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Helin Vakifahmetoglu

Karolinska Institutet

Karolinska Institutet

Institute of Environmental Medicine
Division of Toxicology
Karolinska Institutet, Stockholm, Sweden

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To all My Beloved ones

ABSTRACT

Activation of a family of cysteine proteases, called caspases, is an important event during apoptosis. In comparison to other caspases, less is known about regulatory functions of caspase-2. Previous studies from our group established caspase-2 as an essential apical regulator of apoptosis triggered by DNA damage. In addition, a primary role of caspase-2 has been implicated in DNA damage-induced mitotic catastrophe (MC). p53 family proteins have been suggested to play an important role in the activation of caspase-2, although the precise mechanism(s) is controversial. Despite considerable evidence indicating that caspase-2 in response to DNA damage engages a nuclear-mitochondrial pathway, assigning a distinct function to this protease has been difficult. Therefore, the main goal of this thesis was to investigate and understand the role of caspase-2 in different DNA damage-induced cell death scenarios.

We address the question of potential caspase-2 regulators in DNA damage-induced cell death pathways and provide data concerning caspase-2 activation mechanisms. We show that the presence of functional p53 is needed in order to complete the apoptotic process mediated through the mitochondrial pathway. Both caspase-8 and caspase-2 act as *bona fide* initiator caspases and p53 is fundamental for their activation. While no direct interaction between p53 and caspase-2 was observed in the cell systems used, we clearly demonstrate that a functional connection between these two proteins is essential to initiate an apoptotic process. We further demonstrate the significance of p53 for caspase-2 activation in apoptotic cell death triggered by PRIMA-1^{MET}-induced mutant p53 reactivation. In addition, as suppression of caspase-2 expression affected the p53 protein level, possibilities of a reciprocal interaction between these proteins are discussed.

Our results reveal the participation of endogenous caspase-2 with PIDD and RAIDD in the PIDDosome complex and the significance of this complex for caspase-2 activation in some cellular systems. However, our results also question its role as sole mediator of caspase-2 activation. Thus, we report that the latter is able to utilize the CD95-DISC as an activation platform. Our findings confirm a direct interaction between caspase-2 and -8 upstream of the mitochondria. Moreover, the ability of caspase-8 to cleave caspase-2 is demonstrated. Thus, the observed functional link between caspase-8 and -2 within the DISC complex represents an alternative mechanism to the PIDDosome for caspase-2 activation in response to DNA damage.

Here, we also investigated the phenomenon of MC induced by DNA damage. A role for p53 as a negative regulator of MC is suggested, in a process where neither processing nor activation of caspase-2 is required. Instead caspase-2 and caspases in general, are essential for the termination of MC, suggesting that MC-related morphological changes are followed by activation of the apoptotic machinery. Apoptosis, however, is not always required for MC lethality since necrosis-like lysis of cells was also observed following MC. Thus, we propose that MC is not a specific type of cell death but rather a pre-stage preceding cell death. The latter is determined by the cellular protein profile involved in the regulation of the cell cycle, such as p53 and Chk2. As a result, the Nomenclature Committee on Cell Death (NCCD) recommends the use of terminology such as ‘cell death preceded by multinucleation’ or ‘cell death occurring during metaphase’, when describing MC.

LIST OF PUBLICATIONS

I. **Vakifahmetoglu H,** Olsson M, Orrenius S, Zhivotovsky B.
Functional connection between p53 and caspase-2 is essential for apoptosis induced by DNA damage.
Oncogene. 2006; 25(41):5683-92.

II. Shen J**, Vakifahmetoglu H,** Stridh H, Zhivotovsky B, Wiman KG.
PRIMA-1(MET) induces mitochondrial apoptosis through activation of caspase-2
Oncogene. 2008; 27(51): 6571-80

III. Olsson M, **Vakifahmetoglu H,** Abruzzo PM, Grandien A, Zhivotovsky B.
DISC-mediated activation of caspase-2 in DNA damage-induced apoptosis
[*Under revision in Oncogene*]

IV. **Vakifahmetoglu H,** Olsson M, Tamm C, Heidari N, Orrenius S, Zhivotovsky B.
DNA damage induces two distinct modes of cell death in ovarian carcinomas
Cell Death Differ. 2008; 15(3):555-66.

Additional Publications (not included in the thesis)

I. Robertson JD, Gogvadze V, Kropotov A, **Vakifahmetoglu H**, Zhivotovsky B, Orrenius S.
Processed caspase-2 can induce mitochondria-mediated apoptosis independently of its enzymatic activity
EMBO Rep. 2004; 5(6):643-8.

II. **Vakifahmetoglu H,** Olsson M, Zhivotovsky B.
Death through a tragedy: mitotic catastrophe
Cell Death Differ. 2008; 15(7):1153-62.

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List of Abbreviations

$\Delta\psi_m$	mitochondrial membrane potential
AIF	apoptosis-inducing factor
Apaf-1	apoptosis protease activating factor 1
ASPP	apoptosis-stimulating protein p53
ATM	ataxia telangiectasia mutated
ATP	adenosine tri-phosphate
ATR	ATM and RAD-3 related
Bad	Bcl-2-associated death promoter
Bak	Bcl-2 homologous antagonist / killer
Bax	Bcl-2 associated X protein
Bcl	B cell lymphoma
Bcl-X_L	Bcl-2 related gene, long isoform
BH	Bcl-2 homology
Bid	BH3-interacting-domain death agonist
Bim	Bcl-2 interacting mediator of cell death
Bik	Bcl-2 interacting killer
Bmf	Bcl-2 modifying factor
CAD	caspase-activated DNase/DFF40
CARD	caspase requirement domain
Calpain	calcium-activated neutral protease
Caspase	cysteine-dependent aspartate-specific protease
Cdc	cell division cycle/control
CDDP	cisplatin, [*cis*-diamminedichloroplatinum(II)] or *cis*-DDP
Cdk	cyclin dependent kinase
Chk	checkpoint kinases
CICD	caspase-independent cell death
CRADD	CASP2 and RIPK1 domain containing adaptor with death domain
DAPI	4′, 6-diamidino-2-phenylindole dihydrochloride
DED	death effector domain
DFF	DNA fragmentation factor
DIABLO	direct IAP-binding protein with low p*I*
DISC	death-inducing signaling complex
DR	death receptor
DNA-PK	DNA-dependent protein kinase
DSB	double strand break
Endo G	Endonuclease G
ER	endoplasmic reticulum
FADD	Fas-associated death domain
FasL	Fas ligand
FLICE	FADD-like IL-1 converting enzyme (caspase-8)
FLIP	FLICE (caspase-8) like inhibitor protein
GADD	growth arrest and DNA-damage inducible gene
HMW	high molecular weight
HR	homologues recombination
HtrA2	high temperature requirement protein A2
IAP	inhibitor of apoptosis protein

ICAD	inhibitor of caspase-activated DNase/DFF40
ICE	interleukin-1β-converting enzyme
IMS	inter membrane space
JNK	c-Jun NH_2-terminal protein kinase
Omi/HtrA2	Omi stress-regulated endoprotease/high temperature requirement protein A2
OMM	outer mitochondrial membrane
MAPK	mitogen-activated protein kinase/ERK
MC	mitotic catastrophe
MDM2	mouse double minute 2
MOMP	mitochondrial outer membrane permeabilization
NAIP	neuronal apoptosis inhibitory protein
NALP	NACHT, LRR and PYD containing protein
NHEJ	non-homologues end joining
NFκB	nuclear factor of kappa light polypeptide gene enhancer in B-cells
NLR	NOD-like receptor
NLS	nuclear localization signal
PAK2	p21-activated kinase 2
PARP	poly (ADP-ribose) polymerase
PCD	programmed cell death
PI	propidium iodide
PIDD	p53-induced protein with a death domain
PIGs	p53-inducible genes
PIKK	phosphoinositide 3-kinase (PI3K)-related protein kinase
PK	protein kinase
PML-NBs	promyelocytic leukemia protein nuclear bodies
PRIMA	p53 reactivation and induction of massive apoptosis
PS	phosphatidylserine
PUMA	p53-upregulated modulator of apoptosis
RAIDD	RIP associated ICH/CED3 homologues protein with death domain
RIP	receptor-interacting protein
RNS	reactive nitrogen species
ROS	reactive oxygen species
siRNA	small/short interfering RNA
Smac	second mitochondrial-derived activator of caspases /DIABLO
SSB	single strand break
STS	staurosporine
tBid	truncated Bid
TNF	tumor necrosis factor
TNF-R	TNF receptor
TM	transmembrane
TOM	translocase of the outer membrane
TRADD	TNF receptor-associated death domain
TRAF2	TNFR-associated factor-2
TRAIL	TNF-related apoptosis inducing ligand
XIAP	X chromosome-linked IAP
VDAC	voltage dependent anion channel
WARTS	WTS/large tumor-suppressor
zVDVADfmk	benzyloxycarbonyl–Val–Asp–Val–Ala–Asp–fluoromethyl ketone

General Introduction

Historical remarks

Death is a universal event that all living organisms must go through. For every cell and organisms there is "a time to live" and regardless of its nature (programmed or accidental), "a time to die".

Freud elaborated the "death instinct" in his paper "Beyond the Pleasure Principle" published in German in 1920 as *Jenseits des Lustprinzips*. He came to the conclusion that all living beings have two primary instincts, the life-favoring instinct *Eros* (from the Greek, love), and the death instinct *Thanato* (from the Greek, death). The latter was described as a biological perception directed toward the organism's return to the inorganic state. According to Freud, an instinct or tendency toward own death is inherent to all living creatures, thus he was the first to propose that death was an active and physiological process.

Cell death was observed and recognized as an important biological process since the 19th century, while studying metamorphosis (larval and pupa development) in insect physiology. However, even after the discovery of phagocytosis at the beginning of 20th century, there was little attempt to define the mechanisms of cell death. The experimental examination of cell death was not demonstrated until the mid-20th century. In 1950´s, A. Glücksmann recognized the importance of cell death for morphogenetic (embryonic), histiogenetic (as in metamorphosis) and phylogenetic processes and attempt to illustrate different modes of cell death, such as an apoptotic appearance of nuclei (karyopyknosis) and nuclear fragmentation (karyorrhexis).[1] Furthermore, Viktor Hamburger and Rita Levi Montalcini demonstrated that, the degeneration and death of specific cells were an integral part of embryonic development at the cellular level.[2]

In the beginning of 1960s, several laboratories demonstrated that cell death is biologically controlled (programmed) and that the morphology of some dying cells was common. In 1963, J.W. Saunders and R.A. Lockshin independently utilized the expression "Programmed Cell Death" (PCD) by referring specifically to instances in which a sequence of events could be established to any form of cell death mediated by an intracellular program. In 1965, they suggested that cell death is regulated and published series of publications describing its molecular pathways.[3, 4] In the late 1960s, John Kerr, an Australian pathologist, observed that cells were dying by a consistent but inexplicable mechanism. He demonstrated that this distinct type of cell death, resulting from noxious stimuli, was different from necrosis, and suggested "shrinkage necrosis" or "programmed cell necrosis" as a name for this process.[5]

In the 1970s, the importance of lysosomal activity was implicated for morphological changes associated with cell death process.[6, 7] At first, this suggestion was criticized. However, the scientific community accepted the fact that programmed or genetically controlled cell death appears in different forms, hence may follow different pathways. In the seminal paper by J. Kerr, A. Wyllie and A.R. Currie in 1972, the term "apoptosis" was coined to describe a characteristic, identical sequence of events which they had observed in many different types of cells.[8] They describe the mechanism of cell elimination control which seemed to play a role complementary but opposite to that of mitosis in the regulation of animal cell population. The concept that cell death was as much a part of cell biology as life, according to Wyllie "went against twentieth century philosophy", and was neglected for several years.[8]

By 1990, the genetic basis of programmed cell death had been established, and the first components of the cell death machinery (caspase-3, Bcl-2, and Fas) were identified, sequenced, and recognized as highly conserved in evolution. During the last decade there has been a great interest in cell death research. The comprehension of cell death processes made it possible to identify apoptosis as a type of programmed cell death and that multiple pathways exist for genetically controlled cell death. The cells commitment to undergo death is distinguished from the actual execution pathway of cell death. Still, at present the knowledge concerning the signaling mechanisms of different cell death processes and how they are regulated is limited.

Programmed cell death

As mentioned above, the concept of PCD was introduced in the middle of the 1960's,[4] but attracted researchers only after apoptosis was verified as "a basic biological phenomenon with wide-ranging implication in tissue kinetics".[8] Initially, PCD was referred as apoptosis, but since that time, many different cell death pathways have been described, and it became clear that apoptosis is not the only cell death program. Recently, the Nomenclature Committee on Cell Death (NCCD) suggested definitions for eight mechanism-based types of cell death.[9] Yet, some researchers depicts up to 11 pathways of cell death in mammals.[10] However, an agreement whether all of these pathways illustrate examples of PCD is far to be achieved. According to the seminal publications, PCD refers specifically to a cell autonomous genetic developmental death program. Some of them will be described in this thesis.

Apoptosis

Among the different cell death mechanisms defined using morphological and biochemical criteria apoptosis (type I), autophagy (type II) and necrosis (type III) are the most extensively studied processes by which cells die.

The term Apoptosis, although first coined by Kerr, Wylie and Currie in 1972,[8] was a reintroduction for medical use. *Apoptōsis* (from the Greek, *apo* - from, *ptosis* – falling, or *apopiptein* to fall off), "dropping off" of petals or leaves from plants or trees, had a medical meaning to the Greeks over two thousand years ago. Hippocrates (460-370 BC) used the term to mean, "The falling off of the bones" and Galen extended its meaning to "the dropping of the scabs". Apoptotic cell death is involved in a wide range of physiological and pathological processes. It is essential for embryonic development, maintenance of tissue homeostasis, metamorphosis, maturation of the immune system and a defense mechanism to eliminate damaged and potentially dangerous cells.[11, 12] Dysregulation and/or failure to induce apoptosis or excessive apoptotic cell death are associated with a number of diseases, including cancer, rheumatoid arthritis and neurodegenerative diseases.[13, 14]

Apoptosis is an active and energy-dependent process that takes place at single cell level. It occurs in a controlled, well-regulated fashion, which requires RNA and protein synthesis.[11] Morphological changes are characterized by membrane blebbing, cytoplasmic shrinkage and reduction of cellular volume, condensation of the chromatin (pyknosis), and fragmentation of the nucleus (karyorrhexis), all of which ultimately lead to formation of apoptotic bodies, a prominent feature of apoptotic cell death and rapid phagocytosis by neighboring cells.[8, 15] Cells undergoing apoptosis are also characterized by several biochemical changes including the activation of members of a cysteine protease family called caspases,[16] and the selective cleavage of their cellular substrates,[17, 18] permeabilization of the mitochondrial outer membrane with subsequent release of pro-apoptotic factors into the cytosol,[19] the degradation of DNA by endogenous DNases, which cut the internucleosomal regions into double-stranded DNA fragments of 180–200 base pairs (bp),[20] changes in the phospholipid

content of the plasma membrane with subsequent exposure of phosphatidylserine (PS) to the outer leaflet.

The apoptotic machinery is highly conserved among species. Studies' using the model organism *Caenorhabditis elegans* first initiated by the cell lineage study by Sulston and Horvitz in 1977 and subsequently followed up by genetic and molecular studies by Horvitz and colleagues in early 1990s, provided a genetic framework for the cell death program, and was awarded by the Nobel Prize in Physiology or Medicine in 2002.[21-23]

Autophagy

When nutrients or growth factors are restricted, cells sequester cytoplasmic components into phagosome vesicles to be targeted for lysosomal degradation, in a process called autophagy. Autophagic cell death or autophagy (from the Greek, 'self eating') is morphologically defined as a type of cell death that occurs in the absence of chromatin condensation but accompanied by massive autophagic vacuolization of the cytoplasm. Under normal physiological conditions, autophagy is involved in the turnover of proteins and organelles. It has been shown to involve in cellular remodeling during differentiation, development and neurodegeneration, metamorphosis, aging and in pathogenesis of several diseases, such as cancer and muscular disorders.[24-26]

Like apoptosis, autophagy is an evolutionarily conserved process which occurs in all eukaryotic cells.[27] It can be activated in response to nutrient starvation, differentiation and developmental factors. In contrast to apoptosis, cells that die with an autophagic morphology have little or no association with phagocytes. The most prominent feature is the appearance of double- or multiple membrane enclosed vesicles, so called autophagosomes or autophagic vacuoles, in the cytoplasm, which sequester cytoplasmic components and organelles such as mitochondria and ER. These vesicles fuse with the lysosomal membrane, resulting in degradation of their content and recycling by catabolic enzymes.

Excessive autophagy may lead to collapse of cellular functions and promote cell death directly. Alternatively, autophagy can lead to the execution of apoptotic or necrotic cell death programs. Although autophagy and apoptosis are markedly different processes, several pathways regulate both autophagic and apoptotic machinery, presumably via common regulators such as proteins from the Bcl-2 family.[28] It has also been suggested that autophagy may be a process to reduce the cellular volume prior to apoptosis.[29] On the contrary, inhibition of apoptosis has been shown to induce autophagic cell death, and vice versa.[30] Thus conditions that promote both autophagy and apoptosis may provide the cell with a decision between the two pathways. Moreover, some reports indicate that cells undergoing 'autophagic cell death' recover upon withdrawal of the death-inducing stimulus.[31] In cases in which autophagy is suppressed by genetic knockout/knockdown of essential autophagy-related genes (atg), cell death was not inhibited but rather accelerated,[32] indicating that the prominent role of autophagy may be a pro-survival mechanism. Accordingly it is still debated whether autophagy represent either an alternative pathway of cell death or an ultimate attempt for cells to survive by adapting to stress.

Necrosis

Necrotic cell death or necrosis (from the Greek, 'dead body') also referred to as accidental cell death, is distinguished from apoptosis, and is considered as cell death following rapid loss of cellular homeostasis. This type of cell death is characterized by depletion of intracellular ATP stores, swelling of the cell (oncosis) due to accumulation of water and ion influx leading to disruption of cellular organelles and plasma

membrane rupture. Budding and formation of apoptotic bodies are absent and the nuclear chromatin is irregularly clumped. Leakage of intracellular contents induces an inflammatory response. Phagocytosis of the remnants of dead cells is delayed until accumulation of inflammatory cells.[33]

For a long time necrosis was an alternative to the apoptotic process as an uncontrolled, passive and energy-independent cell death. Indeed, as described above, necrosis has other distinct morphological features compared to apoptosis. However, recently it was suggested that, in contrast to necrosis caused by severe damage, there are various examples when this mode of cell death may be classified as a normal physiological and regulated (programmed) event.[34, 35] Some authors have even proposed the term 'necroptosis' to indicate the regulated necrosis. Although the precise mechanism of programmed necrosis is not elucidated, several cellular processes have been implicated in necrotic cell death, including signal transduction pathways and catabolic mechanisms, such as RIP kinase, Ca^{2+}, JNK/p38, and PARP activation.[36, 37] Thus far, however, there is no consensus on the biochemical changes that may be used to unequivocally identify necrosis.

Mitotic catastrophe

A delayed cell death, referred to as mitotic catastrophe (MC) was originally described as the main form of cell death induced by ionizing radiation.[38] However, it is shown to be triggered by exposure to microtubule stabilizing or destabilizing agents, various anticancer drugs and mitotic failure. The morphology of MC is different from apoptosis. It is characterized by multinucleated (the presence of several nuclei with similar or heterogeneous sizes) or micronucleation (resulting from chromosomes and/or chromosome fragments unevenly distributed between the two daughter nuclei) giant cells with the formation of nuclear envelopes around individual clusters of missegregated and uncondensed chromosomes.[39, 40]

MC has been defined in morphological terms as a mechanism of cell death occurring during or after dysregulated/failed mitosis, fundamentally different from apoptosis.[39] Alternatively, MC has been classified not as a mode of cell death but as a special case of apoptosis. The latter classification is based on the observation that MC shares several biochemical hallmarks with apoptosis, i.e., mitochondrial membrane permeabilization and caspase activation.[41, 42] In addition, MC is defined as a cell survival mechanism of tumors.[43] In this cases MC represent a process through which cells switch from an abnormal to mitotic cell cycle.[44] Despite, or maybe due to, these various definitions until recently there was no generally accepted classification of MC. However, in this thesis, we suggest that, despite its distinctive morphology, death-associated MC may present a "pre-stage" of apoptosis or necrosis,[45] that is determined by the molecular profile of the cells.[45-48] As a result, the Nomenclature Committee on Cell Death, recommends the use of terminology such as 'cell death preceded by multinucleation' or 'cell death occurring during metaphase', when describing MC.[49]

Crosstalk between different cell death modes

Under physiological conditions cells display adaptability with respect to how they die upon different stimuli. Although a particular cell death program may preferential be triggered in distinct circumstances, multiple pathways may be activated simultaneously or sequentially in individual dying cells. Furthermore, there seems to be a cross-talk existing between cell death pathways. In fact, low or moderate doses of some agents have been shown to trigger apoptosis, but increasing doses of the same agent, induces necrosis. The challenge is, therefore, not only to understand the mechanisms leading to cell death but to identify at the molecular level the connection between the different modes of cell death.

The mechanisms of apoptosis

Apoptosis can be triggered by different stimuli, such as reactive oxygen species (ROS), chemical exposure, cytotoxic agents, DNA damage, ionizing and gamma radiation, and viral infections. Depending on the cell type, stimuli and environmental factors, different apoptotic pathways are activated. The two best described pathways leading to apoptosis in the mammalian system are: the extrinsic (receptor-mediated) and the intrinsic (mitochondria-mediated) pathway. The extrinsic pathway depends on binding of appropriate exogenous ligands to death receptors at the cell surface. In contrast, the intrinsic pathway responds to signals from within the cell to induce apoptotic process via the release of mitochondrial proteins.[50, 51] These two pathways give rise to the same morphological changes in apoptotic cell.

The death receptor-mediated extrinsic pathway

The extrinsic apoptotic signaling pathway is initiated when death receptors at the cell surface encounter specific cognate "death ligands", inducing a conformational change that is transmitted through the cell membrane. The three major death receptors described are all members of the Tumor Necrosis Factor (TNF) Receptor Superfamily and include CD95 (Fas/Apo-1) with the appropriate ligand (CD95L), the death receptors, DR4 and DR5, with the TNF-related apoptosis inducing ligand (TRAIL) and the TNFα and TNF receptor (TNF-R1).[52, 53]

CD95 and the TNF receptors are integral membrane proteins containing a receptor domain exposed at the surface of the cell membrane and an intracellular death domain.[54] Binding of these receptors to a complementary death activator, CD95L and TNFα, respectively, causes aggregation and trimerization of the receptors, leading to transition of a signal to the cytoplasm.[54] Bringing together the intracellular death domain (DD) of three receptors appear to be the critical feature for signaling by these receptors. The 80 amino acid DD of these receptors, recruits adaptor proteins, such as TRADD, through homophilic interactions to form a platform for caspase activation, the death inducing signaling complex (DISC) on the cytosolic surface of the receptor. Caspase activation occurs within seconds following ligand binding and leads to apoptotic cell death. Through the DISC, the initiator caspases are activated by autoproteolytic cleavage.[55] These in turn activate a second group of caspases, known as effector caspases, by proteolytic cleavage at specific sites. Upon activation of the latter, the apoptotic process is culminated through cleavage and/or degradation of intracellular proteins.

Among the death receptors, CD95 is the most extensively studied. The DISC is composed by the assembled receptor CD95, the adaptor-accessory protein called Fas-associated death domain (FADD) and pro-caspase-8.[50] FADD harbors an N-terminal death effector domain (DED) along with a C-terminal DD. In response to receptor aggregation, FADD is recruited by CD95 through the highly conserved DD motif found in both proteins. The interaction of FADD and CD95 through their C-terminal DDs unmasks the N-terminal DED of FADD, allowing it to recruit pro-caspase-8 to the signaling complex.[56] Formation of the DISC triggers the self-cleavage of pro-caspase-8 into active caspase-8 (also called FADD-like IL-1 converting enzyme (FLICE)), which cleaves the downstream effector caspase-3, -6, and -7.

The caspase-8 activating capacity of DISC complex is regulated mainly by an inhibitory protein FLIP (FLICE-like inhibitory protein).[57] FLIP exists in several isoforms that are structurally similar to caspase-8, but lacks the enzymatic activity. Incorporation of FLIP into the DISC disables the DISC-mediated processing, thus preventing the activation caspase-8.

Molecules other than FADD and pro-caspase-8 or FLIP may also be involved in CD95-mediated apoptosis. Receptor-interacting protein (RIP) and the RIP-associated ICH (ICE and ced-3 homolog)/CED-3-homologous protein with a DD (RAIDD) form another signaling cascade of the receptor-mediated pathway.[58] The RIP-RAIDD pathway might serve as a backup for the FADD-caspase-8 system, but it does not represent a major CD95-mediated cell death pathway. Another DD-containing adaptor/signaling molecule, CASP2 and RIPK1 domain containing adaptor with death domain (CRADD), structurally similar to that of FADD was also suggested to induce DISC-mediated apoptosis. CRADD has an N-terminal caspase homology domain that interacts with caspase-2 and a C-terminal DD that interacts with RIP.[59]

The mitochondria-mediated intrinsic pathway

Mitochondria play an important role in the regulation of cell death,[60-62] and mitochondrial membrane permeabilization is considered a pivotal event that leads to disruption of the inner and/or outer mitochondrial membrane (OMM). Permeabilization of the mitochondrial membrane can be followed by the loss of $\Delta\Psi m$ and, depending on the stimulus, the subsequent release of various pro-apoptotic molecules which can initiate apoptosis through either caspase-dependent or -independent mechanisms.

The OMM contains anti-apoptotic members of the Bcl-2 family proteins, such as Bcl-2 and Bcl-X_L.[63] A balance between members of this family is considered to tightly determine whether mitochondria remain intact or become permeabilized, thus regulating the release of proteins.[64] The precise molecular mechanisms by which Bcl-2 family proteins regulate mitochondrial permeability are still being investigated.

Upon apoptotic stimuli, such as cytotoxic insults including viral infection, DNA damage and growth-factor deprivation several proteins, normally present in the mitochondrial intermembrane space (IMS), are released into the cytosol. These proteins include the apoptosis-inducing factor, AIF,[65] second mitochondrial activator of caspases (Smac)/direct IAP binding protein with low pI (DIABLO),[66] Omi stress-regulated endoprotease/high temperature requirement protein A2 (Omi/HtrA2), Endonuclease G (Endo G) and cytochrome *c*.[51] In particular, the release of cytochrome *c*, an essential component of the respiratory chain, is the most extensively studied and considered as an important step in apoptosis.[67] Although, the precise mechanism of cytochrome *c* release is still unknown, several models have been proposed.[68, 69] In the pore formation model, cytochrome *c* that normally resides on the outer surface of the inner mitochondrial membrane, is detached from the cardiolipin and is released to the cytosol through the pores formed by oligomerization of the pro-apoptotic Bcl-2 family proteins Bax or Bak.[70] The translocase of the outer membrane (TOM) complex,[71] or cardiolipin[72], an anionic phospholipid at the mitochondrial contact sites (where the outer and inner membranes are close to each other) have been proposed to be the positions for mitochondrial translocation of Bax.

Several apoptotic stimuli cause conformational change and/or translocation of Bax and Bak, from the cytosol to the OMM leading to cytochrome *c* release.[73] Once in the cytosol cytochrome *c* binds to the apoptotic protease-activating factor-1 (Apaf-1), inducing a conformational change allowing Apaf-1, in presence of dATP, to aggregate and assemble the apoptosome complex, a heptameric platform for pro-caspase-9 activation.[74] Pro-caspase-9 is recruited to the apoptosome complex through its caspase activation and recruitment domain (CARD) mediating homotypic interactions with Apaf-1 and is activated by oligomerization within this complex triggering auto cleavage, possibly by simply bringing pro-caspase-9 molecules into the close proximity, without the need for processing. Active caspase-9 cleaves and activates the

effector pro-caspase-3 and –7, responsible of cleaving various proteins leading to biochemical and morphological features characteristic for apoptosis described above.

Activities of caspase-3, -7 and -9 can be modulated by caspase-binding proteins of the inhibitor of apoptosis proteins (IAPs) family, including X-linked IAP (XIAP), c-IAP1, and c-IAP2.[75, 76] Smac/DIABLO and OmiI/HtrA2, being released from the mitochondria upon apoptotic stimuli relieves the inhibition of these caspases by binding to the IAPs, especially through disrupting the association of IAPs with caspase-9 and displacing XIAPs from its interaction with activated caspase-3.[66, 77] Thus, both activation and function of caspases can be regulated through binding of proteins released from mitochondria. Other mitochondrial components with regulatory roles in apoptosis such as AIF and Endo G will be described later.

Crosstalk between the apoptotic pathways

There is a crosstalk between the above described apoptotic pathways. In so-called "type I" cells, the apical initiator caspase-8 commences the activation of the effector caspases-3 and -7. Whereas in "type II" cells where there is no sufficient amount of activated caspase-8 allowing a direct activation of effector caspases, cleavage of the pro-apoptotic Bcl-2 family member Bid might become a major pathway.[78] The cleaved fragment of Bid, tBid, induces release of mitochondrial factors with subsequent activation of caspase-9 (discussed below).[79] Caspase-9, in turn, not only cleaves and activates downstream effector caspases but also the initiator caspase-8, thus forming a positive feedback loop that amplifies the original apoptotic signal.

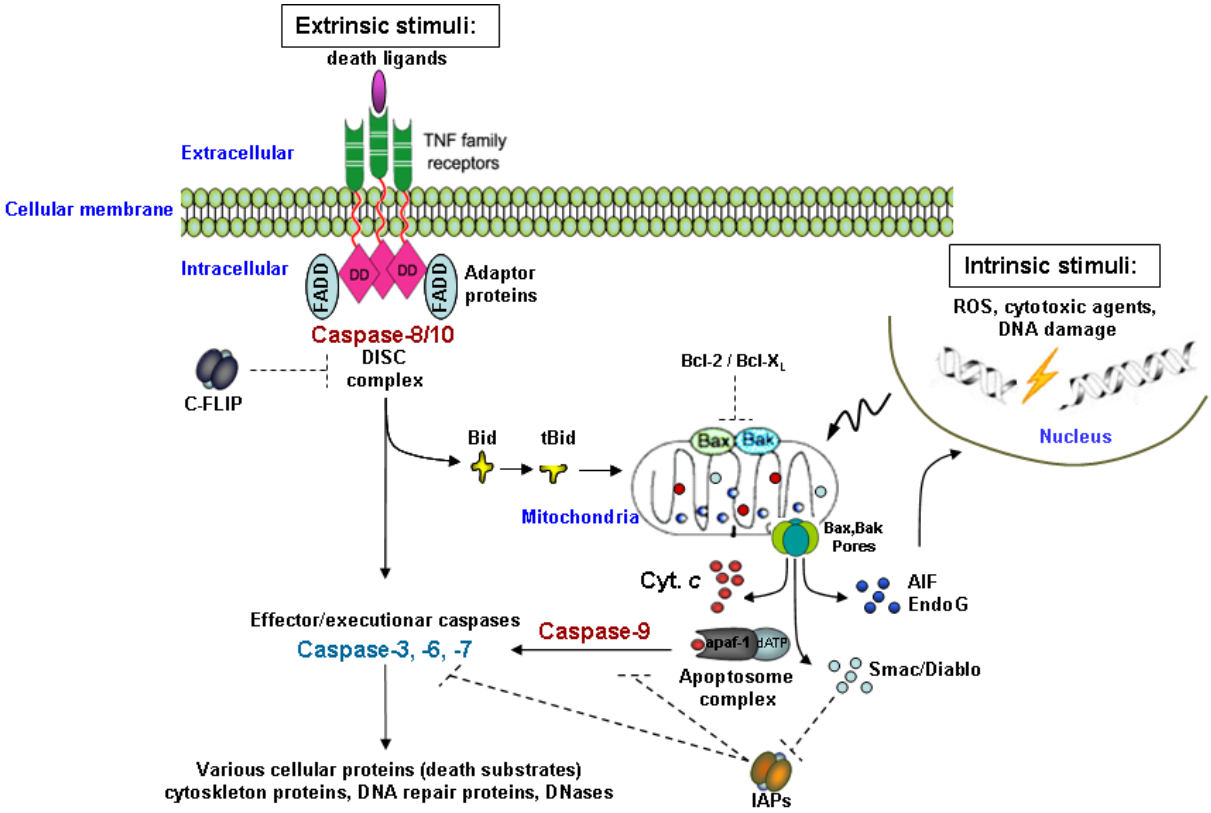

Figure 1

General overview of the extrinsic and intrinsic apoptotic cell death pathways

Caspases

A family of cysteine aspartate proteases known as caspases is typically activated in apoptotic cells and are one of the main regulators of apoptosis, involved in both initiation and execution of the death process.[16]

The first known member of the caspase family was caspase-1, initially known as interleukin-1β-converting enzyme (ICE), an enzyme required for the maturation of IL1β.[80] Caspases are a group of evolutionarily conserved proteases with a critical cysteine residue in the active site, which leads to loss of caspase activity if mutated.[16, 81] Although different caspases have different cleavage specificities involving recognition of unique amino acid sequences, the proteolytic activity of caspases is characterized by their ability to cleave proteins at the aspartic acid residues. The preferred cleavage site for the known caspases is at the C-terminal side of a four amino-acid motif, X-X-X-Asp (where X can be any amino acid).

Thus far, 14 members of the caspase family have been identified in mammals.[17] Caspases share a number of common features and are regulated at the post-translational level. They are widely expressed within the cell as inactive proforms or zymogens with regulatory N-terminal prodomains. There are three basic domains in the immature form: the prodomain, the large subunit (p20), and the small subunit (p10). Following induction of apoptosis the inactive pro-caspases undergo selective proteolytic processing (two cleavages) at specific aspartic acid residues.[82] The prodomain and the linker region between the large and small unit is cleaved. The active mature form of caspases consists of a tetramer containing two large and two small subunits as heterodimers (heterodimers of homodimers).[82] The zymogen processing, however, is not an indicator for initiator caspase activation. For example, the fully processed caspase-9 is only marginally active, similar to its unprocessed zymogen. However, only after recruitment of caspase-9 into the apoptosome complex leads to a dramatic increase (up to 2000-fold) in the catalytic activity.[83, 84] Thus, for caspase-9, activation has no relation with its cleavage; rather, it refers to the apoptosome-mediated enhancement of the catalytic activity of caspase-9.

According to the length of the prodomain caspases can be divided into two major groups.[18] The pro-inflammatory caspases (caspase-1, -4, -5, -11, -12, -13 and -14) called group-I caspases involved in activation/maturation of cytokines and the apoptotic initiator caspases (caspase-2, -8, -9, -10) called group-II caspases contain a long prodomain. Protein-protein interaction motifs, such as the DED (caspase-8 and -10) or the CARD (caspase-2, -9), located in the long prodomain mediates interaction between these caspases with adaptor molecules. In contrast, the apoptotic executioner caspases, also called effector or group-III caspases (caspase-3, -6, -7) are characterized by the presence of a short prodomain lacking the protein-protein interaction motifs. The downstream effector caspases are more abundant and active than the upstream initiator caspases.

Caspases are mainly localized in cytosol, but they also exist in the nucleus, ER and in the Golgi apparatus. The central function of caspases is to cleave a subset of specific cellular proteins. More than 400 substrates have been identified for caspases. Based on their function, the target proteins can be divided into different categories: mediators and regulators of apoptosis (effector caspases, Bid, Bcl-2 and Bcl-X_L, IAPs and $FLIP_L$), structural proteins, (α-fodrin, gelsolin, nuclear lamins and DNA fragmentation factor 45 kDa subunit (DFF45/ICAD), DNA-repair proteins (DNA-dependent protein kinase (DNA-PK), Rad51 and poly(ADP-ribose) polymerase (PARP)), cell cycle-related proteins (RB, Wee1, p21 and p27), and protein kinases (PKCs, MAPK, AKT, ERK, RIP, ROCK1, PAK2 (p21-activated kinase 2) and MEKK1). Upon cleavage, these proteins are responsible for morphological changes associated with apoptosis and the disassembly of a cell into apoptotic bodies.[85-87]

Mechanisms of caspase activation

Initiator and effector caspases are activated by different mechanisms. The zymogens of the initiator caspases exist within the cell as inactive monomers and require dimerization to assume an active conformation. The initiator caspase monomers are autoproteolytically activated when brought into close proximity of each other, which is called the 'induced proximity', later defined as the proximity-induced dimerization model.[88, 89] Based on this model, dimerization event occurs at multiprotein activating complexes, to which the caspase zymogens are recruited. Cleavage of initiator caspases is neither required nor sufficient for their activation, rather, this cleavage event is thought to provide stability to the dimer generated during complex formation, and the fundamental activation event is the actual dimerization of the monomers. Following dimerization to the catalytically active form, the N-terminal domain is removed, allowing the activated caspase to be released into the cytosol.[90]

The activating complexes for initiator caspases involved depends on the origin of the death stimulus, either extrinsic or intrinsic. The homo-dimerization of caspase-9 is promoted by the apoptosome complex whereas the DISC induces dimerization and subsequent auto-activation of caspase-8 or -10. Reminiscent of the DISC and the apoptosome, the caspase-activating complex inflammasome, has been proposed to be the platform for the activation of the inflammatory caspases.[91] Inflammasomes are multiprotein complexes that are responsible for the activation of caspase-1 and caspase-5. Three types of inflammasomes have been identified: the NALP-1 inflammasome, composed of the CARD-containing protein NACHT, LRR and PYD containing protein-1 (NALP-1), ASC, caspase-1 and caspase-5, the NALP3 inflammasome contains NALP3, CARDINAL, ASC and caspase-1, whereas the IPAF inflammasome contains IPAF, neuronal apoptosis inhibitory protein (NAIP) and caspase-1. NALPs, IPAF and NAIPs belongs to a family of intracellular receptors structurally related to Apaf-1 named NOD-like receptors (NLRs).[92] The formation of inflammasome complexes results in the processing and activation of the cytokines IL-1α and IL-18, which play a central role in the immune response to microbial pathogens.[93, 94]

Caspase-2 is also recruited into a protein complex similar to the Apaf-1/caspase-9 apoptosome.[95] Dimerization and activation of pro-caspase-2,[96] facilitated by its interaction with CARD-containing adaptor protein RAIDD and PIDD have been identified as members of this large complex designated the PIDDosome. It has also been suggested that caspase-2 associate with CD95 DISC and is activated in that complex, but apparently not required for CD95-induced cell death.[97] The exact mechanism of caspase-2 activation is still a matter of considerable debate. It is possible that caspase-2 activation occurs by more than one mechanism.

Similar to the initiator caspases, effector caspases can also be autoactivated when overexpressed in bacteria, likely through "Induced Proximity"; yet in mammalian cells they are activated specifically by active initiator caspases. In contrast to initiator caspases, the zymogens of executioner caspases exist as preformed dimers. Their zymogen latency is maintained by steric hindrances imposed by the interdomain linker region. Cleavage of this linker by active initiator caspases permits translocation of the activation loop, facilitating formation of the active site. Notably, the fundamental process of activation, translocation of the activation loop, is conserved for both the initiator and the executioner caspases.

Bcl-2 family of proteins

In addition to caspases, the Bcl-2 (B-cell lymphoma 2) family of proteins represents another group of evolutionarily conserved regulators of apoptosis. Functionally, the Bcl-2 proteins either inhibit or promote apoptosis and are the major

regulators of the mitochondrial events that activate the intrinsic pathway. They are also involved in ER-mediated regulation of the intracellular Ca^{2+} level.[98]

Currently more than 30 Bcl-2 related proteins have been identified in mammalian cells. Most of them possess Bcl-2 homology (BH1–4) domains and a C-terminal transmembrane (TM) domain allowing them to insert into membranes, such as the OMM, the nuclear envelope and the endoplasmic reticulum.[99] Based on their roles in apoptosis and the BH regions, Bcl-2 family members are divided into three subgroups: one anti-apoptotic and two pro-apoptotic groups. The anti-apoptotic Bcl-2 proteins including Bcl-2, Bcl-X_L (Bcl-2 related gene, long isoform), Mcl-1 (Myeloid cell leukemia 1), Bcl-B, Bcl-w and Bf1-1/A1 contain BH domains 1–4 and are generally integrated within the OMM.[100] They could also be found in cytosol, endoplasmic and nuclear membranes. The pro-apoptotic members lack the BH4 domain and are divided into two groups, the "BH3-only" proteins which include, Bad (Bcl-2 antagonist of cell death), Bid (Bcl-2 interacting domain death agonist), Bim (Bcl-2 interacting mediator of cell death), Bik (Bcl-2 interacting killer), Bmf (Bcl-2 modifying factor), Noxa and PUMA (p53-upregulated modulator of apoptosis), and the effector multidomain BH1-3 pro-apoptotic proteins, such as Bax (Bcl-2 associated x protein)], Bak (Bcl-2 antagonist killer 1), Bcl-X_s, Bok and Bcl-G_L.

BH3 only proteins function as sensors to distinct apoptotic stimuli.[101] Upon stimulation, activated BH3-only members mediate a cellular stress signal through protein–protein interactions with other Bcl-2 family proteins. The combined signaling within this family dictates the immediate fate of the affected cell to either initiate the intrinsic pathway by mitochondrial outer membrane permeabilization (MOMP) or not. Thus, a cells decision to undergo apoptosis would rely on the balance between the anti-apoptotic and the pro-apoptotic Bcl-2 family proteins (The rheostat model).[64]

Among the pro-apoptotic Bcl-2 proteins, Bax and Bak appear to be prerequisite for MOMP.[102] They normally exist as inactive monomers in cells and are activated by BH3-only proteins, such as Bid. Upon an appropriate signal, Bak, normally bound to the OMM, undergoes a conformational change and oligomerize in the membrane leading to pore formation in the lipid bilayer through which mitochondrial proteins can released into the cytosol.[103] Unlike Bak, Bax is mainly found in the cytosol, and translocates to the surface of the mitochondria where the anti-apoptotic proteins are located.[104] Once translocated, Bax also oligomerizes to form pores in the OMM. It is still unclear how BH3 only proteins stimulate the activation of Bak and Bax. Two models have been proposed to explain their activation mechanism.

The anti-apoptotic protein neutralization model, which is considered as a modern interpretation of the rheostat model, is based on the hypothesis that the pro-apoptotic protein function overcomes inhibition by the anti-apoptotic proteins. This model suggests that anti-apoptotic proteins continually inhibit the function of Bax and Bak to ensure mitochondrial integrity and survival. MOMP is indirectly promoted by Bax and/or Bak oligomerization, when all anti-apoptotic proteins are functionally neutralized by activated (either transcriptional or post-translational) BH3-only proteins. Accordingly, this mechanism is also referred to as the 'indirect' model.[105]

In contrast, the direct activation model refers to a direct Bak/Bax conformation change and/or translocation to mitochondria by the BH3-only proteins through a transient protein-protein interaction.[106, 107] In this model, BH3-only proteins are divided into two groups: the direct activators and de-repressors/sensitizers. Following stress, the sensitizers, such as Puma and Noxa (BH3-only proteins that cannot induce a direct activation of Bax or Bak alone) are induced, (either by transcriptional up-regulation or by post-translational modification), and bind to the anti-apoptotic Bcl-2 proteins, promoting the release of sequestered, direct activator BH3-only proteins (e.g. Bid or Bim). The interaction between the released direct activators with Bax or

Bak is the key event necessary to engage MOMP in this model. On the contrary, preventing this interaction by the anti-apoptotic Bcl-2 proteins is crucial for mitochondrial integrity and cellular survival.

Other apoptotic regulators

The expression, processing, activation/inactivation of caspases are strictly regulated by different mechanisms within the cells. In addition to the transcriptional and post-translational regulation of pro-caspase genes, the activity of caspases is controlled both by blocking the activation process of caspases, such as by FLIP at the DISC, or through inhibiting the enzymatic activity of caspases by IAPs. Nonetheless, there are a number of other mechanisms through apoptotic cell death can be regulated. The cleavage and activation of executioner caspases, as well as their substrates, can be commenced by proteins, such as granzymes, cathepsins or calpains via direct or indirect mechanisms.

Granzymes are a family of serine proteases with substrate specificity similar to caspases. The apoptotic signaling processes triggered by each granzyme species are relatively distinct. Granzyme B triggers apoptosis via both caspase-dependent, through cleavage of caspase-3, -8, and caspase-independent mechanisms that involve direct cleavage of caspase substrates including Bid and ICAD.[55, 108] Whereas granzyme A induces cell death via a caspase-independent pathway by targeting the SET complex (an ER-associated complex) resulting in the activating DNase during CTL-mediated apoptosis.[109]

Calpains (calcium-activated neutral proteases) represent another family of cytoplasmic cysteine proteases and are activated during apoptosis following intracellular Ca^{2+} increase. They are normally bound by the calpain-specific inhibitor protein calpastatin, which is cleaved by caspases during apoptosis. Calpains share common substrates with caspases, including fodrin, Ca^{2+}-dependent protein kinase and ADP ribosyltransferase/PARP.[110] They have also been shown to cleave Bcl-X_L and Bid, leading to activation of Bax, thus engaging the intrinsic pathway.[111]

Cathepsins are a group of aspartic (cathepsin D and E), serine (cathepsin G) and cysteine proteases that are largely found in lysosomes, where they participate in many biological processes. Recently, their role in apoptosis was demonstrated.[112] To exert their pro-apoptotic function, cathepsins are released from the lysosomes into the cytosol prior to mitochondrial membrane-potential changes.[113, 114] Bid seems to have a central role in lysosomal cathepsin-mediated apoptosis. Among all cathepsins, the aspartic protease cathepsin D is linked to the Bid-signaling pathway with subsequent activation of caspase-9 and -3.[115]

Caspase-independent cell death

As described above, apoptosis is generally dependent upon caspase activation with subsequent substrate cleavage ultimately leading to cell death. However, cells have been observed to frequently die in conditions when caspase activity is inhibited. This process is termed caspase-independent cell death (CICD) and depends on the release of certain mitochondrial proteins, such as AIF, HtrA2/Omi, and Endo G, in response to apoptotic stimuli.

Upon certain stimuli, AIF translocates from mitochondria to the cytosol and together with its obligate cofactor (cyclophilin A), it is imported into the nucleus. Compared to caspase-dependent apoptosis, which leads to extensive chromatin condensation and oligonucleosomal DNA fragmentation, AIF is involved in inducing peripheral chromatin condensation and large-scale DNA fragmentation.[65, 116] Due to these differences, the morphology of dying cells in AIF induced cell death is distinct

from caspase-dependent apoptosis, suggesting that AIF might cause a unique type of death via a yet unknown intracellular pathway.

The regulation mechanism of AIF release is unclear. It is proposed that its release may require caspase activity and depend on upstream mediators such as Bcl-2-family proteins or PARP-1.[117] In contrast, recently AIF release was shown to depend on its processing by a mitochondrial calpain during cell death in a caspase-independent manner.[118]

AIF does not have DNase function itself, thus the DNA fragmentation that occurs following its nuclear translocation may involve other factors.[116] It has been proposed that following MOMP, EndoG, a nuclear DNA-encoded nuclease normally present in IMS, sufficient to generate DNA fragmentation, translocates to the nucleus and cooperates together with AIF to induce cell death.[119, 120] However, the specific nature of this relationship has yet to be revealed.

The stress-activated endoprotease Omi, also known as high temperature requirement protein A2 (HtrA2), is a serine protease that following MOMP, releases into cytosol and promotes apoptosis via both caspase-dependent and independent mechanisms.[121]As previously described, Omi/HtrA2 indirectly initiate the activation of caspases by sequestering and cleaving IAPs, but also contributes to apoptosis via cleavage of cytoskeleton proteins that are non caspase targets. In addition, it can negatively regulate cell cycle progression during interphase, presumably through the proteolytic processing of the mitotic kinase WTS/large tumor-suppressor 1 (WARTS).[122, 123]

Cancer is a complex disease. Carcinogenesis involves a multistep process through which a succession of genetic alterations, each conferring a type of growth advantage, leads to the progressive conversion of normal cells into rapidly growing cancer cells (tumors).[124] The consequence of this uncontrolled cell division can cause significant damage to surrounding tissue and organs, eventually leading to death of the organism.[124,125] There is a close relation between cell death machinery and tumor progression, cancer therapeutics and tumor resistance to treatment.[124, 126] The ability to evade cell death is a major characteristic of uncontrolled cell growth.[127] Consequently, most if not all cancers develop mechanisms to abolish or circumvent this genetic program.[124, 126]

Apoptosis is a key tumor suppressor mechanism. Several chemotherapeutic agents aim to selectively trigger the apoptotic pathways in cancer cells while sparing normal tissue.[127] Accordingly, defects in apoptotic signaling often results in multi-drug resistance and reduce or abrogate treatment response. Mechanisms for inducing apoptosis in cancer cells differ for agent stimuli and depend on the molecular background of different cancer cells.[128] The majority of chemotherapeutic agents function through damaging DNA or interfering with DNA replication. The cellular responses to DNA damage, especially related to repair or tolerance of the damage as well as the activation of apoptosis, are critical elements in determining the effectiveness of most cancer therapies.[129, 130] Thus, identifying and understanding the roles of key proteins in DNA damage response pathways may lead to more effective conventional cancer therapy.

DNA damage

DNA damage or lesions can lead to various modifications of DNA that changes its property or function in transcription or replication. It include DNA adducts, single-strand breaks (SSBs), double strand breaks (DSBs), DNA cross-links and insertion/deletion mismatches.[131] DSBs constitute one of the most severe forms of DNA damage, as they affect both strands of DNA. DNA lesions can be generated in cells by exposure to various endogenous and environmental agents, including ROS and reactive nitrogen species (RNS), errors in DNA replication and repair, chemotherapeutic drugs, ultraviolet and ionizing radiation and heavy metals. The cellular response to DNA damage is a complex process that involves a network of interacting signal transduction pathways. It is initiated by proteins that detect or sense DNA damage and subsequently transmit a signal by activating a cascade of phosphorylation events. This ultimately results in the initiation of a number of cellular responses including cell-cycle transitions, DNA replication, DNA repair and apoptosis, which ensure the maintenance of integrity of the transcribed genome (see figure 2).

The precise mechanism of how DNA damage is sensed in cells is unclear. A group of fission yeast proteins, Rad and Hus, as the human homologue ATRIP, have been implicated as DNA damage sensors.[132, 133] It is proposed that these proteins form a trimeric complex, similar to DNA polymerase, called MRN (Mre11/Rad50/Nbs1) complex, which is recruited to the sites of DNA damage through binding to the broken DNA ends. Upon binding to DNA, MRN complex recruits signal tranducer kinases: DNA-dependent protein kinase (DNA-PK) and the phosphoinositide 3-kinase (PI3K)-related protein kinases (PIKKs), ataxia-telangiectasia mutated (ATM) and ATM/Rad3-related kinase (ATR). MRN complex plays a key role in the efficient and rapid activation of these kinases to propagate a DNA damage response.[134] The transducer kinases respond to different types of stimuli. DNA-PK and ATM mainly

function in response to DSBs and disruptions in chromatin structure, whereas, ATR primarily responds to stalled replication forks and SSBs. In general, ATM and ATR are considered as proximal components of independent pathways in which they function as an integrated molecular circuit that process diverse types of signals effectively linking the DNA replication apparatus with DNA damage response. Recent data, however, indicate that ATM could indirectly cause ATR activation through the MRN complex.

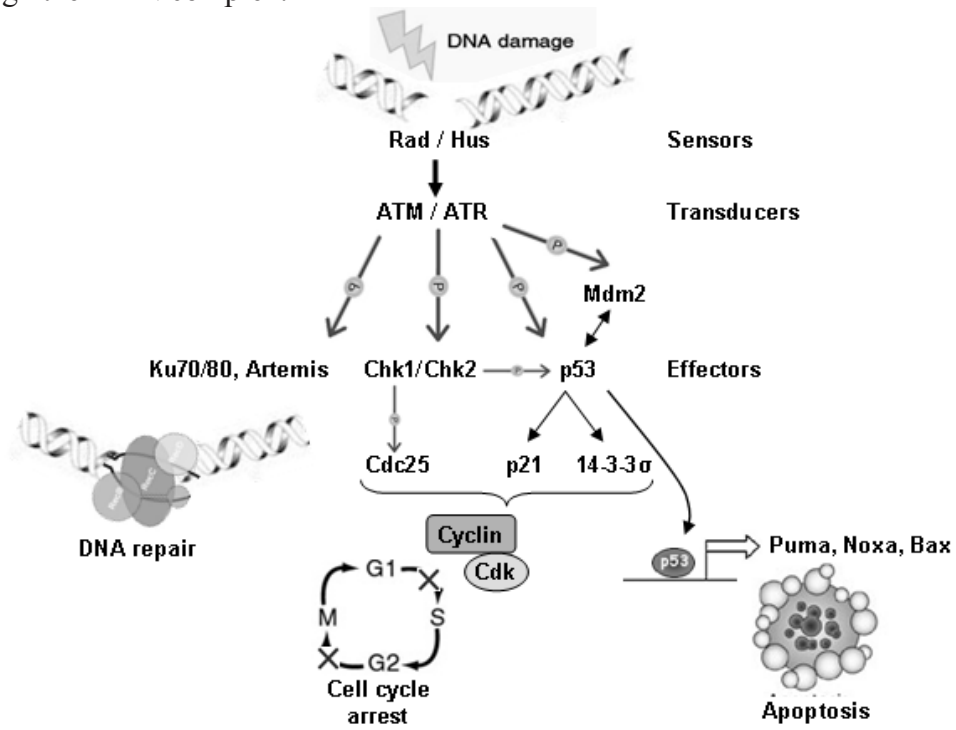

Figure 2
General overview of different cellular responses to DNA-damage

Once activated, ATM/ATR, which possesses serine/threonine kinase activity, phosphorylates downstream key DNA damage response (H2AX, BRCA1, MDC1 and C-Abl) and/or repair proteins (Ku70/80, Artemis and Rad51-like proteins) by recruiting them to the site of DSBs. Through interaction with ATR and the MRN complex, ATM mediates DNA damage repair via two major mechanisms, non-homologues end joining (NHEJ) and homologous recombination (HR). In addition, to provide time for DNA repair, ATM temporarily arrests the cell cycle by phosphorylation and activation of cell cycle checkpoints.

Checkpoint regulations have evolved to coordinate the response to different types of DNA damage.[135] Upon activation, checkpoint proteins pauses the cell cycle at the G_1/S and G_2/M boundaries regulating the transition into S-phase or mitosis, respectively. An intra-S and M-phase checkpoint also exists that regulates the progression through these cell cycle stages. The alert signal is modulated through the action of the so-called checkpoint mediators, including BRCA1, MDC1, and claspin and further transduced via phosphorylation of effector checkpoint kinases (Chk1 and Chk2).[136] Thus, arrest at the different phases of the cell cycle (G_1, S or G_2) are mediated by the ATM-Chk2/ATR-Chk1 responses and requires different downstream key substrates.

Both G_1/S and G_2/M arrest can be mediated by ATM either through ATM-p53/Chk2-p21-Cdk or ATM-ATR-Chk1-mediated pathways, respectively. Following DSBs, ATM directly phosphorylates the tumor suppressor p53 on Ser15, and Chk2, which further phosphorylates p53 on Ser20. These phosphorylation steps stabilize p53

by interrupting its association with its negative regulator, mouse double minute 2 (Mdm2). In addition, ATM can directly phosphorylate Mdm2, which also interfere its binding ability to p53. Upon activation, p53 transcriptionally induces $p21^{waf1/cip1}$, a cyclin-dependent kinase (cdk) inhibitor expression, which leads to inhibition of Cdk2-cyclin E, Cdk2-cyclin A, and Cdk4/6-cyclin D complexes. Inhibition of these complexes prevents E2F-dependent transcription of genes, required for an S-phase progression. In addition to p21, stabilized p53 can transcriptionally upregulate 14-3-3σ, which together with p21 and GADD45 (growth arrest and DNA-damage inducible gene) activate the G_2-M checkpoint directly by binding and inhibiting the required mitotic kinase Cdk1-cyclin B1 complex. Moreover, activation of ATR by ATM, with subsequent activation of Chk1 leads to phosphorylation and inactivation of Cdc25 family of phosphateses. Inhibition of the activity of this family remains the Cdk1-cyclin B1 phosphorylated and inactive. Thus, pathways initiating G_2/M arrest result in the inactivation of Cdk1-cyclin B1 complex, which targets proteins regulating transcription of genes necessary for initiation of mitosis.[137-139]

When repair processes are accomplished, cells reenter the cell cycle. Alternatively, if cellular machinery is not able to repair the injury during the arrest, damage can lead to irreversible cell cycle arrest, termed cellular senescence or different death-associated consequences.

p53, the guardian of the genome

Following exposure to genotoxic stress, depending on the severity of the DNA damage, cells, such as lymphocytes, may undergo apoptosis instantly without arresting the cell cycle and implementing repair mechanisms. These cells are particularly sensitive to apoptosis due to rapid induction of Bax in response to stimuli, such as irradiation. Other cells, especially those of fibroblast origin, do not undergo death immediately and become arrested in G_1 or G_2.[140] Only in cases of impair or deficient DNA repair processes these cells undergo apoptosis by engaging molecular cascades involving expression or activation of p53-family of proteins.

The p53 protein was first described in 1979, as a transformation-related protein associating with the SV40 DNA tumor virus large T antigen.[141, 142] It is known today as a tumor suppressor gene, and its inactivation is implicated in more than 50% of all human cancers.[143] Induction of apoptosis is the most conserved function of p53 and vital for tumor suppression. p53 belongs to a multigene family of proteins that also includes p73 and p63.[144, 145] Both p73 and p63 share 60% sequence homology with the DNA binding region of p53 and can induce cell cycle arrest and apoptosis in a similar way. These proteins, however, differ in their signaling pathways.[146] Moreover, although the members of the p53 family of transcription factors have overlapping functions, knockout studies have demonstrated that each of these proteins might play distinct biological roles.

p53 is a key molecule in response to genotoxic stress and is activated transcriptionally and/or by posttranslational modification, including phosphorylation, acetylation and methylation at multiple sites, which contribute to its stabilization.[137] Several kinases are demonstrated to initiate signaling pathways through p53 phosphorylation in response to DNA damage. The ATM kinase phosphorylates p53 at Ser^{15} in response to ionizing radiation, either directly or indirectly via Chk1-2, whereas ATR plays a role in activation of p53 on Ser^{37} upon exposure to UV-light. In addition, DNA-PK, casein kinase 1, p38 mitogen-activated kinase (MAPK) and Jun N-terminal-kinase (JNK) phosphorylate p53 at different serine or tyrosine residues leading to its stabilization. p53 modifications and chromatin remodeling, through p53 interactions with transcriptional co-activators such as acetyltransferases (p300/CBP, PCAF), methyltransferases (SET9), JMY and/or apoptosis-stimulating protein p53

(ASPP), further activate p53 and increase its DNA binding specificity, allowing selective transcription of p53-dependent genes.[147] In general, DSB-induced phosphorylation on Ser^{46} is assumed to specifically promote the induction of apoptosis inducing genes.

p53 is primarily a sequence–specific transcriptional activator.[148, 149] By binding to p53 responsive elements within the genome and activating transcription of various genes, it can trigger the onset of various cellular processes including gene transcription, DNA synthesis, DNA repair, cell cycle arrest, differentiation, senescence and apoptosis. Hence, it is called "the guardian of the genome" owing to its ability to integrate many signals that control cell survival and death.[150] Both the expression and the activation of p53 are strictly regulated. Under normal conditions, the p53 protein level is low due to Mdm2-mediated ubiquitination and subsequent proteasomal degradation. Mdm2 is an ubiquitin ligase that both blocks p53 transcription and activity (sterically) and mediates the degradation of p53 protein. Upon phosphorylation (of both p53 and Mdm2), p53 interaction with Mdm2 is inhibited, resulting in its stabilization and rapid protein level increase.[151]

Unlike the cell cycle inhibitory capacity of p53, which is mediated primarily by p21, the p53-dependent apoptotic response is more complex and involves activation of multiple pro-apoptotic proteins and engages both transcription-dependent and/or independent mechanisms.[152] In response to genotoxic insult, these pathways are assumed to act together, thereby amplifying the apoptotic signal. Upon activation, p53 induces transcriptional upregulation of various pro-apoptotic target genes, including Bax, DR4, DR5/KILLER, p53AIP1, PIDD, Bid, CD95, Bid, PIGs, Apaf1, Scotin, Puma and Noxa.[153-155] In addition, p53 represses transcription of anti-apoptotic genes, including survivin, Bcl-2 and Bcl-X_L. Transactivation of pro-apoptotic genes initiates apoptosis by different mechanisms. In particular, Bid, Bax, PUMA and Noxa, the Bcl-2 family proteins are of importance for the pro-apoptotic function of p53 mediated through the mitochondrial pathway. In addition, p53-inducible genes (PIGs) encoding redox-regulating enzymes, whose function is connected to membrane integrity, mediate apoptosis through generation of ROS leading to mitochondrial degeneration. CD95 and DR5 are components of the death receptor-mediated apoptotic pathway.[156] p53 also induces apoptosis via an ER-dependent mechanism by transactivating the expression of Scotin, a protein located in the ER that binds to the calcium-binding apoptotic protein ALG-2. PIDD, p53 upregulated protein with a death domain, appears to be a crucial target gene in a signalling pathway that is initiated by p53 and ultimately leads to either activation of a NF-κB-dependent cell survival pathway, or apoptosis. The regulation of these alternate signaling pathways is dependent on the modulation of PIDD autoprocessing. Hence, introduction of a limited amount of DNA damage leads to the formation of a NF-κB-activating fragment of PIDD, PIDD-C, whereas more severe damage triggers formation of PIDD-CC and a strong apoptotic response.[157] The apoptotic response includes activation of caspase-2 within the PIDDosome complex,[95, 158] with subsequent release of cytochrome *c*.[159-161] Thus the PIDDosome-mediated caspase-2 activation may be an important link between DNA damage and the engagement of the mitochondria-mediated apoptotic pathway.[95, 162]

The transcription-independent pro-apoptotic function of p53 involves translocation of p53 to the mitochondria upon DNA damage. The precise signal for this translocation is unclear. However, upon translocation, p53 is suggested to directly bind Bcl-2 and Bcl-X_L, liberating pro-apoptotic proteins Bax and Bak resulting in their activation and oligomerization with subsequent MOMP. p53 is also suggested to directly bind Bak by disrupting the inhibitory Bak/Mcl1 complex, enabling Bak to ultimately undergo oligomerization. In addition, in cells with a cytosolic Bcl-X_L fraction, p53 may activate Bax through a 'hit and run' mechanism

involving an intermediate cytosolic p53/Bcl-X_L complex that is disrupted by cytosolic Puma, which results in Bax conformational change, mitochondrial translocation, oligomerization and MOMP.[163, 164]

The great majority of p53 mutations result in inactivation or impairment of p53 DNA binding and transcriptional activity. Thus, mutant p53-carrying tumors are usually more resistant to currently used anticancer therapy. Reconstitution of wild-type p53 function in human tumors is, therefore an important therapeutic goal. Several low molecular weight compounds that reactivate mutant p53 have been identified, including, CP-31398, MIRA-1 and PRIMA-1.[165-168] PRIMA-1 (p53 reactivation and induction of massive apoptosis) restores wild-type conformation of mutant p53 and its binding to DNA, and induces apoptosis in a broad range of human tumor cell lines. PRIMA-1^{MET}, a methylated form of PRIMA-1, appears to be an even more potent in inducing mutant p53-dependent apoptosis than the original compound.[169] However, the mechanism(s) by which PRIMA-1^{MET} induces apoptosis is currently not elucidated.

Although p53 plays a central role in DNA damage-induced apoptosis, cells lacking p53 or carrying p53 mutations can still die from DNA lesions. Delayed death, commonly related to MC (described above), can occur in cells with impaired/lost checkpoint functions in response to DNA-damage. MC as a consequence of cell failure to arrest in the G_2 phase in response to DNA damaging agents has been described in cells with deficient, down-regulated, or inhibited G_2 checkpoint proteins, including not only p53 but also p21, Chk2 and 14-3-3σ.[138, 139, 170] Given that p53 or its related pathways are inactivated, abrogation of the G_2 checkpoint leads to entrance of cells into premature mitosis in the presence of unrepaired DNA.[138, 171-173] Abnormal mitosis due to premature mitotic entry may lead to genomic instability or cell death through either p53-independent apoptosis or necrosis based on the molecular profile of the cells. [46, 47]

Caspase-2

Previous studies both from our group and others, verify that in various cell lines, as a result of DNA damage induced by different apoptotic stimuli, including cisplatin, etoposide, UV-light and gamma-irradiation, pro-caspase-2 is activated. This activation leads to subsequently release of cytochrome *c*, indicating that caspase-2 acts through the mitochondrial pathway [159-161]. In addition, in cells pretreated with the caspase-2 inhibitor, benzyloxycarbonyl–Val–Asp–Val–Ala–Asp–fluoromethyl ketone (z-VDVAD-fmk), or stably transfected with pro-caspase-2 antisense, blocked cytochrome *c* release and attenuation of downstream events, including caspase-9 and -3 activation, PI exposure and DNA fragmentation was observed. Furthermore, neurons, germ cells and oocytes from caspase-2 knockout mice are shown to be more resistant to β-amyloid (Aβ)- and doxorubicin-induced cell death compared to germ cells and oocytes of caspase-2 wild-type mice.[174] Taken together, these data suggest that caspase-2 provides an important link between DNA damage and the engagement of the apoptotic pathway.

Caspase-2 (Ich-1/ NEDD-2) was initially described as a neuronaly expressed caspase that was downregulated during brain development.[175] It was one of the first mammalian caspases to be identified and is the most conserved caspase across species.[175, 176] This suggested that caspase-2 may comprise a critical role during PCD in mammals, however, the lack of a profound phenotype in caspase-2 deficient mice made this implication difficult to assign for caspase-2. Therefore, until recently, interest has been focused on other members of the caspase family.

Phylogenetically, caspase-2 belongs to the Ich-1 subfamily of IL-1β converting enzyme (ICE) cysteine proteases. It shares sequence homology with the initiator caspases, especially caspase-9 and -1.[177] Similar to other initiator caspases, pro-

caspase-2 contains a long prodomain, containing a CARD domain. In addition, pro-caspase-2 contains two subunits, p19 and p12, which are important for processing and activation of this enzyme. Cleavage specificity of caspase-2 is closer to that of effector caspases, caspase-3 and -7.[178, 179] Consequently, caspase-2 is unique among all caspases displaying features of both initiator and effector caspases. The gene encoding caspase-2 produces two isoforms derived from alternative splicing. The main splicing isoform, caspase-2L mRNA transcript encodes the full-length pro-caspase-2 (435 amino acids), whereas a shorter truncated version caspase-2S (312 amino acids) lacks the enzyme active domain, and functions as a inhibitor of apoptosis.[180] Recently, RBM5 was identified as a protein interacting with caspase-2 pre-mRNA and regulate the caspase-2 alternative splicing.[181] Like other caspases, caspase-2L (referred to here as caspase-2) exists in cells as an inactive pro-enzyme (51 kDa), which is processed in response to apoptotic stimuli. The proform of caspase-2 is found in the cytoplasm and the Golgi complex, and is the only pro-caspase constitutively present in the nucleus[182-184]. Nuclear import of pro-caspase-2 is regulated by two nuclear localization signals (NLS) located in the prodomain. Both the cytosolic and the nuclear form of caspase-2 have been demonstrated to induce apoptosis,[161, 182, 185] whereas localization of caspase-2 in the Golgi complex is assumed to contribute to the fragmentation of this organelle during apoptosis.[183, 184]

In contrast to other known caspases, which require tetrapeptide specificity for cleavage, the preferred substrate for caspase-2 is the pentapeptide VDVAD. Among more than 400 proteins known to be cleaved by different caspases, only very few serve as target proteins for caspase-2. In addition to itself, active caspase-2 can cleave Bid, PARP, Plakin, HDAC4, Huntingtin, Golgin-160, PKCδ, αll-spectrin and DNA fragmentation factor (DFF45)/ICAD.[183, 186-192] Cleavage of these proteins by caspase-2 indicates that caspase-2 can act both as an initiator signaling caspase (cleavage of itself, Bid and PKCδ) and an effector executioner caspase (cleavage of αII-spectrin, PARP, DFF45/ICAD).

Caspase-2 interacts with various proteins, including RAIDD, death effector filament-forming Ced-4 like apoptosis proteins (DEFCAP), pro-apoptotic caspase adaptor protein (PACAP), apoptosis repressor with caspase recruitment domain (ARC), glucocorticoid modulatory element-binding protein 1 (GMEB1), TNFR-associated factor-2 (TRAF2) and RIP1, the serine-threonine kinase containing death domain.[193] Among these proteins the association with RAIDD seems to be particularly important for caspase-2. RAIDD contains both a CARD that interacts with the prodomain of caspase-2 and a DD which bind to DD of other proteins. Through these homotypic interaction, RAIDD connects caspase-2 to different proteins, thus assembling caspase-2 into high molecular weight complexes.[194-196] Prevention of the caspase-2 interaction with RAIDD, though phosphorylation of caspase-2 prodomain by casein kinase-2 is suggested to negatively regulate its activation.[197, 198]

The crystal structure of the CARD-deleted activated caspase-2 revealed that for caspase-2, as other initiator caspases, dimerization (and not processing) is the key event for its activation. However, although the caspase-2 zymogen does not require cleavage for the initial activation, dimerization is followed by autocatalytic cleavage of the prodomain at D333 and at the linker region (D347), connecting the large and small subunits, of each participating monomer of pro-caspase-2. The catalytic activity, determined by C320, seems to be important for the processing of caspase-2 by an unknown mechanism.[96] This processing promotes further stabilization of the dimer formation and is essential to enhance the catalytic activity of caspase-2 required to induce cell death. Thus, both dimerization and cleavage are required for cell death-induced by activated caspase-2. Unlike caspase-8 and -9, fully processed

caspase-2 forms a stable $(p19 + p12)_2$-dimer that contains two active sites, one on each monomer. The two caspase-2 monomers are covalently linked by a central disulfide bridge, a unique feature of caspase-2.[96, 199]

Although dimerization is prerequisite for caspase-2 activation, the precise molecular mechanism of its activation upon apoptotic stimuli, in contrast to other initiator caspases, is poorly defined. It is unclear whether pro-caspase-2 needs to be recruited into a complex with other protein(s) to become activated. In fact, upon overexpression caspase-2 activation seems to occur by an autoproteolytic mechanism, without the requirement of any adaptor protein to oligomerize. Thus it is suggested that, in response to apoptotic signals, pro-caspase-2 molecules that normally exist as monomers in a cell are recruited into close proximity and aggregated spontaneously via interactions mediated through its prodomain. Brought into close proximity, the protease domains of caspase-2 can interact to form transient dimers, which is sufficient to impart some intrinsic catalytic activity to the unprocessed zymogens.[96, 200]

On the contrary considering the sequence similarity with caspase-9, the activation of pro-caspase-2 has been proposed to require the formation of a complex, similar to the apoptosome. Caspase-2 was shown to shape elaborate filamentous and dot-like structures through its CARD domain when expressed in high concentration.[182] Indeed, caspase-2 has been demonstrated to be assembled into a large protein complex with a molecular weight of 670-kDa in cells.[96] Characterization of the PIDDosome complex revealed the presence of an adaptor protein RAIDD and the p53-induced protein with a death domain (PIDD) with pro-caspase-2.[95] The crystal structure revealed that within the PIDDosome complex seven pro-caspase-2 molecules are brought into close proximity for their activation. The identification of PIDDosome indicated a role for p53 and/or p53-related proteins, in caspase-2 activation, since PIDD seems to function in a p53-dependent manner. Thus, the complex assembled by PIDD and caspase-2 are likely to regulate apoptosis induced by DNA damaging agents. However, the molecular mechanism by which the PIDDosome facilitates the activation of caspase-2 remains unknown. It is also unclear whether any additional cofactors are involved in the assembly of PIDDosome. Most importantly, the exact role of PIDDosome in apoptosis remains to be elucidated.

In addition, pro-caspase-2 is suggested to be recruited to the CD95/DISC in some human cell lines, and activated at the DISC on CD95 stimulation. Despite its presence at the DISC, caspase-2 was not involved in the apoptotic response upon CD95 stimulation. Moreover, activation of caspase-8 within the DISC was independent from caspase-2. Thus, the role of DISC for activation of caspase-2 in CD95-mediated apoptosis needs further clarification.

Caspase-2 is also suggested via interactions with RIP1 and TRAF2 to be recruited into a large and inducible 670-kDa complex, involved in signaling pathways leading to both NF-κB and p38 MAPK activation. However, in contrast to apoptosis signaling, NF-κB and p38 MAPK activation seems independent of the proteolytic activity of caspase-2. The association with RIP1 kinase is also connecting caspase-2 with TRADD (TNF-R1 associated DD protein), implicating a role for caspase-2 in TNF-R signaling cell death pathway. It seems that neither of these complexes require caspase-2 activity. Hence, although an association of caspase-2 with RIP1-linked complexes could be detected, their physiological relevance for caspase-2 activation remains obscure.

Furthermore, caspase-2 is found to localize to the promyelocytic leukemia protein nuclear bodies (PML-NBs) that are nuclear macro-molecular complexes whose function is implicated in DNA damage-induced apoptosis. Localization of caspase-2 in PML-NBs is suggested to require both the prodomain and protease domain but appears to be independent of adaptor proteins. However, the recent

identification of a potential CARD in SP100, a resident protein of the PML-NB complexes, indicated a possible mechanism for the recruitment of CARD-containing proteins into PML-NB structures.[201] Co-localization of caspase-2 within the PML-NB complex could be indicative of this recruiting mechanism and might suggest a novel pathway for caspase-2 activation in the nucleus.

In summary, caspase-2 activation has been observed in many experimental systems and seems to be important for cell death in many cell types. In addition to DNA damage, caspase-2 appears to be necessary for the onset of apoptosis triggered by several insults, including, cytoskeletal disruption, trophic factor withdrawal, cytokine deprivation, ROS, administration of TNF, and different pathogens and viruses.[202-206] Although caspase-9 is recognized as the initiator of the intrinsic pathway, it appears that caspase-2 can serve as an apical caspase in the proteolytic cascade triggered in response to a subset of intrinsic stimuli.[207] Upon activation, caspase-2 as an initiator caspase is responsible for engaging the mitochondrial apoptotic pathway through either directly initiating cytochrome c release or indirectly by generation of tBid.[185, 208] Thus, a caspase-2-mediated nuclear-mitochondrial apoptotic pathway exists. In addition, a primary role for caspase-2 as an apical sensor of signals in ER-mediated cell death has been suggested. However, in some cellular systems, depending on the damaging agent and cell type used, caspase-2 may be activated downstream of mitochondrial events, participating in an apoptotic feedback loop as a death signal amplifier caspase. Consequently, further understanding of caspase-2 activation mechanism(s) and enlightening of its role in different mechanisms of cell death is required.

Goal of the research

Mutations in the p53 gene is one of the most frequently observed genetic alterations during tumorigenesis, and it is likely that pathways that require the activity of p53 are also altered in many tumors. Accumulating evidence has suggested that p53 family proteins might play an important role in activation of caspase-2, although the precise mechanism of its activation is still controversial. Upon activation, caspase-2 appears to be important for successful initiation of apoptosis in tumor cells in response to DNA damage, induced by various chemotherapeutic agents. In addition, a primary role of caspase-2 has also been implicated in DNA damage induced mitotic catastrophe. Despite considerable evidence indicating that caspase-2 activation in response to DNA damage engages a nuclear-mitochondrial pathway, assigning a distinct function to this protease during cell death has been difficult. Therefore, the **main goal** of this thesis was to investigate and understand the role of caspase-2 in different DNA damage-induced cell death scenarios.

The **specific questions and aims** of this research project were as follows:

What is the signal linking DNA damage to caspase-2 activation?

Q1. to investigate the mechanism of caspase-2 activation in mammalian cells in response to DNA damage.
Q2. to investigate the role of p53 in caspase-2 activation.
Q3. to assess the mechanism(s) of PRIMA-1^{MET} -induced apoptosis.

What is the role of caspase-2 in apoptotic cell death and in MC?

Q4. to investigate the role of caspase-2 in the initiation of the intrinsic apoptotic pathway.
Q5. to understand the possible role of caspase-2 in DNA damage-induced MC.

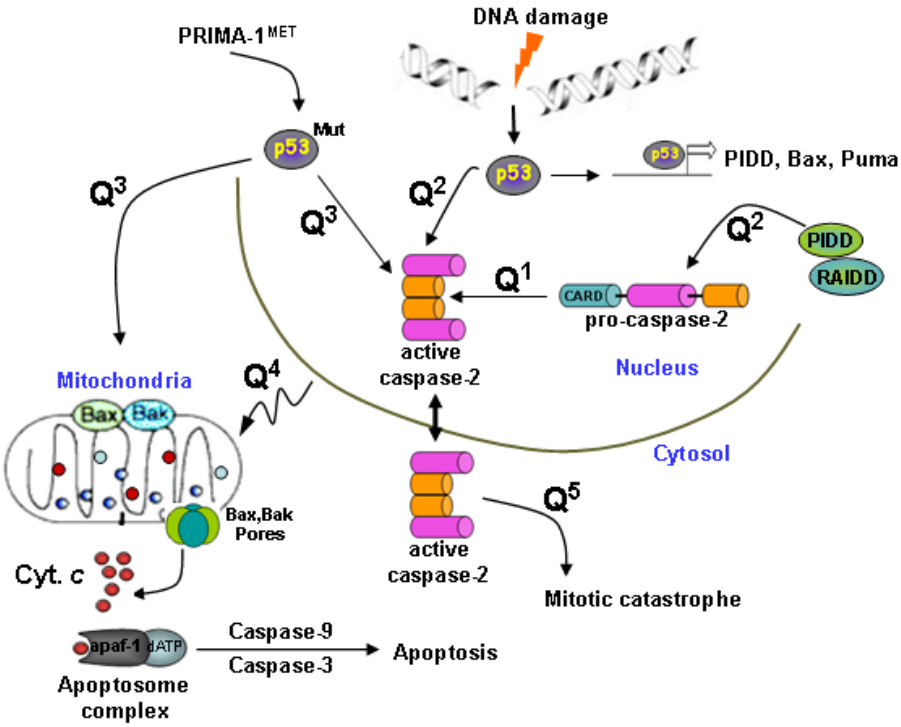

Figure 3

The hypothetical model for this study

Detailed descriptions of all the techniques used, can be found in Papers I-IV presented in this thesis. Here is a brief list and description with commentaries on the materials and methods.

Cell culture

Data presented in this thesis is based on experiments performed on various cancer cell lines of different origin. Throughout the experiments, cells were grown in a humidified 5% CO_2-atmosphere at 37°C and maintained in a logarithmic growth phase.

In **papers I** and **III**, the parental human colon cancer, HCT116 cell line, and its derivate, deficient in p53 mRNA expression, were used. In **paper II**, the following cell lines were used. The p53 null cell lines: H1299 human lung adenocarcinoma, Saos-2 human osteosarcoma, SKOV-TA human ovarian carcinoma. The sublines carrying exogenous mutant p53: H1299-His175, Saos-2-His273 and SKOV-His175. The KRC/Y renal carcinoma cell line carrying endogenous Phe176 mutant p53. In **paper IV**, the human epithelial ovarian cancer cell lines, Caov-4 and SKOV-3, and the 14-3-3$\sigma^{-/-}$ HCT116 cells were used.

HCT116 cell line was cultured in DMEM medium and SKOV-3 and Caov-4 cells in RPMI 1640 complete medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM), while the 14-3-3$\sigma^{-/-}$ HCT116 cells were cultured in McCoy's medium supplemented as above but with additional 0,4 mg/ml geneticin. All cells used in **paper II** were cultured in Iscove's modified Dulbecco's medium supplemented as above but with additional 2.5 mg/ml plasmocin.

Drugs and chemicals

Throughout the experiments cell lines were exposed to different drugs to induce cell death. In **papers I** and **III,** cells were treated with 5-fluorouracil (5-FU). 5-FU is one of the most effective chemotherapeutic agents for gastrointestinal tumors, and belongs to a group of chemicals known as antimetabolites. 5-FU is an analogue of uracil and rapidly enters the cell, in which it is converted to several active metabolites. By incorporation of its active metabolites into RNA and DNA, and by inhibition of thymidylate synthase (TS), 5-FU causes severe DNA damage and activates p53 by more than one mechanism.[209]

In **paper II**, cells were treated with different concentrations of PRIMA-1^{MET}. PRIMA-1^{MET} is a methylated form of PRIMA-1, which is a low-molecular-weight compound capable of inducing both p53 transcription-dependent and independent apoptosis in human tumor cells expressing mutant p53. The mechanism of its action is through restoration of the transcriptional transactivation function to mutant p53, by re-establishing its sequence-specific DNA binding and the active conformation, both *in vitro* and in living cells. PRIMA-1^{MET}, is more active than the original compound.[167, 169]

In paper IV, cells were treated in addition to 5-FU either with cisplatin, STS or doxorubicin. Cisplatin, [*cis*-diamminedichloroplatinum(II)] or *cis*-DDP (CDDP), is highly effective in the treatment of ovarian cancers. DNA is the main target of its mechanism of action and following binding of CDDP to DNA, it forms intrastrand, interstrand, protein-DNA cross-links and monofunctional adducts. The non-DNA target of cisplatin includes thiol-containing biomolecules, such as tripeptide glutathione (GSH), and metalothioneins (MT), and *in vitro* the heat shock protein 90 (Hsp90). CDDP induces apoptosis upon generation of ROS and increase in $[Ca^{2+}]_i$. It

has also been reported to induce clustering of CD95 in the plasma membrane. In response to cisplatin-induced DNA damage, cell can also undergo premature cell senescence and mitotic catastrophe.[210, 211]

Staurosporine, indolo[2,3-alpha]carbazole, is an microbial alkaloid with a potent, albeit non selective, protein kinase C (PKC)-inhibitory activity. It acts by competing at PKC's conserved ATP-binding sites. This agent can inhibit both serine/threonine and tyrosine protein kinases.[212] The members of the PKC family of proteins play important role in the signal transduction pathways important for cellular processes, such as proliferation and differentiation. Thus, inhibition of these kinases using staurosporine initiates apoptotic cell death, through, however, by yet unknown mechanism.[213]

Doxorubicin (Adriamycin), also called as hydroxydaunorubicin, is a cytotoxic anthracycline antibiotic possessing strong antineoplastic activity. This drug possess an ability to intercalate into DNA and block DNA topoisomerase II activity, thus cause DNA double strand breaks. It is also involved in free radical generation.[214]

In all papers, for selected samples, the irreversible caspase-2 inhibitor z-VDVAD-fmk, the caspase-3 inhibitor z-DEVD-fmk, or the selective Chk2 inhibitor, DBH, the specific inhibitor of the nuclear export, leptomycin B were used simultaneously with the chemotherapeutic drug.

Gel electrophoresis and immunoblotting

The **Western blot** (alternatively, **immunoblot**), is an analytical technique for detection of specific proteins in a given sample. It was used in **all papers** to detect and compare the level of proteins involved in apoptosis signaling and the specific cleavage products of some caspase target proteins. Equal amounts of proteins from each sample are size-separated by SDS-PAGE and electroblotted to nitrocellulose membranes. Detection of distinct proteins was made by the use of specific antibodies. Thus, the sensitivity and specificity of this method is mainly determined by the quality of the antibodies used. With the use of appropriate horseradish peroxidase-conjugated secondary antibodies, protein bands were visualized by chemiluminescence (ECL) on X-ray autoradiography films.

Immunocytochemistry

This technique refers to a method for detection of individual protein locations in *situ* by visualizing an antibody-antigen interaction. It was used **in all papers** to study the distribution and co-localization or to investigate the spatio-temporal translocation of proteins of interest. Yet again the sensitivity and specificity of this method is determined by the quality of the antibodies used. Briefly, cells seeded on coverslips were fixed and permeabilized. Cells were fixed to immobilize the antigens, while retaining the cellular and subcellular structure, whereas permeabilization permits the access of antibodies into the cells. Throughout the experiments, fixation was performed in 4% paraformaldehyde (PFA) at 4^{o}C, for 15-20 min. Following incubation with primary antibodies at 4ºC overnight in a humid chamber, appropriate fluorophore-conjugated secondary antibodies were used to imagine the location of proteins examined under either a confocal laser scanner microscope or fluorescence microscope.

Co-Immunoprecipitation

Immunoprecipitation (IP) is a technique used for isolation or concentrating out of a particular protein from crude lysate usually containing many different proteins, using an antibody specifically binding to the protein of interest. Co-immunoprecipitation (Co-IP) is a type of IP applied for analyzing protein-protein interactions. Using an antibody targeting a known protein that is believed to be a

member of protein complex, the entire protein complex can be separated and the unknown protein members of the complex thereby identified. This method was used in **papers I** and **III.** Briefly, Antibodies specific for a particular protein (or group of proteins) are added directly to the protein mixture to bind their targets over night. Beads coated in Protein A/G are added to the antibody protein mixture for 3 hr and the antibodies (which are now bound to their targets) will stick to the beads. The bead-antibody-protein composite is separated from the sample by centrifugations and analyzed by SDS-PAGE.

RT-PCR

RT-PCR (reverse transcription-polymerase chain reaction) is the most sensitive technique for mRNA detection and quantization currently available. This method was used in **papers I** and **IV** for detection and comparison of p53 and PIDD, and in **paper III**, of Fas, mRNA levels. Briefly, total RNA was isolated from induced and non-induced cells by using RNAeasy mini kit. RNA quality was controlled by separation of 18S and 28S ribosomal RNA in denaturing agarose gel electrophoresis and by visualization through ethidium bromide staining. For first strand cDNA synthesis, the RevertAid™ M-MuL V RT enzyme was used in combination with an oligo(dT)18 primer. The PCR was performed using specific primers described in each paper. The conditions for all PCR reactions were as follows: 94°C for 1 min (hot start) followed by 95°C for 15s, 60°C for 30s and 68°C for 50s. Samples were removed every fifth cycle to ensure exponential product growth. PCR yield was analyzed on a 2% agarose gel.

Transfection Methods

a) Transfection of expression cDNA vector

Cell transfection is a method of gene delivery that introduces foreign DNA into cells using plasmid DNA vectors. Cells incorporate the introduced DNA into their genome and starts to efficient express the protein encoded by that gene. It is used to obtain cell lines with desired genetic background. In **paper IV**, SKOV-3 cells were transiently transfected with a plasmid encoding functional p53. As control, cells were also transfected with an empty vector. Transfections were performed with LipofectAMINE PLUS.

b) Retroviral transduction

In **paper III**, another transfection method was used for expression of proteins Bcl-X_L and $FLIP_L$ into HCT116 cell line. The expression vectors pLXIN-hBcl-X_L and pXIN-h$FLIP_L$ were introduced into cells by retroviral transduction.[215] Retroviral particles were produced by transient transfection of the Phoenix-Ampho packaging cell line (kindly provided by Dr. G. P. Nolan, Stanford University, Stanford, CA, USA). Production of viral particles and retroviral transductions were performed as described previously.[215] Transduced cells were selected with 0.8 mg/ml of geneticin.

c) siRNA methodology

Small/short interfering RNA (siRNA) transfection technique is used for introducing foreign genetic information by inducing RNA interference into mammalian cells. The transfected siRNA specifically and efficiently knockdown the gene expression by binding to the key sequences on messenger RNA of proteins of interest. This method was used in **paper I**, for efficient silencing of caspase-2, PIDD and RAIDD, in **paper II** for caspase-2 and PUMA, in **paper III**, for caspase-2, -3, -8, -9, and in **paper IV** for caspase-2 and p53. In all experiments, scrambled siRNA was used as a negative control and transfection was performed using the Lipofectamine

2000 reagent, while transfection of anti-p53 siRNA was carried out using the HiPerFect transfection. 10 nmol of double-stranded siRNA was used in all experiments except for p53, where 5 nmol of double-stranded siRNA was used. The level of protein expression in targeted cells was monitored by SDS–PAGE.

Apoptosis assessment

Apoptosis was originally described as a morphological definition of cell death. Hence, detection of apoptosis was based on morphological features of the cell. Eventually, with the discovery of the biochemical events involved in apoptosis, detection of apoptotic cell death is currently achieved by measurement of caspase activity, subsequent cleavage of target proteins and appearance of DNA laddering (fragmentation). In addition flow cytometry permits rapid and quantitative measurements of various apoptosis related parameters described below.

a) Morphological analysis

To visualize apoptotic cell death or mitotic catastrophe and evaluate the nuclear morphology, cells were stained with either Hoechst (**papers I, III** and **IV**) or DAPI (**papers I** and **II**). In paper I, number of apoptotic cells was measured by assessing the percentage of cells displaying fragmented or condensed nuclei (chromatin condensation) compared to the total amount of cells. Briefly, cells were collected, washed and allowed to dry on slides. Fixation of cells was performed in ice cold ethanol:acetone (1:1) for 5 min. Genomic DNA was stained with Hoechst for 20 min. The cover slips were mounted with glycerol:PBS (1:1) and sealed. Approximately 300 nuclei were counted per sample using a fluorescence microscope. Counterstaining of nuclei in **papers III** and **IV** was performed by incubation with Hoechst, and in **paper I,** with DAPI solution. In **paper II,** cells**,** were mounted using Vectashield H-1000 containing DAPI stain.

b) DNA fragmentation assay

Apoptosis is characterized by the endogenous endonucleases activation with subsequent cleavage of chromatin DNA into internucleosomal fragments with various lengths of 180 to 200 bp. This process was investigated in **papers I** and **IV**. Briefly, DNA precipitated from lysed cells by adding an equal volume of isopropanol, was dissolved in TE buffer containing RNase A, and incubated at 37°C for 30 min in order to degrade mRNA. DNA samples were separated in a 2% agarose gels containing ethidium bromide and visualized by UV light. The DNA ladder VI served as a molecular marker.

c) Caspase processing/activity assessment

The involvement of caspases during cell death processes can be monitored by a number of methods. In this thesis both the processing and the activation of caspases were assessed. In the substrate cleavage assay, used in **papers I, II** and **IV,** the maximum linear rate of cleavage-release of fluorochrome 7-amino-4-methylcoumarin (AMC) coupled to a caspase substrate (e.g. a tetrapeptide), was calculated from multiple measurements in a microplate reader. As applied in combination with specific inhibitors of caspases, this method was used for detection of specific caspase activity. Detection of the pro and processed fragments, as well as the active fragments of some caspases were detected using Western blot analysis, in **papers I, III** and **IV.**

d) Mitochondrial pro-apoptotic protein release

The release of cytochrome *c* and other proteins from the mitochondria into the cytosol can be detected by Western blot technique or by immunohistochemistry. Both of these techniques were used in **papers II** and **IV**. Whereas, only immunoblotting was performed in **papers I** and **III**. Briefly, cells resuspended in 100 µl of buffer (5 mM Tris-HCl, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 5 mM succinate, 1 mM KH_2PO_4, 140 mM manitol), were permeabilized with digitonin (0,01%) for 5 min at RT, and the mitochondria and nuclei were removed from the soluble cytosolic fraction by centrifugation. Pellets, containing mitochondrial, and supernatants, containing released proteins, were subjected to SDS-PAGE gel electrophoresis.

In **paper II**, the mitochondrial membrane potential ($\Delta\psi m$) was assessed by cell permeable cationic fluorochrome dye, tetramethylrhodamine ethyl ester (TMRE) using FACS. TMRE accumulates in mitochondria with intact $\Delta\psi m$ and causes a red shift in the fluorescence emission spectra of TMRE. Upon depolarization of mitochondria, TMRE is released resulting in a decrease in the red fluorescence, which can be quantified by FACS analysis.

e) Flow cytofluorometric analysis of the cell cycle

A population of cells' replication state (DNA content) can be analyzed by flow cytometry using propidium iodide (PI). The PI intercalates into double-stranded DNA and produces a highly fluorescent adducts that can be excited at 488 nm with a broad emission around 600 nm. This method was used to determinate the distribution of cells in the subG_1, G_1, S and G_2/M cell cycle phases by flow-cytometry in **paper IV**. The apoptotic cells with degraded DNA appear as cells with hypodiploid DNA content and are represented in so-called "sub-G_1" peaks on DNA histograms. Briefly, cells were washed in PBS, fixed in ice-cold 50-70% ethanol and stained with PI solution in presence of RNase A (0.5 mg/ml at 4°C overnight) and Triton X-100. The latter allows PI to penetrate into the cells, which is normally membrane impermeant. Flow cytometric analysis was carried out using a FACScan flow cytometer equipped with CellQuest software.

f) Analysis of PS exposure and PI staining

Exposure of phosphatidylserine (PS) on the outer leaflet of the plasma membrane is a key feature of apoptosis. Using Annexin V-FITC staining, which binds to PS, and PI that binds to DNA, cells undergoing either apoptosis or necrosis can be distinguished by FACS analysis. Compared to necrotic cells, in which the damaged cell membrane allows binding of both stains, apoptotic cells bind to annexin V only. This method was used in **papers I** and **IV**. In brief, following treatment cells were washed and resuspended in 100 ml binding buffer containing an Annexin V-FITC-PI mixture. FACS analysis was performed using the FACScan system and the Cell Quest pro software. Annexin V-FITC Apoptosis Detection Kit II was used according to the manufacturer's instructions.

g) Assessment of Bax activation

The pro-apoptotic Bcl-2 family members Bak and Bax undergo conformational changes during their activation. By flow-cytometric analysis, Bax-associated conformational changes, indicative of its activation, was assessed using antibody recognizing the N-terminal epitopes of Bax. This method was used in **paper IV**. Briefly, cells were washed in PBS, fixed 5 min in 0,25% PFA and incubated for 30 min in the presence of digitonin (100 µg/ml). Following incubation with an appropriate FITC-conjugated secondary antibody for 30 min at RT, cells were analyzed on a FACS-Calibur flow cytometer, using Cell Quest software.

Summary of the Papers

- **Paper I**

Vakifahmetoglu H, Olsson M, Orrenius S, Zhivotovsky B. Functional connection between p53 and caspase-2 is essential for apoptosis induced by DNA damage. *Oncogene*. 2006; 25(41):5683-92

Although the identification of the PIDDosome complex suggested a link between caspase-2 and p53, a requirement of p53 for caspase-2 activation was not addressed. In paper I, we investigated the possible functional connection between p53 and caspase-2 activation in DNA damage-induced apoptosis. In order to address the question of the importance of p53 for caspase-2 activation we examined the consequences of 5-FU -induced DNA damage by using the wt p53 containing colon cancer cell line HCT116 and its isogenic subline, deficient in p53 protein expression.

Results in paper I clearly indicate that the apoptotic effect of genotoxic stress induced by 5-FU in HCT116 cells depends on their p53 status, as shown by a lack of apoptosis in $p53^{-/-}$ cells. In contrast, apoptotic features, such as cytochrome *c* release, oligonucleosomal DNA fragmentation, chromatin condensation, accumulation of sub-G_1/apoptotic population and PS exposure were observed in wt p53 cells treated with 5-FU. However, when cells were pretreated with the caspase-2 inhibitor z-VDVAD-fmk, a suppression of these apoptotic parameters was detected. This indicated that caspase-2 might be an important apoptotic mediator of this particular pathway. Indeed, p53 was shown to be required for a time-dependent increase in both activation and processing of caspase-2 in HCT116 cells during 5-FU-induced apoptosis. In agreement with our previous observations in Jurkat cells,[161] caspase-2 appeared to be activated before caspase-3, as both caspase-2 and -3 activities were blocked by z-VDVAD-fmk, but only the latter was blocked using the caspase-3 inhibitor z-DEVD-fmk. Hence, findings in paper I confirmed a role of caspase-2 upstream of mitochondrial events and suggested that p53 is critical for its activation. To further substantiate this, caspase-2 expression was suppressed by a siRNA approach. In cells with downregulated caspase-2 level, a decrease in 5-FU-induced cytochrome *c* release, PS exposure and oligonucleosomal DNA fragmentation was observed, when compared to control cells. These observations indicate that both p53 and caspase-2 are needed for efficient induction of apoptotic cell death in HCT116 cells.

Although we were unable to detect a direct interaction between p53 and caspase-2, our data clearly demonstrate a functional connection between these proteins in order to complete the apoptotic process. Proposed mediators of caspase-2 activation include the PIDDosome complex proteins, PIDD and RAIDD. Through immunoprecipitation studies, a direct interaction between endogenous PIDD, RAIDD and caspase-2 was detected. However, the formation of this complex did not depend on p53 status or 5-FU treatment since complex formation also was detected in $p53^{-/-}$ cells and in untreated $p53^{+/+}$ cells. Moreover, downregulation of PIDD or RAIDD by siRNA technology did not interfere with caspase-2 activation or progress of apoptosis. Although, results confirmed the presence of PIDD and RAIDD in the PIDDosome complex together with pro-caspase-2, it seems that additional factor(s) are required for caspase-2 activation. Our findings also demonstrate that the suppression of caspase-2 by siRNA in 5-FU-treated cells promoted p53 accumulation, when compared to samples treated with 5-FU alone, although the precise mechanism of this effect remains unknown. In conclusion, our results in paper I support the hypothesis of p53 as an upstream regulator of caspase-2 activation mechanisms, and that a reciprocal interaction between these proteins exists.

- **Paper II**

Shen J**, Vakifahmetoglu H**, Stridh H, Zhivotovsky B, Wiman KG. PRIMA-1(MET) induces mitochondrial apoptosis through activation of caspase-2. *Oncogene.* 2008; 27(51): 6571-80

PRIMA-1^{MET} is known to restore the wt conformation and the DNA binding property to mutant p53 and induces apoptosis in a broad range of human tumor cell lines in a mutant p53-dependent manner. Yet, the mechanism(s) by which PRIMA-1^{MET} induces apoptosis was not well studied. Accordingly, in paper II, the detailed mechanisms underlying PRIMA-1^{MET}-induced apoptosis were investigated. It was also of interest to determine whether the reactivation of mutated, nonfunctional p53 influenced caspase-2 activity.

We first examined the kinetics of PRIMA-1^{MET}-induced cell death using p53$^{-/-}$ H1299 human lung adenocarcinoma and its derivate carrying exogenous His175 mutant p53. The results revealed that in mutant p53-expressing cells, PRIMA-1^{MET} induces accumulation of sub-G_1 apoptotic cell population and loss of mitochondrial membrane potential ($\Delta\Psi_m$) in a dose- and time-dependent manner. Similar response was seen in two other human tumor cell lines of different origin, namely Saos-2 osteosarcoma and SKOV ovarian carcinoma. The involvement of mitochondria in PRIMA-1^{MET}-induced apoptosis was further assessed by studying the status of several Bcl-2 family proteins and cytochrome *c* release. Compared to the p53$^{-/-}$ cells, cleavage of Bid, increased Bax and PUMA expression and a redistribution of cytochrome *c* from mitochondria to the cytoplasm were detected in mutant p53 cells after treatment. Although caspase-8-mediated cleavage of Bid plays an important role in the activation of Bax during receptor-mediated apoptosis in type II cells, caspase-8 activity was not detected in these cells. Instead, caspase-2, -9 and -3 seemed to be involved in PRIMA-1^{MET}-induced apoptosis, suggesting that activation of the death receptor-mediated pathway was not required in our experimental system.

Next, the relationship between these caspases in PRIMA-1^{MET}-induced apoptosis was studied. Although, no effect of caspase-2 downregulation on PRIMA-1^{MET}-induced loss of $\Delta\Psi_m$ was observed, a significant reduction in cytochrome *c* release and caspase-3 activation as well as a reduction of sub-G_1 population were detected in cells with suppressed caspase-2 level or activation. On the contrary, using caspase-3 inhibitor no significant effect was seen on caspase-2 activation, whereas both caspase-2 and -3 inhibitors blocked caspase-9 activation. These findings are in line with the results from paper I, and suggest a primary role for caspase-2 in the apoptotic response to PRIMA-1^{MET}. These results also demonstrate that the reactivation of mutant p53 is essential for caspase-2 activation. In addition, although no significant increase in the protein level of PIDD was detectable, a time-dependent accumulation of a band corresponding to the cleavage fragment PIDD-CC was visible upon PRIMA-1^{MET} treatment. This was not seen in p53$^{-/-}$ cells, which, however, exhibited the appearance of the PIDD-C fragment. Furthermore, suppression of PIDD significantly inhibited the accumulation of sub-G_1 DNA content, suggesting that the PIDDosome was of critical importance for the observed caspase-2 activation in cells treated with PRIMA-1^{MET}.

To determine the significance of upregulated PUMA for the apoptotic response to PRIMA-1^{MET}, the protein level of PUMA was decreased using siRNA. Upon PUMA knockdown, a reduction in the sub-G_1 fraction was observed following treatment. However, the combination of both PUMA and caspase-2 suppression did not result in any additional protection from cell death, indicating that although both PUMA and caspase-2 are involved in the progression of the apoptotic process, other p53-dependent pathways may be activated upon PRIMA-1^{MET} treatment. In summary, findings in paper II highlight a chain of cellular events triggered by PRIMA-1^{MET}-induced mutant p53 reactivation leading to mitochondria-mediated apoptotic cell death.

- **Paper III**

Olsson M, **Vakifahmetoglu H**, Abruzzo PM, Grandien A, Zhivotovsky B. DISC-mediated activation of caspase-2 in DNA damage-induced apoptosis. [*Under revision in Oncogene*]

The results in paper I demonstrated the presence of caspase-2 within the PIDDosome complex. However, since the elimination of complex constituents PIDD or RAIDD did not influence caspase-2 activation or processing, the precise mechanism of caspase-2 activation in HCT116 cells was still unclear. The possible existence of an alternative activation mechanism for caspase-2 remained. In paper III, we investigated the link between p53 and caspase-2 in further detail and found initial activation of caspase-8 in addition to caspase-2, upon 5-FU treatment. Moreover, functional p53 was required for the activation of both caspase-2 and -8, and interestingly, the activation of caspase-2 seemed to depend on caspase-8.

Next, we characterized the nature of the caspase-2 and -8 interaction using a set of molecular and biochemical methods as well as image analysis. In 5-FU-treated HCT116 cells, the DISC was found to be assembled through p53-dependent upregulation of CD95. Caspase-8 was able to utilize the CD95-DISC as an activation platform. The recruitment of caspase-8 to this complex seemed to be a prerequisite for activation of caspase-2. Further, investigating the components of DISC by immunoprecipitation, caspase-2 was detected to co-immunoprecipitate with caspase-8, FADD and CD95 receptor in HCT116 cells upon 5-FU treatment. Thus, a direct interaction between the two caspases was demonstrated. Suppression of caspase-8 by siRNA almost completely inhibited caspase-2 processing. However, this effect seemed unidirectional since downregulation of caspase-2 failed to inhibit 5-FU-induced caspase-8 cleavage, suggesting that caspase-8 is acting upstream of caspase-2 in 5-FU-treated HCT116 cells. Moreover, downregulation of caspase-3, -7 and -9 did not affect the activation of caspase-2 or -8; thus, the possibility that an apoptotic amplification loop could lead to the activation of caspase-2 and -8 was ruled out. In addition to inhibition of effector caspases in response to ectopic expression of Bcl-X_L, caspase-2 and -8 remained partially unprocessed in Bcl-X_L overexpressing cells.

To further confirm the requirement of caspase-8 for caspase-2 activation, we performed retroviral transduction of $FLIP_L$ protein and generated a stable HCT116 cell line containing elevated level of this protein. Given that c-$FLIP_L$ localizes to the CD95-DISC, we expected the activation of caspase-8 to be inhibited in these cells. Indeed, overexpression of c-$FLIP_L$ efficiently blocked apical caspase-8 and caspase-2 activities as well as downstream activation of effector caspases. The ability of caspase-8 to cleave caspase-2 was also tested in vitro by incubating [^{35}S]-Methionine-labeled caspase-2 and -8 with recombinant, active enzymes. Like caspase-8, active caspase-2 was able to cleave its own precursor but did not cleave pro-caspase-8. In contrast, caspase-8 directly processed the caspase-2 proform, although caspase-2 seemed not to be completely processed and only fragments originating from cleavage events at D333 and D347, but not of D169, were identified.

Finally, we show that caspase-2 and -8, but not caspase-9, mediated cleavage of Bid, indicating that both caspases act upstream of mitochondrial cytochrome *c* release, eventually leading to the activation of effector caspases through this pathway.

Taken together, the findings in paper III demonstrate that 5-FU-induced DNA damage can lead to assembly of the DISC complex with subsequent caspase-8 and caspase-2 activation. A crosstalk between the two apical caspases exists upstream of the mitochondria, indicating that the activity of these two proteolytic enzymes significantly contributes to the apoptotic response. In conclusion, the functional link between caspases-8 and -2 within the DISC complex represents an alternative to the PIDDosome mechanism for caspase-2 activation in response to DNA damage.

- **Paper IV**

Vakifahmetoglu H, Olsson M, Tamm C, Heidari N, Orrenius S, Zhivotovsky B. DNA damage induces two distinct modes of cell death in ovarian carcinomas. Ce*ll Death Differ.*2008;15(3):555-66

In papers I-III, we demonstrated the importance of p53 for caspase-2 activation and for the apoptotic response initiated either by the DNA-damaging agent 5-FU or by restoration of mutant p53. The possible role, however, of caspase-2 in DNA damage-induced mitotic catastrophe (MC), remained unanswered. Hence, in paper IV the involvement of p53 and caspase-2 in DNA damage-induced MC was investigated in two ovarian cancer cell lines, namely Caov-4 and SKOV-3. We first compared the susceptibility of these cell lines to treatment with the DNA crosslinking agent, cisplatin. Analysis of nuclear morphology using Hoechst staining revealed that Caov-4 and SKOV-3 cells responded differently to cisplatin exposure. Caov-4 cells were characterized by an arrest in the G_2 phase of the cell cycle that followed accumulation of a discrete sub-G_1 population, oligonucleosomal DNA fragmentation, formation of pyknotic nuclei and appearance of apoptotic bodies, well-known apoptotic features. In contrast, following treatment SKOV-3 cells predominantly accumulated in S-phase and displayed characteristics of MC, as they became significantly larger, and appearance of multinucleated cells with decondensed chromatin was observed. At longer treatment time points, these cells started to collapse and detach from the culture plate, indicative of necrosis-like lysis.

Next, we investigated the role of caspases in the response to cisplatin. A time-dependent increase in caspase-2, -3 and -9 activation and processing was seen in Caov-4 cells, whereas no apparent increase was observed in SKOV-3 cells. No caspase-8 activity or processing were detected in either one of the cell lines. In cells with reduced level of caspase-2 using siRNA, a decrease in cisplatin-induced cytochrome *c* release and a reduction in both caspase-3 and -9 activities and PARP cleavage were shown, indicating a significant role of caspase-2 in cisplatin-induced apoptosis. Moreover, a time-dependent translocation of nuclear caspase-2 to the cytosol was seen in apoptotic Caov-4 cells. Such a translocation was not detected in SKOV-3 cells, where caspase-2 remained in the nucleus following treatment. However, caspase-2 was processed and translocated into the cytosol in staurosporine (STS)-treated SKOV-3 cells, which underwent apoptosis upon such treatment. Notably, the cytosolic translocation of nuclear caspase-2 upon apoptotic stimulation was also observed in Caov-4 cells pretreated with leptomycin B, which blocks the active transport via direct interaction with nuclear pore complex protein CRM1.

Our findings suggested that cisplatin treatment resulted in MC without the requirement of caspase activation. Still, in order to investigate any possible involvement of caspase-2 in MC, we suppressed caspase-2 level in SKOV-3 cells using siRNA. The occurrence of the MC phenotype was, however, still detectable upon cisplatin treatment, demonstrating that caspase-2 is not required for cisplatin-induced MC in SKOV-3 cells.

Since the presence of functional p53 was crucial for the activation of caspase-2 by DNA damage, demonstrated in papers I-III, we reasoned that the absence of caspase-2 activation following cisplatin treatment in SKOV-3 cells might be a consequence of a lack of p53. Surprisingly, p53 was found to be mutated in both cell lines. However, in Caov-4 cells p53 was expressed and the protein level accumulated upon treatment and p53 was transcriptionally active, whereas no detectable amount of p53 was found in SKOV-3 cells, irrespectively of treatment. The presence of p53 increased the amount of p21, but only a minor increase in PIDD was seen both at protein and mRNA levels in Caov-4 cells. Notably, a time-dependent accumulation of

PIDD-CC was found in these cells. SKOV-3 cells, however, demonstrated the appearance of the PIDD-C fragment.

The importance of p53 in apoptosis and MC was investigated by suppressing the level of p53 protein in cells by siRNA transfection. In contrast to control cells, the downregulation of p53 in Caov-4 cells led to MC following cispaltin treatment. These observations demonstrate that DNA damage triggers MC in p53-deficient Caov-4 cells, like it does in p53-deficient SKOV-3 cells. Moreover, in SKOV-3 cells, apoptotic functions were restored by transient expression of p53. Hence, p53 appears to act as a switch between apoptosis and MC in ovarian cancer cells.

It was previously reported that the inactivation of Chk2 sensitizes cells to undergo MC in presence of DNA damaging agents.[216] To investigate whether Chk2 inhibition also triggered MC in response to cisplatin in our experimental system, we chemically inhibited Chk2 in Caov-4 cells. Chk2 inhibition in combination with DNA damage led initially to the formation of multinucleated giant cells with chromosome vesicles, indicative of MC also in our experimental system. However, at later time points these cells underwent apoptosis.

Treatment of 14-3-3$\sigma^{-/-}$ HCT116 cells with doxorubicin was reported to lead to MC,[139] in a process that involved caspase activation and chromatin condensation.[216] In light of our findings, it was of interest to investigate the effect of doxorubicin in 14-3-3$\sigma^{-/-}$ cells at later time points. In agreement with the observations using Chk2-inhibited Caov-4 cells, apoptotic cell death was the final outcome also of doxorubicin treatment of 14-3-3$\sigma^{-/-}$ cells. Thus, we confirmed that the inhibition and/or downregulation of the cell cycle proteins Chk2 and 14-3-3σ in cells, although expressing wt p53, sensitize these cells to undergo MC upon treatment with DNA-damaging agents. Nevertheless, in contrast to cells lacking p53, cells undergoing MC upon Chk2 and 14-3-3σ inhibition die through apoptosis due to the presence of functional p53.

In summary, results in paper IV confirmed our findings that both p53 and caspase-2 are required for the apoptotic cell death response to DNA damage. In contrast, a role of caspase-2 in MC was excluded. However, p53 seems to be an important regulator of both initiation and the outcome of MC.

As mentioned above, a series of previously published papers from our group demonstrated a crucial role of caspase-2 upstream of the mitochondria in etoposide-mediated cell death.[161, 193, 208, 217] This was supported by several other groups, verifying a primary caspase-2 activation in various human tumor cell lines in response to treatment with DNA damaging agents.[159, 160, 185] Despite these findings, the exact activation mechanism and the precise function of caspase-2 during DNA damage-induced cell death signaling remained enigmatic. In this thesis, the mechanism(s) of caspase-2 activation and its role in DNA damage-induced cell death signaling pathways were investigated.

The role of caspase-2 in DNA damage-induced apoptosis

Induction of apoptosis is recognized as a major outcome of DNA damage in mammalian systems. Following DNA damage, mitochondrial events such as cytochrome *c* release with subsequent caspase-9 activation, are essential for downstream apoptotic signaling. There are multiple independent routes by which DNA damage can be linked to the mitochondria. These include p53- or p73-dependent transcriptional activation, less well-characterized direct mitochondrial effect of p53, as well as p53-independent pathways and caspase-2 activation.

In the present study, caspase-2 was found to be both processed and activated in response to various DNA damaging agents, such as 5-FU, cisplatin and doxorubicin, in the cancer cell lines used. Following apoptotic stimuli, activation of caspase-2 induced the release of apoptotic effectors from mitochondria. By experiments using chemical inhibitors as well as siRNA approach, we demonstrate the significance of caspase-2 activation for DNA damage-induced apoptosis. Cells with suppressed caspase-2 level were refractory to cytochrome *c* release as well as multiple downstream events, such as caspase-9 and -3 activations, PARP cleavage, PS exposure on the plasma membrane and DNA fragmentation. These data suggested that caspase-2 is an upstream caspase in the proteolytic cascade initiated by DNA damage and that it plays a key role in transduction of the damage signal to the mitochondria. In paper IV, caspase-2 was found to be the most apical caspase activated upon cisplatin treatment in Caov-4 cells. Downregulation of caspase-2 completely abolished the apoptotic response of these cells. Hence, DNA damage induced apoptosis in a caspase-2–dependent manner without the requirement of other initiator caspases. In contrast, in HCT116 cells, caspase-8 was detected as the proximal caspase activated upon 5-FU treatment (papers I and III). In this case, caspase-2 activation was dependent on the activity of caspase-8, since inhibition of DISC by FLIP overexpression, or suppression of caspase-8 level by siRNA, blocked both caspase-2 processing and activation. The apoptotic circuit, however, still required the activities of both caspase-8 and -2 in order to efficiently kill HCT116 cells. Caspase-8 was not required for caspase-2 activation, or for the apoptotic outcome in PRIMA-1^{MET}-induced apoptosis (paper II), as no caspase-8 activity was observed in this study. The identification of caspase-2 as an apical caspase activated upon this treatment further supported the pre-mitochondrial function of this enzyme. Although PRIMA-1^{MET}-induced apoptosis can not be considered a DNA damage response, the activation of caspase-2 occurred in a reactivated mutant p53-dependent manner, thus mediated by nuclear events. However, even though caspase-2 was the apical caspase activated in this system, chemical inhibition or siRNA knockdown of caspase-2 only partially blocked PRIMA-1^{MET}-induced apoptosis, and did not have

any effect on loss of $\Delta\Psi_m$. In this scenario, a full apoptotic response required both the activation of caspase-2 and an upregulation of pro-apoptotic p53 targets, such as Bax and PUMA. However, since no further reduction in cell death was detected upon inactivation of both caspase-2 and PUMA, results (paper II) suggest that the apoptotic signaling induced by PRIMA-1^{MET} also triggers activation of additional caspase-2-independent pathway(s) for execution of apoptosis. It is possible that p53 directly targets mitochondria and activates transcription-independent apoptotic pathways. It is also possible that the PRIMA-1^{MET} treatment *per se* induces the engagement of the mitochondrial pathway, by directly targeting mitochondria and inducing loss of $\Delta\Psi_m$.

In addition to our findings, a large number of other studies support the hypothesis that caspase-2 acts upstream of mitochondria as an initiator caspase in genotoxic cell death, as suggested by findings in various human tumor cell lines, including colon adenocarcinoma H1299 cells, lung epithelial A549 cells, and human prostate cancer cell lines, but also in germ cell apoptosis during the first phase of spermatogenesis.[218-222] In these studies siRNA against caspase-2 was partially, if not completely, protective against apoptosis induced by DNA damaging agents, such as irradiation, exposure to ceramide or cisplatin, as well as the histone deacetylase inhibitor, trichostatin A. However, so far, there is no evidence relating caspase-2 activation to specific type of DNA lesion. Thus, overall, our results suggest that caspase-2 might be considered as a link between primary DNA damage and downstream apoptotic players. Recently, another apoptotic pathway involving ATM/ATR-dependent caspase-2 activation in p53-deficient zebrafish embryos was identified.[223] This pathway is triggered by IR-induced DNA damage in cells in which Chk1 activity is simultaneously compromised. In embryos with impaired Chk1 function, apoptotic cell death in response to radiation was specifically blocked by concomitant ablation of caspase-2 expression, but not by any other evolutionarily conserved cell death mediator. This indicates that a Chk1-suppressed ATM/ATR-dependent apoptotic pathway involves a conserved role for caspase-2 in the DNA damage response.

Once activated, how does caspase-2 act in the sequence of apoptotic events? Although, caspase-2 has been demonstrated to induce the release of proteins from mitochondria directly,[159, 208] in the experimental systems used (papers I-IV), caspase-2 appeared to do so by cleavage of Bid followed by translocation of Bax to the mitochondria, leading to mitochondrial permeabilization and subsequent cytochrome *c* release. These results are consistent with studies showing that suppression of caspase-2 leads to inhibition of Bax translocation and of the release of mitochondrial proteins. Moreover, caspase-2 overexpression-induced cell death was abrogated in HeLa cells with silenced Bid and in murine embryonic fibroblasts (MEF) deficient for Bax/Bak.[160, 161, 224] As mentioned above, compared to other human caspases, caspase-2 is characterized by a unique intracellular distribution, as it is present in the cytosol, the Golgi complex and the nucleus.[182-184] Although a mitochondrial caspase-2 translocation was described in Birc-6 deficient MEF, investigations using a set of antibodies raised to different epitopes of caspase-2 failed to provide clear evidence for its presence in the mitochondria.[225, 226] In addition, based on the observation that mitochondrial events occurred in the lack of apparent nuclear-cytoplasmic transport, nuclear caspase-2 might trigger mitochondrial dysfunction without relocalizing into the cytoplasm.[185] Consistent with these studies, a primarily nuclear localization of pro-caspase-2 was found in Caov-4 cells (paper IV). However, in this study, we show for the first time a nuclear-cytoplasmic translocation of constitutive caspase-2 during apoptosis. Although caspase-2 is transported into the nucleus through interaction with the *α/β*-importin heterodimer, which is a constituent of the active nuclear import machinery,[227] its export into the cytoplasm seems to be independent of the active

transport system via direct interaction with nuclear pore complex protein CRM1. Another mechanism regulating protein export from the nucleus is passive export. In fact, caspase-2 has been reported to diffuse out of the nucleus in late stages of apoptosis,[185] when the size limit for passive diffusion through nuclear pores had increased. However, it should be noted that the nuclear pore membrane protein POM121 is specifically degraded by caspase-3 during apoptosis.[228] Thus, the observed passive diffusion of caspase-2 out of the nucleus during late stages of apoptosis might be a consequence of degraded nuclear pore proteins. However, our data reveal that there is also an early nuclear-cytosolic translocation of caspase-2. Although the precise mechanism responsible for this event is unclear, early translocation of caspase-2 might be a crucial for apoptosis. The question, however, whether caspase-2 activation takes place within the nucleus, and is required for its translocation, or if its translocation precedes activation needs to be further investigated.

In HCT116 cells, caspase-2 remained mainly cytosolic irrespective of induction of cell death. However, it should be noted that in this case caspase-8, and not caspase-2, was the most upstream caspase activated upon apoptotic stimuli. Thus, it is tempting to speculate that the subcellular distribution of caspase-2 in cells might play a significant role for its activation order. On the other hand, although caspase-2 activation occurred in a caspase-8 dependent manner in HCT116 cells, its activity was still required for Bid cleavage and the engagement of the mitochondrial pathway.

In contrast to our data, other studies assign a role for caspase-2 as a downstream caspase in genotoxic stress without the ability to induce mitochondrial dysfunction.[229, 230] In addition, based on the absence of an apparent phenotype in caspase-2 knockout mice, a critical role for caspase-2 in apoptotic signaling has been questioned. Further, caspase-2 silencing in etoposide- or STS-treated Jurkat and MCF-7 cells, as well as primary MEF and lymphocytes from caspase-2 knockout mice, responded similarly to genotoxic stress as the wild-type cells.[174, 231] However, some of these studies are based on UV-irradiation-induced cell death that primarily mediates an ATR-dependent DNA damage response, and, hence, may engage an apoptotic signaling pathway differently from the ATM-p53-caspase-2 circuit. Moreover, it is likely that in caspase-2 knockout cells another initiator caspase is able to balance the lack of caspase-2. Compensatory mechanism, as observed in hepatocytes derived from caspase-$9^{-/-}$ or caspase-$3^{-/-}$ mice, may account for this suggestion.[232] In fact, experiments using caspase-2 and -9 double knockout cells demonstrated that these cells still undergo apoptosis, when triggered by various stimuli, suggesting a high probability of involvement of other caspases in cell death.[231, 233] Thus, caspase-2 may be significant in a much broader context than suggested by caspase-2 knockout mice studies. Furthermore, the role of caspase-2 in apoptosis seems cell type-specific. Studies performed using neurons derived from caspase-2 knockout mice are more prone to an apoptotic response than neurons from wild-type mice, whereas caspase-2-deficient germ cells and oocytes, as well as lymphoblasts, are more resistant to chemotherapeutic drugs than germ cells and oocytes of caspase-2 wild-type mice.[174]

Taken together, despite the observed differences in the hierarchical position of caspase-2 activation in the apoptotic caspase cascade induced by DNA damage in the experimental systems used, our findings indicate that caspase-2 is an important upstream component of the DNA damage response leading to apoptosis.

The role of p53 in caspase-2 activation

It is well established that the p53 tumor suppressor gene is critical for the cellular response to DNA damage and its apoptotic outcome. Its significance for the activation

of caspases in general, and caspase-8 in particular, has previously been shown.[234, 235] The identification of the PIDDosome complex suggested a link between caspase-2 and p53, a possibility which also was suggested by Ren *et al* in their report on Birc6 (Bruce), an upstream regulator of p53.[225] Silencing of Bruce leads to upregulation of p53 protein level which was shown to be requisite for the activation of Bax, Bak, and of PIDD/caspase-2. Additionally, in colorectal cancer cells downregulation of Bcl-2 level induced massive p53-dependent apoptosis, in which caspase-2 and Bax functioned as essential apoptotic mediators.[236] Furthermore, p53-dependent apoptosis in HOXA5 overexpressing MCF-7 cells was shown to involve mechanism(s) mediated by caspase-2.[237, 238] Although these observations suggested a role for p53, or p53-related proteins, in caspase-2 activation, a requirement of p53 for caspase-2 activation was not demonstrated.

In the present study, we found that p53 is fundamental for both caspase-2 activation and for further completion of the apoptotic process. In cells lacking p53, neither caspase-2 processing nor activation were shown, demonstrating the importance of the presence of p53 for caspase-2-mediated apoptosis. Our results did not support the idea of a direct interaction between p53 and caspase-2, suggesting that the apoptotic response was mediated via transitional molecules. In paper I, however, only an insignificant elevation of PIDD, both at transcriptional and protein levels, was observed in response to 5-FU treatment. Thus, the question how this minor transcriptional activation could have such a dramatic impact on apoptotic behavior in HCT116 cells remained to be addressed. Although, it was previously shown that autoprocessing of PIDD, rather than its upregulation, was essential for stimulation of cell death,[157] our results (paper I) argued for a scenario where the p53-caspase-2 apoptotic circuit was mediated by a still unidentified molecule in the PIDDosome complex, or by an alternative pathway of caspase-2 activation. This assumption was primarily supported by siRNA targeting of PIDD or RAIDD, but also by data showing complex formation between PIDD, caspase-2 and RAIDD irrespective of 5-FU treatment. Our results in paper III, revealed that in HCT116 cells a DISC- and caspase-8-mediated activation of caspase-2 occurred in a p53-dependent manner. Induction of CD95 has been observed in a wide range of cells treated with various DNA damaging agents.[239] In fact, the CD95 death receptor gene contains a p53-responsive element, but the transactivation mechanism does not discriminate between wild-type and p53 mutants, which are unable to induce apoptosis.[239, 240] It was reported that in paclitaxel-treated Y79 cells, a marked increase in both CD95 and CD95L proteins was detected in high molecular weight complexes, likely reflecting their oligomerization and activation. In this system, however, caspase-2 activation takes place without a requirement of caspase-8.[241] Furthermore, incubation with a specific inhibitor of JNK, partially suppressed caspase-2 activity, suggesting that the CD95/CD95L interaction was accompanied by JNK-mediated caspase-2 activation. In contrast to this study, we were unable to detect any upregulation of the CD95 ligand. It is, therefore, questionable whether the p53-dependent upregulation of CD95 *per se* induces apoptosis, or if it sensitizes cells to other pro-apoptotic signals.

The importance of p53 for caspase-2 activation was further demonstrated in paper II, in which p53 functions were restored by treatment of cells with PRIMA-1^{MET}. This induced an apoptotic signaling cascade that targeted mitochondria and engaged the intrinsic apoptotic pathway in a caspase-2 dependent manner. In this experimental system, autoprocessing of PIDD to form the PIDD-CC fragment was strongly correlated with the apoptotic response of cells, for which caspase-2 activation is a prerequisite.[95, 157] Furthermore, in contrast to the findings in papers I and III, silencing of PIDD partially inhibited the apoptotic outcome upon PRIMA-1^{MET} treatment, suggesting that PIDD was responsible for the observed activation of caspase-2 upon

such treatment. However, further studies are required to examine the effect of PRIMA-1^{MET} on the PIDDosome in mutant p53-expressing cells.

Further evidence for a significant role of p53 in caspase-2 activation is presented in paper IV. Although p53 was found to be mutated in the cell lines employed in this study, caspase-2 was both processed and activated upon cisplatin treatment in Caov-4 cells, where the mutant p53 was still functional. In contrast, caspase-2 activation was absent in SKOV-3 cells subjected to the same treatment, as a consequence of the lack of functional p53 due to a hotspot mutation. Like in paper II, the autoprocessing of PIDD was found to generate the PIDD-CC fragment in the p53 expressing cells. In the absence of functional p53, PIDD was found to autoprocess only into the PIDD-C fragment, which has been associated with NF-κB-dependent cell survival. p53 transduction markedly enhanced the susceptibility of SKOV-3 cells to cisplatin-induced caspase-2 activation and apoptosis. Similarly, suppression of p53 by siRNA transfection diminished caspase-2 activation. These observations demonstrate a strong correlation between the expression of functional p53 and caspase-2 activation and cellular susceptibility to cisplatin-induced apoptosis. In a recent publication, p53-mediated cell death in H1299 cells expressing tetracycline-repressible wild-type p53, or carrying a p53 mutation at two residues in the transactivation domain I (TAD I), p53$^{Q22/S23}$, was investigated. Although, this mutant is severely impaired with regards to activation of the commonly studied p53 target genes, it induced expression of pro-apoptotic targets including PIDD and AIP1 at least to the some extent as wild-type p53. Similar to our results (paper IV), the mutant versions of p53 in this study caused p53-mediated apoptosis that required the activation of caspase-2 in a PIDD-dependent manner upon treatment. Thus, the results of this study suggest that there might be two separate pathways by which p53 is capable of inducing cell death through mitochondria. One relies on the complete repertoire of wt p53 transactivation functions and involves parallel pathways activating multiple initiator factors, whereas the other pathway depends on restricted transcription, or even non-transactivation functions of mutant p53.

Taken together, our findings place p53 as a fundamental upstream regulator of caspase-2 activation. In addition, we provide data indicating a possible reciprocal functional connection between these proteins. This is based on observations that suppression of caspase-2 expression and activity in 5-FU-treated cells also affected the level of the p53 protein. Interestingly, reduction of the caspase-2 level in treated cells led to even higher amounts of p53 as compared to cells treated with 5-FU alone. This effect was parallel to an increase in PUMA and a decrease in Bax expression, indicating a bidirectional functional connection between p53 and caspase-2, which also affects the expression of p53-inducible genes. Although, there is no evidence for transcriptional activation of the caspase-2 gene in response to 5-FU, or to any other genotoxic agents, our findings point towards the importance of a balance between genes involved in the apoptotic process. Further, they question the current assumption of a unidirectional p53-caspase cascade. Suppression of Bruce, an IAP, that antagonizes cell death by suppressing caspases, and Bcl-2, was reported to be sufficient to activate p53.[225] Thus, a possibility that caspase-2 could be linked to such regulating factors upstream of p53 exists. Alternatively, our results may be explained by involvement of caspase-2 in the turnover of p53. Interestingly, a caspase-2 inhibitor has been shown to block silibinin-induced phosphorylations of Ser139 on H2AX and Ser15 on p53, without having any observable effect on ATM (Ser1981) phosphorylation.[242] Therefore, a possible regulatory effect of caspase-2 on phosphoinositide-3-kinase (PI3K)-related kinase(s) might also be suspected. However, at present there are no reports of p53 regulation by caspase-2. Thus, the physiological context for our findings (paper I) is unclear and needs further investigation.

Another pathway by which p53 expression influences caspase-2 has been described. Caspase-2 was found to be negatively regulated by p53 indirectly via an intact p21/Rb/E2F pathway.[219] Furthermore, complete inactivation of p21, or E2F-1, resulted in the stimulation of caspase-2 expression above basal levels. Since p21 has a well-characterized role as a cell cycle inhibitor, and is not a known apoptotic factor, p53 may repress caspase-2 via p21 in order to facilitate arrest rather than apoptosis.

The mechanism of caspase-2 activation

Overexpression of PIDD has been used to demonstrate the requirement of the PIDDosome platform for caspases-2 activation.[95,243,244] However, the presence of PIDD, RAIDD and caspase-2 in the PIDDosome complex in p53-deficient cells, as well as in untreated p53 parental cells, is demonstrated in paper I.[95,162] In our experimental system, suppression of either PIDD or RAIDD did not significantly influence caspase-2 activation, suggesting that, in some situations, caspase-2 might use another activation platform. Indeed, in paper III we present an alternative mechanism, where DISC formation and caspase-8 activity promote processing and activation of caspase-2.

Several reports have demonstrated a relationship between caspase-2 and -8. In fact, it has been reported previously that caspase-8 can directly cleave and activate pro-caspase-2 *in vitro*, but not vice versa.[245] In HOXA5-induced apoptosis of breast cancer cells, both caspase-2 and caspase-8 were found to be activated prior to mitochondrial events, although the sequential activation of these two caspases was not demonstrated.[237] In T cell apoptosis induced by ceramide and etoposide, activation of caspase-2 was requisite for caspase-8 activation prior to the permeabilization of mitochondria, indicating that activation of effector caspases in ceramide-induced neuronal apoptosis is not caspase-8-dependent.[246, 247] However, both FADD and caspase-8 were shown to be required for ceramide generation in response to CD95 activation using Jurkat T cells, also indicating that caspase-8 might act upstream of ceramide and caspase-2.[248] These observations suggest that caspase-8 might act both upstream and downstream of ceramide and caspase-2. Indeed, in several systems caspase-2 has been shown to cleave pro-caspase-8. The marine alkaloid ascididemin was shown to induce Jurkat T cell apoptosis that requires caspase-2 activation upstream of mitochondria and activation of caspase-8 as an effector caspase downstream of mitochondria.[249] In addition, in silibinin-treated RT4 cells, apoptosis was induced by activation of both caspase-2 and -8.[242] In this case, activated caspase-2 cleaved caspase-8 and Bid, while activated caspase-8 could only partially cleave caspase-2 and Bid, suggesting another bidirectional regulatory mechanism between caspase-2 and -8 and their different role in Bid activation. All these results indicate that the hierarchical position of caspase-2 or -8 activation might be cell type-specific and needs further investigation.

An association between caspase-2 and DISC-mediated apoptosis has also been suggested previously. For example, caspase-2 was shown to be required for Bid cleavage and optimal development of TRAIL-induced apoptosis,[250] and leukemic caspase-2–knockdown cell lines were characterized by a reduced sensitivity to CD95-mediated apoptosis.[251] However, a direct interaction was not described until caspase-2 was found to be recruited to the DISC in T and B cells upon CD95 stimulation.[97] Yet, despite its presence at the DISC, caspase-2 activation was not detected in response to CD95 stimulation of a caspase-8–deficient Jurkat T cell line. This is in line with our finding that DISC- and caspase-8-mediated activation of caspase-2 might occur in response to p53-dependent DNA damage-induced signaling

Our data clearly establish that DISC formation and caspase-8 activation are essential for cleavage of pro-caspase-2 in 5-FU-treated HCT116 cells. Both caspases

then promote apoptosis in a parallel manner. FADD serves as an adaptor protein by linking caspase-8 to the CD95 receptor through homotypic domain interaction. The existence of an analogous protein that might connect caspase-2 to the DISC remains elusive. It is, however, important to note that both PIDDosome and DISC platforms exist in HCT116 cells. It is still unclear whether they contribute jointly to caspase-2 activation, or if they exert activation specificity to different stimuli. It is possible that induction of two death platforms by a single stimulus occurs and that one pathway is active and the other is dormant. Thus, our observations in papers I and III might be similar to the situation seen in trophic factor deprivation-induced death in neurons. In TFD-treated neurons the caspase-2 pathway is predominant and responsible for cell death, but apoptosome activation also occurs although it is suppressed by XIAP. In caspase-2-deficient neurons, the increased abundance of caspase-9 and Smac/Diablo can overcome the inhibition of caspase activity by XIAP and restore sensitivity to TFD.

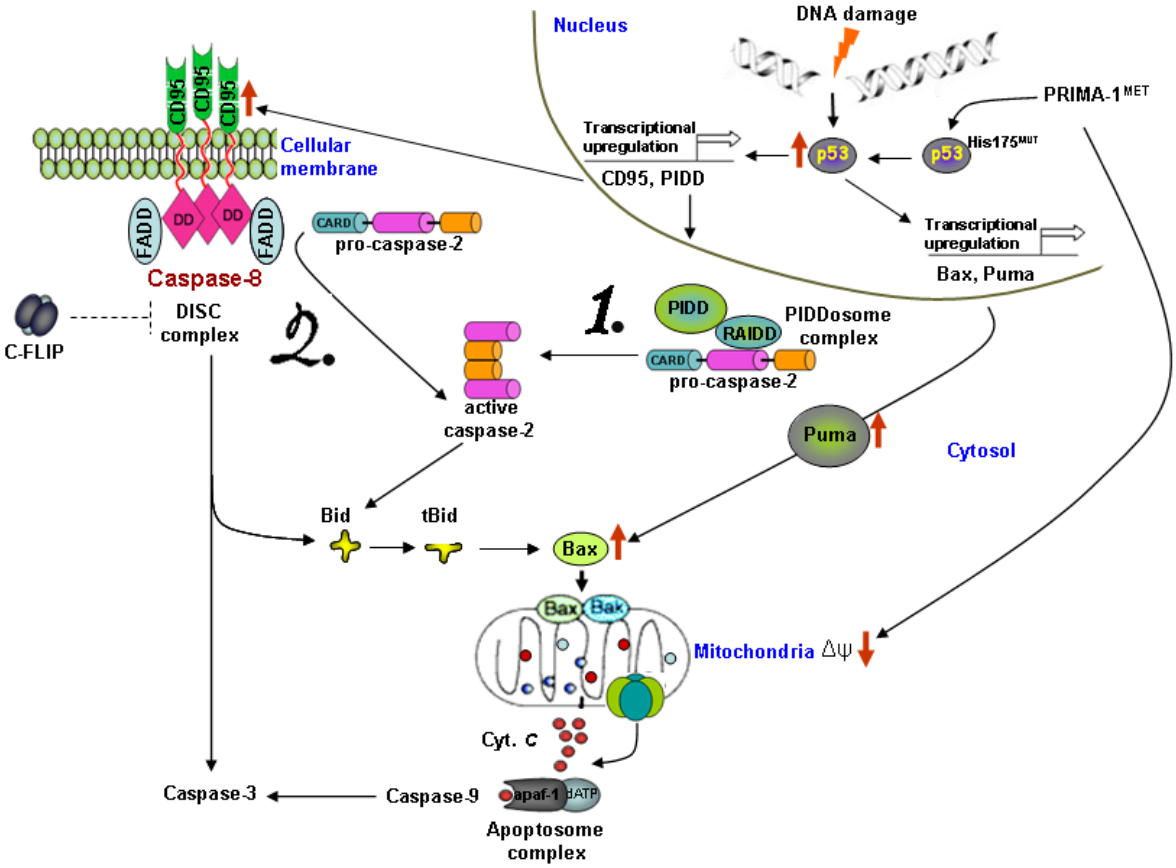

Figure 4

Summary of the findings in papers I –IV

A unique feature of the PIDDosome is that its formation appears to depend on the severity of the apoptotic stimulus. It was proposed that the PIDD-CC fragment formed by autoproteolysis is capable of interacting with RAIDD and caspase-2 only during apoptosis, whereas the PIDD-C fragment was generated also by autoprocessing in response to low-dose DNA damage when cell repair mechanisms are switched on.[157, 252] In addition, cellular responses may depend on the expression levels of PIDD isoforms that either possess or lack the critical cleavage site required for the formation of PIDD-CC.[244] However, it should be noted that caspase-2 knockout MEF were only partially resistant to PIDD overexpression, whereas RAIDD knockout cells were fully protected, suggesting that PIDD may induce cell death independent of caspase-2. Although, the presence of caspase-2 in the PIDDosome was not investigated in papers

II and IV, differences in PIDD autoprocessing were observed between the cell lines tested. Compared to non-apoptotic cells, the cleaved fragment of PIDD, PIDD-CC, was detected in all cell lines undergoing apoptosis following treatment. Moreover, inhibition of PIDD expression (paper II) resulted in decreased apoptotic cell death. This indicated that PIDD may acts as a "death switch" and that PIDD-CC plays a role in caspase-2 activation.

In conclusion, our results imply that PIDD plays an essential role in activating caspase-2, demonstrating the importance of the PIDDosome in the regulation of stress-induced apoptosis in some cells. In these cells, caspase-2 is mainly nuclear. In other cells, the PIDDosome-mediated caspase-2 activation pathway appears to be overtaken by p53-dependent DISC formation required for caspase-8 activation that in turn activates caspase-2 (see figure 4). In this case, caspase-2 is found in the cytosol. These observations may indicate that the subcellular localization of caspase-2 might be of importance for the determination of its activation platform.

What is mitotic catastrophe?

Mitotic catastrophe (MC) has long been considered as a mode of cell death which results from premature or inappropriate entry of cells into mitosis caused by chemical or physical stresses and is associated with various morphological and biochemical changes.[39] The final step of MC is almost always characterized by the formation of nuclear envelopes around individual clusters of missegregated chromosomes eventually leading to cell death. It has also been described as a delayed form of reproductive death based on observations that the multinucleated giant cells can be temporarily viable.[253, 254] However, since most cells undergoing MC eventually die, cellular processes that lead to irreversible growth arrest and are termed reproductive death may better fit to conditions such as senescence.[255] Alternatively, MC has been classified as a special form of apoptosis. This classification is based on the observation that MC shares several biochemical hallmarks with apoptosis, such as MOMP and caspase activation.[41, 42] In addition, there are observations that define MC as a cell survival mechanism of tumors.[43] In some cases MC may represent a process through which cells switch from an abnormal to a mitotic cell cycle.[44] Despite, or maybe due to, these various definitions there is at present no generally accepted classification of MC. In fact, arguments have raised the more general question of whether MC is a mode of cell death or if this is a process that leads to apoptosis or necrosis.[46-48, 256] In paper IV, we present evidence indicating that death-associated MC is not a separate mode of cell death, but rather a process ("pre-stage") preceding cell death, which can then occur by either necrosis or apoptosis.

It is well-known that tumors differ in their sensitivity to treatment. Sensitive cells are dying before entering mitosis when exposed to various chemotherapeutic agents. Cells that do not die during interphase may become arrested in G_1 or G_2. Mitotic phase entry is directed by the cyclin B/Cdk1 kinase and Cdc25C which also is a critical target of checkpoint control.[257] Induced expression of cell cycle inhibitors, such as the Cdk inhibitor p21 and the 14-3-3σ protein, by p53 and checkpoint mediator BRCA1 probably affect duration of the G_2/M arrest. Cells with impaired or lost checkpoint functions, such as p53 deficient SKOV-3 and 14-3-3σ $^{-/-}$ HCT116 cells, are unable to maintain this arrest and enter mitosis prematurely in the presence of unrepaired DNA. Thus, the abrogation of G_1 and/or G_2 checkpoints is essential for MC.[138, 171-173] Importantly, without premature entering mitosis, a cell cannot undergo MC. This suggests that MC is the outcome of DNA damage only when multiple central checkpoint proteins, such as ATR, ATM, Chk1-2, 14-3-3σ and p53 are suppressed and other options for cell death and survival are minimized.[256, 258, 259-260] There are several experimental models that have been utilized to clarify biochemical changes associated

with MC. In our experimental model the lack of p53, Chk2 and 14-3-3σ sensitized cells to undergo MC upon treatment with DNA-damaging agents. These proteins acted as negative regulators of MC. In addition, p53 appeared to act as a switch between apoptosis and the development of MC.

The fate of MC cells depends on multiple parameters and different outcomes are possible. Dying cells are characterized by multiple mitotic abnormalities, including multipolar meta- or anaphase, random distribution of condensed chromosomes throughout the cells, etc. Cell death might occur either during, or after, dysregulated mitosis. Biochemical mechanisms responsible for these differences still require additional investigation. Several possible scenarios for the death of MC cells have been suggested. According to one of them, death is a direct consequence of MC and it is distinct from apoptosis.[39] This conclusion was based on dissimilarities in morphology between MC cells compared with apoptotic cells.[8] Despite their distinct morphology, some authors have suggested that both processes share several biochemical hallmarks. There is no consensus concerning the requirement of caspases for MC.[41, 256, 261] We show that the progression of MC is caspase-independent, since neither inhibition nor down-regulation of caspases influence the appearance of MC cells. However, caspases seem to be essential for termination of MC, suggesting that MC-related morphological changes are followed by activation of the apoptotic machinery.[48, 256] Thus, we propose that MC is not an ultimate manifestation of cell death but rather a process leading to cell death.

The exact molecular mechanism of this association remains to be elucidated, [262, 263] since premature mitosis leading to an arrest during the metaphase-anaphase transition is a p53-independent event. Apoptosis in metaphase has also been suggested to occur independently from p53 but as a consequence of caspase-2 activation.[264] However, since caspase-2 activation itself is dependent on p53,[95, 162] it is unlikely that apoptosis induced by mitotic arrest would be p53-independent. Indeed, our observations showed that the apoptotic outcome of MC is p53-dependent (paper IV). During mitotic arrest, activation of p53 occurs in an ATM/ATR-independent fashion, suggesting that other mechanisms for p53 activation exist.[265] [266] Interestingly, p53 can localize to centrosomes during mitosis but is displaced upon spindle damage, indicating that p53 can sense mitotic failure and initiate apoptosis.[267] In addition to metaphase arrest-induced apoptosis, tetraploid cells that are generated through catastrophic mitosis followed by mitotic slippage undergo p53-dependent apoptosis. An immediate induction of p21 after mitotic slippage is an indicator of a p53-dependent checkpoint response in G_1, which would act as a second "fail-system" after an aberrant mitosis.[266, 268] Furthermore, p53 has been suggested to be responsible for initiating apoptosis after endomitosis and endoreplication.[269, 270] Thus, besides its central role in apoptosis induced by DNA damage, p53 appears to be an important regulator of MC.[271, 272]

Apoptosis, however, seems not always required for MC lethality since SKOV-3 cells, as well as p53-deficient Caov-4 cells, underwent slow death in a necrosis-like manner, characterized by loss of nuclear and plasma membrane integrities. Indeed, mitotic arrest in docetaxel-treated tumor cells was followed by massive cell destruction by means of cell lysis.[273] Necrosis following MC could be an effect of genetic instability caused by aneuploidy and/or polyploidy.[274] Taken together, it is important to note that cells that are facing a MC-linked cell death can die by either apoptosis or necrosis.

In conclusion, in our opinion MC represents a pre-stage of apoptosis or necrosis (see figure 5). This suggestion is based on our and other studies indicating that apoptosis might follow, rather than precede, MC.[254, 275, 276] In DNA-damaged cells, the absence of p53 and down-regulation of Chk-2-related proteins drive cells from MC to necrosis. On the other hand, down-regulation of Chk-2-related proteins in the presence

of functional p53 allows MC cells to die by apoptosis, which can be initiated either in the metaphase-arrested cells or after exiting the aberrant mitosis. The increased p53 expression, followed by caspase activation, occurs subsequently to the morphological changes that characterize MC, indicating that drug-induced MC precedes apoptosis, which is delayed due to a primary mitotic arrest. The final outcome of MC depends on the molecular profile of the cells, which might be of potential relevance for tumor treatment.

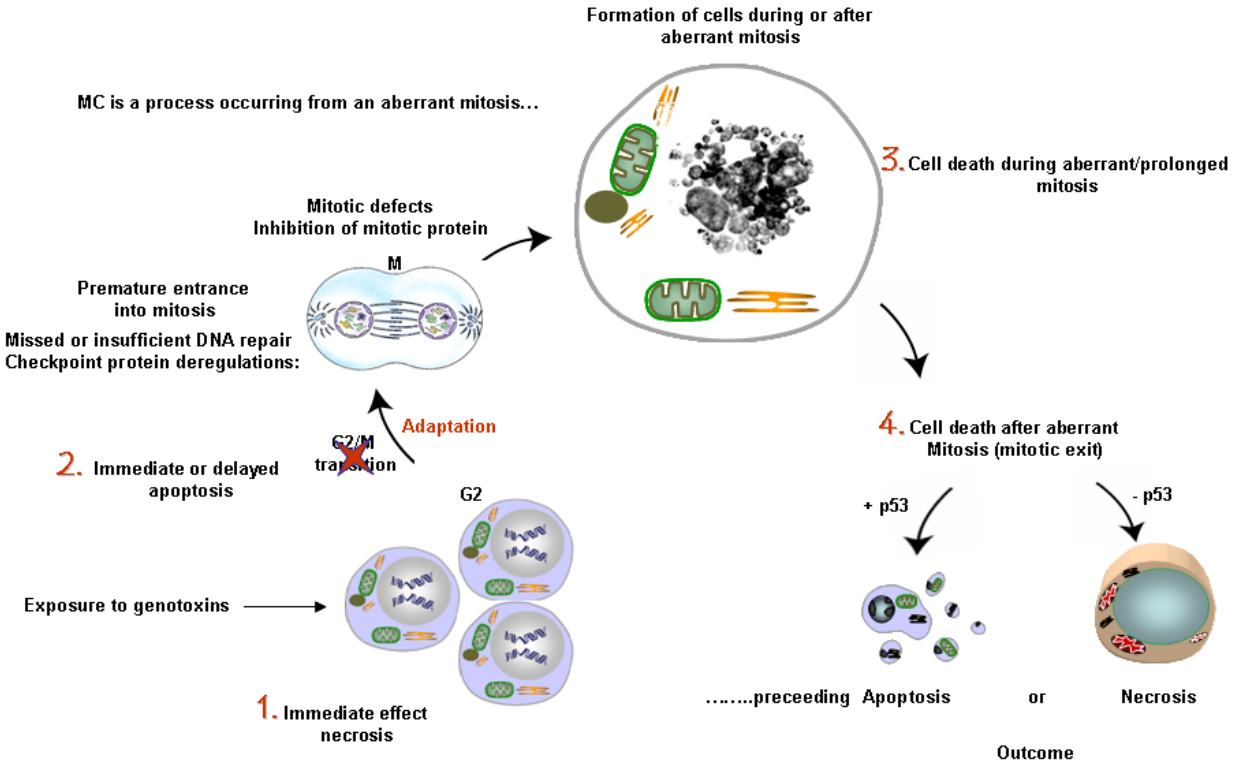

Figure 5
Mitotic catastrophe is not a mode of cell death, but a "pre-stage" preceding cell death, which can occur through necrosis or apoptosis

Conclusion

In general, our results characterize a caspase-dependent DNA damage-induced apoptotic response. The presence of functional p53 is needed in order to complete the apoptotic process mediated through the mitochondrial pathway. Both caspase-8 and caspase-2 act as *bona fide* initiator caspases, and p53 is fundamental for their activation. Hence, p53 acts as an upstream regulator of the apoptotic caspase cascade. In addition, we demonstrate that a bi-directional, functional connection exists between caspase-2 and p53.

Even though a complex containing RAIDD, PIDD and caspase-2 was present in p53 expressing and deficient cells, irrespective of treatment, our findings verify a requirement of p53-dependent pathways for activation of caspase-2. In some cells this pathway relies on DISC-mediated caspase-8 activation. In other cells, the PIDDosome is sufficient for nuclear caspase-2 activation, which then acts as an initiator caspase and translocates from the nucleus into cytosol. Thus, either a direct or sequential caspase-2 activation, occurring upstream of mitochondria, is demonstrated. Hence, the activation of caspase-2 seems to be an intricate process presumably involving several mechanisms depending on the apoptotic trigger and cell type.

Regardless of its activation hierarchy, our results assign a role for caspase-2 upstream of mitochondrial events. In this study, activated caspase-2 was able to induce MOMP and subsequent cytochrome *c* release through Bid cleavage and Bax activation/translocation. Moreover, depending on the stimulus and cell type, caspase-2-dependent nuclear-mitochondrial engagement might occur in parallel with other proapoptotic proteins, such as PUMA.

Finally, we also investigated the phenomenon of MC induced by DNA damage. Inhibition and/or downregulation of p53 and Chk-2 sensitized cells to undergo MC. A role for p53 as a negative regulator of MC was suggested, in a process where caspase activation was not required. Instead, caspases seem to be essential for the termination of MC, suggesting that MC-related morphological changes are followed by activation of the apoptotic machinery. Thus, we propose that MC is not a specific type of cell death but rather precedes cell death. The mode of subsequent cell death appears to be determined by the cellular profile of proteins involved in the regulation of the cell cycle, such as p53 and Chk2.

Significance of the study

It is well known that chemotherapeutic drugs of different structure and specificity can induce the characteristic morphological changes associated with apoptosis, and that the intrinsic apoptotic pathways contribute to the cytotoxic action of DNA damage. In recent years, it has become evident that caspase-2 is a key mediator of the intrinsic apoptotic process. However, the knowledge concerning this enzyme and its function has been limited. Through the experiments performed in papers I-IV, we attempted to illustrate the molecular map bringing the embarking stimuli of genotoxic stress to caspase-2 activation and the downstream molecular event in apoptosis. It is our hope that results outlined in this thesis will help to resolve these issues and to clarify current problems, such as dysfunction of apoptosis in cancer cells, and expose molecular contexts leading to new therapeutic approaches.

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