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# **5-Fluorouracil mediated cell death signaling in colon carcinoma cells**

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## ABSTRACT

Colorectal cancer (CRC) is the third most common form of cancer in the world. 5-Fluorouracil (5-FU) belongs to the fluoropyrimidine-type of drugs and is commonly used in the treatment of several solid tumors. It has become the mainstay of CRC treatment at stage III and high risk stage II, either alone or in combination with other drugs. Primary drug effects include both DNA damage and RNA stress but the relative importance of each triggering points remains elusive.

In Paper I we investigated the signaling cascade leading to 5-FU-induced cell death in the colon carcinoma cell line HCT116 in more detail; especially, with respect to the early response and ensuing apoptosis. Upon 5-FU treatment, the death-inducing signaling complex (DISC) was formed on the plasma membrane and this process was facilitated by p53. Increase in intracellular levels of  $\text{Ca}^{2+}$ , at least partially via L-type channels, was an early response which led to phosphorylation of at least three p53 serine sites (S15, S33, S37) upstream of caspase-8. Mutational analysis concluded that S15 phosphorylation was necessary for the processing of caspase-8 and PARP. Analyses using small molecule inhibitors indicated that  $\text{Ca}^{2+}$ -dependent calmodulin served as an intermediate factor preceding p53 phospho-activation.

Altogether, obtained results present the evidence for a novel apoptotic signaling mechanism induced by 5-FU, dependent on extracellular  $\text{Ca}^{2+}$ , involving DR-DISC and regulated by p53, p53 phosphorylations and calmodulin.

In Paper II we focused on cell death signaling pathways in 5-FU-stressed  $p53^{-/-}$  cells. Using the human colon carcinoma parental cell line HCT116 and its variant lacking p53, we found that the cell death *per se* induced by 5-FU is independent of p53. However, in the absence of the tumor suppressor, the apoptotic response is delayed. In addition, the lack of p53 was associated with the formation of reactive oxygen species (ROS) in mitochondria which resulted in necrotic characteristics. Co-treatment with zVAD-fmk and 5-FU revealed that DNA damage, reflected in phosphorylation of the histone H2AX ( $\gamma\text{H2AX}$ ), is a consequence rather than a cause of apoptosis. Finally, our data suggested that silencing of PARP-1 function may be used as an approach to selectively sensitize p53-deficient tumor cells to 5-FU.

In Paper III we examined the possible crosstalk between apoptosis and autophagy upon 5-FU treatment. In contrast to cells in which apoptosis was blocked, either at the DISC or the mitochondrial level, p53 deficiency was associated with deregulation of autophagy in response to 5-FU. Disruption of lysosomal function with chloroquine (CQ) caused a profound reduction in the appearance of apoptotic markers, as a consequence of death receptor (DR) accumulation in lysosomes and autophagosomes.

Since RNAi targeting of critical regulators of autophagy or inhibition of lysosomal cathepsins reversed apoptosis in different manners, it is unlikely that autophagy *per se*, but rather correct receptor transport is an important factor for 5-FU-induced cell death. Interestingly, apoptosis activated via TRAIL, the cognate ligand for DR5, remained unaffected in the presence of CQ, indicating that 5-FU activates the receptor by a discrete mechanism. Through depletion of membrane cholesterol or inhibition of cholesterol transport, the cytotoxicity of 5-FU was drastically reduced, thereby supporting the idea that correct trafficking of the receptor is important for 5-FU-mediated elimination of cells.

In conclusion, this study indicates a novel chemotherapy-induced mechanism for activation of DR5, which may have important ramifications on research conducted in the apoptosis and tumor treatment field.

# LIST OF PUBLICATIONS

**I. Can G<sup>1</sup>, Akpinar B<sup>1</sup>**, Baran Y, Zhivotovsky B, Olsson M

(<sup>1</sup> denotes equal contribution).

**5-Fluorouracil signaling through a calcium–calmodulin-dependent pathway is required for p53 activation and apoptosis in colon carcinoma cells.**

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**5-Fluorouracil-induced RNA stress engages a TRAIL-DISC-dependent apoptosis axis facilitated by p53.\***

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Aberrant DR5 transport through disruption of lysosomal function suggests a novel mechanism for receptor activation.

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## LIST OF ABBREVIATIONS

$\Delta\psi_m$	mitochondrial membrane potential
$\gamma$ H2AX	gamma H2A histone family, member X
5-FU	5-fluorouracil
AIF	apoptosis-inducing factor
Apaf-1	apoptotic protease activating factor-1
APC	adenomatous polyposis coli
ATG	autophagy-related
ATM	ataxia telangiectasia mutated
ATR	ATM and RAD-3 related
ATP	adenosine triphosphate
AXIN1	axis inhibition protein 1
AXIN2	axis inhibition protein 2
Bad	Bcl-2 associated death promoter
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-X <sub>L</sub>	Bcl-2 related gene, long isoform
BH	Bcl-2 homology
Bid	BH3- interacting-domain death agonist
Bim	Bcl-2 interacting mediator of cell death
BRAF	B-Raf proto-oncogene, serine/threonine kinase
Ca <sup>2+</sup>	calcium ion
CARD	caspase associated recruitment domain
caspase	cysteine-dependent aspartate-specific protease
CDK8	cyclin-dependent kinase 8
cFLIP	FLICE-like inhibitory protein
Chk	checkpoint kinase
CH <sub>2</sub> THF	5,10-methylenetetrahydrofolate
CIMP	CpG island methylator phenotype
CQ	chloroquine
CRC	colorectal cancer
CTNNB1	cadherin-associated protein beta 1
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
dATP	deoxyadenosine triphosphate

DCC	deleted in colon cancer
DD	death domain
DED	death effector domain
DHFU	dihydrofluorouracil
DISC	death inducing signaling complex
DR	death receptor
DR3	death receptor 3 (TRAMP)
DR4	death receptor 4 (TRAIL-R1)
DR5	death receptor 5 (TRAIL-R2)
DR6	death receptor 6
DMEM	Dulbecco's modified eagle medium
DNA-PK	DNA-dependent protein kinase
DPD	dihydropyrimidine dehydrogenase
DSB	double strand break
dTMP	deoxythymidine monophosphate
dTTP	deoxythymidine triphosphate
dUMP	deoxyuridine monophosphate
dUTP	deoxyuridine triphosphate
dUTPase	deoxyuridine triphosphate nucleotidohydrolase
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
FADD	FAS-associated death domain
FAS	cluster of differentiation 95 (CD95)
FasL	FAS ligand (CD95L)
FBS	fetal bovine serum
FdUDP	fluorodeoxyuridine diphosphate
FdUMP	fluorodeoxyuridine monophosphate
FdUTP	fluorodeoxyuridine triphosphate
FUDP	fluorouridine diphosphate
FUDR	fluorodeoxyuridine
FUMP	fluorouridine monophosphate
FUR	fluorouridine
FUTP	fluorouridine triphosphate
FLICE	FADD-like IL-1 converting enzyme (caspase-8)
FLIP	FLICE like inhibitor protein

GTP	guanosine triphosphate
IAP	inhibitor of apoptosis protein
IKK	inhibitor of nuclear transcriptional factor kappa B
IMM	inner mitochondrial membrane
JNK	c-Jun NH <sub>2</sub> -terminal protein kinase
KRAS	Kirsten rat sarcoma viral oncogene
LDH	lactate dehydrogenase
LOH	loss of heterozygosity
MAPK	mitogen-activated protein kinase/ERK
Mcl-1	myeloid cell leukemia sequence 1
MDM2	mouse double minute 2
MLH1	mutL homolog 1
MMR	mismatch repair
MOMP	mitochondrial outer membrane permeabilization
MSH2	MutS protein homolog 2
MSH6	MutS protein homolog 6
MSI	microsatellite instability
MSI-H	microsatellite instability-high
MSI-L	microsatellite instability-low
MSS	microsatellite stable
mTOR	mammalian target of rapamycin
NCCD	Nomenclature Committee on Cell Death
NF-κB	nuclear transcriptional factor kappa B
NRAS	neuroblastoma RAS viral oncogene homolog
OMM	outer mitochondrial membrane
OPRT	orotate phosphoribosyltransferase
p14 <sup>ARF</sup>	cyclin-dependent kinase inhibitor 2A
PARP	poly (ADP-ribose) polymerase
PI	propidium iodide
PI3K	phosphatidylinositol 3-kinase
PIDD	p53-induced protein with a death domain
PLAD	pre-ligand assembly domain
PMS2	postmeiotic segregation increased 2
PRPP	phosphoribosyl pyrophosphate
PTEN	phosphatase and tensin homolog

Puma	p53-upregulated modulator of apoptosis
RAIDD	RIP associated ICH/CED3 homologous protein with death domain
RIP	receptor-interacting protein
RIP1	receptor-interacting protein 1
RR	ribonucleotide reductase
rRNA	ribosomal ribonucleic acid
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
siRNA	small/short interfering RNA
SMAD2	SMAD family member 2
SMAD4	SMAD family member 4
tBid	truncated Bid
TGF	transforming growth factor-beta
TGF $\beta$ IIIR	TGF-beta type II receptor
TK	thymidine kinase
TNF	tumor necrosis factor
TNF-R	TNF receptor
TNF-RSC	TNF-R1 signaling complex
TNM	tumor-node-metastasis
TOM	translocase of the outer membrane
TP	thymidine phosphorylase
TRADD	TNF receptor-associated death domain
TRAF2	TNF-R-associated factor 2
TRAIL	TNF-related apoptosis inducing ligand
tRNA	transfer ribonucleic acid
TS	thymidylate synthase
UK	uridine kinase
UP	uridine phosphorylase
zVAD-fmk	N-benzyloxycarbonyl-Val-Ala-Asp (O-Me) fluoromethyl ketone

# **1. GENERAL INTRODUCTION**

## **1.1 Cancer**

Cancer is one of the leading causes of death worldwide and the prevalence of the disease is increasing<sup>1</sup>. Genetic susceptibility, mutations, environmental factors, diet and life style behaviors have been associated with the risk of cancer development, but a detailed description of how and why cancer is formed remains to be established. In an attempt to describe the distinguishing properties that enable tumor growth and metastatic dissemination, Hanahan and Weinberg introduced the “ten hallmarks of cancer” in the second edition of the “millennium review”. These are sustaining proliferative signaling, resisting cell death, evading growth suppressors, inducing angiogenesis, enabling replicative immortality, activating invasion, metastasis, deregulating cellular energetics, avoiding immune destruction, genome instability and mutation and tumor promoting inflammation<sup>2</sup>.

## **1.2 Modes of cell death**

Depending on the triggering stimulus and the microenvironment, cells can die by several mechanisms including apoptosis, necrosis and autophagy. The Nomenclature Committee on Cell Death (NCCD) proposed recommendations to classify types of cell death according to their well-defined morphological characteristics and biochemical features<sup>3-5</sup>. Regulation of cell death pathways is important for embryonic development and maintenance of normal tissue homeostasis of adult organisms. In addition, dysregulation of cell death is associated with several diseases. It is therefore vital to investigate the mechanisms of cell death modalities in order to develop novel therapeutic approaches.

### **1.2.1 Apoptosis**

Apoptosis is the first described and most studied mode of cell death<sup>6</sup>. Through this programmed elimination of damaged or unwanted cells, tissue homeostasis is maintained without invoking inflammation. Dysregulation of apoptosis has been implicated in the development of several diseases, including cancer, autoimmune diseases, and neurodegenerative disorders<sup>7,8</sup>. Eventually, an improved comprehension of key molecular components and apoptosis regulatory mechanisms can help to develop targeted therapeutic strategies.

Apoptosis is executed by a family of cysteinyl aspartate proteinases, caspases, which cleave their specific substrates next to an aspartate residue during the caspase signaling cascade<sup>9</sup>.

They are synthesized as inactive zymogens containing an N-terminal prodomain followed by the appearance of a p20 (large) and a p10 (small) subunit<sup>10</sup>. Caspases may be classified into two subgroups on the basis of the length of their prodomain: 1) Initiator caspases containing long prodomains; 2) Effector caspases containing short prodomains.

Long prodomains of initiator caspases harbor either a death effector domain (DED) (caspase-8 and -10) or a caspase-associated recruitment domain (CARD) (caspase-2 and -9). Since initiator caspases require dimerization and autoproteolysis for their activation occurring in response to multiprotein complex formation, these domains mediate caspase-interactions with adaptor molecules (such as Apaf-1, FADD and RAIDD)<sup>11</sup>.

Effector caspases (caspase-3, -6 and -7) are characterized by the presence of a short prodomain and are expressed as inactive dimers which require cleavage by upstream caspases for their activation<sup>12</sup>. In consequence, they cleave multiple down-stream substrates, a process leading to the characteristic morphological features of apoptosis, such as cell membrane blebbing, cell shrinkage, chromatin condensation, and DNA fragmentation<sup>13</sup>.

Various types of cellular stress (DNA damage, growth-factor deprivation) can activate apoptosis through two main signaling mechanisms: the extrinsic (death receptor-mediated) and the intrinsic (mitochondria-mediated) pathways.

### **1.2.1.1 The extrinsic pathway**

The extrinsic apoptotic pathway is initiated by the binding of tumor necrosis factor (TNF) superfamily ligands to their cognate death receptors (DRs). DRs are type I transmembrane proteins, characterized by the presence of cysteine rich domains in their extracellular portion which mediate ligand binding<sup>14</sup>. Furthermore, the cytoplasmic region contains approximately 80 amino acids long death domains (DDs) which enable DRs to induce cell death through homotypic domain interactions<sup>15-17</sup>. At present six death receptors have been described: TNF-R1<sup>18, 19</sup>, FAS (CD95)<sup>20,21</sup>, DR4 (TRAIL-R1)<sup>22</sup>, DR5 (TRAIL-R2)<sup>23-27</sup>, DR3 (TRAMP)<sup>28-31</sup> and DR6<sup>32</sup>. TNF-R1 mainly controls inflammatory, prosurvival IKK/NF- $\kappa$ B or JNK/c-Jun pathways whereas DR3 controls noncanonical NF- $\kappa$ B signaling<sup>33-36</sup>. FAS, DR4 and DR5 are the main mediators of the extrinsic pathway.

Most of the TNF superfamily ligands are synthesized as stable homotrimeric type II transmembrane proteins. The specific biophysical context of the ligand can determine the outcome of the intracellular signaling. For example, soluble TNF $\alpha$  activates the TNF-R1

mediated NF- $\kappa$ B pathway, whereas membrane-displayed TNF $\alpha$  activates the TNF-R1 mediated extrinsic apoptotic pathway<sup>37, 38</sup>.

#### **1.2.1.1.1 DISC formation**

The binding of a homotrimeric death inducing ligand to its cognate homotrimeric DR results in further conformational change, clustering of multiple ligand-receptor complexes at the plasma membrane, and ultimately formation of a death-inducing signaling complex (DISC) (Fig. 1). Preassembly of DRs through their extracellular pre-ligand assembly domain (PLAD) is critical for ligand binding<sup>39</sup>. Ligation to DR results in recruitment of the adaptor protein FAS-associated death domain (FADD) by means of death domain (DD) interactions and in turn, recruitment of procaspase-8 to the DISC occurs through homotypic death effector domain (DED) interactions<sup>40,41</sup>.

Caspase-8 predominantly exists as a monomeric cytoplasmic protein. After recruitment to the DISC, the monomers adapt a dimeric conformation which in turn triggers catalytic activity and autocleavage<sup>42</sup>. In addition to interdomain cleavage, which alone can activate executioner caspases, the activation of caspase-8 requires dimerization and further posttranslational modifications such as ubiquitination<sup>43-45</sup>. Active caspase-8 initiates the caspase cascade by cleaving downstream effector caspases such as caspases -3, -6 and -7<sup>46-48</sup>. Once effector caspases are activated they cleave downstream substrates and thereby cause the characteristic biochemical and morphological hallmarks of apoptosis discussed above<sup>49</sup>.

Besides FADD and caspase-8, the FLICE-like inhibitory protein (cFLIP) and caspase-10 are also recruited to the DISC. Although caspase-10 is highly homologous to caspase-8 it cannot trigger apoptosis<sup>50</sup>. Its function in the DISC remains unknown. cFLIP is one of the major regulators of caspase-8 activation at the DISC level and has two main mRNA splice variants; cFLIP<sub>L</sub> and cFLIP<sub>S</sub> (long and short, respectively). Furthermore, cFLIP contains an N-terminal DED similar to the one of caspase-8 but the protein lacks a catalytic cysteine residue<sup>51, 52</sup>. Both cFLIP variants are capable to dimerize at the DISC either with itself or with caspase-8/-10<sup>53, 54</sup>. The role of cFLIP variants is controversial. It has been reported that these isoforms may inhibit or activate apoptosis depending on their expression levels and according to their formation of heterodimers with procaspase-8<sup>53,55-57</sup>.

In TNF $\alpha$ -induced TNF-R1 activation a different adaptor molecule, the TNFR-associated death domain (TRADD), enables the recruitment of the DD containing TNF receptor-associated factor 2 (TRAF2) and the additional signaling molecule receptor-interacting

protein 1 (RIP1) to form the TNF-R1 signaling complex (TNF-RSC) which results in activation of IKK/NF- $\kappa$ B or JNK/c-Jun pathways<sup>58</sup>.

The FAS DISC was first described in 1995, yet the exact stoichiometry of DISC components was not clear at the time<sup>59</sup>. The conventional model suggests that one complex includes one ligand trimer, 2-3 FADD, 2-3 caspase-8 and/or cFLIP monomers. Recently it was proposed that one ligand-receptor trimer recruits one FADD molecule which in turn recruits 6 to 10 DED-containing proteins<sup>41,60,61</sup>.

### **1.2.1.2 The intrinsic pathway**

The intrinsic apoptotic pathway is initiated by internal stimuli and activated on the mitochondrial level. Initiator signals include irradiation-induced DNA damage, chemotherapeutics, growth factor deprivation, oxidative stress as well as other stress stimuli<sup>62</sup>. The intrinsic pathway is controlled and regulated by the Bcl-2 gene family proteins which share structural Bcl-2 homology (BH) domains<sup>63</sup>. Depending on the number of BH domains, this family can be divided into three subgroups: BH1-4, BH1-3 and, BH3-only. BH1-4 containing proteins (Bcl-2, Bcl-X<sub>L</sub>, Mcl-1) serve anti-apoptotic functions, whereas BH1-3 (Bax, Bak) and BH3-only proteins (Bid, Bad) serve pro-apoptotic functions<sup>64,65</sup>.

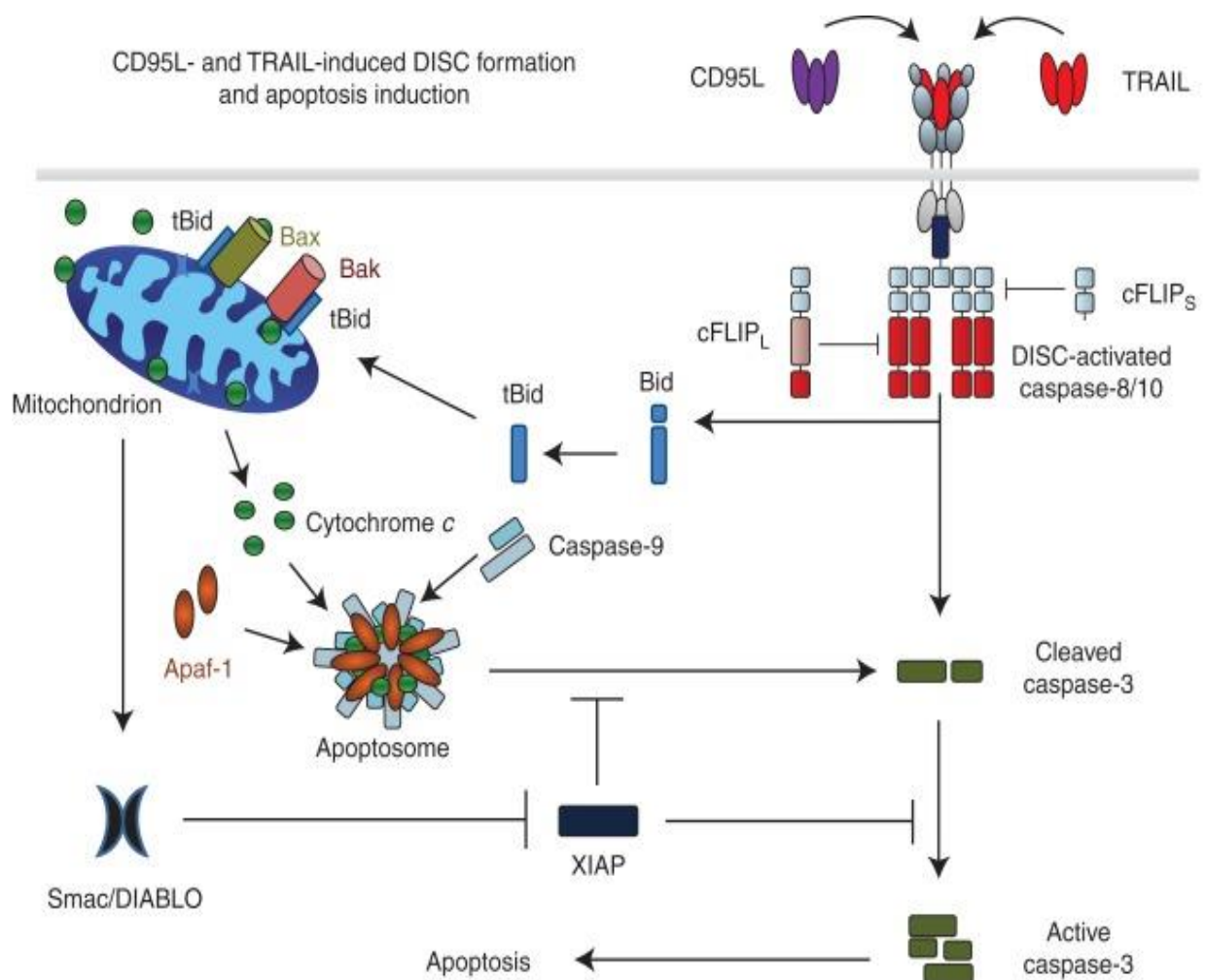
Upon stress signals BH3-only proteins are activated. As a result, Bax and Bak are oligomerized and localized to the outer mitochondrial membrane (OMM) causing disruption of OMM<sup>66</sup>. Mitochondrial outer membrane permeabilization (MOMP) triggers the release of cytochrome *c* from the intermembrane space of mitochondria to the cytoplasm. Subsequently, cytosolic cytochrome *c* binds to the apoptosis protease-activating factor 1 (Apaf-1), causing its conformational change and together with procaspase-9, in presence of dATP, an intracellular high molecular weight complex known as the apoptosome is generated<sup>67</sup>. Procaspase-9 has a caspase-associated recruitment domain (CARD) which mediates its interaction with the CARD-containing adaptor protein Apaf-1. Through this interaction procaspase-9 is recruited to the apoptosome<sup>68</sup>. Finally, the proteolytic activity of caspase-9 leads to processing of caspase-3<sup>69</sup>.

Although the initial phases are characterized by distinct features, extrinsic and intrinsic pathways converge in the activation of effector caspases (caspases -3, -6 and -7), which in turn target a broad spectrum of cellular proteins, thereby, predestining cells to irreversible cell death.



Based on the requirement of the mitochondrial pathway for apoptosis induced by DRs, cells can be divided into type I and type II. In type I cells, DR-mediated caspase-8 activation is adequate to induce apoptosis whereas in type II cells (like HCT116), less caspase-8 activity is generated and involvement of the intrinsic pathway is needed for efficient apoptosis to occur. In this case, caspase-8 cleaves the cytosolic BH3-interacting-domain death agonist (Bid). Myristoylation of cleaved Bid is followed by its activation and translocation to mitochondria to form truncated Bid (tBid), which triggers the cell intrinsic apoptotic pathway through MOMP, augmenting the activation of executioner caspases and committing the cell to death (Fig. 1)<sup>70</sup>.

Finally, cell fragments or apoptotic bodies, generated from apoptotic cells, are recognized through “find me” signals by the cell surface receptors in phagocytes and rapidly cleared by “eat me” signals before loss of membrane integrity. Therefore, inflammation and autoimmune reactions can be avoided and tissue homeostasis can be maintained<sup>71</sup>.



**FIGURE 1.** CD95L- and TRAIL- induced DISC formation and apoptosis induction.

Walczak H<sup>58</sup> Cold Spring Harb Perspec Biol 2013; 5: a008698, copyright Cold Spring Harbor Laboratory Press (reused and reprinted with the permission).

### **1.2.2 Necrosis**

Necrosis can occur during normal physiological processes or as a consequence of pathological conditions<sup>72</sup>. This process can also be induced by microbial infections, reactive oxygen species (ROS) or by inhibition of TNF- or FAS-induced apoptosis by pan-caspase inhibitors<sup>73</sup>. Hallmarks of necrosis are vacuole formation, cell swelling and loss of plasma membrane integrity, the latter which results in an uncontrolled release of the cellular content into the cell's environment and consequent damage of surrounding cells, promoting an inflammatory response in the corresponding tissues<sup>74</sup>.

### **1.2.3 Autophagy**

Autophagy or “self-eating” is both a survival and a cell death mechanism which occurs in lysosomes. The process of autophagy catalyzes the degradation and subsequent recycling of proteins and organelles in order to maintain cell and tissue homeostasis<sup>75</sup>. It can act as a survival mechanism during periods of nutrient stress to enable energy production<sup>76</sup>. The process can also be induced in response to oxidative stress or accumulation of misfolded proteins<sup>77</sup>. It is mediated and regulated by autophagy-related (ATG) proteins. Capturing of intracellular material in double membrane-structures termed as autophagosomes is followed by the fusion with lysosomes and further degradation of autophagosomal content<sup>75</sup>. The effect of autophagy in a disease situation is proposed to be dual since suppression as well as promotion of tumorigenesis has been reported<sup>78</sup>.

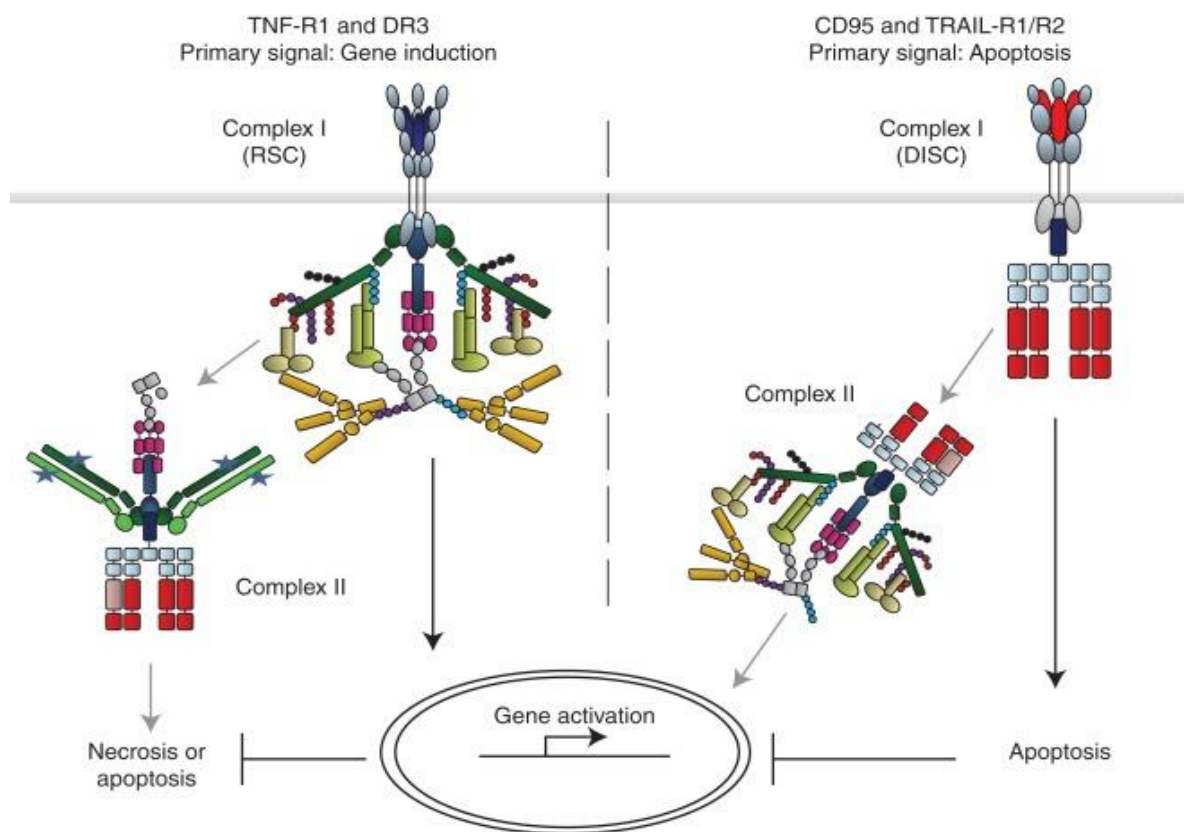
### **1.2.4 Crosstalk between modes of cell death**

Depending on the stimulus and environmental factors a discrete cell death mechanism is often activated. Inhibition of the primarily preferred cell death mechanism, however, can lead to the activation of a secondary pathway. Although FAS, DR4 and DR5 primarily mediate extrinsic apoptosis, they can also induce alternative pathways when apoptosis is inhibited. In addition, TNF-R1, which mainly controls inflammatory, prosurvival pathways, can activate apoptosis when NF- $\kappa$ B activation is blocked. Apart from the capability to generate a complex in plasma membranes FAS/DR4/DR5 as well as TNF-R1/DR3 can form secondary complexes in the cytosol, referred to as complex II, when the respective formation of complex I is inhibited. These complexes trigger a secondary pathway; the DR4/DR5 complex II triggers survival pathways, whereas the TNF-RSC complex II mediates induction of necrosis or apoptosis (Fig. 2)<sup>58</sup>.

Apoptosis and autophagy have been reported to act in synergy but also to counteract each other<sup>79</sup>. It seems that apoptosis and autophagy can act as co-partners, meaning that if one

program is blocked, the other may become dominant. However, autophagy can inhibit apoptosis to promote cell survival<sup>80</sup>. Some of the hallmarks of autophagy can also be observed in cells which undergo necrosis<sup>81</sup>. Inhibition of caspases often fails to block cell death *per se*, but instead converts it from apoptosis to necrosis. Moreover, mitochondria can act as a switchboard in the regulation of several cell death modes, including autophagy, apoptosis and necrosis<sup>81</sup>.

Altogether, even if the currently defined modes of cell death can be divided into subgroups according to their morphological and biological features, their potential crosstalk necessitates general consideration in any given experimental system.



**FIGURE 2.** Comparison of CD95/TRAIL-R1/R2 and TNF-R1/DR3 signaling.

Walczak H<sup>58</sup> Cold Spring Harb Perspec Biol 2013; 5: a008698, copyright Cold Spring Harbor Laboratory Press (reused and reprinted with the permission).

## 2. INTRODUCTION TO THE STUDY

### 2.1 Colorectal cancer

Colorectal cancer (CRC), the third most common form of cancer in the world, is estimated to cause 12.8% of all cancer-related deaths in the European Union in 2016, killing 173,400 individuals<sup>82</sup>. Its predicted death rate is the second highest of all cancers in men (16.2/100,000) and third highest in women (9.3/100,000), but these rates have dropped by 4.6% and 7.0%, respectively, since 2011 due to improved screening strategies and early diagnosis<sup>83</sup>. According to the tumor-node-metastasis (TNM) system, CRC is classified into four distinct stages<sup>84</sup>. Depending on the stage, where I is reflecting the early onset and IV an advanced disease, the five-year relative survival of patients with CRC is ranging between 90.3 and 12.5%<sup>85</sup>. Metastasis remains the main cause of poor prognosis and mortality<sup>86,87</sup>.

CRC is a heterogeneous disease with epigenetic changes and gene alterations in tumor suppressor and/or oncogenes. Since the presence or absence of these markers in many cases relate to treatment outcome they are currently evaluated for their prognostic and predictive values.

#### 2.1.1 Microsatellite instability (MSI)

Due to the mutations in mismatch repair (MMR) genes such as *MLH1*, *MSH2*, *MSH6* and *PMS2*, base-base mispairs in microsatellite regions cannot be repaired which in turn result in changes in the length of repetitive DNA nucleotide sequences. Depending on the frequency, MSI can be divided into three subclasses: microsatellite instability-low,-high and stable (MSI-L, MSI-H, MSS)<sup>88</sup>. Fifteen percent of CRCs harbor MSI and the prevalence in sporadic CRCs varies between 4-20% depending on the stage; 4% in stage IV, 12% in stage III and 20% in stage II<sup>89,90</sup>. The results from different studies have shown that patients with MMR-deficient tumors had a better prognosis compared to patients with an intact MMR<sup>91-93</sup>.

#### 2.1.2 CpG island methylator phenotype (CIMP)

A tumor suppressor gene can be silenced via methylation of CG-rich domains referred to as CpG islands in their promoter regions leading to CIMP and carcinogenesis<sup>94,95</sup>. The global hypomethylation in CRC and adenomas attracted most of the attention in the early years of molecular research. However, the CIMP is also associated with CRCs characterized by methylation of the *MLH1* gene that will further cause MSI<sup>96-98</sup>. Yet, since current data are

inconsistent, further studies are needed in order to evaluate the CIMP as a prognostic and a predictive marker<sup>99,100</sup>.

### 2.1.3 Loss of heterozygosity (LOH)

Genomic loss of one of the alleles in tumor cells is termed loss of heterozygosity (LOH) and occurs in 70% of all CRCs analyzed<sup>101,102</sup>. LOH on chromosome 5q occurs as an early event during the tumor transition stage. LOH on 18q and on 17p, on the other hand, occur during later tumor stages. These loci are corresponding to the localization of adenomatous polyposis coli (*APC*), deleted in colon cancer (*DCC*) and *p53* genes, indicating their important role in tumor progression<sup>103,104</sup>. Currently, the association between LOH status and survival or treatment outcome is elusive<sup>105-108</sup>.

### 2.1.4. Other common mutations

Somatic mutations in the *APC* tumor suppressor gene cause dysregulations in cell migration,  $\beta$ -catenin-dependent Wnt signaling and apoptosis, and account for 70-80% of sporadic CRCs<sup>109-111</sup>. Furthermore, mutations in genes in the Wnt pathway such as *CTNNB1*, *AXIN1*, *AXIN2* and *TCF4* have also been identified in CRC<sup>112</sup>.

The RAS proteins are members of the guanosine-5'triphosphate (GTP)ase-binding protein family. (GTP)ase-binding proteins promote intracellular signaling downstream of the epidermal growth factor receptor (EGFR) associated with the mitogen-activated protein kinase (MAPK) signal transduction pathway. The Kirsten rat sarcoma viral oncogene (*KRAS*) gene is mutated in approximately 30-40% of all CRCs and 85-90% of these mutations occur in exon 2 (codons 12 and 13)<sup>104,113,114</sup>. Due to these mutations, the MAPK pathway is constantly activated, resulting in non-responsiveness to EGFR-targeting drugs such as cetuximab and panitumumab<sup>115,116</sup>.

Phosphatidylinositol-3-kinases (PI3Ks) belong to a family of lipid kinases which control cell survival, proliferation and differentiation by binding to and phosphorylating the phosphatidylinositol (PI3P) intracellular signaling proteins. The phosphatidylinositol 3-kinase catalytic subunit, *PIK3CA*, was reported to be mutated in 15-25% of all CRCs leading to activation of the PI3-K-AKT survival pathway and thus playing a critical role in CRC pathogenesis<sup>114</sup>. In addition, oncogenes such as *NRAS*, *BRAF*, *EGFR*, *CDK8* and tumor suppressor genes such as *PTEN*, *SMAD4*, *SMAD2*, *TGF $\beta$ IIIR* are also mutated in CRC with variable frequencies<sup>109,117</sup>.

The treatment strategies of CRCs are closely related to the stage of the disease. Further studies on identification of potential prognostic and predictive makers in CRC will help to

personalize the treatment by identifying the patients who will benefit from a particular drug and also, eventually, generate more targeted therapies.

## 2.2 p53

The *p53* gene, also known as the guardian of the genome, is a tumor suppressor gene which contributes to several facets of cell homeostasis including DNA repair, cell proliferation and differentiation, cell death and survival pathways. Activation of p53 through DNA damage, aberrant growth signals or lesions caused by chemotherapeutics drugs leads to stabilization of the protein, DNA binding and expression of p53-targeted genes, consequently promoting cell death or cell cycle arrest<sup>118-121</sup>. The expression level of the p53 protein is controlled by MDM2 via ubiquitination and/or inhibition of MDM2 by p14<sup>ARF</sup><sup>122</sup>. p53 exerts its anti-tumorigenic effects via transcriptional activation of pro-apoptotic genes, cell cycle-regulatory genes or DNA-repair genes<sup>118</sup>. The *p53* gene is mutated in about 50% of all human cancers with a variety depending on the tumor type. In addition, several mutations have been associated with tumorigenesis as well as to the tumor treatment response<sup>123</sup>.

As a transcription factor, p53 can regulate both the extrinsic and the intrinsic apoptotic pathways. It can induce the expression of death receptors (DR5, DR4 and FAS) as well as the expression of pro-apoptotic members of the Bcl-2 family, including Bax, Bid, Noxa and Puma<sup>124</sup>. It can also suppress the expression of decoy receptors and anti-apoptotic proteins<sup>124</sup>. Upon DNA-damage the activation of p53 occurs via post-translational modifications which lead to the regulation of DNA repair pathways in transactivation-dependent and independent ways. The tumor suppressor is also implicated in mechanism of cell cycle arrest which allows for DNA-repair<sup>124</sup>. In situations of excess DNA-damage, p53 functions switch from pro-survival to pro-apoptotic<sup>124</sup>. A more detailed description of this event has, however, not yet been presented.

Similar to other carcinomas, the *p53* gene is mutated in more than 50% of all CRCs although with a higher frequency in distal colon tumors<sup>125</sup>. The *p53* gene is located on 17q and LOH on this chromosome region is, as discussed above, a common trait in CRC. Eighty-five percent of these mutations are missense mutations at 'hotspot' codons 175, 245, 248, 249, 273 and 282, which all are located in the DNA binding domain of the protein<sup>126</sup>. Studies evaluating the p53 status as a prognostic and a predictive marker are contradictory, possibly due to the differences in their methodological approaches<sup>125</sup>. Some studies have reported that patients with mutated p53 have an increased risk of death, poor prognosis, as well as lower disease free and overall survival<sup>127-129</sup>. In addition, p53 mutations and gene

overexpression were associated with shorter survival in patients receiving 5-FU adjuvant therapy<sup>129-131</sup>. Remarkably, other studies have not found any supporting evidence that p53 mutations in CRCs can be used as a prognostic marker<sup>128,132-134</sup>.

### 2.3 5-Fluorouracil

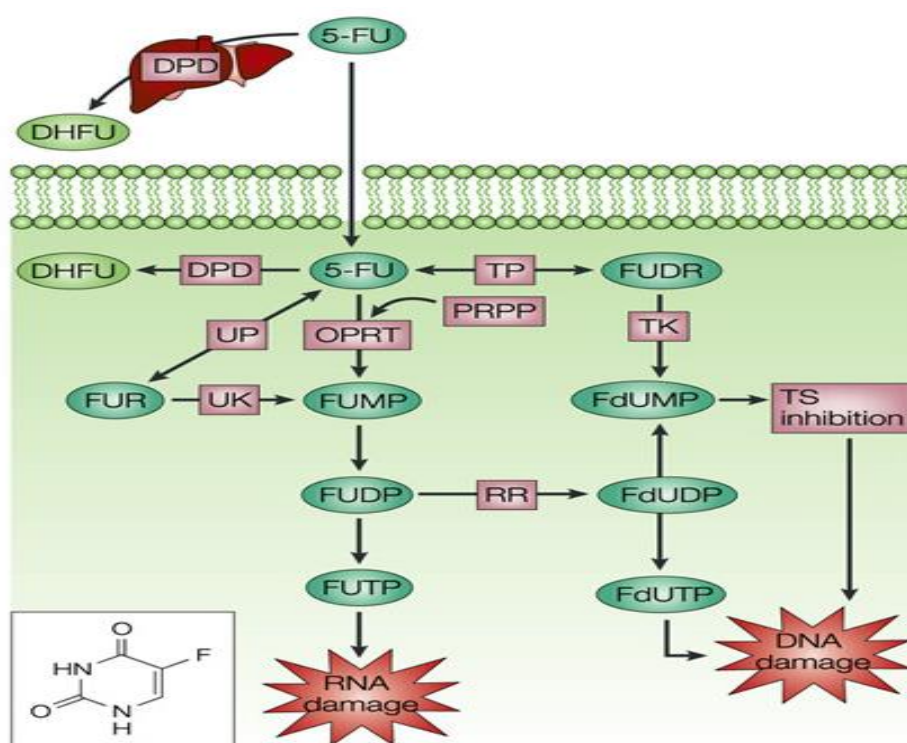
5-Fluorouracil (5-FU) belongs to the fluoropyrimidine type of drugs and is commonly used in the treatment of solid tumors. 5-FU was originally generated by Heidelberger *et al.* in 1957 based on the finding that rat hepatomas utilized uracil more than normal liver cells. Ever since then it has become the mainstay of CRC treatment at high risk stage II, stage III and stage IV, either alone or in combination with other drugs<sup>135</sup>. Depending on the dose and schedules, it can exert different toxicities such as myelosuppression, mucositis and diarrhea<sup>136</sup>. For this reason, the third generation of fluorouracil derivatives was introduced to lessen the drug toxicity.

5-FU is a uracil analogue which has a fluorine atom at the C5 position instead of a hydrogen atom. It enters the cells via membrane carrier-mediated transport in the same way as uracil<sup>137</sup>. The half-life of 5-FU in the plasma is 10 to 20 minutes and approximately 80% of 5-FU is catabolized, primarily in the liver, while the rest is excreted in the urine unchanged<sup>137</sup>. The first reaction and rate-limiting step in this process is the conversion of 5-FU to non-active dihydrofluorouracil (DHFU) by dihydropyrimidine dehydrogenase (DPD) to reduce the pyrimidine ring which is followed by further enzymatic reactions<sup>138</sup>.

5-FU is converted to three active metabolites: fluorodeoxyuridine monophosphate (FdUMP) fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). The first two cause DNA damage while the latter exerts its effect on RNA (Fig. 3)<sup>139</sup>.

The thymidylate synthase (TS) enzyme converts deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) using reduced folate 5, 10-methylenetetrahydrofolate (CH<sub>2</sub>THF) as a cofactor. Irreversible binding of FdUMP to TS together with CH<sub>2</sub>THF results in a stable ternary complex. Inactivation of TS leads to imbalance in deoxynucleotide pools and dTTP depletion<sup>140</sup>. As a result, DNA synthesis is stopped and consequently stalled replication forks are formed<sup>141</sup>. In addition, FdUTP can misincorporate into the DNA directly instead of dTTP. This causes activation of uracil glycosylases and subsequently fragmentation of DNA<sup>142</sup>. Incorporation of FUTP into RNA can affect RNA processing which in turn leads to formation of immature ribosomal RNAs (rRNAs) and misfolded tRNAs<sup>143</sup>.

Conversion of 5-FU to fluorouridine monophosphate (FUMP) can either be catalyzed directly by orotate phosphoribosyl transferase (OPRT) or indirectly through the sequential action of uridine phosphorylase (UP) and uridine kinase (UK) (Fig. 3). If phosphorylation of FUMP to fluorouridine diphosphate (FUDP) is followed by another phosphorylation it forms fluorouridine triphosphate (FUTP)<sup>139</sup>. Alternatively, it can be converted to fluorodeoxyuridine diphosphate (FdUDP) by ribonucleotide reductase (RR). Further phosphorylation or dephosphorylation leads to the formation of FdUTP and FdUMP, respectively. An alternate pathway catalyzes the conversion of 5-FU to fluorodeoxyuridine (FUDR) which is then phosphorylated to FdUMP by the enzymatic actions of thymidine phosphorylase (TP) and thymidine kinase (TK)<sup>139</sup>.



**FIGURE 3.** 5-FU metabolism.

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Treatment strategies of CRCs differ according to the stage of the disease. For stages I-II the predominant approach is surgical resection, for high-risk stage II or stage III - adjuvant chemotherapy, and for stage IV is chemotherapy. The response rate to 5-FU alone as an adjuvant chemotherapy is 10-15%<sup>144</sup>. However, combinatory treatment with oxaliplatin and irinotecan increases the response rates to 40-50% with a better overall survival<sup>145,146</sup>. The catalyzing efficiency and alterations of enzymes responsible for 5-FU metabolism have been investigated deeply in order to identify predictive and prognostic markers. Studies



showed that low TS expression was associated with an improved response rate to 5-FU and acute increase in TS enzyme levels can be responsible for drug resistance<sup>147-150</sup>. High levels of DPD and dUTPase correlated with resistance to TS inhibitors<sup>151,152</sup>. Moreover, increased levels of TP, UP, UK and OPRT are also coupled with 5-FU sensitivity<sup>153-155</sup>. It should be noted that primary tumors and metastases may differ in their sensitivity to chemotherapy.

### **3. AIM OF THE STUDY**

The main aim of this thesis was to investigate in detail still poorly characterized stress signaling pathways elicited by 5-FU metabolites.

The specific aims were as follows:

1. To characterize the DISC formation induced by 5-FU in order to investigate initial apoptotic triggering points and define potential upstream regulatory factors of the caspase cascade.
2. To study the involvement of the p53 protein and its phosphorylations in 5-FU-mediated cell death and identify upstream regulators of p53 phospho-activations including calcium ( $\text{Ca}^{2+}$ ) signaling.
3. To examine reciprocal or unilateral cross-talk between autophagy and apoptosis with respect to 5-FU generated stress.
4. To analyze the mechanism of DR activation in tumor cells treated with 5-FU.

## 4. MATERIALS AND METHODS

A detailed description of the materials and methods used in this thesis can be found in papers I-III. The section below provides a brief summary.

### 4.1 Cell culture

The cell lines used in Papers I-III are summarized in Table 1.

**Table 1.** Cell lines employed in the study.

Cell line	Growth medium	Paper I	Paper II	Paper III	Source
HCT116 <i>wt</i>	DMEM	✓	✓	✓	a
HCT116 <i>wt</i> c-FLIP <sub>L</sub> overexpressing	DMEM			✓	a-c
HCT116 <i>wt</i> BclX <sub>L</sub> overexpressing	DMEM			✓	a-c
HCT116 <i>wt</i> FADD-DN	DMEM		✓	✓	a-c
HCT116 Chk2 <sup>-/-</sup>	DMEM	✓			b
HCT116 <i>p53</i> <sup>-/-</sup>	DMEM	✓	✓	✓	b
HCT116 <i>p53</i> <sup>-/-</sup> c-FLIP <sub>L</sub> overexpressing	DMEM		✓		b-c
HCT116 <i>p53</i> <sup>-/-</sup> FADD-DN	DMEM		✓		b-c
A549	RPMI-1640	✓			a
RKO	DMEM		✓	✓	a
HT-29	DMEM			✓	a

**a** Cell lines purchased from American Type Culture Collection (ATCC, Manassas VA).

**b** Cell lines provided by Professor Bert Vogelstein (Department of Oncology, John Hopkins University, USA).

**c** Phoenix-Ampho packaging cell line was provided by Dr. Garry Nolan, Stanford University, USA).

Growth mediums (DMEM, RPMI-1640, GIBCO) were supplemented with 10% heat-inactivated fetal bovine serum (FBS) (v/v) (GIBCO) and PenStrep (100 U/mL penicillin, 100 µg/mL streptomycin, Sigma-Aldrich). Throughout the experiments cells were maintained in a logarithmic growth phase at 37°C in an atmosphere containing 95% humidity and 5% CO<sub>2</sub>. All experiments were performed using cell lines between passages 4 and 20. Cells were seeded at a density of 40,000-50,000 cells/cm<sup>2</sup> and then allowed to adhere for 24 h before being exposed to treatments. For each experiment, the treatment conditions and incubation times are indicated in the corresponding figures.

### 4.2 Drugs and chemicals

The drugs, chemical inhibitors, antioxidants and metabolites used in Papers I-III are summarized in Table 2. All inhibitors were added to cell cultures at least 1 h prior to drug

treatment, unless otherwise stated. Further details regarding the use of drugs and chemicals can be found in the papers included in the thesis.

**Table 2.** The drugs and chemical inhibitors employed in the study.

Name	Source	Final concentration
<b>Drugs</b>		
5-Fluorouracil (5-FU)	Teva-Accord	10-768 $\mu$ M
Camptothecin	Sigma-Aldrich	600 nM
Cisplatin	Ebewe	40 $\mu$ M
Doxorubicin	Sigma-Aldrich	2 $\mu$ M
Etoposide	Ebewe	20 $\mu$ M
Leucovorin (Leu)	Hospira	4.9-78.2 $\mu$ M
<b>Metabolites</b>		
Uridine	Sigma-Aldrich	375 $\mu$ M
Thymidine	Sigma-Aldrich	375 $\mu$ M
<b>Recombinant Proteins</b>		
Recombinant TRAIL	Thermo Fisher Sci.	10-100 ng/mL
Recombinant soluble DR5	Sigma-Aldrich	2 $\mu$ g/mL
<b>Inhibitors</b>		
BAPTA	Invitrogen	10 $\mu$ M
Thapsigargin	Invitrogen	1-5 $\mu$ M
Verapamil	Sigma-Aldrich	10-60 $\mu$ M
N-benzoyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone (zVAD-fmk)	Peptide	10 $\mu$ M
Olaparib	Selleck Chemicals	500 nM
E64d	Sigma-Aldrich	10 $\mu$ M
Pepstatin A	Sigma-Aldrich	7-10 $\mu$ M
Leupeptin	Sigma-Aldrich	100 $\mu$ M
CA-074 methyl ester	Merck	10 $\mu$ M
Calmidazolium chloride	Santa Cruz	2.5-5 $\mu$ M
Fluphenazine-N-2-chloroethane	Santa Cruz	2.5-10 $\mu$ M
PKC412	Novartis	0.001-5 $\mu$ M
KU55933	Selleck Chemicals	7.5-10 $\mu$ M
SB203580	Selleck Chemicals	10 $\mu$ M
Brefeldin A (BFA)	Sigma-Aldrich	2-1000 nM
Chloroquine (CQ)	Sigma-Aldrich	10-100 $\mu$ M
Bafilomycin A (Baf A)	Sigma-Aldrich	100 nM
3-methyladenine (3-MA)	Sigma-Aldrich	5 mM
U18666A	Sigma-Aldrich	0.1-5 $\mu$ g/mL
Methyl- $\beta$ -cyclodextrin (M $\beta$ CD)	Sigma-Aldrich	2.5-5 mM
PUGNAc	Sigma-Aldrich	25-100 $\mu$ M
Benzyl- $\alpha$ -galNAc	Sigma-Aldrich	2.5 mM
Necrostatin-1 (Nec-1)	Merck	100 $\mu$ M
Necrostatin-1, inactive control	Merck	100 $\mu$ M
<b>Antioxidants</b>		
Trolox	Merck	50 $\mu$ M
N-acetylcysteine (NAC)	Merck	5 mM

### **4.3 Gel electrophoresis and immunoblotting**

Floating and attached cells were harvested by trypsinization, centrifuged at 1300 rpm for 5 min, washed in phosphate-buffered saline (PBS) and then centrifuged at 2000 rpm for 10 min. The cell pellet was subsequently lysed for 10 min at room temperature (RT) in cOmplete Lysis-M containing cOmplete protease and phosphatase inhibitor cocktails (Roche Diagnostics). The BCA Protein assay (Thermo Fisher Scientific) was used to determine protein concentration. Samples were mixed with 5X Laemmli sample buffer and after denaturation for 10 min at 95°C, 20-80 µg of each sample was subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in cold Tris-glycine-SDS running buffer at 80-120 V and electroblotted to 0.45 µM nitrocellulose membranes (Bio-Rad Laboratories) using Tris-glycine transfer buffer containing 20% methanol (v/v) for 2 h at 90 V in a wet blotting system (Bio-Rad Laboratories). Membranes were then blocked with 5% non-fat milk in Tris-buffered saline with Tween-20 (TBST) for 1.5 h at RT and probed with the primary antibody of interest diluted in PBS containing 1% BSA, 0.05% Tween-20 and 0.01% NaN<sub>3</sub> at 4°C overnight (Table 3). Membranes were washed 3 times in TBST for 5 min and 3 times in TBS for 10 min each and then incubated with horseradishperoxidase-conjugated secondary antibodies, which were diluted in blocking solution for 2 h at RT. After washing, all steps were repeated. Finally, signals were revealed by ECL (GE Healthcare Biosciences) and membranes were exposed to SuperRX-N X-ray films (Fujifilm Corporation).

### **4.4 Isolation of membrane proteins**

Cytosolic and membrane fractions were isolated using the Qproteome Cell Compartment Kit (Qiagen) in accordance with the manufacturer's instructions and, following denaturation, subjected to SDS-PAGE.

### **4.5 DR5 dimerization**

To investigate DR5 dimers cells were treated as indicated in the related paper. After lysing for 20 min on ice in non-reducing Triton buffer (20 mM Tris base, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton-X100 and 1 mM EDTA) containing cOmplete phosphatase and protease inhibitors, cells were treated with benzonase nuclease (Sigma-Aldrich) for 15 min at RT. After centrifugation at 16,000 g at 4°C samples were mixed with 3X Laemmli sample buffer with or without β-mercaptoethanol. The samples containing reducing agent were denatured at 95°C for 10 min while the non-reduced samples kept at RT before subjected to SDS-PAGE, as described above.

**Table 3.** Antibodies used in western blotting (WB), immunofluorescence (IF), immunoprecipitation (IP) and flow cytometry (FCM).

Name	Source	Method-Dilution
p53 (DO-1), mAb	Santa Cruz	WB 1:5000, IF 1:400
phospho-p53 (Ser 6,9,15,33,37,46,382), pAb	Cell Signaling	WB 1:1000
DR5, pAb	Sigma-Aldrich	WB 1:5000
DR5 (F2/B4), mAb	a	IF 1:100, IP 1:, FCM 1:100
DR4, pAb	Sigma-Aldrich	WB 1:1000
DR4, mAb	a	IF 1:100, FCM 1:100
FAS pAb	Santa Cruz	WB 1:1000, IF 1:100
Transferrin (MEM-75), mAb	Abcam	IF 1:100, FCM 1:100
FADD, pAb	Upstate Biotechnologies	WB 1:1000, IF 1:100
TRAIL, mAb	BD Biosciences	WB 1:1000
cFLIP	b	WB 1:10
caspase-8 (C15), mAb	b	WB 1:50
caspase-7 (B94-1), mAb	BD Biosciences	WB 1:1000
cleaved-caspase-3, pAb	Cell Signaling	WB 1:1000
caspase-2 clone 35, mAb	BD Biosciences	WB 1:5000
Bcl-x <sub>L</sub> , pAb	Santa Cruz	WB 1:1000
PARP (4C10-5), mAb	BD Biosciences	WB 1:1000
cleaved PARP (Asp214), mAb	Cell Signaling	WB 1:1000
cleaved lamin A, mAb	Cell Signaling	WB 1:1000, IF 1:200
ATF-3 pAb	Santa Cruz	WB 1:1000
anti-PAR mAb	Trevigen	WB 1:1000
ATM (D2E2), mAb	Cell Signaling	WB 1:1000
γH2AX (Ser139), pAb	Cell Signaling	WB 1:1000
PERK,	Cell signaling	WB 1:3000
SQSTM1 (p62) mAb	Santa Cruz	WB 1:2000
Atg5, pAb	Cell Signaling	WB 1:1000
Atg7, pAb	Cell Signaling	WB 1:1000
beclin, pAb	Cell Signaling	WB 1:1000
LC3, pAb	MBL	WB 1:5000, IF 1:100
anti-AIF (E-1), mAb	Santa Cruz	WB 1:1000
cytochrome <i>c</i>	BD Biosciences	WB 1:2000, IF 1:400
Tom-40 pAb	Santa Cruz	WB 1:10000
GAPDH pb	Trevigen	WB 1:5000
tubulin (B-5-1-2), mAb	Sigma-Aldrich	WB 1:10000
Ku80, mAb	BD Biosciences	WB 1:10000
<b>Secondary antibodies</b>		
Anti mouse, rabbit, goat	Thermo Fischer	WB 1:5000-10000
Alexa 488 anti-mouse, rabbit, goat	Molecular Probes	IF 1:200, FCM 1:200
Alexa 594 anti-mouse, rabbit, goat	Molecular Probes	IF 1:200, FCM 1:200

**a** Provided by Dr. L Andêra, Academy of Sciences, Prague, Czech Republic

**b** Provided by Prof. PH Krammer and Dr. I Lavrik, German Cancer Research Center, Heidelberg, Germany

## 4.6 Release of mitochondrial AIF and cytochrome *c*

To detect the release of mitochondrial AIF and cytochrome *c*, cells were treated, harvested by trypsinization, centrifuged at 1300 rpm for 5 min and washed in PBS. Subsequently, cells were permeabilized with digitonin (0.01% w/v) in fractionation buffer (150 mM KCl, 10 mM Tris, 1 mM MgCl<sub>2</sub>) for 15 min at RT, centrifuged at 13,000 g to separate the cytosol from pelleted nuclear/mitochondrial/membrane proteins. After determination of protein concentration by the BCA protein assay, samples were subjected to SDS-PAGE as described above.

## 4.7 Analysis of the mitochondrial membrane potential and reactive oxygen species (ROS)

Cells were incubated for 20 min in Hank's balanced salt solution (Life Technologies) containing 0.1  $\mu$ M tetra-methyl-rhodamine-ethyl ester (TMRE, Life Technologies). Loss of mitochondrial membrane potential ( $\Delta\Psi_m$ ) in live cells was then examined by flow cytometric analysis of mitochondrial accumulation of TMRE.

For assessment of mitochondrial ROS, cells were stained with MitoSOX™ Red (Life Technologies) in accordance with the manufacturer's instructions. Analyses were performed with a FACScan Becton Dickinson flow cytometer and the accompanying software.

## 4.8 Expression vectors and retroviral transduction

Retroviral particles were produced by transient transfection of the Phoenix-Ampho packaging cell line (kindly provided by Dr. GP Nolan, Stanford University, USA) using the retroviral expression vectors pLXIN-hBcl-X<sub>L</sub>, pLXIN-hFADD and pXIN-hFLIP<sub>L</sub> which have been described previously<sup>156</sup>. Transduced cells were selected by treatment with Geneticin (1 mg/mL, Invitrogen) for one week.

## 4.9 Vectors and cloning

Wild type (*wt*) p53 and p53 (S15A) cDNA's were kindly provided by Prof. Moshe Oren (Weizmann Institute of Science, Rehovot, Israel). Subsequent to deletion of the stop codons to allow GFP expression, *wt* and mutated p53 sequences were subcloned into the XbaI-BamHI site of the *pCDH-CMV-MCS-EF1-copGFP* expression vector (System Biosciences). Lentiviral packaging was accomplished using the LentiSuite™ in accordance with the manufacturer's instructions (System Biosciences). Then, transductions of HCT116 *p53*<sup>-/-</sup> cells were performed by incubation under normal culturing conditions in the presence

of virus for 24 h. Transfected cells were then sorted using the FACSVantage/DiVa system (Becton Dickinson) together with the accompanying software.

#### 4.10 RNA isolation and RT-PCR

Total RNA was isolated with the RNAeasy mini kit (Qiagen) in accordance with the manufacturer's instructions. The Maxima First Strand cDNA Synthesis Kit (Thermo Scientific) or Transcriptor First Strand cDNA Synthesis Kit (Roche) were used for cDNA synthesis. Gene expression was measured using the Maxima SYBR Green qPCR Master Mix (Thermo Scientific) or the FastStart SYBR Green Master (Roche) together with the Applied Biosystems 7500 Real-time PCR technology (Applied Biosystems). 50 ng cDNA and 300 nmol primers were used (Table 4) in each reaction. Relative gene expression was calculated by the  $2^{-\Delta\Delta C_t}$  procedure using expression of actin and GAPDH for normalization.

**Table 4.** Primers employed in the study.

Primers	Forward sequence	Reverse sequence
DR5	TCAGGTGAAGTGGAGCTAAGTC	GTGTACAATCACCGACCTT
DR4	ACTCGCTGTCCACTTTCGTCTCTGA	AGGCATCCCCTGGGCCTGCTGTA
Actin	GCTGTGCTATCCCTGTACGC	GAGGGCATACCCCCTCGTAGA
GAPDH	CCTGGCCAAGGTCATCCATG	TGAGGTCCACCCTGTTG

#### 4.11 RNA interference

Silencing of protein expression in cells was performed using the Lipofectamine RNAiMAX (Life Technologies) or INTERFERin transfection reagent (Polyplus transfection) in accordance with the manufacturer's instructions.

In brief, when reaching 60-80% confluence, cells were transfected in complete cell culture medium without PenStrep using 10-20 nM final concentration of siRNA and 3.5  $\mu$ L/mL transfection reagent (Table 5). Depending on the experiment, target protein suppression 24-72 h post-transfection was confirmed by SDS-PAGE as described above.



**Table 5.** The siRNAs employed in the study.

Name	Source
Human TNFSF10, Silencer Select	Thermo Fischer Scientific
Hs.TNFSF10.6, FlexiTube	Qiagen
Hs.TNFSF10.7, FlexiTube	Qiagen
Human TNFRSF10A (8797), ON-TARGET plus, SMARTpool	Dharmacon
Human TNFRSF10A, Silencer Select	Thermo Fischer Scientific
Human TNFRSF10B (8795), ON-TARGET plus, SMARTpool	Dharmacon
Human TNFRSF10B, Silencer Select	Thermo Fischer Scientific
siDR5(h)	Santa Cruz
Human FAS (355), ON-TARGET plus, SMARTpool	Dharmacon
Human CASP8 (841), ON-TARGET plus, SMARTpool	Dharmacon
Hs.TP53.9, FlexiTube	Qiagen,
Hs.ATM.8, FlexiTube	Qiagen,
Hs.ATM.9, FlexiTube	Qiagen,
Hs.ATM.12, FlexiTube	Qiagen,
siPARP1, HP Custom siRNA	Qiagen,
Human BCN1, ON-TARGET plus, SMARTpool	Dharmacon
SQSTM1, Silencer Select	Thermo Fischer Scientific
Human ATG5, ON-TARGET plus, SMARTpool	Dharmacon
Human ATG7, ON-TARGET plus, SMARTpool	Dharmacon
Negative Control No. 1, Silencer Select	Thermo Fischer Scientific
Human Non-Targeting Pool 1, siGENOME	Dharmacon

#### 4.12 Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) was used to determine changes in the morphology of detached (paper II) and attached (paper III) cells in response to treatment. Cells were trypsinized, spun down, resuspended in fixing solution (2.5% w/v, glutaraldehyde in 0.1 M phosphate buffer, pH 7.4) for 30 min at RT. After washing in 0.1 M phosphate buffer, cells were centrifuged, pellets post fixed in 2% (w/v) osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 2 h at 4°C, dehydrated in ethanol followed by acetone, and embedded in LX-112 (Ladd). Serial ultrathin sections (~40–50 nm) were cut using a Leica EM UC 6 ultramicrotome. The contrast of the sections was subsequently enhanced with uranyl acetate followed by lead citrate and examined in a Tecnai 12 Spirit Bio TWIN transmission electron microscope (FEI) at 100 kV. Digital images were recorded using a Veleta camera (Olympus Soft Imaging Solutions).

### **4.13 Immunofluorescence**

Cells were seeded onto coverslips and treated as indicated in the papers. After fixation for 10 min at 4°C in 4% formaldehyde, the cells were washed in PBS. For proteins other than transmembrane proteins, cells were permeabilized in PBS containing 0.1% Triton X-100 for 10 min at RT, blocked with 1% BSA in PBS for 1 h at RT and incubated with the primary antibodies of interest (previously blocked with 1% BSA for 2 h at RT) at 4°C overnight in a humidity chamber (Table 3). After washing the samples, secondary antibodies diluted in 1% BSA were added for 2 h at RT and protected from light. Nuclei were counterstained with Hoechst 33342 (10 µg/mL in PBS). Between all steps, the cells were washed 3 times in PBS for 5 min each. Finally, the coverslips were mounted using Vectashield H-1000 (Vector Laboratories) or Prolong Gold antifade reagent with DAPI (Life Technologies).

Plasma membrane co-staining was achieved by incubating fixed cells in the presence of FITC-conjugated cholera toxin B (CTB; 0.2 µg/mL; Sigma-Aldrich) for 15 min at RT in the dark. Similarly, free cholesterol was detected by Filipin III (50 µg/mL, Sigma Aldrich) in PBS supplemented with 10% FBS and incubation for 2 h at RT in the dark.

Lysosomes were visualized with LysoTracker® Red DND-99 (Molecular probes™) in accordance with the manufacturer's instructions. In brief, 100 nM of this dye was added to the media of control or treated cells for 30 min in advance of washing and fixing procedures. All samples were examined under a Zeiss LSM 510 META confocal laser scanner microscope (Carl Zeiss).

### **4.14 Measurement of caspase-3/-7-like activities**

After washing, cells were suspended in PBS and mixed with peptide substrate diluted in 100 mM HEPES, 10% sucrose, 5 mM DTT, 0.001% NP-40 and 0.1% CHAPS, pH 7.3. Cleavage of the caspase-3/-7-like substrate Ac-DEVD-AMC (acetyl Asp-Glu-Val-Asp 7-amido-4-methylcoumarin, Peptide Institute) was monitored in a Fluoroscan II plate reader (Labsystems) at excitation and emission wavelengths of 355 nm and 460 nm, respectively.

### **4.15 Colony assay**

Eighteen cells per cm<sup>2</sup> were seeded in 100 mm cell culture dishes one day in advance of experiments and then treated for 48 h. Drug-containing media were then replaced with normal media and cell colonies were allowed to form over 10 days before fixation in 100% methanol on ice for 10 min and staining in 0.04% (w/v) crystal violet solution.

#### **4.16 Calcium measurements**

Intracellular calcium levels were monitored using the Fluo-4 AM fluorescent indicator (Invitrogen). In brief, 4  $\mu$ M of the calcium probe was added to cells 30 min in advance of treatments. Time-lapse analysis of living cells was then performed using the Zeiss LSM 510 META confocal laser scanner microscope or the FACS Calibur system in combination with the CellQuest v.3.3 software (Becton-Dickinson).

#### **4.17 Measurement of lactate dehydrogenase (LDH) release**

The amount of lactate dehydrogenase (LDH) released, a marker for plasma membrane integrity, or necrosis, was determined using the fluorometric Cyto Tox-ONE Homogeneous Membrane Integrity Assay (Promega) in accordance with the manufacturer's instructions.

#### **4.18 Sub-G1 analysis**

Cells were harvested and fixed in 70% ethanol for 1 h at 4°C. Repeated washes in cold PBS and RNase A treatment (100  $\mu$ g/mL, Invitrogen) for 1 h at 37°C were followed by propidium iodide staining (40  $\mu$ g/mL, Sigma-Aldrich). Analysis in the FL3 channel in DDM mode was performed using the BD Accuri C6 system in combination with the BD CSampler software (Becton-Dickinson).

#### **4.19 Quantification of plasma membrane receptors**

The level of plasma membrane receptors was detected in cells after incubation with specific antibodies using flow cytometry analysis of live cells. Cells were treated, harvested by trypsinization, centrifuged at 1300 rpm for 5 min, washed in ice cold PBS supplemented with 5% FBS and centrifuged at 2000 rpm for 10 min. Cells were incubated with primary antibodies (anti-DR4, -DR5, and -transferrin) diluted 1:100 in washing solution (Table 3). Cells were then washed twice, and incubated at 4°C for 30 min with the secondary antibody (AlexaFluor-488-conjugated donkey anti-mouse-IgG). After washing twice, the cells were stained at 4°C for 15 min with 7-AAD (1  $\mu$ g/mL, Molecular Probes<sup>TM</sup>), which allowed for the gating of living cells, and analyzed by flow cytometry (FACScan, Becton Dickinson). The 7-AAD-negative cells were subjected to receptor analysis (Cell Quest software). Green fluorescence indicating the amount of the receptor present at the plasma membrane versus cell counts are visualized in histograms, enabling comparisons of control and treated cell populations, and related to control cell samples lacking the specific primary antibody.

## **4.20 Immunoprecipitation of DR5**

Cells were lysed on ice for 15 min in NP-40 lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40) supplemented with PMSF 0.1 µg/mL, cOmplete protease and phosphatase inhibitor cocktails (Roche). After sonication and centrifugation 1 mg of the protein extract was incubated with 2 µg DR5 mAb (clone F2/B4) at 4°C overnight. Proteins were then bound to Protein-G Sepharose 4 Fast Flow (GE Healthcare) and the beads washed 3 times in lysis buffer for 5 min each, diluted 1:1 in reducing 2xLaemmli buffer, and subjected to SDS-PAGE after denaturation.

## **4.21 Statistical analysis**

The results are expressed as means  $\pm$  standard deviations (SD). The GraphPad Prism 5.02 software and the t-test were used for analysis.

## 5. SUMMARY OF THE PAPERS

### Paper I:

Can G<sup>1</sup>, Akpınar B<sup>1</sup>, Baran Y, Zhivotovsky B, Olsson M

(<sup>1</sup> denotes equal contribution).

5-Fluorouracil signaling through a calcium–calmodulin-dependent pathway is required for p53 activation and apoptosis in colon carcinoma cells.

Oncogene 2013 Sep 19; 32(38): 4529-38.

5-Fluorouracil (5-FU), a uracil analogue, is used to treat various solid cancers, including colon carcinoma. Active metabolites of this compound develop their effects via DNA damage and RNA stress, but the relative importance of each triggering points remains elusive.

In Paper I we investigated the effects of 5-FU-induced cell death in the colon carcinoma cell line HCT116 in more detail, especially with respect to initial targets and ensuing death signaling. Upon 5-FU treatment, not only DR5 but also the FAS receptor accumulated in the plasma membranes. However, only DR5 is required for effective formation of the death-inducing signaling complex (DISC) and initiation of apoptosis as confirmed by siRNA silencing, immunostaining and fractionation of the membranes of control and treated cells. In addition, the process is facilitated by p53.

To identify initial factors important for stabilization of p53 and formation of the DISC, the Ca<sup>2+</sup>-chelator BAPTA, the RIP1-kinase inhibitor NEC1, the antioxidant Trolox, the acid protease inhibitor pepstatin A or the cathepsin B inhibitor CA-072 were used in combination with 5-FU. We found that lysosomal proteases promote effector caspase activity downstream of the DISC formation, while Ca<sup>2+</sup>-dependent calmodulin-mediated phosphorylation of at least three p53 serine phosphorylation sites (S15, S33, S37) act upstream of caspase activation.

Since the level of phospho-p53 activation at the serine 15 (S15) site was significantly decreased in the presence of BAPTA, we reintroduced *wt* and p53 mutated at the S15 phospho-site into HCT116 *p53*<sup>-/-</sup> cells using lentiviral transfection technology and found that the S15 phosphorylation is necessary for activation of caspase-8 and cleavage of PARP. Indeed, intracellular levels of Ca<sup>2+</sup> rose during the early hours of 5-FU treatment and remained for as long as 13 h. Ca<sup>2+</sup> was found to enter the cells at least partially via L-type channels and Ca<sup>2+</sup> originating from the endoplasmic reticulum contributed neither to the

phospho-p53 status nor the cell death. In line with these observations, HCT116 cells harboring a mutant S15 p53 showed a 50% reduction in their apoptotic response to 5-FU.

Altogether, the obtained results present the evidence of a novel apoptotic mechanism induced by 5-FU, dependent on extracellular  $\text{Ca}^{2+}$ , involving DR-DISC and regulated by p53, p53 phosphorylation and calmodulin.

## Paper II:

**Akpınar B**, Bracht EV, Reijnders D, Safarikova B, Jelinkova I, Grandien A, Vaculova AH, Zhivotovsky B, Olsson M.

5-Fluorouracil-induced RNA stress engages a TRAIL-DISC-dependent apoptosis axis facilitated by p53.

Oncotarget 2015 Dec 22; 6(41): 43679-43697.

Although 5-fluorouracil (5-FU) was discovered more than 50 years ago it is still commonly used for the treatment of several solid tumors. Primary drug effects relate to both DNA and RNA damage. The tumor suppressor protein p53 is mutated or not functional in most human cancers. Therefore, in Paper II we focused on cell death signaling pathways in 5-FU-stressed *p53*<sup>-/-</sup> cells.

Using the human colon carcinoma parental cell line HCT116 and its variant lacking p53, we found that the cell death *per se* induced by 5-FU is independent of p53. However, in the absence of the tumor suppressor, the appearance of all apoptotic markers examined, including loss of the mitochondrial membrane potential (MMP,  $\Delta\Psi_m$ ), release of cytochrome *c* into the cytosol, DEVDase (caspase-3/-7-like) activity and poly (ADP-ribose) polymerase-1 (PARP-1) cleavage was delayed.

Notably, in contrast to its *wt* counterpart, the HCT116 *p53*<sup>-/-</sup> cell line exposed to 5-FU exhibited a necrotic morphology and prominent release of lactate dehydrogenase (LDH). However, since these necrotic features were eliminated by a pan-caspase inhibitor (zVAD-fmk), they were concluded to be caspase-dependent. In addition, suboptimal apoptotic conditions due to lack of p53 were associated with the formation of ROS in mitochondria. Similar to the parental cell line, 5-FU treatment of p53-deficient cells led to formation of DISC in a TRAIL-dependent manner. Co-treatment with zVAD-fmk and 5-FU revealed that DNA damage reflected in phosphorylation of the histone H2AX ( $\gamma$ H2AX) is a consequence of apoptosis. Finally, our data suggest that silencing of PARP-1 function can sensitize the p53-deficient cells to 5-FU.

In conclusion, the data presented here enhance our understanding of factors that limit the efficacy of 5-FU. By excluding DNA as the main stress target in at least certain types of cells, our observations suggest alternatives to currently used synergistic treatment regimens.

### Paper III:

**Akpınar B**, Safarikova B, Lauková J, Debnath S, Vaculova AH, Zhivotovsky B, Olsson M.

Aberrant DR5 transport through disruption of lysosomal function suggests a novel mechanism for receptor activation.

Manuscript, re-submitted after revision.

Autophagy and apoptosis determine the turnover of cytosolic organelles and intact cells, respectively. In Paper III, reciprocal and/or unilateral interactions between these processes were examined in 5-FU-treated tumor cells employing a combination of chemical inhibitors, RNAi and genetic approaches. In contrast to cells with blocked apoptosis, either at the DISC or the mitochondrial level, p53 deficiency was associated with signs of deregulation of autophagy in response to 5-FU, including failure to induce degradation of p62 and elevated expression of LC3. Of the strategies utilized to prevent autophagy, disruption of lysosomal function with chloroquine (CQ) caused a profound reduction in the levels of apoptotic markers in 5-FU treated cells, with DR5, a death receptor member of the TNF-family essential for 5-FU-induced apoptosis, accumulating in lysosomes and autophagosomes upon treatment.

Since 3-MA, RNAi targeting of critical regulators of autophagy or inhibition of lysosomal cathepsins reversed apoptosis in different manners, it is unlikely that autophagy *per se*, but rather correct receptor transport is an important factor for 5-FU-induced cell death. Interestingly, apoptosis activated via TRAIL, the cognate ligand for DR5, remained unaffected in the presence of CQ, indicating that 5-FU activates the receptor by a discrete mechanism. A distinction between ligand- and chemotherapy-induced apoptosis could also be confirmed by introducing malfunction of protein transport from the ER to the Golgi with brefeldin A. Through depletion of membrane cholesterol or inhibition of cholesterol transport with methyl- $\beta$ -cyclodextrin or U18666A, respectively, the cytotoxicity of 5-FU was drastically reduced. Moreover, as with CQ, treatment with U18666A alone or in combination with 5-FU led to the accumulation of DR5 in the cytosol, thereby supporting the idea that correct trafficking of the receptor is important for 5-FU-mediated elimination of cells.

In conclusion, this study indicates a novel chemotherapy-induced mechanism for activation of DR5, which may have important ramifications on research conducted in the fields of apoptosis and tumor treatment.



## 6. DISCUSSION

DR5, one of the TRAIL receptors, has a prospective therapeutic application in cancer therapy since it was reported that TRAIL has the ability to kill cancer cells selectively<sup>157</sup>. Therefore, a better understanding of DR5 signaling pathways can help to develop new targeted therapeutic approaches. Analysis of tumor samples revealed that expression of DR5 is downregulated progressively with increased stage in CRC and mutations in DRs have been associated with tumor progression<sup>158-162</sup>. 5-FU can trigger the extrinsic apoptotic pathway, however detailed information related to how the signaling reaches to the DRs is still missing.

In the present study we investigated the 5-FU-mediated cell death signaling in HCT116 cells.

We have shown that upon 5-FU treatment, both FAS and DR5 localized to the plasma membrane but only the latter promoted caspase processing. In paper I, we concluded that a significant increase in intracellular calcium levels particularly through the L-type voltage-dependent calcium channels is an early response to 5-FU.

Stabilization and function of p53 is regulated not only by MDM2 but also by posttranslational modifications including phosphorylations. In humans, p53 can be phosphorylated on several serine and threonine residues upon DNA damage, ionizing radiation and endoplasmic reticulum stress leading to different molecular outcomes<sup>163</sup>. In support of our observations, alterations in the intracellular  $\text{Ca}^{2+}$  level have previously been linked to the regulation of apoptosis and p53 phosphorylations<sup>164</sup>.

We confirmed that p53 is stabilized in a time-dependent manner and also phosphorylated on several sites (ser15, 33, 37, 46, 6, 9, 392) upon 5-FU treatment as another early response. Therefore, we speculated that the  $\text{Ca}^{2+}$  response may be associated with p53 phosphorylations. There are several kinases that may promote p53 phospho-activation but due to the increase in intracellular  $\text{Ca}^{2+}$  levels we focused on those that are  $\text{Ca}^{2+}$ -related. Indeed, inhibition of  $\text{Ca}^{2+}$  by the chemical inhibitor BAPTA revealed that at least three serine residues (ser15, 33, 37) were affected. Out of three, only ser15, upstream of DISC formation, had an important role in caspase-8 and -3 activities and PARP cleavage. It should also be noted that mutation of the ser15 position did not affect DR-5 dimerization indicating that 5-FU-induced DISC formation is indeed p53-independent.

In agreement with our data, it has been reported that the ser15 phosphorylation site is important for efficient p53 stabilization and apoptosis<sup>165-167</sup>. Inhibition of ATM and

p38MAP kinase using chemical inhibitor or siRNA did not alter ser15 phosphorylation. Although this is in contrast with previous studies, suggesting that ATM, Chk2 and p38MAP kinases phosphorylate ser15, it is in support of the fact that alternative routes do exist<sup>168-170</sup>. Despite the discrepancy, we do not ignore that these factors can be important in 5-FU-mediated cell death by other mechanisms. Moreover, 5-FU-treatment of Chk2-deficient cells caused slower processing of caspase-8 and -3 compared to the parental cell line without affecting ser15 phosphorylation, demonstrating that this kinase may have a role in 5-FU induced apoptosis separated from p53.

Ligation of TRAIL to its cognate receptors DR4 and DR5 leads to DISC formation and apoptosis<sup>157</sup>. Thus, we subsequently tested whether 5-FU-induced DISC formation involves TRAIL. A soluble recombinant DR5 lacking the intracellular domain did not alter the apoptotic response to 5-FU whereas downregulation of the TRAIL protein by siRNA in the same treatment condition abrogated caspase-3 and PARP cleavage, indicating that 5-FU mediated apoptosis depends on intracellular TRAIL. Interestingly, ER stress-induced ligand independent DR5 activation has also been described<sup>172</sup>. In addition, co-treatment with recombinant TRAIL and 5-FU sensitized the HCT116 cells to apoptosis (Fig. 4A). Similar to our findings, Ganten *et al.* reported that 5-FU-induced DISC formation increased the apoptotic response to TRAIL by means of cFLIP downregulation<sup>171</sup>.

There are conflicting data regarding p53 status and the tumor response to chemotherapy. Studies in breast and colon cancer cell lines and xenograft tumors revealed that loss of p53 reduced chemosensitivity to 5-FU<sup>130,150,173</sup>. In addition, p53 point-mutations and overexpression in CRC patients at stage III and IV were associated with poor prognosis and resistance to chemotherapy<sup>130,131,174</sup>. On the other hand, Paradiso *et al.* could not find any correlation between p53 status and 5-FU sensitivity<sup>175</sup>. However, it should be noted that in 40-50% of all p53 overexpression incidents there is no correlation with mutations of the corresponding gene. In consideration of reported data, it seems that p53 can affect the response in a disease stage-dependent manner.

In paper II, we performed an evaluation of sensitivity to 5-FU with respect to p53 cell status, which, given that the tumor suppressors can be activated both upon DNA and RNA damage, is of particular importance<sup>176</sup>. In the absence of p53, DR4 and DR5 were still upregulated both at the protein and the mRNA levels, although to a lesser extent since they are p53 targeted genes. For this reason, the DR5-DISC was still formed in a slow manner. Downregulation of caspase-8, DR5, DR4 or TRAIL abrogated cell death independently from the p53 status. A 5-FU-induced cell cycle arrest in G1-S phase and all apoptotic

markers that were identified in *p53 wt* cells were also present in *p53<sup>-/-</sup>* cells but less pronounced and at later time points, indicating that absence of p53 does not change the cell death mode. Altogether, 5-FU-induced formation of the DISC should not be considered as a p53-dependent, but rather a p53-facilitated event.

Involvement of ROS in apoptosis and its relation to p53 were analyzed in several studies in order to cope with drug resistance<sup>177-180</sup>. HCT116 cells are type II cells which require involvement of mitochondria during apoptosis. We confirmed that absence of p53 caused an inefficient apoptosis coupled with ROS formation. Based on the TEM images, we observed that even if the cell death mode in *p53<sup>-/-</sup>* cells was apoptosis, they displayed features of necrotic morphology and was further characterized by release of LDH. We next questioned if this slow pace apoptosis can force *p53<sup>-/-</sup>* cells to die in other ways. Co-treatment with zVAD-fmk, uridine and Trolox abrogated the LDH release. However, formation of mitochondrial ROS was caspase-independent and thus may serve to mediate necrotic features. In reverse, 5-FU co-treatment with Trolox did not affect caspase processing. We also ruled out the programmed necrosis due to the absence of mitochondrial AIF release which can be invoked by Ca<sup>2+</sup> overload<sup>181</sup>. In conclusion, under the experimental conditions used, formation of mitochondrial ROS did not contribute to overall caspase processing in *p53<sup>-/-</sup>* cells but did contribute to release of LDH.

It has been suggested that p53 can both induce and suppress autophagy depending on the type of stress<sup>182,183</sup>. In paper III, we tested whether there is a cross-talk between autophagy and apoptosis upon 5-FU treatment. We observed signs of autophagy deregulation in *p53<sup>-/-</sup>* cells. Inhibition of apoptosis at DISC or mitochondria level, on the other hand, did not interfere with 5-FU-induced autophagy. In reverse, downregulation of autophagy related proteins by siRNA did not reduce 5-FU generated apoptosis. However, CQ and 5-FU co-treatment caused a prominent decrease in the apoptotic response at the caspase-8 level without altering the cFLIP isoforms thus positioning the effect upstream of DISC formation. As 5-FU-induced apoptosis was maintained in cells where key autophagy factors had been targeted by siRNA, we concluded that the effect of CQ is independent from autophagy. Moreover, CQ as a single agent increased p53 and DR5 protein levels without any apparent induction of cell death. At the time, we assumed this to be an effect of a dramatic change in receptor localization. Related to this observation, Park *et al.* reported that CQ can up-regulate DR5 mRNA levels via reduction in the E3 ligase of DR5<sup>184</sup>.

However, CQ treatment did not alter the levels of DR5 in the plasma membrane, nor did it alter TRAIL induced apoptosis, indicating that the receptor can be activated by at least two

different mechanisms. Accordingly, we speculated that there can be a functional link between apoptosis and lysosome functions, since we observed that CQ treatment led to the accumulation of DR5 in lysosomes and autophagosomes. Akazawa *et al.* showed that internalization of DR5 promotes lysosomal permeabilization and apoptosis<sup>185</sup>. However, co-treatment with cathepsin inhibitors rather promoted the 5-FU toxicity through p53 stabilization. Likewise, co-treatment with brefeldin A (BFA) increased PARP cleavage upon TRAIL-induced apoptosis but not upon 5-FU treatment, supporting that inhibition of ER to Golgi transport affected TRAIL- and 5-FU-induced apoptosis differently.

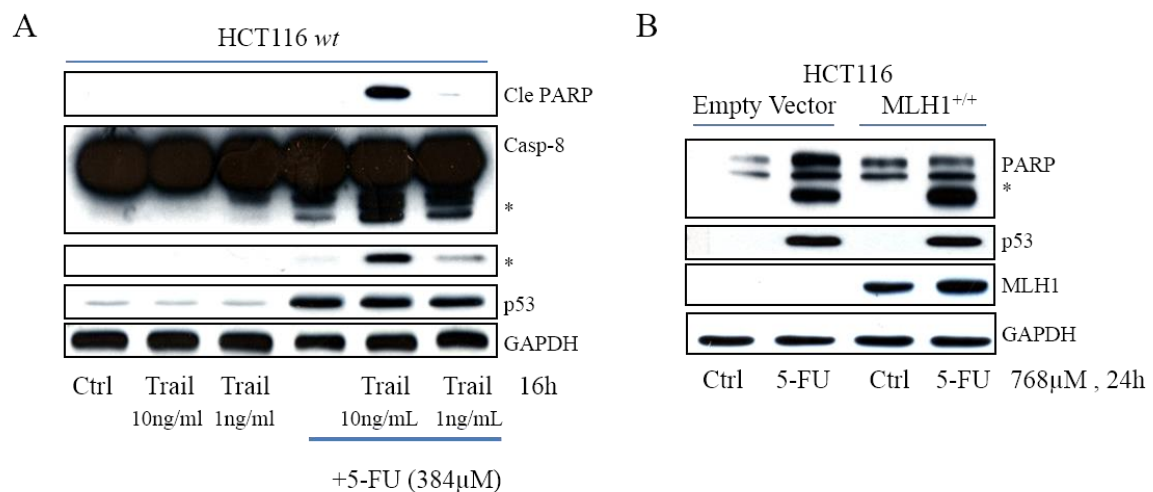
Cholesterol is an important component for membrane integrity, signal transduction and lipid raft formation, the latter has been associated with DR activation and formation of the DISC<sup>186</sup>. Indeed, inhibition of cholesterol trafficking, causing an increase in intracellular levels of soluble cholesterol, served to verify that 5-FU induced apoptosis requires cytoplasmic transportation of DR5.

MSI and components of the MMR system have been investigated as predictive and prognostic markers. Results of retrospective studies and meta-analyses suggested that patients with a deficient MMR system had better prognosis compared to those with an intact MMR system<sup>91,93,187,188</sup>. Investigations of MMR deficiency and resistance to 5-FU have presented conflicting results which can be due to analysis of different CRC stages between studies<sup>117</sup>. HCT116 is a MLH1 deficient cell line which can affect the sensitivity to 5-FU. However, we did not see any difference in generated apoptotic markers though Meyers *et al.* showed that reintroduction of MLH1 sensitized HCT116 cells to 5-FU (Fig. 4B)<sup>189</sup>. The disparity of the results can be an outcome of varying drug concentrations, exposure times, and exposure schedules. Clinical studies have also shown that 5-FU toxicity may vary between treatment regimens<sup>136</sup>.

The TS enzyme has two binding sites; one for the nucleotide and one for CH<sub>2</sub>THF. Irreversible binding of FdUMP to TS together with CH<sub>2</sub>THF results in a stable ternary complex leading to depletion of cellular thymidine pools and DNA damage<sup>139</sup>. In clinics, leucovorin is used in combination with 5-FU to increase the toxicity and drug response<sup>139,150</sup>. Leucovorin can stabilize the ternary complex by increasing the intracellular levels of reduced folate. To investigate the link between DNA and RNA damage and 5-FU-mediated apoptosis, we treated the cell together with leucovorin, uracil or thymidine. Co-treatment of leucovorin and thymidine together with 5-FU did not increase the apoptotic response whereas addition of excess uridine rescued the cells regardless of p53 status. Therefore, we speculated that 5-FU is not exerting its effect through TS inhibition in some

tumor cell lines. In support to this, MLH1 reintroduced HCT116 cells had the same sensitivity to 5-FU as their MLH1-deficient parental cell line (Fig. 4B). In addition, inhibition of caspase activity abolished the formation of  $\gamma$ H2AX and PAR which are regarded as DNA damage markers, indicating that DNA damage is a consequence of apoptosis and not *vice versa*. In fact, ATM inhibition in p53 *wt* as well as PARP1 inhibition in the absence of p53 increased the 5-FU toxicity, indicating that modulation of DNA repair systems can generate a synergistic effect with 5-FU-induced RNA stress.

Taken together, we concluded that in our experimental settings the effects of 5-FU is more directed to RNA rather than to DNA damage. In agreement with our findings, 5-FU and its metabolites have shown to affect proteins related to RNA metabolism. Moreover, 5-FU metabolites have been implicated in the downregulation of ribosomal proteins (Fig. 5)<sup>190</sup>.



**FIGURE 4.** TRAIL sensitizes HCT116 cells to 5-FU (A) and presence of the MLH1 protein does not affect drug-induced apoptosis (B).

HCT116 cells were either treated with 1-10 ng/mL TRAIL or 384  $\mu$ M 5-FU alone or with combination of both for 16 h. p53, processing of PARP and caspase-8 were analyzed by western blot (4A). HCT116 *wt* and HCT116 MLH1<sup>+/+</sup> cells were treated with 768  $\mu$ M 5-FU for 24 h and PARP, p53, MLH1 were analyzed by western blot (4B). GAPDH served as loading control (Fig. 4A-4B).

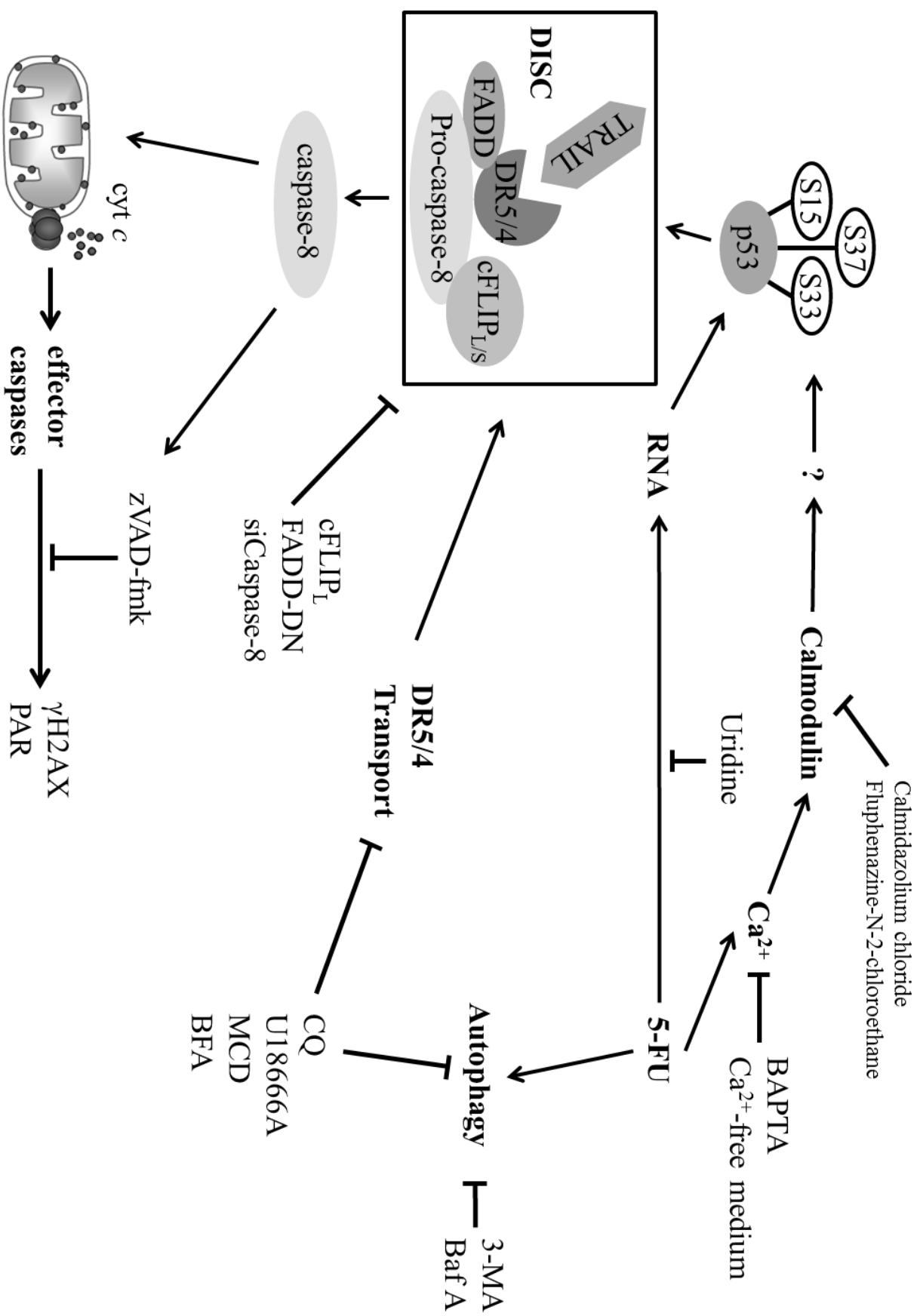


Figure 5. Summary of the findings in Paper I-III.

## 7. CONCLUSION

5-FU-induced apoptosis, in the human colon carcinoma cell line HCT116, depends on p53-facilitated formation of DR-DISCs. This process is, at least partly, regulated by an increase in intracellular  $\text{Ca}^{2+}$  levels via L-type channels and successive  $\text{Ca}^{2+}$ -calmodulin-kinase regulated phosphorylation of at least three p53 serine phosphorylation sites (S15, S33, S37). Mutation of the serine 15 phosphorylation site significantly reduces the apoptotic response to 5-FU.

The absence of p53 results in mitochondrial ROS formation, necrotic morphology and disruptions in autophagic induction. In addition, silencing of PARP-1 function sensitizes p53-deficient cells to 5-FU.

5-FU induces primarily RNA stress which involves a TRAIL-DISC-dependent apoptosis axis facilitated by p53.

Disruption of lysosomal function with chloroquine reduces the levels of apoptotic markers in 5-FU treated cells and leads to accumulation of DR5 in lysosomes and autophagosomes. Based on these findings, we disclosed that activation of DR5 is regulated by separate mechanisms in TRAIL- and 5-FU-induced apoptosis and that correct trafficking of the receptor is important for 5-FU- but not TRAIL-mediated tumor cell elimination.

## **8. SIGNIFICANCE OF THE STUDY**

5-FU has been the mainstay of CRC treatment for over 50 years. However, due to the frequent occurrence of p53 mutations, response rates for 5-FU in advanced CRC are modest. Although combinatorial treatments have improved survival rates, there is still a need for new therapeutic strategies potent enough to compensate or bypass cell death defects and improve disease prognosis. The data relating to mechanisms of 5-FU-mediated RNA stress and death receptor activation that are presented in this thesis will hopefully contribute to a better molecular understanding of drug toxicity, which is of the utmost importance for the development of future treatment approaches.



## 9. FUTURE PERSPECTIVES

The new mechanisms for p53 and DR5 activations indicated in paper I-II and III raise questions that may be addressed in future scientific projects.

1. With respect to DR5, how important are the processes of lysosomal exocytosis and intracellular receptor transport for oligomerization and activation?

We noticed that lysosomal accumulation of death receptors is a consequence of CQ or Baf A treatments. As we believe that a general membrane and receptor protein migration route, important for DR activation, occurs through lysosomes, our aim is to analyze the role of lysosomal exocytosis (LE) in DR activation. Exocytosis of secretory lysosomes is known to be present in all cell types and is important for cellular processes such as membrane remodeling and secretion. Quantification of LE occurring in response to DR activating toxicants will be accomplished through preloading (pinocytosis) of the cells with FITC-dextran. Loss of the dextran from the cells over time in control and treated cells will then be monitored by FACS. DR oligomerization/activation analysis will be accomplished by SDS-PAGE under non-reducing conditions. Compared with synaptic vesicle exocytosis, the molecular mechanisms underlying lysosomal exocytosis are much less understood. However, distinct sets of exocytosis machinery are employed, that will be used to specifically reduce LE in our experimental settings to further analyze the effect on DR activation and apoptosis.

2. With respect to calmodulin and post-translational modifications of p53, what is the role of  $\text{Ca}^{2+}$  in DR activation mechanisms?

The main purpose of this project is to define the molecular map indicated in paper I, leading from cytoplasmic  $\text{Ca}^{2+}$  influx via calmodulin to p53 phospho-activation, DR5 dimerization and apoptosis. The goal is to identify global phosphorylation changes between proteomes isolated from control cells, 5-FU-treated cells and treated cells where calmodulin has been silenced by means of RNAi. This will be accomplished by 2D DIGE (two-dimensional difference gel electrophoresis) followed by phosphorylated protein profiling. The experimental set up enables identification of phosphorylation events in general but also those specifically regulated by calmodulin.

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**Cevat**'a, Kardeş candır. Hayatın hep çalışmak olmadığını hatırlattığın için teşekkürler.

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Thank you,

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