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Effet des facteurs sécrétés par les cellules sénescentes sur la transformation néoplastique et la sensibilisation à TRAIL

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	5-FU	5-fluorouracil
	5mC	5-methylcytosine
A	AA	Amino Acid
	AAV	Adeno-associated Virus
	ALL	Acute Lymphoblastic Leukemia
	AML	Acute Myeloid Leukemia
	APAF1	Apoptotic Protease Activating Factor 1
	APC/C	Anaphase-promoting Complex /Cyclosome
	APL	Acute Promyelocytic Leukemia
	ARF	Alternate Reading Frame
	ATM	Ataxia-telangiectasia mutated
	ATR	Ataxia-telangiectasia Rad3 related
	ATRA	all-*trans* retinoic acid
B	Bcl-2	B cell lymphoma 2
	BMDC	Bone Marrow Derived Cells
	BER	Base Excision Repair
	BET	Bromodomain and Extra Terminal
	bHLHLZ	basic Helix-Loop-Helix-Leucine Zipper
	BRCA1	Breast Cancer 1
	BrdU	Bromodeoxyuridine
C	CAD	Caspase-like Domain
	CAF	Cancer-associated Fibroblast
	CD95	Cluster of Differentiation 95

	CDK 4, 6	Cyclin-Dependent Kinase 4, 6
	CDKIs	Cyclin-Dependent Kinase Inhibitors
	CFLAR	CASP8 and FADD-like Apoptosis Regulator
	c-FLIP	cellular FLICE-inhibitory protein
	CHK 1, 2	Checkpoint Kinases 1, 2
	CIS	Cisplatin
	CLR	Crosslink Repair
	CML	Chronic Myeloid Leukemia (CML)
	CMS	Conditioned Medium of Senescent cells
	CRD	Cysteine Rich Domain
	CTD	C-Terminal Domain
	CTGF	Connective Tissue Growth Factor
D	DcR	Decoy Receptors
	DD	Death Domain
	DDR	DNA Damage Response
	DED	Death Effector Domain
	DISC	Death Inducing Signaling Complex
	DNA	Deoxyribonucleic Acid
	DNMT	DNA methytransferase
	DOX	Doxorubicin
	DR 4, 5	Death Receptor 4, 5
	DSBs	Double Strand Breaks
E	ECM	Extracellular Matrix
	EGFR	Epidermal Growth Factor Receptor
	EMT	Epithelial - Mesenchymal Transition

	ERK	Extracellular-signal-regulated Kinases
	EZH2	Enhancer of zeste homologue 2
F	FADD	Fas-associated Death Domain
	FDA	Food and Drug Administration
G	GAPs	GTP-ase Activating Proteins
	GDP	Guanosine Diphosphate
	GEFs	Guanosine nucleotide Exchange Factors
	GFP	Green Fluorescent Protein
	GSK3β	Glycogen Synthase Kinase 3 β
	GTP	Guanosine Triphosphate
H	H3K4, 27, 79	Histone 3 Lysine 4, 27, 79
	HAT	Histone Acetyl Transferase
	HDAC	Histone deacetylase
	HDM	Histone demethylase
	HFG	Hepatocyte Growth Factor
	HLH	Helix-Loop-Helix
	HMT	Histone Methyl Transferase
	HR	Homologous recombination
	hTERT	human Telomerase Reverse Transcriptase
I	IFN-β	Interferon beta
	IGFBPs	Insulin-like Growth Factor Binding Proteins
	IL 1, 6, 8	Interleukin 1, 6, 8
	iPS cells	induced Pluripotent Stem cells
	IRI	Irinotecan
J	JAK	Janus Kinase

	JNK	c-JUN N-terminal Kinase
K	kDa	kilo Dalton
L	LZ	Leucine Zipper
M	MAPK	Mitogen-activated Protein Kinase
	MAX	MYC-associated factor X
	MDS	Myelodysplastic Syndrome
	MEFs	Mouse Embryonic Fibroblasts
	MEK	MAP kinase kinase
	MLL	Mixed Lineage Leukemia
	MMPs	Matrix Metalloproteinases
	MMR	Mismatch Repair
	MMTV	Mouse mammary tumor virus
	mTOR	Mammalian Target of rapamycin
	MTX	Methotrexate
N	NER	Nucleotide Excision Repair
	NF1	Nuclear Factor 1
	NFκB	Nuclear Factor κB
	NHEJ	Non-Homologous End Joining
	NK cells	Natural Killer cells
	NM	Normal Medium
	NO	Nitric Oxide
	NTD	N-Terminal Domain
O	OHT	Hydroxytamoxifen
	OIS	Oncogene Induced Senescence
	OPG	Osteoprotegerin

P	PAI-1	Plasminogen Activator Inhibitor-1
	PARP	Poly (ADP-ribose) Polymerase
	PDGF	Platelet Derived Growth Factor
	PI3K	Phosphoinositide 3 Kinase
	PIN	Prostatic Intraepithelial Neoplasia
	PLAD	Pre-ligand-binding Assembly Domain
	PML	Promyelocytic Leukemia
	PP2A	Protein Phosphatase 2A
	pRb	protein Retinoblastoma
	PRC	Polycomb Repressive Complex
	PTEN	Phosphatase and Tensin homolog
Q	q-PCR	quantitative Polymerase Chain Reaction
R	rhTRAIL	recombinant human TRAIL
	RIP	Receptor-Interacting Kinase
	RNA	Ribonucleic Acid
	ROS	Reactive Oxygen Species
	RT	Reverse Transcriptase
S	SAHF	Senescence-associated Heterochromatic Foci
	SASP	Senescence-Associated Secretory Profile
	SA-β–Gal	Senescence Associated β-Galactosidase
	shRNA	short hairpin RNA
	SIPS	Stress-induced Premature Senescence
	siRNA	small interfering RNA
	SMAC/Diablo	Second Mitochondria-derived Activator of Caspases
	STAT	Signal Transducers and Activators of Transcription

	SV40ER	Simian Virus 40 Early Region
T	TAD	Transactivation Domain
	TF	Transcription Factor
	TGFβ	Transforming Growth Factor β
	TIS	Therapy Induced Senescence
	TNF	Tumor Necrosis Factor
	TNFRSF	Tumor Necrosis Factor Receptor Superfamily
	TPA	12-O-tetradecanoylphorbol-13-acetate
	tPA	tissue-type Plasminogen Activator
	TRAIL	TNF-Related Apoptosis Inducing Ligand
U	uPA	urokinase Plasminogen Activator
	uPAR	uPA Receptor
	UV	Ultraviolet
V	VEGF/R	Vascular Endothelial Growth Factor/Receptor
	VHL	Van Hippel Landau
W	WCE	Whole Cell Extract
Z	Zn	Zinc

INTRODUCTION: Cancer

1. General introduction and definition

Cancer is by definition a progressive multistep disorder that results in abnormal proliferation of cells that have lost the control mechanisms and acquired the abilities to invade other tissue, disseminate in the organism and form metastases. The cellular transformation usually involves accumulation of both genetic and epigenetic changes.

2. Hallmarks and open questions of cancer

The immense complexity of cancer is evident in terms of heterogeneity of its causes and the diversity of cell origin, with more than 100 types and subtypes of tumors from varied tissues. Still, there are general principles that cells have to fulfill in order to be successful cancer cells. In a landmark paper Hanahan and Weinberg summarized the traits common for the vast majority of (or even all) cancers and thus defined the hallmarks of cancer (Hanahan and Weinberg 2000). The defined traits: self-sufficiency in growth signals, insensitivity to growth inhibitory signals, limitless proliferative potential, resistance to apoptosis, acquisition of angiogenesis and finally the ability to invade tissue and form metastases. These, together with the recent addition of: evasion of immune destruction and efficient changes in their metabolism (Hanahan and Weinberg 2011) (Fig. 1), describe a cohesive cancer phenotype.

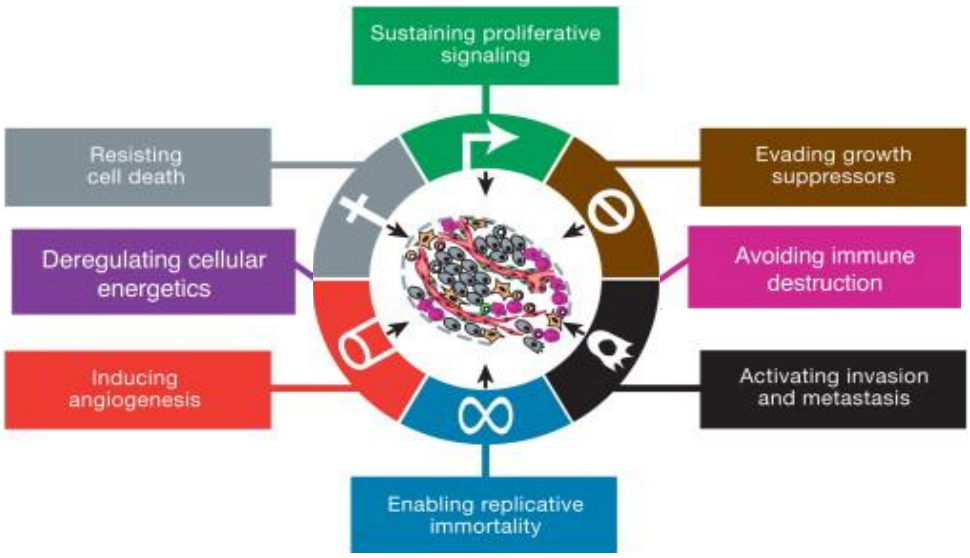

Figure 1. The hallmarks of cancer.
The defining cancer characteristics, common for all types of cancer.
Adapted from Hanahan and Weinberg, 2011.

Despite the increased understanding of cancer biology many of the still open questions include the involvement of the environmental factors, organism lifespan or pre-existing conditions (such as obesity and certain chronic diseases), that change the risk of various cancers. So far, we know these processes are tightly correlated and that advanced age is the highest risk factor for cancer (DePinho 2000). Better understanding of the molecular mechanisms underlying these diseases/phenomena that influence cancer development, can lead to novel therapeutic strategies. For example, it seems that processes that cause inflammation (seen with ageing and chronic conditions) increase the risk of cancer and not surprisingly, anti-inflammatory drugs have been shown to protect against it (Trinchieri 2011).

With the improved genomic and epigenetic tools it is now possible to identify critical changes in cancer development, by discriminating between the actual "driver" mutations, necessary for the tumor growth, and secondarily formed “passengers” events. Several large-scale studies have provided us with information on this dichotomy (Sjoblom et al. 2006; Greenman et al. 2007) and identification of novel driver mutations (Banerji et al. 2012; Stephens et al. 2012), can further be applied for the design of novel anti-cancer therapies.

Another line of questions is based on the identification of very small clusters of tumor cells or potential eradication of cells at the initial steps of the transformation process. Are there malignant properties that can be utilized for specific elimination of these cells from the tissue and could this prevent formation of metastases and higher rates of secondary tumors observed in patients? Development of more specific and preferentially preventive strategies would represent a substantial success in cancer therapy.

3. Governing principles of cell transformation

From early on, it was known that more than one modification was necessary for the cell transformation and the exact number of changes required varied between species, as human cells needed more than rodent ones (Sager et al. 1983). Soon it was discovered that cancer is achieved through gradual accumulation of genetic changes. General estimation of the number of these events is around 4-6 (Renan 1993) and since the rate of mutations in normal human cells is extremely low, some of the mutations may actually target regulators of the genome stability. In some cases, such as for colon carcinoma, studies detailed the order in which the mutations appear, suggesting that each one of them is necessary for the next step of cell transformation (Fearon and Vogelstein 1990). However, more

recent reports describe the alternative mechanism of chromothripsis, whereby massive chromosomal rearrangements are created in a single catastrophic event (Rausch et al. 2012).

Besides genetic changes, cancers are also characterized by epigenetic alterations; which are heritable gene expression modifications that do not involve changes in the DNA sequence. In general, cancer cells exhibit enhanced global DNA hypomethylation, gene-specific promoter hypermethylation and altered functions or recruitment of epigenetic modulators. Each of these contributes to global genome instability, repression of tumor suppressors and other cancer-specific changes (Feinberg and Vogelstein 1983; Weber et al. 2005). The genetic and epigenetic processes can act in concert whereby epigenetic changes can have genetic repercussions. For example, deamination of 5-methylcytosine (5mC) creates a T:G mismatch which is a hotspot for somatic mutations (You and Jones 2012). The inverse also applies where somatic mutations give rise to future epigenetic changes, seen in the case of mutations in genes coding for some of the epigenetic enzymes, such as DNA methytransferase (DNMT3A), commonly found in AML patients (Ley et al. 2010).

Understanding cancer was for a long time limited to purely correlative observations and cancer heterogeneity mainly remained unexplored. However, recent technological advances and the genomic revolution have allowed an insight into the functional complexity of cancer and the first molecular portraits of cancer have started to emerge (Perou et al. 2000). The main focus in cancer research is now shifting to the field of cancer genomics, epigenetics and other approaches such as high-throughput genome-wide translocation sequencing (Chiarle et al. 2011), offering a view into the complex molecular architecture of cancers. A large amount of data obtained using these approaches impacts our understanding of the (molecular and cell) origins of cancer (Gilbertson 2011) and novel cancer therapy design (Dawson et al. 2011). The transcriptome comparison has already yielded several unexpected results, where BRCA1 basal-like breast cancers were shown to arise not from the basal progenitor cells, but the luminal stem cell population (Molyneux et al. 2010).

Importantly, (epi)genetic changes are either repaired or harmful cells are eliminated by surveillance mechanisms - cellular death (apoptosis) or growth arrest (senescence). Therefore, to proliferate, cancer cells need to disable and overcome these failsafe mechanisms that will be discussed later.

4. Genetic regulators of cell transformation

Genes that regulate cell transformation are divided into two distinct groups based on their function as oncogenes or tumor suppressor genes. The underlying difference between these classes was made by Knudson's two hit hypothesis. The observation that hereditary cases of retinoblastoma disease appeared earlier and in both eyes as compared to sporadic patients, was explained by the fact that mutations were necessary in both copies of the Rb gene, a typical tumor suppressor responsible for the disease. Accordingly, in patients that had already inherited a mutated allele, only one more mutation was necessary for the disease to develop, which as expected took less time. While tumor suppressors, molecular brakes of tumor development, require "loss of function" mutations on both alleles in order for an effect to be produces, oncogenes require only one-hit that provides them with a "gain of function" mutation, sufficient for tumor development. This concept was used to identify and classify the first genes involved in tumor formation (Fig. 2).

4.1. Oncogenes

The first oncogenes were discovered through studies of acutely transforming retroviruses (such as Rous sarcoma virus), when it was revealed they contained a viral version of the host cellular proto-oncogene (c-*Src*), obtained through the process of recombination with host DNA (Varmus 1982; Varmus 1988). Subsequently, others were identified in the case of weakly oncogenic retroviruses, where the tumor formation occurred due to the proviral integration in the vicinity of a proto-oncogene (Jiang et al. 1997; Sanchez-Beato et al. 2003), causing their activation.

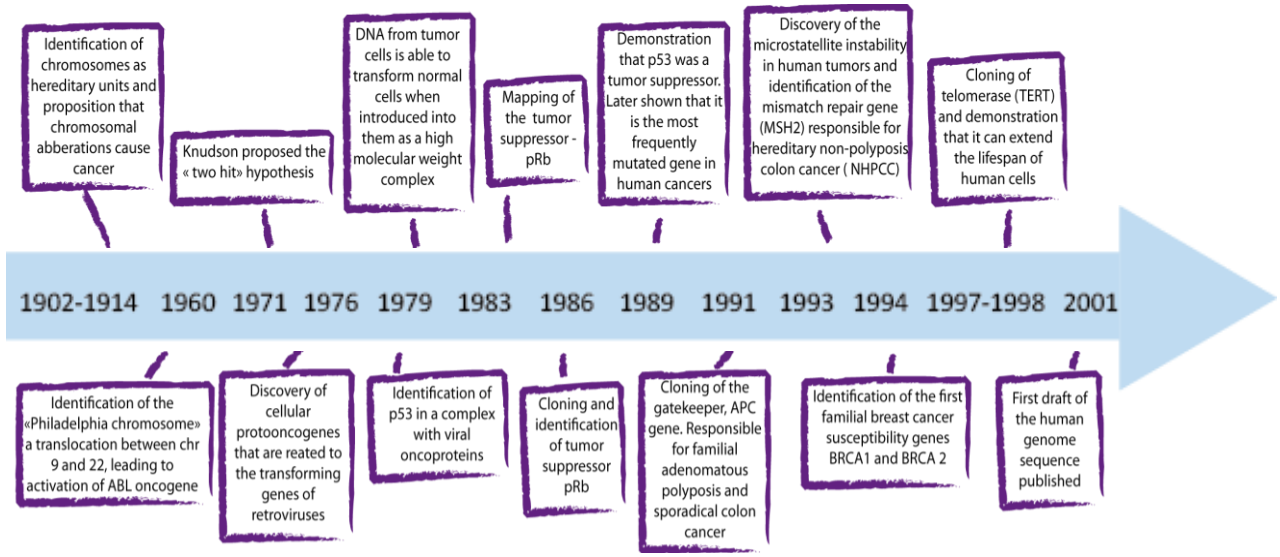

Figure 2. Initial landmark discoveries in cancer research.
Chronological overview of the milestone discoveries in identification of cancer- related genes.
Adapted from Balmain, 2001.

The confirmation of oncogene function came with DNA transfer experiments where DNA content of tumor cells efficiently transformed immortalized rodent cell line and soon after the first human oncogenes were isolated (Shih et al. 1979; Shih et al. 1981; Parada et al. 1982; Pulciani et al. 1982). Chromosomal translocations served as a location guide for the discovery of many new oncogenes (Tomescu and Barr 2001; Falini and Mason 2002) and the first karyotypic abnormality studied in detail was the "Philadelphia chromosome" (Rowley 1973; Nowell and Croce 1986), a 9:22 chromosomal translocation which creates BCR-ABL fusion protein (Groffen et al. 1984), associated with chronic myeloid leukemia (CML) and acute lymphoblastic leukemia (ALL). Fusion oncogenes generated by chromosomal translocations are hallmarks of human leukemias and other prominent examples include *AML1-ETO*, involved in acute myeloid leukemia (AML) and RARα–PML, which results in acute promyelocytic leukemia (APL). Moreover, leukemias tend to exhibit a higher genetic stability than solid tumors and therefore provide an insight into the precise genetic events leading to the development of cancer.

Proto-oncogenes normally exist in the genome and code for proteins that promote cell proliferation and growth, but due to mutations and/or overexpression their function becomes uncontrolled and contributes to cancer. Based on their function, they can be divided into several categories: growth factors, growth factor receptors, signal transducers (such as tyrosine kinase *Src*, serine/threonine kinase *Raf-1* or *Ras* family), transcription factors (*Fos, Jun, Myc, Myb*) and programmed cell death regulators (like *Bcl-2*) (Kufe et al. 2003). Two of the most prominent oncogenes in humans - *RAS* and *MYC* are discussed here in more detail.

4.1.1. Ras family of oncogenes

From the first gene transfer studies, oncogenes of the *RAS* family (consisting of three isoforms *H-RAS, N-RAS* and *K-RAS)* were isolated from various human tumors (Parada et al. 1982; Santos et al. 1982). RAS proteins are small GTP-ases involved in signal transduction and they switch between inactive (GDP-bound) and active (GTP-bound) states. They hydrolyze bound GTP with the help of GTP-ase activating proteins (GAPs, for example NF1), which brings them to the inactive GDP-bound form. Point mutations in Ras usually affect the position 12 (close to the finger loop for GAPs) or position 61 (crucial for hydrolysis) which results in impaired GTPase activity (Malumbres and Barbacid 2003). Mitogen binding to the tyrosine kinase membrane receptors confers a signal to RAS via guanosine nucleotide

exchange factors (GEFs, such as SOS) which facilitate GDP release in exchange for GTP. Thus, mitogens signal to formation of the active GTP-bound form of RAS, which can then specifically activate myriad of signaling pathways including Mitogen-activated protein kinase (MAPK), PI3K pathway and RAL-GDS (Fig. 3). They affect multiple cell activities such as proliferation, growth arrest, differentiation, but also senescence and apoptosis (Malumbres and Barbacid 2003).

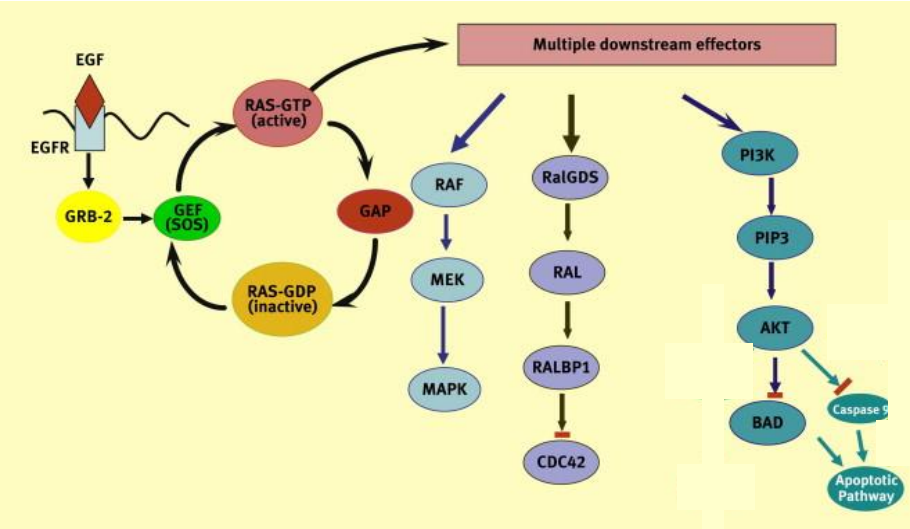

Figure 3. Activated Ras pathways.
Mitogen signaling to formation of active RAS-GTP and three main signaling pathways are activated (MAPK, GI3K and Ral-GDS) that can lead to multiple outcomes. *Adapted from Ibrahim and Arends, 2011.*

In Ras-free mice, the ability to proliferate and migrate only occurs upon reactivation of the Ras/MEK/ERK and not other pathways, indicating that each of the pathways downstream of Ras has specific functions (Drosten et al. 2010).

Ras isoforms are mutated with different efficiency in a tissue-specific manner. While N-Ras is the main cause of myeloid malignancies, K-Ras mutations are the most common ones in pancreatic cancer (Parikh et al. 2007). Gene knockout studies have shown that K-Ras loss is embryonic lethal, but N- or H-Ras deficient mice do not show any obvious defects (Koera et al. 1997; Esteban et al. 2001). Ras involvement in the tumor initiation has been shown with chemical carcinogens (Zarbl et al. 1985; Wiseman et al. 1986) that directly led to Ras mutations. Further study of Ras action in greater detail and in specific cellular and tissue context was achieved by generation of mouse models with tissue-specific promoters such as MMTV (Sinn et al. 1987), Elastase-I (Quaife et al. 1987) or Tyrosinase. The importance physiologic context for tumor development was seen when $KRas^{v12}$ expression at endogenous level in mice somatic cells only affected lung bronchiole-alveolar cells and caused their

malignant progression while the other cell types remained unaffected and embryonic stem cells retained the ability to proliferate and differentiate (Guerra et al. 2003).

4.1.2. Myc oncogenes

The *c-Myc* oncogene, a member of the *Myc* family, with *N-Myc* and *L-Myc*, was discovered in patients with Burkitt's lymphoma due to chromosomal translocations (Hecht and Aster 2000; Boxer and Dang 2001). *c-Myc* codes for a transcription factor, with the basic helix-loop-helix-leucine zipper (bHLHLZ) structure and regulates up to 15% of the complete genome (Klapproth and Wirth 2010). As a large number of its target genes suggests MYC regulates varied cellular processes, with an important role in the promotion of cell growth via stimulation of protein synthesis and blocking the cell cycle arrest through inhibition of CDKIs transcription. MYC also blocks cell adhesion, cell-cell communication as well as terminal differentiation and influences apoptosis (Eilers and Eisenman 2008) through transcriptional activation of TRAIL receptor DR5 (Wang et al. 2004) or by downregulating the transcription of apoptosis inhibitor FLIP (Ricci et al. 2004).

- Myc structure and interactions

MYC contains several conserved elements MYC boxes - MBI, MBII, MBIIIa, MBIIIb and MBIV, with the special involvement of the first two at the N-terminus in the processes of transactivation of MYC target genes, thus comprising the transactivation domain (TAD).

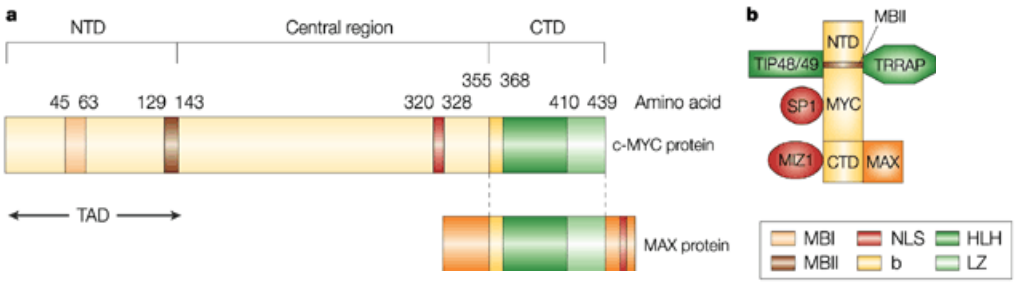

Figure 4. MYC structural elements and important interactions.
a) N-terminal and C-terminal domains of MYC (NTD, CTD) with their components **b)** Model of Myc interaction with different remodeling components – TRRAP and Tip or other transcription factors (MAX and MIZ1). *Adapted from Pelengaris et al., 2002.*

This region also participates in the interactions with various proteins involved in chromatin and histone remodeling. The C- terminal region of MYC contains helix-loop-helix (HLH) and leucine zipper (LZ) structures, responsible for its binding to the DNA and interaction with another bHLHLZ proteins, such as MAX or MIZ1 (Fig. 4).

- Myc – a dual transcriptional regulator

Heterocomplex of MYC and MYC-associated factor X (MAX), enhances transcription by binding to the Enhancer Box (E-Box) sequence of specific genes. They recruit additional transcriptional activators such as histone acetyl transferases (GCN5, TIP48), which leads to transcriptional upregulation of target genes. This action of MYC is antagonized by formation of a second type of MAX complex (MAD-MAX or MNT-MAX), which also bind the E-box elements, but instead recruit co-repressors and lead to decrease in gene transcription. It seems that MAX is essentially a stable, loosely regulated protein, and that its well-regulated heteroduplex partners determine its function (Fig. 5).

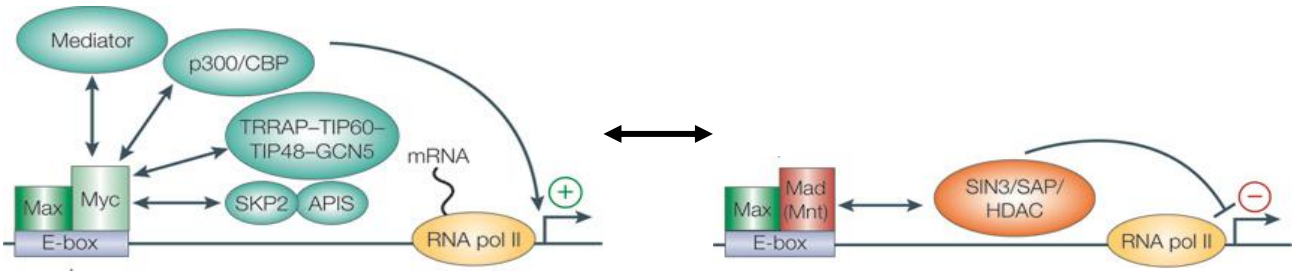

Figure 5. MYC-MAX gene activation model.
MAX heterocomplexes bind to E-box elements and due to recruitment of different coregulators lead to either gene activation (in presence of MYC) or gene repression (in presence of MAD or MNT).
Adapted from Adhikary and Eilers, 2005.

Myc also acts as a transcriptional repressor of multiple target genes (*p15*, p*21*, *p27)* by blocking the action of the appropriate transcription factors (such as SMAD, YY-1, SP1, MIZ-1) (Gartel and Shchors 2003). Perhaps its best studied role is in blocking transcription of MIZ-1 regulated genes. In these cases MYC does not bind the DNA directly, but instead binds MIZ1 at the site of the core promoter, as in the case of FLIP (Ricci et al. 2004).

Essentially, gene repression is achieved through competition of MYC and coactivator p300 for binding to MIZ-1, but also through MYC's ability to recruit the DNA methyltransferase co-repressors (Dnmt3a) to MIZ-1 regulated genes (Brenner et al. 2005) (Fig. 6).

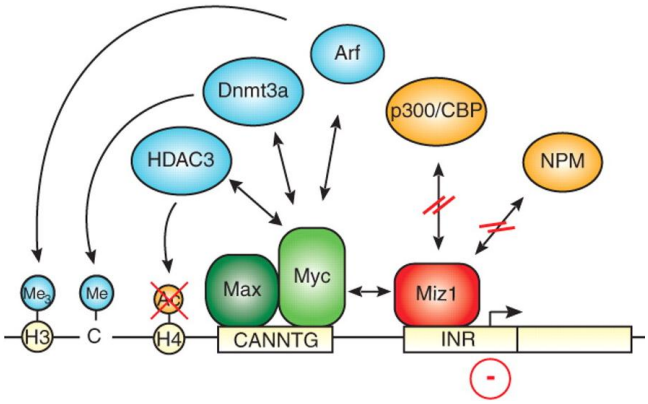

Figure 6. Model of Myc gene repression.
Myc binding to Miz-1 leads to decrease in the preexisting transcription rate and is achieved through competitive inhibition with p300/CBP complex and additional recruitment of co-repressors.
Adapted from Herket and Eilers, 2010.

- Myc regulation

MYC is a short lived protein ($t_{1/2}$ ~ 20 min), but controls a significant number of genes. This is achieved with apparently lower levels of MYC than expected, which makes MYC-responsive gene expression sensitive to slight changes in its amounts that are accompanied by the changes in the co-regulators present. The model of MYC action suggests that it does not bind all targets at the same time, but that they all ultimately become transiently occupied in a certain short period (Eilers and Eisenman 2008).
Due to its importance, MYC is tightly controlled on multiple levels. Various signaling cascades, such as WNT, RAS/RAF/MAPK, JAK/STAT, TGFβ and others, contribute to increases in *MYC* transcription (Liu and Levens 2006). Aditionally MYC is controlled at the posttranscriptional level through phosphorylation, ubiquitinylation and acetylation, which affect its stability and activity. Phosphorylation sites are distributed throughout the MYC protein, with perhaps the best studied ones in the TAD, where MYC is stabilized by the S62 phosphorylation via various kinases (MAPK, JNK, CDK1 or RAS signaling) (Vervoorts et al. 2006). This is a prerequisite for the subsequent T58 phosphorylation, achieved by GSK3β and the double phosphorylated MYC (pS62/pT58) is first dephosphorylated at the S62 residue by PP2A, and then targeted for ubiquitination and proteosomal degradation (Fig. 7). This

ubiquitination involves ubiquitin ligase Fbw_7 (Welcker et al. 2004; Yada et al. 2004) and ubiquitin-dependant protease USP28 (Popov et al. 2007), both commonly found deregulated in cancers.

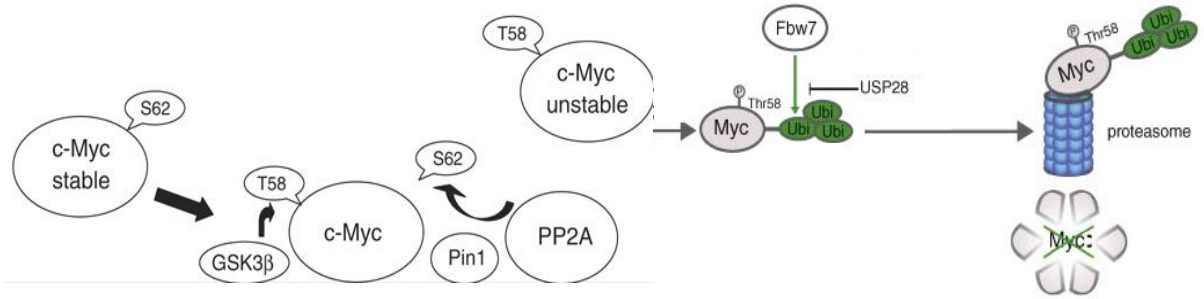

Figure 7. Myc posttranslational modifications and stability.
Myc's stability is regulated through processes of phosphorylation and is finally degraded by ubiquitin-dependent proteosomal degradation. *Adapted from Laurenti et al., 2009 and Schülein and Eilers, 2009.*

Action of other ubiquitin-ligases (such as SCF-SKP2 or HECTH9) has additional consequences (Kim et al. 2003; Adhikary et al. 2005), such as more efficient MYC-dependent gene transcription, probably by affecting its cofactor recruitment.

Importantly MYC stability is altered by other oncogenes and Ras/Raf/ERK pathway through phosphorylation (Sears et al. 2000), which suggests oncogenic synergy in signaling, where MYC probably acts as a central regulator of cellular transformation (Wang et al. 2005). In support of this, the crucial role of Myc signaling is seen *in vivo* in mice models of Ras-induced lung adenocarcinoma and SV-40 driven pancreatic tumor model, where systemic Myc inhibition by dominant negative Omomyc, led to tumor regression (Soucek et al. 2008; Sodir et al. 2011). These mice also showed profound changes in the proliferating tissue which is in accordance with the well described central role of c-Myc in cell pluripotency. Myc is a part of the main quartet of transcription factors and with Oct4, Sox2 and Klf4 it reprograms somatic cells to induced pluripotent stem cells (iPS) (Okita et al. 2007; Takahashi et al. 2007).

4.2. Tumor suppressor genes

Based on the role they perform, tumor suppressor genes can be divided into two categories, namely caretakers and gatekeepers. In principle, this division also follows the lines of Knudson's hypothesis. Due to their different functions, the incidence with which these two classes of tumor suppressors are mutated in hereditary and sporadic forms of cancer vary, and this can help distinguish between the two. For example, inherited mutation in gatekeeper genes requires only one additional mutation in the second allele to produce an effect. Thus, mutations in gatekeepers greatly increase the risk of cancer and these genes are relatively often found in sporadic mutations. On the other hand, a single mutation in a caretaker gene first needs mutation in the second copy of the gene and yet does not leads to neoplasia, but only to higher incidence in acquisition of other mutations, and thus is very rarely seen in sporadic cancers (Kinzler and Vogelstein 1997).

- ***Caretakers*** – have a role in maintaining the genome integrity and preventing the formation of mutations. They are generally involved in DNA repair and can be either the sensors of the DNA lesions (like ATR or BRCA1 or 2) or part of the repair machinery. Depending on the type and size of the damage of the DNA, several types of repair mechanisms exist: Base Excision Repair (BER) or Nucleotide Excision Repair (NER) for relatively small DNA lesions, Non-Homologous End Joining (NHEJ) or Homologous recombination (HR) for double strand breaks (DSB) or Mismatch (MMR) or Crosslink repair (CLR).

- ***Gatekeepers*** – sense stress or damage within a cell that represents a threat to the fidelity of replication and act to halt proliferation. Once gatekeeper pathways are activated, cell can either be physically removed by apoptosis or permanently growth arrested by becoming senescent. Key regulators of these two processes are the same and the main two are p53 and pRb. The tumor suppressor p53 is a transcription factor that is stabilized upon DNA damage and other stress, and acts as a transcriptional repressor of anti-apoptotic genes like *BCL-2* and a transcriptional activator of pro-apoptotic genes, therefore leading to apoptosis induction. Conversely, activation of p53 can also favor senescence via induction of the cyclin-dependent kinase inhibitor (CDKI) p21, which blocks cell proliferation. The other major tumor suppressor, pRb is active in its hypophosphorylated state and functions to block the progression of the cell cycle from G1 to S phase. In the presence of stress or DNA damage signals, p16 interacts with CDK4 and CKD6, blocking their phosphorylation of pRb, thus keeping it in its active state.

4.3. Stepwise transformation model systems

Despite the multiple levels of tumor complexity mentioned before, a reductionist approach has helped us understand the basic principles of cancer development. It consists of identification of the minimal fundamental changes required in different cell types for their transformation. Determining these common mechanisms has enabled us to study cancer at its core.

In their landmark paper Hahn and Weinberg described a stepwise tumorigenic model system in which defined genetic changes had been introduced into several normal cell types in order to generate cancer cells. Successful transformation of normal cells was achieved by expression of catalytic subunit of telomerase hTERT (which prevents telomere shortening), the oncoprotein of Simian virus early region (SV40 ER) and an overexpressed oncogene. The genetic elements introduced allow cells to bypass several pre-existing barriers in cancer development. Blocking tumor suppressors by SV40 expression among other things blocks p53, pRb and PP2A and prevents cells from entering senescence additional expression of hTERT enables cells to surpass cell crisis (Fig. 8). Upon addition of all these elements transformation was achieved and in addition, cells at different stages of transformation were obtained which contain only some of the genetic changes.

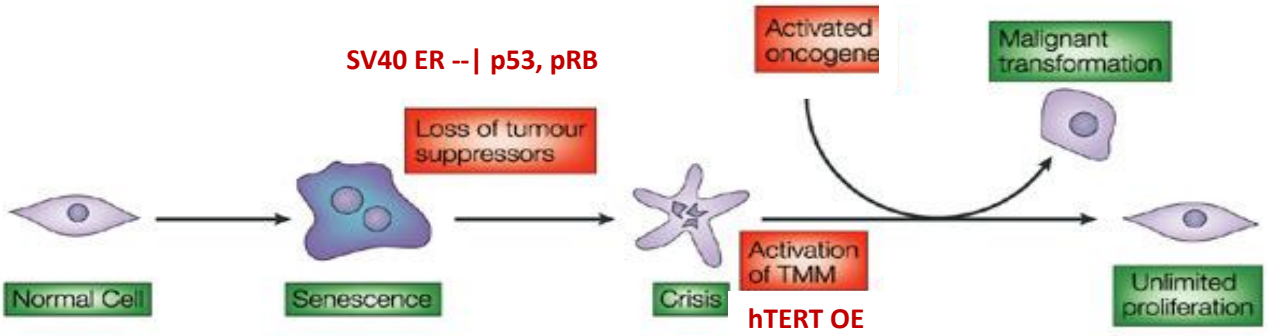

Figure 8. Processes of cell transformation, senescence and crisis.
Several processes separate normal cells from becoming transformed. Senescence (permanent growth arrest), the first barrier to tumor development, is achieved through activity of tumor suppressors .The next barrier – crisis is achieved through short telomeres and both can be surpassed by genetic modifications.
Adapted from Neumann and Reddel, 2002.

Full transformation was achieved in this stepwise manner in multiple cell types, confirming that the rules of tumorigenic transformation are somewhat universal and that despite the heterogeneity of

cancer, there are basic mechanisms that govern the formation of cancer cell. The fully transformed cells obtained exhibit cancer-specific characteristics such as anchorage-independent growth, tumor formation in nude mice (Hahn et al. 1999; Hahn et al. 2002) and are sensitive to TRAIL-induced apoptosis (Nesterov et al. 2004; Wang et al. 2004).

These systems provide a valuable tool in studying the processes of transformation and the transformation-related characteristics.

5. Principles of cancer therapy

As cancer cells are endowed with a limitless replicative potential, it is easy to see why they represent a threat to other cells, tissue structure and the organism in general. The intrinsic mechanisms of protection against tumor development often prove to be insufficient, as cancer represents the second leading cause of death in the world (Lehmann et al. 2008). External modes of tumor elimination are thus developed in the form of anti-tumor therapy. The successful cancer therapeutic should assure two main conditions, firstly to eliminate the cancer cells with the highest possible efficiency and to specifically target cancer cells without damaging the other components and structures in the tissue. This combination of high efficiency and low toxicity is the main paradigm of anti-cancer drug design and will be used in the following text as a decisive quality when the therapies available are discussed.

5.1. Conventional cancer therapy

The main approaches in treating cancer are still radio- and chemotherapy along with surgical removal of the tumor, when possible. Cancer surgery, the oldest approach in treating cancers, dating from thousands of years ago, is not always an option depending on the tumor size and position. The following development of radiotherapy (with the discovery of X rays at the end of 19th century) and the chemotherapy (cytotoxic drugs, from the mid 20th century) presented novel ways of treating cancer. Basically, radiotherapy causes DNA damage in the cell, while chemotherapeutics hamper the mechanisms of mitosis, essential for fast-dividing cancer cells, and also cause DNA damage which is then detected by the intrinsic cellular mechanisms that activate apoptosis. In some cases however, the mechanisms that govern cancer development, for example loss of p53 (Lowe et al. 1993; Lowe et al. 1993) or bcl-2 overexpression present in many cancers (Schmitt et al. 2000) also provide the mechanisms for therapy-resistance. In addition, and consistent with the previously mentioned central

paradigm of successful tumor therapy, classical therapy is not specific, as it targets the complete organism and causes severe toxic side-effects. Despite this it is still widely used and can successfully cure some forms of childhood leukemia.

5.2. Chemotherapy

Chemotherapeutics can be divided either by the phase of the cell cycle in which they are active: S phase-specific (Doxorubicin, Fluorouracil, Methotrexate), M phase-specific (Paclitaxel, Etoposide), G_2 phase-specific (Bleomycin, Irinotecan) and G_1 phase-specific (Corticosteroids) or by the mechanism of action, where the main groups are:

- **Topoisomerase inhibitors** – mainly interfere with the action of topoisomerase I (Irinotecan and Topotecan) and II (Etoposide) and thus deregulate processes of DNA replication and transcription.
- **Alkylating agents** - act by alkylating and damaging biologically important molecules by forming covalent bonds with the amino, carboxyl and phosphate groups (Cisplatin, Carboplatin) or they chemically modify DNA (Cyclophosphamide).
- **Antimetabolites** - act as structural analogues of the building blocks of the DNA (purines and pyrimidines) and thus block the proliferation in the S phase of the cell cycle (5 FU or Methotrexate).
- **Plant alkaloids -** these plant extracts obstruct the microtubule function and thus block cell proliferation (Paclitaxel also known as Taxol).
- **Intercalating agents** - interact with the DNA and intercalate between two bases inducing a structural change altering functionality of this molecule (Adriamycin).
- **Cleaving agents** - that break the DNA molecules (Bleomycin).

Novel cytotoxic agents are continuously being produced and their combinatorial application, within the toxicity tolerated for each drug, is becoming more important. Combinatorial therapies provide maximal cell elimination with a broader range of treatment in a heterogeneous tumor population and it slows or even prevents the development of new drug-resistant cells. In some instances cytotoxic agents are combined with other therapies, as in the example of neoadjuvant or adjuvant therapy where chemotherapy is administered before or after surgical treatment, respectively.

5.3. Targeting the hallmarks of cancer

As mentioned before, a single set of characteristics is apparently shared across different (or even all) types of cancer. These cancer cell hallmarks can be used as a basis for drug development and targeting each of these cancer-specific characteristics will mainly affect the cancer cells and not the normal ones. In this context, among others, it is possible to target the cancer-specifically:

- *Self-sufficiency for growth factors.* These drugs (Cetuximab, Panitumumab) mainly bind tyrosine kinase receptors and inhibit their signaling, either by preventing the receptor dimerization or promoting their internalization. However, they have shown to be ineffective in cells with K-Ras, b-Raf and other mutations.

- *Sustained angiogenesis.* Vascular endothelial growth factor (VEGF), usually used by cancer cells as an angiogenic switch that leads to formation of new blood vessels, acts by signaling through its tyrosine kinase receptors (VEGF and VEGFR). Agents like Bevacizumab bind to the VEGF, sequester and remove it from circulation. Nevertheless, there are side-effects to this, as VEGF is also involved in other types of signaling in the organism.

- *Evasion from apoptosis.* In order to form tumors, cells overcame the self-surveillance systems that upon detection of unrepairable anomaly which activate cell death program or apoptosis. These systems (that will be discussed in more detail later) are regulated by multiple components that either have a pro-apoptotic or anti-apoptotic role. In order to restore the balance in cancer cells, different types of inhibitors namely against Bcl-2, that block the action of antiapoptotic molecules can be used (Hu et al. 2008; Masood et al. 2011).

5.4. Targeting the cancer-initiating principle

In some cases the specific drivers that orchestrate development of particular cancer can be recognized and in these cases therapeutics that specifically target the cause of cancer, have been used highly successfully for therapy.

One example is CML, which originates from the fusion oncoprotein BCR-ABL with constitutive tyrosine kinase activity, and causes the disease (Lugo et al. 1990). One of the first drugs developed by rational drug design, Imatinib mesylate (Gleevec) acts as a tyrosine kinase inhibitor and has been a powerful tool in blocking the activity of such fusion oncoprotein (Druker et al. 2001).

In the case of acute promyelocytic leukemia (APL), chromosomal translocations result in the formation of a specific fusion oncoprotein PML-RARA (de The et al. 1990), disturbing the RARα dimerization process, p53 interaction and its DNA binding capacity (Di Croce et al. 2002; Bernardi et al. 2004). The treatment of APL involves application of all-trans Retinoic acid (ATRA) leading to terminal differentiation of the cells and oncoprotein degradation (Nasr et al. 2008). Combinatorial treatments of APL with ATRA and arsenic trioxide (As_2O_3) are shown to be even more efficient and lead to complete remission (Shen et al. 2004).

These types of therapies lead to high percent of cured patients, 90 % of APL patients were in complete remission (Hu et al. 2009) and 70-80% had a complete cytogenetic response in case of CML (Druker et al. 2006), but are possible only for a small portion of well characterized tumors, as majority of cancers have multiple (epi)genetic changes associated with their development.

5.5. The large scale studies in drug screenings

Genome-wide analysis of various tumor samples of different origin epithelial, mesenchymal or hematopoietic tissue, from patients of different age as well as multiple cancer cell lines, have resulted in identification of new cancer genes (Banerji et al. 2012; Stephens et al. 2012) and importantly enabled functional re-grouping of cancers (Curtis et al. 2012). The new division into tumor subsets was based on changes in DNA sequence (mutations) and structure (translocations), copy-number alterations, epigenetic modifications or the transcriptome profiles, allowed their further association with drug sensitivity (Ellis et al. 2012). These methodologies reconfirmed some of the links previously established, such as *BRAF* mutation provides sensitivity to MEK1/2 inhibitors and *ERBB2* mutation to inhibitors of EGFR. Some have become evident for the first time - sensitivity of Ewing's sarcoma tumors with the *EWS-FLI1* rearrangement to PARP inhibitors (Garnett et al. 2012) or a general sensitivity of myeloma cell lines to IGF1R inhibitors (Barretina et al. 2012). These results identify new therapies for some forms of tumors and also give new targets for rational drug design.

5.6. Other antitumor agents

- ***Epidrugs.***

The enzymes that are involved in establishing and maintaining the cell epigenome and its functional consequences can be classified in three groups, which mutually interact. The "writers", such as histone methyl- and acetyl-transferases (HMTs and HATs) are responsible for generating the epigenetic modifications. The "erasers" - histone demethylases and deacetylase enzymes (HDMs and HDACs) remove them. While the third group of epigenetic enzymes - "readers" (that contain bromodomain, plant homeodomain finger or other modules) transform the information present into a precise epigenetic code to a specific biological output. The improper functioning of a member of either of these groups can have profound consequences on cell regulatory processes and provides a basis for novel drug design (Chi et al. 2010).

Two classes of epidrugs, the DNMT inhibitors azacitidine (Silverman et al. 2002; Silverman et al. 2006) and decitabine (Blum et al. 2007), as well as the HDAC inhibitors vorinostat (Duvic et al. 2007; Olsen et al. 2007) and romidepsin (Piekarz et al. 2011) have been approved by the FDA for the treatment of the myelodysplastic syndrome (MDS) and T cell lymphoma, respectively. Combining these two classes of drugs (Cameron et al. 1999; Gore et al. 2006), provides synergy of their action and this concept has now been extended to the design of the newest generation of triple action epidrugs, such as UVI5008 which targets HDACs, sirtuins, and DNMTs (Nebbioso et al. 2011).

Other breakthroughs include the design of novel small inhibitors that displace the bromodomain and extra terminal (BET) family of proteins from the chromatin (Dawson et al. 2011) in mixed lineage leukemia (MLL) patients or the inhibitor of H_3K_{27} – specific methyltransferase, Enhancer of zeste homologue 2 (EZH2), used to treat acute myeloid leukemia (AML) patients (Fiskus et al. 2009). These drugs have a large potential as it seems that they specifically target transformed cell. However their precise action remains mainly elusive and their transient use can lead to resistance.

- ***Oncolytic viruses.*** Some forms of naturally existing (Parvoviruses, unable to replicate in quiescent cells) or engineered viruses (so that they bind only the proteins present on the membrane of transformed cells) have been developed for cancer treatments (Alemany et al. 2000; Kirn 2000). These viruses generate proteins that activate cell death in the infected cells. The clinical trials with oncolytic viruses have reached phase I and II, and have shown that the viruses can be administered systemically with limited toxicity and latency (Ferguson et al. 2012). However there are many obstacles in terms of

activation of host defence mechanisms and nonspecific uptake by lung, liver and spleen, which limit the virus ability to infect tumor cells after systemic delivery. Further improvements that lead to higher and longer virus presence in the blood could improve efficiency of these treatments (Liu et al. 2007).

5.7. Tumor – cell specific targeting

Agents that target the tumor cells specifically and efficiently, regardless of the exact tumor-inducing mechanism, without affecting the neighboring normal cells, have been the ultimate goal in the search for cancer therapeutics. Some of the naturally existing molecules might be able to fulfill these requirements and if applied exogenously lead to programmed cell death (apoptosis) of cancer cells specifically. TNF-related apoptosis inducing ligand (TRAIL) that will be discussed here in detail represents a promising candidate for this kind of therapy and is in the focus of my research project.

TRAIL

1. General introduction

TRAIL (TNF-related apoptosis inducing ligand, Apo2L, TNFSF10) was discovered independently by two groups (Wiley et al. 1995; Pitti et al. 1996), while searching for TNF related sequences. It is a 281 AA, type II transmembrane glycoprotein, with an extracellular C-domain that can be cleaved to create a soluble ligand form. TRAIL belongs to the Tumor Necrosis Factor (TNF) family of cytokines (along with TNFα, CD95 and others) and its binding to the cognate Death Receptors (DRs) on the cell membrane leads to programmed cell death - apoptosis. Apoptosis is a functional way in which multicellular organisms deal with cells damaged beyond repair or undesired cells, and it plays a critical role in cellular homeostasis, functioning of the immune system and inflammation responses, and even in organ development.

The death receptors to which TRAIL binds belong to the TNFR superfamily and have a specific structure with cysteine rich extracellular domains (CRDs), a transmembrane domains and Death Domains (DDs) in their intracellular regions (Fig. 9). So far, six distinct death receptors and the corresponding ligands (that can be promiscuous) have been discovered. The ligand for DR6 has not yet been determined, although the extracellular fragment of the β-amyloid precursor protein (N-APP), present in Alzheimer affected brains, has been shown to bind and lead to neuron degeneration (Nikolaev et al. 2009).

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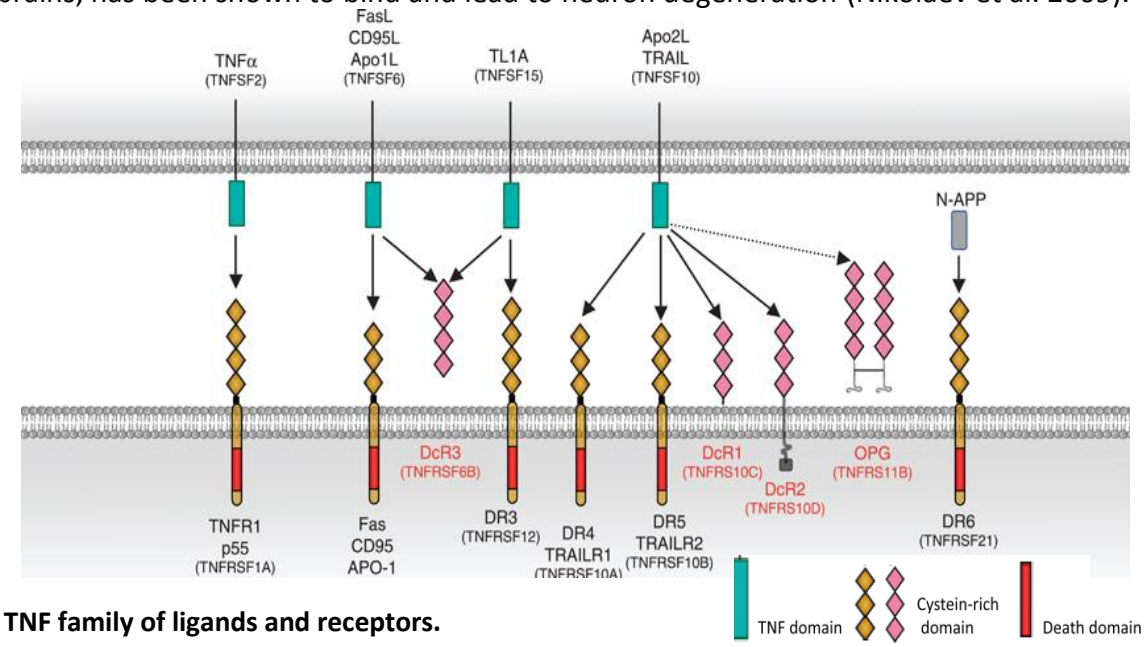

Figure 9. TNF family of ligands and receptors.
Several members of TNF family of ligands (TNFα, Fas, TL1A and TRAIL) and their cognate receptors, shown with their structural component - Death domains (DD) in red, Cystein rich domains in yellow and pink and TNF ligand domains in blue. *Adapted from Gonzalvez and Ashkenazi, 2010*

1.1. TRAIL receptors and signaling

TRAIL ligand can bind to five receptors: DR4 (TRAILR1, TNFRSF 10A), DR5 (TRAILR2, TNFRSF10B), DcR1 (TRAILR3, TNFRSF10C), DcR2 (TRAILR4, TNFRSF10D) and osteoprotegerin (OPG), but only the first two are death receptors (DR4 and DR5) and signal to apoptosis. The other three are Decoy Receptors that lack the intracellular domain (DcR1), have it truncated (DcR2) or are soluble proteins (OPG) (Fig. 9).
TRAIL binds its cognate receptors as a homotrimer (Hymowitz et al. 1999) and initially it seemed that it stimulated receptor trimerization upon binding. However, newer reports suggest that receptor oligomerization can occur even in the absence of ligand through pre-ligand-binding assembly domain (PLAD) (Chan et al. 2000; Clancy et al. 2005). A Zn atom found in the centre of the ligand trimer (Fig. 10) binds to a cysteine side chain of each trimer subunit and regulates it stability, solubility, orientation for receptor interaction and hence its biological activity (Ashkenazi et al. 2008).

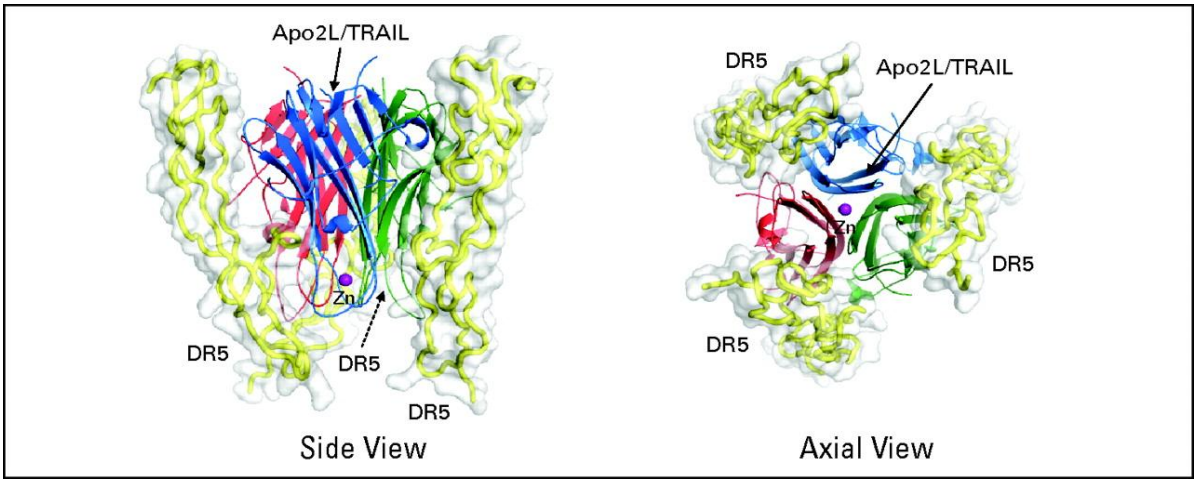

Figure 10. TRAIL ligand - receptor interactions.
Side and Axial view of TRAIL ligand – DR5 receptor interaction, represented with a ribbon structure, shows a homotrimeric ligand with a Zn atom in the center (represented in purple).
Adapted from Ashkenazi et al., 2008.

Upon ligand-receptor binding, the complex recruits the DD containing adaptor protein FADD (Fas-associated DD), that in turn via its Death Effector Domain (DED) recruits the pro-caspases (-8 or-10) or c-FLIP. This completes the formation of Death Inducing Signaling Complex (DISC) leading to pro-caspase self-processing and activation, release of active caspase-8/10 heterotetramers, caspase-cascade signaling and results in apoptosis (Fig. 11).

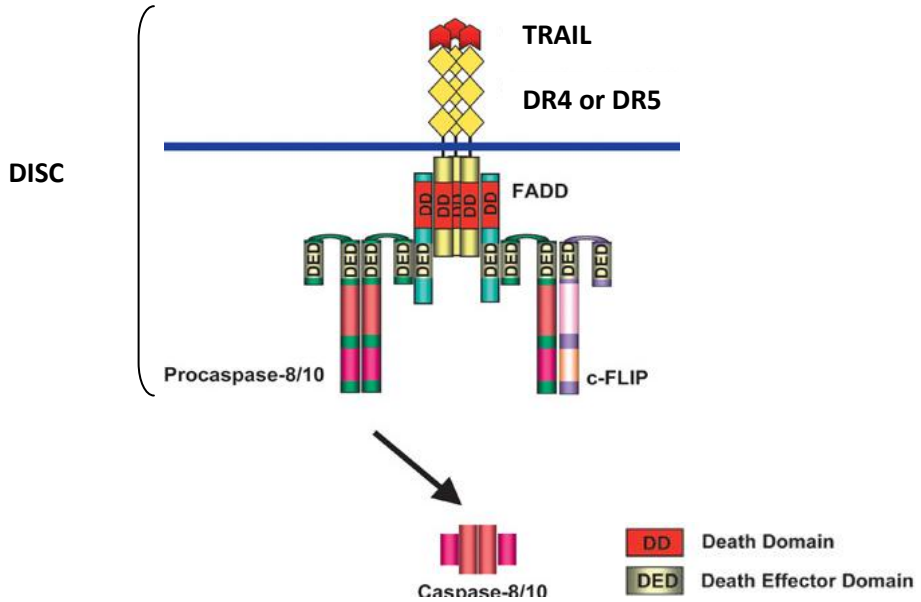

Figure 11. DISC formation, components and signaling.
DISC is formed based on homotypic interactions between its components. DRs and FADD interact via Death Domains (DD) (in red) and FADD and procaspase-8 interact via Death Effector Domains (DED) (in grey). After the complete DISC formation, apoptosis signaling heterotetramer is formed.
Adapted from Lavrik and Krammer, 2009.

Diverse cellular signals, either extracellular or intracellular in nature, control the process of apoptosis. The two types of apoptosis (intrinsic and extrinsic) can sometimes converge, depending on whether they are present in Type I or Type II cells (Hao and Mak 2009) and probably the intensity of their initial activation (Scaffidi et al. 1998). The "intrinsic pathway" is activated upon the detection of intracellular stress (DNA damage, hypoxia, membrane damage and other signals) and with the help of the pro-apoptotic Bcl-2 family members the pro-apoptotic signals, cytochrome c and Smac/DIABLO are released from the mitochondria. Released cytochrome c then binds the adaptor protein Apaf-1, which leads to apoptosome formation and finally cell death (Fig. 12).

The pro-caspases, aspartate-specific cysteine proteases, are synthesized in the inactive form and are involved in the pro-inflammatory (casp-1, -4, -5, -11, -12, -13 and -14) or pro-apoptotic signaling. Pro-apoptotic caspases can be divided into two groups based on the hierarchy of their activation: the initiator caspases (-8, -10, -2 and -9), that directly activate the downstream effector caspases (-3, -6, -7) in some cells. In type II cells, the initiator caspase-8 activates the intrinsic apoptosis pathway via Bid cleavage leading to activation of other downstream caspases (Fig. 12). Other proteins can also

incorporate into DISC (like FLIP - based on its structural similarity to caspases) and their involvement results in altered DISC activity.

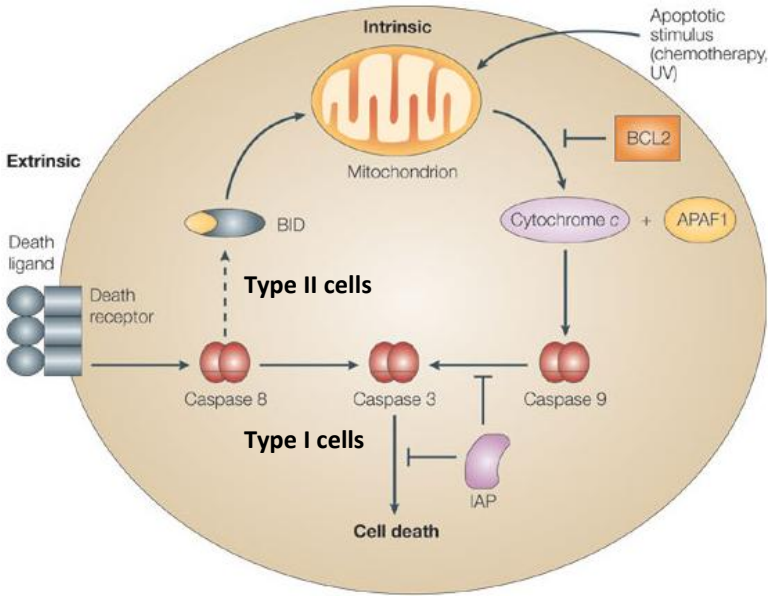

Figure 12. Intrinsic apoptotic pathway.
Several stimuli lead to mitochondrial membrane permeabilisation and cytochome c release, which together with Apaf1 and pro-caspase-9 forms apoptosome and leads to apoptosis.
Adapted from Andersen et al., 2005.

1.2. TRAIL signaling in physiological conditions and in mouse models

In physiological conditions TRAIL has been implicated in shaping and restricting immune responses and has a specific function in the cells of the immune system, such as natural killer (NK), dendritic, cytotoxic T cells and macrophages (Falschlehner et al. 2009). TRAIL has been involved in the process of hematopoiesis, especially in the maturation of erythroblasts (Zamai et al. 2000) and T cells (Janssen et al. 2005). The best characterized function of TRAIL is its anti-tumor activity. Multiple lines of evidence suggest that TRAIL has a tumor protective role, specifically against metastatic tumor cells (Grosse-Wilde et al. 2008). TRAIL and TRAIL receptor loss-of-function models show higher incidence of spontaneous or experimental metastases (Takeda et al. 2001; Cretney et al. 2002; Finnberg et al. 2008). Although some reports show that loss of TRAIL receptor did not affect the development of intestinal tumors (Yue et al. 2005), spontaneous lymphoid malignancies developed in an accelerated manner in TRAIL-deficient mice (Zerafa et al. 2005). A recent study has, for the first time,

demonstrated that TRAIL overexpression had a protective role in cancer *in vivo*. Specific TRAIL overexpression in mouse keratinocytes delayed the onset of tumors in TPA induced chemical carcinogenesis (Kedinger et al. 2011). In addition, the anti-cancer role of TRAIL was inferred from gene expression profiles of breast cancer patients that revealed correlation between TRAIL downregulation and presence of brain metastases (Bos et al. 2009).

1.3. Tumor cell-specificity of TRAIL action

The most remarkable characteristic of TRAIL action is its tumor cell-specificity, even though the exact regulatory mechanism for it is still under investigation. Some reports describe higher levels of the decoy receptors (DcR) in normal cells (Pan et al. 1997), others focus on the increased levels of DR4 and DR5 in cancer cells (Sayers and Murphy 2006), or attribute it to higher levels of O-glycosyltransferase, present in some cancer tissues that hyperglycosylates DRs and promotes their clustering (Wagner et al. 2007). Other forms of posttranslational DR modifications like N-glycosylation (Yoshida et al. 2007) and palmitoylation (Rossin et al. 2009), constitutively present in cancer cells, can also affect TRAIL signaling efficiency. All these modulations (Fig. 13) affect lipid raft formation, described as necessary for the ability of DRs to transduce extrinsic apoptotic signals (Gonzalvez and Ashkenazi 2010). Recently, ubiquitylation of caspase-8 was also recognized as one of the TRAIL-sensitivity determining mechanisms (Jin et al. 2009), while other reports show that, in fact, the activity of oncogenes usually present in cancer cells, Ras and Myc (Nesterov et al. 2004; Wang et al. 2005) or their downstream targets FLIP, sensitizes these cells to TRAIL-induced apoptosis (Ricci et al. 2004).

Whatever the specific mechanism is the cancer cell selectivity of TRAIL action is undisputable, and it makes TRAIL one of the most promising anti-cancer therapeutic molecules. Although some studies have pointed out the possibly harmful consequences of TRAIL-systemic treatments, it seems the negative side effects were a consequence of specific recombinant forms of TRAIL used (Lawrence et al. 2001; Ganten et al. 2006) and that their possible hazardous effect in patients with liver inflammation (Volkmann et al. 2007) are not observed in healthy patients (Hao et al. 2004).

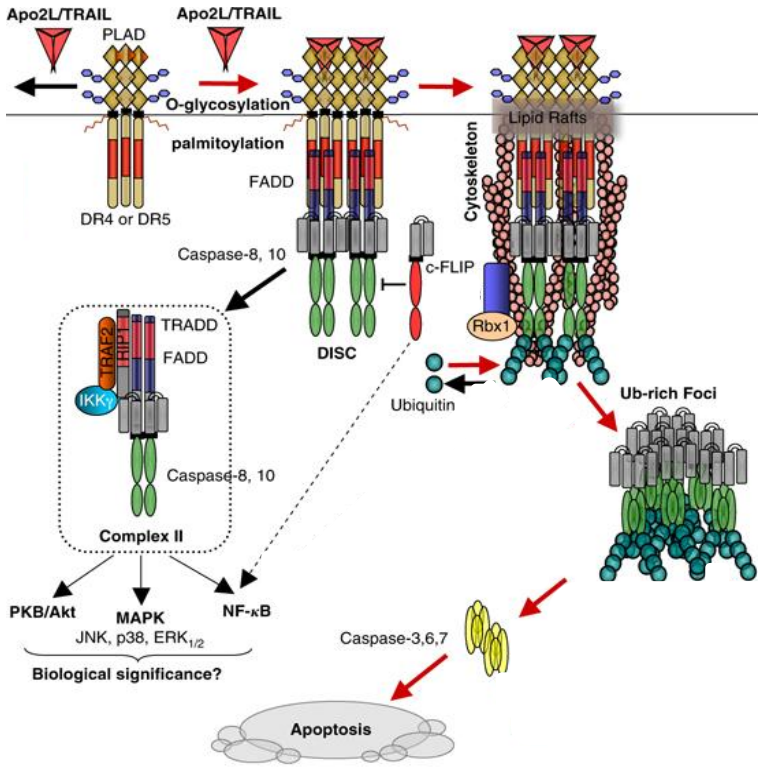

Figure 13. Model scheme of TRAIL action with the regulatory posttranslational modifications.
Posttranslational modifications like a DR O-glycosylation (in blue) or palmytoliation (in red) and caspase-8 ubiqutination (in light blue) are significant regulators of TRAIL sensitivity in cells. TRAIL can also signal to non-apoptotic outcomes via Complex II. *Adapted from Gonzalvez and Ashkenazi, 2010.*

1.4. Other TRAIL signaling pathways and types of apoptosis

Besides the pro-apoptotic DISC signaling ("type I complex"), TRAIL can lead to formation of a "type II complex", with the involvement of FADD, caspase-8 and additional proteins like RIP1, TRAF2 or IKKγ, that can activate different pathways including those governed by JNK, p38 or NFκB (Fig. 13). The role of this complex and subsequent NFκB signaling is not quite clear as it could lead to both increased transcription of some apoptosis inhibitors (c-FLIP, cXIAP$_{1/2}$) or it could serve to upregulate IL-8 and MCP1 production and attract the cells of the immune system to the apoptotic, dying cell (Varfolomeev et al. 2005).

2. Apoptosis in cancer treatments

By the virtue of its life or death controlling role the importance of apoptosis is quite clear. The proper regulation and dosing of apoptosis pathways are essential for multicellular organisms. Multiple

diseases, such as neurodegenerative disorders, AIDS and infertility can develop due to excess or deficient apoptosis, as in case of autoimmune diseases and cancer.

In cancer, the process of apoptosis is deregulated, and damaged, mutated and hyperproliferative cells are not removed from the tissue. Thus apoptosis re-activation represents a powerful tool for cancer treatment. Even classical cancer treatments like chemotherapy and radiation aim at activating deregulated apoptotic pathways (Fig. 12). These mechanisms usually employ the intrinsic apoptotic signaling with the involvement of p53, which is often mutated and leads to acquisition of chemotherapy resistance in cancer cells (Breen et al. 2007). Crucially, the conventional therapies, despite their success in some cases (including childhood leukemia), are often associated with systemic (geno) toxicities, asking for the development of conceptually different approaches.

2.1. TNF ligand based cancer therapies

Identification of the TNF family of small proapoptotic ligands provided an exciting and promising opportunity to treat various types of tumors in a p53 independent manner, by activating the extrinsic apoptotic signaling, as opposed to standard chemotherapy (Ashkenazi and Herbst 2008). Although initial studies with TNFα and Fas ligand were successful in some cases of combination therapies on soft tissue sarcoma (Grunhagen et al. 2006) and B cell chronic lymphocytic leukemia (Bremer et al. 2008) respectively, these ligands exert their pro-apoptotic activity unselectively on any cell type, and lead to massive hepatocyte apoptosis and general toxicity. This result emphasized the importance of the central paradigm of cancer therapy, which is to provide the most efficient clearance of tumor cells without affecting the rest of the normal cells in the organism. As opposed to other apoptosis-inducing ligands, TRAIL has a cancer cell-specific action, which brings it into the main focus in the search for future effective cancer treatments.

2.2. TRAIL- based cancer therapies

The main focus of research and drug development involves either recombinant human TRAIL (rhApo2L/TRAIL) that binds to both DR4 and DR5 or development of receptor-specific TRAIL agonists (monoclonal antibodies) such as Lexatumumab, Apomab and AMG-655 (DR5 specific) or Mapatumumab (DR4 specific). The individual contribution of each receptor is probably tissue and tumor-specific, as some reports show that DR5 plays the main apoptosis inducing role (Kelley et al.

2005), while others point to a more important function of DR4 (MacFarlane et al. 2005). Both these lines of ligands developed are currently in clinical trials (phase I or phase II), either as single agents or in combinatorial treatments with either conventional or other cancer therapies (Gliniak and Le 1999; Walczak et al. 1999; Jin et al. 2004; Muhlethaler-Mottet et al. 2004; Marini et al. 2005; Ashkenazi and Herbst 2008). Most commonly in preclinical cell and animal models, TRAIL is combined with conventional chemotherapeutics such as methotrexate (MTX), doxorubicin (DOX), 5-fluorouracil (5-FU), cisplatin (CIS) or irinotecan (IRI) (Gliniak and Le 1999; Lacour et al. 2001; Naka et al. 2002), irradiation (Shankar et al. 2004), synthetic triterpenoids (Hyer et al. 2005), IAP antagonists (Fulda et al. 2002) and different types of proteasome (Brooks et al. 2005), Bcl-2 (Ray et al. 2005), HDAC (Nakata et al. 2004; Bangert et al. 2011) or kinase inhibitors (Ricci et al. 2007).

These studies have shown that TRAIL has relatively low toxicity and as a single agents lead to disease stabilization, which is positive considering the fact that phase I trial patients usually have advanced malignant diseases, with no response to other therapeutics. However, the main observation has been that TRAIL yields the best results in combination with other therapeutics, with mostly partial or complete responses (Ashkenazi and Herbst 2008). The rationale behind the increased efficiency of combination-treatments is that it involves activation of the intrinsic apoptotic pathways that can reinforce TRAIL-initiated caspase signaling. Secondly, chemotherapeutics can lead to p53-induced increase in DR5 transcription (Wu et al. 1997) or on the contrary TRAIL treatment can remove pre-existing resistance to chemotherapy (Mitsiades et al. 2001). Novel combinatorial approaches, with some unconventional cancer treatments or novel molecules, could further increase TRAIL effectiveness and perhaps broaden the targets of TRAIL action. While the phase I and phase II clinical trials are still ongoing, for both rhApo2L/TRAIL and the monoclonal antibodies (http://clinicaltrials.gov/), an important focus in the future could be the identification of novel biomarkers of TRAIL responsiveness in tumor tissues.

3. Apoptosis inhibitors

As the vital role of apoptosis suggests, it is tightly regulated with the involvement of several (endogenous) inhibitors on multiple levels. First, it can be blocked at the level of the intrinsic mitochondrial apoptotic pathway by several inhibitory proteins such as Mcl-1, Bcl-2 and Bcl-xL (Martinou and Green 2001); or further downstream at the level of activated caspases-3 and 8, where a

family of Inhibitors of Apoptosis (IAPs) blocks caspase activity by binding to their substrates (Liston et al. 1996; Deveraux et al. 1998). Apoptosis signaling can also be controlled right at the initial level of extrinsic apoptosis signaling – at the DISC complex itself. In this case, the control and regulation of the apoptosis signaling is established by FLIP, one of the most potent apoptosis inhibitors.

3.1. FLIP – discovery, structure and isoforms

In search for novel apoptosis inhibitors, data-base mining for the sequences with homology to DED resulted in identification of a new viral apoptosis inhibitor v-FLIP (Fas-associated death domain-Like interleukin-1beta-converting enzyme Inhibitory Protein). This novel type of inhibitor, with high structural similarity to procaspase-8, was very potent in blocking the early events in apoptosis signaling from several death receptors (Thome et al. 1997).

The cellular homologue c-FLIP, also known as CASH, Casper, CASJ, CLARP, FLAME-1, iFLICE, MRIT or Usurpin, was discovered soon afterwards (Irmler et al. 1997). c-FLIP protein originates from the CFLAR (Caspase8 and FADD-like apoptosis Regulator) gene located within the same 200kb cluster as caspase-8 and -10, and their high degree of similarity suggests that they were probably formed through the process of gene duplication (van Noesel et al. 2003). Till date, thirteen splice isoforms of FLIP have been discovered, but only three of them have been detected at the protein level in humans (Micheau 2003). The three isoforms detected in humans are FLIP long (c-FLIP$_L$, 55kDa), FLIP short (c-FLIP$_S$, 27 kDa) and FLIP Raji or c-FLIP$_R$ (25 kDa). All of these isoforms contain a double Death Effector Domain (DED), located in their N-terminal, but differ at their C-terminal domains (Fig. 14). As opposed to shorter isoforms FLIP$_S$ and FLIP$_R$ which have only few extra amino acids at their C-terminus, FLIP$_L$ contains an additional domain. This caspase-like domain (CAD), located at the C-terminus of FLIP$_L$ entails a catalytic active site with mutations that render it inactive.

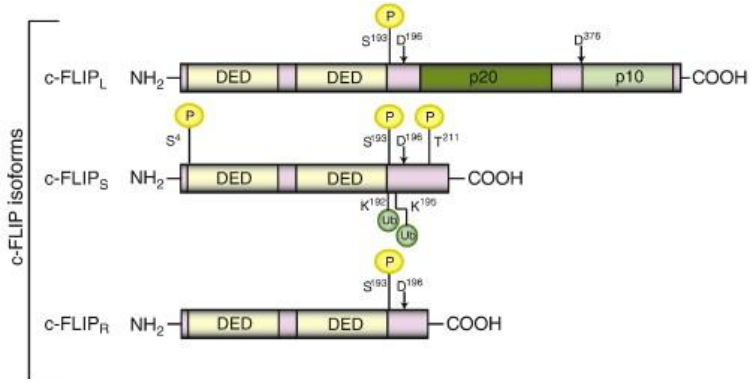

Figure 14. FLIP isoformes and structure.

Three FLIP isoforms present in humans are depicted with the specific DED domains and the isoform-specific post-translational modification sites. *Adapted from Ozturk et al., 2012.*

3.2. FLIP signaling

In the absence of FLIP, two pro-caspase-8 molecules bind to the DISC, which leads to their auto-proteolytic activity and through subsequent cleavages p10 and p18 fragments are generated. These fragments in duplicates form a tetramer, responsible for the downstream apoptosis signaling. Due to a high structural similarity, FLIP competes with procaspase-8 for binding to the DISC and since all three isoforms possess the DED domain that enables their binding to FADD, all of them can be successfully recruited to the DISC (Scaffidi et al. 1999). However, once bound their mechanisms of action vary. While the short isoforms, that lack the catalytic domain, do not permit any proteolytic activity of procaspase-8 and no cleaved fragments are formed, binding of $FLIP_L$ to the DISC and interaction with procaspase-8, allows only the incomplete procaspase-8 cleavage.

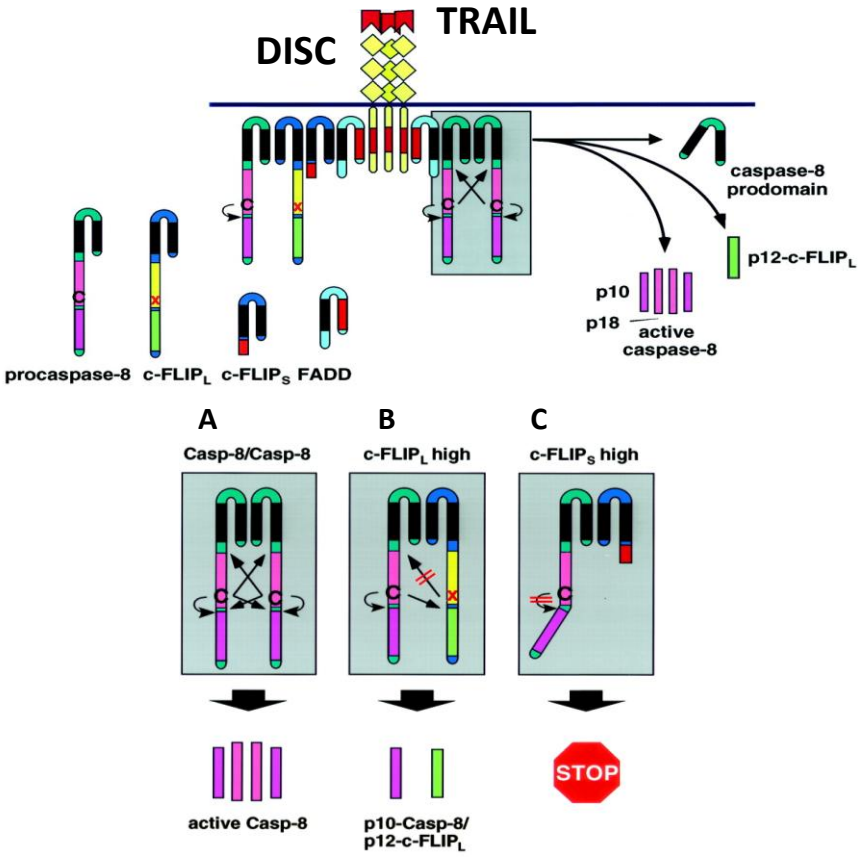

Figure 15. Mechanism of FLIP action
Upper panel: TRAIL DISC formation with all the members involved and the mechanistic model of FLIP inhibition Lower panel: The predicted apoptosis outcomes after (A) only caspase-8, (B) $FLIP_L$ or (C) $FLIP_S$ binding to the DISC. Catalytic active site in procaspase-8 (marked with C), inactive site in $FLIP_L$ (marked with X). *Adapted from Krueger et al., 2001.*

The incomplete cleavage observed, results in the formation of initially cleaved fragment (p10) and the remaining DISC-bound caspase-8 component (p43/41). Importantly, due to the inactive catalytic site present on the $FLIP_L$, full procaspase-8 cleavage does not occur and the production of p18 fragment is abolished (Fig. 15). This prevents the formation of the key downstream apoptosis-signaling tetramer, thus the apoptosis signaling is blocked (Krueger et al. 2001).

The remaining DISC bound caspase-8 fragment does not signal to apoptosis, however some reports suggest that it does retain some catalytic activity restricted to the membrane region and some local substrates (like Receptor-Interacting Kinase, RIP) which could lead to other apoptosis-unrelated downstream effects and activation of NFκB signaling (Micheau et al. 2002).

3.3. Transcriptional and post-translational regulation

Control over cellular FLIP levels is accomplished at multiple levels and with a variety of molecules (Shirley and Micheau 2010). Various transcription factors have a role in the regulation of FLIP levels in a cell. Some of them, such as NFκB (Kreuz et al. 2001; Micheau et al. 2001), p53 (Bartke et al. 2001), p63 (Borrelli et al. 2009) and Sp1 (Ganapathy et al. 2009) upregulate FLIP transcription, while others like Myc (Ricci et al. 2004; Bangert et al. 2011), cFos (Li et al. 2007), Foxo3a (Skurk et al. 2004) and Sp3 (Ganapathy et al. 2009) downregulate FLIP.

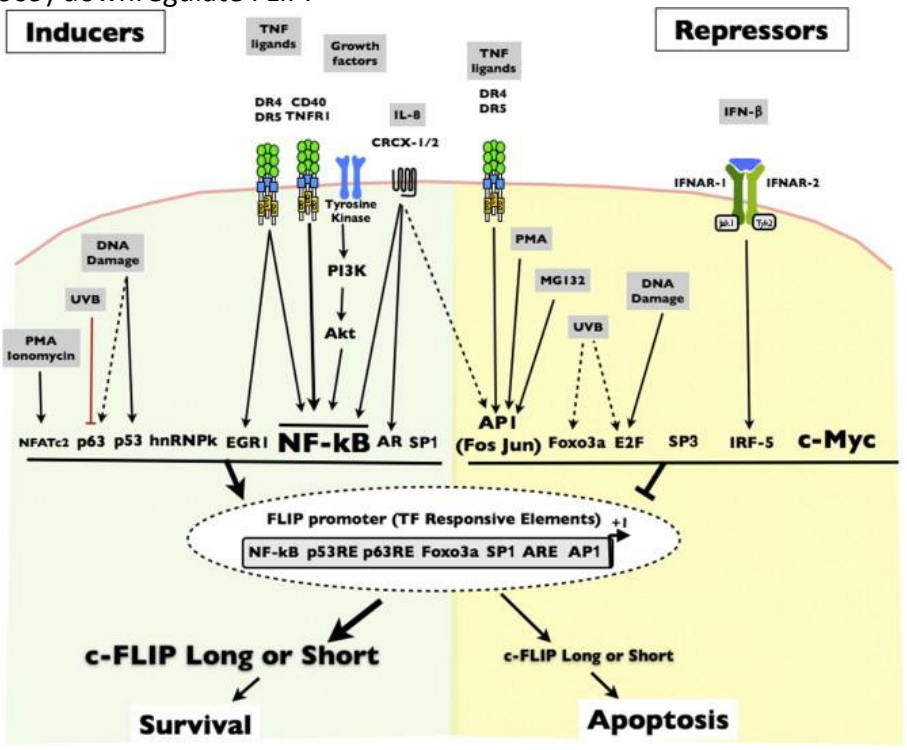

Figure 16. Multifaceted regulation of FLIP transcription.
The scheme represents plethora of factors, signaling pathways and transcription factors that act as positive or negative regulators of FLIP transcription. *Adapted from Shirley and Micheau, 2010.*

A variety of factors, such as chemokines, interleukins, growth factors and DNA damaging agents can activate these TFs and thereby influence FLIP transcription and expression (Fig. 16).

Another important aspect of FLIP regulation is its control at the posttranslational level and it seems that processes of ubiquitination and subsequent proteosomal degradation play a significant role in determination of cellular FLIP levels. Site-specific phosphorylation, that can be isoform-specific, in general provides additional control over proteosomal degradation (Kaunisto et al. 2009). A specific stretch of amino acids present at the very C-terminus of shorter FLIP isoform ($FLIP_S$) leads to its shorter half-life (Poukkula et al. 2005).

3.4. Role of FLIP in physiological conditions and tissues

Initially, v-FLIP was recognized as responsible for the transient resistance to Fas-L in early T cell activation, as its elevated levels were detected in T lymphocytes. Sensitivity that was regained after couple of days correlated well with the decrease in v-FLIP (Klas et al. 1993; Irmler et al. 1997).

FLIP is also upregulated in numerous different cancer tissue and primary cell culture samples from cancer patients (Shirley and Micheau 2010). High FLIP levels detected, probably protect cancer cells from apoptosis and are correlated with the cancer aggressiveness and poor treatment outcome (Ullenhag et al. 2007).

$FLIP_L$ in most of the cases primarily has an anti-apoptotic role (Galligan et al. 2005; Sharp et al. 2005). However, there are certain ambiguities about the role of $FLIP_L$ in apoptosis as some findings point to a pro-apoptotic role at low physiological levels, where it aids the initial cleavage of procaspase-8 and its further anti-apoptotic role at high levels (Chang et al. 2002). This is further supported by the fact that $FLIP_L$ deficiency, as well as deficiency of pro-apoptotic molecules (FADD or caspase-8) leads to *in utero* death caused by cardiac malformations in mice (Yeh et al. 2000). Some reports even suggest that a pro-apoptotic role of $FLIP_L$ could only be observed at supra-physiological levels, upon overexpression in the cells. (Goltsev et al. 1997; Han et al. 1997; Inohara et al. 1997; Shu et al. 1997). In these conditions $FLIP_L$, in excessive amounts could bind to DISC-free procaspase-8 and cytosolic activity of the heterocomplex, usually restricted to the membrane, could be observed (Micheau et al. 2002).

Senescence

1. Introduction to senescence

The term senescence is derived from a Latin word "*senex*" which refers to old age, thus senescence is associated with aging and terms can be used interchangeably in some contexts. Organisms with renewable tissues that have an opportunity to repair and replace their cells are at the same time under the risk of developing hyperproliferative diseases, such as cancer. In addition to the previously mentioned apoptosis, permanent cell growth arrest (senescence) serves as an additional tumor suppressive mechanism. Senescence is also linked to tissue repair and surprisingly, can exert tumor promoting effects.

2. Types of senescence

2.1. Replicative senescence

More than half a century ago, senescence was first described by Hayflick and Moorhead (Hayflick and Moorhead 1961), in a context of limited cell growth of fibroblasts in culture conditions. Details were discovered later, when it was shown that the cause of this limited proliferative potential was in fact telomere shortening during each round of cell replication (Hastie et al. 1990). Fibroblasts, and the majority of other human cells, lack the telomerase enzyme and thus face the end-replication problem, which renders their telomere regions shorter with time. The loss of telomeric sequences, although initially does not cause a loss of genetic information, eventually poses a threat to DNA maintenance, and in essence is detected as a double stranded break (DSB) thus provoking a DNA damage response (DDR). The DDR signaling involves the activation of DNA damage kinases (ataxia-telangiectasia Rad3 related and ataxia-telangiectasia mutated) ATR and ATM, that phosphorylate the H2AX histone variant, forming the γH2AX. This signaling is amplified by a positive feedback loop formed in collaboration with many other so-called DDR mediators, MDC1, 53BP1, RPA, RAD9, RAD1, HUS1, TOPBP1, and leads to activation of checkpoint kinases 1 and 2 (CHK1 and 2) and in final instances activates the key regulator p53. Activated and stable p53 consequently induces transcription of cyclin-dependent kinase inhibitor p21 and steady cell cycle arrest (Fig. 17). As a confirmation, studies have shown that the addition of catalytic subunit of telomerase (human telomerase reverse transcriptase, h-TERT) led to senescence

bypass and prolonged cell life (Bodnar et al. 1998). Importantly short telomeres are found to induce senescence *in vivo* (Cosme-Blanco et al. 2007; Feldser and Greider 2007).

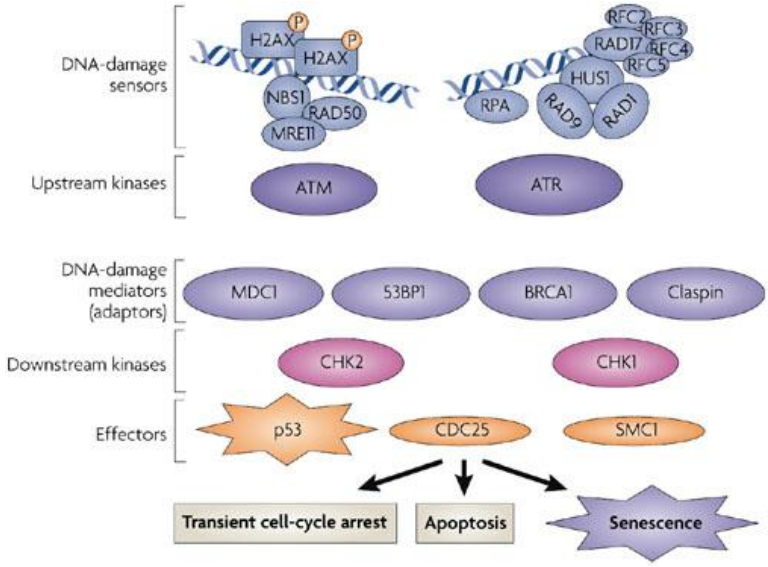

Figure 17. Model of replicative senescence caused by DDR
Short telomeres activate DDR and lead to induction of senescence with participation of numerous DDR-mediators. *Adapted from Campisi and d'Adda di Fagagna, 2007.*

In many other cases overexpression of h-TERT does not block senescence induction, as numerous stimuli that lead to senescence induction do not involve telomerase signaling. All these other types of senescence, caused by various inducers are known as premature senescence.

2.2. Premature senescence

Multiple factors, not directly connected to aging and telomere shortening, can cause a block in cell proliferation and senescence induction. They can be divided into several categories:

a) Cellular stress - various stress factors from inappropriate culturing conditions (Coppe et al. 2010), absence of layers of feeders cells (Ramirez et al. 2001), and most importantly oxygenic stress (Parrinello et al. 2003) can cause shock and lead to stress-induced premature senescence (SIPS) (Toussaint et al. 2000). Sublethal levels of hydrogen peroxidase (H_2O_2) have also been shown to be powerful senescence inducers (Chen and Ames 1994) and in some cases absence of oxygen that impedes the normal proliferation rate can actually serve as a block to replicative senescence (Chen et al. 1995).

b) γ-irradiation (Di Leonardo et al. 1994), UV (Lewis et al. 2008) and DNA damaging agents can act as senescence inducers. Importantly, the same was observed for chemotherapeutics, in culture and *in vivo* and this is phenomenon is known as Therapy Induced Senescence (TIS) (Roninson 2003). These senescent cells, newly formed within the tumor tissue, might have an important role in regulating tumor maintenance due to the fact that a subset of tumorigenic cells becomes growth-arrested and secretes factors that can affect their environment.

c) Oncogene induced senescence (OIS) - it has been well established that oncogene activation and overexpression in normal cells does not lead to transformation, but instead to induction of senescence. A large list of oncogenes can serve as senescence inducers: RAS (Serrano et al. 1997; Di Micco et al. 2006), RAF1 (Zhu et al. 1998), BRAF (Michaloglou et al. 2005), MEK (Lin et al. 1998), MOS (Bartkova et al. 2006), RAC1 (Debidda et al. 2006), MYC (Grandori et al. 2003), PML (Pearson et al. 2000; Bischof et al. 2002) and β-catenin (Xu et al. 2008). Many of these inducers activate the mitogen-activated protein kinase (MAPK) pathway and lead to a biphasic response, with an initial transient burst in proliferation followed by a cell-cycle arrest (Fig. 18).

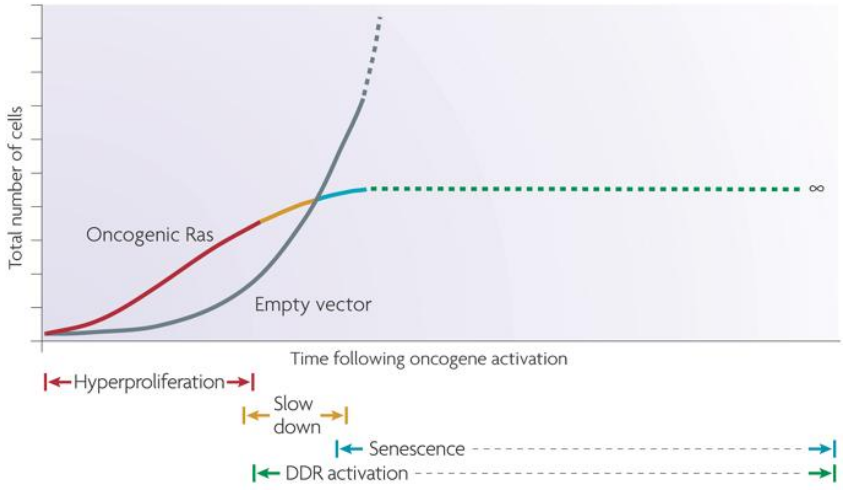

Figure 18. Biphasic response present in some cases of OIS
After initial hyperproliferation (depicted in red), proliferative slow down is detected (in yellow) and finally complete cell growth arrest is achieved (in blue). Adapted from *d'Adda di Fagagna, 2008.*

Oncogene-induced senescence could be cell type specific or depend on the intensity of the oncogene signal. Rather weak Ras activation in MEFs leads to proliferation and tumor formation (Tuveson et al. 2004), while strong signaling induces senescence in fibroblasts (Serrano et al. 1997). This dose-dependent oncogene signaling was observed also in mammary tissue (Sarkisian et al. 2007) and could

perhaps be explained by the initial increase in proliferation due to mild Ras signaling. Thus, more frequent mutations, that with time accumulate, encompass the tumor suppressors and finally lead to tumor formation. This is avoided in the case of initially strong oncogene signaling (or in a specific cellular setting), where it induces senescence at once (Sarkisian et al. 2007; Courtois-Cox et al. 2008).

Another interesting model of OIS involves a negative-feedback signaling loop, formed immediately downstream of an activated oncogene that serves to actually shut down the oncogene signal (Courtois-Cox et al. 2006). The mechanism of OIS still remains to be elucidated, as other studies point out to the opposite strong oncogene signaling network (Chen et al. 2005).

It appears that oncogene activation leads to a DNA damage response, either through ROS formation (Lee et al. 1999) or due to excessive replication, with multiple replication forks and their defective progression (Bartkova et al. 2006; Di Micco et al. 2006; Mallette et al. 2007), but DDR is not always observed (Hemann and Narita 2007; Efeyan et al. 2009) and other factors may be necessary to enable permanent and stable growth arrest (Courtois-Cox et al. 2008). Finally, oncogene signaling activates pRb and p53 which induces senescence, but there are probably other regulators involved, as both pRb and p53 are also active during quiescence.

d) Tumor suppressors can also serve as senescence inducers. Oncogene activation and other previously mentioned factors lead to senescence through activation or stabilization of tumor suppressors. Thus, it is not surprising that ectopic expression of tumor suppressors INK4a/p16 or p21 also leads to senescence (Coppe et al. 2011) and their increased expression correlates well with aging (Zindy et al. 1997; Krishnamurthy et al. 2006).

In contrast to this, loss of some other tumor suppressors (PTEN or NF1) can also cause senescence. It has been proposed that loss of PTEN, infact, drives senescence by both activation of p53 via mTOR1 and INK4A/p16 upregulation via the APC/C-CDH1 complex (Nardella et al. 2011). In the first study of such kind, the tumor suppressor PTEN was specifically deleted in mouse prostate and its role in tumorigenesis was studied by combining it with the activity of the other tumor suppressor - p53. The impressive results showed that the most dramatic effect of PTEN loss, namely aggressive and fast-growing tumors, were only observed once the PTEN loss was combined with the loss of both p53 alleles (Fig. 19). On its own, PTEN loss was not sufficient for tumor formation, as the tumors formed were growing slowly and non-invasive. In fact, when p53 was still present in the cell, loss of PTEN led to

senescence induction, thus explaining the formation of only prostatic intraepithelial neoplasia (PIN) (Chen et al. 2005).

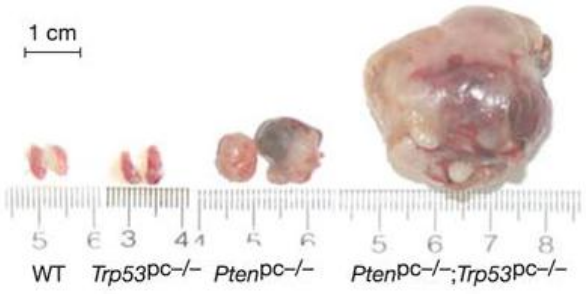

Figure 19. Prostate tumors developed in PTEN deficient mice.
PTEN deficient mice developed either small neoplasia, or full blown malignancies depending on their p53 status. *Adapted from Chan et al., 2005.*

In support of this, senescent cells were readily observed only within the prostate tissue of mice expressing p53. This has shed light on a completely new role of tumor suppressors in the interplay between the cancer formation and senescence induction. The observations were confirmed by examining the human prostate samples with early-stage lesions, where numerous senescent cells were detected in the PIN regions.

Similarly, loss of other tumor suppressors like NF1 (Courtois-Cox et al. 2006) or VHL (Young et al. 2008), was found to be responsible for senescence induction.

e) In recent years it has become evident that senescence inducers could also be signaling molecules with paracrine activities secreted from senescent cells themselves (Fig.20), such as IL6, IL8, IGFBP-7 or other anti-proliferative cytokines like IFN-β (Moiseeva et al. 2006) and TGF−β (Katakura et al. 1999; Reimann et al. 2010).

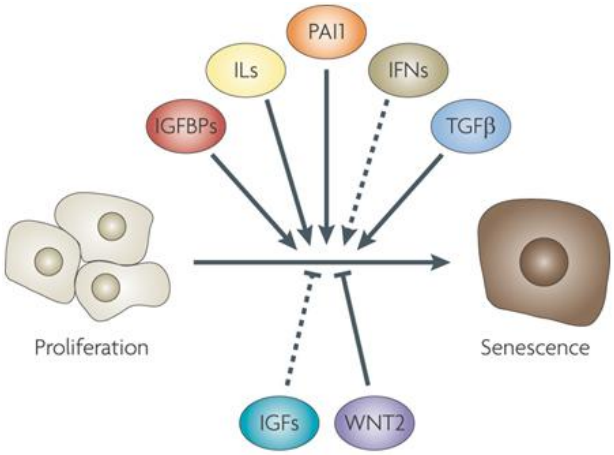

Figure 20. Senescent secreted factors as senescence inducers.
Different types of molecules that belong to SASP can, through their paracrine action induce senescence in normal proliferating cells. *Adapted from Kuilman and Peeper, 2009.*

In this way, once senescence has been induced in some of the cells in the tissue, the senescent cell populations can expand by affecting neighboring cells. They also secrete molecules that attract components of the immune system (discussed below) and thus, stimulate their own clearance. Perhaps the ratio between the two actions or the tissue specificity determines whether senescent cells will remain in the tissue or are eliminated.

3. Key pathways of senescence induction and regulation

Two major axes of tumor suppressors are responsible for senescence induction and maintenance. One branch is composed of p53 and its downstream target 21, while the other one entails INK4a/p16 and pRb (Fig. 21). Even though these regulators can achieve senescence on their own, an extensive interplay between the pathways exists. The activation of either of these regulators seems to be tissue-specific, species-specific and inducer-specific and there are even cases of senescence induction independent of both (Olsen et al. 2002; Campisi and d'Adda di Fagagna 2007).

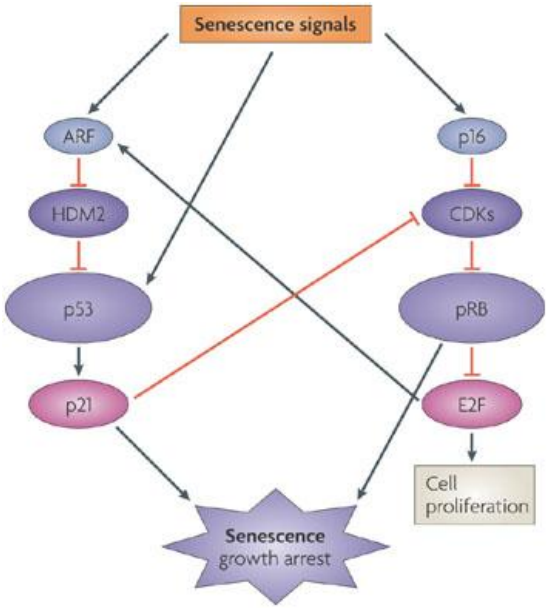

Figure 21. Main regulators and inducers of senescence
Network of tumor suppressors activated in response to multiple senescence inducers.
Adapted from *Campisi and d'Adda di Fagagna, 2007.*

Tumor suppressor p53 is probably the main player responsible for senescence induction especially in cases of telomere attrition and ionizing radiation. p53 is under the direct control of Mdm2 (Hdm2 in humans) and its levels can be controlled by regulation of stability and degradation in multiple site-specific phosphorylations. On its own, p53 acts as a direct transcriptional upregulator of p21, a potent cyclin-dependent kinase inhibitor (CDKI). Together with it p16, the CDKI from the other axis, it mainly signals to block the phosphorylation of pRb and keep it in permanently hypophosphorylated, active form. As a major consequence of these signals, transcription of E2F genes is suppressed. It seems that INK4a/p16 is usually involved in senescence induction of epithelial cells and has a crucial role in SAFH formation (Narita et al. 2003). In many cases loss of p53 function is followed by the escape from senescence (Brown et al. 1997) especially when the additional control is not provided by p16. Since these signals intertwine, in many cases blocking one part of signaling network is not sufficient to escape senescence or even more repression of one part of the network only increases the signaling of the other. For example E2F inversely controls the p53 levels, by regulating ARF and thus E2F repression will only lead to more stable p53 (Beausejour et al. 2003).

4. Senescent cell characteristics and markers

Senescent cells exhibit a number of traits which can be used for their identification.

- The first described characteristic of senescent cells was their **growth arrest.** Senescent cells are usually arrested in G1 phase, unable to divide in presence of mitogens and can be detected by a lack of Ki67 staining or low BrdU incorporation. The growth arrest of senescent cells is essentially permanent, but can be abrogated by specific genetic manipulations (Beausejour et al. 2003; Dirac and Bernards 2003) or depletion of signaling interleukins (Kuilman et al. 2008). Some reports have confirmed irreversible growth arrest and lack of proliferation of senescent cells *in vivo*; although once the oncogene inducer is removed the senescent cells were lost, probably through processes of immune clearance (Sarkisian et al. 2007). However, new findings by Peeper and colleagues (Vredeveld et al. 2012), in which same rare mutations were found in contiguous nevus-melanoma specimens from patients, suggest that senescent cells in some rare cases could progress to melanomas.

- Senescent cells are usually characterized by striking **morphological changes.** Cells are usually enlarged and flattened (Serrano et al. 1997; Parrinello et al. 2003) (Michaloglou et al. 2005) (Fig. 22) with high numbers of endoplasmatic reticulum vacuoles (Denoyelle et al. 2006).

- The other common senescent cell marker is senescence associated β-Galactosidase staining (**SA-β–Gal**) (Dimri et al. 1995), where at the suboptimal pH6 blue perinuclear staining is observed in senescent cells (Fig. 22), suggesting the increased activity of lysosomal β-galactosidase enzyme. This type of enzymatic staining can be performed in both cell culture and on tissue cryosections (Debacq-Chainiaux et al. 2009) and increased staining of cells is also observed in aged tissue (Dimri et al. 1995).

- Another specific feature of senescent cells is presence of **SAHFs** (Senescence-associated Heterochromatic Foci). Discovered by Lowe and collaborators (Narita et al. 2003), SAHFs can be visualized by simple DNA staining using DAPI, while the proliferating cells show homogenous nuclear staining (Fig. 22). These foci contain several gene repression marks and were implicated in the repression of E2F target genes, although the necessity of their presence and their actual function are disputed in recent reports (Kosar et al. 2011). More recently a distinct type of senescence-specific nuclear structures, DNA scars, have been described (Rodier et al. 2010).

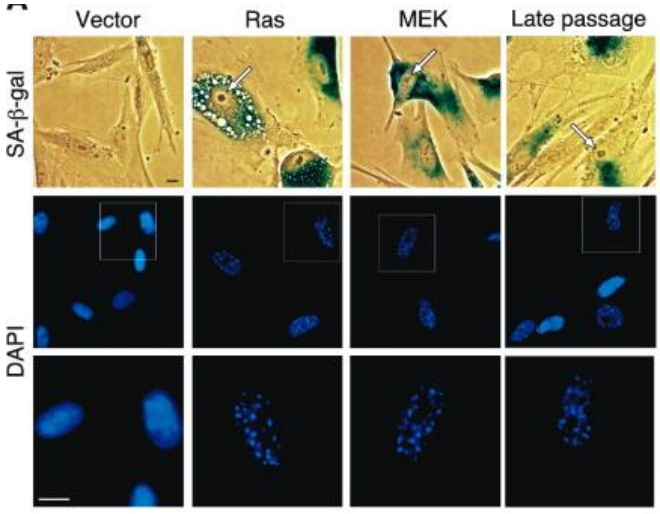

Figure 22. Several senescent - specific characteristics.
Oncogene (Ras or MEK) overexpression or short telomeres generate senescent cells that exhibit specific traits – altered morphology, presence of SA-β Gal and SAHFs. *Adapted from Narita et al., 2003.*

- The changes in **levels of expression** of specific CDKIs (p21, INK4a/p16) (te Poele et al. 2002; Collado et al. 2005; Baker et al. 2011), secreted factors, p53 accumulation (Chen et al. 2005), hypophosphorylated form of pRb (Serrano et al. 1997) and noticeable repression of cell cycle genes are also used as markers of senescent cell. Recently, action of microRNAs was implicated in the stable repression of these proliferation promoting genes (Benhamed et al. 2012).

- One of the most prominent changes in the transcriptome of the senescent cells is the upregulation of the secreted factors that compose the characteristic **Senescence-associated secretory profile (SASP)** (Coppe et al. 2008) also known as Senescence Messaging Secretome (SMS) (Kuilman and Peeper 2009) with diverse and complex function that will be discussed in more detail later. Levels of mRNA transcripts of most commonly senescent secreted proteins (Acosta et al. 2008; Kuilman et al. 2008) can be used to detect senescent cells or immuno-stained for the presence of these secreted factors (Coppe et al. 2008).

However, none of the fore mentioned characteristics is exclusively or uniquely restricted to senescent cells. For example, proliferative arrest is also observed in quiescent or differentiated cells, while the presence of positive β-gal staining can be also seen in some culturing conditions in which cells are highly confluent or in some cases *in vivo* (Severino et al. 2000). On the other hand, it is common that senescent cells do not possess all of the characteristics mentioned above and so far senescent cells that do not have SASP (Coppe et al. 2011) do not show presence of SAHF (Astle et al. 2011; Kosar et al. 2011) or lack the tumor suppressor INK4a/p16 upregulation (Beausejour et al. 2003) have all been described. Finally, it seems that clearly defining senescent cells is more difficult that initially thought. Not all specific characteristics have to be present in each type of senescence, but simultaneous detection of several markers significantly improves the characterization.

5. SASP analysis

As mentioned before SASP is one of the defining characteristics of senescent cells and several groups have focused on its regulation, composition and signaling potential.

5.1. SASP composition

5.1.1. Proteomic composition

In one of the first attempts to explore the composition of senescent secretory profiles, a DNA microarray analysis showed that senescent fibroblasts have a transcriptional profile quite similar to the pro-inflammatory profile seen during wound-healing (Shelton et al. 1999). A proteomic study of different types of senescence, using two-dimensional gel electrophoresis (Dierick et al. 2002), observed a partial overlap of proteins present in all types of senescent cells, while the remaining were specific for a type of senescence or the specific senescence-inducer used.

An extensive study performed by Campisi and colleagues (Coppe et al. 2008), quantitatively compared secretomes of different cells (fibroblasts, epithelial cells and even transformed epithelial cells) treated by various senescence inducers, using antibody arrays. The results showed that DNA damage was a requirement for SASP induction and that several different cell types tested, including tumor cells, were able to produce SASP upon DNA damage of sufficient extent and duration.

Even though there were notable quantitative and qualitative differences, there was a high degree of similarity in SASPs of different cell lines. In addition, SASP does not appear to be species-specific since murine fibroblasts, under appropriate conditions, can develop a human-like SASP (Coppe et al. 2010).

These studies have revealed that complex panel of molecules that forms SASP is relatively well defined and secreted at high levels from senescent cells. The SASP can be divided into 3 major classes of molecules (Fig. 23) (Coppe et al. 2008; Kuilman and Peeper 2009; Freund et al. 2010).

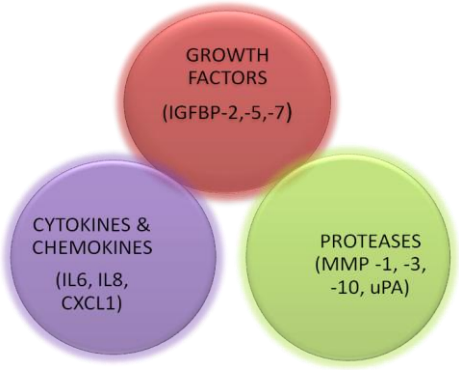

Figure 23. Major components of SASP.
Complex and versatile SASP consists of three major groups of factors: cytokines and chemokines, growth factors and extracellular matrix-modulating proteases.

Immunomodulatory cytokines and chemokines, with the most prominent ones being IL6, which has a wide variety of different signaling functions (Lu et al. 1992; Lu et al. 1996; Ancrile et al. 2007; Kuilman et al. 2008); IL8 which has an auto- and paracrine activity (Sparmann and Bar-Sagi 2004; Acosta et al. 2008) and GRO-1 (Yang et al. 2006). Additionally, IL6 is regulated by IL1α and in turn controls IL8 and has an extensive signaling network.

1) Growth factors - among which the most important ones are insulin-like growth factor binding proteins - IGFBPs (-2, -3, -4, -5, -6, -7)(Kim et al. 2007; Kim et al. 2007; Wajapeyee et al. 2008), connective tissue growth factor (CTGF) that promotes angiogenesis and tumorigenesis in prostate tissue (Yang et al. 2005), as well as VEGF (vascular endothelial growth factor) (Coppe et al. 2006) and HGF (hepatocyte growth factor) (Ohuchida et al. 2004).

2) Proteases such as matrix metalloproteinases, among which are collagenase-1, stromelysin-1 and -2 (MMP-1, -3, and -10, respectively), that modulate the extracellular matrix and can promote tumorigenic growth (Parrinello et al. 2005; Liu and Hornsby 2007) but also, due to their proteolytic function regulate other SASP factors (McQuibban et al. 2002). Other class of serine-proteases includes urokinase- and tissue-type plasminogen activators (uPA and tPA), uPA receptor (uPAR) and inhibitors of these serine-proteases (PAI-1) (Kortlever et al. 2006) which regulate plasminogen activation pathway.

5.1.2. Non-proteomic composition

Senescent cells have been associated with an increase in the release of exosome-like microvesicles that can carry immuno-modulatory proteins (B7-H3 protein) and genetic information in the form of exosomal shuttle RNA (Lehmann et al. 2008). Several other interesting non-protein molecules, present in an increased fashion in senescent cells and found elevated in aged tissue are for example nitric oxide (NO) and reactive oxygen species (ROS) (Lee et al. 1999; van der Loo et al. 2000). These biologically relevant molecules, formed due to differences in activities of nitric oxide synthase and superoxide-dismutase, could significantly affect the environment of senescent cells.

Recently, a detailed comparative metabolic and bioenergetics study (Quijano et al. 2012), revealed a large panel of metabolites, increased in senescent cells with particularly altered levels and synthesis of components of lipid metabolism. Differences in the metabolites were also observed between OIS and replicative senescence. These metabolic signatures could point to the overall changes in lipid metabolism and perhaps infer to differences in the secretome as well.

These novel discoveries further support the notion that senescent cells, can through their versatile secretory profile, affect and alter their microenvironment in perhaps ways even more complex than we currently anticipate. Proteins secreted from senescent cells have been studied more extensively than the other types of molecules, but the multifaceted nature of SASP has yet to be fully examined, with possible surprises still to come.

5.2. Regulation of SASP formation

Although SASP is not a compulsory component of senescent cells (Coppe et al. 2011), it is a key mediator of senescent cell signaling in the tissue. This secretory phenotype is a consequence of the

modified transcriptome of a senescent cell and several pathways are responsible for its formation. The two main regulators are C/EBPβ and NFκB which in turn can be regulated by variety of molecules and signals.

In general, it appears that DNA-damage response (DDR) is the prerequisite for the production of SASP (Rodier et al. 2009) with NFκB signaling being activated via ATM (Fig. 24). In support of this, downregulation of ATM or other cell cycle control factors (NBS1, CHK2) prevents SASP formation.

Some cell cycle regulators seem not only to be dispensable for SASP formation, such as p16, (Coppe et al. 2011), but actually act as a molecular brake, as their downregulation leads to increased production of secreted factors, like in the case of p53 (Coppe et al. 2008).

Several other factors have been shown to play a role in the upregulation of specific secreted factors. p38 MAPK signaling regulates IL8 production in MEK-induced senescence (Acosta et al. 2008). In addition, IL1α (Orjalo et al. 2009) and two microRNAs (miR-146a/b) (Bhaumik et al. 2009) have also been described as regulators of IL6 and IL8, and it seems that they also act through NFκB either by its repression (miR-146a/b) or activation (IL1α). Additionally, non-coding RNAs have been recently implicated in the induction of DNA-damage and OIS via DDR activation (Francia et al. 2012)

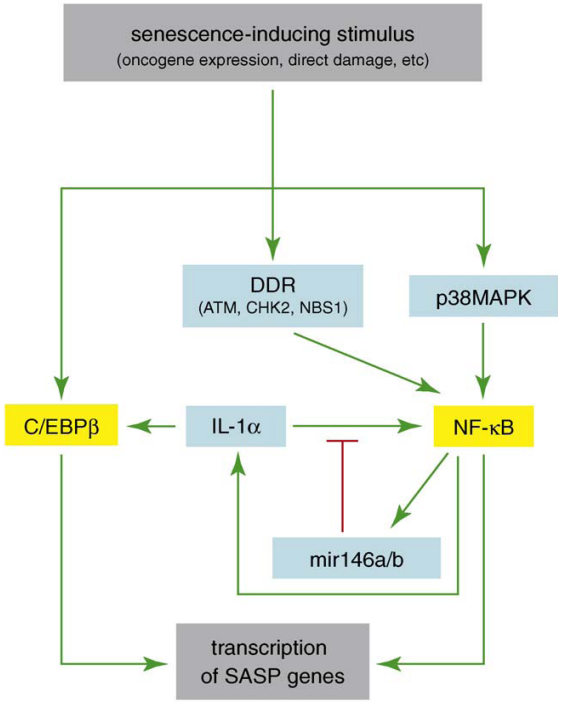

Figure 24. Signaling network responsible for SASP formation

Multiple factors are involved in regulation of SASP, with a major role of C/EBPβ and NFκB.

Adapted from Freund et al., 2010.

5.3. SASP signaling

It is important to distinguish between the cell-autonomous and cell non-autonomous functions of the senescence secretome. While cell-autonomous functions of SASP are both enabled and displayed within senescent cells, and essentially represent autocrine signaling of SASP, the non-autonomous, paracrine actions of SASP involve various other cell types, such as normal (primary) cells, pre-transformed and transformed cells. Importantly, the final outcome of SASP signaling is ultimately determined by the type of target cell and its molecular composition, and the same signaling molecules can lead to very different consequences.

5.3.1. Autocrine (cell-autonomous) action of SASP

Senescence-associated secreted factors can affect these senescent cells themselves in an autocrine mode of action, usually leading to reinforcement of senescence. This observation has been made with several different cytokines IL6 (Kuilman et al. 2008), IL8 (Acosta et al. 2008), a serine protease inhibitor PAI-1 (Kortlever et al. 2006) or an insulin-like growth factor, IGFBP5 (Kim et al. 2007) and IGFBP7 (Wajapeyee et al. 2008), although there is some controversy relating to the last case (Wajapeyee et al. 2008; Schrama et al. 2009; Scurr et al. 2010).

Autocrine signaling is necessary and in many cases sufficient for the maintenance of senescence. In this way, a positive feedback loop is established, increased amounts of signaling molecule are present over time and senescent cells are able to sustain their own presence. Blocking the autocrine signaling of specific SASP factors led to escape of senescence (Acosta et al. 2008; Kuilman et al. 2008). It is important to add that besides their role in maintaining the senescent state, the same factors signal to the “normal” neighboring cells.

5.3.2. Paracrine (cell non-autonomous) action of SASP

In this type of signaling senescent cells affect neighboring cells that are usually normal, pre-transformed, fully transformed or even cells of the immune system. The effect of senescent signaling in this case can be either anti-tumorigenic or pro-tumorigenic, depending on multiple factors.

- **Pro-tumorigenic signaling of secreted factors**

Several publications have shown that senescence-secreted molecules affect some transformed or pre-transformed cells by promoting their tumor-specific characteristics. Some of the pro-tumorigenic characteristics that are augmented by SASP include increased proliferation (Krtolica et al. 2001) and the early growth of xenografted tumors (Liu and Hornsby 2007). Other characteristics include increased angiogenesis (Coppe et al. 2006), anchorage-independent growth (Rinehart et al. 1999), invasiveness and promotion of Epithelial to Mesenchymal Transition (EMT) (Fig. 25) (Coppe et al. 2008), chemoresistance (Canino et al. 2011) and the loss of tissue structure and function (Parrinello et al. 2005), which could lead to other non-cancer related consequences, usually seen in aged tissue.

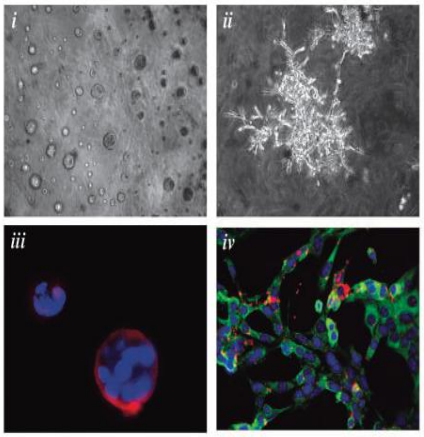

Figure 25. Senescent secreted factors stimulate pro-tumorigenic traits of pre-malignant cells.
i and *ii* - Morphology of SCp2 cancer cells before and after being exposed to SASP signaling.
ii and *iv* - SCp2 cells show EMT like changes due to SASP signaling (decrease of cytokeratin- red, increase of vimentin-green). *Adapted from Parinello et al., 2004.*

- **Anti-tumorigenic effect of secreted factors**

One could consider the senescence-activated immune-clearance of tumor cells as the anti-tumorigenic function of the SASP. As mentioned before, from the initial microarray analysis it was clear that the senescence-secretome resembles the inflammatory signaling network, especially that present during wound healing (Shelton et al. 1999). Recent reports have shown that the secretory profile of senescent cells not only contains immunomodulatory components, but that indeed *in vivo*, it can activate the immune system. This leads to clearance of senescent cells themselves and also leads to eradication of the tumor present in the tissue.

This phenomenon has been observed in many examples; one of the most compelling results was reported by Lowe and colleagues (Xue et al. 2007), where upon doxycycline stimulated p53 reactivation in murine liver carcinomas senescent cells are formed and the secreted factors attracted macrophages, neutrophils and NK cells. The presence of these immune cells was rapidly detected in the tissue and it lead to complete tumor clearance within days of initial p53 activation (Fig. 26).

Tumor regression after p53 restoration by Cre-loxP based system, was also seen with other types of tumors (Ventura et al. 2007) and while it was a consequence of induced apoptosis in lymphomas, senescence induction was observe

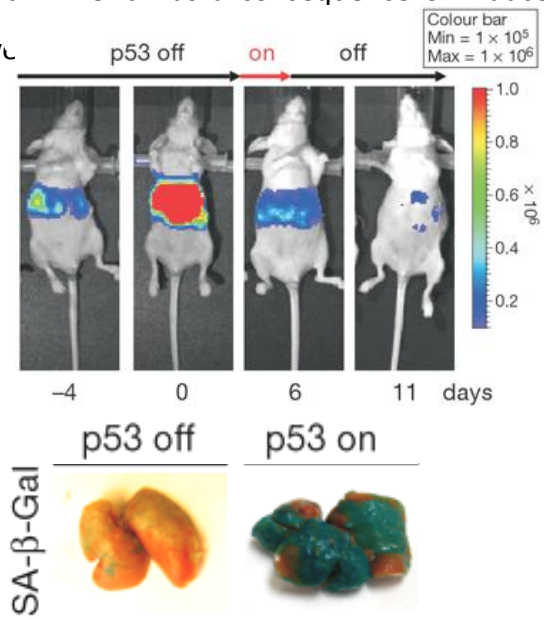

Figure 26. Tumor clearance due to SASP formation.
Senescence that develops in murine liver carcinoma after p53 reactivation, leads to complete tumor clearance within days. *Adapted from Xue et al., 2007.*

Similarly, senescent cells can protect against other cancer non related pathologies. Liver damaging agent induced fibrosis and senescent cell formation, while more severe cirrhosis was observed in p53 $^{-/-}$; INK4a/ARF $^{-/-}$ double knock-out mice, incapable of senescence. Thus, senescence formation restricted fibrosis and in addition led to fibrosis reversion by NK cells activation and immune surveillance (Krizhanovsky et al. 2008).

5.3.3. The context dependent SASP signaling

It is important to stress that the consequence of the SASP signaling is determined by the target cell. Cells, depending upon their repertoire of receptors and signaling pathways, would differentially

process this signaling. This general concept, clearly seen in the case of TGFβ–activated signaling of cells at different steps of transformation (Massague 2008), could also be well applied to SASP signaling.

In one case, described by Peeper and colleagues (Kuilman et al. 2008), IL6 secreted from senescent cells could differently affect three cell types. Firstly, in an autocrine manner it promotes maintenance of senescence and by paracrine signaling it promoted growth arrest of normal fibroblasts and proliferation of transformed cells (Fig. 27).

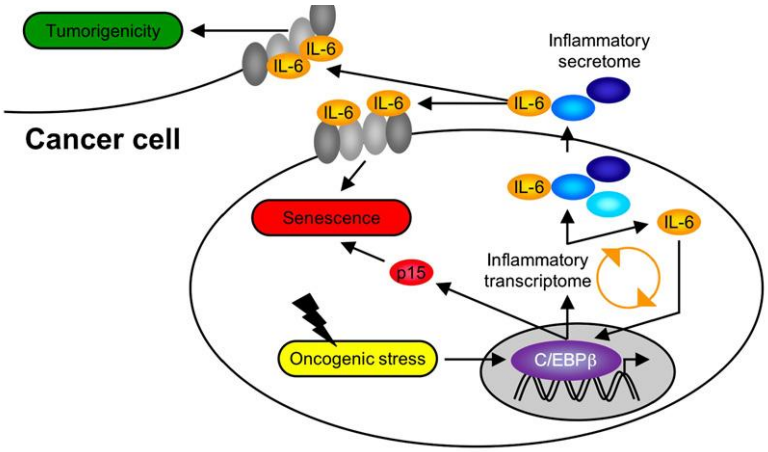

Figure 27. Context-dependent IL6 signaling.
IL6 secreted from senescent cells can induce or reinforce senescence or stimulate proliferation, depending on the type of the cell that receives the signal. *Adapted from Kuilman et al., 2008.*

Another example of context-dependent signaling is seen when, once secreted from normal fibroblast, IL6 can efficiently stimulate proliferation of melanoma cells from metastatic lesions, but inhibits the growth of those from primary lesions (Lu et al. 1992).

6. Senescence *in vivo* and its role in aging

Many reports indicate that senescence comes with a "Janus face" where in the beginning it prevents tumors from forming but later on, through its secretory profile, it serves as a tumor-promoting factor. Evolutionary these kinds of mechanisms that have a positive effect on survival in early life, yet a hazardous effect that appears after the progeny formation, are known as "antagonistic pleiotropy".

6.1. Senescence as a tumor suppressive mechanism

Senescent cells are formed once a cell has been extensively damaged and these cells are removed from the replicating pool, so the organism is essentially protected from their hazardous proliferation and in final instances cancer development. Several lines of evidence indicate that senescence can be viewed

as a potential barrier to tumor development and a tumor suppressive mechanism, together with apoptosis (Campisi 2001; Coppe et al. 2010).

First, most common mutations found in cancers are the mutations in tumor suppressor genes INK4a/p16 and p53, which are essential for induction of senescence (Shay et al. 1991; Beausejour et al. 2003). Second, a number studies in mice and humans showed that senescent cells are found in sites of benign lesions (Braig et al. 2005; Chen et al. 2005; Collado et al. 2005; Michaloglou et al. 2005) and oncogene overexpression in normal cells leads senescent cells induction. The essentially tumor protective role of senescence was confirmed in cases where abrogation of senescence resulted in tumor development or its augmentation (Braig et al. 2005; Chen et al. 2005) and similarly, reactivation of senescence in transformed cells led to tumor reduction (Ventura et al. 2007).

6.2. Senescence and aging

It seems likely that senescent cells accumulate in organisms with age, due to the gradual shortening of telomeres and increased duration to acquire damaging insults. The first connection between senescence and aging was made on the cellular level, but the molecular mechanisms that govern the process of cellular aging could also be responsible for the overall aging observed in tissue and organisms. The connection between senescence and aging has been emphasized by the fact that senescent cells accumulate in mitotic tissue of humans (Dimri et al. 1995), and other primates (Herbig et al. 2006; Jeyapalan et al. 2007) and that they are found at sites of age-related pathologies such as atherosclerosis (Minamino et al. 2002; Foreman and Tang 2003) and osteoarthritis (Price et al. 2002). At these sites, SASP could play an important role in tissue remodeling through its matrix-modulating enzymes and other signaling molecules, in the observed age-associated phenotypes. Previous observations that reactivation of telomerase in mice led to reversion of tissue degradation and other degenerative traits (Jaskelioff et al. 2010) were confirmed in a recent study where removal of INK4a/p16 overexpressing senescent cells led to delay of aging-associated disorders in the eye, muscles and fat tissue (Baker et al. 2011). Thus, senescent cells if not directly causing, at least contribute to development of these age-associated traits. Additional support has come from a recent publication where AAV (adeno-associated virus)-based TERT expression in adult mice, led to overall beneficial effects, increased their lifespan without affecting the incidence of cancer and reduced several biomarkers of aging, such as osteoporosis and insulin sensitivity (Bernardes de Jesus et al. 2012).

Objectives of the thesis

The senescencence-associated secretory profile (SASP), also known as Senescence Messaging Secretome (SMS), has been extensively studied in terms of its composition, regulation and signaling consequences. Studies performed have mostly focused on the pro-tumorigenic role of its components in various settings and have shown that it can enhance and support multiple pro-tumorigenic traits. However, none of them explored the possible effects of SASP on TRAIL sensitivity, yet another tumor cell-specific trait.

TRAIL, a naturally existing ligand, represents a powerful apoptosis inducer and specifically targets fully transformed cells while sparing the rest. Thus, it is recognized as a promising anti-cancer therapeutic agent. The potential modifications of TRAIL sensitivity in the tumor and normal cell compartments as well as in the intermediate populations of pre-transformed cells, could lead to important therapeutic consequences. Previous studies showed that SASP can promote tumor-specific characteristics of pre-malignant and malignant cells. Our goal was to explore if SASP/SMS could affect TRAIL responsiveness and would suffice for the acquisition of TRAIL sensitivity by pre-transformed cells of the stepwise tumorigenesis model system.

Our results, indeed, show that senescence-secreted factors specifically sensitize pre-transformed cells but not other cell types to TRAIL-induced apoptosis and that the sensitization is mediated through Myc-$FLIP_L$ axis. Further dissection of the senescence secretome and characterization of the factor(s) responsible will reveal the signals that lead to the activation of the TRAIL sensitivity in pre-malignant cells. The knowledge derived from studies of the TRAIL-sensitization by factor(s) of the senescence secretome may lead to novels therapeutic paradigms for intervention at benign tumor stages.

Appendix I: Manuscript

Discussion

Discussion

1. Synergy of cell types and interactions in cancer

The complexity of cancer is not only based on the diverse underlying genetic and epigenetic changes, but also on the observation that multiple cell types are present and functionally involved in its growth and progress. Cancer cells, cancer-associated fibroblasts, cells of the immune system, endothelial cells and importantly senescent cells mutually interact together with the extracellular matrix (ECM), creating a specific microenvironment, affecting cancer development and growth (Fig. 28). In the interaction between tumor cells and their environment, both sides shape and modulate each other via a plethora of signals. As our understanding of the organization and function of these cell types in a tumor context increases we start to see the resemblance of organ structure and function (Hanahan and Weinberg 2011).

On a similar note, it also leads to the idea that tumor development occurs through mutual co-evolution of tumor cells and their microenvironment (Sung et al. 2008; Polyak et al. 2009)

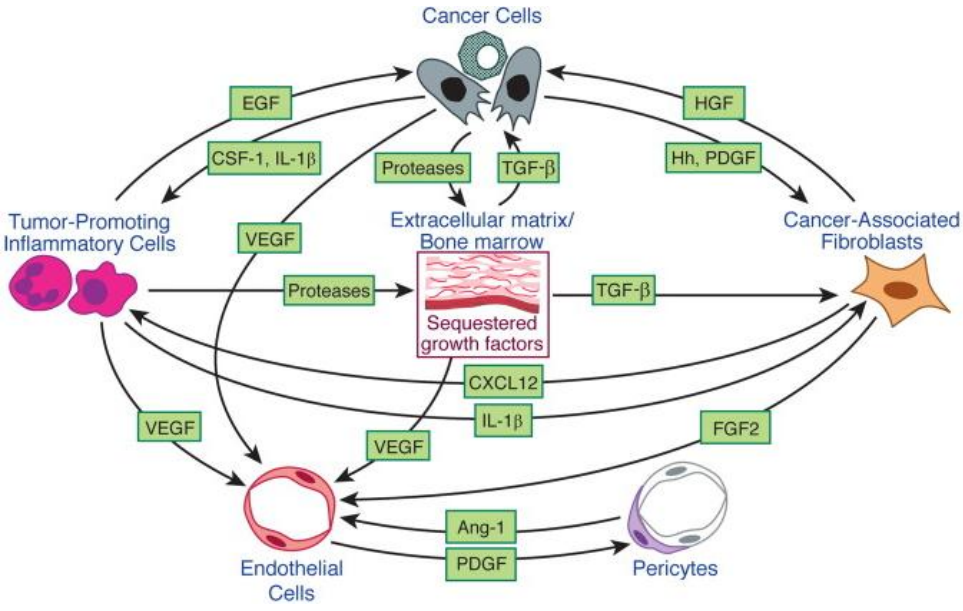

Figure 28 Complexity of signaling between different cell types within cancer.
Crosstalk of multiple signals from various cell types present within the cancer tissue modulate its growth and invasion.
Adapted from Hanahan and Weinberg, 2011.

Several studies show the decisive role of the local environment, in the growth of the initially identical populations of cells (Dawe et al. 1966; Barcellos-Hoff and Ravani 2000; Hayward et al. 2001). Tumors also secrete factors that enable their own growth by modulating the paracrine action of the neighboring cell types. In the case of IL6 secreted from the Ras-driven tumors, although the direct target of the IL6 paracrine signaling is unknown, the final endothelial cell effectors are found in increased number leading to enhanced tumorigenesis. Blocking IL6 in these settings has been proposed as a therapeutic approach (Ancrile et al. 2007).

For quite some time it is clear that secretome of the fibroblasts adjacent to tumors, termed cancer-associated fibroblasts (CAFs), powerfully enhances cancer growth. These cells can originate locally or from epithelial cells after EMT or even from bone marrow derived cells (BMDC) (Anderberg and Pietras 2009). Their formation is supported either by paracrine action of locally present transformed cells and secreted factors such as PDGF (Anderberg et al. 2009) and TGFβ (Vaughan et al. 2000); or by processes of immune response and irradiation (Barcellos-Hoff and Ravani 2000). Once generated, CAFs form their own GF-rich secretome, which supports tumor growth (Orimo et al. 2005). In some cases the exact mechanism of action has been delineated (Bhowmick et al. 2004) and now forms the basis for novel cancer-therapeutic approaches (Gonda et al. 2009).

Another important component of cancer reactive stroma that has received increasing attention, are the senescent cells. These cells express a secretome highly similar to the one present during wound healing (Shelton et al. 1999) and resembles that of CAFs (Campisi 2005). It is thus not surprising that several reports show a pro-tumorigenic role of senescent cells both *in vitro* and *in vivo* (Lu et al. 1996; Krtolica et al. 2001; Parrinello et al. 2005). Contrastingly, process of senescence and the SASP also has well established anti-tumor functions (see Introduction “The pro- and anti-tumorigenic action of SASP”). Thus, a precise understanding of the interactions between the cancer and the senescent cells is important for their exploitation in cancer therapies.

2. Anti-tumorigenic potential of SASP

Tumor cell specificity is a hallmark of TRAIL activity, however it comes with limitations. Not all tumor cells are TRAIL sensitive and the inability of TRAIL to induce apoptosis in other cell types like the pre-transformed cells could lead to tumor recurrence and therefore, diminish its potency.

We observed that senescent cells, through their secretome, are able to specifically sensitize pre-transformed cells and some TRAIL insensitive cancer cell lines to TRAIL-induced apoptosis in cell culture conditions (Fig. 1 and Fig. A1). Thus, we show for the first time that pre-transformed cells can be rendered sensitive to TRAIL apoptosis. This suggests that SASP secreted factors would not harm or alter normally existing cells in the tissue, but due to the preexisting differences in their genetic makeup, selectively target the pre-transformed and/or TRAIL insensitive transformed cells. This fits well with the context-dependent SASP signaling previously mentioned and observed in many cases (Lu et al. 1992; Kuilman et al. 2008).

One of the direct mechanisms of the anti-tumorigenic action is the ability of SASP to arrest cell proliferation and induce senescence (Acosta et al. 2008; Kuilman et al. 2008). Secondly, in some settings specific components of the SASP have been shown to activate the immune signaling and recruit specific components of the immune system, which leads to clearance of senescent and tumor cells from the tissue (Xue et al. 2007; Kang et al. 2011). Our data reveal a novel mechanism of the anti-tumorigenic signaling of SASP that affects several potentially harmful cell populations. This is distinct from the other two anti-tumorigenic actions of SASP, as the senescence induction via SASP seems to be reserved for normal cells and immune clearance seems to occur only in fully formed tumors.

There are several ways in which our findings can complement the understanding and application of tumor-senescence interaction.

A. Senescence within tumors

Utilization of SASP in any type of treatment would require the senescent secretome either to be produced in the vicinity of the targeted cells or the specific factor(s) to be exogenously administered. In fact, senescent cells are already found within or in the immediate vicinity of tumors due to several distinct mechanisms. First, multiple evidence show that senescent cells spontaneously form an initial barrier to the tumor development in many different tissues in response to diverse initial stimuli (Braig

et al. 2005; Chen et al. 2005; Collado et al. 2005; Michaloglou et al. 2005). Secondly, as will be discussed in more detail later, many classical anticancer treatments like chemotherapy and irradiation commonly induce senescence within tumor cells.

Senescent cells can also be artificially induced in tumors via tumor suppressor reactivation (Ventura et al. 2007), loss of second tumor suppressor (Chen et al. 2005), or by the use of multiple chemical inhibitors (Acosta and Gil 2012). These circumstances that position senescent cells in immediate vicinity of tumors and importantly pre-neoplastic cells (PINs and nevi) make the therapeutic use of SASP realistic.

B. Therapeutic implications

1) Combining Therapy-induced senescence (TIS) and TRAIL treatment

Even though cancer cells have activated mechanisms to evade senescence (Beausejour et al. 2003), many of them can still be induced into senescence either by transfection of growth-inhibitory genes p53, p63, pRb, p21, INK4a/p16 into cancer cell lines (Roninson 2003). More promising than these, are various agents commonly used in cancer treatments, chemotherapy or ionizing radiotherapy. Besides their genotoxic effects that lead to apoptosis, these treatments are known to induce senescence (termed “therapy induced senescence”, TIS) within a tumor cell population (Wang et al. 1998; Chang et al. 1999; Michishita et al. 1999; Elmore et al. 2002; Han et al. 2002). Importantly both of these mechanisms, apoptosis and senescence, contribute to efficient anti-cancer treatment (Schmitt et al. 2002). Senescence induction in tumors could be used to treat cancers (Alimonti et al. 2010) and the ability of cancer cells to go into senescence upon chemotherapy can be used as prediction marker for the treatment outcome (Haugstetter et al. 2010). In breast cancer patients that have received chemotherapy TIS has been detected *in vivo*; importantly SA-β-Gal staining was confined to tumor cells and the rest of the tissue remained unaffected (te Poele et al. 2002).

The senescent cells produced via TIS exhibit the characteristics of regular senescent cells (see *Fig. A2*). Our results offer a new treatment perspective by combining the well described mechanism of TIS with TRAIL treatment. Senescent cells formed from the tumor cells would provide necessary TRAIL sensitizing factor(s) to their neighboring tumor cells and addition of TRAIL to these mixed populations could enhance the tumor clearance and lead to better treatment outcome.

2) Combining SASP factor(s) and TRAIL

An alternative therapeutic potential of our discoveries is the exogenous application of the senescent factor(s) responsible for sensitization in combination with TRAIL itself, or perhaps design of improved novel TRAIL receptor-activating compounds. This would enable us to broaden the targets of TRAIL therapy and for example, to design TRAIL-based therapies for pre-transformed cells for the first time.

Many additional tests need to be performed both *in vitro* and importantly *in vivo* before this possibility is even considered. One of the most important aspects of such screens would be to dissociate pro- and anti-tumorigenic aspects of SASP and its factors. As we have seen so far the total SASP does sensitize cells to TRAIL and leads to increased apoptosis of pre-transformed cells, but it also supports other, essentially pro-tumorigenic traits, such as tumor formation and EMT *(Fig. S2 and Fig A8).* Once the responsible senescent secreted factor(s) is identified, testing each of these properties separately will enable us to dissociate these effects.

The TRAIL-sensitizing factor(s) could be exogenously applied for improved TRAIL treatment. The results obtained so far imply that the TRAIL-sensitizing factor seems to be highly stable, as the activity of the compound seemed to be unaffected by several freeze-thaw cycles and active, which makes it an attractive candidate for therapeutic use.

3) Predicting TRAIL sensitivity of a tumor

Previous observations associate stroma-related signaling to certain forms of chemotherapy resistance (Farmer et al. 2009) and similar resistance-predictive correlations have been made for some components of CAF secretome and specific therapeutic approaches, such as anti-VEGF treatment (Crawford et al. 2009) and receptor tyrosine kinase inhibitors (Wang et al. 2009). In a similar way, the observation we have made about the anti-tumorigenic action of SASP, by sensitization of (pre)transformed cells to TRAIL-induced apoptosis, offers an opportunity to use senescence-specific signaling within a tumor and its specific paracrine signaling as a predictive marker for the success of TRAIL therapy. The capability of tumors to generate senescent cells has already been used as a negative marker for lung cancer reoccurrence (Sporn et al. 2009) or a positive marker for the treatment outcome in patients (Haugstetter et al. 2010).

Additionally, in the case of pro-tumorigenic CAF signaling, once the transducers of tumor growth were identified, they also became the targets of novel therapeutic design that provided more effective

chemotherapy, as in the case of PDGF inhibition (Pietras et al. 2002). It is possible that once we identify the factor(s) responsible for the TRAIL sensitization, prediction will be more direct, and importantly, druggable. As we have not seen differences in the TRAIL-sensitizing action of various types of senescent cells (Replicative, OIS, Etoposide-induced; see *Fig. 1D* and *Fig. A2C*) it would seem irrelevant whether the senescent cells are spontaneously present in the tumor or are generated as a consequence of previously mentioned mechanisms of irradiation and chemotherapy. Their presence or the percentage of the senescent cells in the tumor could be a positive marker for TRAIL therapy.

C. TRAIL signaling in the immune system?

As previously mentioned, senescent cells secrete factors that lead to senescent or malignant cell removal, and in this context some of the studies give more importance to the elements of the innate immune system (Xue et al. 2007); while others show that the clearance is a CD4+ dependent mechanism, with a necessary presence of macrophages and monocytes (Kang et al. 2011). Indeed, the mechanisms regulating senescence surveillance are only partially understood. It would be interesting to see if the signaling that we describe, sensitization to TRAIL and apoptosis execution, might be in any way involved in these processes. TRAIL is a naturally existing ligand produced by several types of cells of the immune system (NK, macrophages, dendric cells and T lymphocytes among others) (Zerafa et al. 2005), and is involved in the naturally occurring immunoregulatory functions, including antimetastatic function in mice (Takeda et al. 2001; Cretney et al. 2002). One could imagine that senescence is probably not simultaneously induced in all tumor or pre-transformed cells and that perhaps senescent cells formed signal to their neighboring pre-transformed or tumor cells, rendering them TRAIL sensitive and causing their removal from the tissue. Thus, the mechanism that we describe could already exist within the interactomes of senescent, (pre-) transformed and immune cells. This hypothesis would of course need further supporting evidence.

3. Understanding and circumventing the mechanisms of TRAIL resistance

The action of several different therapeutic approaches and how they fit with the central paradigm of cancer treatment has already been mentioned in detail (see Introduction – Principles of cancer

therapy). In the quest for the most specific and least toxic therapeutics, TRAIL has attracted attention due to its potent and specific mechanism of action (Gonzalvez and Ashkenazi 2010).

Even though TRAIL is selectively active in tumor tissue, not all cancer cells respond to TRAIL treatment or at least not to the same extent. Cases of TRAIL resistance are common in cancer cell lines and different types of tumors, due to multiple levels at which it can be regulated. Some studies suggest that the expression levels of DR4 and DR5 do not play a role in determining the final response to TRAIL treatments and novel parameters like levels of O-glycosylation enzyme (Wagner et al. 2007) are given more attention. The role of Myc oncogene and its downstream target FLIP have also been given a direct role in predicting the TRAIL sensitivity of a tissue (Ricci et al. 2004; Bangert et al. 2011). Interestingly, our data with the stepwise transformation model system support their importance, showing that transitory increase in Myc levels and downregulation of FLIP that play a crucial role in sensitization to TRAIL.

With the undeniable potential of its application the question remains how can TRAIL action be further improved? In fact, there are two basic types of TRAIL resistance observed. First is a primary resistance and in this case cancer cells are resistant to TRAIL since their formation. In the second case, tumor cell resistance to TRAIL is acquired secondarily (Zhang and Fang 2005).

A. Blocks that can be removed

It would be worth investigating whether both categories of non-responsive cells can be sensitized to TRAIL via SASP action.

An even more important aspect would be to delineate which molecular players responsible for TRAIL resistance can be altered by SASP action. So far, we have recognized the MYC-$FLIP_L$ signaling as the main axis of SASP activity in sensitization of pre-transformed cells to TRAIL. This would suggest that potentially in all other types of TRAIL non-responsive cells in which FLIP represents the main block to successful TRAIL signaling, SASP could reverse TRAIL resistance.

Furthermore, other factors involved in TRAIL signaling such as DR5, that are direct targets of Myc repression (Wang et al. 2004) could also be affected by SASP. If SASP efficiently leads to transitory increase of multifaceted transcription factor- Myc, it would be of great importance to explore the other Myc-related functions and their potential changes in association to SASP action. This would increase the number of cases in which SASP-action would be possible. Additional insight into how the SASP

signal is transmitted to the cell and the initial steps of the SASP signaling with the type of the receptor necessary, would give us better understanding of the requirements for the efficient SASP signaling.

B. Mechanism of SASP action

The question remains whether the mechanism we observed via the Myc-$FLIP_L$ axis is the only "TRAIL-sensitizing" action of SASP? With its complex composition, activation of several simultaneous mechanisms would not be surprising. Our results, using chemical inhibitors, implicate p38 signaling in cell sensitization to TRAIL (Fig. 4), with this action seemingly unrelated to Myc. Whether these two mechanisms are in any way related will be interesting to investigate further. Perhaps p38 signaling due to SASP factors is present and responsible for sensitization to TRAIL in a larger set of cell types. In that way, the involvement of p38 would not only lead to CMS effects in pre-transformed cells, but perhaps plays a role in determining the TRAIL responsiveness in the panel of cancer cell lines used and the other members of the stepwise system.

Additionally, while the cells of the transformed stepwise system show a clear correlation between the Myc and $FLIP_L$ levels present and their sensitivity to TRAIL, we could not efficiently sensitize immortalized (BJEH) cell by $FLIP_L$ downregulation, as we did in the case of pre-transformed cells *(Fig. A3)*. While this could be a consequence of different $FLIP_L$ levels both initially present and obtained after transfections, we could not exclude the existence of other TRAIL- resistance mechanisms in immortalized cells that perhaps involve defective p38 signaling. As these cells could also not be sensitized to TRAIL-induced apoptosis by SASP, they could probably possess critical determinant(s), which cannot be overcome by SASP action.

4. FLIP – an important therapeutic target

With its crucial role as a regulator of death ligand induced-apoptosis, FLIP represents a promising therapeutic target and indeed, in our experiments, downregulation of FLIP proved to be a crucial step in the sensitization of pre-transformed cells to TRAIL. A plethora of exogenous compounds (Fig. 29) have shown to be able to inhibit FLIP on both transcriptional level, namely chemotherapy, DNA damaging agents, HDAC (Kauh et al. 2010; Bangert et al. 2011) and post-translational level, for example cyclohexamide, bortezomib (Seki et al. 2010), doxorubicin and MG132 or Compound C an inhibitor of

AMP-activated protein kinase (Jang et al. 2010). The importance of the signaling cascade that we report here and its decisive role in establishing the TRAIL-sensitivity of the cells, has been emphasized in many different cancer cell lines (Ricci et al. 2004; Bangert et al. 2011; Yerbes et al. 2012), while in some cases combinatorial treatments of $FLIP_L$ downregulation are necessary. Recent work with colorectal cancer cell lines in APC deficient mice showed that only combined action of retinoic acid-induced DR4/DR5 upregulation and $FLIP_L$ downregulation made these cells TRAIL-sensitive (Zhang et al. 2010).

Inhibitors of c-FLIP and their main modes of action.

	Primary mode of action	Agent
Transcriptional	Alkylating agent	Cisplatin, oxaliplatin
	Intercalating of DNA	Doxorubicin
	Histone deacetylase inhibitor	Vorinostat, trichostatin, droxinostat, valproic acid
	Topoisomerase I inhibitor	Camptothecin, 9-NC, irinotecan
	Modulation of Ras/PI3K/NF-κB pathway	Lupeol (triterpene)
	Suppression NF-κB pathway	Celastrol, zerumbone (sesquiterpene)
		Withaferin A (steroidal lactone), quinacrine
	Decreases TNFα mediated NF-κB activation	Chrysin (flavanoid)
	Inhibition of STAT3 activation	CDDO–imidazolide – synthetic triterpenoid
Postranscriptional	RNA interference	siRNAs
	? – Phosphorylation of long form	Cisplatin
	RNA synthesis inhibitor	Actinomycin D
	Protein synthesis inhibitor	Cyclohexamide, anisomycin
	Thymidylate synthase inhibitor	Fluorouracil (5-FU)
	Proteasome inhibitor	PS-34 (bortezomib)
	Small molecule proteasome inhibitor	MG-132
	PPARγ modulating agent	Troglitazone
	Multikinase inhibitor	Sorafenib
	Antimicrotubule agent	Taxol (paclitaxel), nocodazole
	Downregulation Akt and NF-κB	Genistein (isoflavone)
	?	Silibinin (flavonoid)
	COX-2 inhibitor	Celecoxib
	?	CDDO–Me

Figure 29. Various FLIP inhibitors and their mode of action
List of chemical compounds and agents commonly used for FLIP downregulation on both transcriptional and posttranscriptional level. *Adapted from Shirley and Micheau, 2010.*

In addition, increases in $FLIP_L$ synthesis and subsequent loss of TRAIL sensitivity was shown to occur in several myeloma cell lines as a consequence of soluble secreted factor(s) from the tumor microenvironment, more specifically by myeloma stromal cells (Perez et al. 2008).

Development of novel approaches to regulate its levels could be of great therapeutic use and for this a better understanding of the determinants of FLIP regulation is necessary. Our work suggests that an administration of FLIP inhibitors in combination with TRAIL may provide improved treatment outcomes.

5. Identification of a TRAIL-sensitizing SASP factor(s)

Our initial attempts to identify the TRAIL-sensitizing component(s) of SASP by specifically targeting the most prominent members of SASP, proved to be inefficient (see Fig. S1). Loss of the specific SASP component IL6, IL8, MMP, CXC did not affect the senisitization phenomenon. Further exploration showed that the pre-transformed (BJEL) cells, successfully sensitized to TRAIL-induced apoptosis, did not respond to recombinant IL6, and showed reduced levels of the IL6 receptor, compared to the control HEPG2 cell population (see Fig. S1).

This raised questions on the basic nature of the sensitizing compound(s). While we still have not identified the SASP factor(s) responsible for the observed sensitization to TRAIL, our study identified certain relevant features of the component(s). In coherence with previously observed results that none of the major SASP factors are involved in the process, we observed that the component is a molecule of low molecular weight (smaller than 3kDa), not of polypeptide nature and is thermostable. Further, it seems that the factor is highly hydrophilic as it does not bind to various reverse phase matrices.

The majority of the results obtained so far suggest that the sensitizing factor is a highly polar molecule with biological activity and signaling properties. Phospholipids represent one of the most interesting classes of molecules that would satisfy all these criteria. This group is composed of many molecules such as phosphatidylcholine (Lecithin, PC), phosphatidylserine (PS), various phosphoinositides, sphingomyelin (SM), ceramide, sphyngosine and their derivatives, with important roles in cell growth, apoptosis, senescence and inflammation (Hannun and Obeid 2008). Ceramide signaling seems to be specifically interesting and some of its protein interactors have been recognized, such as Ser-Thr phosphatases PP1 and PP2A (Chalfant et al. 2004). In comparison to other phospholipids and in contrast to both quiescent cells and normal cells, ceramide levels were found specifically increased in senescent cells (Venable et al. 1995). Additionally, exogenous ceramide was a potent senescent inducer (Venable et al. 1995; Venable and Yin 2009), although in some cases it rather increases chemo-sensitivity and apoptosis while promoting escape from senescence (Modrak et al. 2009). Some forms of ceramide can induce apoptosis through DNA fragmentation on their own (Obeid et al. 1993), or can regulate processes of TRAIL-induced apoptosis (White-Gilbertson et al. 2009). Additional metabolomic studies are necessary to identify the factor(s) responsible and whether it belongs to a class of lipids or another category of bioactive polar molecules.

Perspectives and Conclusion

Perspectives and Conclusion

W have identified a novel mechanism of action of senescence secreted factors which sensitizes pre-transformed cells to TRAIL apoptosis. This permits the extension of the apoptotic repertoire of TRAIL to these normally unaffected cells. Further, the SASP can also be used to sensitize other TRAIL resistant cancer cell lines. This important finding has implications in developing novel molecular targets. The two main future directions of the study are to confirm our observations of TRAIL-sensitizing action of the SASP *in vivo*, and identify and potentially therapeutically apply the senescent secreted factor(s) responsible for this action.

Our study of the SASP sensitization to TRAIL-induced apoptosis covers a relatively small subset of pre-transformed cells. A systematic collection and testing of cells from adenomas, papilomas and other hyperproliferative sites (such as prostate intraepithelial neoplasias, PINs) that are the *in vivo* equivalent of pre-transformed cells in our stepwise model system, would improve our understanding of the signaling. These naturally existing pre-transformed cells are currently not targeted by TRAIL-based cancer treatments and the synergistic action of SASP and TRAIL could offer the opportunity for their elimination. The real challenge would be the demonstration of its effects *in vivo.* Our pilot studies suggest that senescent cells are able to stimulate the *in vivo* proliferation of pre-transformed cells of the stepwise system (Fig. S2), in accordance with the previously described action of SASP (Krtolica et al. 2001). The next phase would be to study the combined action of SASP and TRAIL in a therapeutic context and assess their joint contribution and the prevailing outcome ie. does SASP render these tumors sensitive to TRAIL apoptosis?

The additional line of work is the identification of the mediator(s) of the reported TRAIL-sensitization. Our results show that this small, highly hydrophilic compound is not a polypeptide, which makes its identification rather difficult as only the protein composition of the SASP has been explored in significant detail. Without identifying the specific component(s) it will be difficult to transfer the findings to the clinical use and future work in those terms will be based on metabolomic and specifically lipid studies. Many small molecules have been shown to act in a powerful manner and recently in the senescence field more attention has been given to the non-protein fraction of SASP. These discoveries hold a great deal of therapeutic potential and in the years to come we will see if their application becomes reality.

Appendix II: French abstract

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Effet des facteurs sécrétés par les cellules sénescentes sur la transformation néoplastique et la sensibilisation à TRAIL

Résumé

Malgré la complexité du processus de transformation, plusieurs systèmes modèles ont été développés dans lesquels les cellules normales se transforment d'une manière progressive par l'introduction d'éléments génétique. Ici, seules les cellules transformées rentrent en apoptose induite par TRAIL. Comme le milieu des cellules sénescentes a déjà été impliqué dans certaines caractéristiques tumorales (la prolifération, l'invasion et d'autres), notre objectif était d'évaluer son effet potentiel sur l'acquisition de la sensibilité à TRAIL. Lorsque toutes les cellules de ce système de transformation sont incubés avec le milieu conditionné de cellules sénescentes (CMS) la sensibilisation à TRAIL a été observée dans les cellules pré-transformées mais pas dans les cellules immortalisées ou normales. Ainsi, nous avons conclu que les différentes étapes de la transformation fournissent un contexte cellulaire et moléculaire particulier qui agit en synergie avec le CMS afin de promouvoir la sensibilisation à TRAIL. Ces observations mettent l'accent sur le rôle spécifique des cellules sénescentes et leur phénotype sécrétoire. Nous avons ensuite étudié les mécanismes activés dans les cellules pré-transformées par le CMS et responsables de leur sensibilisation à TRAIL. Nos résultats suggèrent un rôle clé de l'axe Myc-$FLIP_L$ dans la signalisation activée par le CMS.

Mots clés : cellules sénescentes, sécrétome, TRAIL, apoptose, signalisation, tumeur

Résumé en anglais

Despite the complexity of the transformation process, several model systems have been developed in which normal cells are transformed in a step-wise manner by introduction of genetic elements (telomerase, SV40ER viral genes and oncogenes). In this system, only the transformed cells go into TRAIL-induced apoptosis. As senescent-secreted factors have already been involved in promoting some of the tumor characteristics (proliferation, invasion and others), our objective was to assess its potential effect on the acquisition of sensitivity to TRAIL. When all the cells of the transformation system were incubated with conditioned medium of senescent cells (CMS) the sensitization to TRAIL was observed only in the pre-transformed, but never in normal or immortalized cells. Thus, we concluded that the different steps of transformation provide a cellular and molecular context that acts in synergy with the CMS to promote TRAIL sensitivity. These observations emphasize the specific role of senescent cells and their secretory phenotype. We then studied the mechanisms activated in the pre-transformed cells by the CMS, responsible for their sensitization to TRAIL. Our results suggest a key role of the Myc-$FLIP_L$ axis in signaling activated by the CMS.

Key words: Senescence, secretome, TRAIL, apoptosis, signaling, tumor