually, X-CTAs are families clustered along the X chromosome (like MAGE but also NY-ESO-1, GAGE and BAGE families) while non-X-CTAs are generally lonely genes scattered on other chromosomes [Simpson *et al*., 2005].

The biological functions of CTAs are poorly understood. Nevertheless, concerning the MAGE family, increasing evidences indicate that they could modulate gene transcription by favoring or inhibiting the recruitment of cofactors. Moreover, it seems more and more clear that they may have a role in tumorigenesis.

[10] http://www.cta.incc.br/

Subfamily	Gene name	Subfamily	Gene name
MAGE-A	MAGE-A1 MAGE-A2 MAGE-A3 MAGE-A4 MAGE-A5 MAGE-A6 MAGE-A7 MAGE-A8 MAGE-A9 MAGE-A10 MAGE-A11 MAGE-A12 MAGE-A13 MAGE-A14 MAGE-A15	**MAGE-B**	MAGE-B1 MAGE-B2 MAGE-B3 MAGE-B4 MAGE-B5 MAGE-B6 MAGE-B7 MAGE-B8 MAGE-B9 MAGE-B10 MAGE-B11 MAGE-B12 MAGE-B13 MAGE-B14 MAGE-B15 MAGE-B16 MAGE-B17
MAGE-C	MAGE-C1 MAGE-C2 MAGE-C3 MAGE-C4 MAGE-C5 MAGE-C6 MAGE-C7	**MAGE-D**	MAGE-D1 MAGE-D2 MAGE-D3
MAGE-E	MAGE-E1 MAGE-E2 MAGE-E3	**MAGE-F**	MAGE-F1
MAGE-G	MAGE-G1	**MAGE-H**	MAGE-H1
MAGE-I	MAGE-I1 MAGE-I2	**MAGE-J**	MAGE-J1
MAGE-K	MAGE-K1	**MAGE-L2**	MAGE-L2
Necdin	NECDIN		

Table 2 : Known human members of MAGE family. Genes are grouped by homology and localization on the chromosome. Today, more than 50 MAGEA genes are identified. However, some of them are only pseudogenes and therefore not expressed (notably MAGEA7, -A13, -A14 and -A15). Class I MAGE genes (MAGEA, -B and -C) are only expressed in cancer or in normal testis, while Class II MAGE genes (all others) are expressed in normal somatic cells. Modified from Chomez *et al.*, 2001.

4.2.1. MAGE Family

Among CTAs, the MAGE family is one of the most studied lately. Following the discovery of MAGEA genes, sequence analysis of the Xp21 region led to the discovery of a second cluster of MAGE genes: hMAGE-B [Dabovic *et al*., 1995; Lurquin *et al*., 1997; Muscatelli *et al.,* 1995]. Finally, a third group was discovered, hMAGE-C [Lucas *et al*., 1998; Lucas *et al.,* 2000], while previously identified MAGE genes were renamed hMAGEA. Nevertheless, in 1999, Lucas *et al*. and Pold *et al.* published the very same year the sequence of a new MAGE gene that was ubiquitously expressed in normal somatic cells. This finding was the basis of a fourth cluster of human MAGE genes: hMAGE-D [Põld *et al*., 1999; Lucas *et al*., 1999].

In 2001, Chomez *et al.* published a list of human MAGE genes coding or not for antigens potentially interesting for cancer immunotherapy. Based on hMAGE-D2 sequence, a database screening was undergone. Table 2 presents the current members of the MAGE family [Chomez *et al*., 2001]. Nowadays, two types of MAGE genes are defined. The class I MAGE genes are only expressed in cancer or normal testis, and class II MAGE are expressed ubiquitously.

4.2.2. MAGE structure

All class I MAGE genes are located on the X chromosome while class II are scattered throughout the genome. MAGE genes are characterized by a large third and terminal exon carrying the complete open reading frame, an observation first made by E. De Plaen and later confirmed by Chomez *et al.*. This feature is shared by all MAGE genes to the exception of MAGE-D subfamily. The MAGE-D genes contain 13 exons, 11 of which encode for the protein.

Concerning the structure of MAGE proteins, three main regions are defined. A N-terminal region where most of the divergence among members is recorded [De Plaen *et al.,* 1994; Chomez *et al*., 2001]. The central region of the protein contains a Mage Homology Domain (MHD) of approximatively 200 amino acids and is composed of two winged helix motifs. Structurally, the Winged helix motif is a compact α/β structure composed by two wings (W1 and W2), three α helix (H1, H2 and H3) and three β strands (S1, S2 and S3) arranged in a specific order (H1-S1-H2-H3-S2-W1-S3-W2). Although, it is thought to contact DNA [Gajiwala and Burley, 2000] to our knowledge, no evidence has been recorded of MAGE proteins directly binding DNA. Nevertheless, the MHD is thought to be responsible for protein-protein interactions [Doyle *et al*., 2010], feature also predicted for Winged motif [Gajiwala and Burley, 2000]. Finally, a C-terminal region is defined, usually shorter than the N-

terminal region. The exact role of this region is not well known. The C-terminal part of MAGEA4 is thought to confer a pro-apoptotic activity to the fragment *in vitro*.

4.2.2.1. MAGEA subfamily

One of the main known functions of MAGEA proteins is the interaction and regulation of different pathways. Here are a few examples.

4.2.2.1.1. MAGEA1 and SKIP

In 2004, Laduron *et al*. showed that MAGEA1 was able to directly bind SKI Interacting Protein (SKIP) which acts in several molecular pathways such as the NOTCH1 pathway. NOTCH1 pathway is a cell signaling cascade active among others during neuronal development [Gaiano and Fishell, 2002; Aguirre *et al*., 2010], differentiation processes during embryonic life [Murtaugh *et al*., 2003; Grego-Bessa *et al*., 2007] and expansion of stem cell population [Aguirre *et al*., 2010, Dontu *et al*., 2004]. There are five canonical Notch ligands in mammals: Jagged-1, Jagged-2, DLL1, DLL3 and DLL4. Once bound to the transmembrane receptor NOTCH1, a two-step cleavage of NOTCH1 is observed. First, A Disintegrin And Metalloproteinase (ADAM) protease cleaves the

extracellular domain of NOTCH1. Afterwards, the remaining internal domain is further cleaved by a gamma-secretase ensuing its release into the cytosol[11]. In the cytosol, the Notch intracellular domain (NICD) relocates in the nucleus and modulates the expression of numerous genes such as those encoding for $p21^{WAF-1}$, p27, NFKB1, NFKB2… Mechanistically, NICD interacts with C-promoter binding factor-1 (CBF-1). CBF-1 inhibits the transcription of genes by binding SKIP and the SMRT co-repressor complex containing HDACs. Repression is released when NCID binds SKIP and CBF-1 and forces the recruitment of HATs and other co-activators. MAGEA1 was found to directly interact with SKIP, to displace NICD and to recruit HDAC1. Since NOTCH1 pathway is impaired in presence of MAGEA1, MAGEA1 participates to tumor cell growth by inhibiting expression of genes such as $p21^{WAF-1}$ [Laduron *et al*., 2004].

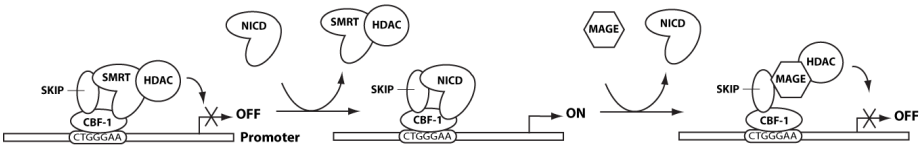

Figure 16 : MAGEA1 counteracts NICD transactivation by recruiting HDAC1. By binding to SKIP and recruiting HDAC, MAGEA1 counteracts Notch1-IC transactivation and thereby represses transcription of genes. Modified from Laduron *et al*., 2004.

[11] http://www.adipogen.com/media/Catalogs/PDFs/Notch_NP_final_2011.pdf

4.2.2.1.2. MAGEA11 and androgen receptor modulation

Androgen receptor (AR) signaling has an important role in sex male development and in normal prostate function. On the other hand, it is also a major actor in prostate cancer [Sharifi and Auchus, 2012; Mateo *et al*., 2014]. In normal epithelial and stromal prostatic cells, ARs are located in the cytosol. They are bound to Heat Shock Proteins (HSPs) that act as repressors. Androgens, displace HSP proteins [Pratt and Toft, 1997; Mateo *et al*., 2014] and allow the nuclear translocation and the binding of ARs to DNA. Androgen-dependent genes include IGF-1 (insulin like growth factor-1), CDK1, CDK2, prostate transmembrane protein-1 (PMEPA1) and TMPRSS2 [Jariwala *et al*., 2007] (Figure 17).

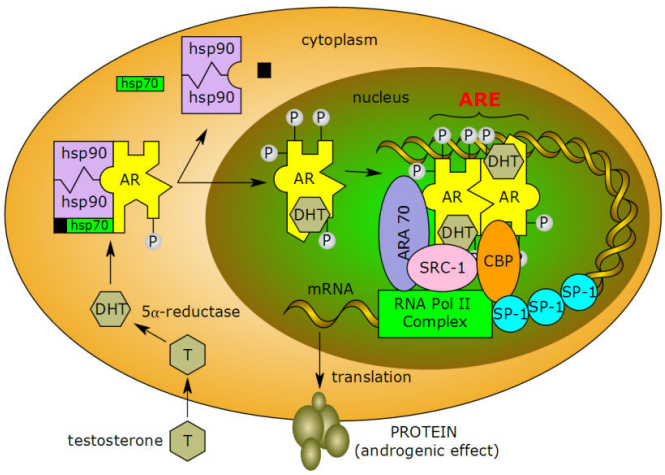

Figure 17: Androgen receptor modulation. Androgens are generated from cholesterol to form testosterone (T) and are further transformed in dyhydrotestosterone (DHT) by the 5α-reductase. DHT possesses a higher affinity for androgen receptor (AR). Once binded to AR, DHT induces a conformational change destabilizing the inhibitory complex formed by Heat Shock Protein 90

(HSP90) and AR. AR translocates to the nucleus where it undergoes dimerization. AR dimers bind DNA and recruit the transcription machinery. CBP (CREB binding protein); SRC-1 (steroid receptor coactivator 1); AR-associated protein 70 (ARA70). Modified from Meehan and Sadar, 2003 [12].

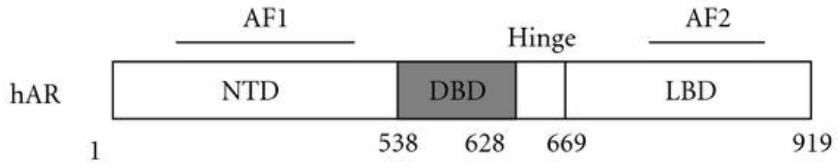

Figure 18 : Human androgen receptor structure. The protein is composed by a N-terminal domain (NTD) which contains activation function-1 (AF1), a central DNA-binding domain (DBD) responsible for androgen response elements binding and a C-terminal domain (LBD) responsible for ligand-binding activity and containing the ligand-dependent transactivation domain, AF2. From Lavery and Bevan, 2011.

The AR is composed by a N-terminal domain called activation function-1 (AF1), a C-terminal domain called activation function 2 (AF2) and a central DNA binding domain (DBD) (Figure 18). While, AF2 is responsible for the interaction with the co-activator SRC1 (p160), AF1 contains a FXXLF motif (^{23}FQNLF^{27}) that interacts with AF2 to mediate the so called "N/C interaction" required for the stabilization and the expression of many androgen-dependent genes [Wilson, 2010; Mateo *et al*., 2014].

In 2005, Bai *et al*. showed that MAGEA11 was able to regulate AR activity. MAGEA11 disrupts the N/C interaction by interacting with the FXXLF motif responsible for the AF1/AF2 interaction. Instead of weakening AR-mediated transcription, this will increase it by enhancing the binding of SRC/p160 coactivator to the AF2 region. Moreover, Askew

12 http://upload.wikimedia.org/wikipedia/commons/thumb/e/e7/Human_androgen_receptor_and_androgen_binding.svg/2000px-Human_androgen_receptor_and_androgen_binding.svg.png

et al. in 2009 described that MAGEA11, once bound to AR, recruits TIF2, another coactivator important for the transcription of AR-dependent genes. Thus, both mechanisms lead to a better activation of AR-dependent genes.

In their articles, Bai *et al*. also describe a potential post-translational modification of MAGEA11. The protein may be phosphorylated on Thr360 probably by CHK1. This induces its ubiquitination on Lys240 and 245 resulting in an enhanced binding to AR. Additionally, Askew *et al*. described that Ser174 may be phosphorylated by ERK1 [Bai and Wilson, 2008; Askew *et al*., 2009].

Our in silico analysis also indicate that Ser168, Ser174 but also Lys225, Lys236, Lys 240 and Lys245 are conserved among all MAGEA proteins, while Thr360 becomes a methionine in MAGEA2. This suggests that phosphorylations / ubiquitinations could play an important role in the regulation of MAGEA activity. These different conserved amino acids have yet to be investigated.

4.2.2.1.3. MAGEA2 and Estrogen receptor modulation

Recently, Wong et al. linked the expression of MAGEA2 to tamoxifen resistance in breast cancer [Wong *et al*., 2014]. Estrogen receptors (ERs) are transcription factors that transduce hormone signaling and trigger cell proliferation in breast and ovarian tissues

following oestradiol (E_2) signaling [Helguero *et al.*, 2005]. Two different ERs have been identified: ERα and ERβ. Like androgen receptors, they both possess a DNA binding domain, a C-terminal region responsible for ligand binding and a N-terminal part where the most divergence is observed. Two activation domains are determined: the N-terminal AF-1 and the C-terminal AF-2. Both AFs recruit coregulator proteins to the DNA bound ER receptors[13]. Like androgens receptors, ERs are sequestrated in the cytosol by Heat Shock Proteins (notably HSP90). In response to oestradiol binding, conformational changes abolish the sequestration by HSP90, allowing ERs to relocate in the nucleus, bind their response elements and recruit transcriptional co-activators such as p300/CBP histone acetyl transferases (HAT).

Tamoxifen inhibits transcription of ERs target genes since the complex tamoxifen-ERs binds DNA but fails to undergo the conformational change required for co-activators recruitment. Instead, repressors are recruited (Figure 19).

Wong and colleagues analyzed gene expression of tamoxifen resistant breast cancer and compare it to tamoxifen sensitive breast cancer. Results lead to the identification of several genes among which stands MAGEA2. They next tested a wide range of tamoxifen resistant breast cancer cell lines and confirmed the expression of MAGEA2 in each of them. Besides, by overexpressing MAGEA2 in MCF-7 tamoxifen

[13] http://physrev.physiology.org/content/87/3/905

sensitive cells, they showed a significant difference in cell growth compared to untransfected control cells unable to proliferate in presence of tamoxifen. Annexin V/IP labelling coupled to flow cytometry analysis established that MCF-7 cells overexpressing MAGEA2 were 5 to 10 times less sensitive to tamoxifen-induced apoptosis. By investigating the impact of MAGEA2 on ERα activity with a luciferase reporter gene under the control of an ER dependent promoter, they found that the inhibition by tamoxifen of genes normally induced by E2 was abolished. The underlying mechanism seems to rely on MAPK activity and correlates with an increased ER phosphorylation [Wong *et al*., 2014].

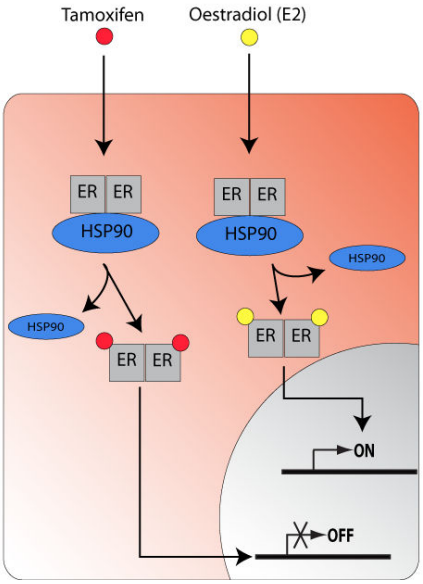

Figure 19 : Comparison between oestradiol and tamoxifen mode of action. Oestradiol binds ER and disrupts the inhibitory complex existing between HSP90 and ER. Free, ER translocates to the nucleus. Oestradiol-ER complex induces a conformational change of ER allowing co-activator recruitment. Tamoxifen acts in a similar way, except that it fails to induce the conformational change. Modified from Ratanaphan, 2012.

4.2.2.1.4. MAGEA and p53

MAGEA proteins have been shown to inhibit cellular growth and apoptosis in several models [Monte et al., 2006; Yang et al., 2007]. In 2006, a link between MAGEA proteins and the tumor suppressor p53 has been suggested. Since, the relation between those two proteins is subject to active research. Here, the suggested mechanisms will be discussed.

4.2.2.1.4.1. HDAC Recruitment

Monte et al. in 2006 published for the first time that a correlation exists between MAGEA1, -A2 and -A6 expression and a reduced p53 activity. They used a plasmid containing a synthetic p53 promoter followed by a luciferase reporter gene, and co-expressed p53 and MAGEA proteins in H1299 p53 null cells. They observed that MAGEA expression decreased p53 transcriptional activity. Moreover, they treated with etoposide a melanoma cell line expressing MAGEA genes (13923M) and another one that did not (15392M) and established a link between a high MAGEA expression and cell survival. Using quantitative CHiP assays (BAX promoter), they evidenced a complex composed of MAGEA2, p53 and Histone Deacetylase III (HDACIII) that deacetylates surrounding histones and consequently reduces gene transcription by

favoring chromatin condensation (Figure 20). They also evidenced the direct binding of MAGEA2 to p53 DNA binding domain, without inhibition of p53 binding to its RE [Monte *et al*., 2006].

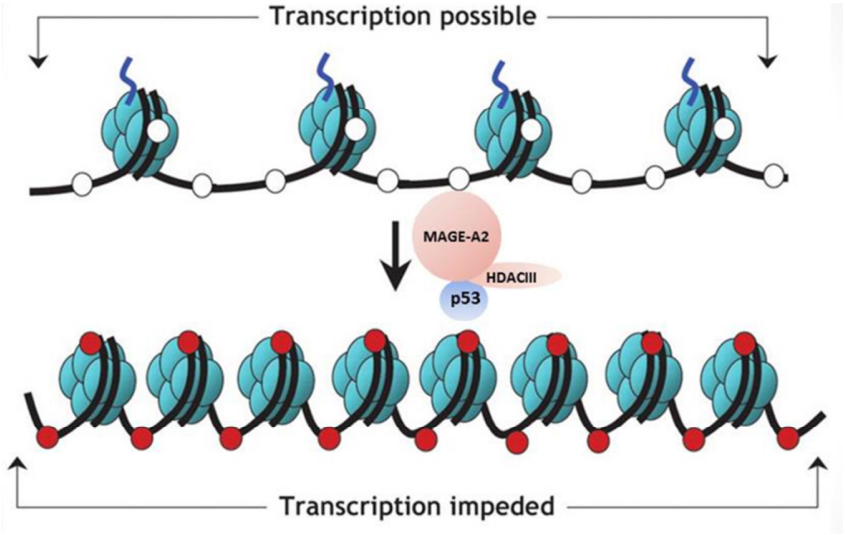

Figure 20 : Recruitment of HDACIII by MAGEA2 protein. MAGEA2 binds p53 at the p53-dependent promoter. MAGEA2-p53 complex recruits HDACIII that inhibits transcription by de-acetylating surrounding histones. Modified from Monte *et al.,* 2006.

4.2.2.1.4.2. Enhanced degradation

Yang et al. in 2007 and, in 2010, Doyle et al. described a relation between MAGEA expression and an impaired p53 function. The suggested mechanism, nonetheless, is dissimilar and some inconsistences with Monte et al. are observed. Here, MAGEA2, but also -A3, -A6 and -C2 do not directly bind p53 but rather bind to the E3 ubiquitin ligase TRIM28 (aka KAP1). This heterodimer will enhance the ubiquitination of natural TRIM28 substrates among which stand p53. Although TRIM28 is not the major ubiquitin ligase targeting p53, it is suggested to enhance p53 degradation and decrease p53 intracellular levels. Therefore, in stress condition, p53-dependent cell cycle arrest or apoptosis are compromised. Up to now, the exact mechanism enhancing TRIM28 activity is not understood. It may either be an enhanced recruitment of the E2 ubiquitin-conjugating enzyme by the MAGE protein or an increased ubiquitin transfer from E2 to TRIM28. Interestingly, Doyle *et al*. described for the first time the crystal structure of MAGEA4. It appears that the MAGEA MHD is composed of two winged helix motifs (WH-A and WH-B). Winged helix motifs are known to recognize DNA, raising the possibility that MAGEA proteins may directly bind DNA [Yang *et al*., 2007; Doyle *et al*., 2010] (Figure 21).

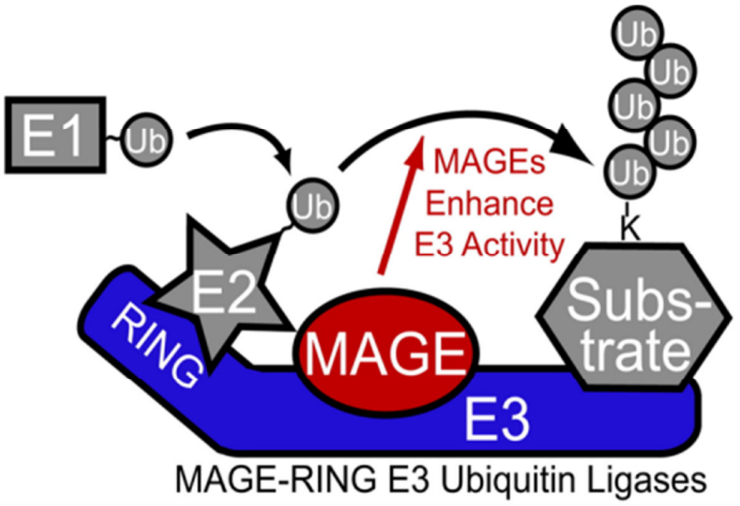

Figure 21 : MAGEA2 enhancement of TRIM28 activity towards p53. TRIM28 is an ubiquitin E3 ligase that naturally targets p53. MAGEA2 complexes with TRIM28 and enhances its activity. p53 is degraded and unable to induce pathways preventing transformation. Modified from Doyle *et al.*, 2010.

4.2.2.1.4.3. Inhibition of p53 binding to RE

In 2010, another mechanism was published by Marcar and collaborators. Here again, the link between MAGEA proteins and p53 was highlighted. They describe a direct binding between MAGEA2 and p53 involving p53 DNA binding domain and an undefined part of MAGEA2, as previously published by Monte *et al*. However, in this model, interaction with MAGEA2 renders p53 unable to regulate the transcription of its target genes. Marcar *et al.* used a pepscan assay to decipher the region of p53 interacting with MAGEA2. Biotynilated oligopeptides of 15 amino acids derived from the p53 DBD were synthetized and incubated with MAGEA2. Immunoprecipitation of MAGEA2 using streptavidin-conjugated beads allowed the characterization of the interaction [Marcar *et al*., 2010]. To our opinion, although attractive, this method cannot take into account the complexity of the 3D structure of the DNA binding domain of the tumor suppressor. Nevertheless, these results are in accordance with Monte *et al.* since they both emphasize a direct interaction of MAGEA2 with p53 DBD (Figure 22).

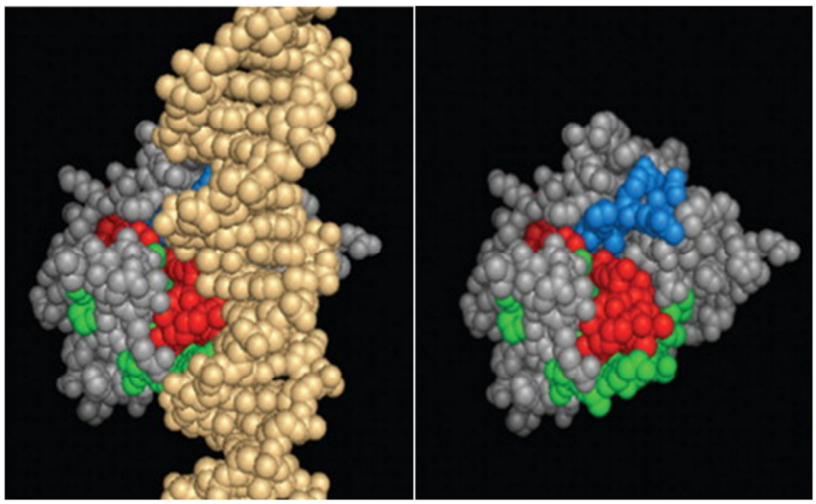

Figure 22 : MAGEA2 inhibits p53 binding to DNA. Left panel: the DNA binding domain of p53 interacting with DNA. Right panel: Region of p53 binding MAGEA2. Marcar *et al*. suggest a mechanism in which MAGEA2 binds p53 residues also important for interaction with DNA. The interaction with MAGEA2 results in impaired DNA binding and the inhibition of p53-dependent transcription. Red: amino acids 261–280: high affinity for MAGEA2, blue amino acids 247–257: moderate affinity for MAGEA2 and green amino acids 117 – 141: moderate affinity for MAGEA2. Modified from Marcar *et al*., 2010.

4.2.2.1.4.4. Lack of acetylation

Finally, in 2012, Peche *et al.* described that MAGEA2 could contribute to cancer early progression by interfering with Promyelocytic leukemia (PML) Nuclear bodies (NB) and consequently inhibiting the senescence program required for cell transformation prevention. Although *pml-/-* mice develop normally and live well [Lallemand-Breitenbach and de Thé, 2010], PML-NBs are platforms facilitating protein interactions and seem important for apoptosis and senescence induction [Zhang *et al*., 2005; Bernardi and Pandolfi, 2003]. PML-NBs possess a diameter range varying from 200nm to 1µm and are mainly composed of two kind of proteins: the SP100 protein and the PML proteins (seven isoforms of PML are described so far: PML-I to VII) [Bernardi and Pandolfi, 2007; Lallemand-Breitenbach and de Thé, 2010]. These proteins possess a SUMO interacting motif (SIM) and are able to be sumoylated. For example, PML proteins contain three different sumoylation sites required for protein recruitment and for interaction with SP100 via the SIM domain [Zhong *et al.,* 2000]. The SUMO-SIM interaction is the key to PML-NBs stabilization. For instance, desumoylation during mitosis breaks down the structure of PML-NBs [Lang *et al*., 2009].

Authors demonstrate that MAGEA2 colocalizes in the nucleus with PML-NBs and this correlates with the depletion of p53 acetylated forms. MAGEA2 inhibits PMLIV sumoylation, necessary for the PML-NBs stabilization. Several papers describe the relative importance of PML-NBs for p53 post-translational modification. Moreover, several proteins known to interact with and modify p53 such as HIPK2, HAUSP, MDM2, CBP are found along with p53 on PML-NBs [Pearson *et al.,* 2000; de Stanchina *et al*., 2004]. By abolishing those platforms, MAGEA2 could prevent different p53 post-translational modifications and therefore inhibit senescence and apoptosis induction [Peche *et al*., 2012].

4.2.2.2. MAGEA regulation

MAGEA gene expression, like other CTAs, seems to be regulated by epigenetic events such as DNA methylation. Commonly, DNA methylation leads to the silencing of gene expression. It consists in the addition of a methyl group by specific enzymes called DNA methyltransferases. This will either inhibit the binding of cofactors that would normally favor gene expression or will recruit chromatin remodeling co-repressor complexes.

In 1996, De Smet *et al*. hypothesized that the genome-wide demethylation was responsible for the reactivation of MAGEA1 expression during cancer progression [De Smet *et al*., 1996]. In 1999,

they described that all CTAs possess a promoter methylated on several cytosine-guanine dinucleotides (CpG) in normal somatic tissues [De Smet *et al*., 1999]. Previously, in 1994, Weber et al. demonstrated that DNA hypomethylating agents were able to re-activate the expression of MAGEA1 [Weber *et al*., 1994]. Further evidence demonstrating the importance of methylation status has been given by Sigalotti *et al.* (2002). Using a reporter gene under the control of MAGEA2, -A3 and -A4 promoters methylated or un-methylated *in vitro,* they showed that the major element driving MAGEA expression was the methylation status of their promoter [Sigalotti *et al.*, 2002].

Even if methylation seems capital for MAGEA gene expression, histone modifications also appear to play a role. In 2006, Wischnewski *et al.* described that the association of a HDAC inhibitor with a DNA methylase inhibitor induces a MAGEA gene expression three times higher than with the DNA methylase inhibitor alone (relative expression of mRNA). However, it is important to note that the HDAC inhibitor alone had no effect [Wischnewski *et al.,* 2006].

4.2.2.2.1. BORIS

The BrOther of the Regulator of the Imprinted Site (BORIS or CTCF Like protein) is a transcription factor protein containing eleven Zn fingers and known to bind multiple DNA sequences [Martin-Kleiner, 2012]. Only expressed in primary spermatocytes and silenced in normal adult tissues, it has been considered as an oncogene since BORIS is reactivated in cancer. BORIS is a protein that shares high homology with CTCF, the second member of the family. Both proteins bind *in vitro* the same DNA sequences but diverge in their N- and C-terminal region [Klenova *et al*., 2002]. It has been demonstrated that BORIS acts as an antagonist of CTCF in normal and in cancer cells by binding to the same response element. Moreover, in normal condition, the expression of BORIS correlates with a decrease in CTCF levels and a global reduction of DNA methylation [Klenova *et al*., 2002; Recillas-terga *et al*., 2006].

In 2011, Boris has been identified, by ChIP, on CTAs promoter and its presence was correlated to an increased expression MAGEA1 and NY-ESO-1 [Bhan *et al*., 2011]. BORIS is also known to modify the methylation status of genes like the one coding for bag-1 [Sun *et al*., 2008]. BORIS switches the histones methylation status from an inhibitory (Histone H3 trimethylated on lysine 9: H3K9) to a permissive one (Histone H3 trimethylated on Lysine 4: H3K4) by recruiting the methyltransferase SET1A. Experiments undertaken by Bhan *et al*. showed that BORIS was

present on MAGEA promoters (-A2, -A3 and -A4) and it is thought to lead to increased MAGEA1 expression through an enhancement of activating histone methylations and acetylations. Concerning DNA methylation itself, the mechanism seems to vary among MAGEA genes. The presence of BORIS is correlated with an unmethylated promoter of MAGEA3 but no difference in CpG methylation was observed for MAGEA2 and -A4 promoters, suggesting that an alternative mechanism of regulation could exist. How BORIS mediates DNA demethylation is controversial and four hypotheses are currently under investigation [Roldán-Arjona and Ariza, 2009]. Finally, BORIS expression has been shown to be repressed by p53 suggesting a role of p53 in inhibiting MAGEA expression [Necochea-Campion *et al.,* 2011].

4.2.2.2.2. Mi-RNA34a

Micro RNAs seem to play a role in MAGEA expression. By analyzing several miRNA targets with prediction algorithms, MAGEA family members such as -A2, -A3, -A6 and -A12 were found to possess a miR-34a binding site in their 3' untranslated regions (UTRs). They were further confirmed as direct targets of miR-34a. The hypothesis suggests that miR-34a directly targets MAGEA genes and allows p53 levels to increase. In turn, p53 acts as a transcriptional activator for miR-34A thus

inducing a positive feedback loop. This is suggested to enhance sensitivity to chemotherapy [Weeraratne *et al*., 2011].

4.2.2.3. MAGEA and cancer therapy

Correlation between MAGEA expression and a poor prognosis is well documented [Zhou *et al*., 2011; Lian *et al.,* 2012; Mengus *et al*., 2012; Laban *et al*., 2014]. Additionally, some studies suggest that MAGEA expression could be used as a new diagnostic tool for patient care. In 2012, Shin *et al.* successfully detected MAGEA expression, by RT-nested PCR in bronchial washing fluids, as cancer marker in the case of lung cancer invisible to bronchoscopy [Shin *et al.*, 2012]. Similar results were obtained in gastric carcinoma [Jeon *et al*., 2010]. Recently, Sang *et al.* used blood samples to detect circulating MAGEA proteins. Results demonstrated that blood detection of MAGEA proteins was technically possible and synonym of a poor outcome for the patient. Since the only reason for MAGEA expression and detection is the presence of cancer cells, it is conceivable that MAGEA proteins may be used as an accurate marker for cancer detection or surveillance, and could even be more reliable than carcinoembryonic antigen (CEA) [Lee *et al.,* 2014], CA15-3 [Ali *et al*., 2013] or CA27-29 [Brooks, 2009] currently used for breast cancer. Taken together, those data underline the clinical relevance of

MAGEA proteins in the cancer field as well as their role in cancer progression.

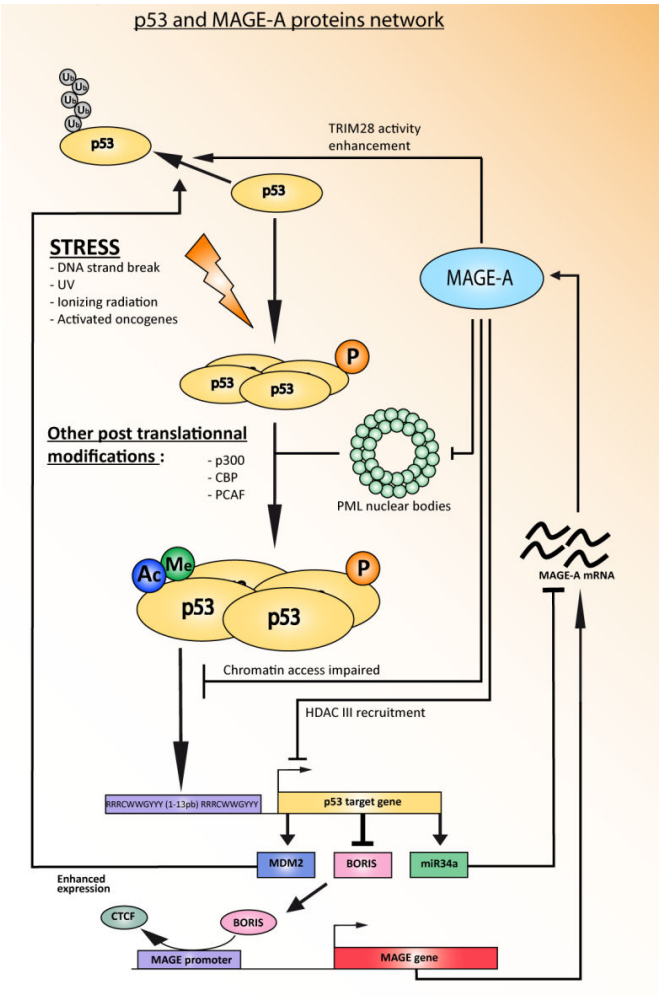

Figure 23 : Cellular effects of MAGEA proteins. MAGEA are implicated into p53 destabilization and inhibition of transcription. MAGEA2 expression leads to an enhanced ubiquitination of p53 (Doylet *et al.*), to the inhibition of PML-NBs leading to a decreased p53 acetylation (Peche *et al.*), to the recruitment of HDAC on p53 promoters or to the inhibition of p53 binding to DNA (HDACI, Laduron *et al.*; HDACIII, Monte *et al.*). On the other hand, p53 also modifies MAGEA activity: p53 induces miR34a expression that directly targets MAGEA mRNA. Moreover, p53 also inhibits the expression of BORIS, responsible for the demethylation of MAGEA promoter.

Chapter 5: Resveratrol

5.1. Natural products as anti-cancer drugs

Different natural molecules are currently among the most potent anti-cancer agents. Vincristine, etoposide and paclitaxel are examples of plant-derived compounds while actinomycin D, mitomycin C, doxorubicin and l-asparaginase are derived from bacteria. Today, even oceans are sources of anti-cancer drugs (e.g. cytarabine). These drugs induce anti-cancer effects by varied mechanisms of action: interaction with microtubules, inhibition of topoisomerases I or II, alkylation of DNA, and interference with tumour signal transduction[14, 15] [Nobili *et al.,* 2009].

5.1.1. Alkylating agents

These molecules are able to add an alkyl group to DNA bases, leading to DNA strand breaks when repair enzymes proceed, or to create crossbridges between base pairs inhibiting the separation of both strands during replication. A famous member of this family is mitomycin C that induces crossbridges in the DNA [Colvin, 2003].

[14] www.cancer.org
[15] http://www.elmhurst.edu/~chm/vchembook/655cancer2

5.1.2. Antimetabolites

Antimetabolites are molecules that inhibit RNA or DNA replication. For example, cytarabine (1β-arabinofuranosylcytosine, Ara-C), a pyrimidine nucleoside analogue, is the first anti-cancer agent coming from the sea (sponge). In the cytosol, cytarabine is rapidly phosphorylated into tri-phospho-cytarabine (Ara-CTP) and competes with dCTP. DNA and RNA synthesis was shown to be impaired [Giles *et al*., 2012].

5.1.3. Anti-tumor antibiotics

Two classes of anti-tumor antibiotics exist and were isolated from *Streptomyces*: the anthracycline antibiotics such as doxorubicine or idarubicin and non-anthracycline antibiotics such as actinomycin-D. Anthracyclines are intercalating drugs able to induce double strand breaks in the DNA and therefore to trigger p53. On the other hand, Actinomycin D, first discovered in 1940 while studying *Actinomyces antibioticus,* binds DNA and inhibits RNA elongation. It thus triggers transcription arrest [Nobili *et al*., 2009].

5.1.4. Topoisomerase inhibitors

TOPOisomerases eliminate DNA supercoiling appearing during DNA replication. Two classes of TOPOisomerases (TOPOI and TOPOII) exist based on mechanisms resolving supercoiling. TOPOI cleaves one strand of the DNA, eliminates supercoiling and religates. Camptothecin stabilizes the TOPOI-DNA cleavable complex. DNA single strand breaks are formed and activate p53. Camptothecin was purified from a Chinese tree (*Camptotheca acuminate*) but was too toxic for a therapeutic use. In 1996, two camptothecin-derived molecules were designed to overcome such drawbacks. TOPOII removes supercoiling by creating a double strand break, passes another DNA molecule through the gap and religates (Figure 24). Doxorubicin but also etoposide (derived from the roots of the Indian *Podophyllum peltatum*) are TOPOII inhibitors. They stabilize the TOPOII-DNA covalent intermediate in the open conformation thus inhibiting relegation (Figure 24). It generates double strand breaks in the DNA leading to apoptosis [Nobili *et al*., 2009].

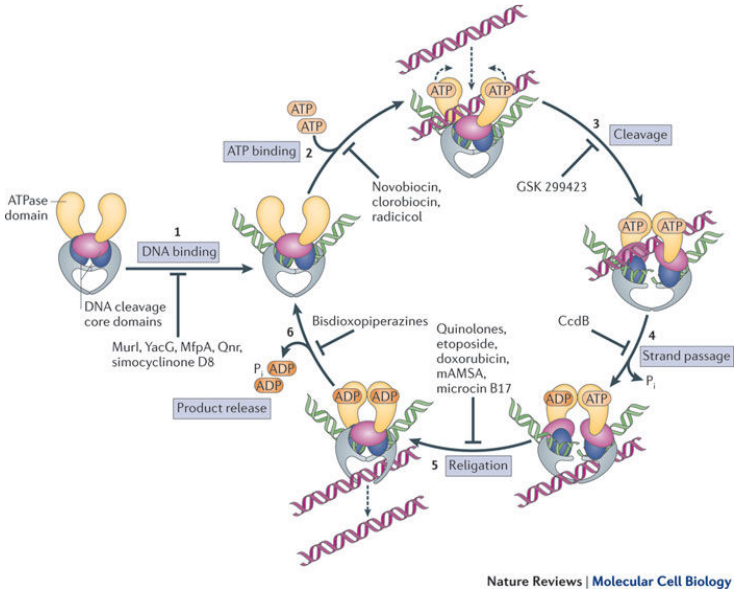

Figure 24 : The Topoisomerase II cycle with, for each step, inhibitory molecules. 1. TOPO II binds a DNA molecule. 2) ATP binding stimulates the ATPase domain of TOPOII and a second DNA molecule is recruited. 3) Once recruited the ATPase domain cleaves the first DNA molecule. 4) Through the opening TOPOII passes a second molecule. 5) The cleaved molecule is religated. 6) DNA is released. Doxorubicin and etoposide inhibits the fifth step of this cycle leading to double strand breaks. From Vos *et al*., 2011.

5.1.5. Mitotic inhibitors

Vinblastine and vincrinstine are alkaloïds both isolated from extracts of *Catharanthus roseus*. They bind tubulin and prevent microtubules polymerisation[16,17]. The taxane paclitaxel (taxol) is a molecule found in the bark of *Taxus brevifolia* that stabilizes formed microtubules by binding to tubulin. Consequently, cytoskeleton disassembly and flexibility is lost and mitosis impaired.

5.2. Resveratrol

Resveratrol (*trans*-3, 4', 5-trihydroxystilbene; RSV; Figure 25) is a polyphenol notably found in fruits (grapes) or nuts (peanuts). The molecule raises a lot of interest from the scientific community for several reasons. Some are described here below.

5.2.1. RSV and caloric restriction

When animals are exposed to a moderate reduction of their caloric intake (20%), but provided with correct amounts of minerals and vitamins, their lifespan increases. This concept, called "caloric

[16] www.cancer.org
[17] http://www.elmhurst.edu/~chm/vchembook/655cancer2

restriction" is observed in a wide range of eukaryotes such as yeast, worms but also more advanced forms of life such as insects, fishes, rodents and monkeys [Kulkarni and Cantò, 2014]. Increased lifespan, reaching up to 50%, is observed in association with prevention of age-related physical deterioration, prevention of tissue damages and a lower cancer incidence [Mattison *et al*., 2012; Colman *et al*., 2009].

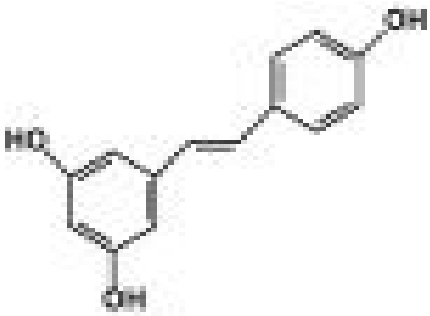

Figure 25 : Molecule of resveratrol.

In 1999, Kaeberlein *et al.* associated the protein Silent information regulator 2 (Sir2) to yeast aging. Interestingly, by increasing Sir2 expression, yeast lifespan was enhanced by 30%, while deletion of Sir2 reduced lifespan by 50% [Kaeberlein *et al*., 1999]. Moreover, Lin and collegues demonstrated that caloric restriction advantages were lost when Sir2 was deleted [Lin *et al*., 2000]. Quickly, seven mammalian isoforms of Sir2 were identified: SIRT1 to 7 [Kulkarni and Cantò, 2014]. SIRT1, the most studied was shown to recreate some caloric restriction characteristics when overexpressed in mouse: decreased DNA damage, improved glucose tolerance, fewer carcinomas and sarcomas. However,

SIRT1 overexpression was not sufficient to generate an increase in lifespan [Herranz *et al*., 2010; Bordone *et al*., 2007].

Molecular analyses of SIRT1 demonstrate that FOXO transcription factors are SIRT1 targets. Moreover, SIRT1 activates PPAR-γ-coactivator-1α (PGC-1α). PGC-1α regulates genes located both in the nuclear and mitochondrial genomes to induce pathways involved in mitochondrial DNA replication, fatty acid oxidation and import as well as electrons transport chain assembly [Rowe *et al.,* 2010].

Based on these studies, *in vitro* screening for SIRT1 potentiators began with the hope to delay aging. The most promising compound identified is RSV. Mice on a high fat diet supplemented with RSV, are protected against body weight gain, have a better metabolism and [Lagouge *et al.,* 2006] an increased lifespan, as if RSV mimicked some aspects of caloric restriction. Effects on osteoporosis, cataract and vascular dysfunction were also observed [Pearson *et al*., 2008]. Nevertheless, some wondered if RSV was indeed responsible of the enhanced lifespan or if metabolites of RSV were responsible. Evidences behind this suggestion are the poor bioavailability of RSV due to its rapid transformation in the body and the rather long time observed between RSV treatment and actual SIRT1 activation [Kulkarni and Cantò, 2014].

Identification of other proteins involved in caloric restriction led to the identification of the AMP-activated kinase (AMPK). AMPK is activated by a high AMP/ATP ratio, itself reflecting the energy status of

the cell. AMPK becomes activated under energy stress such as hypoxia, exercise or fasting. It induces pathways such as lipid oxidation or glycolysis, rapidly replenishing cellular energy [Hardie *et al*., 2012]. In 2006, RSV was reported to induce AMPK activation within minutes in several cell lines [Breen *et al*., 2008; Zang *et al*., 2006]. The mechanism behind RSV-dependent AMPK activation seems to rely on RSV ability to inhibit the F1FO-ATPase/ATPsynthase. Therefore, ATP formation is inhibited and the AMP/ATP ratio drastically increases leading to AMPK activation [Zheng and Ramirez, 2000]. Interestingly, AMPK also activates PGC-1α suggesting an overlapping role with SIRT1 [Jäger *et al.,* 2007]. Moreover, studies report that AMPK may activate SIRT1 by enhancing NAD+ cell concentration [Cantò *et al*., 2009] and since AMPK activation by RSV is more rapid than SIRT1 activation, that would suggest a direct downstream activation. However, some contradictory reports demonstrate that SIRT1 may activate AMPK. Indeed, Liver Kinase B1 (LKB1), an upstream kinase of AMPK, is a direct SIRT1 target. Therefore, the exact mechanism is under construction. Nevertheless, what is certain is that RSV action requires both AMPK and SIRT1 activation to mimic caloric restriction.

5.2.2. Resveratrol and cancer

In addition to its effects on the energy metabolism, RSV was shown to be a cancer chemopreventive polyphenol. In 1997, Jung *et al.* demonstrated that mice on a RSV-containing diet were less prone to develop breast cancer when treated with carcinogen injections in the mammary gland. Similar results were observed in a skin cancer model. Moreover, RSV treatment is able to inhibit growth and elicit autophagy or apoptosis in multiple cellular models [Garcia-Zepeda *et al.,* 2013; Rojas *et al*., 2014; Wu *et al*., 2015]. Mechanistically, different hypothesis have been raised for RSV anti-cancer effects. Mostly, RSV induces cancer regression by promoting senescence or apoptosis. In presence of RSV, cyt C release is observed, BAX and p53 expression is enhanced and levels of anti-apoptotic BCL-2 members are decreased. However the exact mechanism triggering cell death or cell cycle arrest remains a subject of intense debate [Udenigwe *et al*., 2008]. Hwang *et al.* reported that RSV induced production of reactive oxygen species (ROS) mediating release of cyt C [Hwang *et al*., 2007]. Interestingly, opposite effects were observed in normal cardiac cells: RSV protects against ROS related to cardiovascular diseases [Hwang *et al*., 2008]. The exact mechanism of RSV-dependent ROS production in cancer cells is still non-elucidated.

Clinical studies on healthy individuals demonstrate that RSV is generally well tolerated. This makes RSV suitable for therapeutic use. For

example, a one year administration of 16mg of RSV per day appears to be safe. No severe side effects were observed and a protection against cardiovascular diseases observed [Tomé-Carneiro *et al*., 2013]. RSV does not have side effects at a dose of 1g per day. However, diarrhea or vomiting are recorded when the daily uptake reaches 2.5g per day [Singh *et al*., 2014].

Major drawbacks of RSV reside in its poor water solubility, its high instability and its low oral bioavaibility. Those issues make difficult the use of RSV as a potent therapy. [Singh *et al*., 2014; Kulkarni and Cantò, 2014]. Indeed, oral intake of RSV rapidly leads to its metabolization mainly into glucuro- and sulfo-conjugates in the liver [Cottart *et al*., 2010]. Therefore, strategies are currently developed to overcome these issues. For example, combinations with alkaloids such as piperin or with the polyphenolic compound curcumin increase RSV plasma concentration by inhibition of its glucuronidation [Johnson *et al*., 2011; Basu *et al*., 2007]. Finally, it could be possible to generate RSV derivatives that will be less sensitive to the hepatic metabolism.

AIMS OF THE STUDY

It is commonly accepted that the tumor suppressor p53 is crucial for the prevention of cancer development. The fact that p53 is mutated in more than 50% of all cancer reinforces this statement. The apoptosis induced by p53 is one of the pathways triggered following an acute stress. Therefore, in tumor bearing the wt form of the tumor suppressor, a better knowledge of the mechanisms that impair its activity could be important for the development of new strategies to fight cancer. In addition, the identification of small natural molecules able to trigger p53-dependent apoptosis in cancer cells as well as the understanding of their mechanisms of action, could also lead to new therapies. This is why, during this four year project, my goal was to determine the effects of two different actors on p53 dependent apoptosis. Therefore, this work is divided into two parts. First, I analyzed the inhibitory effects of MAGEA family members on p53-dependent apoptosis. Second, I focused on the polyphenol resveratrol in order to understand how this molecule triggers the p53 pathway.

1) MAGEA project

In 2006, Monte *et al.* found that MAGEA2 is able to complex with wt p53 and recruit the histone deacetylase III on p53-dependent promoters. This report was the first clue on the impact of CTAs on p53. In 2010, Doyle *et al.* hypothesized that MAGEA proteins interact with E3 ubiquitin ligases such as TRIM28 and enhance p53 degradation. Another paper by Marcar *et al.* suggested that MAGEA proteins bind the DBD of p53 preventing it to interact with the promoter of its target genes. Finally, it has been suggested that MAGEA proteins inhibit the formation of the PML-NBs and reduce p53 acetylation. These publications raised the attention on MAGEA functions in cancer cells.

However, these different mechanisms are in opposition on some points. For example, Marcar *et al*. and Monte *et al.* described a direct binding between MAGEA2 and the tumor suppressor while Doyle *et al.* demonstrated the contrary. Another stumbling block lies on the effects of MAGEA on p53 cellular levels. Doyle *et al*. described an increased degradation of p53 (although they used cell lines expressing mutant p53). In the meanwhile, Monte *et al.* did not observed an effect of MAGEA expression on wt p53 levels. There is also a disagreement on the consequences of this interaction between MAGEA proteins with the tumor suppressor. Both Monte *et al.* and Marcar *et al.* agree on the binding between these proteins, however they disagree on the ability of

the complex MAGEA-p53 to still interact with DNA. Finally, only MAGEA2 has been clearly analyzed since based on the work of Monte *et al.*, it was thought to be the most active member of the family. Other MAGEA genes were slightly analyzed.

Therefore, since MAGE-A protein are CTAs that target p53 and since only MAGEA2 has been clearly analyzed, our objectives were to analyze how six members of the MAGEA family (MAGEA1, -A2, -A3, -A4, -A6 and -A12) influence p53 activity. MAGEA members selection has been based on the work of De Plaen *et al.* After studying a large range of cancer samples and cancer cell lines, they demonstrated that these six MAGEA genes were frequently expressed in cancer. More precisely, we aimed to analyze the impact of each of these six MAGEA on cell resistance to genotoxic stresses and p53-dependent apoptosis. We also aimed at determining the interactions with the tumor suppressor and to characterize the phosphorylation status of p53 in presence of MAGEA during a genotoxic insult. This is an original hypothesis that has not been investigated before.

2) Resveratrol project.

RSV has been shown to have beneficial effects on various pathologies. For example, type 2 diabetes may be treated in parts with RSV since it notably enhances glucoregulation and increases sensitivity to insulin. It has also effects on obesity by reducing adipocyte size and by enhancing adiponectin expression, as well as on cardiovascular diseases by reducing plasma lipids or cholesterol levels and by reducing blood pressure. Moreover, RSV has cancer chemopreventive properties. It has been shown to reduce the proliferation of cancer cells, to enhance caspase-3 activation and to reduce inflammation.

Research on the mechansims of actions of RSV on cancer cells has led to the identification of notably the generation of ROS that triggers a senescent phenotype [Heiss *et al.*, 2007]. However, the mechanism by which RSV induces apoptosis was still unclear. Before my arrival in the laboratory of P. Dumont, M. Hermant did a wonderful master thesis on the effects of RSV on cancer cells. She demonstrated that treatment of HCT-116 colon cancer cells with RSV induced chromatin condensation, formation of γ-H2AX foci and PARP cleavage. Moreover, C. Castrogiovanni used HCT-116 cells wt and KO for p53 to demonstrate that RSV-induced apoptosis was p53-dependent, at least partly. Finally, M. Hermant concluded that ROS formation was not involved in formation of γ-H2AX foci and apoptosis induction.

Therefore, our main goal was to identify the mechanism by which RSV induces DNA damage in colon cancer cells. Several mechansims could be responsible. Among them, we decided to analyze the following three: DNA intercalation, TOPOI inhibition and TOPOII inhibition. In addition, we also aimed at determining the pathway leading to p53 activation and more specifically the kinases involved.

RESULTS

Preamble

An early study from De Plaen *et al.* indicates that MAGEA1, -A2, -A3, -A4, -A6 and -A12 are commonly expressed in various cancer cell lines as well as tumor samples. In contrast, MAGEA5, -A8, -A9, -A10 and -A11 are rarely expressed [De plaen *et al*. 1994]. Therefore, we selected the first group of genes for our study.

To determine the impact of MAGEA on p53 functions, we first selected a cellular model suitable for our strategy. We wanted to express separately these six members of the MAGEA family in a cancer cell line wt for p53 that does not express endogenously the different MAGEA. RT-Q-PCR data on four p53 wt cancer cell lines available in our laboratory allowed us to determine that the MCF-7 (breast cancer) cell line was suitable for our project since it only expresses very low levels of some of the MAGEA (Supplemental data: Figure 34).

To overexpress the different MAGEA in MCF-7 cells, the cDNA of the corresponding MAGEA was cloned in the V1899-pGATEWAY-5'CMV-Triple-FLAG expression vector. The MAGEA cDNAs were cloned in fusion with a triple-FLAG tag since no specific antibodies were available at that time. By analyzing MAGEA structure and since Doyle *et al*. suggested that the C-terminally located MHD was responsible for MAGE activity, we choose to flag our proteins on the N-terminal end. Finally, and since

no antibiotic resistance gene were present on the V1899-pGATEWAY-5'CMV-TripleFLAG vector, we also inserted a puromycin resistance cassette derived from the pPur vector. By the end of this preparation phase, six expression vectors V1899-pGATEWAY-5'CMV-Triple FLAG-Puro^{R} each containing a specific MAGEA cDNA were created.

To analyze the effects of MAGEA expression, MCF-7 stable clones were established following transfection of the different vectors and selection with puromycin. We selected clones based on western blot analysis using an anti-FLAG antibody (Supplemental data: Figure 35).

We first identified which members significantly enhanced cell resistance to a genotoxic stress and, then focused on p53-dependent apoptosis. We also analyzed whether a direct binding between MAGEA and the tumor suppressor existed. Ultimately, we aimed to identify a new mechanism responsible for the reduced activity of the tumor suppressor in presence of MAGEA.

Chapter I:

MAGEA1 and -A2 impair the stabilization and activation of p53 in presence of a genotoxic stress

MAGEA1 and -A2 impair the stabilization and activation of p53 in presence of a genotoxic stress

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Running title: MAGEA inhibits p53 activation

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Abstract

MAGEA (Melanoma AntiGEn-A) genes are members of the cancer testis antigens (CTAs). They are silenced in healthy human adult tissues to the exception of male germ cells. However, a re-expression is commonly observed in various types of cancer. Several studies demonstrated a correlation between MAGEA levels and advanced cancer stages, malignant transformation as well as increased resistance to chemotherapy. These observations emphasize that MAGEA proteins could have oncogenic properties and favor cancer development. However, the functions of MAGEA proteins in cancer cells remain largely unknown. In order to study the functions of the MAGEA commonly expressed in cancer, we used the MCF-7 breast cancer cell line to derive clones stably expressing them. Here, we present evidence that MAGEA1 and MAGEA2 (but not -A3, -A4, -A6 and -A12) diminish the sensitivity of breast cancer cells to the chemotherapeutic agent doxorubicin. We link this resistance to reduced DNA damage-induced apoptosis in presence of MAGEA expression. Furthermore, we demonstrate that the mechanism is p53-dependent. MAGEA1 and -A2 proteins directly interact with the DNA binding domain of the tumor suppressor and inhibit the stabilization and activation of p53 in cells exposed to a genotoxic stress. We link the decreased activation and lowered half-life of stress-induced p53 to an impaired phosphorylation of its N-terminal region in presence

of MAGEA1 or -A2. Our data suggest that preventing MAGEA proteins to bind and inhibit p53 could be an interesting strategy to combat cancers that harbor the wt version of the tumor suppressor.

Introduction

Cancer-testis antigens (CTAs) are silenced in healthy human adult tissus, to the exception of male germ cells. In addition, they are abnormally expressed in different cancers. Even if the normal functions of these genes seem to be related to embryonic development and more specifically to lineage differentiation [1], a clear identification of the molecular mechanisms involved remains to be performed.

Currently, the CTAs family emcompasses more than an hundred different genes. The first clue on their existence was described by Van der Bruggen in 1991 [2]. Back then, a specific antigen (MZ2-E) carried by melanoma cells was shown to activate cytolytic T-lymphocytes. It turned out that this antigen was encoded by a portion of 2.4kb gene that was later named MAGEA1, the first CTA identified. This study also gives evidence that MAGEA1 is part of a bigger family and that the third exon of the gene encodes for the entire protein [2]. This breakthrough on the existence of tumor specific antigens raised the hope for a cancer specific vaccine. [3,4,5].

The MAGEA family contains eleven genes and four pseudo genes. Those genes are located in the q28 region of the X chromosome and are characterized by a MAGE Homology Domain (MHD) of approximately 200 amino acids [6,7,8]. As members of the cancer testis antigens (CTAs), those genes are silenced in the vast majority of healthy human adult

tissues [9]. However, they are expressed in various types of cancer such as breast cancer, melanoma, lung cancer or colon cancer [10]. The main reason for this re-expression in cancers seems to be the demethylation of the promoter [11,12]. Several studies have demonstrated a correlation between MAGEA expression and an overall shorter survival of patients in different cancers such as breast cancer [13], head and neck cancer [14], lymphoma [15] and lung cancer [16]. Moreover, MAGEA expression correlates with advanced cancer stages [17], malignant transformation [18] and increased resistance to chemotherapeutic treatments [15,19,20,21].

Taken together, these data emphasize that MAGEA proteins could have oncogenic properties and favor cancer development. Surprisingly, the cellular functions of MAGEA remain largely unknown. Recently, several studies described that they could target the tumor suppressor p53 [19,22,23].

The tumor suppressor p53, also known as “guardian of the genome”, is a transcription factor that sets up the cellular response to numerous stresses. It is a major regulator of cell cycle arrest, replicative senescence and apoptosis [24]. It has been assessed that more than 50% of human cancers carry a mutation of the *TP53* gene [25]. Although defined as a transcription factor, p53 is also able to induce apoptosis in a transcriptional independent manner. This activity of p53 involves its

translocation to the mitochondria and interactions with BCL-2 family members, notably BAK [26,27,28,29,30].

In the absence of stress, the intracellular concentration of p53 is permanently kept at a low levels due to its degradation by the ubiquitin E3 ligase MDM2 [31] but also PIRH2 [32], ARF-BP1 [33] and COP1 [34]. Following specific stimuli such as DNA damage or oncogene activation, p53 is stabilized. This step involves expression of the tumor suppressor p14ARF, an inhibitor of MDM2, as well as several post-translational modifications of p53 including phosphorylations and acetylations [35]. These post-translational modifications occur on specific amino acids mainly located in the N- and C-terminal part of the p53 protein and modulate for instance the recruitment of transcriptional co-activators, the affinity of p53 for target promoters or the cellular localization of the tumor suppressor [36,37,38].

In 2006, Monte *et al*. described a mechanism in which MAGEA2 directly interacts with p53 on the BAX promoter and recruits HDAC3 [19]. This allows the deacetylation of surrounding histones and by consequence decreases the expression of p53 target genes. A second hypothesis suggested by Yang *et al.* (2007) and developed by Doyle *et al.* (2010) is that MAGEA proteins could interact with KAP-1 (TRIM28), an ubiquitin E3 ligase, and act as co-repressors of p53 activity by enhancing its degradation [20,22]. In this study a direct binding of p53 to MAGEA proteins was not described. In 2010, Marcar *et al.* demonstrated that

MAGEA2 was able to directly interact with the DNA binding domain of p53 and inhibit its access to target promoters [23].

Here, we present evidence that, among the MAGEA genes commonly expressed in cancer, only MAGEA1 and MAGEA2 bear the ability to reduce the sensitivity of breast cancer cells to the chemotherapeutic agent doxorubicin. We link this resistance to reduced DNA damage-induced apoptosis in presence of these MAGEA proteins. Furthermore, we demonstrate that the mechanism is p53-dependent, p53 function being altered in presence of MAGEA1 or -A2. We also describe a direct binding of MAGEA1 and -A2 to the DNA binding domain of p53. Remarkably, in cells exposed to a genotoxic stress, p53 stabilization and activation is altered. This decreased activation of p53 appears to be linked to an impaired phosphorylation of its N-terminal region in response to the genotoxic stress.

Materials and Methods

Plasmids

The pDEST-V1899-FLAG plasmid (Invitrogen) was first modified by insertion of a multiple cloning site and the puromycin resistance cassette from pPur plasmid (Clontech Laboratories). MAGEA cDNAs, a gift from Pr. E De Plaen (Ludwig Institute for Cancer Research, UCL, Belgium), were then inserted into the modified pDEST-V1899-FLAG vector through the Gateway method (Invitrogen) following manufacturer instructions. The pCR2.1-MAGEA1 and -A2 vectors were obtained by insertion of FLAG-MAGEA1 and -A2 sequences amplified by PCR from the previously created vectors. The pGEX-3X plasmids containing GST-p53, GST-N-terminal p53 (1-92) and GST-p53 DBD (102-292) were generously given by Pr. M. Murphy (Wistar Institute, Philadelphia PA, USA).

Cell Culture and Treatments

The PA-1 human teratocarcinoma cell line (ATCC, CRL-1572) and the MCF-7 breast cancer cell line (ATCC, HTB-22) were grown in DMEM (Gibco, 42430-082) supplemented with 10% foetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S) (Gibco, 15140-163). MCF-7 stable clones expressing MAGEA proteins were obtained by transfection of the pDEST-V1899-FLAG plasmids containing the different MAGEA cDNAs. Transfections were performed with FuGENE HD Transfection Reagent (Promega, E2311) following manufacturer instructions. Cells were

selected with puromycin (0.5µg/ml) before being characterized by western blot for MAGEA expression. Cells were treated with nutlin-3a (Sigma, N6287), doxorubicin (Sigma, 038K1349) and camptothecin (Sigma, C9911) as indicated in the figures.

MTT Assays

MTT assays were used to determine global cell growth after treatment. Briefly, cells were plated in 96-well plates at 5,000 cells per well and treatments started 48h later. After treatments (72h), cells were incubated for 3h with DMEM (10% FBS, 1% P/S) containing 0.5 mg/ml MTT (Sigma, M2128). Plates were then centrifuged (2100rpm) and the supernatants discarded. We added 200µl DMSO to dissolve the formazan precipitate. We then read the absorbance at 560 nm and subtracted that at 670 nm.

Flow Cytometry

LDS751/PI labelling assays (Guava ViaCount, Millipore 4000-0041) and Annexin V-PE/7-AAD labelling assays (Guava Nexin, Millipore 4500-0455) were realized to respectively determine the overall cell death after treatment and the proportion of apoptotic cells. Analysis was performed on a Guava easyCyte 6HT-2L flow cytometer (Millipore, USA). For both tests, cells were seeded in 25cm² flasks and treated 24h later. Following treatment, cells were harvested and labelled according to manufacturer instructions.

Immunoprecipitations

Cells were harvested, washed (PBS, 4°C) and lysed with NP-40 lysis buffer (50mM Tris pH8, 5mM EDTA pH8, 150mM NaCl, 0.5% NP-40) supplemented with protease inhibitors. After removal of cell debris by centrifugation, protein extracts were incubated O/N at 4°C with protein-A sepharose beads covalently bound to the anti-p53 FL-393 polyclonal antibody (Santa Cruz, SC-6243). Antigens were then eluted following manufacturer instructions (Thermo scientific, 26147) and analyzed by western blot.

Expression and purification of GST-p53 constructs

GST-p53 and deletion constructs were prepared from E. *Coli* BL21 after induction of protein expression with 1mM IPTG (Sigma, I6758) for 3h at 37°C. Bacteria were centrifuged, washed with STE buffer (10mM Tris pH8, 150mM NaCl, 1mM EDTA) supplemented with protease inhibitor and resuspended in lysis buffer consisting of STE buffer supplemented with 100µg/ml Lysozyme (Sigma-Aldrich, L3790), 1mM PMSF (Sigma-Aldrich, 78830), 10µg/ml aprotinin (Sigma-Aldrich, 10820), 5 mM DTT (Sigma-Aldrich, 0632) and 1.5% sarkozyl. After sonication and removal of debris by centrifugation, proteins were bound to gluthathione-sepharose 4B beads during 3h. Beads were washed 4 times with PBS and proteins were elutated O/N with 5mM reduced glutathione.

GST pull-down

In vitro translated (IVT) MAGEA1 and -A2 were produced using the TNT quick coupled Transcription/Translation system (Promega, L1170) following manufacturer instructions. We incubated for 2h at 4°C, 1µg of IVT MAGEA1 or -A2 proteins with 1µg of GST-p53, GST-p53 1-92 or GST-p53 102-292 in binding buffer (20mM HEPES pH8, 150mM KCl, 1mM EDTA, 4mM $MgCl_2$, 1mM DTT, 0.02% NP-40, 10% Glycerol, 0.5mM PMSF). Glutathione-sepharose 4B beads were then added for 30 min. After washes and centrifugation, pellets were resuspended in Laemmli buffer for western blot analysis.

Western Blot

Total proteins were extracted using RIPA buffer (150mM NaCl, 0.5% sodium deoxycholate, 50mM Tris-HCL pH 8.0, 0.1% SDS, 0.1% Triton X-100) supplemented with protease and phosphatase inhibitors (Complete Mini EDTA Free, ROCHE 11836170001 ; Phosphatase Inhibitor Cocktail 2 and 3, Sigma-Aldrich P0044 and P5720). Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked and probed overnight. The following antibodies were used: mouse anti-□-actine (Sigma A1978), mouse anti-FLAG M2 (Sigma-Aldrich, F3165), mouse anti-p53 (Calbiochem, OP43), rabbit anti-pSer15 p53 (Cell Signaling, 9284S), rabbit anti-pThr18 p53 (Cell Signaling, 2529S), rabbit anti-pSer20 p53 (Cell Signaling, 9287S) and rabbit anti-pSer46 p53 (Cell Signaling, 2521S). After 3 washes, membranes were incubated with the

appropriate peroxydase-conjugated secondary antibodies: goat anti-mouse (Dako, P0447) and goat anti-rabbit (Dako, P0448). Immunoreactive bands were detected using the Western Lightning kit (Perkin Elmer, NEL104001EA) and signal intensity quantified using Image J V1.41 software (NIH, USA).

Results

MAGEA1 and -A2 but not -A3, -A4, -A6 and -A12 protect cells from the chemotherapeutic agent doxorubicin

As described in the preamble, we generated MCF-7 stable clones expressing separately the different MAGE-A genes selected based on the early study of E. De Plaen [10]. For each MAGEA, three clones were treated with increasing concentrations of doxorubicin for 72h (0, 10, 25, 50, 100, 250, 500nM). We measured global cell growth by MTT assays after treatment (Figure 26). A marked reduction of sensitivity to doxorubicin was observed at all concentrations in clones expressing MAGEA1 or -A2 (Figure 26 part 1 and 2). IC50s (concentrations that inhibits 50% of global cell growth) were determined. They indicate that clones overexpressing MAGEA1 and -A2 are 5-fold more resistant to doxorubicin than clones transfected with the empty vector (Figure 27). On the other hand, MAGEA3, -A4, -A6 and -A12 did not protect cells from the chemotherapeutic agent since IC50s were not significantly different from control clones (Figure 27)

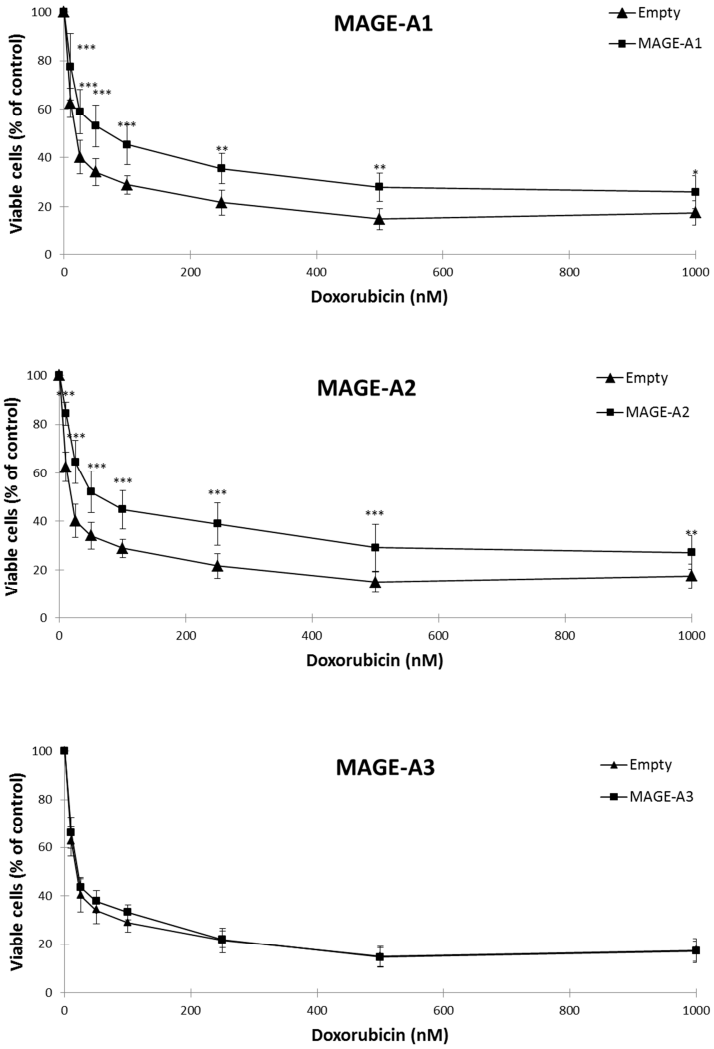

Figure 26 (Part1) : Expression of MAGEA1 and -A2 enhances resistance to the genotoxic agent doxorubicin. (A) Curves represent the percentage of viability of MCF-7 stable clones expressing or not the MAGEA proteins. Cells were treated for 72h with 0, 10, 25, 50, 100, 250, and 500 nM doxorubicin. Untreated controls represent 100% viability. After treatment, viability was measured by MTT assays as described in the Materials & Methods. Data are means of three different clones expressing MAGEA1, -A2, -A3 or transfected with the empty vector (Empty). Statistical test: Mann-whitney. ***: p-val < 0, 0001; **: p-val < 0,001; *: p-val < 0,05) (N=5, n=6).

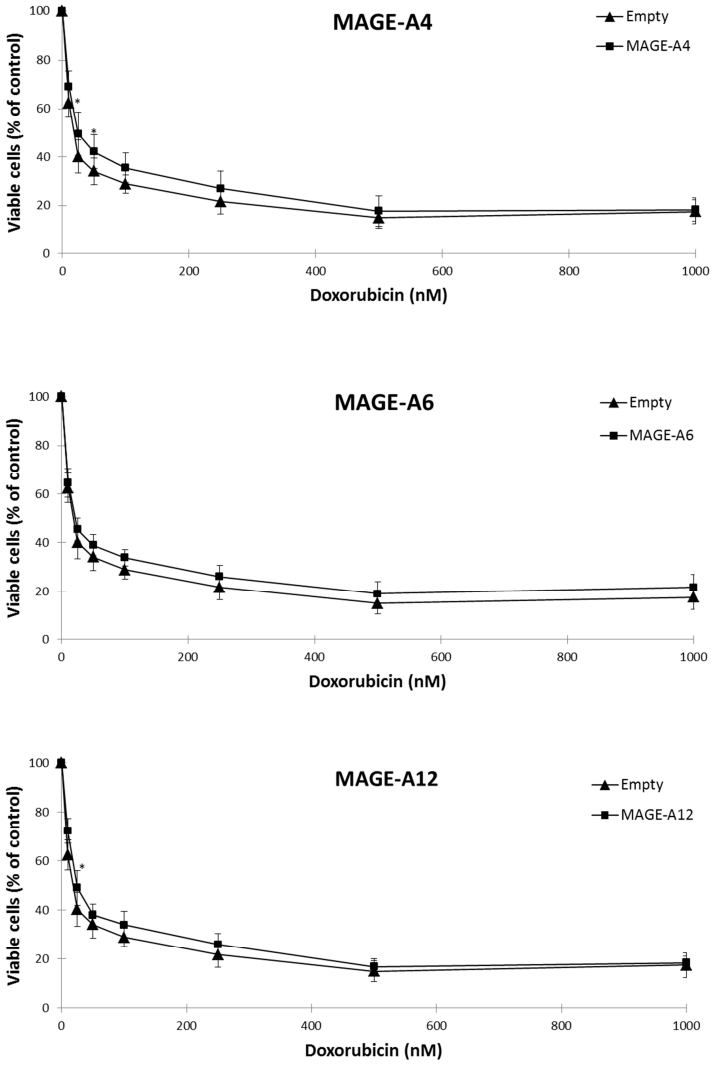

Figure 26 (Part2): Expression of MAGEA1 and -A2 enhances resistance to the genotoxic agent doxorubicin. (A) Curves represent the percentage of viability of MCF-7 stable clones expressing or not the MAGEA proteins. Cells were treated for 72h with 0, 10, 25, 50, 100, 250, and 500nM doxorubicin. Untreated controls represent 100% viability. After treatment, viability was measured by MTT assays as described in the Materials & Methods. Data are means of three different clones expressing MAGEA4, -A6, -A12 or transfected with the empty vector (Empty). Statistical test: Mann-whitney. ***: p-val < 0, 0001; **: p-val < 0,001; *: p-val < 0,05) (N=5, n=6).

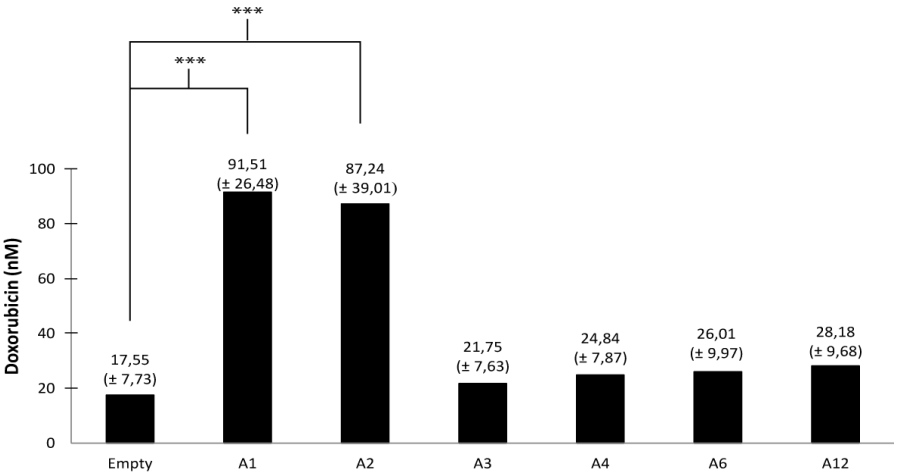

Figure 27: Expression of MAGEA1 and -A2 enhances resistance to the genotoxic agent doxorubicin. IC50s (Doxorubicin concentration inhibiting 50% of global growth) were determined based on the viability curves. Values represented are the mean of three clones expressing a MAGEA protein or transfected with the empty vector. Standard deviations are indicated between brackets. Statistical test: Kruskal-Wallis coupled with a multiple comparison of Dunn. ***: p-val < 0, 01. (N=5).

MAGEA1 and -A2 protect cells from camptothecin-induced apoptosis

Since MTT assays are only able to measure global growth, we next sought to determine whether MAGEA1 and -A2 could have an impact on apoptotic cell death in presence of a genotoxic agent. For this purpose, we treated MCF-7 clones stably expressing MAGEA1 and -A2 as well as clones transfected with the empty vector with 1µM camptothecin for 24h. Cell death was assessed by flow cytometry analysis after labelling with LDS751/PI. We found that MAGEA1 and -A2 decreased cell death by respectively 60% and 44% (Figure 28a). Apoptosis was next quantified by flow cytometry after labelling with annexin V-PE/7-AAD. As shown in Figure 28b, after treatment, control clones exhibit a proportion of apoptotic cells reaching 25.8%. By comparison, clones expressing MAGEA1 -and -A2 display a marked reduction of apoptosis, with respectively 11.1% and 13.9% of annexin V positive cells. Altogether, the above data allowed us to conclude that an elevated expression of MAGEA1 and -A2 protects MCF-7 breast cancer cells from cell death triggered by genotoxic agents.

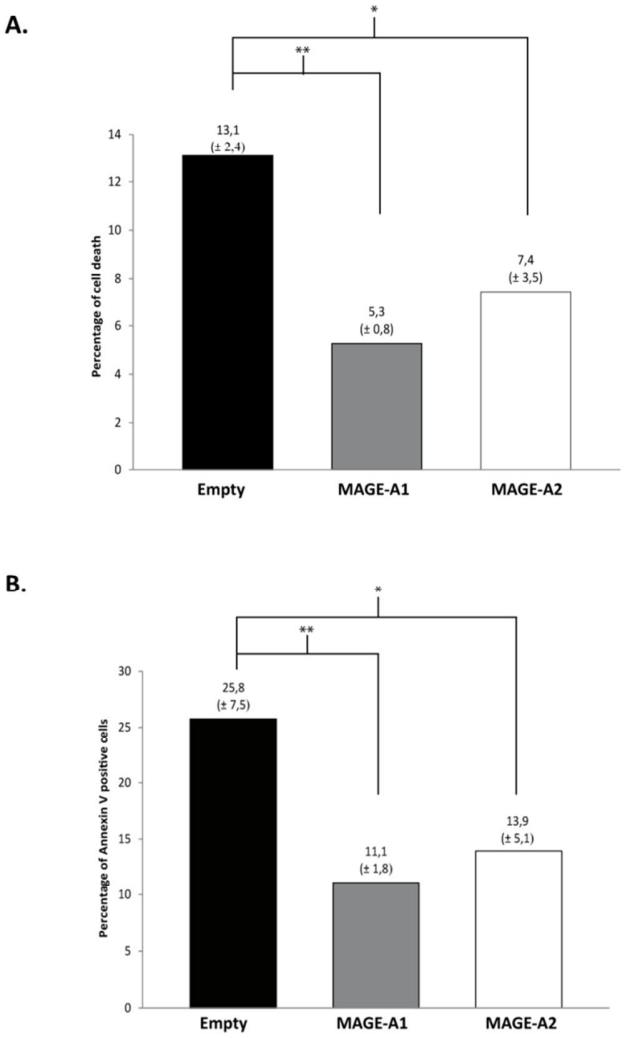

Figure 28 : MAGEA1 and -A2 expression reduces campthotecin-induced apoptosis. (A) LDS751/PI staining followed by flow cytometry analysis allowed the quantification of cell death. MCF-7 stable clones expressing or not MAGEA1 or -A2 were treated with 1µM camptothecin for 24h. Data are means of three clones expressing MAGEA1, -A2 or transfected with the empty vector. Statistical test: Kruskal-Wallis coupled with a multiple comparison of Dunn. ** : p-val < 0,001 ; * : p-val < 0,05) (N=4). (B) MCF-7 stable clones expressing or not MAGEA1 or -A2 were treated with 1µM camptothecin for 24h and thereafter labelled with annexin V-PE/7-AAD. Data were obtained in quadruplicate and mean values with associated standard deviations are shown. Statistical test: Kruskal-Wallis coupled with a multiple comparison of Dunn. **: p-val < 0,001; *: p-val < 0,05 (N=4).

MAGEA1 and -A2 expression enhances resistance to the p53 activator nutlin-3a

A hypothesis to the reduction of sensitivity to doxorubicin and camptothecin observed in clones expressing MAGEA1 and -A2 involves inhibition of p53 function. Indeed, the tumor suppressor is activated following genotoxic stresses and is important for induction of apoptosis [39,40]. We treated the MCF-7 stable clones expressing or not MAGEA1 or -A2 with increasing concentration of nutlin-3a (0-20µM) and measured overall cell growth by MTT assays after 72h of treatment (Figure 29). Nutlin-3a is a specific activator of p53. It interacts with the ubiquitin E3 ligase MDM2 and prevents it to bind and ubiquitinate p53, thus allowing stabilization of the tumor suppressor [41]. Data revealed that at every tested concentration, except 20µM, cells overexpressing MAGEA proteins were significantly more resistant to nutlin-3a than control cells (Figure 29). These data support the premises that MAGEA1 and -A2 target p53 function, protecting cells from different apoptotic stimuli such as genotoxic stresses.

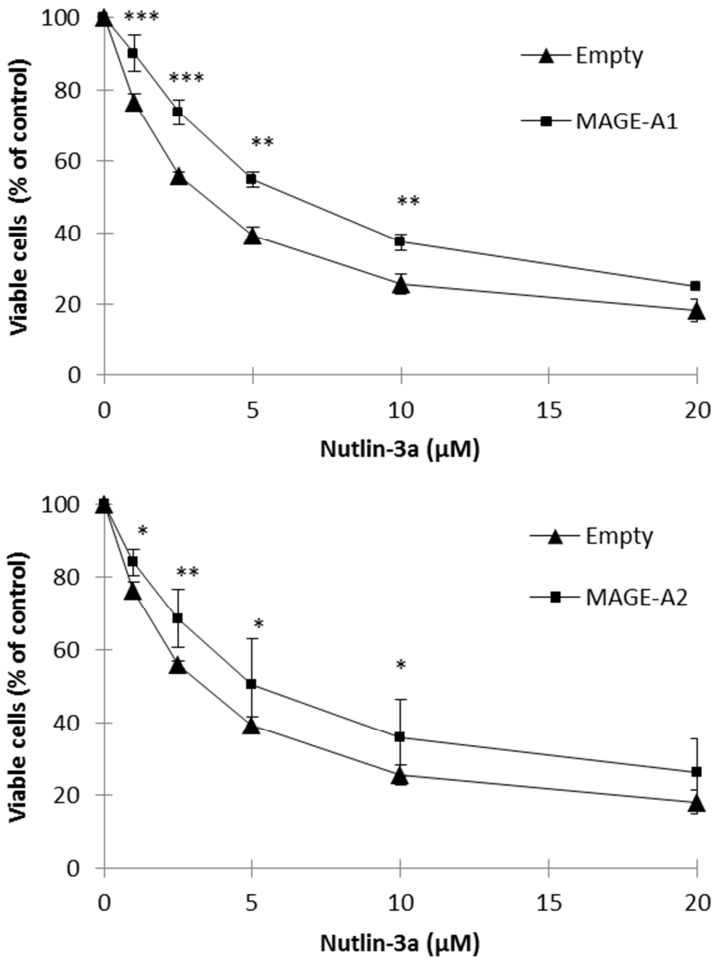

Figure 29 : Expression of MAGEA1 and -A2 protects against the p53 activator nutlin-3a. MCF-7 clones expressing MAGEA1, -A2 or transfected with the empty vector were treated with increasing concentration of nutlin-3a (0, 1, 2, 5, 10 and 20 µM). After 72h of treatment, viability was measured by MTT assays. Data are means of three different clones. Statistical test: Student T-test. ***: p-val < 0, 0001; **: p-val < 0,001; *: p-val < 0, 05 (N=4, n=6).

MAGEA1 and -A2 interact with the DNA binding domain of p53

Considering the above data indicating that MAGEA1 and -A2 enhance cell resistance to genotoxic stresses as well as the MDM2 inhibitor nutlin-3a, we tested for a possible interaction between these proteins and the tumor suppressor. We treated cells with 500nM doxorubicine for 18h and prepared total proteins extracts. A subsequent immunoprecipitation-western blot analysis revealed that stress-activated p53 interacts with MAGEA1 and -A2 (Figure 30a).

To determine whether the interaction between p53 and MAGEA1 or -A2 was direct, we next performed GST pull-down assays (Figure 30). In vitro translated (IVT) FLAG-MAGEA1 and -A2 were incubated with GST-p53 produced in E. coli. Western blot analysis after pull-down of the complex with glutathione sepharose beads indicates a direct binding between the tumor suppressor and both MAGEA proteins. Furthermore, the N-terminal region (amino acids 1-92) as well as the DNA binding domain (DBD, amino acids 102-292) of p53 were examined for their ability to bind MAGEA1 and -A2. GST-pull down assays show that the DBD of p53 is responsible for the direct binding. We also observed an interaction with the N-terminal part of p53 (Figure 30b). Although weak, this signal was consistent through all experiments realized, while the GST control was totally free from signal. These results allow us to say that MAGEA proteins bind directly to the DBD of the tumor suppressor and possibly its N-terminal region (Figure 30b).

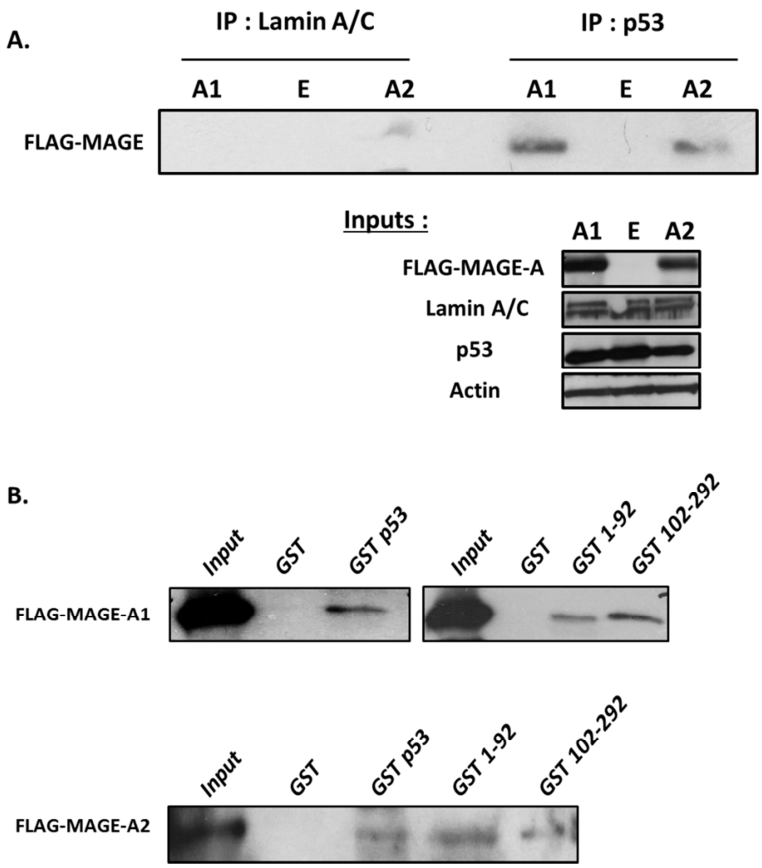

Figure 30 : MAGEA1 and -A2 directly interacts with the DNA binding domain of p53. (A) MCF-7 clones expressing MAGEA1 (A1) or -A2 (A2) as well as clones transfected with the empty vector (E) were treated with 500nm of doxorubicin for 18h to induce p53 expression. After treatment, total protein extracts were prepared and for each sample, 500µg proteins were immunoprecipitated with an anti-p53 polyclonal antibody. As negative control, an anti-lamin A/C antibody was used. After SDS-PAGE, interactions were revealed by western blot using an antibody targeting the N-terminal FLAG tag of the MAGEA proteins. The expression of p53, lamin A/C and MAGEA are also shown in the total protein extracts before immunoprecipitation (Inputs). (B) GST pull-down assays using on one hand in vitro translated (IVT) MAGEA1 or -A2 and on the other hand GST (negative control), GST-p53 wt, GST p53 1-92 (N-terminal region) or GST p53 102-292 (DNA Binding Domain) purified from E. Coli. GST-fusion constructs and IVT translated MAGEA proteins were incubated for 1h and interactions were isolated with gluthation-sepharose beads. A western blot analysis using anti-FLAG antibody revealed the direct interaction of MAGEA1 and -A2 with the tumor suppressor.

MAGEA1 and- A2 expression correlates with decreased p53 levels in cells exposed to a genotoxic stress

We next sought to determine what would be the impact of these interactions on p53 stabilization and induction during a stress. In MCF-7 stable clones treated with 500nM doxorubicin during 24h, we found a markedly decreased p53 levels in presence of MAGEA1 and -A2 (Figure 31a). Densitometry analyses of three different experiments demonstrate that the expression of MAGEA1 and MAGEA2 reduces p53 levels by respectively 25% and 35% (Figure 31b). Moreover, we repeated the experiments in a second model. We transiently transfected PA-1 p53 wt ovarian carcinoma cells with MAGEA1 and MAGEA2 and treated the cells with 500nM doxorubicin. The levels of p53 were examined 24h later. Again, both MAGEA1 and -A2 markedly decreased p53 levels (Figure 31c). To summarize, our data show that expression of MAGEA1 or -A2 reduces total p53 levels in cells exposed to a genotoxic agent.

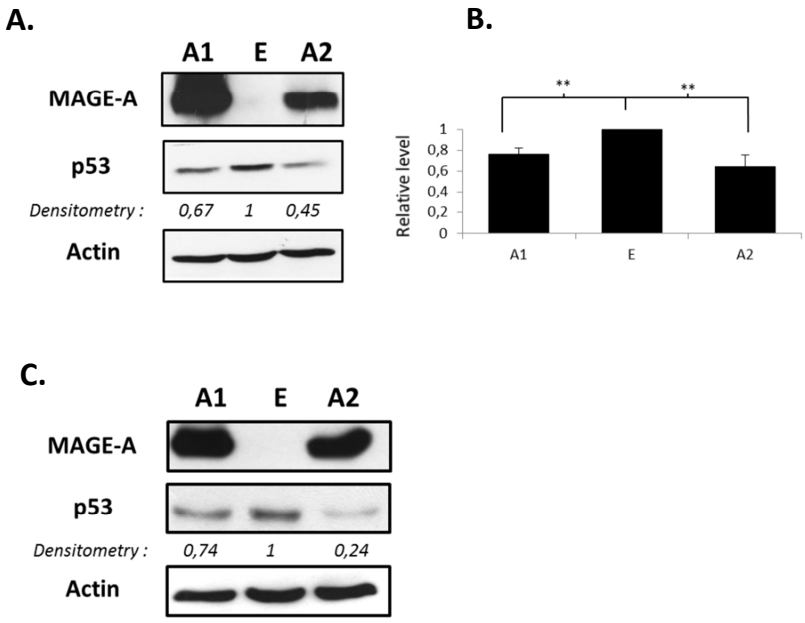

Figure 31: MAGEA1 and -A2 expression reduces p53 levels in cells exposed to doxorubicin. (A) MCF-7 cells stably expressing MAGEA1 or -A2 as well as cells transfected with the empty vector (E) were treated with 500nM doxorubicin for 24 h and total proteins were extracted. Levels of p53 were analyzed by western blot using a mouse monoclonal anti-p53 antibody. Levels of p53 in the different clones were quantified by a densitometry analysis of the corresponding bands and normalization to the loading control (Actin). (B) Graph depicting the relative p53 levels in cells expressing or not the MAGEA proteins. The p53 levels in cells transfected with the empty vector was set to 1. The graph is based on three independent experiments. Statistical test: Student T-test. **: p-val < 0,001 (N=3) (C) PA-1 ovarian cancer cells (p53 wt) were transiently transfected with MAGEA1, -A2 or the empty vector (E). The next day, cells were treated with 500nM doxorubicin for 24h. The levels of p53 were then analyzed by western blot using a mouse monoclonal anti-p53 antibody. The levels of p53 were quantified by a densitometry analysis of the corresponding bands and normalization to the loading control (Actin).

MAGEA1 and -A2 expression correlates with a decreased phosphorylation of p53 N-terminal region

A reduced p53 levels in presence of MAGEA1 and -A2 could be the consequence of an impaired stabilization and activation of p53 during the early response phase to the genotoxic stress. A crucial step for the activation of p53 involves different post-translational modifications. For example, phosphorylation of Thr18 and Ser20 are essential to abrogate MDM2 dependent inhibition of p53 since they abolish the docking of MDM2 on the N-terminal region of p53 [37,42]. In addition, phosphorylation of the adjacent Ser15 enhances p53 transcriptional activity by favoring the recruitment of the transcriptional co-activator CBP/P300 [43].

We examined whether the altered stabilization of p53 could be linked to a deficient phosphorylation of its N-terminal region. MCF-7 stables clones expressing or not MAGEA1 and -A2 were treated with 500nM doxorubicin for 18h and total protein extracts analyzed thereafter by western blot using antibodies specifically targeting phosphorylated forms of p53 (Figure 32). Since MAGEA1 -and -A2 reduce total p53 levels (Figure 31), we calibrated each sample in order to load the same amount of “total p53” in each well. We found that, in presence of an overexpression of MAGEA1 or -A2, the abundance of p53 Ser15 phosphorylation was markedly decreased (Figure 32a). Phosphorylation of Ser20 is thought to be dependent on the prior phosphorylation of

Ser15 [43]. Data were consistent since Ser20 also displayed a reduced phosphorylation in presence of MAGEA1 and -A2 (Figure 32b). Additionally, phosphorylation of Thr18 was also impaired in presence of MAGEA proteins (Supplemental data: Figure 37).

We next determined the levels of phosphorylation of Ser46. Ser46 phosphorylation has been described as crucial for p53-dependent apoptosis. For instance, in MCF-7 cells, mutation of Ser46 into alanine markedly reduces UV light-induced apoptosis and prevents induction of p53 target genes such as PUMA and p53AIP1 [45,46]. When MCF-7 clones expressing MAGEA1 and -A2 were examined after treatment with doxorubicin, we found a reduction of Ser46 phosphorylation (Figure 32c). This observation is also in accordance with the increased resistance of these cells to apoptotic stimuli.

Altogether, our data support the hypothesis that an elevated expression of MAGEA1 and -A2 impact on different post-translational events occurring in the early phase of p53 activation following a stress. A deficient N-terminal phosphorylation could explain the decreased p53 levels in presence of MAGEA1 and -A2. Moreover, a decreased phosphorylation of Ser46 is known to compromise the expression of several p53 pro-apoptotic target genes and therefore p53-dependent apoptosis.

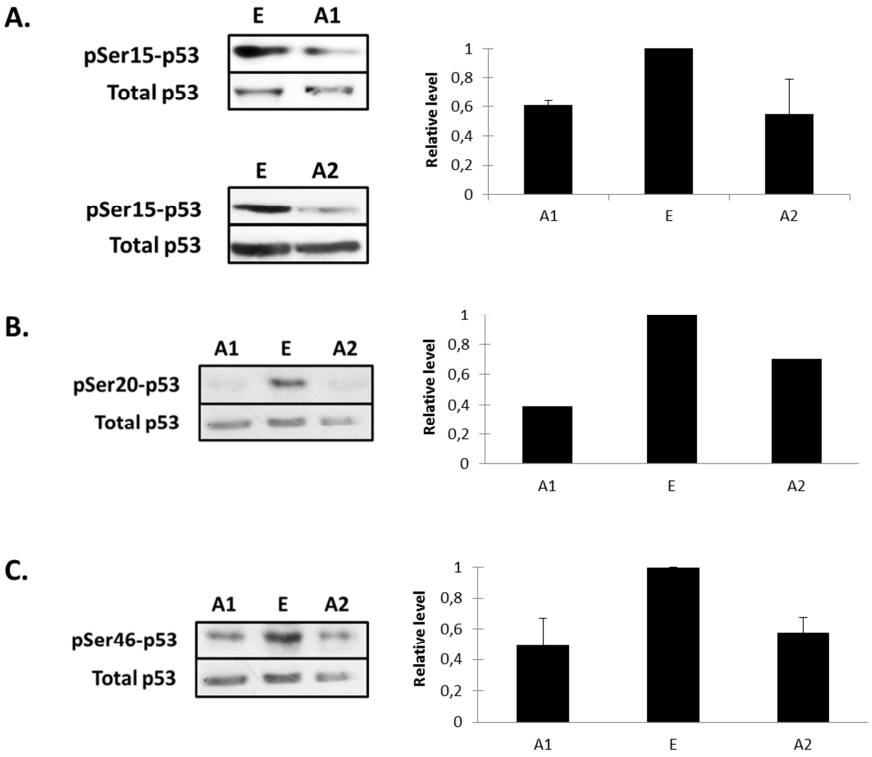

Figure 32: MAGEA1 and -A2 expression impairs p53 N-terminal phosphorylation. MCF-7 stable clones expressing MAGEA1, -A2 or transfected with the empty vector (E) were treated with doxorubicin for 24 h. Proteins extracts were standardized in order to load the same amount of total p53 in each well for western blot analysis. Western blot were realized with antibodies (Cell Signaling) targeting the phosphorylated forms of p53. (A) Left, western blot analysis showing the reduced phosphorylation of p53 Ser15 in presence of MAGEA1 or -A2. Right, Graph depicting the relative levels of pSer15-p53 in MCF-7 cells expressing or not the MAGEA proteins. The p53 levels in cells transfected with the empty vector was set to 1. Results are based on densitometry analysis from 4 independent experiments (N=4). (B) and (C) Analysis of respectively pSer20-p53 and pSer46-p53 using the same strategy (N=3).

MAGEA1 and -A2 expression reduces p53 stability in cells exposed to doxorubicin

As mentioned above, following a stress, different N-terminal phosphorylation events lead to a drastic enhancement of p53 half-life. Since we observed an impaired phosphorylation of p53 in presence of MAGEA1 and -A2, we examined whether p53 stability could be affected (Figure 33). MCF-7 clones expressing or not MAGEA1 and -A2 were treated with 500nM doxorubicine for 18h. Thereafter, protein synthesis was blocked with cycloheximide and total protein extracts prepared at different time points in order to evaluate the stability of stress-activated p53. As shown in Figure 33, the stability of the tumor suppressor was altered in presence of MAGEA proteins. After 8 hours of incubation with cycloheximide, densitometry analysis revealed a reduction of p53 level of 70% and 71% in presence of respectively MAGEA1 and -A2. These observations correlate with our previous data showing an impaired N-terminal phosphorylation of p53 and overall decreased p53 levels in presence of MAGEA1 and -A2.

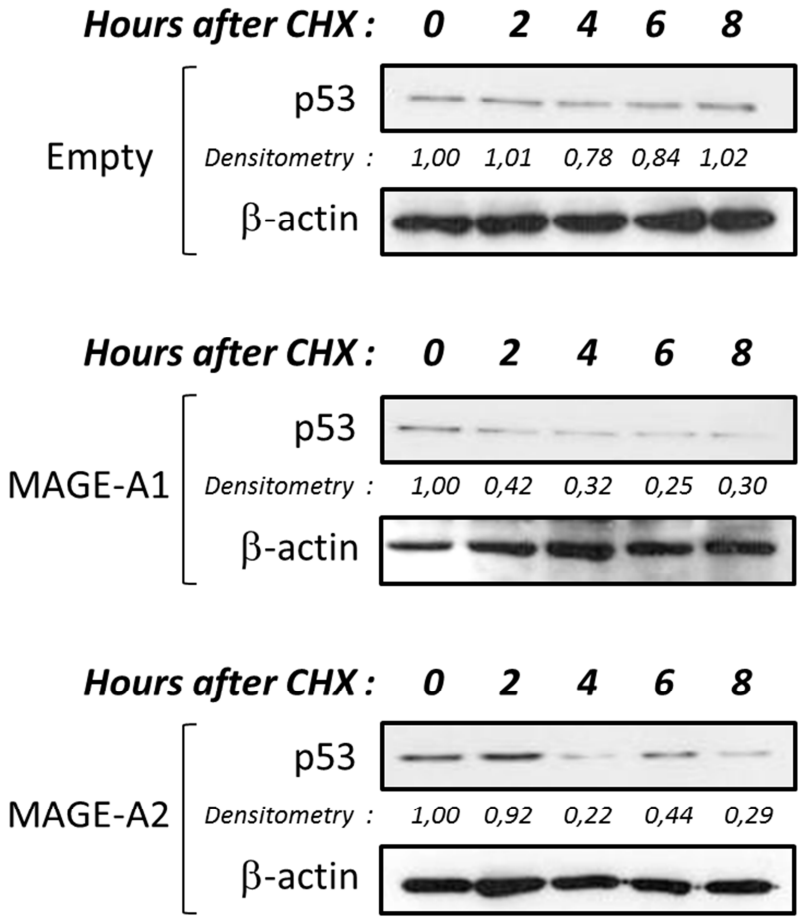

Figure 33 : MAGEA1 and -A2 expression reduces p53 half-life. MCF-7 stable clones expressing MAGEA1, -A2 or transfected with the empty vector (E) were treated with 500nM doxorubicin for 24h. We next incubated cells with 30µg/ml cycloheximide (CHX) for 0, 2, 4, 6 and 8 hours and measured p53 levels by western blot. Half-life of p53 (T1/2) is reduced when MAGEA1 or -A2 is expressed as demonstrated by the densitometry. (N=3).

Discussion

In this study, we demonstrate that among the MAGEA commonly expressed in cancer, only MAGEA1 and -A2 are able to increase the resistance of MCF-7 cells to the genotoxic agent doxorubicin. We further analyzed the effects of MAGEA1 and -A2 and found that they decrease apoptosis in presence of camptothecin, another genotoxic agent. Using nutlin-3a, an inhibitor of the ubiquitin E3 ligase MDM2, we also evidenced that cell growth inhibition triggered by this direct activator of p53 is reduced in MCF-7 clones stably expressing MAGEA1 or -A2, indicating that the tumor suppressor p53 could be a target of these proteins. Again, no significant protective effect was demonstrated for the other MAGE-A proteins when the clones were treated with 10 µM nutlin-3a (supplemental data: Figure 36).

Whether MAGE-A proteins directly bind to p53 is controversial, therefore we aimed to study these interactions. In MCF-7 stable clones, we immunoprecipitated p53 and found a robust interaction with MAGEA1 and -A2. Moreover, GST-pull down experiments clearly indicated that the interaction is direct and involves the DNA binding domain of p53, as well as its N-terminal region.

Moreover, in cells exposed to a genotoxic stress, we consistently observed markedly lower p53 levels in presence of MAGEA1 or -A2. This was observed in the MCF-7 stable clones and also in PA-1 cells

transiently transfected with MAGEA1 and -A2. Following a genotoxic stress, the classical model for wt p53 activation involves different N-terminal phosphorylations that stabilize the tumor suppressor [37], allowing its intracellular concentration to increase. Our data indicate that Ser-15 phosphorylation is impaired in presence of MAGEA1 and -A2. This phosphorylation is an early post-translational event following a stress and is carried out by the ATM kinase as well as others. Ser-15 phosphorylation is known to favor the recruitment of the acetyl-transferase CBP/p300 and the subsequent phosphorylation of Thr-18 and Ser-20 [42]. Our data indicate that these two phosphorylations of p53 are also altered in presence of MAGEA1 and -A2. Thus, MAGEA1 and -A2 prevent three N-terminal phosphorylations of p53 that act together to inhibit MDM2 binding and therefore to promote stabilization of the tumor suppressor. Subsequent experiments where we measured the half-life of stress-activated p53 in presence or not of the MAGEA proteins confirmed that p53 was destabilized in cells expressing MAGEA1 or -A2.

Finally, we examined the phosphorylation of Ser-46 and found that this was also altered in presence of MAGEA1 or -A2. This post-translational modification is of interest since it is important for the ability of the tumor suppressor p53 to upregulate the expression of different pro-apoptotic genes such as those coding for PUMA and p53AIP1. Therefore, phosphorylation of Ser-46 is associated with enhanced p53-

dependent apoptosis. Indeed, the S46A p53 mutant is markedly impaired for apoptosis induction [45,46,48].

Based on our different data, we propose a whole new mechanism to describe the impact of MAGEA expression on p53 function. MAGEA1 and -A2 are able to directly interact with p53. In addition, MAGE-A expression has a destabilizing effect on p53. In cells exposed to a genotoxic stress, lower p53 levels are observed in presence of MAGEA1 and -A2. This is correlated to an impaired phosphorylation of p53 N-terminal region, notably the phosphorylation of Ser15, Thr18 and Ser20. Phosphorylation of Ser46 is also affected in presence of MAGEA1 and -A2. The absence of this post-translational modification is known to reduce p53 ability to trigger apoptosis.

Several teams highlighted a relation between a high expression of type I MAGEs in cancer and disease aggressiveness, formation of metastasis, resistance to chemotherapeutic treatments and an overall poor patient prognosis [13,14,17,47]. Although their re-expression in cancer is known to be linked to the hypomethylation of their promoter, the biochemical functions of these proteins within cancer cells remained largely a mystery. The protective effect of MAGEA proteins against DNA damage-induced apoptosis was already described by Monte et al. (2006). For instance, using a siRNA pool able to target simultaneously different MAGEA, they evidenced in p53 wt U2OS cells an enhancement of apoptosis in presence of etoposide [19]. We here provide additional

information by pointing out that only MAGEA1 and -A2 protect from the DNA damaging agent doxorubicin while MAGEA3, -A4, -A6 and -A12 do not.

The relation between MAGEA and p53 has been first identified by Monte *et al*. and later confirmed by other teams [22,23]. As far as we know, the destabilizing effect of MAGEA1 and -A2 on stress-activated wt p53 was never reported before. However, it was already observed that MAGE-C2 or mMAGE-b siRNA enhanced wt p53 levels in unstressed cells. Knock-down of the expression of MAGEA was also shown to increase the intracellular levels of mutant p53 in different cell lines [22]. Moreover, expression of MAGEA2 or -C2 in HCC143 cells that express the p53 R248Q mutant results in a decreased expression of mutant p53 [22].

Further studies are now required to understand why the N-terminal phosphorylation of p53 is altered in presence of these MAGEA proteins. Hypothesis are numerous, one of them could be that MAGEA interaction prevents access to p53 N-terminal region. In favor of this, we detected a binding of MAGEA to the N-terminal (amino acids 1-92) of the tumor suppressor. On the other hand, it is also conceivable that MAGE-A proteins directly or undirectly regulate upstream regulators of p53 such as the kinase ATM, ATR, CHK1 or CHK2. This would explain why after acute DNA damage stress, p53 post translational modification is impaired. Finally, another hypothesis would involve the recruitment of phosphatases by MAGEA proteins.

In addition to deciphering the mechanism of p53 destabilization by MAGEA, it would be interesting to determine in different cancer types whether there is a strong association between wt p53 and MAGEA1 and -A2. In that case, preventing MAGEA proteins to bind and inhibit p53 could be an interesting strategy to sensitize cancer cells that retain the wt version of the tumor suppressor. To our knowledge, no such molecule exists. However, Bhatia et al. identified three compounds able to inhibit the binding of MAGEA to KAP1 (aka TRIM28) a RING domain protein tought to enhance p53 degradation in presence of MAGE-A proteins [49].

In summary, we describe a novel mechanism that shed light on the reduced activity of the tumor suppressor p53 in presence of MAGEA: MAGEA expression destabilizes p53 by impairing phosphorylation of its N-terminal region in cells exposed to a genotoxic stress.

Acknowledgements

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Supplemental data

Cell lines	MAGE-A1	MAGE-A2	MAGE-A3	MAGE-A4	MAGE-A6	MAGE-A12
WM-115	1	1	1	1	1	1
PA-1	Not detected	0,0304 (0,023-0,039)	Not detected	0,001 (0,0008-0,002)	Not detected	Not detected
MCF-7	0,017 (0,014-0,020)	Not detected	Not detected	0,0014 (0,0008-0,003)	Not detected	Not detected
HCT-116	0,71 (0,5 -1,0)	30,21 (20,8-43,8)	1,84 (1,3-2,6)	32,84 (22,7-47,6)	1,09 (0,8-1,6)	43,36 (32,0-58,8)

Figure 34 : Relative expression of MAGEA1, -A2, -A3, -A4, -A6 and -A12 in different cancer cell lines harboring wt p53: WM-115 (melanoma), PA-1 (ovarian teratocarcinoma), MCF-7 (breast adenocarcinoma) and HCT-116 (colorectal carcinoma). RNA was extracted using Trizol method and a reverse transcription was performed. Expression data were obtained by qPCR and allow a relative quantification by comparing the Ct (Cycle threshold). Results are depicted as 2-ΔΔCt form. The ΔCt has been calculated by the substraction of the geometrical mean of housekeeping genes Ct (SP1 and TBP) by the means Ct of each of the MAGEA genes. The ΔΔCt is determined by the substraction of calibrator ΔCt (WM-115 cell line ; ATCC ref : CRL-1675 ; human melanoma cell line) from the MAGEA specific ΔCt. By consequence, in this figure, the expression of MAGEA mRNA is equal to 1 (20) for the WM-115 cell line. A comparison is established between the expression of MAGEA mRNA in this cell line and the others : MCF-7 (ATCC ref : HTB-22 ; adenocarcinoma derived cell line from human mammary gland), PA-1 (ATCC ref : CRL-1572 ; human ovarian teratocarcinoma derived cell line) and HCT-116 (ATCC ref : CCL-247 ; human colorectal carcinoma derived cell line).

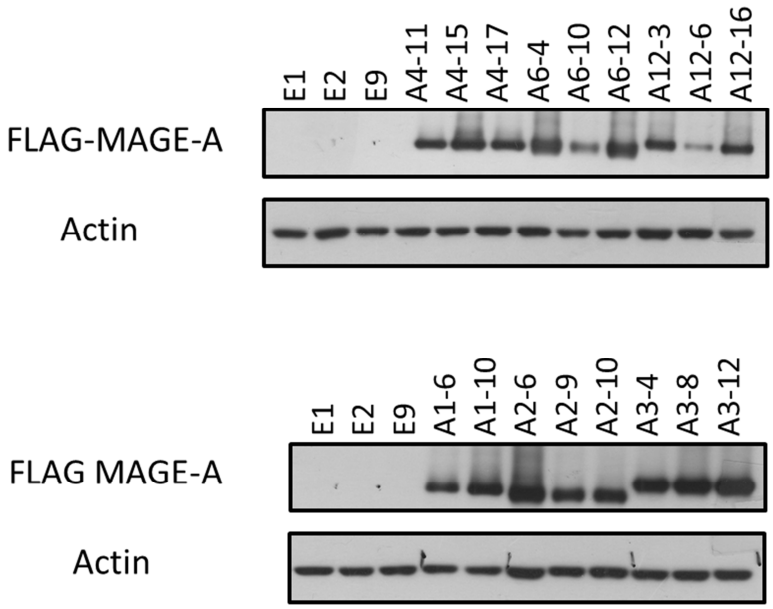

Figure 35 : Expression of the different MAGEA proteins in MCF-7 stable clones. Following puromycin selection, each MCF-7 clone was analyzed for its MAGEA expression. The plasmid pDEST-v1899-FLAG allows the fusion of the MAGEA protein with a N-terminal triple FLAG tag. Therefore, we extracted proteins and check FLAG-MAGEA expression by western blot using the M2 anti-FLAG antibody (Sigma-Aldrich). Data show that clones express MAGEA proteins while control clones (transfected with the empty vector) do not. It is interesting to notice that MAGEA proteins migrate to approximatively 45kDa in 12% polyacrylamide gels when they predicted molecular weight is about ~34-35kDa (Expasy). Moreover, differences in migration may be observed among MAGEA proteins.

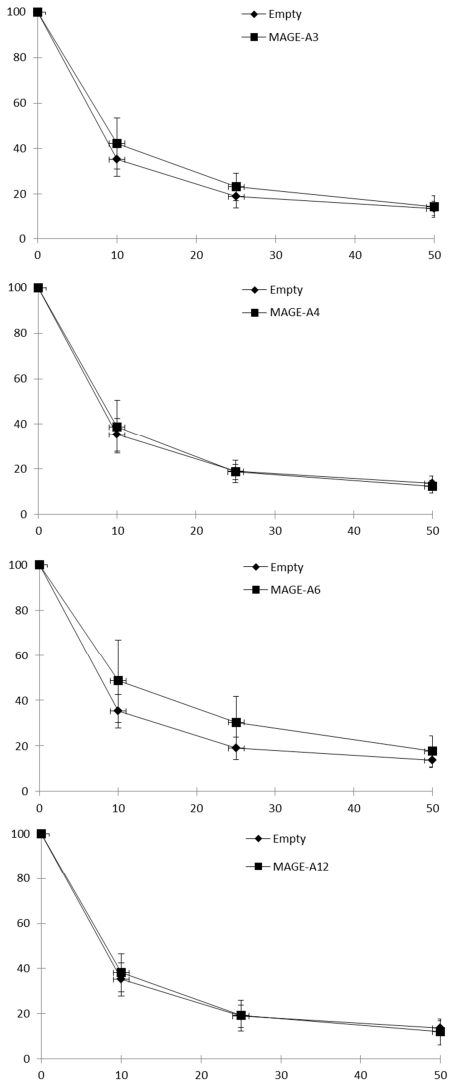

Figure 36 : Expression of MAGEA3, -A4, -A6 and -A12 does not protect against the p53 activator nutlin-3a. MCF-7 clones expressing the different MAGEA or transfected with the empty vector were treated with increasing concentration of nutlin-3a (0, 10, 25 and 50 µM). After 72h of treatment, viability was measured by MTT assays. Data are means of three different clones. Statistical test: Student T-test. ***: p-val < 0, 0001; **: p-val < 0,001; *: p-val < 0, 05 (N=4, n=3).

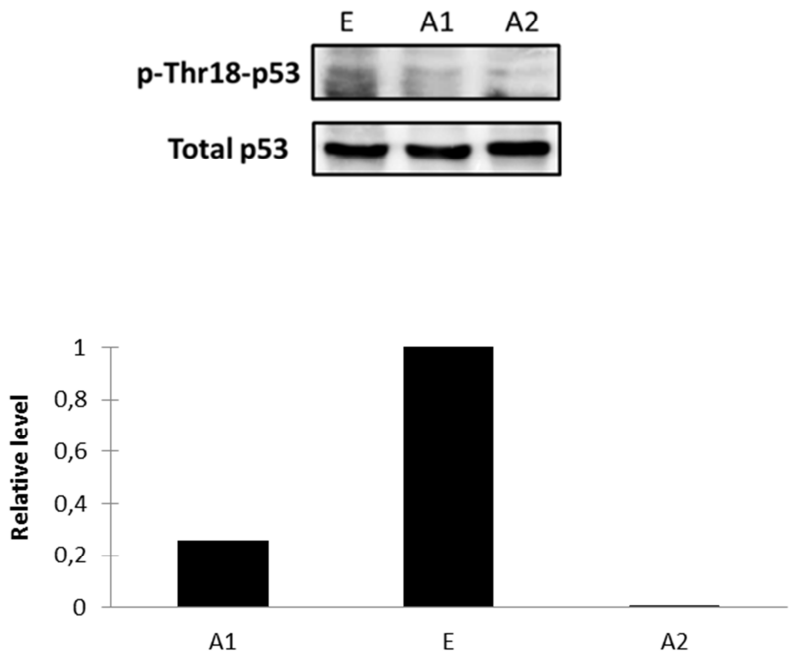

Figure 37 : MAGEA1 and -A2 expression decreases p53 phosphorylation on Thr18. MCF-7 clones expressing MAGEA1, -A2 or transfected with the empty vector were treated with doxorubicin for 24h. Protein extracts were standardized in order to load the same amount of "total p53" for western blot analysis with an antibody (Cell signaling) targeting the Thr18 phosphorylated form of p53. For this post-translational modification, only one experiment was performed.

Supplemental data: Materials and Methods

Cell culture. The human colorectal carcinoma cell line HCT-116 (ATCC number: CCL-247), the human teratocarcinoma cell line PA-1 (ATCC number: CRL-1572), the breast cancer cell line MCF-7 (ATCC number : HTB-22) and melanoma cell line WM-115 (ATCC number : CRL-1675) were grown in DMEM (Gibco 42430-082) supplemented with 10% foetal bovine serum (FBS) and 1% Penicillin-Streptomycin (P/S) (Gibco 15140-163).

RT-qPCR. Cell pellets were obtained by centrifugation and RNA was extracted using Trizol (Life Technologies). RNA extracts were reverse transcribed using Quantitect Reverse Transcription kit following manufacturer instructions (Qiagen, 205311). Primers were designed to amplify specific regions of the different MAGEA genes. Triplicate samples were prepared for qPCR with SYBR®Green PCR master Mix (Applied Biosystems N°. 4309155). Reactions contained 100ng of cDNA and 200nM of each primer. Reactions were submitted to the following amplification program: 95 °C for 2min and then 40 cycles of 95 °C for 30 sec, 60 °C for 30sec. The following primers pairs were used:

MAGEA1 : F: 5' – GTAGAGTTCGGCCGAAGGAACCT – 3' / R: 5' – TGATGACTCTCGTCAGGGCAG – 3'

MAGEA2 : F : 5' – GAGGACAGTGTCTTCGCAC – 3' / R : 5' – GGTTCTCCACCGATCTTTAGTG -3'

MAGEA3 : F : 5' – GAGATTCTCGCCCTGAGCAACGAG – 3' / R : 5' – CAGATCTTCTCCTTCAGTGCTCCTCC – 3'

MAGEA4 : F : 5' – GCGTGAAGCAGCTTTGTTAGAGGA – 3' / R : 5' – AAGCTGCTGCACAGGGCTGTTAG – 3'

MAGEA6 : F : 5' – GCTGAGTGTGTTAGAGGTGTT – 3' / R : 5' – CAGGAGTGGGTAGGAAATGC – 3'

MAGEA12 : F : 5' – CTGAGTGTGTTGGAGGCATC – 3' / R : 5' – GGTGGGTAGGAAATGTGAGGT – 3'

SP1 : F : 5' – CCGCTCCCAACTTACAGAAC – 3' / R : 5' – ATGATGTTGCCTCCACTTCC – 3'

TBP : F : 5' – TTCGGAGAGTTCTGGGATTG – 3'/ R : 5' – AATCAGTGCCGTGGTTCGT – 3'

Western Blot. Total proteins were extracted from cells using RIPA buffer (150mM NaCl, 0.5% sodium deoxycholate, 50mM Tris-HCl pH 8.0, 0.1% SDS, 0.1% Triton X-100) supplemented with protease and phosphatase inhibitors (Complete Mini EDTA Free,

ROCHE 11836170001 ; Phosphatase Inhibitor Cocktail 2 and 3, Sigma-Aldrich P0044 and P5720). Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked and then probed overnight with the following antibodies: mouse anti-p53 (dilution 1/1000; Calbiochem OP43) and rabbit anti-pThr18 p53 (dilution 1/1000; Cell Signaling 2529S). After 3 washes, membranes were incubated with the appropriate peroxydase-conjugated secondary antibodies. Immunoreactive bands were detected using the Western Lightning kit (Perkin Elmer, NEL 104001EA) and signal intensity quantified using ImageJ V1.41 software (NIH, USA)

MTT assays. Briefly, cells were plated in 96-well plates at 5,000 cells per well and treatments started 48h later. After treatments (72h), cells were incubated for 3h with DMEM (10% FBS, 1% P/S) containing 0.5 mg/ml MTT (Sigma, M2128). Plates were then centrifuged (2100rpm) and the supernatants discarded. We added 200µl DMSO to dissolve the formazan precipitate. We then read the absorbance at 560 nm and subtracted that at 670 nm.

Chapter II:

Resveratrol induces DNA damage in colon cancer cells by poisoning topoisomerase II and activates the ATM kinase to trigger p53-dependent apoptosis

Resveratrol induces DNA damage in colon cancer cells by poisoning topoisomerase II and activates the ATM kinase to trigger p53-dependent apoptosis

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Running title: Resveratrol poisons topoisomerase II and induces p53

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Key words: Resveratrol, p53, apoptosis, DNA damage, colon cancer, ATM, topoisomerase II

Abstract

Resveratrol (trans-3,4',5-trihydroxystilbene) is a natural polyphenol synthesized by various plants such as grape vine. Resveratrol (RSV) is a widely studied molecule, largely for its chemopreventive effect in different mouse cancer models. We propose a mechanism underlying the cytotoxic activity of RSV on colon cancer cells. Our data show that resveratrol induces apoptosis, as observed by the cleavage of PARP-1 and chromatin condensation. We show that the tumor suppressor p53 is activated in response to RSV and participates to the apoptotic process. Additionally, we show that HCT-116 p53 wt colon carcinoma cells are significantly more sensitive than HCT-116 p53 -/- cells to RSV. RSV induces DNA damage including double strand breaks, as evidenced by the presence of multiple γ-H2AX foci in 50% of cells after a 24h treatment with 25 µM RSV. The formation of DNA damage does not appear to rely on a pro-oxidant effect of the molecule, inhibition of topoisomerase I, or DNA intercalation. Rather, we show that DNA damage is the consequence of type II topoisomerase poisoning. Exposure of HCT-116 cells to RSV leads to activation of the Ataxia Telangiectasia Mutated (ATM) kinase, and ATM is required to activate p53.

Introduction

Resveratrol is a natural polyphenol found in the skin of various fruits including grapes and berries (1). A first study published in 1997 described that the molecule prevents the development of pre-neoplastic lesions in cultured carcinogen-treated mammary glands and exerts protection against skin cancer in a mouse model (2). Since then, resveratrol (RSV) has been widely studied for this now fairly well evidenced chemopreventive properties. In addition, a potential use of RSV or derivatives in cancer chemotherapy is investigated. Indeed, reduction of tumor growth has been observed in different mouse cancer models including neuroblastoma, colon, prostate, liver and breast cancer (3-5). RSV acts as a suppressor of inflammatory processes that can influence cancer progression (6). The activity of RSV is also linked to its ability to inhibit tumor invasion, metastasis formation as well as neo-angiogenesis. Finally, other properties of RSV relevant to cancer prevention or therapy include modulation of cell redox status, inhibition of cell proliferation and induction of apoptosis (5-8).

Among various mechanisms proposed to explain the apoptosis inducing properties of RSV on cancer cells, the molecule is known for its modulation of BCL-2 family members expression. RSV is able to upregulate the expression of pro-apoptotic proteins such as BAX, BAK, PUMA and NOXA while decreasing that of anti-apoptotic members like BCL-2, MCL1 and BCL-XL. Therefore, RSV could favor outer mitochondrial membrane (OMM) permeabilization and release

of the transmembrane apoptosis effectors in the cytosol (9, 10). RSV also modulates the extrinsic pathway of apoptosis that relies on binding of ligands such as TNF-α, FASL or TRAIL on their specific membrane receptor. RSV drives redistribution of the receptor FAS/CD95 to lipid rafts, therefore facilitating activation of this pathway (5). It also sensitizes cells to TRAIL-induced apoptosis presumably through similar mechanisms (5).

The tumor suppressor p53 also appears to be a target of RSV since the molecule is able to activate p53 in a variety of cancer cell lines including breast, colon and prostate cancer cells as well as osteosarcoma and B-cell lymphoma (5). However, how RSV signals to p53 is unknown. Alteration of pathways regulating the apoptotic process and direct mutation of genes involved in apoptosis is a hallmark of cancer (11). Central to apoptosis, the tumor suppressor p53 is the most frequently mutated gene in human cancer, with an overall mutation rate over 50% (12-15). As a transcription factor, p53 induces or represses the expression of a variety of genes which products have respectively a pro-apoptotic (BAX, NOXA, PUMA, KILLER/DR5, FAS/CD95,…) or a pro-survival role (BCL-2, SURVIVIN, MDR1,…) (16, 17). Additionally, p53 can trigger apoptosis by acting directly at the mitochondria. Indeed, in response to an apoptotic insult, p53 translocates to mitochondria and induces OMM permeabilization. This activity requires direct interaction of p53 with BCL-2 family members, and importantly BAK (18-23).

The aim of this study is to investigate the mechanism(s) of RSV-induced cell death in colon cancer cells, and the involvement of p53

in this process. We show that RSV is able to induce extensive DNA damage and more specifically DNA double strand breaks in human colon carcinoma cells, leading to p53-dependent apoptosis through the activation of the Ataxia Telangiectasia Mutated (ATM) kinase. We also provide evidence that DNA damage upon RSV treatment is due to topoisomerase II poisoning, and not an elevation of the intracellular levels of reactive oxygen species (ROS).

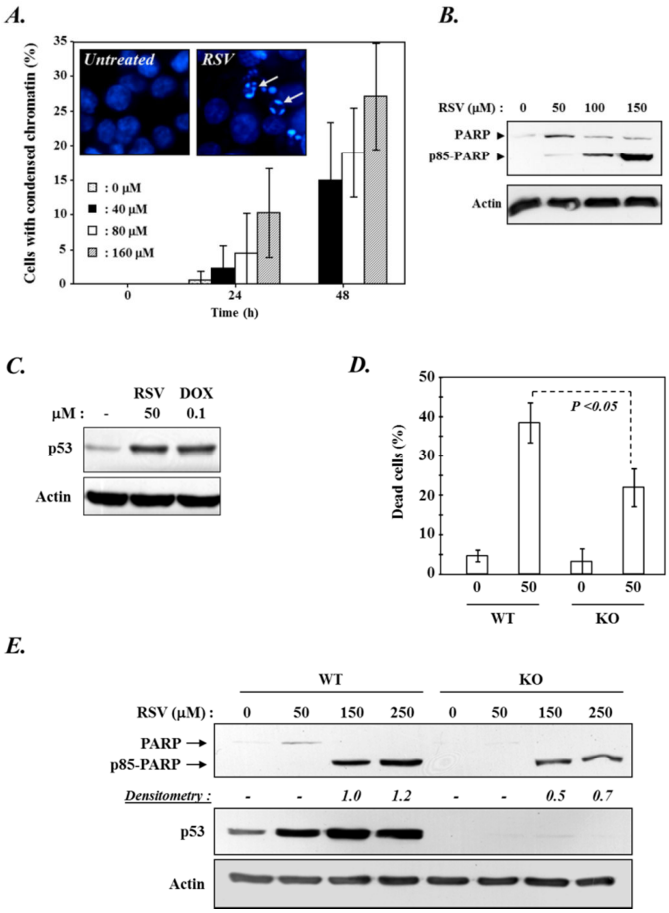

Figure 38: Resveratrol induced apoptosis is p53-dependent. (A) DAPI staining reveals that RSV induces chromatin condensation. HCT-116 p53 wt cells were treated with different concentrations of RSV (0, 40, 80 and 160 µM) during 0, 24 or 48h. The graph represents the analysis of the percentage of cells presenting a nucleus with a condensed chromatin. In each experimental condition, countings were based on 12 images (20-100 cells per image). Data are presented as mean percentage ± SD of cells with a condensed chromatin. Photos are representative fluorescence microscopy images of DAPI stained cells. The white arrows indicate nuclei showing a condensed chromatin. (B) Analysis of the cleavage of PARP-1 by western blot in HCT-116 cells treated for 24h with 0, 50, 100 and 150 µM RSV. (C) Western blot analysis of the levels of p53 in untreated HCT-116 cells and cells exposed for 24h to 50 µM RSV or 0.1 µM DOX. (D) Percentages of cell death in populations of p53 wt and knockout (KO) HCT-116 cells exposed to RSV. Cells were treated with 0 or 50 µM RSV during 24h and thereafter analyzed by flow cytometry (co-labeling with LDS751 and propidium iodide). Data are mean of 4 independent experiments ± SD. Statistical differences between means were calculated by analysis of variance, followed by the unpaired Student's t test. (E) Western blot analysis of the cleavage of PARP-1 and the levels of p53 in HCT-116 p53 wt and KO cells treated for 24h with 0, 50, 150 and 250 µM RSV. The cleavage of PARP-1 was quantified by a densitometry analysis of the corresponding bands and normalization to the loading control (Actin).

Results

Resveratrol induced apoptosis is enhanced in the presence of wt p53.

Cultures of HCT-116 p53 wt colon carcinoma cells were exposed, for 0, 24 or 48h, to increasing concentrations of RSV and labeled with DAPI at the end of treatments in order to measure chromatin condensation, a morphological characteristic of the apoptotic nucleus. Following this treatment, the percentage of cells containing condensed chromatin increased in a time- and dose-dependent manner (Figure 38A). PARP cleavage was also clearly observed in cultures incubated for 24h to concentrations of RSV equal or above 50 µM (Figure 38B). In addition, RSV triggered a marked induction of the tumor suppressor p53 (Figure 38C) as well as its translocation to the mitochondria, concomitant to cytochrome c release (Data not shown). We next examined whether RSV induced cell death was dependent on p53 function. HCT-116 p53 wt cells and their somatic-cell knock-out derivative HCT-116 p53 -/- (24) were incubated for 24h in the presence of 50 µM RSV and cell viability was thereafter quantified by flow cytometry (co-labeling with LDS751 and propidium iodide). Data show that the percentage of death is significantly higher in p53 wt cell populations (38.4 ± 5.1%) as compared to their null derivative (21.9 ± 4.8%) ($p<0.05$, Figure 38D). PARP cleavage was also assessed by western blotting in HCT-116 p53

wt and p53 -/- cells exposed to different concentrations of RSV for 24h. A marked induction of p53 was observed in wt cells. Moreover, densitometry analysis of the bands revealed that PARP cleavage was 2-fold more abundant in wt cells, indicating that presence of the tumor suppressor indeed enhances apoptosis (Figure 38E). A similar dependence of cell death on p53 function was observed using the PA1 ovarian teratocarcinoma cell line (p53 wt) and its derivative expressing the human papillomavirus (HPV) E6 protein (Figure 44).

Resveratrol induces formation of multiple γ-H2AX foci.

RSV was previously shown to damage plasmid DNA in vitro in the presence of copper cations, by an oxidative mechanism that requires generation of peroxides (25, 26). We therefore sought to determine whether RSV is able to induce DNA damage in HCT-116 colon carcinoma cells and whether this is dependent on the generation of reactive oxygen species (ROS). At first, we monitored the formation of γ-H2AX foci. In response to DNA damage and more specifically DNA strand breaks, ATM and other kinases such as ATR and DNA-PK are known to phosphorylate the histone variant H2AX on Ser-139 (γ-H2AX) (27, 28). Very few cells presented with γ-H2AX foci in untreated controls (2.9 ± 3.4 %) or after a 24h exposure to the MDM2 inhibitor nutlin-3a (NUT) (0.3 ± 0.4 %), as expected (Figure 39A). Treatment of cells with doxorubicin (DOX) at 3 μM for 24h triggered formation of γ-H2AX foci (51.7 ± 10 %), in accordance with its described mechanism of action (29). Interestingly, we found a high

proportion of γ-H2AX positive cells in the presence of RSV (Fig. 2A). Formation of γ-H2AX foci was directly proportional to the concentration of the polyphenol, with 50% of cells displaying multiple foci after a 24h treatment in the presence of 25 μM RSV (Figure 39B).

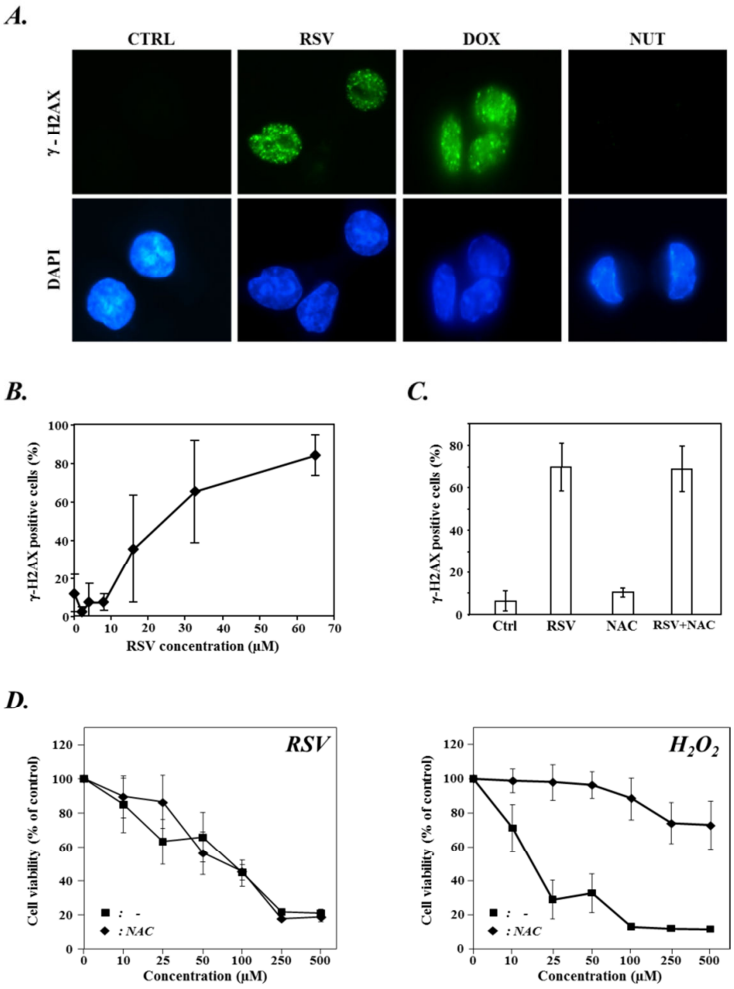

Figure 39: Resveratrol triggers formation of DNA damage. (A) Representative immunofluorescence microscopy images of p53 wt HCT-116 cells treated or not (CTRL) for 24h with 150 µM RSV, 35 µM NUT or 3 µM DOX. Formation of DNA damage was assessed by analyzing the presence of γ-H2AX foci, using an antibody that specifically recognizes the histone variant H2AX phosphorylated on Ser-139 (γ-H2AX). Nuclei are labeled with DAPI. (B) Percentage ± SD of cells presenting multiple γ-H2AX foci in cultures of HCT-116 p53 wt cells exposed for 24h to increasing concentrations of RSV (0, 2, 4, 8, 16, 32 and 64 µM). Countings were based on 6 different images for each experimental condition (100-200 cells/image). (C) Effect of the anti-oxidant NAC on RSV induced DNA damage. HCT-116 p53 wt cells were treated for 24h with 0 or 30 µM RSV in the presence or not of 5 mM NAC. Determination of the proportion of cells showing γ-H2AX foci was assessed by immunofluorescence microscopy. (D) Analysis of the effect of NAC on global cell growth. HCT-116 p53 wt cells were exposed for 72h to concentrations of RSV and H2O2 ranging from 0 to 500 µM, in the presence or not of 5 mM NAC. Global cell growth was determined by MTT assays at the end of treatments. Results are expressed as percentage of the control (untreated cells). Data are means of sextuplicates ± SD.

Formation of γ-H2AX foci and cell death are not inhibited by the anti-oxidant N-acetyl cysteine.

As a polyphenol, RSV displays anti-oxidant properties (30, 31). However, some reports also suggest a pro-oxidant action of RSV. For instance, chronic exposure of HCT-116 cells to RSV was reported to induce senescence and this was associated to an elevation of the intracellular levels of reactive oxygen species (ROS), including hydrogen peroxide and superoxide anion (32). To determine whether the cell death and γ-H2AX foci were the result of pro-oxidant activity of RSV, we analyzed the impact of the anti-oxidant N-acetyl cysteine (NAC). We found that NAC is consistently unable to inhibit formation of γ-H2AX foci in HCT-116 cells treated for 24h in the presence of RSV (Figure 39C). We also tested whether NAC was able to improve the viability of cell populations exposed to RSV. HCT116 cells were incubated for 72h to increasing concentrations of RSV (from 0 to 500 µM) in the presence or not of 5 mM NAC. We found that cell viability, as determined by MTT assays, was not rescued by NAC (Figure 39D). In contrast, NAC was highly protective against hydrogen peroxide, thus verifying the efficiency of the antioxidant (Figure 39D). The combined data support the premise that RSV-induced cell death is not due to its pro-oxidant function.

Resveratrol is not a DNA intercalating agent and does not inhibit topoisomerase I.

To determine the mechanism by which RSV induces DNA damage, we next analyzed whether RSV acts as a DNA intercalating agent or affects the activity of topoisomerases (TOPO). We first performed DNA unwinding tests in the presence of TOPO I, an enzyme able to negatively supercoil a relaxed circular DNA plasmid in the presence of an intercalating agent (33) (Figure 40A). We compared RSV to doxorubicin (DOX), a known DNA intercalator. Relaxed circular pRc/CMV plasmid was incubated in the presence of TOPO I, and increasing concentrations of RSV or DOX. The effect of DOX was readily observed at 5 μM, as noted by a shift of the topoisomers towards the supercoiled form. Our data indicate that RSV, even up to concentrations of 500 μM, does not intercalate into DNA (Fig. 3A and data not shown). Notably, the failure of RSV to affect DNA was not the consequence of a direct inhibition of TOPO I enzymatic activity by RSV, as evidenced by our finding that TOPO I was able to relax a supercoiled plasmid in the presence of 200 μM RSV (Figure 40B).

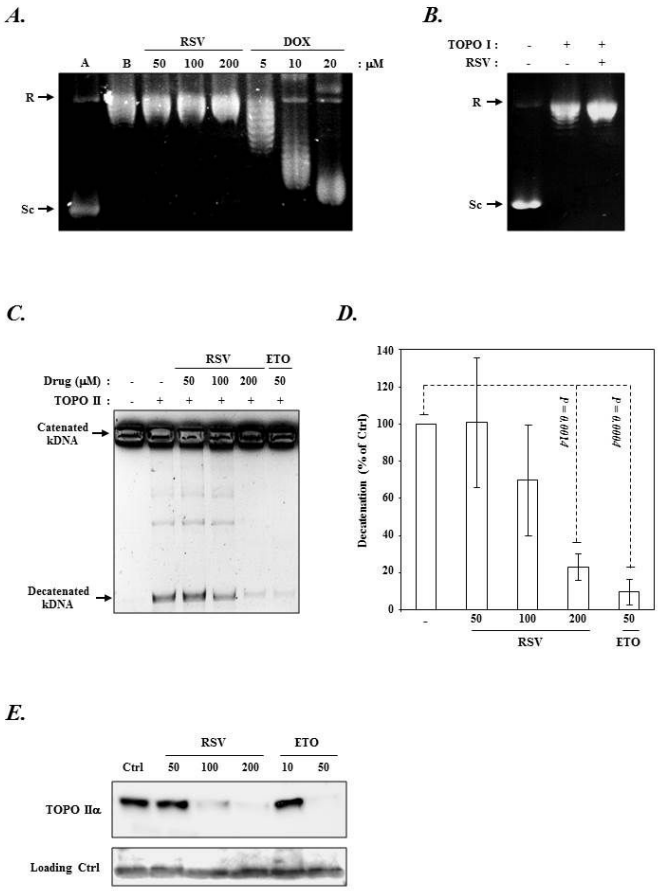

Figure 40: Resveratrol acts as a topoisomerase II poison. (A) DNA intercalation was monitored by conversion of relaxed pRc/CMV plasmid to supercoiled molecules in the presence of TOPO I. RSV was used at concentrations of 50, 100 and 200 µM. DOX is used as positive control for DNA intercalation at concentrations of 5, 10 and 20 µM. A: pRc/CMV plasmid purified from E. coli and showing a mix of the relaxed (R) and supercoiled (Sc) forms. B, substrate of the reaction: relaxed pRc/CMV plasmid. (B) RSV does not inhibit TOPO I. Supercoiled pRc/CMV was relaxed in the presence of TOPO I and either RSV at 200 µM or its solvent alone. Sc, supercoiled pRc/CMV; R, relaxed pRc/CMV. (C) Inhibition of TOPO II activity was analyzed by a kDNA decatenation assay. Catenated kDNA fails to migrate during electrophoresis due to the large size of the network, while decatenated kDNA migrates to generate a major band at 2.5 kb. RSV was used at concentrations of 50, 100 and 200 µM. Etoposide was used as positive control for TOPO II inhibition at a concentration of 50 µM. Left, ethidium bromide stained agarose gel showing the inhibition of kDNA decatenation by RSV and etoposide. Right, graph presenting a quantification of the data based on a densitometry analysis of the bands corresponding to decatenated kDNA. Data are mean of 3 independent experiments and are expressed as percentage ± SD of decatenation relative to the control (reaction in the presence of TOPO II but without drug). Statistical differences between means were calculated by analysis of variance, followed by the unpaired Student's t test. (D) TOPO II band depletion assay. HCT-116 p53 wt cells were exposed for 24h to 0, 50, 100 and 200 µM RSV as well as etoposide at concentrations of 10 and 50 µM. At the end of treatments, the soluble nuclear fraction was isolated and the levels of free TOPO II was determined by western blot. The loading control is a non specific band detected at a MW of 75 kDa.

Resveratrol triggers DNA damage by poisoning topoisomerase II.

The enzyme topoisomerase II (TOPO II) regulates chromatin topology by operating a breakage-reunion cycle that involves formation of an intermediate, the so-called cleavable complex, in which each subunit of the TOPO II homodimer is covalently linked to the newly created 5' phosphate ends (34). To test the possibility that RSV was a TOPO II poison, we compared the effect of RSV to that of etoposide, a reference TOPO II poison (34). Kinetoplast DNA, which consists of a large network of small interlocked circular DNA molecules, is too large to enter an agarose gel. However, in the presence of TOPO II, monomers are released from the network and migrate into the gel generating a major band at 2.5 kb. We observed that both etoposide (50 µM) and RSV (100 and 200 µM) are able to inhibit TOPO II-mediated kDNA decatenation (Figure 40C). Quantifications obtained by measuring the densitometry of the bands indicate that 200 µM RSV causes 80% inhibition ($p=0.0014$, Figure 40D), which is comparable to the effect of 50 µM etoposide (90% inhibition) ($p=0.0004$, Figure 40D). Next, we tested whether RSV stabilizes the cleavable complex. We used the well-known 'band depletion' assay that allows determination of the relative amount of chromatin-bound (cleavable complex) and free TOPO II in a nuclear fraction. We found that a 24h treatment of HCT-116 p53 wt cells with 100 or 200 µM RSV leads to the depletion of TOPO II from the soluble nuclear fraction, indicating that the enzyme is trapped on chromatin. A similar effect was observed in the presence of 50 µM etoposide (Figure 40E). The combined data are consistent with the premise that

RSV acts as bona fide TOPO II poison, by stabilizing a covalent enzyme-DNA intermediate. This correlates with the presence of γ-H2AX foci in RSV-treated cells since the histone variant H2AX is indeed phosphorylated on Ser-139 by different kinases including ATM following DNA double strand breaks.

The tumor suppressor p53 is phosphorylated on serine 15 by the kinase ATM in response to resveratrol.

In order to probe the RSV signaling pathway upstream of p53, we next monitored phosphorylation of this protein. After a 24h treatment of HCT-116 cells with RSV, we observed phosphorylation of p53 on Ser-15, Ser-33 and Ser-37 (Figure 41A). Ser-15 phosphorylation constitutes an early post-translational modification of p53 following DNA damage and can be performed by different kinases including ATM, ATR and DNA-PK (35, 36). To understand the signaling pathway triggering p53 activation in response to RSV, we aimed to identify the protein kinase responsible for Ser-15 phosphorylation. Since TOPO II poisoning and formation of γ-H2AX foci occur in the presence of RSV, we first investigated the possible role of ATM. When HCT-116 cells were treated with RSV for 24h in the presence of caffeine, an ATM kinase inhibitor (37), we observed a reduced stabilization of p53, as well as reduced Ser-15 phosphorylation (Figure 41B). We repeated the experiments using KU55933, a specific ATM kinase inhibitor (38). Similar to caffeine, KU55933 prevented the stabilization of p53 after a 24h treatment with RSV and decreased p53 Ser-15 phosphorylation (Figure 41C). To

confirm the specificity of the phospho-specific antibody, SAOS-2 cells (p53 -/-) were transiently transfected with wt p53 or the p53 mutant S15A, where Ser-15 has been replaced by an alanine. An antibody against “total p53” detected both the mutant and wt forms of the protein, while the phospho-specific antibody only recognized p53 wt (Figure 45). We also found that caffeine can markedly reduce p53 activation in the presence of RSV or DOX, but fail to do so in the presence of the MDM2 inhibitor, nutlin-3a (NUT) (Figure 41D). The combined data indicate that RSV-induced DNA damage leads to the activation of ATM and that this kinase participate to stabilization of p53.

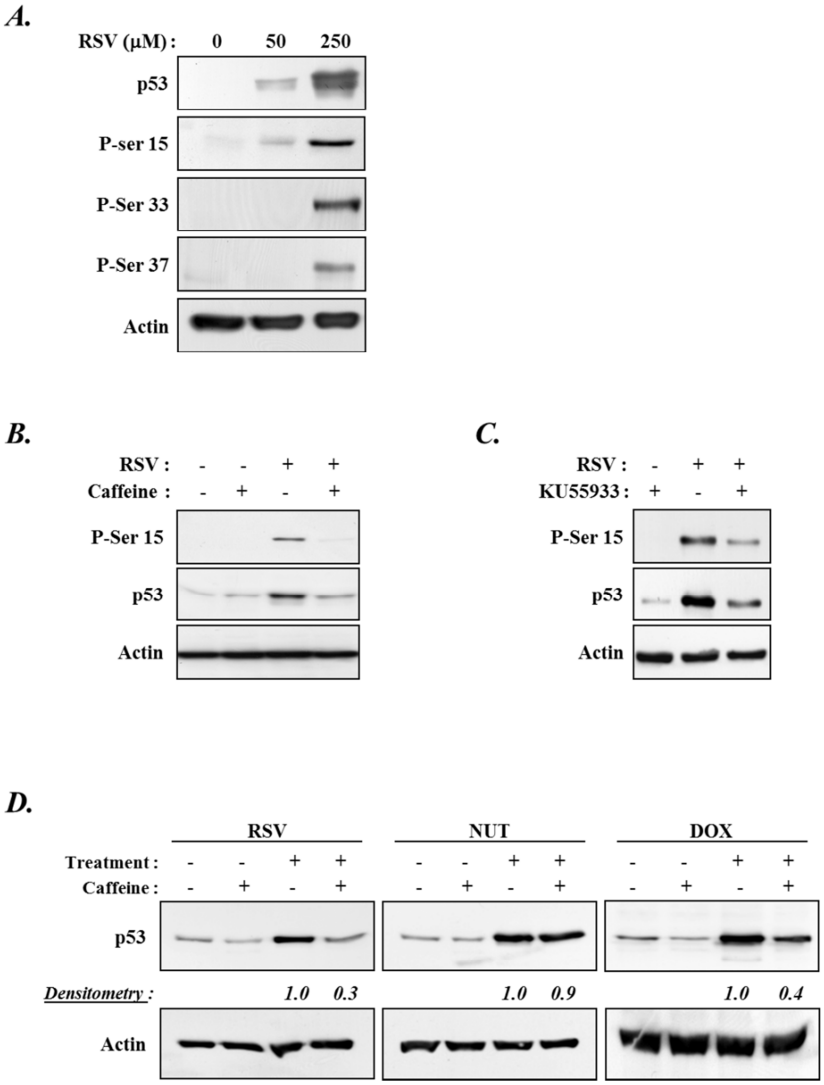

Figure 41: The ATM kinase phosphorylates p53 on Ser-15 in response to RSV treatment. (A) Western blot analysis of different N-terminal phosphorylations of p53 after a 24h treatment of HCT-116 cells with increasing concentrations of RSV. Membranes were probed with primary antibodies specifically recognizing the p53 forms phosphorylated on serines 15, 33 and 37. Actin was used as loading control. (B) Caffeine inhibits p53 stabilization and its phosphorylation on Ser-15. HCT-116 cells were treated or not for 24h with 250 µM RSV. (C) The ATM inhibitor KU55933 decreases p53 induction and Ser-15 phosphorylation in HCT-116 cells exposed or not for 24h to 250 µM RSV. (D) Effect of caffeine on p53 stabilization following an apoptotic stimulus. HCT-116 cells were treated for 24h with 250 µM RSV, 60 µM NUT or 750 nM DOX, in the presence or not of 4 mM caffeine. The effect of caffeine was quantified by a densitometry analysis of the p53 bands and normalization to the loading control (Actin). The value of 1.0 was attributed to the levels of p53 in cells exposed to the apoptotic agent in the absence of caffeine.

p38MAPK and ERK1/2 are not involved in serine 15 phosphorphorylation of p53.

It has been described that p38MAPK and ERK1/2 are activated in the presence of RSV and may play a role in stabilization of the tumor suppressor p53 (39). HCT-116 p53 wt cells were treated with DOX, RSV or NUT in the presence or not of the well-described p38MAPK inhibitor, SB203580 (40). We found that the inhibition of p38MAPK has no effect on the intracellular concentration of the p53 protein or its phosphorylation on Ser-15 (Figure 42A, top). In order to monitor the efficiency of SB203580, we studied its effects on Ser-46 phosphorylation since this residue is also the target of p38MAPK: we previously published that SB203580 inhibits p53 phosphorylation on Ser-46, as well as apoptosis, in MCF-7 breast carcinoma cells exposed to UV light (41). We found that SB203580 was able to prevent DOX-induced Ser-46 phosphorylation of p53 (Figure 42A, bottom). Next, similar experiments were performed to test for a role of ERK1/2. HCT-116 p53 wt cells were treated for 24h with RSV, DOX or NUT in the presence or not of the MEK1 inhibitor PD98059 (42). As shown in Figure 42B, this compound does not affect the overall levels of p53 protein or its phosphorylation on Ser-15, suggesting that ERK1/2 is not involved.

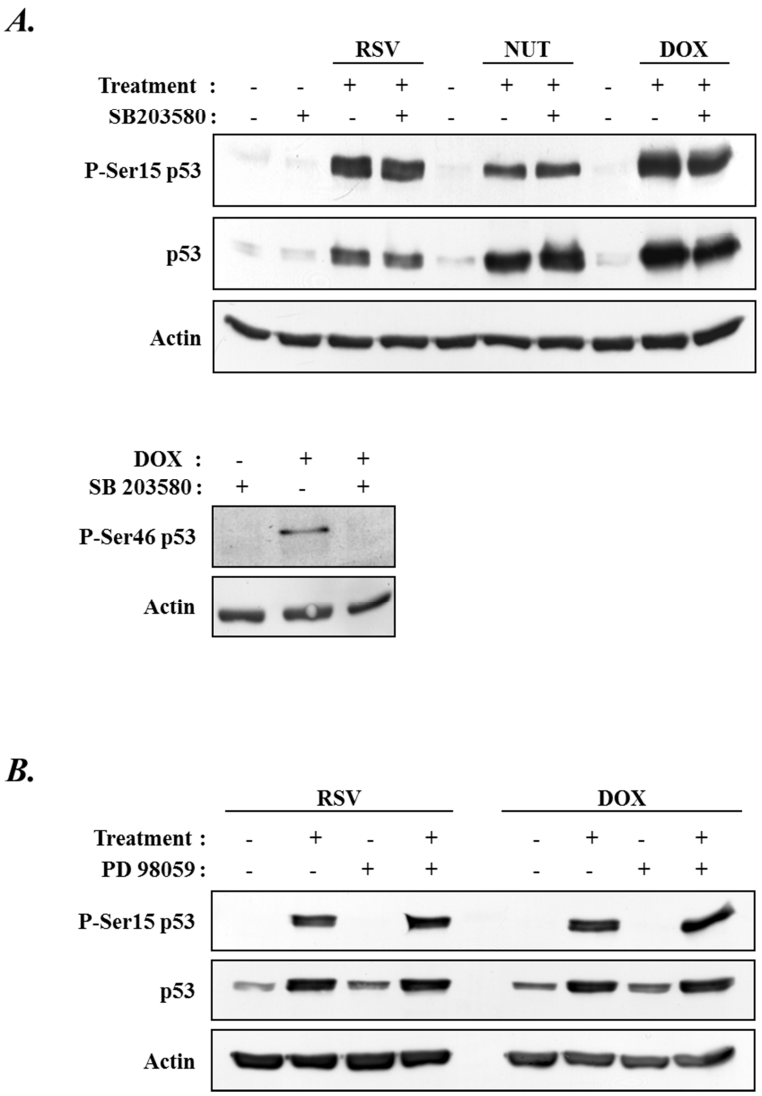

Figure 42: p38MAPK and ERK1/2 are not involved in p53 Ser-15 phosphorylation. (A) HCT-116 colon carcinoma cells were treated for 24h with 250 µM RSV, 60 µM NUT or 750 nM DOX in the presence or absence of the p38MAPK inhibitor SB203580. The molecule was added 30 min before the apoptotic agents at a final concentration of 20 µM. An analysis of the levels of p53 as well as of its phosphorylations on Ser-15 and -46 was performed by western blot. (B) HCT-116 cells were treated as in (A) in the presence or not of the MEK1 inhibitor PD98059 used at a concentration of 35 µM. We analyzed by western blot the levels of p53 and its form phosphorylated on Ser-15.

Discussion

Here we provide data that RSV is cytotoxic to colon cancer cells and that it induces apoptosis at least partly in a p53-dependent manner. Specifically, we find that p53 is stabilized and activated in the presence of RSV, as noted by the increase of its intracellular level and presence of different phosphorylations such as P-Ser15, P-Ser33 and P-Ser37. These phosphorylation events are part of an initializing cassette of N-terminal post-translational modifications that allow p53 activation in response to detrimental stimuli. Within this cassette, Ser-15 phosphorylation is believed to be one of the earliest events in response to DNA damage but also hypoxia or nutrient deprivation. Depending on the stimulus, different kinases including ATM, ATR, DNA-PK, mTOR, AMPK, RSK and CDK5 can phosphorylate the Ser15 of p53 (35-36). Since we observed extensive development of γ-H2AX foci in cells exposed to RSV, we first investigated for a role of ATM, the major kinase involved in C-terminal Ser139 phosphorylation of histone variant H2AX in response to DNA double strand breaks (43) Using two different ATM kinase inhibitors, caffeine and KU55933, we demonstrate that Ser15 phosphorylation of p53 is highly dependent on the activity of this enzyme (Figure 43).

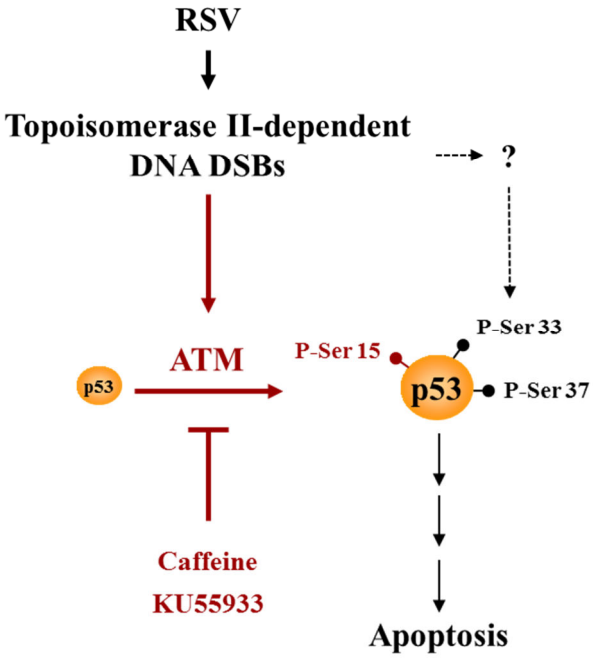

Figure 43: Model depicting the mechanism of RSV induced apoptosis. RSV acts as a genuine TOPO II poison, converting the enzyme into an intracellular toxin that generates DNA double strand breaks. This results in ATM kinase activation and phosphorylation of its substrates, including Ser139 of the histone variant H2AX and the tumor suppressor p53. In response to RSV, p53 is phosphorylated on Ser15 by the ATM kinase and is stabilized. Its intracellular levels increases markedly and this is blocked by ATM kinase inhibitors such as caffeine and KU55933. Other post-translational modifications can also be detected on p53, including Ser33 and Ser37 phosphorylation. Ultimately, RSV triggers apoptosis in a p53-dependent manner.

Extensive DNA damage in presence of RSV as well as p53 and ATM activation led us to analyze whether the polyphenol could act as a DNA intercalating agent or modulate the activity of type I and II TOPO. The experiments described here allow concluding that RSV does not show the features of a DNA intercalator. Moreover, concentrations of RSV as high as 500 µM do not affect type I TOPO. Interestingly, in vitro kDNA decatenation assays revealed inhibition of type II TOPO. In correlation, band depletion assays performed in

colon cancer cells indicate that the enzyme is depleted from the soluble nuclear fraction in presence of RSV. Thus our data suggest that, similary to etoposide, RSV behaves as a TOPO II poison, stabilizing a covalent DNA-protein intermediate that is transiently formed during the catalytic cycle of the enzyme. Resolution of this intermediate ultimately generates DNA double strand breaks, a stimulus sufficient to trigger ATM activation and p53-dependent apoptosis (Figure 43). Our study is not the first to report that stilbenes target TOPO II. It has been described since several years that polyphenolic fractions from grape cell cultures exhibit inhibition of TOPO II catalytic activity (46). Among this fraction, RSV was identified (47). Similar properties have also been reported for nepalensinol (resveratrol oligomers) (48). More recently, similar activity of RSV towards TOPO II was documented by Leone *et al*. in glioblastoma cells (49). In addition, when RSV is combined to doxorubicin, a reduction of the TOPO II poisoning effects of doxorubicin is observed, suggesting a competition between the two molecules (50). TOPO II poisoning is a common and well known mechanism of action among drugs used in cancer chemotherapy. Further studies are now needed to precisely quantify the effect of RSV by comparison to a panel of well described TOPO II poisons. Our data already suggest that RSV is less efficient than etoposide: depending on the assay employed, we found that a 2- to 4-fold higher RSV concentration was required to match the effect of etoposide.

In this study, formation of DNA damage or cell death induced by the dietary polyphenol was not affected, even marginally, by co-incubation with the general antioxidant NAC. This strongly suggests that RSV does not cause an increase, even at high doses, of intracellular ROS such as H_2O_2. Our results are in apparent contradiction with previous work. For instance, RSV was recently shown to trigger the senescence of non-small cell lung cancer (NSCLC) cell lines. This was associated with presence of DNA damage and ROS production. Moreover, DNA damage (formation γ-H2AX foci) and entry into premature senescence (SA-β-gal activity) induced by RSV were both significantly decreased in the presence of NAC (44). An earlier study, in 2007, described that chronic exposure of HCT-116 cells to low doses of RSV leads to development of a senescent phenotype. Again, this was associated with an elevation of the intracellular level of ROS, including hydrogen peroxide and superoxide anion and inhibited by NAC (32) Thus, depending on different parameters such as the cell line employed, expression of identified or yet unknown intracellular targets and likely the dose applied, RSV could trigger different responses in the target cells. One response is cell death by apoptosis and appears to be independent of ROS generation whereas a second involves entry into premature senescence and is tightly linked to ROS production. Indeed, exposure of cancer or normal cells, such as human fibroblasts, to sublethal doses of oxidants are known to cause a senescent phenotype characterized among others by an irreversible cessation of cell division and presence of the SA-β-gal activity (45).

To conclude, it is reasonable to believe that usage of RSV as a cancer chemotherapeutic drug is conditioned to the development of analogs with an improved bioavailability and/or TOPO II poisoning activity, a strategy that we are currently exploring. In addition, cancers from the gastrointestinal tract and more specifically colorectal cancer, an important cause of death in developed countries, could be a good target for RSV. Recently, Patel *et al*. (2010) measured the RSV concentrations in tumor tissues of patients suffering from colon cancer and given 1g of the polyphenol daily for 8 days (51). They found mean concentrations of 2.07 and 94.1 nmol/g (or 94.1 µM assuming that 1g of tissue represents 1 ml) in respectively left (descending colon) and right (ascending colon) sided tumors (51). Thus, it can reasonably be hypothesized that high doses of RSV or derivatives given orally could locally achieve concentrations high enough to trigger inhibition of tumor cell proliferation or apoptosis induction.

Materials and Methods

Cell culture and treatments. We used the p53 wt HCT-116 human colorectal carcinoma cell line (ATCC CCL-247) and its knockout derivative (p53 -/-) described by Bunz *et al.* (1998) (24), as well as the p53 null Saos-2 human osteosarcoma cell line (ATCC HTB-85). All cells, routinely tested for mycoplasma contamination, were grown in DMEM (Gibco 42430-082) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Gibco 15140-163). Resveratrol (RSV) (Sigma-Aldrich R5010), nutlin-3a (NUT) (Sigma-Aldrich N6287) and doxorubicin (DOX) (Sigma-Aldrich 038K1349) were used as indicated in the figures. The following protein kinase inhibitors were employed: caffeine (4 mM) (Sigma-Aldrich C0750), KU55933 (10 μM) (Tocris Bioscience), SB203580 (20 μM) (Sigma-Aldrich S8307) and PD98059 (35 μM) (Cell Signaling CST9900). The inhibitors were applied 30-60 min before treatments of cells with the apoptosis inducers. The anti-oxidant N-acetyl cysteine (NAC) was used at a concentration of 5 mM (Sigma-Aldrich A9165).

***Western blot*.** Proteins were extracted with ice-cold radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 0.1% Triton X-100) supplemented with protease (Complete Protease Inhibitor Cocktail, Roche Diagnostics 11836170001) and phosphatase (Phosphatase Inhibitor Cocktail 2 & 3, Sigma Aldrich P5726 & P0044) inhibitors. Protein concentration of the lysates was determined using the Dc

Protein Assay Kit (Bio-Rad Laboratories). For each sample, equal amounts of proteins were resolved on polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked for 30 min with PBS containing 0.2% Tween-20 (PBS-T) and 5% non fat dry milk or alternatively 3% BSA. After blocking, membranes were incubated with the primary antibodies either 1h at room temperature or overnight at 4°C. The following primary antibodies were used: mouse anti-actin antibody (ab) (dilution of 1:5,000; Sigma-Aldrich A1978), mouse anti-p85-PARP ab (dilution of 1:2,000; Cell Signaling 9546), mouse anti-p53 ab (dilution of 1:1,000; Millipore OP43), rabbit anti-pSer9 p53 ab (dilution of 1:1,000; Cell Signaling 9288); rabbit anti-pSer15 p53 ab (dilution of 1:1,000; Cell Signaling 9284); rabbit anti-pSer20 p53 ab (dilution of 1:1,000; Cell Signaling 9287); rabbit anti-pSer33 p53 ab (dilution of 1:1,000; Cell Signaling 2526); rabbit anti-pSer37 p53 ab (dilution of 1:1,000; Cell Signaling 9289); rabbit anti-pSer46 p53 ab (dilution of 1:1,000; Cell Signaling 2521) and rabbit anti-pThr18 p53 ab (dilution of 1:1,000; Cell Signaling 2529). After 3 washes with PBS-T, membranes were incubated with the appropriate peroxydase-conjugated secondary antibodies at a 1:2,500 dilution: goat anti-mouse ab (Dako P0447); goat anti-rabbit ab (Dako P0448). Immunoreactive bands were detected using the Western Lightning Kit (Perkin Elmer). Densitometry analysis of the bands was performed with Image J 1.47v software (NIH, USA).

***MTT assay*.** Cells seeded in 96-well plates (5,000 cells / well) were treated for 72h as described in the figures, each experimental condition being performed in sextuplicates (6 wells). After treatment, cells were incubated for 3h in culture medium supplemented with 0.5 mg/ml 3-(4,5-dimethylthiazolyl-2)-2,5 diphenyltetrazolium bromide (MTT) (Sigma-Aldrich M2128). Then, plates were centrifuged at 1,000g and the supernatant was discarded. We added 200µl DMSO (Sigma 154938) to dissolve the formazan precipitate. Global cell growth was assessed by measuring the absorbance at 560 nm and subtracting for each well that at 670 nm.

***Immunofluorescence*.** Cells growing on glass coverslips in 12-well plates (20,000 cells/well) were treated as described in the figures. After treatment, cells were washed with PBS, fixed for 20 min with PBS supplemented with 4% formaldehyde and permeabilized for 15 min with PBS containing 0.2% Triton X-100. After blocking at 37°C for 10 min with PBS supplemented with 0.05% Tween 20 and 3% BSA, cells were incubated overnight at 4°C with a primary antibody directed against γ-H2AX (dilution of 1:400; Cell Signaling 2577). After washing, cells were incubated with a goat anti-rabbit ALEXA Fluor488-conjugated secondary antibody (dilution of 1:400; Invitrogen A11034). After three additional washes, cells were mounted in Vectashield (Vector laboratories INCH1000) / DAPI (Roche 10236276001) (9:1 v/v).

Flow cytometry. Cell viability was determined by co-labeling with LDS751 and propidium iodide, using the Guava Viacount reagent (Millipore, USA) according to the instructions of the manufacturer.

After 5 min of incubation, cells were analysed on a Guava easyCyte 6HT-2L flow cytometer (Millipore, USA).

***DNA unwinding assay*.** Circular pRC/CMV plasmid (0.8 µg) (Invitrogen) purified from E. coli and predominantly supercoiled was first relaxed with 2U of topoisomerase I (TOPO I) during a 20 min reaction at 37°C, in 30µl of 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 0.5 mM dithiothreitol, 50 µM EDTA and 30 µg/ml BSA. Molecules were then added at concentrations of 50, 100 and 200 µM for RSV or 5, 10 and 20 µM for DOX. After 1h of incubation at 37°C, the reactions were stopped by addition of SDS to a final concentration of 0.2% (wt/v) and digestion of TOPO I with proteinase K (50 µg/ml, 30 min at 37°C). The plasmid was extracted using phenol/chloroform (1:1 v/v) and a 1% agarose gel electrophoresis was performed overnight at 30 mV. Gels were stained the next day with ethidium bromide to visualize distribution of the pRC/CMV topoisomers.

***Topoisomerase II kDNA decatenation assay*.** Effect of RSV on topoisomerase II (TOPO II) catalytic activity was assessed using kinetoplast DNA (kDNA) from C. fasciculata as substrate**.** Briefly, 250 ng of kDNA (Vaxron Corp., USA) were incubated with 1U of TOPO II in the presence or not of RSV and etoposide in 10 µL of 0.5 M Tris-HCl (pH 8.0), 1.0 M NaCl, 10 mM dithiothreitol, 0.1 M $MgCl_2$, 20 mM ATP and 200 µg/ml BSA. After 15 min at 37°C, reactions were stopped by addition of SDS. TOPO II was then digested with proteinase K and the entire reaction was resolved on a 1% agarose gel.

Topoisomerase II band depletion assay. Cells were treated for 24h with 0, 50, 100 or 200 µM RSV. Etoposide at 10 and 50 µM was used as positive control for TOPO II poisoning. After treatment, cells were collected, centrifuged and washed with NB buffer (2 mM KH2PO4, 5 mM MgCl2, 150 mM NaCl, 1 mM EGTA, 0.2 mM dithiothreitol, pH 6.5). The cell pellet was resuspended in 5 ml NB buffer supplemented with 0.35% Triton X-100, incubated for 5 min on ice and then centrifuged at 1,000g for 10 min. The obtained nuclear pellet was washed once and proteins were extracted with NB buffer containing 0.35 M NaCl (4°C, 30 min). Chromatin and non soluble material were pelleted during a 10 min centrifugation at 17,000g. The supernatant (soluble nuclear fraction) was then collected and stored. For each sample, 50 µg of proteins were resolved on a 6% polyacrylamide gel and transferred to nitrocellulose membranes. A western blot analysis with an antibody against TOPO II allowed to detect the presence of the enzyme in the different samples.

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Conflict of interest

The authors report no financial or other conflict of interest.

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Supplemental data.

	PA1	*PA1-E6*
Resveratrol	***21.0 ± 8.0 µM***	***54.0 ± 24.0 µM***
Nutlin-3a	***6.1 ± 4.6 µM***	***24.0 ± 5.1 µM***
Doxorubicin	***60 ± 40 nM***	***231 ± 10 nM***

Figure 44 : Inhibition of global cell growth by RSV is p53-dependent. To confirm in a second model that the activity of RSV relies at least partly on the tumor suppressor p53, we used the PA-1 ovarian teratocarcinoma cell line (p53wt) and its derivative overexpressing the HPV E6 protein. The E6 protein binds and recruits E6-AP (E6-Associated Protein) that has an ubiquitin ligase activity. This results in ubiquitination of p53 and its targeting for proteasomal degradation. Thus, these cells fail to stabilize p53 in the presence of an apoptotic stimulus. The two cell lines were exposed during 72h to increasing concentrations of three different apoptotic agents: DOX, NUT and RSV. After treatment, MTT assays were performed as described in the material and methods. The table shows the calculated IC50s concentration that inhibits global cell growth by 50%) for each drug in both cell lines. Data indicate that the cell line harboring an active p53 is more sensitive to the three apoptotic stimuli.

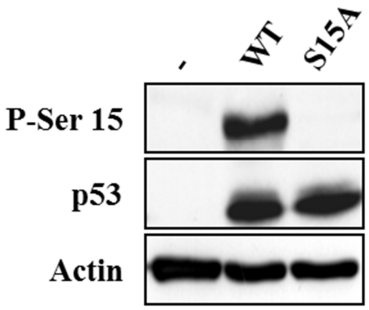

Figure 45 : Specificity of the antibody directed against the Ser-15 phosphorylated form of p53. SAOS-2 cells (p53 -/-) were transiently transfected with pRc/CMV p53 wt, the p53 S15A mutant as well as the empty pRC/CMV (-). An antibody against "total p53" detected both the mutant and wt forms of the protein, while the phospho-specific antibody only recognizes p53 wt.

GENERAL DISCUSSION

AND PERSPECTIVES

1. MAGEA

1.1. Main findings of the study

By the end of this work, we identified a new mechanism of p53 inhibition by MAGE-A proteins. We demonstrate that the presence of MAGEA1 and -A2, but not -A3, -A4, -A6 and -A12, is correlated with a reduced sensitivity of MCF-7 cells to DNA damage. Moreover, we show that this reduced sensitivity is, at least in part, due to lower apoptosis induction.

Cells expressing MAGE-A1 or -A2 display lower intracellular levels of the tumor suppressor p53 after exposure to the genotoxic agent doxorubicin. By looking for potential causes, we identified that major stabilizing post translational modifications such as phosphorylation of Ser15, Thr18 and Ser20 were impaired. Moreover, phosphorylation of Ser46, important for apoptosis induction, was also reduced in cells expressing MAGEA1 and -A2. Our data (immunoprecipitation-western blot analysis) also indicate that stress-activated p53 interacts with MAGEA1 and -A2. GST pull down experiments show that both the DNA binding domain and the N-terminal region of p53 interact with MAGEA proteins. To our knowledge, it is the first time that MAGEA expression is associated to an impaired phosphorylation of the p53 N-terminal region and correlated to reduced p53 levels in presence of DNA damage.

1.2. Integration to the current knowledge

In 2012, Ladelfa *et al*. proposed a unified model, combining preexisting models, to described the complex relations between MAGEA and p53 (Figure 46). In absence of stress, two mechanisms co-exist. First, the E3 ubiquitin ligase TRIM28 sees its action enhanced by MAGEA2. This leads to an enhanced degradation of p53 either by a better recruitment of the E2 enzyme on the E3 ubiquitin ligase or by an optimized transfer of ubiquitin to the substrate [Doyle *et al*.]. On the other hand, in absence of stress, MAGEA proteins directly bind the DNA binding domain of p53 impairing its access to DNA [Marcar *et al*., 2010].

In stress condition, MAGEA proteins action switches to the recruitment of HDAC3 to p53-dependent promoters [Monte *et al*.]. In the same time, MAGEA causes impaired p53 acetylation by inhibition of PML-NBs formation [Peche *et al*.].

Our results give a new dimension to this unified model. During a genotoxic stress, cells display reduced p53 levels when MAGEA1 or-A2 are present. This is concomitant to a decreased phosphorylation of Ser15, Thr18 and Ser20. Absence of these phosphorylations compromises stabilization of the tumor suppressor. Moreover, phosphorylation of Ser46 is also impaired, partially explaining the reduced apoptosis. Our results are not in opposition to this unified model but rather complementary.

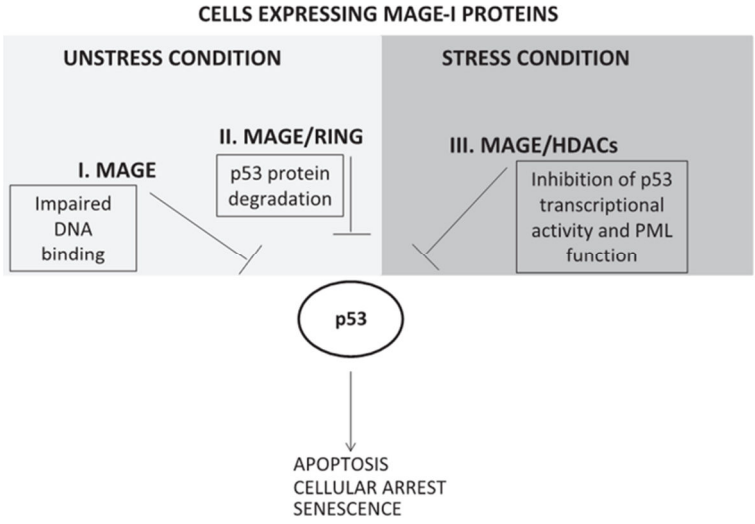

Figure 46: Ladelfa *et al*. model for MAGEA inhibition of p53 function: (I and II) In transformed unstressed cells expressing MAGEA, a low activity of p53 may be the results of the combined mechanisms of Marcar *et al.* and Doyle *et al.* causing a low concentration of p53 in the cell and an impairment of p53 binding to DNA. (III) In stress condition, such as chemotherapy, p53 levels and activity is modulated by the recruitment to p53 target promoters of HDACs by MAGEA proteins. This reduces histones acetylation and also probably p53 acetylation. Inhibiton of PML function by MAGEA could also participate to the inhibition of p53 acetylation.

Therefore, our mechanism may be integrated to the current knowledge on the relations between MAGEA and p53. In Figure 47, the p53-MAGEA network is extended with the new mechanism.

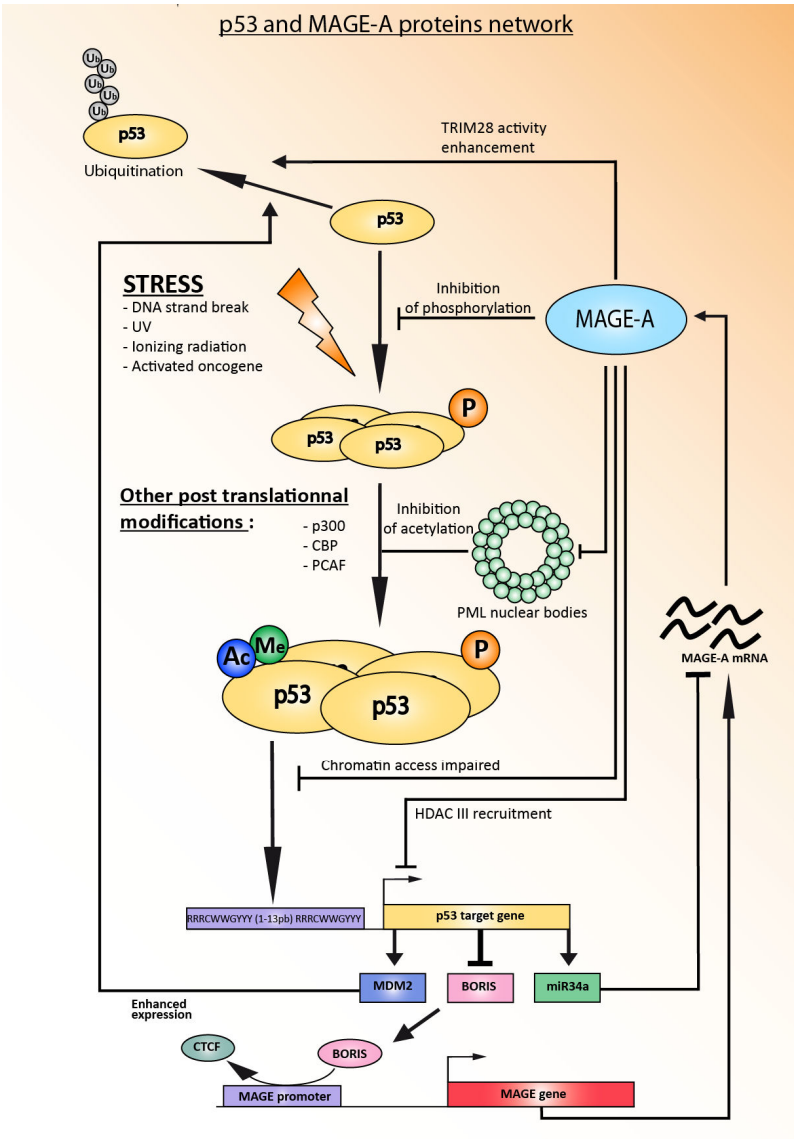

Figure 47 : Updated version of MAGEA activities. MAGEA are implicated into p53 destabilization and inhibition of transcription. MAGEA2 expression leads to an enhanced ubiquitination of p53 (Doylet *et al.*) and to the inhibition of PML-NBs leading to decreased p53 acetylation (Peche *et al.*), to the recruitment of HDAC on p53 promoters or to the inhibition of p53 binding to DNA (HDACI, Laduron *et al.*; HDACIII, Monte *et al.*). On the other hand, p53 also modifies MAGEA activity: p53 induces miR34a expression that directly targets MAGEA mRNA. Moreover, p53 also inhibits the expression of BORIS, responsible for the demethylation of MAGEA promoter. Here, we add a layer of complexity to this network by showing that phosphorylations important for p53 stabilization (Ser15, Thr18 and Ser20) and apoptosis induction (Ser46) are impaired in presence of MAGE-A proteins.

1.3. Discussion

1.3.1 MAGEA family and resistance of cancer cells to genotoxic stress

In 2006, Monte *et al*. showed the protective effect of MAGEA by comparing cell lines expressing various MAGEA genes (13923M, 879M and 20706M) with cell lines that do not express MAGEA genes (15392M,18732M and3962M). They treated cells with increasing concentrations of etoposide and show that 13923M, 879M and 20706M cell lines were more resistant. However, in their experiments, the MAGEA genes responsible for the reduced sensitivity to etoposide were not identified [Monte *et al*., 2006]. Therefore, we created clones expressing separately the different MAGEA commonly expressed in cancer to assess their effects on cell resistance to genotoxic stresses. MTT assays determined that only MAGEA1 and -A2 diminish the sensitivity of MCF-7 breast cancer cells to doxorubicin while MAGEA3, -A4, -A6 and -A12 do not. Let's continue our analysis for each of them.

Concerning MAGEA1 overexpression, our results clearly show an increased resistance of MCF-7 cells to doxorubicin as well as camptothecin. Other studies linked the expression of MAGEA1 to an enhanced resistance to paclitaxel and docetaxel [Suzuki *et al.,* 2007]. On the other hand, in 2007, Yang *et al*. specifically knocked down MAGEA1 in the Hs-294T cell line without seeing a clear drop in cell

survival [Yang *et al*., 2007] suggesting that MAGEA1 is not required to ensure cancer cell viability in the absence of a stress. Our results show for the first time the importance of MAGEA1 expression to maintain viability in presence of a genotoxic stress.

MAGEA2 also shows protective effects in our model. Several studies described the effect of MAGEA2 on cellular growth and resistance to genotoxic drugs [Yang *et al*. 2007; Duan *et al*., 2003, Nardiello *et al*., 2011]. For example, Duan *et al.* transfected cells originally sensitive to doxorubicin with the MAGEA2 cDNA and observed a clear increase in the resistance to the drug thereafter. Our results are concordant and further emphasize the importance of this member of the family.

MAGEA3, -A6 and -A12 are phylogenetically close since they share 94% (A3 vs A6) and 84% (A3/A6 vs A12) of homology for the MHD [Doyle *et al*., 2010]. In the literature, Yang *et al*. mentioned a stop of cell growth when MAGEA3 and -A6 expression were knocked down and Nardiello *et al*. correlated the expression of MAGEA3 with disease progression although MAGEA3 silencing did not affect the cell cycle [Nardiello *et al*., 2011]. Our data demonstrate that MAGEA3, -A6 and -A12 are not able to reduce the sensitivity of breast cancer cells to doxorubicin.

Finally, in the literature, MAGEA4 is clearly different from the other family members since two different teams described that MAGEA4, and more precisely a cleaved formed of MAGEA4, is able to induce apoptosis instead of preventing it [Sakurai *et al.,* 2004; Peikert *et al*., 2006]. Our results are in opposition since no drop in cell

survival has been observed when we overexpressed MAGEA4 in MCF-7 breast cancer cells. On the other hand, Peche *et al*., demonstrated that MAGE-A2 was able to reduce p53 acetylation while MAGE-A4 has no effects on the tumor suppressor [Peche *et al*., 2012]. This lack of activity corroborates our results. In addition, it is interesting to note that MCF-7 cells lack caspase-3 [Wesierska-Gadek *et al*., 2011]. It is possible that the absence of a pro-apoptotic effect in our model is due to a failure to cleave MAGEA4 since it seems to be caspase-dependent [Sakurai *et al.,* 2004]. However, MCF-7 cells do express caspase-7 which shares almost the same substrates and inhibitors specificity than caspase-3 [Fuentes-Prior and Salvesen, 2004].

In summary, our work analyzed for the first time, the individual impact of each MAGEA, commonly expressed in cancer, on cell resistance to a genotoxic stress. We thus, provide additional information on the MAGEA family by pointing out that only MAGEA1 and -A2 are able to increase resistance to doxorubicin in the MCF-7 cell line. Differences in activity towards p53 between MAGEA family members, despite the close homology, raise questions. By analyzing the amino acids sequences, no particular features could be identified to explain the specific activity of MAGEA1 and -A2 on cell resistance to a genotoxic agent. As proposed by Ladelfa *et al*., it seems obvious that the cellular context may play a critical role by determining the MAGEA interactions with other proteins [Ladelfa *et al*. 2012]. Moreover, it has been recently shown that MAGEA proteins could be post-transcriptionally modified. Bai *et al.* demonstrated that MAGEA11 may be phosphorylated on residues like Thr360 by CHK1

[Bai and Wilson, 2008]. Thus, it is plausible that post-translationnal modifications may also modulate the interactions of MAGEA proteins and their activity.

1.3.2 Connection of MAGEA to the p53 pathway

After treatment with camptothecin, we observed a reduced apoptosis in cells expressing MAGEA1 or -A2 as compared to control cells. Altogether, our data suggest that MAGEA1 and -A2 have an inhibitory effect on the apoptotic process induced by genotoxic agents. We next examined the relationship between MAGEA and the p53 pathway. When cells overexpressing MAGEA1 or -A2 were treated with nutlin-3a, we observed a reduction of cell growth inhibition, suggesting a connection between some MAGEA family members and the p53 pathway.

Since a conflict exists in the literature regarding the direct binding of p53 to MAGEA proteins, we examined potential interactions by immunoprecipitation and GST-pull down assays. Our data are concordant with these of Monte *et al.* and Marcar *et al.* When p53 is immunoprecipitated with the FL-393 polyclonal antibody, MAGEA1 and -A2 are co-immunoprecipitated. Moreover, data from GST-pull down assays show a direct binding of MAGEA1 and -A2 to the DNA binding domain of p53. This is in opposition with the studies of Yang *et al.* and Doyle *et al*. since both studies do not evidence a direct binding. The reason for this difference remains

unknown since we also used GST-pull down assays with *in vitro* translated tagged-MAGEA proteins.

1.3.3 MAGE-A and p53 activation following a genotoxic stress

We next analyzed p53 levels in MCF-7 clones expressing or not MAGEA1 or -A2, following a treatment with doxorubicin. Our data show that when MAGEA1 and -A2 are expressed, the cellular levels of p53 is reduced in stressed cells.

Two hypotheses were proposed: either p53 is more degraded or its stabilization following a stress is impaired. In the literature, the classical model for p53 activation asserts that p53 requires post-translational modifications in order to be stabilized [Riley *et al*., 2008; Lavin and Gueven, 2006]. Therefore, we sought to analyze different N-terminal phosphorylations of p53 known to impact on its stability. Data identified a reduction of the phosphorylation of p53 Ser15 in presence of MAGEA. Since phosphorylation of Ser15 favors the subsequent phosphorylation of Thr18 and Ser20, we next analyzed these two post-translational modifications. We found that both Thr18 and Ser20 phosphorylations were decreased in cell expressing MAGEA as compared to control cells transfected with the empty vector. Phosphorylation of Ser15, Thr18 and Ser20 are reported to be crucial to inhibit MDM2/MDMX binding to p53. Ser15 seems to be more important for the subsequent phosphorylation of Thr18 and Ser20 rather than for p53 stabilization itself [Dumaz and Meek, 1999; Saito *et al*. 2003; Lavin and Gueven, 2006]. Phosphorylation of Thr18

and Ser20, on the other hand, is essential to prevent MDM2 binding. Lee *et al.* demonstrated that Thr18 phosphorylation impairs MDM2 binding to p53 by more than 20 fold while the double phosphorylation of Ser15 and Thr18 inhibits it even more.

Our results are original since Monte *et al.* did not observe differences in total p53 or its Ser15 phosphorylated form. Doyle *et al.* for their part, detected a reduced p53 level in presence of MAGEA but measures were done in unstressed cells expressing mutant p53. According to Doyle *et al*., in unstressed cells expressing MAGEA, p53 levels is very low due to its enhanced degradation by KAP1 [Doyle *et al*., 2010]. Therefore, in presence of a stress such as oncogene activation, cells will be unable to activate p53. We demonstrate by western blot that wt p53 is detectable and activated after a stress in presence of MAGEA proteins. Therefore MAGEA expression is not sufficient to totally prevent p53 induction following a stress. We propose that the impaired phosphorylation of p53 in presence of MAGEA leads to an unstable p53 protein prone to be degraded. This leads to reduced intracellular p53 levels as observed in stressed cells.

Phosphorylation of Ser46, on the other hand, is important for apoptosis induction [Oda *et al*., 2000]. We shown that this phosphorylation is also decreased in presence of MAGEA1 or -A2. This gives a new insight into the mechanism by which MAGEA proteins decrease p53-dependent apoptosis. Ser46 has been shown to be phosphorylated by DIRK2 (following DNA damage [Ollson *et al.,* 2007]) but also by HIPK2 (following UV light exposure [Ollson *et al*., 2007]) and p38MAPK (following UV light exposure [Li *et al*. 2005])

and is considered as a switch increasing p53 affinity for the promoter of its apoptotic targets such as the genes coding for NOXA or PUMA.

1.3.4 Perspectives

The mechanism responsible for the impaired phosphorylation of Ser15, thr18, Ser20 and Ser46 remains to be elucidated. Hypotheses are numerous. Interestingly, Peche *et al.* suggested a mechanism in which MAGEA proteins inhibit PML-NBs formation [Peche *et al*., 2012]. PML-NBs are platforms thought to play a role in p53 post-translational modification by recruiting in a same place modifiers and the tumor suppressor. Proteins recruited on PML-NBs are numerous and HIPK2 phosphorylation of p53 has been shown to depend on PML-NBs formation [Moller *et al*., 2003]. Therefore impairment of PML-NBs formation could be a hypothesis to explain the impaired p53 Ser46 phosphorylation. To our knowledge, no evidence of the presence of DYRK2 or p38MAPK on PML-NBs has been published. Could ATM (ser15), CK2 (Thr18), CHK1 (Ser20), CHK2 (Ser20)… be recruited on such platforms? As far as we know, there is no evidence in the literature of a presence of these kinases on PML-NBs. Moreover, it is ignored whether PML-NBs are required or not to ensure the phosphorylation of p53 by ATM or other kinases. This requirement has only been shown for HIPK2. To our opinion, this hypothesis deserves a priority in further investigations.

Another hypothesis is that MAGEA proteins could target kinases involved in the post-translational modification of p53. For example,

in presence of DNA damage, Ser15 phosphorylation is performed by DNA-PK or ATM [Ollson *et al*., 2007]. It would be interesting to determine if MAGEA proteins could directly interact with them and inhibit their action. Alternatively, MAGEA could also inhibit upstream signals necessary for ATM or DNA-PK activation.

In addition, MAGEA proteins interacting with p53 may inhibit the recruitment of protein kinases by steric hindrance. Therefore, MAGEA proteins could be directly responsible for the impaired N-terminal phosphorylations of p53. Finally, the activation by MAGEA of specific phosphatases, like PP1 that would dephosphorylate p53 on Ser15 [Haneda *et al*., 2004]) could also result in a decreased p53 stabilization.

These different hypotheses also require further investigations in order to identify the mechanism(s) responsible for the impaired N-Terminal phosphorylations of p53 in presence of MAGEA. This could bring useful informations on how to fight cancers expressing MAGEA1 and -A2.

1.4. CTAs: past, present and future.

Cancer testis antigens were first analyzed for their potential use in cancer immunotherapy. Targeting antigens that are only expressed by cancer cells may represent the best anti-cancer therapy so far. The enthusiasm was so strong that it was published in Nature Medecine in 2011 that “wiping out cells expressing CT antigens should theorically cause no side effects, no-off target effects on normal tissues, none at all.” [Scudellari, 2011]. However, clinical trials based on strategies targeting well characterized CT antigens have shown limited success [Suri *et al*., 2012]. A good example is the recent failure of the vaccine targeting MAGEA3 (GSK1572932A), designed to treat non-small cell lung cancer. By compiling evidences, it seems that the expression of MAGEA1 but also -A2 and -A3 is responsible for resistance to TNF-α cytoxicity, one of the main death pathways induced by NK and LTc to elicit cell death [Parc *et al*., 2002].

However, the use of CTAs as therapy is not withdrawed. A technology, named CAR-T Cell, recently reaches clinical trials. CAR-T cell, for Chimeric Antigen Receptor T cells, is based on the use of genetically engineered T-Cell lymphocytes that express a single-chain variable fragment (scFv) (derived from monoclonal antibodies) fused to CD3-zeta transmembrane- and endo-domain. More precisely, the external part of this receptor is composed by the variable part of the heavy and light chains of the antibody. Both parts are fused by a linker to form a scFv. This construction keeps the specificity toward

the antigen and LTc expressing such construction will be sensible to the antigen normally targeted by the antibody. Following binding, the CD3-zeta chain will trigger a signal activating LTc toxicity. For example, using a construction based on antibodies recognizing MAGEA1, Willemsen *et al*. demonstrated a drastic increase of the cytolytic capacity of cells expressing their scFv [Willemsen *et al*. 2005]. Today, clinical trials evaluating the potential of this technology are currently ongoing.

For the future, CTAs analysis will be compulsory. It becomes more and more obvious that multiple cancer testis antigens and in particular several MAGEA are implicated in cellular pathways that reinforce cell resistance to death signals or increase cell growth. For example, in 2014, a team also demonstrated that in presence of growth factors, CT45A1 overexpression (Cancer Testis Antigen-45 family member) activated ERK and CREB signaling, promoted epithelial to mesenchymal transition and increased stem cell characteristics [Shang *et al*., 2014]. Moreover, recent publications linked MAGEA expression to EMT and the generation of cancer stem cells [Yang *et al.,* 2015]. Consequently, in addition to an increased resistance to classic chemotherapies, CTAs favor cancer cell dedifferentiation, migration and invasion of other tissues. In the light of these diverse properties conferred, a systematic analysis of all CTAs should be undertaken. The goal would be to determine their functions and to identify the underlying mechanisms. With this knowledge, it must be possible to develop new therapies and with the rising of the personalized medicine, a screening and identification

of the cancer testis antigens expressed may help the physician in choosing the best treatment. For example, we describe here the effects of MAGEA1 and -A2 expression on the inhibition of p53-dependent apoptosis. Since MAGEA1 and -A2 increase resistance to DNA damage-based chemotherapies, the physician could in the future prefer to use alternative treatments. On the other hand, when new mechanisms of CTAs actions are identified, research could focus on finding molecules inhibiting the gain of function induced by CTAs. Such molecules would restore a less aggressive behavior to the cancer as well as sensitize it to regular chemotherapies. Ultimately, this will decrease the disease burden and hopefully lead to a better outcome for the patient.

2. Resveratrol

2.1. Main findings of the study

In this work, we shown that RSV induces p53-dependent apoptosis in colon cancer cells and identified the molecular mechanism involved. After cell treatment, we observed a chromatin condensation that was dose- and time-dependent. We further confirmed the apoptotic phenomenon by analyzing PARP cleavage, p53 intracellular levels and the morphological features of the treated cells. Moreover, by measuring the presence of γ-H2AX foci, we determined that RSV causes DNA damage. We tested whether ROS could be responsible for the observed loss of cell viability and DNA damage formation. However, we failed to observe differences in cell growth or formation of γ-H2AX foci when combining RSV treatment with the anti-oxidant NAC, suggesting that ROS are likely not responsible for DNA damage and apoptosis induction in RSV-treated HCT-116 cells.

We next wondered if RSV could act as a DNA intercalating agent or modulate the activity of type I and II TOPO. Our data revealed that RSV is neither a DNA intercalating drug nor a TOPOI inhibitor. By checking for TOPOII inhibition in presence of kinetoplast DNA, we demonstrated that RSV inhibits TOPOII activity. We further demonstrate by performing “band depletion assays” that the polyphenol presumably stabilizes the so-called “cleavable complex”,

a covalent DNA-TOPOII intermediate. The hypothesis is that RSV prevents the religation step, leading to the formation of DNA double strand breaks.

Thus, RSV acts as a TOPOII poison and does not seem to possess other activity that may lead to DNA damage such as ROS production, DNA intercalation or TOPOI inhibition. Moreover, it is interesting to notice that this mechanism of action of RSV is valid for different cellular models [Leone *et al.,* 2012]. Our study also indicates that RSV, at similar concentrations, is less efficient than the well-known TOPOII poison etoposide.

We further analyzed the molecular mechanism associated to RSV-induced apoptosis. Using two different ATM kinase inhibitors, caffeine and KU55933, we show that p53 induction in cells treated with RSV relies on the activity of the ATM kinase. Moreover, we demonstrate that p38MAPK and ERK1/2 are not involved in p53 stabilization in HCT-116 colon cancer cells.

2.2. Discussion

Benefic effects of resveratrol have been identified in several domains. For example, RSV shows activity against cardiovascular diseases [Park and Pezzutto, 2015] and inflammation [Ma *et al*., 2015]. In a narrower point of view, RSV also seems to display anti-cancer activities [Wang *et al*., 2015; Khan *et al*., 2014].

Previous observations reported an increased ROS level in HCT-116 cells treated with RSV, concomitant to cell cycle arrest and the development of a senescent phenotype [Heiss *et al*., 2007]. Using NAC, an antioxidant molecule, that was previously shown to be protective on RSV-induced entry into senescence, we failed to observe differences in cell growth or γ-H2AX foci formation when combining RSV treatment with NAC. Similarly, at 25µM of RSV, almost the concentration used by Heiss *et al*. (30µM), no differences were observable. In the opposite, NAC was highly protective against H_2O_2 treatment. To conclude, our experiments suggest that ROS are not responsible for inhibition of cell growth or DNA damage formation in RSV-treated HCT-116 cells.

RSV is neither a DNA intercalating drug nor a TOPOI inhibitor. This is an original result since, to our knowledge, this was not analyzed before. However, the activity of RSV toward TOPOII was previously reported by an Italian team [Leone *et al*., 2012]. Here we confirm those results obtained in glioblastoma cells.

Results concerning p38MAPK and ERK1/2 are in opposition with previous publications [She *et al*., 2001]. In 2001, She *et al.* used

inhibitors of ERK1/2 and p38MAPK to demonstrate the implication of these two kinases in the stabilization of p53 as well as its phosphorylation on Ser15 in cells treated with RSV. Our data suggest the opposite : these two kinases are not involved in p53 stabilization in presence of RSV. However, differences with our work could lie in the cellular model used. In their study, She *et al*. used a normal mouse skin epidermal cell line (JB6 Cl41)[18]. Therefore, variations observed using the same P38MAPK inhibitor (PD98059) could be the consequence of a particular HCT-116 cellular context. However, Olsson *et al*. described that P38MAPK and ERK1/2 phosphorylate Ser15 of p53 only following UV light exposure [Olsson *et al*., 2007]. Finally, ATM implication in the phosphorylation of Ser15 has also been identified by Heiss *et al*. Here, we confirm the role of ATM in p53 Ser15 phosphorylation and further emphasize it since kinases previously thought to mediate RSV-dependent effects are not involved.

[18] http://www.lgcstandards-atcc.org/products/all/CRL-2010.aspx?geo_country=be

2.3. Conclusion and future of resveratrol in cancer therapy

RSV has been shown to induce apoptosis in cancer cells, to reduce angiogenesis by suppressing VEGF expression [Bishayee *et al*., 2010] and has little side effects in human, even at high doses. All these characteristics make RSV a good candidate for chemotherapy. However, some important issues must be faced.

First RSV has a very poor oral bioavailability due to its rapid metabolization. New formulation could improve this. A rapidly dismissed solution came from oligomers of RSV. It consists in the polymerization of two to eight RSV units. Several studies demonstrated *in vitro* the enhanced activity of those forms compared to monomeric RSV [Xue *et al*., 2014]. The hypothesis was that the metabolism would be less active on such molecules and that these molecules would progressively release free RSV. However a recent publication determined that the intestinal absorption rate of two RSV oligomers, ε-viniferin and hopeaphenol, is low and negligible when compared to RSV itself [Willenberg *et al*., 2015]. Therefore, to overcome this problem, injection in the bloodstream may be a solution. Nevertheless, analyses of such compounds are at the early stages. Basic toxicological studies are still ongoing.

Another solution may come from a recent rapport in Science. This study describes that metabolized forms of RSV circulating in the blood can be absorbed by cells, transiently stored and converted

again into RSV. Indeed, monosulfate metabolites were shown to be re-converted into RSV in human colorectal cancer cells and toparticipate to the *in vivo* effects of RSV [Patel *et al*., 2013]. This is particularly interesting since it raises the possibility to create modified forms of RSV that may act as a Trojan horse. Conjugated forms of RSV, designed to be easily absorbed by the gut epithelium may safely travel to the cancer cells through the circulation. Ideally, the conjugated form would be cancer specific and would depend on enzymes expressed by the cancer cell for its activation. Theorically, after absorption, the cancer cell would naturally regenerate RSV and senescence or apoptosis could be induced.

Another issue lies in the fact that it is not clear whether RSV metabolites play a role in the effects attributed to RSV. To add a layer of complexity, it seems that the type of metabolites formed is related to the RSV dose. For example, at low doses, sulfation represents the main form of metabolization while at higher doses, glucuronidation prevails [Szekeres *et al*. 2011]. A clear understanding of the activity, toxicity and behaviour of each metabolite will be important for the further evaluation of RSV in clinical trials.

In conclusion, a lot of work on RSV and its metabolization remains to be done prior to a use in chemotherapy. However, our work emphasizes the pro-apoptotic and anti-cancer potential of this polyphenol and the hope it raises for the development of future therapies.

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