Genetic Analysis of Caspase-6 and Caspase-7 Function in Vertebrate DT40 Cell Line

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Abbreviations

°C	degrees Celsius
aa	amino acid
AIF	apoptosis inducing factor
AIP	apoptosis inhibitory protein
Apaf-1	apoptotic protease-activating factor 1
APS	ammonium persulphate
ATP	adenosine-5′-triphosphate
BCR	B-cell receptor
BLAST	basic local alignment search tool
bp	base pair(s)
BSA	bovine serum albumin
Bsr	blasticidin resistance cassette
cDNA	complementary DNA
c-Flip	cellular Flip
CAD	caspase activated DNase
CIP	calf intestinal alkaline phosphatase
CLAP	chymostatin, leupeptin, antipain, pepstatin A
Coomassie	Coomassie Brilliant Blue R
DAPI	4'6'-diamidino-2-phenylindole
DD	death domain
Database	NCBI sequence database
DED	death effector domain
DEPC	diethylpyrocarbonate
dH_2O	de-ionised water
DISC	death-inducing signalling complex
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide-5′-triphosphate
DTT	1,4-dithiothreitol
E. coli	*Escherichia coli*
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme Linked ImmunoSorbent Assay
EST	expressed sequence tag
Et	Etoposide
FADD	Fas associated death domain
FBS	fetal bovine serum
FLIP	FLICE(caspase-8)-inhibitory protein
Fmk	fluoromethyl ketone
g	gravity
GFP	A. *victoria* green fluorescent protein
H	hour
HEPES	*N*-[2-Hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]

HisD	histidinol resistance cassette
HRP	horseradish peroxidase
ICAD	Inhibitor of CAD
JNK	Jun N-terminal kinase
kb	kilobase pair(s)
KDa	kilodaltons
LB	Luria-Bertani medium
M	molar, (moles/litre)
mRNA	messenger RNA
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PARP	poly(ADP-ribose) polymerase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming unit
PIPES	1,4-piperazinediethanesulfonic acid
PMSF	phenylmethylsulfonyl fluoride
Puro	puromycin resistance cassette
RIP	receptor intercating protein
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease
rpm	revolutions per minute
RT-PCR	reverse transcriptase PCR
SDS	sodium dodecyl sulphate
TAE	tris acetate EDTA
TEMED	*N,N,N′,N′* - tetramethylethylenediamine
TNF	tumor necrosis factor
TNFR	TNF receptor
TRADD	TNFR-associated death domain
TRAIL	TNF-related apoptosis-inducing ligand
Tris	Tris (hydroxymethyl)aminomethane
TRIzol	Total RNA Isolation Reagent
Tunel	Terminal deoxynucleotidyl transferase-mediated dUTP nick labeling
UTR	untranslated region
UV	ultraviolet
V	volt
Z-EK(bio)D-aomk	N-(Nα-benzyloxycarbonylglutamyl-N-ε-biotinyl-lysyl aspartic acid ((2,6-dimethylbenzoyl)oxy)methyl ketone

Standard prefixes used:

k	kilo (10^3)
m	milli (10^{-3})
μ	micro (10^{-6})
n	nano (10^{-9})
p	pico (10^{-12})

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'Avant Propos'

It is common knowledge of the 21st century that metozoan cells have the capability to undergo programmed cell death (PCD) and that cellular survival is sometimes dependent on the repression or activation of this self-destruction program. It is exactly four decades ago, in 1963, that Richard Lockshim first reported the term "programmed cell death", nine years later the Greek term APOPTOSIS[1] was adopted to designate PCD.

I became very interested in the study of apoptosis while pursuing my Masters studies at Bradford University. One of our assignments was to write a review about a recent booming topic in molecular biology and it was then that my attention was directly caught by the amazing field of apoptosis. Soon after I felt that I would like to grasp a deeper understanding in the field of cell death and especially its molecular mechanism. I was fortunate enough to apply and to be accepted for a Ph.D. in Professor William Earnshaw's laboratory. Apoptotic research in the Earnshaw research group started with the findings of Dr. Edgar Wood, a former postdoc in Professor Earnshaw's laboratory in 1989. Dr Wood's so called "mitotic" extracts prepared for a cell free experiment had a rather unusual effect when added to isolated nuclei. These nuclei were undergoing apoptotic morphology as recognised by Dr Yuri Lazebnik. Since then, work conducted in the Earnshaw laboratory has provided deeper understanding in the molecular, genetic and cellular mechanisms underlying cell death. My work began with an ongoing *caspase*-6 knockout project and in my second year of graduate studies I started a *caspase*-7 knockout project which constituted the major part of my Ph.D. research studies.

This thesis, divided into five chapters, is a portrayal of the generation and characterisation of caspase-6 and caspase-7 deficient DT40 cells. Chapter I is an overview of apoptosis starting by a brief historic synopsis on the discovery of cell death proceeding to a summery on the progress made so far in the understanding of the molecular basis of apoptosis and moving on to introduce the Ph.D. projects and the DT40 system used for the generation of deficient cells. Chapter II is a detailed description of the materials, methods and protocols used. Chapter III begins with the characterisation of the *caspase*-6 and *caspase*-7 loci and ends with the design of replacement vectors. Chapter IV is allocated to examination of *caspase*-$6^{-/-}$ cells and chapter V to the analysis of caspase-$7^{-/-}$ cells. Finally, this thesis ends by a general conclusion and future recommendations.

[1] The word apoptosis "falling off" or "dropping off" , in Greek (apó = from, ptósis = a fall), previously used to describe the dropping off of the leaves from trees in the autumn was suggested by James Cormac, Professor of Classical Greek at the University of Aberdeen (reviewed in Cummings et al., 1997)

Abstract

Apoptosis, also known as programmed cell death, is an evolutionarily conserved mechanism by which the organism removes unwanted cells. The process is common to somatic as well as germ cells. It is actively involved in development and morphogenesis, cell number control and removal of infected, mutated or damaged cells. Many insights on the activation of final triggers and pathways in apoptosis came from genetic studies of apoptosis using the worm, *C.elegans* This work showed that the worm Ced-3 is a protease of pivotal role in apoptosis of *C.elegans.* The mammalian counterparts of CED-3 have been identified as members of a family of intracellular proteases that form the core of the apoptotic machinery. Since these are cysteine proteases that cleave cellular substrates at specific aspartate residues, they were all termed **caspases.**

In the last ten years natural and synthetic inhibitors and genetic approaches have been used to study elucidate specific caspase function. An alternative system is gene disruption in the chicken DT40 cell line. These cells have a high homologous recombination rate that facilitates the isolation of knockout alleles. The aim of my project is the genetic analysis of caspase-6 and -7 function. My work started with an ongoing *caspase-6* knockout project along with a senior postdoctoral scientist in our lab, Dr Sandrine Ruchaud. Subsequently, I concentrated my work on the caspase-7 knockout poject. I generated DT40 cell line where both alleles of *caspase-6* and *-7* were disrupted independently.

Caspase-6: No obvious morphological differences were observed in the apoptotic process in caspase-6 deficient cells compared to the wild type. However, examination of apoptosis in a cell free system revealed a block in chromatin condensation and apoptotic body formation when nuclei from HeLa cells expressing lamin A or lamin A-transfected Jurkat cells were incubated in caspase-6 deficient apoptotic extracts. Transfection of exogenous caspase-6 into the clone reversed this phenotype. Lamins A and C, which are caspase-6-only substrates, were cleaved by the wild type and heterozygous apoptotic extracts but not by the extracts lacking caspase-6. Furthermore, the caspase-6 inhibitor z-VEID-fmk mimicked the effects of caspase-6 deficiency and prevented the cleavage of lamin A. Taken together these observations indicate that caspase-6 activity is essential for lamin A cleavage and that when lamin A is present, it must be cleaved in order for the chromosomal DNA to undergo complete condensation during apoptotic execution.

Caspase-7: Viability assays showed that *caspase-7*$^{-/-}$ clones are more resistant to common apoptosis-inducing drugs such as etoposide and staurosporine. Further examination of the apoptotic process revealed that *caspase-7*$^{-/-}$ cells show a delay in phosphatidylserine externalization and DNA fragmentation as well as cleavage of the caspase substrates PARP and lamins B1 and B2. Caspase affinity labeling and activity assays indicated that deficient cells exhibit a delay in caspase activation when compared to wild type DT40 cells, providing an explanation for the differences in apoptotic execution between *caspase-7* null and wild type DT40 cells. These results strongly suggest that caspase-7 is involved earlier than other effector caspases in the apoptotic execution process in DT40 B lymphocytes.

My results have provided important new insights into the function of caspase-6 and caspase-7 during apoptotic execution.

Chapter I: Introduction

Apoptosis, also known as programmed cell death, is an evolutionarily conserved mechanism by which the organism removes unwanted cells. The process is common to somatic as well as germ cells (Kerr et al., 1972; Print and Loveland, 2000). It is actively involved in development and morphogenesis, cell number control and removal of infected, mutated or damaged cells (Guo and Bruce, 1999). Thus, apoptosis is a critical process for the health of every multicellular organism. Disregulation in programmed cell death can cause many diseases. Too little apoptosis may lead to cancer or autoimmune diseases; too much can induce neurodegenerative diseases such as Alzheimer's (LeBlanc et al., 1999).

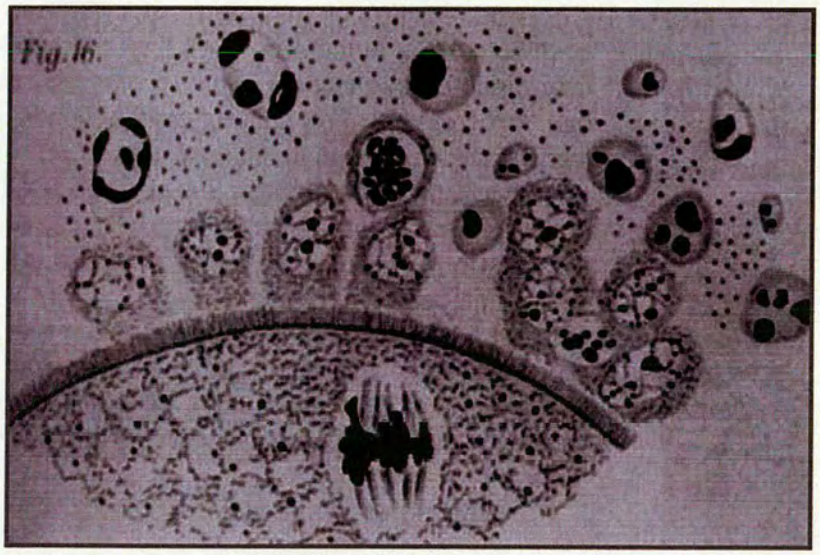
I A. A brief history of programmed cell death

The first reference to developmental cell death dates back to the mid 19th century when Vogt, in 1842, reported death of notochord and adjacent cartilage of metamorphic toads (Vogt, 1842). However, Vogt abandoned his curiosity in the field of cell death and for decades this new oddity remained silent until Flemming, in 1885, presented the first morphological descriptions of naturally occurring cell death (Fig 1.1A) (Flemming, 1885). Flemming's description was the first to argue that death involved chemical changes within the cell. Studying dying granulosa cells of rabbit Graafian follicles, Flemming noted an "ill-defined nucleus containing several small, heavily stained lumps, and a pale, homogeneous cytoplasm containing what appeared to be fine fat droplets". Chromatolysis was the term he proposed and introduced to describe cell death observed (Flemming, 1885). Soon after in 1889, Weissmann wrote that "higher organisms contain within themselves the germs of death" and by the beginning of the 20th century the concept of intrinsic cell death machinery was already acknowledged by many scientists (Weissmann, 1889). Yet, the pioneer for the

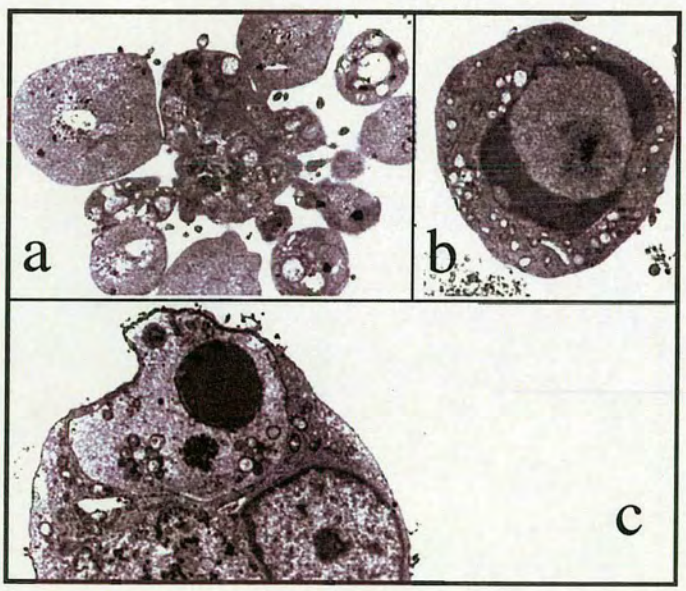
genetic basis of cell death was the German anatomist Ludvig Gräper. In 1914 Gräper published a paper titled "A new point of view regarding the elimination of cells" with the idea that mechanisms must exist to counterbalance mitosis and referring to Flemming's chromatolysis (Gräper, 1914). Sadly, Gräper's publication was overlooked and the atypical concept of cell death almost perished (Majno and Joris, 1995). However, chromatolysis survived among embryologists such as Glücksmann and Saunders who once more stressed on the importance of cell death in development such as digit shaping and removal of useless structures (Glücksmann, 1951).

The first evidence for the existence of two morphologically distinct types of cell death came from an Australian pathologist, John Kerr. In 1965, Kerr was a graduate student who focused his Ph.D. studies on hepatocyte atrophy. Whilst studying cytotoxicity in the liver, the structural changes he noted did not conform to the established notions of cell death. Cells did not display a watery cytoplasm, the intracellular material was not released and organelles did not rupture. Instead, the cell lost contact with its neighbors, shrunk, nuclear chromatin condensed, membrane bound organelles were formed, efficiently phagocytosed and degraded (Kerr et al., 1965). This curious and distinct form of liver cell death was first designated as "shrinkage necrosis". Subsequently, Kerr and his collaborators Alastair Currie and Andrew Wyllie introduced the term apoptosis (see 'Avant Propos') and defined it as a "morphologically distinctive, active, inherently controlled form of cell death, complementary to mitosis in the regulation of animal cell populations both in physiological and pathological conditions" (Fig 1.1B) (Kerr et al., 1972). In 1976 and 1984, studies on irradiated lymphoid tissues led to the notion that during apoptotic cell death chromatin broke down into fragments that produced a DNA ladder (Wyllie et al., 1984; Yamada et al., 1981). Since then, studies on apoptosis witnessed an explosion and apoptosis research became one of the fastest evolving fields in biology. The exploitation of molecular biological techniques led to rapid advances in knowledge about programmed cell death in all its aspects including biochemical, cellular, genetic and developmental.

Figure 1.1: Early observations of opoptotic morphology



A



B

Fig 1: **A,** Apoptosis of an overian follicle as figured by Flemming in1880s (Flemming, 1889) **B**, (a)Transmission electron micrograph of a late-stage apoptotic fibroblast. (b) chromatin condensation of an apoptotic fibroblast (c) phagocytosed apoptotic body within a phagosome (Wyllie, 1992).

I B. Apoptosis research in the molecular era

I B1. Morphological changes during apoptosis

Most of the morphological changes of cell death were observed, characterized and described by Kerr over four decades ago in the seminal paper (see above) (Kerr et al., 1972). As other scientists fascinated by this new phenomenon carried on observing dying cells additional characteristics of apoptosis were identified. As a consequence, it became widely accepted that at least two types of cell death, distinct in morphology and consequences exist, apoptosis, a natural program which is normally silent within the host, and necrosis, a form of death that occurs in response to external injury, and that leads to exposure of normally sequestered molecules and inflammation (Chautan et al., 1999; Fiers et al., 1999; Hacker, 2000; Lincz, 1998; Lopez et al., 2003; Matsumura et al., 2000; Scheffer et al., 2003; Schwartz et al., 1995). The classical morphological characteristics linked to apoptosis are portrayed in Figure 1.2A and include:

- Cell detachment by the loss of microvilli and gap junctions
- Aggregation of compacted chromatin against the nuclear membrane
- Chromatin DNA fragmentation and break-up of the nucleus into discrete, membrane-enclosed fragments in which the segregation of compacted chromatin is maintained
- Cell shrinkage associated with marked convolution of the cell surface to produce membrane-bounded apoptotic bodies
- Budding and dispersion of apoptotic bodies
- Functional and intact mitochondria until the late stages

Figure 1.2: Apoptotic morphology

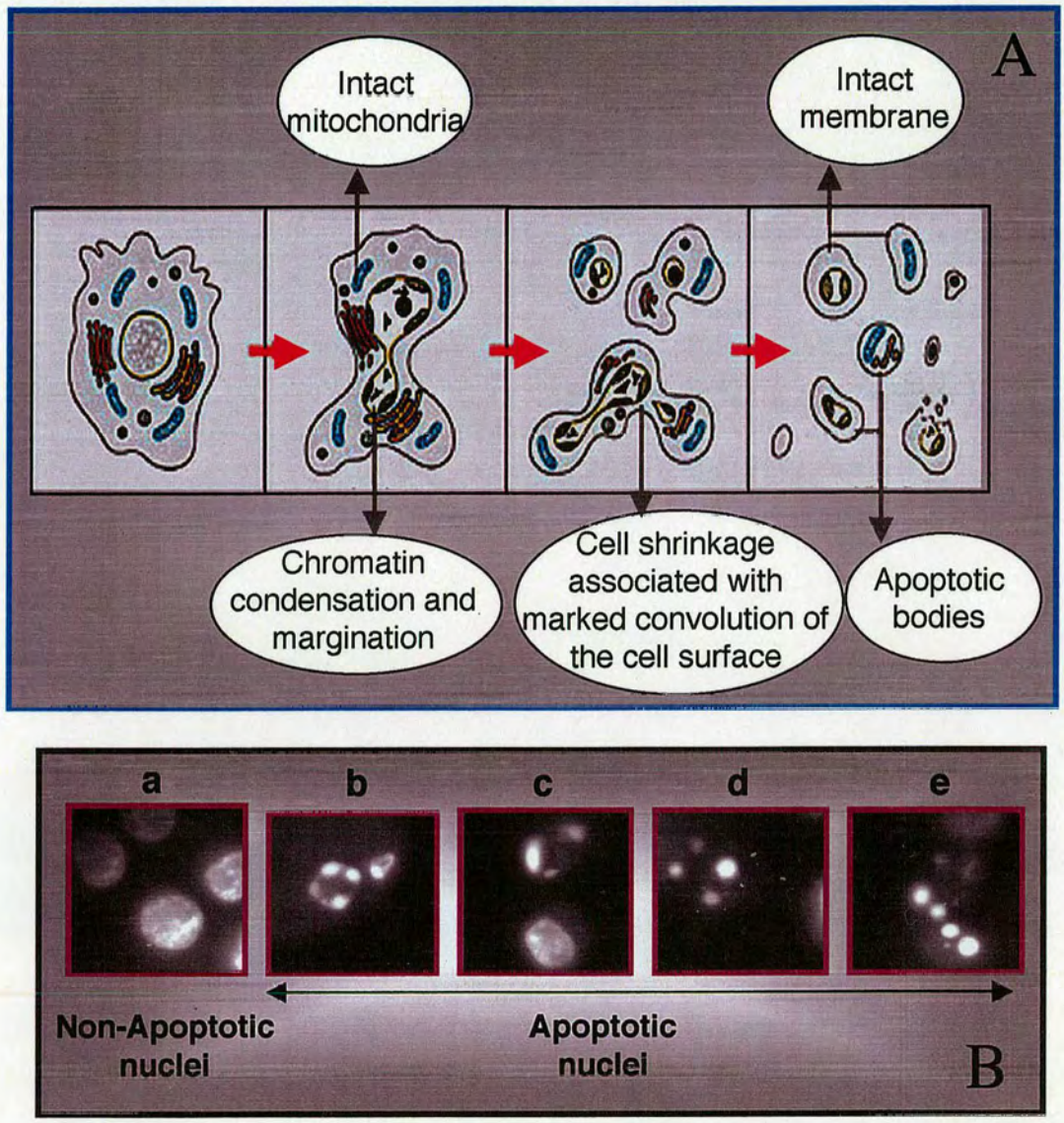


Fig 1.2: **A**, Schematic representation of the typical morphological characteristics of a dying cell. **B**, DAPI stained apoptotic DT40 nuclei representing the nuclear apoptotic events. (a) non-apoptotic. (b) chromatin condensation and margination. (d,e) breaking up of the nucleus into discrete, membrane-enclosed fragments

I B2. Molecular and Cellular Changes

The observed morphological changes described above are a direct result of biochemical modifications occurring in a dying cell. These alterations were revealed with the advancement of molecular biology techniques that allowed the characterization and identification of apoptotic cells on the molecular level. Two most important changes used in the identification of apoptotic cells are the loss of plasma membrane asymmetry, leading to phosphatidylserine externalization, and DNA fragmentation.

I B2a. Phosphatidylserine externalization

In non-apoptotic cells phosphatidylserine is located in the inner leaflet of the plasma membrane. However, early in apoptotic execution, phosphatidylserine becomes randomly distributed between the two leaflets (Koopman et al., 1994; Martin et al., 1995). This event may be a signal to phagocytes (Fadok et al., 1992), yet it is not an absolute requirement for macrophage recognition and apoptotic body clearance. Phosphatidylserine acts as a pro-coagulant, an effect that is reversed when bound to annexin V. Annexin V, belonging to the annexin family of proteins which are characterized by their anticoagulant properties, preferentially binds to negatively charged phospholipids including phosphatidylserine in the presence of Ca^{2+}. Annexin V binding to the cell membranes can be detected before other apoptotic morphological changes take place and before the loss of membrane integrity. Therefore, Annexin V bound to a fluorochrome, was established as a useful tool in detecting apoptotic cells and is used in quantitative and qualitative studies (Koopman et al., 1994; Vermes et al., 1995).

I B2b. DNA condensation and fragmentation

In apoptotic cells the nuclear DNA condenses and marginates to the inside of the nuclear membrane (Hollis et al., 1987; Lincz, 1998; Morey et al., 1993). Initially, in most (but not all cell types) DNA is cleaved into 300 kb and 50 kb fragments, then to 180-200 bp internucleosomal fragments which result in a characteristic DNA laddering effect

observed by agarose gel electrophoresis (Brown et al., 1993; Wyllie et al., 1987). However it is important to point out that, whilst DNA fragmentation is a classical marker of apoptosis, it is not required for its execution, for example, apoptosis in the MCF-7 breast carcinoma cell line can proceed with no DNA fragmentation (Kagawa et al., 2001; Nagata, 2000).

DNA fragmentation can be easily detected by agarose gel electrophoresis or by a readily available and commonly used technique, Terminal deoxynucleotidyl transferase-mediated dUTP nick labeling (Tunel) staining. The tunel technique detects apoptotic cells by using the enzyme terminal deoxynucleotidyl transferase (TdT) to directly label the 3'ends of broken DNA strands with a fluorescent base analogue. Similar to annexin V staining, Tunel can be used for both quantitative and qualitative analysis (Gavrieli et al., 1992).

I B2c. Acidification of the cytoplasm

A third cellular change observed in apoptotic cells is cytoplasmic acidification, which is recognized as a feature of apoptosis in a variety of systems (Gendron et al., 2001; Meisenholder et al., 1996). However, the exact function of this event is still unknown and it has not been extensively studied. Some research groups suggested that the change in cytoplasmic pH is involved with Bax function(Thangaraju et al., 1999) and caspase activation (see Section I H1) (Furlong et al., 1997; Matsuyama et al., 2000).

The morphological changes in apoptosis are consequences of interaction, activation and inhibition of numerous apoptotic effectors, that interact within and between each other. Some effectors have the responsibility to promote cell death whilst others have the task of inhibiting apoptosis. Among the most important of the latter is a family of proteolytic enzymes known as caspases.

In the following part of this Introduction, the discussion of apoptosis will focus first on caspases and their role in apoptotic execution and second on pathways of caspase activation.

I C. The biochemistry of cell death: apoptotic execution

I C1. Common mechanisms in apoptotic execution

It is well established that apoptosis occurs in two phases: an initial commitment phase followed by an execution phase which involves cytoskeleton distortion, membrane blebbing, condensation and fragmentation of chromatin and the formation of apoptotic bodies (Boulares et al., 1999; Earnshaw et al., 1999). Significant progress in understanding the molecular and biochemical aspects of apoptosis has come from genetic studies of developmental cell death in the nematode *Caenorhabditis elegans* (Yuan, 1996). In the course of *C. elegans* development, 131 of the 1090 cells are eliminated in a spatially and temporally organized manner. Initial genetic analysis revealed three worm death genes, namely, CED-9 which encode an anti-apoptotic regulator, and CED-3 and CED-4, the two worm pro-apoptotic genes (Hengartner, 1995). Vertebrates have evolved multiple gene families that resemble the *C. elegans* death machinery, each of the nematode genes possesses at least one functional equivalent in vertebrates. Indeed, the cell death pathway in *C. elegans* can be considered as a simple model for apoptosis in higher organisms (Hengartner, 1998).

Mammalian counterparts of CED-3 have been identified as members of a family of intracellular proteases called caspases that form the core of the apoptotic machinery (Alnemri et al., 1996; Yuan, 1995). These proteolytic enzymes are not only responsible for some of the characteristic patterns of cell destruction that mark apoptosis, but also for activating themselves, and many other cell death effectors. It is their auto-catalytic ability that ensures a prompt and rapid apoptotic execution phase (Earnshaw et al., 1999; Fischer et al., 2003; Hengartner, 2000). The only mammalian CED-4 homologue identified to date is (Apaf-1) whereas, the Bcl-2 protein-related family was found to be the counterpart for the nematode anti-apoptotic protein CED-9. In higher eukaryotes Bcl-2 members can either promote or inhibit apoptosis (see Section I H).

I C2. The hallmarks of apoptotic execution

Studies over the last decade indicate that proteolytic cleavages of key proteins by activated caspases are responsible for the accomplishment of the apoptotic morphology. In spite of the fact that the exact mechanism of how degradation of these proteins results in apoptotic morphology remains unknown, many proteins targeted by caspases participate in the formation and regulation of the membrane-associated cortical microfilament cytoskeleton, an important determinant of apoptotic cell shape (Brancolini et al., 1997; Brown et al., 1997; Mashima et al., 1997; Vanags et al., 1996; Wang et al., 1998b). Caspases also cleave protein kinases at the cell-to-cell and cell-to-matrix attachment sites enhancing pro-apoptotic signaling and possibly cell detachment from surrounding tissue (Cardone et al., 1997; Kwan et al., 2001; Rudel and Bokoch, 1997; Wen et al., 1998). In addition, caspases and their activated substrates are involved in the regulation of chromatin condensation and DNA fragmentation. Topoisomerase II alpha (Durrieu et al., 2000) and acinus (Sahara et al., 1999) have been shown to have roles in apoptotic chromatin condensation. The DNAse enzymes responsible for DNA fragmentation during apoptosis include, Caspase Activated DNAse (CAD) (Enari et al., 1998; Sakahira et al., 1998) and Endonuclease G (Li et al., 2001; Parrish et al., 2001). CAD is an apoptosis-specific endonuclease that cleaves DNA into the observed oligonucleosomal fragments. CAD is found in the nucleus in an inactive complex bound to its inhibitor ICAD (Samejima and Earnshaw, 2000). However, ICAD is not just an inhibitor but also chaperone for CAD, since active CAD is only expressed in the presence of ICAD. The cleavage of ICAD by caspases results in dissolution of the complex, CAD activation and DNA degradation (Enari et al., 1998; Sakahira et al., 1998). However, CAD seems to be required for oligonucleosomal fragmentation but not essential for certain classes of high molecular weight DNA cleavage and the early stages of apoptotic chromatin condensation in DT40 cells (Samejima et al., 2001).

A large number of important observations have been made in the field of cell death, some of which are stated above, yet the greatest impact on the study of apoptotic execution has been the identification of caspases as the executioners of cell death.

I D. What are Caspases?

As stated in Section I C1, in nematodes CED-3 is critical for *C. elegans* cell death. Cloning and sequencing of CED-3 indicated that it is related to a mammalian protease known as interleukin-1-β-converting enzyme (ICE) (Yuan et al., 1993). ICE was recognized for its role in inflammation and studies of ICE concentrated on the need to clarify mechanisms for conversion of Pro IL-1β to IL-1β, the highly potent cytokine (March et al., 1985; Mosley et al., 1987; Wilson et al., 1994). The discovery of ICE homology to nematode CED-3 encouraged scientists to inquire about potential roles for ICE in apoptosis, however resulting reports showed that it was not primarily involved in cell death, rather ICE main role remained its inflammatory regulatory function (Zeuner et al., 1999). On the other hand, subsequent investigation revealed the existence of multiple vertebrate CED-3-like homologues, most of which were later shown to function primarily in apoptosis (see following sections). Soon after the identification of a number of this family of proteases it became apparent that they all share similar functions and structure (Earnshaw et al., 1999; Hengartner, 2000).

A unifying nomenclature was adopted for convenience and to provide a definition for this family of proteases. Consequently, the enzymes were designated by 'caspases', an abbreviation for **c**ysteine dependent **asp**artate directed prote**ases**. At present, the proteins of this family are individually named *caspase*-n, where n= the number of the caspase in question. Thirteen caspases have been identified to date in humans (Fischer et al., 2003; Hu et al., 1998; Koenig et al., 2001), many of them are found to be implicated in the apoptosis process (Earnshaw et al., 1999; Fischer et al., 2003; Hengartner, 2000; Humke et al., 1998). Consequently, many interesting questions concerning their structure, function, inter and intra interactions, regulation by different effectors and exact contributions to cell death pathways arose.

I D1. Caspase structure

Caspases are constitutively present in healthy cells as proenzymes. Biochemical studies of procaspases have identified three basic domains, the pro-domain, the large subunit

(p20), and the small subunit (p10). Caspases share a high substrate specificity whereby they cleave target proteins just after an N-terminal Asp residue (P1site). The domains of caspase zymogens are partitioned by Asp-X bonds (Green and Amarante-Mendes, 1998; Howard et al., 1991). Caspase activation results after proteolysis and processing of the two subunits followed by their association to form a heterodimer (Figure 1.3). The crystal structure of active caspases, such as caspase-1, -3,-7, -8 and –9, showed that they exist as a tetrameter of the two heterodimers (Blanchard et al., 1999; Renatus et al., 2001; Riedl et al., 2001; Wei et al., 2000). The groove resulting from the intimate association of the large and small subunits constitute the cysteine-dependent active site (Blanchard et al., 1999; Wei et al., 2000) (Fig 1.3)

I D2. Caspase families

Although members of the family of caspases share a common structure, there are differences that reflect their slightly different roles in apoptosis (could be removed for the page break). Depending on the primary structure of their N-terminus, apoptotic caspases were grouped into initiator caspases and effector caspases, yet not all caspases are involved in apoptosis and a third family exist, mainly involved in inflammation (Earnshaw et al., 1999; Lincz, 1998; Raff et al., 1998; Raff, 1992).

Cytokine processor caspases family (ICE):

Family members (caspase-1, -4, -5 and –11) are mainly involved in inflammation.

Initiator caspase family:

Caspases in this group contain an extended pro-domains, which allow association with receptor complexes for receptor-stimulated apoptosis. Caspase-2 and -9 contain Caspase Recruitment Domains (CARDs) and caspase-8 and -10 contain Death effector domains (DEDs). Initiator caspases generally act upstream of effector caspases.

Execution caspase family:

Caspases (caspase-3, -6 and -7) in this group have short, non-functional (except for maintaining inactivity) pro-domains. They are responsible for interacting with other caspases and non-caspase molecules to induce apoptosis

Figure 1.3: Caspase activation

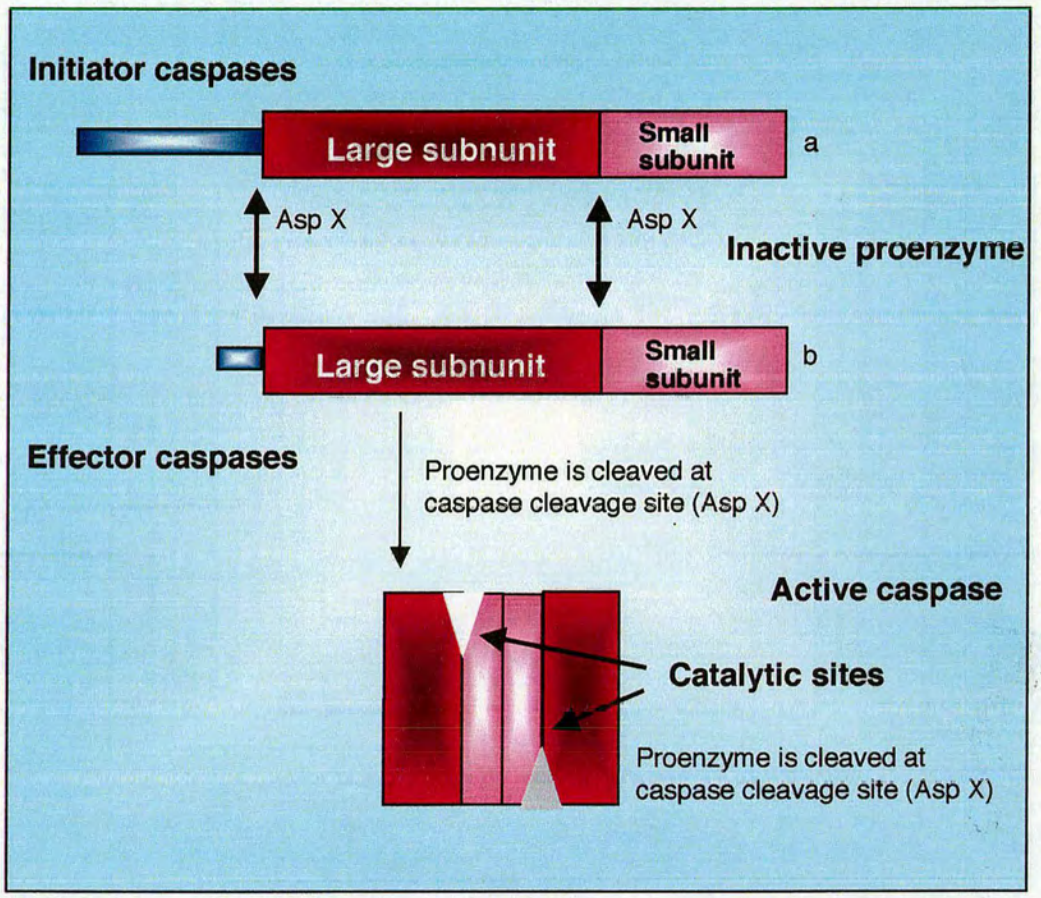


Fig 1.3: Schematic representation of caspase activation. (a) caspases with a substantial prodomain *(caspase-2, 8, 9, 10),* (b) caspases with short prodomain *(caspase-3, -6 and -7).* (see text for detail)

I D3. Caspase localization

Caspases have different subcellular localizations depending on their state of activation as reported by many research groups (Chandler et al., 1998; Kim et al., 2000a; Zhivotovsky et al., 1999; Baliga et al., 2003; Nakagawa et al., 2000; Yoneda et al., 2001) and summarized in Table 1.1.

Table 1.1: *Caspase localization*

Caspase identity	Procaspase localization	Active caspase localization
Caspase-2	Mitochondial and cytosolic	Microsomal and nuclear
Caspase-3	Mitochondial and cytosolic	Mitochondrial, nuclear and cytosolic
Caspase-6	Cytosolic and nuclear	Nuclear
Caspase-7	Cytosolic	Cytosolic and nuclear
Caspase-8	Cytosolic	Cytosolic
Caspase-9	Cytosolic and mitochondial	Cytosolic and mitochondrial
Caspase-12	Microsomal	Microsomal

I E.The role of caspases in apoptosis

I E1. The role of caspases in the execution phase of apoptosis

Inhibition of caspase function by genetic and biochemical means has confirmed that caspases play an important role in apoptosis. In particular, effector caspases are reported to cleave several types of protein during apoptotic execution (for review see Earnshaw et al., 1999; Fisher et al., 2003) which include:

- Abundant cytoplasmic proteins such as adherans junction components, the intermediate filament proteins and RNA-binding and ribosomal proteins
- Abundant nuclear proteins, such as the lamins and the nuclear mitotic apparatus protein. In particular, it is known that caspase-6 cleaves Lamin A, B and C in the mammalian system (Takahashi et al., 1996).
- Proteins involved in DNA metabolism and repair such as PARP-1 (Poly (ADP-ribose) Polymerase-1) and DNA topoisomerase II (Kaufmann, 1989b; Nakajima et al., 1995).
- Protein kinases, for example, PKCs, focal adhesion protein kinase, the MAPK/ERK kinase MEKK1, Raf1 and Akt1 (Wen et al., 1998; Widmann et al., 1998).

- Proteins involved in the regulation of the cell cycle and proliferation such as the retinoblastoma protein (pRB) and p21/Waf1 (Gervais et al., 1998).
- Proteins whose cleavage plays a direct role in apoptosis such as caspases themselves and members of the Bcl2 protein family such as Bcl-2, $BclX_L$, Bid and Bax (Fujita and Tsuruo, 1998).

Without a doubt the most studied effector caspase is caspase-3 and it is clear that caspase-3 plays a substantial role in cleaving numerous substrates in the apoptotic process. Yet, caspase-7 is another caspase with a short prodomain that shares the same substrate specificity as caspase-3. In fact, many of the substrates are cleaved by caspase-3 can also be efficiently cleaved by caspase-7, suggesting at least partial redundancy of the two caspases. In caspase-3-deficient mice caspase-7 activity is reported to be upregulated, suggesting that the absence of caspase-3 can be compensated by caspase-7 (Fischer et al., 2003). Interestingly, caspase-7 was shown to cleave substrates at atypical glutamate motifs. This occurs during cleavage of the cytoplasmic tail of TNF-R1, the cardiac myosin light chain vMLC, connexin 45.6 and the transcription factor Max (Ethell et al., 2001) (Krippner-Heidenreich et al., 2001). In addition, caspase-7 can be autoactivated in an unconventional processing manner where the cleavage specificity at aspartic acid is not strictly required for caspase activation. For example, procaspase-7 can be activated by serine proteases, for example cathepsin G activates caspase-7 by cleaving at a glutamate bond (Zhou et al., 1997).

The first substrates of Caspase-6 to be identified were the nuclear lamins (Orth et al., 1996; Takahashi et al., 1996). Both chemical inhibitor studies and a follow-up study in which caspase-6 activity was selectively inhibited with the serpin SPI-2 suggested that lamin cleavage is required for nuclear disassembly during apoptotic execution (Lazebnik et al., 1995a; Takahashi and Earnshaw, 1996). Other substrates reported to be cleaved by caspase-6 include cytokeratin 18 (Caulin et al., 1997), focal adhesion kinase (Gervais et al., 1998), nuclear mitotic apparatus protein (NuMA) (Hirata et al., 1998), the beta-amyloid precursor protein (Pellegrini et al., 1999), topoisomerase I (Samejima et al., 1999), nuclear matrix protein SATB1 (Galande et al., 2001), transcription factor AP-2alpha (Nyormoi et al., 2001), vimentin (Byun et al., 2001) and huntingtin (Wellington

et al., 1998). However as implied above, these substrates are also cleaved by other caspases, typically caspase-3 and -7, at other cleavage sites. In fact, the only substrate presently thought to be cleaved exclusively by caspase-6 is lamin A/C (Takahashi et al., 1996).

I E2. Specific function of some individual caspases

Several methods were adopted to elucidate caspase function, yet the most commonly used approach to examine the exact physiological function of specific caspases is gene targeting. Several groups have generated knockout mice, Table 1.2 is a summary of some of their findings:

Table 1.2: Caspase mouse-knockout

Caspase	Development	Phenotype	References
Caspase-1	Normal	Immune system resistance to a variety of pro-inflammatory treatments, reduced IL 1α and IL 1β processing, renal and liver failure.	(Kuida et al., 1995; Li et al., 1995; Melnikov et al., 2001; Rowe et al., 2002)
Caspase-2	Normal	Neuronal defects (delay in apoptosis), germ cells	(Bergeron et al., 1998; Troy et al., 2000)
Caspase-3	Postnatal lethal (1-3 weeks)	Impaired brain development, lack of DNA fragmentation and delay in apoptosis, hepatocyte cell impairment and ovarian problems	(Carambula et al., 2002; Kuida et al., 1996; Pompeiano et al., 2000; Woo et al., 1999; Woo et al., 1998)
Caspase-8	Embryonic lethal	Heart development failure, impairment of death receptor pathway (Fas, TNF and DR3)	(Varfolomeev et al., 1998)
Caspase-9	Embryonic lethal	Brain defect (reduced apoptosis, delayed DNA fragmentation), fibroblast resistance to UV and gamma irradiation, reduced cytochrome c-mediated apoptosis[1]	(Hakem et al., 1998; Kuida et al., 1998)
Caspase-11	Normal	resistance to LPS-induced shock, Lymphocytes show a delay in caspase-3/-7 activation, defects in thymocytes apoptosis and caspase-1 activation is impaired	(Kang et al., 2002; Wang et al., 1998c)
Caspase-12	Normal	Resistance to ER mediated apoptosis after treatment with befeldin A, tunicamycin and thapsigargin	(Nakagawa et al., 2000)

I F. Caspase activation

Caspases are enzymes that must be kept under tight control, yet need to be quickly activated. Therefore, caspases are regulated at the post-translational level and require

[1] According to Hakem et al., cytochrome c is translocated to the cytosol of Casp9-/- ES cells upon UV stimulation of fibroblasts but not after UV irradiation of brain cells (Hakem *et al.*, 1998).

processing for their activation. Activation of initiator caspases is mediated by binding and interaction with adapter molecules via their prodomain. These prodomains function by protein-protein interactions mediating the recruitment of initiator caspases into death complexes (Singh et al., 1998). Two types of activation, with independent signaling cascades, have been reported. In both pathways the prodomain binds to an adapter molecule containing a similar domain. The first, 'induced proximity model' includes pro-caspase-8 and -10 activation via the death induced signaling complex in the receptor-mediated pathway (Krueger et al., 2001b; Muzio et al., 1998). The second, 'association with regulatory subunit' involves pro-caspase-9 activation through the association with Apaf-1 in the mitochondrial stress-mediated pathway (Li et al., 1997; Rodriguez and Lazebnik, 1999).

I F1. Major pathways of caspase activation

I F1a. Death receptor mediated apoptosis

Vertebrates have evolved a variety of mechanisms enabling them to direct individual cells to activate their death pathways. Death receptors, embedded in the plasma membrane, can transmit an apoptotic signal that leads to caspase activation within seconds (Thornberry, 1998; Thornberry and Lazebnik, 1998). Death receptors are members of the tumor necrosis factor (TNF) receptor superfamily that are defined by similar, cysteine rich extracellular domains and a cytoplasmic sequence of 80 aa known as the death domain (DD). The best characterized death receptors are CD95/Fas and TNFR1 (Williams and Smith, 1993; Yonehara et al., 1989). Other death receptors have also been identified such as death receptor 3 (DR3), DR4, DR5 and DRb (Chaudhary et al., 1997; Schneider et al., 1997; Wiley et al., 1995; Wu et al., 2003). Ligands that bind to these death receptors belong to the TNF gene superfamily. Consequently, FasL (Fas ligand) binds to Fas and TNF binds to TNFR1 resulting in the initiation of the cell death cascade (Ashkenazi and Dixit, 1998).

Death signaling by Fas

This pathway is typically engaged in the immune system and is activated to delete activated T-cells at the end of an immune response (O'Connell et al., 2002). Fas/CD95 is a 45 kDa protein expressed in activated T cells and NK cells. FasL binding to its receptor stabilizes its trimerization resulting in the clustering of the intracellular DD followed by the recruitment of Fas-associated death domain (FADD). FADD also contains a DD which mediates its association with the oligomerised Fas. This complex is called the DISC (Death Inducing Signaling Complex) (Medema et al., 1997). The DISC recruits procaspase-8. As more procaspase-8 molecules are clustered, they undergo transcatalysis, producing active caspase-8 (Ducoroy et al., 2003). The active caspase-8 then activates downstream caspases such as procaspase-3, -6 or –7 which in turn dismantle the cell (Walczak and Krammer, 2000) (see Fig. 1.4). Recently it was suggested that Fas signaling and the formation of the death inducing signaling complex is instigated by two adjacent trimeric Fas ligands (Holler et al., 2003).

Death signaling by TNFR1

Signaling through TNFR1 is more complex than Fas and involves the activation of multiple factors that lead to apoptosis through branched signaling cascades (see Fig 1.4) (McFarlane et al., 2002). The apoptotic cascade through TNFR Associated Death Domain is very similar to the one through Fas and also involves the formation of a DISC. However, TRADD can also recruit 'Receptor Interacting Protein' (RIP) that leads to the activation of NF-kB which protects the cell from apoptosis (Wang et al., 1998a). TNF Receptor Associated Factor 2 (TRAF2) can also bind to TNFR1 and activate the c-Jun N-terminal kinase (JNK). Active JNK induces apoptosis indirectly by activating downstream caspases leading to apoptotic cell death (Liu et al., 1996b) (see Fig. 1.4).

Death signaling via TRAIL/ Apo-2L

TNF-Related Apoptosis-Inducing Ligand (TRAIL/Apo2L), was identified as the ligand that shares the most similarity to FasL (Wiley et al., 1995). TRAIL, which is constitutively expressed in many human tissues, induces apoptosis in a number of tumor

cell lines and surprisingly is relatively non-toxic to normal cells (Lacour et al., 2003; Uno et al., 2002). The reason behind the selective toxicity may be due to the existence of a family of four membrane-bound TRAIL receptors (TRAIL-R1-R4). All of the receptors can bind TRAIL, yet they differ in their ability to transduce the death signal (Griffith et al., 1999; LeBlanc et al., 2003). Recently, this hypothesis has been challenged based on poor correlations between TRAIL receptor expression and TRAIL sensitivity. This suggested that other factors, such as TRAIL-induced NF-κB activation (Eid et al., 2002; Rivera-Walsh et al., 2001) or death inhibitors including c-FLIP (see section I G1) (Kreuz et al., 2001; Siegmund et al., 2002), are involved in determining differential sensitivity to TRAIL (MacFarlane et al., 2003).

Among the initiator caspases processed at the DISC, caspase-8 activation in particular has been extensively studied (Boatright et al., 2003; Krueger et al., 2001a; Krueger et al., 2001b; Medema et al., 1997; Muzio et al., 1997; Srinivasula et al., 1996). Results showed that, caspase-8 can only be cleaved when bound to the DISC (see above). Binding of caspase-8 to the DISC induces conformational changes in the enzyme (Medema et al., 1997) whereby the intrinsic enzymatic activity of the zymogen is sufficient to allow the reciprocal activation of recruited procaspase molecules (Boatright et al., 2003; Muzio et al., 1997). Most likely caspase-2 and caspase-10 are activated at the DISC by a similar mechanism to the one described above. (Ducoroy et al., 2003; Fernandes-Alnemri et al., 1996; Sprick et al., 2002).

Figure 1.4: Receptor mediated pathway

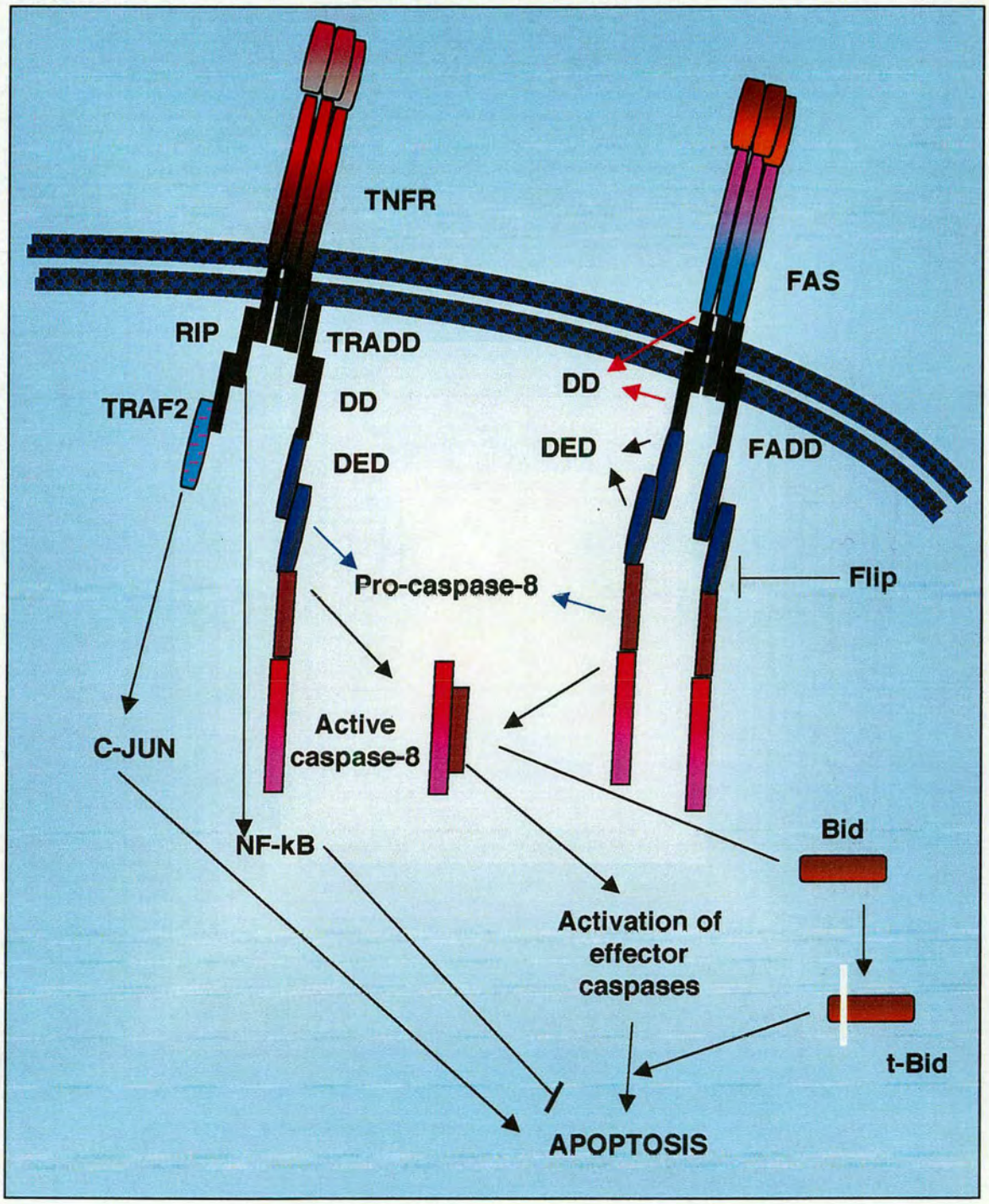


Fig 1.4: Schematic representation of *Receptor mediated pathway*, trimerization of FAS and TNFR receptors initiates recruitment of various adaptor proteins (see text) through homophilic DD interactions leading to the binding of pro-caspase-8 (via its DED domain) and its transcatalytic activation

I F1b. Mitochondrial-stress induced signaling pathway

It is now well established that mitochondria undergo major changes in membrane integrity before manifestation of the classical morphological apoptotic signs. The first report on the involvement of mitochondria in programmed cell death came from a study showing that the induction of apoptosis in cell free extracts requires cytochrome c and dATP (Liu et al., 1996a). During apoptosis the mitochondrial membrane is permeabilised and cytochrome c is released to the cytoplasm. In the cytosol, cytochrome c interacts with Apoptosis activating factor-1 (Apaf-1), dATP and procaspase-9 to form a complex called the apoptosome (Cain et al., 2000; Cecconi et al., 1998). Cytochrome c is required for Apaf-1 activation since cytochrome c null mice fail to activate the stress induced caspase cascade (Li et al., 2000).

Apoptosome formation and Caspase-9 activation

Similar to caspase-8, caspase-9 activation was studied in detail. Caspase-9 exists *in vivo* primarily as an inactive monomer and its activation requires dimer formation (Renatus et al., 2001). Although caspase 9 in solution is predominantly a monomer, the high protein concentrations used for crystal growth compels its dimerization. Hence, the crystal structure of caspase-9 contain four catalytic domains, consisting of the two caspase dimers. What is interesting about caspase-9 is that unlike other caspases, proteolysis is not sufficient or even unnecessary for its activation (Rodriguez and Lazebnik, 1999; Stennicke et al., 1999). As stated above, dimer formation is a crucial step in its activation, yet the dimerization cannot occur with the *in vivo* concentration of procaspase-9 (20 nM) (Stennicke et al., 1999). This obstacle is overcome by the formation of the apoptosome, where procaspase-9 molecules are recruited and stabilized on Apaf-1 oligomers by homophilic interaction between Caspase Activated Recruitment Domains (CARDs) (Cain et al., 2002; Cain et al., 2000).

To clarify, the CARD of caspase-9 can interact with other CARDs found in various apoptotic proteins, most importantly, Apaf-1 (Bratton et al., 2000). The structure of Apaf-1 revealed that it is composed of three domains, an N-terminal caspase recruitment

domain (CARD), a central CED-4 homology domain, and 12–13 repeats of WD-40[2] in the C-terminal half (Qin et al., 1999; Zou et al., 1997; Zou et al., 1999). The WD-40 repeats are thought to interact with cytochrome c whereas the CED-4 homology domain mediates the oligomerization of Apaf-1 in the presence of dATP. Following the release of caspase-9, in the cytoplasm Apaf-1 binds cytochrome c and alters the conformation of the WD-40 repeat region. This, in the presence of dATP, enables Apaf-1 oligomerization via a mutual interaction of the CED-4-like regions. In addition, the association with cytochrome c and dATP induces a conformation change in Apaf-1, enabling it to recruit caspase-9. The oligomerized Apaf-1 recruits procaspase-9 via a homophilic CARD/CARD interaction (Hu et al., 1998; Hu et al., 1999; Lauber et al., 2001; Qin et al., 1999). In the stabilized apoptosome, caspase-9 can form dimer molecules and dimer formation may activate the caspase. Theses dimers are active in an unprocessed form, and each heterotetramer has a single active site. Thus, in a unique manner, the activation of caspase-9 zymogen is reliant on cytosolic factors where the apoptosome is an essential regulatory subunit (Chen et al., 2002).

The final outcome of either 'stress-induced' or 'receptor mediated apoptosis' is ultimately cell death, since both pathways lead to caspase activation bringing the cell to the final execution phase (Green and Amarante-Mendes, 1998). The two pathways are not totally independent and it has been well established that pathway 'cross talk' exists. For example, it is well established that caspase-8 is indirectly involved in cytochrome c release by cleaving the pro-apoptotic Bcl2-family members such as Bid (See section I H) (Gross et al., 1999; Luo et al., 1998; Sarig et al., 2003). Bid, is cleaved by caspase-8 after Fas/TNF-R1 engagement (Moreau et al., 2003; Nechushtan et al., 2001). Specifically, in type II cells[3], cell death triggered by Fas ligation must proceed through a mitochondrial amplification step in order to cause cell death (Scaffidi et al., 1999).

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[2] Loosely conserved set of sequences found in many regulatory proteins, such as beta-subunits of heterotrimeric G proteins.

[3] Flowing the initiation of the apoptotic process via the death receptor pathway, the amount of active caspase-8 generated at the DISC determines whether cells are of type I or type II. In type I cells, induction of apoptosis is accompanied by activation of large amounts of caspase-8 at the DISC, whereas in type II cells DISC formation and caspase-8 activation are strongly reduced. The small amounts of activated caspase-8 is responsible for cleaving a molecule called Bid (see section I H2) which induces cytochrome c release from the mitochondria and generate the amplification loop for the death receptor induced apoptosis via the activation of caspase-9.

Figure 1.5 : Stress-induced pathway

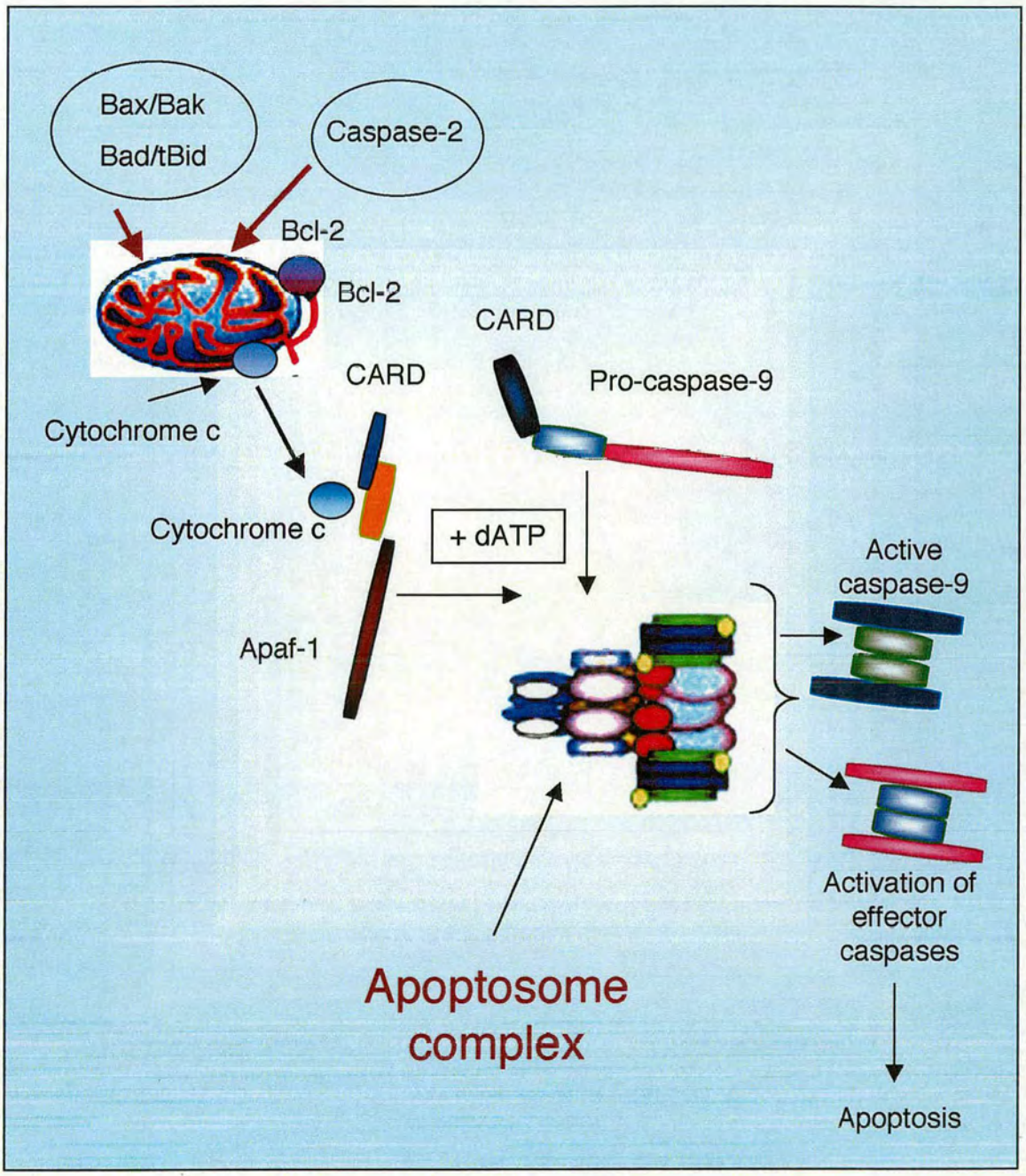

Fig 1.5:: Schematic representation of *Stress induced pathway,* Mitochondrial stress (via caspase-2 and Bcl-2, see txt section I H for more details) induce the release of *cytochrome* c into the cytoplasm. Cytochrome c along with dATP induces conformational changes in Apaf-1 allowing its oligomerization into a complex capable of recruiting and activating caspase-9 and other effector caspases.

Upon activation, initiator caspases proteolytically cleave effector caspases through a regulated sequence of reactions that separate the large and small subunit, and then detach the short prodomain (Earnshaw et al., 1999; Earnshaw et al., 2000). Before proceeding any further it is important to point out that, other than caspases themselves, the only mammalian protease known to cleave and activate caspases is granzyme B which has also been implicated in apoptosis (Galvin et al., 1999; Zapata et al., 1998). (Fig. 1.5).

Apoptosis Initiating Factor (AIF), Smac/Diablo,Omi/HtrA2 and EndonucleaseG (EndoG)

Cytochrome c is not the only apoptotic factor released from the mitochondria during apoptosis. Recently four proteins, normally located in mitochondria, with a function in apoptotic execution were identified. They include, AIF (Daugas et al., 2000; Lorenzo et al., 1999), EndoG (Li et al., 2001; Parrish et al., 2001), Smac/Diablo (Wu et al., 2000) and Omi/HtrA2 (see section I G3 (Hegde et al., 2002; van Loo et al., 2002). Smac/Diablo and Omi/HtrA2 will be described in Section I G3.

AIF was identified as a novel mitochondrial protein restricted to the intermembrane space responsible for chromatin condensation and DNA fragmentation in apoptotic nuclei (Susin et al., 1999). Crystal structure analysis indicated that AIF possesses an oxidoreductase enzyme activity and contains a DNA binding site in a groove on the surface of the molecule (Mate et al., 2002; Ye et al., 2002). Under normal conditions, AIF is a flavoprotein that acts as an electron acceptor/donor with oxidoreductase activity. However, in response to apoptotic stimuli in certain cell types, AIF is released from the mitochondria and translocates to the nucleus, where it binds DNA and triggers caspase-independent cell death (Susin et al., 1999; Ye et al., 2002). AIF apoptotic function is under the regulation of the Bcl-2 family of proteins (see section I H). Bcl-x_L blocks both AIF and cytochrome c release from mitochondria. Analogous to cytochrome c, AIF-mediated cell death is independent of its redox function (Mate et al., 2002; Miramar et al., 2001). The exact mechanism of AIF function is still not of completely understood. Investigation of the pro-apoptotic function of AIF suggests that

it is most likely to be mediated via its DNA binding activity (Wang et al., 2002; Zhang et al., 2002).

EndoG, translated in the cytosol, and subsequently imported into the mitochondria, was identified as a nuclease that can induce DNA degradation in both worms and mammals after apoptosis induction (Li et al., 2001; Parrish et al., 2001). The release of EndoG from apoptotic mitochondria occurs at comparable rate to that of cytochrome c. When released EndoG is able to induce nucleosomal DNA fragmentation. EndoG activity is independent of caspase activation (van Loo et al., 2002). However, EndoG activity is not redundant with CAD in all cell types since in chicken B lymphocytes deficient for CAD, EndoG is unable to cleave the nuclear DNA (Samejima et al., 2001).

I G. Regulation of caspase activation

I G1. Death receptor pathway regulation

Upstream caspase-8 activation is regulated at the receptor level through a specific group of inhibitors collectively named FLIPs (FLICE Inhibitor Protein) (Irmler et al., 1997; Keppler et al., 1999; Thome et al., 1997). FLIPs are Death Effector Domain (DED) containing proteins. The first to be identified were viral FLIPs (v-FLIP) (Hu et al., 1997a; Thome et al., 1997). Characterization of v-FLIPs showed that they were capable of inhibiting the induction of apoptosis by several death receptors (CD95, TNF-R1, DR3, and DR4), suggesting that these receptors use similar signaling pathways (Grundhoff and Ganem, 2001; Meinl et al., 1998; Renne et al., 2001). Soon after, many research groups discovered a human homologue of v-FLIPs, cellular-FLIP (c-FLIP[4]) (Goltsev et al., 1997; Hu et al., 1997b; Inohara et al., 1998; Shu et al., 1997). Two splice variants of c-FLIP were described, c-$FLIP_S$ and c-$FLIP_L$ (Scaffidi et al., 1999). c-$FLIP_S$ is more related to its viral counterparts and is composed of two DEDs and a short C-terminal extension. c-$FLIP_L$ resembles caspase-8 by containing tandem DEDs and a caspase-like domain. However the latter lacks amino acid residues that are critical for

[4] c-FLIP has many names including FLAME-1, I-FLICE, Casper, CASH, MRIT, CLARP, and usurpin.

caspase activity (Irmler et al., 1997; Scaffidi et al., 1999). The exact mechanism of c-FLIP action is still under investigation. Elaborate work from various studies strongly suggests that c-FLIPs are potential competitive inhibitors of caspase-8, preventing its activation at the DISC (Aron et al., 2003; Lens et al., 2002; Micheau et al., 2002; Rasper et al., 1998). However, c-FLIP does not abolish the recruitment of caspase-8. On the contrary, in the presence of both $FLIP_S$ and c-$FLIP_L$ caspase-8 still associates with the DISC. Hence, the mode of action of c-FLIPs is not inhibition of caspase recruitment but rather an inhibition of caspase activation (Krueger et al., 2001b; Xiao et al., 2002). Indeed, $FLIP_S$ and $FLIP_L$ are reported to form a caspase-8/ FLIP heterodimers (Krueger et al., 2001b; Perez and White, 2003). Interestingly, $FLIP_L$ forms a caspase-8/heterodimer in which caspase-8 along with $FLIP_L$ are partially processed in the presence of high concentrations of $FLIP_L$ (Krueger et al., 2001b; Scaffidi et al., 1999). The cleavage of both protein might be the basis of caspase inhibition. It has been suggested that in the caspase-8/ $FLIP_L$ complex, the initial step of caspase-8 cleavage proceeds autocatalytically. However this processed caspase domain formed could be inactive, consequently the second transcatalytic step of caspase-8 activation can not take place (Krueger et al., 2001a; Krueger et al., 2001b).

In addition to inhibiting caspase-8 activation, FLIPs can transmit a signaling cascade that promotes cell survival. Through the Fas signaling pathway, $FLIP_L$ recruits RIP, TRAF1, and TRAF2 to the DISC, consequently activating NF-κB and ERK signaling cascades and regulating not only cell survival but cell proliferation and differentiation (Bortul et al., 2003; Chaudhary et al., 2000; Kataoka et al., 2000; Liu et al., 2002).

I G2. Regulation of caspase activation by Inhibitor of Apoptosis Proteins (IAPs)

IAP genes were first discovered in the genomes of viruses. By expressing their viral IAP (v-IAP), viruses have evolved mechanisms that block the host cells from destroying themselves before viral production has been completed (Deveraux et al., 1999). Recently, endogenous mammalian inhibitors homologous to v-IAP have been identified (Deveraux et al., 1999; Tamm et al., 1998). To date seven human members of this

family have been characterized (XIAP, c-IAP-1, c-IAP-2, NIAP, BRUCE and ML-IAP), three of which are involved in caspase inhibition (Holcik et al., 2000; Holcik et al., 2001; Salvesen et al., 2002). Specifically, human c-IAP-1 and c-IAP-2 have been shown to inactivate effector caspases such as active caspase-3, and –7 and XIAP is shown to inhibit, in addition to caspase-3 and -7, the upstream caspase-9 (Huang et al., 2001). To date, none of the IAPs seem to have any effect on the upstream initiator caspase-8 (Devereaux et al., 1997).

IAPs are a family of proteins are characterized by one or more characteristic BIR Baculovirus IAP Repeat (BIR) domains, Zn^{++} fingers that promote protein-protein interaction. In addition to the BIR domain, some family members contain CARD and/or RING domains (see Table 1.3 and Fig. 1.6). c-IAP1, cIAP2, and XIAP have three BIRs near the N-terminus of the molecule and a RING finger at the C-terminus. Through their BIR domains, IAPs bind and inhibit active caspases. In addition to binding and inhibiting caspases, IAPs have multiple biological activities that include regulating cell cycle progression, and modulating receptor-mediated signal transduction (Chai et al., 2001; Huang et al., 2001; Riedl et al., 2001; Roy et al., 1997; Sun et al., 1999; Sun et al., 2000) (Table 1.3).

Table 1.3: *IAP proteins*

IAP gene	BIR	RING	CARD	inhibition
XIAP	3	1	0	*caspase-3* *caspase-7* *caspase-9*
c-IAP1 and c-IAP2	3	1	1	*caspase-3* *caspase-7*
survivin	1	0	0	*caspase-3* ? *caspase-7*?
Livin/ML-IAP	1	1	0	*caspase-3* *caspase-7* *caspase-9*

Figure 1.6: Inhibitors of apoptosis (IAP)

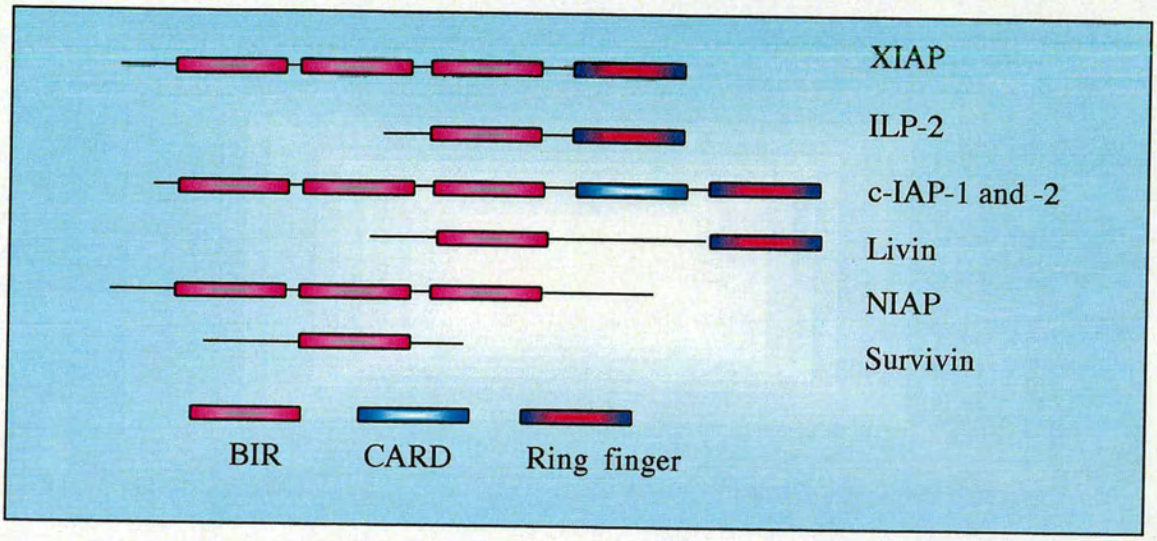

Fig 1.6: Schematic representation of mammalian IAP.

The structural basis of caspase-3 and –7 inactivation has been extensively studied and seems to function through a similar mechanism. The linker region between the BIR1 and BIR2 domains is responsible for caspase binding (in their active sites) and inactivation (Chai et al., 2001; Riedl et al., 2001). The BIR1/BIR2 linker segment is involved in competitive inhibition of both caspase-3 and –7. However, the BIR2 is responsible for non competitive inhibition of caspase-7 (Suzuki et al., 2001). The nature of inhibition by cIAP-1 and 2 is less understood. On the other hand, sequence alignment among IAPs suggests that the mode of binding and inactivation of caspase-3 and –7 is conserved (Chai et al., 2001).

The mechanism of effector caspase-3 and –7 inhibition by XIAP differs from that of initiator caspase-9 (Huang et al., 2001). Caspase-9 can only be effectively inhibited by XIAP (Deveraux et al., 1999; Suzuki et al., 2001). The BIR3-RING fragment, but not the BIR1-BIR2 fragment of XIAP, binds and inhibits caspase-9. It has been recently shown that the surface of caspase-9 that interacts with BIR3 also mediates its homodimerization. As stated previously, dimerization is an essential step in caspase-9 activation and monomeric caspase-9 is catalytically inactive. It was shown that XIAP sequesters caspase-9 in a monomeric state and ensures that it remains in a catalytically inactive state (Shiozaki et al., 2003).

I G3. Indirect caspase activation via Smac/ DIABLO and Omi/HtrA2

Second Mitochondria-derived Activator of Caspases (Smac/DIABLO) and Omi/HtrA2 are two mitochondrial proteins that are proteolytically processed and released into the cytosol during apoptosis (Du et al., 2000; Martins, 2002). In the cytosol, processed Smac/DIABLO and Omi/HtrA2 bind and inhibit a variety of IAPs (Du et al., 2000; Srinivasula et al., 2001). Specifically, Smac/DIABLO and Omi/HtrA2 recognize and bind to the surface groove on BIR3 of XIAP, competing with caspase-9 for the same binding site (Wu et al., 2000). In addition, Smac can also bind to the linker peptide between the BIR1 and BIR2 domains, sterically inhibiting caspase-3 and caspase-7 binding (Huang et al., 2001). Since Smac/DIABLO and Omi/HtrA2 release can trigger

the apoptotic cascade, these proteins are kept under tight control to prevent any unnecessary or unwanted cell death. Smac/DIABLO and Omi/HtrA2 release is regulated by the Bcl-2 family of proteins (see Section I H). In addition, the degradation of Smac/DIABLO is also effected by its binding to IAPs (Springs et al., 2002; Wu et al., 2000). The RING domain of XIAP possesses ubiquitin-protein ligase activity and XIAP binds tightly to mature Smac. Further investigations revealed that XIAP functions as a ubiquitin-protein ligase (E3) in the ubiquitination of Smac (MacFarlane et al., 2002). Whether these two proteins have additional roles in the regulation of apoptosis is still under investigation. Recently, it was suggested that Omi/HtrA2, similar to AIF, can induce apoptosis in human cells in a caspase-independent manner through its protease activity (Hegde et al., 2002).

I H. Control of apoptosis by the Bcl-2 family

The first report on Bcl-2 dates back to 1985 by virtue of its involvement in a chromosome translocation associated with follicular lymphoma (Tsujimoto et al., 1985). In addition to lymphomas, increased expression of Bcl-2 was also observed in several other tumors, including prostate, breast, lung and colorectal cancers (Reed, 1997). Subsequent studies indicated that Bcl-2 (and other related famility members (see below)) not only contribute to tumour progression, but may also confer tumor-resistance to apoptosis induced by conventional anti-cancer treatments (Naik et al., 1996). At least 30 proteins belonging to the Bcl-2 family have been identified to date. They can be divided into two subgroups, antiapoptotic and proapoptotic. Pro and antiapoptotic members can dimerize in order to control each other's function. The characteristic variable numbers of Bcl-2 Homology (BH) regions found in both subgroups (BH1-BH4) determine their capacity to interact with each other or with other unrelated proteins (Oltvai et al., 1993) (Fig 1.7). The BH3 domain is thought to be crucial for pro-apoptotic interactions since proteins where the only Bcl-2 related sequence is the BH3 prodomain are all pro-apoptotic (Bouillet et al., 2002; Chao et al., 1998; Fitch et al., 2000; Kawatani and Imoto, 2003; Zong et al., 2001).

Figure 1.7 Bcl-2 family

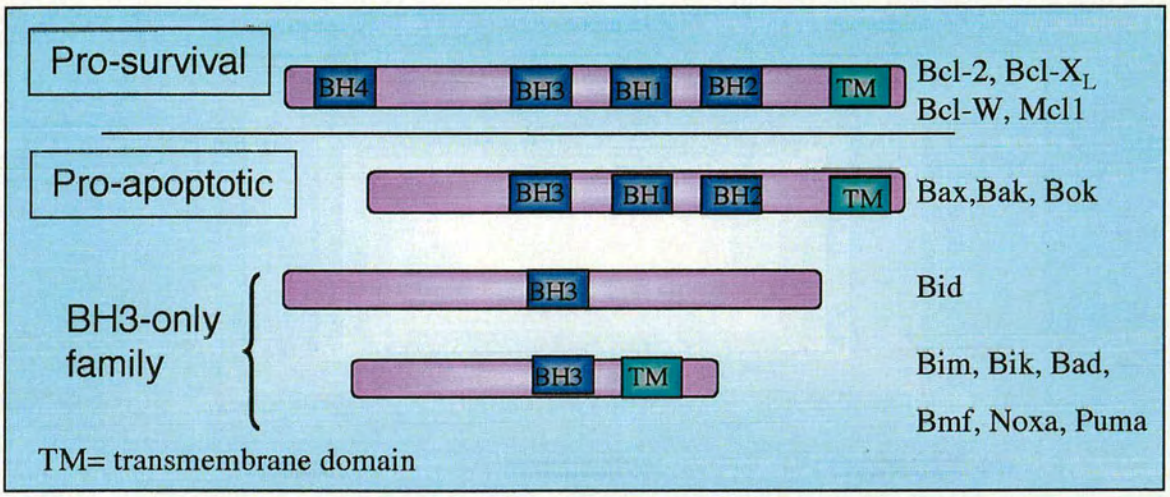

Fig 1.7: Schematic representation of the three Bcl-2-related proteins family.

I H1. Bax and Bak

Structural similarity of some of the Bcl-2 family proteins to diphteria toxins and subsequent studies showing that they can form channels in artificial membranes suggests that they regulate apoptosis by influencing ion or protein transport (Minn et al., 1995; Muchmore et al., 1996; Schendel et al., 1997). Bak resides on the outer mitochondrial membrane oriented toward the cytosol, while Bax is normally located in the cytosol (Adams and Cory, 1998; Hausmann et al., 2000; Jia et al., 1999; Suzuki et al., 2001). When stimulated, Bax translocates to the mitochondria where it undergoes a conformational change, exposing the N-terminus (Desagher and Martinou, 2000). This conformational change is thought to allow oligomerization and insertion into the outer mitochondrial membrane. Once located in the outer membrane, Bax and/or Bak can oligomerize forming channels that are large enough to allow cytochrome c to pass through (Godlewski et al., 2001; Ikemoto et al., 2000; Yi et al., 2003; Zhao et al., 2001). Interestingly, studies showed that individual Bak or Bax loss has no drastic effect on apoptotic execution. On the other hand, inactivation of both proteins has dramatic effects. Cells lacking both Bax and Bak are resistant to mitochondrial and endoplasmic reticulum stress-induced apoptosis and are completely resistant to tBid-induced cytochrome c release and apoptosis (see below) (Cheng et al., 2001; Lindsten et al., 2000; Wei et al., 2001).

I H2. BH3-only proteins

In C. elegans all programmed cell death is orchestrated by one BH3-only protein, EGL-1 (Conradt and Horvitz, 1998; del Peso et al., 1998). Higher organisms possess multiple BH-3 only proteins including Bid, Bim, Bad, Bik, Bmf, Hrk, Bnip3, Nix, Noxa and Puma (Cheng et al., 2001; Marani et al., 2002; Moreau et al., 2003; Zong et al., 2001). The mode of BH3-only protein function is by insertion of their BH-3 domain into a groove of other pro- and anti-apoptotic Bcl-2 members. Activation of BH-3 proteins results in rapid cell death, therefore these proteins are keep under tight restraint. For example, some mammalian BH3-only proteins, such as Noxa and Puma, are regulated at the transcriptional level (Han et al., 2001; Nakano and Vousden, 2001; Yu et al., 2001)

whilst others are regulated post-transcriptionally. For example, Bim and Bid, which are sequestered to microtubules or the actin cytoskeleton (Puthalakath et al., 1999; Puthalakath and Strasser, 2002; Puthalakath et al., 2001).

Among the BH3-only proteins, Bid was extensively studied and found to be exceptionally important in Type II cells (see Section I F1b). Activated caspase-8 cleaves the inactive form of Bid into two parts. The truncated Bid, tBid (15 KDa) translocates to the mitochondria, (Grinberg et al., 2002; Kim et al., 2000b; Li et al., 1998; Lutter et al., 2001; Yi et al., 2003). There, tBid binds Bax, ion channels are formed and cytochrome c is released (Brenner et al., 2000; Madesh and Hajnoczky, 2001; Vieira et al., 2002; Xie et al., 1998).

BH3-only proteins seem to exert their effects in a tissue-specific manner as suggested by mice knockout studies. Bid-deficient mice appear normal with the single observation that their hepatocytes are resistant to Fas-induced cell death (Wang et al., 1998). Bim knockout mice studies showed that it is required for haematopoietic cell homeostasis and prevention of autoimmune diseases as well as neuronal apoptosis (Bouillet et al., 1999; Bouillet et al., 2002; Putcha et al., 2002). Interestingly, it seems that the balance between the pro-survival and pro-apoptotic proteins dictates the fate of the cell. In Bcl-$2^{-/-}$ mice the removal of a single Bim allele can restore almost all the physiological effects associated with Bcl-2 loss (Bouillet et al., 2002).

Without a doubt, the extensively studied pathways of apoptosis are far from being fully mapped. It is one widely accepted view that Bcl-2 family members are the upstream effectors in the mitochondrial induced apoptosis and that mitochondrially released proteins, rather than caspases, are the active players in cell dismantling. In that context, caspases were thought that to be just amplifiers and Bcl-2 members key effectors. However, this belief was challenged recently by the finding that the down-regulation of caspase-2 by siRNA blocks apoptosis induced by DNA damaging agents, yet the loss of caspase-2 function had no effect on receptor-induced apoptosis (Lassus et al., 2002). Ironically, this finding implies that the two modes of cell death might operate through the same mechanism where caspases are the apical players.

I I. Endoplasmic reticulum (ER), golgi and lyzosome specific cell death

Although most intracellular membranes are permeablised during apoptosis, the main focus in organelle-specific apoptosis has always concentrated on the role of mitochondria. However, other organelles such as the ER, Golgi apparatus and lysosomes also play an a role in cell death (Chen et al., 2002; Hacki et al., 2000; O'Reilly et al., 2002; Scorrano et al., 2003; Trapani et al., 1998; Zang et al., 2001).

I I1. The role of the ER in apoptosis

The most established role of the ER is the post-translational processing of newly synthesized peptides from ribosomes, as well as synthesis and metabolism of lipids. High levels of sustained ER stress induced by interference with protein secretion can lead to apoptosis. (Zinszner et al., 1998). Specifically, caspase-12 is an ER-associated cell death effector that is activated by proteolytic processing during ER stress and mobilization of Ca^{++} stores (Mandic et al., 2003; Nakagawa et al., 2000; Yuan and Yankner, 2000). In addition, caspase-12 knockout mouse studies showed that the kidney and neurons of caspase-$12^{-/-}$ mice are resistant to ER-stress, suggesting that caspase-12 may mediate an ER-specific apoptosis pathway (Nakagawa et al., 2000).

I I2. The role of the Golgi apparatus and lysosomes in apoptosis

The Golgi apparatus is the major site for sorting and modifications of proteins and lipids. The main function of lysosomes is degrading various macromolecules. However, both organelles are also associated with apoptotic functions. Several apoptosis-inducing factors have been associated with the Golgi apparatus including. These include *caspase-2*, TNFR-1, TRAIL-R1 and TRAIL-R2 (Bennett et al., 1998; Mancini et al., 1998; Wang et al., 2003; Zhang et al., 2000). The role of lysosomes in apoptosis is linked to the activation and cytosolic translocation of cathepsins (Madge et al., 2003; Zang et al., 2001). Specifically, the release of cathepsin B and D release is associated with

cytochrome c activation (Guicciardi et al., 2000; Katz et al., 2001; Nakanishi et al., 2001; Roberg and Ollinger, 1998) yet Bid cleavage and activation of pro-*caspase-9* following lysosomal photodamage seems to be independent of cathepsins (Reiners et al., 2002). To date, the exact contributions of the Golgi apparatus and lysosomes to apoptosis are still under investigation.

I J. Methods employed to study caspase function

The highly important role of caspases in the apoptotic process was documented by several findings and using different methodologies, including:

- Overexpression of caspases
- Using synthetic or natural inhibitors of caspases
- Using the techniques of protein chemistry, enzymology, crystallography, and recombinant DNA methodologies
- Using knock-out animal models to study the individual and specific function of caspases
- Using RNA interference

Among these, it is important to stress the valuable role played by gene knockouts in the elucidation of specific caspase function. The primary structure of caspases are very similar and conclusions drawn from using specific inhibitors are not completely reliable. Therefore, targeted gene disruption has been widely used to determine the individual functions of caspase genes in mice (Table 1.2). These studies have identified differential involvement for caspases -1, -2, -3, -8, -9, -11 and -12 in development, homeostasis and disease (Nakagawa et al., 2000; Zheng et al., 1999). However, this powerful approach is less useful when the gene of interest is essential for early embryonic development.

I K. The advantages of the DT40 system

Targeted gene disruption is a very powerful approach to study gene function. However, it is limited by low frequency of homologous recombination in most cells. DT40, the avian virus-induced bursal lymphoma cell line provides an alternative to this problem. These cells retain the ability to diversify their rearranged IgL gene during *in vitro* passages, and this apparently leads to a high rate of homologous recombination rate (Kim et al., 1990).

Similar to avian bursal B cells (Reynaud et al., 1987; Thompson et al., 1987), DT40 cells continue to diversify their immunoglobulin (Ig) light chain locus by gene conversion with pseudogenes serving as donors. It has been demonstrated that DT40 cells' light chain diversification occurs by gene conversion in a comparable way to cells derived from the bursal B cells. Transfection of DNA constructs into DT40 chicken cells has proven to be highly successful, with targeting efficiencies reaching up to 90% (Dieken et al., 1996). Moreover, DT40 cells show an increased ratio of targeted to random integration after transfection (Buerstedde and Takeda, 1991). In addition to the gene targeting benefit, DT40 cells possess further advantageous characteristics. Cells have an invariant karyotype and phenotype and a doubling time of 8-10 hours at 39 °C. Due to the very high cloning efficiency, isolation of stably transfected cells and subcloning of DT40 cells is easily done. Furthermore, a new chicken EST database is available at the following web sites: htpp://genetica.hpi.uni.hamburg.de/dt40.html (Buerstedde et al., 2002) http://www.chicken.umist.ac.uk (Sonoda et al., 2001b). Therefore, the DT40 cell line represents a powerful system for generation of knockout cell lines. DT40 cells can be further modified, using tetracycline-responsive promoters allowing the expression of wild type cDNAs. This can be of great importance when the studied gene is essential for cell survival. The cell can be rescued by transfection of the wild type cDNA under the control of the tet-operator system (Gossen and Bujard, 1992). Using this system, the gene can be turned on or off by removal or addition of tetracycline (Lahti, 1999). Given the availability of multiple selectable markers and vectors in which the markers

multiple selectable markers and vectors in which the markers are flanked by loxP sites, DT40 cells offer another great advantage, namely the possibility to perform multiple gene knockouts. Use of multiple knockouts can lead to the exact elucidation of signal pathways resulting from the loss of genes involved in one cellular process, such as apoptosis. More than 60 genes have been targeted to date in DT40 cells. These include, the cell cycle regulator cyclin D1 (Lahti et al., 1997), the DNA repair proteins including RAD51 (Sonoda et al., 1998) and RAD52 (Yamaguchi-Iwai et al., 1998), centromere component CENP-C (Fukagawa and Brown, 1997), Scc1 (Sonoda et al., 2001a), the cohesin subunit SCII (Hudson et al., 2003, University of Edinburgh, personal communication), the chromosomal passenger protein survivin (Carvalho, University of Edinburgh, personal communication) as well as proteins involved in the apoptotic process such as CAD (Samejima et al., 2001) and MEKK1 (Kwan et al., 2001). In each case, stable deficient cell lines were used to characterize the phenotype and elucidate the role of the gene in question.

I L. Purpose of this study

Many previous elaborate studies have been conducted to unravel the mechanisms and cell signaling pathways that govern caspase activation as well as to understand the specific enzyme function and mode of activation in apoptotic execution. Yet, the activation and individual roles of particular caspases are not completely understood. Three caspases are classified as effector enzymes, namely, caspase-3, -6 and –7. Caspase-3 has long been the focus of many investigators, whereas the specific roles of caspase-6 and caspase-7 remain to be determined. In this study, a gene knockout approach in the DT40 cell line was used to elucidate caspase-6 and caspase-7 function in apoptosis. The aim of this thesis is to seek a deeper understanding for specific caspase-6 and caspase-7 functions.

Chapter II: Material and Methods

II A. Materials

Analytical and reagent grade chemicals were purchased from Amersham-Pharmacia, BDH, Bio-Rad, Boehringer Manheim and Promega. Hybond, Hybond N+ and Hybond C membranes were obtained from Amersham-Pharmacia. Radioactive isotopes were supplied by Amersham and oligodeoxynucleotides primers were supplied by Oswell and Sigma-Genosys.

Restriction enzymes were purchased from New England Biolabs (NEB) and Roche. The Klenow Fragment was obtained from Amersham-Pharmacia. T4 DNA ligase and T4 DNA polymerase were obtained from NEB. Taq polymerase was obtained from Roche and Takara. *Pfu* polymerase was purchased from Stratagene. Purchased kits used were Qiagen Mini, Midi and Maxi Kit for plasmid DNA preparation.

II B. Tissue culture techniques

II B1. Cell culture

II B1a. DT40 cells

DT40 cells (Avian Leukosis Viral- induced bursal lymphoma cell line) were grown in suspension in RPMI medium supplemented with 10% foetal bovine serum, 1% chicken serum and Penicillin (100 U/ml)/ Streptomycin (100 U/ml) (GibcoBRL) at 37°C or 39°C.

II B1b. Hela cells

HeLa cells (Henrietta Lacks cervical carcinoma cell line) were grown attached in RPMI medium supplemented with 10% foetal bovine serum and Penicillin (100 U/ml)/

Streptomycin (100 U/ml) (GibcoBRL) at 37°C. The cells were harvested by addition of 1% trypsin and incubation at 37°C for the desired time.

II B1c. Jurkat cells

Jurkat cells (human leukemia T cell line) were grown in suspension in RPMI medium supplemented with 5% foetal bovine serum and Penicillin (100 U/ml)/Streptomycin (100 U/ml) (GibcoBRL) at 37°C.

II B2. Stable transfection of DT40 cells

$1x10^7$ cells were centrifuged (3000 x g for 5 min at room temperature) and washed once with 25 ml ice-cold Optimem (Gibco-BRL). The cells were centrifuged again, resuspended in 0.5 ml ice-cold Optimem and transferred to Gene Pulser cuvettes (Bio-Rad 0.4 cm electrode gap). 20-25 µg of linearized vector was added to the cells and mixed by pipetting, then samples were left on ice for 10 minutes. Transfection was done by electroporation (300 V 950 µF) with the Gene Pulser apparatus (Bio-Rad). After electroporation, the cells were incubated on ice for 10 minutes, then transferred to 10 ml of pre-warmed (37°C) RPMI supplemented with 10% foetal bovine serum, 1% chicken serum and Penicillin (100 U/ml)/ Streptomycin (100 U/ml) (GibcoBRL) and incubated at 39°C for at least 24 hours. Cells were expanded to 40 ml of appropriate selective media in RPMI (supplemented with 10% foetal bovine serum, 1% chicken serum and Penicillin (100 U/ml)/ Streptomycin (100 U/ml) (GibcoBRL) and seeded into 96 well plates (100 µl per well) and grown at 39°C.

II B3. DT40 drug selection

Transfected cells seeded into 96 well plates were incubated in appropriate drug selective medium from 6-10 days depending on the type of drug used. The selection drugs were used at the following concentration:

- Blasticidin : 25 µg/ml
- Histidonol: 1 mg/ml
- Hygromycin B: 2.5 mg/ml
- Neomycin: 2 mg/ml

- Hygromycin B: 2.5 mg/ml
- Neomycin: 2 mg/ml
- Puromycin: 0.5 μg/ml

Only single colonies were picked into non-selective RPMI media (supplemented with 10% foetal bovine serum, 1% chicken serum and Penicillin (100U/ml)/ Streptomycin (100U/ml) (GibcoBRL) and placed at 39°C.

II B4. Tetracycline regulated system for gene expression (tTA)

The tTA (Tetracycline regulated system for gene expression) was developed by Gossen and Bujard in 1992 (Gossen and Bujard, 1992). In this system, the tetR (tetracycline repressor) system described in *E.coli* was modified to create a chimeric transcriptional activator domain that allows its own expression along with the target gene in cultured cells.

The tTa system was developed by fusing the full-length tetR to the transcriptional activation domain of the herpes simplex virus VP-16. In the absence tetracycline the tTa is associated with the tetO DNA sequence causing the recruitment of RNA polymerase, hence allowing transcription. However, when tetracycline is added tTA dissociates from the tetO. As a consequence, the transcpitional complex is released from the DNA and transcription is abrogated. The advantage in using tetracycline as a repressor is its low toxicity to mammalian cells and ready absorption across the plasma membrane. The tTa 2/3/4 plasmids express tetracycline-controlled transactivators containing minimal VP16 activation domains. These mutated VP16 domains do not bind as efficiently to the target thus reducing the relative transient activation to 39% and 14% for tTA 3 and 4 respectively (Baron et al., 1997).

Vector construction: The full length gene of interest, caspase-6 in this case, was cloned into the PUHD10-3 vector (Manfred Gossen-unpublished, ZMBH, Heidelberg), downstream the *EcoRI/XbaI* multiple cloning site. The resulting plasmid was digested to completion with *XbaI* and used along with tTA3 and tTA4 (caspase-6) and contransfected into DT40 cells by electroporation at a molar ratio of 1/10 respectively.

II C. Common molecular biology methods

II C1. Restriction enzyme digestion

II C1a. Complete digestion

Restriction enzyme digestion was carried out in recommended buffers, at temperatures and conditions according to the manufacturer's intructions. On average, a 5-fold excess of enzyme was used. For plasmid DNA, digestion was carried out for 2 hours. For phage DNA, the incubation was carried out for at least 3 hours. For genomic DNA incubations were performed overnight, for 14-16 hours.

II C1b. Partial digestion

Partial digestion was performed by serial dilution of the enzyme. The DNA was quantified by measuring the OD (optical density) at 260 nm and 280 nm. 10 µg of DNA were completely digested by *NotI* in a final concentration as follows: 1x of *NotI* buffer and 15 U of the assayed enzyme in 30 µl total volume. Afterward, the volume was increased to 160 µl containing 1x of the restriction-enzyme buffer to be tested and divided into 5 samples, on ice, as follows: 30 µl, 20 µl, 20 µl, 20 µl, and 10 µl in the first, second, third, fourth and the fifth tube, respectively. 15 U of the enzyme was added to the first tube, and mixed by pipeting. Working on ice, 10 µl of the first tube mixture was transferred to the second performing serial dilutions. The final amounts of the enzyme per reaction tube were 10 u, 3.3 u, 1.1 u, 0.3 u and 0.1 u, against 2 µg of DNA in each of the five tubes. The reaction was further incubated for 15 minutes at 37°C and immediately transferred to 70°C for 10 minutes or frozen on dry ice in cases where the enzyme can readily not be heat inactivated. The reactions were loaded on a 0.6% agarose gel containing ethidium bromide (1 µg/ml) to visualise the DNA, and electrophoresed for 3 hours at 100 V. Then, the picture was taken under UV against a marker ruler.

II C2. Agarose gel electrophoresis

Agarose gels were run in 1x TAE buffer (0.04 M Tris-acetate, pH 7.5, 0.001 M EDTA). Gel concentrations ranged from 0.6% to 3% depending on the size of band to be resolved. Gels were supplemented with ethidium bromide to a final concentration of 1 µg/ml. DNA bands were visualised under UV light and photographed with a digital camera.

II C3. Agarose gel blotting on nylon membrane

The gel was depurinated by soaking it in 0.25 N HCl for 30 min and rinsed with dH_2O. Then the gel was denatured in 1.5 M NaCl, 0.5 N NaOH for 2 x 15 minutes, rinsed with dH_2O again, and neutralised for 20 min with 0.5 M Tris-Cl pH 7.5, 1.5 M NaCl, 0.01 M EDTA). The gel was transferred to a pre-wet nylon membrane (*HybondN, Amersham*) by upward capillary transfer (Sambrook J. and Russel D.W, 2001). The transfer was performed with 10 x SSC (10 x stock: 1.5 M NaCl, 0.15 M sodium citrate, pH adjusted to 7.0 with citric acid) overnight; alternatively the gel was transferred using the VacuGene™ Vacuum blotting system (*Pharmacia Biotech*) for 2 hours. The membrane was dried at room temperature and the DNA was UV crosslinked at 1200 J.

II C4. Purification of DNA from agarose gel

DNA fragments from digested plasmids or PCR products were separated on agarose gels. The fragments were visualised under UV and the band of interest cut out with a thin scalpel blade. DNA fragments were purified from the agarose gel using the Qiagen gel extraction kit according to the manufacturer's recommendations.

II C5. DNA sequencing

Sequencing was performed at the ICMB (Institute of Cell and Molecular Biology, University of Edinburgh) sequencing facility using on ABI 3100 sequencing machine. 200-500 ng of DNA, 3.2 pmole of primers and 4 µl of ABI big dye mix (Applied Biosystems) were mixed with water in a final volume of 10 µl. 25 Cycle sequencing

Biosystems) were mixed with water in a final volume of 10 µl. 25 Cycle sequencing reactions (95°C 1, min 50°C 15 sec, 60°C 4 min) were amplified following manufacturer's recommendations. The DNA pellet was then precipitated at room temperature for 15 minutes by adding 1 µl of 3 M of sodium acetate and 25 µl of 100% ethanol per reaction. The DNA was then centrifuged at 20,000 x g for 15 minutes, washed with 70% ethanol and then re-centrifuged and air dried. Sequences were analysed using computer programs, including Editview (ABI Prism, Perkin Elmer), Gene Jockey (BioSoft) and Sequencher (Gene Codes Corporation). Plasmid maps were constructed using DNA Strider.

II C6. DNA sequence labeling and membrane hybridization

II C6a. Oligo nucleotide probe preparation

20 pmol of DNA oligonucleotide was added to a 20 µl total reaction mixture containing 1 x kinase buffer, 5 µl of γ-^{32}P dATP (5000 Ci/mmol) and 10 U of T4 polynucleotide kinase. The reaction mixture was incubated at 37°C for 45 minutes. After inhibiting the reaction at 65°C for 10 minutes, 80 µl of TE buffer plus 1 µl of 1 M EDTA (Na^+) were added to the mixture, which was then passed through a 10 cm G-25 Sephadex column by centrifugation at 1800 x g, for 4 minutes. The purified probe was added to the hybridisation buffer (6x SSC, 5x Denhardt's, 0.2% SDS) and incubated overnight at 30°C. The membrane was washed at 30°C in 3x SSC, 0.2% SDS until the desired signal strength remained.

II C6b. DNA probe preparation

DNA sequences to be used as probes were labelled using the Megaprime DNA labelling system (Amersham), following the manufacturer's protocol using 1 µg of DNA. After labelling, the reaction mixture was passed through an 8 cm G-50 Sephadex column or ProbeQuant™ G-50 Micro columns (Amersham Pharmacia), which were centrifuged at 1800 x g, for 2 minutes. The purified probe was added to the hybridisation buffer,

Church buffer (0.5 M NaPi (Disodium hydrogen orthophosphate dehydrate pH 7.5), 7% SDS, 1 mM EDTA) and incubated overnight at 65°C. The membrane was washed at 65°C in 40 mM NaPi, 2% SDS.

The radioactive signal on the membrane was revealed using Kodak MS-1 film and intensifying screens at –80°C.

II D. DNA preparation

II D1. DNA amplification

II D1a. PCR

PCR primers were commercially prepared either at Oswel or Sigma Genosys. The following Table 2.1 shows the specific primers sequences designed for particular DNA amplifications.

The PCR reaction was performed under standard conditions as recommended by the manufacturers. The conditions used for each enzyme are summarised in Table 2.2.

Table 2.1: *Primers sequence and usage*

Primer name	Sequence 5′ - 3′	Use
KO Eco3F	GGGGAATTCCAACCAGTAATGTGAGATCG	3' arm of KO vector
KO Hind3R	GGGAAGCTTGCACAAGAGGAAGTCTGCTC	
KO Bam5R	GAAGGATCCCTGCAGTCAACTGCGATATC	5' arm of KO vector
KO SacII5F	GTTCCGCGGCACTGGGTATCAGAGAAGCA	
NP3	TCGAATTCCTCACTGAAGCGTGGATC	C-terminal 73 amino acids of *caspase-7* used for RT-PCR
NP5	AGGGGATCCCTGTTTGATATTCCACA	
NKC73	TCGGATCCCAGAAGTAAAGTTCCTTA	Cloning *caspase-7* ORF in pEGFPN-1
NKC75	CCGAATTCATGTCAGGAGATCAGCAT	
Probe 3F	GAAAGATAACTTTGAGAG	3' external probe
Probe 3R	CATATCCACTCGTCATTG	
Probe 5F	GTCATGCTGCTCCTGTTC	5' external probe
Probe 5R	AATGTGAGTGTCTCTGCC	

***Table 2.2:** PCR conditions*

Enzyme used	Denaturation	Cycle	Amplification	Final concentration	
				Primer	dNTP
Taq polymerase (Roche)	95°C, 2 min	30	95°C, 45 sec 55 °C-65 °C, 45 sec 72 °C ,30 sec/kb	500 nM	200 nM
TaKaRa LA Taq) (TaKaRa Shuzoco, Ltd)	94°C, 5 min	30	94°C, 1 min 55°C-65°C, 72 °C 30 sec/ kb	200 nM	200nM
pfuTurbo (Stratagene)	95°C, 5 min	35	95°C, 1 min 55 °C-65 °C, 1min 76 °C , 2 min/kb	250 ng	250 nM

PCR fragments were analysed by agarose gel electrophoresis. Desired DNA fragments were gel purified using a Qiagen gel extraction kit or cleaned up using an UltraClean PCR cleanup kit (MoBio). Taq (Roche or Takara) amplified DNA fragments were cloned into PEGM-T vector (Promega), TopoII or Topo2.1 vectors (Invitrogen). For *pfuTurbo* amplified DNA fragments to be used in cloning experiments the primers were designed to contain restriction sites specific for cloning in the desired vectors. Except when PCR was used as a screening method, amplified PCR fragments were cloned and sequenced fully on both the 5' and 3' strands. If the DNA fragment accuracy was not crucial or critical for its subsequent experimental use, only one step of sequencing for each strand was performed for each strand using the vector specific primers.

II D2. RT-PCR

cDNA was synthesised using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen) following the manufacturer's recommendations. The total or polyA mRNA was prepared (as described in section II H2) and the first strand cDNA reaction was primed using oligo (dT) primers to hybridise to the 3' polyA tail. 1 µg of total RNA or 100 ng of polyA(+) RNA were used per reaction. The cDNA obtained was amplified directly by PCR using 2 µl per reaction (which would correspond to 10% of the first

strand cDNA obtained). The PCR reaction was performed as described in the previous section.

II D3. Mini scale DNA preparation

A single colony was picked and placed in 2 ml LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH adjusted to 7.0 with 5 N NaOH) containing the appropriate antibiotics and left shaking at 37°C for a minimum of 8 hours. Plasmid DNA was then isolated and purified using the UltraClean mini plasmid kit (MoBio) according to the manufacturer's recommendations.

II D4. Large scale DNA preparation

A single colony or 100 µl of previously grown bacterial culture were placed in buffle flasks containing 50 ml (medium scale) or 100 ml (large-scale) of LB medium plus the appropriate antibiotics and left shaking for 14-16 hours at 37°C. Plasmid DNA was isolated and purified using a QIAfilter plasmid midi or maxi kit (Qiagen) according to the manufacturer's recommendations. The plasmid DNA thus obtained was quantified by reading the absorption at 260 and 280 nm and subsequently analysed by gel electrophoresis.

II E. Protein analysis

II E1. Whole cell extract preparation (cultured cells)

Cell number was determined using a haemocytometer. The desired number of cells was taken, centrifuged at 800 x g for 5 min and washed once with 1x PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4). Cells were lysed in sample buffer (150 mM Tris:HCl pH 6.8, 45% sucrose, 6 mM K-EDTA pH 7.4, 9% SDS, 0.03% bromophenol blue) and boiled for 5 minutes at 100°C. Extracts were either used directly or stored at -20°C.

II E2. DT40 cytosolic extract preparation

Cytosolic extracts were mainly used in *in vitro* experiments for the induction of apoptotic execution. Hence, cytosolic extracts were prepared from untreated and apoptotic DT40 cells (Lazebnik et al., 1993). Cells were induced to undergo apoptosis either by 10 μM etoposide treatment for 5-6 h or 1 μM staurosporine treatment for 7-8 h. Cells were collected, centrifuged at 800 g and washed once in 25 ml PBS and then once in 10 ml KPM buffer (50 mM Pipes, pH 7.0, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT, 50 μM cytochalasin B, PMSF, chymopain, leupeptin, antipain and pepstatin). Cells were finally resuspended in 1 ml of KPM buffer transferred to a 1.5 ml eppendorf tube and centrifuged at 250 x g. The supernatant was aspirated and the cell pellet was frozen in a mixture of dry ice/isopropanol. Cells were lysed by three cycles of freeze/thaw, then sonicated and centrifuged at 190,000 x g for 2 h at 4°C. Keeping the samples on ice, the clear supernatant was collected, aliquoted and frozen at -80°C. The amount of protein was quantified using the Bradford assay (Bio-Rad).

II E3. SDS –Polyacrylamide gel electrophoresis of protein samples

Proteins were separated on 1-D SDS-polyacrylamide gels. The composition of resolving gels and the duration of electrophoresis were dependent on the protein size. Table 2.3 shows the correlation between the concentration of acrylamide and the molecular weigh of protein to be separated. Table 2.4 and 2.5 shows the composition of resolving gels and stacking gels when preparing 20 ml and 5 ml respectively.

Proteins were detected either with Coomassie blue or silver staining or transferred to a nitrocellulose membrane for immunoblotting experiments.

Table 2.3

Acrylamide gel concentration in correlation to the size of protein to be resolved

Acrylamide concentration (%)	Linear range of separation (kDa)
16	< 10
15	10-45
12	15-60
10	20-80
7.5	35-95
5	60-200

Table 2.4: *Resolving gel composition*

	7.5%	10%	12.5%	13%	13.5%	15%	16%
1.5 M Tris pH 8.8	5 ml	5	5	5	5	5	5
30% acrylamide/bis stock (BioRad)	5.4	6.7	8.3	8.65	9	10	10.3
20% SDS	0.1	0.1	0.1	0.1	0.1	0.1	0.1
H_2O	9.4	8.1	6.5	6.15	5.8	4.8	4.5
10% APS	0.2	0.2	0.2	0.2	0.2	0.2	0.2
TEMED	0.02	0.02	0.02	0.02	0.02	0.02	0.02

Table 2.5: *Stacking gel composition*

Staking gel composition	Amount used
1.5 M Tris pH 6.8	0.4ml
30% acrylamide/bis stock (BioRad)	0.8
20% SDS	0.05
H_2O	3.75
TEMED	0.005
10 % APS	0.05

Gels were run in tank buffer (25 mM Tris, 250 mM glycine, 0.1% SDS). The duration of the runs depended on the percentage of the gel and the size of the protein to be analyzed. In general, Mini gels (8 cm x 5cm) were run at 200 V for 45 min to 1 hour and large gels were run for a total of 80 mA to 120 mA for variable lengths of time.

II E4. Immunoblotting

Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose membranes was carried out using a Bio-Rad transfer apparatus according to the manufacturer's instruction in transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol and 0.0375% SDS) at 4°C for 1.5 hours at 250 mA for mini gels and 3 hours at 400 mA for large gels. After transfer, membranes were washed with PBS, stained with PonceauS (Sigma) to visualize the proteins and blocked either with 5% low fat dry milk or 3% BSA in PBS. The primary antibody was added in blocking solution and incubated for 1-3 hours at room temperature or 16 hours at 4°C depending on the antibody used (Table 2.6). Membranes were washed three times for 10 min with blocking solution, then the appropriate, horseradish peroxidase-conjugated secondary antibody was applied at a dilution according to the manufacturer's recommendations. The signal was revealed using ECL solutions (Amersham-Pharmacia).

Table 2.6: Working dilutions of antibodies used for immunoblotting

Antibody	Working dilution and incubation time	Reference
Caspase-6	1:500/ 3 hours RT	Ruchaud et al., 2002
Lamin B1 (E-3)	1:500 / 3 hours RT	Zymed laboratories
Lamin B2 (L-5)	1:500 / 3 hours RT	Zymed laboratories
Lamin A (Clone 4B4-11)	1:1000 / 3 hours RT	Provided by Dr. Reimer Stick
Monoclonal anti-tubulin (B512)	1:10000/ 1 hour RT	Sigma
PARP (9542)	1:250 / 4 hours RT	Cell Signaling (NewEngland biolabs)

II E5. Immunofluorescence analysis

Cultured cells were adhered to slides either by incubating on polylysine coated slides (BDH) for 30 min at 37 or 39°C or by cytospinning 500 µl at 500 x g for 5 min using the

Cytospin3 apparatus (Shandon). Slides were washed twice with PBS, fixed in 4% formaldehyde in PBS solution for 5 min at room temperature, stained with DAPI (5 μg/ml) and mounted with Vectashield (Vector). Images were captured on a Zeiss Axioplan II epifluorescence microscope using a Princeton Instruments Micromax cooled CCD camera and processed using IP lab Spectrum software.

II F. Cloning experiments

II F1. Bacterial host strains

DH5α (Gibco-BRL) and Top 10 (Invitrogen) cells were used for all standard DNA cloning.

II F2. Plasmid vectors

- pBluescript (KS or SK) (Stratagene) for standard cloning
- pGEM-T (Promega), Topo II and Topo 2.1 (Invitrogen) for Taq amplified PCR fragments
- pUHD10-3 (Manfred Gossen-unpublished, ZMBH, Heidelberg) for tetO-dependent expression
- pCDNA3 (Invitrogen) for mammalian expression

The DNA used in cloning was purified plasmid or phage DNA or a PCR product.

II F3. Dephosphorylation of the end of a DNA sequence

Dephosphorylation was performed using calf intestinal alkaline phosphatase (CIP) (NEB) in the appropriate restriction buffer. 0.5 U CIP/μg of digested DNA were mixed and incubated for 1 hour at 37°C. The enzyme was then heat inactivated at 70°C for 15 min. The DNA was purified using the UltraClean PCR cleanup kit (MoBio) according to the manufacturer's recommendations.

II F4. Blunting the end of a digest DNA fragment

Overhangs were blunted using T4 DNA polymerase (NEB). 2 U of the enzyme were added to the reaction mixture in the presence of 1x T4 polymerase buffer and 250 µM dNTP. The reaction was incubated for 5 min at 37°C. The enzyme was heat inactivated at 70°C for 20 min. The DNA fragment was then purified using the UltraClean PCR cleanup kit (MoBio) according to the manufacturer's recommendations.

II F5. Ligation

For both 'sticky' and blunt end ligation the reaction was performed in a total volume of 20 µl at a 3/1 ratio of insert/vector DNA amount in 1x NEB ligation buffer, 250 U of concentrated T4 DNA ligase (NEB). The tubes were placed into the PCR machine for 6-12 hours and exposed to a temperature gradient cycling between 16°C and 22°C with a rate of 1 min/°C.

II F6. Transformation of Competent bacteria

II F6a. Preparation and transformation of chemical competent E. coli

Preparation: E. coli were grown in 100 ml of LB at 37°C to an OD_{600} of 0.5, then transferred to ice for 5 minutes. Cells were pelleted by centrifugation at 3300 x g for 15 minutes and then re-suspended in 40 ml TfbI (30 mM potassium acetate, 100 mM $RbCl_2$, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% glycerol. pH to 5.8 with dilute acetic acid, filter sterilised and stored at 4°C). Thereafter, cells were centrifuged and re-suspended in 4 ml TfbII (10 mM MOPS, 75 mM $CaCl_2$, 10 mM $RbCl_2$, 15% glycerol. pH to 6.5 with KOH, filter sterilised and stored at 4°C) and incubated on ice for 15 minutes. Cells distributed into 100 µl aliquots were snap frozen in liquid nitrogen and stored at -80°C.

Transformation: Competent bacteria were thawed on ice. Plasmid DNA was added then the mixture was left on ice for 15-45 min. The cells were heat shocked at 42°C for 90 sec then returned on ice for 1-2 min. Four volumes Luria-Bertani (LB) medium were

added and the samples incubated with gentle shaking at 37°C for 45-60 min. The cells were plated on appropriate selective media and incubated overnight at 37°C.

II F6b. Preparation and Transformation of electrocompetent bacteria

Preparation: *E. coli* were grown overnight in 50 ml of LB at 37°C. The next day 5 ml of the overnight culture were added to 2 L of LB and left to grow until the cell density reached A_{550}, then the culture was transferred to ice for 5 min. Cells were centrifuged at 4000 x g at 4°C. Cells were washed twice in 100 ml of ice cold 1 mM Hepes pH 7.0 plus ice-cold 10% (v/v) glycerol. Cells were collected by centrifugation at 4000 g for 15 min at 4°C, resuspended in 2-3 ml in ice-cold 10% (v/v) glycerol, distributed into 100 µl aliquots and stored at -80°C.

Transformation: 1-5 µl of DNA were placed in a sterile microcentrifuge tube and chilled on ice. Bacterial cells were thawed gently and placed on ice. 50 µl of bacterial cells were transferred to the pre-chilled the microcentrifuge tube containing DNA. The mixture was left on ice for 1 min and then transferred to a chilled cuvette (Bio-Rad), gently shaken, and placed in into the sliding cuvette holder (Bio-Rad Gene Pulser™). A pulse of 25 µF, 200 Ω and 2.5 kV was applied and immediately 450 µl of LB were added, mixed with the cell suspension, transferred to a 1.5 ml eppendorf tube and placed at 37°C for 30 min. . The cells were plated on appropriate selective media and incubated overnight at 37°C.

II G. Genomic phage library screening and phage DNA preparation

II G1. Lambda FixII library screening

The Lambda FixII vector is a replacement vector used for cloning large fragments of genomic DNA (up to 28 kb in length). This vector, with appropriate inserts, is able to grow on strains lysogenic for P2 such as XL1-blue MRA-P2 (the host cell used in our

laboratory), a P2 lysogen of XL1-blue MRA strain. The *red* and *gam* genes in the Lambda FixII DNA are located on the stuffer fragment; therefore the wild type phage can not grow on P2 cells. When the stuffer fragment is replaced by an insert, the recombinant Lambda FixII phage becomes Red-/Gam-, and the phage is able to grow on the P2 lysogenic strain. Therefore, by plating the library on P2 lysogenic cells, only the recombinant Lambda FixII phages will grow.

For plating, MRA-P2 Cells are grown in LB supplemented with $MgSO_4$ (10 mM) and maltose (0.2%) to an OD_{600} of 0.5 then centrifuged down and re-suspended in 10 mM $MgSO_4$ to an OD_{600} of 0.5. Plating cells (200 µl) are mixed with the phage and incubated at 37°C for 15 minutes. To this Top agarose (previously melted and kept at 50°C until required) is added and the mixture is poured quickly onto an LB agar plate, which had been previously dried and warmed at 37°C.

II G2. Performing plaque lifts

1st round screening: 0.8 µl of phage stock (3.5 x 10^8 µl plaque forming units/plate) were adsorbed to MRA-P2 cells, then plated on four large 150 mm-agar plates and incubated at 37°C for 10 hours. The plates were then chilled and phages transferred onto nylon membranes. Duplicate plaque lifts were made. A needle and ink were used to prick and mark through the agar for orientation. The phage DNA was denatured by soaking the membrane for 2 min with 1.5 M NaCl, 0.5 M NaOH, neutralized for 3 min in 1.5 M NaCl, 0.5 M Tris-Cl/pH 7.5, and washed with 0.2 M Tris/pH 7.5, 2xSSC. The DNA was UV crosslinked to the membrane and hybridised with the desired probe prepared as discussed in section II C6. Double positive phages (signals present on both plaque lifts) were picked. The phages were stored at 4°C in 500 µl of SM buffer plus 30 µl of chloroform.

Further screening: Four sets of dilutions (10^{-1}-10^{-4}) of the phage stocks were mixed with freshly prepared MRA-P2 cells and plated on 9 cm LB agar plates. Plaque lifts were performed following the same protocol used for the 1st round of screening. Pure plaques were obtained after 4 rounds of hybridisation screening. A high titer stock of

pure plaques was prepared according to the manufacturer's recommendation (Stratagene) and kept at 4°C. Two aliquots were placed in 7% DMSO (Sigma) and stored at -80°C.

II G3. Phage DNA preparation

Phage DNA was prepared according to the *Qiagen Lambda phage maxi prep* protocol.

II H. Genomic DNA and mRNA preparation

II H1. Genomic DNA preparation

Long method: 10 million cells were centrifuged at 800 x g for 5 min. The supernatant media was discarded and the cells were lysed in the DNA lysis buffer by vortexing (10 mM Tris pH=7, 100 mM EDTA, 0.5% SDS). RNase A was added to a final concentration of 20 µg/ml and the samples were incubated at 37°C for 2 hours. Proteinase K was then added to a final concentration of 100 µg/ml and the samples were incubated overnight at 37°C. The next day 1 ml of phenol (pH 8) was added, mixed thoroughly at first then left to mix gently for 10 min at 4°C. Afterward, the samples were centrifuged for 15 min at 1800 x g at 4°C. The upper phase was recovered and the DNA was precipitated by adding $1/10^{th}$ volume of ammonium acetate (10 M) and 2 volumes of absolute ethanol. The DNA was washed with 70% ethanol and the pellet was dried and re-suspended in sterile water. The DNA was left to dissolve overnight at 4°C.

Short method: 2 million cells were centrifuged at 800 x g for 5 min. The supernatant media was then removed by aspiration and the cells were lysed in TAIL buffer (50 mM Tris/HCl (pH 8.8), 100 mM EDTA, 100 mM NaCl, 1% SDS) previously warmed to 37 °C to facilitate the lysis process. Proteinase K was added to a final concentration of 100 µg/ml and the samples were incubated overnight at 37°C. The next day the samples were mixed by shaking for 5 minutes. Then 500 µl of 6 M NaCl were added and the samples were mixed again by shaking for another 5 minutes. The tubes

were centrifuged at maximum speed (20, 000 x g) for 5 minutes at 4°C. The supernatant was transferred to a new tube and 250 µl of isopropanol was added. The samples were mixed by inverting and centrifuged for 5 minutes at 20, 000 x g. The resulting DNA pellet was washed with 500 µl of 70% ethanol, air-dried and re-suspended in 100 µl sterile water. The DNA was either left to dissolve overnight at 4°C or was incubated at 37°C for 20 min (in cases where the DNA was to be used the same day).

II H2. mRNA preparation

Long method: 10-20 million cells were centrifuged at 1200 rpm for 5 min. The supernatant media was then removed and the cells were lysed in RNA lysis buffer (4 M guanidium thiocyanate, 25 mM Sodium citrate, 0.5% sarkosyl, 0.1 M ß mercaptoethanol) while vortexing. The lysate was poured into a 2 ml eppendorf tube, 90 µl of sodium acetate (2 M; pH 4) and 0.9 ml of water was added and mixed thoroughly. Subsequently, 180 µl of chloroform were added and mixed again and centrifuged for 20 min at 10, 000 x g at 4°C. The upper phase was recovered to a new eppendorf tube. The RNA was precipitated by adding 1 volume of isopropanol, placing the tubes at -20°C for 1 h and centrifuging for 30 min at 20, 000 rpm at 4°C. The RNA pellets were washed with 1 ml 70% ethanol. The pellet was left to air dry and resuspended in 30-50 µl RNase free water. The RNA was quantitated by diluting 4 µl of stock RNA in 1 ml of water and measuring the OD at 260 and 280 nm. OD 260 nm x 10 = RNA concentration in µg/µl.

Short method: total mRNA was prepared using the TRIzol reagent (Invitrogen) according to the manufacturer's recommendations.

Oligo(A) mRNA preparation: Oligo(A) mRNA were prepared from 1 mg of total RNA using Oligotex mRNA Mini or Midi kits following the manufacturer's recommendations.

II I. Apoptosis experiments

II I1. Apoptosis induction

Apoptosis was induced in cultured cells by either etoposide (Sigma and Calbiochem) treatment (5 μM-100 μM) for 3-5 hours or staurosporine treatment (0.1 μM –10 μM) for 5-7 hours.

II I2. Loss of plasma membrane assymetry : Annexin V staining

Annexin V staining was used when investigating for phosphatidylserine (PS) exposure (see Chapther I section I B2a). Annexin V staining was carried out using either the Annexin-V-Fluos Staining Kit (Roche) or the Annexin V- PE Apoptosis detection kit (BioVision) according to the manufacturer's recommendations.

II I3. DNA fragmentation analysis

DNA fragmentation analysis was assessed either by Tunel staining or DNA laddering (see Chapter I section I B2b).

II I3a. Terminal deoxynucleotidyl transferase-mediated dUTP nick labeling (Tunel) staining

Tunel staining was carried out using either Tunel Label (Boehringer Mannheim) or *In situ* cell death detection kit, TMR red (Roche) according to the manufacturer's recommendations.

II I3b. DNA laddering

Cells were induced to undergo apoptosis for 5-7 hours as described above. At various time points cells were centrifuged at 800 x g and immediately lysed in DNA lysis buffer. DNA was extracted as described in section II D4, quantified and 5 μg was loaded and electrophoresed on a 1.5 % agarose gel.

II I4. Detection of apoptosis by flow cytometric analysis

PS exposure and DNA fragmentation were quantified, as described above, by Annexin V and Tunnel labelling. After staining, cells were transferred to a 5 ml Polystyrene Round-Bottom Tube (Falcon) and, when required, diluted in PBS. Cells were analysed using a Becton Dickinson FACS Calibur flow cytometer. A total of 15,000 cells were counted at maximum rate of 500 events per second. Data was acquired and analysed using the Cell Quest software.

II I5. Cell viability assays on DT40 cells

Cell viability assays were carried out in flat bottom 96 well microtiter plates as follows:

A 1	2	3	4	5	6	7	8	9	10	11	12
B											
C	LIVE CELLS	Cell death-inducing drug added by serial dilution from rows 3 to 12									
D											
E	RPMI										
F											
G											
H											

The 96 wells were filled first with 50 µl of RPMI supplemented with 10% FBS and 1% chicken serum. To column 1 an additional 50 µl of medium were added. The cell death inducing drugs etoposide or staurosporine were added to row 3 at final concentrations of 100 µM and 1 µM respectively and in a final volume of 100 µl. 50 µl of column 3 was then taken and mixed with row 3. This was repeated from row 3 to row 12, diluting the drug by two-fold each time. A 96 well plate usually served for two assays which were conducted in triplicate. Consequently, to rows B-d 50 µl of cells *X, eperiment 1* were added and to rows E-G 50 µl of cells *Y, experiment 2* were added. Plates were left at 39°C for 24 hours. After incubation, 10 µl of the cell proliferation reagent WST-1[1] (Roche) was added to each well and incubated for an additional 3 hours at 39°C. The

[1] Cell proliferation and viability assays use the tetrazolium salt (WST-1). Tetrazolium salts are cleaved to formazan by the "succinate-tetrazolium reductase" system which belongs to

absorbance was measured using an ELISA plate reader at 420 nm with a reference wavelength of 600 nm and the data was analysed using *KC*^{4} software.

II I6. Cell free system

In the cell free system, isolated HeLa or Jurkat nuclei were incubated with cytosolic extracts from caspase-6 or caspase-7 cells (prepared as described in section II E4) and analysed either by immunoblotting, or fluorescence microscopy.

HeLa and Jurkat nuclei were prepared as follows: Cells were centrifuged at 800 x g, washed once in PBS, and once in nuclei buffer (10 mM Pipes/pH 7.4, 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 20 µM cytochalasin B, 1 mM CLAP and 10 µg/ml PMSF). Cells were transferred to a Dounce homogenizer, allowed to swell by incubating on ice for 20 min then lysed by douncing. The state of lysis was assessed by a phase contrast microscope (Olympus CH2). When 90% of cells were judged to be lysed, the nuclei were layered over a 30 % sucrose bed and centrifuged at 800 g for 10 min. Finally, the nuclei were resuspended in storage buffer (10 mM Pipes, pH 7.4, 80 mM KCl, 20 mM NaCl, 250 mM sucrose, 5 mM EGTA, 1 mM DTT, 0.5 mM spermidine, 0.2 mM spermine, protease inhibitors and 50 % glycerol).

Apoptosis induction: Nuclei were washed twice in MDB buffer (10 mM Pipes/pH 7.0, 50 mM NaCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT), resuspended in MDB buffer and divided equally between the samples to be analysed. Apoptotic extracts and an ATP regenerating system (2 mM ATP, 10 mM creatine phosphate, 50 µM creatine kinase) were added and the mixtures were incubated at 37°C for 1 to 3 hours. 1 µl of nuclei were fixed in 2 % paraformaldehyde and stained with DAPI. Images were captured using a Zeiss Axioplan II microscope and analysed using the IP Lab 3.2.3 software. The remainder of each sample was lysed in sample buffer for immunoblotting analysis.

II I7. Caspase affinity labelling experiments

25-30 μg of protein from cytosolic extracts were added to 1 μM zEK(bio)D-aomk (Peptide Institute, Kyoto Japan) and incubated for 15 min at 37°C. 5 μl of Laemmli buffer were added and samples were boiled for 5 min then separated on a 12% SDS/PAGE and proteins were transferred to a nitrocellulose membrane as described in section II E4. After transfer, the membrane was blocked in PBS/5% milk for 45 min, washed thoroughly and incubated with horseradish peroxidase-linked streptavidin (Sigma) for 3 h at 25°C. The signal was revealed by ECL.

II I8. Caspase activity assays

Caspase activity assays were performed in Professor Scott Kaufmann's laboratory at the Mayo Clinic, Rochester, MN USA. Cleavage of DEVD-AFC (Biomol, Plymouth Meeting, PA), VEID-AFC (Enzyme Systems Products, Dublin, CA) and LEHD- AFC (Enzyme Systems Products, Dublin, CA) was assayed as previously described (Kottke et al., 1999; Martins et al., 1997a). DEVD-AFC, VEID-AFC and LEHD-AFC are synthetic tetrapeptide fluorogenic substrates that are used to identify and quantitate caspase-3,- 6 –7 activity respectively in apoptotic cell lysates. Caspase-3, -6 –9 cleave selectively their appropriate tetrapeptide between D and AFC releasing the fluorescent AFC (7-amino-4-trifluoromethyl-coumarin dye) which can be measured in cell lysates by spectrofluorometry.

In brief, 50 μg of cytosolic protein in 50 μl buffer A (25 mM HEPES/pH 7.5 at 4°C, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin) or 0 μg protein (as a blank) was diluted with 225 μl of freshly prepared buffer B (25 mM HEPES/pH 7.5, 0.1% (w/v) CHAPS, 10 mM DTT, 100 U/ml aprotinin, 1 mM PMSF) containing 100 μM substrate and incubated for 2 h at 37°C. After reactions were terminated by addition of 1.225 ml ice cold buffer B, fluorescence was measured using an excitation wavelength of 360 nm and emission wavelength of 475 nm. Standards containing 0-15 nmoles of AFC were utilized to determine the amount of fluorochrome released.

Chapter III: Generation of caspase-6-/- and caspase-7-/- DT40 cells

III A. Introduction

In the last ten years several natural caspase inhibitors were discovered and used to study protease functions in apoptotic execution (reviewed in (Rudel, 1999). In addition, genetic approaches were used to knockout caspase genes in mice (see Chapter 1 section IE2). The results showed that caspases-3 and -9 are crucial to mouse brain development and that caspase-8 plays an essential role in heart development (Earnshaw et al., 1999; Zheng and Flavell, 2000). An alternative system that we have used to study the role of caspases in apoptosis is gene targeting using the chicken DT40 lymphoma cell line.

The aim of my project is the genetic analysis of caspase function by gene knockout in the DT40 cell line. No DT40 caspase knockout had been published when my work started with participation in an ongoing *caspase-6* knockout project. Dr. Pascal Villa, a former postdoctoral in the Earnshaw laboratory, had screened a genomic Lambda FixII DT40 library and isolated several phages containing the chicken *caspase-6* coding region. I started my work in collaboration with Dr. Sandrine Ruchaud, another postdoc in the Earnshaw laboratory, who had followed up the *caspase-6* knockout project. An interesting second knockout that we decided to work on was *caspase-7* since it is also involved in the execution phase of apoptosis. I spent the last two years on that project.

As discussed in the Introduction to this thesis, both caspases-6 and -7 have an active role in the execution of the death pathway. Knocking out the genes responsible for their production gives us additional insights into their role in apoptosis. We aimed for the elimination of the gene responsible for the production of caspase-6 and –7 and subsequently to study the morphological and biochemical characteristics of apoptosis

in *caspase-6*$^{-/-}$ and -*7*$^{-/-}$ cells. The work on *caspase-6* started with mapping of previously isolated phages containing the genomic locus and the *caspase-7* project started with chicken *caspase-7* (Expressed sequence tag) EST sequencing and screening a lambda FixII genomic library to isolate phages containing the *caspase-7* locus. Since some of the initial steps of the *caspase-6* and *–7* knockout project were similar, this chapter will discuss the two projects simultaneously and later Chapters IV and V will be allocated to the description and characterisation of each of the, caspase-6 and -7, knockout cell lines.

III B. Isolation of lambda phages containing *caspase-6* and *caspase–7* loci

III B1. Chicken caspase-6 cDNA

As stated above *caspase-6* genomic phages had been isolated when I joined the *capase-6* knockout project. Sequencing of the *caspase-6* cDNA revealed that it is 67% homologous to human *caspase-6* (GeneJockey, sequencing processor).

III B2. Chicken *Caspase-7* cDNA

A chicken *caspase-7* EST was obtained from university of Delaware (Newerk, Delaware Newark, USA). The first step was to amplify the plasmid in order to have sufficient amounts of DNA to sequence and use for future studies. Top10 competent *E.coli* cells were transformed with the EST-containing plasmid (see Chapter II section II F6a). A 2.2 Kb insert was removed by a double digest with restriction enzymes *NotI/EcoRI* (see Chapter II Section II C1a). The EST was sequenced by automatic sequencing using the ABI system (*Biosystem*) (see Chapter II section II C5). The resulting sequences were analyzed by nucleotide sequence comparisons using the NCBI/BLASTN advanced search engine. The results showed that the EST contains the complete open reading frame (ORF) and the 3′ untranslated region (UTR). Alignment with human caspase-7 showed the two proteins are 66% homologous (GeneJockey, sequencing processor).

III B3. Restriction enzyme digestion of *caspase-7* EST

A series of restriction digests were performed in order to map restriction sites within the *caspase-7* cDNA in order to design probes for genomic library screening. Figure 3.1 is a preliminary map of the *caspase-7* cDNA. Accordingly, a 5′ probe was chosen for screening the genomic DT40 lambda FixII library. The vector was digested with *KpnI* and the resulting 1.2 kb band was removed and purified using the *Qiagen gel purification kit* (Fig 3.1).

Figure 3.1: Caspase-7 cDNA

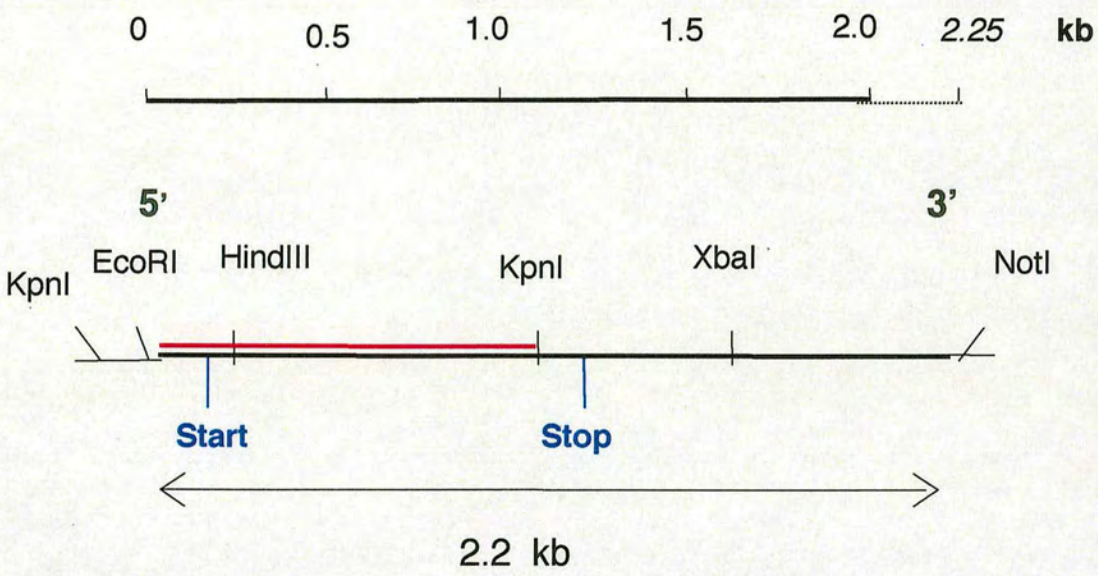

Fig 3.1 : Schematic representation of caspase-7 cDNA cloned into pCDNA3 (Invitrogen). The line in red represents the probe used to screen the lambda FixII library

III B4. Lambda FixII library screening for *caspase-7* gene locus

I next screened a DT40 genomic lambda FixII library in order to isolate clones covering the *caspase-7* genomic locus. My aim was to use 5′ and 3′ genomic DNA fragments flanking the caspase gene to subsequently construct the *caspase-7* targeting vector. The genomic library was titered according to the *Stratagene* instruction manual. The library titer was 4.48 x 10^8 plaque forming units/ml. Approximately two million phages were screened from the library.

After the first screening, using the 1.2 kb cDNA fragment as a probe, we isolated fourteen positive phage plaques. After four rounds of screening five pure plaques were isolated and, among the five phages, three were amplified, titred and stored at -80°C.

III C. Mapping and sequencing of the *caspase-6* and *-7* genes

At first, the *caspase-6* and *-7* genes were mapped by performing a series of partial digestions with restriction enzymes of interest followed by Southern analysis and hybridisation with oligo nucleotide probes to T3 and T7 promoters in the λ phage arms containing the inserts (see Chapter II Sections II C1b and II C6a). A representative result of such a restriction digest is shown in figure 3.2 where *SacI* was the enzyme of choice. Partial mapping was performed on four *caspase-6* phages and three *caspase-7* phages. The partial mapping allowed phages to be selected for sequencing at GATC (GATC Biotech, Germany). One phage for each caspase was selected. The requested amount of genomic DNA was prepared and sent for sequencing.

The entire loci, 18 kb for *caspase-6* and 19 kb for *caspase-7*, were sequenced and the position of the exons determined by comparison with the cDNA sequence using GeneJockey, sequencing processor. The *caspase-6* gene appeared to be at least 8800 bp in length and contains 8 exons (Fig. 3.3A). the *caspase-7* sequence covered the last 5 exons of the gene locus, excluding the 5' end of the gene (Fig 3.4A).

Figure 3.2: Mapping of caspase-6 locus by partial digest

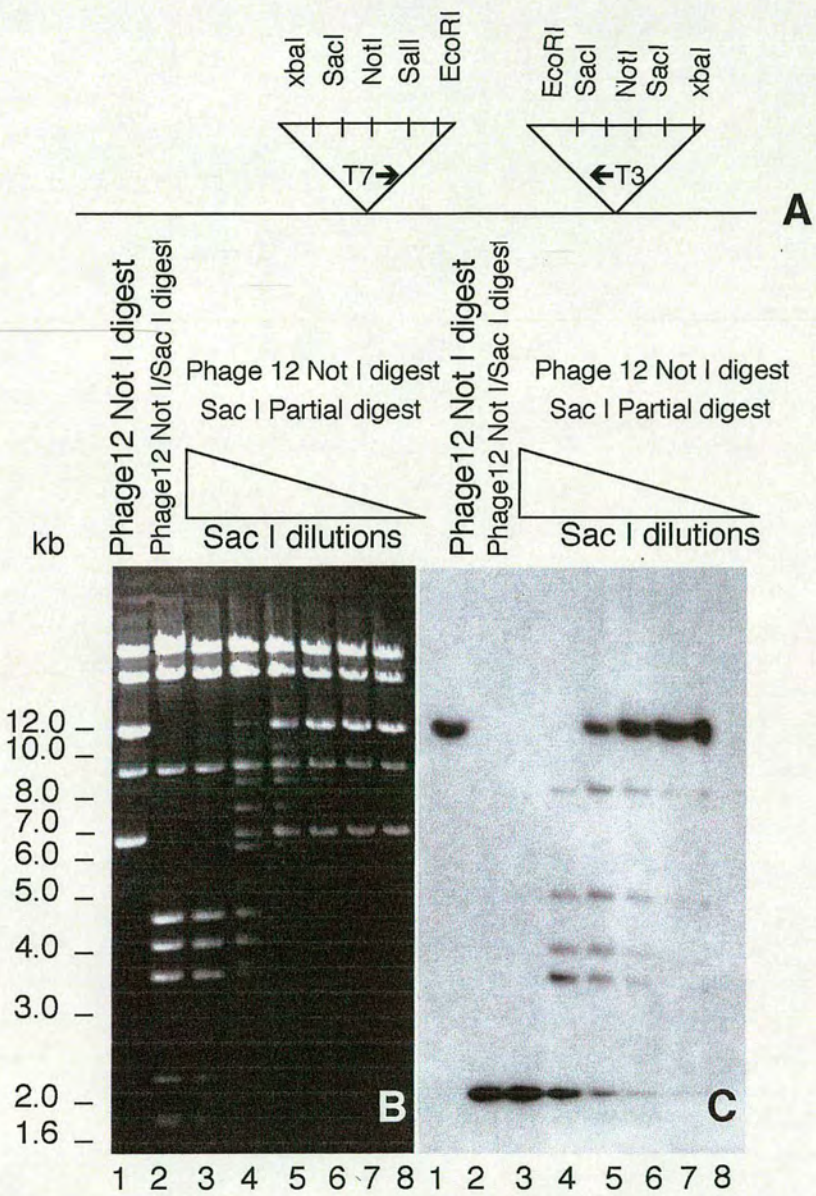

Fig 3.2 : Caspase-6 gene locus mapping on phage 12, **A,** Schematic representation of Lamda FixII showing the multiple cloning site and the T3 and T7 DNA sequences flanking the insertion region **B,** Ethidium Bromide picture of the gel under UV. **C,** Southern blot of the gel presented in B. The membrane was hybridised with a radiolabelled T3 oligo nucleotide primer.

III D. Knockout vectors

The caspase replacement vectors were designed to contain two regions of homology to the targeted gene with the 5' end upstream and 3' end downstream to the coding genomic locus. These two genomic fragments were located on either side of selectable markers, which gives histidinol, blasticidin or puromycin resistance. Both of the genomic DNA fragments were cloned in the same orientation, 5'-3', with respect to the gene (Fig 3.3 and 3.4).

III D1. *Caspase-6* knockout vectors

To disrupt the *caspase-6* gene we constructed a targeting vector in which a resistance cassette (puromycin or histidinol) was flanked by a 5' genomic arm situated upstream from exon 2 and a 3' genomic arm situated downstream of the stop codon (Fig. 3.3B). Targeted integration of these constructs Casp6*puro* and Casp6*his* would remove a 7211 bp gene fragment containing the majority of the open reading frame (888 bp of 915 bp) and part of the 3' untranslated region.

A 3' screening strategy was designed to screen for proper targeting events. As shown in Figure 3.3B, a *BamHI* restriction digest of genomic DNA followed by Southern analysis using a 3' external probe would recognises a 7.7 kb band for wild type DT40 cells and a 5.3 kb or 2.7 kb band after integration of the Casp6*puro* or Casp6*his* constructs respectively.

III D2. Caspase-7 knockout vectors

To disrupt the *caspase-7* gene, we constructed targeting vectors in which a resistance cassette (blasticidin or histidinol) was flanked by a 5' genomic arm situated upstream of the first exon found in the phage clone and a 3' genomic arm situated downstream of the stop codon (Fig 3.4B). Targeted integration of these constructs, termed Casp7*blast* and Casp7*his*, would remove a 3700 bp gene fragment containing the majority of the open reading frame (786 bp of 930 bp). Following insertion of these vectors, only the first 13

Figure 3.3: Structure and targeting strategies of the Gallus gallus caspase-6 gene

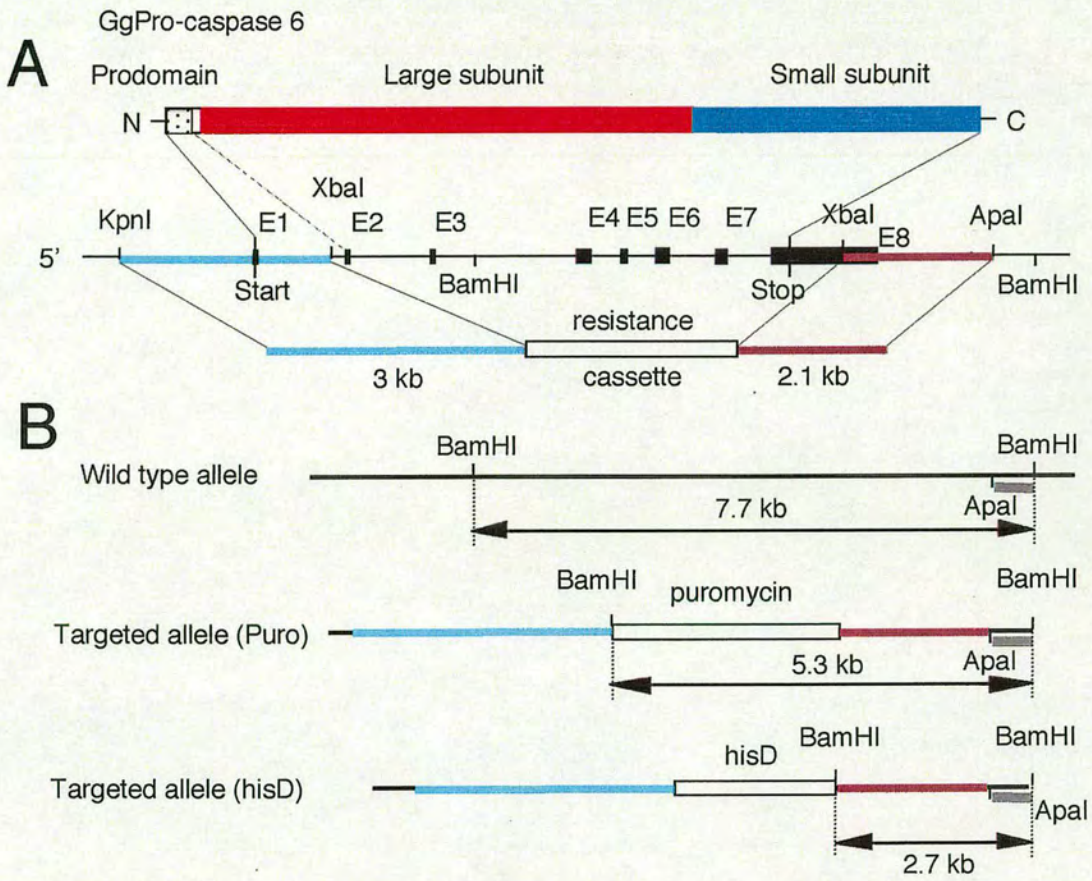

Fig 3.3: **A,** Structure of the chicken *caspase-6* gene together with the targeting vectors and homologous recombinants containing either the *Puromycin* or the *Histidinol* cassette. **B,** Schematic representation of 3' screening strategy using BamHI digest. Line in grey represents the 3' external probe.

Figure 3.4: Structure and targeting strategies of the Gallus gallus caspase-7 gene

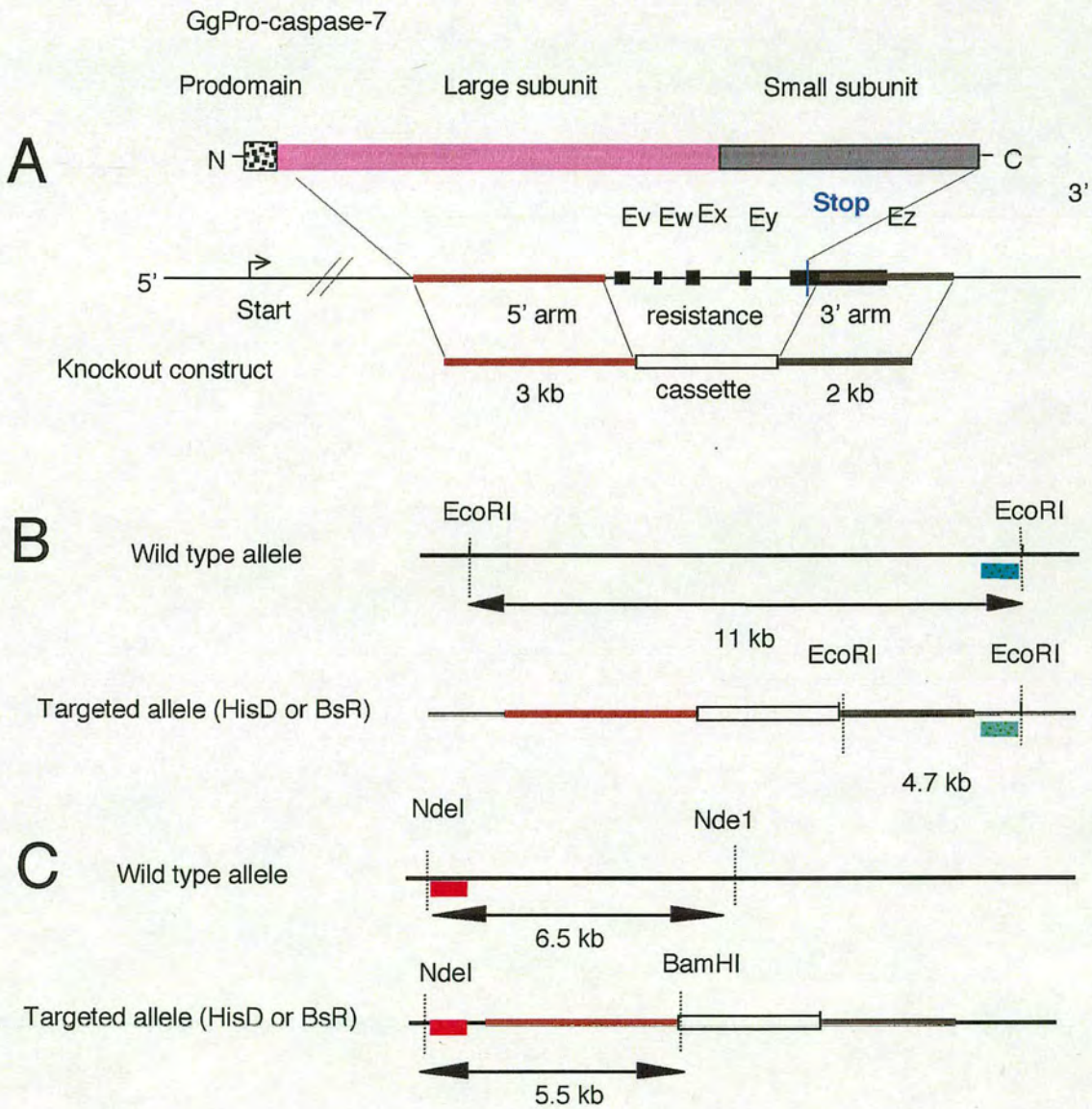

Fig 3.4: **A,** Partial structure of the chicken *caspase-7* gene and the knockout constructs containing the 5' and 3' arms separated by a resistance cassette (Blasticidin or Histidinol). **B,** Schematic representation of 3' screening strategy using EcoRI digest. Line in green represents the 3' external probe. **C,** Schematic representation of 5' screening strategy using a double BamHI and NdeI digest. Line in red represents the 5' external probe.

amino acids of the enzyme prodomain and 3 amino acids of the large subunit could potentially be expressed. However, since caspase are enzymes and their activation requires proteolysis followed by processing and association of two subunits to form the active protease (see Chapter I Section I D), it is highly unlikely that the short peptide, if expressed, would be functional.

A 3' and a 5' screening strategy were designed to screen for proper targeting events. As shown in Figure 3.4, an *EcoRI* restriction digest on genomic DNA followed by Southern analysis using a 3' external probe would recognise an 11 kb band for wild type DT40 cells and a 4.7 kb band after integration of the Casp7*puro* or Casp7*bsr*. A double *BamHI/NdeI* restriction digest followed by Southern analysis using a 5' probe would recognise a 6.5 kb for wild type cells and a 5.5 kb band for targeted alleles (Fig 3.4 C).

III E. Generation of *caspase-6*$^{-/-}$ and *caspase-7*$^{-/-}$ cells

III E1. Generation of *Caspase-6* deficient DT 40 clones

In order to isolate heterozygous *caspase-6* clones, wild type DT40 cells were transfected with the Casp6*puro* or Casp6*his* constructs and puromycin or histidinol resistant clones were isolated. As explained in Section III D1, targeted events were recognised by BamHI digestion followed by Southern analysis using a 3' probe. As shown in figure 3.5, the 3' external probe recognised a 7.7 kb band corresponding to the wild type allele and a 5.3 kb band after targeted integration of the Casp6*puro*. The targeting efficiency for the first allele was 8% . For some unknown reasons, we were unable to isolate heterozygous clones using the Casp6*his* construct. Several attempts were made to generate a caspase-6 deficient cell line using different knockout constructs but after screening 350 clones no double targeting events were isolated.

These results implied that *caspase-6* might be an essential gene in DT40 cells. Thus we decided to transfect *caspase-6* heterozygote clones with the *caspase-6* cDNA under control of the TetA tetracycline repressable system (see Material and Method Section II B4). The cloning was done by Dr. Sandrine Ruchaud. At the end we obtained several clones expressing caspase-6:GFP under tetracycline control.

The clones obtained contained the following expression plasmids:

The clones obtained contained the following expression plasmids:

- 7x tet casp6 + tTA3 (1 clone selected called I)
- 7x tet casp6 + tTA4 (1 clone selected called II)
- 3x tet op-casp6-EGFP + tTA3 (2 clones selected called III and IV)
- 7x tet casp6:EGFP + tTA3 (1 clone selected called V)

For clones I-IV, Dr Sandrine Ruchaud checked the expression of exogenous caspase-6 by caspase activity affinity labelling (Fig 3.6) and by immunofluorescence for clone V. She then confirmed that the repression of the caspase-6 transcript was effective after the addition of 1 μM doxycycline for 48 hours. Clones I to V were transfected with a second caspase-6 knockout vector encoding histidinol resistance. The results of transfection are represented in Table 3.1:

Table 3.1: *Screening results for second caspase-6 allele targeting*

Clone ID	Resistant clones obtained	Clones screened	*Caspase-6* deficient cells
Clone I	55	30	1, (lost)
Clone II	106	44	1, **II 26**
Clone III	130	28	0
Clone IV	85	34	1, IV35
Clone V	92	40	2, V63 and V40

The efficiency of second allele targeting was 4% as expected (half of that obtained for first allele targeting). Subsequently, clones II26 and V63 were used to investigate of the role of caspase-6 in apoptotic execution of DT40 cells.

Following closer inspection we were surprised to find out that clone II26 had lost the *caspase-6* cDNA. Since I was involved in the analysis of that particular clone, the following sections will be focused on the analysis of *caspase-6* deficiency in clone II26 (Fig 3.7A).

In this clone, loss of caspase-6 expression was confirmed by Dr Ruchaud by Northern blot analysis and by immunoblotting analysis (Fig 3.7B, C).

Figure 3.5: First allele targeting of Gallus gallus caspase-6 gene

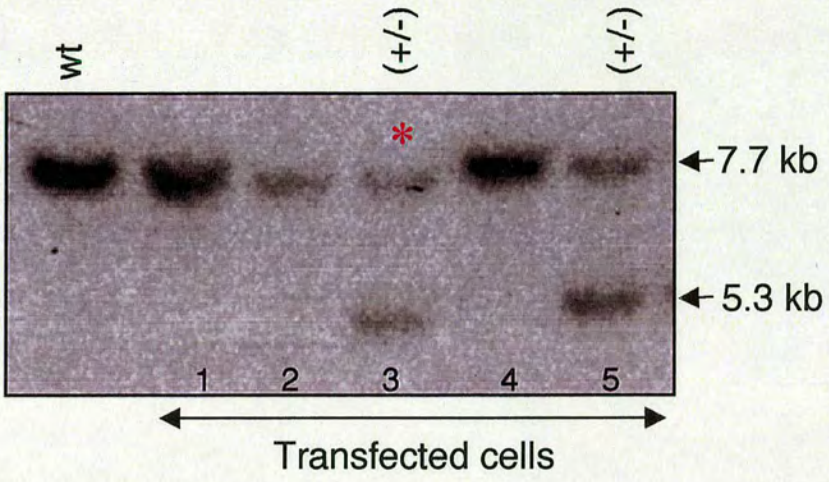

Fig 3.5: Southern blot analysis of DNA digested with BamHI using the 3' genomic external probe represented in 3.3 B. Lanes 3 and 5 are examples of first allele replacement. (*) Heterozygote clone used for second allele targeting.

Figure 3.6: Caspase-6$^{-/+}$:casp-6:cDNA and Caspase-6$^{-/+}$:casp-6:EGFP stable cell lines

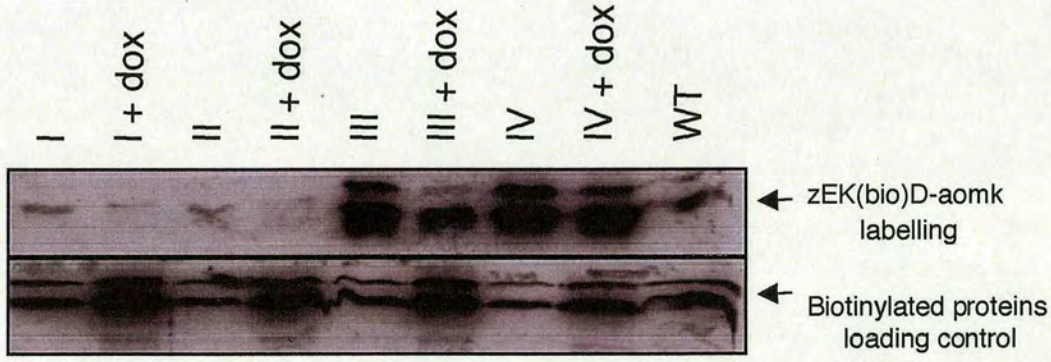

Fig 3.6: Labelling of active caspases by zEK(bio)D-aomk and separation on SDS-PAGE. Apoptotic extracts were prepared form clones (I-IV) treated with 10 μM etoposide for 4 hours in the presence or absence of 1 μM doxycycline. Endogenous biotinylated protein expression is used as gel loading control.

Figure 3.7: Targeting of Gallus gallus caspase-6 gene

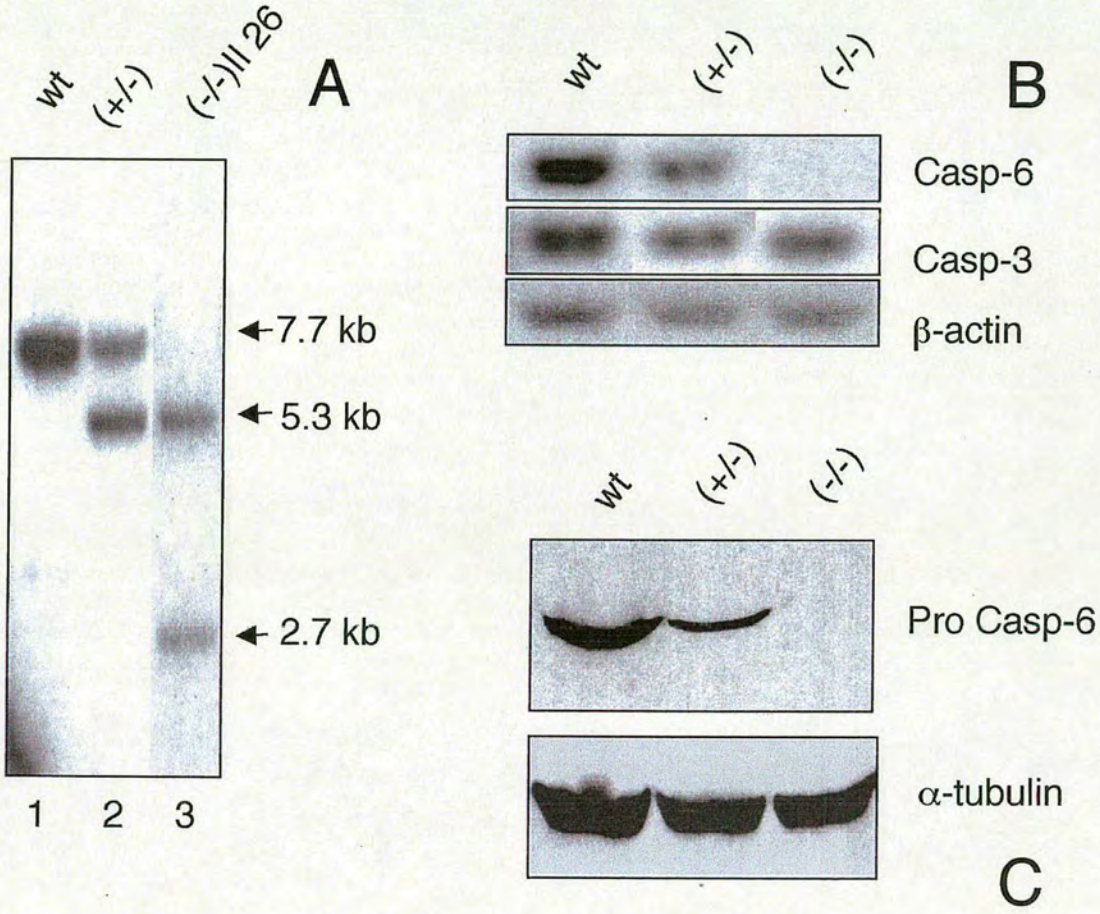

Fig 3.7 : **A,** Southern blot analysis of DNA digested with Bam HI, using the 3' genomic external probe Apa I-Bam HI represented in 3.3B. Lane 2 shows the replacement of one of *caspase-6* alleles with the targeting construct, lane 3 shows the disappearance of the WT alleles replaced by the proper targeting of homologous knockout constructs. **B,** Northern blot analysis showing chicken *caspase-6 and -3* mRNA expression.Caspase-3 mRNA expression levels are not affected by the loss of caspase-6. Caspase-6 mRNA could not be detected in null cells. **C,** Immunoblotting of caspase-6 using a polyclonal antibody directed against the large subunit of the enzyme (R549) . Heterozygote clones show a diminished caspase-6 protein expression. In null cells no caspase-6 protein could be detected .

III E2. Generation of caspase-7 deficient DT40 clones

The caspase-7 gene deletion was performed by homologous recombination in the chicken B cell lymphoma cell line DT40. Wild type DT40 cells were transfected with the Casp7*his* and Casp7*brs* constructs and histidinol and blasticidin resistant clones were isolated. Homologous targeting events were recognised by Southern blot analysis after an *EcoRI* or a double *BamHI/NdeI* restriction digestion of genomic DNA digest using a 3' or 5' probe respectively (see above Section III D3). As shown in Figure 3.8A, the 3' probe recognised an 11 kb band corresponding to the wild type allele and a 4.7 kb band after targeted integration of the Casp7*blast* or Casp7*his* constructs. The 5' probe detected a 6.5 kb in WT cells and a 5.5 kb band for targeted alleles (Fig 3.8 B). The targeting efficiency for the first allele of 1.4% was similar for both knockout constructs. The targeting efficiency for the second allele was an unexpectedly high 10%, Several caspase-7 deficient clones were isolated (Fig 3.8A, B).

Our aim was to confirm caspase-7 deficient clones by Northern analysis (see Chapter II section or by RT-PCR (see Chapter II Section II D2) . Therefore Northern blot analysis and RT-PCR was performed on total and polyA mRNA (see Chapter II section II H2) from wild type and caspase-7 deficient clones. After several attempts using the complete *caspase-7* cDNA or two other different probes amplified by PCR from *caspase-7* cDNA using NKC75 and NKC73 or NP5 and NP3 (see table 2.1 in chapter II, material and methods) we were unable to detect any *caspase-7* mRNA in wt DT40 cells. The possibility that we failed to detect any *caspase-7* mRNA due to the probes we used was ruled out by using DU249 and MSB, two other chicken cell lines, as positive controls (Fig 3.9A). Our RT-PCR attempts using primers NKC75 and NKC73 or NP5 and NP3 as primers gave exactly the same results (Fig 3.9B).

Figure 3.8: Targeting of Gallus gallus caspase-7 gene

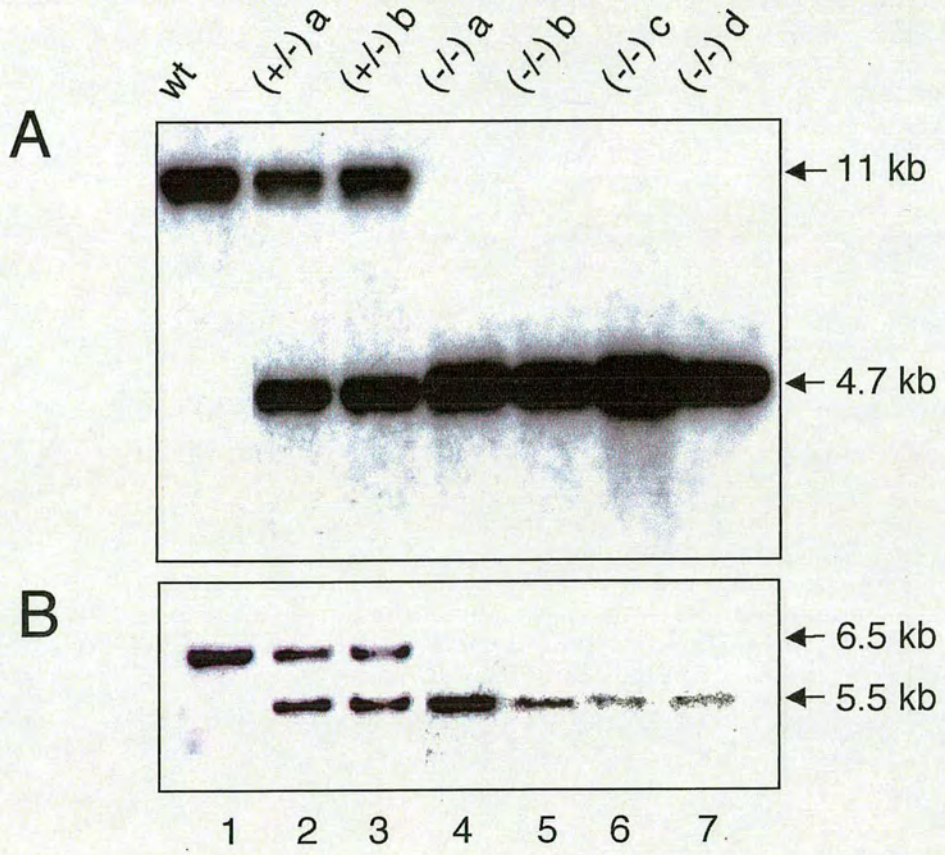

Fig 3.8: **A,** Southern blot analysis of DNA digested with EcoRI, using the 3' genomic external probe represented in 3.4B. **B,** Southern blot analysis of DNA digested with BamHI and NdeI, using the 5' genomic external probe represented in 3.4C

Lanes 2 and 3 are examples of heterozygote clones where one of the *caspase-7* alleles has been properly targeted. Lanes 4-7 are examples of null clones. WT alleles are not present anymore and only targeted alleles are detected.

Figure 3.9: Caspase-7 mRNA expression in DT40 and other cell lines

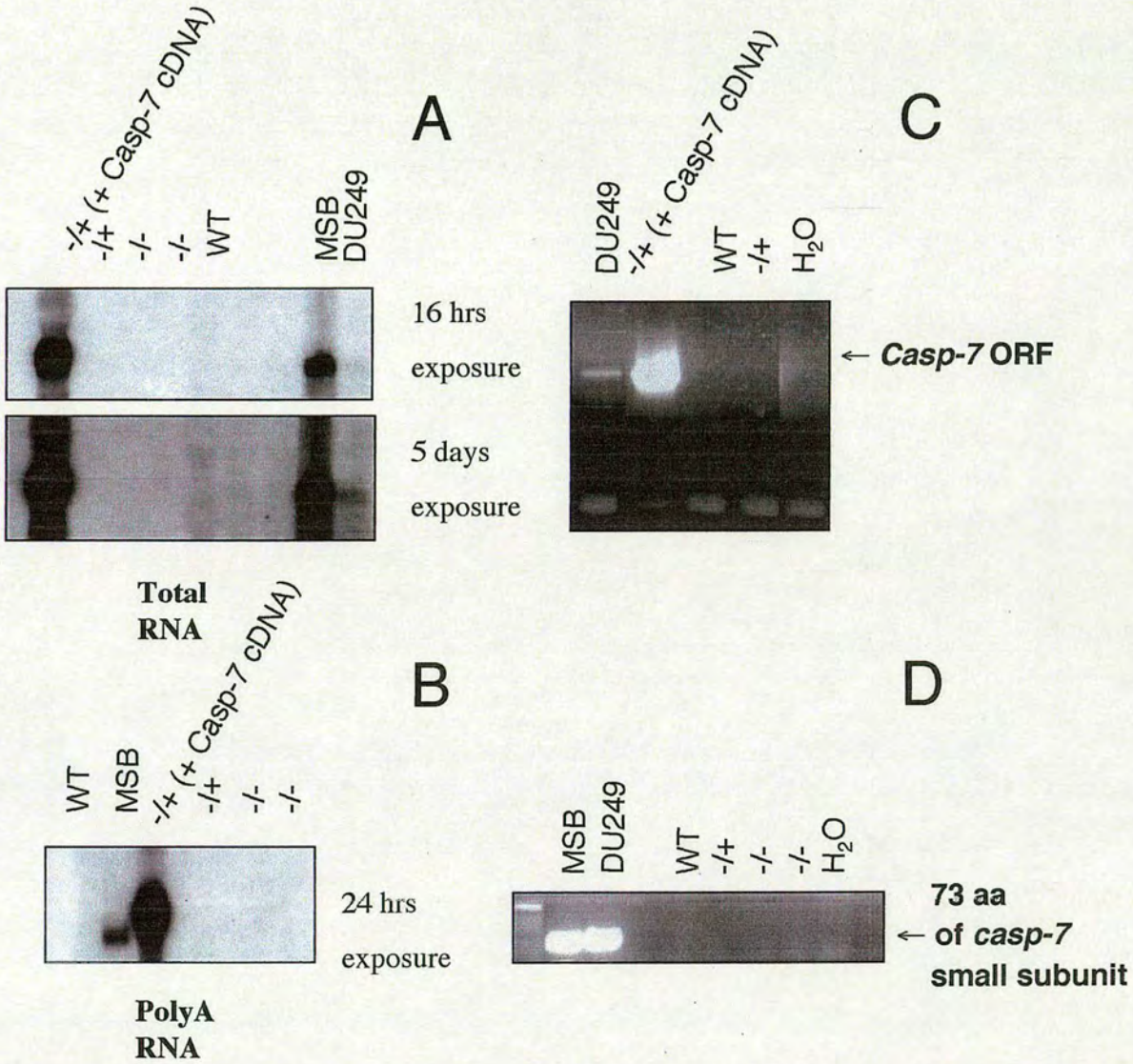

Fig 3.9: **A,B,** Northern blot analysis using total RNA (A) or PolyA RNA (B) from WT, heterozygous, *caspase-7* $^{-/-}$, *caspase-7* $^{-/-}$*:casp-7:cDNA,* MSB and DU249 cells. The probe used was *caspase-7* ORF. **C,D,** RT-PCR on RNA from WT, heterozygous, *caspase-7* $^{-/-}$, *caspase-7* $^{-/-}$*:casp-7:cDNA,* MSB and DU249 cells. Primers used were NKC73/NKC75 (C) and NP3/NP5 (D) to amplify the *caspase-7* ORF and *caspase-7* small subunit respectively .No *caspase-7* mRNA could be detected, by Northern blot analysis or RT-PCR in WT DT40 cells. Caspase-7 mRNA was detected in MSB, DU249 and *caspase-7* $^{-/-}$*:casp-7:cDNA* cells.

III F. Discussion

III F1. Generation of a caspase-6 deficient DT40 cell line

The results of this chapter report the isolation of a *caspase-6* deficient DT40 cell line. The targeting efficiency of *caspase-6* allele was not as expected. The first allele targeting occurred with a frequency of 8 %, therefore we expected a 4% efficiency of targeting for the second *caspase-6* allele. However, when the second knockout construct was transfected into *caspase-6*$^{+/-}$ cells and 350 clones were screened, it was not possible to isolate any clone where the second *caspase-6* allele was replaced by knockout constructs. When the same experiment was repeated in the absence of selection for the first marker, gene targeting was possible and occurred at a frequency of 16 %, however, all recombinants had re-targeted the first allele.

This strongly suggested that the caspase-6 gene is essential in DT40 cells. Therefore, heterozygotes were transfected with cDNAs expressing caspase-6 or caspase-6:EGFP and stable cell lines were isolated. These *caspase-6* heterozygous clones expressing exogenous caspase-6 were transfected with the second replacement vector. Only then it was possible to target the second allele (7.5% targeting efficiency). This was consistant with the notion that *caspase-6* might be an essential gene. Surprisingly, one of the caspase-6 deficient clones isolated, subsequently clone II 26, initially expressing caspase-6, lost the exogenous sequences. Clone II 26 was confirmed to be a caspase-6 deficient clone by Southern, Northern and Immunoblotting analysis, as well as caspase-6 activity assay. The fact that clone II26 grew normally indicated that *caspase-6* was not essential as first thought but suggests that caspase-6 might possibly have a role in homologous recombination (see Chapter VI Section VI C1 for further details).

III F2. Generation of a caspase-7 deficient DT40 cell line

The results of this chapter also report the isolation of *caspase-7* deficient DT40 cell lines. The targeting efficiency for the first allele was 1.4%, lower than expected when compared to first allele replacement of caspase-6 (8%) and CAD (~ 10%) (Samejima et al., 2001). Surprisingly, targeting the second allele of caspase-7 using the same replacement vectors with different selectable markers occurred at a high

frequency of 10% (see Chapter V and VI Section VI C3 for further discussion). Targeted events were verified for proper integration of the replacement vector at the 3' and 5' ends of the locus. When Northern and RT-PCR analysis were subsequently conducted to confirm the loss of *caspase-7* mRNA , the caspase-7 transcript could not be detected in wild type DT40 cells (see above section III D2). The logical explanation behind this result is that caspase-7 is expressed at very low levels and below the threshold of sensitivity for the techniques used. We were aware that the inability to verify that caspase-7 is expressed in DT40, and confirm the transcript is lost in caspase-7 deficient cells, could impose a problem in subsequent analysis of caspase-$7^{-/-}$ cells. To overcome this problem, caspase-$7^{-/-}$ cells were transfected with a construct containing the *caspase-7* cDNA fused to EGFP. We then isolated stable *caspase-*$7^{-/-}$:casp-7:EGPF-expressing cell lines. These cell lines were then used to confirm that phenotypes observed after the loss of caspase-7 are rescued by expressing exogenous caspase-7, providing the proof that the phenotypes observed were due to loss of caspase-7.

III F3. The choice of the DT40 knockout system to study caspase functions

Genetic knockout is an attractive approach to study caspase function since the thirteen caspases identified are highly homologous and the use of specific inhibitors is not completely reliable to study specific caspase functions. *Caspase-6* and *caspase-7* knockout mice have been generated, however, it was apparently problematic to draw any conclusion regarding specific caspase function (see review (Zheng et al., 1999)). *Caspase-*$6^{-/-}$ mice had no phenotype and *caspase-*$7^{-/-}$ mice were very early embryonic lethal. Thus the choice of gene knockout in DT40 cells is ideal, since it allows the exploration of the contribution of these caspases to apoptotic execution at a cellular level. As discussed in greater detail in the introduction to this thesis, the high homologous recombination, stable genotype, small genome size, 9-12 hour doubling time and the possibility to generate multiple knockouts persuaded us to adopt this system for the study of caspase-6 and caspase-7 functions.

At this point, one unavoidable question is why the use of a time consuming knockout approach when protein knockdown by RNA interference (RNAi) and small

the same replacement vectors with different selectable markers occurred at a high frequency of 10% (see Chapter V and VI Section VI C3 for further discussion). Targeted events were verified for proper integration of the replacement vector at the 3' and 5' ends of the locus. When Northern and RT-PCR analysis were subsequently conducted to confirm the loss of *caspase-7* mRNA , the caspase-7 transcript could not be detected in wild type DT40 cells (see above section III D2). The logical explanation behind this result is that caspase-7 is expressed at very low levels and below the threshold of sensitivity for the techniques used. We were aware that the inability to verify that caspase-7 is expressed in DT40, and confirm the transcript is lost in caspase-7 deficient cells, could impose a problem in subsequent analysis of caspase-$7^{-/-}$ cells. To overcome this problem, caspase-$7^{-/-}$ cells were transfected with a construct containing the *caspase-7* cDNA fused to EGFP. We then isolated stable *caspase-*$7^{-/-}$:casp-7:EGPF-expressing cell lines. These cell lines were then used to confirm that phenotypes observed after the loss of caspase-7 are rescued by expressing exogenous caspase-7, providing the proof that the phenotypes observed were due to loss of caspase-7.

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Chapter IV: Analysis of caspase-6$^{-/-}$ DT40 cells

IV A. Introduction

Caspase-6[1], first identified in 1996 as the novel cysteine protease (Fernandes-Alnemri et al., 1995a), was classified as a member of the effector caspase family because of its short prodomain. As described in further detail in the Introduction to this thesis, the first substrates identified for caspase-6 were the nuclear lamins (Orth et al., 1996; Takahashi et al., 1996). Analysis of caspase-6 function showed that this enzyme is involved in lamin disassembly and nuclear envelope breakdown during apoptotic execution (Cross et al., 2000; Duband-Goulet and Courvalin, 2000). Subsequent studies identified other caspase-6 substrates, however, in general, these proteins are also cleaved by other caspases, typically caspase-3, and the only substrate thought to be cleaved exclusively by caspase-6 remains the one of the first identified, lamin A/C (Slee et al., 2001; Takahashi et al., 1996).

As described in Chapter III, a DT40 cell line in which both *caspase-6* alleles were targeted allowed the *in vivo* and *in vitro* analysis of the role of caspase-6 role in apoptosis. Although no *caspase-6* knockout had been published, one review referred to *caspase-6* null mice (Zheng et al., 1999). In this review the authors reported that *caspase-6*$^{-/-}$ mice are no different than wild type mice in all the cell death aspects investigated and concluded that there is no phenotype linked to disruption of the enzyme. The powerful DT40 knockout system provided the advantage of analysing the role of caspase-6 at a cellular level and allowed a detailed study of the enzyme functions in apoptosis, possibly revealing details that could pass unnoticed while analysing knockout animals.

This chapter focuses on the characterisation of caspase-6 deficient DT40 cell lines.

[1] Caspase-6 was originally named Mch2 (mammalian ced-3 homologue 2)

Before proceeding any further, I would like to point out once more that Dr. Ruchaud was the main person involved in the analysis of the phenotype. I worked under Dr. Ruchaud's supervision while analysing the phenotype of caspase-6 deficient DT40 cell lines.

IV B. Phenotypic analysis of *caspase-6*$^{-/-}$ DT40 cells

IV B1. Apoptotic morphology

To address whether caspase-6 deficient cells display any differences in apoptotic morphology when compared with wild type DT40 cells, *caspase-6*$^{-/-}$ and wild type cells were induced to undergo apoptosis by incubation with 10 μM etoposide for five hours. Surprisingly, no differences were noted in the aspects of apoptosis studied. The timing and extent of chromatin condensation (Fig. 4.1A), as well as DNA fragmentation into a nucleosomal ladder (Fig. 4.1B), were similar.

As stated above, lamins are the best known substrates for caspase-6, especially lamin A, which is thought so far to be cleaved only by this caspase. Many lymphoid cells, however, do not express lamin A (Guilly et al., 1987; Kaufmann, 1989a), providing a potential explanation for the lack of an effect on nuclear apoptosis when *caspase-6* was deleted in DT40 cells (a B lymphocyte derived cell line)

IV B2. Lamin A, B1 and B2 expression in DT40 cells

In order to verify the possibility that DT40 cells lack lamin A, a polyclonal chicken anti-lamin A antiserum (provided by Dr. Reimer Stick) was used to assay for the expression of the protein by immunoblot analysis. As shown in Figure 4.2A, chicken lamin A antibody failed to detect lamin A in DT40 cells. Immunoblots with a monoclonal anti-human lamin A antibody detected a protein migrating at around 66 kDa in DT40 cells while nothing was detected by using this antibody in chicken muscle tissue. On the other hand a strong expression of lamin B2 in DT40 cells was detected when compared to chicken muscle tissue. This highly expressed lamin B2 (migrating at 66 kDA) might be recognized by the anti-human lamin A antibody giving this cross reacting band. In control experiments, lamins A and C were readily detected in HeLa cells, but not in Jurkat T-lymphoma cells (Fig. 4.2A).

Figure 4.1: Apoptosis in caspase-6 deficient DT40 cells

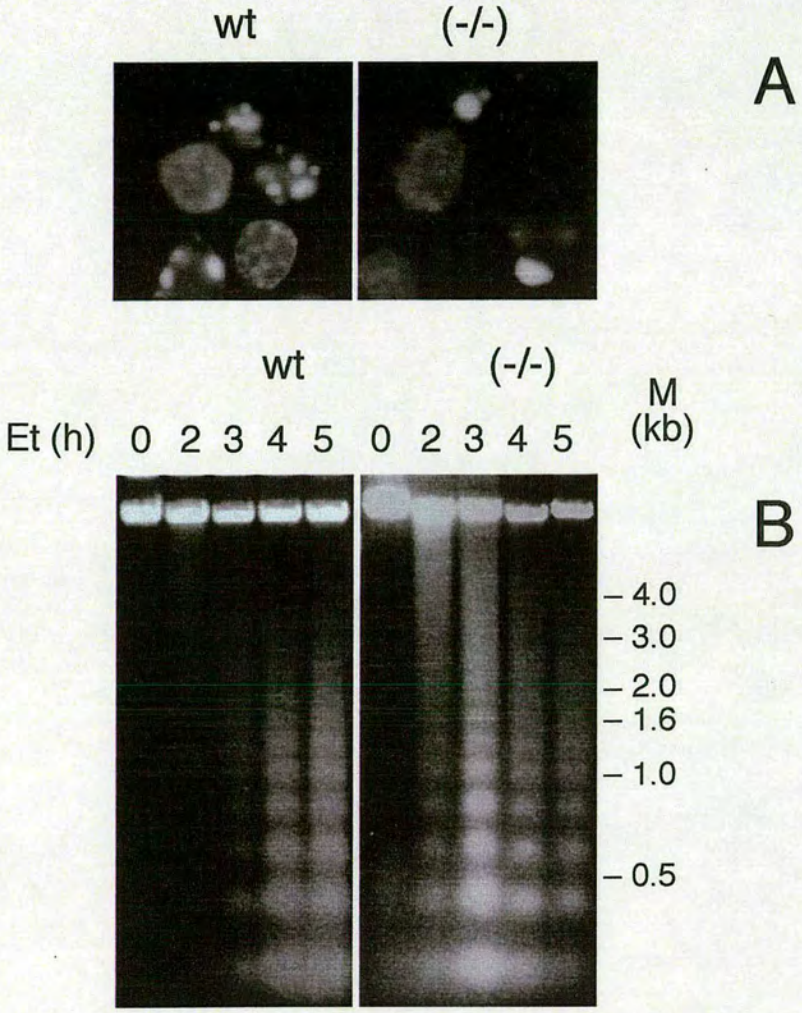


Fig 4.1: **A,** DAPI staining of wild type and *caspase-6*$^{-/-}$ cells treated with 10 μM etoposide for 4 hrs. **B,** DNA fragmentation analysis during 10 μM etoposide time course on wild type (WT) and *caspase-6*$^{-/-}$ (-/-) cells. No difference in the timingand extent of DNA laddering were noted between WT and caspase-6 deficient cells.

IV B2a. Lamin B1 and B2 cleavage in apoptotic caspase-6$^{-/-}$ cells

Lamin B1 and B2 cleavage during apoptotic execution was assessed after incubating *caspase-6*$^{-/-}$ cells and wild type DT40 cells with 10 μM etoposide. As shown in Figure 4.2B, lamin B1 and B2 were cleaved when apoptosis was induced by etoposide in both wild type and *caspase-6*$^{-/-}$ cells but not to the same extent. Lamin B2 is almost completely cleaved in wild type, but only cleaved to about 50% in *caspase-6*$^{-/-}$ cells (Fig. 4.2B). This later observation is consistent with the fact that *caspase-6* is known to be partly involved in the cleavage of B-type lamins.

Was the lack of lamin A in DT40 the reason why *caspase-6* deficeint cells displayed no diffrence in apoptosis when compared to wild type DT40 cells? Ideally we wanted to transfect caspase-6 deficient DT40 cells with chicken lamin A cDNA and assess *in vivo* the role of caspase-6 in lamin A cleavage. However, no chicken lamin A was available and, after an attempt of transfecting DT40 cells with human lamin A, the localisation and processing of human lamin A in chicken cells was not convincing. Therefore, to answer this question we turned to use of an *in vitro* cell-free system (see below and Chapter II Section II E2 of Chapter II).

IV C. Analysis of apoptosis in *caspase-6*$^{-/-}$ cells in a cell free system

In order to further examine the role of lamin cleavage in nuclear disassembly, a cell-free system in which HeLa cell nuclei were induced to undergo apoptotic morphological changes in the presence of extracts from wild type and *caspase-6*$^{-/-}$ DT40 cells was used. To perform this analysis, cytosolic extracts (Lazebnik et al., 1993) were prepared from wild-type, *caspase-6*$^{+/-}$ and *caspase-6*$^{-/-}$ DT40 cells following exposure to etoposide or staurosporine for varying lengths of time.

Figure 4.2: Lamin B1 and B2 cleavage

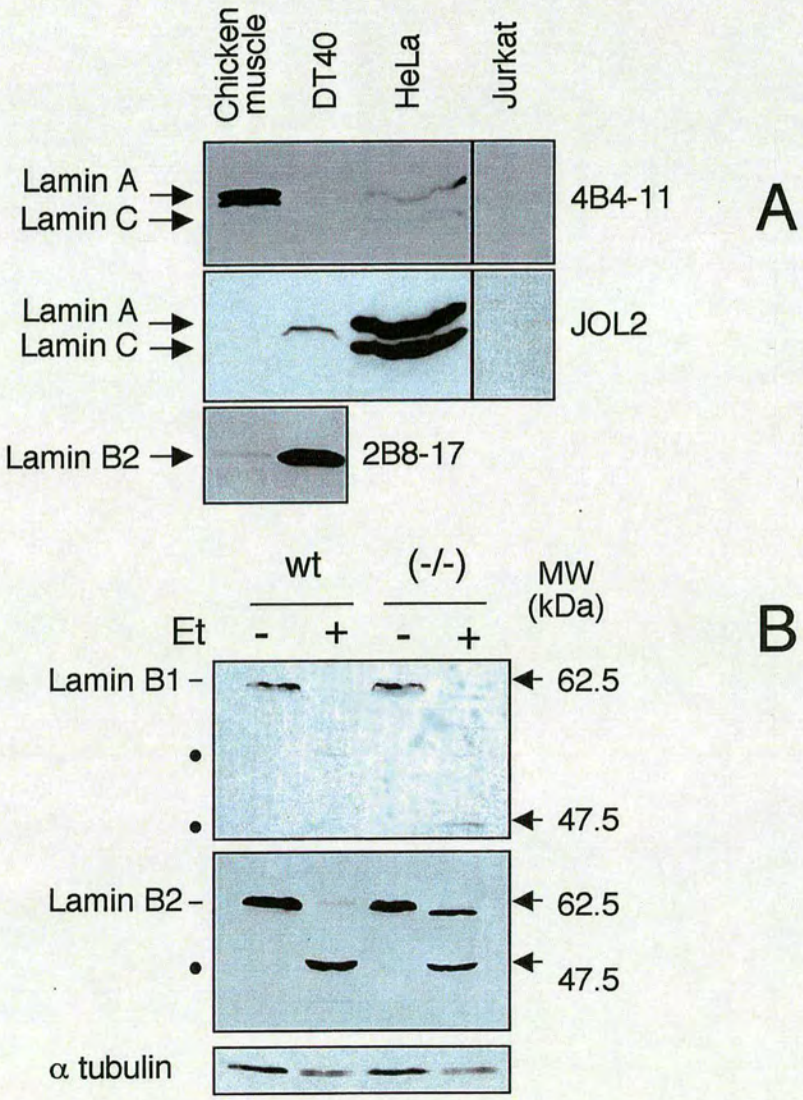


Fig 4.2: **A,** Immunoblot analysis of DT40, Jurkat and HeLa lysates together with chicken muscle tissue lysate using the monoclonal antibody to human laminA/C (JOL2), chicken lamin a (4B4-11) or chicken lamin B2 (2B8-17), ***B,*** Immunoblot analysis of wild type DT40 and *caspase-6*$^{-/-}$ cells treated or not treated with 10 μM etoposide at for 4h, using monoclonal antibodies to chicken lamin B1 and B2 (clone L-5 and 2B8-17 respectively). The cleavage products are shown by black dots.

IV C1. Characterization of cytosolic extracts

Extracts were characterized in terms of caspase-6 expression and activation, and for caspase activity. Immunoblotting analysis revealed that the caspase-6 in apoptotic extracts is processed: active caspase-6 (large subunit) appeared at 20 kDa in both the wild type and heterozygous extracts (Fig. 4.3A). Labeling of caspases using zEK(bio)D-aomk confirmed the strong activation of several caspase species in these extracts, with a major labeled band appearing at ~20 kDa. Caspase-6 and caspase-3 have been shown to be the major effector caspases activated during apoptosis (Faleiro et al., 1997; Martins et al., 1997b). Interestingly, the overall level of caspase labeling with zEK(bio)D-aomk was significantly reduced in *caspase-6*$^{-/-}$ extracts relative to that seen in wild type and *caspase-6* $^{+/-}$ extracts (Fig. 4.3A). This could indicate either that *caspase-6* is the major caspase activated in DT40 cells under these conditions, or that *caspase-6* is required for the activation of other caspases in these cells. In an independent approach, *caspase-6*-like activity was assessed in the laboratory of Professor Scott Kaufmann at the Mayo Clinic, Rochester, MN, USA, by measuring VEID-AFC cleavage (Fig 4.3B). This activity was substantially decreased in extracts from the *caspase-6*$^{-/-}$ cells. Although a low level of activity remained, control experiments (Fig. 4.3C) indicated that cleavage of VEID-AFC by caspase-3 is sufficient to account for this. Interestingly, extracts from the *caspase-6*$^{-/-}$ cells also showed a modest reduction in the ability to cleave DEVD-AFC, a substrate usually considered to be specific for caspase-3-like enzymes. Control experiments (Fig. 4.3C), indicated that loss of cleavage of DEVD-AFC by caspase-6 might account for this decrease in activity, although the results shown in Fig 4.3A raise the possibility that deletion of *caspase-6* also results in a decrease in the level of *caspase-3* activation in these cells.

Figure 4.3: Characterization of caspase-6 apoptotic extracts

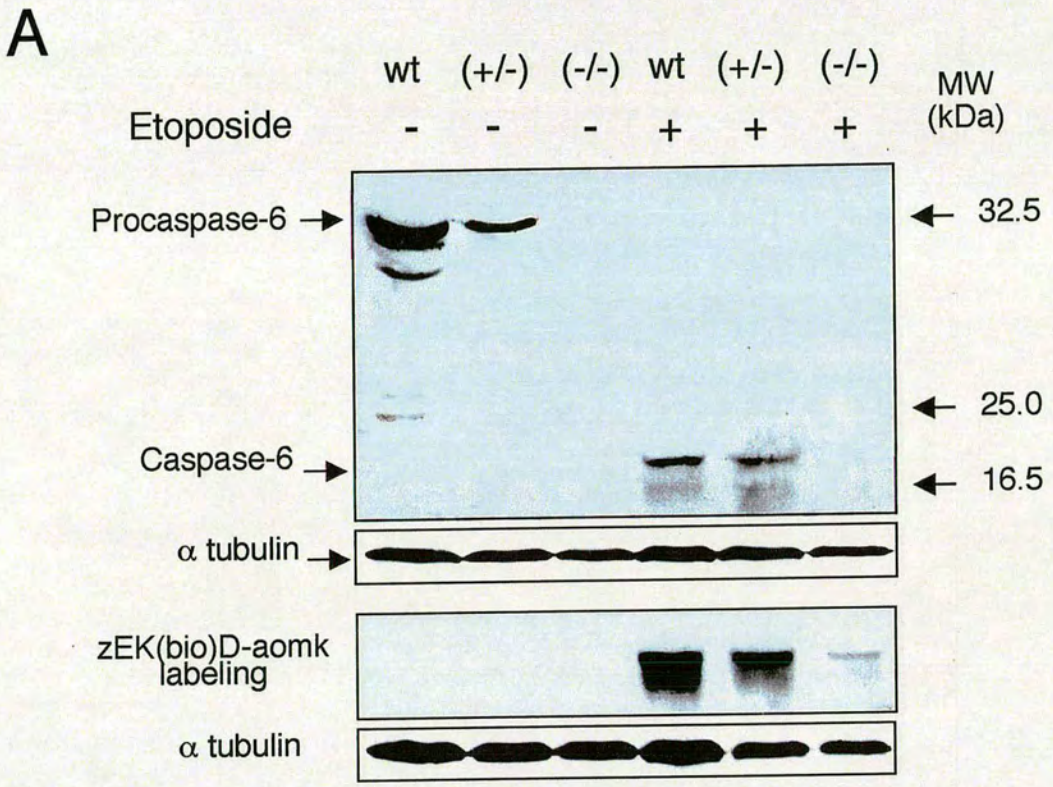


Fig 4.3 : **A,** Caspase-6 expression analysis by immunoblotting in extracts from wt, (+/-) and (-/-) cells treated with 10 μM etoposide or diluent for 5 h. When caspase-6 in the apoptotic extracts is processed, the antibody (R549) recognises the large subunit at around 20 kDa. The lower panel shows the labeling of active caspases by zEK(bio)D-aomk. α-tubulin expression is used as a gel loading control.

Figure 4.3(continued): Characterization of caspase-6 apoptotic extracts

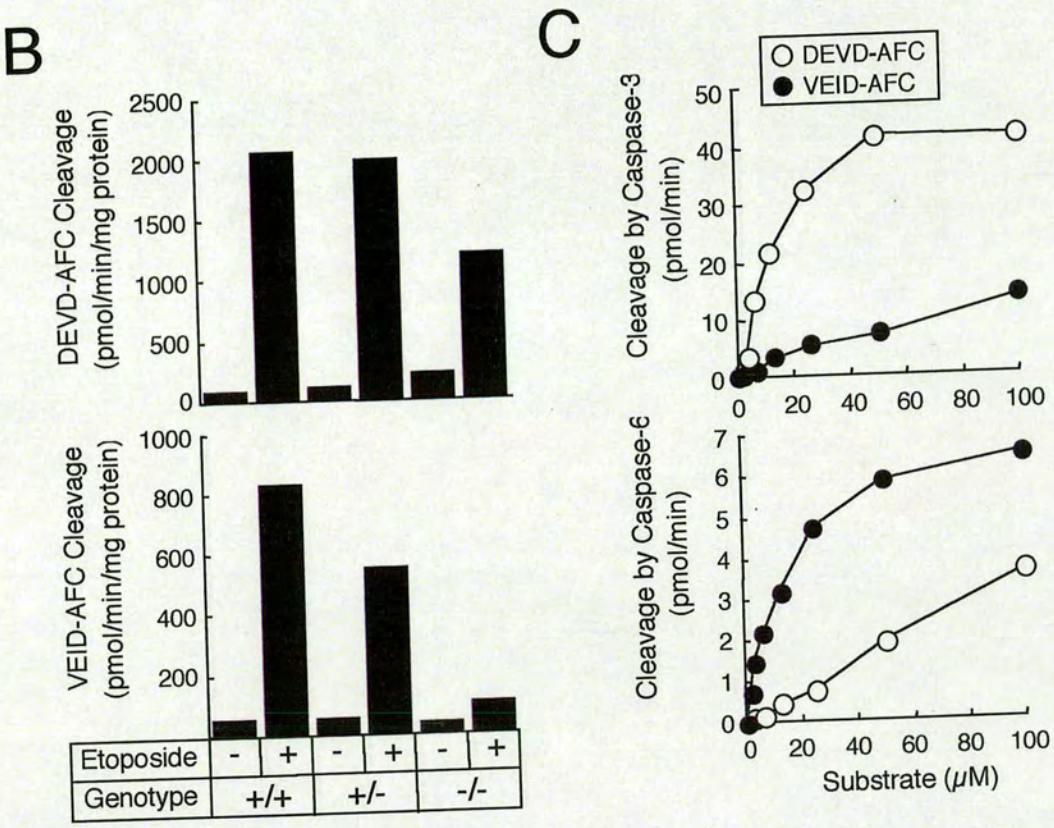


Fig 4.3: **B,** Measurement of DEVD-AFC and VEID-AFC cleavage activity in cytosol from 10 μM etoposide-treated WT, caspase-$6^{+/-}$ and caspase-$6^{-/-}$ DT40 cells. Similar results were obtained when cytosol was prepared from staurosporine-treated DT40 clones (data not shown). **C,** Evaluation of the selectivity of recombinant caspases-3 and –6 toward DEVD-AFC and VEID-AFC. Note the substantial cleavage of VEID-AFC by caspase-3 and DEVD-AFC by caspase-6 under widely used reaction conditions despite the relatively low affinity of the enzymes for these non-preferred substrates.

IV C2. Classification of apoptotic nuclei according to their apoptotic morphology

To determine whether apoptotic extracts lacking caspase-6 were able to induce apoptotic morphology, first, apoptosis in HeLa nuclei was defined as four sequential stages (I-IV) depending on the chromatin condensation stage (Fig 4.4A). Second, the morphology of nuclei following exposure to extracts from etoposide-treated wild type, heterozygote and *caspase-6*$^{-/-}$ cytosolic extracts, after 2 hours of incubation at 37°C was scored according to the chromatin condensation stage (Fig. 4,4A, left). After incubation in extracts from apoptotic wild type cells, 22 ± 11% of HeLa nuclei displayed condensation of chromatin into discrete peripheral clumps (stage III) and 78 ± 14% had been fragmented into small membrane bound vesicles (stage IV). Similar results were observed in extracts from apoptotic *caspase-6*$^{+/-}$ cells, except that more nuclei displayed discrete clumps of peripheral chromatin and fewer had fragmented into apoptotic bodies. On the other hand, the *caspase-6*$^{-/-}$ extracts revealed clear defects in chromatin condensation and formation of apoptotic bodies in HeLa nuclei. In addition to a low percentage (15 ± 2%) of non-apoptotic nuclei, the great majority (75 ± 2%) of HeLa nuclei incubated in the *caspase-6*$^{-/-}$ apoptotic extracts appeared to be blocked in an early stage of chromatin condensation (stage II) after 2 h incubation and remained in this stage for up to 5 h (data not shown). The same effect was observed when a relatively selective inhibitor of caspase-6, z-VEID-fmk, was added to wild type apoptotic extracts: 70 % of HeLa nuclei were blocked at a similar early stage of chromatin condensation (Fig 4.4A). Despite these changes in chromatin condensation, this experiment failed to detect any defect in oligonucleosomal DNA fragments in *caspase-6*$^{-/-}$ extracts (Fig. 4.4B).

Figure 4.4 :Cell free system/Scoring of apoptotic nuclei and lamin cleavage

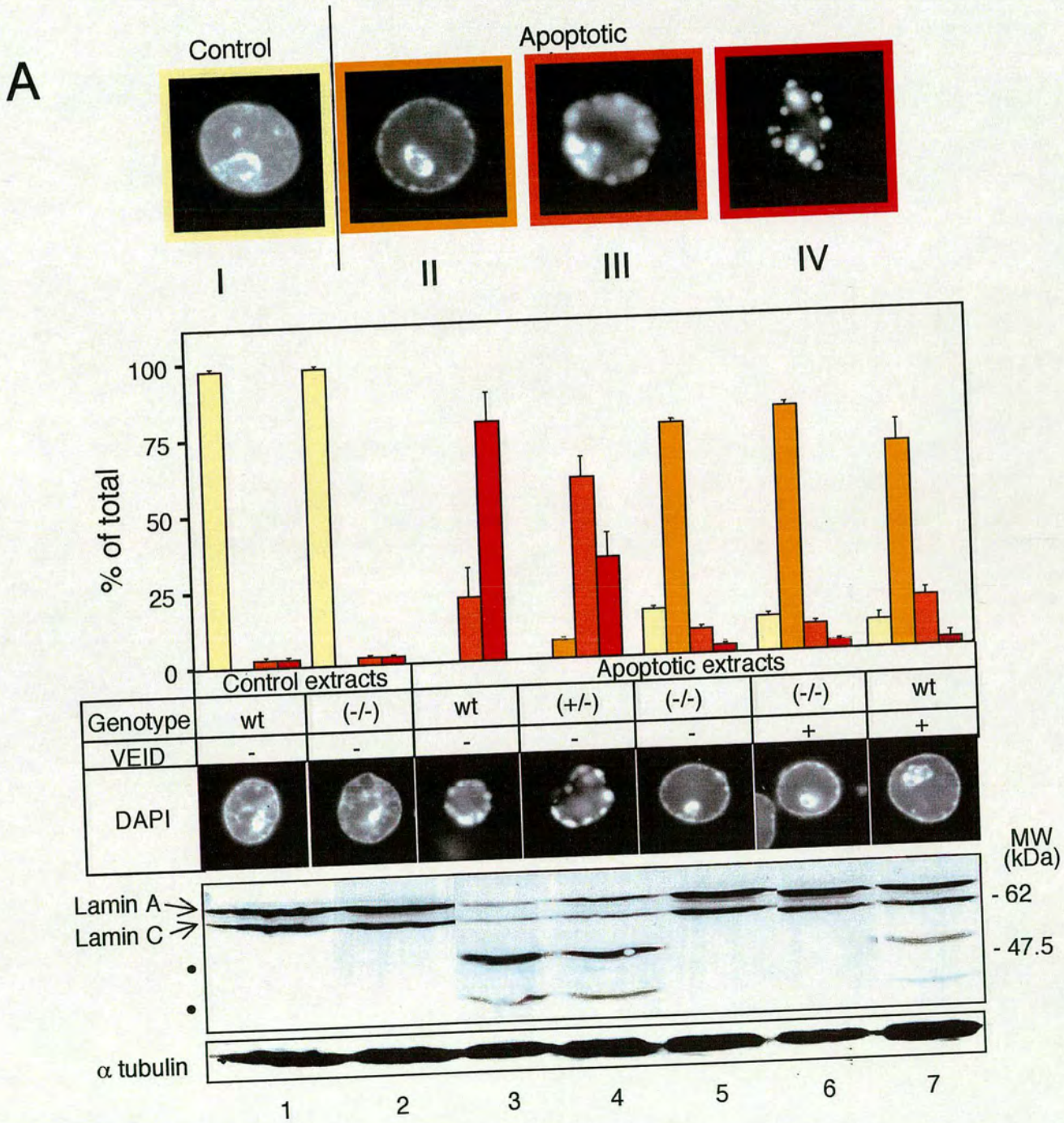


Figure 4.4 (continued) :Cell free system/DNA fragmentation in HeLa nuclei

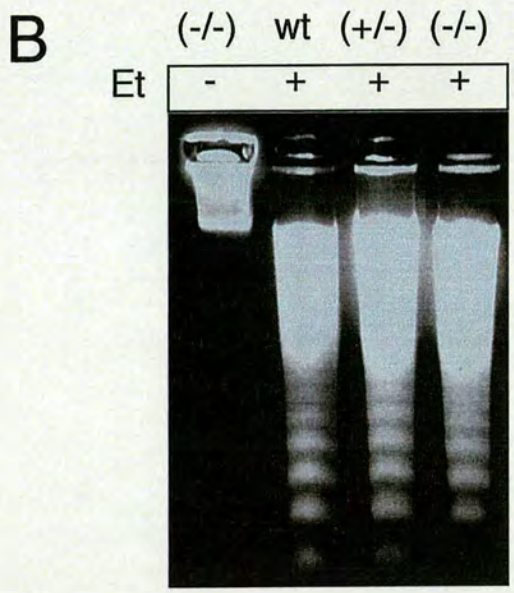


Fig 4.4: Isolated HeLa nuclei were incubated for 2 h in the apoptotic extracts previously characterized from wt, (+/-) and (-/-) cells (see Fig. 4.3) in the presence (+) or absence(-) of the caspase-6 specific inhibitor VEID-fmk at 1 μM. **A,** For each extract, the number of nuclei in apoptotic stages (I to IV) was determined. The stages were defined following DAPI staining of HeLa nuclei incubated in etoposide-treated DT40 apoptotic extracts or control DT40 extracts. HeLa nuclei incubated in control extracts define stage I. After addition to apoptotic extracts, the chromatin begins to condense against the nuclear periphery and nucleoli (stage II). Next, the peripheral chromatin ring condenses into discrete masses that separate from one another while the condensed chromatin from the nucleolus migrates to the nuclear periphery (stage III). Finally, the chromatin masses form discrete apoptotic bodies and the nuclear shape is lost (stage IV). A nucleus representative of the major population is shown for each stage. Data shown represent three independent experiments with an average of 300 nuclei counted per condition. After the 2 h incubation in the extracts, lamin A/C cleavage from HeLa nuclei was assessed by immunoblotting using the monoclonal antibody to human lamin A/C, JOL2. The cleavage products are shown by black dots. α-tubulin expression is used as a gel loading control. Controls in this experiment include extracts from untreated wild type cells, which gave 98% of uncondensed (non-apoptotic) nuclei upon incubation. The low (2%) frequency of apoptotic nuclei seen in controls reflects apoptotic cells in the HeLa population used to prepare nuclei, as this low percentage is always observed even at early incubation times. **B,** Analysis of DNA fragmentation in the HeLa nuclei after a 2 h incubation in the extracts.

IV C3. Lamin A cleavage and DNA fragmentation

Following a 2 hour incubation with cytosolic extracts from wild type and *caspase-6*$^{-/-}$ cells, HeLa nuclei were as also assessed for the state of lamin cleavage by immunoblot analysis using the JoL-2 monoclonal anti-human lamin A (Fig 4.4A). Lamin A cleavage product was present after incubation of the nuclei in cytosolic extracts with wild type and *caspase-6*$^{+/-}$ cells, but not following incubation of nuclei in the *caspase-6*$^{-/-}$ extracts.

These results strongly implied that caspase-6 is essential for the complete disassembly of HeLa cell nuclei in the *in vitro* apoptosis system.

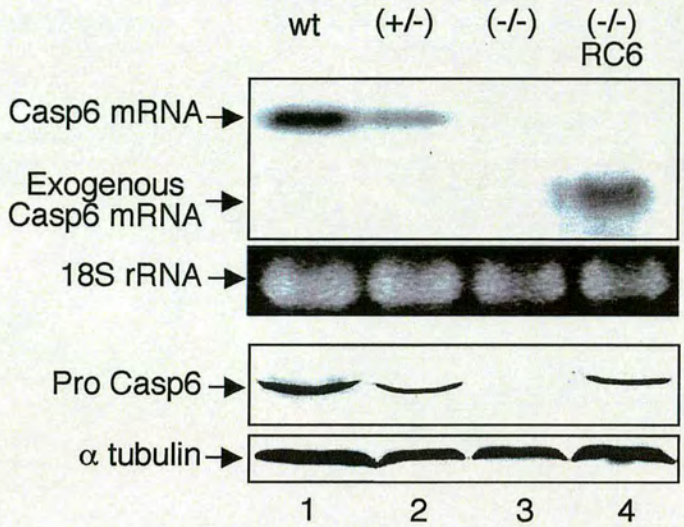
On the other hand, further investigations revealed that, unlike chromatin condensation, oligonucleosomal fragmentation of the DNA was not affected by the loss of caspase-6 (Fig 4.4B).

IV D. Apoptotic morphology of the nucleus and lamin cleavage in *caspase-6*$^{-/-}$ expressing *caspase-6* cDNA

IV D1. Generating *caspase-6*$^{-/-}$:casp-6:EGFP stable cell line

In order to confirm that the effects observed above were linked to the loss of caspase-6 function, *caspase-6*$^{-/-}$ cells were transfected with *caspase-6* cDNA. The *caspase-6* ORF was cloned into pEGFP-N1 vector, in which caspase-6:EGFP expression is under the control of a CMV promoter. Since caspase-6$^{-/-}$ cells (II26) were resistant to neomycin (see chapter III section III D1), the pEGFP-N1 vector was modified by replacing its neomycin resistant cassette by a blasticidin resistance cassette. This construct was transfected into transfect *caspase-6*$^{-/-}$ cells and blasticidin-resistant clones were isolated. More than 12 clones were picked and the expression of exogenous caspase-6 was assayed by Northern and immunoblot analysis. One of these clones (RC6) showing a level of exogenous caspase-6 expression relatively close to wild type was selected for further study (Fig 4.5).

Figure 4.5 : Transfection of caspase-6$^{-/-}$ cells with caspase-6 cDNA



Fir 4.5: Analysis of the exogenous caspase-6 expression in one transfected clone RC6. Upper panels, mRNA expression analysed by Northern blotting; lower panels, protein expression analysed by immunoblotting using polyclonal anti-caspase-6 (R549).

Figure 4.6 : Analysis of nuclear morphology in a cell free system using caspase-6$^{-/-}$:casp6:EGFP apoptotic extracts

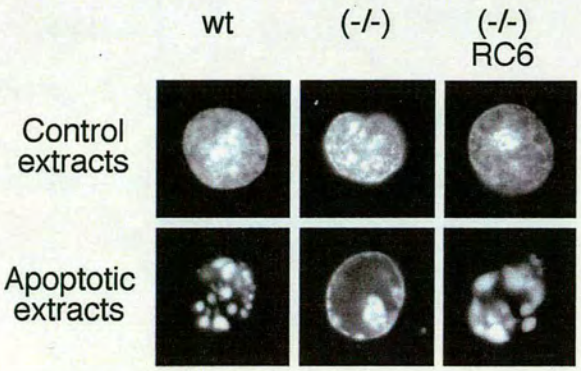


Fig 4.6: In vitro changes induced in HeLa nuclei induced after 2 h incubation with control and apoptotic extracts from wt, *caspase-6$^{-/-}$* and *caspase-6$^{-/-/RC6}$* cells

IV D2. Apoptosis analysis in the *cell free system*

Apoptotic extracts were prepared from clone RC6 following induction of apoptosis by etoposide and assayed for the ability to induce apoptotic morphological changes in HeLa nuclei *in vitro* (Fig. 4.6). After 2 h incubation, nuclei in RC6 extracts showed a level of chromatin condensation similar to those incubated in the wild type extracts, whereas the chromatin condensation was still blocked in nuclei incubated with *caspase-6*$^{-/-}$ extracts. These results showed that the exogenous caspase-6 transfected into the *caspase-6*$^{-/-}$ clone totally reversed the nuclear disassembly defects due to caspase-6 deficiency.

Results so far showed that caspase-6 is required for the completion of chromatin condensation and formation of apoptotic bodies in nuclei containing lamin A; on the other hand they do not answer the question of weather lamin A cleavage in critical for nuclear apoptosis.

IV E. Lamin A cleavage and its implication in the apoptotic morphology of the nucleus

IV E1. Apoptotic morphology of Jurkat nuclei incubated in apoptotic extracts from wild type and *caspase-6*$^{-/-}$ extracts

A final series of experiments was performed to determine whether the chromatin condensation defect observed in HeLa nuclei was due to the presence of uncleaved lamin A or to a requirement for caspase-6 action on some other substrate. As mentioned previously, Jurkat cells are a T lymphoma cell line, and therefore, do not express lamin A. As an initial step we compared the induction of apoptosis *in vitro* in Jurkat nuclei using extracts prepared from control or staurosporine-treated wild type and *caspase-6*$^{-/-}$ cells. As expected, after 2 h incubation in apoptotic extracts from wild type cells, Jurkat nuclei showed normal condensed chromatin and formation and the fragmentation of apoptotic nuclei. Strikingly, Jurkat nuclei incubated in *caspase-6*$^{-/-}$ apoptotic extracts also showed essentially normal chromatin condensation and nuclear disassembly (Fig 4.7) similar to the apoptotic morphology of the nuclei of caspase-6 deficient DT40 cells (see Fig 4.1).

Figure 4.7: Analysis of the morphology of Jurkat nuclei in cell free system

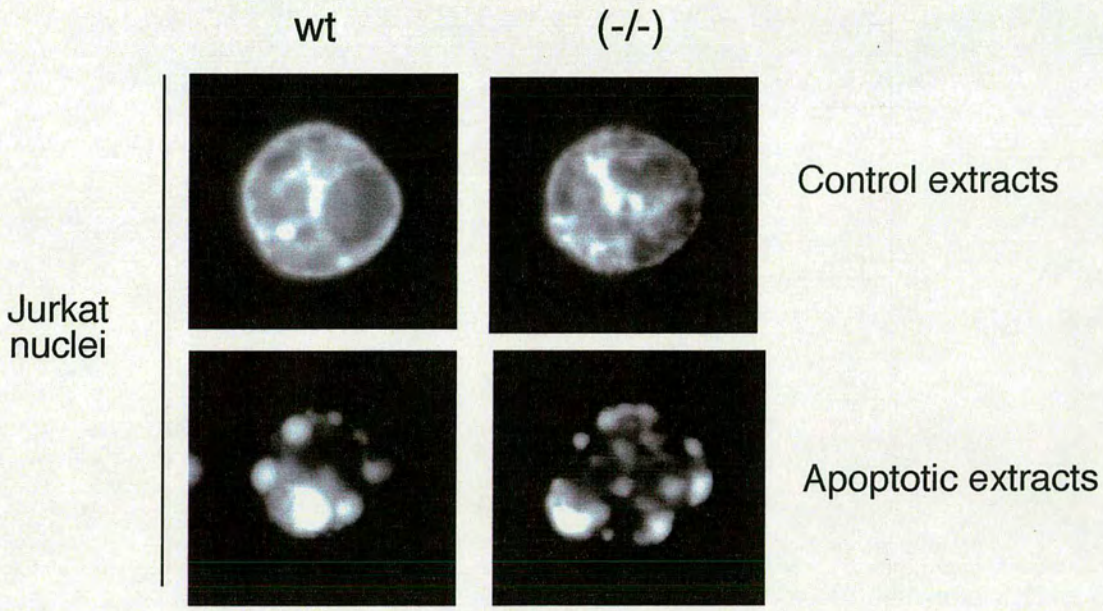


Fig 4.7: In vitro changes induced in Jurkat nuclei after 2 h incubation with control and apoptotic extracts from wild type and *caspase-6*$^{-/-}$ cells

These results indicated that a critical substrate of caspase-6 required for nuclear disassembly is not present in Jurkat cell nuclei, suggesting that this substrate could be lamin A.

IV E2. Apoptotic analysis of Jurkat nuclei expressing GFP-Lamin A incubated in apoptotic extracts from wild type and *caspase-6*$^{-/-}$ extracts

IV E2a. Nuclear morphology

To confirm the specific requirement for lamin A cleavage in apoptotic chromatin condensation and nuclear disassembly, Jurkat cells were stably transfected with a cDNA expressing GFP-lamin A (gift from Professor Larry Karnitz). This fusion protein showed the proper localization to the nuclear periphery. Jurkat nuclei expressing GFP-lamin A were then isolated and assayed, along with HeLa and normal Jurkat nuclei, for their ability to undergo chromatin condensation and nuclear disassembly in extracts from wild type and *caspase-6*$^{-/-}$ DT40 cells (Fig. 4.8B). Interestingly, nuclei of Jurkat cells expressing GFP-lamin A behaved like HeLa nuclei in the extracts. They underwent normal disassembly accompanied by cleavage of GFP-lamin A in apoptotic extracts from wild type DT40 cells, but exhibited an early block in chromatin condensation and nuclear disassembly in the *caspase-6*$^{-/-}$ extracts. Furthermore, the GFP-lamin A appeared to remain in a normal rim structure.

IV E2b. Lamin A and B1 cleavage

Similar to the HeLa assays described previously, after two hours of incubating Jurkat nuclei with the various extracts, lamin A cleavage was inspected by immunoblotting using the JoL-2 monoclonal anti-human lamin A (Fig 4.8C). As shown in Figure 4.8C Jurkat nuclei ordinarily lack lamin A (lane 4-6). When GFP:lamin A was present (lanes 7-10), it was cleaved only in the wild type apoptotic extracts (lane 8) but not in the *caspase-6*$^{-/-}$ extracts (lane 9). As expected, both HeLa and Jurkat nuclei contain lamin B1. This protein was completely cleaved in the wild type apoptotic extracts, but only partially cleaved in the *caspase-6*$^{-/-}$ extract.

Figure 4.8: Lamin A cleavage and its implications for nuclear apoptosis

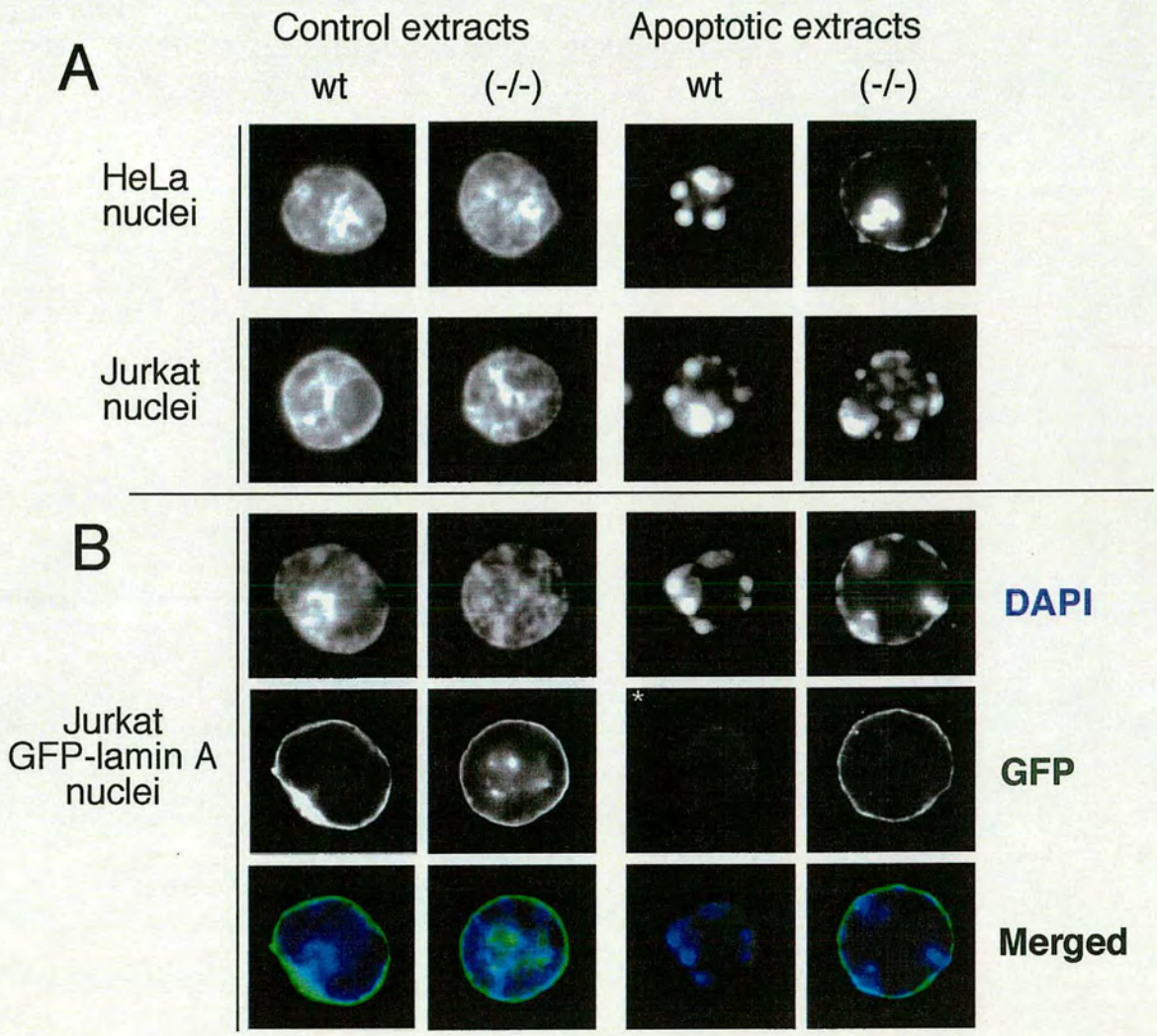


Fig 4.8: **A**, Isolated HeLa, Jurkat or Jurkat:GFP-lamin A nuclei were incubated for 2 h in extracts from wt and *caspase-6*$^{-/-}$ cells treated with 1 μM staurosporine or diluent for 8 h. **B**, A nucleus representative of the major population is shown for each condition (DAPI) along with the GFP in the Jurkat:GFP-lamin A nuclei. The signal in the panel indicated by a white star was enhanced relative that in the other panels in order to see the residual GFP-lamin A fluorescence

Figure 4.8 (continued): Lamin A cleavage and its implications for nuclear apoptosis

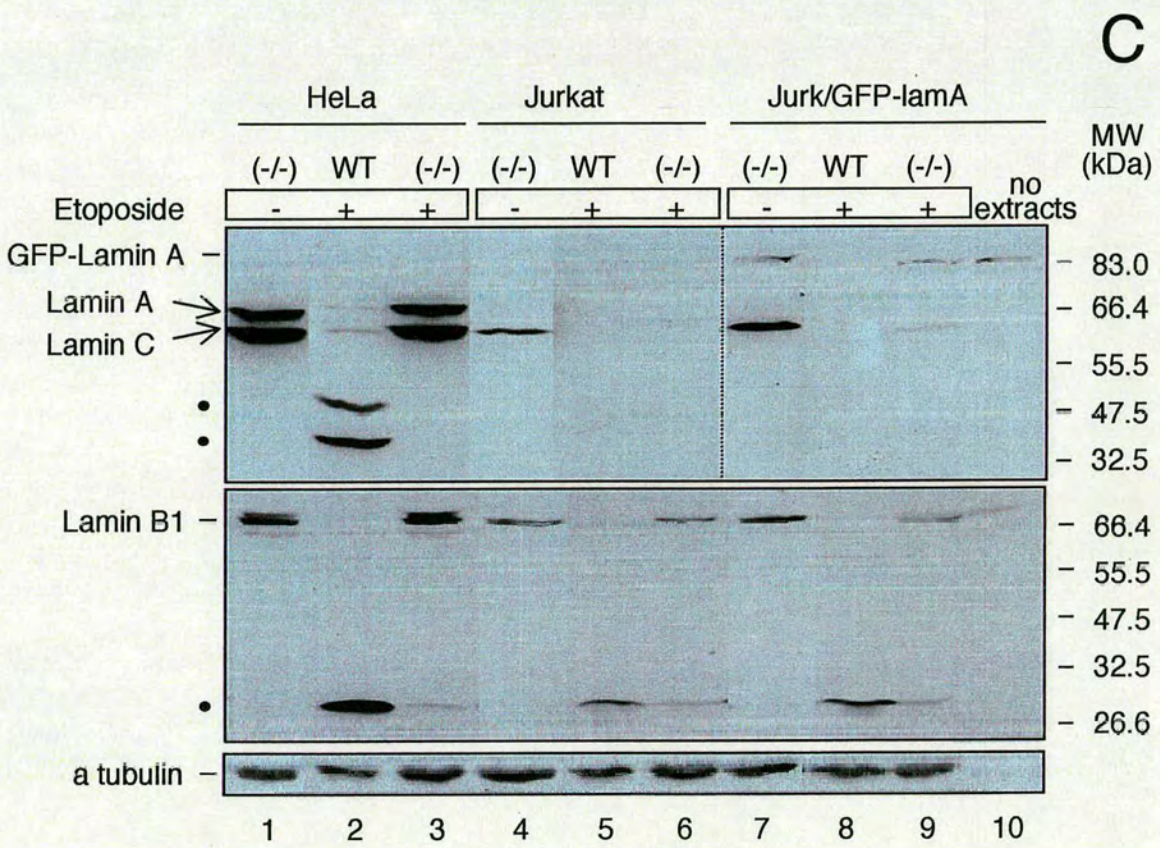


Fig 4.8 : **C,** After the 2 h incubation in the extracts, the integrity of lamin A/C and B1 from HeLa nuclei was assessed by immunoblotting using the antibody to human lamin A/C (JOL2) and the antibody to lamin B1 (clone L-5). The cleavage products are indicated by black dots. Nonadjacent lanes on the same blot have been juxtaposed to compose each panel in this figure. The signal in lanes 7-10 was enhanced relative that in the other panels in order to see the GFP-lamin A band.

IV F. Discussion

IV F1. Role of caspase-6 during apoptotic execution

Initial quests to discover the role of caspase-6 in cell death showed that the enzyme can cleave lamin A adjacent to the sequence VEID (Takahashi et al., 1996; Takahashi et al., 1997). The results presented above describe the characterisation of caspase-6 deficient cells. This study revealed that in cells lacking lamin A (DT40 and Jurkat cell lines), caspase-6 seems to be dispensable for apoptotic execution *in vivo* and *in vitro*. Previous work showed that with the exception of lamin A, which appears to be the only caspase-6 specific substrate, all other caspase-6 substrates are also cleaved by other caspases. Surprisingly, apoptotic extracts from the *caspase-6*$^{-/-}$ cells showed a reduction in the level of DEVD-AFC cleavage activity, implying a decrease in the level of activation of *caspase-3*-like enzymes. This suggests that caspase-6 in DT40 cells might act upstream to activate *caspase-3* as has already been shown in another system (Allsopp et al., 2000). To further study the role of caspase-6 in apoptotic execution we decided to investigate the effects of the loss of caspase-6 in cells that normally express lamin A

IV F2. Role of *caspase-6* in apoptotic chromatin condensation

Our *in vitro* studies using the cell free system on isolated Hela nuclei show that caspase-6 is required for chromatin condensation and proper dismantling of apoptotic nuclei in expressing lamin A. Yet, previous studies have yielded conflicting results concerning the role of caspase-6 in apoptotic chromatin condensation. The specific role of caspase-6 in chromatin condensation was stressed in one *in vitro* study when caspase-6 along with acinus were factors promoting apoptotic chromatin condensation in nuclei of permeabilized HeLa cells in the presence of active caspase-3 (Sahara et al., 1999). In contrast, examination of nuclei isolated from apoptotic 293T cells did not reveal any significant role for caspase-6 in the process (Slee et al., 2001). This finding is not contradictory with our results since it is not clear whether 293T cells express lamin A.

IV F3. Block in chromatin condensation and nuclear disassembly during apoptotic execution when lamin A is uncleaved

The major conclusion drawn from the present study is that lamin A is cleaved only by caspase-6, as opposed to other substrates that could be cleaved by caspase-6 as well as other caspases. Additionally, these results also provide new information about the role of lamin A in nuclear structure. It is documented that expression of dominant negative mutants of lamin A can perturb DNA replication (Moir et al., 2000; Spann et al., 1997) and transcription (Lourim and Lin, 1992), and models in which lamin A has a role in organising the chromatin at the nuclear periphery have become popular. However, evidence supporting these models comes primarily from *in vitro* studies, and to date, no evidence has been obtained for a role of lamin A in organising the peripheral chromatin in intact nuclei.

Published studies have suggested two possible mechanisms by which lamin A could have a role in organising the peripheral chromatin. A number of biochemical studies suggested that the tail and rod domains of lamins can bind directly to DNA, chromatin and core histones (Burke, 1990; Collard et al., 1990; Glass et al., 1993; Rzepecki et al., 1998; Taniura et al., 1995). More recent work has suggested that lamin A might make indirect interactions with the chromatin through a chain of interactions involving the LEM-family proteins emerin and LAP 2 (Lin et al., 2000). Emerin and LAP 2 interact strongly with A-type lamins (Clements et al., 2000; Foisner and Gerace, 1993; Sakaki et al., 2001). Both emerin and LAP 2 bind a recently discovered protein called BAF (barrier to autointegration factor) through interactions with their LEM domains (Furukawa, 1999; Haraguchi et al., 2001; Shumaker et al., 2001). BAF is a highly conserved general dsDNA-binding protein that forms complexes *in vitro* containing multiple BAF subunits and multiple DNA molecules (Zheng et al., 2000a). Thus, it has been proposed that BAF may serve as a DNA bridging factor. Elimination of BAF by RNAi in C. elegans gives rise to chromosome segregation defects (Zheng et al., 2000a). BAF is also required for the proper targeting of Lamin A, emerin and LAP2 to the nuclear envelope following mitosis (Haraguchi et al., 2001). Emerin binds to lamin A and BAF using distinct sites, and it has been proposed that a putative ternary complex of lamin A-emerin-

BAF could serve to tether the chromatin at the nuclear periphery (Haraguchi et al., 2001; Lee et al., 2001).

To sum up, when lamin A is present, its cleavage is required for the completion of apoptotic chromatin condensation and apoptotic body formation. In the absence of lamin A cleavage, the chromatin collapses against the nuclear periphery, but it is unable to then fully condense further into membrane bound vesicles. This behaviour of the chromatin is consistent with it remaining tethered against the nuclear envelope, and suggests that one role of lamin cleavage in its rod domain during apoptosis may be to free the chromatin from the lamina.

Therefore, lamin A cleavage appears to be essential for nuclear disassembly. Even when lamin B1 is cleaved, the uncleaved lamin A maintains a peripheral structure that tethers the chromatin. This first evidence for lamin A-dependent tethering of the chromatin at the nuclear periphery supports recent proposals that this protein may have an important role in the establishment or maintenance of nuclear architecture.

Chapter V: Analysis of *caspase-7*$^{-/-}$ DT40 cells

V A. Introduction

In parallel to the phenotypic analysis of caspase-6 deficient cell lines I started my main Ph.D. project which aimed for the elimination of the gene responsible for the production of caspase-7 in DT40 cells. As a whole, the *caspase-7* knockout project followed the approach adopted to generate caspase-6 deficient DT40 cells. Again, the targeted disruption of the *caspase-7* gene provided the tools necessary to study its particular role in the apoptotic process.

The first reports on caspase-7 date back to 1995 when Fernandes-Alnemri *et al.*, identified and characterized a CPP32/Mch2 homologue, Mch3, that was later named caspase-7 (Fernandes-Alnemri et al., 1995b). Caspase-7, like caspase-6, possesses a short prodomain and is most closely related to caspase-3, the best known player in apoptosis execution. Therefore, caspase-7 along with caspase-3 and –6, were classified as effector caspases.

Caspase-7 plays an important role in nuclear apoptotic events. First, it was reported that caspase-7 is involved in the cleavage of ICAD thus, indirectly is involved in DNA fragmentation (Nagata, 2000). Secondly, it was proposed that caspase-7, in addition to caspase-3, is responsible for PARP cleavage (Germain et al., 1999). Caspase-7 was also reported to cleave focal adhesion kinase and kinectin, the receptor for the molecular motor kinesin which is involved in microtubule-based vesicle transport and membrane trafficking (Machleidt et al., 1998; Wen et al., 1997).

Despite all the studies on caspase-7, caspase-3 is still by far the most extensively studied effector caspase, and has been shown to cleave numerous cytoplasmic and nuclear proteins (Earnshaw et al., 1999; Faleiro et al., 1997; Fernandes-Alnemri et al., 1995a). It was initially claimed that caspase-3 is the major caspase activated in dying cells, and the enzyme was placed at the apex of the apoptotic execution pathway (Nicholson et al., 1995). However, subsequent studies placed caspase-6, a

second effector caspase upstream of caspase-3 in at least one experimental system (Allsopp et al., 2000). As discussed in chapter IV, we have found that when *caspase-6*$^{-/-}$ cells were exposed to etoposide, the overall level of caspase activation was substantially decreased, stressing the point that in the DT40 B lymphoma cell line caspase-3 might not be the most apical effector caspase. Therefore, the specific role of each caspase is apparantly more complicated than first expected and having successfully disrupted the *caspase-7* gene in DT40 cells as described in chapter III we could now dissect its specific role.

V B. Phenotypic analysis of caspase-7 deficient DT40 cells

V B1. Proliferation rates

First, proliferation rates of *caspase-7*$^{+/-}$, *caspase-7*$^{-/-}$ and *caspase-7*$^{-/-}$*:casp-7:EGFP* (see below) cells were compared. As shown in Figure 5.1 heterozygote, *capase-7* deficient cells and *caspase-7*$^{-/-}$*:casp-7:EGFP* cells proliferated at a rate similar to wild type DT40 cells.

V B2. Resistance of caspase-7 deficient cells to apoptosis inducing drugs

To examine whether deletion of the *caspase-7* gene affected the resistance of the cells to proapoptotic stimuli, cell viability assays (see chapter II section II I5) were conducted after 24 h treatment using different doses of etoposide on wild type cells and two independent clones of both *caspase-7* $^{+/-}$ and *caspase-7* $^{-/-}$ cells. Both caspase-7 deficient clones were 3.5 to 4.5 times more resistant to the drug than wild type cells. The IC_{50} for wild type, *caspase-7*$^{+/-}$ and *caspase-7*$^{-/-}$ clones was 1.5, 5.2 and 7.9 μM, respectively (Fig. 5.2). Surprisingly, heterozygous clones showed a phenotype similar to the knockout cells. This resistance could explain the higher targeting efficiency obtained for the second allele if heterozygous cells were also more resistant to apoptosis during electroporation. On the other hand, it is possible it might be an outcome of clonal variability. Consequently, viability assays on two independent *caspase-7* heterozygous clones were conducted. Results of these viability assays (Fig 5.2) showed that these two *caspase-7* $^{+/-}$ clones behave similarly. This ruled out the possibility of clonal variability and confirmed that *caspase-7* is haplo-insufficient.

Figure 5.1: Growth curves of wild type, caspase-7$^{+/-}$, caspase-7$^{-/-}$ and caspase-7$^{-/-}$:casp-7:EGFP DT40 cells

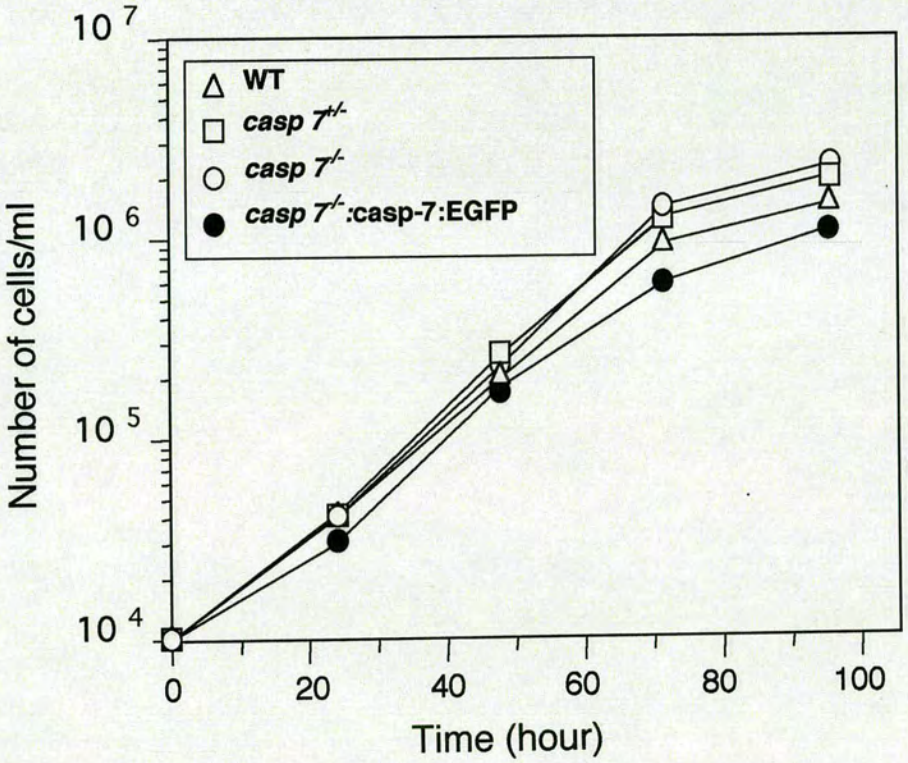


Fig 5.1: Growth curves of wild type, *caspase-7* $^{+/-}$, *caspase-7* $^{-/-}$ and *caspase-7* $^{-/-}$:casp-7:EGFP DT40 cells. Doubling time for the three cell lines was 8 h at 39°C.

Figure 5.2: Viability assays of wild type, caspase-7$^{+/-}$ and caspase-7$^{-/-}$ DT40 cells

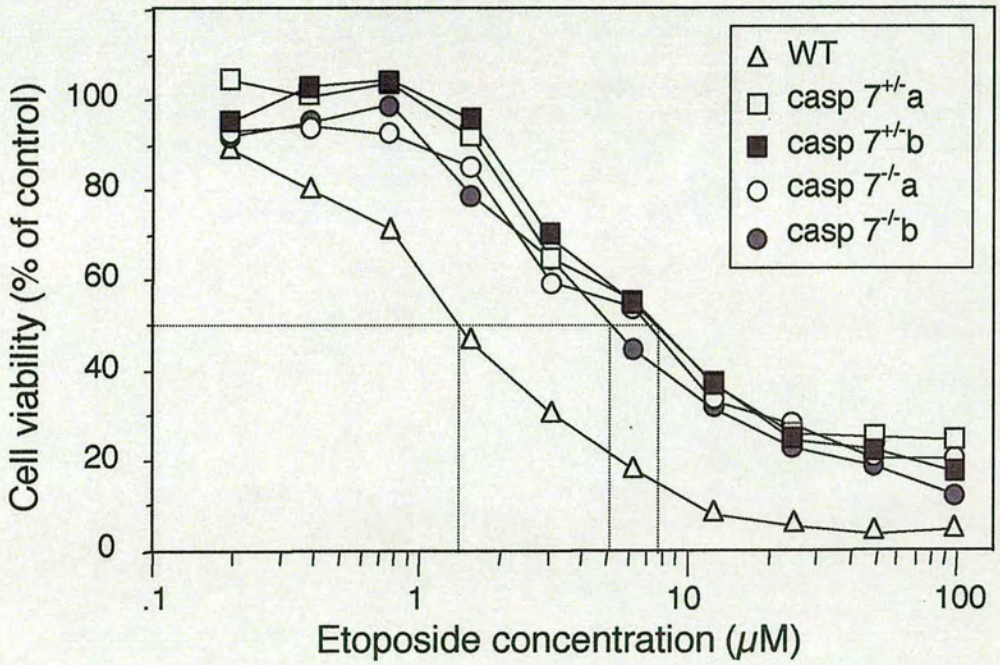


Fig 5.2: Viability of different DT40 clones 24 h after incubation with increasing doses of etoposide. (a) and (b) represent two different clones for *caspase-7$^{+/-}$* and *caspase-7$^{-/-}$* cells.

In order to confirm that the drug resistance phenotype was a direct result of the *caspase-7* gene deletion, *caspase-7*$^{-/-}$ cells were transfected with a construct containing the chicken caspase-7 cDNA fused at its C-terminus to EGFP and under the control of a CMV promoter. Clones were isolated, and expression of the exogenous caspase-7 was visualized by fluorescence microscopy (Fig. 5.3).

Cell viability assays were then performed by comparing *caspase-7*$^{-/-}$:casp-7:EGFP clones with wild type and *caspase-7*$^{-/-}$ cells (Fig. 5.4). Transfection with *caspase-7*$^{-/-}$:casp-7:EGFP restored the wild-type sensitivity to the drug treatments. The rescue of the phenotype by expression of an exogenous caspase-7 in *caspase-7*$^{-/-}$ cells confirms that the drug-resistance phenotype results from the loss of caspase-7 expression.

V C. DNA fragmentation analysis

V C1. DNA ladder formation

Since caspase-7 has been viewed as an effector caspase, terminal events in the apoptotic process in wild type and *caspase-7*$^{-/-}$ cells were examined. At various times after treatment with etoposide, cellular DNA was extracted and assessed for internucleosomal cleavage (Fig. 5.5A), a classical feature of apoptotic execution (Wyllie, 1980). The onset of DNA fragmentation was delayed approximately 2 h after the addition of etoposide in two different knockout clones relative to wild type cells. This delay was no longer visible by 3 h after the addition of etoposide.

Figure 5.3: Caspase-7$^{-/-}$:casp-7:EGFP cell line

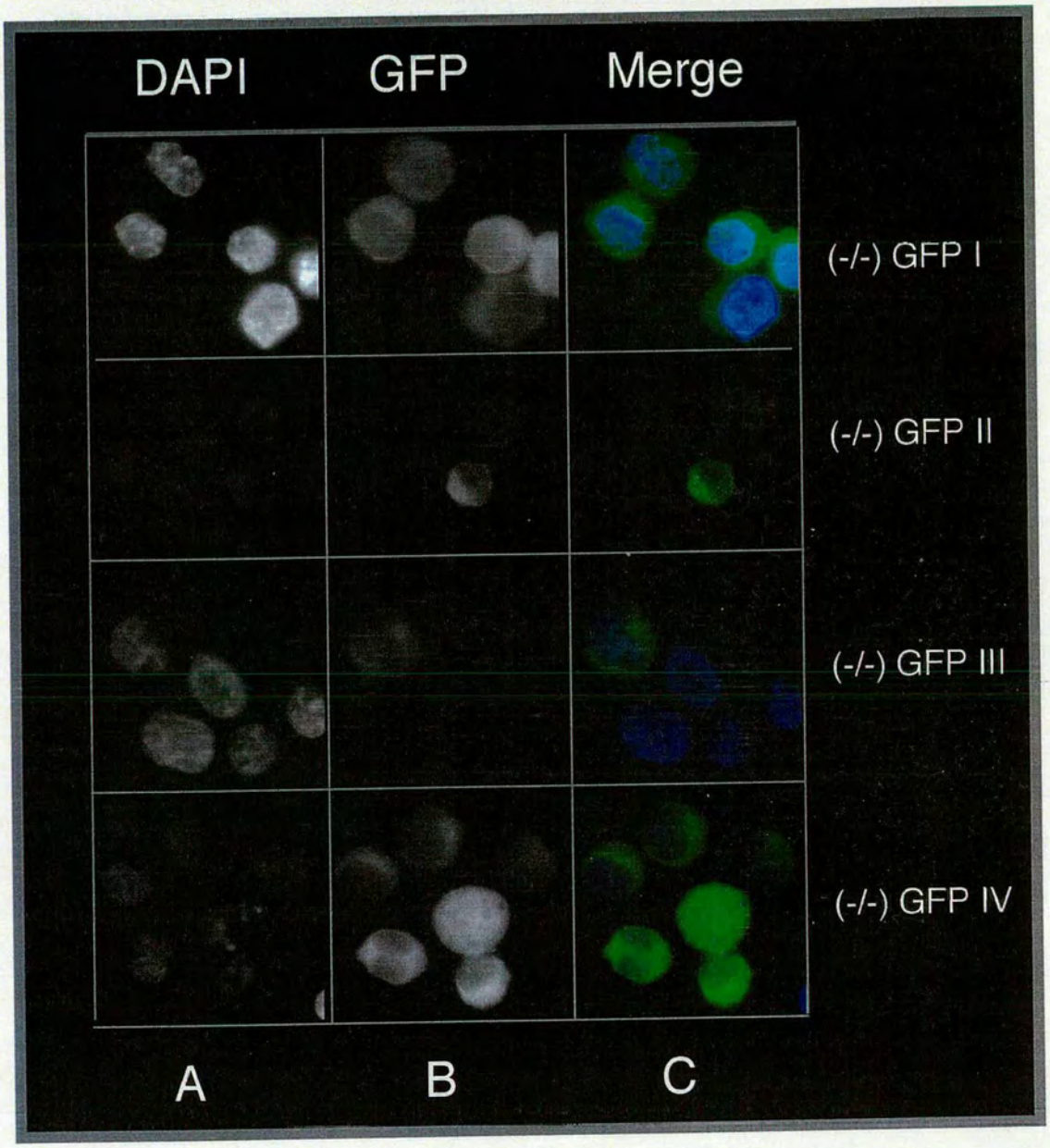


Fig 5.3: Immunofluorescence of stable *caspase-7$^{-/-}$*. casp-7:EGFP expressing cell lines. *Caspase-7$^{-/-}$*cells were transfected with *caspase-7*:EGFPN-1 (Invitrogen). Four neomycin resistant clones expressing caspase-7-EGFP with different levels are represented. (A)chromatin stained with DAPI. (B) caspase-7-:EGFP. (C) Merged pictures.

Figure 5.4: Viability assays of WT, caspase-7$^{-/-}$ and caspase-7$^{-/-}$:casp-7:EGFP DT40cells

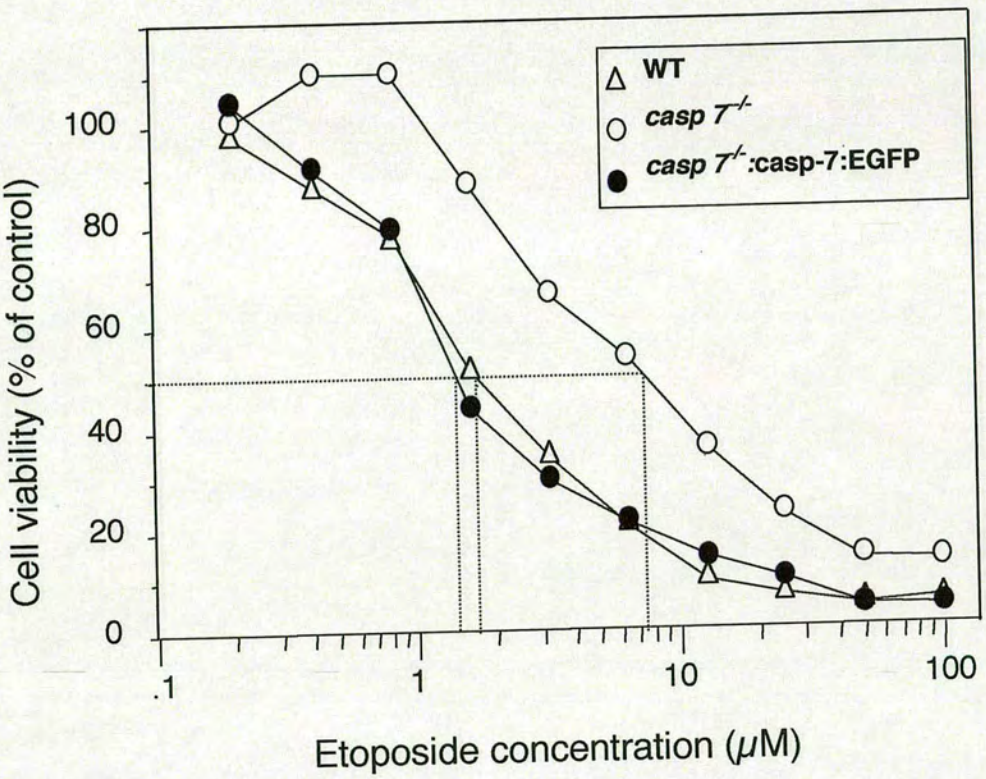


Fig 5.4: Viability assays of *caspase-7*$^{-/-}$, *caspase-7*$^{-/-}$:casp-7:EGFP clones and wild type cells 24 h after incubation with increasing doses of etoposide.

gene, as expression of the caspase-7:EGFP fusion protein in the *caspase-7*$^{-/-}$ cells largely eliminated the delay (Fig. 5.5B).

Previously it has been shown that it is the CAD/DFF40/CPAN nuclease (Enari et al., 1998; Halenbeck et al., 1998; Liu et al., 1997) and not endonuclease G (Li et al., 2001; Parrish et al., 2001) that is responsible for oligonucleosomal DNA fragmentation during etoposide-mediated apoptosis of DT40 cells (Samejima et al., 2001). Consequently, the observed delay in the nucleosomal DNA fragmentation process strongly suggests a direct or indirect involvement of caspase-7 in the cleavage of ICAD, yet caspase-7 fails to cleave ICAD (McIlroy et al., 2001) .

V D. Substrate cleavage

As stated previously in the introduction to this chapter, caspase-7 cleaves several substrates during apoptotic execution. However, it has been argued that it is caspase-3 and not caspase-7 which cleaves PARP and the lamins *in vivo*, since in apoptotic Jurkat cells, active caspase-7 localised only to the mitochondrial and microsomal fractions, however caspase-3 could be detected in the nuclear fraction (Zhivotovsky et al., 1999). Therefore, the kinetics of cleavage of lamin B1, B2 and PARP, in *caspase-7*$^{-/-}$ and wild type cells were compared.

V D1. Lamin B1 and B2 cleavage

Results of lamin B1 and B2 cleavage showed that caspase-7 is required for the normal kinetics and extent of lamin B1 and B2 cleavage in DT40 cells (Fig 5.6). In wild type cells, both lamins were totally cleaved after 3 h of 10 μM etoposide treatment. However, in the *caspase-7*$^{-/-}$ cells, lamin B1 was partially cleaved after 3 h and lamin B2 cleavage began only after 4 h. For both lamins, an uncleaved pool remained for as long as 5 h. These results could reflect either a direct involvement of caspase-7 in lamin cleavage, or they could be a result of delayed activation of other caspases that normally function downstream of caspase-7 in DT40 cells.

Figure 5.5: DNA fragmentation analysis on WT and caspase-7$^{-/-}$ cells

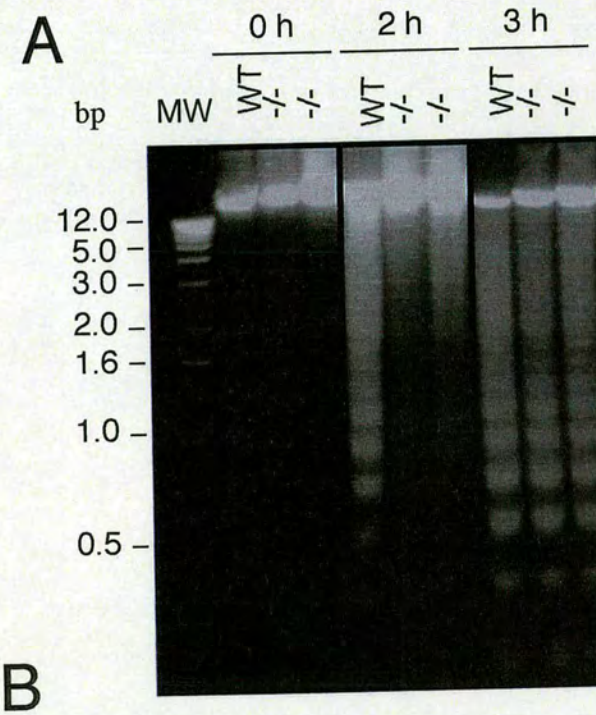


Fig 5.5: **A**, DNA nucleosomal laddering analysis during an etoposide time course on wild type cells and two different *caspase-7*$^{-/-}$ clones. **B,** Quantitative analysis of DNA fragmentation by Tunel staining measured by flow cytometry. Comparison of wild-type, *caspase-7*$^{-/-}$ and *caspase-7*$^{-/-}$:casp-7:EGFP cells following exposure to 10 μM etoposide for varying lengths of time. Data shown represent three independent experiments. For each sample, 15,000 cells were counted

Figure 5.6: Lamin B1 and B2 in apoptotic WT and caspase-7$^{-/-}$ DT40 cells

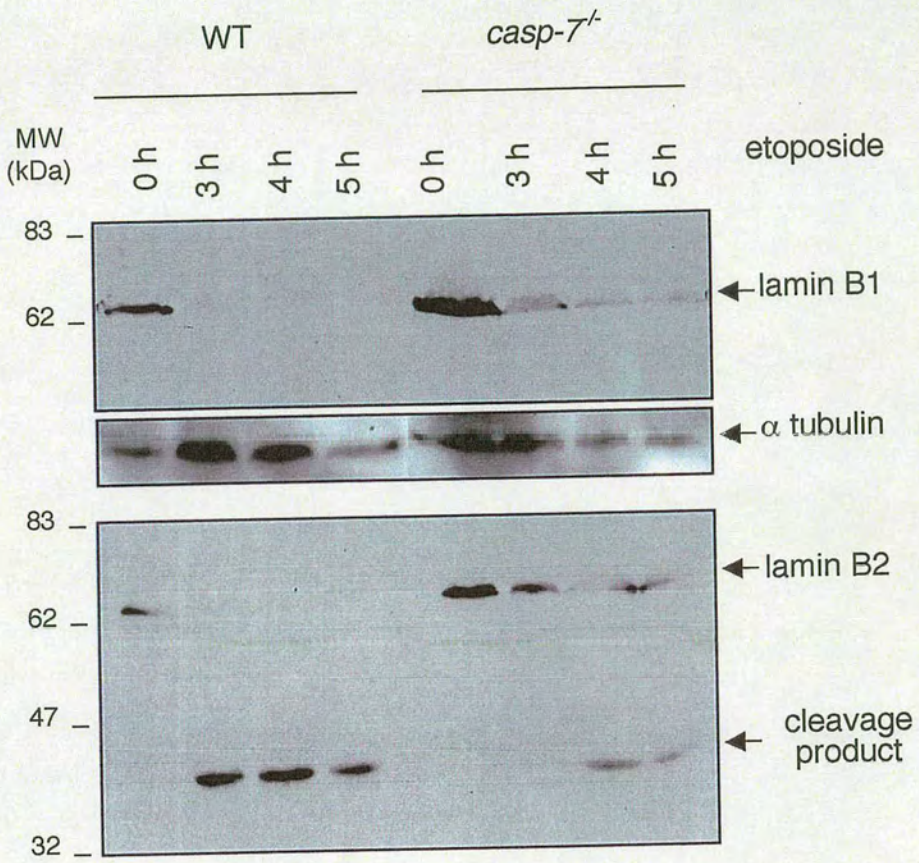


Fig 5.6 : Analysis of lamin B1 and B2 cleavage kinetics by immunoblotting of cell lysates from wild-type and *caspase-7*$^{-/-}$ cells treated with 10 μM etoposide for up to 5 h, using monoclonal antibodies to chicken lamin B1 (Zymed laboratory clone E-3) and B2 (Zymed laboratory clone L-5).

In the former case, one would expect that the slower cleavage might be a result of sub-optimal recognition of the binding site by a redundant caspase, such as caspase-3, that might recognize the binding site with lower affinity. In the latter case, it would be expected that once the downstream caspase were activated, the cleavage would proceed with normal kinetics.

V D2. PARP cleavage

To distinguish between these two models, the cell-free system was used to examine the cleavage of PARP, a classical substrate of both caspases –3 and –7 *in vitro* (Fernandes-Alnemri et al., 1995a; Kaufmann et al., 1993; Salvesen and Dixit, 1997). PARP cleavage typically takes place before lamin cleavage (Lazebnik et al., 1993; Zhivotovsky et al., 1999). Unfortunately, none of antibodies tested recognized chicken PARP. Therefore, we employed the cell-free system in which HeLa nuclei were induced to undergo apoptotic biochemical changes in extracts prepared from apoptotic wild type and *caspase7*$^{-/-}$ DT40 cells (as described in material and methods section II E2).

HeLa nuclei were added to cytosolic extracts prepared from wild-type and *caspase-7*$^{-/-}$ DT40 cells following exposure to etoposide for 1, 3 and 6 hours, these will be referred to as 1 h, 3 h and 6 h extracts. Following a 3-hour incubation at 37°C, PARP cleavage was assessed by immunoblotting (Fig. 5.7). When HeLa nuclei were incubated with 1h-extracts from wild-type cells, the majority of PARP was cleaved. Cleavage was complete in the 3h-extracts and 6h-extracts. In contrast, when HeLa nuclei were incubated in 1h-extracts from *caspase7*$^{-/-}$ cells, only a small fraction of PARP was cleaved, and some uncleaved 116 kDa protein could still be seen following the incubation in 3h-extract from *caspase7*$^{-/-}$ cells. PARP cleavage was complete following incubation of the HeLa nuclei in 6h-extract from *caspase7*$^{-/-}$ cells.

Figure 5.7: PARP cleavage kinetics in apoptotic WT and caspase-7 $^{-/-}$ DT40 cells

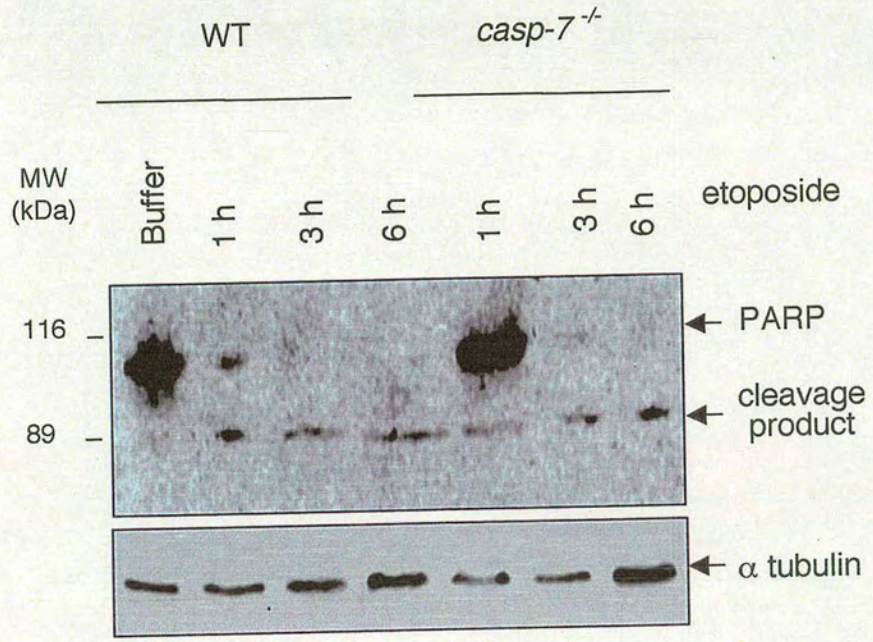


Fig 5.7: Cleavage kinetics of PARP following incubation of HeLa nuclei with extracts prepared from etoposide-treated wild-type and *caspase-7* $^{-/-}$ cells. PARP cleavage was assessed by immunoblotting with a polyclonal antibody (Cell Signalling, 9542),

V E. Loss of plasma membrane asymmetry during apoptotic execution in *Caspase-7* deficient cells

One of the hallmarks, and considered an early marker, of apoptosis is the loss of plasma membrane asymmetry which can be assessed by the externalisation of phosphatidylserine. In the presence of Ca^{++} Annexin V binds to phosphatidylserine, which is usually restricted to the inner leaflet of the plasma membrane, but which becomes randomly distributed early in apoptotic execution (Koopman et al., 1994). Although some studies have suggested that this occurs in a caspase-dependent manner (Martin et al., 1996; Vanags et al., 1996; Frasch et al., 2000) others have raised the possibility that the event can be independent of caspase activation (Ferraro-Peyret et al., 2002). The involvement of caspase-7 in this event was assessed by measuring Annexin V labeling after addition of etoposide or staurosporine to the culture. After 3 h 15 mins of etoposide induction, 50% of the wild type cells were annexin V-positive whilst knockout cells reached this level only after 5 h of etoposide treatment (Fig. 5.8A) [1]. This delay in membrane reorganisation was confirmed to result from loss of caspase-7 expression. The expression of exogenous caspase-7 rescued the phenotype, and *caspase-7* $^{-/-}$*:*casp-7:EGFP cells expressed surface phosphatidylserine with kinetics similar to wild type cells. Analogous results were obtained when the same experiment was repeated using staurosporine to induce cell death (Fig. 5.8B). However, in this latter assay, where the overall kinetics of phosphatidylserine exposure were substantially delayed, the expression of an exogenous caspase-7 in the knockout cells gave only a partial rescue of the phenotype, suggesting that the caspase-7:EGFP fusion protein may not be fully functional.

[1]PI staining was conducted, as recommended, to discriminate between apoptotic and necrotic cells. However, we were unable to get any significant results for the PI staining by FACS analysis. Therefore, for each time point, prior to collecting cells for annexin V staining/FACS analysis, cells were carefully examined under the microscope. We are confident that the annexin V positive cells, after etoposide and staurosporine treatment, were apoptotic and not necrotic DT40 cells.

Figure 5.8: Analysis of phosphatidylserine exposure at the cytoplasmic membrane

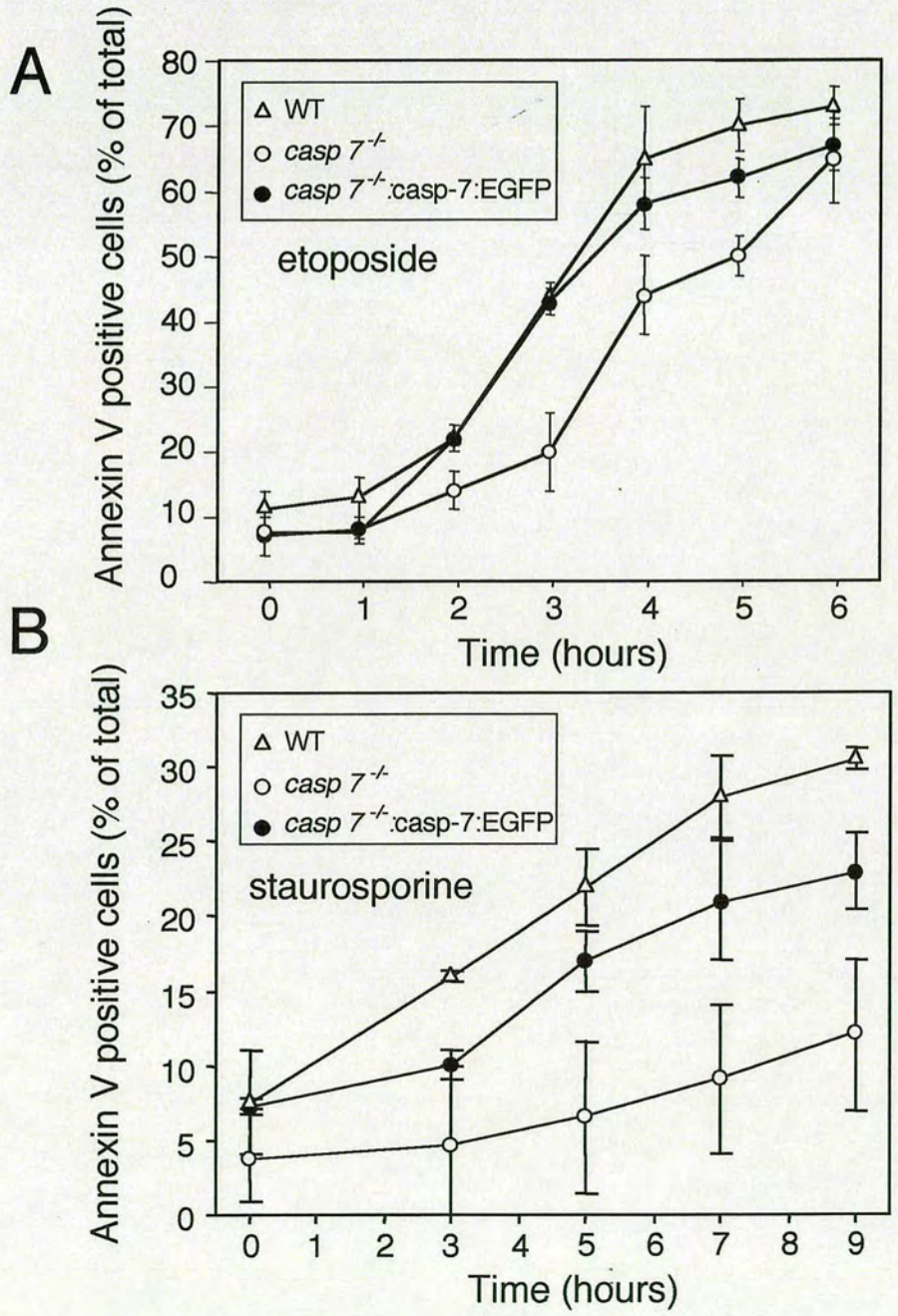


Fig 5.8: Quantitative analysis of phosphatidylserine exposure at the cytoplasmic membrane by annexin V staining measured by flow cytometry. Data shown represent three independent experiments. For each sample, 15,000 cells were counted. Wild-type, *caspase-7*$^{-/-}$ and *caspase-7*$^{-/-}$:casp-7:EGFP cells were compared following exposure to : **A,** etoposide at 10 μM for up to 6 hours; **B,** staurosporine at 1 μM for up to 9 hours.

V F. Caspase activation in caspase-7 deficient cells

To better understand the reasons behind the delays in apoptosis observed in the analysis of caspase-7 deficient cells and to further assess the possibility that caspase-7 deficiency results in altered activation of other apoptotic caspases, cytosolic extracts (see Chapter II section II E2) were prepared from wild-type, *caspase-7*$^{-/-}$ and *caspase-7*$^{-/-}$:casp-7:EGFP DT40 cells at varying times following addition of 10 μM etoposide.

Caspase activity was first assayed in extracts prepared 3 and 6 hours after addition of etoposide (Fig. 5.9). These two time points were used because, when checked using the inverted phase contrast microscope, DT40 cells started displaying an apoptotic morphology not earlier than 3 hours after etoposide addition.

Extracts were characterised in terms of overall caspase activation, and also for specific caspase activity. Broad spectrum labelling of caspases using zEK(bio)D-aomk showed the activation of several caspase species in these extracts, with a major labelled band appearing at ~20 kDa (Fig. 5.9A).

Wild type and *caspase-7* $^{-/-}$ cells exhibited no significant differences in caspase labelling after induction of apoptosis. In an independent approach, caspase-6, -3/7, and –9 -like activities were assessed in these extracts by measuring VEID-AFC, DEVD-AFC and LEHD-AFC cleavage respectively, in the laboratories of Professor Scott Kaufmann, Rochester, USA (Fig. 5.9B). Again, no significant differences were detected between wild type and *caspase-7*$^{-/-}$ cells.

Figure 5.9: Caspase affinity assays and caspase labelling assays after 3 and 6 hours of 10 μM etoposide treatment

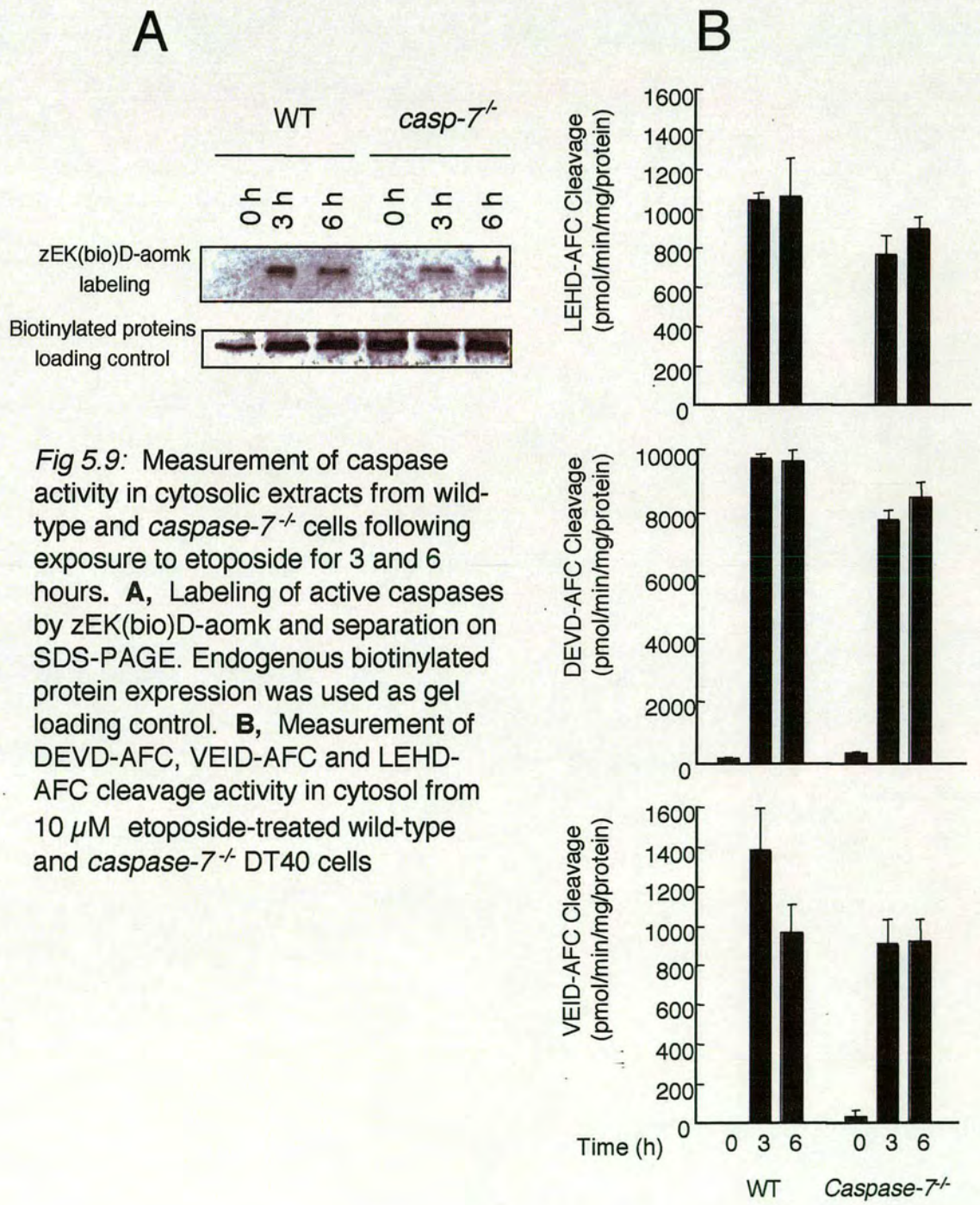


Fig 5.9: Measurement of caspase activity in cytosolic extracts from wild-type and *caspase-7*$^{-/-}$ cells following exposure to etoposide for 3 and 6 hours. **A,** Labeling of active caspases by zEK(bio)D-aomk and separation on SDS-PAGE. Endogenous biotinylated protein expression was used as gel loading control. **B,** Measurement of DEVD-AFC, VEID-AFC and LEHD-AFC cleavage activity in cytosol from 10 μM etoposide-treated wild-type and *caspase-7*$^{-/-}$ DT40 cells

Upon closer inspection, a slight decrease of about 15% was observed for the DEVD-AFC cleavage activity in the *caspase-7* $^{-/-}$ cells when compared to the wild type. We hypothesised that this might be the result of a delay in caspase activation occurring earlier than 3 h in *caspase-7* $^{-/-}$ cells. Therefore, caspase activity and caspase labelling assays using apoptotic extracts from cells treated for 30 min or 1 hour with 10 μM etoposide were repeated. Interestingly, both the caspase labelling and activity assays showed clearly that caspase activation was delayed in caspase-7 deficient cells (Fig. 5.10). In wild type cells, caspases were activated after one hour of etoposide treatment, whereas no detectable caspase activity was observed in caspase-7 deficient cells (Fig. 5.10A). Quantitative assays of caspase-6, -3/7, and -9-like activity confirmed this result. One hour after apoptosis induction with 10 μM etoposide, VEID-AFC, DEVD-AFC and LEHD-AFC cleavage was readily detected in wild type apoptotic extracts, but this was almost totally absent in *caspase-7* $^{-/-}$ extracts (Fig. 5.10B).

These results provide strong evidence for an upstream role for caspase-7 in apoptosis induction in the DT40 B lymphoma cell line.

Figure 5.10: Caspase affinity assays and caspase labelling assays after 30 min and 1 hour of 10 μM etoposide treatment

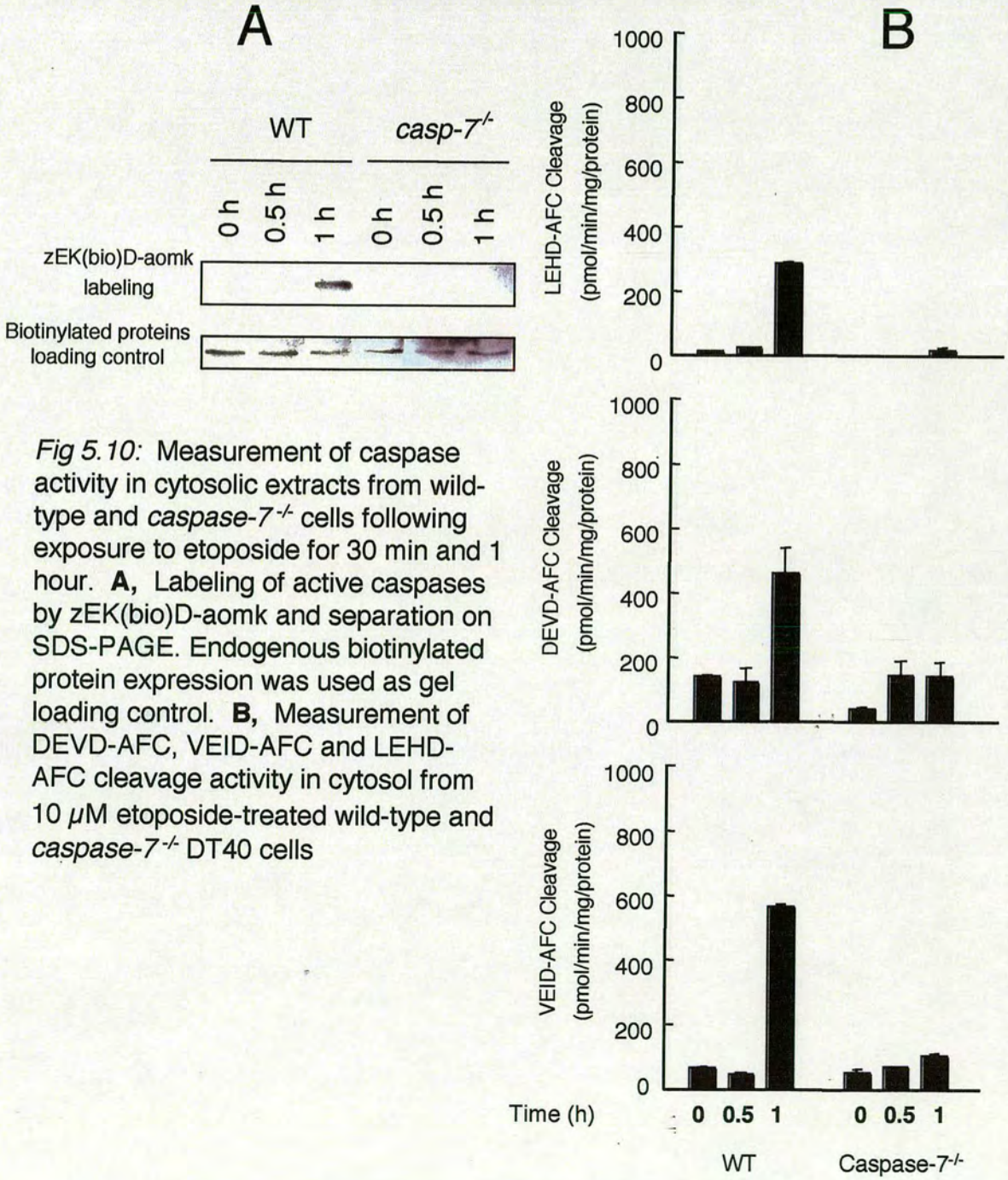


Fig 5.10: Measurement of caspase activity in cytosolic extracts from wild-type and *caspase-7*$^{-/-}$ cells following exposure to etoposide for 30 min and 1 hour. **A,** Labeling of active caspases by zEK(bio)D-aomk and separation on SDS-PAGE. Endogenous biotinylated protein expression was used as gel loading control. **B,** Measurement of DEVD-AFC, VEID-AFC and LEHD-AFC cleavage activity in cytosol from 10 μM etoposide-treated wild-type and *caspase-7*$^{-/-}$ DT40 cells

V G. Discussion

Many research groups believe that caspase-3 is the main executor caspase responsible for almost all aspects of apoptotic morphology, and that it is even required for caspase-6 and –7 activation (Bratton et al., 2000; Wolf et al., 1997). Alternatively, a variety of studies have raised the possibility that caspase-3 and caspase-7 might have redundant roles. This view is based on the similar ability of these two enzymes to cleave tetrapeptide-based synthetic substrates (Fernandes-Alnemri et al., 1996; Fernandes-Alnemri et al., 1995a; Talanian et al., 1997) and on the ability of caspase-9 to activate caspases-3 and –7 directly (Slee et al., 2001; Srinivasula et al., 1997). On the other hand, differences in the abilities of caspases-3 and –7 to cleave polypeptide substrates have been extensively described (Ghayur et al., 1996; Kottke et al., 2002; Lane et al., 2001; McIlroy et al., 1999; Slee et al., 2001). Moreover, *caspase-3* gene deletion results in viable mice that have neurological defects in some genetic backgrounds, whereas *caspase-7* gene deletion reportedly is lethal in mice (Zheng et al., 1999; Zheng et al., 2000b). These results raise the possibility that caspase-3 and caspase-7 have separate and distinct functions in a cellular context.

Results presented in this study show that *caspase-7* functions early in the apoptotic pathway induced by drugs such as etoposide and staurosporine. Because caspase-7 deficiency results in a delay in activation of caspase-3, caspase-6 and caspase-9, the results described above place caspase-7 upstream of these executioner caspases during apoptosis.

V G1. Caspase-7 is required for timely execution of apoptosis in DT40 cells

The characterisation of caspase-7 deficient cells revealed a clear delay in DNA fragmentation as concluded from DNA ladder formation and quantitative analysis of DNA fragmentation by Tunel staining. Almost certainly these results reflect a delay in the activation of DFF40/CAD/CPAN following cleavage of DFF45/ICAD, as it has been shown previously that endonuclease G has no significant role in apoptotic

DNA fragmentation in DT40 cells (Samejima et al., 2001). Moreover, the widely accepted conviction is that caspase–7, with a short prodomain, is an effector caspase activated downstream the apoptosis cascade with a role in the later stages of apoptotic execution. However, this belief has been challenged by the experiments presented here since deficient cells showed that the enzyme functions early in the apoptotic pathways induced by drugs such as etoposide and staurosporine, possibly even prior to the commitment of cells to death.

Caspase-7 deficient cells show not only a delay in loss of plasma membrane asymmetry, but were also more resistant to the common apoptosis inducing drugs. In more detail, the requirement for caspase-7 in the initiation of the apoptotic process was evident in the phophatidylserine (PS) redistribution after apoptosis induction, an early marker of apoptosis (Koopman et al., 1994). Published evidence has disagreed over whether caspases are (Martin et al., 1996), or are not (Ferraro-Peyret et al., 2002) required for phosphatidylserine exposure. Annexin V staining of caspase-7 deficient cells showed a delay of at least 90 min after 10 μM etoposide or 1 μM staurosporine treatment.

Further evidence for an important role of caspase-7 in the coordination of cell death came from the viability assays conducted. After 24 hours of etoposide treatment, at least 50% of the population of *caspase-7*$^{-/-}$ cells was still viable at drug concentrations where >90% of wild type cells and cells expressing a *caspase-7*$^{-/-}$:casp-7:EGFP construct were dead. *Caspase-7*$^{-/-}$ and *caspase-7*$^{+/-}$ cells also seemed to survive the process of electroporation and cloning better than their wild-type counterparts.

Surprisingly, *caspase-7*$^{+/-}$ cells showed the same phenotype as *caspase-7*$^{-/-}$ cells in all assays. This could be explained if caspase-7 locus is haplo-insufficient (NK & SR, unpublished data). Alternatively, one allele of the caspase-7 gene in DT40 cells may be hypermethylated, as gene imprinting has been demonstrated in chicken (McQueen et al., 2001). This is less likely, as several independently obtained heterozygotes gave identical phenotypes. However, we cannot exclude the additional possibility that targeting of the two alleles might not be random.

Importantly, all phenotypes observed in *caspase-7*$^{+/-}$ and *caspase-7*$^{-/-}$ cells were reversed upon transfection with a cDNA encoding chicken caspase-7 fused to EGFP.

In addition, further support to the requirement of caspase-7 for the timely execution of late apoptotic process came form analysis of substrate cleavage lamin B1 and B2. These results clearly demonstrate that caspase-7 is required for the timely execution of this important stage of the apoptotic pathway.

The observed delays could be due to slower rates of cleavage by a caspase with a lower affinity for each substrate if, for example, this cleavage were normally executed by caspase-7. To assess this possibility, nuclei were exposed to cytosolic extracts prepared from wild-type and *caspase-7*$^{-/-}$ cells following exposure to etoposide for 3 h, at which time the caspase cascade appeared to be equally active in both cell types. If caspase-7 were particularly effective at cleaving substrates, we would have observed diminished cleavage in caspase-7-deficient extracts in this experiment. Although previous findings have indirectly implicated caspase-7 in PARP cleavage (Germain et al., 1999; Woo et al., 1998) our observations revealed no difference in PARP cleavage in the 3 h extracts. These results support and extend a previous study in which caspase-7 was immunodepleted from Jurkat extracts (Slee et al., 2001). Results of both studies argue against the possibility that caspase-7 is required for particular apoptotic substrate cleavages. Instead, our further experiments suggest that the delayed cleavage of substrates and CAD activation observed in *caspase-7*$^{-/-}$ cells reflect an overall delay in activation of the executioner caspases.

V G2. Caspase-7 is required for early events of apoptosis in DT40 cells

Examination of the kinetics of caspase activation revealed a delay in activation of caspases-3 and –6 in *caspase-7*$^{-/-}$ cells. This delay was evident when active caspases were analyzed by affinity labeling and also when individual caspase activities were assayed using tetrapeptide-based substrates. In particular, activities that cleave DEVD-AFC, VEID-AFC and LEHD-AFC, classical substrates for caspases-3, -6 and –9, were all elevated in < 1 h in parental cells but not *caspase-7*$^{-/-}$ cells. The delay in activation of caspases-3 and –6 clearly places caspase-7 upstream of the abundant executioner caspases; and the delay in detection of caspase-9-like activity might seem to place caspase-7 upstream of caspase-9 activation as well. Consistent with this possibility, Marsden et al. recently suggested that active caspase-7 can be

detected by affinity labeling as Apaf-1-deficient cells, which lack the ability to activate caspase-9, undergo apoptosis (Marsden et al., 2002). On the other hand, the fact that the level of active caspases in *caspase-7* $^{-/-}$ cell extracts ultimately reached that in wild type cell extracts, reveals that a slower alternative mechanism of caspase activation remains intact in these cells.

V G3. An emerging view of caspase-7 function in apoptosis

A combination of direct experimental evidence and primary structure analysis has led to the view that there are two classes of caspases. Initiator caspases contain long prodomains with protein-protein interaction motifs that are responsible for targeting them to scaffolding complexes where induced proximity enables the weakly active zymogens to auto- and trans-activate (Earnshaw et al., 1999). Effector caspases lack extended prodomains, and have been thought to be activated *en masse* by the initiator caspases. The experiments described above show that caspase-7 may function much closer to the apex of the cascade than implied by this dogma.

The results presented above suggest that caspase-7 is activated before caspase-3, and may even be involved in caspase-3 activation in DT40 cells. Several other research groups have also documented similar observations. Studies of the death of MOLT-4, human leukemia cells, following exposure to ionizing radiation suggested that caspase-7 is activated prior to caspase-3 (Coelho et al., 2000), and after B cell receptor crosslinking, B lymphocytes activate selectively caspase-7. Likewise, caspase-7 (but not caspase-3) has been implicated in the cleavage of caspase-12 during endoplasmic reticulum stress (Rao et al., 2001) and in cell death occurring in the absence of Apaf-1 and caspase-9 activation (Marsden et al., 2002). Finally, a form of the caspase cascade has recently been proposed in which the down-regulation of the anti-apoptotic protein Bcl-2 leads to initiator caspase activation followed by a selective activation of caspase-7 and dismantling of the cell (Le et al., 2001).

The overall picture from the analysis of caspase-7 deficient cells strongly suggests that caspase-7 is involved earlier than other effector caspases in the apoptotic execution process in DT40 cells. However, more experiments need to be conducted to confirm that caspase-7 is indeed activated prior to any 'conventional' initiator

caspase in DT40 cells. For instance, the *caspase-7*$^{-/-}$:Casp-7:EGFP cell line could be used to assess the kinetics of caspase-7 cleavage. A time course experiment could be conducted using varied apoptosis inducing drugs, such as etoposide or staurosporine, and the activation of caspase-7 could assessed by monitoring, by western analysis, using an anti-GFP antibody. In parallel apoptotic extracts would be prepared form the same samples and the activation of initiator caspases (caspase-2, -8 and -9) would be assessed by activity assays and compared to the timing of caspase-7 activation.

The results of the experiments explained above could provide more proof to our belief that caspase-7 role in DT40 cells is rather an 'initiator caspase' contrary what has been thought. We hypothesis that DT40 cells may have two sequential pathways leading to caspase activation in response to etoposide and staurosporine treatment. The first pathway leads to the activation of caspase-7, and this leads to activation of the other caspases. In the absence of caspase-7, the second pathway is eventually activated, with a 1-2 hour delay in the amplification of the caspase cascade (Fig 5.11).

Figure 5.11: Proposed model for caspase-7 activation cascade in DT40 cells

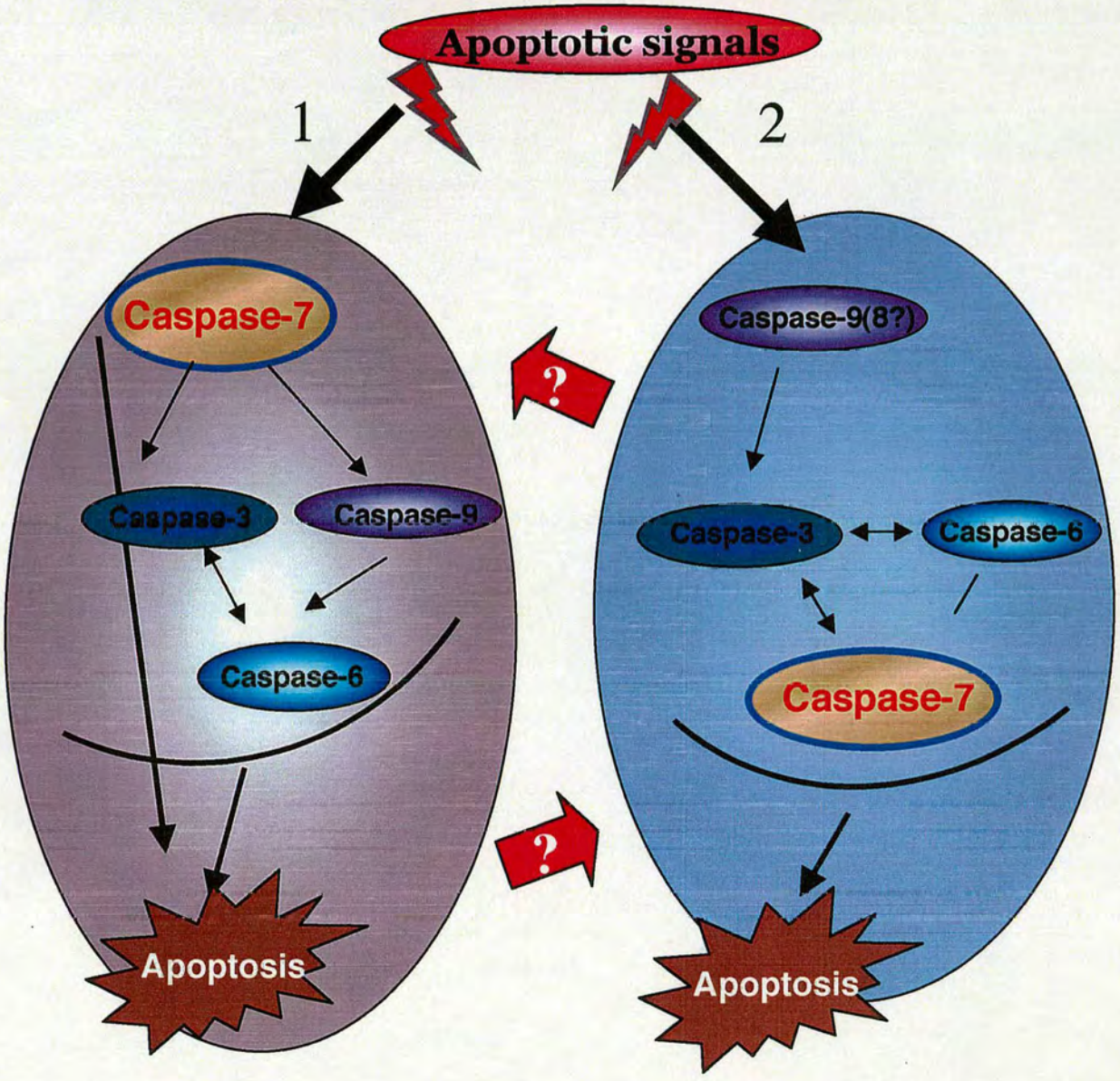


Fig 5.10: Proposed model, DT40 cells may have two sequential pathways leading to caspase activation. In the first pathway (**1**,left) caspase-7 is the most apical effector caspase capable of apoptotic execution dependently or independently of downstream capases it activates. The second pathway (**2,** right) is the conventional caspase signalling cascade . Pathway **1** is the first to be activated in normal apoptotic cells. In the absence of caspase-7, the second pathway is eventually activated, with a 1- to 2-hour delay in the amplification of the caspase cascade. Further studies are required to determine the presence and relative importance of these two pathways in other cell types.

Chapter VI: Conclusions and future directions

VI A. Aim of this thesis

The aim of this thesis was to seek a deeper understanding of specific caspase-6 and caspase-7 functions in apoptotic execution. The particular contributions, of each of these two enzymes, to apoptotic cell death had not been fully characterized. A gene knockout approach, in the DT40 cell line, was adopted to elucidate the role played by caspase-6 and caspase-7 in apoptotic cell death.

VI B. Conlusions

VI B1. Generation of *caspase-6*$^{-/-}$ and *caspase-7*$^{-/-}$ DT40 cell lines

To investigate the roles of caspase-6 and caspase-7 in apoptosis, the aim was to generate null DT40 cell lines. The two projects were conducted independently, however the strategies adopted were similar. Chicken DT40 genomic lambda FixII library was screened using parts of *caspase-6* and *caspase-7* chicken cDNA as probes. Several phages were isolated and one phage was chosen for each caspase and sent for sequencing. Targeting vectors, for the caspase in question, were generated and transfected into DT40 cells. Using Southern blot analysis as a screening strategy to identify proper targeting events, caspase-6 and caspase-7 deficient DT40 cells were isolated. The loss of caspase-6 expression was confirmed by Northern and Western blot analysis. However, unexpectedly, when Northern blot assays and RT-PCR experiments were conducted to verify the loss of *caspase-7* mRNA in caspase-7 targeted cells, they failed to detect *caspase-7* mRNA in wild type cells. The most probable explanation is that caspase-7 expression in DT40 cells is very low. Consequently, in order to confirm that the conclusions drawn from analysis of *caspase-7*$^{-/-}$ cells are indeed a result of the loss of this enzyme, caspase-7 deficient cells expressing caspase-7 fused to EGFP were used as controls in subsequent experiments.

VI B2. Analysis of Caspase-6 deficient DT40 cells

The first assays conducted were to examine and compare apoptosis of *caspase-6*$^{-/-}$ cells to wild type DT40 cells. However, when apoptosis was induced, no difference between wild type and caspase-6 deficient cells was noted in any of the apoptotic processes investigated (see chapter IV). Further characterisation of DT40 cells revealed that the B lymphoma cell line does not express lamin A. As discussed in the Introduction to this thesis and in further details in chapter IV, caspase-6 is reported to cleave lamin A specifically (Lazebnik et al., 1995b; Takahashi et al., 1996). Therefore, the next step of analysis was to assess the role of caspase-6 in apoptotic execution in cells that express lamin A, such as HeLa cells. An *in vitro* approach was adopted where cytosolic extracts were prepared from non-apoptotic and apoptotic *caspase-6*$^{-/-}$ and wild type cells and subsequently incubated with isolated HeLa nuclei. The results of this assay showed that lamin A cleavage and chromatin condensation were blocked when HeLa nuclei were incubated with *caspase-6*$^{-/-}$ cytosolic extracts, whereas nuclei were properly dismantled when incubated with wild type apoptotic extracts. Therefore, the first conclusion drawn was that caspase-6 is required for completion of chromatin condensation and formation of apoptotic bodies in nuclei containing lamin A. Logically, the next question asked was "is lamin A cleavage required for chromatin condensation and nuclear disassembly?" To answer this query Jurkat cells expressing a GFP-lamin A were used.

Jurkat cells (a human T lymphoma cell line), like DT40 cells, do not express lamin A and, therefore apoptosis proceeded normally when *caspase-6*$^{-/-}$ apoptotic extracts were incubated with isolated Jurkat nuclei. However, when Jurkat nuclei expressing GFP-lamin A were used, lamin A cleavage and chromatin condensation were blocked. These results showed that, when cells express lamin A, it must be cleaved in order for nuclear apoptosis to go to completion.

To sum up, characterisation of deficient cells indicates that caspase-6 is dispensable for the induction of apoptosis, but is required for the completion of chromatin condensation and formation of apoptotic bodies when nuclei express lamin A.

VI B3. Analysis of Caspase-7 deficient DT40 cells

To investigate the impact of lack of caspase-7 in DT40 cells, viability assays were conducted using the apoptosis-inducing drugs etoposide and staurosporine. The results of these experiments showed that *caspase-7*$^{-/-}$ cells are more resistant to apoptosis. Further examination of the apoptotic process by annexin V staining, to assess the loss of plasma membrane integrity, and Tunel staining and DNA laddering, to inquire about DNA fragmentation, revealed that *caspase-7*$^{-/-}$ cells are delayed by at least 90 min in all aspects of apoptotic execution investigated. In addition, lamin B1, B2 and PARP cleavage were delayed in *caspase-7*$^{-/-}$ cells. These results showed that caspase-7 is required for timely execution of apoptosis in DT40 cells. Surprisingly, *caspase-7*$^{+/-}$ cells showed the same phenotype as *caspase-7*$^{-/-}$ cells in all assays conducted and using several heterozygous clones to rule out the possibility of clonal viability. This result could be explained if caspase-7, which is expressed at extremely low levels in DT40 cells, is haplo-insufficient. The fact that caspase-7 and its mRNA could not be detected in wild type DT40 cells and that all phenotypes observed in *caspase-7*$^{+/-}$ and *caspase-7*$^{-/-}$ cells were reversed upon transfection with a cDNA encoding chicken caspase-7 fused to EGFP suggest that caspase-7 might well be haplo-insufficient in DT40 cells. Finally, to assess the possibility that the observed apoptotic delay in *caspase-7*$^{-/-}$ cells is a result of altered activation of other apoptotic caspases, caspase affinity labelling and caspase activity assays performed. The results of these assays showed that deficient cells exhibit a delay in caspase activation when compared to wild type DT40 cells, providing an explanation for the differences in apoptotic execution between *caspase-7* null and wild type DT40 cells.

In conclusion, the inability of any other caspase to substitute for the loss of caspase-7 underlines its non-redundant function and even implies that caspase-7 is rate limiting for the early stages of apoptosis. The results presented in this study strongly suggest that caspase-7 is involved earlier than other effector caspases in apoptotic execution.

VI C. Future directions

VI C1. A non-apoptotic role for Caspase-6

Initial gene targeting experiments suggested strongly that caspase-6 is an essential gene. The first allele targeting of the caspase-6 gene occurred with a frequency of 8 %. When the same knockout construct (with a different selectable marker) was introduced into the heterozygotes, no homologous recombinants were obtained among the 306 clones screened (all had acquired the second drug resistance marker). Remarkably, when the same experiment was performed in the absence of selection for the first marker, specific gene targeting was obtained in 16 % of the clones. In this case, all recombinants had re-targeted the first allele. When heterozygotes were transfected with cDNAs expressing caspase-6 or caspase-6: EGFP, the homologous targeting efficiency was 4% as expected. Normally, these data would be taken as rigorous evidence that caspase-6 is an essential gene, however, one of the caspase-6 deficient clones isolated (clone II26) that initially expressed caspase-6, apparently later lost the exogenous sequences. This clone was confirmed to be caspase-6 null by Southern, Northern and Immunoblotting, as well as caspase-6 activity assay but grows normally, confirming conclusively that caspase-6 is not essential for life. Therefore, the next step in the analysis of caspase-6 function would be to explore the interesting possibility that this enzyme may be essential either for homologous recombination or for some other aspect of the growth of DT40 cells under the conditions used to obtain homologous recombinants. To test this hypothesis, *caspase-6*$^{-/-}$ and wild type DT40 cells will be transfected with the ovalbumin replacement vectors (generous gift of Dr Takeda, Japan). It has been shown previously that these vectors have a remarkable 90% efficiency for proper integration when transfected to DT40 cells (Buerstedde and Takeda, 1991). Therefore, a comparison of ovalbumin targeting efficiency between *caspase-6*$^{-/-}$ cells and wild type DT40 cells might reveal a role for caspase-6 in homologous recombination.

VI C2. Caspase-7 and alternative signalling cascades during apoptotic execution

Studies of clonal deletion of autoreactive B cells by BCR (B Cell Receptor) cross-linking suggest that caspase-7 is a key player of this process (Nitta et al., 2001). IgM

treatment of immature B lymphocytes causes growth arrest and caspase-7 activation independently of caspase-8 and cytochrome c release (Ruiz-Vela et al., 1999). In addition, BCR cross-linking induces the selective activation of the effector caspase-7, but not of caspase-3 (Bras et al., 1999). DT40 cells are ideal for the study of apoptosis induced by BCR cross-linking. Ironically, clone 18, the wild type DT40 clone used in the generation of caspase-7 deficient cells, was selected as an IgM^- clone, and therefore, the apoptotic pathway in clone 18 could not be induced through the BCR pathway. However, after several rounds of cell passages, IgM^- could revert and re-express their absent surface IgM (Buerstedde et al., 1990). It will be challenging to establish the optimal conditions, if possible, for clone 18 apoptotic response to anti-IgM application and consequently assess the effect of BCR crosslinking in *caspase-7*$^{-/-}$ cells.

Likewise, caspase-7 (but not caspase-3) has been implicated in the cleavage of caspase-12 during endoplasmic reticulum (ER) stress. Therefore, caspase-7 deficient DT40 cells could be subjected to ER stress-inducing agents and apoptotic extracts would be prepared to assess for caspase activation and evaluate the role of the ER and more specifically, to comprehend the status of caspase-7 in this novel signalling cascade.

In addition to surface IgM engagement and the ER's role in apoptosis, it would be interesting to assay for a role of caspase-7 in conventional receptor mediated apoptosis cascades. Unfortunately, tools available for chicken systems are limited and human FasL or TNF are reported to be functional in chicken cell lines. Nevertheless, available ligands could be tested since it is important to query whether caspase-7 activation precedes caspase-8 activation in the receptor mediated pathway redefining the role of caspase-7, in particular the classification of effector caspases as proteins with short prodomains.

VI C3. A non apoptotic role for caspase-7

As discussed, caspase-6 may play a role in recombination (see above). Strikingly, similar but opposite to caspase-6, caspase-7 gene replacement did not follow the predicted mathematical rules which predicts that the efficiency of targeting for the

second allele should be half that of the first. The targeting efficiency for the first allele was 1.4% for both knockout constructs used (chapter III). We predicted a 0.7% efficiency for the second allele, consequently more than 200 clones were screened for second allele replacement. Surprisingly, the second allele targeting was 10% and many independent caspase-7 deficient clones were isolated. The most possible explanation for this high targeting efficiency is, if caspase-7 is haplo-insufficient in DT40 cells, *caspase-7*$^{+/-}$ are more resistant to cell death than wild type DT40 cells and therefore can better withstand electroporation procedure.

However it was interesting and unforeseen to have opposite results for *caspase-6* and *caspase-7* gene targeting and it would be challenging to investigate whether caspase-7 has any role in homologous recombination. Consequently, replacement vectors with low targeting efficiency could be transfected into *caspase-7*$^{-/-}$ and wild type DT40 cells to compare the ratios of proper integration between caspase-7 deficient and normal DT40 cells.

VI D. Final remarks

In addition, caspase-7 deficient cells could be used for subsequent biochemical assays to elucidate the functions of different domains or the effects of modifications on the enzyme function, for example to study the importance of caspase-7 phosphorylation and its impact on caspase-7 activation in the apoptotic process. Initial experiment conducted in the Earnshaw and Kaufmann laboratory showed that human caspase-7 is a phosphoprotein. Therefore, when the caspase-7 phosphorylation site is mapped, caspase-7 deficient cells could be used to reintroduce caspase-7 mutated at the phosphorylation site(s) into these cells and assess the effects on caspase-7 activation and trafficking and on the induction of apoptosis.

Finally, The results of this thesis showed that in DT40 cells caspase-6 is dispensable for apoptotic cell death and caspase-7 might be involved earlier than other caspases in apoptotic execution, yet caspase-7 is not required for cell death. We suggested that two sequential signalling cascades are activated in dying DT40 cells (see Chapter V Fig 5.11). It would be challenging to investigate whether in signalling

cascade 1 (Chapter V Fig 5.11) caspase-7 functions directly in inducing apoptotic execution or if instead its role is only to activate downstream caspases. Moreover, it will be interesting to investigate if caspase-7 could substitute for the loss of caspase-3 in both cascades 1 and 2 (Chapter V Fig 5.11). The answer to these questions requires the inhibition if caspase-3 function. Ideally this could be done using caspase-3 specific inhibitors. However, to date, it is not possible to selectively inhibit caspase-3 without affecting caspase-7. Consequently, generating caspase-$3^{-/-}$ DT40 cell lines would provide a better understanding of activation mechanisms, interaction and the role of the 'effector caspase' family (caspase-3, -6 and –7). Finally, we could take advantage of the fact that DT40 cells are ideal to perform multiple gene knockouts to further dissect the particular function of each of these caspases during the apoptotic process.

VI D. Closure

The pathways of apoptosis are constantly being redefined. This complex evolutionary mechanism is still far from being completely understood. Clearly, components of signalling cascades and other key players in apoptotic execution function in a stimulus and context dependent manner, and the specific roles of these proteins are still under rigorous investigation. The results presented in this thesis provided important new insights into the understanding of caspase-6 and caspase-7 functions during apoptotic execution.

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