Molecular Characterization of a Novel, Aberrant Type of Apoptosis Induced by the Adenoviral Death Protein E4orf4

By

Adam Szymborski
B.Sc.

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Biochemistry
McGill University
Montreal, Quebec, Canada

Your file Votre référence
ISBN: 0-612-85828-6
Our file Notre référence
ISBN: 0-612-85828-6

Abstract

The adenoviral Early Region 4 open reading frame 4 (E4orf4) protein has been shown to induce p53-independent apoptosis in transformed human cell lines. In rodent cells, E4orf4 has been shown to mediate apoptosis in the presence of a caspase inhibitor. These early results suggested that E4orf4 triggers a novel, non-classical form of apoptosis that may be independent of the core death machinery. A series of experiments were performed to analyze the activation of apoptotic machinery in response to the expression of E4orf4 in human cell lines. The analysis of E4orf4-induced cell death focused on a detailed examination of molecular mechanisms involved in the induction and execution of classical apoptosis. The results showed that E4orf4 induces moderate levels of cell killing of human cancer cells and causes some externalization of phosphatidylserine. However, E4orf4-mediated cell death is not associated with the activation of caspases or with the release of cytochrome c from mitochondria. Furthermore, poly(ADP-ribosyl) polymerase (PARP) is not proteolytically processed, mitochondrial membranes are not depolarized, and reactive oxygen species (ROS) are not generated. Thus, E4orf4 seems to induce an aberrant type of apoptosis characterized by no apparent activation of the classic molecular apoptotic machinery.

Résumé

La protéine virale E4orf4 est capable de causer l'exécution de l'apoptose dans des lignées de cellules humaines immortalisées indépendemment de la protéine p53. De plus, la protéine E4orf4 peut induire l'apoptose dans des cellules d'ovaires de hamsters (CHO) et ce malgré la présence d'un inhibiteur de caspase. Les résultats préliminaires indiquent que la protéine E4orf4 cause une nouvelle forme d'apoptose qui est possiblement indépendente de l'activation de mécanismes typiquement associés avec la mort cellulaire programmée. De nombreuses expériences ont été entreprise dans des lignées cellulaires humaines afin de déterminer l'effet de l'expression de la protéine E4orf4 sur les mécanismes intrinsèques de l'apoptose dite "*classique*". Les caractéristiques biochimiques de l'apoptose furent analysées pour étudier la mort cellulaire produite par la protéine E4orf4. Les résultats démontrent que la protéine E4orf4 cause un niveau modéré d'apoptose dans des cellules cancéreuses, de même qu'une faible externalisation de phosphatidylsérine au feuillet externe de la membrane plasmique. Par contre, la mort cellulaire induite par la protéine E4orf4 n'est pas associée avec l'activation des caspases ou de la liberation de cytochrome C des mitochondries. De plus, l'apoptose observée n'est pas accompagnée d'une dépolarisation de la membrane des mitochondries, ni d'une production de radicaux d'oxygène libres et la protéine poly(ADP-ribosyle) polymérase (PARP) n'est pas modifiée par des protéases. En conclusion, les résultats obtenus démontrent que la protéine virale E4orf4 orchestre l'exécution d'une apoptose abbérante qui n'est pas accompagnée de l'activation de mécanismes généralement observés avec cette dernière.

Acknowledgements

I would like to thank my supervisor Dr. Philip E. Branton for his support and guidance during the course of my graduate studies and for his helpful suggestions in the writing of this thesis.

I would like to thank Richard Marcellus for his original ideas relating to my project and for his assistance with my experiments.

I also acknowledge members of the Branton lab: Diana, Claudine, Patrick-Jean, Marie-Joelle, Paola, Suiyang, Scott, J.S., Olivier, Dennis and Chu for their help and support, both in and out of the lab.

I would also like to thank my parents for being there for me for the past 25 years. I could not have done it without them.

I also thank Sandi for her love, caring, and unconditional support she has given me in the last year. I want to thank her for making my life more enjoyable.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

243R	243 residue E1A protein from the 12S mRNA
289R	289 residue E1A protein from the 13S mRNA
a.a.	amino acid
Ad5	Adenovirus type 5
AIDS	Acquired Immunodeficiency Syndrome
AIF	Apoptosis Inducing Factor
ANT	Adenine nucleotide translocator
APAF1	Apoptosis Protease Activating Factor 1
AR1	Auxiliary Region 1
AR2	Auxiliary Region 2
ARF	Alternate Reading Frame
ATM	Ataxia-Telangiectasia Gene
ATP	Adenosine Triphosphate
BH	Bcl-2 Homology Region
Bp	base pair
cAMP	Cyclic Adenosine Monophosphate
CAD	Caspase-activated DNase
CAR	Coxsackievirus and Adenovirus Receptor
CARD	Caspase Recruitment Domain
Caspase	Cysteinyl Aspartate-Specific Proteinase
CBP	CREB binding protein
Cdk	Cyclin-dependent kinase
cDNA	complementary DNA
Ced	Cell Death Gene
C.elegans	*Caenorhabditis elegans*
CR1	Conserved Region 1
CR2	Conserved Region 2
CR3	Conserved Region 3
CREB	Cyclic AMP Responsive Element Binding Protein
C-terminal	carboxy terminal
CTL	Cytotoxic T lymphocyte
dATP	deoxyadenosine triphosphate
DBP	DNA Binding Protein
DcR1	Decoy receptor R1
DcR2	Decoy receptor R2
DD	Death Domain
DED	Death Effector Domain
DFF40	DNA Fragmentation Factor 40
DNA	Deoxyribonucleic Acid
DNA PK	DNA-dependent Protein Kinase
ds	double-stranded
Dox	Doxycycline

E1A	Early region 1A
E1B	Early Region 1B
E2	Early Region 2
E3	Early Region 3
E4	Early Region 4
ECL	Enhanced Chemiluminescence
EndoG	Endonuclease G
Egl-1	Egg Laying Defective-1
eIF2	Elongation Initiation Factor 2
ER	Endoplasmic Reticulum
FACS	Fluorescence Activated Cell Sorting
FADD	Fas-associated death domain
FLIP	Fas-associated death domain-like ICE inhibitory proteins
Fmk	Flouromethylketone
GC	Guanine/Cytosine
HSP	Heat Shock Protein
IAP	Inhibitor of Apoptosis
ICAD	Inhibitor of caspase-activated DNase
ICE	Interleukin-1beta-converting enzyme
I-kappaB	Inhibitor of NFkappaB
IKK	Inhibitor of I-kappaB kinase complex
IF	Immuonfluorescence
ITR	Inverted terminal repeat
JNK	Jun N-terminal kinase
kb	kilo base
kD	kilo Dalton
MDM2	Murine Double Minute 2
MHC	Major Histocompatibility Complex
mRNA	messenger RNA
NIK	NFkappaB kinase inducing kinase
NK cells	Natural Killer cells
N-terminal	amino terminal
orf	open reading frame
p53AIP1	p53 regulated apoptosis inducing protein 1
PCD	Programmed Cell Death
PARP	Poly-ADP Ribose Polymerase
PKA	Protein Kinase A
PKR	Protein Kinase R
PP2A	Protein Phosphatase 2A
PS	Phosphatidylserine
PT	Permeability Transition
PTP	Permeability Transition Pore
PTPC	Permeability Transition Pore Complex
pTP	Preterminal Protein
Rb	Retinoblastoma Susceptibility Gene Product
RAIDD	RIP-associated ICH-1 homologous protein with DD

RGD	Arginine, Glycine, Aspartic acid
RID	Receptor Internalization and Degradation Complex
RIP	Receptor Interacting Protein
RNA	Ribonucleic Acid
ROS	Reactive Oxygen species
SDS-PAGE	Sodium dodecyl Sulphate Polyacrylamide Gel Electrophoresis
Ser	Serine
Serpin	Serine Proteinase Inhibitor
ss	single stranded
TAF	TBP-associated factor
tBid	truncated Bid
TBP	TATA-binding protein
TF	Transcription factor
TNF	Tumor Necrosis Factor
TNFR	Tumor necrosis Factor Receptor
TP	Terminal Protein
TRADD	TNFR-associated death Domain
TRAF2	TNFR-associated factor 2
TRAIL	TNF-related apoptosis inducing ligand
UPR	Unfolded Protein Response
UTR	Untranslated Region
VDAC	Voltage-dependent Anion Channel
YY1	Yin Yang protein 1

1. INTRODUCTION

1.1. Apoptosis

1.1.1. Definition and historical perspective

Apoptosis, or programmed cell death (PCD), is a genetically controlled and biochemically executed process of self-destruction of metazoan cells. Apoptotic death occurs in a series of uniform events in response to various stress signals that activate an intrinsic, highly conserved cell death apparatus. Following the activation of apoptotic machinery (core death machinery), cells die within 30 minutes and are consumed by macrophages and surrounding cells (section 1.1.2).

Although the occurrence of PCD was observed numerous times over the last 150 years in studies of tissue development (Vogt 1842, Flemming, 1885), molecular mechanisms involved in regulation and execution of apoptosis have not been characterized until recently. PCD was first described three decades ago by an Australian pathologist John F. Kerr following a series of observations in his studies of hypertrophy and ischemic injury of rat livers. Kerr initially noted that dying hepatocytes did not exhibit necrotic histology, but instead shrunk and transformed into round cytoplasmic masses (Kerr, 1965). Using electron microscopy, Kerr later observed the conversion of damaged liver cells into small vesicles containing intact organelles and condensed chromatin (Kerr, 1969), which were quickly engulfed by adjacent cells, leaving no trace of the dead cell's prior existence (Kerr, 1972a). Initially, Kerr used a term "shrinkage necrosis" to describe the novel type of cell death (Kerr, 1971, 1972b), but he later replaced it with the term "apoptosis" (Kerr, 1972a) to completely differentiate between the novel physiological form of cell death and necrosis. The word "apoptosis" ("to detach and fall" in Greek) referred to the fall of leaves from flowers, which coincidentally turned out to be a programmed death process as well. Molecular mechanisms involved in the regulation and execution of mammalian apoptosis were elucidated in the last decade, following genetic

characterization of the equivalent process in a nematode organism *C.elegans* (Ellis and Horvitz, 1986, described in section 1.1.6).

1.1.2. Overview of apoptotic hallmarks

In years following Kerr's early discoveries, apoptosis has been thoroughly studied and characterized on both molecular and morphological levels. The morphological hallmarks of apoptosis were found to include chromatin condensation, cytoplasmic shrinkage, cytoplasmic membrane blebbing (Wyllie et al., 1981, Kerr, 1972a) and swelling of the outer mitochondrial membrane (Vander Heiden et al., 1997). Molecular events occurring during apoptosis include degradation of nuclear DNA (Wyllie, 1981, Brown and Rose, 1992, Oberhammer et al., 1993), release of cytochrome c from mitochondria (Kluck et al., 1997, Yang et al., 1997), proteolysis of several structural and regulatory proteins (Brancolini et al., 1995, Los M. et al., 2002, Cryns and Yuan, 1998)), depolarization of the outer mitochondrial membrane (Green and Reed, 1998), production of Reactive Oxygen Species (ROS) (Shoji et al, 1995, Jacobson, 1996), and externalization of phosphatidylserine (PS) (Fadok and Henson, 1998). End-stage apoptosis is characterized by the enclosure of cell fragments into membrane-bound vesicles termed apoptotic bodies (observed by Kerr in 1969) and their engulfment by macrophages or neighboring cells (Savill et al., 1993). Recognition and phagocytosis of apoptotic cells is mediated by binding of macrophages to several ligands on the cell surface (Savill 1997, Savill and Fadok, 2000), one of them being the externalized PS (Fadok, et al., 1998, Chimini 2001). PS is normally restricted to the inner leaflet of the membrane bilayer (Zachowski 1993, Williamson and Schlegel 1994), but translocates to the outer layer of the membrane during apoptosis and becomes exposed on the cell surface as a molecular phagocytosis marker (Schlegel and Williamson., 2001, Fadok et al., 1998, Fadok et al., 2001).

1.1.3. Distinction of apoptosis from necrosis

Necrosis is a form of cell death that lacks the well-defined morphological hallmarks and regulatory complexity seen in apoptosis (Fiers et al., 1999, Granville et al., 1998, Los et al., 2001). Necrotic cell death is a passive, non-physiological process triggered in extreme circumstances such as physical assault on the cell or severe disruption of cellular metabolism. Typical morphological features of necrosis include complete destruction of organelles, non-specific degradation of chromatin (Sun et al., 1994), and swelling of the cell membrane. The ultimate consequence of necrosis is the rupture of the cell, resulting in the release of cellular contents into the extracellular environment. The presence of released proteins in the blood stream generates a strong immune response involving the incursion of macrophages to the site of damage. Necrosis is therefore an inflammatory form of cell death that is neither genetically controlled nor biochemically executed.

1.1.4. Significance of apoptosis

The interest in the study of apoptosis grew rapidly in the last 15 years when it was realized that programmed cell death plays a very critical role in normal physiology. Physiological processes that are dependent on apoptosis include maintenance of tissue homeostasis and development of tissues and organs. Prominent examples of apoptosis in development include involution of the tadpole tail, involution of interdigital cells in the primitive limb paddle, formation of luminae and cavities, as well as deletion of neuronal cells during self-assembly of the central nervous system (reviewed in Holbrook et al., 1996). Another important role of apoptosis involves the destruction of dangerous and injured cells that would otherwise compromise the integrity of the whole organism. The elimination of non-reactive or auto-reactive T cells during the negative selection of lymphocytes proceeds via apoptosis and prevents immunological destruction of healthy tissues by self-directed cytotoxic T cells. Cells with damaged DNA and overexpressed oncogenes are removed by apoptosis to prevent neoplastic transformation leading to tumorigenic states. Similarly, cells infected with viruses are deleted to terminate viral growth and prevent potential virally-induced pathogenesis.

Deregulation of apoptotic processes has been shown to lead to some serious pathology (Thompson, 1995). Diseases linked with increased rates of apoptosis resulting in unwarranted cell death include AIDS (Hellerstein and McCune, 1997), neurodegenerative disorders such as Alzheimer's and Parkinson's diseases (Iwahash et al., 1997), ischemic injury (e.g. stroke), and some autoimmune disorders (Benoist and Mathis, 1997). On the other hand, suppression of apoptosis is associated with viral infections, some autoimmune disorders (systemic lupus erythematosus), and cancer.

1.1.5. Apoptosis in cancer

It has become clear, that together with deregulated growth, inhibition of apoptosis is a necessary condition for tumorigenesis (reviewed in Zornig et al, 2001, Green and Evan, 2002). Factors that drive cell proliferation and have a potential of triggering aberrant growth are also potent inducers of PCD (Evan and Littlewood, 1998). These include oncogenes such as Myc (Alarcon et al., 1996, Juin et al., 1999) and E1A, which readily induce apoptosis when overexpressed (section 1.1.7.2.). Apoptosis is also triggered as a result of disruption of cell-to-cell contacts, lack of sufficient nutrition, and cell detachment from the matrix (anoikis), all common to developing tumors. The suppression of apoptosis is therefore a necessary condition for both the initial neoplastic transformation of the cell as well as the expansion of the transformed clone of cells.

The evidence obtained in the study of human and mouse carcinogenesis shows that acquired resistance to apoptosis is a hallmark of most types of cancer (Strasser et al., 1997, Jaattela, 1999, Hanahan and Weinberg, 2000). Cancers have been associated with deregulation of both the inducers and inhibitors of cell death. As many as 70% of human cancers have been associated with inactivating mutations of p53 (Evan GE et al., 1995), a tumor suppressor protein involved in cell cycle arrest and induction of programmed cell death (Choisy-Rossi and Yonish-Rouach, 1998, section 1.1.7.3.). Other pro-apoptotic proteins functionally inactivated in cancers include Bax (Rampino et al., 1997, Shibata et al., 1999, Zhang et al., 2000), Bak

(Krajewska et al., 1996), CD95 death receptor (Zornig et al., 1995, Gronbaek et al., 1998, Park et al., 2001), and caspases (Teitz et al., 2000, Juin and Evan, 2000, Winter et al., 2001, section 1.1.9.). Cancers are also associated with overexpression or increased activity of endogenous cellular inhibitors of apoptosis, including Bcl-2 (Bruckheimer et al., 2000), Bcl-XL (Naik et al, 1996), and a group of caspase inhibitors termed IAP's (Ambrosini et al., 1997, Adida et al., 2000, Islam et al., 2000, section 1.1.9.4.). The overexpression of Bcl-2 due to a t(14:18) chromosomal translocation has been reported in 80% of patients with human follicular B-cell lymphoma (Strasser et al. 1997). The above-mentioned regulators of apoptosis will be discussed in section 1.1.7.

1.1.6. Evolutionary conservation of apoptotic machinery

Programmed cell death has been observed in all studied multicellular organisms, including plants, slime molds, nematodes, and insects (Vaux et al. 1994). Genetic studies of apoptosis in a worm nematode *C.elegans* have led to a complete characterization of the developmental lineage of all *C.elegans* cells as well as to the identification of key components of its apoptotic machinery. During development, 131 of the 1090 *C.elegans* progenitor cells undergo apoptotic cell death and 959 are retained in the adult organism. The study of mutations in the 131 cells committed to apoptosis has led to the discovery of several genes involved in the regulation and execution of nematode cell death. Three most important apoptotic genes include *ced* (cell death defective)-*3*, *ced-4*, and *ced-9*. Ced-3 and Ced-4 gene products are both required for apoptosis (Ellis and Horvitz, 1986), and their inactivation inhibits cell death. Ced-9 gene product is anti-apoptotic, acting to antagonize the killing activity of Ced-3 and Ced-4. The inactivation of Ced-9 leads to the widespread, lethal embryonic cell death, whereas gain-of-function mutagenesis of Ced-9 blocks apoptosis (Hengartner et al., 1992).

The functions of *C.elegans Ced* genes have been characterized (Vaux, 1997). Ced-3 protein has been shown to be a protease with a conserved cysteine residue in its active site and proteolytic specificity for aspartate residues. Ced-4 is an adapter

protein that physically interacts with both Ced-3 (Irmler et al., 1997a, Chinnaiyan et al., 1997), and Ced-9 (James et al., 1997, Spector et al., 1997, Wu et al., 1997). Binding of Ced-4 to Ced-3 promotes auto-catalytic activation of Ced-3 that is required for the induction of cell death, whereas binding of Ced-4 to Ced-9 prevents the activation of Ced-3 and blocks apoptosis (Xue and Horvitz, 1997).

Mammalian homologues of *C.elegans* Ced proteins have also been discovered. Ced-3, a highly conserved enzyme among metazoans, has been found to be homologous to mammalian ICE (interleukin 1B-converting enzyme)-like protease caspases 1, a critical player in the execution phase of apoptosis. To date, 13 other caspases have been identified (Wolf and Green, 1999, Cohen, 1997, discussed in section 1.1.9.). Ced-9 has been shown to be a homologue of mammalian proto-oncogene Bcl-2, the key inhibitor of cell death whose overexpression leads to cell transformation and tumorigenesis (section 1.1.5). The mammalian homologue of Ced-4 has been shown to be Apaf-1 (apoptotic protease activating factor), an adapter protein involved in cytochrome c-mediated auto-activation of caspases 9 (section 1.1.8.1.3.). Additionally, *C.elegans* protein egl-1 has been linked to mammalian pro-apoptotic BH3-only members of the Bcl-2 family, such as Bad or Bid, and shown to interact with Ced-9 and likely prevent Ced-9-mediated inhibition of Ced-3 (Conradt and Horvitz, 1998, section 1.1.7.1.). Similarly, the human homologue of *C.elegans* Ced-6 protein has recently been shown to promote phagocytosis of apoptotic cells (Liu and Hengartner, 1999, Smits et al., 1999).

1.1.7. Regulators of apoptosis

1.1.7.1. Bcl-2 family of proteins

Bcl-2-related proteins are the main cellular integrators of death and survival upstream signals, and perform critical functions in inhibition and activation of core death machinery. The Bcl-2 family consists of a number of proteins with structural similarity to Bcl-2, the anti-apoptotic homologue of *C.elegans* Ced-9 (section 1.1.6), and includes both pro- and anti-apoptotic members. The inhibitors of apoptosis

include Bcl-2, Bcl-Xl, Bcl-w, Bfl-1, Brag-1, Mcl-1, A1, while the promoters of apoptosis comprise Bax, Bak, Bcl-Xs, Bad, Bid, Bik, Hrk/DP5, Bim, Blk, and Bok. The relative ratio between these two subsets of proteins has been shown to control the cell's decision to survive or self-destruct (Oltvai et al., 1993). The deregulation of Bcl-2 members has been linked to a number of pathological states: overstimulation of anti-apoptotic members and inhibition of pro-apoptotic members of the Bcl-2 family has been implicated in tumorigenesis (section 1.1.5.).

1.1.7.1.1. Structure and localization of Bcl-2 family members

The defining structural feature of the Bcl-2 family members is the presence of four alpha-helical Bcl-2 homology (BH) domains. All four BH domains, designated BH1, BH2, BH3, and BH4 are conserved in the anti-apoptotic members, whereas the pro-apoptotic members possess only the first three domains and exhibit less conservation in the BH4 domain (Cory and Adams, 1998, Kelekar and Thompson 1998, Reed, 1998). The first three BH domains are involved in homo- and hetero-dimerization (Yin et al., 1994), while BH4 is not (Huang et al., 1998). Interestingly, the most potent inducers of apoptosis in the Bcl-2 family possess only the BH3 region and otherwise bear no resemblance to other members of Bcl-2 family (Strasser et al., 2000). These proteins include Bad, Bik, Bid, Hrk/DP5, Bim/Bod, Blk, and *C.elegans* egl-1. All "BH3-only" members characterized to date are pro-apoptotic.

Most members of the Bcl-2 family have a conserved C-terminal transmembrane region, which localizes these proteins to the outer leaflets of the mitochondrial membrane, nuclear envelope, or endoplasmic reticulum (Krajewski et al., 1993, de Jong et al., 1994). Cellular fractionation studies and electron microscopy analysis have shown that Bcl-2, Bcl-Xl, A1, Mcl-1, Bax, and Bak all localize to the cytosolic leaflet of these membranes (Borner et al., 1994, Zha et al., 1996). Although some of these proteins, like Bcl-2 and Bcl-Xl, are constitutively targeted to outer membranes, others are redistributed from the cytoplasm to the membrane only in response to apoptotic stimuli. The potent pro-apoptotic member Bax, for example, is normally

localized in the cytoplasm, but translocates to the surface of the mitochondrial membrane in response to stress signals (Nguyen et al., 1994, Gross et al., 1998, section 1.1.7.1.3.).

1.1.7.1.2. Post-translational modifications

An important characteristic of proteins of the Bcl-2 family is their ability to homo- or hetero-dimerize. The dimerization of Bcl-2 proteins has been shown to be mediated by the first three BH domains (Yin et al., 1994, section 1.1.7.1.1.). It has been proposed that the tendency of Bcl-2 proteins to hetero-dimerize is a mechanism for binding and inhibition of Bcl-2 proteins mediating opposite effects. This assumption has led to the "rheostat" model for Bcl-2 and Bax function, which states that the onset of cell death is dependent of the relative amounts of Bax and Bcl-2 present in the cell (Kroemer , 1997). However, recent reports show that Bax can promote apoptosis and Bcl-2 can suppress it by heterodimerization-independent mechanisms (Cheng et al., 1996, Zha and Reed, 1997).

The activity of Bcl-2 proteins is also regulated by phosphorylation. The BH3-domain-only protein Bad is phosphorylated on two Serine residues (Ser 112 and Ser 136) and sequestered to the cytosol by the 14-3-3 protein (Zha et al., 1996). Dephosphorylation of Bad following a death stimulus targets Bad to the mitochondria, where it associates with Bcl-2 and Bcl-Xl via its BH3 domain (Kelekar et al., 1997, Ottilie et al., 1997, Zha et al., 1997) and mediates its pro-apoptotic effects. Phosphorylation of Bad likely prevents the exposure of its BH3 domain and thus inhibits dimerization with anti-apoptotic members. The kinases implicated in phosphorylation of Bad include Akt and protein kinase A (PKA) (del Peso et al., 1997, Datta et al., 1997, Harada et al., 1999). Bcl-2 and Bcl-Xl, two anti-apoptotic members of the family, are also subject to phosphorylation (Maundrell et al., 1997, Ito et al 1997, Chang et al., 1997). The phosphorylation of Bcl-2 and Bcl-Xl has been shown to increase the pro-survival activity of these proteins (Ito et al., 1997, Chang et al., 1997, Poommipanit et al., 1999), although the mechanism has not been characterized to date.

Proteolysis plays a key role in regulation of one of the BH3-only members of the Bcl-2 family, Bid. The activation of Bid proceeds via caspase 8-mediated cleavage of full-length p22 Bid pre-cursor into its truncated form of 15 KD, tBid (Luo et al., 1998, Li et al., 1998). Cleavage of Bid into tBid results in the translocation of the cleaved product from the cytosol to the outer mitochondrial membrane and tBid-mediated release of cytochrome c from mitochondria (section 1.1.8.1.).

1.1.7.1.3. Bcl-2 members and mitochondria

In addition to generation of energy needed to sustain cellular functions, mitochondria are also implicated in controlling the onset of PCD (section 1.1.8.1.). Many known regulators and effectors of PCD either translocate to, or are released from mitochondria. Most Bcl-2 family members have been shown to act on mitochondria to either prevent or induce programmed cell death. The pro-apoptotic effects of tBid, described above, are mediated following its interaction with integral, anti-apoptotic mitochondrial membrane proteins Bcl-2 and Bcl-Xl. Other pro-apoptotic members, including Bax and Bak, have also been shown to translocate to the mitochondria during apoptosis (Wolter et al. 1997), where they appear to form pores and facilitate permeability transition of the outer mitochondrial membrane (section 1.1.8.1). Homodimerization of two Bax molecules and hetero-dimerization of Bax and Bak molecules is a necessary step for the insertion of these pro-apoptotic Bcl-2 family members into the mitochondrial membrane. It is unclear, however, whether Bax and Bak induce the permeability transition autonomously (Martinou JC and Green DR, 2001), or if they require interaction with the permeability transition pore complex (PTPC) (Zamzami and Kroemer, 2001, section 1.1.8.1.). In addition to their pro-apoptotic counterparts, most anti-apoptotic members of the Bcl-2 family, including Bcl-2 and Bcl-Xl, are also targeted to the mitochondrial membrane (Kroemer, 1997). The inhibition of cell death by these mitochondrial anti-apoptotic members is likely related to their ability to inhibit Bax-mediated mitochondrial membrane permeability transition (Kroemer, 1997).

□□

1.1.7.2 Oncoprotein-induced apoptosis

Another group of proteins involved in regulation of PCD includes a number of oncogene products. Overexpression of oncoproteins has been shown to be associated with increased rates of apoptotic cell death (Harrington et al., 1994, Green and Evan 2002). Oncoproteins involved in the induction of cell death include c-Myc (Evan et al., 1992), adenovirus protein E1A (White, 1993, Teodoro et al., 1995, section 1.2.6.1.), c-Jun (Bossy-Wetzel et al., 1997), and c-Fos (Preston et al., 1996), while other oncoproteins such as Raf and Ras were shown to trigger permanent growth arrest (Lloyd et al., 1997, Serrano et al., 1997).

The finding that proteins involved in driving cell proliferation also trigger an opposite process of cell death led to the hypothesis that PCD acts as an inbuilt failsafe to prevent inappropriate cell growth and division (Harrington et al., 1994, Evan and Littlewood, 1998). The coupling of contradictory pathways of cell proliferation and cell death downstream of proteins like c-Myc is considered a potent safety mechanism for the suppression of carcinogenesis (Hueber and Evan, 1998). Since the apoptosis-inducing activity of oncoproteins hinders the expansion of potentially malignant cells, c-Myc, E2F, and other oncogenes can be regarded as tumor suppressors. Myc appears to sensitize cells to a wide-range of insults on cell integrity, including nutrient deprivation, hypoxia, and DNA damage (Alarcon et al., 1996, Juin et al., 1999).

The mechanisms of oncogene-mediated apoptosis have not yet been fully elucidated. The induction of apoptosis and suppression of growth by some oncogenes, however, is known to operate through the ARF/Mdm2/p53 pathway (Sherr, 2001). Myc, E1A, Ras, and E2F all induce the expression of ARF, which binds and inactivates Mdm-2, an inhibitor of p53 (Lowe, 1999, section 1.1.7.3.). E2F-1 has also been shown to induce apoptosis by upregulation of the p53 homologue p73 (Irwin et al., 2000, section 1.1.7.3.4.), whereas Myc has been shown to transcriptionally upregulate Bax, a pro-apoptotic member of the Bcl-2 family.

1.1.7.3. Role of p53 in regulation of apoptosis

The p53 tumor suppressor protein has emerged as the key cellular sensor and integrator of a wide variety of apoptotic signals (Giaccia and Kastan, 2000, Caspari , 2000). The best characterized activities of p53 are the induction of apoptosis and inhibition of the cell cycle in challenged cells (Choisy-Rossi and Yonish-Rouach, 1998, Levine, 1997). P53 is considered to be the main guardian of cellular integrity, and not surprisingly has been found to be functionally inactivated in as many as 70% of human tumors (Evan et al., 1995, section 1.1.5.). It is also a common target for inactivation by a number of viral products during viral infections, as the induction of p53-mediated cell death would interfere with viral replication and spread (Teodoro and Branton, 1997, Roulston et al., 1999).

P53 is a short-lived nuclear protein that is continuously targeted for degradation, but becomes stabilized and abundant in response to cellular stresses such as DNA damage, viral infections, hypoxia (lack of oxygen in body tissue or tumor mass), ionizing radiation, or oncogene expression (Evan and Littlewood., 1998). P53 functions as a DNA-binding transcription factor that drives the expression of genes involved in the induction of PCD or cell cycle arrest. The suppression of the cell cycle by p53 is dependent on p53-mediated transcription of p21 (Waf1/CIP1), an inhibitor of cyclin-dependent kinases involved in driving cell proliferation (May and May, 1999). Similarly, the induction of apoptosis by p53 is mediated by a number of pro-apoptotic, p53-responsive genes (section 1.1.7.3.3.).

1.1.7.3.1. Structure of p53

P53 is a 393 a.a. DNA-binding transcription factor composed of several functional domains (Funk et al., 1992, Kern et al., 1992). The N-terminal region is involved in transactivation of p53 target genes (Unger et al., 1992, Raycroft et al., 1990), binding to the TATA-associated factors TAFII70 and TAFII31 and both subunits of TFIID (Lu and Levine, 1995, Thut et al., 1995), and in phosphorylation-mediated regulation of p53 activity (section 1.1.7.3.2.). The DNA binding domain is

located in the central part of the protein from residues 102 to 292. It is composed of a loop-sheet-helix motif and two beta-sheets that support two alpha-helical loops (Cho et al., 1994). The alpha-helical loops are stabilized by a zinc atom and form the DNA-binding surface of p53. p53 contacts two specific sets of inverted repeats of 5bp in target promoters (Kern et al., 1991). The C-terminal domain of p53, connected to its DNA binding domain by a 37 a.a.-long linker region, is involved in tetramerization of p53 molecules (Wang et al., 1994, Pavletich et al., 1993). P53 binds DNA only in its tetrameric form (Wang et al., 1995). The C-terminal domain appears to regulate DNA binding activity (Hupp et al., 1992), although this function has not been characterized in detail.

1.1.7.3.2. Regulation of p53

The regulation of p53 activity occurs at many different levels (Lakin and Jackson, 1999, Vogelstein et al., 2000). P53 regulates its own stability by a negative feedback loop involving p53-mediated expression of the Mdm2 protein (Wu et al., 1993), which binds to the p53 transactivation domain and limits p53 activity in two ways. Firstly, Mdm2 binding to the p53 N-terminal domain prevents interaction of p53 with the transcriptional machinery, thereby inhibiting the activation of p53-responsive genes (Momand et al., 1992, Lin et al., 1994). Secondly, Mdm2 targets bound p53 for ubiquitin-mediated degradation (Haupt et al., 1997). Following DNA damage, however, Mdm2 liberates p53 via three independent mechanisms. One involves the reduction in sumoylation of Mdm2 (decrease in binding of Mdm2 to protein SUMO-1), leading to the increased levels of Mdm2 self-ubiquitination (Buschmann et al., 2000). The other mechanism involves the oncoprotein-mediated upregulation of p19ARF, the product of an alternate reading frame in the INK4a locus encoding the Cdk inhibitor p16. ARF binds Mdm2 and promotes its degradation (Zhang et al., 1998), thereby preventing Mdm2-mediated proteolysis of p53. The third mechanism involves the phosphorylation in the p53 N-terminal domain at Serine 15 that reduces affinity of Mdm2 for p53 (Shieh et al., 1997).

Phosphorylation is an important mechanism of regulating p53 activity. As mentioned above, phosphorylation of p53 on Ser15 reduces Mdm2-mediated degradation of p53. Phosphorylation of Ser15 is mediated by ATM, a Serine/Threonine kinase expressed from a gene mutated in ataxia telangiectasia (hence ATM) in response to ionizing radiation (Barlow et al., 1997, Banin et al., 1998). Phosphorylation of Ser15 is also mediated by DNA-dependent protein kinase DNA-PK (Shieh et al., 1997). In addition, phosphorylation of Ser-46 controls transcriptional activation of the pro-apoptotic p53-target gene p53AIP1 (Oda et al., 2000). Apart from phosphorylation, acetylation and deacetylation have been shown to regulate p53-mediated inhibition of the cell cycle (Luo et al., 2000).

The upregulation of p53 activity also proceeds via the stabilizing interactions of p53 with other cellular proteins. The accumulation of p53 in cells deprived of oxygen (hypoxia) occurs as a result of HIF (hypoxia-inducible factor)-1 alpha-mediated stabilization of p53 (An et al., 1998). HIF-1 alpha binding to p53 has a stabilizing effect, but does not appear to affect the transcriptional activity of p53.

1.1.7.3.3. Mechanisms of apoptosis induction by p53

Transcriptional activation and repression of genes appears to be the main mechanism by which p53 induces apoptosis. P53 has been shown to transactivate as many as 107 genes and to repress 54 or more other genes that code for proteins involved in apoptosis (Zornig et al., 2001). One of the identified p53-responsive genes codes for Bax, a potent pro-apoptotic member of the Bcl-2 family (section 1.1.7.1.). The expression of Bax is transcriptionally upregulated by p53 in response to variety of stresses including DNA damage (Miyashita and Reed, 1995). Other pro-apoptotic genes transcriptionally activated by p53 include: "BH-3-only" member of the Bcl-2 family Noxa (Oda et al., 2000), Apaf-1 (Moroni et al., 2001), PUMA (Yu et al., 2001), TRAIL death receptor DR5 (Takimoto and El-Deiry, 2000), CD95 death receptor (Aragane et al., 1998, Gutierrez del Arroyo et al., 2000), MCG10 RNA binding protein (Zhu and Chen, 2000), a death domain-containing protein Pidd

(Lin et al., 2000), and a mitochondrial protein p53AIP1 (p53-regulated Apoptosis Inducing Protein 1) (Oda et al., 2000).

A non-transcriptional mechanism of induction of apoptosis by p53 may involve its shuttling properties. P53 has been shown to be involved in the transport of CD95 death receptor from the Golgi apparatus to the cell surface (Bennett et al. 1998), thereby sensitizing cells to external apoptotic stimuli.

Translocation of p53 from nucleus to mitochondria also promotes apoptosis (Marchenko et al., 2000). P53 has been shown to localize both to the surface of and within mitochondria, where it interacts with a mitochondrial protein heat shock protein Hsp70 (Marchenko et al., 2000). However, the contribution of the mitochondrion-targeted p53 to PCD is yet to be determined.

1.1.7.3.4. p53 homologues: p51/p63 and p73

Two p53-related genes, p51 (also known as p63) and p73, were recently identified as structural homologues of the p53 tumor suppressor gene (Ishida et al., 2000). The sequence similarities in DNA binding domains between p51 and p73, p51 and p53, and p73 and p53 were shown to be 87%, 60%, and 63%, respectively (Shimada et al., 1999). P51 and p73 have been shown to share similar transcriptional targets as p53, and to be able to induce apoptosis (Jost et al., 1997). The transcriptional targets of p53 homologues include 14-3-3a (Zhu J et al., 1998), Bax, p21/WAF1, and MdM2 (Shimada et al., 1999).

As with p53, the p53 homologues have been shown to accumulate in cells subjected to stress. p51 has been shown to be stabilized in response to DNA damage in a time course similar to that of p53 (Katoh et al., 2000), whereas p73 has been shown to accumulate in response to ionizing radiation (Lohrum and Vousden, 2000, Gong et al., 1999, Yuan et al., 1999).

The presence of p53 homologues in the cell may serve as a way to replace damaged p53 and thus provide an additional layer of defense against tumors. Both p51 and p73 have been shown to activate p53-responsive promoters and induce apoptosis in some tumor cells lacking wild-type p53 (Kaghad et al., 1997, Osada et

al., 1998, Jost et al., 1997, Prabhu et al., 1998). Some reports suggest that in a number of cell lines, p51 and p73 induces apoptosis even more extensively than p53 itself (Ishida et al., 2000). However, the loss of the functional p53 is still associated with a great number of human cancers (section 1.1.5.), suggesting that p73 and p51 do not complement p53 at all times. The exact roles of p53 homologues in growth suppression and cell death induction are still to be determined.

Interestingly, recent evidence suggests that viral oncoproteins such as adenoviral E1B55K, SV40 large T antigen, and papillomavirus E6, which bind and inactivate p53, do not interact with p73 (Marin et al., 1998, Roth et al., 1998, Bobblestein and Roth, 1998). Thus, p73 may serve to complement the inactivated p53 in termination of virally-infected cells.

1.1.8. Molecular apoptotic pathways

1.1.8.1. The mitochondrial pathway

In normal cells, mitochondria function as cellular power plants involved in generation of energy in the form of ATP. Recently, these organelles were also implicated in the induction of cell death. The key apoptotic events that focus on mitochondria include the regulatory activity of Bcl-2 related proteins and the release of pro-apoptotic proteins that normally reside inside the organelle (Green and Reed 1998). The characteristics of mitochondria in apoptosis include the disrupted electron transport and the loss of mitochondrial transmembrane potential (reviewed in Green and Reed 1998, Wang, 2001).

1.1.8.1.1. Apoptogenic mitochondrial proteins

The best characterized protein released from mitochondria during apoptosis is cytochrome c (Kroemer and Reed 2000, Martinou et al., 2000). Cytochrome c is normally confined to the intermembrane mitochondrial space, where it functions as an electron carrier in oxidative phosphorylation (Reed 1997). In cells exposed to

apoptotic stimuli, however, cytochrome c translocates from mitochondria to the cytosol and participates in the activation of caspase 9 (Slee et al., 1999, section 1.1.8.1.2.).

Other proteins released from mitochondria during apoptosis include Smac/Diablo, which bind and inhibit cytosolic inhibitors of apoptosis called IAPs (Verhagen et al., 2000, Du et al., 2000). Cytoplasmic Smac/Diablo thus relieve IAP-mediated inhibition of the proteolytic activation of procaspases. Homologues of Smac/Diablo, proteins Reaper, Grim, and Hid, were identified in *Drosophila* and shown to function in a similar way as Smac/Diablo (Silke et al., 2000).

Interestingly, a group of caspases, both in their pro- and cleaved forms, has been found to localize to mitochondria and to exit the organelle during apoptosis (Costantini P et al., 2002). These include caspase 2 (Susin et al., 1999a), caspase 3 (Mancini et al, 1998), caspase 8 (Qin et al., 2001), and caspase 9 (Susin et al., 1999a, Krajewski et al., 1999, Costantini, 2002). Caspases are key effectors of cell death and will be discussed in section 1.1.9.

AIF (Apoptosis Inducing Factor) is another pro-apoptotic protein that resides in, and is released from, mitochondria. The non-apoptogenic AIF precursor of 67 KD is cleaved to a 57 KD form which is imported to the intermembrane mitochondrial space. In normal circumstances, AIF may function in oxidative phosphorylation, given its sequence homology with bacterial oxidoreductases (Susin et al., 1999b). In cells exposed to apoptotic stimuli, however, AIF translocates from mitochondria to the nucleus, where it mediates apoptosis independently of caspases (Daugas et al., 2000, Joza et al., 2001, section 1.1.9.5.). Typical apoptotic hallmarks Induced by nucleus-targeted AIF include chromatin condensation, large-scale DNA fragmentation, loss of the mitochondrial membrane potential, and exposure of phosphatidylserine (Susin et al., 1999b).

Lastly, Endonuclease G (EndoG) is released from mitochondria during apoptosis (Li et al., 2001). Endo G appears to be conserved among metazoans. Cps-6 protein recently characterized in *C.elegans* localizes to the mitochondria and is structurally and functionally homologous to mammalian EndoG (Parrish et al., 2001). In normal cells, EndoG is likely involved in the replication of the mitochondrial DNA (Cote et

al., 1993). In apoptotic cells, however, Endo G translocates from mitochondria to the nucleus where it induces nucleosomal DNA fragmentation, a typical hallmark of apoptosis. Interestingly, unlike DFF40/CAD-mediated DNA fragmentation (Enari et al., 1998, section 1.1.9.3.), EndoG-induced fragmentation occurs in a caspase-independent manner (Li et al., 2001). Thus, together with AIF, EndoG contributes to the induction of cell death independently of caspases (caspase-independent apoptosis is discussed in section 1.1.9.5.).

1.1.8.1.2. The role of cytochrome c

The best-characterized mechanism of apoptosis induction through the mitochondrial pathway involves the activity of released cytochrome c. Cytoplasmic cytochrome c binds to Apaf-1, dATP, and pro-caspase 9 to form a complex referred to as apoptosome. Apaf -1 (apoptotic protease activating factor -1) is a mammalian homologue of *C.elegans* Ced-4 product (section 1.1.6), although it is larger and structurally more complex than Ced-4 (Li et al., 1997). The binding of cytochrome c to Apaf-1 is thought to induce conformational change in this protein, allowing Apaf-1 to bind and activate caspase 9 (Srinivasula et al., 1998). In the absence of cytochrome c, the interaction of Apaf-1 and caspase 9 is repressed by Apaf-1 C-terminal WD-40 repeats. The binding of Apaf-1 to caspase 9 is mediated by CARD (caspase recruitment domain) motifs present on both Apaf-1 and the pro-domain of caspase 9 (Duan and Dixit, 1997, Hofmann et al., 1997, Zou et al., 1997). Binding of dATP to the complex completes the apoptosome and triggers the autocatalytic activation of caspase 9 (Li et al., 1997). The processed caspase 9 activates downstream effector caspases involved in execution of apoptosis (Slee et al., 1999, Thornberry and Lazebnik, 1998, section 1.1.9.).

1.1.8.1.3. Mitochondrial membrane permeability transition

As mentioned, one of the most notable features of apoptotic mitochondria is membrane depolarization and a general increase in membrane permeability (Vander

Heiden et al., 1997). Membrane permeability transition allows the release of the pro-apoptotic proteins from mitochondria, and therefore constitutes a critical event in the induction of cell death. The changes in permeability are attributed to the opening of the permeability transition pore (PTP), a conductance channel spanning both the outer and inner mitochondrial membranes (Qian et al., 1997). PTP consists of two main components: the adenine nucleotide translocator (ANT) in the inner membrane and the voltage-dependent anion channel (VDAC) in the outer membrane (Petit et al., 1996, Bernardi et al., 1994). Both channels operate in synchrony and exclude proteins larger than 1.5 KD. Opening of PTP leads to the efflux of protons from the matrix to the intermembrnae space, abolishing the proton gradient and causing membrane depolarization (Green and Reed 1998). PTP opening also results in the expansion of the matrix, leading to the rupture of the outer mitochondrial membrane and release of pro-apoptotic proteins.

1.1.8.1.4. Regulation of mitochondrial apoptotic pathway

The events pertaining to the opening of the PTP pore are regulated by the Bcl-2 family of proteins (section 1.1.7.1.). Two anti-apoptotic members of the Bcl-2 family, Bcl-2 and Bcl-Xl, have been shown to prevent permeability transition and hyperpolarization of mitochondrial membranes (Zamzami et al., 1996, Green and Reed, 1998). On the other hand, the pro-apoptotic member Bax has been shown to induce permeability transition and membrane depolarization by binding to the ANT component of PTP (Marzo et al. 1998). The ability of Bax to oligomerize and form large conductance channels (Martinou et al., 2000) has led to a hypothesis that membrane permeability transition is potentially caused by the formation of a pore by Bax molecules in the mitochondrial outer membrane, rather than Bax-mediated opening of the PTP. This model predicts no pronounced damage to the outer mitochondrial membrane other than puncturing of the membrane with Bax oligomers, which serve as channels for the release of the pro-apoptotic proteins. This model is supported by evidence that Bax added to isolated mitochondria will trigger the release of cytochrome c in the presence of PT blockers.

A pro-apoptotic "BH-3 only" member of the Bcl-2 family Bid has also been implicated in the induction of mitochondrial membrane permeability. Proteolytically-activated Bid (tBid) (section 1.1.7.1.2.) facilitates oligomerization of Bax (Eskes et al., 2000, Korsmeyer et al., 2000) and Bak (Wei et al., 2000), two pro-apoptotic members of the Bcl-2 family of proteins, thereby promoting their insertion into the mitochondrial outer membrane.

The Bcl-2 family members have also been shown to regulate apoptosome activity. The mammalian homologue of Ced-9, Bcl-2, appears play a role in the regulation of Apaf-1-mediated activation of caspase 9. Bcl-2 present in the outer mitochondrial membrane binds Apaf-1 and blocks the activation of caspase 9. The targeting of pro-apoptotic molecules like Bax, Bak, tBid, and Bik, potentially promotes apoptosis by displacing Bcl-2 from Apaf-1 (Zornig et al., 2001).

Considering the functions described above, mitochondria constitute an important control point in the induction of apoptosis. Firstly, mitochondria contain pro-apoptotic molecules, which when released, mediate cell death irreversibly. Secondly, mitochondria are responsive to upstream stress signals relayed by the members of the Bcl-2 family. They therefore serve as transmitters of apoptotic signals to the death execution machinery. The mitochondrial apoptotic signaling is depicted in Figure 1.1

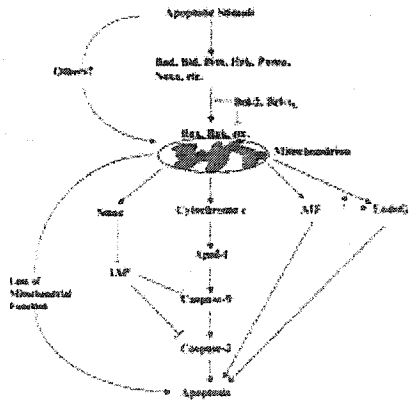


Figure 1.1. Mitochondrial apoptotic pathway. The Bcl-2 family of regulators as well as the released apoptogenic proteins are indicated. (Adapted from Wang, 2001)

1.1.8.2. Apoptosis mediated from other organelles

1.1.8.2.1. Endoplasmic reticulum

Unfolded protein response (UPR) (Patil and Walter, 2001) and calcium depletion (Kaufman, 1999, Jamora et al., 1996) constitute two main mechanisms of cell death initiation from the ER. UPR is triggered by conditions that compromise proper protein folding. Protein misfolding activates numerous ER transmembrane kinases, including PERK and AFT-6. PERK phosphorylates and inactivates the translation initiation factor eIF2-alpha, shutting off protein synthesis. Activated AFT-6, on the other hand, is severed from the ER membrane and translocates to the nucleus, where it functions as a transcription factor of the ATF/CREB family. Genes transactivated by AFT-6 include ER chaperones (Bip/Grp78), and an apoptosis regulator CHOP/GADD153, a transcription factor involved in downregulation of Bcl-2 expression (McCullough et al., 2001). The ER chaperone Bip/Grp78 localizes to the lumen of the ER and functions in preserving ER calcium stores, thus preventing the induction of apoptosis (Miyake et al., 2000). The details of how ER calcium regulates apoptosis have not been elucidated.

The ER also contributes to apoptosis by releasing activated caspase 12. Procaspase 12 is localized on the cytoplasmic side of the ER membrane and is proteolytically activated by calcium responsive protease m-calpain (also localized to the outer side of the ER membrane) (Nakagawa and Yuan, 2000). ER signaling is therefore connected to the caspase cascade.

Interestingly, Bcl-2, the anti-apoptotic protein localized in the outer mitochondrial membrane, has also been found to reside in the ER membrane. A Bcl-2 variant (Bcl-2/cb5) is targeted exclusively to the ER and has been shown to inhibit Myc-induced cytochrome c release from mitochondria, depolarization of mitochondrial membrane, and the ensuing death of the cell (Hacki et al., 2000, Annis et al., 2001). Other reports have shown that Bcl-2/cb5 helps to retain calcium in the ER, thus inhibiting the induction of ER-mediated apoptosis (Distelhorst et al., 1996, He et al., 1997).

Bcl-2 has also been shown to bind an ER membrane spanning protein Bap31 (Ng et al., 1997). Bap31 is proteolytically processed during apoptosis to a p20 form (p20Bap) and as such promotes apoptosis (Ng et al., 1997). The interaction of Bcl-2 with Bap31 prevents cleavage of Bap31 and generation of the p20 apoptogenic form. Bcl-2-mediated inhibition of Bap31 cleavage is relieved by Bax in apoptotic cells. It is not clear, however, how p20Bap31 mediates apoptosis, although it could be implicated in preservation of calcium in the ER (Kroemer et al., 1997).

1.1.8.2.2. Golgi apparatus and endosomes

The finding that inhibition of protein glycosylation by alpha-mannosidase II inhibitors leads to apoptosis (Goss et al., 1995) has implicated Golgi organelles as potential mediators of cell death. Although it is not clear how Golgi functions as a stress sensor, the organelle is known to harbor some pro-apoptotic molecules. These include caspase 2 (Mancini et al. 2000), death receptors TNF-R1, CD95 (Bennett et al. 1998), TRAIL receptors 1 and 2 (Zhang et al., 2000), as well as PI(3)K, Beclin, and GD3 synthase (DeMaria et al., 1997). Recent data indicate that the CD95 receptor localized in Golgi translocates to the cell surface in response to p53 activation (Bennett et al., 1998). Furthermore, CD3 synthase-catalyzed conversion of ceramide to GD3 has been shown to result in the release of GD3 from Golgi and its targeting to mitochondria, where it induces mitochondrial membrane depolarization (DeMaria et al., 1997, Rippo et al., 2000).

Endosomes contain a RhoB GTPase, which appears to play a role in regulation of apoptosis. Inhibition of RhoB farnesylation has been shown to result in its dissociation from the endosomal membrane and induction of apoptosis (Prendergast, 2000).

1.1.8.2.3. Lysosomes

Lysosomes contain several proteases, termed cathepsins, which are implicated in the induction of apoptosis (Deiss et al., 1996). The release of cathepsin D from

lysosomes has been shown to be linked with the release of cytochrome c from mitochondria, although the cross-talk pathway is unknown (Roberg, 2001). Cathepsin B appears to mediate caspase-independent cell death in transformed WEHI-S cells (Foghsgaard et al., 2001, section 1.1.9.5.), and has been implicated in the induction of oligonucleosomal DNA fragmentation and proteolytic activation of pro-inflammatory caspase 11 (Schotte et al., 1998). A cathepsin-l-related lysosomal protease has been shown to cleave Bid at a slightly different site than caspase 8 to generate a modified form of tBid capable of inducing mitochondrial membrane depolarization and cell death (Stoka et al., 2001). Lysosomal proteases clearly play a role in the induction of apoptosis. However, more research has to be done to determine their exact roles.

1.1.8.3. The death receptor pathway

The death receptor pathway is generally recognized as the link between extracellular stress signals and the intracellular apoptotic machinery. Death receptors are cell surface receptors that transmit apoptosis signals after binding of specific death ligands (reviewed in Ashkenazi and Dixit, 1998). Death receptors are type I membrane proteins of homo-trimeric structures stabilized by disulphide bonds, with a conserved cysteine-rich region in the extracellular domain (Hughes and Crispe, 1995, Smith et al., 1995). Death receptor ligands, which include Fas, CD95L, and TNF-alpha, are generally released from immune cells or virally-infected somatic cells. Binding of ligands to death receptors causes clustering of cytoplasmic receptor domains that initiates signaling cascades involving caspases (section 1.1.9), although caspase-independent death pathways may also be triggered (Charette et al., 2001, section 1.1.9.5.). The ligands, like their corresponding receptors, are also homo-trimeric in structure (Smith et al., 1994).

1.1.8.3.1. CD95/Fas signaling

CD95 signaling is important for killing of virally-infected cells and cancer cells by cytotoxic T cells or Natural Killer (NK) cells, as well as for deletion of activated mature T cells at the end of an immune response (Nagata, 1997). CD95 receptor is therefore expressed in activated lymphocytes as well as in all other somatic cells. CD95 ligand (CD95L), on the other hand, is expressed predominantly in cells of the immune system, including lymphocytes, NK cells, and erythroblasts.

The binding of the CD95L trimer to the CD95 receptor induces the clustering of the receptor cytoplasmic domains (Huang et al., 1996). The cytoplasmic domains of death receptors contain 65 amino acid long death domains (DD) involved in the recruitment of downstream effector molecules (Fraser and Evan, 1996). Death domains are also present on FADD (Fas-associated death domain) molecules (also called Mort 1), which are adapter proteins that link the receptor with caspase 8 (Chinnaiyan AM et al., 1995). The interactions of CD95/Fas and FADD occur via death domains present on both molecules. The interaction of FADD with caspase 8 is mediated by DED (death effector domains) present on both FADD and caspase 8 (Muzio et al., 1996, Boldin et al., 1996). Linking of caspase 8 to FADD by the interaction of analogous DED domains leads to the proteolytic autoactivation of caspase 8 (Muzio M, 1998), and ultimately commits the cell to apoptosis. Caspase 8 is involved in the activation of downstream effector caspases, but it also cleaves Bid to tBid, which translocates to the mitochondria and induces cytochrome c release (Li et al., 1998, Luo et al, 1998, section 1.1.7.1.2.). The death receptor pathway is therefore linked with the mitochondrial pathway and the two seem to cooperate in the induction of cell death.

Recently, it has been shown that CD95/Fas receptor also binds Daxx protein (Yang et al, 1997). Linking of Daxx to the receptor activates Jun N-terminal kinase (JNK) and promotes CD95-induced apoptosis, although the mechanism has not been fully characterized. In a separate study Daxx has been shown to translocate from the nucleus to the cytoplasm in response to the activation of Fas receptor (Charette et al., 2000, Charette and Landry, 2000, Charette et al., 2001). The Fas-mediated relocalization of Daxx leads to the induction of a novel type of apoptosis independently of caspases (Charette et al., 2001, section 1.1.9.5.). Thus, CD95/Fas

receptor seems to induce two distinct cell death pathways, one involving caspase activation, the other being caspase-independent (section 1.1.10).

To prevent the induction of apoptosis, some viruses produce v-FLIPs (Fas-associated death domain-like ICE inhibitory proteins) which contain death effector domains (DED) similar to those on FADD and caspase 8 (Thome et al, 1997, Hu et al., 1997). The role of v-FLIPs is to bind and sequester FADD and caspase 8, thus interfering with the induction of receptor-mediated cell death (Thome et al., 1997). Cellular homologues of v-FLIPs (c-FLIPs) were also identified, but their role in regulation of cell death is not clear (Irmler et al., 1997, Han et al., 1997, Rasper et al., 1998). To further evade the induction of cell death, viruses have also evolved CD95 decoy receptors (DcR3), which are released to bind CD95L, thereby inhibiting CD95/CD95L-triggered apoptosis (Pitti et al., 1998).

1.1.8.3.2. TNF receptor signaling

TNF (tumor necrosis factor) ligand is produced mainly in activated T cells and macrophages in response to infection (Tartaglia and Goeddel, 1992). Binding of TNF trimerizes the TNF receptor (TNFR1) and induces the aggregation of the cytoplasmic domains of the receptors. There are two identified signaling cascades Induced by activated TNFR1. One signaling pathway leads to apoptosis, while the other pathway promotes cell survival by activating Nf-kappaB transcription factor and JNK (c-Jun N-terminal kinase) (Lee and Choi 1997). The TNFR1-induced apoptotic pathway is similar to that induced by CD95 ligation (section 1.1.8.3.1.), and involves FADD-mediated activation of caspase 8 (Hsu et al., 1996a). In the TNFR1 pathway, however, FADD does not directly bind to TNFR1 but rather associates with TRADD (TNFR-associated death domain). TRADD, acting like a bridge between TNFR1 and FADD, binds to the cytoplasmic DD of TNFR1 via its own death domain and recruits FADD. It also recruits RIP (receptor interacting protein) by interacting with a DD on RIP (Hsu et al., 1996b, Ting et al., 1996). RIP binds to two proteins, RAIDD (RIP-associated, ICH-1-homologous protein with DD) and TRAF2 (TNFR-associated factor 2) (Rothe et al., 1996, Hsu et al., 1996a). RAIDD binds caspase 2

via DED dimerization, thereby activating caspase 2 and initiating caspase-mediated apoptosis (Ahmad et al., 1997, Duan and Dixit, 1997). TRAF2, on the other hand, activates NF-kappaB kinase-inducing kinase NIK, which in turn activates IKK (Malinin et al., 1997, Zandi et al., 1997), the inhibitor of I-kappaB kinase complex. IKK phosphorylates I-kappaB (inhibitor of NF-kappaB), leading to its degradation. The elimination of I-kappaB activates the transcription factor Nf-kappaB, which drives the expression of pro-survival genes (Van Antwerp et al., 1996, Wang et al., 19). RIP-bound TRAF2 also mediates the activation of JNK involved in the suppression of cell death (Natoli G et al., 1997). Interestingly, TRAF2 also binds c-IAPs (cellular inhibitors of apoptosis) (Shu et al., 1996), which may be involved in the inhibition of TNFR1-mediated apoptosis. Thus, TNFR1 signaling promotes both cell death and cell survival mechanisms. The factors controlling the decision whether to proliferate or die remain largely uncharacterized. Recently identified protein TRIP has been shown to bind TRAF2 and inhibit TRAF-induced activation of the apoptosis suppressor Nf-kappaB (Lee and Choi, 1997). Thus, the outcome of TNFR1 signaling may depend on relative concentrations of pro-apoptotic TRIP and anti-apoptotic cIAPs in the cell, and their affinities for the receptor (Zornig et al., 2001).

1.1.8.3.3. TRAIL receptor signaling

TRAIL (TNF-related apoptosis inducing ligand) receptors DR4 and DR5 are expressed in most human tissues and some tumor cell lines. TRAIL (the ligand) is also broadly expressed, although at higher levels in activated T cells (Screaton et al., 1997, Jeremias et al., 1998). Similarly to CD95L, TRAIL drives apoptosis through FADD interaction with DR4/5 via their cytoplasmic death domains and subsequent activation of caspase 8 (Bodmer et al., 2000). However, two other identified TRAIL receptors, DcR1 and DcR2, do not possess functional cytoplasmic domains and are therefore unable to mediate any cellular signaling (Golstein, 1997). DcR1 and DcR2 are decoy receptors that bind TRAIL and prevent TRAIL-mediated activation of DR4 and DR5, thereby blocking DR4/5-induced apoptosis (Pan et al., 1997). The role of endogenous decoy receptors for inhibition of apoptosis is not known.

Death receptor signaling is very often a target of viral products. A number of viral homologues of cFLIPs involved in sequestering of FADD and caspase 8 were identified (Thome et al., 1997, Goltsev et al., 1997). Adenoviruses produce RID protein that downregulates Fas expression (Tollefson et al., 1998) and E1B-19K protein, which sequesters FADD (Perez and White, 1998).

Activation of death receptors can also be of therapeutic use (section 1.1.9.6.). Death receptor-mediated apoptosis is independent of p53, which is mutated in most types of human cancer. Thus, targeting death receptors may prove to be an efficient means of blocking tumorigenic progression. Apoptotic pathways initiated from death receptors are summarized in Figure 1.2.

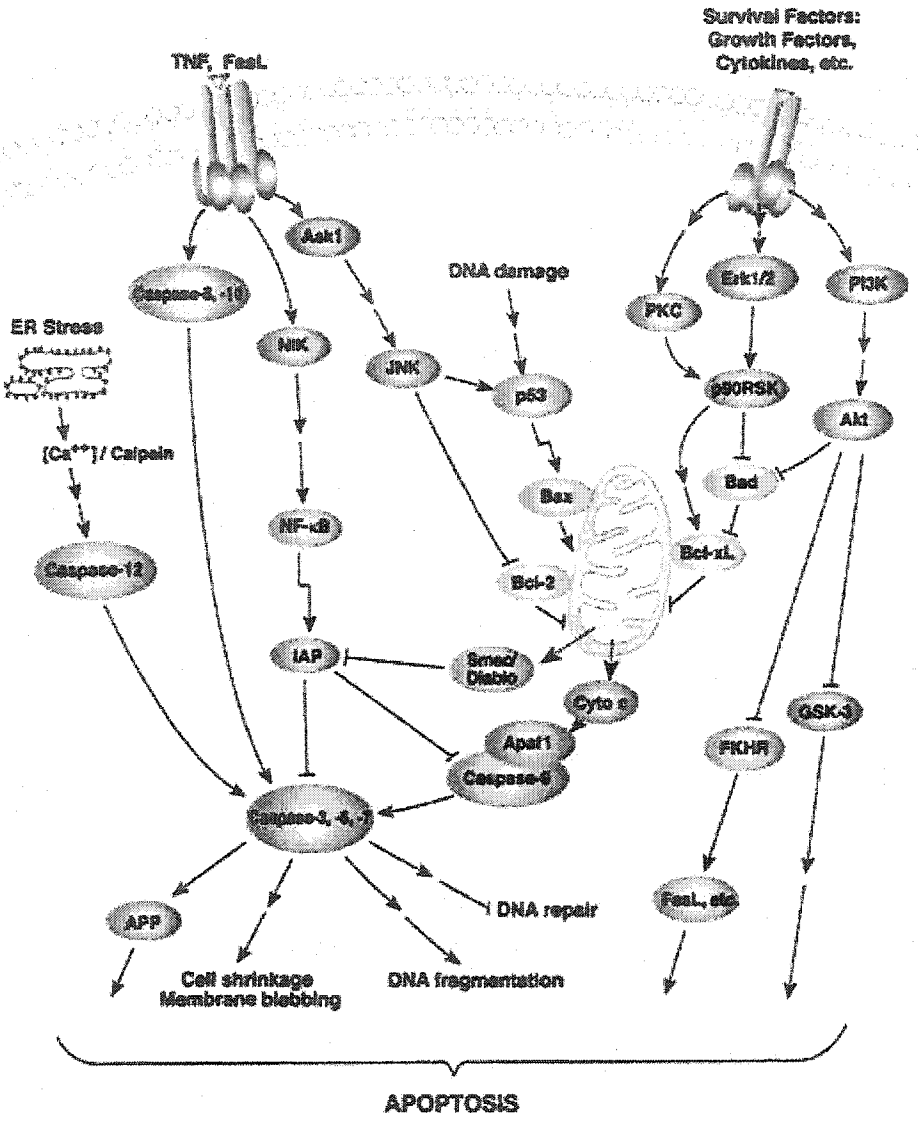


Figure 1.2. Diagram showing apoptotic pathways initiated from cell surface death receptors. The mitochondria and the ER are also incorporated in the schematic. Death receptor-mediated signaling culminates in activation of caspases, the central effectors of the execution phase of apoptosis (Adapted from Cell Signaling Technology Inc.)

1.1.9. Caspases: the effectors of cell death

Both the mitochondrial and death receptor apoptotic pathways converge on an effector phase of apoptosis that involves activation of a set of cellular proteases, termed caspases (reviewed in Cryns and Yuan, 1998, Thornberry and Lazebnik, 1998, Zornig et al., 2001). Activated caspases cleave a number of cellular proteins and essentially mediate the disassembly of a cell. The first identified caspase, referred to as ICE or caspase 1, is a homologue of *C.elegans* Ced-3 protease (Yuan J et al., 1993, section 1.1.6.). To date, 13 other caspases were identified. Caspase 1, 4, 5, and 11 are all involved in proteolytic maturation of cytokines, including interleukin-1beta (Li P et al., 1995, Wang S et al., 1998). All other caspases, namely 2, 3, 6, 7, 8, 9, 10, 12, 13, and 14, are involved in the execution of apoptosis (Wolf and Green, 1999).

1.1.9.1. Structural features of caspases

The caspase catalytic site is highly conserved among metazoans and consists of the QACXG sequence, where centrally-positioned cysteine is directly involved in catalysis. The caspase recognition site in substrates consists of four amino acids, with aspartate being first in the sequence (Thornberry et al., 1997). The cleavage of substrates by caspases occurs specifically on the carboxy end of the aspartate residue present in the 4-a.a. recognition sequence (Cryns and Yuan, 1998).

Caspases can be subdivided into three groups based on substrate specificities. The first group consists of ICE caspases implicated in cytokine processing and includes caspases 1, 4, 5, and 11, all with WEHD recognition sequence specificity. The second Ced-3/CPP32 group of caspases includes caspases 3, 6, and 7, which recognize DEXD sequences in the substrates and play a role in the execution phase of PCD. The last group, ICH-1/Nedd-2, contains caspases 2, 8, 9, and 10, which recognize (I/L/V)EXD sequences, and are primarily involved in the initiation of apoptosis (Thornberry et al, 1997).

All active caspases exist in hetero-dimeric forms. Larger subunits contain the catalytic cysteine residue, whereas smaller subunits determine substrate specificity. Two caspase heterodimers bind to one another to ultimately form a tetramer consisting of two large and two small subunits (Walker et al., 1994, and Wilson et al., 1994).

1.1.9.2. Caspase activation

Caspases are synthesized as inactive pro-enzymes with one regulatory pro-domain and two effector domains. The pro-domain is located on the N-terminus whereas the large and small subunits comprising the effector domain are localized on the C-terminus. Caspases are activated by proteolytic removal of the pro-domain, which is cleaved off specifically at caspase substrate cleavage sites. This implies that caspases are themselves targets of other caspases, or are auto-activated, and this is precisely the case. Procaspase 8 cleaves and activates itself upon binding to the death receptor complex (Muzio et al., 1998, section 1.1.8.3.), whereas caspase 9 proteolytically self-activates as part of the apoptosome complex (Wang, et al., 2001, section 1.1.8.1.). Caspases 8 and 9 are the two best characterized upstream caspases, which upon activation, cleave a number of downstream effector caspases, thus initiating a caspase cascade. Downstream effector procaspases 3 and 7 are proteolytically processed by caspases 8 and 9, and as active caspases execute apoptotic death. The execution phase involves cleavage of a number of structural and regulatory proteins by the effector caspases.

The pro-domains of the upstream caspases are long and contain regulatory regions (Fraser and Evan, Hofmann et al., 1997). The pro-domain of caspase 8 contains the DED motif involved in caspase 8 interaction with death receptor complexes (section 1.1.8.3.), while caspase 9 contains the CARD motif mediating caspase 9 interaction with Apaf-1 (section 1.1.8.1.). In contrast, the effector caspases contain short prodomains of maximum 40 a.a. in length.

1.1.9.3. Cellular targets of activated caspases

Caspase substrates comprise a large group of proteins with different functions (Cryns and Yuan 1998, Nicholson, 1999). They include structural proteins, both pro- and anti-apoptotic regulatory proteins, repair enzymes, as well as signaling molecules. Downstream procaspases also constitute a group of substrates for their upstream counterparts.

Some well characterized caspase targets include Bcl-2 related proteins. Caspase 8 cleaves Bid, generating tBid, a potent inducer of cytochrome c release (Li et al., 1998, Luo et al., 1998, section 1.1.7.1.2.). Caspase 3 cleaves Bcl-2, thus inactivating its anti-apoptotic function and actually generating a pro-apoptotic C-terminal Bcl-2 fragment (Cheng et al., 1997, Clem et al., 1998). Caspase 3 also cleaves ICAD/DFF45, an inhibitor of CAD (caspase-activated DNase), thus promoting the translocation of CAD from the cytoplasm to the nucleus, and CAD-mediated degradation of DNA (Sakahira et al., 1998, Enari et al., 1998). The nuclear DNA repair enzyme PARP (Poly ADP-ribose polymerase) is also cut and inactivated (Casciola-Rosen et al., 1996), possibly to facilitate DNA degradation (Cohen, 1997). Two structural targets of caspase 6 and caspase 3 include lamins and gelsolin (Takahashi et al., 1996, Kothakota et al., 1997). Lamins constitute the major component of the nuclear envelope, and their cleavage by caspase 6 causes nuclear changes that are hallmarks of apoptosis (section 1.1.2.). Gelsolin, on the other hand, is critically involved in regulation of actin activity, and its proteolysis by caspase 3 has drastic effects on the integrity of the entire cell. Cleavage of PAK2/hPAK65 and FAK kinases induces cellular detachment and phosphatidylserine flip, and is thought to promote phagocytosis of apoptotic bodies (Brancolini et al., 1997, Wen et al., 1997).

1.1.9.4. Caspase inhibitors: cellular, viral, and synthetic

A number of viruses have evolved mechanisms to inhibit caspases, and thus prevent the induction and execution of cell death (Roulston et al., 1999). Endogenous caspase inhibitors also exist to perform regulatory functions and prevent unnecessary

caspase activation. Furthermore, there are a number of synthetic caspase inhibitors used in research and medical settings (reviewed in Ekert et al., 1999).

Cellular inhibitors of apoptosis, known as cIAPs, constitute a group of evolutionarily conserved caspase blockers found in yeast, *C.elegans*, *Drosophila*, and mammals (Clem and Duckett, 1997, Uren et al, 1998, LaCasse et al., 1998). IAPs appear to bind to both activated caspases (Ekert PG et al., 1999) as well as inactive procaspases (Deveraux et al., 1998) and block their activation, although the mechanism of inhibition is not clear. cIAPs are themselves inhibited by Smac/Diablo proteins released from mitochondria during apoptosis (Verhagen et al., 2000, Du et al., 2000, section 1.1.8.1.2.). IAP overexpression is associated with several types of cancers including that of lung, colon, prostate, and breast, as well as 50% of non-Hodgkin's lymphomas (Ambrosini et al., 1997, section 1.1.5).

Several viral caspase inhibitors were identified (reviewed in Roulston et al., 1999). Baculoviruses and poxviruses produce functional homologues of cIAPs. Poxviruses also produce serpins, homologs of mammalian Serine proteinases inhibitors (Potempa et al., 1994). CrmA, the best characterized viral serpin, inhibits Fas- and TNF-induced apoptosis by binding to caspases 1 and 8 (Tewari et al., 1995, Zhou et al., 1997). CrmA acts as a pseudosubstrate that is recognized and bound by active caspases, thereby neutralizing them (Zhou et al., 1997, Garcia-Clavo et al., 1998, Komiyama, 1994). Baculoviruses, on the other hand, encode p35 protein that can inhibit mammalian caspases 1, 3, 6, 7, 8, and 10 as well as *C.*elegans Ced-3 (Xue and Horvitz, 1995, Ahmad et al., 1997). Like CrmA, p35 is a caspase pseudosubstrate, which is recognized and cleaved by caspases. Unlike CrmA, however, p35 does not dissociate from the inhibited caspase, but rather remains bound in an inhibitory complex (Bump et al., 1995).

Several synthetic caspase inhibitors have also been designed. The inhibitors are currently being applied in studies of caspase contribution to cell death. Caspase inhibitors are designed to contain the caspase recognition sequence and chemical groups capable of inhibiting caspase catalytic sites. The most notable synthetic caspase inhibitors are zVAD-fmk, zDEVD-fmk, and Boc-Asp-FMK. All contain one or more amino acids present in the caspase recognition sequence as well as a

fluoromethylketone (fmk) group, which inactivates cysteine in the active site via intermolecular interactions (Thornberry et al., 1994).

1.1.9.5. Caspase-independent apoptosis

The observation that caspase inhibition with zVAD-fmk does not always prevent the induction of PCD has led to the hypothesis that there must exist caspase-independent apoptotic pathways (reviewed in Borner and Monney., 1999, Bidere and Senik, 2001). Although in some cases apoptosis has been shown to be inhibitable by zVAD-fmk and therefore critically dependent on caspases (Hara et al., 1997, Rodriguez et al., 1996), a growing number of reports implicate caspase-independent mechanisms in the induction of PCD. In one experiment, addition of zVAD-fmk to cells with overexpressed Bax inhibited Bax-mediated nuclear fragmentation, but did not rescue cells from apoptosis (Xiang et al., 1996, Miller et al., 1997). In another setting, addition of zVAD-fmk to cells challenged with DNA damaging and oncogenic apoptotic stimuli abolished cleavages of caspase substrates, chromatin condensation, and DNA degradation, but did not block cell shrinkage, membrane blebbing, or the occurrence of cell death (McCarthy et al., 1997). Similarly, zVAD-fmk failed to inhibit cytotoxic T cell-mediated cell shrinkage, membrane blebbing, PS externalization, depolarization of mitochondrial membrane, and the onset of cell death, although it did prevent nuclear damage (Sarin et al., 1997, Trapani et al., 1998). These observations suggest that caspases are required for mediation of apoptotic nuclear events, but may be dispensable for the induction of other apoptotic hallmarks and cell death in general. Similar effects were observed in studies with the following stimuli: GD3 ganglioside (DeMaria et al., 1997), class 1 MHC antibodies (Woodle et al., 1997), Fas and puromycin (Schlapbach and Fontana, 1997), polyamine analogues (Ha et al., 1998), anti-CD2 and staurosporine (Deas et al., 1998, Weil et al., 1998), the retinoid AHPN (Adachi H et al., 1998), VP-16 (Benson et al., 1998), actinomycin D, PML (Quignon et al., 1998), nitric oxide (Okuno et al., 1998), and adenoviral death protein E4orf4 (Lavoie et al., 1998, Szymborski et al., publication in preparation, E4orf4 is discussed in detail in section 1.2.11.). All of the

above stimuli appear to mediate a novel, caspase-independent form of apoptosis that is clearly distinct from necrosis, as there is no cell swelling, cell rupture, or organelle disintegration.

The mechanism of caspase-independent PCD has yet to be characterized. Apoptogenic proteins residing in mitochondria are good candidates for mediators of apoptosis without caspases. The onset of mitochondrial depolarization and permeability transition, as well as pro-apoptotic activity of Bax appear not to be affected by zVAD-fmk, implying that the release of mitochondrial proteins does not require caspases. Upon their release to the cytoplasm, some pro-apoptotic mitochondrial molecules mediate apoptotic effects in the presence of zVAD. AIF and EndoG nuclease (van Loo et al., 2001, described in section 1.1.8.1.1.) are examples of mitochondrial proteins implicated in the induction of apoptotic hallmarks and cell death in the presence of zVAD-fmk. Organelles other than mitochondria also harbor molecules capable of inducing apoptosis independently of caspases. One example is cathepsin B present in the lysosomes (discussed in section 1.1.8.2.3.). Lastly, nuclear, PML-associated protein Daxx activated by Fas receptor signaling has also been implicated in the induction of a novel type of cell death independently of caspases (discussed in section 1.1.8.3.1.). Daxx-mediated cell death is characterized by crumpled nuclei (Charette et al. 2001) and requires interaction of Daxx with Ask1 kinase. This interaction is regulated by HSP27 (heat shock protein 27), which prevents Daxx from binding Ask1 (Charette and Landry, 2000), although the mechanism of inhibition is not known. HSP27-mediated inhibition of Daxx is relieved following phosphorylation of HSP27 in response to cellular stresses. Daxx-mediated apoptotic pathway is diagrammed in Figure 1.3.

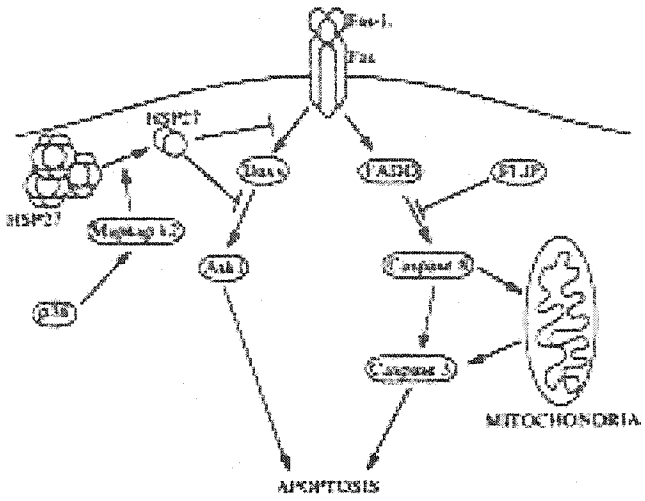


Figure 1.3. A model for Fas-induced and Daxx-mediated mechanism of apoptosis induction. Both caspase-dependent and independent pathways are diagrammed (Adapted from Charette SJ and Landry J, 2000).

1.1.9.6. Caspase inhibition and activation in therapy

The use of caspase inhibitors can potentially be extended from research laboratories to the clinical setting. Inappropriate activation of caspases has been linked to many medical conditions, including Alzheimer's disease, stroke, and heart attacks. Targeting of overactive caspases with caspase inhibitors may cure or alleviate some of these diseases. Recent data indicate that baculoviral p35 expression prevents blindness due to retinal degeneration in *Drosophila* (Davidson and Steller, 1998), although no data were reported from mammalian models. In the future, application of caspase inhibitors will inevitably be extended to humans, although research in the field is still in the initial stages.

Treatment of cancers, on the other hand, may potentially involve activation of caspases as a means of inducing cell death in cancerous cells. Since p53 is mutated and inactive in most cancers, caspases should ideally be targeted for activation through p53-independent channels, such as the death receptor pathway (Ashkenazi and Dixit, 1998), or p53-homologues (section 1.1.7.3.4.).

1.2. Adenovirus

1.2.1. Discovery and Medical Perspective

Adenoviruses were discovered in 1953 by Rowe, who retrieved them from adenoid and tonsil cell cultures during his studies of the common cold (Rowe et al., 1953). Although adenoviruses turned out not to be the main causative agents of the common cold, various adenovirus serotypes were linked to a number of other diseases, including acute febrile respiratory disease (subgroup B), infantile gastroenteritis (subgroup F), and pneumonia (subgroup E) (reviewed in Horvitz, 1996). Adenoviruses were also shown to induce malignant transformation in rodent cells (Trentin et al, 1962), although their oncogenic potential has never been demonstrated in humans, as to date no human cancers have been causatively associated with adenoviral infection. Since most individuals acquire neutralizing antibodies against at least one adenoviral serotype within the first year of life, adenoviruses are not regarded as serious pathogenic agents in humans (Huebner et al., 1954).

Adenoviruses have proven to be excellent models for study of cellular processes that govern cell proliferation and cell death. Molecular examination of the viral infectious cycle has advanced our understanding of cellular gene expression, cell cycle regulation, DNA replication, as well as control of apoptosis. Adenoviruses are also successfully used as targeting vectors in research and gene therapy (reviewed in Shenk, 1996).

1.2.2. Classification

Adenoviruses belong to the Adenoviridae family which contains over 100 members and is subdivided into two genera. Mastadenovirus genus includes adenoviruses that infect mammals, while Aviadenovirus genus contains adenoviruses that infect birds (Norrby et al., 1976). Human viruses of the Mastadenovirus genus

are further categorized into six groups based on their ability to agglutinate red blood cells (Rosen, 1960). All members within the same group exhibit similarities in cell and tissue tropisms, oncogenic potential in mammals (Green et al., 1980), guanine/cytosine (GC) content (Pina and Green, 1965), and electrophoretic mobility of virion proteins (Waddell et al, 1979). Members of the six groups are further classified into 47 serotypes based on their reactivity with neutralizing antibodies. The classification of adenoviruses is depicted in Table 1.1.

			Haemagglutination:	
Subgroup:	Serotypes:	Oncogenic Potential:	Rhesus:	Rat:
A	12, 18, 31	HIGH	-	+ / -
B	3, 7, 11, 14, 21, 34, 35	Weak	+	-
C	1, 2, 5, 6	None	-	+ / -
D	8-10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39	None	+ / -	+
E	4	None	-	+ / -
F - G	40, 41	?	-	-

Table 1.1: Classification of human adenoviruses (Adapted from Shenk et al., 1996, Fields' Virology)

1.2.3. Structure of the Virion

Adenoviruses shell is composed of the capsid that lacks membrane envelope and is icosahedral in shape (Horne et al., 1959). The iscosahedron measures 70-100nm in diameter and consists of 20 triangular planes and 12 vertices. The capsid shell is made up of 252 units termed caposmeres, of which 240 are hexons at the edges of the icosahedron, and 12 are pentons at the vertices (Ginsberg et al., 1966). Hexons are composed of protein II trimers stabilized by three other viral proteins, proteins VI, VIII, and IX (Everitt et al., 1973). Protein IIIa bridges capsid to the core of the virus as well as joins pentons and hexons (Devaux et al., 1987). Each penton, composed of protein III pentamers, is surrounded by five hexons. The fiber protein, which is based on each of 12 pentons, is composed of three copies of protein IV and

together with the knob at its apex is important for recognition of and interaction with cell surface receptors.

The viral core contains one double stranded and supercoiled DNA molecule. Viral DNA is complexed with proteins V, VII, terminal protein, and mu protein. Protein V links viral DNA with the penton base is required for the entry of viral genome into the host's nucleus. Protein VII is an arginine rich histone-like protein which assembles viral DNA into nucleosomes (Vayda and Flint, 1987). The terminal protein is covalently bound to the 5' ends of the genome and is involved in the replication of viral DNA (Enomoto et al., 1981, Tamanoi and Stillman, 1982). Adenovirus structure is summarized in Figure 1.4 and in Table 1.2.

Protein Name:	Location/Type:	Known Functions:
II	Hexon monomer	Structural
III	Penton base	Penetration
IIIa	Associated with penton base	Penetration
IV	Fibre	Receptor binding; haemagglutination
V	Core: associated with DNA & penton base	Histone-like; packaging?
VI	Hexon minor polypeptide	Stabilization/assembly of particle?
VII	Core	Histone-like
VIII	Hexon minor polypeptide	Stabilization/assembly of particle?
IX	Hexon minor polypeptide	Stabilization/assembly of particle?
TP	Genome - Terminal Protein	Genome replication

Table 1.2. List of adenovirus structural proteins (adapted from Shenk, 1996).

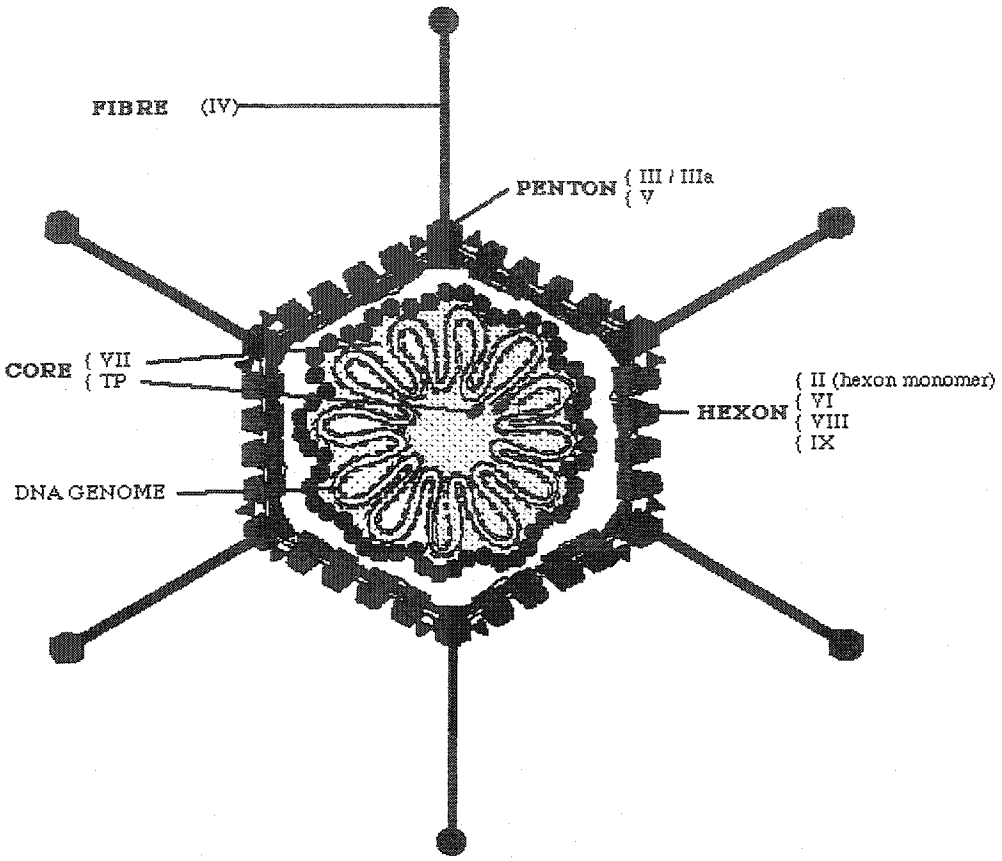


Figure 1.4. 2-D diagram of adenoviral capsid. Structural components and viral genome are indicated. (Adapted from Alan Cann)

1.2.4. Adenoviral Genome

Adenovirus genome consists of one double stranded DNA molecule of 30 to 36 kilo bases (kb) in length, depending on the Serotype (reviewed in Petterson and Roberts, 1986, Chroboczek et al., 1992). The inverted terminal redundancy regions (ITR) of 140 kb present at two ends of viral DNA contain the adenoviral origin of replication and are capable of forming panhandle structures necessary for the initiation of viral replication (Wolfson and Dressler, 1972, Steenberg et al., 1977). ITRs also contain cis-acting packaging sequences which are relevant for packaging of viral DNA into virions (Hearing et al., 1987).

Adenoviral genome is conventionally divided into 100 map units. Transcription of genes proceeds in both directions. The temporal expression of genes is the basis for their classification into early, delayed early, and late genes. The early transcriptional units include: E1A, E1B, E2, E3, and E4, each coding for multiple proteins. There are also two delayed-early units (IX and IVa2) and one major late unit coding for five products (L1-L5). All adenoviral genes are subject to alternative splicing, and are transcribed by RNA polymerase II. Adenovirus genome is depicted in Figure 1.5.

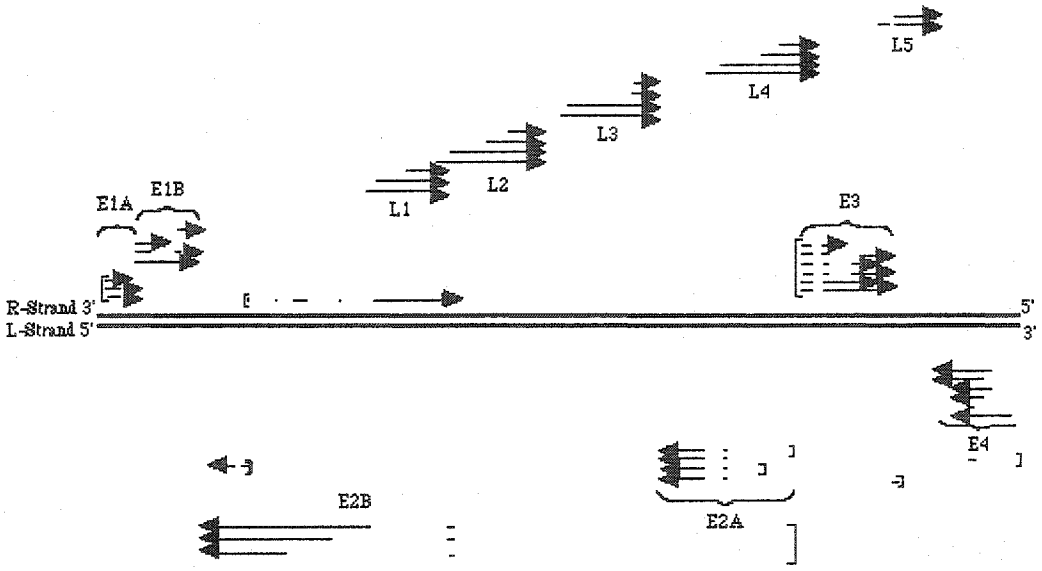


Figure 1.5. The structure of adenoviral genome. Genes are transcribed from both DNA strands. (adapted from Alan Cann)

1.2.5. Adenoviral DNA Replication

Adenovirus DNA replication begins at either end of the DNA molecule by 6 hours after infection and peaks at 18 hours post-infection (Horwitz et al., 1990). The terminal protein (pTP) initiates replication by binding to ITR sequences at a 5' end

of viral DNA and associating with E2-DNA Polymerase (Rekosh et al., 1977). E2-DNA polymerase initiates DNA synthesis from a deoxy-CMP (dCMP) primer bound to pTP. Initiation of replication also engages two host proteins, nuclear factor I and III (NF-I and NF-III) (Chen et al., 1990, Temperly and Hay, 1992). NF-I is involved in stabilization of the preinitiation complex (Chen et al., 1990), whereas NF-III promotes separation of DNA strands (Verrijzer et al., 1991). DNA replication proceeds in one direction, displacing the non-template strand. Elongation is aided by cellular NF-2 topoisomerase, which relieves torsional strain Induced in the DNA molecule during replication (Nagata et al., 1983), and by adenoviral DNA binding protein stabilizing the single strand of template DNA. The displaced strand is eventually replicated by forming a panhandle structure by ITR-mediated hybridization of its 5' and 3' ends, resulting in its association with pTP and E2-DNA polymerase. Replication of adenovirus DNA is depicted in Figure 1.6.

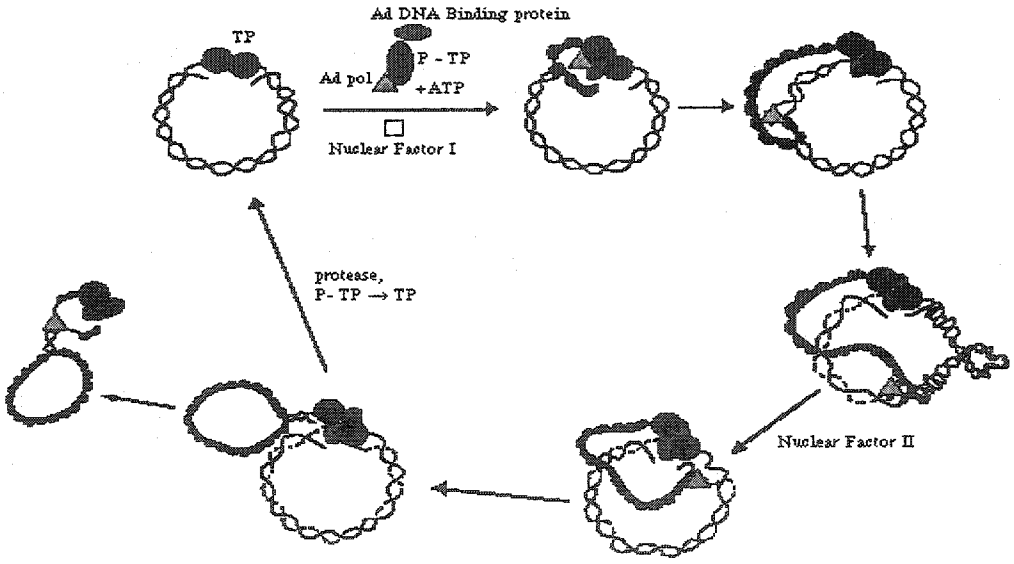


Figure 1.6. Replication of adenoviral DNA. Both viral and cellular factors are involved in the process. At the end of the cycle, a segment of the pre-terminal protein is cleaved off while the remaining 55 kD fragment, termed the terminal protein (TP) stays attached to the core DNA in mature virions (Adapted from Alan Caan)

1.2.6. Adenovirus Infection

Human type 5 adenovirus primarily targets terminally differentiated human epithelial cells of the upper respiratory tract (reviewed in Horwitz, 1996). The adenoviral infectious cycle generally lasts from 24 to 48 hours and culminates in the release of about 10,000 progeny virions. Initial stages of the infectious cycle involve adsorption of virus to the target cell, virus entry into the host cell, uncoating of viral DNA, and expression of early viral genes. Later stages involve DNA replication, late gene expression, virion packaging, and virion release from the host cell.

The adsorption of adenovirus to target cells is mediated by the interaction of the carboxy (C-) terminal knob of the penton fiber protein with coxsackievirus adenovirus receptor (CAR) (Bergelson et al., 1997). Adenoviral fiber knob also interacts with the alpha2 domain of the MHC (major histocompatibility complex) class I receptor expressed on the surface of epithelial cells (Hong et al., 1997).

Internalization of attached virus is mediated by the interaction of viral protein III of the penton base with the integrin family of cell surface receptors. The binding of protein III to cellular integrins occurs via an RGD (arginine, glycine, aspartic acid) sequence present in protein III (Wickham et al., 1993). RGD recognition sequence is also present in the extracellular adhesion molecules that are normal ligands of integrin receptors (Bai et al., 1993). Internalization of virions proceeds via endocytosis following fiber knob-mediated adsorption (Greber et al., 1993). Disassembly of the capsid leading to the release of viral core DNA occurs within endososmes as well as in the cytoplasm. Penton capsomeres are removed in endosomes due to a decrease in pH (Greber et al., 1993), whereas hexon capsomeres are proteolytically digested in the cytoplasm.

Upon capsid dissociation, the viral DNA core is transported to the nucleus through nuclear pores (Fredman and Engler, 1993). The transport of viral core is catalyzed by microtubules (Luftig and Weihig, 1975) and involves an interaction of viral protein V with cellular protein p32. p32 is known for its association with nuclear splicing factors ASF/SF2 (Krainer et al, 1991), but its function in the transport of viral DNA is not clear. Following its entry into the nucleus, viral DNA

associates with the nuclear matrix via the terminal protein (Bodnar et al., 1989) which results in the initiation of early gene expression.

1.2.7. Early Gene Expression

Adenoviral early genes are expressed within the first six hours of the infection (Nevins et al., 1979). The products of early transcriptional unit include E1A, E1B, E2, E3, and E4. E1A protein, the first expressed viral product and a major regulator of early events in the infection, is responsible for activation of transcription of the remaining early genes and stimulation of viral DNA replication.

1.2.7.1 Early Region 1A

1.2.7.1.1. mRNA and Protein Structure

The E1A transcription unit encodes five mRNAs of different Svedberg sedimentation coefficients. The mRNAs, which include 13S, 12S, 11S, 10S, and 9S molecules, arise as a result of alternate splicing of one large E1 mRNA transcript. The functions of products of three smallest mRNAs are not known (Stephens and Harlow, 1987). The products of the 13S and 12S transcripts, 289R and 243R proteins respectively, are major regulators of the adenoviral life cycle (Pericaudet et al, 1980 a,b). The two proteins are identical except for a 46 a.a. internal segment present only in the 289R protein. The 46 a.a. segment corresponds to conserved region 3 (CR3) and is absent from the E1A 12S product. Both 13S and 12S E1A products, however, contain conserved sequences CR1 and CR2. All conserved regions are involved in mediating E1A binding to other proteins (Van Ormondt et al, 1980, Kimelman et al., 1985).

E1A proteins are highly acidic and linear in structure due to a high proline content (Howe et al., 1990, Barbeau et al., 1994). The 289R and 243R E1A proteins, each with a half-life of 20-30 minutes (Spindler and Berk, 1984, Branton and Rowe, 1985) localize to the nucleus (Douglas and Quinlan, 1996). In addition to the three

conserved regions, E1A protein contains auxiliary region 1 (AR1) and auxiliary region 2 (AR2), two non-conserved elements located on the C-terminus of the protein (Bondesson et al., 1992).

1.2.7.1.2. E1A-mediated gene transactivation

E1A activates transcription of genes primarily by binding and interacting with a number of cellular DNA-binding transcription factors and targeting them to the viral promoters (Fergusson et al., 1985). E1A itself does not possess any DNA binding domains and therefore is not a classical transcription factor (Hearing and Shenk, 1985). The transactivational activity of E1A is attributed to CR3 in the 13S product (Berk, 1986), which contains two critical subdomains: the N-terminal transactivation domain and the C-terminal promoter targeting region (Webster and Ricciardi, 1991). The transactivation domain is responsible for binding of E1A to the TATA box binding protein (TBP), a DNA-binding component transcription factor IID (TFIID) involved in the formation of the transcription initiation complex. This interaction allows for activation of viral as well as some cellular promoters containing TATA box promoters (Wu et al, 1987). The mechanism of E1A CR3-mediated targeting of TBP to the promoter may involve the displacement of Dr1 and p53 from TBP by E1A. Both p53 and Dr1 interact with TBP and repress its activity (Horikoshi et al., 1995). The C-terminal region of CR3 mediates binding of E1A to several promoter-bound transcription factors including ATF-2, YY1, and AP-1. The E1A, E2, E3, and E4 promoters all contain ATF binding sites and are therefore responsive to E1A transactivational activity (Lee et al., 1987). By binding to ATF-2 and other DNA-bound factors, E1A is thought to bring TBP to the site of transcription initiation and thereby promote the formation of the initiation complex (Lee et al., 1991). The presence of ATF sites within the E1A promoter allows E1A to transactivate its own transcription in a positive feedback loop. E1B is expressed by read-through from the E1A promoter (Nevins, 1989).

The AP-1 family consists of heterodimeric transcription factor complexes, some of which are found to be the binding targets of E1A CR3. AP-1 dimers vary in content and may contain transcription factors such as Fos, Jun, and ATF. E1A promotes the binding of cJun/ATF heterodimers to the ATF sites in the viral promoters, stimulating their transcription (Benbrook and Jones, 1990), although the mechanism of AP-1 action is not fully understood. Another target of E1A CR3, the Yin Yang (YY1) protein, is a repressor of several promoters containing its binding sites. Binding of E1A to YY1 relieves the YY1-mediated repression, although the precise mechanism has not been characterized to date.

Furthermore, E1A is able to activate gene transcription through E2F binding sites present in the E2 viral promoter as well as in a number of cellular promoters. E2F is a transcription factor that induces the expression of cellular factors involved in the induction of cell cycle progression, including c-myc, N-myc, cdc2, and DHFR gene. Since the targets of adenoviral infection are non-dividing cells, the transcriptional activity of E2F is normally inhibited by physical interaction with the retinoblastoma (Rb) tumor suppressor protein (Sellers and Kaelin, 1997). E1A relieves Rb-mediated suppression of E2F by binding and dissociating Rb from the transcription factor. E1A interacts with Rb via its CR1 and CR2 domains, thus both 289R and 243R E1A products can activate E2F. The adenoviral E4orf6/7 product also aids the E2F-mediated gene transcription by binding E2F and stabilizing E2F-containing complexes (Hardy et al., 1989, Weill et al., 1990).

E1A is also able to stimulate transcription through the E4F binding sites that are present in the E4 transcriptional unit. The binding of E4F, a cellular transcription factor, to the E4 promoters is critically required for their stimulation (Bondesson et al., 1992). E1A promotes E4F binding to its promoters by interacting with the transcription factor via its two auxiliary regions, AR1 and AR2, as well as by stimulation of E4F phosphorylation (Muller et al., 1989). However, the mechanism of E1A-mediated activation of E4F remains uncharacterized.

1.2.7.1.3. E1A-Stimulated DNA Synthesis

As mentioned, adenovirus infects terminally-differentiated, non-dividing cells in which proliferative mechanisms, including replication of DNA, are suppressed. E1A is a viral mitogen that induces cell growth and allows the virus to replicate. The induction of the cell cycle is mediated from the amino terminus as well as CR1 and CR2 domains of E1A. Thus, both 13S and 12S products can induce DNA synthesis (Spindler et al., 1985, Kaczmarek et al., 1986, Smith and Ziff, 1988).

E1A has been shown to stimulate DNA synthesis through its interaction with several cellular proteins. One of its main binding partners is the retinoblastoma (Rb) tumor suppressor protein, a well recognized inhibitor of the cell cycle. Rb arrests cell cycle progression in mid and late G1 phase by binding to the E2F transcription factor and undermining its transactivation functions (Chellappan et al., 1991, Flemington et al., 1993, Helin et al., 1993). Rb is brought to the E2F-responsive promoters in complex with E2F (Sellers et al., 1995) where it mediates its inhibitory effects by interacting with histone deacetylase enzymes (HDACs) (Brehm et al., 1998). HDACs catalyze deacetylation of histones, thereby promoting condensation of the chromatin, making it less accessible to the transcription machinery, as well as by deacetylation of some transcription factors leading to their inactivation (Imoff et al., 1997). HDAC activity is therefore associated with decreased rates of transcription.

Rb activity is regulated by phosphorylation (DeCaprio et al., 1989, Ludlow et al., 1990). Rb tumor suppressor is able to bind and inactivate E2F in a hypophosphorylated state. Rb phosphorylation, mediated by cyclin-dependent kinases (cdk) in late G1 phase, leads to the release of E2F (Helm et al., 1992), thereby disrupting inhibitory effects of Rb and allowing E2F to drive transcription of its target genes (Hinds et al., 1992). 13S and 12S E1A products also act to remove Rb from E2F through binding the Rb protein and disrupting its interaction with E2F factors.

CR1 and CR2 regions of E1A cooperate to inhibit Rb. CR2 region mediates the binding of E1A to Rb (Egan et al., 1988) while CR1 region regulates the displacement of E2F from Rb (Ewen et al., 1991, Kaelin et al., 1990).
The CR2 region contains a leucine-X-cysteine-X-glutamic acid (LXCXE) sequence

(Egan et al., 1988, Whyte et al., 1989) which represents an Rb recognition motif (DeCaprio et al., 1988, Dyson et al., 1990).

E1A activates E2F functions also through cyclin A and cyclin-dependent protein kinase 2 (cdk-2) proteins (Kleinberger and Shenk, 1991, Tsai et al., 1991). Cdk-2, also known as p30, phosphorylates Rb and releases active E2F. Cyclin A (also referred to as p60) forms a complex with cdk-2 and regulates its activity (Hunter and Pines, 1994). E1A binds cyclin A and cdk-2 indirectly through p107 and p130, two E1A-binding proteins (Schwarz et al., 1993, Howe and Bayley, 1992). This interaction is associated with increased levels of cdk-2-mediated phosphorylation of Rb (Barbeau et al., 1994).

E1A also promotes cell growth by associating via the amino terminus and the CR1 region with p300, p400, and CBP proteins (Barbeau et al., 1993, 1994). p300, p400, and CBP (CREB binding protein) are histone acetyl transferases (HATs) and act as transcriptional coactivators of genes involved in cell cycle arrest (Arany et al., 1994). P300 and CBP stimulate gene transcription in response to the accumulation of second messenger cAMP molecules, which activate protein kinase A (PKA). Upon activation, PKA phosphorylates cAMP responsive element (CRE) binding protein (CREB) (Gonzalez and Montminy, 1989). CREB then binds CBP and p300, and the complex activates transcription of genes containing CRE promoters (Lundland et al., 1995). P300 is thought to mediate transcriptional coactivation through its intrinsic HAT activity as well through an associated HAT P/CAF. P/CAF (p300/CBP associated factor) is a histone acetyltransferase that is brought to CRE-containing promoters in complex with p300 and CBP (Yang et al., 1996). The products of CREB responsive genes block cell cycle progression and stimulate the process of differentiation (Moran, 1993). E1A prevents transcription of these genes by binding p300 and CBP (Arany et al., 1995) and inhibiting HAT activity of p300 and P/CAF (Hamamori et al., 1999).

1.2.7.1.4. E1A and Apoptosis

The E1A-mediated induction of unscheduled synthesis of DNA leads to activation of the p53 tumor suppressor protein (discussed in section 1.1.7.3.) involved in the induction of apoptosis and cell cycle arrest (Lowe and Ruley, 1993). E1A increases p53 half life from 20 minutes to 2 hours (Querido et al., 1997b) in part by upregulating p19/ARF (deStanchina et al., 1998), which complexes with Mdm-2 and prevents Mdm-2-mediated degradation of p53 (Pomerantz et al., 1998, discussed in section 1.1.7.3.2.). E1A can also inhibit p53 activation by a mechanism involving binding and sequestering of p300 (section 1.2.6.1.3.) (Gu et al., 1997, Lill NL et al., 1997).

Furthermore, E1A has been shown to sensitize cells to TNF-induced apoptosis (Shister et al., 1996, death receptor-mediated apoptotic pathways are discussed in section 1.1.8.3.). Adenoviral encoded proteins such as early region 3 (E3) 14.7 kD protein, E3 10.4/14.5 heterodimer, and early region 1B (E1B) 19 Kd protein, are later produced to interfere with the TNF-mediated cell death pathway, thus preserving host cell for viral replication (Gooding et al., 1991, Chiou et al., 1994).

Thus, in addition to the induction of cell growth and DNA replication, E1A also transactivates proteins involved in the inhibition of programmed cell death. In later stages of infection E1A-transactivated viral products induce p53-independent, caspase-independent cell death that is thought to be the mechanism of viral exit from infected cells (Marcellus et al., 1996, 1998, Lavoie et al.,1998, Shtrichman and Kleinberger, 1998). Viral mediators of the novel type of apoptosis are discussed later.

1.2.7.2 Early Region 1B (E1B)

The E1B products arise from an alternate splicing of the major E1B mRNA transcript. The E1B transcription unit produces a number of mRNAs as a result of read-through transcription of the E1A region, and is mostly independent of E1A transactivation activity. The major E1B transcript is 22S in size and encodes two critical proteins of 19 and 55 kilodaltons (called E1B-19K and E1B-55K proteins) (Petterson and Akusjarvi, 1983). Other E1B transcripts, including 13S and 14S,

encode several additional products such as 84R, 156R, and 93R proteins (Montell et al., 1984, Takayesu et al., 1994) whose functions have not been fully characterized.

E1B-19K and E1B-55K are the best characterized E1B products and are critical for the neutralization of E1A-induced apoptotic effects (Barker and Berk, 1987, Rao et al., 1992). E1B-19K protein localizes to nuclear and cytoplasmic membranes, intermediate filaments and nuclear lamins (Grand et al., 1985, White and Cipriani, 1989) and is essential for inhibition of apoptosis Induced in response to adenoviral infection (White et al., 1984, 1991, 1992). Mutational inactivation of E1B-19K has been shown to result in apoptotic death of infected cells and poor viral yields (Ezoe et al., 1981). The mechanism of E1B-19K-mediated suppression of apoptosis involves binding and neutralizing the pro-apoptotic members of the Bcl-2 family of proteins, including Bax, Bak, and Bik (Farrow et al., 1995, Han et al., 1996). The interaction of E1B-19K with these proteins is achieved through two Bcl-2 homology (BH) domains present in the 19K and Bcl-2-like proteins (discussed in section 1.1.7.1.). Thus, E1B-19K acts like an anti-apoptotic member of the Bcl-2-like family of proteins. Interestingly, E1B-19K has also been shown to prevent TNF and Fas-mediated apoptosis (Perez and White, 1998), and E1A-induced p53-independent cell death (Teodoro et al., 1995).

E1B-55K, the second major product of the E1B promoter, is a highly stable viral protein regulated by phosphorylation (Branton and Rowe, 1985, Rowe et al., 1983). E1B-55K inhibits p53-induced apoptosis (Marcellus et al., 1996a) by binding to the p53 transactivation domain (Lin et al., 1994) and repressing p53-initiated transcription (Yew and Berk, 1992). By binding to p53, E1B-55K also increases p53 affinity for DNA (Martin and Berk, 1998), further promoting its own inhibitory action. In addition, E1B-55K, in complex with E4orf6, targets p53 for degradation (Moore et al., 1996, Querido et al., 1997a), thereby eliminating this potent inducer of apoptosis. The roles of E1B-55K extend to the selective stabilization and transport of viral mRNA as well as the shut-off of host cell functions (discussed later).

1.2.7.3. Early Region 2 (E2)

The products of the E2 transcription unit are involved in the regulation of viral DNA replication. The E2 unit produces two different mRNA species, E2A and E2B, as a result of alternative splicing and different polyadenylation sites (Berk and Sharp, 1977). The E2A mRNA encodes the E2 DNA binding protein (E2-DBP) whereas the E2B mRNA encodes the DNA polymerase (E2-DNA pol) and the pre-terminal protein (E2-pTP). E2-DNA pol is involved in the replication of viral genome. It possesses a 5' to 3' DNA polymerase activity for DNA replication and a 3' to 5' exonuclease activity for proofreading (Field et al., 1984). E2-pTP is covalently bound to the 5' end of viral genome and promotes the initiation of DNA replication (Stillman et al., 1981). E2-pTP acts as a primer of DNA synthesis and directs the formation of the initiation complex at the viral origin of replication (Rekosh et al., 1977, Challberg et al., 1980). At the end of the replicative cycle, pTP is cleaved by L4-protease, but the generated 55 kD fragment remains attached to viral genome in mature virions (Challberg and Kelly, 1981). E2-DBP binds and stabilizes ssDNA intermediates that arise during adenoviral replication (Field et al., 1984). It is therefore important at the elongation stage of replication (Van der Vliet et al., 1975).

1.2.7.4. Early Region 3 (E3)

The E3 transcription unit produces 9 mRNA families (Chow et al., 1980). The products of the E3 region are implicated in downregulation of the host's immune response directed against the virus (Gooding, 1992). The E3 region is therefore essential in *in vivo* infections (reviewed in Ploegh, 1998), but has no role and is often deleted for in-vitro studies (Cladaras and Wold, 1985). Several E3 products have been identified and their functions characterized.

The E3-19 kD protein (gp19K) acts to protect virally-infected cells from cytotoxic T-lymphocyte (CTL)-mediated cell lysis (Andersson et al., 1987). Gp19K localizes to the endoplasmic reticulum (ER) membranes (Wold et al., 1985) where it binds and sequesters major histocompatibilty complex (MHC) class I (Kvist et al., 1978). MHC class I molecules are involved in presentation of viral antigens on the host's cell surface and are therefore markers of infection for the CTLs. Gp19K serves to block

their relocalization to the cell surface, thereby preventing CTL-mediated cell death (Paabo et al., 1987).

The E3-14.7 kD protein localizes to the cytoplasm and nucleus and acts to inhibit tumor necrosis factor (TNF)-mediated cytotoxicity (Tollefson and Wold, 1988, Horton et al., 1991). TNF-alpha cytokine, released from activated lymphocytes and macrophages, induces cytopathic effects through its receptor, TNF (reviewed in Wallach et al., 1997). Ligation of TNF-alpha to the TNF receptor induces apoptosis via several distinct pathways, one of which was described in section 1.1.8.3.2. The second mechanism involves the induction of calcium-dependent translocation of phospholipase 2 (cPLA2) to the cytoplasmic membrane where it catalyzes the release of arachadonic acid (AA). The dissociation of AA is linked to the onset of apoptosis in infected cells. The E3-14.7 kD product blocks this process, inhibiting cPLA2 activity and thereby preventing AA release (Zilli et al., 1992).

Furthermore, the E3-14.5 kD protein inhibits TNF-mediated cell death in complex with E3-10.4 kD product. These two proteins form heterodimers that localize to the plasma membranes (Tollefson et al., 1990). The heterodimers, also referred to as the receptor internalization and degradation (RID) complex, prevents cPLA2 translocation to the membranes, thus blocking TNF-induced apoptosis (Krajicsi et al., 1996). RID also mediates internalization and degradation of cell surface Fas receptors (Shisler et al., 1997) and epidermal growth factor receptors (EGFR) (Stewart et al., 1995). The elimination of Fas blocks Fas-initiated apoptosis (discussed in detail in section 1.1.8.3.1.). The purpose of EGFR degradation is not as clear.

In contrast to other characterized E3 products, the E3-11.6 kD protein does not appear to be involved in modifying the host immune responses. A deletion of the E3-11.6 kD coding region has been shown to result in defective virion release and improper host cell lysis (Tollefson et al., 1996). Thus, this particular E3 product may promote cell lysis in the final stages of infection, although the mechanism of its action is not known. The pattern of expression of E3-11.6 kD supports this hypothesis. The protein is present at very low levels in the initial stages of infection, but is upregulated 400-fold in late stages (Tollefson et al., 1992). Also in the late

infection, E3-11.6 kD protein translocates from the ER and Golgi apparatus to the nuclear membrane (Scaria et al., 1992), although the significance of this movement is not clear.

1.2.7.5. Early Region 4 (E4)

The E4 transcriptional unit produces 12 unique mRNA species that encode at least seven products from seven different open reading frames (orf) (Virtanen et al., 1984).

The E4orf1 product of 14 kD is possibly a transforming protein. E4orf1 from several adenovirus Serotypes has been shown to induce mammary carcinomas in rats (Weiss et al., 1996) and transform human cells (Weiss et al., 1997a). The oncogenic potential of E4orf1 protein is ascribed to its C-terminal PDZ domain (Weiss et al., 1997b) involved in binding of a number of cellular proteins. One of the characterized E4orf1 binding proteins is the *Drosophila* disc large tumor suppressor (Lee et al., 1997), but the significance of this and other interactions is not clear.

The function of E4orf2 is equally elusive. The E4orf2 region encodes a protein of 14.6 kD that localizes to the cytoplasm (Dix and Leppard, 1995), but its role has not been characterized.

The E4orf3 product of 11 kD localizes to the nucleus (Sarnow et al., 1982) where it associates with promyelocytic leukemia oncogenic domains (PODs). PODs are multi-protein complexes involved in regulation of gene expression and DNA replication. E4orf3 induces the relocation of POD proteins to the site of viral replication and to viral late promoters (Doucas et al., 1996), thereby enhancing the expression of viral genes. In addition, E4orf3 product contributes to transcription of the late viral genes by bringing cellular transcription regulators to late viral promoters.

E4orf4 viral protein is involved in the induction of apoptosis in later stages of infection and is discussed in more detail in section 1.2.11.

The E4orf6 product of 34 kD is involved in the selective transport of viral mRNA from nuclei to the cytoplasm as well as in host cell shut-off. In complex with E1B-

55K, the E4orf6 product functions to prevent p53-mediated apoptosis Induced in response to the viral infection. E4orf6 binds to the C-terminus of p53 and may inhibit p53 gene transactivation ability by displacing TAF 31 (Dobner et al., 1996, Nevels et al., 1997). Furthermore, E4orf6-E1B55K complex reduces p53 activity by inducing turnover of p53 (Querido et al., 1997a) by targeting it to the ubiquitin degradation complex (Boivin et al., 1999, Tauber and Dobner, 2001, Querido et al., 2001).

E4orf6/7 is a 19.5 kD hybrid of E4orf6 and E4orf 7 sequences that arises as a result of alternative mRNA splicing. E4orf6/7 stimulates the expression of E2 products, which are involved in the synthesis of viral DNA (section 1.2.6.2.). The mechanism of upregulation of E2 genes by E4orf6/7 involves E4orf6/7 mediated stabilization of transcription machinery at E2 promoters (Obert et al., 1994).

E4orf3/4 product is a 7.1 kD hybrid of E4orf3 and E4orf4 mRNA due to the alternative splicing of mRNA. Its function has not been characterized.

1.2.8. Late Gene Expression

The shift from the expression of early genes to the expression of late genes occurs at the onset of replication of viral genome. One single late primary transcript 29 kilo bases (kb) in length is expressed from the major late promoter (MLP). Five late mRNA transcripts, L1-L5, are produced from the primary transcript as a result of alternative splicing and different polyadenylation signals (Chow et al., 1977). The splicing of late viral mRNA is regulated by E4orf3 and E4orf6 (Nordqvist, 1994). The products of five late mRNAs are structural proteins that compose the adenovirus virion.

1.2.9 Host cell shut-off

At the time of viral DNA replication, host protein synthesis is completely turned off by the virus (Beltz and Flint, 1979). The inhibition of the translation of host proteins is achieved by two mechanisms. First, viral mRNA is selectively transported from the nucleus to the cytoplasm, while cellular mRNA is retained in the nucleus

(Bridge and Ketner, 1990). Selective mRNA transport is mediated by the protein complex composed of E1B-55K and E4orf6 that shuttles between the cytoplasm and the viral transcription sites in the nucleus (Sarnow et al., 1984, Ornelles and Shenk, 1991). The binding and sequestering of E1B-AP5, a nuclear RNA binding protein, by E1B-55K also contributes to the selective transcription of viral mRNAs (Gabler et al., 1988).

The second mechanism involves the protection of viral mRNA from the interferon response (reviewed in Zhang and Schneider, 1993). Normally, viral infection activates interferon-inducible protein kinase R (PKR) (O'Malley et al., 1986), which phosphorylates and inactivates the translation initiation factor eIF-2alpha (deHaro et al., 1996). Translation of viral mRNA does not require this factor, thus the synthesis of viral proteins is not inhibited. Furthermore, the mRNA cap binding translation initiation factor eIF-4F is also inactivated during adenoviral infection, so that translation of cellular mRNA is suppressed (Huang and Schneider, 1991). EIF-4F helicase activity is required to overcome the secondary structures at the site of translation origin. Translation of viral mRNA proceeds efficiently in the absence of eIF-4F because the 5'-untranslated region (UTR) of viral mRNA is free of secondary structure (Dolph et al., 1990).

1.2.10. Virion Assembly and Release

The virions are assembled upon completion of viral DNA replication and translation of the late structural proteins (reviewed in Philipson, 1984). Hexon formation is mediated by the L4-100 kD protein which supports the trimerization of polypeptide II (Cepko and Sharp, 1982). Pentons are formed from polypeptide III which pentamerizes and then associates with the fiber composed of three polypeptide II molecules (Velicer and Ginsberg, 1970). Hexon and penton capsomeres form an empty capsid which is then filled with one copy of the viral genome. The encapsidation of the genome is mediated by two late viral proteins, L1-52/55 kD and L3-coded viral protease. The first protein acts as a scaffold component (Hasson et al., 1989), whereas the second protein catalyses the digestion of some viral proteins

that is required for stability and infectivity of the mature virions (Webster et al., 1993).

The release of adenovirus from infected cells does not appear to involve cell lysis. Instead, adenoviral infection is associated with cytoskeletal breakdown and membrane disruption leading to an apoptotic type of cell death (Defer et al., 1990, Chen et al., 1993). Recent studies involving E4orf4 support the hypothesis that adenoviruses kill the host cell by apoptosis to evade the immune response and efficiently infect new cells. Since inactivating mutation of the E3-11.6 kD product also promotes cell survival, this protein is thought to cooperate with E4orf4 in the induction of apoptosis at the end of the infectious cycle.

1.2.11. E4orf4: overview

The adenoviral E4orf4 product is a small 14 kD protein and is produced in the early phase of infection (reviewed in Branton and Roopchand, 2001, Kleinberger T, 2000). E4orf4 localizes primarily to the nucleus, although significant amounts are also present in the cytoplasm (Miron et al., unpublished). The exact role of E4orf4 is not known. A number of studies have linked E4orf4 to the suppression of E1A-promoted gene transcription. E4orf4 has been shown to negatively regulate the expression of several E1A-activated promoters, including AP-1-responsive genes JunB and c-Fos (Muller et al., 1989), E4 adenoviral genes (Bondesson et al., 1996), as well as E2F-responsive genes from the adenoviral E2 region (Mannervik et al., 1999). These effects were attributed to E4orf4-induced reduction of phosphorylation levels of E1A and AP-1 (Muller et al., 1992).

E4orf4 is also involved in regulation of alternative splicing. E4orf4 modulates the activity of SR splicing factors involved in spliceosome assembly and recognition of splice sites (Zahler et al., 1992). E4orf4 protein has been linked to splicing of adenoviral L1 mRNA products through dephosphorylation of SR products (Kanopka et al., 1998).

Most recently, E4orf4 protein has been implicated in killing of infected cells by apoptosis in end stages of the viral cycle (Marcellus et al., 1998). The killing ability of E4orf4 also appears to involve dephosphorylation of cellular proteins.

1.2.11.1. E4orf4 and Apoptosis

The induction of apoptosis is generally associated with most viral infections (Roulston et al., 1999, Teodoro and Branton, 1997). Apoptotic mechanisms are triggered in metazoan cells in response to viral infections in order to prevent viral growth and spread to other cells and tissues. To counter host-mediated PCD, viruses have evolved mechanisms to block this apoptotic response (Roulston et al., 1999, section 1.1.9.4.). Adenoviral E1B 19K, E1B 55K, and E4orf6 proteins, discussed in previous sections, are all involved in the inhibition of the apoptotic machinery that is activated by E1A. Other viruses produce similar proteins that inhibit apoptosis to ensure viral survival (reviewed in Roulston et al., 1999).

Unlike E1A-induced apoptosis, however, E4orf4-mediated apoptosis is likely not an unwanted by-product of the infection, but a deliberate mechanism used by virus to kill the cell and promote viral exit. Virally-mediated apoptosis in end stages of infection confers several advantages for the virus. Firstly, this form of cell death protects the virus from the host's immune response as apoptosis is a non-inflammatory process, while cell lysis induces a potent immune response against the virus. Secondly, the engulfment of apoptotic bodies by surrounding cells facilitates viral entry into these cells and therefore promotes the viral infectious cycle.

The identification of E4orf4 as the adenoviral inducer of apoptosis was preceded by the finding that the expression of full-length E1A in p53-null cells resulted in the induction of cell death with some apoptotic features such as DNA laddering and chromatin condensation (Teodoro et al., 1995). Since E1A-mediated apoptosis critically relies on functional p53, this finding implied that late adenoviral apoptosis was caused by another viral product. Infections of cells with adenovirus mutants lacking various regions of the genome showed that the E4 region is essential for the induction of p53-independent apoptosis (Marcellus et al., 1996). The killing activity

was later mapped to E4orf4 following the analysis of several E4 mutants (Marcellus et al., 1998). It has also been affirmed that E4orf4 can induce cell death when expressed alone, and therefore does not need to cooperate with other viral products to mediate cytotoxic effects (Marcellus et al., 1998). In Chinese Hamster Ovary (CHO) cells, E4orf4 has been shown to induce apoptosis in the presence of zVAD-fmk caspase inhibitor (discussed in section 1.1.9.4.). Thus, E4orf4 appears to trigger apoptosis in a p53- and caspase-independent manner. Most recent data suggest, however, that in some mammalian cell lines E4orf4-induced apoptosis may be associated with the activation of caspase 3, caspase 8, and the release of cytochrome c from mitochondria (Livne, 2001).

E4orf4-mediated cell killing and other functions discussed earlier appear to depend on dephosphorylation of various proteins. Incidentally, the best characterized binding partner of E4orf4 is protein phosphatase 2A (PP2A). PP2A consists of three main subunits. A and B subunits perform regulatory functions, whereas the C subunit is catalytic (Janssens and Goris, 2001). The B subunit defines substrate specificity of PP2A (Kremmer et al., 1997). PP2A substrates include proteins involved in cell cycle regulation (e.g. cyclin-dependent kinases), DNA replication and cell metabolism (Janssens and Goris, 2001). It has been shown that interaction of E4orf4 with the B-alpha subunit of PP2A is absolutely required for the induction of cell death, both in mammalian cells (Marcellus et al., 2000, Shtrichman et al., 1999) and in yeast (Roopchand et al., 2001). The significance of E4orf4/PP2A interaction for the induction of apoptosis is not clear. It has been proposed that E4orf4 could alter substrate specificity of PP2A towards proteins involved in apoptosis, or binds and targets these proteins towards PP2A. Possible targets for E4orf4/PP2A-mediated dephosphorylation include Bcl-2 and Bcl-Xl, two anti-apoptotic members of the Bcl-2 family of proteins, as well as Bad, the pro-apoptotic member of the same family (section 1.1.7.1.). The activity of Bcl-2 and Bcl-Xl is suppressed by dephosphorylation (Yamamoto et al., 1999), whereas the activity of Bad is stmulated by it (section 1.1.7.1.2.). Thus, dephosphorylation of these proteins by PP2A would promote apoptosis. In fact, PP2A has been shown to associate with phosphorylated

Bcl-2 while the AC dimer has been shown to dephosphorylate Bcl-2 *in vitro* (Deng et al., 1998).

E4orf4 has also been found to associate with Src family kinases and contribute to extracellular apoptosis, or anoikis (Lavoie et al., 2000). Anoikis is a form of apoptosis Induced in response to detachment of cells from the extracellular matrix (ECM). The attachment of cells to the ECM is promoted by Src-mediated survival signaling, and the association of E4orf4 with Src appears to interfere with Src-initiated survival cascades (Lavoie et la., 2000). The consequences of the E4orf4 interaction with Src kinases are not entirely clear. Binding of E4orf4 to Src has been shown to increase E4orf4 affinity for the cytoskeletal proteins and to increase the amount of membrane blebbing and nuclear condensation due to E4orf4 expression. Furthermore, the inhibition of Src family kinases with PP2 chemical inhibitor reduced the number of apoptotic E4orf4-expressing cells (Lavoie et al., 2000). Although the mechanism of E4orf4-mediated anoikis is not known, E4orf4 is thought to disrupt Src signaling, leading to reorganization of actin cytoskeleton, induction of apoptotic features such as membrane blebbing, and triggering of cell death.

The ability of E4orf4 to induce p53-independent apoptosis presents a great potential in cancer therapy. As mentioned, most human cancers are associated with inactivating mutations in p53. Thus, the use of E4orf4 in gene therapy or as a model for drug development could facilitate the design of effective cancer treatment. In addition, the selectivity of E4orf4 killing for transformed cells would ensure that normal cells be spared from E4orf4's cytotoxic effects.

1.3. Thesis proposal

Adenoviral protein E4orf4 has been shown to induce programmed cell death independently of p53 (Marcellus et al., 1998, Shtrichamn and Kleinberger, 1998) and caspases (Lavoie et al., 1998). E4orf4 therefore seems to trigger a novel, non-classical form of apoptosis without core death machinery. The goal of this project was to analyze in detail the molecular hallmarks of E4orf4-induced cell death in

mammalian cells. The delivery and expression of E4orf4 in human transformed cell lines was followed by the examination of the molecular events that are generally associated with apoptosis. The activation of several mechanisms involved in the induction and execution of the classical apoptosis was studied by molecular and cell biology methods. In addition, changes in cellular morphology in response to E4orf4 expression were also examined and compared with those occurring in classical apoptosis.

2. Materials and Methods

2.1 Cell culture

293T cells are derived from human embryonic kidney cells and express Ad5 E1A and E1B proteins as well as SV40 Large T Antigen (Graham et al., 1977). H1299 are $p53^{-/-}$ human non-small-cell lung carcinoma cells. Both cell lines were cultured in α-minimal essential (α-MEM) medium supplemented with 10% fetal bovine Serum, 100 units/ml penicillin, 100 units/ml streptomycin, and 0.292mg/ml L-glutamine in a humidified 5% CO_2 atmosphere at 37° C.

2.2. Plasmids and Transient Transfections

293T cells were plated in 60-mm dishes and transfected using Lipofectamine PLUS reagent (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instructions. For caspase cleavage assessment, cells were transfected with 2.0 ug of empty vector pCDNA3 (Invitrogen, Burlington, ON, Canada), pFLAG-E4orf4 (Marcellus et al., manuscript in preparation) or pCDNA3-EGFP-p15BID (donated by Dr. Gordon Shore's lab, McGill University, Montreal, Canada). For luciferase assay, cells were co-transfected with 1.0 ug of pCDNA3, pFLAG-E4orf4 or pCDNA3-EGFP-p15BID, and 1.0 ug of pGL-2 vector expressing firefly luciferase (Promega Corporation, Madison, WI).

2.3. Adenovectors and Infections

Ad5 rtTA and Ad ind HA-tagged E4orf4 vectors were obtained from GeminX Biotechnologies Inc. and used to deliver and express E4orf4 in H1299 cells (Marcellus et al., 1998).

Ad5CMVp53 vector expressing wild-type human p53 protein from the CMV promoter (Bachetti and Graham, 1993, Marcellus et al., 1996 or Sandig et al., 1997) and Ad5pm2/3, a 19K deficient adenovirus (Boulakia et al., 1996, McLorie et al.,

1991), and Ad5tBid expressing the pro-apoptotic fragment of Bid (Szymborski et al., in preparation) were used to induce classical apoptosis in H1299 control cells.

Doxycycline (Sigma Diagnostics, St.Louis, MO) was added to cells following the infection at a concentration of 1 ug/ml.

Z-Val-Ala-Asp(OMe)-FMK Inhibitor (zVAD-fmk) (Enzyme Systems Products, Livermore, CA), a wide-range caspase inhibitor, was added to cells at a concentration of 40 uM immediately after addition of doxycycline.

2.4. Cell death assays: Trypan Blue and luciferase assay

Cell death in H1299 cells was analyzed by 0.4% Trypan Blue dye exclusion. Cells were plated in 24-well dishes, infected with adenovectors expressing HA-E4orf4 or p53 or with 19K- adenovirus, and treated with 1 ug/ml of doxycycline in the presence or absence of 20 uM z.VAD.fmk (Enzyme Systems Products, Livermore, CA) for 24, 48 or 72 hours. Adherent and non-adherent cells were collected and the aliquots were mixed with an equal volume of 0.4% Trypan Blue (GIBCO BRL, Gaithersburg, MD). Cells that absorbed Trypan Blue were considered to be dead.

Cell death in 293T cells was analyzed using the luciferase assay. Cells were plated and transfected as described above. Cells were harvested 48 hours post-transfection, resuspended in assay buffer (50mM ATP, 1M Tris-HCl pH=7.8, 1M MgCl2), lysed by three 4-minute freeze-thaw cycles, resuspended in luciferin solution (10mM luciferin, 1M Tris-HCl pH=7.8), and assayed for luciferase activity using Lumat LB 9507 luminometer (EG&G Berthold, Bundoora, Australia).

2.5. Protein extraction

To perform immunodetection of proteins other than cytochrome c, AIF, or PARP, H1299 and 293T cells were harvested and lysed in SDS Lysis Buffer (1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M NaP, 2 mM EDTA, 2ug/ml leupeptin, 2ug/ml aprotinin, 1mM pafabloc). Protein extracts were subjected

to quantification, SDS-PAGE, electrotransfer, and immunoblotting, as described below.

2.6. Cellular Fractionation

For immunodetection of cytochrome c and AIF, H1299 cells were harvested, washed in PBS, and resuspendended in HIM buffer (200mM mannitol, 70 mM sucrose, 10mM HEPES, 1mM EGTA, 2ug of leupeptin per ml, 2 ug of aprotinin per ml, 1mM pafabloc). Cells were homogenized on ice in a 2 ml motorized teflon homogenizer by crushing 40 times with a pestle rotating at 1,500 rpm. The homogenates were centrifuged at 2,500 rpm for 10 minutes at 4° C to remove the nuclei. The supernatants were further centrifuged at 11,000 rpm for 20 minutes at 4° C to isolate mitochondrial (pellet) and cytosolic (supernatant) fractions. Mitochondrial (P10) extract fraction was subjected to quantification, SDS-PAGE, electrotransfer, and immunoblotting, as described below.

2.7. PARP extraction

For immunodetection of PARP, H1299 cells were harvested, resuspended in SDS sample buffer (50 mM Tris-HCl, pH 6.8, 6% B-mercaptoethanol, 3% SDS, 0.0003% bromophenol blue, 6M Urea), and sonicated on ice 3 times for 15 seconds. Protein amounts were standardized based on cell number. Extracts from 40,000 cells were subjected to SDS-PAGE, electrotransfer and immunoblotting.

2.8. SDS-PAGE and Immunoblotting

Protein concentrations, with the exception of PARP extracts, were quantified using the Bio-Rad (Biorad Laboratories, Hercules, CA). Equal amounts of protein were loaded on 8-15% acrylamide gels and subjected to SDS-PAGE. Proteins were electrotransferred onto PVDF membranes (Millipore Corporation, Bedford, MA) and detected immunologically. To detect protein expression, the following antibodies

were used: mouse anti-HA (HA.11, BabCO Richmond, CA), mouse anti-Flag (Sigma Diagnostics, St-Louis, MO), mouse anti-caspase 1 (Pharmingen, San Diego, CA), mouse anti-caspase 2 (Pharmingen, San Diego, CA), mouse anti-caspase 3 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-caspase 6 (Pharmingen, San Diego, CA), mouse anti-caspase 7 (Pharmingen, San Diego, CA), mouse anti-caspase 8 (Oncogene Research Products, Cambridge, MA), and mouse anti-caspase 9 (Oncogene Research Products, Cambridge, MA), rabbit anti-cytochrome c (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-AIF (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-PARP (C-2-10) (BIOMOL Research Laboratories, Plymouth Meeting, PA). Anti-p53 mouse monoclonal antibody Ab1801 was purified by precipitation using ammonium sulfate from supernatants obtained from hybridoma cell cultures grown in Dulbecco's modified Eagle's medium supplemented with 10% low-immunoglobulin G (IgG) fetal calf Serum (Bethesda Research Laboratories). The rabbit anti-actin antibody was a generous gift from Dr. Gordon Shore (McGill University, Montreal, Canada).

The antigen-antibody complexes were detected using horseradish peroxidase-linked goat anti-mouse or anti-rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at a dilution of 1/10000, and visualized using the enhanced chemiluminescence (ECL) detection system (Amersham Corporation, Arlington Heights, IL)

2.9. Cell Viability Testing by ATP quantitation

H1299 lung carcinoma and C33A cervical carcinoma cells were plated in 96 well dishes at densities of 2000 and 4000 cells/well respectively. The following day the cells were infected at an MOI of 50 (in replicates of six) with adenovectors expressing HA tagged E4orf4, p53, a control vector (AdrtTA) or were mock infected. Cellular ATP levels were measured at time zero, and at 24, 48 and 72 hours post-infection using the ViaLight HS detection system (Bio-Whittaker Molecular Applications). ATP levels at time zero were set at 100 and other values are plotted

relative to that. In brief, the ViaLight protocol involves detergent lysis of the cells followed by the addition of luciferase enzyme, which consumes ATP in the process of light emission. Light production was measured using an EG & G Berthold Lumat LB 9507 luminometer.

2.10. Immunofluorescence (IF) and Fluorescence Activated Cell Sorting (FACS)

2.10.1. Detection of mitochondrial membrane depolarization by FACS

JC-1 is a lipophylic, cationic fluorescent dye that exhibits potential-dependent accumulation on negatively charged mitochondria to form "J" complexes that emit red fluorescence at 585nm wavelength. When mitochondria are depolarized, JC-1 cannot aggregate and thus remains in its monomeric form that emits green fluorescence at 530nm. The stock of JC-1 dye was prepared in high purity DMSO at a concentration of 5 mg/ml. Cells were infected with adenovectors expressing HA-E4orf4 or p53, and incubated for 48 hours. Cells were then harvested, resuspended in a-MEM media at a concentration of 1,000,000 cells/ml and incubated with JC-1 antibody at a concentration of 10 ug/ml for 15 minutes at 37 C. Following the incubation, mitochondrial membrane polarity was analyzed using flow cytometry. Data acquisition was done on a Coulter Epics XL 4 colors (Miami, Florida) equipped with a 488nm argon air-cooled laser. Emission was measured using the FL1 (BP 525nm filter) and FL2 (BP 575nm filter) channels. The analysis was performed using the SYSTEM II software.

2.10.2. Detection of ROS by FACS

The stock of 2,7-dihydrodichlorofluorescin (DFC) dye was prepared in high purity DMSO at a concentration of 5 mM. H1299 cells were infected with adenovectors expressing E4orf4 or p53. After 16 hours of incubation, cells were harvested, washed with PBS, and resuspended in a-MEM media at a concentration of 1,000,000 cells/ml. Cells were treated with 5 uM of DFC and incubated at room

temperature for 15 minutes. Following the incubation, cells were analyzed by flow cytometry. Samples were run on a BD Biosciences Facscan (San Jose, CA) equipped with a 488 nm air-cooled laser. Emission was measured using FL1 channel (530/30 filter). Analysis was done with CellQuest Pro software.

2.10.3. Detection of phosphatidylserine exposure by FACS and IF

H1299 cells were infected with adenovectors expressing E4orf4 or p53. After 48 hours, cells were harvested, washed with PBS, and resuspended in Annexin-Binding Buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2, pH 7.4) at a concentration of 1,000,000 cells/ml. The aliquots of 100uL of cell suspension were treated with 5uL of annexin V alexa fluor 488 conjugate or annexin V alexa fluor 594 conjugate (Molecular Probes, Eugene, OR) and incubated for 15 minutes at room temperature. Cells treated with alexa fluor 488 conjugates were analyzed by flow cytometry. Samples were run on a BD Biosciences Facscan (San Jose, CA) equipped with a 488nm argon air-cooled laser. Emission was measured using FL1 channel (530/30 filter). Analysis was done with Cellquest Pro software.
Samples treated with alexa fluor 594 conjugates were mounted on cover slips and analyzed by fluorescence microscopy (see Fluorescence Microscopy).

2.10.3.1. Fluorescence Microscopy

The binding of annexin V probe were visualized using a Nikon TE-300 inverted microscope using the 40X objective. The images were captured with a CCD camera driven by Act-1 software.

3. Results

3.1 E4orf4 induces cell death in human H1299 cells

To assess the cytotoxicity of E4orf4 in mammalian cells, H1299 cells were infected with adenovector expressing E4orf4. An HA-tagged version of E4orf4 was used to facilitate the detection of the expressed product. E4orf4 expression, as assessed by western blot, was evident after 24 hours after exposure to doxycycline, and peaked at 48 hours (Figure 3.1B). Cytotoxicity, measured by uptake of Trypan Blue, was not very pronounced at 24 hours post-infection, but became evident 48 hours of exposure to doxycycline. The levels of membrane permeability to Trypan Blue were about 35% of the total cell population at 72 hours of infection, clearly higher than those in non-expressing cells (Figure 3.1A). These results imply that E4orf4 induces moderate levels of cell death in H1299 cells. Ad p53 and Ad pm2/3, the inducers of classical apoptosis, induced higher levels of cell death than E4orf4 (Fig 3.1A).

3.2. zVAD-fmk does not inhibit E4orf4-mediated apoptosis in H1299 cells

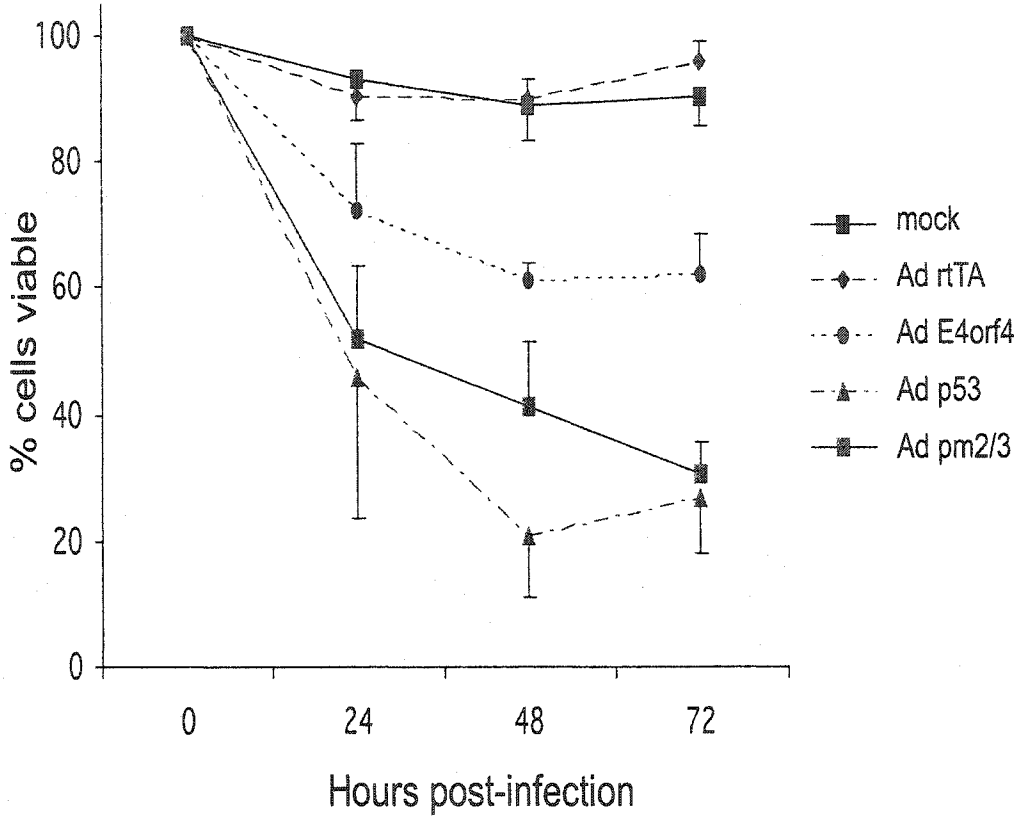
The classical apoptotic pathway implicates a group of cysteine-aspartate proteases known as caspases (section 1.1.9.). To examine caspase requirement in the induction of apoptosis by E4orf4, H1299 cells were treated with a wide-spectrum caspase inhibitor zVAD-fmk shortly after infection. Addition of zVAD-fmk has effectively impaired p53-mediated apoptosis, but it had no effect on E4orf4-mediated cytotoxicity. Expression of E4orf4 induced the same amount of cytotoxicity in the presence of zVAD-fmk as in its absence, in both cases reaching about 35% of the total cell population (Figure 3.2.A). However, addition of zVAD-fmk to cells treated with p53 caused an almost 50% decrease in cell killing, from 73% in the absence of the drug, to 26% in its presence (Figure 3.2.A). Since p53 is a known mediator of the

Figure 3.1. Viability of E4orf4-expressing H1299 cells

A. Cells were infected with indicated adenovectors and treated with 1ug/ml of doxycycline. Cell viability was analyzed by Trypan Blue exclusion. As indicated, Ad p53 and Ad pm2/3 induce high levels of cytotoxicity, while E4orf4 mediates moderate cytotoxicity. The results are representative of three separate experiments.

B. The expression of E4orf4 was confirmed by western blot. E4orf4 is expressed at high levels throughout the course of the experiment.

A. TRYPAN BLUE EXCLUSION ASSAY



B.

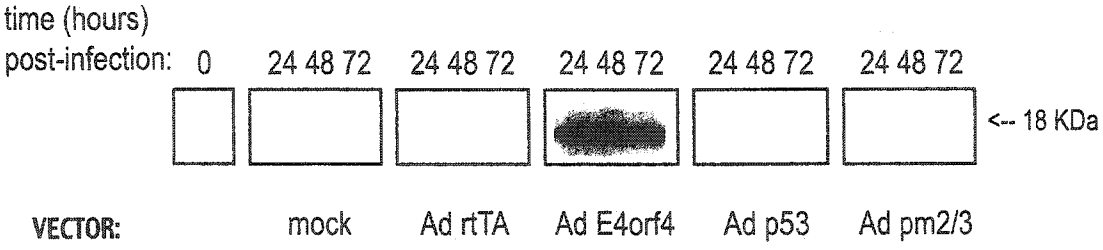
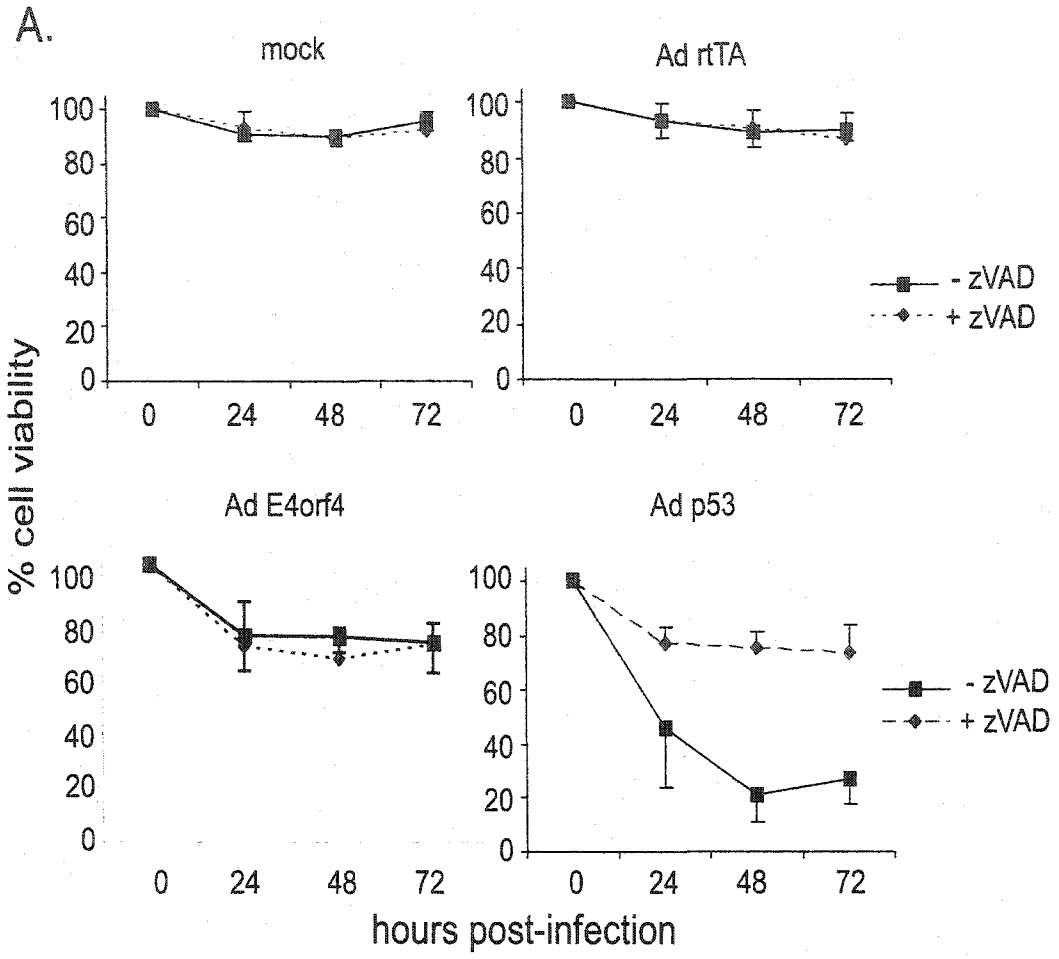
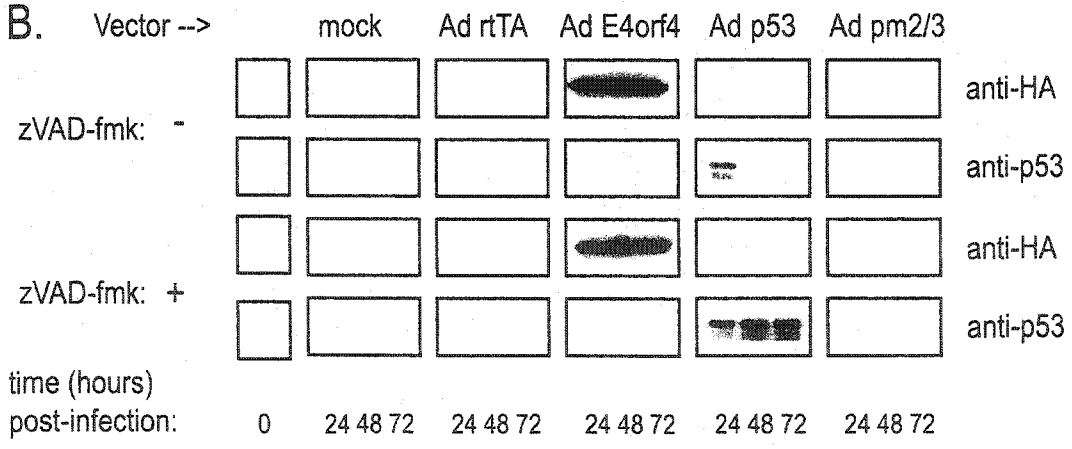


Figure 3.2. The effect of caspase inhibition on E4orf4-mediated cytotoxicity.

A. H1299 cell were infected with indicated vectors for 24, 48 and 72 hours. Cells were either treated with 40uM of zVAD-fmk, or left untreated. Cell viability was assessed by 0.4% Trypan Blue exclusion. Results show that caspase inhibition does not affect the cytotoxic potential of E4orf4, but reduces that of p53. Data are representative of three separate experiments.
B. The expression of E4orf4 and p53 was analyzed by western blot. The expression of p53 decreases in cells undergoing classical apoptosis, but is stable in cells rescued with zVAD-fmk. The expression of E4orf4 is high in the presence and absence of the drug.


A.
mock
Ad rtTA
Ad E4orf4
Ad p53
% cell viability
100
80
60
40
20
0
0
24
48
72
- zVAD
+ zVAD
hours post-infection


B.
Vector -->
mock
Ad rtTA
Ad E4orf4
Ad p53
Ad pm2/3
zVAD-fmk: -
zVAD-fmk: +
anti-HA
anti-p53
anti-HA
anti-p53
time (hours)
post-infection:
0
24 48 72
24 48 72
24 48 72
24 48 72
24 48 72

classical apoptotic pathway, it is not surprising that its killing efficiency was diminished by the addition of zVAD-fmk. Failure of zVAD-fmk to hinder E4orf4-mediated cytotoxicity suggests that E4orf4 triggers cell death in a caspase-independent manner. The expression of E4orf4 and p53 over the course of 72 hours was monitored by western blots (Figure 3.2.B). The expression of E4orf4 is stable over the duration of the experiment (Fig. 3.2.B). The expression of p53, on the other hand, is detectable for only 24 hours due to the instability of p53 in apoptotic cells. In cells rescued with zVAD-fmk, however, the expression of p53 is stable over 72 hours (Fig. 3.2 B).

3.3 E4orf4 does not induce caspase activity in H1299 cells

To further analyze the involvement of caspases in E4orf4-induced apoptosis, the activity of seven known caspases was specifically examined. It was recognized that procaspases undergo cleavage from full-length forms into shorter forms upon activation (section 1.1.9). Western blots were used to assess the processing of pro-caspases 1, 2, 3, 6, 7, 8, and 9 over 96 hours after exposure of H1299 cells to E4orf4. No loss of the pro-form was detected in any of the studied caspases in E4orf4-expressing cells (Figure 3.3.), therefore implying that none of these caspases were activated. This result is consistent with the fact that caspase inhibition with zVAD-fmk does not exert any detrimental effect on E4orf4-mediated apoptosis (Figure 3.2.A). E4orf4, therefore, seems to induce cell death via a caspase-independent pathway.

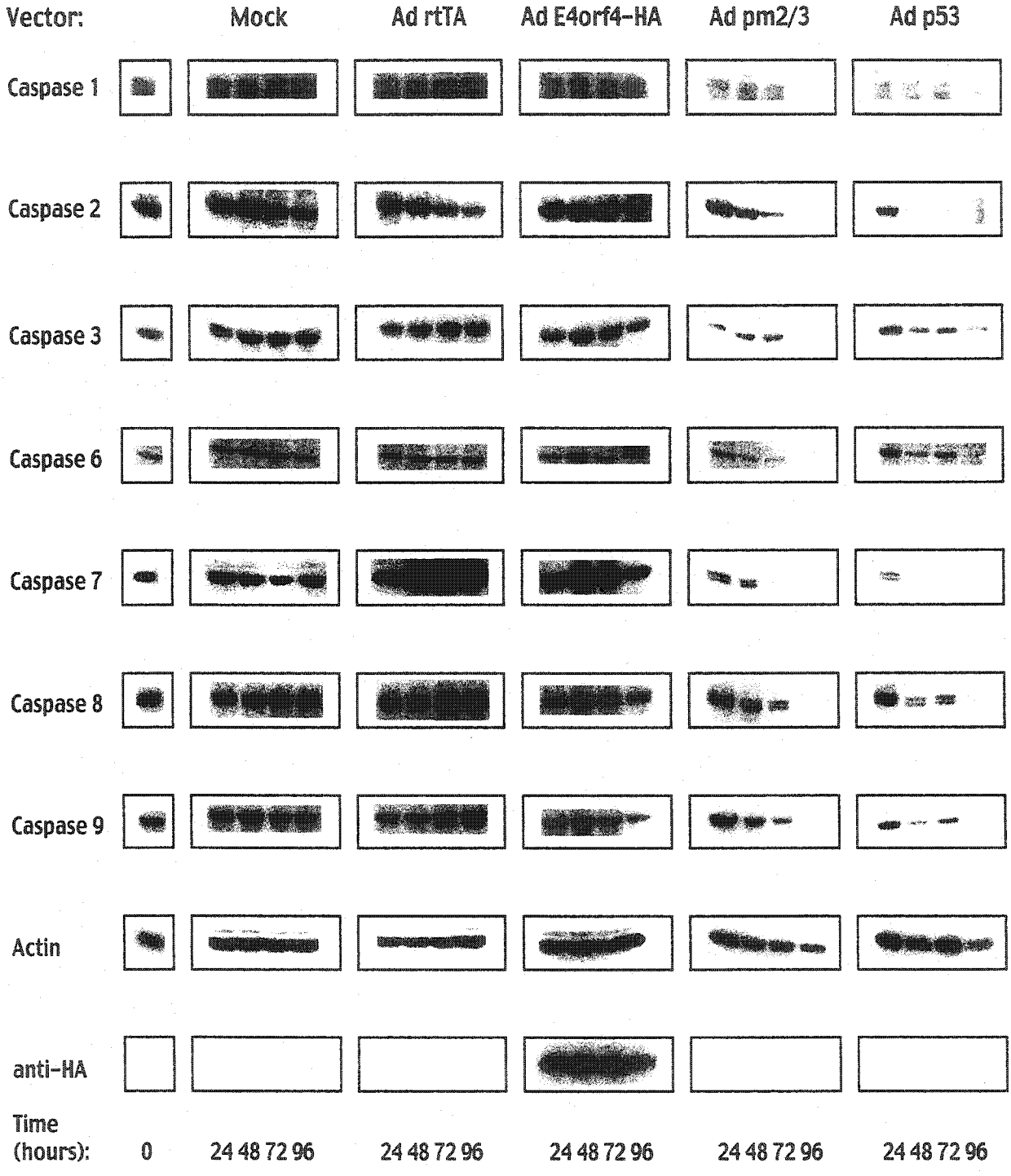
3.4. E4orf4 induces cell death but no caspase activity in 293T cells

It has been previously shown (Livne et al, 2001) that in mammalian 293T cells E4orf4 induces apoptosis through the death receptor pathway which involves the activation of caspase 8, but not caspase 9. Furthermore, caspase inhibition with a

Figure 3.3. Analysis of caspase activation by E4orf4 in H1299 cells.

H1299 cells were infected with indicated adenovectors. Caspase activation was analyzed by western blot. E4orf4 does not induce activation of caspase 1, 2, 3, 6, 7, 8, and 9, which remain in their inactive pro-form state. Massive activation of all studied caspases occurs in cells infected with Ad pm2/3 and Ad p53. The expression of E4orf4 was confirmed by immunoblotting with anti-HA antibody.

ANALYSIS OF CASPASE ACTIVATION BY E4orf4



known viral inhibitor CrmA impaired E4orf4-induced apoptosis in this cell line (Livne et al., 2001). To verify these results, 293T cells were transfected with a pcDNA3 vector expressing E4orf4 and analyzed for caspase activation by western blotting. As in H1299 cells, no loss of the pro-form of caspases 3, 8, and 9 was observed (Figure 3.4A). The expression of E4orf4 was confirmed by immunoblotting against the Flag tag (Figure 3.4A). To analyze the cytotoxic effects of E4orf4 in 293T cells, luciferase assays were performed. The levels of luciferase activity correlate positively with cell viability, so that apoptotic cells exhibit less luciferase activity. The results showed that at 48 hours post-transfection the viability of cells expressing E4orf4 is significantly lower than that of cells transfected with the pcDNA3 vector alone (Figure 3.4B). It is therefore clear that E4orf4 kills 293T cells efficiently, yet it fails to activate caspases. Thus, as in H1299 cells, a caspase-independent apoptotic pathway seems to be at work.

3.5. PARP is not processed in E4orf4-expressing H1299 cells

Poly-ADP Ribose Polymerase (PARP) is a DNA repair enzyme that is cleaved and thereby inactivated during apoptosis (Los et al. 2002) and is one of many known substrates of downstream caspases (section 1.1.9.3). The cleavage of PARP was analyzed by western blotting with an antibody that detects both its full-length form (116 kD) as well as the cleaved product (85 kD). The results show that PARP is not processed in response to E4orf4 expression 48 hours after addition of doxycycline (Figure 3.5). After 72 hours, a slight processing of the enzyme can be seen, however not as pronounced as in cells undergoing classical apoptosis in cells expressing 19K-adenovirus. In these cells, PARP is cleaved very efficiently within 48 hours of infection (Figure 3.5).

Figure 3.4. Analysis of caspase activation in E4orf4-expressing 293T cells.

A. Caspase activation in 293T cells was analyzed by western blots. Cells were transfected with indicated vectors expressing E4orf4 or tBid. At 48 hours post-transfection, the expression of E4orf4 did not induce cleavage of procaspases 3,8, and 9, suggesting that these caspases remain inactive. In contrast, the expression of tBid, a known mediator of classical apoptosis, induced activation of caspases 3 and 9, but not 8.

B. Cytotoxicity of E4orf4 in 293T cells was measured by luciferase assay. Cells were co-transfected with vectors expressing E4orf4 or tBid, and pGL-2 vector expressing firefly luciferase. The expression of E4orf4 reduced the transcription of the luciferase enzyme by 50%, implying that E4orf4 effectively killed 50% of E4orf4-expressing cells. The expression of tBid led to higher levels of cytotoxicity, killing 80% of tBid-expressing cells. Data are representative of three separate experiments.

Analysis of caspase activation by E4orf4 in 293T cells.

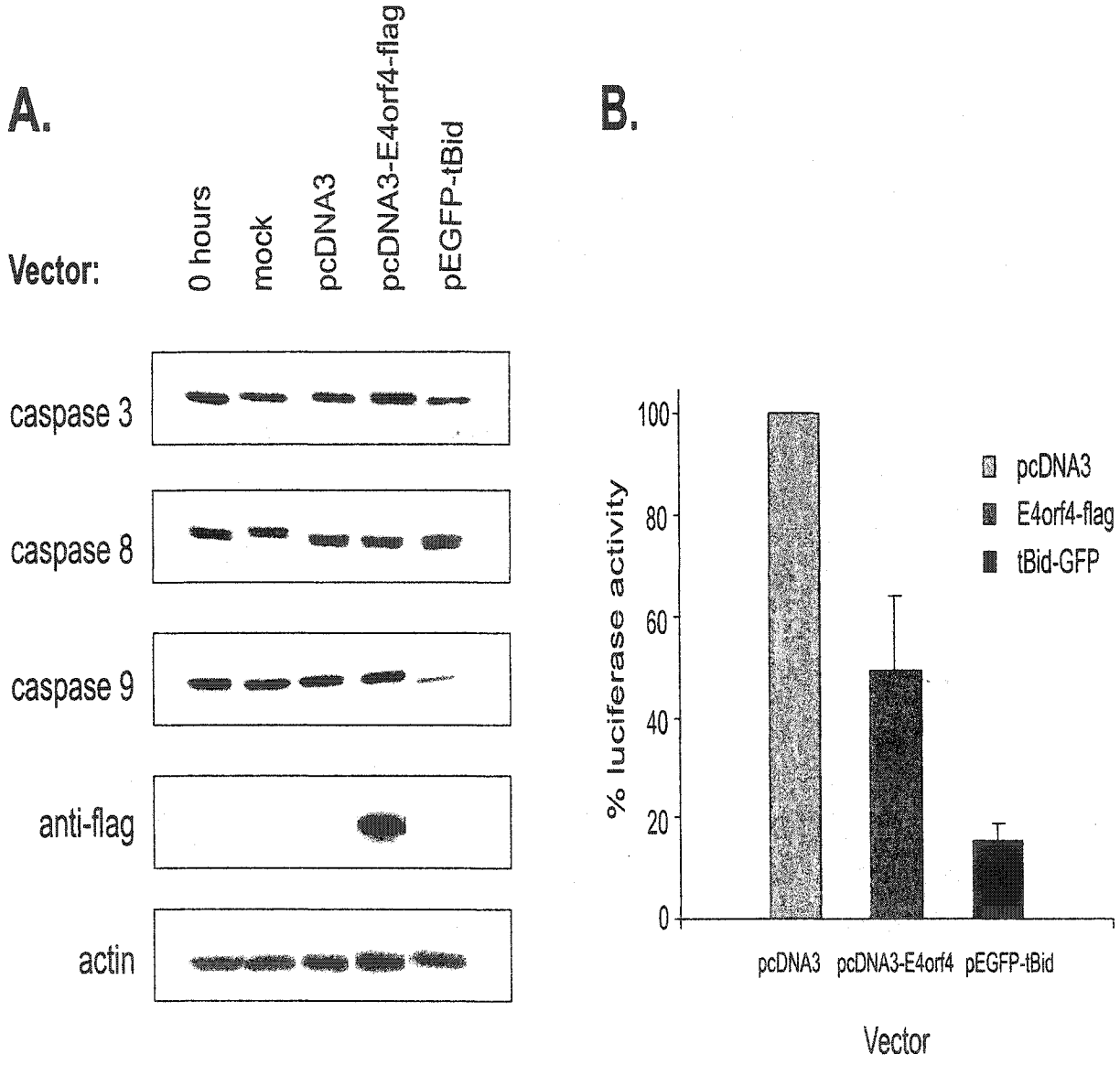


Figure 3.5. Analysis of PARP cleavage in H1299 cells expressing E4orf4.

H1299 cells were infected with indicated vectors. PARP cleavage was analyzed by immunoblotting with C-2-10 antibody that detects both full-length and the cleaved product of PARP. E4orf4 induced a weak processing of PARP 72 hours post-infection. In contrast, Ad pm2/3, which triggers cell death in a caspase-dependent manner, induced cleavage of PARP within 48 hours.

Analysis of processing of Poly-ADP Ribose Polymerase (PARP)

3.6 E4orf4 does not induce release of cytochrome c from mitochondria.

The release of cytochrome c from mitochondria is a molecular hallmark of the classical apoptosis. Cytochrome c released into the cytoplasm binds to APAF-1, dATP, and caspase 9 to form apoptosome (Zou H. et al., 1997, section 1.1.8.1.). Caspase 9 is then activated by auto-proteolysis to initiate the caspase cascade that culminates in apoptotic death of the cell. The release of cytochrome c from mitochondria in H1299 cells was studied by fractionation of cells into microsomal and cytoplasmic fractions and subsequent analysis of the protein content of the mitochondrial fraction . No loss of cytochrome c from the mitochondrial fraction was detected in cells infected with Ad E4orf4 after 48 hours, at which time it was depleted from mitochondria in cells expressing p53 and tBid (Figure 3.6). The lack of release of cytochrome c from mitochondria was also confirmed with immunofluorescence analysis (Miron MJ et al., unpublished data). Thus, E4orf4 does not seem to operate through the mitochondrial pathway, as was suggested elsewhere (Livne et al, 2001). The reasons for this discrepancy are still to be determined.

3.7. AIF does not mediate killing of H1299 cells by E4orf4.

AIF is located in the intermembrane mitochondrial space and has been proposed to have an electron donor/acceptor function based on its shared homology with bacterial oxidoreductases (Susin et al., 1999). Interestingly, in apoptotic cells, AIF has also been shown to translocate from mitochondria to the nucleus in order to induce oligonucleosomal DNA degradation and chromatin condensation, two hallmarks of classical apoptosis (Daugas et al., 2000, 1999, section 1.1.8.1.2.). Therefore, like cytochrome c, AIF seems to be a bifunctional protein with a role in cellular respiration and in the induction of apoptosis. To assess whether AIF is

relevant in E4orf4-mediated apoptosis, fractionation of cells expressing E4orf4 was performed and the content of the mitochondrial fraction was analyzed. After 48 hours of infection with AdE4orf4, no AIF has been released from mitochondria (Figure 3.6.). At the same time, exposure of cells to p53 and tBid efficiently depleted AIF from mitochondria. This result rules out the involvement of AIF in E4orf4-induced apoptosis, and further suggests that the mitochondrial pathway is not implicated in this process.

3.8. Mitochondrial membrane is not depolarized in E4orf4-expressing cells.

To further examine the involvement of mitochondria in E4orf4-mediated cell death, measurements of the mitochondrial membrane potential were carried out. In healthy cells mitochondrial membrane is negatively charged due to an electron gradient. In classical apoptosis, mitochondria undergo pronounced changes in membrane permeability, which results in the dissipation of the proton gradient and depolarization of the membrane (Kroemer and Reed, 2000, section 1.1.8.1.4.). The polarity status of mitochondria can be detected with cationic dyes that bind intact mitochondria through interactions with the negatively charged membranes (Kroemer G. and Reed J.C. 2000). JC-1 is a lipophilic, cationic dye that is monomeric in the cytoplasm but dimerizes upon binding to intact mitochondrial membranes. In its monomeric form it emits green fluorescence, while in its dimeric form it emits red fluorescence. In cells undergoing apoptosis, the mitochondrial electron gradient is abolished, so that JC-1 monomers cannot bind mitochondria and are therefore unable to dimerize. Thus, the concentration of monomeric forms increases and results in a shift in JC-1 emission spectra. Cells undergoing mitochondrial depolarization exhibit higher levels of green fluorescence detectable by flow cytometry (Cossarizza A. et al 1993). As can be seen in Figure 3.7A, E4orf4-expressing cells do not exhibit a pronounced shift in green fluorescence that is clearly evident in p53-expressing cells. The magnitude of JC-1 signal emitted from E4orf4-expressing cells does not differ from the one emitted from control non-

Figure 3.6. Analysis of cytochrome c and AIF release from mitochondria in response to E4orf4 expression in H1299 cells.

Cells were infected with indicated vectors for 48 hours. The microsomal fraction was obtained by fractionation and immunoblotted for cytochrome c, AIF, and TOM20. E4orf4 did not induce any release of cytochrome or AIF from mitochondria as both proteins are clearly retained in the mitochondrial fraction. In contrast, tBid and p53 triggered the release of both cytochrome c and AIF. TOM20 is an integral mitochondrial protein that served as loading control.

Microsomal fraction
Western blot
48 hours post-infection

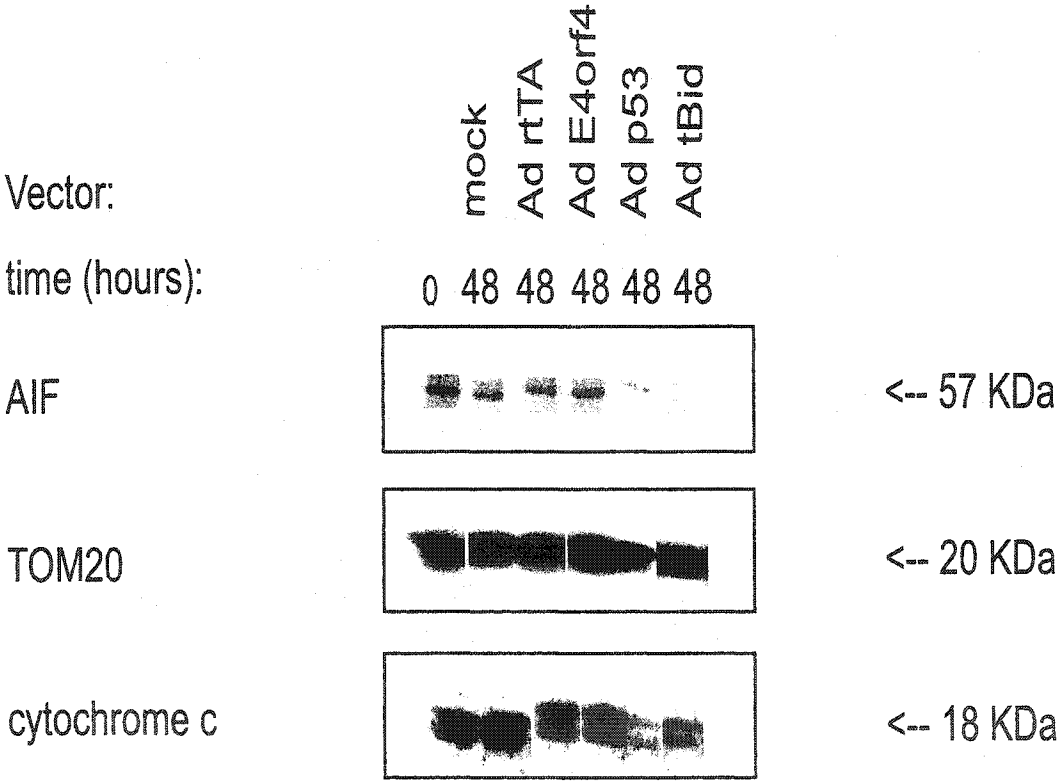
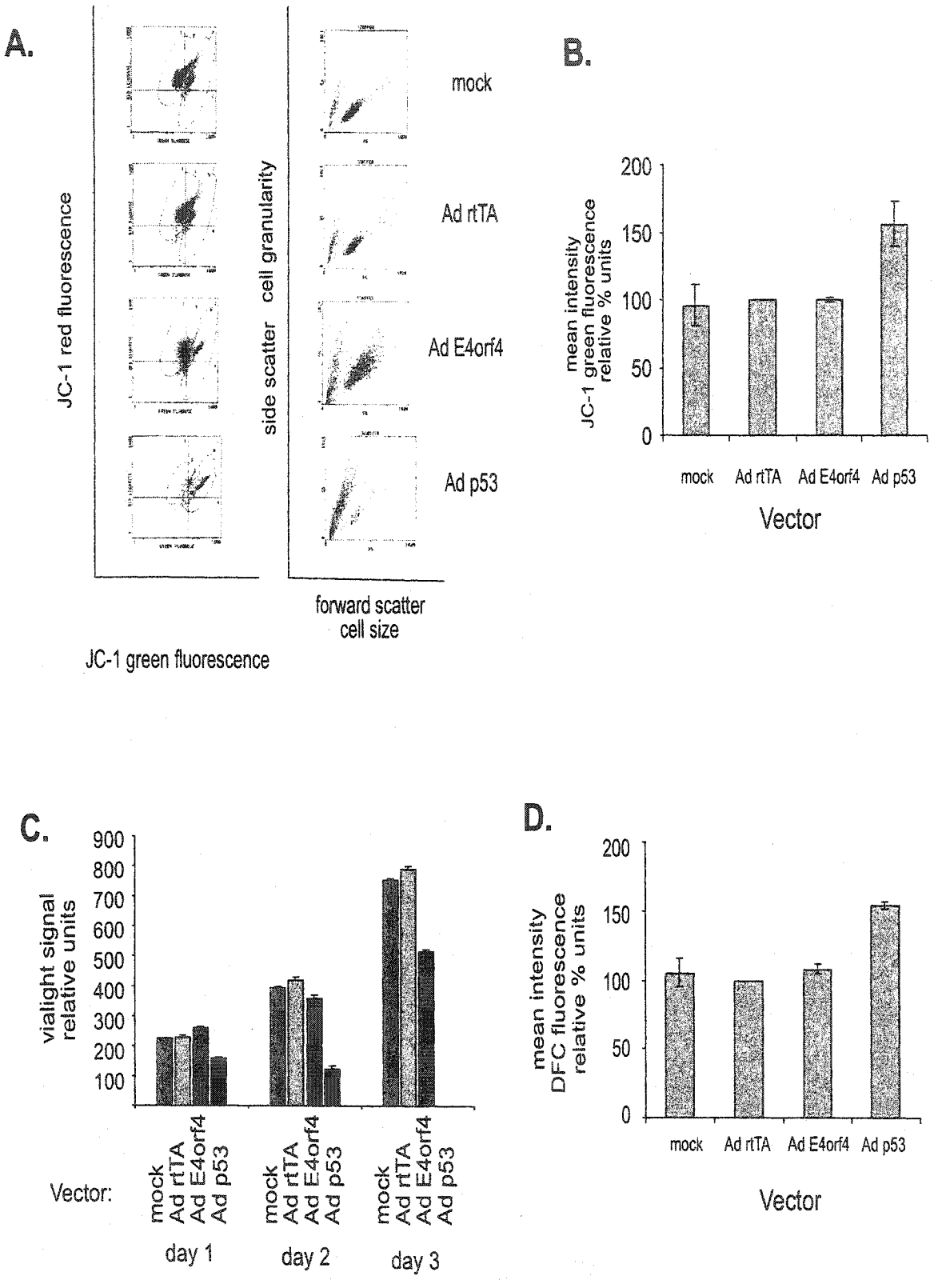


Figure 3.7. Analysis of mitochondrial membrane depolarization, ATP synthesis, and formation of Reactive Oxygen Species (ROS) in E4orf4-expressing H1299 cells.

A. Mitochondrial membrane depolarization was analyzed 48 hours after exposure of cells to E4orf4. First panel shows relative emission of green and red fluorescence from Ad rtTA, Ad E4orf4, and Ad p53-infected cells, as well as from uninfected mock population. A pronounced shift in green fluorescence can be seen with p53-expressing cells, but not with E4orf4-expressing cells. Second panel depicts morphology of analyzed cells. Only p53-expressing cells exhibit rounding and shrinkage typical of apoptosis, while other cells retain normal morphology.
B. Graph showing the mean intensity of emission of green fluorescence. E4orf4-expressing cells emit green fluorescence at levels comparable to those seen in mock and Ad rtTA-infected cells. Data are representative of three separate experiments.
C. ATP synthesis was measured using vialight assay. E4orf4 does not significantly affect the production of ATP, while p53 induces a massive drop in ATP synthesis.
D. Formation of ROS was measured using DFC dye 16 hours post-infection. The expression of E4orf4 in H1299 cells did not result in any increase in ROS production.


A.
JC-1 red fluorescence
JC-1 green fluorescence
side scatter cell granularity
forward scatter
cell size
mock
Ad rtTA
Ad E4orf4
Ad p53
B.
mean intensity
JC-1 green fluorescence
relative % units
200
150
100
50
0
mock
Ad rtTA
Ad E4orf4
Ad p53
Vector
C.
vialight signal
relative units
900
800
700
600
500
400
300
200
100
Vector:
mock
Ad rtTA
Ad E4orf4
Ad p53
day 1
day 2
day 3
D.
mean intensity
DFC fluorescence
relative % units
200
150
100
50
0
mock
Ad rtTA
Ad E4orf4
Ad p53
Vector

expressing cells (Fig. 3.7 B). Cells expressing p53, on the other hand, produce a stronger signal, 50% higher than that in the control population.

3.9. E4orf4 does not affect ATP production in H1299 cells

In normal, healthy cells mitochondria function as cellular "power-plants", which generate energy in the form of ATP. ATP production is diminished in apoptotic cells due to the disruption of mitochondria (Wang, 2001). The effects of E4orf4 expression on synthesis of ATP in H1299 cells were studied using the vialight method, which correlates the intensity of produced light to the amount of ATP produced in the cell (section 2.9., Szymborski et al., in preparation). Analysis of vialight intensity indicates that ATP production levels seem unaffected by E4orf4 (Figure 3.7.C). Levels of ATP increase in E4orf4-expressing cells as in control cells over the course of 72 hours. In p53-expressing cells, however, production of ATP is hindered and drops significantly (Fig 3.7.C). Thus, the energy status of cells committed to death by E4orf4 does not seem to be affected.

3.10. E4orf4 does not induce accumulation of ROS in H1299 cells

The production of ROS, such as H2O2 and O2-, is a hallmark of classical apoptosis (section 1.1.2.). The accumulation of H2O2 and O2- has been shown to play an important role in the induction of p53-mediated classical apoptotic cell death (Li et al., 1999). The amounts of hydrogen peroxide produced in response to E4orf4 expression were measured using fluorescent die 2,7-dihydrodichlorofluorescin (DFC). These results clearly indicate that there is no accumulation of H2O2 in H1299 cells in response to E4orf4 expression. As shown in Figure 3.7.D, DFC signal emitted from E4orf4-expressing cells does not differ significantly from the signal emitted from non-expressing control cells. At the same time, p53 induces a 50% increase in the magnitude of DFC signal, implying a half-fold increase in the

production of H2O2. This result shows that E4orf4-induced cell death does not seem to be associated with elevated levels of ROS.

3.11. E4orf4 induces some externalization of phosphatidylserine

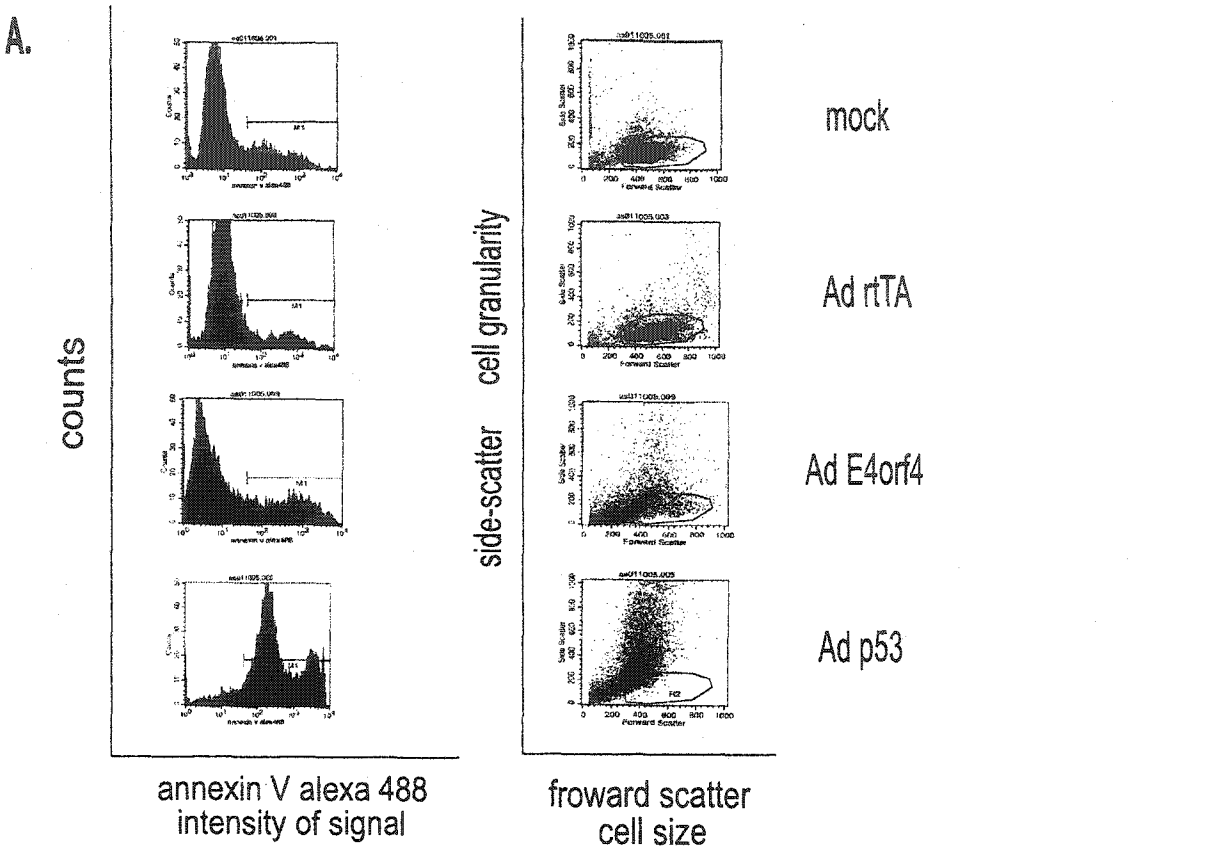
Phosphatidylserine is a fatty acid localized in the inner leaflet of the cytoplasmic membrane that translocates to the outer leaflet of the membrane in cells undergoing apoptosis and becomes exposed on the extracellular surface of the cell (Fadok et al, 1998, section 1.1.2.). Phosphatidylserine-specific antibody annexin V was used to study levels of phosphatidylserine exposure induced by E4orf4. Cells were infected with adenovectors expressing E4orf4 or p53 for 48 hours and treated with two types of annexin V antibody. Alexa Fluor 488 conjugate of annexin V was used to perform cytometric analysis, whereas alexa fluor 594 was used for immunofluorescence analysis. In flow cytometry, population of 10000 cells was analyzed by cell sorting for the amounts of emitted fluorescence, which correlates positively with the amount of antibody bound to the externalized PS. As shown in Figure 3.8.A, p53-expressing apoptotic cells exhibit very high intensity of alexa 488 fluorescence, whereas AdE4orf4-infected cells exhibit intensity of lesser magnitude. As shown in Fig 3.8.B, E4orf4 induces a 4-fold increase in the alexa 488 signal as compared to that in non-expressing cells. However, the signal emitted from p53-treated cells is 15 times stronger than that in non-expressing cells. This suggests that E4orf4 induces some exposure of phosphatidylserine, although significantly less than is seen in classical apoptosis.

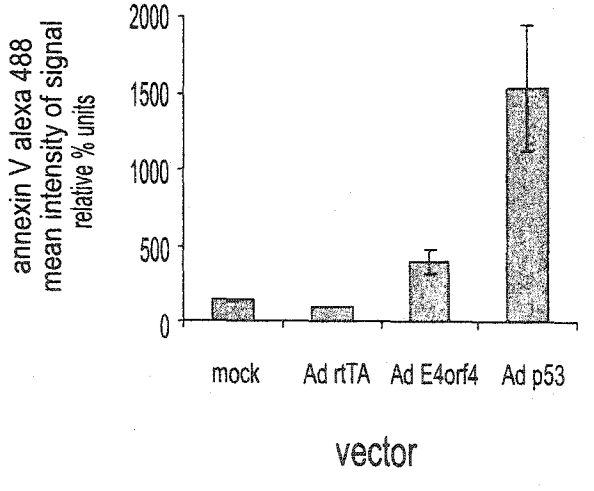
Moderate externalization of phosphatidylserine by E4orf4 was also observed when cells were analyzed by immunofluoresce. Cells were infected for 48 hours and incubated in the presence of alexa fluor 594 conjugate of annexin V. Pictures of selected fields in Figure 3.8.C depict annexin V-positive cells in the row on the right and the entire analyzed field (phase contrast) shown on the left. In order to quantify the externalization of phosphatidylserine, the number of fluorescent cells in a given field was compared to the total number of cells in that field. Roughly one third (32%) of cells infected with Ad E4orf4 stained red (data not shown), as compared to

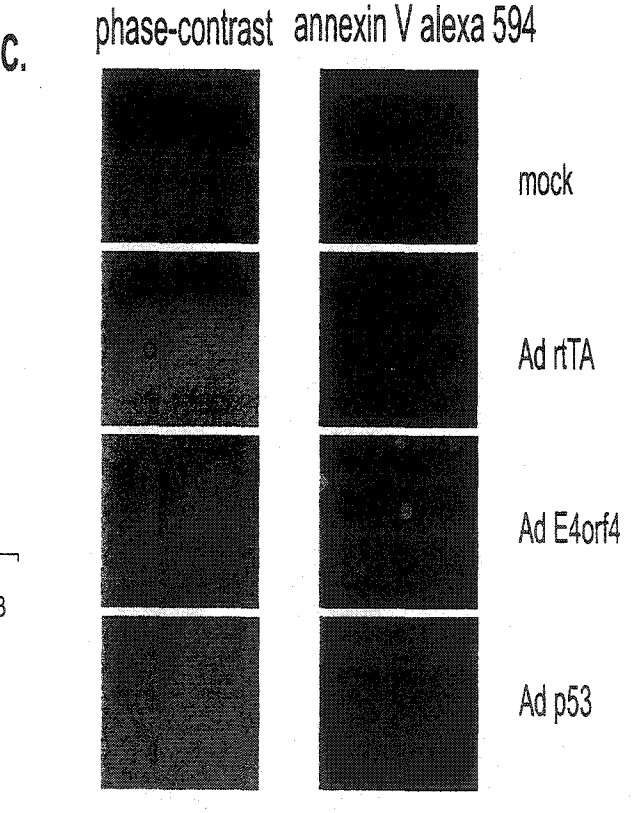
one tenth (10%) of cells infected with vector alone (data not shown). The ratio of red, annexin V-positive, cells to the total cell population was significantly higher (95%) in Ad p53-infected population (data not shown).

Figure 3.8. Externalization of phosphatidylserine in response to E4orf4 expression.

A. H1299 cells were infected with indicated vectors and treated with annexin V alexa fluor 488 conjugate 48 hours later. The intensity of emitted fluorescence was examined by FACS. First panel shows the intensity of alexa 488 signal and corresponding cell numbers. Second panel depicts cellular morphology.
B. Mean of alexa 488 intensity is shown for each vector. Cells expressing E4orf4 emit slightly higher levels of fluorescence than cells infected with a control vector. P53-expressing cells undergoing classical apoptosis produce a much stronger signal.
C. Cells were treated with annexin V alexa 594 conjugate 48 hours post-infection. Binding of annexin V was analyzed by immunofluorescence. Pictures of selected fields are shown. Cell treated with E4orf4 and p53 exhibit more binding of alexa 594 than non-expressing cells.


A.
counts
annexin V alexa 488
intensity of signal
side-scatter cell granularity
froward scatter
cell size
mock
Ad rtTA
Ad E4orf4
Ad p53


B.
annexin V alexa 488
mean intensity of signal
relative % units
2000
1500
1000
500
0
mock
Ad rtTA
Ad E4orf4
Ad p53
vector


C.
phase-contrast
annexin V alexa 594
mock
Ad rtTA
Ad E4orf4
Ad p53

4. Discussion and future work

The mechanism of induction of classical apoptosis through two distinct pathways has been characterized in detail. The upstream and downstream mediators of both the mitochondrial and death receptor pathways were identified and described (Introduction, figure 1.1. and 1.2.). In recent years, however, some studies have implied that there may be other molecular mechanisms that trigger programmed cell death (section 1.1.10). These mechanisms have not been characterized to date.

It has been suggested that adenoviral death protein E4orf4 does not require activation of core death machinery to induce apoptosis in rodent cells, and may utilize a yet uncharacterized apoptotic pathway (Lavoie et al, 1998). In this study we examined E4orf4-mediated apoptosis in transformed mammalian cell lines. The major focus was to analyze the effects of E4orf4 on known elements of the classical apoptotic pathway in order to verify whether or not they are dispensable in the induction of cell death by the adenoviral death protein. The results indicate that even though E4orf4 induces cell death with some, but not all, hallmarks of apoptosis, it does not activate any of the studied components of core death machinery. We therefore hypothesize that in transformed mammalian cells E4orf4 operates through a pathway that is distinct from the classical one, yet yields a similar end-result. The characterization of that pathway is the next step.

Proteins regulating and executing nuclear apoptotic changes are possible mediators of E4orf4-related cell death. Results from our lab indicate that E4orf4 induces massive condensation of nuclear chromatin (Miron, unpublished data), suggesting the involvement of DNA nucleases. A closer look at the mechanisms regulating the processing of DNA might therefore provide some insights about E4orf4 mode of action. A promising candidate to study is a mitochondrial protein Endonulease G (Endo G), which has been shown to mediate DNA degradation independently of caspases (van Loo et al, 2001, section 1.1.8.1.2.). It is therefore

possible that Endo G is part of a caspase-independent apoptotic pathway that is induced by the adenoviral death protein. The key experiment that must be done is to track the localization of EndoG throughout the course of Ad E4orf4 infection. The translocation of EndoG to the nucleus would implicate the nuclease as the key player in E4orf4-induced apoptosis. The identification of proteins interacting with EndoG during its translocation from mitochondria to the nucleus would provide further insights into the mechanism of caspase-independent apoptosis induced by E4orf4.

Signal transduction pathways have been shown to play a key role in the characterized apoptotic pathways, as in the death receptor pathway (Ashkenazi A. and Dixit V.M. 1998, section 1.2.7.11.1.). It is therefore possible that E4orf4 regulates some uncharacterized signaling cascade through its binding partner Protein Phosphatase 2A (PP2A). It has been shown that E4orf4 binding to PP2A is essential to cell killing, as the mutants that are unable to bind PP2A are defective at killing (Marcellus et al., 2000, Shtrichman and Kleinberger, 1998, Shtrichman R. et al. 1999). Thus, it is possible that E4orf4 mediates signaling events through regulation of PP2A specificity by selectively targeting PP2A to its substrates. A group of potential phosphatase substrates might include heat shock proteins that are implicated in the regulation of some apoptotic pathways. It has been reported that in its phosphorylated form, heat shock protein 27(HSP 27) inhibits caspase-independent apoptosis mediated by Daxx (section 1.1.8.1.2.). Dephosphorylation of HSP 27 relieves inhibition of Daxx and leads to the induction of aberrant, non-classical cell death (Charette et al., 2000). The contribution of Daxx to E4orf4-mediated apoptosis can be confirmed co-immunoprecipitation of Daxx and PP2A in the presence and absence of E4orf4. Interaction of PP2A and Daxx in the presence of E4orf4, but not in its absence, would suggest that PP2A is in fact targeted to Daxx by E4orf4. Inhibition or knock-out of Daxx would allow to definitively implicate Daxx as a critical player in E4orf4-mediated apoptosis.

Other potential mediators of E4orf4-induced apoptosis are non-caspase proteases. Cathepsin B and calpains are two examples of proteases that have been previously

implicated in the induction of caspase-independent cell death (section 1.1.8.2.). Similarly to E4orf4, cathepsin B has been shown to induce non-classical type of apoptosis in transformed cells while eliciting no apparent effects in primary cells (Foghsgaard et al. 2001). Cathepsin B is therefore potentially involved in E4orf4-induced apoptosis. The involvement of cathepsin B in E4orf4-mediated cell death can be verified by inhibition of the protease with CA-074-Me, a protease inhibitor specific for cathepsin B. The same effect can be achieved by RNA anti-sensing. Decrease of apoptotic rates in E4orf4-expressing cells due to the inhibition of cathepsin B would implicate cathepsin B as the key mediator of cytotoxicity of E4orf4. The activation of cathepsin B can also be verified by immunofluorescence that allows to track the release of the protease from lysosomes to the cytoplasm, as well as by the use of synthetic cathepsin B-specific substrates.

Although the mechanism of E4orf4-induced apoptosis remains largely uncharacterized, the clinical potential of E4orf4 is widely recognized. Apoptosis triggered by E4orf4 is unique in that it is specific to transformed cell lines (Marcellus, in preparation, Shtrichman et al, 1999) and is induced independently of p53 (Marcellus, 1998). Since most human tumors arise as a result of mutations in the p53 gene, the elucidation of a mechanism that triggers p53-independent cell death might provide insights into treatment of most human cancers. The ability of E4orf4 to induce cell death only in transformed cells could also provide ideas on selective elimination of tumor cells. The work on E4orf4 is on-going in academic and industrial settings and will hopefully yield results that will benefit scientific research as well as the medical field.

5. List of References

Adachi H., Adams A., Hughes F.M., Zhang J., Cidlowski J.A., Jetten A.M. (1998) Induction of apoptosis by the novel retinoid AHPN in human T-cell lymphoma cells involves caspase-dependent and independent pathways. Cell Death Differ 5(11):973-83

Adams J.M., Cory S. (1998) The Bcl-2 protein family: arbiters of cell survival. Science 281(5381):1322-6

Adida C, Haioun C, Gaulard P, Lepage E, Morel P, Briere J, Dombret H, Reyes F, Diebold J, Gisselbrecht C, Salles G, Altieri DC, Molina TJ. (2000) Prognostic significance of survivin expression in diffuse large B-cell lymphomas. Blood 96(5):1921-5

Ahmad M., Srinivasula S.M., Wang L., Litwack G., Fernandes-Alnemri T., Alnemri E.S. (1997) Spodoptera frugiperda caspase-1, a novel insect death protease that cleaves the nuclear immunophilin FKBP46, is the target of the baculovirus antiapoptotic protein p35. J Biol Chem 272(3):1421-4

Alarcon R.M., Rupnow B.A., Graeber T.G., Knox S.J., Giaccia A.J. (1996) Modulation of c-Myc activity and apoptosis in vivo. Cancer Res 56(19):4315-9

Ambrosini G., Adida C., Altieri D.C. (1997) A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat Med 3(8):917-21

An W.G., Kanekal M., Simon M.C., Maltepe E., Blagosklonny M.V., Neckers L.M. (1998) Stabilization of wild-type p53 by hypoxia-inducible factor 1alpha. Nature 392(6674):405-8

Andersson M., McMichael A., and Peterson P.A. (1987). Reduced allorecognition of adenovirus 2 infected cells. J. Immunol. 138:3960-3966.

Annis M.G., Zamzami N., Zhu W., Penn L.Z., Kroemer G., Leber B., Andrews D.W. (2001) Endoplasmic reticulum localized Bcl-2 prevents apoptosis when redistribution of cytochrome c is a late event. Oncogene 20(16):1939-52

Aragane Y., Kulms D., Metze D., Wilkes G., Poppelmann B., Luger T.A., Schwarz T. (1998) Ultraviolet light induces apoptosis via direct activation of CD95 (Fas/APO-1) independently of its ligand CD95L. J Cell Biol 140(1):171-82

Arany Z., Newsome D., Oldread E., Livingston D.M., and Eckner R. (1995). A family of transcriptional adaptor proteins targeted by the E1A oncoprotein. Nature 374:81-84.

Arany Z., Sellers W.R., Livingston D.M., and Eckner R. (1994). E1A-associated p300 and CREB-associated CBP belong to a conserved family of coactivators. Cell 77:799-800.

Ashkenazi A., Dixit V.M. (1998) Death receptors: signaling and modulation. Science 281(5381):1305-8

Bacchetti S., and Graham F.L. (1993). Inhibition of cell proliferation by an adenovirus vector expressing the human wild type p53 protein. Inter. J. Oncol. 3:781-788.

Bai M., Harfe B., and Freimuth P. (1993) Mutations that alter an Arg-Gly-Asp (RGD) sequence in the adenovirus type 2 penton base protein abolish its cell-rounding activity and delay virus reproduction in flat cells. J. Virol. 67:5198-5205.
Banin S., Moyal L., Shieh S., Taya Y., Anderson C.W., Chessa L., Smorodinsky N.I., Prives C., Reiss Y., Shiloh Y., Ziv Y. (1998) Enhanced phosphorylation of p53 by ATM in response to DNA damage. Science 281(5383):1674-7

Barbeau D., Charbonneau R., Whalen S.G., Bayley S.T., and Branton P.E. (1994). Functional interactions within adenovirus E1A protein complexes. Oncogene 9:359-373.

Barbeau D., Marcellus R.C., Bacchetti S., Bayley S.T., and Branton P.E. (1993). Quantitative analysis of regions of adenovirus E1A products involved in interactions with cellular proteins. Biochem. Cell Biol. 70:1123-1124.

Barker D., and Berk A.J. (1987). Adenovirus proteins from both E1B reading frames are required for transformation of rodent cells by viral infection and DNA transfection. Virology 156:107-121.

Barlow C., Brown K.D., Deng C.X., Tagle D.A., Wynshaw-Boris A. (1997) Atm selectively regulates distinct p53-dependent cell-cycle checkpoint and apoptotic pathways. Nat Genet 7(4):453-6

Beltz G.A., Flint S.J. (1979). Inhibition of Hela cell protein synthesis during adenovirus infection. J. Mol. Biol. 131:353-373.

Benbrook D.M., and Jones N.C. (1990). Heterodimer formation between CREB and Jun proteins. Oncogene 5:295-302.

Bennett M., Macdonald K., Chan S.W., Luzio J.P., Simari R., Weissberg P. (1998) Cell surface trafficking of Fas: a rapid mechanism of p53-mediated apoptosis. Science 282(5387):290-3

Benoist C. and Mathis D. (1997) Cell death mediators in autoimmune diabetes—no shortage of suspects. Cell 89(1): 1-3

Benson R.S., Dive C., Watson A.J. (1998) Comparative effects of Bcl-2 over-expression and ZVAD.FMK treatment on dexamethasone and VP16-induced apoptosis in CEM cells. Cell Death Differ 5(5):432-9

Bergelson J.M., Modlin J.F., Wieland-Alter W., Cunningham J.A., Crowell R.L., Finberg R.W. (1997) Clinical coxsackievirus B isolates differ from laboratory strains in their interaction with two cell surface receptors. J Infect Dis 175(3):697-700

Berk A.J., and Sharp P.A. (1977). Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease digested hybrids. Cell 12:721-732.

Bernardi P., Broekemeier K.M., Pfeiffer D.R. (1994) Recent progress on regulation of the mitochondrial permeability transition pore; a cyclosporin-sensitive pore in the inner mitochondrial membrane. J Bioenerg Biomembr 26(5):509-17

Bidere N., Senik A. (2001) Caspase-independent apoptotic pathways in T lymphocytes: a minireview. Apoptosis 6(5):371-5

Bodmer J.L., Holler N., Reynard S., Vinciguerra P., Schneider P., Juo P., Blenis J., Tschopp J. (2000) TRAIL receptor-2 signals apoptosis through FADD and caspase-8. Nat Cell Biol 2(4):241-3

Bodnar J.W., Hanson P.I., Polvino-Bodnar M., Zempsky W., and Ward D.C. (1989). The terminal regions of adenovirus and minute virus of mice DNAs are preferentially associated with the nuclear matrix in infected cells. J. Virol. 63:4344-4353.

Boivin D., Morrison M.R., Marcellus R.C., Querido E., Branton P.E. (1999) Analysis of synthesis, stability, phosphorylation, and interacting polypeptides of the 34-kilodalton product of open reading frame 6 of the early region 4 protein of human adenovirus type 5. J Virol 73(2):1245-53

Boldin M.P., Goncharov T.M., Goltsev Y.V., Wallach D. (1996) Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. Cell 85(6):803-15

Bondesson M., Svensson C., Linder S., and Akusjärvi G. (1992). The carboxy-terminal exon of the adenovirus E1A protein is required for the E4F-dependent transcription activation. EMBO J. 11:3347-3354.

Borner C., Martinou I., Mattmann C., Irmler M., Schaerer E., Martinou J.C., Tschopp J. (1994) The protein bcl-2 alpha does not require membrane attachment, but two conserved domains to suppress apoptosis. J Cell Biol 126(4):1059-68

Borner C., Monney L. (1999) Apoptosis without caspases: an inefficient molecular guillotine? Cell Death Differ 6(6):497-507

Bossy-Wetzel E., Bakiri L., Yaniv M. (1997) Induction of apoptosis by the transcription factor c-Jun. EMBO J 16(7):1695-709

Boulakia C.A., Chen G., Ng F.W., Teodoro J.G., Branton P.E., Nicholson D.W., Poirier G.G., and Shore G.C. (1996). Bcl-2 and adenovirus E1B 19 kDa protein prevent E1A-induced processing of CPP32 and cleavage of poly(ADP-ribose) polymerase. Oncogene 12:529-535.

Brancolini C., Benedetti M., and Schneider C. (1995). Microfilament reorganization during apoptosis: The role of Gas2, a possible substrate for ICE-like proteases. EMBO J. 14:5179-5190.

Brancolini C., Benedetti M., and Schneider C. (1995). Microfilament reorganization during apoptosis: The role of Gas2, a possible substrate for ICE-like proteases. EMBO J. 14:5179-5190.

Branton P.E., and Rowe D.T. (1985). Stabilities and interrelations of multiple species of human adenovirus type 5 early region 1 proteins in infected and transformed cells. J. Virol .56:633-638.

Branton P.E., Roopchand D.E. (2001) The role of adenovirus E4orf4 protein in viral replication and cell killing. Oncogene 20(54):7855-65

Brehm A., Miska E.A., McCance D.J., Reid J.L., Bannister A.J., and Kouzarides T. (1998). Retinoblastoma protein recruits histone deacetylase to repress transcription. Nature 391:597-601.

Bridge E., and Ketner G. (1990). Interaction of adenoviral E4 and E1B products in late gene expression. Virology 174:345-353.

Brown D.A. and Rose J.K. (1992) Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. Cell. 68(3):533-44.

Bruckheimer E.M., Brisbay S., Johnson D.J., Gingrich J.R., Greenberg N., McDonnell T.J. (2000) Bcl-2 accelerates multistep prostate carcinogenesis in vivo. Oncogene 19(46):5251-8

Bump N.J., Hackett M., Hugunin M., Seshagiri S., Brady K., Chen P., Ferenz C., Franklin S., Ghayur T., Li P. (1995) Inhibition of ICE family proteases by baculovirus antiapoptotic protein p35. Science 269(5232):1885-8

Buschmann T., Fuchs S.Y., Lee C.G., Pan Z.Q., Ronai Z. (2000) SUMO-1 modification of Mdm2 prevents its self-ubiquitination and increases Mdm2 ability to ubiquitinate p53. Cell 101(7):753-62

Casciola-Rosen L., Nicholson D.W., Chong T., Rowan K.R., Thornberry N.A., Miller D.K., Rosen A. (1996) Apopain/CPP32 cleaves proteins that are essential for cellular repair: a fundamental principle of apoptotic death. J Exp Med 183(5):1957-64

Cepko C.L., and Sharp P.A. (1982). Assembly of adenovirus major capsid protein is mediated by a non-virion protein. Cell 3:407-414.

Challberg M.D., and Kelly T.J.Jr. (1981). Processing of the adenovirus terminal protein. J. Virol. 38:272-277.

Chang B.S., Minn A.J,. Muchmore S.W., Fesik S.W., Thompson C.B. (1997) Identification of a novel regulatory domain in Bcl-X(L) and Bcl-2. EMBO J 16(5):968-77

Charette S.J., Lambert H., Landry J. (2001) A kinase-independent function of Ask1 in caspase-independent cell death. J Biol Chem 276(39):36071-4

Charette S.J., Landry J. (2000) The interaction of HSP27 with Daxx identifies a potential regulatory role of HSP27 in Fas-induced apoptosis. Ann N Y Acad Sci 926:126-31

Charette S.J., Lavoie J.N., Lambert H., Landry J. (2000) Inhibition of Daxx-mediated apoptosis by heat shock protein 27. Mol Cell Biol 20(20):7602-12

Chellappan S.P., Siebert S., Mudryj M., Horowitz J.M., and Nevins J.R. (1991). The E2F transcription factor is a cellular target for the RB protein. Cell 65:1053-1061.

Chen M., Mermod N., and Horwitz M.S. (1990). Protein-protein interactions between adenovirus DNA polymerase and nuclear factor-I mediate formation of the DNA replication preinitiation complex. J. Biol. Chem. 265:18634-18642.

Chen P.H., Ornelles D.A., and Shenk T. (1993). The adenovirus L3 23-kilodalton proteinase cleaves the amino-terminal head domain from cytokeratin 18 and disrupts the cytokeratin network of HeLa cells. J. Virol. 67:3507-3514.

Cheng E.H, Levine B., Boise L.H., Thompson C.B., Hardwick J.M. (1996) Bax-independent inhibition of apoptosis by Bcl-XL. Nature 1996 379(6565):554-6

Cheng E.H.Y., Kirsch D.G., Clem R.J., Ravi R., Kastan M.B., Bedi A., Ueno K., and Hardwick J.M. (1997). Conversion of Bcl-2 to a Bax-like death effector by caspases. Science 278:1966-1968.

Chimini G (2001) Engulfing by lipids: a matter of taste? Cell Death Differ. 8(6):545-8.

Chinnaiyan A.M., Chaudhary D., O'Rourke K., Koonin E.V., Dixit V.M. (1997) Role of CED-4 in the activation of CED-3. Nature 388(6644):728-9

Chinnaiyan A.M., O'Rourke K., Tewari M., Dixit V.M. (1995) FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell 81(4):505-12

Chiou S.-K., Rao L., and White E. (1994). Bcl-2 blocks p53-dependent apoptosis. Mol. Cell. Biol. 14:2556-2563.

Cho Y., Gorina S., Jeffrey P.D., and Pavletich N.P. (1994). Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. Science 265:346-355.

Choisy-Rossi C., Yonish-Rouach E. (1998) Apoptosis and the cell cycle: the p53 connection. Cell Death Differ 5(2):129-31

Chow L.T., Roberts T.R., Lewis J.B., and Broker T.R. (1977). A map of cytoplasmic RNA transcripts from lytic adenovirus type 2, determined by electron microscopy of RNA:DNA hybrids. Cell 11:819-836.

Chroboczek J., Bieber F., and Jacrot B. (1992). The sequence of the genome of adenovirus type 5 and its comparison with the genome of adenovirus type 2. Virology 186:280-285.

Cladaras C., and Wold W.S. (1985). DNA sequence of the early E3 transcription unit of adenovirus 5. Virology 15:28-43.

Clem R., Cheng E., Karp C., Kirsch D., Ueno K., Takahashi A., Kastan M., Griffin D., Earnshaw W., Veliuona M., and Hardwick J. (1998). Modulation of cell death by Bcl-xL through caspase interaction. Proc. Natl. Acad. Sci. USA 95:554-559. Cohen G.M. (1997) Caspases: the executioners of apoptosis. Biochem J 326 (Pt 1):1-16

Conradt B., Horvitz H.R. (1998) The *C. elegans* protein EGL-1 is required for programmed cell death and interacts with the Bcl-2-like protein CED-9. Cell 93(4):519-29

Cossarizza A., Baccarani-Contri M., Kalashnikova G., Franceschi C. (1993) A new method for the cytofluorimetric analysis of mitochondrial membrane potential using the J-aggregate forming lipophilic cation 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1). Biochem Biophys Res Commun 197(1):40-5

Costantini P, Bruey JM, Castedo M, Metivier D, Loeffler M, Susin SA, Ravagnan L, Zamzami N, Garrido C, Kroemer G. Pre-processed caspase-9 contained in mitochondria participates in apoptosis. Cell Death Differ 9(1):82-8

Cote J., Ruiz-Carrillo A. (1993) Primers for mitochondrial DNA replication generated by endonuclease G. Science 261(5122):765-9

Cryns V., Yuan J. (1998) Proteases to die for. Genes Dev 112(11):1551-70

Datta S.R., Dudek H., Tao X., Masters S., Fu H., Gotoh Y., Greenberg M.E. (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 91(2):231-41

Daugas E., Susin S.A., Zamzami N., Ferri K.F., Irinopoulou T., Larochette N., Prevost M.C., Leber B., Andrews D., Penninger J., Kroemer G. (2000)

Mitochondrio-nuclear translocation of AIF in apoptosis and necrosis. FASEB J 14(5):729-39

Davidson F.F., Steller H. (1998) Blocking apoptosis prevents blindness in Drosophila retinal degeneration mutants. Nature 391(6667):587-91

de Haro C., Mendez R., and Santoyo J. (1996). The eIF-2alpha kinases and the control of protein synthesis. FASEB J. 10:1378-1387.
de Jong D., Prins F.A., Mason D.Y., Reed .C., van Ommen G.B., Kluin P.M. (1994) Subcellular localization of the bcl-2 protein in malignant and normal lymphoid cells. Cancer Res 1994 54(1):256-60

De Maria R., Lenti L., Malisan F., d'Agostino F., Tomassini B., Zeuner A., Rippo M.R., Testi R. (1997) Requirement for GD3 ganglioside in CD95- and ceramide-induced apoptosis. Science 277(5332):1652-5

de Stanchina E., McCurrach M.E., Zindy F., Shieh S.Y., Ferbeyre G., Samuelson A.V., Prives C., Roussel M.F., Sherr C.J., and Lowe S.W. (1998). E1A signaling to p53 involves the p19(ARF) tumor suppressor. Genes Dev. 12:2434-2442.

Deas O., Dumont C., MacFarlane M., Rouleau M., Hebib C., Harper F., Hirsch F., Charpentier B., Cohen G.M., Senik A. (1998) Caspase-independent cell death induced by anti-CD2 or staurosporine in activated human peripheral T lymphocytes. J Immunol 161(7):3375-83

DeCaprio J.A., Ludlow J.W., Figge J., Shew J.-Y., Huang C.-M., and Lee W.-H., Marsillo E., Paucha E., and Livingston D.M. (1988). SV40 large tumor antigen forms a complex with the product of retinoblastoma gene. Cell 54:275-283.

DeCaprio J.A., Ludlow J.W., Lynch D., Furukama Y., Griffin J., Piwinica-Worms H., Huang W.-M., and Livingston D.M. (1989). The product of the retinoblastoma susceptibility gene has properties of a cell-cycle regulatory element. Cell 58:1085-1095.

Defer C., Belin M.T., Caillet-Boudin M.L., and Boulanger P. (1990). Human adenovirus-host cell interactions: comparative study with members of subgroups B and C. J. Virol. 64:3661-3673.

Deiss L.P., Galinka H., Berissi H., Cohen O., Kimchi A. (1996) Cathepsin D protease mediates programmed cell death induced by interferon-gamma, Fas/APO-1 and TNF-alpha. EMBO J 15(15):3861-70

del Peso L., Gonzalez-Garcia M., Page C., Herrera R., Nunez G. (1997) Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science 278(5338):687-9

Deng X., Ito T., Carr B., Mumby M., May W.S. Jr. (1998) Reversible phosphorylation of Bcl2 following interleukin 3 or bryostatin 1 is mediated by direct interaction with protein phosphatase 2A. J Biol Chem 273(51):34157-63

Devaux C., Caillet-Boudin M.L., Jacrot B., and Boulanger P. (1987). Crystallization, enzymatic cleavage and the polarity of the adenovirus type 2 fiber. Virology 161:121-128.

Deveraux Q.L., Roy N., Stennicke H.R., Van Arsdale T., Zhou Q., Srinivasula S.M., Alnemri E.S., Salvesen G.S., Reed J.C. (1998) IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. EMBO J 17(8):2215-23

Distelhorst C.W., Lam M., McCormick T.S. (1996) Bcl-2 inhibits hydrogen peroxide-induced ER Ca2+ pool depletion. Oncogene 12(10):2051-5

Dix I., and Leppard K.N. (1995). Expression of adenovirus type 5 E4 Orf2 protein during lytic infection. J. Gen. Virol. 76:1051-1055.

Dobbelstein M., Roth J. (1998) The large T antigen of simian virus 40 binds and inactivates p53 but not p73. J Gen Virol 79 (Pt 12):3079-83

Dobner T., Horikoshi N., Rubenwolf S., and Shenk T. (1996). Blockage by adenovirus E4orf6 of transcriptional activation by the p53 tumor suppressor. Science 272:1470-1473.

Dolph P.J., Huang J., and Schneider R.J. (1990). Translation by the adenovirus tripartite leader: elements which determine independence from cap-binding protein complex. J. Virol. 64:2660-2677.

Doucas V., Ishov A.M., Romo A., Juguilon H., Weitzman M.D., Evans R.M., and Maul G.G. (1996). Adenovirus replication is coupled with the dynamic properties of the PML nuclear structure. Genes and Dev. 10:196-207.

Douglas J.L., and Quinlan M.P. (1996). Structural limitations of the Ad5 E1A 12S nuclear localization signal. Virology 220:339-349.

Du C., Fang M., Li Y., Li L., Wang X. (2000) Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell 102(1):33-42

Duan H., and Dixit V.M. (1997). RAIDD, a novel death adaptor molecule. Nature 385:86-89.

Dyson N., Bernards R., Friend S.H., Gooding L.R., Hassell J.A., Major E.O., Pipas J.M., Vandyke T., and Harlow E. (1990). Large T antigens of many poliomaviruses are able to form complexes with the retinoblastoma protein. J. Virol. 64:1353-1356.

Egan C., Jelsma T.N., Howe J.A., Bayley A.T., Ferguson B., and Branton P.E. (1988). Mapping of cellular protein-binding sites on the products of early-region 1A of human adenovirus type 5. Mol. Cell. Biol. 8:3955-3959.

Ekert P.G., Silke J., Vaux D.L. (1999) Caspase inhibitors. Cell Death Differ 6(11):1081-6

Ellis H.M., Horvitz H.R. (1986) Genetic control of programmed cell death in the nematode *C. elegans* Cell 44(6):817-29

Enari M., Sakahira H., Yokoyama H., Okawa K., Iwamatsu A., Nagata S. (1998) A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature 391(6662):43-50

Enomoto T., Lichy J.H., Ikeda J., and Hurwitz J. (1981). Adenovirus DNA replication in vitro: purification of the terminal protein in a functional form. Proc. Natl. Acad. Sci. USA 78:6779-6783.

Eskes R., Desagher S., Antonsson B., Martinou J.C. (2000) Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. Mol Cell Biol 20(3):929-35

Evan G,, Littlewood T. (1998) A matter of life and cell death. Science 281(5381):1317-22

Evan G., Littlewood T. (1998) A matter of life and cell death. Science 281(5381):1317-22

Evan G.I., Wyllie A.H., Gilbert C.S., Littlewood T.D., Land H., Brooks M., Waters C.M., Penn L.Z., Hancock D.C. (1992) Induction of apoptosis in fibroblasts by c-myc protein. Cell 69(1):119-28

Evan GI, Brown L, Whyte M, Harrington E. (1995) Apoptosis and the cell cycle. Curr Opin Cell Biol 7(6):825-34

Everitt E., Sundquist B., Pettersson U., and Philipson L. (1973). Structural proteins of adenoviruses. X. Isolation and topography of low molecular weight antigens from the virion of adenovirus type 2. Virology 62:130-147.

Ewen M.E., Xing Y., Lawrence J.B., and Livingston D.M. (1991). Molecular cloning, chromosomal mapping, and expression of the cDNA for p107, a retinoblastoma gene product-related protein. Cell 66:1155-1164.

Ezoe H., Lai Fatt R.B., and Mak S. (1981). Degradation of intracellular DNA in KB cells infected with cyt mutants of human adenovirus type 12. J. Virol. 40:20-27.

Fadok V.A., Bratton D.L., Henson P.M. (2001) Phagocyte receptors for apoptotic cells: recognition, uptake, and consequences. J Clin Invest 108(7):957-62

Fadok V.A., Henson P.M. (1998) Apoptosis: getting rid of the bodies. Curr Biol 1998 8(19):R693-5

Fadok VA, Bratton DL, Frasch SC, Warner ML, Henson PM. (1998) The role of phosphatidylserine in recognition of apoptotic cells by phagocytes. Cell Death Differ 5(7):551-62

Farrow S.N., White J.H.M., Martinou I., Raven T., Pun D.-T., Grinham C.J., Marinou J.-C., and Brown R. (1995). Cloning of a bcl-2 homologue by interaction with adenovirus E1B 19K. Nature 374:731-733.

Ferguson B., Krippl B., Andrasani O., Jones N., and Westphal H. (1985). E1A 13S and 12S mRNA products made in Escherichia coli both function as nucleus-localised transcription activators but do not directly bind DNA. Mol. Cell. Biol. 5:2653-2661.

Field J., Gronostajski R.M., and Hurwitz J. (1984). Properties of the adenovirus DNA polymerase. J. Biol. Chem. 259:9487-9495.
Fiers W, Beyaert R, Declercq W, Vandenabeele P. (1999) More than one way to die: apoptosis, necrosis and reactive oxygen damage. Oncogene 18(54):7719-30

Flemington E.K., Speck S.H., and Kaelin W.G. (1993). E2F-1 mediated transactivation is inhibited by complex formation with the retinoblastoma suceptibility gene product. Proc. Natl. Acad. Sci. USA 90:6914-6918.

Flemming W. (1885). Über die Bildung von Richtungsfiguren in Säugethiereiern beim Untertang Graaf'scher Follikel. Arch Anat EntwGesch XX:221-224.

Foghsgaard L., Wissing D., Mauch D., Lademann U., Bastholm L., Boes M., Elling F., Leist M., Jaattela M. (2001) Cathepsin B acts as a dominant execution protease in tumor cell apoptosis induced by tumor necrosis factor. J Cell Biol 153(5):999-1010

Fraser A., Evan G. (1996) A license to kill. Cell 85(6):781-4

Fredman J.N., and Engler J.A. (1993). Adenovirus precursor to termianl protein interacts with the nuclear matrix in vivo and in vitro. J. Virol. 67:3384-3395

Funk W.D., Pak D.T., Karas R.H., Wright W.E., and Shay J.W. (1992). A transcriptionally active DNA-binding site for human p53 protein complexes. Mol. Cell. Biol. 12:2866-2871.

Giaccia A.J., Kastan M.B. (1998) The complexity of p53 modulation: emerging patterns from divergent signals. Genes Dev 12(19):2973-83

Ginsberg H.S., Pereira H.G., Valentine R.C., and Wilcox W.C. (1966). A proposed terminology for the adenovirus antigens and virion morphological subunits. Virology 28:782-783.

Golstein P. (1997) Cell death: TRAIL and its receptors. Curr Biol 7(12):R750-3

Goltsev Y.V., Kovalenko A.V., Arnold E., Varfolomeev E.E., Brodianskii V.M., Wallach D. (1997) CASH, a novel caspase homologue with death effector domains. J Biol Chem 272(32):19641-4

Gong J.G., Costanzo A., Yang H.Q., Melino G., Kaelin W.G. Jr., Levrero M., Wang J.Y. (1999) The tyrosine kinase c-Abl regulates p73 in apoptotic response to cisplatin-induced DNA damage. Nature 399(6738):806-9

Gonzalez G.A., and Montminy M.R. (1989). Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell 59:675-680.

Gooding L.R. (1992). Virus proteins that counteract host immune defenses. Cell 71:5-7.

Gooding L.R., Ranheim T.S., Tollefson A.E., Aquino L., Duerksen-Hughes P., Horton T.M., and Wold W.S. (1991). The 10,400- and 14,500-dalton proteins encoded by region E3 of adenovirus function together to protect many but not all mouse cell lines against lysis by tumor necrosis factor. J. Virol. 65:4114-4123.

Goss P.E., Baker M.A., Carver J.P., Dennis J.W. (1995) Inhibitors of carbohydrate processing: A new class of anticancer agents. Clin Cancer Res 1(9):935-44

Graham F.L., Smiley J., Russell W.C., and Nairn R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. 36:59-72.

Graham F.L., Smiley J., Russell W.C., and Nairn R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. 36:59-72.

Grand R.J.A., Roberts C., and Gallimore P.H. (1985). Acylation of adenovirus type 12 early region 1B 18-kDa proteins. Further evidence for its localizion in the cell membrane. FEBS Lett. 181:229-235.

Granville D.J., Carthy C.M., Hunt D.W.C., and McManus B.M. (1998). Apoptosis: Molecular aspects of cell death and disease. Lab. Inves. 78(8): 893-913

Greber U.F., Willetts M., Webster P., and Helenius A. (1993). Stepwise dismantling of adenovirus 2 during entry into cells. Cell 75:477-486.

Green D.R., Evan G.I. (2002) A matter of life and death. Cancer Cell. 1(1):19-30

Green D.R., Reed J.C. Mitochondria and apoptosis. Science 281(5381):1309-12

Green M., Wold W.S.M., Brackmann K.H. et al. (1980). Human adenovirus transforming genes: group relationships, integration, expression in transformed cells and analysis of human cancers and tonsils. In Viruses in Naturally Occurring Tumors: Seventh Cold Spring Harbor Conference on Cell Proliferaion. M. Essex, G. Todaro, H. zurHausen, eds. (New York: Cold Spring Harbor Press) pp. 373-397.
Gronbaek K., Straten P.T., Ralfkiaer E., Ahrenkiel V., Andersen M.K., Hansen N.E., Zeuthen J., Hou-Jensen K., Guldberg P. (1998) Somatic Fas mutations in non-Hodgkin's lymphoma: association with extranodal disease and autoimmunity. Blood 1998 92(9):3018-24

Gross A., Jockel J., Wei M.C., Korsmeyer S.J. (1998) Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis. EMBO J 1998 17(14):3878-85

Gu W., Shi X.-L., and Roeder R.G. (1997b). Synergistic activation of transcription by CBP and p53. Nature 387:819-823.

Gutierrez del Arroyo A., Gil-Lamagniere C., Lazaro I., de Marco M.C., Layunta I., Silva A. (2000) Involvement of p53 and interleukin 3 in the up-regulation of CD95 (APO-1/Fas) by X-ray irradiation. Oncogene 19(32):3647-55

Ha H.C., Woster P.M., Casero R.A. Jr. (1998) Unsymmetrically substituted polyamine analogue induces caspase-independent programmed cell death in Bcl-2-overexpressing cells. Cancer Res 58(13):2711-4

Hacki J., Egger L., Monney L., Conus S., Rosse T., Fellay I., Borner C. (2000) Apoptotic crosstalk between the endoplasmic reticulum and mitochondria controlled by Bcl-2. Oncogene 19(19):2286-95

Han D.K., Chaudhary P.M., Wright M.E., Friedman C., Trask B.J., Riedel R.T., Baskin D.G., Schwartz S.M., Hood L. (1997) MRIT, a novel death-effector domain-containing protein, interacts with caspases and BclXL and initiates cell death. Proc Natl Acad Sci U S A 94(21):11333-8

Han J., Sabbatini P., and White E. (1996). Induction of apoptosis by human Nbk/Bik, a BH3 containing E1B 19K interacting protein. Mol. Cell. Biol. 16:5857-5864.

Hanahan D., Weinberg R.A. (2000) The hallmarks of cancer. Cell 100(1):57-70

Hara H., Friedlander R.M., Gagliardini V., Ayata C., Fink K., Huang Z., Shimizu-Sasamata M., Yuan J., Moskowitz M.A. (1997) Inhibition of interleukin 1beta converting enzyme family proteases reduces ischemic and excitotoxic neuronal damage. Proc Natl Acad Sci U S A 94(5):2007-12

Harada H., Becknell B., Wilm M., Mann M., Huang L.J., Taylor S.S., Scott J.D., Korsmeyer S.J. (1999) Phosphorylation and inactivation of BAD by mitochondria-anchored protein kinase A. Mol Cell 3(4):413-22

Harrington E.A., Fanidi A., Evan G.I. (1994) Oncogenes and cell death. Curr Opin Genet Dev 4(1):120-9

Hasson T.B., Soloway P.D., Ornelles D.A., Doerfler W., and Shenk T. (1989). Adenovirus L1 52- and 55-kilodalton proteins are required for assembly of virions. J. Virol. 63:2612-3621.

Haupt Y., Maya R., Kazaz A., and Oren M. (1997). Mdm2 promotes the rapid degradation of p53. Nature 387:296-299.

He H., Lam M., McCormick T.S., Distelhorst C.W. (1997) Maintenance of calcium homeostasis in the endoplasmic reticulum by Bcl-2. J Cell Biol 138(6):1219-28

Hearing P. and Shenk T. (1985). Sequence-independent autoregulation of the adenovirus type 5 E1A transcription unit. Mol. Cell Biol. 5:413-421.

Hearing P., Samulski R., Wishart W., and Shenk T. (1987). Identification of a repeated sequence element required for efficient encapsidation of the adenovirus type 5 chromosome. J. Virol. 61:2555-2558.

Helin K., Harlow E., and Fattaey A. (1993). Inhibition of E2F-1 transactivation by direct binding of the retinoblastoma protein. Mol. Cell. Biol. 13:6501-6508.
Hellerstein M.K., McCune J.M. (1997) T cell turnover in HIV-1 disease. Immunity 7(5):583-9

Hengartner M.O., Ellis R.E., Horvitz H.R. (1992) *Caenorhabditis elegans* gene ced-9 protects cells from programmed cell death. Nature 356(6369):494-9

Hinds P.W. Mittnacht S., Dulic V., Arnold A., Reed S.I., and Weinberg R.A. (1992). Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. Cell 70:993-1006.

Hofmann K., Bucher P., and Tschopp J. (1997). the CARD domain: a new apoptotic signalling motif. Trends Biochem. Sci. 257:155-156.

Holbrook N.J., Liu Y., Fornace A.J. Jr. (1996) Signaling events controlling the molecular response to genotoxic stress. EXS 77:273-88

Hong S.S., Karayan L., Tournier J., Curiel D.T., Boulanger P.A. (1997) Adenovirus type 5 fiber knob binds to MHC class I alpha2 domain at the surface of human epithelial and B lymphoblastoid cells. EMBO J 16(9):2294-306

Horne R.W., Bonner S., Waterson A.P., and Wildy P. (1959). The icosahedral form of an adenovirus. J. Mol. Biol. 1:84-86.

Horton T.M., Ranheim T.S., Aquino L., Kusher D.I., Saha S.K., Ware C.F., Wold W.S., Gooding L.R. (1991). Adenovirus E3 14.7K protein functions in the absence

of other adenovirus proteins to protect transfected cells from tumor necrosis factor cytolysis. J. Virol. 65:2629-2639.

Horwitz M.S. (1996). Adenoviruses. In Virology (3rd edition) B.N. Fields, D.M. Knipe, P.M. Howley et al., eds. (Philidelphia: Lippincott-Raven Publishers) pp. 2149-2171.

Horwitz, M.S. (1990). Adenoviridae and their replication. In Virology (2nd Edition) B.N. Fields, D.M. Knipe, P.M. Howley et al., eds. (New York: Raven Press) pp. 1679-1721.

Howe J.A., and Bayley S.T. (1992). Effects of Ad5 E1A mutant viruses on the cell cycle in relation to the binding of cellular proteins including the retinoblastoma protein and cyclin A. Virology 186:15-24.

Howe J.A., Mymryk J.S., Egan C., Branton P.E., and Bayley S.T. (1990). Retinoblastoma growth suppressor and a 300-kDa protein appear to regulate cellular DNA synthesis. Proc. Natl. Acad. Sci. USA 87:5883-5887.

Hsu H., Huang J., Shu H.B., Baichwal V., Goeddel D.V. (1996b) TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. Immunity 4(4):387-96

Hsu H., Shu H.B., Pan M.G., Goeddel D.V. (1996a) TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell 84(2):299-308

Hu S., Vincenz C., Buller M., Dixit V.M. (1997) A novel family of viral death effector domain-containing molecules that inhibit both CD-95- and tumor necrosis factor receptor-1-induced apoptosis. J Biol Chem 272(15):9621-4

Huang B., Eberstadt M., Olejniczak E.T., Meadows R.P., Fesik S.W. (1996) NMR structure and mutagenesis of the Fas (APO-1/CD95) death domain. Nature 384(6610):638-41

Huang J., and Schneider R.J. (1991) Adenovirus inhibition of cellular protein synthesis involves inactivaiton of cap binding protein. Cell 65:271-280.
Hueber A.O., Evan G.I. (1998) Traps to catch unwary oncogenes. Trends Genet 14(9):364-7

Huebner R.J., Rowe W.P., Ward T.G., Parrott R.H., and Bell J.A. (1954). Adenoidal-pharyngeal conjunctival agents. N. Engl. J. Med. 251:1077-1086.
Hughes D.P, Crispe I.N. (1995) A naturally occurring soluble isoform of murine Fas generated by alternative splicing. J Exp Med 182(5):1395-401

Hunter T., and Pines J. (1994). Cyclins and Cancer II: Cyclin D and CDK inhibitors come of age. Cell 79:573-582.

Hupp T.R., Meek D.W., Midgley C.A., and Lane D.P. (1992). Regulation of the specific DNA function of p53. Cell 71:875-86.

Imhof A., Yang X.J., Ogryzko V.V., Nakatani Y., Wolffe A.P., and Ge H. (1997). Acetylation of general transcription factors by histone acetyltransferases. Curr Biol. 7: 689-692.

Irmler M., Hofmann K., Vaux D., Tschopp J. (1997) Direct physical interaction between the Caenorhabditis elegans 'death proteins' CED-3 and CED-4. FEBS Lett 406(1-2):189-90

Irmler M., Thome M., Hahne M., Schneider P., Hofmann K., Steiner V., Bodmer J.L., Schroter M., Burns K., Mattmann C., Rimoldi D., French L.E., Tschopp J. (1997b) Inhibition of death receptor signals by cellular FLIP. Nature 388(6638):190-5

Irwin M, Marin MC, Phillips AC, Seelan RS, Smith DI, Liu W, Flores ER, Tsai KY, Jacks T, Vousden KH, Kaelin WG Jr. (2000) Role for the p53 homologue p73 in E2F-1-induced apoptosis. Nature 407(6804):645-8

Ishida S., Yamashita T., Nakaya U., Tokino T. (2000) Adenovirus-mediated transfer of p53-related genes induces apoptosis of human cancer cells. Jpn J Cancer Res 91(2):174-80

Islam A., Kageyama H., Takada N., Kawamoto T., Takayasu H., Isogai E., Ohira M., Hashizume K., Kobayashi H., Kaneko Y., Nakagawara A. (2000) High expression of Survivin, mapped to 17q25, is significantly associated with poor prognostic factors and promotes cell survival in human neuroblastoma. Oncogene 19(5):617-23

Ito T., Deng X., Carr B., May W.S. (1997) Bcl-2 phosphorylation required for anti-apoptosis function. J Biol Chem 272(18):11671-3

Iwahashi H., Eguchi Y., Yasuhara N., Hanafusa T., Matsuzawa Y., Tsujimoto Y. (1997) Synergistic anti-apoptotic activity between Bcl-2 and SMN implicated in spinal muscular atrophy. Nature 390, 413 – 417

Jaattela M. (1999) Escaping cell death: survival proteins in cancer. Exp Cell Res 1999 248(1):30-43

Jacobson M.D.. (1996) Reactive oxygen species and programmed cell death. Trends Biochem Sci., 21(3):83-6

James C., Gschmeissner S., Fraser A., Evan G.I. (1997) CED-4 induces chromatin condensation in Schizosaccharomyces pombe and is inhibited by direct physical association with CED-9. Curr Biol 7(4):246-52

Jamora C., Dennert G., Lee A.S. (1996) Inhibition of tumor progression by suppression of stress protein GRP78/BiP induction in fibrosarcoma B/C10ME. (1996) Proc Natl Acad Sci U S A 93(15):7690-4

Janssens V., Goris J. (2001) Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. Biochem J 353(Pt 3):417-39

Jeremias I., Herr I., Boehler T., Debatin K.M. (1998) TRAIL/Apo-2-ligand-induced apoptosis in human T cells. Eur J Immunol 28(1):143-52

Jost C.A., Marin M.C., Kaelin W.G. Jr. (1997) p73 is a simian [correction of human] p53-related protein that can induce apoptosis. Nature 389(6647):191-4

Joza N., Susin S.A., Daugas E., Stanford W.L., Cho S.K., Li C.Y., Sasaki T., Elia A.J., Cheng H..Y, Ravagnan L., Ferri K.F., Zamzami N., Wakeham A., Hakem R., Yoshida H., Kong Y.Y., Mak T.W., Zuniga-Pflucker J.C., Kroemer G., Penninger J.M. (2001) Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. Nature 410(6828):549-54

Juin P., Evan G. (2000) Caspase 8: the killer you can't live without. Nat Med 6(5):498-500

Juin P., Hueber A.O., Littlewood T., Evan G. (1999) c-Myc-induced sensitization to apoptosis is mediated through cytochrome c release. Genes Dev 13(11):1367-81

Kaczmarek L., Ferguson B., Rosenberg M., and Baserga R. (1986). Induction of cellular DNA synthesis by purified adenovirus E1A proteins. Virology 152:1-10.

Kaelin W.G., Ewen M.E., and Livingston D.M. (1990). Definition of the minimal simian virus 40 large T antigen- and adenovirus E1A-binding domain in the retinoblastoma gene product. Mol. Cell. Biol. 10:3761-3769.

Kaghad M., Bonnet H., Yang A., Creancier L., Biscan J.C., Valent A., Minty A., Chalon P., Lelias J.M., Dumont X., Ferrara P., McKeon F., Caput D. (1997) Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. Cell 90(4):809-19

Kanopka A., Mühlemann O., Petersen-Mahrt S., Estmer C., Öhrmalm C., and Akusjärvi G. (1998). Regulation of adenovirus alternative RNA splicing by dephosphorylation of SR proteins. Nature 393:185-187.

Katoh I., Aisaki K.I., Kurata S.I., Ikawa S., Ikawa Y. (2000) p51A (TAp63gamma), a p53 homolog, accumulates in response to DNA damage for cell regulation. Oncogene 19(27):3126-30

Kaufman R.J. (1999) Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. Genes Dev 13(10):1211-33

Kelekar A., Chang B.S., Harlan J.E., Fesik S.W., and Thompson C.B. (1997). Bad is a BH3 domain-containing protein that forms an inactivating dimer with Bcl-XL. Mol. Cell. Biol. 17:7040-7046.

Kelekar A., Chang B.S., Harlan J.E., Fesik S.W., Thompson C.B. (1997) Bad is a BH3 domain-containing protein that forms an inactivating dimer with Bcl-XL. Mol Cell Biol 17(12):7040-6

Kelekar A., Thompson C.B. (1998) Bcl-2-family proteins: the role of the BH3 domain in apoptosis. Trends Cell Biol 8(8):324-30

Kern S.E., Kinzler K.W., Bruskin A., Jarosz D., Friedman P., Prives C., and Vogelstein B. (1991). Identification of p53 as a sequence-specific DNA-binding protein. Science 252:1708-1711.

Kern S.E., Pietenpol J.A., Thiagalingam S., Seymour A., Kinzler K.W., and Vogelstein B. (1992). Oncogenic forms of p53 inhibit p53-regulated gene expression. Science 256:827-830.

Kerr J.F. (1965). A histochemical study of hypertrophy and ischaemic injury of rat liver with special reference to changes in lysosomes. J. Pathol. Bacteriol. 90:419-435.

Kerr J.F. (1969). An electron-microscope study of liver cell necrosis due to heliotrine. J. Pathol. 96:557-562.

Kerr J.F. (1971). Shrinking necrosis: A distinct mode of cellular death. J. Pathol. 105:13-20.

Kerr J.F. (1972b). Shrinkage necrosis of adrenal cortical cells. J Pathol. 107(3):217-9.

Kerr J.F., Wyllie A.H. & Currie A.R. (1972a). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Brit. J. Cancer 26:239-257.

Kimelman D.J., Miller J.S., Porter D., and Roberts B.E. (1985). E1a regions of the human adenoviruses and of the highly oncogenic simian adenovirus 7 are closely related. J. Virol. 53:399-409.

Kleinberger T. (2000) Induction of apoptosis by adenovirus E4orf4 protein. Apoptosis 5(3):211-5

Kleinberger T., and Shenk T. (1991). A protein kinase is present in a complex with adenovirus E1A proteins. Proc. Natl. Acad. Sci USA 88:11143-11147.

Kluck R.M., Bossy-Wetzel E., Green D.R., Newmeyer D.D. (1997). The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science. 275(5303):1132-6.

Komiyama T., Ray C.A., Pickup D.J., Howard A.D., Thornberry N.A., Peterson E.P., Salvesen G. (1994) Inhibition of interleukin-1 beta converting enzyme by the cowpox virus serpin CrmA. An example of cross-class inhibition. J Biol Chem 269(30):19331-7

Korsmeyer S.J., Wei M.C., Saito M., Weiler S., Oh K.J., Schlesinger P.H. (2000) Pro-apoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in the release of cytochrome c. Cell Death Differ 7(12):1166-73

Kothakota S., Azuma T., Reinhard C., Klippel A., Tang J., Chu K., McGarry T.J., Kirschner M.W., Koths K., Kwiatkowski D.J., and Williams L.T. (1997). Caspase-3-generated fragment of gelsolin: Effector of morphological change in apoptosis. Science 278:294-298.

Krajcsi P., Dimitrov T., Hermiston T.W. Tollefson A.E., Ranheim T.S., Vande Pol S.B., Stephenson A.H., and Wold W.S. (1996). The adenovirus E4-14.7K protein and the E3-10.4K/14.5K complex of proteins, which independently inhibit tumor necrosis factor (TNF)-induced apoptosis, also independently inhibit TNF-induced release of arachidonic acid. J. Virol 70:4904-4913.

Krajewska M., Moss S.F., Krajewski S., Song K., Holt P.R., Reed J.C. (1996) Elevated expression of Bcl-X and reduced Bak in primary colorectal adenocarcinomas. Cancer Res 56(10):2422-7

Krajewski S., Krajewska M., Ellerby L.M., Welsh K., Xie Z., Deveraux Q.L., Salvesen G.S., Bredesen D.E., Rosenthal R.E., Fiskum G., Reed J.C. (1999) Release of caspase-9 from mitochondria during neuronal apoptosis and cerebral ischemia. Proc Natl Acad Sci U S A 96(10):5752-7

Krajewski S., Tanaka S., Takayama S., Schibler M.J., Fenton W., Reed J.C. (1993) Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. Cancer Res 1993 53(19):4701-14

Kremmer E., Ohst K., Kiefer J., Brewis N., and Walter G. (1997). Separation of PP2A core enzyme and holoenzyme with monoclonal antibodies against the regulatory A subunit: Abundant expression of both forms in cells. Mol. Cell. Biol. 17:1692-1701.

Kroemer G. (1997) The proto-oncogene Bcl-2 and its role in regulating apoptosis. Nat Med 3(6):614-20

Kroemer G. (1997). The proto-oncogene Bcl-2 and its role in regulating apoptosis. Nat. Med. 3:614-620.

Kroemer G., Reed J.C. (2000) Mitochondrial control of cell death. Nat Med 6(5):513-9

Kvist S., Ostberg L., Persson H., Philipson L., and Peterson P.A. (1978). Molecular association between transplantation antigens and cell surface antigen in adenovirus-transformed cell line. Proc. Natl. Acad Sci. USA 75:5674-5678.

LaCasse E.C., Baird S., Korneluk R.G., MacKenzie A.E. (1998) The inhibitors of apoptosis (IAPs) and their emerging role in cancer. Oncogene 17(25):3247-59

Lakin N.D., Jackson S.P. (1999) Regulation of p53 in response to DNA damage. Oncogene 1999 Dec 13;18(53):7644-55

Lavoie J.N., Champagne C., Gingras M.C., Robert A. (2000) Adenovirus E4 open reading frame 4-induced apoptosis involves dysregulation of Src family kinases. J Cell Biol 150(5):1037-56

Lavoie J.N., Nguyen M., Marcellus R.C., Branton P.E., and Shore G.C. (1998) E4orf4, a novel adenovirus death factor that induces p53-independent apoptosis by a pathway which is not inhibited by zVAD-fmk. J. Cell Biol. 140:637-645.

Lee K.A., Hai T.Y., SivaRaman L., Thimmappaya B., Hurst H.C., Jones N.C., and Green M.R. (1987). A cellular protein, activating transcription factor, activates transcription of multiple E1A-inducible adenovirus early promoters. Proc. Natl. Acad. Sci. USA 84, 8355-8359.

Lee S.Y., Lee S.Y., Choi Y. (1997) TRAF-interacting protein (TRIP): a novel component of the tumor necrosis factor receptor (TNFR)- and CD30-TRAF signaling complexes that inhibits TRAF2-mediated NF-kappaB activation. Exp Med 185(7):1275-85

Lee W.S., Kao C.C., Bryant G.O., Liu X., and Berk A.J. (1991). Adenovirus E1A activation domain binds the basic repeat in the TATA box transcription factor. Cell 67:365-376.

Levine A.J. (1997). p53, the cellular gatekeeper for growth and division. Cell 88:323-331.

Li H., Zhu H., Xu C.J., Yuan J. (1998) Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 1998 94(4):491-501

Li L.Y., Luo X., Wang X. (2001) Endonuclease G is an apoptotic DNase when released from mitochondria. Nature 412(6842):95-9

Li P., Allen H., Banerjee S., Franklin S., Herzog L., Johnston C., McDowell J., Paskind M., Rodman L., Salfeld J., et al. (1995) Mice deficient in IL-1 beta-converting enzyme are defective in production of mature IL-1 beta and resistant to endotoxic shock. Cell 80(3):401-11

Li P., Nijhawan D., Budihardjo I., Srinivasula S.M., Ahmad M., Alnemri E.S., Wang X. (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91(4):479-89

Li P.F., Dietz R., von Harsdorf R. (1999) p53 regulates mitochondrial membrane potential through reactive oxygen species and induces cytochrome c-independent apoptosis blocked by Bcl-2. EMBO J 18(21):6027-36

Lill N.L., Grossman S.R., Ginsberg D., DeCaprio J., Livingston D.M. (1997). Binding and modulation of p53 by p300/CBP coactivators. Nature 387:823-827.

Lin J., Chen J., Elenbaas B., and Levine A.J. (1994). Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptional activation, binding to mdm-2 and the adenovirus 5 E1B 55-kD protein. Genes Dev. 8:1235-46.

Lin Y., Ma W., Benchimol S. (2000) Pidd, a new death-domain-containing protein, is induced by p53 and promotes apoptosis. Nat Genet 26(1):122-7

Liu Q.A., Hengartner M.O. (1999) Human CED-6 encodes a functional homologue of the *Caenorhabditis elegans* engulfment protein CED-6. Curr Biol 9(22):1347-50

Livne A., Shtrichman R., Kleinberger T. (2001) Caspase activation by adenovirus e4orf4 protein is cell line specific and Is mediated by the death receptor pathway. J Virol 75(2):789-98

Lloyd A.C., Obermuller F., Staddon S., Barth C.F., McMahon M., Land H. (1997) Cooperating oncogenes converge to regulate cyclin/cdk complexes. Genes Dev 11(5):663-77

Lohrum M.A., Vousden K.H. (2000) Regulation and function of the p53-related proteins: same family, different rules. Trends Cell Biol 10(5):197-202

Los M., Mozoluk M., Ferrari D., Stepczynska A., Stroh C., Renz A,. Herceg Z., Wang Z.Q., and Schulze-Osthoff K. (2002) Activation and Caspase-mediated Inhibition of PARP: A Molecular Switch between Fibroblast Necrosis and Apoptosis in Death Receptor Signaling. MBC 13(3): 978-988
Lowe S.W. (1999) Activation of p53 by oncogenes. Endocr Relat Cancer 6(1):45-8

Lowe S.W., and Ruley H.E. (1993). Stabilization of the p53 tumor suppressor is induced by adenovirus E1A in mouse thymocytes. Genes Dev. 7:535-545.

Lu H., and Levine A.J. (1995). Human TAF-31 is a transcriptional coactivator of the p53 protein. Proc. Natl. Acad. Sci. USA 92:5154-5158.

Ludlow J.W., Shon J., Pipas J.M., Livingston D.M., and DeCaprio J.A. (1990). The retino-blastoma susceptibility gene product undergoes cell cycle-dependent dephosphorylation and binding to and release from SV40 large T. Cell 60:387-396.

Luftig R.B., and Weihing R.R. (1975). Adenovirus binds to rat brain microtubules in vitro. J. Virol. 16:696-706.

Luo J., Su F., Chen D., Shiloh A., Gu W. (2000) Deacetylation of p53 modulates its effect on cell growth and apoptosis. Nature 408(6810):377-81

Luo X., Budihardjo I., Zou H., Slaughter C., Wang X. (1998) Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell 1998 94(4):481-90

Malinin N.L., Boldin M.P., Kovalenko A.V., Wallach D. (1997) MAP3K-related kinase involved in NF-kappaB induction by TNF, CD95 and IL-1. Nature 385(6616):540-4

Mancini M., Nicholson D.W., Roy S., Thornberry N.A., Peterson E.P., Casciola-Rosen L.A., Rosen A. (1998) The caspase-3 precursor has a cytosolic and mitochondrial distribution: implications for apoptotic signaling. J Cell Biol 140(6):1485-95

Mannervik M., Fan S., Strom A.C., Helin K., Akusjarvi G. (1999) Adenovirus E4 open reading frame 4-induced dephosphorylation inhibits E1A activation of the E2 promoter and E2F-1-mediated transactivation independently of the retinoblastoma tumor suppressor protein. Virology 256(2):313-21

Marcellus R.C., Chan H., Paquette D., Thirlwell S., Boivin D., Branton P.E. (2000) Induction of p53-independent apoptosis by the adenovirus E4orf4 protein requires binding to the Balpha subunit of protein phosphatase 2A. J Virol 74(17):7869-77

Marcellus R.C., Lavoie J.N., Boivin D., Shore G.C., Ketner G., and Branton P.E. (1998). The early regionr orf4 protein of human adenovirus type 5 induces p53-independent cell death by apoptosis. submitted to J. Virol.

Marcellus R.C., Teodoro J.G., Charbonneau R., Shore G.C., and Branton P.E. (1996a). Expression of p53 in Saos-2 osteosarcoma cellls induces apoptosis which can be inhibited by Bcl-2 or the adenovirus E1B-55 kDa protein. Cell. Growth Differ. 7:1643-1650.

Marcellus R.C., Teodoro J.G., Wu T., Brough D.E., Ketner G., Shore G.C., and Branton P.E. (1996). Adenovirus 5 early region 4 is responsible for E1A-induced p53-independent apoptosis J. Virol. 70:6207-6215.

Marchenko N.D., Zaika A., Moll U.M. (2000) Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling. J Biol Chem 275(21):16202-12

Marin M.C., Jost C.A., Irwin M.S., DeCaprio J.A., Caput D., Kaelin W.G. Jr. (1998) Viral oncoproteins discriminate between p53 and the p53 homolog p73. Mol Cell Biol 18(11):6316-24

Martin M.E.D., and Berk A.J. (1998). Adenovirus E1B 55K represses p53 activation in vitro. J. Virol. 72:3146-3154.

Martinou J.C., Desagher S., Antonsson B. (2000) Cytochrome c release from mitochondria: all or nothing. Nat Cell Biol 2(3):E41-3

Martinou J.C., Green D.R. (2001) Breaking the mitochondrial barrier. Nat Rev Mol Cell Biol 2(1):63-7

Marzo I., Brenner C., Zamzami N., Jurgensmeier J.M., Susin S.A., Vieira H.L., Prevost M.C., Xie Z., Matsuyama S., Reed J.C., Kroemer G. (1998) Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. Science 281(5385):2027-31

Maundrell K., Antonsson B., Magnenat E., Camps M., Muda M., Chabert C., Gillieron C., Boschert U., Vial-Knecht E., Martinou J.C., Arkinstall S. (1997) Bcl-2 undergoes phosphorylation by c-Jun N-terminal kinase/stress-activated protein kinases in the presence of the constitutively active GTP-binding protein Rac1. J Biol Chem 272(40):25238-42

May P., May E. Twenty years of p53 research: structural and functional aspects of the p53 protein. Oncogene 18(53):7621-36

McCarthy N.J., Whyte M.K.B., Gilbert C.S., and Evan G.I. (1997). Inhibition of Ced-3/ICE-related proteases does not prevent cell death induced by oncogenes, DNA damage, or the Bcl-2 homologue Bak. J. Cell Biol. 136:215-227.
McCullough K.D., Martindale J.L., Klotz L.O., Aw T.Y., Holbrook N.J. (2001) Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. Mol Cell Biol 21(4):1249-59

McLorie W., McGlade C.J., Takayesu D., Branton P.E. (1991) Individual adenovirus E1B proteins induce transformation independently but by additive pathways. J Gen Virol 72 (Pt 6):1467-71

Miller T.M., Moulder K.L., Knudson C.M., Creedon D.J., Deshmukh M., Korsmeyer S.J., Johnson E.M. Jr. (1997) Bax deletion further orders the cell death pathway in cerebellar granule cells and suggests a caspase-independent pathway to cell death. J Cell Biol 139(1):205-17

Miron MJ, unpublished results

Miyake H., Hara I., Arakawa S., Kamidono S. (2000) Stress protein GRP78 prevents apoptosis induced by calcium ionophore, ionomycin, but not by glycosylation inhibitor, tunicamycin, in human prostate cancer cells. J Cell Biochem 77(3):396-408

Miyashita T., Reed J.C. (1995) Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 80(2):293-9

Momand J., Zambetti G.P., Olson D.C., George D., and Levine A.J. (1992). The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell 69:1237-1245.

Moore M., Horikoshi N., and Shenk T. (1996). Oncogenic potential of the adenovirus E4orf6 protein. Proc. Natl. Acad. Sci. USA 93:11295-11301.

Moroni M.C., Hickman E.S., Denchi E.L., Caprara G., Colli E., Cecconi F., Muller H., Helin K. (2001) Apaf-1 is a transcriptional target for E2F and p53. Nat Cell Biol 3(6):552-8

Müller U., Kleinberger T., and Shenk T. (1992). Adenovirus E4orf4 protein reduces phosphorylation of c-Fos and E1a proteins while simultaneously reducing the level of AP-1. J. Virol. 66:5867-5878.

Müller U., Roberts M.P., Engel D.A., Doerfler W., and Shenk T. (1989). Induction of transcription factor AP-1 by adenovirus E1A protein and cAMP. Genes Dev. 3:1991-2002.

Müller U., Roberts M.P., Engel D.A., Doerfler W., and Shenk T. (1989). Induction of transcription factor AP-1 by adenovirus E1A protein and cAMP. Genes Dev. 3:1991-2002.

Muzio M. (1998) Signalling by proteolysis: death receptors induce apoptosis. Int J Clin Lab Res 28(3):141-7

Muzio M., Chinnaiyan A.M., Kischkel F.C., O'Rourke K., Shevchenko A., Ni J., Scaffidi C., Bretz J.D., Zhang M., Gentz R., Mann M., Krammer P.H., Peter M.E., Dixit V.M. (1996) FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death--inducing signaling complex. Cell 85(6):817-27

Nagata K., Guggenheimer R.A., and Hurwitz J. (1983). Adenovirus DNA replication in vitro: synthesis of full-length DNA with purified proteins. Proc. Natl. Acad. Sci. USA 80:4266-4270.

Nagata S. (1997) Apoptosis by death factor. Cell 88(3):355-65

Naik P., Karrim J., Hanahan D. (1996) The rise and fall of apoptosis during multistage tumorigenesis: down-modulation contributes to tumor progression from angiogenic progenitors. Genes Dev 10(17):2105-16

Nakagawa T., Yuan J. (2000) Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. J Cell Biol 150(4):887-94

Natoli G., Costanzo A., Ianni A., Templeton D.J., Woodgett J.R., Balsano C., Levrero M. (1997) Activation of SAPK/JNK by TNF receptor 1 through a noncytotoxic TRAF2-dependent pathway. Science 1997 Jan 10;275(5297):200-3

Nevels M., Rubenwolf S., Spruss T., Wolf H., and Dobner T. (1997). The adenovirus E4orf6 protein can promote E1A/E1B-induced focus formation by interfering with p53 tumor suppressor function. Proc. Natl. Acad. Sci. USA 94:1206-1211.

Nevins J. R., Ginsberg H. S., Blanchard J. M., Wilson M. C., and Darnell, J. E. (1979). Regulation of the primary expression of the early adenovirus transcription units. J. Virol. 32:727-733.

Nevins J.R. (1989). Mechanisms of viral-mediated trans-activation of transcription. Advances in virus research 37:35-83.

Ng F.W., Nguyen M., Kwan T., Branton P.E., Nicholson D.W., Cromlish J.A., Shore G.C. (1997) p28 Bap31, a Bcl-2/Bcl-XL- and procaspase-8-associated protein in the endoplasmic reticulum. J Cell Biol 139(2):327-38

Nguyen M., Branton P.E., Walton P.A., Oltvai Z.N., Korsmeyer S.J., Shore G.C. (1994) Role of membrane anchor domain of Bcl-2 in suppression of apoptosis caused by E1B-defective adenovirus. J Biol Chem 269(24):16521-4

Nicholson D.W. (1999) Caspase structure, proteolytic substrates, and function during apoptotic cell death. Cell Death Differ 6(11):1028-42

Nordqvist K., Öhman K., and Akusjärvi G. (1994). Human adenovirus encodes two proteins which have opposite effects on accumulation of alternatively spliced mRNAs.

Norrby E., Bartha A., and Boulanger P. (1976). Adenoviridae. Intervirology 7:117-125.

O'Malley R.P., Mariano R.M. Siekierka J., Merrick W.C., Reichel P.A., and Mathews M.B. (1986). The control of protein synthesis by adenovirus VA RNA. Cancer Cells 4:291-301.

Oberhammer .F, Wilson J.W., Dive C., Morris I.D., Hickamn J.A., Wakeling A.E., Walker P.R., Sikorska M. (1993) Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. EMBO J. 12(9):3679-84.

Obert S., O'Connor R.J., Schmid S., and Hearing P. (1994). The adenovirus E4-6/7 protein transactivates the E2 promoter by inducing dimerization of a heterodimeric E2F complex. Mol. Cell. Biol. 14:1333-1346.

Oda E., Ohki R., Murasawa H., Nemoto J., Shibue T., Yamashita T., Tokino T., Taniguchi T., Tanaka N. (2000) Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Science 288(5468):1053-8

Oda K., Arakawa H., Tanaka T., Matsuda K., Tanikawa C., Mori T., Nishimori H., Tamai K., Tokino T., Nakamura Y., Taya Y. (2000) p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. Cell 102(6):849-62

Okuno S., Shimizu S., Ito T., Nomura M., Hamada E., Tsujimoto Y., Matsuda H. (1998) Bcl-2 prevents caspase-independent cell death. J Biol Chem 273(51):34272-7

Oltvai Z.N., Milliman C.L., Korsmeyer S.J. (1993) Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 74(4):609-19

Ornelles D. and Shenk T. (1991). Localization of the adenovirus early region 1B 55-kilodalton protein during lytic infection: association with nuclear viral inclusions requires the early region 4 34-kilodalton protein. J. Virol. 65:424-439.

Osada M., Ohba M., Kawahara C., Ishioka C., Kanamaru R., Katoh I., Ikawa Y., Nimura Y., Nakagawara A., Obinata M., Ikawa S. (1998) Cloning and functional analysis of human p51, which structurally and functionally resembles p53. Nat Med 4(7):839-43

Ottilie S., Diaz J.L., Horne W., Chang J., Wang Y., Wilson G., Chang S., Weeks S., Fritz L.C., Oltersdorf T. (1997) Dimerization properties of human BAD. Identification of a BH-3 domain and analysis of its binding to mutant BCL-2 and BCL-XL proteins. J Biol Chem 1997 272(49):30866-72

Pääbo S., Bhat B.M., Wold W.S.M., and Peterson P.A. (1987). A short sequence in the COOH-terminus makes an adenovirus membrane glycoprotein a resident of the endoplasmic reticulum. Cell 50:311-317.

Pan G., Ni J., Wei Y.F., Yu G., Gentz R., Dixit V.M. (1997) An antagonist decoy receptor and a death domain-containing receptor for TRAIL. Science 277(5327):815-8

Park W.S., Oh R.R., Kim Y.S., Park J.Y., Lee S.H., Shin M.S., Kim S.Y., Kim P.J., Lee H.K., Yoo N.Y., Lee J.Y. (2001) Somatic mutations in the death domain of the Fas (Apo-1/CD95) gene in gastric cancer. J Pathol 193(2):162-8

Parrish J., Li L., Klotz K., Ledwich D., Wang X., Xue D. (2001) Mitochondrial endonuclease G is important for apoptosis in C. elegans. Nature 412(6842):90-4

Patil C., Walter P. (2001) Intracellular signaling from the endoplasmic reticulum to the nucleus: the unfolded protein response in yeast and mammals. Curr Opin Cell Biol 13(3):349-55

Pavletich N.P., Chambers K.A., and Pabo C.O. (1993). The DNA-binding domain of p53 contains the four conserved regions and the major mutation hot spots. Genes Dev. 7:2556-2564.

Perez D., and White E. (1998). E1B 19K inhibits Fas-mediated apoptosis through FADD-dependent sequestration of FLICE. J.Cell. Biol. 141:1255-1266.

Perez D., and White E. (1998). E1B 19K inhibits Fas-mediated apoptosis through FADD-dependent sequestration of FLICE. J.Cell. Biol. 141:1255-1266.

Perricaudet M., Le Moullec M. J., and Pettersson, U. (1980a). Predicted structure of two adenovirus tumor antigens. Proc. Natl. Acad. Sci. USA 77:3778-82.

Perricaudet M., Le Moullec M. J., Tiollais P., and Pettersson, U. (1980b). Structure of two adenovirus type 12 transforming polypeptides and their evolutionary implications. Nature 288:174-6.

Petit P.X., Susin S.A., Zamzami N., Mignotte B., Kroemer G. (1996) Mitochondria and programmed cell death: back to the future. FEBS Lett 396(1):7-13

Pettersson U., and Roberts R.J. (1986). Adenovirus gene expression and replication: a historical review. Cancer Cells 4:37-57.

Philipson L. (1984). Adenovirus assembly. In The adenoviruses, Ginsberg H., ed. (New York: Plenum Press).

Pina M., Green M.(1965) Biochemical studies on adenovirus multiplication. IX. Chemical and base composition analysis of 28 human adenoviruses. Proc Natl Acad Sci U S A 54(2):547-51

Pitti R.M., Marsters S.A., Lawrence D.A., Roy M., Kischkel F.C., Dowd P., Huang A., Donahue C.J., Sherwood S.W., Baldwin D.T., Godowski P.J., Wood W.I., Gurney A.L., Hillan K.J., Cohen R.L., Goddard A.D., Botstein D., Ashkenazi A. (1998) Genomic amplification of a decoy receptor for Fas ligand in lung and colon cancer. Nature 396(6712):699-703

Ploegh H.L. (1998). Viral strategies of immune evasion. Science 280: 248-253.

Pomerantz J., Schreiber-Agus N., Liegeois N.J., Silverman A., Alland L., Chin L., Potes J., Chen K., Orlow I., Lee H.W., Cordon-Cardo C., and DePinho R.A. (1998). The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. Cell 92:713-723.

Poommipanit P.B., Chen B., Oltvai Z.N. (1999) Interleukin-3 induces the phosphorylation of a distinct fraction of bcl-2. J Biol Chem 274(2):1033-9

Potempa J., Korzus E., Travis J. (1994) The serpin superfamily of proteinase inhibitors: structure, function, and regulation. J Biol Chem 269(23):15957-60

Prabhu N.S., Somasundaram K., Satyamoorthy K., Herlyn M., El-Deiry W.S. (1998) p73beta, unlike p53, suppresses growth and induces apoptosis of human papillomavirus E6-expressing cancer cells. Int J Oncol 13(1):5-9
Prendergast G.C. (2000) Farnesyltransferase inhibitors: antineoplastic mechanism and clinical prospects. Curr Opin Cell Biol 12(2):166-73

Preston G.A., Lyon T.T., Yin Y., Lang J.E., Solomon G., Annab L., Srinivasan D.G., Alcorta D.A., Barrett J.C. (1996) Induction of apoptosis by c-Fos protein. Mol Cell Biol 16(1):211-8

Qian T., Nieminen A.L., Herman B., Lemasters J.J. (1997) Mitochondrial permeability transition in pH-dependent reperfusion injury to rat hepatocytes. Am J Physiol 273(6 Pt 1):C1783-92

Qin Z.H., Wang Y., Kikly K.K., Sapp E., Kegel K.B., Aronin N., DiFiglia M. (2001) Pro-caspase-8 is predominantly localized in mitochondria and released into cytoplasm upon apoptotic stimulation. J Biol Chem 276(11):8079-86

Querido E., Marcellus R.C., Lai A., Charbonneau R., Teodoro J.G., Ketner G., and Branton P.E. (1997a). Regulation of p53 levels by the E1B 55-kilodalton protein and E4orf6 in adenovirus-infected cells. J. Virol. 71:3788-3798.

Querido E., Teodoro J.G., and Branton P.E. (1997b). Accumulation of p53 induced by the adenovirus E1A protein requires regions involved in the stimulation of DNA synthesis. J. Virol. 71:3526-3533.

Quignon F., De Bels F., Koken M., Feunteun J., Ameisen J.C., de The H. (1998) PML induces a novel caspase-independent death process. Nat Genet 20(3):259-65

Rampino N., Yamamoto H., Ionov Y., Li Y., Sawai H., Reed J.C., Perucho M. (1997) Somatic frameshift mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype. Science 275(5302):967-9

Rao L., Debbas M., Sabbatini P., Hockenbery D., Korsmeyer S., and White E. (1992). The adenovirus E1A proteins induce apoptosis, which is inhibited by the E1B 19-kDa and bcl-2 proteins. Proc. Natl. Acad. Sci. USA 89:7742-7746.

Rasper D.M., Vaillancourt J.P., Hadano S., Houtzager V.M., Seiden I., Keen S.L., Tawa P., Xanthoudakis S., Nasir J., Martindale D., Koop B.F., Peterson E.P., Thornberry N.A., Huang J., MacPherson D.P., Black S.C., Hornung F., Lenardo M.J., Hayden M.R., Roy S., Nicholson D.W. (1998) Cell death attenuation by 'Usurpin', a mammalian DED-caspase homologue that precludes caspase-8 recruitment and activation by the CD-95 (Fas, APO-1) receptor complex. Cell Death Differ 5(4):271-88

Raycroft L., Wu H.Y., and Lozano G. (1990). Transcriptional activation by wild-type but not transforming mutants of the p53 anti-oncogene. Science 249:1049-1051.

Reed J.C. (1997) Bcl-2 family proteins: regulators of apoptosis and chemoresistance in hematologic malignancies. Semin Hematol 34(4 Suppl 5):9-19
Reed J.C. (1997) Cytochrome c: can't live with it--can't live without it. Cell 91(5):559-62

Rekosh D.M.K., Russell W.C., Bellet A.J.D., and Robinson A.J. (1977). Identifiation of a protein linked to the ends of adenovirus DNA. Cell 11:283-295.

Rippo M.R., Malisan F., Ravagnan L., Tomassini B., Condo I., Costantini P., Susin S.A., Rufini A., Todaro M., Kroemer G., Testi R. (2000) GD3 ganglioside directly targets mitochondria in a bcl-2-controlled fashion. FASEB J 14(13):2047-54

Roberg K. (2001) Relocalization of cathepsin D and cytochrome c early in apoptosis revealed by immunoelectron microscopy. Lab Invest 81(3):149-58

Rodriguez I., Matsuura K., Ody C., Nagata S., Vassalli P. (1996) Systemic injection of a tripeptide inhibits the intracellular activation of CPP32-like proteases in vivo and fully protects mice against Fas-mediated fulminant liver destruction and death. J Exp Med 184(5):2067-72

Roopchand D.E., Lee J.M., Shahinian S., Paquette D., Bussey H., Branton P.E. (2001) Toxicity of human adenovirus E4orf4 protein in Saccharomyces cerevisiae results from interactions with the Cdc55 regulatory B subunit of PP2A. Oncogene 20(38):5279-90

Roth J., Konig C., Wienzek S., Weigel S., Ristea S., Dobbelstein M. (1998) Inactivation of p53 but not p73 by adenovirus type 5 E1B 55-kilodalton and E4 34-kilodalton oncoproteins. J Virol 72(11):8510-6

Rothe M., Xiong J., Shu H.B., Williamson K., Goddard A., Goeddel D.V. (1996) I-TRAF is a novel TRAF-interacting protein that regulates TRAF-mediated signal transduction. Proc Natl Acad Sci U S A 93(16):8241-6

Roulston A., Marcellus R.C., Branton P.E. (1999) Viruses and apoptosis. Annu Rev Microbiol 53:577-628

Rowe D.T., Graham F.L., and Branton,P.E. (1983). Intracellular localization of adenovirus type 5 tumor antigens in productively infected cells. Virology 129:456-468.

Rowe W.P., Huebner R.J., Gilmore L.K., Parrott R.H., and Ward T.G. (1953). Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. Proc. Soc. Exp. Biol. Med. 84:570-573.

Sakahira H., Enari M., and Nagata S. (1998). Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature 391:96-99.

Sandig V., Brand K., Herwig S., Lukas J., Bartek J., Strauss M. (1997) Adenovirally transferred p16INK4/CDKN2 and p53 genes cooperate to induce apoptotic tumor cell death. Nat Med 3(3):313-9

Sarin A., Williams M.S., Alexander-Miller M.A., Berzofsky J.A., Zacharchuk C.M., Henkart P.A. (1997) Target cell lysis by CTL granule exocytosis is independent of ICE/Ced-3 family proteases. Immunity 6(2):209-15

Sarnow P., Hearing P., Anderson C.W., Halbert D.N., Shenk T., and Levine A.J. (1984). Adenovirus early region 1B 58,000-dalton tumor antigen is physically associated with an early region 4 25,000-dalton protein in productively infected cells. J. Virol. 49:692-700.

Sarnow P., Hearing P., Anderson C.W., Reich N., and Levine A.J. (1982). Identification and characterization of an immunologically conserved adenovirus early region 11,000 Mr protein and its association with the nuclear matrix. J. Mol. Biol. 162:565-583.

Savill J. (1997) Recognition and phagocytosis of cells undergoing apoptosis. Br Med Bull 53(3):491-508

Savill J., Fadok V. (2000) Corpse clearance defines the meaning of cell death. Nature 407(6805):784-8

Savill J., Fadok V., Henson P, Haslett C. (1993) Phagocyte recognition of cells undergoing apoptosis. Immunol Today 14(3):131-6

Scaria A., Tollefson A.E., Saha S.K., and Wold W.S. (1992). The E3-11.6K protein of adenovirus is an Asn-glycosylated integral membrane protein that localizes to the nuclear membrane. Virology 191:743-753.

Schlapbach R., Fontana A. (1997) Differential activity of bcl-2 and ICE enzyme family protease inhibitors on Fas and puromycin-induced apoptosis of glioma cells. Biochim Biophys Acta 1359(2):174-80

Schlegel R.A., Williamson P. (2001) Phosphatidylserine, a death knell. Cell Death Differ; 8(6):551-63

Schotte .P, Van Criekinge W., Van de Craen M., Van Loo G., Desmedt M., Grooten J., Cornelissen M., De Ridder L., Vandekerckhove J., Fiers W., Vandenabeele P.,

Beyaert R. (1998) Cathepsin B-mediated activation of the proinflammatory caspase-11. Biochem Biophys Res Commun 251(1):379-87

Schwarz J.K., Devoto S.H., Smith E.J., Chellappan S., Jakoi L., and Nevins J.R. (1993). Interactions of the p107 and Rb proteins with E2F during the cell proliferative response. EMBO J. 122:1013-1020.

Screaton G.R., Mongkolsapaya J., Xu X.N., Cowper A.E., McMichael A.J., Bell J.I. (1997) TRICK2, a new alternatively spliced receptor that transduces the cytotoxic signal from TRAIL. Curr Biol 7(9):693-6

Sellers W.R., and Kaelin W.G.Jr. (1997). Role of the retinoblastoma protein in the pathogenesis of human cancer. J. Clin. Oncol. 15:3301-3312

Sellers W.R., Rodgers J.W., and Kaelin W.G.Jr. (1995). A potent transrepression domain in the retinoblastoma protein induces a cell cycle arrest when bound to E2F sites. Proc. Natl. Acad. Sci. USA 92:11544-11548.
Serrano M., Lin A.W., McCurrach M.E., Beach D., Lowe S.W. (1997) Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell 88(5):593-602

Shenk T. (1996). Adenoviridae: The Viruses and Their Replication. In Fields Virology (3rd edition) B.N. Fields, D.M. Knipe, P.M. Howley et al., eds. (Philidelphia: Lippincott-Raven Publishers) pp. 2111-2148.

Sherr C.J. (2001) The INK4a/ARF network in tumour suppression. Nat Rev Mol Cell Biol 2(10):731-7

Shibata M.A., Liu M.L., Knudson M.C., Shibata E., Yoshidome K., Bandey T., Korsmeyer S.J., Green J.E. (1999) Haploid loss of bax leads to accelerated mammary tumor development in C3(1)/SV40-TAg transgenic mice: reduction in protective apoptotic response at the preneoplastic stage. EMBO J 18(10):2692-701

Shieh S.Y., Ikeda M., Taya Y., and Prives C. (1997). DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. Cell 91:325-334.

Shimada A., Kato S., Enjo K., Osada M., Ikawa Y., Kohno K., Obinata M., Kanamaru R., Ikawa S., Ishioka C. (1999) The transcriptional activities of p53 and its homologue p51/p63: similarities and differences. Cancer Res 59(12):2781-6

Shisler J., Yang C., Walter B., Ware C.F., and Gooding L.R. (1997). The adenovirus E3-10.4/14.5K complex mediates loss of cell surface Fas (CD95) and resistance to Fas-induced apoptosis. J. Virol. 71:8299-8306.

Shoji Y., Uedono Y., Ishikura H., Takeyama N., Tanaka T. (1995) DNA damage induced by tumour necrosis factor-alpha in L929 cells is mediated by mitochondrial oxygen radical formation. Immunology. 84(4):543-8.

Shtrichman R., and Kleinberger T. (1998). Adenovirus type 5 open reading frame 4 protein induces apoptosis in transformed cells. J. Virol. 72:2975-2982.

Shtrichman R., Sharf R., Barr H., Dobner T., Kleinberger T. (1999) Induction of apoptosis by adenovirus E4orf4 protein is specific to transformed cells and requires an interaction with protein phosphatase 2A. Proc Natl Acad Sci U S A 96(18):10080-5

Silke J., Verhagen A.M., Ekert P.G., Vaux D.L. (2000) Sequence as well as functional similarity for DIABLO/Smac and Grim, Reaper and Hid? Cell Death Differ 7(12):1275

Slee E.A., Harte M.T., Kluck R.M., Wolf B.B., Casiano C.A., Newmeyer D.D., Wang H.G., Reed J.C., Nicholson D.W., Alnemri E.S., Green D.R., Martin S.J. (1999) Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. J Cell Biol 144(2):281-92

Smith C.A., Farrah T., Goodwin R.G. (1994) The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. Cell 76(6):959-62

Smith D.H., and Ziff E. (1988). The amino-terminal region of the adenovirus serotype 5 E1a proteins performs two separate functions when expressed in primary baby rat kidney cells. Mol. Cell. Biol. 8:3882-3890.

Smits E., Van Criekinge W., Plaetinck G., Bogaert T. (1999) The human homologue of *Caenorhabditis elegans* CED-6 specifically promotes phagocytosis of apoptotic cells. Curr Biol 9(22):1351-4

Spector M.S., Desnoyers S., Hoeppner D.J., Hengartner M.O. (1997) Interaction between the C. elegans cell-death regulators CED-9 and CED-4. Nature 385(6617):653-6

Spindler K.R., and Berk A.J. (1984). Rapid intracellular turnover of adenovirus 5 early region 1A proteins. J. Virol. 52:706-710.

Spindler K.R., Eng C.Y., and Berk A.J. (1985). An adenovirus early region 1A protein is required for maximal viral DNA replication in growth arrested human cells. J. Virol. 53:742-750.

Srinivasula S.M., Ahmad M., Fernandes-Alnemri T., Alnemri E.S. (1998) Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. Mol Cell 1(7):949-57

Steenberg P.H., Maat J., van Ormondt H., and Sussanbach J.S. (1977). The sequence at the termini of adenovirus type 5 DNA. Nuc. Acids Res. 4:4371-4389.

Stephens C., and Harlow E. (1987). Differential splicing yields novel adenovirus 5 E1A mRNAs that encode 30kd and 35kd proteins. EMBO J. 6:2027-2035.

Stewart A.R., Tollefson A.E., Krajcsi P., Yei S.P., and Wold W.S.M. (1995). The adenovirus E3 10.4K and 14.5K proteins, which function to prevent cytolysis by tumor necrosis factor and to down-regulate the epidermal growth factor receptor, are localized in the plasma membrane. J. Virol. 69:172-181.

Stewart P.L., Fuller S.D., and Burnett R.M. (1993). Difference imaging of adenovirus: bridging the resolution gap between X-ray crystallography and electron microscopy. EMBO J. 12:2589-2599.

Stillman B.W., Lewis J.B., Chow L.T., Mathews M.B., and Smart E.(1981). Identification of the gene and mRNA for the adenovirus terminal protein precursor. Cell 23:497-508.

Stoka V., Turk B., Schendel S.L., Kim T.H., Cirman T., Snipas S.J., Ellerby L.M., Bredesen D., Freeze H., Abrahamson M., Bromme D., Krajewski S., Reed J.C., Yin X.M., Turk V., Salvesen G.S. (2001) Lysosomal protease pathways to apoptosis.

Cleavage of bid, not pro-caspases, is the most likely route. J Biol Chem 276(5):3149-57

Strasser A., Huang D.C., Vaux D.L. (1997) The role of the bcl-2/ced-9 gene family in cancer and general implications of defects in cell death control for tumourigenesis and resistance to chemotherapy. Biochim Biophys Acta 1333(2):F151-78

Strasser A., O'Connor L., Dixit V.M. (2000) Apoptosis signaling. Review. Annu Rev Biochem 69:217-45

Sun D.Y., Jiang S., Zheng L.M. Ojcius D.M., and Young J.D. (1994). Separate metabolic pathways leading to DNA fragmentation and apoptotic chromatin condensation. J. Exp. Med. 179:559-568.

Susin S.A., Lorenzo H.K., Zamzami N., Marzo I., Brenner C., Larochette N., Prevost M.C., Alzari P.M., Kroemer G. (1999a) Mitochondrial release of caspase-2 and -9 during the apoptotic process. J Exp Med 189(2):381-94

Susin S.A., Lorenzo H.K., Zamzami N., Marzo I., Snow B.E., Brothers G.M., Mangion J., Jacotot E., Costantini P., Loeffler M., Larochette N., Goodlett D.R., Aebersold R., Siderovski D.P., Penninger J.M., Kroemer G. (1999b) Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 397(6718):441-6

Szymborski A., Miron M.J, Marcellus R.C., Branton P.E. The adenoviral death protein E4orf4 induces a novel, aberrant type of apoptosis independently of core death machinery (in preparation)

Takahashi A., Alnemri E.S., Lazebnik Y.A., Fernandes-Alnemri T., Litwack G., Moir R.D., Goldman R.D., Poirier G.G., Kaufmann S.H., and Earnshaw W.C. (1996). Cleavage of lamin A by Mch2a but not CPP32: Multiple interleukin 1b-converting enzyme-related proteases with distinct substrate recognition properties are active in apoptosis. Proc. Natl. Acad. Sci. 93:8395-8400.

Takimoto R., El-Deiry W.S. (2000) Wild-type p53 transactivates the KILLER/DR5 gene through an intronic sequence-specific DNA-binding site. Oncogene 19(14):1735-43

Tamanoi F., and Stillman B.W. (1982). Function of adenovirus terminal protein in the initiation of DNA replication. Proc. Natl. Acad Sci. USA 79:2221-2225.
Tartaglia L.A., Goeddel D.V.(1992) Two TNF receptors. Immunol Today 13(5):151-3

Tauber B., Dobner T. (2001) Adenovirus early E4 genes in viral oncogenesis. Oncogene 20(54):7847-54

Teitz T., Wei T., Valentine M.B., Vanin E.F., Grenet J., Valentine V.A., Behm F.G., Look A.T., Lahti J.M., Kidd V.J. (2000) Caspase 8 is deleted or silenced preferentially in childhood neuroblastomas with amplification of MYCN. Nat Med 6(5):529-35

Temperley S.M., and Hay R.T. (1992). Recognition of the adenovirus type 2 origin of DNA replication by the virally encoded DNA polymerase and preterminal proteins. EMBO J. 11:761-768.

Teodoro J.G., and Branton P.E. (1997a). Regulation of apoptosis by viral gene products. J. Virol. 71:1739-1746.

Teodoro J.G., and Branton P.E. (1997a). Regulation of apoptosis by viral gene products. J. Virol. 71:1739-1746.

Teodoro J.G., and Branton P.E. (1997b). Regulation of p53-dependent apoptosis, transcriptional repression, and cell transformation by phosphorylation of the 55-kilodalton E1B protein of human adenovirus type 5. J. Virol. 71:3620-3627

Teodoro J.G., and Branton P.E. (1997b). Regulation of p53-dependent apoptosis, transcriptional repression, and cell transformation by phosphorylation of the 55-kilodalton E1B protein of human adenovirus type 5. J. Virol. 71:3620-3627.

Teodoro J.G., Shore G.C., and Branton P.E. (1995). Adenovirus E1A proteins induce apoptosis by both p53-dependent and p53-independent mechanisms. Oncogene 11:467-474.

Teodoro J.G., Shore G.C., and Branton P.E. (1995). Adenovirus E1A proteins induce apoptosis by both p53-dependent and p53-independent mechanisms. Oncogene 11:467-474.

Tewari M., and Dixit V.M. (1995). Fas- and TNF-induced apoptosis is inhibited by the poxvirus crmA gene product. J. Biol. Chem. 270:3255-3260.

Thome M., Schneider P., Hofmann K., Fickenscher H., Meinl E., Neipel F., Mattmann C., Burns K., Bodmer J.L., Schroter M., Scaffidi C., Krammer P.H.,

Peter M.E., Tschopp J. (1997) Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. Nature 386(6624):517-21

Thompson C.B. (1995) Apoptosis in the pathogenesis and treatment of disease. Science 267(5203):1456-62

Thornberry N.A., Lazebnik Y. (1998) Caspases: enemies within. Science 281(5381):1312-6

Thornberry N.A., Peterson E.P., Zhao J.J., Howard A.D., Griffin P.R., Chapman K.T. (1994) Inactivation of interleukin-1 beta converting enzyme by peptide (acyloxy)methyl ketones. Biochemistry 33(13):3934-40

Thornberry N.A., Rano R.A., Peterson E.P., Rasper D.M., Timkey T., Garcia-Calvo M., Houtzager V.M., Nordstrom P.A., Roy S., Vaillancourt J.P., Chapman K.T., and Nicholson D.W. (1997). A combinatorial approach defines seecificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. J. Biol. Chem. 272:17907-17911.

Thut C.J., Chen J.L., Klemin R., and Tjian R. (1995). p53 transcriptional activation mediated by coactivators TAFII40 and TAFII60. Science 267:100-104.

Ting A.T., Pimentel-Muinos F.X., Seed B. (1996) RIP mediates tumor necrosis factor receptor 1 activation of NF-kappaB but not Fas/APO-1-initiated apoptosis. EMBO J 15(22):6189-96

Tollefson A.E., and Wold W.S. (1988). Identification and gene mapping of a 14,700-molecular-weight protein encoded by region E3 of group C adenoviruses. J. Virol. 62:33-39.

Tollefson A.E., Hermiston T.W., Lichtenstein D.L., Colle C.F., Tripp R.A., Dimitrov T., Toth K., Wells C.E., Doherty P.C., and Wold W.S.M. (1998). Forced degradation of Fas inhibits apoptosis in adenovirus-infected cells. Nature 392:726-730.

Tollefson A.E., Krajcsi P., Yei S.P., Carlin C.R., and Wold W.S. (1990b). A 10,400-molecular-weight membrane protein is coded by region E3 of adenovirus. J. Virol. 64:794-801.

Tollefson A.E., Ryerse J.S., Scaria A., Hermiston T.W., and Wold W.S. (1996). The E3-11.6-kDa adenovirus death protein (ADP) is required for efficient cell death: characterization of cells infected with adp mutants. Virology 220:152-162.

Tollefson A.E., Scaria A., Saha S.K., and Wold W.S. (1992). The 11,600-MW protein encoded by region E3 of adenovirus is expressed early but is greatly amplified at late stages of infection. J. Virol. 66:3633-3642.

Trapani J.A., Jans D.A., Sutton V.R. (1998) Lymphocyte granule-mediated cell death. Springer Semin Immunopathol 19(3):323-43

Trentin J.J., Yabe Y., and Taylor G. (1962). The quest for human cancer viruses. Science 137:835-849.

Tsai L.-H., Harlow E., and Meyerson M. (1991). Isolation of the human cdk2 gene that encodes the cyclin A- and adenovirus E1A-associated p33 kinase. Nature 353:174-177.

Unger T., Nau M.M., Segal S., and Minna J.D. (1992). p53: a transdominant regulator of transcription whose function is ablated by mutations occurring in human cancer. EMBO J. 11:1383-1390.

Uren A.G., Coulson E.J., Vaux D.L. (1998) Conservation of baculovirus inhibitor of apoptosis repeat proteins (BIRPs) in viruses, nematodes, vertebrates and yeasts. Trends Biochem Sci 23(5):159-62

Van Antwerp D.J., Martin S.J., Kafri T., Green D.R., Verma I.M.(1996) Suppression of TNF-alpha-induced apoptosis by NF-kappaB. Science 274(5288):787-9

van der Vliet P.C., Levine A.J., Ensinger M.J., and Ginsberg H.S. (1975). Thermolabile DNA-binding protein from cells infected with a temperature-sensitie mutant of adenovirus defective in viral DNA synthesis. J. Virol. 15:348-354.

van Loo G., Schotte P., van Gurp M., Demol H., Hoorelbeke B., Gevaert K., Rodriguez I., Ruiz-Carrillo A., Vandekerckhove J., Declercq W., Beyaert R.,

Vandenabeele P. (2001) Endonuclease G: a mitochondrial protein released in apoptosis and involved in caspase-independent DNA degradation. Cell Death Differ 8(12):1136-42

van Ormondt H., Maat J., and Dijkema R. (1980). Comparison of nucleotide sequences of the early E1a regions for subgroups A, B and C of human adenoviruses. Gene 12:63-76.

Vander Heiden M.G., Chandel M.S., Williamson E.K., Schumacker P.T., Thompson CB (1997) Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria. Cell. 91(5):627-37.

Vander Heiden M.G., Chandel N.S., Williamson E.K., Schumacker P.T., Thompson C.B. (1997) Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria. Cell 91(5):627-37

Vaux D.L., Haecker G., Strasser A. (1994) An evolutionary perspective on apoptosis. Cell 1994 76(5):777-9

Vaux DL. (1997) CED-4--the third horseman of apoptosis. Cell 1997 90(3):389-90

Vayda M.E., and Flint S.J. (1987). Isolation and characterization of adenovirus core nucleoprotein subunits. J. Virol. 16:3335-3339.

Velicer L.F., and Ginsberg H.S. (1970). Synthesis, transport and morphogenesis of type 5 adenovirus capsid proteins. J.Virol. 5:338-347.

Verhagen A.M., Ekert P.G., Pakusch M., Silke J., Connolly L.M., Reid G.E., Moritz R.L., Simpson R.J., Vaux D.L. (2000) Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell 102(1):43-53

Verrijzer C.P., van Oosterhout A.W.M., van Weperen W.W., and van der Vliet P.C. (1991). POU proteins bend DNA via the POU-specific domain. EMBO J. 10:3007-3014.

Virtanen A., Gilardi P., Naslund A., LeMoullec J.M., Pettersson U., and Perricaudet M. (1984). mRNAs from human adenovirus 2 early region 4. J. Virol. 51:822-831.

Vogelstein B., Lane D., Levine A.J. (2000) Surfing the p53 network. Nature 408(6810):307-10

Vogt C. (1842) Untersuchungen uber die Entwiclunggsgeschichte der Geburshelferkrote (*Alytes obstetricians*), ed. JU Gassman, 130 pp.

Walker N.P., Talanian R.V., Brady K.D., Dang L.C., Bump N.J., Ferenz C.R., Franklin S., Ghayur T., Hackett M.C., Hammill L.D. (1994) Crystal structure of the cysteine protease interleukin-1 beta-converting enzyme: a (p20/p10)2 homodimer. Cell 78(2):343-52

Wallach D., Boldin M., Varfolomeev E., Beyaert R., Vandenabeele P., Fiers W. (1997). Cell death induction by receptors of the TNF family: towards a molecular understanding. FEBS Lett. 410:96-106.

Wang C.Y., Mayo M.W., Korneluk R.G., Goeddel D.V., Baldwin A.S. Jr. (1998) NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science 281(5383):1680-3

Wang P., Reed M., Wang Y., Mayr G., Stenger J.E., Anderson M.E., Schwedes J.F., Tegtmeyer P. (1994). p53 domains: structure, oligomerization, and transformation. Mol Cell. Biol. 14:5182-5191.

Wang S., Miura M., Jung Y.K., Zhu H., Li E., Yuan J. (1998) Murine caspase-11, an ICE-interacting protease, is essential for the activation of ICE. Cell 92(4):501-9

Wang X. (2001) The expanding role of mitochondria in apoptosis. Genes Dev 15(22):2922-33

Wang Y. Reed M., Wang P., Stenger J.E., Mayr G., Anderson M.E., Schwedes J.F., Tegtmeyer P. (1993). p53 domains: identification and characterization of two autonomous DNA-binding regions. Genes Dev. 7:2575-2586.

Webster A., Hay R.T., and Kemp G. (1993). The adenovirus protease is activated by a virus-coded disulphide-linked peptide. Cell 72:97-104.

Webster L.C., and Ricciardi R.P. (1991). trans-dominant mutants of E1a provide genetic evidence that the zinc finger of the trans-activation domain binds a transcription factor. Mol. Cell. Biol. 11:4287-4296.

Wei M.C., Lindsten T., Mootha V.K., Weiler S., Gross A., Ashiya M., Thompson C.B., Korsmeyer S.J. (2000) tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. Genes Dev 14(16):2060-71

Weil M., Jacobson M.D., Raff M.C. (1998) Are caspases involved in the death of cells with a transcriptionally inactive nucleus? Sperm and chicken erythrocytes. J Cell Sci 111 (Pt 18):2707-15

Weiss R.S., Gold M.O., Vogel H., and Javier R.T. (1997b). Mutant adenovirus type 9 E4 ORF1 genes define three protein regions required for transformation of CREF cells. J. Virol. 71:4385-4394.

Weiss R.S., Lee S.S., Prasad B.V., and Javier R.T. (1997a). Human adenovirus early region 4 open reading frame 1 genes encode growth-transforming proteins that may be distantly related to dUTP pyrophosphatase enzymes. J. Virol. 71:1857-1870.

Weiss R.S., McArthur M.J., and Javier R.T. (1996). Human adenovirus type 9 E4 open feading frame 1 encodes a cytoplasmic transforming protein capable of increasing the oncogenicity of CREF cells. J. Virol. 70:862-872.

Wen L.P., Fahrni J.A., Troie S., Guan J.L., Orth K., Rosen G.D. (1997) Cleavage of focal adhesion kinase by caspases during apoptosis. J Biol Chem 272(41):26056-61

White E, and Cipriani R. (1989). Specific disruption of intermediate filaments and the nuclear lamina by the 19-kilodalton product of the adenovirus E1B oncogene. Proc. Natl. Acad. Sci. USA 86:9886-9890.

White E. (1993) Regulation of apoptosis by the transforming genes of the DNA tumor virus adenovirus. Proc Soc Exp Biol Med 204(1):30-9

White E., and Cipriani R. (1990). Role of adenovirus E1B proteins in transformation: altered organization of intermediate filaments in transformed cells that express the 19-kildalton protein. Mol. Cell. Biol. 10:120-130.

White E., Cipriani R., Sabbatini P., and Denton A. (1991). Adenovirus E1B 19-kilodalton protein overcomes the cytotoxicity of E1A proteins. J. Virol. 65:2968-2978.

White E., Grodzicker T., and Stillman B.W. (1984). Mutations in the gene encoding the adenovirus early region 1B 19,000 molecular weight tumor antigen cause the degradation of chromosomal DNA. J. Virol. 42:410-419.

Whyte P., Williamson N.M., and Harlow E. (1989). Cellular targets for transformation by the adenovirus E1A proteins. Cell 56:67-75.

Wickham T.J., Mathias P., and Cheresh D.A. and Nemerow G.R. (1993). Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. Cell 73:309-319.

Williamson P., Schlegel R.A. (1994) Back and forth: the regulation and function of transbilayer phospholipid movement in eukaryotic cells. Mol Membr Biol; 11(4):199-216

Wilson K.P., Black J.A., Thomson J.A., Kim E.E., Griffith J.P., Navia M.A., Murcko M.A., Chambers S.P., Aldape R.A., Raybuck S.A. (1994) Structure and mechanism of interleukin-1 beta converting enzyme. Nature 370(6487):270-5

Winter R.N., Kramer A., Borkowski A., Kyprianou N. (2001) Loss of caspase-1 and caspase-3 protein expression in human prostate cancer. Cancer Res 2001 61(3):1227-32

Wold W.S., Cladaras C., Deutscher S.L., and Kapoor Q.S. (1985). The 19-kDa glycoprotein coded by region E3 of adenovirus. Purification, characterization, and structural analysis. J. Biol. Chem. 260:2424-2431.
Wolf B.B., Green D.R. (1999) Suicidal tendencies: apoptotic cell death by caspase family proteinases. J Biol Chem 274(29):20049-52

Wolfson J., and Dressler D. (1972). Adenovirus DNA contains an inverted terminal repetition. Proc. Natl. Acad. Sci. USA 69:3054-3057.
Wolter K.G., Hsu Y.T., Smith C.L., Nechushtan A. Xi X.G., Youle R.J. (1997) Movement of Bax from the cytosol to mitochondria during apoptosis. J Cell Biol 139(5):1281-92

Woodle E.S., Smith D.M., Bluestone J.A., Kirkman W.M. 3rd, Green D.R., Skowronski E.W. (1997) Anti-human class I MHC antibodies induce apoptosis by a pathway that is distinct from the Fas antigen-mediated pathway. J Immunol 158(5):2156-64

Wu D., Wallen H.D., Inohara N., Nunez G. (1997) Interaction and regulation of the Caenorhabditis elegans death protease CED-3 by CED-4 and CED-9. J Biol Chem 272(34):21449-54

Wu L., Rosser D.S.E., Schmidt M.G., and Berk A.J. (1987). A TATA box implicated in E1a transcriptional activation of a simple adenovirus 2 promoter. Nature 326:512-515.

Wu X., Bayle J.H., Olson D., and Levine A.J. (1993). The p53-mdm2 autoregulatory feedback loop. Genes Dev. 7:1126-1132.

Wyllie A.H., Beattie G.H., Hargreaves A.D. (1981). Chromatin changes in apoptosis. Histochem J. 13(4):681-92.

Xiang J., Chao D.T., and Korsmeyer S.J. (1996). BAX-induced cell death may not require interleukin 1b-converting enzyme-like proteases. Proc. Natl. Acad. Sci. USA 93:14559-14563.

Xue D., Horvitz H.R. (1997) *Caenorhabditis elegans* CED-9 protein is a bifunctional cell-death inhibitor. Nature 390(6657):305-8

Yamamoto K., Ichijo H., Korsmeyer S.J. (1999) BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G(2)/M. Mol Cell Biol 19(12):8469-78

Yang J., Liu X., Bhalla K., Kim C.N., Ibrado A.M., Cai J., Peng T.I., Jones DP,

Wang X (1997) Prevention of apoptosis by Bcl-2: release of cutochrome c from mitochondria blocked, 275: 1129-1132

Yang X., Khosravi-Far R., Chang H.Y., Baltimore D. (1997) Daxx, a novel Fas-binding protein that activates JNK and apoptosis. Cell 89(7):1067-76

Yang X.J., Ogryzko V.V., Nishikawa J., Howard B.H., and Nakatani Y. (1996). A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. Nature 382:319-324.

Yew P.R., and Berk A.J. (1992). Inhibition of p53 transactivation required for transformation by adenovirus early region 1B protein. Nature 357:82-85.

Yin X.M., Oltvai Z.N., and Korsmeyer S.J. (1994). BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. Nature 369:321-323

Yu J., Zhang L., Hwang P.M., Kinzler K.W., Vogelstein B. (2001) PUMA induces the rapid apoptosis of colorectal cancer cells. Mol Cell 7(3):673-82

Yuan J., Shaham S., Ledoux S., Ellis H.M., Horvitz H.R. (1993) The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. Cell 75(4):641-52

Yuan Z.M., Shioya H., Ishiko T., Sun X., Gu J., Huang Y.Y., Lu H., Kharbanda S.,

Weichselbaum R., Kufe D. (1999) p73 is regulated by tyrosine kinase c-Abl in the apoptotic response to DNA damage. Nature 399(6738):814-7

Zachowski A. (1993) Phospholipids in animal eukaryotic membranes: transverse asymmetry and movement. Biochem J; 294 (Pt 1):1-14

Zahler A.M., Lan W.S., Stolk J.A., and Roth M.B. (1992). SR proteins: A conserved family of pre-mRNA splicing facotrs. Genes Dev 6:837-847.

Zamzami N., Kroemer G. (2001) The mitochondrion in apoptosis: how Pandora's box opens. Nat Rev Mol Cell Biol 2(1):67-71

Zamzami N., Susin S.A., Marchetti P., Hirsch T., Gomez-Monterrey I., Castedo M., Kroemer G. (1996) Mitochondrial control of nuclear apoptosis. J Exp Med 183(4):1533-44

Zandi E., Rothwarf D.M., Delhase M., Hayakawa M., Karin M. (1997) The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. Cell 91(2):243-52

Zha H., Reed J.C. (1997) Heterodimerization-independent functions of cell death regulatory proteins Bax and Bcl-2 in yeast and mammalian cells. Biol Chem 1997 272(50):31482-8

Zha J., Harada H., Osipov K., Jockel J., Waksman G., Korsmeyer S.J. (1997) BH3 domain of BAD is required for heterodimerization with BCL-XL and pro-apoptotic activity. J Biol Chem 1997 272(39):24101-4

Zha J., Harada H., Yang E., Jockel J., Korsmeyer S.J. (1996) Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L) Cell 1996 87(4):619-28

Zhang L., Yu J., Park B.H., Kinzler K.W., Vogelstein B. (2000) Role of BAX in the apoptotic response to anticancer agents. Science 290(5493):989-92

Zhang X.D., Franco A.V., Nguyen T., Gray C.P., Hersey P. (2000) Differential localization and regulation of death and decoy receptors for TNF-related apoptosis-inducing ligand (TRAIL) in human melanoma cells. J Immunol 164(8):3961-70

Zhang Y., Xiong Y., and Yarbrough W.G. (1998). ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the RB and p53 tumor suppression pathways. Cell 92:725-734.

Zhou Q., Snipas S., Orth K., Muzio M., Dixit V.M., Salvesen G.S. (1997) Target protease specificity of the viral serpin CrmA. Analysis of five caspases. J Biol Chem 272(12):7797-800

Zhu J., Chen X. (2000) MCG10, a novel p53 target gene that encodes a KH domain RNA-binding protein, is capable of inducing apoptosis and cell cycle arrest in G(2)-M. Mol Cell Biol 20(15):5602-18

Zhu J., Jiang J., Zhou W., Chen X. (1998) The potential tumor suppressor p73 differentially regulates cellular p53 target genes. Cancer Res 58(22):5061-5

Zilli D., Voelkel-Johnson C., Skinner T., and Laster S.M. (1992). The adenovirus E3 region 14.7 kDa protein, heat and sodium arsinate inhibit the TNF-induced release of arachidonic acid. Biochem. Biophys. Res. Comm. 188:177-183.

Zornig M., Grzeschiczek A., Kowalski M.B., Hartmann K.U., Moroy T. (1995) Loss of Fas/Apo-1 receptor accelerates lymphomagenesis in E mu L-MYC transgenic mice but not in animals infected with MoMuLV. Oncogene 10(12):2397-401

Zornig M., Hueber A., Baum W., Evan G. (2001) Apoptosis regulators and their role in tumorigenesis. Biochim Biophys Acta 1551(2): F1-37