Molecular dissection of the role of cell survival and apoptotic signaling in normal and cancer cells

by

Subba Reddy Maddika

A thesis submitted to the Faculty of Graduate Studies of The University of Manitoba

In partial fulfillment of the requirements of the degree of

Doctor of Philosophy

Department of Biochemistry and Medical Genetics

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Abstract

Apoptosis, triggered by various cytotoxic cancer therapies, is a physiological form of cell death and is tightly controlled via complex interactions between cell survival and cell death regulatory molecules. Using Apoptin as a model molecule, we demonstrate the role of different cell survival and cell death pathways during apoptosis in normal and cancer cells. Apoptin, a chicken anemia virus derived protein, induces cell death selectively in cancer cells. The signalling pathways of Apoptin induced cancer cell-selective apoptosis are not well understood. Apoptin triggers caspase dependent apoptosis by activating the mitochondrial/intrinsic pathway, which is regulated by Bcl-2 family members. In addition, Apoptin, via its proline rich motif, interacts with the SH3 domain of p85 regulatory subunit of PI3-kinase leading to the constitutive activation of PI3-kinase and Akt. Downstream of PI3-kinase, Apoptin also interacts with Akt and mediates the nuclear translocation of active Akt. In the nucleus, Akt activates CDK2 and mediates its cytoplasmic translocation, where it phosphorylates Bcl-2 specifically at Thr-56 residue, thus mediating its degradation, which contributes to the activation of the mitochondrial death pathway. In addition, CDK2 phosphorylates Apoptin and regulates its tumor specific nuclear retention. Among additional targets of Apoptin's signaling events in the nucleus, Akt directly phosphorylates $p27^{kip1}$ and enhances its proteosome-dependent degradation. Apoptin also indirectly mediates the cytoplasmic translocation of Nur77. These events are crucial for the activation of mitochondrial apoptotic pathway.

A direct link between Akt and CDK2 in the nucleus has also been demonstrated, which is important both for apoptosis and cell cycle progression. Akt phosphorylates CDK2 at threonine 39 residue, both *in vitro* and *in vivo*. The Akt mediated CDK2

phosphorylation occurs during the specific S/G2 boundary of the cell cycle via a transient nucleo-cytoplasmic shuttling of Akt during S and G2 phases. The phosphorylated CDK2 translocates to the cytoplasm temporarily, which is required for cell cycle progression from S to G2/M phase as the CDK2 T39A mutant lacking the phosphorylation site and defective in cytoplasmic localization severely affects the cell cycle progression at S-G2/M transition. Interestingly, we have also shown that the Akt/CDK2 pathway is constitutively activated by some anticancer drugs, like methotrexate and docetaxel, and under these conditions it promotes-, rather than suppress cell death. Thus, the constitutive activation of the Akt/CDK2 pathway and altered sub cellular localization, promotes apoptosis. In contrast, transient physiologic Akt/CDK2 activation is necessary for cell cycle progression. These results reveal a novel function for the PI3-kinase/Akt pathway during an apoptotic process that could serve as a target for novel strategies for modulating apoptosis in cancer therapies.

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I dedicate this work to my grandparents (the first teachers of my life) and to my wife Srilekha

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List of Abbreviations

A	Alanine
AIDS	Acquired immune deficiency syndrome
AIF	Apoptosis inducing factor
Apaf-1	apoptotic protease-activating factor-1
APC	anaphase promoting complex
ATP	Adenosine triphosphate
BH	Bcl-2 homology
BrdU	5′-bromo-2′-deoxyuridine
BSA	Bovine serum albumin
CaCl2	Calcium chloride
CARD	caspase recruitment domain
CAK	CDK activating kinase
CDK	cyclin dependent kinase
Cyt c	cytochrome c
CLL	Chronic Lymphocytic Leukemia
DD	death domain
DED	death effector domain
DISC	death inducing signaling complex
DMEM	Dulbecco's modified eagle medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
E	glutatmatic acid
ECL	enhanced chemical luminescence
EDTA	ethylenediaminotetraacetic acid
EGF	epidermal growth factor
FACS	fluorescence activated cell sorter
FADD	Fas-associating protein with death domain
FBS	fetal bovine serum
FITC	flourescein isothiocyanate

fmk	flouromethylketone
GFP	green-fluorescent protein
GSK-3β	glycogen synthase kinase-3β
GST	glutathione-S-transferase
HA	hemagglutinin
HRP	horseradish peroxidase
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL2	interleukin 2
JC-1	5,5′, 6, 6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide
JNK	c-Jun-N-terminal kinase
kb	kilobase
kDa	kiloDalton
μg	microgram
μl	microliter
μM	micromolar
MAPK	mitogen activated protein kinase
MEFs	mouse embryonic fibroblasts
ml	milliliter
NES	nuclear export signal
NF-κB	nuclear factor-κB
NLS	nuclear localization signal
nM	nanomolar
NP-40	Nonidet P-40
OD	optical density
P	proline
PAGE	polyacrylamide gel electrophoresis
PBLs	peripheral blood lymphocytes
PBS	phosphate buffered saline
PCD	programmed cell death
PI3-K	phophatidylinositol 3′-kinase
PI	propidim iodide

PMSF	phenylmethylsulfonyl fluoride
PKC	protein kinase C
PT	permeability transition
R	arginine
RIP	receptor interacting protein
Rpm	revolutions per minute
RT	room temperature
S	serine
SDS	sodium dodecyl sulfate
T	threonine
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
UV	ultraviolet
v/v	volum/volume
w/v	weight/volume
wt	wild type
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
$\Delta\Psi m$	mitochondrial membrane potential

1. Introduction

1.1 Apoptosis

Apoptosis or programmed cell death (PCD), first described in 1972, is a genetically controlled physiological process by which multicellular organisms maintain tissue homeostasis during growth and development (Ellis et al., 1991; Kerr et al., 1972). An aberrant regulation of apoptosis is seen in, and contributes to, a myriad of pathophysiological conditions such as cancers, stroke, AIDS, neurological disorders and autoimmune diseases (Orrenius, 1995). In addition to its physiological role, apoptosis is an important mechanism utilized by different cytotoxic therapies currently used for treatment of cancer, for example, chemotherapy, γ-irradiation, immunotherapy and suicide gene therapy (Fulda and Debatin, 2004; Thompson, 1995). Characteristic apoptotic features include cell membrane blebbing, cell shrinkage, chromatin condensation and DNA fragmentation, finally ending with engulfment by macrophages or neighboring cells, thereby avoiding an inflammatory response in surrounding tissues (Darzynkiewicz et al., 1997; Khosravi-Far and Esposti, 2004). Apoptosis is distinct from necrosis in which the cells suffer a major insult, leading to a loss of membrane integrity, swelling and disruption of cells, resulting in a strong inflammatory response in the corresponding tissue. The basic understanding of apoptotic pathways came from genetic studies in the nematode *C. elegans*. Four important genes have been identified in genetic screens that control the cell death program in C. elegans; *Ced-3, Ced-4, Ced-9* and *Egl-1*. The genes *Ced-3, Ced-4* and *Egl-1* are required for the induction of cell death, as the mutants with loss of function were defective in inducing apoptosis. Ced-9 is an anti-apoptotic molecule in C. elegans, as its

mutations with gain of function are embryonic lethal and loss of function mutants prevent the cell death process. *Egl-1* binds to *Ced-9*, displacing *Ced-4*, which in turn activates *Ced-3* to induce apoptosis (Hengartner et al., 1992; Liu and Hengartner, 1999). In mammals, the execution of apoptosis is tightly regulated through complex interactions between apoptosis-promoting and apoptosis-opposing molecular machinery. Homologues of the four cell death regulatory genes of C. elegans have been identified in mammals, though with increased complexity. *Ced-3* is functionally homologous to the mammalian caspase family, *Ced-9* represents the anti-apoptotic Bcl-2 members, *Ced-4* is homologous to Apaf-1 and *Egl-1* is homologous to pro-apoptotic BH-3 only Bcl-2 family members (Yuan et al., 1993; Zou et al., 1997). The induction of apoptosis or programmed cell death occurs via two major pathways, the death receptor-dependent (extrinsic) pathway through TNF family ligands or via the mitochondrial (intrinsic) pathway induced by different factors such as UV radiation, chemotherapeutics, free radicals or DNA damage (Brouckaert et al., 2005; Los et al., 1995b; Maddika et al., 2006). Caspases constitute the key effector molecules in programmed cell death and their activation triggered by either the extrinsic or the intrinsic pathway is central to the process of apoptosis.

1.2 Caspases – The central initiators and executioners of apoptosis

1.2.1 Members of the caspase family

Caspases, the activation of which is a hallmark of apoptosis, are a family of cysteine proteases that specifically cleave their substrates after an aspartate residue. The importance of caspases was originally established through genetic studies in C. elegans, which revealed the existence of the *Ced-3* gene that controls developmental cell death in nematode, a homologue of the interleukin-1β processing enzyme (ICE). This protein was

shown to be sufficient to induce apoptosis when overexpressed in fibroblasts (Miura et al., 1993). To date, about 14 mammalian caspases have been identified (Los et al., 1999). Based on their function, structure and the order of their action in cell death signaling, caspases can be classified into three major groups: (1) The apoptotic initiator caspases, which possess long prodomains containing either a death effector domain (DED) (caspase-8 and -10) or a caspase activation and recruitment domain (CARD) (caspase-2 and -9) that mediate the interaction with upstream adaptor molecules. These apical initiator caspases cleave and activate the downstream effector caspases. (2) The apoptotic effector caspases, characterized by the presence of a short prodomain (caspase-3, -6 and -7) without any CARD or DED motifs, get activated by initiator caspases and perform the downstream execution steps of the apoptotic program by cleaving multiple substrates in the cell. (3) The inflammatory caspases include caspase-1, -4 and -5, which have an N-terminal CARD motif and are involved mainly in the inflammatory response instead of apoptosis. However, caspase-12, -13 and -14 sometimes have been assigned roles in both inflammation and apoptosis, the details of which are less understood (Boyce et al., 2004).

1.2.2 Caspase activation

In general, caspases are synthesized as inactive zymogens containing a prodomain followed by p20 (large) and p10 (small) subunits. These zymogens can be cleaved to form active enzymes following the induction of apoptosis. Two major apoptotic signaling (death receptor and mitochondrial) pathways lead to caspase zymogen processing (More details on the pathways provided in sections 1.3 and 1.4). It was initially proposed that the initiator caspases are autoproteolytically activated when brought into close proximity of each other, which is called the 'induced proximity' model. Later, this model was refined by

the proximity-induced dimerization model, where the homodimerization of caspase-9 and caspase-8 in the apoptosome and DISC complexes lead to their activation due to their increased local concentrations (Boatright et al., 2003). The homophilic interactions of the CARD domain in caspase-9 and -2 and the DED motifs of caspase-8 and -10 with CARD or DED motif containing proteins like Apaf-1 or FADD respectively mediates the adaptor driven caspase activation (Boatright and Salvesen, 2003; Donepudi et al., 2003). Genetic studies from knock-out mice indicate that caspase-8 is required for all known death receptor mediated apoptotic pathways, whereas caspase-9 is mostly involved in the mitochondrial pathway (Hakem et al., 1998; Kuida et al., 1998; Varfolomeev et al., 1998). On the other hand, the effector caspases such as caspase-3, -6 and -7 are normally cleaved at the Asp-X sites and activated by active initiator caspases. Genetic evidence has shown that the loss of caspase-3 results in partial resistance to apoptosis in response to both the extrinsic and the intrinsic pathways (Woo et al., 1998). Caspases serve as signaling mediators that orchestrate apoptotic execution pathways by cleaving various subsets of cellular proteins. So far, more than 100 substrates have been identified for caspases and the list is still growing. Important caspase substrates include cytoskeletal and structural proteins (eg. Fodrin, β-catenin, actin, Gas-2 and lamins), cell cycle proteins (such as pRB, $p27^{kip1}$, $p21^{cip1}$, Cdc27, Wee1 and cyclin A), cellular DNA repair proteins (such as DNA-PK, Rad51, ATM and PARP), apoptotic proteins (such as Bid, ICAD, Bcl-2, Bcl-xL, IAP, $FLIP_L$, Akt, RIP, PAK2, MEKK1, PKC and NF-κB) and other cellular proteins (Nedd4, ataxin-3, atrophin-1, presenilins, Ras Gap, SP1 and STATs) (Stroh and Schulze-Osthoff, 1998).

1.3 Death receptor (Extrinsic) pathway

The death receptor mediated extrinsic apoptotic pathway is activated mainly by ligand bound death receptors mainly including CD95L-CD95, TNF-TNFR-1 and TRAIL-DR4 or DR-5.

1.3.1 The CD95 pathway

The CD95 (Fas/Apo-1) molecule, which has an essential role in various physiological and pathological forms of cell death (Nagata, 1998), was originally identified as a cell surface receptor that mediates apoptotic cell death of transformed cells and causes regression of experimental tumors growing in nude mice (Trauth et al., 1989). The basic understanding of the CD95 receptor and its ligand has greatly been enhanced by the finding that both molecules are mutated in mouse strains suffering from severe autoimmune lymphoproliferative syndrome (ALPS), a disease, which is also reported in humans (Rieux-Laucat et al., 1995). Lpr (lymphoproliferation) mice, which lack a functional CD95 receptor (Watanabe-Fukunaga et al., 1992) as well as gld (generalized lymphoproliferative disease) mice, which bear a mutant CD95 ligand (Takahashi et al., 1994) fail to remove excess lymphocytes and display lymphoproliferative phenotypes including lymphoadenopathy and splenomegaly.

Binding of CD95 ligand or agonistic antibodies promotes CD95 receptor trimerization that in turn results in the intracellular clustering of death domains (DD). This allows an adaptor protein called FADD (Fas-associated death domain containing protein) to associate with the receptor through an interaction between homologous death domains on both molecules. FADD, via its death effector domain (DED), recruits pro-caspase-8

(also known as FLICE) and results in the formation of a protein complex called DISC (Chinnaiyan et al., 1995; Kischkel et al., 1995). The CD95 ligand induced clustering of CD95, FADD and pro-caspase-8 within the DISC leads to autoproteolytic processing of caspase-8 by induced proximity and dimerization. Following the cleavage, caspase-8 is released from the DISC as an active heterotetramer containing two p18 and two p10 subunits (Los et al., 1995b; Medema et al., 1997), although one recent study suggests that all the cleavage products of caspase-8 remain bound to DISC (Lavrik et al., 2003). Studies in FADD and caspase-8 deficient mice indicated that both are required for CD95-mediated apoptosis (Varfolomeev et al., 1998; Zhang et al., 1998). Other DED containing proteins like caspase-10, Daxx, FAP-1, RIP, FLASH and FLIP have also been shown in several studies to be recruited to the DISC. FLIP, homologous to caspase-8, acts a negative regulator of CD95 mediated cell death (Irmler et al., 1997; Sprick et al., 2002; Stanger et al., 1995; Yang et al., 1997b). Cells can be separated into two types according to their requirement for the mitochondrial pathway in CD95 induced apoptosis, which depends on factors like the amount of active caspase-8, the efficiency of DISC formation and the dependency on Bcl-2 proteins. In type I cells, active caspase-8 directly activates executioner caspases like caspase-3 and caspase-7, whose action on defined substrates leads to the execution of apoptosis. In type II cells, caspase-8 cleaves Bid, a pro-apoptotic Bcl-2 member into a truncated Bid (tBID), which results in activation of the mitochondrial pathway to activate caspase-9 and downstream caspases. Caspase-3 and -7 in turn activate caspase-8, thus forming a positive amplification loop. The type II, but not type I cells are sensitive to cell death inhibition by overexpression of Bcl-2 and Bcl-xL (Scaffidi et al., 1998).

1.3.2 The TNF pathway

Tumor necrosis factor is a multifunctional pro-inflammatory cytokine that elicits a broad spectrum of biological responses including chronic inflammation, bone resorption, neutrophil activation, cytokine production and is involved in many diseases like autoimmune disorders, cancer and neurodegenerative diseases (Wajant et al., 2003). There are two major TNF receptors TNF-R1 and TNF-R2. TNF-R1 is ubiquitously expressed in most tissues and is the major mediator of TNF signaling, whereas TNF-R2 is mainly expressed in the immune system and can only be fully activated by membrane bound TNF, but not by soluble TNF (Chen and Goeddel, 2002). Clustering and trimerization of TNF receptors by TNF recruits the death domain containing adaptor protein TRADD to the death domain of TNF-R, which in turn mediates the binding of TNF receptor associated factor (TRAF2) and the death domain containing RIP, a serine/threonine kinase (Devin et al., 2003; Hsu et al., 1996). Subsequently, the TRAF-2/TRADD/RIP complex (complex I) recruits a stable IKK (IkappaB kinase) complex containing IKKα, IKKβ and a regulatory protein NEMO, where activated IKK complex phosphorylates and targets IκB for proteosome-mediated degradation (Hsu et al., 1995). This finally results in the release of NF-κB and allows it to translocate to the nucleus where it can activate genes involved in different cellular functions. Complex I also mediates the TNF induced p38 MAPK (Mitogen activated protein kinase) and JNK (c-Jun-N-terminal kinase) activation, which again results in the activation of gene transcription via the c-Jun-Fos transcription factor complex (Lee et al., 1997; Tournier et al., 2001). Meanwhile, as a second step of the TNF/TNFR pathway, TRADD and RIP1 associate via their death domains and subsequently associate with FADD and caspase-8 forming a cytoplasmic complex II.

Caspase-8 gets activated via the complex II formation and released into the cytosol to mediate the downstream caspase activation followed by apoptosis (Aggarwal, 2003). When NF-κB is activated by complex I, complex II harbors the caspase-8 inhibitor $FLIP_L$ and the cell survives. Otherwise, cells undergo apoptosis through the complex II mediated signaling pathway. Thus, TNF/TNFR-mediated signal transduction includes a checkpoint, resulting in cell death in instances where the initial signal (complex I) fails to be activated (Micheau and Tschopp, 2003). TNF receptor is also able to mediate apoptosis through the recruitment of an adaptor molecule called RAIDD (RIP-associated ICH-1/CED-3 homologous protein with a death domain), which recruits caspase-2 via an interaction motif similar to death effector domain called CARD (caspase recruitment domain). Recruitment of caspase-2 leads to apoptosis (Duan and Dixit, 1997).

1.3.3 The TRAIL pathway

Five distinct TRAIL receptors have been identified: death receptor 4 (DR4/TRAIL-R1), DR5 (TRAIL-R2), DcR1 (TRAIL-R3), DcR2 (TRAIL-R4) and Osteoprotegrin, which elicit either pro-death signaling (TRAIL-R1 and R2) or death inhibitory signaling (TRAIL-R3, R4 and osteoprotegrin) (Emery et al., 1998; Pan et al., 1997a; Pan et al., 1997b; Sheridan et al., 1997; Walczak et al., 1997). All the TRAIL receptors share high sequence homology in their extracellular domains, but differ significantly in their intracellular domains. TRAIL-R1 and TRAIL-R2 contain a C-terminal DED that signals downstream activation of caspases but, TRAIL-R3 and TRAIL-R4 (decoy receptors) lack a functional cytoplasmic death domain. The apoptotic signaling induced by TRAIL is similar to that induced by CD95. Binding of TRAIL to its receptors DR4 or DR5 triggers the formation of the DISC by recruiting FADD, caspase-8 and caspase-10, followed by activation of

caspase-8 and -3 and rapid apoptosis in various cancer cell types (Ashkenazi, 2002). TRAIL induced apoptosis may also involve the activation of the mitochondrial death pathway, similar to CD95 induced cell death in type II cells. It has been reported that the release of Smac/Diablo from mitochondria to block the caspase inhibitory effect of IAPs is required for TRAIL induced cell death (Deng et al., 2002). The effect of Bcl-2 family members on TRAIL induced cell death varies with cell type, as the overexpression of Bcl-2 or Bcl-xL does not block TRAIL induced cell death in lymphoid cells, but significantly inhibits apoptosis in human prostate and lung cancer cells (Keogh et al., 2000; Munshi et al., 2001). In cells dependent on mitochondria for cell death, inhibition of the death pathway may change the sensitivity to TRAIL induced apoptosis. For instance, HCT-116 Bax-null cells are completely resistant to TRAIL induced cell death (Ozoren and El-Deiry, 2002). TRAIL also activates NF-κB under certain circumstances via recruiting the adaptors RIP and TRAF2 and utilizes this activation in the expression of pro-apoptotic genes during the cell death process (Lin et al., 2000). The ability of TRAIL to induce apoptosis in a wide variety of cancer cell lines, while leaving little toxicity towards many types of normal cells, suggests that this molecule may be an ideal agent for cancer therapy (Buchsbaum et al., 2006) (further discussed in section 1.11.2). The high expression of decoy receptors in normal cells, but not in tumor cells was originally proposed as a major mechanism resulting in differential effects of TRAIL. The decoy receptors are highly homologous to the extracellular domains of DR4 and DR5 but either lack the transmembrane domain (DcR1) or have a truncated non-functional death domain (DcR2), thus defective in transmitting the apoptotic signal and act in a dominant negative manner to TRAIL (Ashkenazi and Dixit, 1998). Several other factors have been implicated in selective

toxicity of TRAIL, including the expression levels of caspase-8, caspase-10, FLIP, IAPs, the status of other signaling pathways such as the Akt pathway, NF-κB or c-Myc pathways and aberrant methylation and silencing of tumor suppressor genes, but none of these have been widely accepted (Almasan and Ashkenazi, 2003).

1.4 Mitochondrial (Intrinsic) death pathway

The intrinsic pathway of apoptosis is activated in response to cell death signals such as chemotherapy drugs, DNA damage, and growth factor withdrawal (Reed, 2000). These diverse apoptotic stimuli activate intracellular signals that converge at the level of mitochondria, resulting in the loss of mitochondrial membrane potential ($\Delta\Psi m$) and the release of pro-apoptotic molecules such as cytochrome c, apoptosis inducing factor (AIF), Endonuclease G, Omi/HtrA2 and Smac/Diablo (Green and Reed, 1998). Once cytochrome c is released from the mitochondria, it forms a high molecular weight pro-apoptotic protein complex in the cytosol called the apoptosome. This complex, which contains oligomerized Apaf-1, pro-caspase-9, cytochrome c and dATP, triggers the activation of pro-caspase-9 to caspase-9 (Cohen, 1997; Jin and El-Deiry, 2005). The interaction between Apaf-1 and pro-caspase-9 is mediated by the so-called caspase recruitment domains present in both the proteins, and serves as yet another example of the importance of homotypic protein module interactions during apoptosis signaling. The activated caspase-9 proteolytically activates the downstream effector caspases like caspase-3, -6 and -7 (Los and Walczak, 2002; Slee et al., 1999). *In vivo* studies with mouse embryonic cells derived from Apaf-1, caspase-9 and caspase-3 null mice have shown resistance to various apoptotic stimuli (Los et al., 1999; Yoshida et al., 1998). The second group of proteins released from mitochondria during apoptosis is IAP antagonists including Smac/Diablo and Omi/HtrA2.

Smac/Diablo selectively binds to IAPs (the inhibitory proteins of caspases) via its IAP-binding motif and promotes their auto-ubiquitination and consequent degradation. On the other hand Omi/HtrA2, a serine protease can proteloytically cleave and inactivate the IAP proteins, presumably acting as an efficient IAP suppressor (van Loo et al., 2002; Verhagen and Vaux, 2002). Recent data, however, has shown that the cells from Omi/HtrA2 transgenic mice with a mutation in a non-catalytic serine residue were more sensitive to apoptosis induced by Etoposide, Tunicamycin and hydrogen peroxide (Jones et al., 2003). These conflicting studies may suggest that Omi/HtrA2 may have dual roles, where it promotes cell death during apoptotic conditions, but maintains normal mitochondrial functions in living cells in a similar fashion to cytochrome c.

In addition to proteins that trigger or mediate caspase activation, mitochondria also release pro-apoptotic proteins such as AIF and Endonuclease G that are unrelated to caspase activation. AIF, a flavoprotein, once released to the cytosol translocates to the nucleus and induces peripheral chromatin condensation and large-scale DNA fragmentation (Joza et al., 2001). Endonuclease G, an endonuclease released from the mitochondria also translocates to the nucleus and generates oligonucleosomal DNA fragmentation (Li et al., 2001). In addition to caspase independent cell death, AIF also has other functions in cell survival, as recent studies have suggested its role in maintenance of glutathione levels in conditions of cellular stress and oxidative phosphorylation in mitochondria via its oxidoreductase properties (Cande et al., 2004).

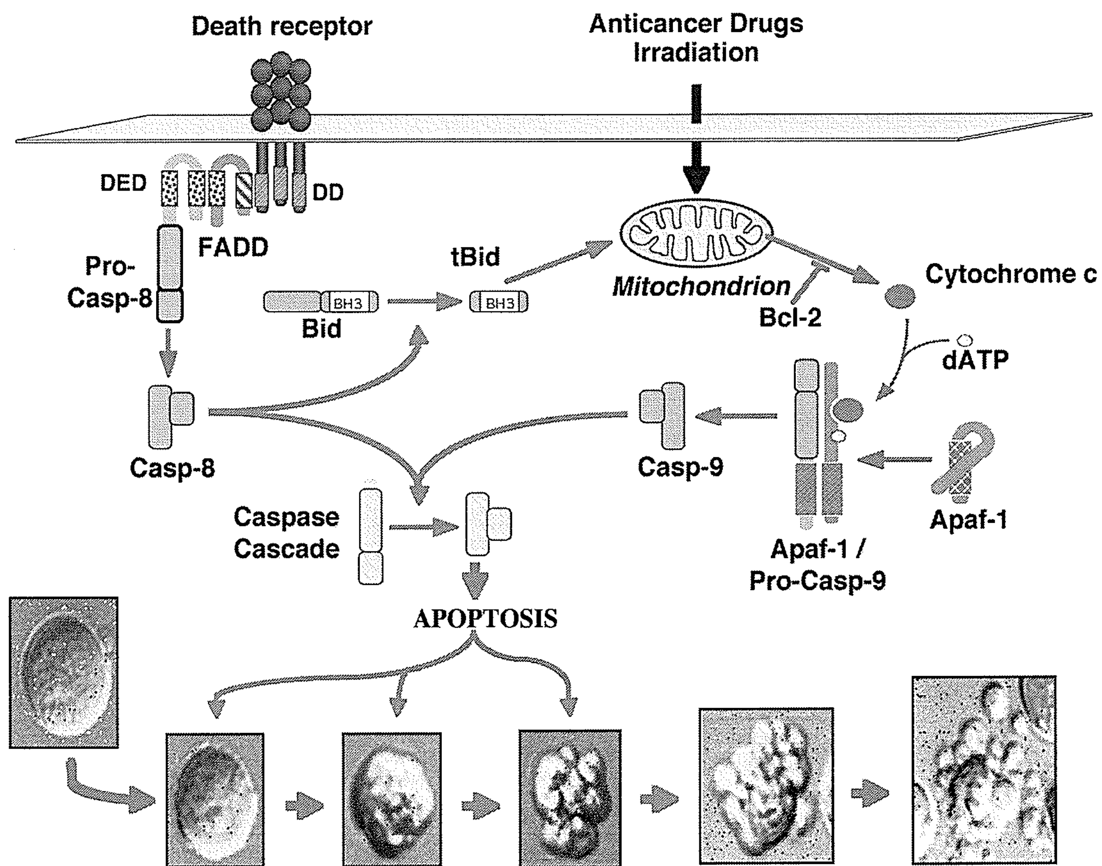


Figure 1: The death receptor (extrinsic) and the mitochondrial (instrinsic) death pathways during apoptosis [Adapted and modified from (Los et al., 1999)]. The details of the two distinct pathways are provided in the text.

1.5 Bcl-2 family proteins - Regulators of cell survival and cell death

1.5.1 Classification of Bcl-2 family

The Bcl-2 family proteins constitute a critical checkpoint in the intrinsic pathway of apoptosis as they regulate mitochondrial outer membrane permeabilization. Bcl-2, a 26 kDa protein that is mainly localized in mitochondria, endoplasmic reticulum and perinuclear membranes was originally identified as an oncogene involved in human follicular lymphoma of B cell origin (Bakhshi et al., 1985; Tsujimoto et al., 1984). It was demonstrated to have an anti-apoptotic activity to various apoptotic stimuli such as IL-3 deprivation, serum deprivation, heat shock and chemotherapeutics (Strasser et al., 1991; Tsujimoto, 2003). Bcl-2 also has the ability to inhibit certain forms of necrotic cell death (Kane et al., 1995; Shimizu et al., 1996). About 20 Bcl-2 family members have been identified in mammals, which contain at least one of the four conserved motifs known as Bcl-2 homology domains (BH1-BH4) (Adams and Cory, 1998). Based on their structure and function, Bcl-2 family members are mainly grouped into three sub-families: (1) The Bcl-2 sub family (Bcl-2, Bcl-xL, Mcl-1 and Bcl-w) members contain at least BH1 and BH2 domains, and those most similar to Bcl-2 have all four BH domains. This subfamily of Bcl-2 proteins contains mainly the pro-survival members, meaning that, in general, they oppose the activation of the mitochondrial death pathway. (2) The Bax subfamily (Bax, Bak and Bok) members contain BH1, BH2 and BH3 domains and are very similar in structure to the Bcl-2 subfamily. They even share a similar 3D confirmation featuring a globular bundle of α-helices with a hydrophobic surface groove: Functionally, however, they oppose the action of anti-apoptotic Bcl-2 subfamily members, and thus act as pro-apoptotic molecules by activating mitochondrial permeabilization. (3) The BH3-only

protein sub family (such as Bik, BNIP3, Bim, Bad, Bid, Blk and Hrk) members, which are pro-apoptotic molecules possess only the central short (9-16 amino acids) BH3-domain that is necessary and probably sufficient to induce apoptosis. In addition most of the Bcl-2 family members except Bad, A1 and Bid contain a C-terminal transmembrane domain which is required for their membrane anchoring to the outer mitochondrial membrane or other cellular organelles such as the endoplasmic reticulum and nucleus (Reed, 1997).

1.5.2 Mechanisms of apoptosis regulation by Bcl-2 family members

Anti- and pro-apoptotic Bcl-2 family members prevent and activate the cell death process respectively by mainly regulating the release of mitochondrial cytochrome c and other pro-apoptotic factors. One of the striking features of Bcl-2 family proteins is their ability to form homodimers and heterodimers (Cory and Adams, 2002; Oltvai et al., 1993). The heterodimerization mediated by the insertion of BH3 region of pro-apoptotic protein member into a hydrophobic pocket composed of BH1, BH2 and BH3 from an anti-apoptotic protein is considered to inhibit the biological activity of their partners (Sattler et al., 1997). Oligopeptides corresponding to the BH3 region of Bax and Bak are able to induce apoptotic cell death. Interestingly, the replacement of the BH3 region of Bcl-2 by the BH3 region of Bax converts Bcl-2 from an anti-apoptotic molecule to a pro-apoptotic molecule (Hunter and Parslow, 1996). Bcl-2 and Bcl-xL prevent the apoptosis-associated release of cytochrome c, AIF and other pro-apoptotogenic factors by binding either directly to pro-apoptotic Bcl-2 members like Bax and Bad or even cytochrome c and Apaf-1 (Adachi et al., 1997; Hu et al., 1998; Yang et al., 1997a). Thus the ratio of anti- to pro-apoptotic Bcl-2 family members constitutes a rheostat that sets the cell's threshold of susceptibility to the apoptotic program (Korsmeyer et al., 1993). Among other

mechanisms, death signals activate some BH-3 only members which in turn either induce direct oligomerization of pro-apoptotic Bcl-2 proteins like Bax and Bak to insert into the outer mitochondrial membrane or can act indirectly to release the pro-apoptotic proteins from the anti-apoptotic Bcl-2 proteins that sequester them (Desagher et al., 1999; Zha et al., 1997; Zhao et al., 2003). Bax is predominantly a cytosolic monomer in healthy cells, but during apoptosis it undergoes a conformational change, translocates to the outer mitochondrial membrane and oligomerizes. Bak is present as a monomer in the mitochondrial membrane in healthy cells, but changes conformation and forms larger aggregates during apoptosis (Antonsson and Martinou, 2000). The pro-apoptotic Bcl-2 proteins engage either permeability transition (PT) pore dependent or independent mechanisms for mitochondrial outer membrane permeabilization to release the apoptogenic factors (Marzo et al., 1998; Shimizu et al., 1999). During PT-dependent permeabilization, apoptotic signals open the PT pore composed of cyclophilin D, peripheral benzodiazepene receptor, ANT protein in the inner mitochondrial membrane and VDAC (voltage dependent anionic channel) in the outer mitochondrial membrane via direct binding with pro-apoptotic Bcl-2 members like Bax and Bik. Opening the PT pore causes ion flux in and out of the mitochondria from the cytosol and thus results in the loss of mitochondrial membrane potential, which further causes the rupturing of the outer mitochondrial membrane to release the proteins of the mitochondrial intermembrane space. In PT independent mitochondrial membrane permeabilization, the pro-apoptotic Bcl-2 proteins like Bax and Bak directly form the channels in the outer mitochondrial membranes by inserting themselves in the membranes thus causes the release of

mitochondrial pro-apoptotic proteins into the cytosol (Antonsson and Martinou, 2000; Jurgensmeier et al., 1998).

1.5.3 Regulation of Bcl-2 family members

The regulation of Bcl-2 family members occurs both at the transcriptional and post-translational levels. Some of the examples of transcriptional regulation of Bcl-2 family members include the regulation of Bax and Bad by different transcription factors. p53 transcriptionally upregulates Bax, whereas NF-κB upregulates Bad, and favours the pro-cell death process (Baetz et al., 2005; Chipuk et al., 2004; Grimm et al., 2005; Lowe et al., 1993). In contrast, NF-κB upregulates anti-apoptotic Bcl-2 family members such as Bcl-2, Bcl-xL, Bfl1/A1 and Nrl3 in the process of mediating its cell survival function under certain conditions (Perkins and Gilmore, 2006). Additionally, many recent studies support a transcriptional and post-translational independent regulation of Bcl-2 family members. For instance, p53 translocates from nucleus to mitochondria where it binds and inhibits Bcl-2/Bcl-xL function or binds to Bax and activates its function (Chipuk and Green, 2004). Among the mechanisms of post-translational regulation, phosphorylation plays a crucial role in regulating Bcl-2 members. Phosphorylation of Bcl-2 at serine 70 residue activates, but the phosphorylation of its several loop sites at residues serine 56, threonine 74 and serine 87 by JNK and related kinases inhibits its anti-apoptotic activity (Deng et al., 2001). Akt phosphorylates Bad and via 14-3-3 proteins sequesters it away from the mitochondria, which precludes its inhibition of Bcl-xL, thus leading to cell survival (Datta et al., 1997).

1.6 PI3-Kinase/Akt survival pathway

The phosphatidylinositol 3` - kinase (PI3-K)/Akt signaling pathway is activated by many types of cellular stimuli like growth factors and hormones thus regulating fundamental cellular functions such as proliferation, growth, survival, transcription, translation, cell cycle and apoptosis (Cantley, 2002; Vanhaesebroeck and Alessi, 2000). The PI3K/Akt signaling pathway is frequently disrupted in human cancers and plays a major role not only in tumor growth but also in the potential response of tumors to cancer treatment (Vivanco and Sawyers, 2002). PI3-Ks are a family of lipid kinases defined by their ability to phosphorylate the 3′-OH group of the inositol ring in phosphotidyl inositols.

Phosphotidyl inositols, a family of phospholipids, despite representing only a small percentage of total cellular lipids, play a crucial role in signal transduction as the precursors of several second messenger molecules (Divecha and Irvine, 1995; Shi et al., 2006). Five free hydroxyl groups are present on the inositol head, which has the potential to get phosphorylated by different PI3-Kinases. To date, the following phosphoinositides have been identified in cells: PtdIns-3-phosphate (PtdIns-3-P), PtdIns-4-phosphate (PtdIns-4-P), PtdIns-5-phosphate (PtdIns-5-P), PtdIns-3,4-bisphosphate (PtdIns-3,4-P_2), PtdIns-3,5-bisphosphate (PtdIns-3,5-P_2), PtdIns-4,5-bisphosphate (PtdIns-4,5-P_2) and PtdIns-3,4,5-triphosphate (PtdIns-3,4,5-P_3). Three families of phosphoinositide kinases were characterized to date: phosphoinositide 3- kinases (PI3-Ks), phosphoinositide 4- kinases (PI4-Ks) and PtdIns-P (PIP) kinases (PIP5Ks) (Fruman et al., 1998). PI3-Ks are well studied and characterized compared to the others and thus will form the major focus of further description in this work.

PI3-Ks are further classified into 3 classes: Class I PI3-Ks, Class II PI3-Ks and Class III PI-3Ks (Figure 2).

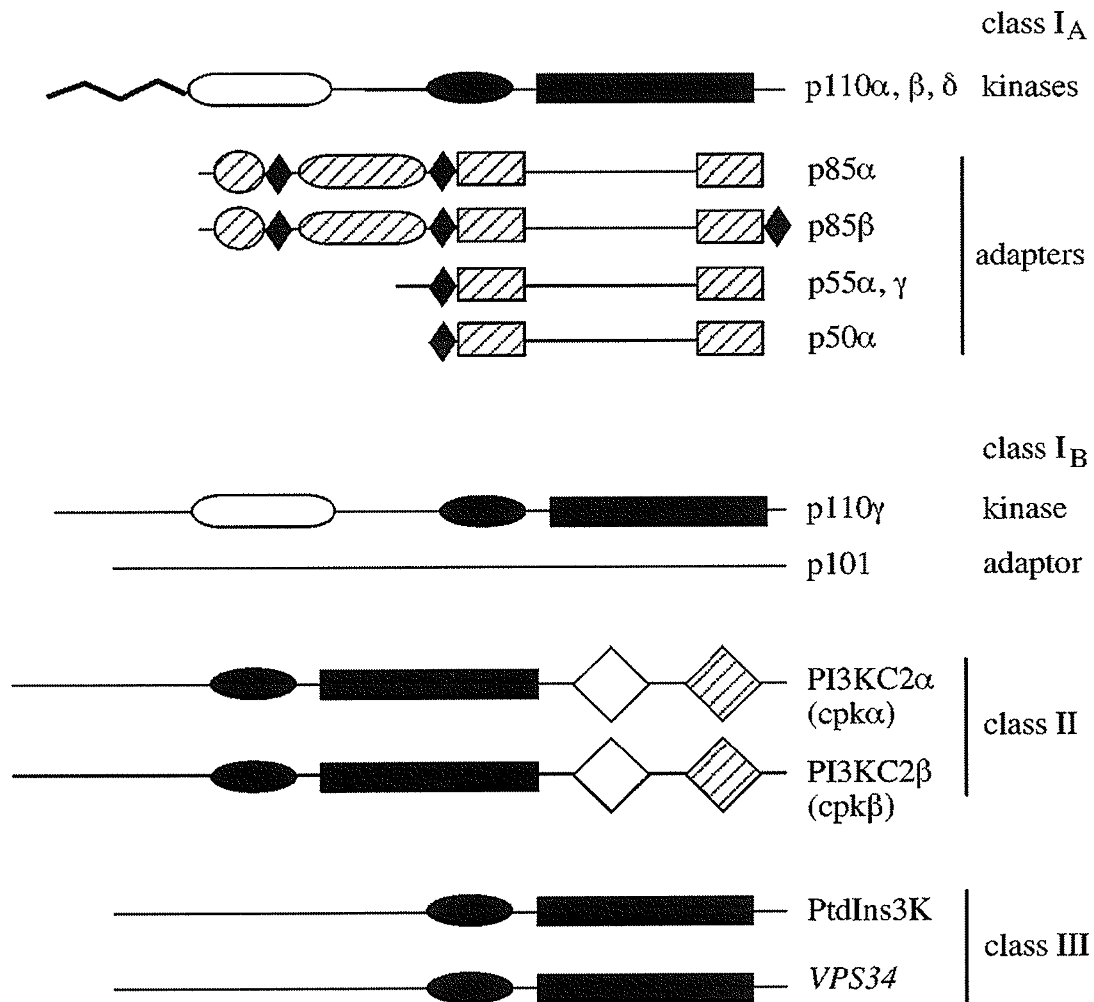


Figure 2: Structure and classification of PI3-Kinases [adapated and modified from (Fruman et al., 1998)]. The protein domains shown are as follows: catalytic domain (solid rectangle), PIK-domain (solid oval), ras-binding domain (open oval), rhoGAP-homology domain (hatched oval), SH2 domain (hatched rectangle), PX domain (open diamond), proline-rich motifs (solid diamonds), SH3 domains (hatched circle), and C2 domain (hatched diamond). Further details are provided in the text.

1.6.1 Class I PI3-Kinases

Class I PI3-Ks are heterodimers composed of a catalytic subunit (p110) and an adapter/regulatory subunit (p85). Three mammalian p110 subunits grouped into Class I_A PI3-Ks (p110α, p110β and p110δ) have been cloned that share nearly 50 % sequence homology. Each of these p110 proteins consists of an N-terminal p85 interaction domain, a Ras binding domain, a PIK domain and a C-terminal catalytic domain (Hiles et al., 1992; Hu et al., 1993; Vanhaesebroeck et al., 1997). The p85 regulatory subunit has no enzymatic activity, but binds to the p85 interaction domain of p110 and thus regulates its function. Two p85 isoforms (p85α and p85β) have been cloned (Escobedo et al., 1991; Otsu et al., 1991). Both p85 isoforms contain and N-terminal src-homology 3 (SH3) domain, two or three proline rich sequences, a BCR homology domain, two src homology-2 (SH2) domains and an inter-SH2 (iSH2) domain that interacts with the p110 subunit. The p85 gene has different splice variants, three of them, p50α, p55α and p55γ have a unique 6-30 amino acid N-terminus and share the C-terminus of p85 including the second proline rich domain and the SH2 domains (Vanhaesebroeck and Alessi, 2000).

Class I_B PI3-K has a p110γ catalytic subunit that shares nearly 40% identity to Class I_A but diverges from the N-terminal p85-binding domain of Class I_A enzymes, and thus does not interact with the p85 proteins. p101 acts a regulatory subunit of p110γ, but has no homology to other regulatory proteins (Stephens et al., 1994; Stephens et al., 1997; Stoyanov et al., 1995).

1.6.1.1 Activation and Regulation of Class I PI3-Kinases

Class I PI3-Ks phosphorylate PtdIns, PtdIns-4-P, PtdIns-5-P and PtdIns 4,5-P2 on the free 3-OH position *in vitro* but *in vivo* they have only shown to selectively increase the levels of PtdIns-3,4,5-P_3 (PIP3) by phosphorylating PtdIns 3,4-P_2 (PIP2) (Stephens et al., 1991). The activation of PI3-Ks occurs via two different mechanisms, a classical pathway involving the receptor protein tyrosine kinase dimerization, autophosphorylation and the other involving the intracellular non-receptor tyrosine kinases (classical pathway shown in figure 3). While receptor protein tyrosine kinases activate Class I_A PI3-Ks, the heterotrimeric G-protein coupled receptors activate the Class I_B PI3-Ks (Stephens et al., 1994; Stephens et al., 1997; Stoyanov et al., 1995; Vivanco and Sawyers, 2002). Several receptor tyrosine kinase families including but not limited to epidermal growth factor receptor, insulin receptor and platelet-derived growth factor receptor activated by their respective ligands have been shown to be responsible for the downstream PI3-K activation. The regulatory p85 subunit directly associates with the active tyrosine kinases via the physical interaction of its SH2 domains selectively with the phosphotyrosine residues in the YXXM motif of the receptor protein tyrosine kinases (Cantley, 2002). Under some circumstances, the kinase and the p85 interaction also occurs indirectly via intermediate phosphoproteins. For instance, the insulin receptor substrates IRS1 and IRS2 bind to the RPTKs (receptor protein tyrosine kinases) with their SH2 domains, which will further interact with the p85 domain via their phosphotyrosine motifs (White, 1998). PI3-K activity is tightly regulated in normal cells by different mechanisms. In a resting cell, the p110 catalytic activity is inhibited by the interaction with its partner subunit p85, which acts as an inhibitory molecule, but once the upstream kinases are activated, the binding of

p85 with the pY residues relieves the inhibition effect on p110 by p85 due to the changes in the conformation of p85 subunit. Interestingly, the catalytic activity of PI3-K requires the stable binding of the p85 subunit to p110 subunit, but in an activatory mode (Cuevas et al., 2001; Yu et al., 1998). Though the interaction of the SH2 domains with the tyrosine-phosphorylated proteins is universally accepted to be responsible for PI3-K activation, there are other studies that reported the activation of PI3-K via alternate mechanisms. For example, the conformational switch within the p85-p110 holoenzymes also occurs via the interactions of SH3 domain/proline rich sequences, BCR-homology domain/GTP loaded adaptor proteins and others. The p85 proline rich sequence interaction with the SH3 domains of src family kinases including src itself, lck, lyn and Fyn or SH3 domain of cytoplasmic tyrosine kinase abl are all known to activate the PI3-Kinase. The members of the Ras super family like Rac and cdc42 also bind to the p85 rho-GAP homology region (also called a breakpoint cluster [BCR] region homology domain) and this interaction is shown to stabilize and activate the p85/p110 complex (Liu et al., 1993; Pleiman et al., 1994; Prasad et al., 1993; Zheng et al., 1994). The BCR homology domain and the SH3 domain were assigned to have a negative regulatory effect on the catalytic activity of PI3-K, as the p55α and p50α, which lack these domains, were more efficient than p85 in activating p110 (Inukai et al., 1997). It has also been shown that Ras activated by various extracellular stimuli regulates the activity of class IA PI3-Ks by directly interacting with the p110 catalytic subunit in a GTP dependent manner via the Ras effector site. The increase in PI3-K activity both *in vitro* and *in vivo* by Ras-GTP is inhibited by the expression of dominant negative Ras mutant (Rodriguez-Viciana et al., 1994).

1.6.2 Class II PI3-Kinases

Class II PI3-Ks identified by sequence homology and PCR cloning are large (170-200 kDa) proteins that contain a PIK domain and a catalytic domain, but are not well characterized functionally. Three members (PI3K-C2α, PI3K-C2β and PI3K-C2γ) have been identified in this class of PI3-Ks. Class II PI3-Ks are not so far known to be associated with any regulatory subunit, but each of these proteins has a C-terminal tandem PX domain (similar to NADPH oxidase associated proteins phox-40 and phox-47), C2 domain (similar to PKC isoforms) and an N-terminal domainless region (Domin et al., 1997; MacDougall et al., 1995). Class II PI3-Ks are mainly associated with membrane structures, including plasma membrane, nuclear membrane and other intracellular membranes unlike class I PI3-Ks, which are mainly cytoplasmic in the resting state and membrane localized when activated (Brown and Shepherd, 2001). Several extracellular signals including growth factors and chemokines have been shown to activate the class-II PI3-K activity, but no clear mechanism has been established. Class-II PI3-Ks phosphorylate PtdIns and PtdIns-4-P, but not PtdIns-4,5-P_2 (class I substrate). The *in vivo* function of Class-II PI3-Ks has not yet been established, though there are some recent reports emerging to suggest that this class of PI3-Ks is required for ATP-dependent priming of neurosecretory granule exocytosis (Meunier et al., 2005).

1.6.3 Class III PI3-Kinases

Class III PI3-Kinases are represented by a sole member, VPS34, that was originally identified in a yeast screen for mutants conditionally defective in vacuolar protein sorting (Volinia et al., 1995). Unlike class I and class II PI3-Ks, VPS34 phosphorylates only PtdIns but not the other phosphotidyl inositol molecules such as PtdIns-4-P, PtdIns-5-P and

PtdIns-4,5-P_2. A 150 kDa regulatory subunit (VPS15 in yeast and PI3KR4 in humans), which has an intrinsic protein serine kinase activity, has been shown to be associated with the VPS34 and is required for its function. Class III PI3-K functions have been implicated in endosome fusion during intracellular trafficking events and are localized mainly in the intracellular membranes. Several recent studies have assigned a functional role for class III PI3-Ks in several intracellular processes including the process of autophagy, phagosome formation, retrograde endosome to Golgi transport and transport at the nuclear membrane (Burda et al., 2002; Kihara et al., 2001; Roggo et al., 2002; Vieira et al., 2001).

1.6.4 Negative regulators of PI3-Kinase

PTEN (phosphatase and tensin homolog deleted on chromosome 10), also known as MMAC1 (mutated in multiple advanced cancers) is a dual specificity phosphatase that has both lipid and protein substrates (Steck et al., 1997). PTEN is considered to be the second most mutated gene in different cancers (Ali et al., 1999). In addition, PTEN germline mutations are associated with disorders like Cowden disease, Bannayan-Zonana syndrome and Lhermitte Duclos disease in which unsystematic hamartomas appear in multiple organs (Pilarski and Eng, 2004). PTEN has two major structural domains, the C2 domain (the lipid binding domain) and the phosphatase domain. In addition, there is a putative PtdIns (4,5) P_2 binding domain at the N-terminus and a PDZ ligand binding consensus motif at the C-terminus (Lee et al., 1999b). PTEN converts PtdIns-3,4,5-P_3 to PtdIns 4,5-P_2 and acts as a negative regulator of the PI3K pathway (Stambolic et al., 1998). In addition, PTEN was reported to dephosphorylate itself and other proteins like focal adhesion kinase and the platelet derived growth factor receptor, but the biological effects of the protein dephosphorylation have yet to be clearly established (Gu et al., 1998;

Raftopoulou et al., 2004; Tamura et al., 1998). The identification of PTEN mutants, which are defective in either lipid phosphatase activity or both lipid and protein phosphatase activities, made it possible to delineate the importance of these mutations in regulating the different aspects of tumor growth. For instance, the PTEN C124S mutation found in many tumors inactivates both the lipid and protein phoshphatase activities. Expression of this mutant in PTEN null cancer cells does not cause growth arrest as the wild type PTEN, indicating that catalytic activity is required for this effect. PTEN G129E mutation in the catalytic domain is another commonly found mutation in Cowens syndrome that has no lipid phosphatase activity, but has an intact protein phosphatase activity indicating that the loss of lipid phosphatase activity is sufficient to cause the cancer phenotype (Vivanco and Sawyers, 2002).

SHIP1 and SHIP2 (SH2 containing inositol-5-phosphatase 1 and 2) also negatively regulate the production of PIP3, but are not as effective as PTEN in regulating the PI3-Kinase pathway. These enzymes are unique phosphatases that remove the phosphate from the 5-position of PIP3 (rather than 3 position as PTEN) thus resulting in the reduction of the lipid secondary messengers involved in calcium signaling, cell migration, cell proliferation and apoptosis (Liu et al., 1999). The activation of many cytokine receptors, as well as B and T cell receptors recruits SHIP1 and SHIP2 to the tyrosine phosphorylated Shc and its associated Grb2-Sos1 complexes (Helgason et al., 2000). SHIP1 contains an N-terminal SH2 domain, two NPXY motifs interacting with PTB domains and a C-terminal Pro-rich region (PxxP motifs) with consensus sites for SH3 domain interactions. Compared to SHIP1, SHIP2 contains a single NPXY site and a C-terminal sterile alpha motif (SAM) domain (Backers et al., 2003). The SAM domain is a widespread domain in signaling

present in many proteins, some receptors (Eph-related tyrosine kinases) and in nuclear proteins. In intact cells, SHIP1 is involved in the control of PtdIns (3,4,5) P_3 levels upon BCR aggregation (Brauweiler et al., 2000) and upon stem cell factor stimulation in mast cells (Scheid et al., 2002). PtdIns (3,4,5) P_3 levels were also affected in SHIP2 transfected cell models (Blero et al., 2001; Taylor et al., 2000; Giuriato et al., 2002). SHIP1 and SHIP2 are quite different enzymes from the tumor suppressor PTEN (Maehama and Dixon, 1998). PTEN-/- mice have a stronger tumor phenotype compared to SHIP1-/- or SHIP2 -/- mice and thus PTEN may have a more critical role than SHIP1 and SHIP2 in cell growth and development (Stambolic et al., 1998). PTEN plays a role in suppressing the PI3 K/ Akt pathway. Depending on the cell type, this inhibitory effect on PKB is not always seen for SHIP1 and SHIP2, e.g., in myeloid cells (Giallourakis et al., 2000) or in tumors from terminal B lymphocytes (Choi et al., 2002). The data could be the consequence of different roles of PTEN and SHIPs reaction products PtdIns (4,5) P_2 and PtdIns (3,4) P_2, respectively. Therefore, the biochemistry of SHIPs is do not always affect PKB/Akt activity.

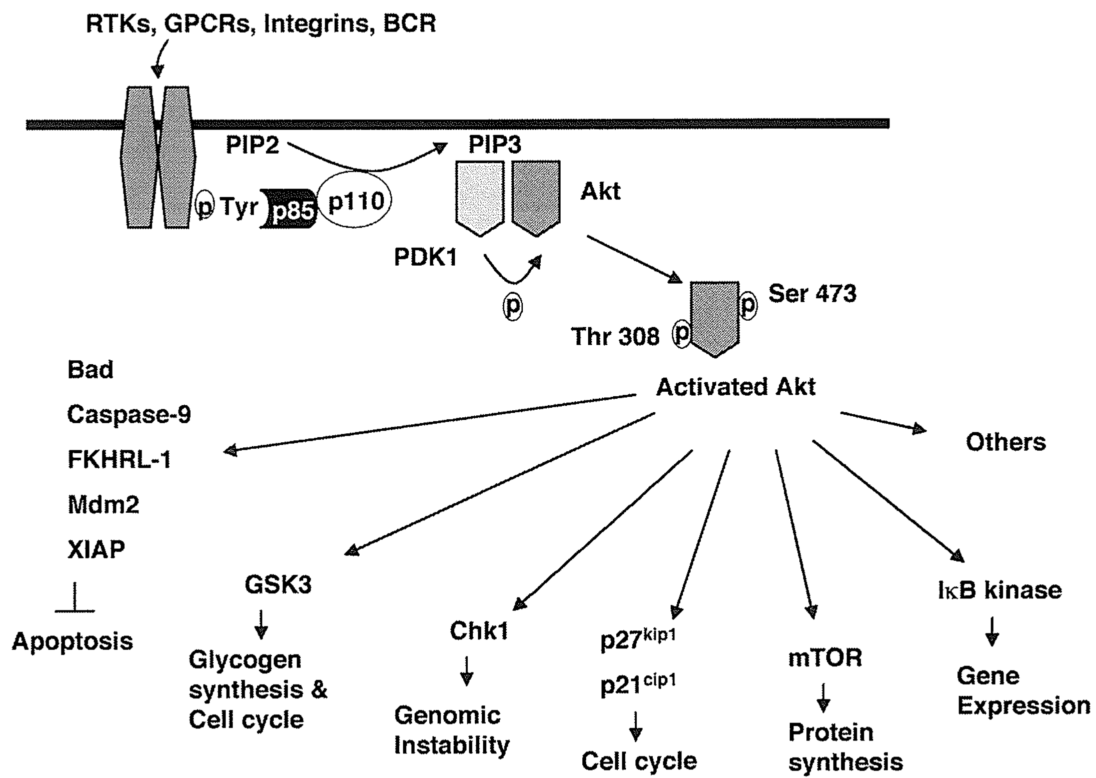


Figure 3: Schematic representation of the PI3-Kinase/Akt pathway. Details of the pathway are provided in the text.

1.6.5 Akt (Protein kinase B): The central mediator of PI3-K signaling

Akt/PKB is a 57 kDa serine/threonine protein kinase and a cellular homologue of the viral onco-protein v-Akt. It belongs to the family of AGC kinases with a high homology to the protein kinases A and C. PKB was first cloned by three independent research groups in 1991, after more than a decade since the original identification of the transforming murine leukemia virus (Coffer and Woodgett, 1991; Jones et al., 1991; Staal, 1987; Staal et al., 1977). The mammalian PKB/Akt subfamily consists of three related genes encoding the isoforms PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3. PKBβ and PKBγ share nearly 80% homology with PKBα and all the isoforms show a broad tissue distribution (with PKBα and PKBβ highest expression in brain, heart, thymus and lung, and PKBγ high in brain and testis but low in heart, lung, spleen and skeletal muscle (Coffer and Woodgett, 1991; Jones et al., 1991). The structure of all the mammalian isoforms contains three functional domains: an N-terminal pleckstrin homology (PH) domain, a central kinase domain and a C-terminal regulatory domain containing the hydrophobic motif except that the γ isoform is devoid of 23 residues on the C-terminus. Two specific sites, one in the kinase domain (Thr 308 residue) and other in the C-terminal regulatory motif (Ser 473 residue) get phosphorylated during the full activation of these kinases. Sequence analysis of Akt and v-Akt revealed that the viral gene is a fusion between a truncated tripartite viral group-specific antigen gag (p12, p15 and Δp30) and Akt. These two domains are joined by a 21 amino acid long peptide encoded by the sequences from the 5′ untranslated regions of Akt and three extra nucleotides at the recombination break point. Other than mammals, Akt homologues have been identified in *Drosophila* (DAkt-1),

Dictyostelium and *C.elegans.* No homologues for Akt have yet been reported for plants and fission yeast (Coffer et al., 1998).

1.6.5.1 PDK1

PDK1 (3`-phosphoinositide dependent kinase 1), also a member of AGC family of kinases is a 63 kDa serine/threonine kinase widely expressed in human tissues (Alessi et al., 1997b). It contains an N-terminal kinase domain and a C-terminal PH domain. The PH domain of PDK1 binds to PtdIns (3,4,5) P_3 with a higher affinity than Akt. PH domain mutants of PDK1 that do not interact with phosphorylated lipids are completely cytosolic, suggesting that the membrane association and further activation of PDK1 is entirely dependent on the functional PH domains. PDK1 was first identified by its ability to phosphorylate Akt at Thr 308 residue *in vitro* and this activity is again dependent on PtdIns (3,4,5) P_3. Overexpression of PDK1 in cells was sufficient to partially activate Akt and mutation of the PH domain in either PH domain of Akt or PDK1 significantly reduce the Akt activation (Alessi et al., 1997a; Stephens et al., 1998).

1.6.5.2 PDK2

The identity of the kinase phosphorylating Akt at Ser 473 residue (presumably PDK2) is unknown yet. Some studies have suggested that PDK1 itself phosphorylates the Ser 473 residue and others suggested that Akt autophosphorylates Ser 473 residue dependent on the Thr 308 phosphorylation. Later it has been shown that the phosphorylation status of Ser 473 residue is unaffected in cells overexpressing PDK1, thus ruling out the possibility of PDK1 role and the autonomous phosphorylation (Coffer et al., 1998). Several kinases have been assigned the role of PDK2 by different groups (Vanhaesebroeck and Alessi, 2000). Mitogen-activated protein kinase-activated protein

(MAPKAP)-kinase-2 phosphorylates Akt at Ser 473 residue *in vitro*, but is unlikely to phosphorylate physiologically as the agents that activate MAPKAP kinase-2, like TNF-α and IL-1 do not induce Akt phosphorylation at Ser 473 site (Alessi et al., 1996). Also, a variety of Akt agonists such as insulin, PDGF and IGF-1 do not significantly activate MAPKAP kinase-2 thus ruling out its role as PDK2. Also, integrin-linked kinase (ILK) has been claimed to be a possible PDK2 as it phosphorylates Akt Ser 473 residue *in vitro* and in over expressed cells (Delcommenne et al., 1998). This hypothesis has been discounted by other studies which showed that ILK indirectly regulates Akt activity (Lynch et al., 1999).

1.6.5.3 Regulation of PKB/Akt activity

In resting cells, PKB/Akt exists in an inactive state and is localized in the cytosol. The Akt activity enhanced by the stimulation of cells with either various growth factors, insulin, G-protein coupled signaling or integrin signaling is a complex process (Andjelkovic et al., 1997; Welch et al., 1998). Several studies have proposed various mechanisms of Akt activation, but here we present the model of Akt activation that is widely studied and accepted. Upon activation of the upstream PI3-K by various ligands, PtdIns (3,4,5) P_3 is synthesized at the plasma membrane and then Akt interacts with these lipids via its PH domain. This interaction mediates the anchoring of Akt at the plasma membrane, where the conformational change of Akt occurs resulting in the exposure of its Thr 308 and Ser 473 phosphorylation sites. PDK1, which is already membrane localized by virtue of its PH domain bound to either the basal levels of PtdIns (3,4,5) P_3/PtdIns (3,4) P_2 or the phosphorylated lipids produced by PI3-K, phosphorylates Akt at the exposed sites. The fully phosphorylated and activated Akt then translocates either to cytosol or the

nucleus and acts on its various downstream targets (Cuevas et al., 2001; Fruman et al., 1998).

Though controversial, in addition to the PI3-K dependent mechanism, several reports have suggested Akt activation independent of PI3-K activity. Konishi et al, reported that Akt is activated by heat shock in 3T3 fibroblasts and this activation is not inhibited by PI3-K inhibitors (Konishi et al., 1996). Also, the agonists that increase Ca2+ levels in cells have been reported to activate Akt in PI3-K independent manner through the Ca2+/calmodulin-dependent protein kinase kinase (Filippa et al., 1999).

1.6.6 Physiological functions of Akt - Role in cell survival and apoptosis

Akt has been estimated to have nearly 9000 potential substrates in the cell and modulates different biological functions both in the cytoplasm and the nucleus (Coffer et al., 1998). We discuss here mainly those substrates related to cell survival, cell cycle and apoptosis. The additional important Akt targets in other cellular processes such as cell growth, protein synthesis and glucose metabolism (Fig.3) are listed in the Table 1. The minimum sequence motif required for efficient phosphorylation of small peptide substrates by Akt is RXRXXS/T, where X is any amino acid. An important function of activated PI3 K/Akt in cells is maintaining the cell survival via inhibition of apoptosis. Akt regulates the process of cell survival by phosphorylating different substrates that either directly or indirectly regulate the apoptotic program. Some of the important targets for Akt during this process involve the phosphorylation of BAD (a pro-apoptotic Bcl-2 family member), caspase-9 (a pro-cell death protease), FKHRL1 (a fork head transcription factor), IKK, Mdm2 and cyclic AMP response binding protein (CREB). Akt mediated phosphorylation of BAD at serine 136 promotes cell survival by inhibiting its interaction with the anti-

apoptotic Bcl-2 family members like Bcl-2 itself and Bcl-xL, further preventing the cytochrome c release (Datta et al., 1997). Akt also phosphorylates Caspase-9 at serine 196 residue thus causing a conformational change and further leading to the inhibition of its proteolytic activity (Cardone et al., 1998). Akt also indirectly regulates apoptosis by phosphorylating a forkhead transcription factor FKHRL1. FKHRL1 is a member of the Forkhead transcription factor family involved in regulating apoptosis and the cell cycle by transcriptionally upregulating either pro-apoptotic genes like FasL, Bim, IGFBP1, GADD45 and Puma or cell cycle regulators like $p27^{kip1}$, cyclin B, cyclin D1, cyclin G2 and PLK1. The phosphorylation at Threonine 32, serine 253 and serine 315 by Akt results in the redistribution of FKHRL1 to the cytoplasm, thus inhibiting nuclear transcriptional activity (Brunet et al., 1999; Guo et al., 1999; Kops et al., 1999; You et al., 2006). In addition, Akt promotes cell survival by activation of NF-κB via phosphorylating IKKα, which in turn mediates the degradation of IκB, an NF-KB inhibitor (Kane et al., 1999; Romashkova and Makarov, 1999). This leads to further elevation in transcription of NF-KB dependent survival genes (Bcl-xL, Bcl-2, c-IAPs, c-FLIP) (Catz and Johnson, 2001; Lee et al., 1999a; Wang et al., 1998). Akt mediated phosphorylation of CREB also enhances cell survival by increasing transcription of pro-survival genes like Bcl-2, Mcl-1 and Akt itself (Pugazhenthi et al., 2000; Reusch and Klemm, 2002; Wang et al., 1999a). Further, Akt also negatively regulates apoptosis by enhancing the degradation of p53, via phosphorylation, enhanced nuclear localization and p53 binding of Mdm2, a negative regulator of p53 (Mayo and Donner, 2001).

Table 1: Partial list of potential Akt substrates

Cell survival targets

- Bad
- Caspase-9
- Mdm2
- TSC1/TSC2
- XIAP
- ASK1
- IKK
- Cot1
- CREB
- E2F
- Yap
- Androgen receptor
- SGK

Cell cycle targets

- GSK3β
- $p27^{kip1}$
- $p21^{cip1}$
- $p70^{S6k}$

Protein synthesis & cell growth

- Raf1
- mTOR
- $p70^{S6k}$
- GSK3β

Genomic instability

- BRCA1
- Chk1

Transcription factors

- FKHRL1
- DAF-16
- Androgen receptor
- BF-1/c-qin
- GATA1/2/3
- HIFβ
- FosB
- MATH1
- GADD151/CHOP

Glucose metabolism

- GSK3β
- Glut4
- PFK2

Synaptic signaling & Neurodegeneration pathway

- GABA
- Huntingtin (Htt)
- Ataxin

Others

- eNOS
- PDE-3B
- PKC-α
- PKC-ς
- JIP1
- RAC1/CDC42
- $p47^{phox}$ and Other proteins

1.6.7 Pro-cell death function of PI3-K/Akt pathway

Although many studies support the positive role of the PI3-K/Akt pathway in promoting cell proliferation and cell survival, there are several exceptions, where the PI3-K pathway is also involved during promotion of cell death pathways. The activation of PI3-K/Akt pathway is shown to be required for the induction of apoptosis in some selected systems under certain apoptotic stimuli. The PI3-K/Akt pathway has been shown to have a positive role in CD95 (Fas) mediated cell death in epidermal C141 cells (Lu et al., 2006). Inhibition of PI3-K/Akt pathway by dominant negative overexpression of PI3-K (Δp85) and Akt (Akt T308A/S473A) protects the epidermal cells from apoptosis, indicating an unexpected pro-apoptotic role of PI3-K/Akt pathway in the Fas signaling process. The binding of Fas receptor with its ligand induces the tyrosine phosphorylation and activates the PI3-K/Akt pathway, which is required for Fas induced cell death. The death promoting influence of PI3-K/Akt pathway is also shown in studies with Arsenite toxicity and TNF-α induced cell death along with caveolin-1. The pre-treatment of cells with Wortmannin, a PI3-K inhibitor or the transfection with Akt dominant negative vector severely affected the cell death induced by arsenite and TNF-α (Ono et al., 2004; Shack et al., 2003). In addition, the cytokine activation of PI3-Kinase sensitized the cells to cisplatin induced cell death in hematopoietic cells (Nimbalkar et al., 2003). In other studies, the treatment of cells with LY294002, a potent PI3-K inhibitor inhibited the activation of p53 by several DNA damage inducing anti-cancer drugs such as cisplatin, 5-flourouracil and camptothecin (a topoisomerase inhibitor) (Bar et al., 2005). The LY294002 mediated attenuation of p53 induction by DNA damage drugs and subsequent inhibition of chemotherapy-induced apoptosis suggest a clear positive role of PI3-K/Akt pathway

during apoptosis. Using H9c2 cells derived from rat cardiomyocytes, PI3-K activation is also shown to accelerate the autophagic cell death during glucose deprivation (Aki et al., 2003) and necrotic cell death during hypoxia (Aki et al., 2001). In both studies, the pretreatment of cells with LY294002 or the transfection of dominant negative PI3-K severely reduced the percentage of cell death. Recently, using cytokine dependent bone marrow derived Ba/F3 cells, in which Akt activation can be specifically induced by the addition of 4-hydroxytamoxifen (4-OHT), van Gorp et al. have shown that the constitutive activation of Akt results in apoptosis whereas the transient activation protects the cells from cell death. The constitutive Akt activation results in increased oxidative stress due to elevated levels of reactive oxygen species and thus results in the enhanced expression of Foxo3a at both the transcriptional and protein levels leading to higher expression of its transcriptional targets $p27^{kip1}$ and Bim (van Gorp et al., 2006).

1.6.8 Role of PKB/Akt in G1/S progression

In addition to its regulatory role in cell survival, the PI3-K/Akt pathway has been attributed to have a key role in cell cycle progression. The PI3-K pathway is activated during two phases of the cell cycle, at first during early G1 phase and the second wave of PI3-K activity is during late S phase (Jones et al., 1999). Many studies have demonstrated the functional significance of PI3-K activation during G1/S transition, but the significance of the second wave of activation is not completely known. During G1/S phase transition, PI3-Kinase/Akt pathway has multiple substrates involving but not limited to cyclin D, Myc, $p27^{kip1}$ and $p21^{waf1}$. Akt regulates the level of cyclin D1 and Myc proteins by stabilizing them against proteosome degradation. GSK3β phosphorylates cyclin D1 at threonine 286 (Diehl et al., 1998) and Myc at threonine 58 (Gregory et al., 2003), which

promotes their degradation via ubiquitin mediated degradation along with their cytoplasmic relocalization. Thus, Akt by phosphorylating and inactivating its substrate GSK3β, prevents the degradation and cytoplasmic relocalization of cyclin D1 and Myc, which in turn facilitates the G1/S progression. Akt also regulates the G1/S transition by controlling the cell cycle inhibitors $p27^{kip1}$ and $p21^{waf1}$ at their transcriptional and the post-translational levels. Akt enhances the proteosome dependent degradation of $p27^{kip1}$ by upregulating SKP2 mRNA levels. SKP2 is a key component of the SCF/SKP2 ubiquitin ligase that mediates $p27^{kip1}$ degradation in a cyclin E/CDK2 dependent phosphorylation (Hara et al., 2001; Mamillapalli et al., 2001; Pagano et al., 1995). Previously it was shown that Akt mediated phosphorylation of $p27^{kip1}$ at Thr 157 also causes the relocalization of $p27^{kip1}$ to the cytoplasm, thus relieving the nuclear substrates from $p27^{kip1}$ inhibition, which subsequently enhances cell cycle progression (Shin et al., 2002). It was also reported that $p27^{kip1}$ is regulated at the transcriptional level by Akt activation. Akt phosphorylates FKHRL1 and inactivates its transcriptional activity, thus leading to the downregulation of $p27^{kip1}$ at the mRNA level (Medema et al., 2000). Akt phosphorylates another cell cycle inhibitor $p21^{waf1}$ at two different residues Thr145 and Ser 146. The Thr145 residue phosphorylation by Akt results in the cytoplasmic localization of $p21^{waf1}$ and thus promotes the cell cycle (Zhou et al., 2001), whereas the Ser 146 site phosphorylation enhances the stability of the protein and further increases the assembly of cyclin D-CDK4 G1/S transition complex (Li et al., 2002). Recently, we also observed the regulation of $p21^{waf1}$ at the transcription level by Akt in a FKHRL1 dependent manner (Maddika S and Los M., unpublished results).

1.6.9 Role of PKB/Akt in G2/M progression

Although, the PI3-K/Akt pathway is mostly reported to be required for G1/S progression, there are a few studies suggesting a role for this pathway in G2/M progression, though the mechanisms are not completely understood (Shtivelman et al., 2002). Inhibition of Akt activation by LY294002 was shown to delay the G2/M progression in Rat-1 cells released from an Aphidicolin mediated S-phase blockade. In addition, MDCK cells synchronized by double thymidine block released from S phase, when added with PI3-K inhibitor led to G2 arrest and further apoptosis (Shtivelman et al., 2002). Also, it was shown that Akt activation could overcome a G2/M cell cycle checkpoint induced by DNA damage (Kandel et al., 2002). In this study, the transfection of constitutively active Akt abolished the cdc2 inhibition and the G2 arrest induced by exposure of Rat1a cells to γ-irradiation or 6-thioguanine, a DNA damaging agent. Recently, it also has been shown that Akt phosphorylates human Chk1 kinase and leads to its inactivation (Shtivelman et al., 2002). Thus, inactivation of Chk1 by constitutively active Akt would impair the Chk1 mediated Cdc25C phosphorylation/inactivation, which in turn, activates Cdc2 resulting in overcoming the DNA damage induced G2/M cell cycle arrest. It should be noted that although PI3-K/Akt activity might be important for G2/M progression, it later should be transiently inactivated for the mitotic exit as the constitutive activation of this pathway leads to G2/M cell cycle arrest in an FKHRL1/cyclinB/PLK dependent manner (Alvarez et al., 2001).

1.6.10 PI3-K/Akt pathway & Cancer

There is now a growing body of evidence, which comes from different biochemical and genetic studies that strongly suggests the role of PI3-Kinase/Akt pathway in various cancers (reviewed in; Vivanco and Sawyers, 2002). Here we list some of the important aspects of the deregulated PI3-K/Akt pathway components in cancer. The gene encoding the p110α catalytic subunit of PI3-K is amplified and overexpressed in some groups of ovarian and cervical cancers (Shayesteh et al., 1999). Also, overexpression of the downstream kinase Akt/PKB has been reported in several breast, ovarian and pancreatic cancers (Bellacosa et al., 1995; Ruggeri et al., 1998). In addition, some primary human colon and ovarian cancers have mutations in the p85α regulatory subunit, which leads to PI3-K activation (Philp et al., 2001). These structural alterations presumably release the p85-p110 complex from negative regulation, bypassing the normal role of RTPK signaling in PI3-K activation. Activating mutations in various RTKs such as EGFR and ErbB2 in breast, ovarian and lung cancers themselves provide additional, although indirect evidence for the important role of PI3-K/Akt pathway in human cancers (Moscatello et al., 1998). As stated earlier, the most compelling evidence for the role of PI3-K/Akt pathway comes from the fact that PTEN, a negative regulator of this pathway is frequently mutated in various types of cancers in particular glioblastomas, prostate and endometrial cancers (Ali et al., 1999). The ultimate proof of the involvement of PI3-K/Akt pathway in malignancy comes from mouse genetic models. The transgenic mice expressing a constitutively active p110α catalytic subunit in the heart results in bigger hearts, whereas the dominant negative p110α gives a smaller heart (Shioi et al., 2000). Very similar phenotype is seen with the constitutively active Akt and dominant negative Akt. Interestingly, specific expression of

activated Akt in T-cells and pancreas increases the cell survival, but in both cases no malignancy is seen (Jones et al., 2000; Tuttle et al., 2001). Mice with a constitutively activated p85 regulatory subunit develop a lymphoproliferative disorder, but progresses to lymphoma only when crossed with p53- knock-out mice (Borlado et al., 2000). Also, mice that express Akt under the control of MMTV-LTR promoter have a delay in postpartum mammary gland involution, but do not develop any mammary tumors (Hutchinson et al., 2004). In further support of this hypothesis, the retroviral transfer of activated Akt and Ras together, but not either gene alone, into the glial progenitor cells in mouse brain produced glioblastomas (Holland et al., 2000). Thus, it can be concluded that the PI3-K/Akt pathway may contribute to malignancies, but is insufficient alone to cause cancers.

1.7 An Introduction to the Cell Cycle

The cell cycle is a universal, complex process involved in the growth and proliferation of cells, as well as in the regulation of DNA damage repair, tissue hyperplasia as a response to tissue injury, organism development and diseases such as cancer (Schafer, 1998). Cell cycle transition is an ordered, tightly regulated process that involves multiple checkpoints that assess extracellular growth signals, cell size and DNA integrity. The molecular machinery of CDK (cyclin dependent kinase) and cyclin complexes tightly controls the progression of the mammalian cell cycle through the G1, S, G2 and M phases. During G1 (Gap 1) phase, the cell prepares itself for DNA synthesis to follow in the S phase by stimulating the early response genes and protein synthesis. The S phase is characterized by DNA duplication, which is followed by G2 (Gap 2) phase, where the cells prepares for Mitosis or M phase. When the cells cease proliferation, due either to the absence of proper mitogenic signals or to specific anti-mitogenic signals, then they exit the cell cycle and enter a non-dividing, quiescent state known as G0 phase (schematically shown in figure 4).

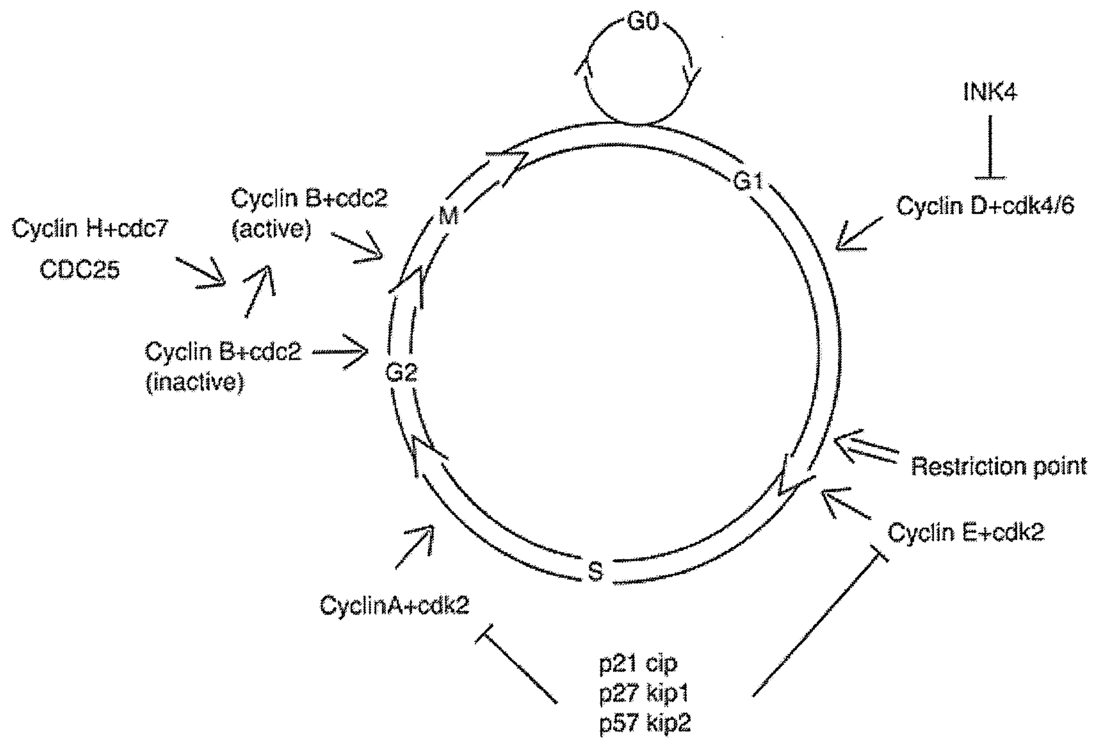


Figure 4: Diagrammatic representation of different phases of the mammalian cell cycle and its regulators

Adapted from http://www.brc.riken.jp/lab/dna/en/GENESETBANK/cell_cycle.png

1.7.1 Cell cycle regulation by CDKs and cyclins

The CDK family of serine/threonine kinases forms the central active regulatory role during cell cycle progression via binding to their cyclin partners (Morgan, 1997). In yeast a single CDK (Cdc28 in the budding yeast *Saccharomyces cerevisiae* and Cdc2 in the fission yeast *Schizosaccharomyces pombe*) is able to regulate the diverse cell cycle transitions at least in part by associating with multiple stage-specific cyclins (Stern and Nurse, 1996). The discovery of nearly 10 Cdc-2 related proteins in vertebrates makes the eukaryotic cell cycle a complex process involving different combinations of CDKs and cyclins and various mechanisms of regulation. However, only CDK1, CDK2, CDK4, CDK6 and CDK7 play a central role in the mammalian cell cycle (Morgan, 1997). CDK4/6 are involved in G1 phase whereas CDK2 and CDK1 are necessary for G1/S phase and G2/M phase transition respectively. CDK7 in complex with cyclin H acts as a CDK activation kinase (CAK) and mediates the activating phosphorylation of CDK1 and CDK2. In addition, the CDK7/cyclin H complex is associated with the general transcription factor TFIIH, where it phosphorylates the C-terminal domain of RNA polymerase II, thus regulating transcription. Other CDKS such as CDK5 and CDK9 have roles outside the cell cycle (reviewed in Kaldis and Aleem, 2005; Vermeulen et al., 2003). CDKs are regulated by different mechanisms during each phase of the cell cycle (Morgan, 1995). The oscillating concentrations of cyclin subunits and their specific binding to specific CDKs underlie the stage-specific timing of CDK activity. The three D type cyclins (Cyclin D1, D2 and D3) bind to CDK4 and CDK6 and are essential for entry into G1 phase (Bartek et al., 1996; Sherr, 1996). Unlike the other cyclins, D type cyclins are not expressed periodically, but are synthesized as long as growth factor stimulation persists. Cyclin E is

associated during G_1 to S phase transition and activates CDK2. Cyclin A gets activated during the S phase transition and binds to CDK1 and CDK2 (Pardee, 1989). In late G2, cyclin A switches from the CDK2 complex to CDK1 complex. B type cyclins are present during G_2 exit and mitosis phase and are associated with CDK1 (Coqueret, 2003). Sixteen cyclins have been identified so far but, like CDKs, not all of them are involved in the cell cycle process. Cyclins C, cyclin G and cyclin T associate with CDK8, CDK5 and CDK9 respectively and function outside the cell cycle progression (Johnson and Walker, 1999). The oscillation of cyclin levels during specific stages of the cell cycle is maintained by their timely *de novo* synthesis and their degradation via ubiquitin mediated proteolysis (Nakayama and Nakayama, 2006).

In addition to cyclin binding, most CDKs (in particular CDK1 and CDK2) require phosphorylation at the conserved residue in the T-loop that stabilizes the neighboring interactions of both CDKs and cyclins (Thr 160 in human CDK2, Thr 161 in human CDK1) and allows them to achieve full kinase activity. CAK (CDK activating kinase), the enzyme responsible for phosphorylating CDKs, has been identified in several organisms (Drapkin et al., 1996; Lolli and Johnson, 2005). The major candidate for higher eukaryotic CAK is the CDK7-cyclin H-Mat1 complex, whose activity is not rate limited and regulated during cell cycle (Nigg, 1996). But, CDK phosphorylation by CDK7 at the activating threonine, which is cyclin dependent, tends to parallel cyclin levels *in vivo.* In addition to the activating phosphorylations, CDKs are also regulated by inhibitory phosphorylations. The Wee1 and Myt1 kinases phosphorylate CDK1 and CDK2 at tyrosine-15 and/or threonine-14, thereby changing the conformation and inactivating the CDKs. Dephosphorylation at tyrosine-15 and threonine-14 by Cdc25 is necessary for the

activation of CDK1 and further progression through the cell cycle. Interestingly, the mitotic CDK1 phosphorylates Wee1 and Cdc25 indicating a positive feedback regulation mechanism (Hoffmann et al., 1993; Lew and Kornbluth, 1996; Mueller et al., 1995).

The intracellular localization of different cell cyle regulatory proteins also contributes to proper cell cycle progression. Cyclins control the function of CDKs by regulating their subcellular localization during specific stages of the cell cycle via their nuclear localization and nuclear export signals, which CDKs lack. For example, cyclin E and A bind to and import CDK2 into the nucleus during G1/S phase. Cyclin B contains an active nuclear export sequence (NES) and is excluded from the nucleus until the beginning of prophase during mitosis. However, the mechanisms of CDK/cyclin complex nucleo-cytoplasmic shuttling during specific stages of the cell cycle are not clearly known. The 14-3-3 groups of adaptor proteins regulate the intracellular trafficking of different proteins involved in cell cycle progression. During certain stages of interphase, cdc25 is kept in the cytoplasm via the interaction with 14-3-3 proteins and thus mediates the inactivation of CDKs at the proper time by the Wee1 and Myt1 kinases. 14-3-3 proteins also mediate sequestration of CDK1-cyclin B complex in the cytoplasm following DNA damage (Obaya and Sedivy, 2002).

CDKs regulate cell cycle progression by phosphorylating different downstream substrates during different phases of the cell cycle. In early G1, CDKs phosphorylate retinoblastoma (pRB) and thus lead to the disruption of interactions of RB-E2F-HDAC and RB-DP1-HDAC complexes, which occur in inactive state. The disruption of the RB complexes releases active E2F and DP1 transcription factors, which then positively regulates the transcription of the genes that are required for the G1/S progression,

including cyclin E, cyclin A and cdc25. CDK2/cyclin E complex maintains the pRB in the hyperphosphorylated state through the remainder of the cell cycle (Kato et al., 1993; Weinberg, 1995). In addition, the G1 CDKs phosphorylate the other retinoblastoma protein family members such as p107 and p130 and regulates their cell cycle inhibitory function. During G1/S progression, CDK2/cyclin E also phosphorylates its inhibitor $p27^{kip1}$ and thus induces its proteosome-dependent degradation. CDK2/cyclin E phosphorylates histone H1 and regulates chromosome condensation, which is required during DNA replication. CDK1/cyclin B also phosphorylates histone H1. Cyclin A bound CDK2 also regulates the initiation of DNA replication by phosphorylating DNA polymerase alpha primase and the components of the ORC complex. In G2 and M phases, the CDKs phosphorylate the cytoskeletal proteins such as nuclear lamins, tubulin and vimentin, which are required for the correct mitosis. Also CDK1 in G2/M phase phosphorylates its own regulators Wee1 and Cdc25, the phosphorylation of which is required for the proper G2/M progression. Other than the above-mentioned targets, there are several other targets for CDK that either regulate cell cycle progression or cell cycle check point activation (John et al., 2001).

1.7.2 Regulation of CDKs by CDK inhibitors

The cell cycle inhibitory proteins, called CDK inhibitors, which bind to CDK alone or CDK/cyclin complexes, can also regulate the activity of CDKs (Lee and Yang, 2001). Two distinct families of CDK inhibitors have been discovered; the INK4 family and the Cip/Kip family. The INK4 family includes p15 (INK4a), p16 (INK4b), p18 (INK4c) and p19 (INK4d), which specifically inactivate the G1 CDKs (CDK4 and CDK6). This INK4 family of CKIs forms stable complexes with the CDK enzyme before cyclin binding, preventing association with cyclin D and blocking the transition from G_1 to the S phase

(Carnero and Hannon, 1998). Moreover, disruption of p16$^{Ink\ 4a}$-mediated CDK4/6-cyclinD binding cancels the Rb-E2F mitogenic signal transduction and cells remain in G_1 phase (Sherr, 2001). The second inhibitory protein from the INK family, p15$^{Ink\ 4b}$ regulates the cell cycle clock by inhibiting the CDK4/6-cyclin D mediated phosphorylation of Rb. The p15$^{Ink\ 4b}$-triggered G_1-phase arrest occurs in response to TGF-β (Hannon and Beach, 1994; Reynisdottir et al., 1995). The other members of INK4 class, p18$^{Ink\ 4c}$ and p19$^{Ink\ 4d}$ are expressed during fetal development and seem to play a key role in terminal differentiation (Phelps et al., 1998; Zindy et al., 1997). The expression of these inhibitors increases during the G_1/S transition, mimicking G_1 arrest caused by pharmacologic CDK inhibition (Hirai et al., 1995). The p18$^{Ink\ 4c}$ inhibitor plays a key role in growth control and the fact that the gene encoded for this protein is widely expressed in variety of tissues supports this hypothesis. It has been suggested that loss of p18 function results in shortening the G_1 phase and driving the cell cycle machinery.

The second family of CKIs, the Cip/Kip family, includes p21 (waf1/cip1), p27 (kip1/cip2) and p57 (Kip2). This family of CKIs inhibit the G1-S CDK/cyclin complexes, in particular the CDK2 complexes and to a lesser extent the CDK1/cyclin B complexes (Mainprize et al., 2001). This family of CKIs function through competing for cyclin binding on the CDKs and thus prevents the CDKs to phosphorylate their downstream substrates during the cell cycle progression. The p21$^{Cip1/Waf1}$ is a member of the Kip/Cip, broad specificity class of CDKIs. This inhibitor was identified simultaneously by independent research groups (el-Deiry et al., 1993; Harper et al., 1993), and named as Waf1 (wild-type p53-activated fragment 1) and Cip1 (CDK-interacting protein 1) . As the name indicates, the p21$^{Cip1/Waf1}$ is under the control of the p53 tumor suppressor, which

drive faulty cells into apoptosis. The main role of $p21^{Cip1/Waf1}$ in cell cycle regulation lies in the ability to inhibit the activity of cyclin A/CDK2 and cyclin E/CDK2 required for G_1/S transition and therefore induces G_1 arrest. The second member of Cip/Kip family of CDKIs, $p27^{Kip1}$ inhibits cyclin E/CDK2 and regulates G_1/S transition (Toyoshima and Hunter, 1994). This inhibition occurs in cells arrested by TGF-β or cell-cell contact. The activity of $p27^{Kip1}$ is regulated by its fluctuations during the cell cycle as the concentration of $p27^{Kip1}$ decreases in response to mitogen stimulation and increases when mitogens are withdrawn (Sherr, 1996). The degradation of $p27^{Kip1}$ mediated by interleukin-2 is required for cell cycle entry (Nourse et al., 1994). $p57^{Kip2}$, another member of the Kip/Cip family of CDK inhibitors, plays a major role in embryonic development and its loss leads to developmental disorders (Yan et al., 1997). Reduction in expression of this inhibitor correlates with tumorigenesis in laryngeal mucosa, which can serve as a good marker for diagnosis. The implication of $p57^{Kip2}$ in the control of cell cycle is not clear. $p21^{waf1}$, but not p27 or p57, also binds and inhibits the proliferating cell nuclear antigen (PCNA), a subunit of DNA polymerase δ and thus regulates DNA replication and different types of DNA repair including nucleotide excision repair, mismatch repair and base excision repair (Li et al., 1994).

1.7.3 Cell cycle regulators and cancer

In recent years it has become apparent that tumorigenesis is frequently associated with mutations or abnormalities in the expression of several cell cycle regulatory proteins such as cyclins, CDKs and CKIs in various types of human cancers (Sherr, 1996).

1.7.3.1 Cyclins in cancer

In the early 90's, a cyclin D1-parathyroid hormone gene (PRAD1) fusion was identified in a human parathyroid adenoma, which gave the first clue that cyclins might be directly involved in human cancers (Motokura et al., 1991). Later a cyclin D1 gene translocation t(11:14) resulting in the overexpression of cyclin D1-immunoglobulin heavy chain fusion protein was found associated with B-cell malignancies. Cyclin D1 gene amplification was found in breast, lung, bladder, esophageal and squamous cell carcinomas (Hunter and Pines, 1994). Cyclin D2 and cyclin D3 also have been reported to be overexpressed in cases of breast and lung tumors (Leach et al., 1993). Cyclin E has been found to be amplified, overexpressed or both in the cases of breast and colon cancers as well as in acute lymphoblastic and acute myeloid leukemias (Iida et al., 1997). Both cyclin E and cyclin A were found overexpressed in lung carcinomas and elevated expression of cyclin A, but not cyclin E correlated with low survival (Dobashi et al., 1998).

1.7.3.2 Alterations in CDKs and CDK activating enzymes in cancer

The involvement of CDKs and CDK regulators have been reported in various cancers, but to a lower degree as compared to other cell cycle proteins. CDK4 overexpression has been reported in some tumor cell lines and primary melanomas, sarcomas and gliomas (Wolfel et al., 1995). CDK1 and CDK2 have been reported to be overexpressed in a subset of colon adenomas (Yamamoto et al., 1998). The Cdc25A and Cdc25B genes of the Cdc25 family, proposed to be oncogenes, co-operate with mutated H-ras, activated Myc and pRb loss during the transformation process (Nilsson and Hoffmann, 2000; Vermeulen et al., 2003). Cdc25B is overexpressed in nearly 30% of breast cancers

and other studies have found higher expression of Cdc25B in various cancer cell lines including the cells transformed by SV40 large T-antigen.

1.7.3.3 CDK inhibitors and cancer

The CKIs, by inhibiting CDK/cyclin complexes, result in growth suppression through activation of pRB and this is responsible for their tumor suppressor function. The p16 gene deletions, point mutations and hypermethylation-mediated inactivation were reported in a high percentage of tumors and cancer cell lines (Kamb et al., 1994). Deletions of p16 have been reported in approximately 50% of gliomas, 40-60% of pancreatic and bilary tract tumors and nearly 30% of acute lymphoblastic leukemias (reviewed in Vermeulen et al., 2003). Large deletion of ARF-INK4 locus, which codes for p16 and p19 proteins, and the simultaneous deletion of the p15 gene on chromosome 9 deregulate p53 function (Harper and Elledge 1996). No mutations in the $p27^{kip1}$ gene have been identified, however reduced $p27^{kip1}$ expression was reported in lung, breast and bladder carcinomas and is correlated with poor prognosis and tumor aggressiveness. Paradoxically, a high expression of $p27^{kip1}$ is found in the series of human esophageal, breast and colon cancer cell lines and small-cell lung carcinomas, despite their high degree of malignancy (Park and Lee, 2003). Alterations in p18 and p21, such as gene deletions and mutations in breast tumors and leukemias have also been reported (reviewed in Vermeulen et al., 2003).

1.7.3.4 Other cell cycle proteins in cancer

Among other regulators of the cell cycle, frequent alterations in various cancers have been associated with p53, pRB and Mdm2 proteins. p53 mutations have been identified in as many as 50% malignancies and is known to be the most frequently mutated gene in human cancers (Miller and Koeffler, 1993). The point and missense mutations in

p53 lead to conformational changes and inactivation of the protein. $p53^{-/-}$ mice develop normally, but are predisposed to a variety of spontaneous tumors, predominately lymphosarcomas, osteosarcomas and pulmonary adenocarcinomas (Donehower et al., 1992). Overexpression by gene amplification and enhanced mRNA translation of Mdm-2, a negative regulator of p53, has been reported in leukemia, lymphomas, breast carcinomas, sarcoma and glioma, which may represent an alternative mechanism to p53 mutations in human cancers (Bueso-Ramos et al., 1996; Moller et al., 1999). Similar to p53 tumor suppressor, pRB was altered in many cancers. Deletions and missense mutations that result in a truncated, nonfunctional pRb or complete lack of detectable pRb have been reported in human neoplasias (Hall and Peters, 1996). The loss of pRb function leads to uncontrolled E2F mediated transcription, which results in the loss of G1/S restriction point and further mediates uncontrolled proliferation during the development of human cancers.

1.8 Cell cycle proteins and apoptosis

In addition to the role of cell cycle proteins in the regulation of cell cycle progression, many studies have suggested a role for these proteins in the regulation of the apoptotic program. Several cell cycle regulators including, but not limited to p53, pRB, E2F family of transcription factors, Myc family of transcription factors, CDKs, CKIs and cyclins have been assigned a dual role in the regulation of cell cycle and apoptosis. Here, we discuss only the role of relevant molecules pertaining to this thesis, specifically; we will discuss the roles of CDKs, cyclins and CKIs in apoptosis.

1.8.1 Cyclin Dependent Kinases and apoptosis

Although CDKs are well known for their role in regulating the cell cycle, several studies using a variety of experimental approaches (including the genetic mutants, siRNA and chemical inhibitors of protein kinase activity) have suggested that CDKs may participate in a subset of apoptotic programs (Golsteyn, 2005). The initial evidence for the role of CDKs came from experiments in which enhanced CDK1 activity associated with cyclin A complexes was observed in YAC lymphoma cells that were committed to enter apoptosis after treatment with death inducing compounds like perforin and fragmentin-2 (Shi et al., 1996). Later, the enhanced CDK1 activity irrespective of the cell cycle phase and its requirement for the apoptotic process in Jurkat cells has been reported during Granzyme B and Camptothecin induced cell death (Borgne and Golsteyn, 2003; Shimizu et al., 1995). Further evidence for the role of CDKs came from the studies showing the increase in the CDK2 activity in HeLa cells treated with staurosporine. Using a CDK2 dominant negative mutant CDK2 D145N, these studies have shown a substantial decrease in sensitivity to staurosporine-induced apoptosis in the absence of CDK2 activity (Harvey et al 2000). Dominant negative forms of other CDKs including CDK1, CDK3, CDK4, CDK5 and CDK6 block apoptosis in other experimental models of cell death by proteosomal inhibitors and c-AMP (Golsteyn, 2005). In non-cycling G_1 CD4+CD8+ thymocytes, during exposure to apoptotic stimuli such as Dexamethasone, heat-shock, γ-irradiation, or Fas/CD95 cross-linking, CDK2 activity was found to be increased indicating its role in thymocyte selection (Hakem et al., 1999). In addition, using CDK specific inhibitors like Roscovitine, Olomoucine and Iso-olomoucine, other studies have shown the role of CDK1 and CDK2 during CD437 induced cell death in both p53 negative Hep3B

and p53 wild-type HepG2 human hepatoma cell lines (Hsu et al., 1999). Several other studies have pinpointed the activation and requirement of specific CDK complexes during apoptosis induced by various agents like TGF-β, DNA damaging agents like Camptothecin, and viral protein E4orf4 (Golsteyn, 2005).

1.8.2 Cyclins and apoptosis

In addition to CDKs, their cyclin partners are also been implicated in the apoptotic process. Several recent studies have revealed a positive role of cyclin E during apoptosis in hematopoietic cells (Mazumder et al., 2002). Cyclin E activity is increased substantially in the apoptotic process triggered by irradiation of RPMI 8226 cells. Overexpression of cyclin E by transient transfection did not directly induce apoptosis, however these cells were sensitized to irradiation. Cyclin E usually exists as a 50 kDa protein (p50-cyclin E). During apoptosis it is cleaved into an 18 kDa fragment (p18-cyclin E) by caspases and it can no longer bind to the CDKs. Overexpression of Bcl-2 protein down regulated the apoptotic process and at the same time reduced the expression of p18-cyclin E (Mazumder et al., 2002). These observations throw some light on the roles of cyclin E in apoptosis. Unexpected evidence came from the experimental results of the same group, which states that p18-cyclin E interacts with Ku-70, a critical component in the non-homologous end-joining repair. It is speculated that interaction of cyclin E might inhibit the activity of Ku-70 and hence augments the apoptotic process by inhibiting the DNA repair mechanism (Mazumder et al., 2004). Cyclin A1 null mice show cell cycle arrest and apoptosis of spermatocytes (Salazar et al., 2005). Apparently, Bax expression is elevated in the spermatocytes of cyclin A1 null mice as compared to normal mice, and hence leads to their apoptotic demise. This clearly shows that cyclin A1 plays a vital role in the regulation of

apoptotic process by inhibiting the expression of Bax gene. Cyclin G overexpression promotes cell growth in RKO colon carcinoma cells. However, it has also been shown that retroviral expression of cyclin G potentiates the TNF-induced apoptotic process in mouse fibroblasts (Okamoto and Prives, 1999).

Cyclin D1 has been shown to induce apoptosis in variety of cancer cells such as NIH 3T3, mouse kidney and R070B cells (Kranenburg et al., 1996). It is also reported that cyclin D1 induces apoptosis in rat fibroblast cell lines under serum depleting conditions (Sofer-Levi and Resnitzky, 1996). Ectopic expression of cyclin D in the above experiments caused apoptosis by deregulating the pRb/E2F pathway. Furthermore, cyclin D3 interacts with caspase-2, and this interaction leads to increased caspase activity and apoptosis in HEK 293 cells (Mendelsohn et al., 2002). While overexpression of caspase-2 induced apoptosis in HEK 293 cells, co-expression of cyclin D3 and caspase-2 potentiated the apoptotic process. Accordingly, co-expression of caspase-2 mutants that do not interact with cyclin D3, together with a wild type cyclin D3 does not increase apoptosis. These results clearly point out the fact that interaction of cyclin D3 and caspase-2 is required for the enhancement of apoptosis (Mendelsohn et al., 2002). Cyclin B1 is also an important regulator of apoptotic cell demise in many cell types. For example, in γ-radiation induced apoptosis of hematopoietic cells, cyclin B1 was necessary and sufficient to induce apoptosis (Porter et al., 2000). Moreover, downregulation of cyclin B1 by antisense RNA rescued cells from apoptosis (Porter et al., 2000). DNA damage-induced apoptosis was strongly dependent on the nuclear localization of cyclin B. This has been proven by ectopic expression of either cytoplasmic or nuclear cyclin B1. Apoptotic death was seen in the nuclear localizing cyclin B1 but not in cytoplasmic localized cyclin B1 (Porter et al.,

2003). In HeLa cells, cyclin B1 is expressed at high levels. Down regulation of cyclin B1 expression by siRNA lead to anti-proliferative effects in the tumor cells. Interestingly, targeting of cyclin B1 by siRNA in primary cells; human umbilical vein endothelial cells (HUVECS) does not decrease their proliferation, (Yuan et al., 2004). Camptothecin treatment of HT29 cells induced apoptosis (Borgne et al., 2006; Huang et al., 2006b), which was accompanied by increased in the expression of cyclin B1 and cyclin E2. siRNA-mediated inhibition of cyclin B1 prevented the cells from undergoing apoptosis, whereas siRNA-targeting of cyclin E2 had no effect (Borgne et al., 2006). Thus, cyclin B1 expression is required for apoptotic induction by camptothecin in HT29 cells. Overexpression of cyclin L2, a recently identified novel human cyclin, induces apoptosis in human hepatocellular carcinoma cell lines (Yang et al., 2004). Apoptosis mediated by cyclin L2 is due to the up-regulation of p53 and Bax and downregulation of Bcl-2.

1.8.3 CDK inhibitors and apoptosis

Beyond the cell cycle, $p21^{Cip1/Waf1}$ can perform as a negative regulator of both p53-dependent and p53-independent apoptosis (Gartel, 2005). Whereas, the p53-dependent cell cycle arrest is primarily mediated by $p21^{waf1}$, the mechanisms of $p21^{waf1}$ role in p53 dependent apoptosis are not clearly understood. There is mounting evidence that $p21^{waf1}$ is a major inhibitor of p53-dependent apoptosis (Brugarolas et al., 1995; Deng et al., 1995), but it is not entirely clear how a cell chooses between apoptosis and $p21^{waf1}$ dependent cell cycle arrest after DNA damage and stabilization of p53. Often high levels of $p21^{waf1}$ mediate cell cycle arrest and protect from p53-mediated apoptosis. Cells treated with various DNA damaging agents undergo cell cycle arrest mediated by $p21^{waf1}$, followed by apoptosis after caspase-3 mediated cleavage of $p21^{waf1}$ (Gartel, 2005). A431 cells

expressing mutant p53 that cannot induce $p21^{waf1}$ undergo apoptosis after exposure to γ- or UV radiation, but induction of $p21^{waf1}$ by mimosine protects the same cells from apoptosis (Bissonnette and Hunting, 1998). Irradiation of baf-3 murine hematopoietic cells with wild type p53 in the presence of IL-3 induces $p21^{waf1}$ and G1 arrest, whereas the radiation in the absence of IL-3 results in much weaker induction of $p21^{waf1}$ and in apoptotic cell death (Canman et al., 1995). Among the p53-independent apoptotic functions of $p21^{waf1}$, TGF-β, TNF, IFN-γ, IL-6 induce p53-independent expression of $p21^{Cip1/Waf1}$ and cause not only cell cycle inhibition but also suppression of apoptosis. The probable mechanism of this action include: (*i*) interaction with pro-apoptotic molecules such as pro-caspase-3, caspase-8 or ASK-1 (apoptosis signal-regulating kinase 1), and (*ii*) induction of G_1 arrest in response to binding to cyclin A, E/CDK2 complexes (Gartel and Tyner, 2002). In addition to its negative role during apoptosis, paradoxically, $p21^{Cip1/Waf1}$ also have been suggested to have a positive pro-apoptotic role under certain conditions. Overexpression of $p21^{waf1}$ in thymocytes led to hypersensitivity to p53 dependent cell death in response to ionizing and UV radiation (Fotedar et al., 1999). Overexpression of $p21^{waf1}$ also promoted C6-ceramide-induced apoptosis in the p53 deficient Hep3B human hepatoma cell line (Kang et al., 1999). In these cells, overexpression of $p21^{waf1}$ resulted in the induction of the pro-apoptotic Bax modulating the ratio of Bcl-2/Bax in favour of apoptosis. Other studies including Genistein induced cell death, CD95/Fas mediated cell death, overexpression of RAD50 (a DNA recombination repair gene) induced cell death, $p21^{waf1}$ has been assigned a positive role during apoptosis (Gartel and Tyner, 2002). The second member of Cip/Kip family of CDKIs, $p27^{Kip1}$ inhibits cyclinE/CDK2 and regulates G_1/S transition. In addition, several studies have suggested a crucial role for $p27^{Kip1}$ either negatively or positively

during apoptosis (Tenjo et al., 2000). The adenoviral $p27^{kip1}$ gene transfer or the overexpression of $p27^{kip1}$ by other transfection methods has been shown to cause apoptosis in several cell lines (Katayose et al., 1997; Wang et al., 1997). However, the molecular targets for $p27^{kip1}$ during apoptosis are not yet clearly known. On the other hand, it has been reported that apoptosis in growth factor deprived human endothelial cells is associated with the cleavage of $p27^{kip1}$ by the caspase pathway. After the cleavage, $p27^{kip1}$ loses its ability to bind and inhibit CDK2/cyclin complexes, thus leading to a marked increase in CDK2 activity, mainly the cyclin A-associated CDK2 activity. Therefore, the inactivation or downregulation of $p27^{kip1}$ might be important for induction of cell death in certain conditions. Indeed, studies on $p27^{kip1}$-deficient mice have revealed an anti-apoptotic role of $p27^{kip1}$, as the cells derived from $p27^{kip1}$ null mice were sensitive to apoptosis by various stimuli compared to p27 wild type cells (Hiromura et al., 1999). Other studies suggested that increased $p27^{kip1}$ confers drug resistance to tumor cells (Eymin et al., 1999). Overexpression of $p27^{kip1}$ in a human leukemia cell line induces resistance to the induction of apoptosis by several cytotoxic agents. Thus, accordingly the suppression of $p27^{kip1}$ expression by siRNA induces apoptosis and/or sensitizes cytotoxic drug induced cell death in several cancer cells (Lee and McCormick, 2005).

1.9 Nur77 – A dual regulator of cell survival and cell death

Nur77 and p53 are the two well established molecules known to transmit the death signals from the nucleus to mitochondria. Nur77 (NGFI-B, TR3. NR4A1 or NAK1), an orphan nuclear receptor, was first identified as an immediate-early gene induced by serum, NGF or in response to seizures and mechanical lesions (Cao et al., 2004; Milbrandt, 1988; Watson and Milbrandt, 1989), and further as a gene induced by T cell receptor signaling in T cell hybridomas and thymocyte apoptosis (Woronicz et al., 1994). It belongs to the NR4A family of orphan nuclear receptors that also encompasses Nurr1 and Nor1. The Nur77 protein has a typical steroid receptor structure composed of an N-terminal transactivation domain, a central DNA binding domain containing two zinc fingers, and a C-terminus with homology to hormone-binding domains (Carson-Jurica et al., 1990). Interestingly, the induction of Nur77 expression by different stimuli varies, where Nur77 expression is transient after treatment with NGF, serum or phorbol esters but T-cell receptor activation leads to the stable expression of the gene. The kinetics of induction was shown to be correlated with the function of Nur77, where stable expression of Nur77 often leads to apoptosis (Woronicz et al., 1995) but the transient expression of Nur77 may not necessarily lead to apoptosis, but in fact promotes cell cycle progression and acts as a survival factor (Katagiri et al., 1997; Kolluri et al., 2003; Suzuki et al., 2003).

Nur77 was initially known for its role in cell proliferation and differentiation, but later paradoxically shown to be a potent pro-apoptotic molecule (Maruyama et al., 1998; Zamzami and Kroemer, 2001). Overexpression of dominant negative Nur77 or antisense inhibition of Nur77 expression inhibits apoptosis whereas constitutive expression of Nur77 induces rapid apoptosis (Li et al., 2000; Li et al., 1998; Weih et al., 1996; Woronicz et al.,

1994). The fact that Nur77 and its family members, Nor-1 and Nurr1, are expressed as immediate early genes in different cell types following various stimuli suggests that they play a role in a diverse set of biological functions. Indeed, Nurr1-deficient mice lack midbrain dopaminergic neurons and die soon after birth (Zetterstrom et al., 1997). Nur77 also acts as a transcription factor and regulates the expression of several genes like CD30, FasL, TRAIL, TGF-β3, NDG1 and NDG2 (Rajpal et al., 2003). Nur77 can bind to a consensus NBRE sequence (AAAGGTCA) as a monomer or to a palindromic DNA binding motif (NurRE, TGATATTTX6AAATGCCA) as a homodimer (Maira et al., 1999; Philips et al., 1997). Expression of Nur77, Nor-1, or Nurr1 alone is sufficient to activate NBRE or NurRE-directed transcriptional activities, suggesting that the Nur77 family members might be "constitutive" orphan steroid receptors that do not require ligands for activation. Nur77, apart from acting as a transcription factor as a monomer, also modulates the transcriptional activity of other steroid receptors. For instance, Nur77 heterodimerizes with retinoid x receptor (RXR) and regulates its transcriptional activity (Perlmann and Jansson, 1995; Wallen-Mackenzie et al., 2003; Zetterstrom et al., 1996). Interestingly, Nur77 has also been reported to bind and alter the activity of COUP-TF, an orphan steroid receptor thought to negatively regulate the activation function of vitamin D receptor, retinoic acid receptor and RXR (Wu et al., 1997).

The other very interesting feature of Nur77 is its ability to act differently based on its localization in the cells. Nur77 in the nucleus acts as a transcription factor and is known to regulate the expression of different genes either involved in proliferation or apoptosis. During certain stimuli like TPA, NGF, and 3-cl-AHPC (Katagiri et al., 2000; Li et al., 2000) the localization of the protein changes to mitochondria where it executes the

mitochondrial apoptotic pathway by the release of cytochrome c. Two different mechanisms have been reported for the regulation of Nur77 translocation to the mitochondria: the first mechanism relies on the phosphorylation of Nur77 at different residues and the second mechanism involves the heterodimerization of Nur77 with RXR. The phosphorylation of Nur77, which results in its predominant localization in the cytoplasm, can be carried out by several kinases depending on the stimuli. These include members of the MAP kinase family and the protein kinase Akt. Akt phosphorylates Nur77 in its DNA binding domain at serine 351, resulting in reduced Nur77 DNA binding activity and predominant localization in the cytoplasm (Masuyama et al., 2001; Pekarsky et al., 2001). In PC12 rat pheochromocytoma cells, phosphorylation of Nur77 in its N-terminal region at serine 105 by members of the MAP kinase family (Trk/Ras/MAP kinase pathway dependent) regulates the ability of Nur77 to be exported to the cytoplasm in response to NGF (Katagiri et al., 2000). JNK also phosphorylates Nur77 and regulates the cytoplasmic translocation and further mediates cell death by mitochondrial activation (Han et al., 2006). Nur77 contains three nuclear export signals located in the ligand binding domain that, when mutated, cause Nur77 to remain in the nucleus despite the presence of NGF and the intact serine phosphorylation site. This data suggests that NGF stimulation results in phosphorylation of Nur77, thus exposing the export signals within the C-terminal ligand binding domain and causing translocation of Nur77 to the cytoplasm. An alternate mechanism proposed recently for the regulation of Nur77 translocation to the mitochondria is dependent on the heterodimerization of Nur77 with RXR. During the process of mitochondrial translocation Nur77 forms heterodimers with RXR via the DNA binding domains and the heterodimerization leads to the exposure of nuclear export sequences on

RXR (but not Nur77) (Cao et al., 2004). In contrast, in the presence of RXR ligands, Nur77 and RXR heterodimerize at their ligand binding domains leading to the masking of RXR nuclear export sequences. Thus, in the presence of RXR ligands, Nur77 translocation and apoptosis is inhibited even though it dimerizes with RXR indicating the crucial role of nuclear export sequences during Nur77 export out of the nucleus to cytosol. It has been further suggested that Nur77 may interact with Bcl-2 at the mitochondria and convert it from an anti-apoptotic to a pro-apoptotic member (Lin et al., 2004).

1.10 Apoptin- A cancer selective killer molecule

1.10.1 CAV (Chicken anemia virus)

CAV is a very small (23-35 nm), single stranded 2.3 kb DNA containing, encaspidated avian-specific virus (circovirus family) that only infects young chickens (highest infectivity rate at the day of hatching) (Noteborn et al., 1991; Yamaguchi et al., 2001). Older chickens, starting at the age of 10-14 days, acquire a rapid resistance against the effects of the virus. The CAV genome contains a 5′ non-transcribed region with the promoter activity. The genome contains three partially overlapping open reading frames, which together produce a single strand of unspliced RNA. It encodes three different viral proteins; VP1 (51.6 kDa structural protein), VP2 (24 kDa non-structural protein) and VP3 (also known as Apoptin), which are found in all the viral infected cells (Phenix et al., 1994). Infection with CAV causes a disease in young chickens that is characterized by generalized lymphoid atrophy, development of subcutaneous haemorrhages, severe anemia, increased incidence of bacterial secondary infections and ultimately mortality (Adair, 2000; Noteborn, 2004). Previous studies have suggested that the hemocytoblasts in the bone marrow and T cell precursors but not B cell precursors in the thymus are important targets for the virus infection. The early infection of hemocytoblasts in the bone marrow results in the depletion of erythrocytes and thromocytes thus leading to anemia and the intramuscular hemorrhages. In the thymus, cortical lymphocytes, in particular the CD3+/TCR+ T-cell precursor cells were among the first cells to be destroyed, whereas non-lymphoid leukocytes and stromal cells were unaffected. Also, the CD8+/Cd4+ mature T cells were greatly reduced by CAV viral infection in these chickens (Taniguchi et al., 1983). Studies have shown that the mechanism of thymocyte and hemocytobalst depletion

during CAV infection is due to apoptosis. Electron microscopic observations of the cells indicated apoptotic bodies and apoptotic nucleosomal laddering in DNA agarose gel after CAV infection. The induction of apoptosis by CAV in these cells has been attributed to its non-structural protein VP3 or Apoptin (Adair, 2000; Maddika et al., 2006; Noteborn, 2004).

1.10.2 Apoptin

Apoptin (VP3) is a 121-amino acid (14 kDa) proline rich protein containing no homology to any known viral or cellular proteins (Noteborn, 1999; Noteborn et al., 1994; Oro and Jans, 2004). The structure of Apoptin (shown in figure 5) contains no distinct cellular domains, but contains two clusters of basic residues near the C-terminus of the protein hypothesized to function as bipartite nuclear localization signals (NLS). Apoptin also contains a stretch of hydrophobic amino acids (33-46), which was originally hypothesized to be the nuclear export sequence. It was later ruled out as an NES, but remained as Leucine rich sequence required for PML protein association. Recently, a CRM1 binding nuclear export sequence (amino acids 97-105) has been mapped on the C-terminus of apoptin's structure, which plays a critical role in the nuclear export of apoptin in the cells (Alvisi et al., 2006). The interesting feature of Apoptin is its selective toxicity towards cancer cells without affecting normal cell types. Apoptin selectively induces apoptosis in various tumor and transformed cell lines, but not in primary, non-transformed cells upon its expression either by transfection, transduction or adenoviral mediated transfer (Danen-Van Oorschot et al., 1997; Maddika et al., 2006; Poon et al., 2005b). The selective toxicity of Apoptin is shown not to be a cell type specific, as the toxicity is tested on many types of cell lines derived from various cancers including breast cancers, prostate

cancers, leukemias, hepatomas, cervical cancers and sarcomas (Oro and Jans, 2004). The non-toxic nature of Apoptin on primary cells has been shown, both by us and others, using a variety of primary cell types including HUVECs, primary fibroblasts, primary lymphoid cells, keratinocytes, epidermal-, endothelial, and smooth-muscle cells (Burek et al., 2006; Danen-Van Oorschot et al., 1997; Danen-van Oorschot et al., 2000). Interestingly, however the co-expression of SV-40 large T-antigen along with Apoptin or pre-treatment of normal cells with UV-irradiation was sufficient to induce cell death, indicating that even brief expression of the viral transforming gene or the brief exposure of cells with transforming stimuli makes normal cells susceptible to Apoptin induced cell death. Apoptin's cellular localization is crucial for its selective toxicity towards transformed cells. In primary cells, it remains in the cytoplasm, whereas in transformed cells it migrates into the nucleus and ultimately kills the cell by activation of the mitochondrial death pathway (Danen-Van Oorschot et al., 2003; Poon et al., 2005a; Rohn and Noteborn, 2004). However, targeted translocation of Apoptin into the nuclei of primary cells is not sufficient for Apoptin's toxicity. Thus additional interaction partners or specific activation of other signalling pathways in the cancer cells preceding the nuclear accumulation of the protein might be necessary for the Apoptin's tumor specific toxicity. By using *in vivo* tumor models, recently Apoptin was also shown to be very effective in inducing tumor specific apoptosis. A single intratumoral injection of adenoviral Apoptin into nude mice with human hepatomas (HEPG2 cells in Balb/$C^{nu/nu}$ mice) resulted in reduction of tumor growth and symptoms of regression within 7 days of Apoptin administration (Pietersen et al., 1999). In this report, toxicity studies with rats showed that either control adenovirus or adenovirus containing Apoptin administered by intraperitoneal, subcutaneous or

intravenous injections have no adverse effects. In another study, the intratumoral injection of fowlfox virus containing apoptin inhibited the growth of the aggressive subcutaneous H22 mouse hepatomas (Li et al., 2006). In these studies, no effect of Apoptin was seen in normal hepatocytes suggesting the tumor specific toxicity of apoptin *in vivo.* Further, a combined administration of Apoptin with IL-18 gene in C57BL/6 mice bearing Lewis lung carcinomas (LLC) significantly inhibited the tumor growth compared to Apoptin alone (Lian et al., 2007). The immunization of mice with Apoptin in conjunction with IL-18 elicited strong natural killer activity and LLC tumor-specific cytotoxic T-lymphocyte responses. In addition, T cells from lymph nodes of mice vaccinated with Apoptin and IL-18 secreted high levels of Th1 cytokine IL-1 and IFN-γ, indicating that the combined administration mediated regression of tumor cells is related to a Th1-type dominant immune response.

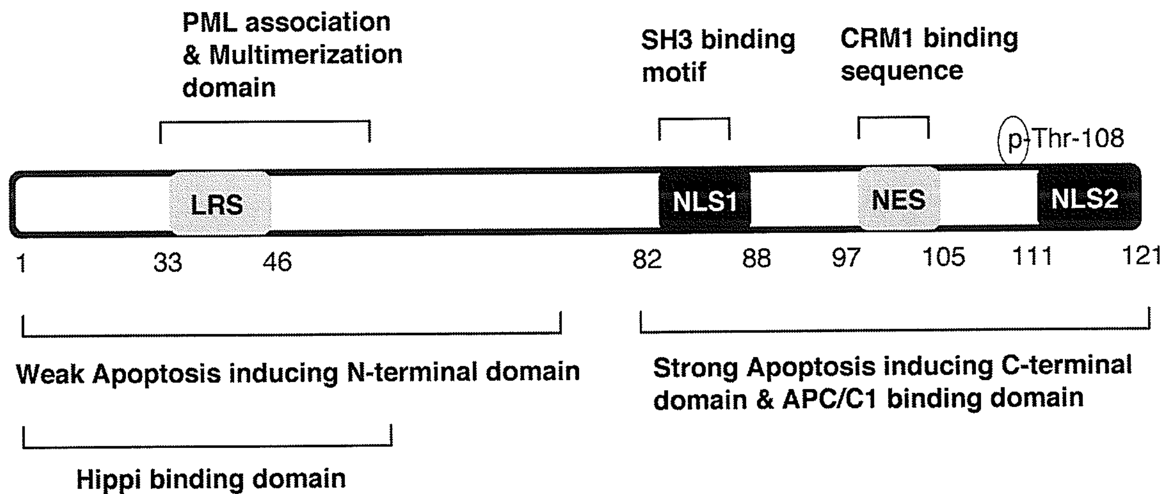


Figure 5: Diagrammatic representation of structural and functional domains of Apoptin (The number of amino acids at each structural motif is indicated).

1.10.3 Apoptin interacting proteins

Several studies using either yeast two-hybrid screening or affinity purification have revealed the interaction of Apoptin with various cellular proteins including DEDAF (death effector domain associated factor) (Danen-van Oorschot et al., 2004), Hippi (the protein interactor of the Huntingtin interacting protein 1) (Cheng et al., 2003), Nmi (N-Myc interaction protein) (Sun et al., 2002) and APC1 (Anaphase promoting complex subunit 1) (Teodoro et al., 2004). DEDAF was identified as a protein associated with the DED containing proteins like procaspase-8 and 10, but does not contain DED itself (Zheng et al., 2001). Others have found DEDAF, also known as YEAF1, as a binding partner for the transcription factor E4TF1/hGABP and other E2F transcription factor family members (Sawa et al., 2002; Schlisio et al., 2002). Apoptin interacts with DEDAF specifically in the

nucleus of tumor cells but not in normal cells. The Apoptin mutants replaced with alanine at the residues Thr 41, Lys 101 and Thr 106 show reduced affinity with DEDAF and accordingly, the Ala 41 mutant is cytoplasmic in its localization and has reduced cell death inducing activity after 3 days but not 5 days of transfection compared to wild type Apoptin (Danen-van Oorschot et al., 2004). Interestingly, DEDAF not only co-colocalized with Apoptin in the nucleus, but also displayed a similar cell death activity as Apoptin when overexpressed in tumor cells. DEDAF expression has been shown to selectively induce apoptosis in cancer cells, but not in normal cells. Co-expression of Apoptin and DEDAF resulted in higher percentage of cell death than expression of either protein alone, but it is not known whether DEDAF is absolutely required for Apoptin induced cell death as no studies were performed to functionally inhibit DEDAF or to inhibit its expression by siRNA studies.

A yeast two hybrid screening by Cheng *et al.*, (Cheng et al., 2003) has identified Hippi as an Apoptin interacting partner, which is also confirmed to associate with Apoptin in mammalian cells. Hippi is the interacting partner for Hip1 (Huntingtin interacting protein), which as a complex is shown to induce apoptosis via the recruitment and activation of pro-caspase-8 (Cheng et al., 2003; Gervais et al., 2002). Under normal circumstances, Hip1 is associated with huntingtin protein Htt as an inactive complex, but upon the expansion of polyglutamine repeats on Htt, the inactive complex dissociates and the released Hip1 now forms a complex with Hippi. The Hippi/Hip complex recruits pro-caspase-8 via the interaction between pseudo DED motif on Hip1 and DED motif of caspase-8. Mapping studies indicated that Hippi interacts with the self-mulitmerization domain of Apoptin via its C-terminal DED like motif. Interestingly, Apoptin interacts with

Hippi and co-localizes in the cytoplasm of normal cells, but no interaction is seen in tumor cells (Cheng et al., 2003). Thus, this selective interaction in normal cells might suggests the possibility that in normal cells, Hippi might be preventing Apoptin's nuclear import and/or conversely Apoptin might be preventing the recruitment of caspase-8 to the Hippi-Hip1 complex. Nonetheless, it would be interesting to study whether the binding of Hippi to Apoptin is indeed inhibiting Apoptin induced cell death by Hip overexpression and mutant Htt expression.

Nmi (N-Myc interacting protein) is another cellular protein shown to be interacting with Apoptin (Sun et al., 2002). Nmi is an interferon inducible protein that interacts with the Myc family of transcription factors (n-Myc, c-Myc and Max), c-fos and STAT family of transcription factors (Bao and Zervos, 1996). Nmi, though it lacks an intrinsic DNA binding or activation domain, enhances the transcriptional activity of Myc and STAT family members, which contain bHLH-Zip, bHLH or Zip domains (Zhu et al., 1999). Nmi is expressed at very low levels in all the normal tissues, but at very high levels in different cancers (Bao and Zervos, 1996). The functional significance of the Apoptin-Nmi interaction is not yet known. Another recent study by Teodoro et al, has shown that Apoptin interacts with the APC1 subunit of the APC/C complex (Anaphase promoting complex/Cyclosome) (Teodoro et al., 2004). APC is a large protein complex of 11 core proteins that can further associate with at least three known different activators that guides the cell through mitosis (though some studies also suggest a role for APC in G1 progression and DNA replication) (reviewed in Castro et al., 2005). APC/C complex catalyzes the ubiquitination of several substrates including cyclin B, cyclin A, Plk1, Aurora A, Geminin, Cdc6 and kinesins (Kip1 and Cin8) thus regulates mainly the anaphase entry,

progression and exit from mitosis. Apoptin, via its C-terminal domain, interacts with the APC1 subunit alone, but not other proteins in the APC complex and leads to cell cycle arrest in the G2/M phase. The interaction of Apoptin and APC1 is reported to occur specifically in cancer cells, but not in normal cells, which might partially explain the selective toxicity of Apoptin. In addition, Apoptin has also been shown to co-localize with FADD (Fas associated death domain containing protein) and Bcl-10, but no studies were done to establish the physical interaction of these proteins with Apoptin in the cells (Guelen et al., 2004).

1.10.4 Apoptin's Nuclear Import and Export

As stated previously, the selective toxicity of Apoptin is mainly attributed to the differential localization of Apoptin in tumor and normal cells. Apoptin mainly localizes to the nucleus of cancer cells, but this is severely impaired in normal cells and is localized in their cytoplasm (Danen-Van Oorschot et al., 2003). Although not completely understood, the molecular basis of Apoptin's tumor specific nuclear localization has been thoroughly investigated. Apoptin is a nucleo-cytoplasmic shuttling protein whose localization is regulated by nuclear localization signals (NLS) and nuclear export signals (NES). Studies using various Apoptin deletion and point mutant derivatives revealed a C-terminal bipartite basic nuclear localization signal (spanning the amino acids 82-88 and 111-121). The C-terminal NLS by binding to the Importin β complex confers the nuclear localization of apoptin transporting via the nuclear pore complex (Poon et al., 2005a). Interestingly the Apoptin (74-121) mutant binds to IMPβ with three times higher affinity compared to full length (1-121) Apoptin, suggesting that the NLS of Apoptin might be regulated by Apoptin's N-terminal intra-molecular masking. The NLS of Apoptin is active both in

normal and cancer cells as Apoptin is shown to translocate to the nucleus in both types of cells though the amount of nuclear Apoptin is significantly lower in normal cells compared to the cancer cells (Poon et al., 2005b; Rohn and Noteborn, 2004; Wadia et al., 2004). Recently, however, a CRM1 recognised nuclear export sequence (NES residues 97-105) has been identified, which is active in normal cells but not in tumor cells, thus determining the tumor cell specific localization of Apoptin (Poon et al., 2005a). The treatment of normal cells with Leptomycin B, a CRM1 specific nuclear export inhibitor significantly enhanced the nuclear localization of Apoptin, but no effect was seen in tumor cells. In addition to the tumor specific nuclear targeting sequences, Apoptin has a phosphorylation site at Threonine 108 residue adjacent to the NES (Rohn et al., 2002). Although the kinase phosphorylating Apoptin ha not been identified yet, it is highly active in tumor cells compared to normal cells. The phosphorylation at Threonine-108 residue is reported to be responsible for tumor specific Apoptin's nuclear accumulation via inactivating the NES in tumor cells thus inhibiting the nuclear export specifically in tumor cells. The phosphomimetic T108E Apoptin mutant shows increased nuclear accumulation in normal cells comparable to that of tumor cells and is not responsive to the leptomycin B (LMB) treatment, supporting the hypothesis that T108 phosphorylation inhibits the NES activity. Interestingly, the Apoptin T108E mutant expression in normal cells shows increased apoptosis in accordance with the increased nuclear accumulation (Rohn et al., 2002). Apoptin also contains a leucine rich sequence (LRS; amino acids 33-46) at the N-terminus, which is important for Apoptin's interaction with promyelocytic leukaemia (PML) proteins (Heilman et al., 2006). The PML nuclear bodies formed via the PML proteins are the structured protein complexes associated with the nuclear matrix and have roles in

regulating apoptosis, DNA replication, repair, transcription and RNA transport (Salomoni and Pandolfi, 2002). Interestingly the inactivating mutations in the LRS sequence not only reduces the ability of Apoptin to associate with PML bodies, but also reduces Apoptin's nuclear accumulation in tumor cells. Recently, Apoptin was also reported to be sumoylated and this modification is required for its association with PML nuclear bodies as the sumoylation deficient mutant fails to bind to PML and associated with nuclear matrix (Janssen et al., 2006). Although, Apoptin sumoylation is required for PML body association, it is not required for apoptin induced cell death.

1.10.5 Mechanisms of Apoptin mediated cell death

Despite several studies, the mechanism of Apoptin induced cell death and its tumor specific toxicity is still not completely understood. Apoptin induces tumor specific cell death independent of the p53 status of the cell (Zhuang et al., 1995b). The p53 tumor suppressor gene plays a very important role in the prevention of oncogenic transformation by facilitating apoptosis (Mowat, 1998). Adenoviral-Apoptin expression in LNCaP prostate cancer cells with wild type p53 did not induce any changes in the p53 Ser15 phosphorylation. However, Apoptin expression in DU145 cells with mutant p53 shows elevated levels of p53 Ser15 phosphorylation compared to adenoviral GFP control expressing cells (Liu et al., 2006a). Nonetheless, Zhuang *et al.*, (Zhuang et al., 1995b) have shown that Apoptin does not require p53 to induce apoptosis as the expression of Apoptin in either cells having functional p53, mutant p53 or no p53 shows the similar amount of apoptosis. Data from our lab also confirms the p53 independent action of Apoptin, since Apoptin was toxic to SV40 T-large antigen transformed murine fibroblasts (T-antigen sequesters and inhibits p53). Interestingly, it has also been reported that Apoptin mediated

cell death is independent of the Bcl-2 status of the cell, in fact the co-expression of Apoptin and Bcl-2 in Saos-2 cells was shown to accelerate the cell death compared to Apoptin alone (Danen-Van Oorschot et al., 1999a; Danen-Van Oorschot et al., 1999b). This is quite surprising since Bcl-2 is an anti-apoptotic molecule and known to inhibit apoptosis, despite some studies suggesting the positive role of Bcl-2 depending on the context and the structural confirmation of Bcl-2 (Lin et al., 2004). Interestingly, using an oligonucleotide microarray, other studies have shown that the Bcl-2 family members like Bad and Bax were upregulated in the presence of Apoptin (Tai and Qian, 2005). Thus, the role of Bcl-2 family members during Apoptin induced cell death is still controversial and needs to be further evaluated in future studies.

The preliminary studies with Apoptin have indicated no role of caspases in Apoptin induced cell death as the peptide based caspase inhibitors and CrmA have no effect on the process of cell death (Noteborn, 1999). Later this has been disproved by many other studies showing the activation of effector caspases (caspase-3 and 7) in the presence of Apoptin. The inhibition of caspases by p35 caspase inhibitor and zVAD-fmk, a broad range caspase inhibitor significantly inhibited Apoptin induced cell death (Burek et al., 2006; Danen-van Oorschot et al., 2000). Although, selective nuclear localization plays a critical role in Apoptin induced cell death, recently Wadia et al,. (Wadia et al., 2004) proposed that the nuclear translocation is based not on the tumorigenic status, but depends on the cellular concentration of Apoptin. Nonetheless, this has been challenged by three independent groups confirming that the higher nuclear localization in cancer cells compared to the cytoplasmic localization in normal cells is based on an unknown tumor specific kinase activity and subsequent nucleo-cytoplasmic shuttling (Heilman et al., 2006;

Poon et al., 2005b; Rohn et al., 2002). The targets for Apoptin in the nucleus are not completely understood. Apoptin has been reported to bind to DNA, but the functional significance of this association is not known (Leliveld et al., 2004). Recently Liu et al., (Liu et al., 2006a; Liu et al., 2006b) have proposed a novel mechanism for Apoptin induced cell death using prostate cancer cells and DU145 xenograft models. These studies have shown that apoptin modulated the sphingolipid-ceramide pathway leading to the increased concentration of lipid ceramide in human prostate cancer cells independent on the expression of p53, Bax, survivin, $Flip_s$, XIAP and CIAP. Ceramide, a second messenger molecule and a central molecule in sphingolipid metabalism, plays a crucial role as a tumor suppressor lipid that mediates an intracellular response to diverse therapeutic agents such as Fas ligand, TNF-α, ionizing radiation and chemotherapeutic drugs. Inhibition of ceramide synthesis is associated with drug resistance (Ogretmen and Hannun, 2004). Ceramide metabolism is regulated by a complex biochemical pathway and the molecular studies of this pathway have identified numerous targets for drug development. Though the exact mechanism is not completely known, Apoptin increases ceramide levels by the activation of acid sphingomyelinase, resulting in increased hydrolysis of sphinogomyelin to ceramide. In addition, Apoptin increases the levels of ceramide by inhibiting acid ceramidase, an enzyme that deacylates ceramide to yield sphingosine. In line with these observations, a combined administration of adenoviral Apoptin and LCL04, an acid ceramidase inhibitor enhanced the anti-tumor effect of Apoptin, resulting in increased animal survival compared to Apoptin alone. Also it is reported that Apoptin activates c-Jun N-terminal kinase (JNK) signaling during its cell death process in U937 human lymphoma cells (Ben et al., 2005), but again the mechanism

of the JNK activation is not known. Thus, deciphering the detailed molecular mechanisms of tumor specific toxicity of Apoptin would greatly enhance the usage of this molecule in future cancer therapies.

1.11 Other selective cancer therapy molecules

Several molecules derived from viruses, blue-green algae, marine fungi or even mammalian cellular proteins have been attributed to have tumor selective killing activity. Here we discuss only two of the examples that have been well studied and established to have cancer selective properties.

1.11.1 E4orf4

E4orf4 is another example of a molecule with a tumor specific toxicity (Mitrus et al., 2005). The E4orf4 (adenovirus type 5 E4 open reading frame 4) protein is a product of the E4 early transcription unit of adenovirus, which encodes seven open reading frames (Freyer et al., 1984). E4orf4 is a small protein of 114 amino acids with no significant homology to any other known proteins.

Similar to apoptin, E4orf4 is reported to have a very intriguing feature of selective toxicity towards cancer cells. E4orf4 is shown to specifically induce apoptosis in a wide range of cancer cells but not in normal primary cells (Marcellus et al., 1998; Shtrichman et al., 1999). The interesting feature of E4orf4 is its ability to induce apoptosis independent of the p53 status of the cell (Marcellus et al., 2000). It was reported that E4orf4 induces cell death both dependent and independent of caspase activation in a cell line specific manner, and the toxicity of E4orf4 is not severely affected by z-VAD-fmk, a broad range caspase inhibitor. Further it was shown that E4orf4 mediates its toxicity through the activation of a

death receptor pathway as the cells expressing either dominant negative caspase-8 mutant or dominant negative FADD mutant show reduced cell death compared to wild type cells (Lavoie et al., 1998; Livne et al., 2001). E4orf4 has also been shown to activate the mitochondrial death pathway and releases cytochrome c, but interestingly does not activate the caspase-9 downstream of cytochrome c. An alternate caspase-9 independent cell death pathway has been proposed, where E4orf4 induces its cytotoxicity through the accumulation of ROS downstream of caspase-8 activation (Livne et al., 2001). E4orf4 requires at least 24-48 hours to induce cell death and this process is inhibited by the anti-apoptotic Bcl-2 family members Bcl-2 itself and Bcl-xL (Lavoie et al., 1998).

In addition, it was also reported that E4orf4 mediated cytotoxicity towards transformed cells absolutely requires the interaction of E4orf4 with a phosphatase PP2A. Accordingly, the mutants lacking the interaction sites with PP2A are unable to induce apoptosis in these cells (Marcellus et al., 2000; Shtrichman et al., 2000). PP2A is a serine/threonine phosphatase and is a holoenzyme made of 3 subunits A, B and C with different isoforms for each subunit (Van Hoof and Goris, 2003). E4orf4 binds mainly to the Bα subunit of PP2A, but the interaction with other PP2A population is also shown. The specific interaction of E4orf4 with B subunit of PP2A, but not with other subunits is essential for the induction of cell death. It is shown that E4orf4 leads to the reduced phosphorylation of different proteins either involved in transcription or splicing (Kleinberger and Shenk, 1993; Mannervik et al., 1999; Muller et al., 1992). E4orf4-PP2A complex regulates the transcriptional activity of c-Fos, E2F and JunB by hypophosphorylating the trancription factors and their regulators; however, the kinase pathways targeted by E4orf4-PP2A complex during this process are not explored.

E4orf4 is mainly localized in the nucleus of the transformed cells, which is mediated by the arginine rich motif, but the localization of the protein in normal cells has not been studied yet. The nuclear E4orf4 is shown to induce irreversible growth arrest in the yeast *Saccharomyces cerevisiae* by physically interacting with APC/C (Kornitzer et al., 2001; Roopchand et al., 2001). Further, the interaction of E4orf4 and APC/C in the nucleus of mammalian transformed cells is confirmed to induce G2/M cell cycle arrest. It is also shown that CDK1 (Cyclin dependent kinase 1, a component of Mitosis promoting factor) activity is elevated in the E4orf4 over expressed cells, which correlates with the APC/C inhibition (Kornitzer et al., 2001). Though E4orf4 is seen in the nucleus of the transformed cells, the cytoplasmic distribution of the protein is also reported and in particular accumulation in the cytoplasmic membranes. Interestingly, the cytoplasmic E4orf4 is also active in inducing cell death, but with a distinct mechanism whereby it interacts with the src family kinases and thus deregulates the survival signals mediated by these kinases (Gingras et al., 2002; Lavoie et al., 2000). E4orf4 gets phosphorylated at tyrosine residues Y26, Y42 and Y59 by src kinases and this phosphorylation and the interaction with the src kinases inhibits the nuclear import of the protein. It is also shown that the E4orf4 arginine rich motif interacts with the kinase domain of the src kinases and regulates the tyrosine phosphorylation of some of the downstream targets like FAK, paxillin (higher levels of phosphorylation) and cortactin (reduced tyrosine phosphorylaton) (Champagne et al., 2004; Lavoie et al., 2000). The downstream effects of the regulation of this phosphorylation of src substrates are the deregulated assembly of the focal adhesions accompanied by changes in the actin cytoskeleton, membrane blebbing and eventually the loss of survival signals. E4orf4 is also shown to activate $p70^{s6k}$ in a mTOR dependent

pathway, but independent of PKB activation (O'Shea et al., 2005a; O'Shea et al., 2005b). The activation of $p70^{s6k}$ and mTOR by E4orf4 even in the absence of nutrient/growth factors signals plays a role in promoting the adenoviral replication, but it is not known whether this activation is required for the induction of cell death by E4orf4.

1.11.2 TRAIL

Based on its ability to induce apoptosis selectively in a wide variety of cancer cell lines and human tumor xenografts, TRAIL has been in clinical trials as a potential biological agent for cancer therapy (Duiker et al., 2006). TRAIL, a member of the TNF family of cytokines, induces cell death via a classical death receptor pathway in a p53 independent manner. TRAIL is a type II membrane protein, which can be cleaved from the cell surface to release a soluble death ligand. Both full-length membrane expressed TRAIL and the soluble ligand can rapidly induce apoptosis in a wide variety of cancer cells but not normal cells, though the mechanism of their tumor specific killing is not completely known yet (Wang and El-Deiry, 2003). TRAIL binds to five different receptors (four membrane bound and one soluble receptor), which gives a varied functional response (Buchsbaum et al., 2006). TRAIL binds DR4 and DR5 as a homotrimer, which results in the trimerisation of the receptors and thus leads to the assembly of the death inducing signaling complexes (DISC) via the recruitment of FADD and pro-caspase-8. The dimerisation of caspase-8 molecules at the DISC leads to the formation of mature caspase-8 via self-cleavage and thus the activated caspase-8 leads to effector caspases (caspase3, 6, and 7) activation that executes apoptosis (discussed in section 1.3.3).

The unraveling of the TRAIL pathway has resulted in many preclinincal studies, which revealed the potential of recombinant human TRAIL (rhTRIAL) for the treatment of

cancer. In early clinical trials, the safety profile of rhTRAIL has been evaluated in non-human primates and no adverse toxicity was observed with administration of rhTRAIL (Ashkenazi et al., 1999; Kelley et al., 2001). The agonistic antibodies directed specifically against DR4 and DR5 are also under clinical trials. Three fully humanized monoclonal antibodies, one directed to DR4 (HGS-ETR1) and two directed against DR5 (HGS-ETR2 and HGS-TR2J) are in clinical trials after their success in tumor regression in xenograft models and pre-clinical studies with a broad range of tumor cell lines (Motoki et al., 2005). Apart from the use of rhTRAIL and agonistic antibodies alone, numerous studies have shown that combination of TRAIL and other agents in several human tumor types were synergistic and effective in overcoming the resistance to either of the agents. TRAIL in combination with either HDAC inhibitors, proteasome inhibitors, heat-shock protein (HSP) inhibitors (Geldamycin), EGFR/Her2 receptor inhibitors (Trastuzumab), Bcl-2 inhibitor or the mTOR inhibitor (rapamycin) enhances apoptosis in several tumor cell lines compared to TRAIL alone (Cuello et al., 2001; Kabore et al., 2006; Ma et al., 2006; Oltersdorf et al., 2005; Panner et al., 2005; Shankar and Srivastava, 2004). Thus the ongoing Phase I and II clinical studies with rhTRAIL and the agonistic antibodies may not only provide the insights into the efficacy and possible side effects, they may also lead to a more profound comprehension of the TRAIL pathway and give rise to a real tumor-tailored therapy.

2. Materials and Methods

2.1 Reagents

All the chemicals were purchased from either Sigma-Aldrich. Inc (St. Louis, MO. USA) or Fisher Scientific Co. (Ottawa, ON) unless otherwise indicated.

2.2 Chemotherapeutics and inhibitors

Methotrexate (10 μM), docetaxel (50 nM), doxorubicin (50 μg/ml) and cisplatin (75 μg/ml) were purchased from Health Sciences Centre pharmacy (Winnipeg, MB) and used at the indicated concentrations. Staurosporine (2.5 μM) from Roche Diagnostics, Mannheim, Germany and activating anti-CD95 (50 ng/ml) from Upstate signaling, Charlottesville, VA were used. The following inhibitors were used: Wortmannin (IC_{50} 5nM), LY294002 (IC_{50} 1.4μM), PD98059 (IC_{50} 2μM), CDK2 specific inhibitor, Roscovitine (Kd 700nM), MG-115 (K_i 35nM) (all from Calbiochem, San Diego, CA, USA) and a caspase inhibitor zVAD-fmk (Enzyme System Products, Aurora, OH, USA).

2.3 Antibodies

The following antibodies were used: murine anti-PI3-K (p85), anti-mouse IgG-HRP, anti-phospho Histone H1, rabbit anti-caspase-3, anti-rabbit IgG-HRP (all from Upstate Cell Signaling Solutions, Charlottesville, VA, USA), rabbit anti-$p27^{kip1}$, murine anti-tubulin, rabbit anti-GFP, rabbit anti-phospho-$p27^{kip1}$-Thr-187, rabbit anti-CDK2, rabbit anti-CDK1, murine anti-cyclin E, murine anti-cyclin A, murine anti-cyclin B1, rabbit anti-Nur77, goat anti-phospho Ser-351 antibody, murine anti-cytochrome c, goat anti-phospho-Bcl-2-Thr-56, goat anti-phospho-Bcl-2-Thr-74, rabbit anti-Bcl-xL, rabbit anti-Bad, murine anti-Bcl-2 antibody, rabbit anti-Bak antibody, murine anti-AIF antibody,

anti-goat IgG-HRP (all from Santa Cruz Biotechnologies, Santa Cruz, CA, USA), rabbit anti-Akt, rabbit anti-phospho-specific Akt-substrate antibody, murine anti-phospho-Akt Ser-473, murine anti phospho- Ser 70 antibody, murine anti-phoshpho-Thr antibody, murine anti-phospho-Thr-Pro antibody, murine anti-caspase-8 antibody (all from Cell Signaling technologies, Danvers, MA, USA), anti-human CD5-FITC, CD19-PerCP, anti-cytochrome c (BD pharmingen, Mississauga, ON), murine anti-Bax antibody, murine anti-HA antibody, murine anti-phospho-Ser antibody, anti-goat Cy3, anti-rabbit Cy3, anti-murine Cy3 (all from Sigma, Oakville, ON), anti-Myc antibody (Invitrogen, Carlsbad, CA, USA), murine anti-p21^{waf1} (Biomeda, CA, USA), anti-phospho-p27^{kip1}-Thr-157 (R&D systems, Minneapolis, MN, USA), anti-CD95 neutralizing antibody (kind gift from Dr. H. Walczak, Heidelberg, Germany) and murine anti-Apoptin antibody (kind gift from Dr. D. Jans, Victoria, Australia).

2.4 Expression plasmids

The following plasmids were used: GFP, GFP-Apoptin (Apoptin cloned into pEGFP-C1 vector, clontech), GST, GST-Apoptin (Apoptin cloned into PGEX-2T vector, Amersham Biosciences), Myr-Akt vector, Akt (K179M) vectors were constructed and established in the lab. pTAT-GFP, pTAT-Apoptin (obtained from Dr. M. Tavassoli, London, UK), PI3-K dominant negative vector, Akt wild type vector [kind gift from Dr. J. Downward, London, UK, (Kauffmann-Zeh et al., 1997)], Apoptin mutant plasmids described earlier (Poon et al., 2005a), p85 deletion mutants [obtained from Dr. T. Mustelin, La Jolla, CA, USA (Jascur et al., 1997)], PDK1 dominant negative and constitutively active vectors (obtained from Dr. A. Halayko, Winnipeg, MB), PTEN WT and PTEN C124S phosphatase dead mutants (gifts from Dr. D.H. Anderson, Saskatchewan Cancer

Agency, Saskatchewan), PKC dominant negative vector (obtained from Dr. E. Kardami, Winnipeg, MB), CDK2 T160A mutant [obtained from Dr. David Morgan, San Franscisco (Gu et al., 1992)], GST-CDK2 WT, GST-CDK2 T160A vectors described earlier (Gu et al., 1992; Poon et al., 1997) (obtained from Dr. Tony Hunter, San Diego, CA, USA), Ad-NLS-Akt (obtained from Dr. M.A Sussman, San Diego, CA, USA) (Shiraishi et al., 2004), and Ad-Akt-dominant negative vector (Luo et al., 2000) (kind gift from Dr. K.Walsh, Boston, MA, USA). All the plasmids were propagated either in BL21(DE3)pLys S or Top10F E.coli bacterial strains, after transforming the respective competent bacterial cells using standard $CaCl_2$ mediated chemical transformation. The plasmid isolation from the positive clones was done using Qiagen Miniprep and Maxiprep kits using their standard protocols.

2.5 Site directed mutagenesis

The site directed mutagenesis in pSR CDK2 T160A vector to generate CDK2 WT, CDK2 T39A, CDK2 T39E and GST-CDK2 WT as well as T160A vectors to T39A was performed according to Stratagene quickchange site directed mutagenesis kit protocol by using the following primers:

A160T primers

5'- GAGTCCCTGTTCGTACTTACACACATGAGGTGGTGACCCTGT-3' and

5'-CACAGGGTCACCACCTCATGTGTGTAAGTACGAACAGGGACT-3'

T39A primers

5'-CTTAAGAAAATCCGCCTGGACGCCGAGACTGAGGGTGTGCCCAG-3' and

5'-CTGGGCACACCCTCAGTCTCGGCGTCCAGGCGGATTTTCTTAAG-3'

T39E primers

5'-GAAAATCCGCCTGGACGAAGAGACTGAGGGTGTGC-3' and

5'-GCACACCCTCAGTCTCTTCGTCCAGGCGGATTTTC-3'.

Briefly, 25 ng of dsDNA template, 5μl of reaction buffer, 125ng of the sense and anti-sense primer, 1μl of dNTP mix and 1μl of Pfu ultra HF DNA polymerase were added to a final volume of 50μl in a tube and incubated in a thermal cycler. Sixteen cycles with the cycling parameters of 95°C-30 sec, 55°C-60 sec and 68°C-5 min were performed. After the temperature cycling, the reaction mix was incubated with 1μl of Dpn-I restriction enzyme for 1h at 37°C and later transformed into XL-I blue competent bacterial cells using chemical transformation. The positive clones with the desired mutation were screened and verified by DNA sequencing.

2.6 Cells and cell culture

Jurkat (T cell leukemia) cells, Jurkat clones stably transfected with FADD-DN, caspase-8 deficient Jurkat cells, BJAB (B cell lymphoma) cells, Jurkat/Bcl-2 overexpressing cells, Jurkat Bcl-xl overexpressing cells, BJAB-FADD DN cells, MCF-7 human breast cancer cells, MCF-7/caspase-3 (provided by Dr. R. Janicke, Dusseldorf, Germany) cells, MCF-7/Bcl-2 overexpressing cells, PC-3 prostate cancer cells, L929 fibrosarcoma cells, transformed Nur77 wild type and Nur77 dominant negative cells (Suzuki et al., 2003) were grown in RPMI-1640 medium (Gibco BRL) supplemented with 10% FBS (Hyclone), 100μg/ml penicillin and 0.1μg/ml streptomycin (Gibco BRL). 293T human kidney embryonic fibroblasts, DU145 prostate cancer cells, DU145/Bax cells, primary mouse embryonic fibroblasts, transformed mouse embryonic fibroblasts, primary human lung fibroblasts (Cambrex technologies) and GL31 primary human diploid

fibroblasts were cultured in DMEM (Gibco BRL) supplemented with 10% FCS and antibiotics. All cells were grown at 37°C with 5% CO_2 in a humidified incubator and maintained in a logarithmic growth phase. Early passage human umbilical vein endothelial cells (HUVECs) were maintained in DMEM medium containing 20% FCS, 2mM glutamine, antibiotics and 10ng/ml basic fibroblast growth factor (Sigma). Primary Apaf-1 (-/-) fibroblasts (provided by Dr. F. Cecconi, Munster, Germany) and the respective wild type control cells, CDK2 (-/-) fibroblasts (Berthet et al., 2003) and their respective wild type control cells (provided by Dr. P. Kaldis, NCI, Frederick, MD) were immortalized by retroviral transduction with a temperature sensitive SV-40 large T-antigen as described (Almazan and McKay, 1992) and maintained in RPMI-1640 medium with 10% FCS and antibiotics.

2.7 Isolation of peripheral blood lymphocytes

The peripheral blood lymphocytes were isolated from chronic lymphocytic leukemia (CLL) patients or normal healthy individuals by Ficoll gradient fractionation, as described previously (Los et al., 1995a). The freshly drawn blood from an individual was diluted with an equal volume of sterile phosphate buffered saline (PBS) and carefully layered onto one-third volume of Ficoll-paque solution. The samples were centrifuged at 400g for 30 minutes at room temperature and the layer of lymphocytes between the plasma and Ficoll solution was collected carefully. Three volumes of PBS was added to the lymphocyte layer and centrifuged twice at 100g for 10 minutes to remove excess platelets, plasma proteins, granulocytes and Ficoll solution. The supernatant rich in peripheral lymphocytes was then added and maintained in RPMI growth medium with 10% FCS and antibiotics.

2.8 B cell staining and FACS analysis

Peripheral blood lymphocytes from normal individuals and CLL patients either left untreated or TAT-Apoptin treated for the indicated times were washed twice with ice cold PBS and then incubated with both CD5-FITC and CD19-PerCP antibodies (each 0.5μg per 10^6 cells) for 30 minutes at 4°C in the dark. The cells were then washed twice with cold PBS and resuspended in 300μl of PBS. Samples were analyzed by flow cytometry by using both FL1 (FITC) and FL2 (PerCP) channels and percentage of B cells was obtained by gating the double positive cells compared to unstained control.

2.9 Transfection of mammalian cells

The transfection of different mammalian primary and cancer cell lines with the desired plasmids was performed using Lipofectamine 2000 (Invitrogen) reagent. 24 h prior to transfection, the cells were plated at 60-70 % confluency in an antibiotic free medium. At the time of transfection, plasmid DNA was added to 100μl of medium in a tube at the recommended concentrations (typically 2μg for a 12 well plate and 5μg for a 6 well plate). In a second tube, Lipofectamine reagent (5μl for 12 well and 10μl for 6-well plate) was diluted in 100μl of medium. After 5 minutes of incubation, the DNA and Lipfectamine reagents were mixed and incubated for 20 minutes at room temperature to allow the formation of DNA-liposome complexes. After the incubation, the DNA-lipid mixture was added dropwise directly to the cells that had previously been rinsed with PBS and replaced with fresh medium. The transfection mix was replaced with fresh growth medium 6 hours post-transfection.

2.10 Small interfering RNA

The plasmids coding for PI3-K shRNA (pKD-PI3-Kinase, p85-V3), CDK2 shRNA (pKD-CDK2-v6) and the negative control siRNA were purchased from Upstate Cell Signaling. The $p27^{kip1}$ siRNA and Bcl-2 siRNA was obtained commercially from Santa Cruz Biotechnologies. The target siRNA for Nur77 (CAG UCC AGC CAU GCU CCU) and the scrambled siRNA were purchased from Dharmacon RNA technologies. The described plasmids or the siRNA sequences were transfected into the cells grown to 70% confluency using Lipofectamine (Invitrogen) according to the manufacturer's protocol. The expression of the proteins before and after siRNA transfection was analyzed by Western blotting after 48 hours of transfection.

2.11 Adenoviral infections

Adenoviral transfections with Akt-dominant-negative mutant vector and nuclear targeted Akt were performed according to the protocol provided by Dr. K. Walsh Lab, Boston University School of Medicine, Boston, MA and described previously (Fujio et al., 1999). Briefly, a 150 mm plate of confluent 293 cells were infected with adenovirus for 72 hours and the cells were harvested in PBS/10% glycerol by centrifugation at 3000 rpm for 10 min. The harvested cells were mixed with 1/10 voume of 5% sodium deoxycholate and incubated for 30 min, mixed and incubated with buffer A (1% 2M $MgCl_2$, 0.5% RNase A and 0.5 % DNase I) for 30 min at 37°C. Subsequently the viruses from the cell lysate were purified by first using a discontinuous $CsCl_2$ gradient and later a continuous $CsCl_2$ gradient. The purified viruses were titered on 293 cells fixed in an agarose medium mixed with growth medium (1:1) using a serial dilution technique and the numbers of plaque forming units (PFU) were counted after 6 days of incubation. Typically, 100 PFU were

used each time for infection of the desired cells to express the protein coded by adenoviral vector during the course of experiments.

2.12 RNA isolation and Northern blotting

Total RNA was isolated from the cells using RNA isolation kit (Qiagen, Mississagua, ON) by following the standard protocol provided by the manufacturer. The detection of specific mRNA species was performed by northern blotting using a non-radioactive digoxigenin (DIG) labeling method (Roche diagnostics, Mannheim, Germany). 10μg of total RNA isolated from the cells either untreated or treated with appropriate stimuli mixed with 1X RNA loading buffer [80% (w/v) deionized formamide, 10mM EDTA pH 8.0, 50% (v/v) glycerol, 0.25% (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol FF], preheated at 65°C for 3 min was loaded and run in 1.2% formaldehyde agarose gel overnight at 15V. The RNA from the gel was transferred onto a 20X SSC (3M NaCl and 300mM sodium acetate pH 7.0) pre-soaked PVDF membrane by capillary transfer using MOPS buffer (200mM MOPS, 50mM sodium acetate and 10mM EDTA). Subsequently, RNA was fixed on the membrane using UV crosslinking and pre-hybridized at 68°C for 30 min in DIG easy hybridization buffer (Roche). The membrane was then hybridized with a DIG labeled DNA probe (using oligonucleotide tailing kit from Roche) at 68°C for 4 h and washed twice each with a low stringency wash buffer (2X SSC, 0.1% SDS) and a high stringency wash buffer (0.1X SSC, 0.1% SDS). The membranes were then incubated with an HRP conjugated anti-DIG antibody (Novus Biologicals, Littleton, CO, USA) diluted in blocking buffer for 1 hour at room temperature and then washed twice with wash buffer for 15 min each. The signal for specific mRNA was detected by using enhanced chemiluminescent (ECL) staining (Amersham, Piscataway, NJ, USA).

The following probes for different genes were used for DIG labeling:

Bad – 5′ -AGT CCA CAA ACT CGT CAC TCA TCC -3′

Bax – 5′ -TGT CCA GCC CAT GAT GGT TCT GAT -3′

Bcl-2 – 5′ -TAC AGT TCC ACA AAG GCA TCC CAG -3′

Bcl-X_L – 5′ -ACT GAA GAG TGA GCC CAG CAG AA -3′

$p27^{Kip1}$ – 5′ -CAA ATG CGT GTC CTC AGA GTT AGC -3′

Cyclin A – 5′ -TTG ACT GTT GTG CAT GCT GTG GTG -3′ .

2.13 Protein purifications

2.13.1 TAT-fusion protein purification

The recombinant TAT-GFP and TAT-Apoptin proteins were expressed in BL21 bacterial cells and purified as described before (Guelen et al., 2004). Briefly, the bacterial clone expressing the recombinant protein was grown overnight in 100 ml of LB and used to inoculate 1 litre of LB medium with 50µg/ml ampicillin. The bacteria were allowed to grow for 4-5 hours at 37°C and harvested by centrifugation at 5000g (Beckman JA-10 rotor) for 10 min. Cell pellets were washed once in PBS, resuspended in 10 ml of Buffer Z (8M urea, 100mM NaCl and 20mM HEPES, pH 7.0) and sonicated by three pulses of 15 seconds each in ice. Cell debris was removed by centrifugation at 10000g for 10 minutes at 4°C. Imidazole was added to the TAT-fusion proteins in Buffer Z to a final concentration of 10 mM. TAT-fusion proteins from the supernatant were isolated by Ni-ion affinity chromatography (Amersham Pharmacia Biotech) with stepwise elutions in 1 ml fractions in buffer Z with increasing imidazole concentrations (100, 250 and 500 mM). Excess urea was removed by elution through PD-10 desalting columns (Amersham Pharmacia Biotech) using sterile PBS with 10% glycerol and protease inhibitors (complete mini, Roche). The

concentration of TAT-fusion proteins was assessed by mixing with Bradford reagent (Biorad) and the absorbance measured at 595nm using a 96 well plate reader.

2.13.2 GST-fusion protein purification

The GST and the recombinant GST-Apoptin proteins were purified by using glutathione sepharose high performance beads (Amersham Biosciences) according to the manufacturer's protocol. Briefly, the bacterial clone expressing the recombinant protein grown overnight in 50 ml of LB was used to inoculate 500 ml of LB medium with 50μg/ml ampicillin. The bacteria were allowed to grow at 37°C until they reached an OD_{600} of 0.6-0.8 and then induced with Isopropyl-β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM for 3 h. Subsequently the cells were harvested by centrifugation at 5000 RPM (Beckman JA-10 rotor) for 10 min. Cell pellets were washed once in PBS, resuspended in 10 ml of lysis buffer (1X PBS, 100 mM EDTA, 0.5 mM DTT and protease inhibitors) and sonicated three times for 45 sec. Triton X-100 was added to the cell lysate to a final concentration of 1% and incubated at 4°C for 15 min on a rotary shaker. The cell lysate was centrifuged for 15 min at 13000 RPM; the supernatant containing the GST fusion proteins was separated and added to the 1 ml of 50% glutathione sepharose beads. After incubating the fusion proteins for 4 hours at 4°C, the beads were washed three times with 50 volumes of 1X PBS/1% Triton X-100 and once with 50 mM Tris (pH 7.5)/150 mM NaCl. Finally the fusion proteins were eluted by using 3 ml of 10mM reduced glutathione in 50mM Tris (pH 8.0) and the excess salt was removed by using PD-10 desalting columns. The recombinant proteins were analysed by SDS-PAGE followed by either Coomassie staining or silver staining.

2.14 TAT-mediated protein transduction

Cells were seeded in either 6 well or 12 well culture plates (Nunc) and grown to 70-80% confluence. TAT-fusion proteins were diluted in culture medium to the desired concentrations (optimum concentration of 1 μM) and added directly to the cells. Cells were incubated with TAT-fusion proteins at 37°C in a 5% CO_2 humidified incubator.

2.15 GST-pull down assay and protein identification

The GST-pull down assay was performed to detect Apoptin's interacting partners. Briefly, either purified GST or GST-Apoptin along with total PC-3 cell lysate was immobilized on glutathione sepharose beads overnight at 4°C. The beads were washed thrice with ice-cold lysis buffer and the bound proteins were isolated on SDS-PAGE. The proteins specific for Apoptin were subjected to in-gel digestion (according to the protocol supplied by University of Manitoba proteomic centre) using trypsin and further identified by MALDI-TOF mass spectometry at the proteomics centre at the University of Manitoba. Alternatively, the proteins from the GST-pull down assay were identified by immunoblotting.

2.16 Immunoblotting

Cells were washed twice with cold PBS, lysed with ice-cold lysis buffer (50 mM Tris, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 1 mM Na_3VO_4, 2 mM EGTA, protease inhibitor cocktail), incubated for 10 min on ice, and centrifuged for 10 min at 4°C. The protein concentration in the supernatant (cell lysate) was estimated by Bradford assay. 30 μg of the protein lysate was mixed with 1X SDS loading dye (100 mM Tris pH6.8, 4% SDS, 0.2% bromophenol blue, 20 % glycerol and 200 mM β-mercaptoethanol), heated for

5 min at 99°C. The protein samples were resolved electrophoretically on SDS-polyacrylamide gels and transferred onto a PVDF-membrane (Amersham biosciences) using a semi-dry transfer apparatus (Biorad) for 1h at constant current of 85 mA. Membranes were blocked for 1h with 5% non-fat dry milk powder in tris-buffered saline (TBS) and then immunoblotted overnight with the appropriate primary antibody diluted in blocking buffer. Membranes were washed three times for 10 min in 0.25% v/v Tween-20 in TBS and later incubated with an appropriate secondary antibody conjugated with horseradish peroxidase (HRP) for 1 h at room temperature. Following the repeated washing steps after secondary antibody, the specific proteins on the membrane were detected by using enhanced chemiluminescent (ECL) staining (Amersham).

2.17 Immunoprecipitation

Depending on the protein, 100-500 μg of cell lysate was added with 2-5 μg of appropriate antibody and incubated for 4 h at 4°C agitating on a rotary shaker. Subsequently, 100 μl of 50% protein-A agarose beads (Amersham Pharmacia Biotech) washed twice with 1X PBS was added to the protein-antibody immune complexes and further incubated for 1 h at 4°C. After the incubation, the beads were washed four times with the lysis buffer, each time centrifuging at 4°C and removing the supernatant. After final wash, the beads were suspended in 50 μl of 2X SDS loading dye, boiled for 5 min at 95°C and the proteins bound to the beads were collected by brief centrifugation at 13000 g. The proteins were resolved on SDS-PAGE and detected by immunoblotting with appropriate antibodies.

2.18 Cell fractionation

The separations of the cellular cytoplasmic, nuclear and mitochondrial fractions were done by using differential centrifugation (Wu et al., 2002). Briefly, the cells were treated with apoptin and then harvested and washed once with PBS after indicated time points. The cells were re-suspended for 5 min on ice in a lysis buffer: 10 mM Tris-HCl (pH 7.8), 1% Nonidet P-40, 10 mM β-mercaptoethanol, 0.5 mM PMSF, 1 mg/ml aprotinin and 1 mg/ml leupeptin. In some experiments, an equal amount of distilled water was added to the cells in order to increase the cell lysis. Cells were then sheared by passing them through a 22-gauge needle. The nuclear fraction was recovered by centrifugation at 600 g for 5 min, and the "low speed" supernatant was centrifuged at 10,000g for 30 min to obtain the mitochondrial fraction (pellet) and the cytosolic fraction (supernatant). The mitochondrial fraction was further lysed in the buffer: 10 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100 and 5 mM EDTA (pH 8.0).

2.19 Immunocytochemistry and confocal imaging

Cells were grown overnight on coverslips and then treated with appropriate stimuli. At the desired time of experiment, the cells were washed with PBS and then fixed in 4% w/v paraformaldehyde in PBS. Then, the cells were permeabilized with permeablization buffer (0.1% triton X-100 in PBS) by incubating for 15 minutes at room temperature. The cells were blocked with 3% BSA/PBS for 30 min and subsequently incubated with an appropriate primary antibody diluted in the blocking buffer. After the incubation, three washes of each 5 min with 1X PBS were performed followed by an appropriate secondary antibody conjugated with FITC, Cy3 or Cy5 depending on the experiment. To visualize nuclei, cells were co-stained with DAPI (10 μg/ml). The mitochondria were stained with

Mitotracker Red CMXRos (Molecular probes) (200 nM in RPMI medium for 15 minutes, prior to fixing), a mitochondrial specific dye. The cells were finally fixed on the slides and the fluorescent images were then analyzed using an Olympus-IX81, a multi-laser confocal microscope.

2.20 Measurement of mitochondrial membrane potential

Mitochondrial permeability transition was determined by staining the cells with 5,5',6,6'-tetrachloro-1,1',3,3'- tetraethyl-benzimidazolyl carbocyanine iodide (JC-1; Molecular Probes) as described previously (Bedner et al., 1999). Briefly, the cells treated with appropriate stimuli were harvested and resuspended in 1 ml of fresh medium. JC-1, the mitochondrial dye was added to the cell suspension at a concentration of 2.5μg/ml and samples were incubated in the dark for 20 min. After the incubation, the cells were washed twice with 1X PBS by centrifuging the samples at 500g for 5 min. After the washes, the cells were resuspended in 300μl of PBS and the mitochondrial membrane potential was quantified by flow cytometric determination of cells with decreased fluorescence in FL-2 channel. Data was collected and analysed using a FACSCalibur (Becton Dickinson) equipped with CELLQuest software. Data was given in percent cells with low $\Delta\Psi m$, which reflects the percentage of cells losing mitochondrial membrane potential.

2.21 Apoptosis and cell proliferation assays

2.21.1 Morphological detection of cell death

Cells were grown on coverslips and treated with appropriate apoptosis inducing agents or apoptotic inhibitors. Cells were then washed twice with PBS, fixed for 15 min in 3.7% v/v paraformaldehyde in PBS and stained with the DNA dye DAPI (10 μg/ml,

Molecular probes, Eugene, OR) in the presence of 0.1mg/ml RNase A. The morphology of the cells was examined by using phase contrast microscopy and the nuclear alterations of apoptosis were visualized by confocal imaging.

2.21.2 Measurement of cell death by propidium iodide uptake

Cells subjected to appropriate experimental conditions of apoptosis were washed once with PBS and collected by centrifugation at 800g for 10 min at room temperature. The cells were suspended in 1X PBS and propidium iodide was added (1 μg/ml for suspension cells and 0.5 μg/ml for adherent cell types) and the PI fluorescence was measured directly by flow cytometry using FL-3 channel. Living cells with intact cell membrane were PI-negative, whereas dead cells with a permeable membrane were PI-positive with a stronger fluorescence signal. The PI-positive cells were gated using BD cellquest pro software and represented in terms of percentage of cell death.

2.21.3 Measurement of apoptotic hypodiploid nuclei

The amount of apoptotic hypodiploid nuclei (<2N) was measured by using the Nicoletti method (Nicoletti et al., 1991). Briefly the cells treated with appropriate apoptotic stimuli for the indicated times were harvested by centrifugation at 800g for 5 minutes. The cells were washed once with PBS, and then resuspended in a hypotonic PI lysis buffer (1% sodium citrate, 0.1% Triton X-100, 0.5 mg/ml RNase A, 40 μg/ml propidium iodide). Cell nuclei were then incubated for 30 minutes at 30°C and were subsequently analyzed by FACS. The histograms of normal DNA content shows typical G1-S-G2/M cell cycle peaks, but the nuclei left of the G1 (2N DNA) peak, which contains hypodiploid DNA (<2N) were considered and represented as percentage of apoptotic cells.

2.21.4 MTT assay

The MTT assay was performed as described previously (Ghavami et al., 2005). Cells grown in 96 well plates were added with 10 μl of 5 mg/ml tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)]. They were then incubated for 4 hours at 37°C. After the incubation, the cells were spun down at 90g for 10 min and the supernatant was discarded by rapid inverting of the plate. The formazon crystals were dissolved by adding150 μl of ethanol:DMSO mix (1:1) to each well. Finally, the absorbance was measured at λ=570nm using a spectrophotometer and the percentage of living cells in experimental wells was calculated by comparing to the control cells.

2.21.5 Determination of cell senescence

The cellular senescence was assessed by using a senescence β-galactosidase staining kit (Cell Signaling technologies) according to the manufacturer's protocol. Briefly, cells treated with appropriate stimuli were washed twice with 1X PBS and fixed in a fixative solution (20% formaldehyde and 2% glutaraldehyde in 10X PBS) for 15 min at room temperature. After incubation, the cells were washed twice with 1X PBS and incubated with 1ml of staining solution (400 mM citric acid/sodium phosphate pH6.0, 1.5M Nacl, 20 mM $MgCl_2$, 500 mM potassium ferrocyanide, 500 mM potassium ferricyanide and 20 mg/ml X-Gal) overnight at 37°C. Next day, the cells were checked under the light microscope (40X magnification) for the development of blue color and the images were captured.

2.22 Cell cycle analysis

The cell cycle analysis was performed as described before (Alvarez et al., 2001). To arrest the cells in G0, cells were grown to confluency and maintained in serum free medium for 36 hours. For G1 phase, cells were collected after 1 h of addition of serum to the serum-starved cells. To arrest in S phase, the cells were treated with 0.5μg/ml of Aphidicolin for 20 h, which gives approximately 80% cells in S phase measured by BrdU pulse labeling. For G2 arrest, the cells were incubated with 5 μM etoposide (Sigma), which yielded around 70% of cells in G2 phase. For M phase arrest, nocodozole (0.8 μg/ml) was used, which yielded nearly 90% of cells in M phase. The quantification of G1/S and G2/M populations was done by hypotonic cell lysis followed by labeling the DNA with propidium iodide and analysis was performed by flow cytometry using FL3-channel. The cell cycle phase analysis for the experiment shown in figure 29C was performed using BrdU-FITC flow staining kit (BD-Pharmingen), according to manufacturer's instructions. Briefly, cells were serum-starved for 36 h. After the release from G0 by addition of serum, they were pulse-labeled at the indicated time points using BrdU, following by labeling with propidium iodide. To analyze the transition from S to G2 phase, the cells were arrested in S phase for 20 hours after transfecting with desired expression vectors and labeled with BrdU-FITC and 7-AAD. Later the cells were released from S phase arrest for the indicated times and percentage of G2 phase cells was quantified by flow cytometry.

2.23 PI3-kinase ELISA

A non-radioactive competitive ELISA based assay was used to assess the PI3-K activity under different conditions according to the manufacturer's protocol (Echleon Biosciences). Briefly, equal amounts of PI3-K from the PC-3 cell lysates were immuno-

precipitated with anti-p85 antibodies overnight at 4°C and then incubated with protein A-Sepharose beads for 1 h at 4°C. The bead-bound enzymes were incubated with 100 pM of phosphatidylinositol (4, 5) bisphosphate (PtdIns (4, 5) P_2) substrate in kinase reaction buffer (4 mM $MgCl^2$, 20 mM Tris, pH 7.4, 10 mM NaCl, and 25 μM ATP) for 2 h at room temperature. The mixtures were then incubated with phosphatidylinositol (3,4,5) trisphosphate (PtdIns (3,4,5) P_3) detector for 1 h at room temperature in the dark, and subsequently added to PtdIns (3,4,5) P_3-coated microplate wells, and incubated for 30 min at room temperature in the dark. After thorough washing, peroxidase-linked secondary detection reagent was added, and PtdIns (3,4,5) P_3 detector protein binding to the plate was assessed by measuring absorbance at 450 nm. The data for the kinase activity are expressed as fold induction in transfected cells compared with the activity in untreated cells.

2.24 *In vitro* kinase assays

The *in vitro* kinase assays were performed using a non-radioactive based method. Briefly, 5 ng of the recombinant CDK2/cyclin A (New England Biolabs), active Akt, inactive Akt, CDK2/cyclin E, CDK1/cyclin B (Upstate) or the immuno-precipitated CDK2, CDK1, cyclin A, cyclin B, cyclin E were used in a kinase reaction with 5μg of Histone H1, GST-Apoptin, TAT-Apoptin or GST-CDK2 as substrates in the presence of 200μM ATP in a kinase assay buffer (25 mM Tris pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na_3VO_4 and 10 mM $MgCl_2$). The kinase reaction was performed at 30°C for 45 min and the end products were resolved by SDS-PAGE (12-15%) and detected by immunoblotting using their respective phospho-specific antibodies.

3. Results

3.1 Apoptin activated death pathways

3.1.1 Apoptin induces cell death specifically in cancer cells but not in normal cells

Apoptin, a CAV derived protein, has been shown to be specifically toxic to cancer cells but not to normal primary cells. To study the selective toxicity of Apoptin in our model cell systems, we have used two different recombinant Apoptin proteins. The first recombinant protein, an N-terminal GFP tagged Apoptin was constructed in a pEGFP-C1 vector and the expression of Apoptin in different cells was achieved by Lipofectamine mediated transfection protocol. The alternative approach to express Apoptin in the cells was by using a recombinant TAT-Apoptin. TAT-Apoptin purified by using Nickel-affinity chromatography can be added to the cells directly in the medium. The TAT peptide acts as a passive transporter for Apoptin and thus Apoptin was expressed in the cell by transduction process. We tested the selective toxicity of GFP-Apoptin by using human diploid fibroblasts as primary cells and Saos-2 osteosarcoma cells as cancerous cells. After 24 and 48 hours, GFP-Apoptin was toxic to Saos-2 cells but not the primary diploid fibroblasts. GFP alone used as a negative control showed no significant toxicity either in primary or cancer cells (Fig. 6A & 6B). In other experiments, the peripheral blood lymphocytic (primary) cells (PBLs) were fully resistant to TAT-Apoptin (Fig. 6C.). TAT-GFP was used as a negative control, whereas actinomycin D was used as positive control. IL-2 (5 U) added to some sets of PBLs to prevent spontaneous cell death didn't affect the cell death induced by Apoptin. In contrast to primary cells, Jurkat T cell leukemia cells were highly sensitive towards TAT-Apoptin treatment (Fig. 6D). In addition, we tested

HUVEC, normal human lung fibroblasts and mouse embryonic fibroblasts as primary cells for Apoptin toxicity. MCF-7, PC-3, 293 cells and transformed mouse fibroblasts were used as cancer/transformed cells for assessing Apoptin mediated cancer specific cell death (Table 2). All the primary cells tested except mouse embryonic fibroblasts showed no sensitivity to cell death with either GFP-Apoptin or TAT-Apoptin. But, all the cancer cells tested show significant sensitivity towards Apoptin induced cell death, thus confirming the previously reported selective toxicity of Apoptin.

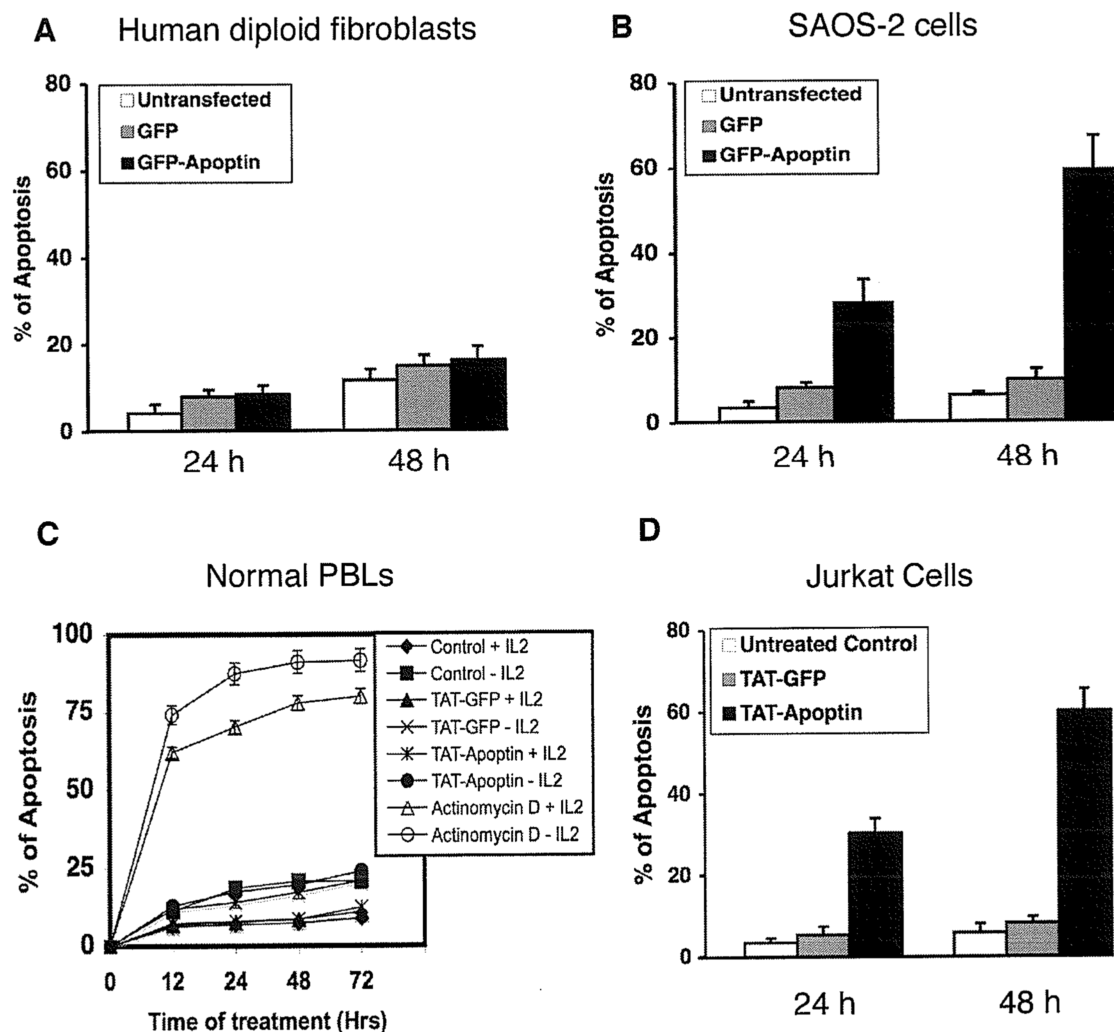


Figure 6: Apoptin is selectively toxic to cancer cells but not normal cells. (A) Primary human diploid fibroblasts or **(B)** Saos-2 osteosarcoma cells were left untransfected or

transfected with 5μg of either GFP or GFP-Apoptin plasmids. The cell death was measured by PI staining of hypo diploid nuclei at the indicated times of 24 and 48 h. **(C)** Normal peripheral blood lymphocytes (PBLs) treated with recombinant TAT-GFP (1μM) or TAT-Apoptin (1 μM) for the indicated times or with 5μg/ml Actinomycin D as a positive control. The cell death was measured as in Figure 6A. IL-2 (5U) was added to some samples to counteract the spontaneous cell death. **(D)** Jurkat cells were left untreated, treated with either TAT-GFP or TAT-Apoptin for the indicated times of 24 and 48 hours and subsequently the cell death was measured by PI uptake. The error bars shown in figures 6A-D represent the standard deviation of the data from three independent experiments. All the data presented in figures 6A-D was statistically significant ($p<0.05$) as measured by student's t-test.

Table 2: Comparison of Apoptin's toxicity in primary and cancer cells

	GFP-Apoptin toxicity*		TAT-Apoptin toxicity*	
Cell type	24 h	48 h	24 h	48 h
Primary cells				
HUVEC	3.33 ± 2.2	5.46 ± 3.7	4.13 ± 2.2	4.70 ± 1.9
Normal Human Lung fibroblasts	5.13 ± 2.6	7.56 ± 3.2	5.10 ± 4.2	4.28 ± 2.2
Mouse Embryonic Fibroblasts	20.2 ± 1.9	27.1 ± 4.8	25.9 ± 5.4	26.8 ± 3.5
Cancer/transformed cells				
MCF-7 cells	22.9 ± 3.2	42.8 ± 3.5	31.7 ± 6.8	53.7 ± 5.1
PC-3 cells	46.8 ± 3.4	58.9 ± 6.0	63.2 ± 4.5	67.1 ± 3.4
293 cells	57.6 ± 2.7	65.4 ± 4.3	63.3 ± 3.7	69.5 ± 1.1
Immortalized Mouse Fibroblasts	31.6 ± 5.1	35.4 ± 5.5	41.6 ± 6.4	47.9 ± 1.7

* Apoptin's toxicity was expressed in the percentage of dead cells measured by PI-uptake. The data presented with +/- indicate the standard deviation of five different experiments.

In addition to cell lines, we compared Apoptin's toxicity using PBLs from CLL patients compared to the PBLs from a normal healthy individual. B cells in CLL and normal PBLs were detected by double staining for the specific cell surface markers CD5 and CD19. In normal PBLs, there was no significant difference in the B-cell population before (6.1%) or after (5.2%) 48 hours of treatment with TAT-Apoptin. In contrast, there was a significant decrease in the CLL PBLs upon TAT-Apoptin treatment (11.2%) compared to the control (40.1%) (Fig. 7). Thus, the data indicates that Apoptin effectively kills the malignant B cells from CLL patients, but not the normal counterparts, therefore confirming Apoptin's selective toxicity. TAT-GFP, used as a negative control for the toxicity, had no significant effect on the survival of both normal and malignant cells.

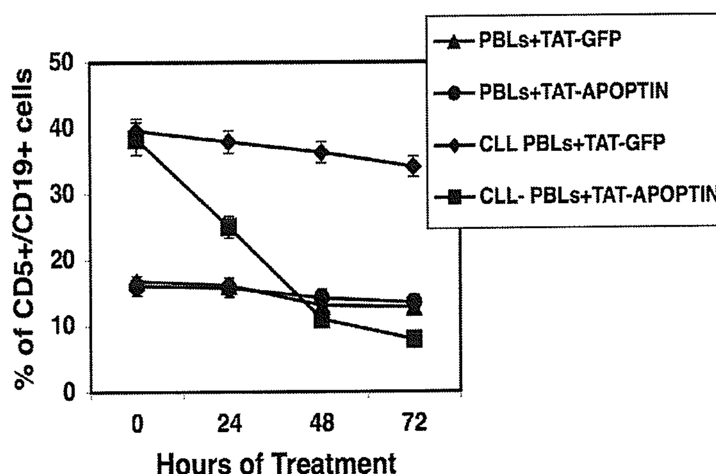
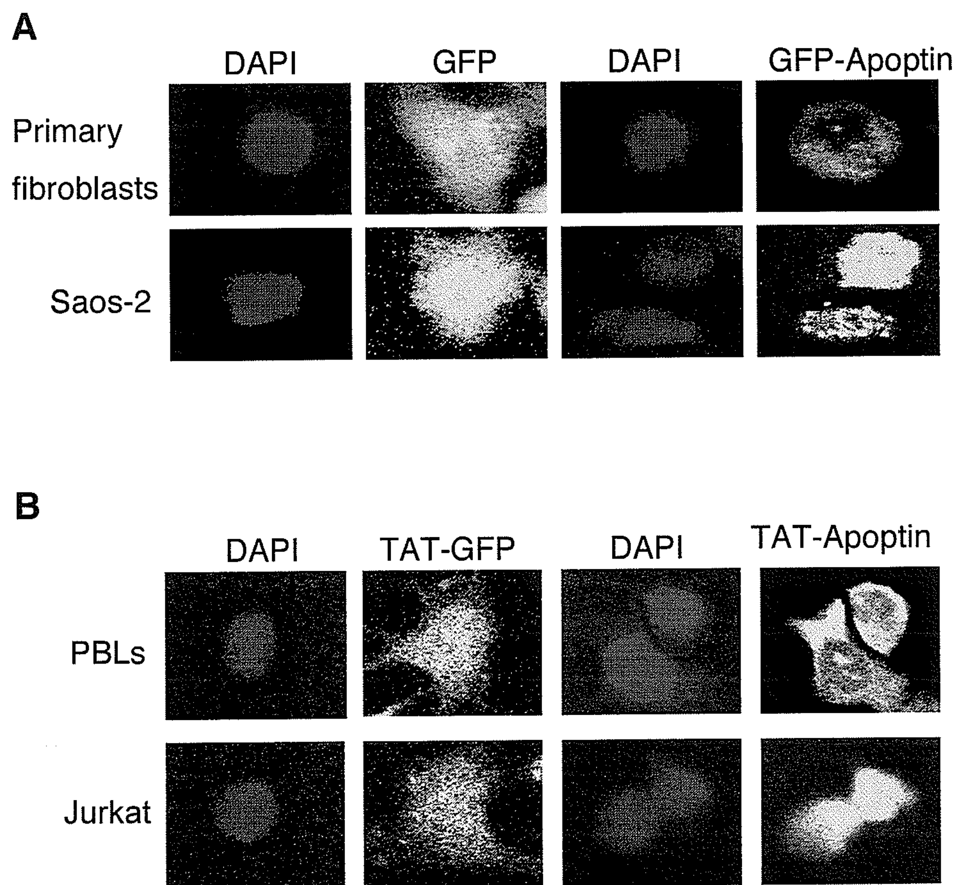


Figure 7: Apoptin kills CLL B lymphocytes but not normal lymphocytes. The B cells from either normal PBLs or the CLL PBLs were double stained for CD5/CD19 surface markers using FITC conjugated CD5 and PerCP conjugated CD19 antibodies at different indicated time points after either TAT-GFP or TAT-Apoptin treatment. The samples were then analysed by flow cytometry and the percentage of CD5/CD19 double positive cells were plotted. The error bars shown in the graph represent the standard deviation of the data from four independent experiments and the data is statistically siginificant ($p<0.02$).

3.1.2 Apoptin is differentially localized in primary and cancer cells

The selective toxicity of Apoptin towards cancer cells has been reported to be dependent on the differential localization of Apoptin in normal and cancer cells (Danen-Van Oorschot et al., 2003). Thus, we tested if Apoptin is differentially localized in primary and cancer cells. GFP control vector transfection either into primary human fibroblasts or into Saos-2 osteosarcoma cell line resulted in even distribution of the GFP, whereas GFP-Apoptin transfection into primary cells resulted in predominantly cytoplasmic distribution of Apoptin, whereas it was mainly localized in the nucleus of Saos-2 cells (Fig. 8A).



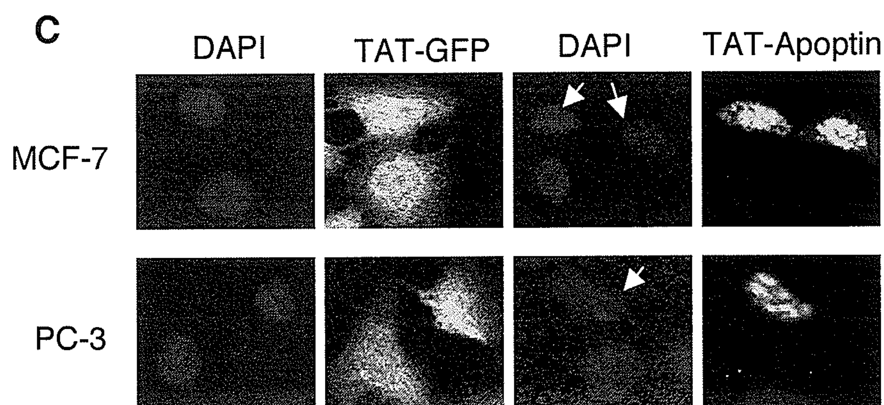


Figure 8: Apoptin is differentially localized in primary and cancer cells. (A) Primary human diploid fibroblasts or Saos-2 osteosarcoma cells were transfected with either GFP or GFP-Apoptin. After 24 h, the cells were fixed and the localization of the GFP and Apoptin was analysed by using confocal imaging. DAPI was used to stain the nucleus. **(B)** Normal PBLs, Jurkat cells, **(C)** MCF-7 and PC-3 cells were treated either with TAT-GFP or TAT-Apoptin for 24 h and the localization of the proteins was detected by immunostaining with anti-HA antibodies (HA tag is incorporated in both the recombinant proteins) followed by confocal microscopy. Cells were counterstained with DAPI. The arrows indicate the nuclei with apoptotic morphology.

We also tested for the differential localization of TAT- tagged GFP and Apoptin, as we will be using these proteins in some of our experimental systems to replace the GFP and Apoptin transfections. In primary PBLs, TAT-Apoptin was localized in the cytoplasm, but in Jurkat cells it was found in the nucleus. TAT-GFP used as a control was distributed evenly in the cells (Fig. 8B). Similarly, MCF-7 and PC-3 cells show nuclear accumulation of TAT-Apoptin and even distribution of control TAT-GFP (Fig. 8C). Thus, the data confirms that Apoptin is differentially localized to be selectively toxic for transformed cells.

3.1.3 Apoptin induced cell death is independent of the death receptor pathway

During apoptosis a cell employs either of the two distinct cell death pathways, the death receptor pathway or the mitochondrial pathway and sometimes both. FADD and caspase-8 are the key downstream signalling components of the TNFR/NGFR receptor family, which initiates apoptotic signalling through the death receptor activation. To investigate the role of the death receptor pathway in Apoptin triggered death we used Jurkat T cells, which lacked the expression of caspase-8 (Jurkat-caspase-8$^{(-/-)}$). The caspase-8-deficient Jurkat cells were equally sensitive to TAT-Apoptin-induced apoptosis as compared with the wild type Jurkat cells (Fig. 9A). Next, we tested the role of the FADD adaptor molecule in the Apoptin-triggered death pathway by using Jurkat cells overexpressing a truncated, dominant-negative form of FADD (FADD-DN). Overexpression of FADD-DN prevents formation of a functional DISC, and thus the activation of caspase-8 is not triggered by CD95-L/Fas-L, TRAIL or anti-APO-1-antibodies (Sprick et al., 2000; Stroh et al., 2002). The cells were treated with TAT-Apoptin and, after the indicated time points; apoptosis was measured by flow cytometry. After 48 hours of Apoptin treatment, about 55% of both the parental Jurkat cells and the cell clones overexpressing FADD-DN were killed (Fig. 9B). The same pattern is observed with the cells treated for 72 and 96 h respectively. In a control experiment, Jurkat cells lacking FADD or caspase-8 activity and the 'wild type' cells were treated with agonistic anti-CD95 antibody that cross-links the CD95/APO-1/Fas receptor molecule and induces cell death via a caspase-8/FADD dependent mechanism. After 8 h of treatment with the anti-CD95 antibody, wild type cells showed an increased amount of cell death, whereas the cells lacking either caspase-8 or overexpressing FADD-DN, were completely resistant

(Fig. 9C). These results suggest that cell death induced by Apoptin does not depend on caspase-8 or FADD.

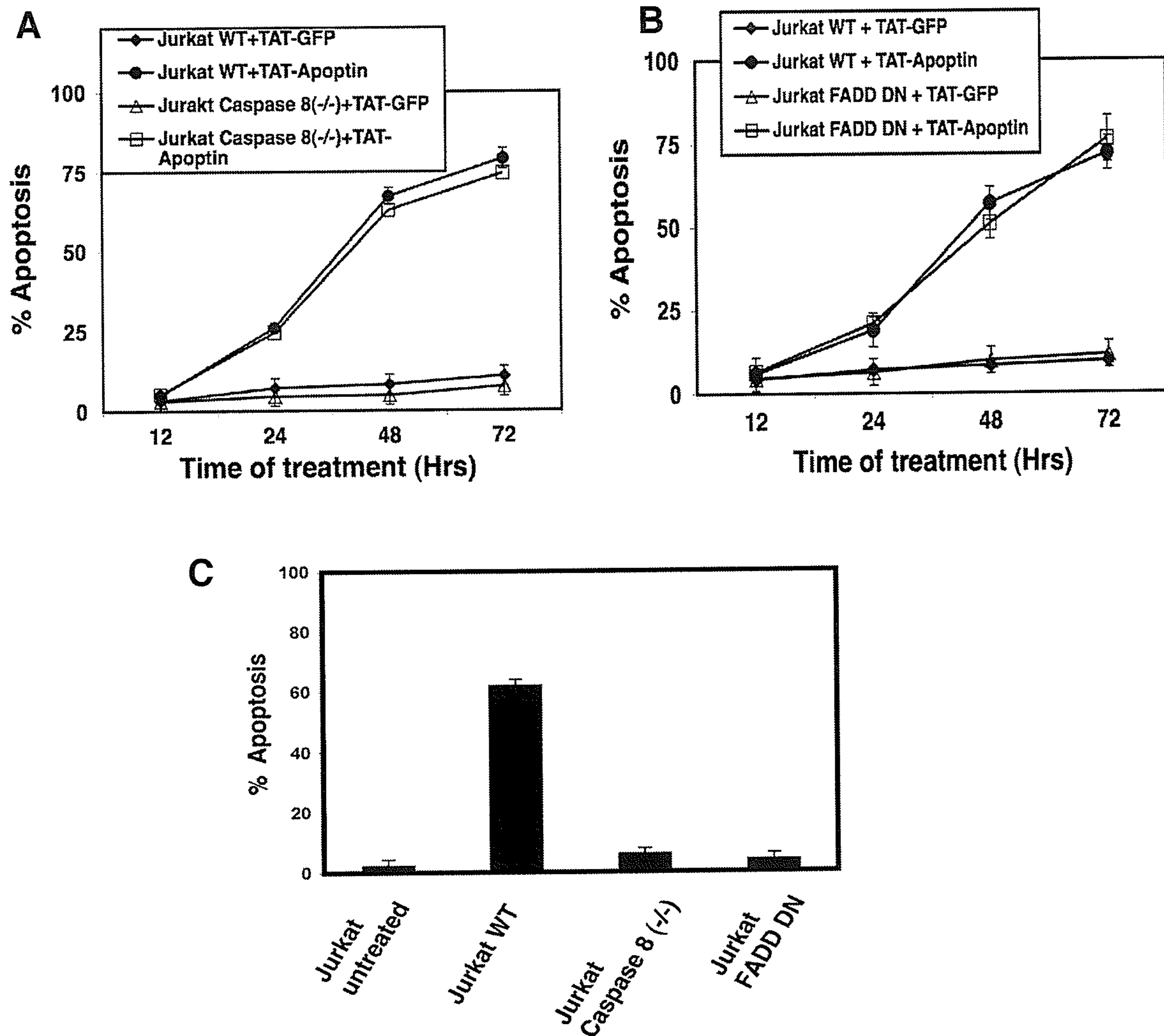


Figure 9: Apoptin induced cell death is independent of caspase-8 and FADD. (A) Jurkat wild type (WT), Jurkat caspase-8 (-/-) cells **(B)** Jurkat cells expressing FADD dominant negative vector (FADD-DN) were either treated with TAT-GFP or TAT-Apoptin for the indicated times. The percentage of cell death was measured by Nicoletti method followed by flowcytometry. **(C)** Jurkat cells either left untreated or treated with agonistic anti-CD95 antibody (0.1μg/ml) for 8 h and apoptosis was measured by flow cytometry. CD95 treated Jurkat caspase-8 (-/-) cells and Jurkat FADD-DN cells are also shown. The error bars shown in figures 9A-C represent the standard deviation of the data from three independent experiments. The data is statistically significant ($p<0.05$) as measured by student's t-test.

In order to further confirm the above findings we inhibited the activation of CD95 by using a neutralizing anti-APO-1 (IgG_1) antibody. Unlike the anti-APO-1 IgG_3 and anti-CD95 IgM antibodies that can exist as polymers and can thus activate the CD95/Fas/APO-1, anti-APO-1 IgG_1 exists only in the monomeric form and thus it fails to induce CD95 trimerization that is required for the activation of CD95-death pathway. The anti-APO-1 IgG_1 antibody strongly reduced cell death induced by the stimulatory anti-CD95 (IgM) antibody (Fig. 10B). By contrast, apoptosis induced by Apoptin was not affected by the neutralizing anti-CD95 antibody (Fig. 10A). Thus, all of the above data indicate that Apoptin acts independently of the death receptor-signalling pathway.

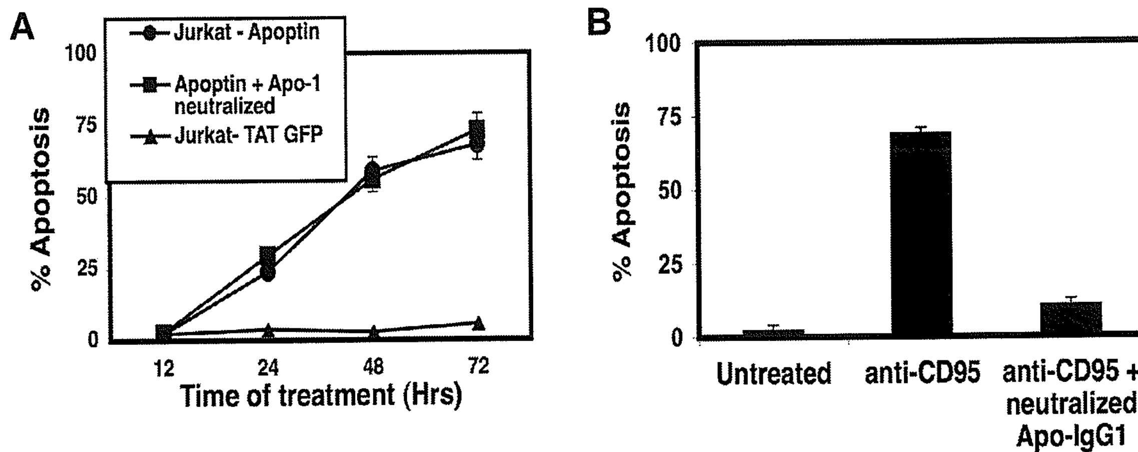


Figure 10: CD95/Fas blocking has no effect on Apoptin induced cell death. (A) Flow cytometry analysis of apoptosis in Jurkat cells, either left in medium or incubated with a human anti-Apo-1-IgG1 (a neutralizing antibody that blocks the interaction of CD95/Apo-1/Fas and its ligand), treated with TAT-Apoptin for the indicated times. To maintain the blockade, incubation with the blocking antibody was repeated every 8 h. TAT-GFP was used as a negative control. **(B)** Jurkar cells were either left untreated or treated with activating CD95 alone or in combination with the neutralizing Apo-IgG, and cell death was measured after 8 hours. The error bars shown in the figures 10A & 10B represent the standard deviation of the data from three independent experiments. The data is statistically significant ($p<0.01$) as measured by student's t-test.

3.1.4 Loss of mitochondrial potential occurs during Apoptin induced cell death

To gain more insight into the mechanism of apoptotic signalling triggered by Apoptin, we investigated the mitochondrial/apoptosome dependent apoptotic pathway. The loss of mitochondrial potential ($\Delta\Psi M$), an early indicator of mitochondria mediated apoptosis, was monitored by JC-1, a mitochondrial dye which shows decreased fluorescence when the mitochondrial membrane potential is lost. Cells treated with Apoptin showed a significant loss of mitochondrial membrane potential as compared to the untreated cells (Fig. 11). Furthermore, the deficiency of either caspase-8 or FADD displayed no effect on the loss of mitochondrial membrane potential caused by Apoptin. This experiment indicates that Apoptin triggered cell death is associated with the loss of mitochondrial membrane potential, and it further suggests that Apoptin kills the cells independently of the death receptor pathway.

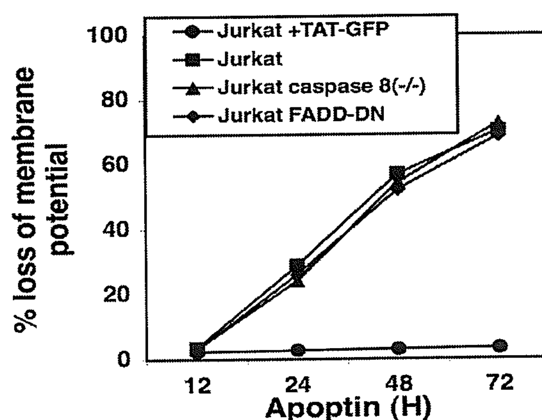


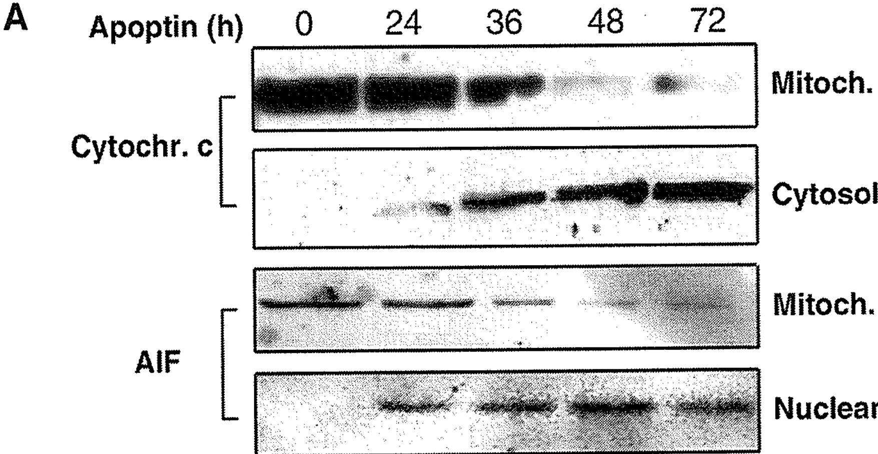
Figure 11: Mitochondrial membrane potential is lost during Apoptin-induced apoptosis. Jurkat wild type cells, caspase-8 (-/-) Jurkat cells and FADD-DN cells were treated with TAT-Apoptin for the indicated times and the mitochondrial membrane potential ($\Delta\psi M$) was measured by using flow cytometry after staining the cells with a cationic carbocyanine dye, 5, 5′, 6, 6′- tetrachloro- 1,1′, 3, 3′- tetraethyl benzimidazolyl carbocyanine iodide (JC-1), which shows a decrease in red fluorescence upon loss of membrane potential. Similar results were seen in three independent sets of experiments.

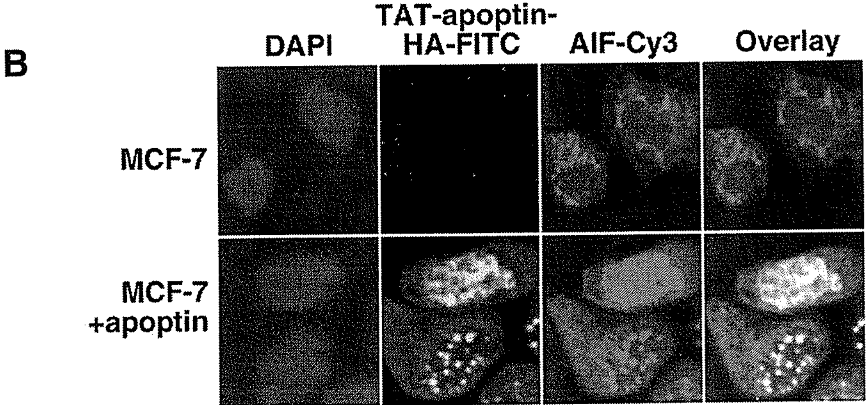
3.1.5 The mitochondrial components cytochrome c and AIF are released upon Apoptin triggered cell death

To further examine the effect of Apoptin on mitochondria, we studied the release of various factors from the mitochondria. To check the release of cytochrome c from mitochondria upon Apoptin induced cell death, we separated various fractions of cells treated with TAT-Apoptin and then detected the protein by Western blot. As shown in figure 12A, after 24-36 hours of Apoptin treatment, significant quantities of cytochrome c are detected in the cytosol. In a similar way, we have studied the release of AIF from mitochondria during Apoptin induced cell death. AIF, similarly to cytochrome c, starts to appear in the cytoplasm around 24-36 h after Apoptin treatment. AIF, unlike cytochrome c, goes to the nucleus after it is released from mitochondria. In the nucleus AIF causes "high-molecular-weight" DNA fragmentation and chromatin condensation (Loeffler and Kroemer, 2000). Using confocal microscopy, we then investigated whether Apoptin caused the nuclear accumulation of AIF. As shown in figure 12B, in the absence of Apoptin, AIF is localized in mitochondria. However, when the cells are transfected with TAT-Apoptin, after 30 hours most of the AIF is released from mitochondria and transferred into the nucleus.

The involvement of the mitochondrial death pathway during Apoptin induced cell death has further been established by testing the role of Apaf-1, a mitochondrial downstream apoptotic-signalling molecule. The transfection of Apoptin or the transduction of TAT-Apoptin into transformed wild type mouse fibroblasts results in significant cell death after 24 to 48 hours, whereas the Apaf-1 null fibroblasts were resistant to Apoptin induced cell death (Fig. 12C). The control GFP or TAT-GFP treated cells showed no

significant cell death either in wild type or Apaf-1 null fibroblasts. These results indicate the requirement of Apaf-1 during Apoptin signalling thus confirming the involvement of the mitochondrial death pathway.





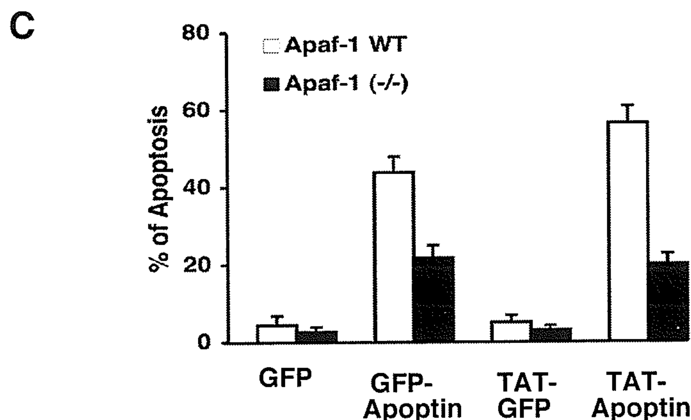
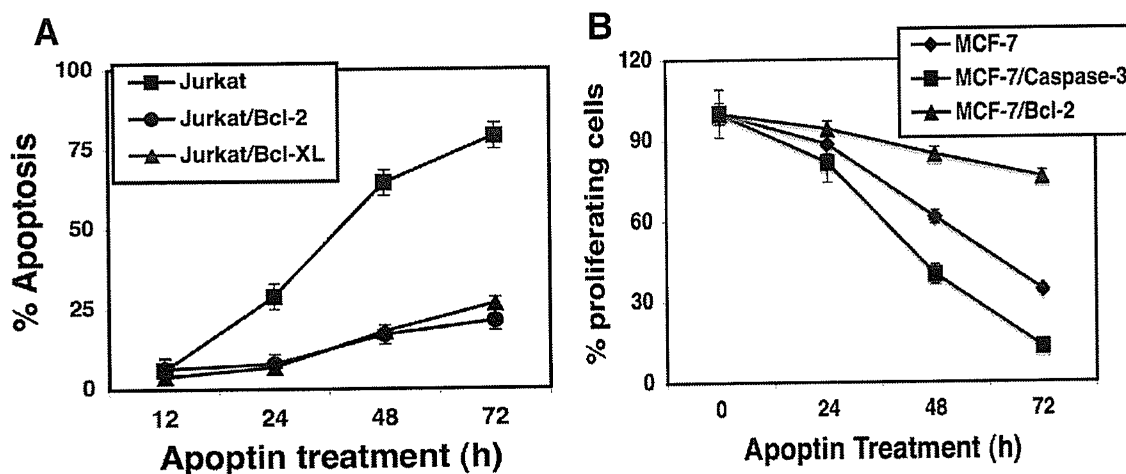


Figure 12: Apoptin triggers the activation of the mitochondrial death pathway and requires the downstream involvement of Apaf-1. (A) Western blot analysis of cytochrome c and AIF in mitochondrial (mitocho.), cytosolic (cytosol) and nuclear fractions of Jurkat cells treated with TAT-Apoptin. **(B)** MCF-7 cells were either left untreated or stimulated with Apoptin. After 36 hours, the cells were immunostained with anti-AIF antibody followed by Cy-3 conjugated secondary antibody and with an anti-HA antibody followed by a FITC conjugated secondary antibody. DAPI was used to counterstain the nuclei. The images were acquired and analysed by using confocal microscope. **(C)** The Apaf-1 wild type (WT) and Apaf-1 knock-out (-/-) fibroblasts were either transfected with GFP, GFP-Apoptin or treated with TAT-GFP and TAT-Apoptin. After 48 h the percentage of apoptosis was measured by PI staining of hypodiploid nuclei. The error bars shown in the graph represent the standard deviation from three indpendent experiments and the data is statistically significant ($p<0.002$) according to the student's t-test.

3.1.6 Bcl-2 family members modulate Apoptin induced cell death

The pro-apoptotic and anti-apoptotic Bcl-2 family members either positively or negatively modulate the mitochondrial cell death pathway. We therefore tested if the over-expression of Bcl-2 or Bcl-X_L, affects the Apoptin triggered death pathway. As shown in figure 13A, both Bcl-2 and Bcl-X_L strongly inhibited Apoptin-induced cell death upon their overexpression in Jurkat cells compared to the wild type Jurkat cells. To broaden the above conclusion, we have tested the effect of Apoptin on the MCF-7 human breast adenocarcinoma cells that lack the expression of caspase-3, a clone re-transfected with the

caspase-3 and an MCF-7 overexpressing Bcl-2. MCF-7 cells showed significant sensitivity towards TAT-Apoptin, which was augmented by the expression of caspase-3 but significantly inhibited by Bcl-2 (Fig. 13B). The pro-apoptotic Bcl-2 family member Bax promotes Apoptin induced cell death, as Bax deficient DU145 prostate cancer cells stably transfected with Bax were more sensitive to Apoptin than the wild type DU145 cells (Fig. 13C). We also have observed that Apoptin modulates the role of Bcl-2 family members by controlling the balance between the pro and anti-apoptotic Bcl-2 family members. Apoptin expression in MCF-7 breast cancer cells leads to the up-regulation of selective pro-apoptotic Bcl-2 family members like Bax and Bad but not Bak (Fig 13D). The northern blot analysis of Bax and Bad mRNA revealed higher levels of their expression in Apoptin transfected cells compared to the control cells indicating that the upregulation of Bax and Bad is due to the increase of their mRNA levels (Fig. 13E). In parallel, the expression levels of anti-apoptotic Bcl-2 family member Bcl-2, but not Bcl-xL were downregulated at the protein level (Fig.13D). There was no difference observed at the mRNA levels of Bcl-2 in Apoptin transfected cells compared to control cells (Fig.13E), thus indicating that the downregulation of Bcl-2 may be at the post-translational level.



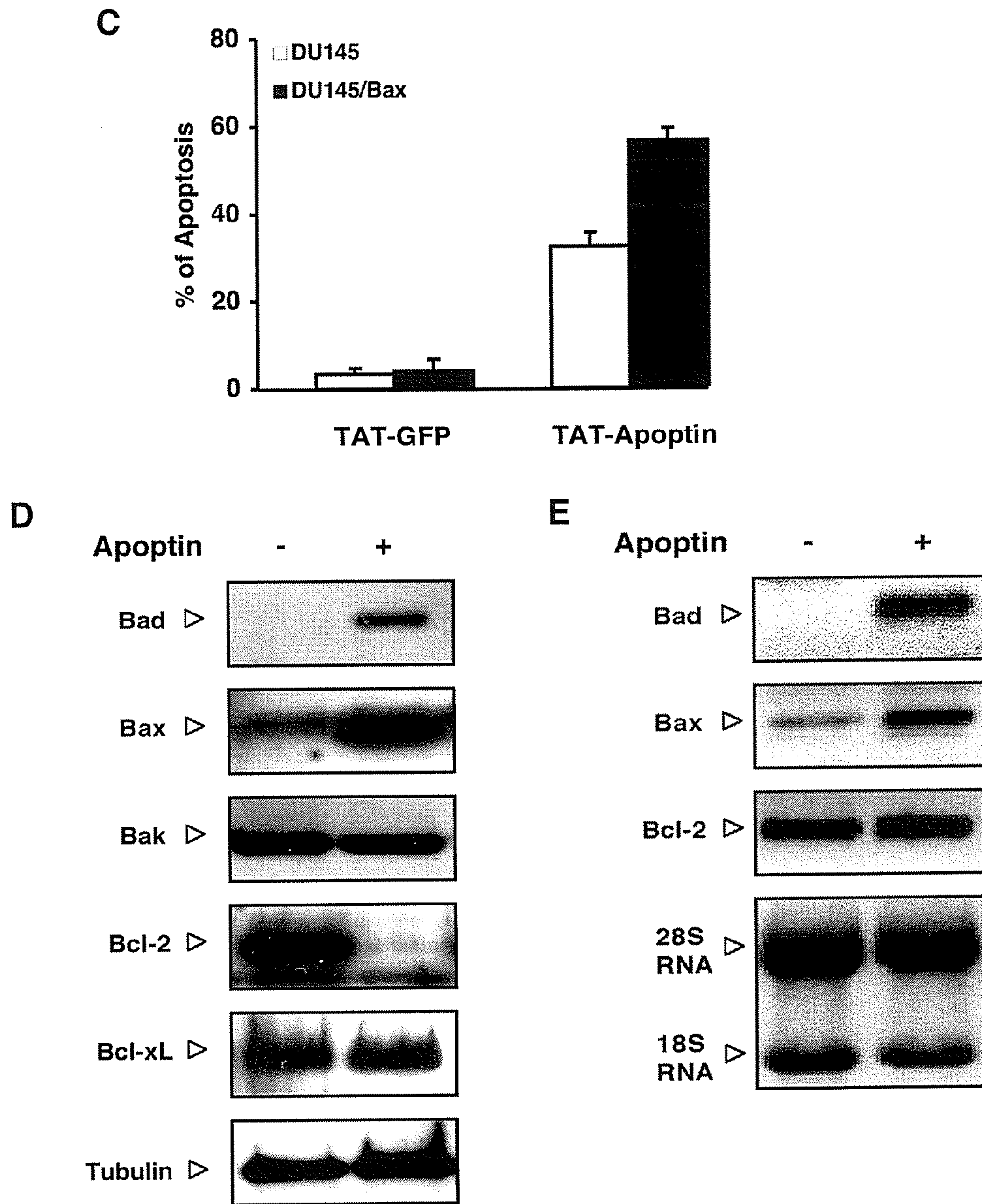


Figure 13: Bcl-2 family members modulate Apoptin induced cell death. (A) Jurkat wild type cells or the clones stably transfected with Bcl-2 and Bcl-xL were treated with TAT-Apoptin for the indicated times and the percentage of apoptosis was measured by using PI staining of hypodiploid nuclei. **(B)** MCF-7 or the clones stably overexpressing Bcl-2 or caspase-3 were treated with TAT-Apoptin and the percentage of proliferating cells were assessed by MTT assay. **(C)** DU145 prostate cancer cells or the clone stably expressing Bax were treated with either TAT-GFP or TAT-Apoptin for 48 hours and apoptosis was measured by PI uptake. The error bars shown in figures 13A-C represent the

standard deviation and the data is statistically significant ($p<0.001$) according to the student's t-test **(D)** MCF-7 cells either left untreated or treated with Apoptin for 24 hours. The expression of the indicated proteins was detected by immunoblotting with their respective antibodies. Tubulin acts a loading control **(E)** MCF-7 cells either untreated or treated with Apoptin for 22 hours and the mRNA expression of the indicated molecules was detected by Northern blot analysis using non-radioactive DIG labelling. 28S and 18S RNA acts as loading control.

3.1.7 Apoptin activates mitochondrial downstream effector caspases

We studied the activation of the downstream effector caspases to further demonstrate the role of the mitochondrial pathway during Apoptin induced cell death. Once the cell death signals reach the mitochondria, cytochrome c is released into the cytosol where it triggers the apoptosome pathway further activating downstream caspases like caspase-3 and -7. The Western blot analysis of Jurkat cells treated with TAT-Apoptin shows that the 32 kDa procaspase-3 molecule was cleaved into an active 17 kDa subunit after 48 hours. The cleavage of pro-caspase-3 was inhibited in the presence of a broad range caspase inhibitor, zVAD-fmk. However, the Western blot analysis of pro-caspase-8 cleavage, which acts proximally at the death receptor pathway, shows no cleavage in the presence of Apoptin (Fig. 14). Using GFP-Apoptin, we also have shown the activation of caspase-3 and caspase-7 and the effect of their inhibition on apoptin induced cell death in other studies (Burek et al., 2006). Thus, these data further confirm that Apoptin triggers the activation of caspases via the intrinsic/mitochondrial death pathway, and not the death receptor/extrinsic pathway.

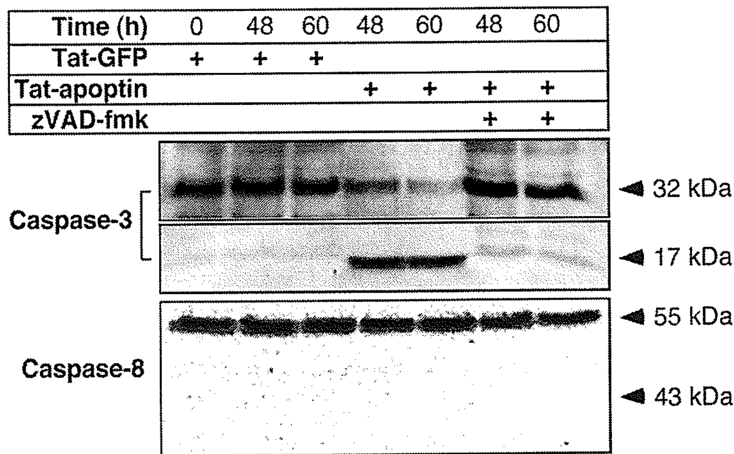


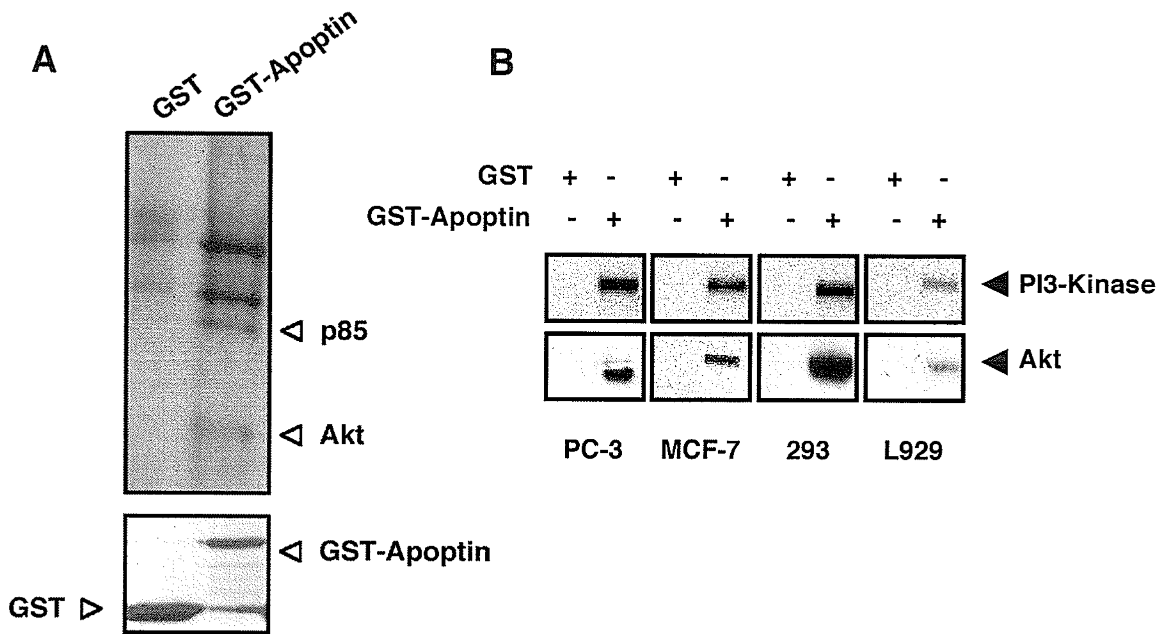
Figure 14: Apoptin activates caspase-3 but not caspase-8. Western blot analysis of the cell lysates from Jurkat cells treated with Apoptin in the presence or absence of a caspase inhibitor zVAD-fmk (40μM) at the indicated times to analyse the expression of caspase 3 uncleaved (32 kDa) and cleaved form (17kDa). Caspase-8 uncleaved (55 kDa) and cleaved forms (43 kDa) were also shown. TAT-GFP treated cells were used as a negative control. The molecular sizes of pro-caspase molecules and the active fragments are indicated.

3.2 Role of PI3-Kinase/Akt pathway during Apoptin induced cell death

3.2.1 Apoptin interacts with PI3-Kinase and Akt

To define the precise mechanisms for the selective toxicity of Apoptin to cancer cells, we tried to identify various interaction partners for Apoptin in cancer cells. As a first step to identify cellular targets of Apoptin action in the cell, we generated an expression construct encoding glutathione-S-transferase (GST) fused to the Apoptin N-terminus. GST-Apoptin and control GST were used in a pull-down assay with cell extracts derived from the MCF-7 breast cancer cells (Fig. 15A). Mass spectrometric analysis of proteins specifically bound to Apoptin identified the two major components of the PI3-K/Akt pathway, the p85 regulatory subunit of PI3-K, and the serine/threonine kinase Akt, a kinase downstream of PI3-K. We confirmed the mass spectrometric data by detecting p85 and Akt by Western blot in the samples from the GST-Apoptin pull-down assay (Fig. 15B), as well as using total cell extracts from PC-3 prostate cancer cells, L929 mouse fibrosarcoma cells, and 293 transformed human embryonic kidney cells. The interaction of Apoptin with PI3-K and Akt was also examined *in vivo* in cultured cells, whereby PC-3 cells were transfected with GFP-Apoptin, and total cell extracts immuno-precipitated with anti-GFP antibody at different time points of post transfection. The composition of the immune complexes was analyzed for PI3-K and Akt by immunoblotting (Fig 15C). In a separate series of experiments, PI3-K (p85), and Akt were immuno-precipitated, and Apoptin detected by Western blot. Figure 15C shows that PI3-K and Akt both interact with Apoptin confirming the data from GST-pull down assays. The interaction of Apoptin with PI3-K could be detected as early as 6-12 hours after Apoptin transfection, thus preceding Apoptin induced cell death, which was initiated at least 18-24 h later. The above

experiment indicates that PI3-K interaction with Apoptin is a very early event in Apoptin induced cell death. We consistently observed strong interaction of the PI3-K with Apoptin within 6-48 hours post-transfection, whereas interaction of Apoptin with Akt seems weaker and is seen only at earlier time-points upon Apoptin treatment.



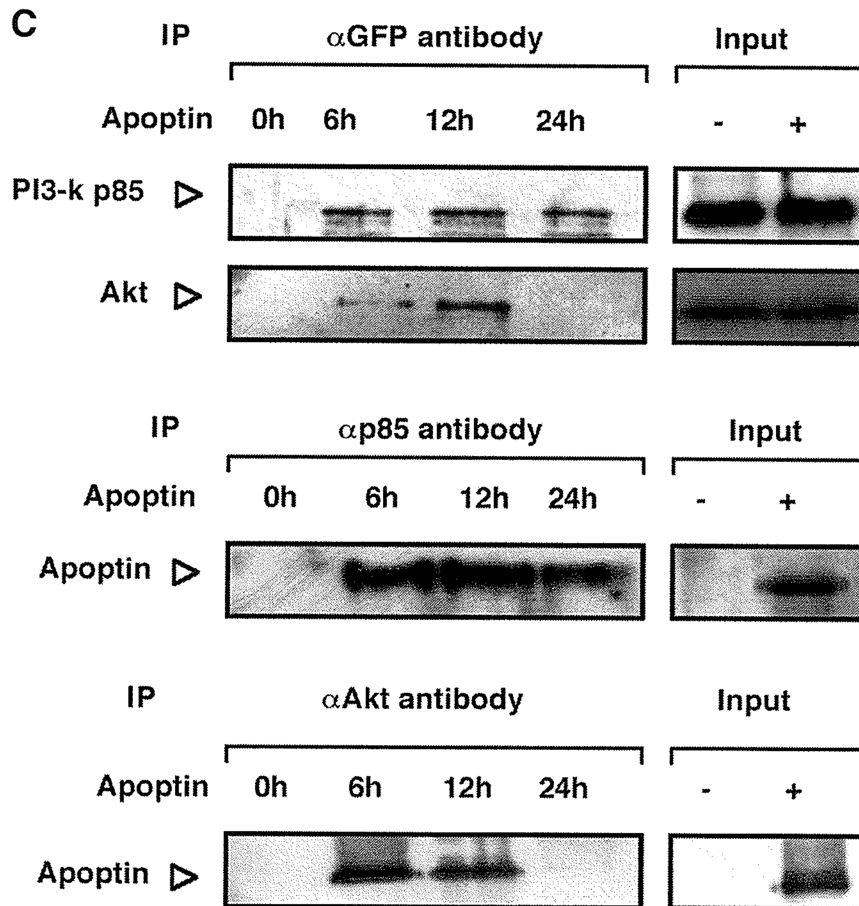


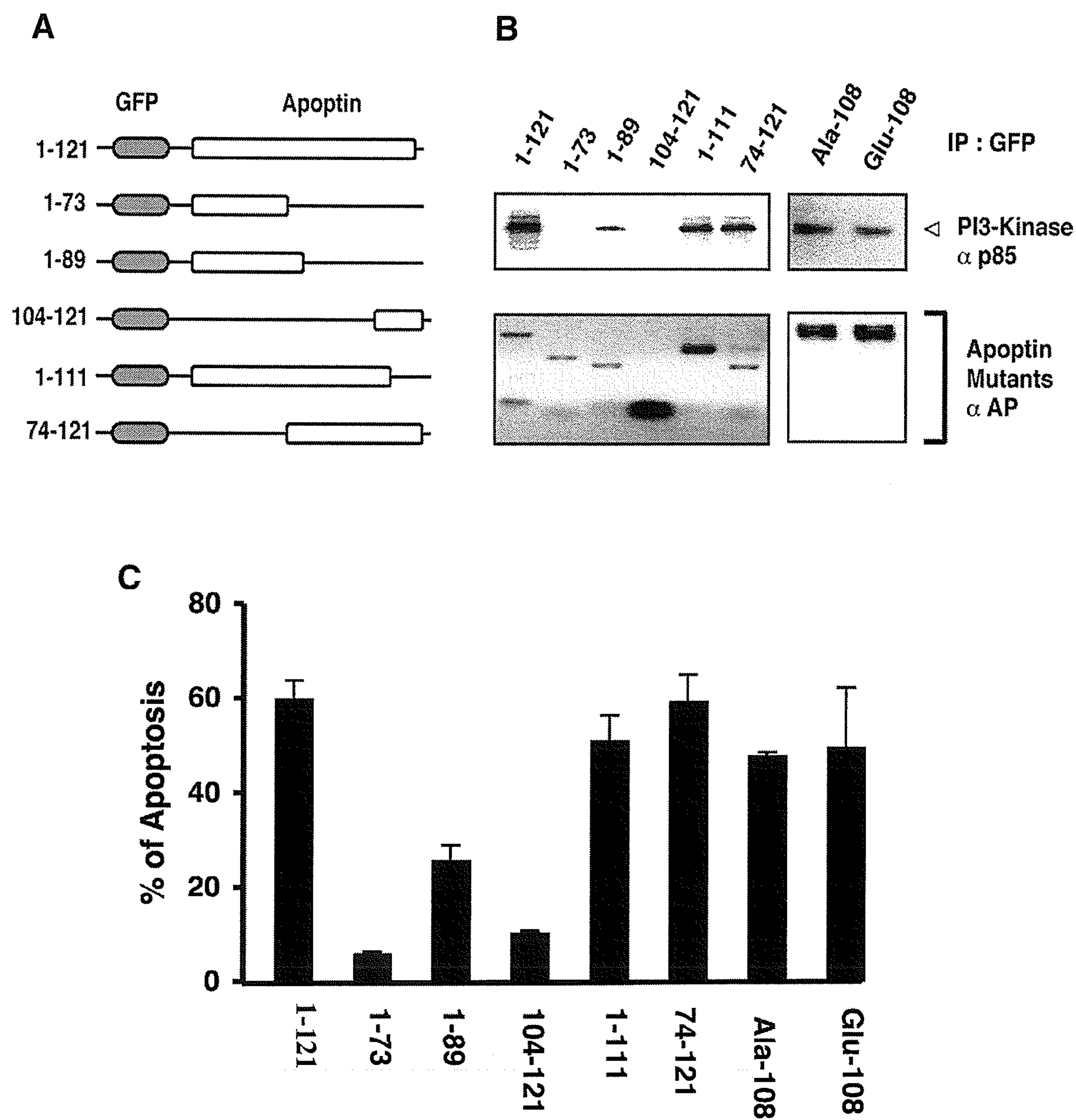
Figure 15: Apoptin interacts with PI3-Kinase and Akt. (A) GST-pull down assay performed with MCF-7 lysate using either GST control or GST-Apoptin and the proteins ($p85^{PI3-K}$ and Akt) specific for Apoptin interaction identified by mass spectrometry are indicated. **(B)** GST pull down assay is performed with PC-3, MCF-7, 293 and L929 cell lysates with either GST or GST-Apoptin and the presence of the p85 subunit of PI3-K and Akt in Apoptin complexes was determined by immuno-blotting with the respective antibodies. **(C)** Co-immuno-precipitation indicates the interaction of Apoptin with p85 and Akt. PC-3 cell lysates were immuno-precipitated with anti-GFP antibody (GFP-Apoptin) at different time-points upon transfection with GFP-Apoptin. The co-immunoprecipitated p85 and Akt was detected by Western blot. Reciprocal immuno-precipitation with either anti-p85- or anti-Akt antibodies and the detection of Apoptin by immuno-blotting is shown at the bottom.

3.2.2 Apoptin interacts with the SH3 domain of p85 regulatory subunit via its proline-rich sequence

To identify and map the sites on Apoptin responsible for interaction with PI3-K, PC-3 cells were transfected to express either full-length GFP-Apoptin, or various deletion mutant derivatives of Apoptin tagged with an N-terminal GFP as listed in figure 16A. Apoptin was immuno-precipitated with anti-GFP antibodies 24 hours post transfection and the immune complexes were analyzed for the interaction with PI3-K by immunoblotting using anti-p85 antibodies. PI3-K was found in the immuno-precipitates of full-length Apoptin and Apoptin derivatives that harbored amino acids from 74-100 (a proline-rich region), implying that this region of Apoptin is important for interaction with PI3-K (Fig. 16B). We then tested if the interaction of PI3-K with Apoptin is essential for Apoptin-induced cell death by assessing the percentage of apoptosis induced by the different mutants. In agreement with the interaction data, only those mutants that had the intact interaction site for PI3-K were able to induce apoptosis (Fig. 16C). Interestingly, in our model system the mutant Ala-108, which is a non-phosphorylatable Apoptin and stated to be non-toxic to the cells in previous reports (Rohn et al., 2002), was still able to induce significant apoptosis. This was dependent on its interaction site with the PI3-K being intact though the cell death by Ala-108 mutant was not up to the level of wild type Apoptin.

Next, we investigated the Apoptin interaction site on PI3-K by co-transfecting with a vector encoding full-length Apoptin, together with full length PI3-K or various deletion derivatives thereof. Some PI3-K deletion mutants were tagged with haemagglutinin tag (HA) at their N-terminus (Fig. 16D). Both full length PI3-K and the mutant lacking the iSH2 domain were immuno-precipitated by anti-p85 antibody, while other mutants were

immuno-precipitated using anti-HA antibodies. Immuno-detection of Apoptin in the immune complexes of PI3-K and its deletion mutant derivatives implied that Apoptin interacts with the intact SH3 domain of PI3-K (Fig. 16E).



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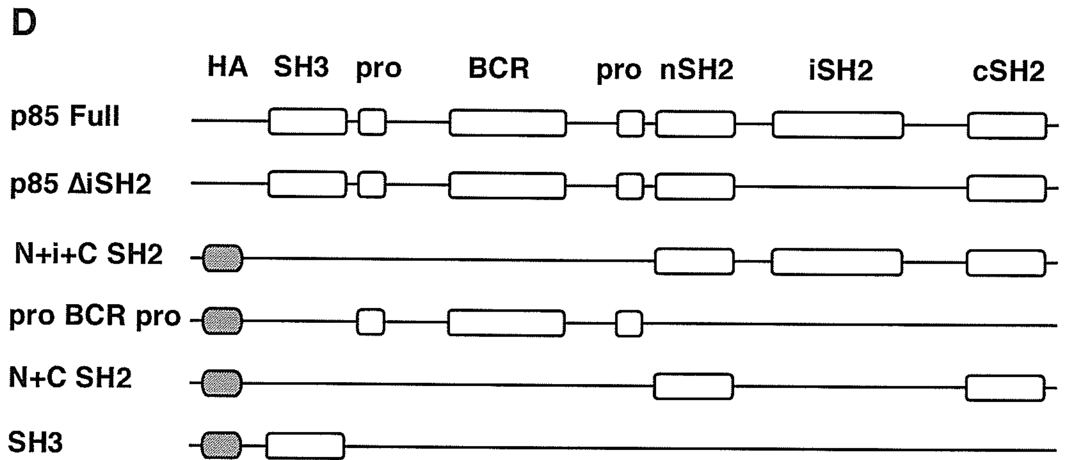

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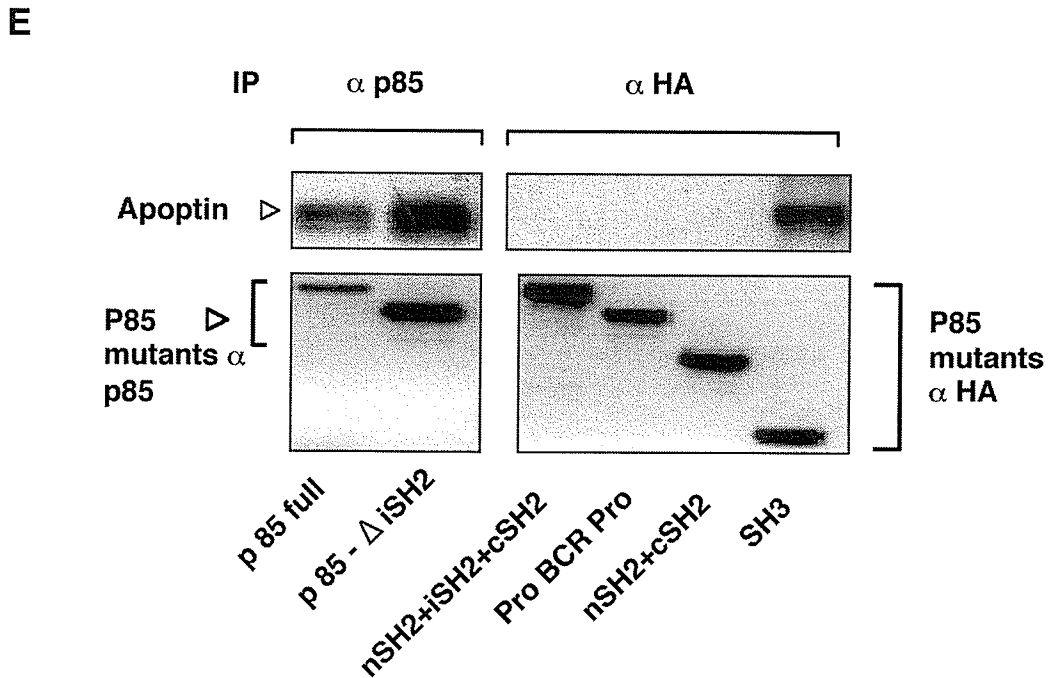


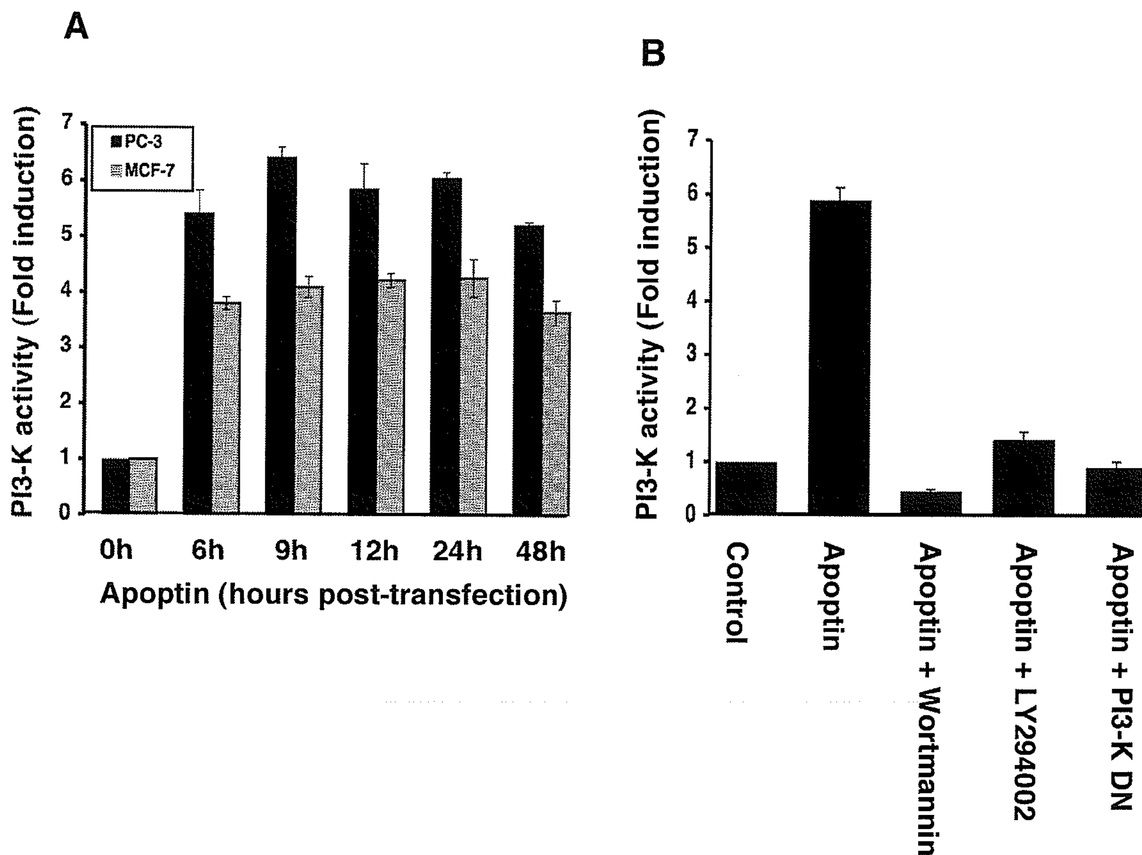
Figure 16: Apoptin interacts with the SH3 domain of p85 regulatory subunit via its proline-rich sequence. **(A)** Schematic representation of Apoptin deletion mutants tagged with N-terminal GFP. **(B)** The deletion mutants were transfected into PC-3 cells, immuno-precipitated with anti-GFP antibody after 18 hours post transfection and the presence (association) of p85 was detected by immuno blotting. **(C)** The percentage of cell death induced by different Apoptin mutants assessed by flow cytometry (Nicoletti method) 24 hours after transfecting the PC-3 cells with them. The error bars shown in the graph represent the standard deviation of the data in triplicates. The data is statistically significant ($p<0.04$) as calculated by student's t-test. **(D)** Schematic representation of the PI3-K p85 deletion mutants indicated with their domains. **(E)** The p85 deletion mutants

were co-transfected together with Apoptin into PC-3 cells. 24 h post-transfection cells were lysed, and p85 was immuno-precipitated with either anti-p85 antibody (the wt, and ΔiSH2 mutants) or with anti-HA antibody for other mutants. Apoptin interaction with the mutants was detected by immuno-blotting with anti-Apoptin antibodies and the expression of deletion mutants was detected by anti-p85 or anti-HA antibodies.

3.2.3 PI3-K is constitutively activated during Apoptin-induced apoptosis

To determine the functional significance of Apoptin's interaction with the p85 regulatory subunit, we measured PI3-K activity using a non-radioactive ELISA-based method. Surprisingly, MCF-7 and PC-3 cells transfected to express GFP-Apoptin revealed constitutive activation of PI3-K in Apoptin-transfected cells (Fig. 17A). PI3-K activity was increased nearly four-fold in Apoptin transfected MCF-7 cells and up to six-fold in PC-3 cells, compared to the GFP-transfected control. PI3-K activation was seen around 6 h after transfection, consistent with the interaction data, with activation retained at a similar level for up to a further ~40 hours. In a control experiment, various PI3-K inhibitors prevented Apoptin-triggered generation of PIP_3, whilst co-transfection of Apoptin with a dominant negative PI3-K vector reduced Apoptin-induced PI3-K activation to basal levels (Fig. 17B). We then tested if the activation of PI3-K is a direct consequence of Apoptin's interaction by monitoring PI3-K activity in cells transfected to express different Apoptin deletion derivatives. As expected, the full-length Apoptin induced six-fold PI3-K activation, while the Apoptin 1-89 derivative, that probably harbors only the partial PI3-K interaction site, was much less active. The Apoptin derivatives 1-111, 74-121, Ala-108 and Glu-108 were all able to activate PI3-K to an extent comparable to full-length Apoptin, whereas the 1-73 and 104-121 derivatives were unable to activate PI3-K. This observation implies that the intact p85 interaction site on Apoptin is crucial for the activation of PI3-K (Fig. 17C). On the other hand, PI3-K activity in cells co-transfected to express full-length

Apoptin and p85 deletion mutants confirmed that the interaction of Apoptin with the SH3-domain of p85 is essential for its activity. The p85 derivatives that retain both SH3 and iSH2 domains have constitutive PI3-K activity in the presence of Apoptin, whereas others lacking these domains are severely impaired in their activity (Fig. 17D). Interestingly, the mutant N+I+C SH2 having only the SH2 domains but lacking the SH3 domain have relatively higher PI3-K activity compared to other deletion mutants and to the control, indicating that the intact SH3 domain in the wild-type p85 protein might have an inhibitory action on its activity. Thus, the interaction of Apoptin with the SH3 domain appears to elicit a conformational change in the p85 protein, converting it from an inhibitory to an active state.



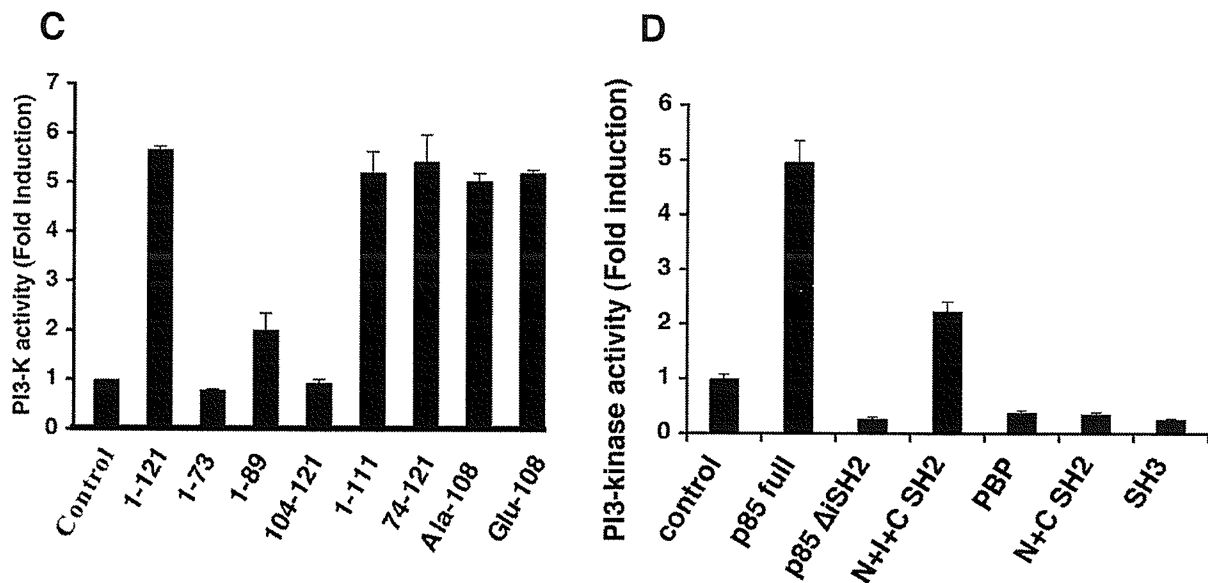


Figure 17: PI3-K is constitutively activated during Apoptin-induced apoptosis. (A) The PI3-K activity was measured by an ELISA-based assay after immuno-precipitating PI3-K from the lysates of PC-3 and MCF-7 cells transfected with Apoptin for the indicated time points, as described in the methods section, and the fold induction was calculated. PI3-K activity in non-transfected cells was considered basal level (1x). The error bars represent the standard deviation of the data in triplicate. The data is statistically significant ($p<0.03$) as measured by student's t-test. (**B**) PC-3 cells were either transfected with Apoptin alone, pretreated with Wortmannin, LY294002 followed by Apoptin transfection, or co-transfected with Apoptin and a PI3-K dominant negative vector. The PI3-K activity was measured 24 h post transfection. The inhibitors were also added during the assay. (**C**) PI3-K activity was measured 24 h after transfecting the cells with different Apoptin deletion mutants. (**D**) PI3-K-mutants described in Fig. 1G were transfected into PC-3 cells. 24 h later, the cells were lysed, and PI3-K forms were immuno-precipitated. The kinase activity was measured by ELISA based method. The error bars shown in figures 17B-D represent the standard deviation in triplicate. The data is statistically significant ($p<0.04$) as measured by student's t-test.

3.2.4 PI3-Kinase inhibition attenuates Apoptin induced cell death

In order to investigate the direct effect of PI3-K inhibition on Apoptin induced cell death, we pre-treated the cells with the PI3-K inhibitors, Wortmannin and LY294002, 30 min before Apoptin transfection and cell death was assayed 24 and 48 h later. To our surprise, both inhibitors afforded significant protection against Apoptin-induced cell death, despite generally being known to enhance the cell death process through inhibition of PI3-

K activity. This effect was further confirmed by co-transfection of cells to express Apoptin together with a dominant-negative derivative of PI3-K, which significantly protected cells from Apoptin-induced cell death (Fig. 18A). To support the data from the inhibitor and transfection experiments, we used siRNA to test whether knocking down endogenous PI3-K would affect resistance to Apoptin-induced cell death. In PC-3 cells, the expression of p85 was completely abrogated by p85-specific siRNA but not by the control siRNA (Fig. 18B), as assayed approximately 30 hours post-transfection. The assessment of cell death revealed that the cells lacking p85 expression were strongly resistant towards Apoptin induced cell death compared to control siRNA-expressing cells (Fig. 18C).

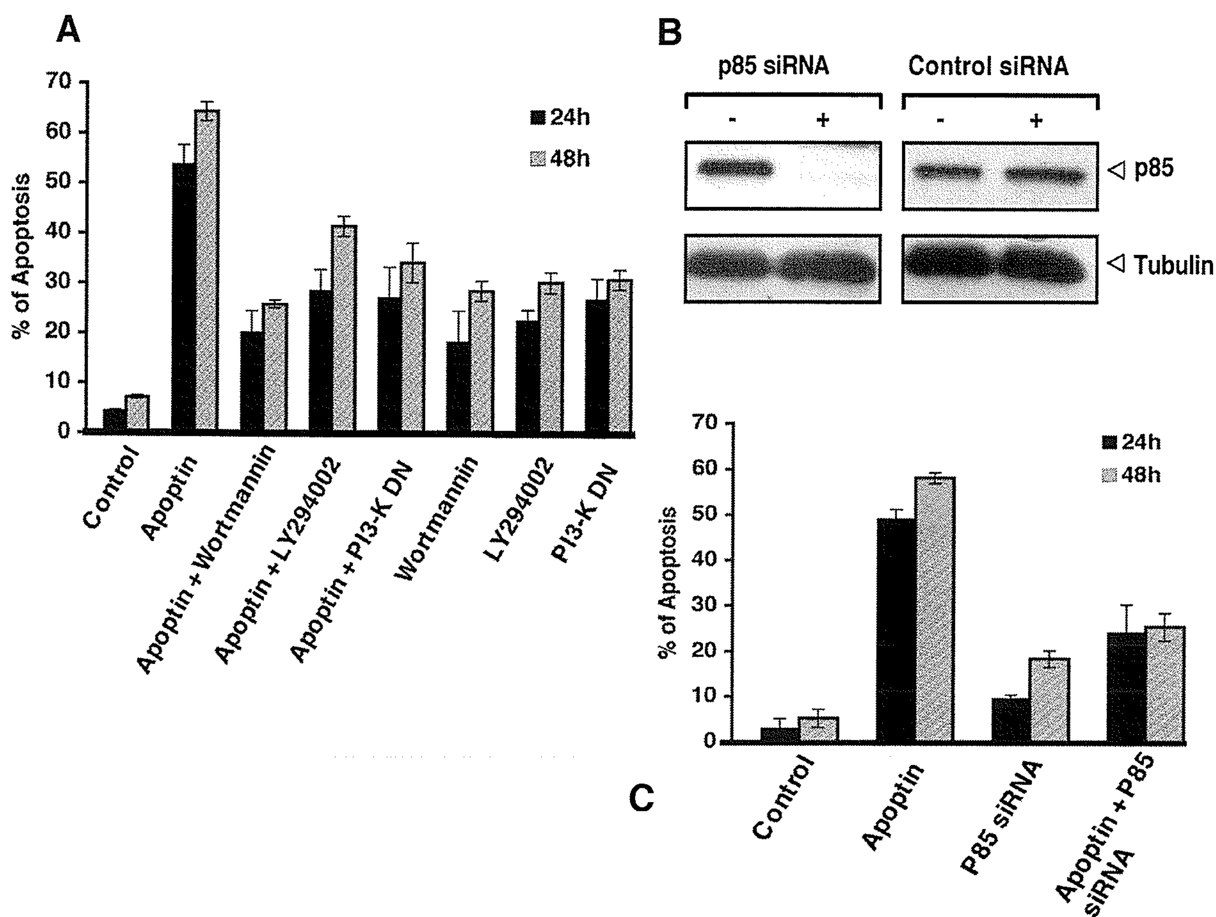


Figure 18: PI3-Kinase inhibition attenuates Apoptin induced cell death. (A) The effect of PI3-K inhibition on Apoptin induced cell death was assessed by flow cytometry (Nicoletti method). The cells were either transfected with GFP control plasmid, or GFP-Apoptin, co-treated with Wortmannin, LY294002 along with Apoptin transfection, co-transfection of Apoptin with PI3-K dominant negative plasmid or Wortmannin, LY294002, or PI3-K-DN. Apoptosis was then measured 24 and 48 hours post transfection. The error bars shown in the graph represent the standard deviation and the data is statistically significant ($p<0.02$) according to the student's t-test **(B)** PC-3 cells were either transfected with p85 siRNA plasmid (Upstate) or the control siRNA plasmid for 48 hours, and the expression of p85 in the presence or absence of siRNA was detected by immuno-blotting. Tubulin was used as a loading control. **(C)** The PC-3 cells were transfected either with Apoptin alone, or with p85-inhibitory siRNA. After 48 h, cells were transfected with Apoptin. Following another 24 or 48 h the amount of cell death was measured by flow cytometry (Nicoletti method). The error bars shown in the graph represent the standard deviation and the data is statistically significant ($p<0.001$) according to the student's t-test

3.2.5 PI3-Kinase activity is required for nuclear localization of Apoptin in cancer cells

Apart from the negative effect of PI3-K inhibition on Apoptin induced cell death, we also tested if PI3-K inhibition affects Apoptin's subcellular localization. Apoptin is mainly localized in the nucleus of PC-3 and MCF-7 cells, but in the presence of Wortmannin, Apoptin was found mainly distributed in the cytoplasm as shown by the immuno-staining followed by confocal laser microscopic imaging (Fig. 19A). The effect of PI3-K inhibition on Apoptin's localization was further studied using p85 siRNA. Upon the inhibition of p85 expression, nuclear localization of Apoptin was almost completely abrogated (Fig. 19B) indicating that PI3-K activity is not only necessary for Apoptin induced cell death, but also for the localization of the protein during its apoptotic action in cells. Previous reports indicated that the tumor-specific phosphorylation of the Thr-108 residue in Apoptin is required for the differential localization of the protein (Rohn et al., 2002). PI3-K is mainly a lipid kinase, though some protein kinase activity has been

reported; the possibility that PI3-K might directly phosphorylate Apoptin was firstly excluded in our preliminary *in vitro* kinase assays (data not shown). It thus seems likely that one of the downstream effectors of the PI3-K pathway phosphorylates Apoptin and controls its localization, but in a PI3-K dependent manner.

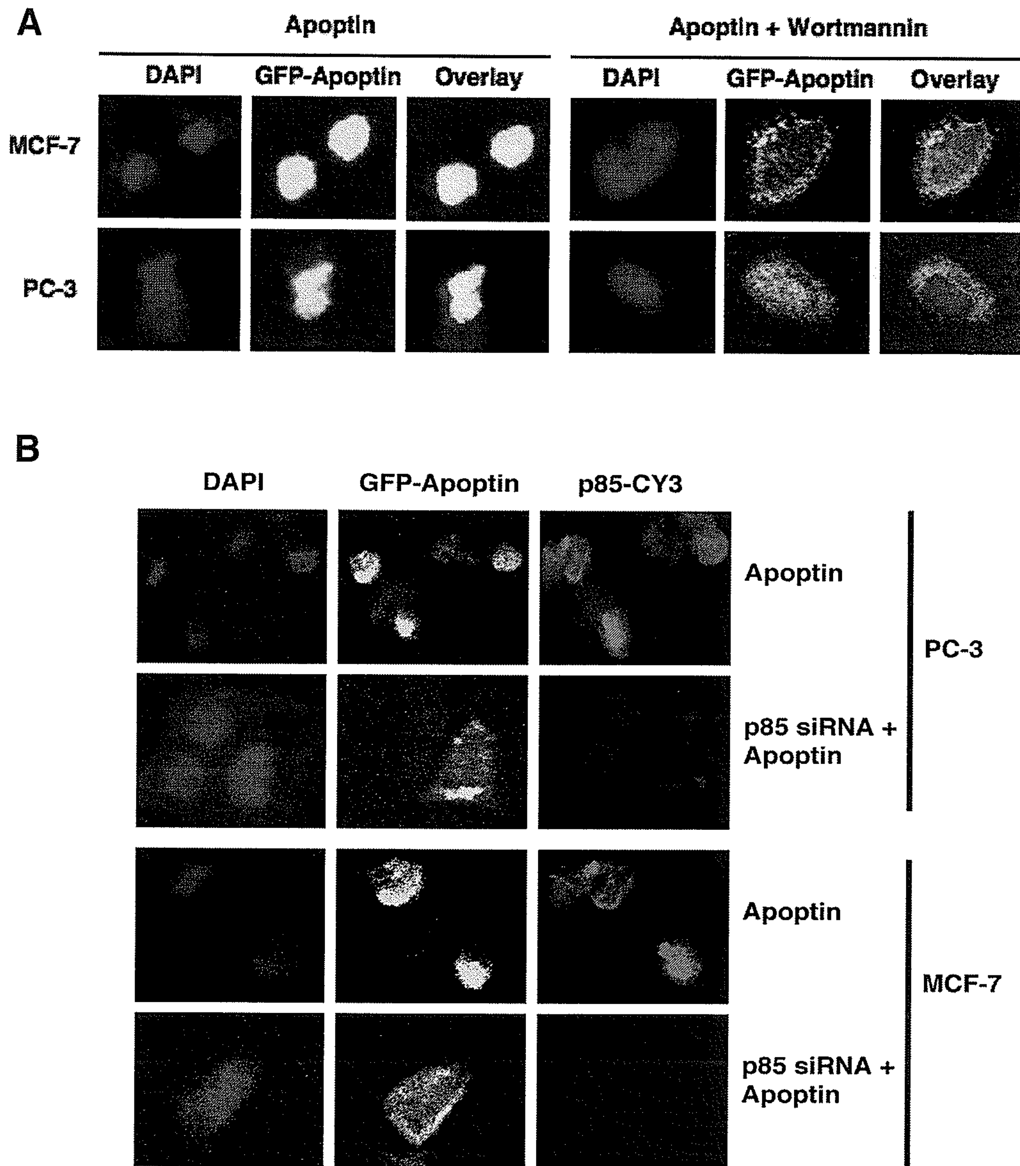


Figure 19: PI3-Kinase activity is required for nuclear localization of Apoptin. (A) The localization of Apoptin in both MCF-7 and PC-3 cells was detected by confocal microscopy upon GFP-Apoptin transfection alone, or in the presence of Wortmannin. **(B)** The effect of p85 inhibition on Apoptin's localization was demonstrated by confocal

microscopy, 24 h upon transfection with either GFP-Apoptin alone or upon co-transfection with p85-targetting siRNA plasmid. The expression of p85 was detected by anti-p85 antibody followed by Cy-3 conjugated secondary antibody.

3.2.6 Akt is activated downstream of PI3-Kinase during Apoptin induced cell death

We next investigated the effect of Apoptin on downstream targets of the PI3-K pathway by transfecting the PC-3 cells to express Apoptin, and assessing Akt activation by Western blotting at different time points post-transfection. In agreement with the results for PI3-K activation, increased levels of activated Akt were seen around 6-12 hours post-transfection with pronounced levels of phosphorylated Akt detectable even 24 hours post-transfection (Fig. 20A). The activation of Akt is downstream of-, and dependent on PI3-K activation; pretreatment of cells with the inhibitors Wortmannin or LY294002, or co-transfection of Apoptin-expressing cells with constructs encoding PI3-K or PDK1 dominant-negative mutants impaired Akt activation. Also, over-expression of wild type PTEN, a negative regulator of Akt activation, severely reduced Apoptin-induced Akt activation, whereas over-expression of a phophatase deficient PTEN mutant had no effect (Fig. 20B). To further confirm the role of PI3-K in Apoptin-induced cell death, and to validate the role of Akt in this process, we transiently over-expressed a dominant negative mutant of PDK1 (PDK1-DN), and/or the dominant negative Akt (Akt-DN) using adenoviral vectors. Both dominant-negative kinase mutants as well as over-expression of wild type PTEN significantly protected against Apoptin induced cell death, thus confirming the key role of the PI3-K/Akt pathway in this process (Fig. 20C).

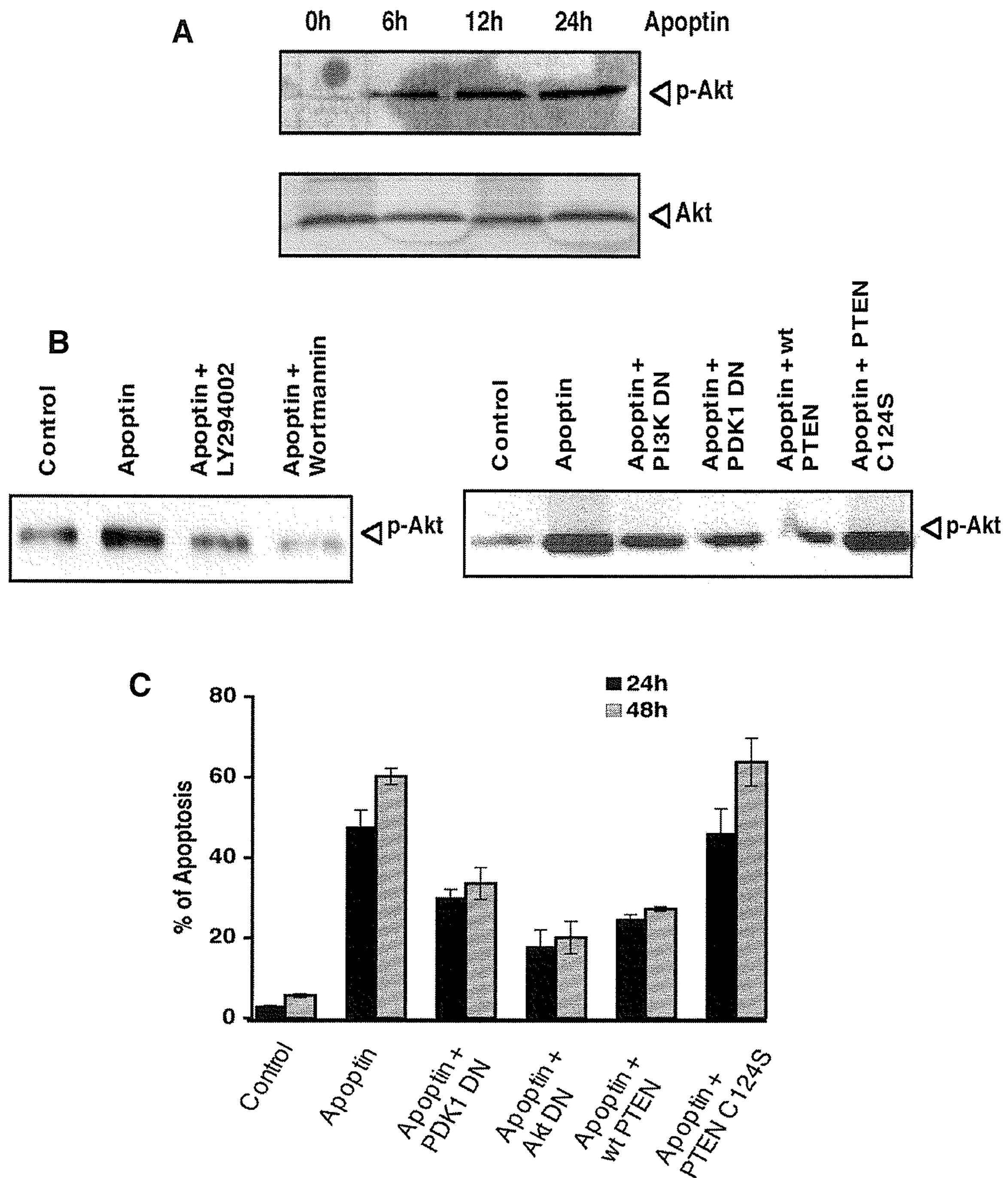


Figure 20: Downstream of PI3-K, Akt is activated and is required for Apoptin induced cell death. (**A**) The activation of Akt by Apoptin was detected in PC-3 cells by immuno-blotting with an antibody against the phosphorylated Akt at Ser-473 at different time points after Apoptin transfection. Total Akt was also detected by immuno-blotting. (**B**) PC-3 cells were transfected with Apoptin. Some samples were co-treated with either Wortmannin, or with LY294002. The phosphorylated Akt was then detected by immuno-

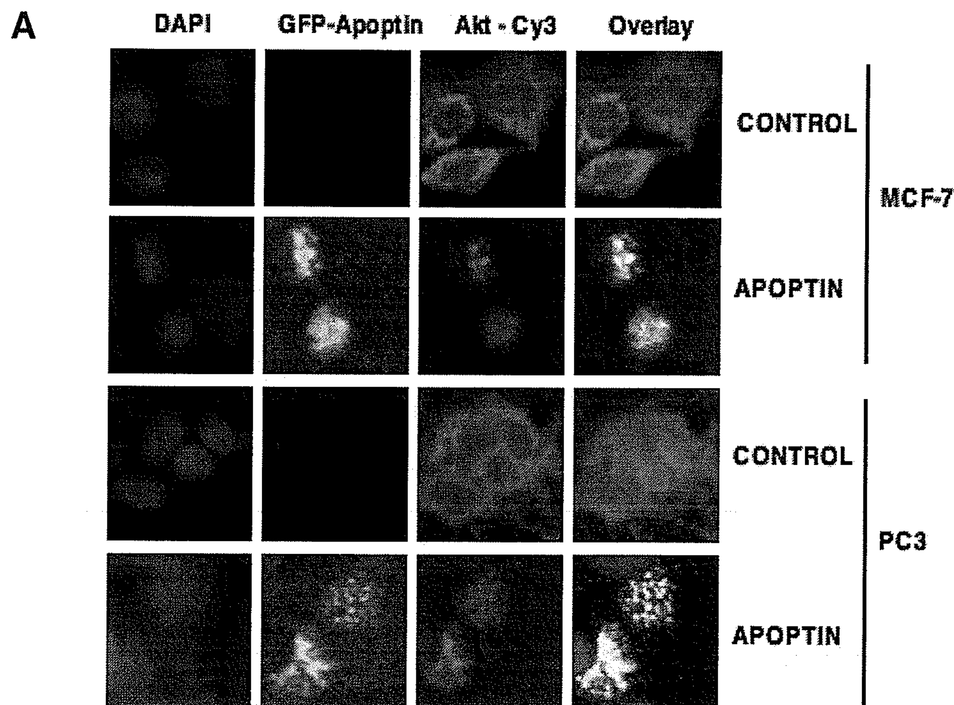
blotting. The activation of Akt was also determined by immuno-blotting in the cell lysates from cells transfected with Apoptin alone or co-transfected with either PI3-K dominant negative vector (DN), PDK1-DN, wild type PTEN, or phosphatase deficient C124S-PTEN mutant. No significant changes in total Akt was observed (data not shown). **(C)** The effect of Akt inhibition on Apoptin's toxicity was assessed in PC-3 cells by Nicoletti method at 24 h and 48 h after either transfection with Apoptin alone, or co-transfection or infection with PDK1-DN, Akt-DN (adeno-viral vector), wild type PTEN, C124S-PTEN. The error bars shown in the graph represent the standard deviation and the data is statistically significant ($p<0.03$) according to the student's t-test

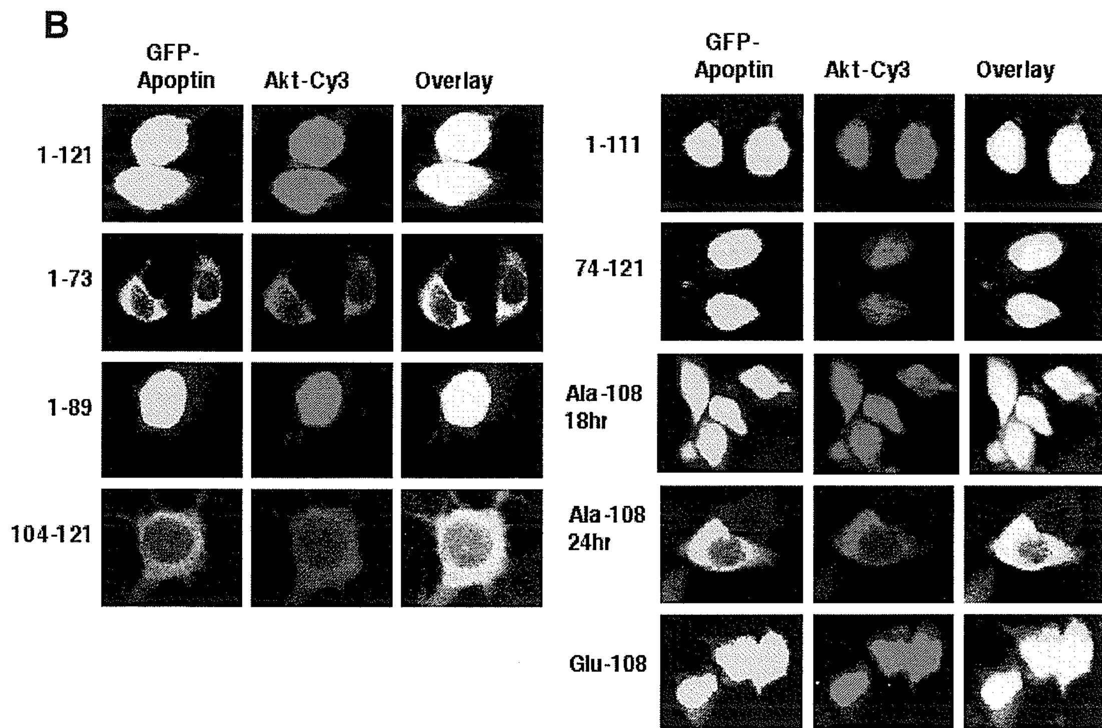
3.2.7 Akt translocates to the nucleus during Apoptin induced cell death

Akt is generally regarded as a survival-, or proliferation-promoting kinase and not at all a pro-apoptotic molecule. However, in the presence of Apoptin, Akt is clearly acting as a pro-cell death molecule as its inhibition severely inhibits the cell death pathways triggered by Apoptin. Next, we asked if Apoptin redirects Akt to different cellular targets by modulating its subcellular localization. As shown in Figure 21A, in the absence of Apoptin expression, Akt is mainly localized in the cytoplasm of both untransfected PC-3 and MCF-7 cells, but becomes clearly nuclear in Apoptin expressed cells. Furthermore, to determine the effect of different Apoptin mutants on Akt translocation and to test if Akt nuclear translocation depends on Apoptin/PI3-K interaction, we investigated the localization of Akt in cells transfected to express different Apoptin deletion mutants. As shown in Figure 21B, Akt translocates to the nucleus only in cells expressing Apoptin mutants 1-121, 1-89, 1-111, 74-121 and the Glu-108 mutant, all which are nuclear localizing, and have an intact PI3-K interaction site. Cells expressing the Apoptin mutants 1-73 and 104-121 did not exhibit Akt nuclear translocation. This is in concordance with their ability both to interact with PI3-K and induce apoptosis. In the presence of the Ala-108 mutant, Akt translocates to the nucleus together with Apoptin around 18 hours post transfection, but later at 24 h, traces of Akt were observed in the cytoplasm along with

Apoptin. Apoptin mutant T108A was still able to enter the nucleus, and facilitate the nuclear translocation of Akt, but it is deficient in nuclear accumulation. This clearly implies that Apoptin might be acting as a carrier molecule for Akt, directly mediating its nuclear transport via a "piggy back" interaction.

To determine if nuclear Akt alone is sufficient to induce cell death even in the absence of an apoptotic stimulus, we infected PC-3 cells with an NLS-Akt adenoviral vector in the absence and presence of Apoptin expression. As shown in Figure 21C, nuclear Akt alone is not very effective at inducing apoptosis, whereas in the presence of Apoptin, it significantly enhanced the cell death. Quantitative analysis using flow cytometry to determine the extent of cell death (Fig. 21D) indicated that NLS-Akt exhibits slightly increased toxicity (13%+/-) compared to the negative (wild type) control (7%+/-). Co-expression of NLS-Akt together with Apoptin highly sensitized the cells towards Apoptin-induced cell death.




B
GFP-
Apoptin
Akt-Cy3
Overlay
1-121
1-73
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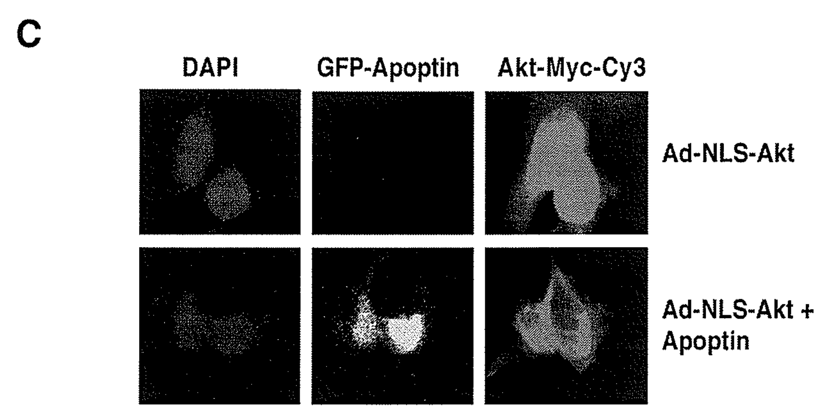
18hr
Ala-108
24hr
Glu-108


C
DAPI
GFP-Apoptin
Akt-Myc-Cy3
Ad-NLS-Akt
Ad-NLS-Akt +
Apoptin

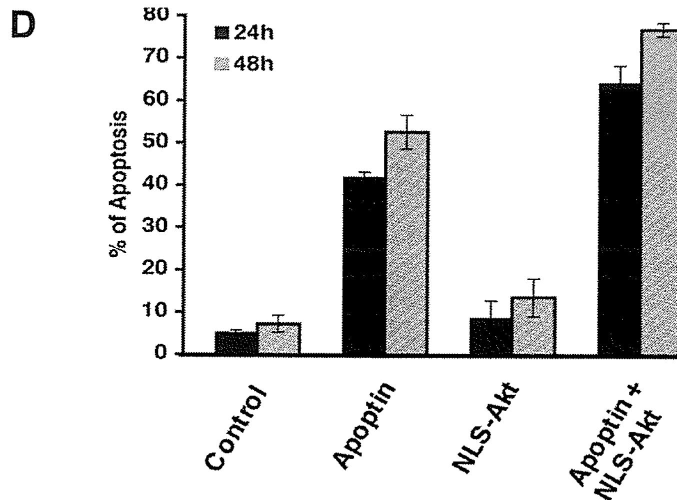


Figure 21: Akt translocates to the nucleus during Apoptin induced cell death. (A) The localization of Akt either in the absence or presence of Apoptin in both MCF-7 and PC-3 cells was detected by confocal microscopy, after immuno-staining with anti-Akt antibody followed by Cy3 conjugated secondary antibody. DAPI was used as counterstain for the nuclei, and the images were overlaid to determine the Akt localization within the cell. **(B)** MCF-7 cells were transfected with Apoptin mutants and the localization of Akt and Apoptin was detected by immunocytochemistry. **(C)** The cells were infected with either adenoviral Myc-tagged NLS-Akt alone or co-transfected with Apoptin and the localization of NLS-Akt was detected by anti-Myc-tag antibody followed by Cy3-marked secondary antibody. **(D)** Quantitative assessment of Apoptin-triggered apoptosis in NLS-Akt expressing cells as compared to controls. Cells were infected with adenovirus encoding NLS-Akt, and/or transfected with GFP-Apoptin expression vector. Apoptotic cell death was detected by flow cytometry (Nicoletti) 24 or 48 h after transfection with Apoptin. The error bars shown in the graph represent the standard deviation of three independent experiments and the data is statistically significant ($p<0.02$) according to the student's t-test.

3.2.8 Apoptin regulated Akt activation leads to senescence in normal cells

We have shown that the constitutive activation of PI3-Kinase/Akt pathway is required for Apoptin induced cell death in cancer cells. Further, we tested if Apoptin activates this pathway in normal cells. After 12 hours, PI3-Kinase activity increased by nearly two-fold in Apoptin transfected primary human diploid fibroblasts compared to control cells (Fig. 22A). Akt was also activated downstream of PI3-K in Apoptin transfected primary cells (Fig. 22B). But interestingly, the activation of PI3-Kinase activity

by Apoptin in primary cells did not lead to apoptosis but rather induced cell senescence. Apoptin induced senescence as determined by β-Galactosidase assay in primary cells is dependent on the PI3-Kinase/Akt pathway, as the cells pre-treated with Wortmannin show no signs of senescence even in the presence of Apoptin (Fig. 22C).

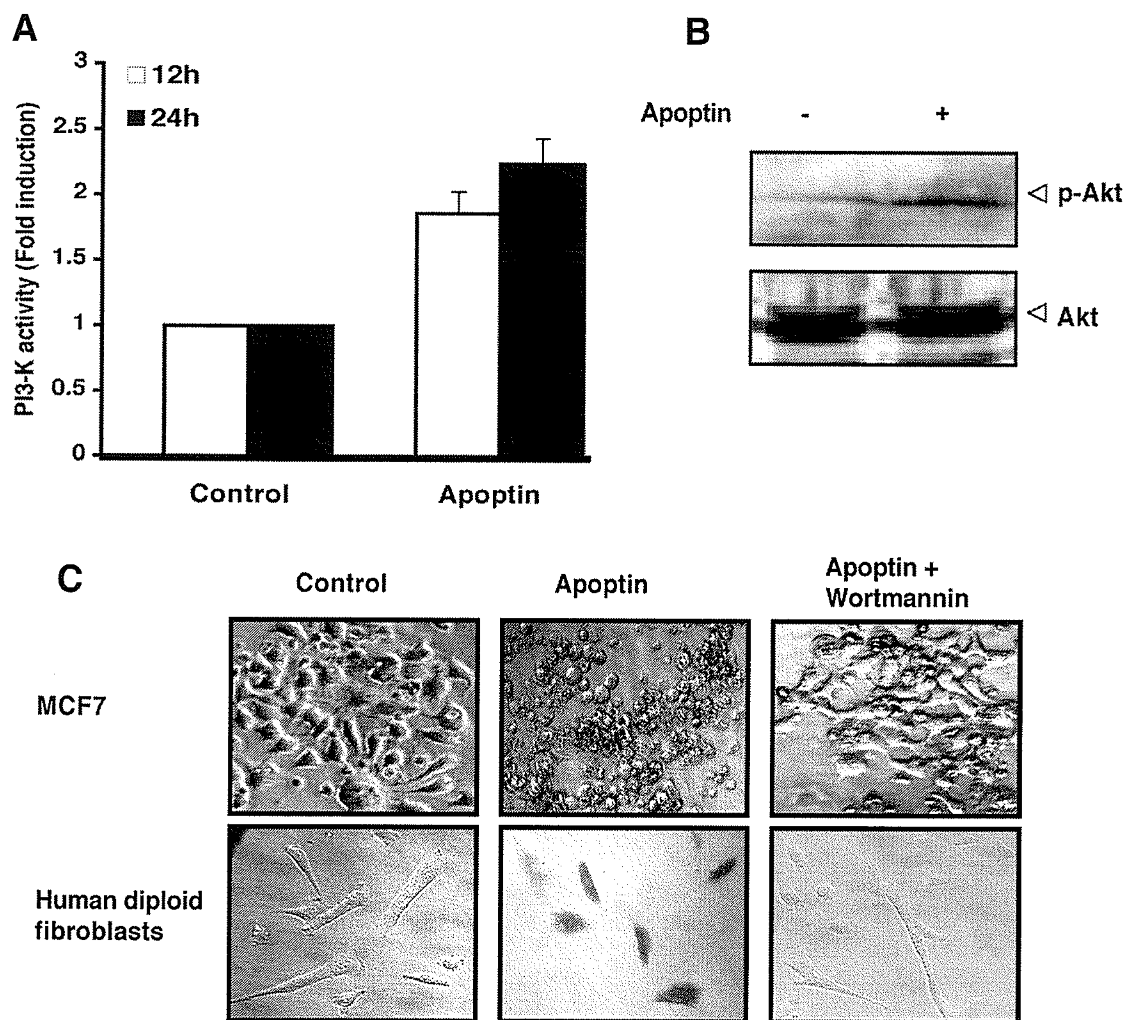


Figure 22: PI3-Kinase activation by Apoptin induces senescence in primary cells. (A) The PI3-K activity was measured by an ELISA-based assay after immuno-precipitating PI3-K from the lysates of primary diploid human fibroblasts transfected with Apoptin for the indicated time points and the fold induction was calculated. The data is statistically significant ($p<0.04$) as determined by student's t-test. **(B)** The activation of Akt in either untransfected or Apoptin transfected primary cells was detected by immuno-blotting with an anti-phospho Akt (Ser-473).Total Akt was also detected by immuno-blotting with anti-Akt antibody. **(C)** MCF-7 breast cancer cells and the human diploid fibroblasts were left untranfected, transfected with Apoptin alone or pretreated with Wortmannin followed by

Apoptin transfection. The senescence (indicated by the development of blue colour) was detected by using a β-Galactosidase staining kit.

3.3 Role of CDK2 during Apoptin induced cell death

3.3.1 CDK2/cyclin A activity is elevated during Apoptin-induced cell death

In the process of identifying the potential nuclear targets for Akt during Apoptin's pro-apoptotic signaling, we measured the activation of two major cyclin-dependent kinases, CDK1 and CDK2, using an *in vitro* kinase assay with their substrate histone H1. PC-3 cells were transfected to express either GFP or GFP-Apoptin and the CDK activity was measured 24 hours post transfection by immuno-precipitating using CDK1 or CDK2 specific antibodies. In the presence of Apoptin expression, CDK2 but not CDK1 activity was elevated as indicated by the increase H1 phosphorylation (Fig. 23A). To determine if the increased CDK2 activity during Apoptin-induced cell death was associated with cyclin E or A, immuno-precipitation was performed using specific antibodies, either in the presence of over-expressed GFP or GFP-Apoptin. Figure 23A, shows that only cyclin A- but not cyclin E-associated CDK2 phosphorylated histone H1, suggesting that only cyclin A/CDK2 has a role during Apoptin-induced cell death. CDK2 activity was detectable at 16 hours and peaked at 24 hours post transfection (Fig. 23B). We also tested if this increase in CDK2 activity during Apoptin-induced apoptosis was dependent on upstream PI3-K/Akt activation. Figure 23C shows that CDK2 is activated only when the PI3-K/Akt pathway is active; inhibition of Akt activation by Wortmannin, Ly294002, PI3-K-DN, PDK1-DN or Akt-DN significantly decreased Apoptin-induced CDK2 activation.

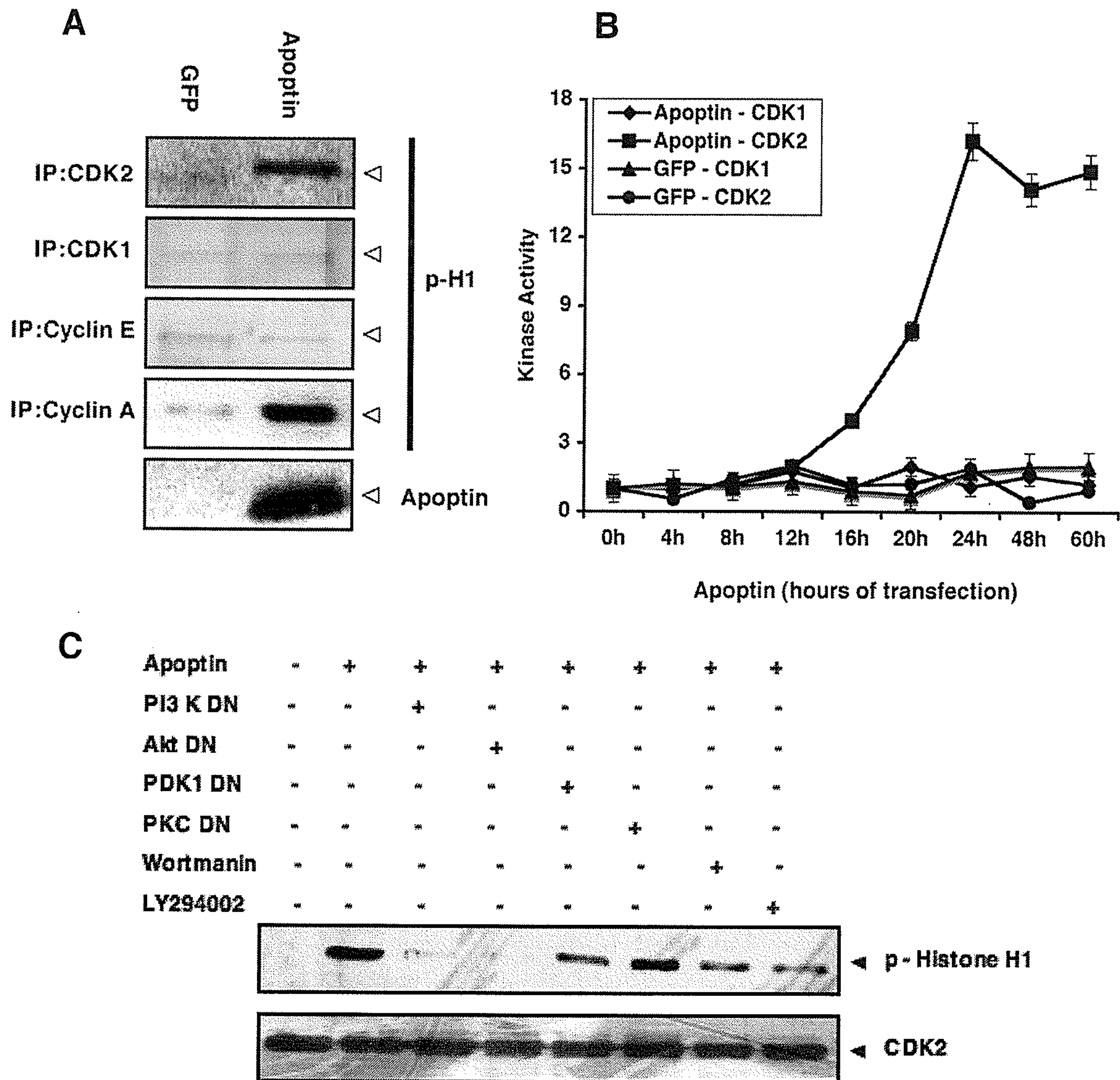


Figure 23: CDK2 is activated during Apoptin induced cell death. (A) CDK2, CDK1, cyclin E and cyclin A immuno-precipitated from the cells transfected with either GFP or GFP-Apoptin 24 hours post-transfection, was used in an *in vitro* kinase assay on Histone H1 substrate. The level of Histone H1 phosphorylation was detected by immuno-blotting with an antibody against phosphorylated Histone. (**B**) The kinase activity of CDK1 and CDK2 at different times of post transfection as indicated with both GFP or Apoptin was measured and the kinase activity is plotted by quantifying the immuno-blot signals against the untransfected control. (**C**) PC-3 cells were transfected with Apoptin alone or in the presence of different mutants or PI3-Kinase inhibitors as indicated and CDK2 activity was measured using Histone H1 substrate. Total CDK2 levels were shown by western blotting.

3.3.2 CDK2/cyclin A activity is required for Apoptin triggered cell death

We used two different approaches to test if CDK2 activity is required for Apoptin-induced cell death. The first was to inhibit CDK2 activity using a CDK2-specific inhibitor Roscovitine. Figure 24A, shows that in the presence-, as compared to the absence of CDK2 inhibitor; PC-3 cells were significantly resistant to Apoptin-induced cell death. The background levels of cell death seen with the combination of CDK2 inhibitor and Apoptin expression may be attributed to the CDK2 inhibitor itself, as the inhibitor itself is slightly toxic. The second approach was to knock down the expression of CDK2 by transfection with a plasmid encoding a CDK2 specific siRNA, and test its effect on Apoptin induced cell death. As shown in Figure 24B, CDK2 expression in PC-3 cells was down regulated by CDK2-specific siRNA after 48 hours of transfection, with the CDK2-negative cells concomitantly highly resistant to Apoptin-induced cell death (Fig. 24C).

We have shown that Apoptin's death signaling converges at the mitochondrial death pathway (Section 3.1). Here, we further investigated whether CDK2 activation is upstream, or if it is a consequence of mitochondrial death pathway activation. As shown in Figure 24D, CDK2 activation is unaffected either by Bcl-2 over-expression or by caspase inhibition using the inhibitor zVAD-fmk, but the prevention of CDK2 activation by CDK2 inhibitor completely blocked release of cytochrome c from the mitochondria to the cytosol (Fig. 24E), indicating that CDK2 activation is upstream of the mitochondrial death pathway.

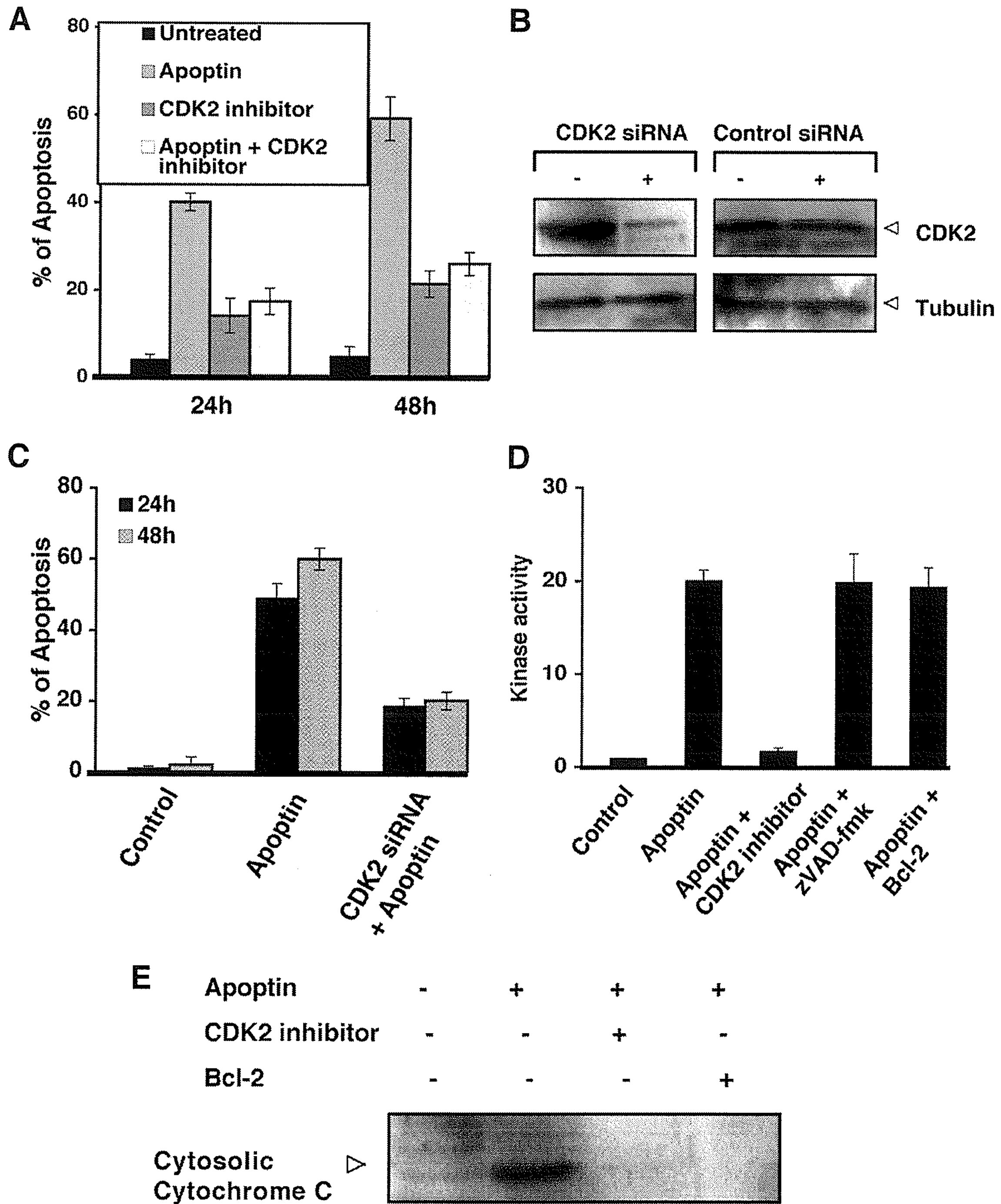


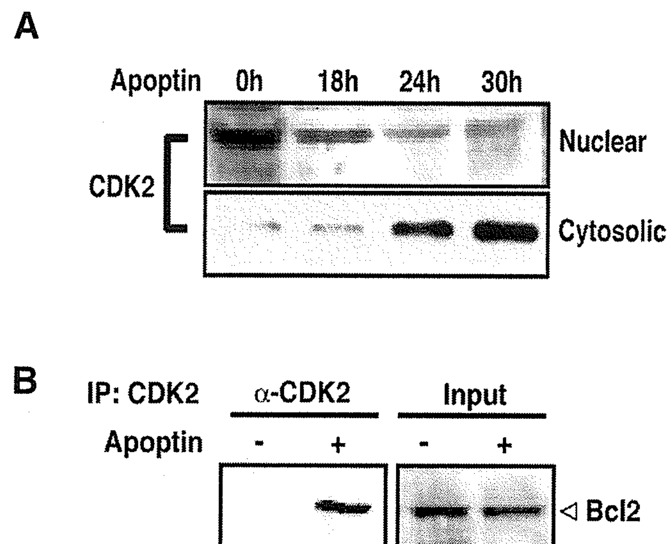
Figure 24: CDK2/cyclin A activity is required for Apoptin triggered cell death. (A) The effect of CDK2 inhibitor on Apoptin's toxicity was measured by transfecting the cells with Apoptin alone or in the presence of CDK2 inhibitor or treated with CDK2 inhibitor alone. Cell death was measured by the Nicoletti method followed by flow cytometry after 24 and 48 h respectively. The error bars shown in the graph represent the standard deviation and the data is statistically significant ($p<0.01$) according to the student's t-test.

(B) PC-3 cells were transfected with either CDK2 siRNA plasmid or control siRNA for 48 h and the CDK2 expression was detected by immuno-blotting. **(C)** The detection of apoptosis in cells transfected with GFP (control), GFP-Apoptin, and co-transfection with Apoptin and CDK2-targetting siRNA at 24 and 48 hours was done by flow cytometry. The error bars shown in the graph represent the standard deviation and the data is statistically significant ($p<0.001$) according to the student's t-test. **(D)** CDK2 activity was measured at 24 h post-transfection with Apoptin alone, cotreatment with CDK2 inhibitor, caspase inhibitor zVAD-fmk or overexpressing Bcl-2 before Apoptin transfection. The error bars shown in the graph represent the standard deviation of the data in triplicate. **(E)** The levels of cytosolic cytochrome c were detected by cellular fractionation followed by immuno-blotting with cells transfected with Apoptin alone, co-treatment with CDK2 inhibitor or Bcl-2 overexpression.

3.3.3 CDK2 translocates to the cytoplasm and downregulates Bcl-2 by its phosphorylation and enhanced degradation

Subcellular localization of CDK2 determines whether it drives either cell proliferation or cell death (Hiromura et al., 2002). CDK2 is localized mainly in the nucleus during the execution of its normal cell cycle regulatory function, but in the presence of Apoptin, CDK2 was observed predominantly in the cytoplasm (Fig. 25A). We tested the functional significance of the cytoplasmic CDK2 by investigating its substrates in the cytoplasm by co-immuno-precipitation, whereby Bcl-2 was detected in the CDK2 immune complexes in the presence of Apoptin expression (Fig. 25B). Bcl-2-phosphorylation is known to facilitate its degradation via the proteosome pathway (Furukawa et al., 2000; Yamamoto et al., 1999); to determine the effect of CDK2 on Bcl-2, we tested the levels of Bcl-2 phosphorylated either at serine- or threonine residues after immuno-precipitation from lysates from cells transfected to express Apoptin. Increased Bcl-2 phosphorylation at threonine but not serine residues was observed. The increased phosphorylation of Bcl-2 appears to occur specifically at Thr-56 (Fig. 25C); significant changes in phosphorylation of Bcl-2 at Ser-70, Thr-74 and Ser-87 were not observed either in the presence or absence

of Apoptin expression (data not shown). Bcl-2 phosphorylation results in a dramatic decrease of Bcl-2 protein, apparently due to proteosome-mediated degradation, since treatment with the proteosome inhibitor MG-115 restored Bcl-2 levels (Fig. 25D). Both Bcl-2 phosphorylation and the decrease in total protein was found to be dependent on CDK2 activity, since down-regulation of CDK2-expression by siRNA prior to transfection to express Apoptin resulted in a significant decrease in Bcl-2 phosphorylation and little change to protein levels observed as determined by quantitation of the signals (data not shown). Bcl-2 phosphorylation by CDK2 is dependent on upstream activation of the PI3-K/Akt pathway. This was indicated by the fact that down-regulation of PI3-K-expression using a specific siRNA showed an effect on Bcl-2 similar to that of the down-regulation of CDK2-expression (Fig. 25D). Thus, PI3-K/Akt mediated activation of CDK2 leads to the Bcl-2-degradation, with the low Bcl-2 level altering the balance between the pro- and anti-apoptotic Bcl-2 family members in favour of pro-apoptotic molecules, and ultimately activating the mitochondrial death pathway.



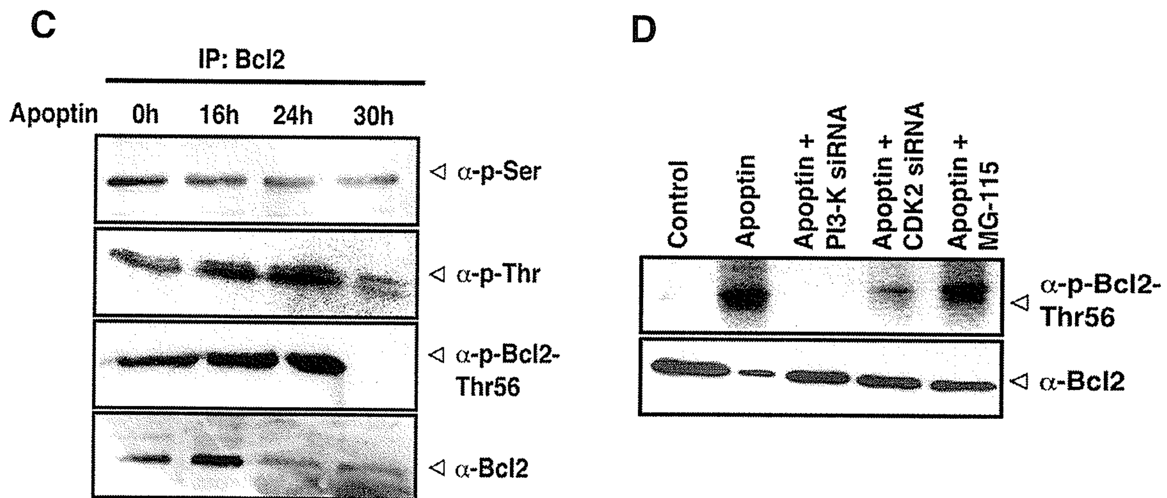


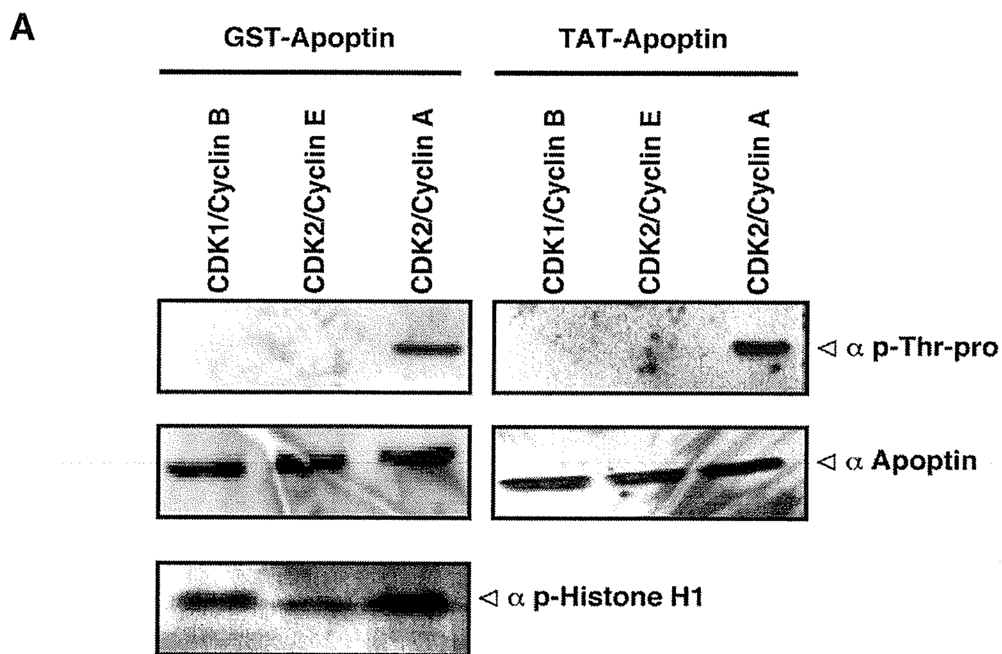
Figure 25: CDK2 translocates to cytoplasm and targets Bcl-2 for degradation. (A) The nuclear or cytoplasmic localization of CDK2 was detected by cellular fractionation followed by western blotting at different indicated times of Apoptin post transfection. **(B)** PC-3 cells were transfected with Apoptin and 20 hours later CDK2 was immunoprecipitated. The precipitates were then resolved on SDS-PAGE, and Bcl-2 was detected in the immune complexes by a specific antibody. **(C)** The phosphorylation levels of Bcl-2 were detected by immunoblotting with anti-phospho-serine, anti-phospho-threonine and anti-phopho-threonine 56 Bcl-2 specific antibodies after immunoprecipitating Bcl-2 at different indicated times of Apoptin transfection. The total Bcl-2 levels were detected by immunoblotting with anti-Bcl-2 antibody. **(D)** PC-3 cells were either transfected with Apoptin alone, co-transfected with Apoptin, PI3-K siRNA, and CDK2 siRNA or pretreated with a proteosome inhibitor MG-115 along with Apoptin. 20 hours later, the level of Bcl-2 phosphorylation at threonine 56 residue and the total Bcl-2 were detected by immunoblotting with anti-phospho-threonine 56 Bcl-2 specific antibody and anti-Bcl-2 antibody respectively.

3.3.4 Activated cyclin A-associated CDK2 is the Apoptin kinase that regulates its nuclear localization in cancer cells

Apoptin phosphorylation at Thr-108 has been previously reported to be critical for its activity and tumor cell-specific nuclear localization (Poon et al., 2005a; Rohn et al., 2002). The Apoptin's threonine-108 residue is a consensus phosphorylation site for the proline-dependent kinases like CDKs. Thus, to test whether CDK2 may directly phosphorylate Apoptin, we performed an *in vitro* kinase assay using recombinant GST-Apoptin and TAT-Apoptin as substrates. As shown in Figure 26A, detection of phosphorylated Apoptin using a phospho-threonine-proline specific antibody revealed that Apoptin can be phosphorylated by active, recombinant CDK2/cyclin A. Interestingly, active, recombinant CDK2/cyclin E was not able to phosphorylate Apoptin *in vitro*, nor was CDK1/cyclin B, although both were able to phosphorylate Histone H1 *in vitro*. We further confirmed the CDK2 phosphorylation of Apoptin using immuno-precipitated CDK2, CDK1, cyclin A, cyclin E, and cyclin B, with inactive CDK2 (CDK2 T160A) as a negative control. Apoptin was phosphorylated only by the immune complex of active CDK2 and cyclin A, but not by other combinations of CDKs and cyclins (Fig. 26B). This data correlates with the activation of cyclin A-associated CDK2, and not other CDKs, in the presence of Apoptin.

We next investigated, if activated CDK2 phosphorylates Apoptin *in vivo* at Thr-108 by two different approaches. First, we inhibited CDK2 activity in PC-3 cells using a CDK2-specific inhibitor and transfected the cells to express Apoptin. 24 h later, Apoptin was immuno-precipitated and its phosphorylation-status assessed using the phospho-specific Thr-Pro antibody. Figure 26C shows that Apoptin is phosphorylated in the

presence of active CDK2, with the level of phosphorylation significantly reduced in the presence of the CDK2 inhibitor. Secondly, we tested the effect on Apoptin phosphorylation of inhibiting the expression of CDK2 using siRNA. Figure 26C shows that Apoptin phosphorylation was severely abrogated by reduced CDK2 expression in the cells, implying that CDK2 is the Apoptin kinase. We next investigated the role of CDK2 in controlling Apoptin's nuclear localization by inhibiting CDK2 expression in PC-3 cells using siRNA. Figure 26D shows that in the presence of CDK2, Apoptin is exclusively in the nucleus, but knock down of CDK2 by siRNA severely impaired Apoptin nuclear accumulation in both cell lines. This suggests strongly that Apoptin-activated CDK2 is the Apoptin kinase controlling Apoptin nuclear accumulation in cancer cells. Furthermore, upstream activation of PI3-K/Akt is necessary for CDK2 kinase activity towards Apoptin, as the phosphorylation of Apoptin by CDK2 is reduced in the presence of PDK1-DN or Akt-DN, but not upon co-expression of an unrelated PKC-DN (Fig. 26E).



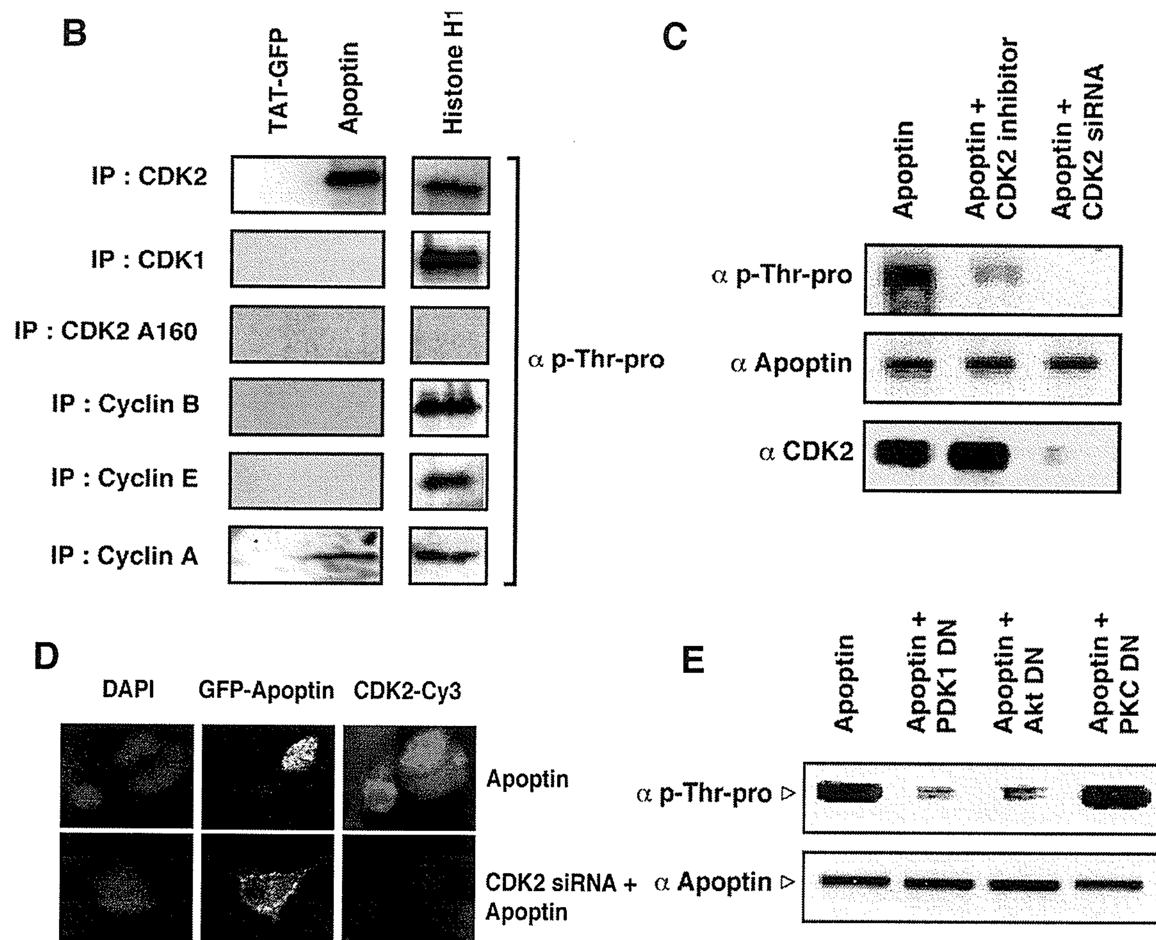


Figure 26: CDK2 is the tumor specific Apoptin kinase. (A) A non-radioactive *in vitro* kinase assay was performed with GST-Apoptin and TAT-Apoptin as substrates using active CDK1/cyclin B, CDK2/cyclin E or CDK2/cyclin A. Apoptin phosphorylation was detected by immuno-blotting using an antibody against phosphorylated threonine-proline residues. Total Apoptin levels were detected by Apoptin antibody. Histone-H1 was used as a positive control. **(B)** The active CDK2, CDK1, cyclin B, cyclin E, cyclin A were immuno-precipitated with their respective antibodies and the CDK2 T160A mutant was immuno-precipitated with anti-HA antibody and used in a kinase assay with TAT-GFP, TAT-Apoptin or H1 as substrates. The phosphorylation was detected as in figure 26A. **(C)** PC-3 cells were transfected with GFP-Apoptin alone, in the presence of CDK2 inhibitor or CDK2-targetting siRNA and GFP-Apoptin was immuno-precipitated 24 hours post-transfection with anti-GFP antibodies. The phosphorylation of Apoptin in the immuno-precipitates was detected by anti-phospho-Thr-Pro antibodies by immuno-blotting. Total Apoptin and CDK2 levels are indicated. **(D)** The effect of CDK2 inhibition on Apoptin's localization was demonstrated in PC-3 cells with GFP-Apoptin alone or co-transfection with CDK2-targetting siRNA plasmid followed by confocal imaging. **(E)** The levels of phosphorylation of Apoptin in the presence of the indicated mutants were determined as in figure 26C. Total apoptin was detected with anti-Apoptin antibodies.

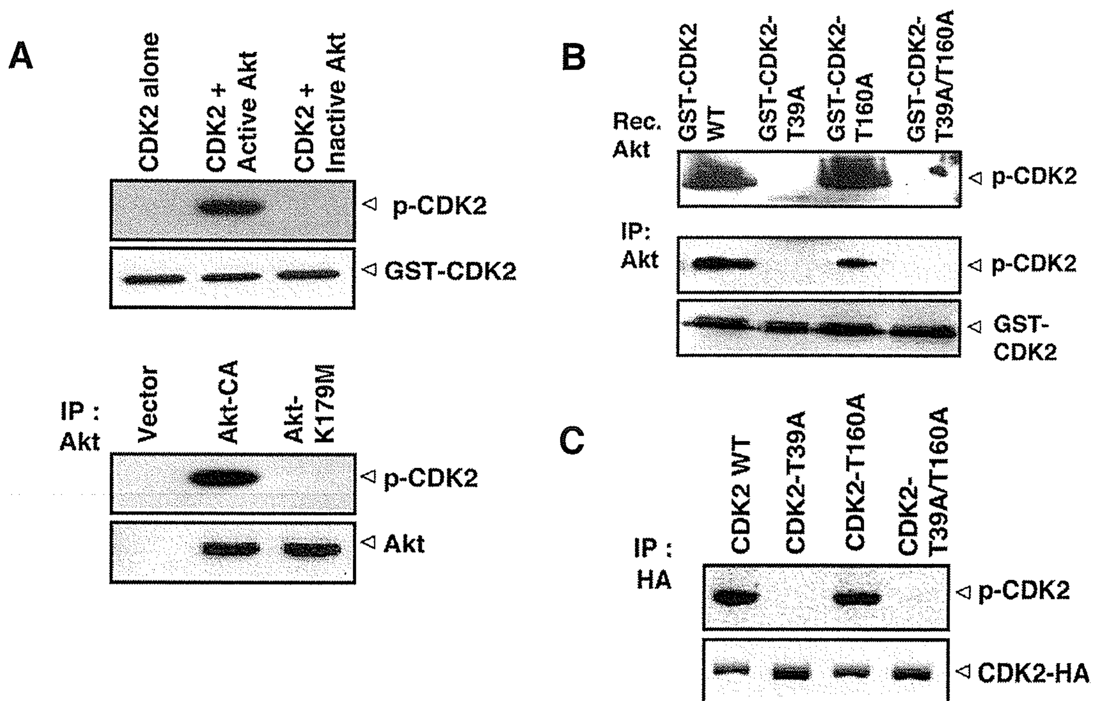
3.4 Physiological role of Akt and CDK2 signaling in cell cycle and cell death

3.4.1 Akt phosphorylates CDK2 both *in vitro* and *in vivo*

In sections 3.2 and 3.3 we have clearly shown that Akt and CDK2 are activated and are interlinked during Apoptin induced cell death. But there were no studies suggesting a direct link between Akt and CDK2 activation. Also, the physiological function of Akt and CDK2 activation together on a single signaling context is not known. Thus, to establish a direct link between Akt and CDK2, we studied whether Akt phosphorylates CDK2. We used a recombinant bacterial expressed GST-CDK2 protein as a substrate in an *in vitro* kinase assay. Akt, if activated, is able to phosphorylate CDK2 (Fig. 27A). These results were confirmed by using a constitutively active form of Akt (Akt-CA), and a catalytically inactive Akt (Akt-K179M) over-expressed in 293T cells and subsequently immuno-precipitated (Fig. 27A, lower panel). A preliminary analysis of CDK2 protein for the putative Akt phosphorylation sites revealed two partial Akt consensus sites at Threonine-39 and Threonine-160 residues. To determine if these residues are the possible Akt phosphorylation sites, the mutants replaced with a non-phosphorylatable alanine instead of the T39, T160 or both residues were used in the kinase assay. Both, active recombinant Akt or immuno-precipitated Akt phosphorylated T160A mutant to the same level as the wild type protein. No phosphorylation was seen with T39A and T39A/T160A mutant (Fig. 27B), indicating that the T39 residue is the only Akt target on CDK2.

To determine whether Akt phosphorylates CDK2 *in vivo*, within cultured cells, we constructed HA-tagged CDK2 wild type, T39A and T160A vectors and transiently

transfected them into 293T cells. In the presence of serum, the phosphorylation of CDK2 revealed that both CDK2 and CDK2 T160A mutants were phosphorylated, but T39A and T39A/T160A mutants were not phosphorylated *in vivo* (Fig. 27C). This was checked using anti-phospho-Akt substrate antibody after immuno-precipitation of CDK2. Further, to determine if Akt is the kinase phosphorylating CDK2 at the T39 site *in vivo*, either CDK2 WT or CDK2 T39A vector was transiently expressed in transformed mouse 3T3 fibroblasts. Serum starvation of cells for 30 h, which inactivated Akt, completely abolished the phosphorylation of CDK2. The activation of Akt, stimulated by either EGF or by reintroduction of 10% serum to the cells restored the CDK2 phosphorylation, which was inhibited in the presence of Wortmannin, a selective PI3-K/Akt pathway inhibitor (Fig. 27D). As expected, no Akt mediated phosphorylation was observed with CDK2 T39A mutant. This indicates that CDK2 phosphorylation at T39 residue occurs *in vivo* and is mediated by PI3-K/Akt pathway.



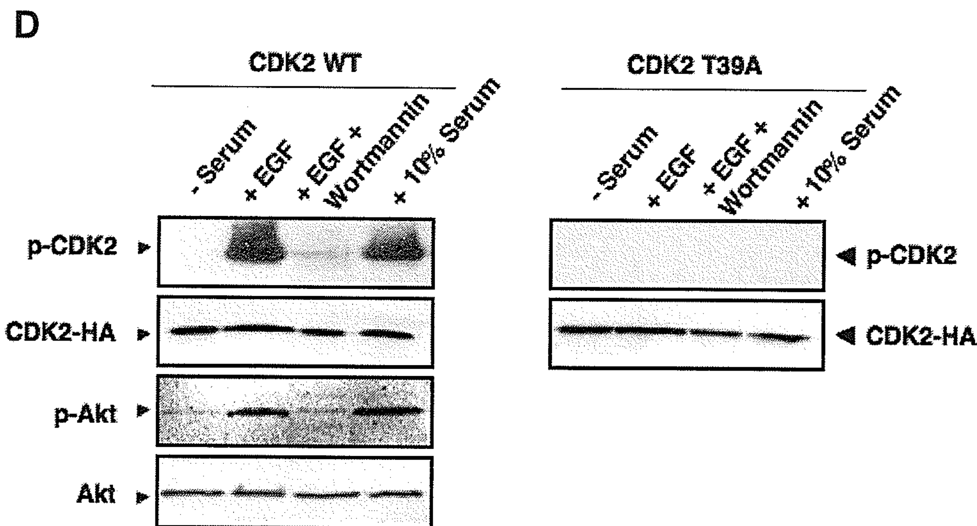


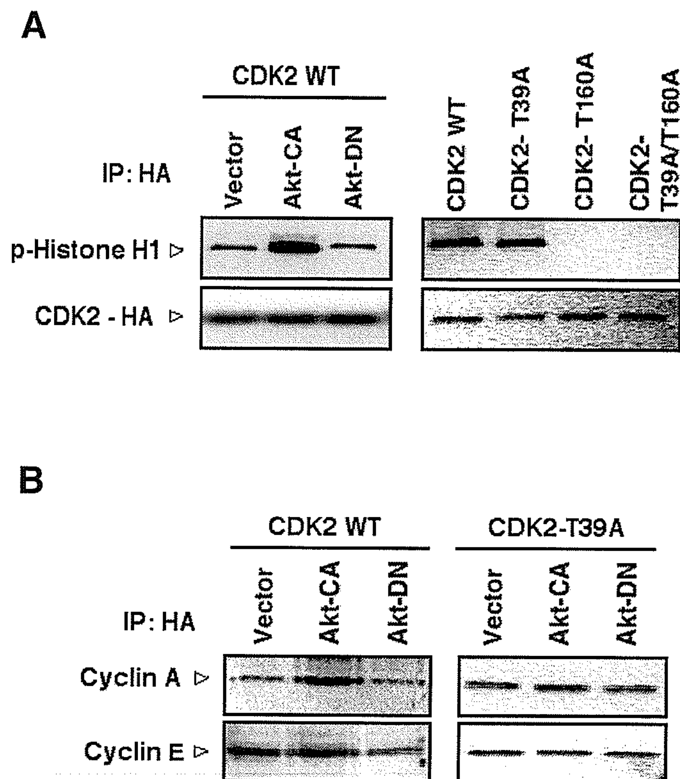
Figure 27: Akt phosphorylates CDK2 both *in vitro* and *in vivo*. **(A)** A non-radioactive *in vitro* kinase assay was performed with GST-CDK2 alone, using a recombinant active Akt or inactive Akt and the phosphorylation of CDK2 was detected by immuno-blotting with an anti-phospho substrate Akt antibody (upper panel). The levels of GST-CDK2 were detected by immuno-blotting with CDK2 antibodies. Constitutively active Akt (Akt-CA) or catalytically inactive Akt (Akt-K179M) immuno-precipitated from 293T cells were used in a similar kinase assay using GST-CDK2 as a substrate and the phosphorylation of CDK2 was detected as above (lower panel). **(B)** CDK2 wild type (WT) protein or mutated at T39, T160 or both were used in a kinase assay with a recombinant active Akt (Rec. Akt) or immuno-precipitated active Akt and the CDK2 phosphorylation was detected by immuno-blotting as in figure 27A. Immuno-blotting with CDK2 antibody monitored the expression of GST-CDK2 wild type and mutant proteins. **(C)** The CDK2 phosphorylation *in vivo* was assessed by transfecting 293T cells with either pSR-CDK2 wild type vector or the mutant proteins all tagged with HA-epitope, immuno-precipitated using anti-HA antibodies after 48 h, and blotted with anti-phospho-Akt substrate antibody. The expression of CDK2 wild type and mutant proteins was detected by anti-HA antibodies. **(D)** Murine 3T3 fibroblasts were transiently transfected with a wild type CDK2 or CDK2-T39A mutant and serum starved for 30 hours, then incubated with 20ng/ml EGF, in combination with EGF and Wortmannin (5 nM) or 10% fetal calf serum. The phosphorylation of CDK2 was detected as in figure 27A. Total CDK2 expression, phosphorylated Akt and total Akt were detected by their respective antibodies.

3.4.2 Akt mediated CDK2 phosphorylation regulates its cellular localization

To test the functional significance of CDK2 phosphorylation by Akt, we checked the CDK2 kinase activity by using Histone H1 as its substrate. The co-transfection of Akt CA, a constitutively active form of Akt along with CDK2 wild type, significantly enhanced the CDK2 kinase activity (Fig. 28A). On the other hand, the expression of dominant negative Akt restored the kinase activity to normal levels similar to the transfection with an empty vector. The expression of active Akt had no effect on total CDK2 protein levels. Interestingly, the kinase activity assessment with the wild type CDK2 along with the non-phosphorylatable T39A mutant revealed that the T39A mutant was able to phosphorylate Histone H1 as effectively as the wild type protein (Fig. 28A). This indicates that although T39 phosphorylation by Akt enhances the CDK2 kinase activity, this is dispensable for its basal kinase activity. As expected, the other CDK2 mutants lacking the phosphorylation site T160 had no kinase activity. Furthermore, to determine the effect of T39 phosphorylation on CDK2, we analyzed the association of cyclin A and cyclin E with CDK2 under different conditions. The expression of active Akt significantly enhanced the binding of cyclin A to CDK2, which was reduced to normal levels with the expression of dominant negative Akt (Fig. 28B). In contrast, the activation of Akt had no effect on the binding of cyclin E to CDK2. Interestingly the T39A mutation on CDK2 again had no effect on the binding of either cyclin A or cyclin E, thus indicating that T39 phosphorylation is not absolutely required for either cyclin A or cyclin E binding.

We next tested whether the activation of Akt and CDK2 T39 phosphorylation affects the intracellular localization of CDK2. As shown in Fig. 28C, the wild-type CDK2 was predominantly localized in the cytoplasm in the presence of Akt-CA, but was

relocated to the nucleus when dominant negative Akt was co-expressed. The mutation of CDK2 T39 to a non-phosphorylatable Alanine abolished the cytoplasmic localization even in the presence of Akt-CA. CDK2 T39A is predominantly nuclear either in the presence of Akt-CA or Akt-DN. The CDK2 T39E mutant, which mimics the phosphorylation status, was predominantly localized in the cytoplasm irrespective of the activation status of the Akt pathway. Thus, these results indicate that the T39 site is important for the regulation of CDK2 intracellular localization and the phosphorylation of this residue by Akt results in CDK2 cytoplasmic localization.



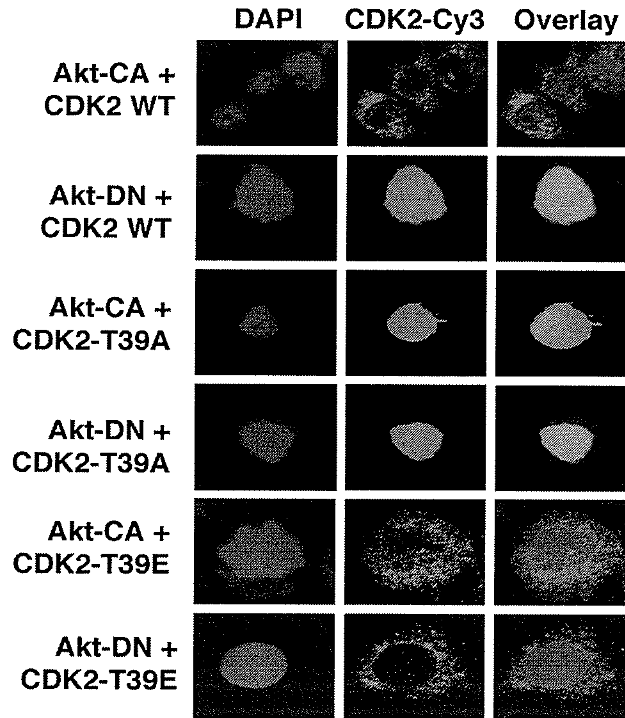


Figure 28: Akt mediated CDK2 phosphorylation regulates its cellular localization but is dispensable for basal CDK2 kinase activity. (A) 293T cells were transiently transfected with HA tagged CDK2, co-transfected with Akt-CA or dominant negative Akt (Akt-DN), or with vectors coding for CDK2-mutants. CDK2 was immuno-precipitated using anti-HA antibody and the kinase activity was assessed using Histone H1 as substrate. The phosphorylated Histone H1 was detected by immuno-blotting with phospho-H1 antibody. The expression of wild type CDK2 and mutant proteins was detected by anti-HA antibody. **(B)** The wild type CDK2 or CDK2-T39A coding vectors were transiently transfected with either vector alone, constitutively-active Akt (Akt-CA) or Akt-DN and CDK2 was immuno-precipitated using anti-HA antibody. The presence of cyclin A and cyclin E co-immuno-precipitated with CDK2 was detected by immuno-blotting with their respective antibodies. **(C)** Wild type CDK2, CDK2-T39A or CDK2-T39E and Akt-CA or Akt-DN vectors were co-transfected into 293T cells. After 24 hours, the localization of CDK2 was detected by confocal microscopy after immuno-staining with anti-CDK2 antibodies followed by Cy3 conjugated secondary antibody. DAPI was used to counter-stain nuclei.

3.4.3 Akt phosphorylates CDK2 in the nucleus during S/G2 boundary of the cell cycle

CDK2 is a nuclear protein and is required for cell cycle progression at the G1/S phase. Akt is known to regulate the cell cycle at various stages, despite the fact that it is primarily a cytoplasmic protein. We thus tested if Akt has differential activation status and further differential localization during various phases of the cell cycle, so that it can act on distinct substrates either in the cytosol or in the nucleus. The murine 3T3 fibroblasts were arrested in different phases of the cell cycle as described in the methods section, and tested for the activation of Akt by immuno-blotting with anti-phospho-Akt antibodies (Fig. 29A, upper panel). Akt, which was inactive in G0 phase, became activated in G1 phase. Akt activity was downregulated in S phase arrested cells, but peaks up again in cells arrested in G2 phase of the cell cycle. The M-phase arrested cells had a lower Akt activity. The arrest in different phases of the cell cycle has no effect on the total Akt levels. Furthermore, we tested the Akt-mediated phosphorylation of CDK2 at T39 by immuno-precipitating CDK2 and blotting with anti-phospho-Akt substrate antibody at different phases of the cell cycle. CDK2 was heavily phosphorylated at T39 residue in G2-phase arrested cells, where weaker phosphorylation was seen during S phase of the cell cycle (Fig. 29A, lower panel). No phosphorylation was seen during other phases.

In addition to the differential Akt activation status, the localization of Akt in cells arrested in different phases of the cell cycle revealed Akt either in the cytosol or nucleus, depending on the phase of the cell cycle. Akt was mainly cytoplasmic during G0 and G1 phases, whereas during G2 phase it was exclusively seen in the nucleus. During S phase, Akt is dispersed both in the cytoplasm and the nucleus (Fig. 29B, upper panel). As Akt

regulates the cellular localization of CDK2, in a separate experiment, we also tested the localization of CDK2 in cells arrested in different phases of the cell cycle. CDK2 is mainly nuclear in G1 phase, whereas exclusively cytoplasmic during G2 phase of the cell cycle. During G0 and S phases, CDK2 was found both in nucleus and the cytoplasm (Fig. 29B, lower panel). This correlates with the Akt nuclear localization at S and G2 phases of the cell cycle. These results indicate that activated Akt may translocate to the nucleus during the S phase, phosphorylate CDK2 and promote cytoplasmic CDK2 localization during late S and G2 phases of the cell cycle. To provide further evidence on the role of Akt mediated phosphorylation on the cellular localization of CDK2, we performed kinetic-studies of Akt activation and CDK2 phosphorylation as the cells progressed through the cell cycle. Murine 3T3 fibroblasts arrested in G0 phase by 30-h serum starvation, were released from G0 and the Akt and CDK2 phosphorylation was assessed at different time points as the cells progressed through the cell cycle. Akt was activated immediately after G0 release as the cells enter G1, but was only activated transiently. As the cells progressed from G1 to S phase, the Akt was deactivated. Once S phase was initiated, as the cells prepare for S to G2 progression, a second peak of Akt activation was seen around 16-18 hours after G0 release in these cells (Fig. 29C). In contrast, CDK2 activation was not seen during G1 or early S phases, but seen only during late S and G2 phases, as it was evident only after 16-18 h of G0 release in correlation with a second peak of Akt activation (Fig. 29C, lower panel). CDK2 was mainly nuclear during G1 and early S phases, but upon Akt-mediated phosphorylation it translocated to the cytoplasm during late S phase and G2 phase around 16-18 h after G0 release (Fig. 29D) and re-localized to the nucleus again during late phases of the cell cycle.

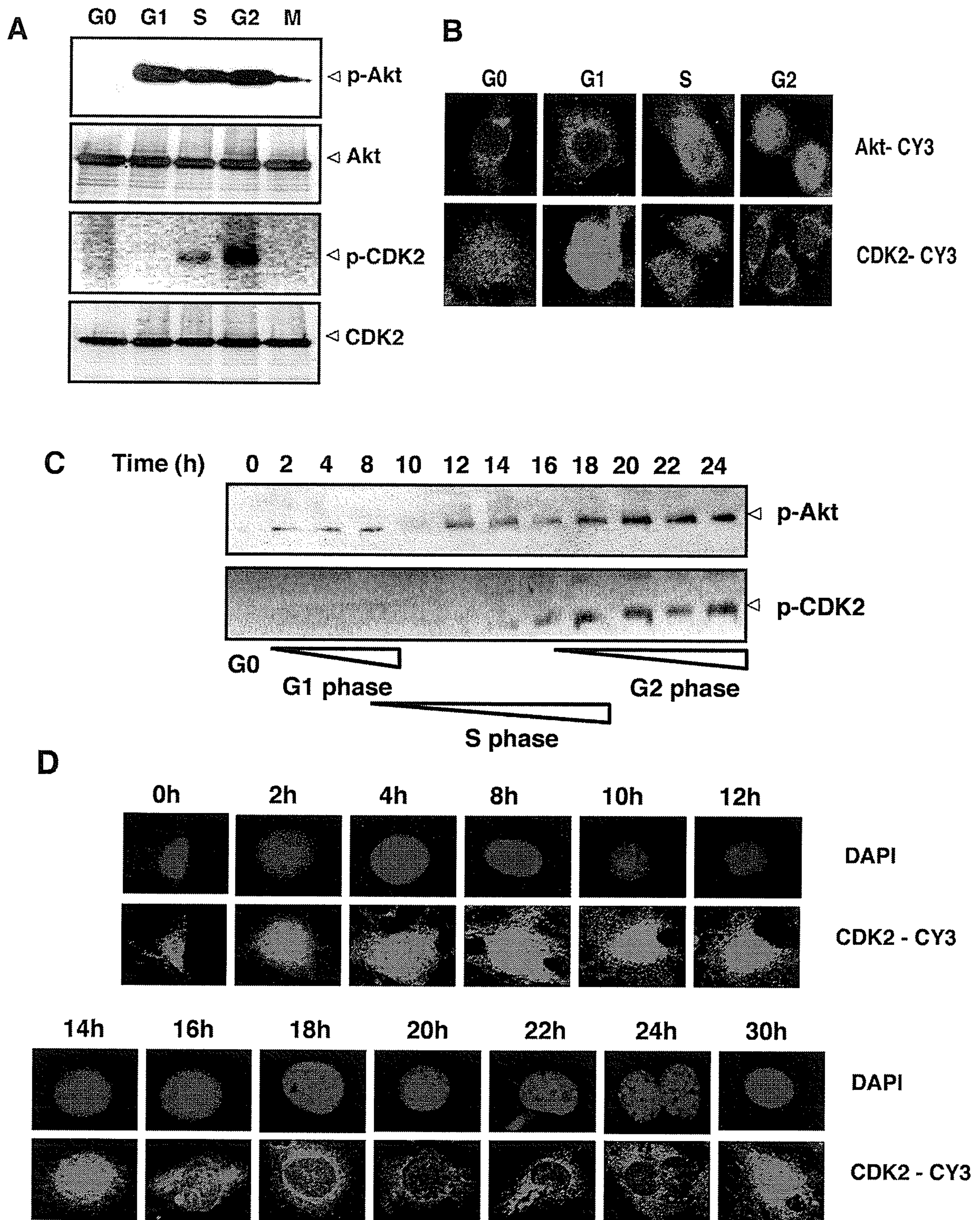


Figure 29: Akt phosphorylates CDK2 in the nucleus during S/G2 boundary of the cell cycle. (A) Murine 3T3 fibroblasts were arrested in G0, G1, S, G2 and M phases of the cell cycle as described in the methods section and the phosphorylated Akt, total Akt, phosphorylated CDK2 and total CDK2 were detected by immuno-blotting with their

respective antibodies. **(B)** The 3T3 fibroblasts were arrested in different phases of the cell cycle and the localization of Akt was detected by using confocal microscopy after immunostaining with Akt antibody followed by Cy3-conjugated secondary antibody (upper panel). In parallel samples, the localization of CDK2 was detected by using CDK2 antibody followed by Cy-3 conjugated secondary antibody (lower panel). **(C)** 3T3 fibroblasts were serum starved for 36 h and then serum was added to allow the cells to progress sequentially through different phases of the cell cycle. At the indicated time points, cells were sampled, the phosphorylated Akt was detected by immuno-blotting and the phosphorylated CDK2 was detected by immuno-precipitation of CDK2 followed by immuno-blotting with anti-phospho-substrate-Akt antibody. The stages of the cell cycle were confirmed by 'FITC-BrdU Flow Kit' staining, followed by flow cytometry at the indicated time points and each cell cycle phase is denoted below the lower immuno-blotting panel. **(D)** The cells grown on cover slips were synchronously put through the cell cycle as described in figure 29C, they were then collected at indicated time points, and the localization of CDK2 was detected by confocal microscopy preceded by immuno-staining with anti-CDK2 antibodies followed by a Cy-3 conjugated secondary antibody. DAPI was used to counterstain the nucleus.

3.4.4 Akt regulated nucleo-cytoplasmic CDK2-relocation is required for cell cycle progression

To determine the effect of CDK2 phosphorylation on the progression of the cell cycle, we assessed both the G1/S and G2/M population under different conditions. The overexpression of a non-phosphorylatable CDK2-T39A mutant, or inactivation of Akt by overexpression of Akt-DN, resulted in an increase in G1/S cell population (Fig. 30A) and a significant decrease of cells in G2/M phase of the cell cycle (Fig. 30B). On the other hand, the transfection of Akt-CA or wild type CDK2 increased the G2/M population. This indicates that Akt activation and CDK2 phosphorylation are important for S to G2/M progression. To determine the role of CDK2 T39 phosphorylation on the progression through the S- and G2 phases of the cell cycle, we examined the progression of S to G2 phase in the presence of both phosphorylation active and inactive CDK2 mutants. The 3T3 fibroblasts arrested in S phase were released and the percentage of cells entering G2 was

assessed. After 3 hours of S phase release, the presence of wild type CDK2 and the CDK2-T160A mutant had no significant effect on the percentage of cells entering G2 phase compared to controls, whereas the CDK2-T39A transfection resulted in the reduction of cells entering from S to G2 (Fig. 30C). Although the CDK2-T39A/T160A has a non-phosphorylatable T39 site, it had no effect on the percentage of G2 cells, as it is inactive as a CDK2 kinase. Thus, these results indicate that Akt mediated transient phosphorylation of CDK2 at the T39 site and the resulting CDK2 cytoplasmic localization occurs in late S and G2 phases and is absolutely necessary for the cells to progress from S to G2/M phases.

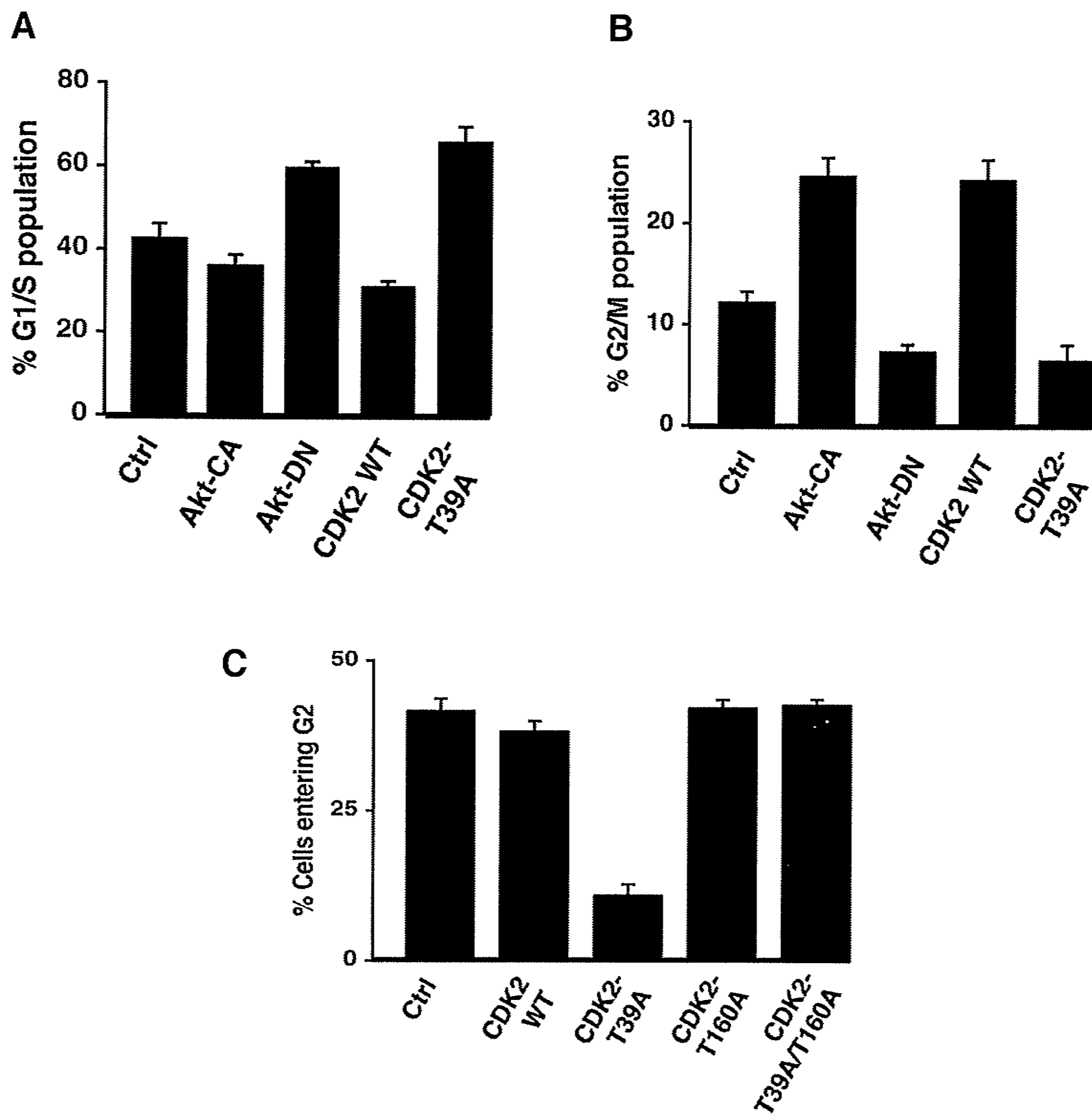
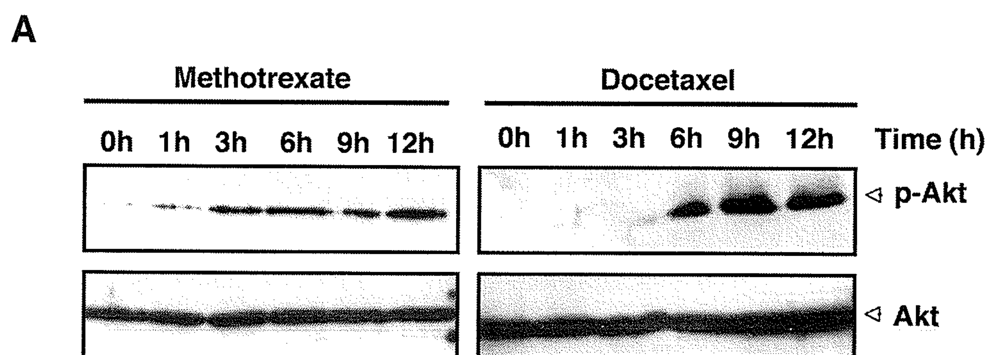


Figure 30: Akt regulated nucleo-cytoplasmic CDK2-relocation is required for cell cycle progression. (A) 3T3 fibroblasts were transfected with Akt-CA, Akt-DN, wild type CDK2, or with CDK2-T39A expressing vectors. 24 h later the percentage of G1/S and **(B)** G2/M populations were gated and analyzed by flow cytometry following propidium iodide staining. **(C)** The cells were transfected with wild type CDK2, CDK2-T39A, CDK2-T160A or CDK2-T39A/T160A expressing vectors, then arrested in S phase using 2μg/ml of Aphidicolin for 20 hours and released from S phase for 3 hours. The percentage of cells entering from S to G2 phase was assessed by BrdU labeling as described in the methods section, followed by flow cytometric analysis. The error bars shown in figures 30A-C represent the standard deviation from three independent experiments and the data is statistically significant ($p<0.002$) according to the student's t-test.

3.4.5 Selected anticancer drugs constitutively activate PI3-K/Akt pathway

Although Akt and CDK2 are required for cell cycle progression, they have also been assigned an important role in regulating apoptosis either positively or negatively by different apoptotic stimuli (Deb-Basu et al., 2006; Shi et al., 1996). In sections 3.2 and 3.3 we have shown that the constitutive activation of PI3-K/Akt-CDK2 pathway is required for Apoptin induced cell death. Further, we also tested if the above-described PI3-K/Akt-CDK2 pathway has a role in apoptosis induced by other selected anticancer drugs. We screened for the activation of PI3K-Akt pathway in MCF-7 cells using several known anti-cancer drugs and found that methotrexate and docetaxel (Fig. 31A) were able to constitutively activate Akt. The activation of Akt by methotrexate occurred very rapidly after around 30 minutes, and the kinase remained constitutively active even upto 12 hours of treatment. Docetaxel-induced Akt activation occurred late, after 6 hours of treatment, and it remained constitutively active. We further tested the localization of Akt in presence of methotrexate and docetaxel. Akt was mainly cytoplasmic (Fig. 31B), but the treatment of MCF7 cells with docetaxel for 6 hours resulted in the nuclear localization of Akt. Also, the treatment of cells with methotrexate for 3 hours induced Akt nuclear localization. The

nuclear localization of Akt was dependent on the upstream activation of the PI3-Kinase, as the inhibition of the PI3-K activity by Wortmannin abrogated the nuclear localization of Akt even in the presence of the chemotherapeutics (Fig. 31B). To further demonstrate the importance of nuclear Akt during cell death induced by these drugs, we compared the cells treated with the anticancer drugs alone to those that were transduced with adenovirus encoding nuclear Akt. The transduction of MCF-7 cells with nuclear Akt enhanced the cell death induced by both methotrexate and docetaxel. Interestingly, the cell death was partly inhibited by pre-treatment of cells with Wortmannin (Fig. 31C), indicating that the PI3-K/Akt activation and the nuclear Akt are important for cell death induced by these drugs. We also observed a similar trend with another chemotherapeutic drug, doxorubicin (data not shown). In a control experiment, we transduced both the 293 and PC-3 cells with a constitutively active Akt, and cell death was assayed 48 h after transfection. The over-expression of Akt-CA alone induced 10-15% cell death, as compared to the control cells (Fig. 31D), preceded by a G2/M cell cycle arrest (Fig. 30B).



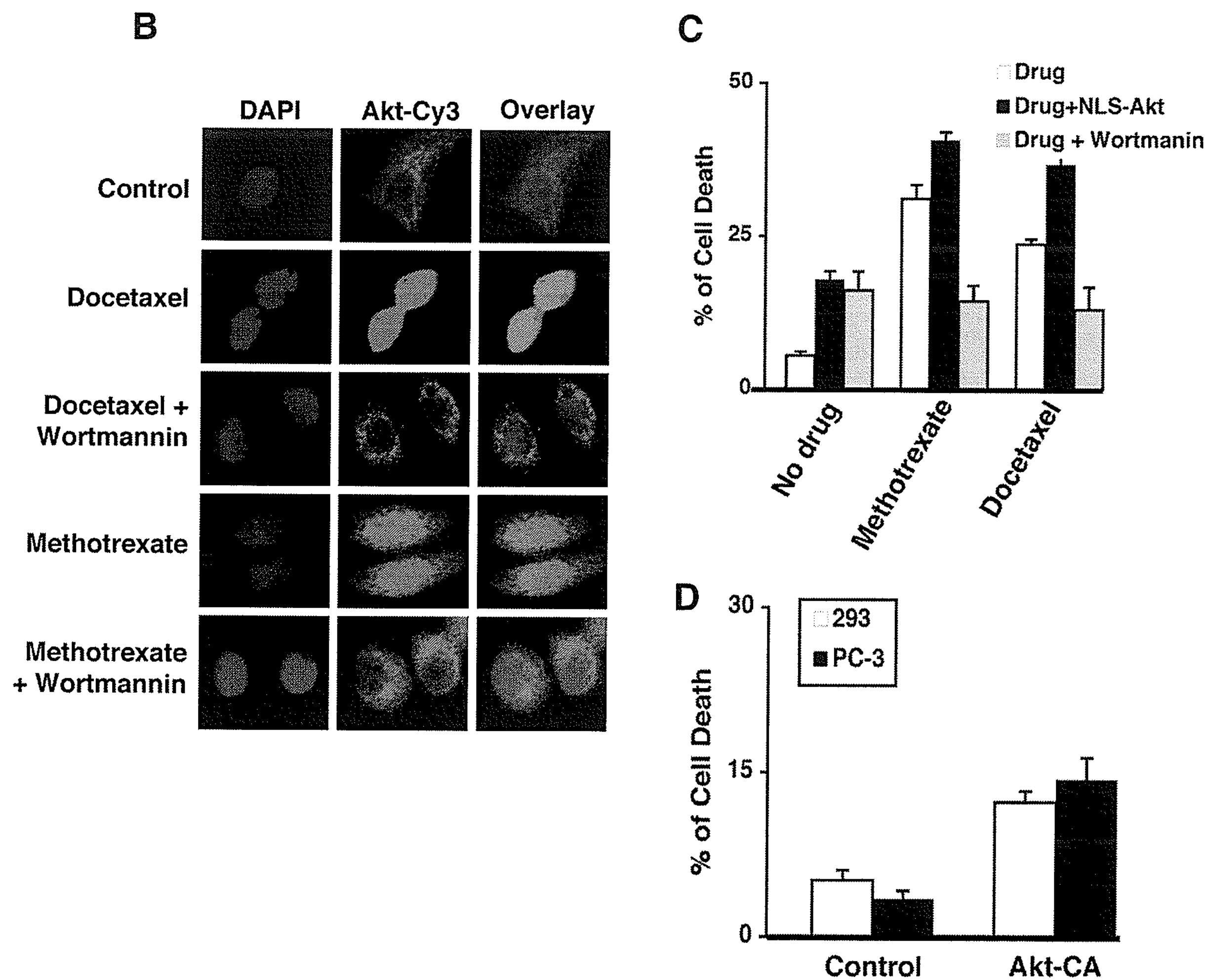


Figure 31: Methotrexate and docetaxel activate Akt and induce its constitutive nuclear translocation. (A) MCF-7 cells were treated with methotrexate (10μM) or docetaxel (0.05μM) for the indicated time, then phosphorylated Akt and total Akt were detected by immuno-blotting with the respective antibodies. **(B)** The localization of Akt in the nucleus in either control untreated cells or the drug treated cells (as indicated) was assessed by immuno-staining followed by confocal microscopy. Six hours after docetaxel treatment or 3 h after Methotrexate treatment, cells were fixed, and the immuno-staining was performed with anti-Akt antibodies followed by secondary Cy-3 conjugated antibody. DAPI was used as nuclear counter-stain. **(C)** 293T cells were left untreated or treated with Methotrexate or Docetaxel alone, or in combination with either adenoviral vector coding for nuclear Akt, or with Wortmannin. 24 h later, the cells were stained using the Nicoletti method, and the percentage of cell death was assessed by flow cytometry. The error bars shown in the graph represent the standard deviation and the data is statistically significant ($p<0.04$) according to the student's t-test. **(D)** 293 T and PC-3 cells were transiently transfected with Akt-CA coding vector, the cell death was then assayed by flow cytometry as described above. Comparison was made between the Akt-CA –transfected and the non-transfected, control cells. The error bars shown in the graph represent the standard

deviation of the data from four different experiments. The significance of the data ($p<0.02$) is statistically evaluated using the student's t-test.

3.4.6 CDK2 is required for Methotrexate and Docetaxel induced cell death

As Methotrexate and Docetaxel activated Akt and induced nuclear Akt localization constitutively, we tested if the Akt/CDK2 pathway was involved in the cell death induced by these drugs. The CDK2 phosphorylation detected by phospho-specific antibody after CDK2 immuno-precipitation revealed that CDK2 is phosphorylated at the T39 site upon treatment of Methotrexate (Fig. 32A, upper panel) for 6 h and Docetaxel (Fig. 32A, lower panel) for 9 h respectively. The CDK2 phosphorylation induced by Methotrexate and Docetaxel was dependent on Akt activation as the pre-treatment of the cells with Wortmannin severely reduced the CDK2 phosphorylation even in the presence of the anticancer drugs. The CDK2 phosphorylation at T39 upon Methotrexate and Docetaxel treatment resulted in the constitutive translocation of CDK2 to the cytoplasm (Fig. 32B). To determine if the CDK2 activity in the cytoplasm is required for cell death induced by these drugs, we used two different approaches. First, we inhibited the CDK2 activity using a pharmacologic CDK2 inhibitor, Roscovatine and treated the cells with anticancer drugs. Roscovatine treatment significantly inhibited cell death induced by both Methotrexate and Docetaxel (Fig. 32C). In the second approach, we compared cell death induced by these anticancer drugs in immortalized CDK2 wild type fibroblasts and the CDK2-deficient fibroblasts. Both Methotrexate and Docetaxel were able to induce cell death efficiently in wild type fibroblasts, whereas cell death was significantly inhibited in CDK2 null cells (Fig. 32D). These results indicate that Akt mediated CDK2 phosphorylation at T39 contributes to cell death induced by Methotrexate and Docetaxel.

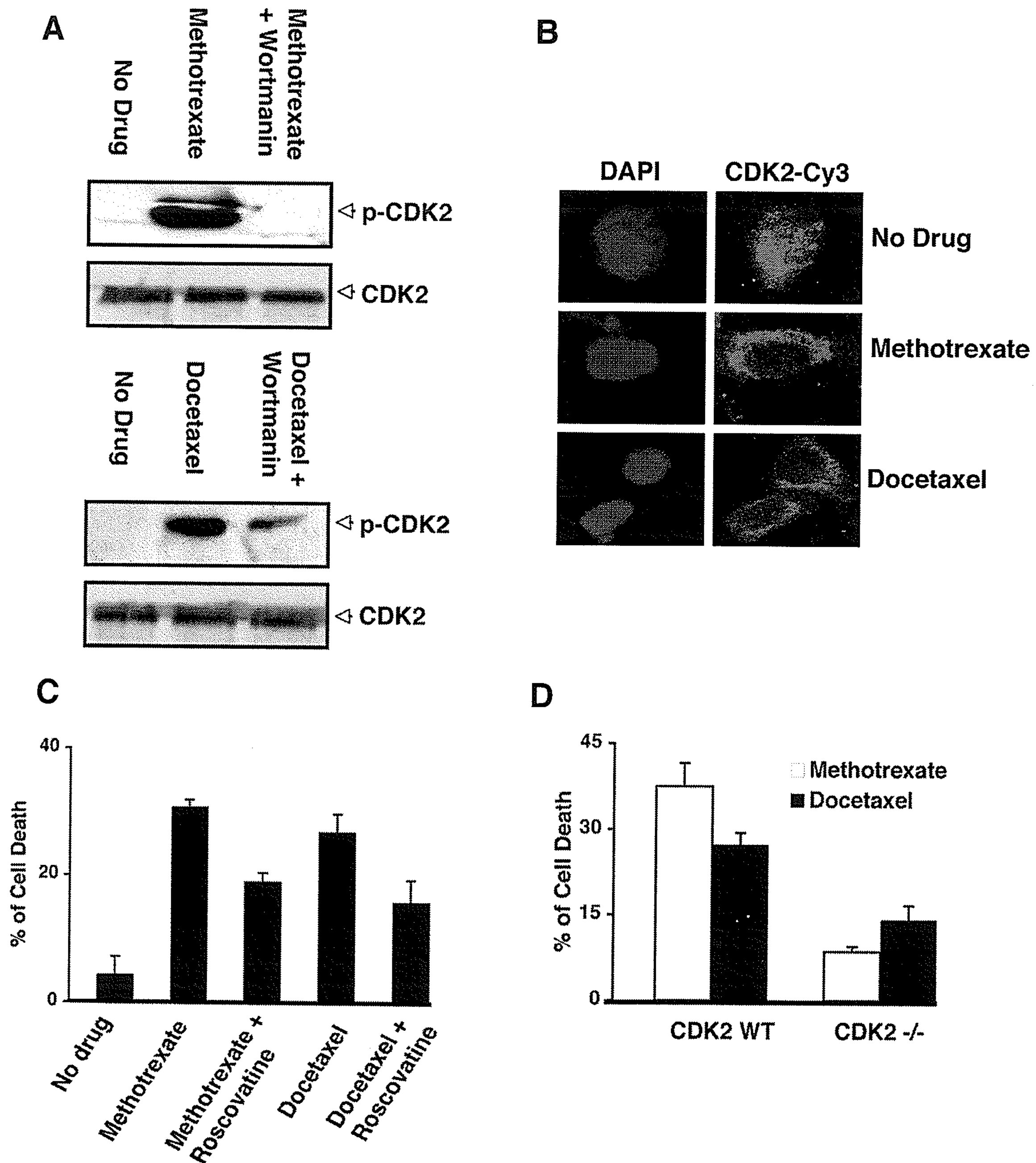


Figure 32: CDK2 is required for Methotrexate and Docetaxel induced cell death. (A) 293T cells were left untreated, treated with Methotrexate alone (upper panel), Docetaxel alone (lower panel) or after 15 min pre-treatment with Wortmannin. CDK2 was immuno-precipitated with anti-CDK2 antibody and the phosphorylation of CDK2 was detected by immuno-blotting with a phospho-specific Akt-substrate antibody. Total CDK2 was detected by a CDK2 specific antibody. **(B)** 293T cells were left untreated, treated with Methotrexate or Docetaxel and the localization of CDK2 was detected by confocal microscopy after immuno-staining with anti-CDK2 antibody followed by a Cy-3

conjugated secondary antibody. **(C)** The cells were left untreated, treated with Methotrexate alone, Docetaxel alone or the drugs were added 15 min after pretreatment with Roscovatine. 24 h later, the percentage of cell death was assayed by flow cytometry (Nicoletti method). The error bars shown in the graph represent the standard deviation and the data is statistically significant ($p<0.04$) according to the student's t-test. **(D)** Immortalized CDK2 wild type (CDK2 WT) and CDK2 deficient fibroblasts (CDK2-/-) were treated with either Methotrexate or Docetaxel and 24 h later the percentage of cell death was assayed by flow cytometry (propidium iodide staining). The error bars shown in the graph represent the standard deviation and the data is statistically significant ($p<0.02$) according to the student's t-test.

3.5 Additional targets in the Akt and Apoptin cell death pathway

3.5.1 Akt phosphorylates $p27^{kip1}$ in the nucleus

As most of the Akt is localized to the nucleus during Apoptin induced cell death, we next attempted to identify other possible nuclear targets for Akt. $p27^{kip1}$, a negative regulator of the cell cycle at G1/S phase, has an Akt consensus phosphorylation motif. In addition $p27^{kip1}$ is a potent inhibitor of CDK2, thus we investigated whether $p27^{kip1}$ is a potential nuclear target of Akt activity. $p27^{kip1}$ was immuno-precipitated from the lysates of untransfected cells and cells transfected to express Apoptin. Phosphorylation of $p27^{kip1}$ was monitored using anti-phospho-serine or anti-phospho-threonine antibodies. $p27^{kip1}$ threonine phosphorylation levels increased in the presence of Apoptin, but Apoptin had no effect on serine phosphorylation (Fig. 33A). We confirmed the phosphorylation of $p27^{kip1}$ at threonine residues by using an antibody against phosphorylated Thr-157 (a potential Akt consensus site). Analysis of the kinetics of phosphorylation of $p27^{kip1}$ at Thr-157 revealed that it occurs soon after Akt nuclear translocation. A significant increase in the phophorylation at Thr-157 of $p27^{kip1}$ was seen 12 hours after transfection to express Apoptin and diminishes after 24-30 hours (Fig. 33B). Phosphorylation of $p27^{kip1}$ at Thr-157 was strongly dependent on Akt activation, which was further dependent on PI3-K and

PDK1 upstream activation. The co-transfection of cells to express Apoptin together with dominant negative PI3-K, PDK1, or Akt mutant derivatives significantly decreased the phosphorylation at Thr-157 of $p27^{kip1}$. In contrast, phosphorylation of the Thr-157 residue was not affected by co-transfection to express a dominant negative form of an unrelated kinase, PKC (Fig. 33C).

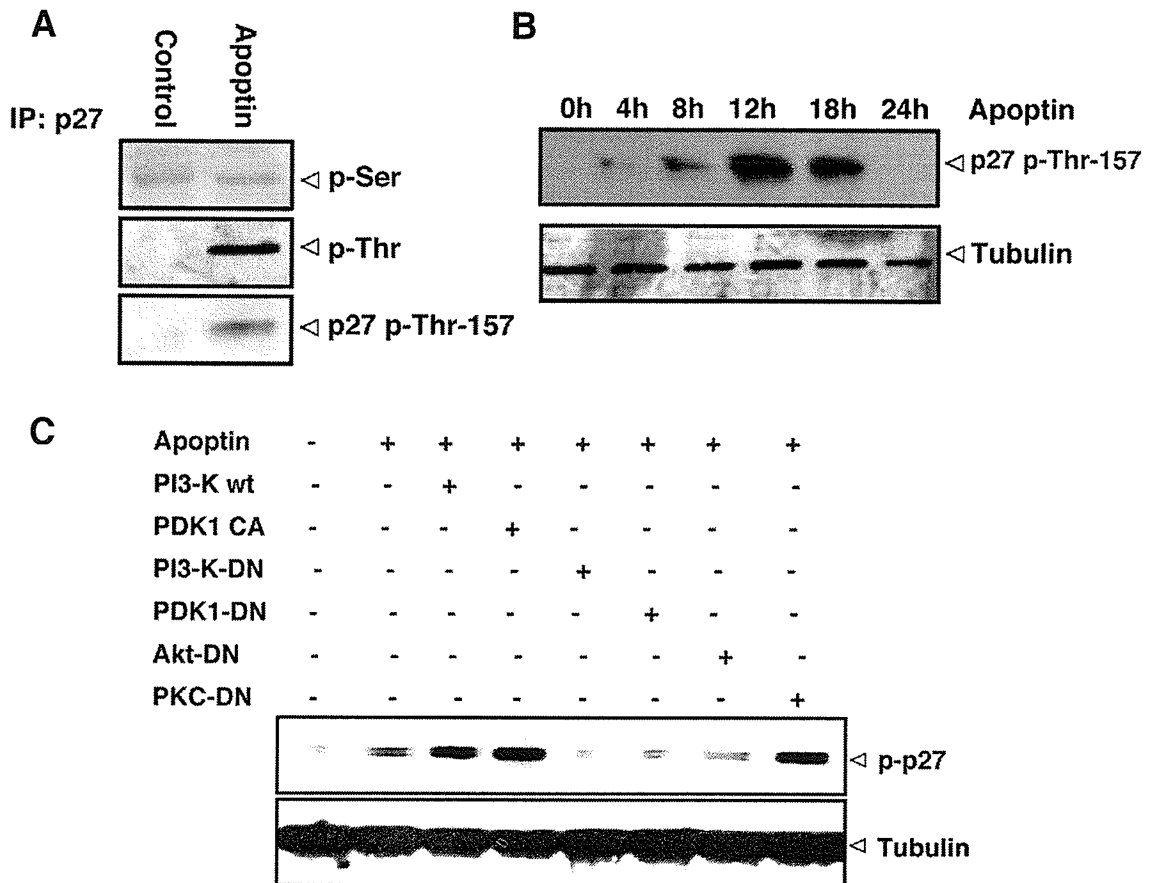


Figure 33: Akt phosphorylates $p27^{kip1}$ in the nucleus. (A) The phosphorylation of $p27^{kip1}$ at serine and threonine residues either in the presence of GFP control plasmid or GFP-Apoptin was detected by phospho-serine or phospho-Thr-specific antibodies after immuno-precipitation with anti-$p27^{kip1}$ antibody. The threonine phosphorylation was also detected by an antibody against phosphorylated $p27^{kip1}$ at Thr-157 residue. **(B)** $p27^{kip1}$ phosphorylation after Apoptin transfection at different time points was detected by immunoblotting with anti-phospho-$p27^{kip1}$-Thr-157 antibody, tubulin was used as a loading control. **(C)** Cells were transfected with Apoptin alone or co-transfected with either 'wild type' PI3-K, constitutively active PDK1 (CA), PI3-K dominant-negative (DN) vector,

PDK1-DN, Akt-DN and PKC-DN vectors. After 16 hours, cells were lysed and the phosphorylated $p27^{kip1}$ was detected by Western blot.

3.5.2 Akt mediated phosphorylation enhances the degradation of $p27^{kip1}$ via proteosomal degradation pathway

Previous studies have shown that $p27^{kip1}$ phosphorylation targets it into the proteosome-dependent degradation pathway (Tsvetkov et al., 1999). To determine the functional significance of $p27^{kip1}$ phosphorylation by Akt, we checked the protein levels of $p27^{kip1}$ before and after transfection to express Apoptin. Strikingly, we found a dramatic decrease in the levels of $p27^{kip1}$ in Apoptin-expressing cells compared to the GFP control expressing cells (Fig. 34A). The kinetics of $p27^{kip1}$ down regulation revealed that about 24 hours post-transfection, $p27^{kip1}$ was completely down regulated in PC-3 cells. While the kinetics were observed to vary somewhat in different cell lines (data not shown), a similar trend of transient phosphorylation followed by down regulation was clearly evident. The decrease in the $p27^{kip1}$ protein levels was entirely dependent on $p27^{kip1}$ phosphorylation by Akt, as the levels of the protein were restored to the control levels in Apoptin expressing cells that have been pre-treated with Wortmannin but not with PD98059 (Fig. 34B). The restoration of $p27^{kip1}$ levels was also seen upon co-expression of PI3-K-DN, PDK1-DN or Akt-DN but not with PKC-DN (Fig. 34C). The decrease in the protein levels of $p27^{kip1}$ was due to proteosome-dependent degradation as the treatment of cells with the proteosome inhibitor MG-115 restored $p27^{kip1}$ levels even in the presence of Apoptin expression (Fig. 34B).

To investigate the role of $p27^{kip1}$ down-regulation during Apoptin-induced cell death, we used an siRNA-based approach. $p27^{kip1}$ expression was down-regulated upon

transfection to express p27^{kip1}-specific but not control siRNA (Fig. 34D). The transfection of Apoptin into p27^{kip1}-siRNA expressing cells revealed that p27^{kip1} down regulation sensitizes the cells to Apoptin-induced cell death (Fig 34E) compared to Apoptin alone.

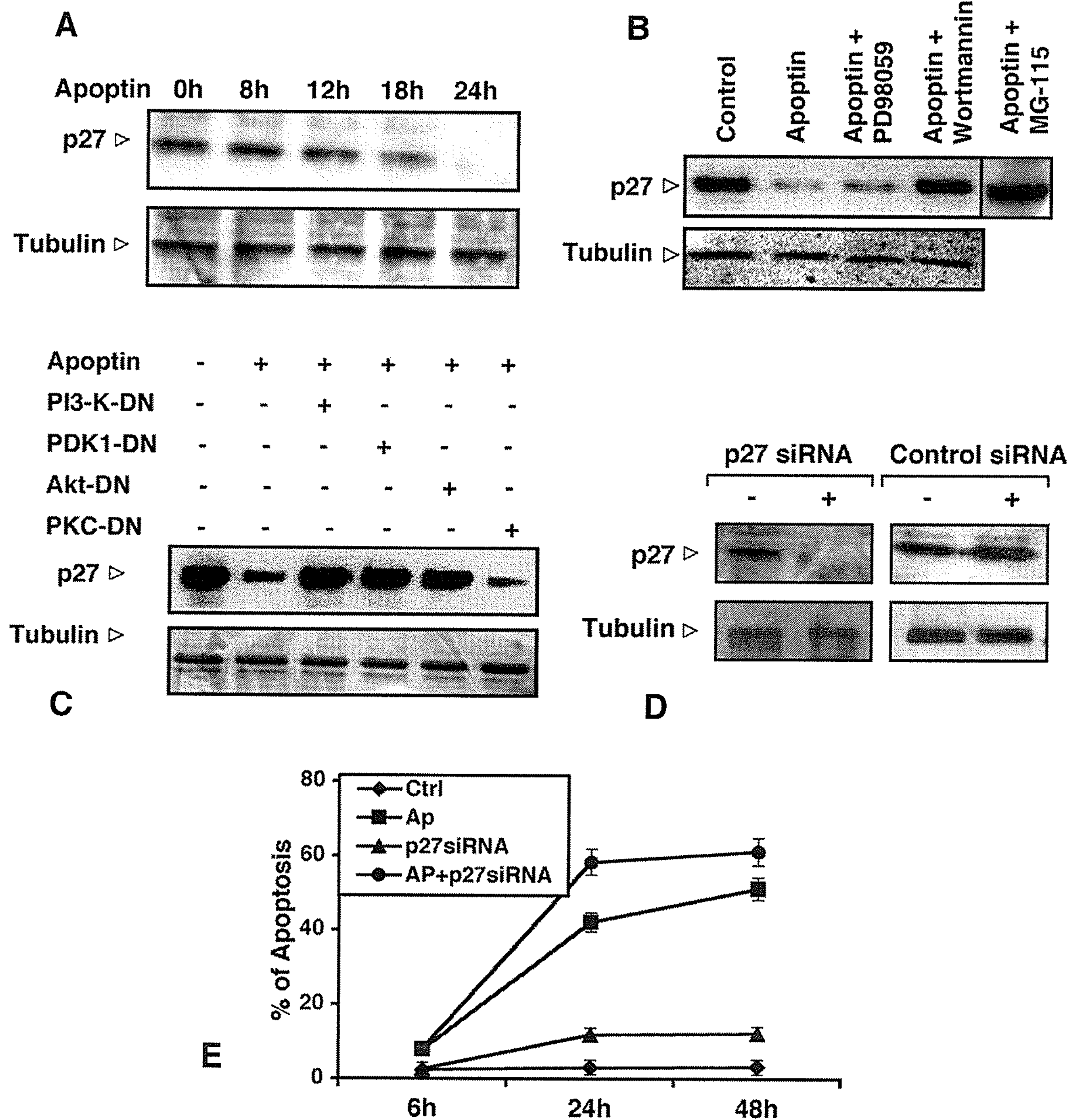


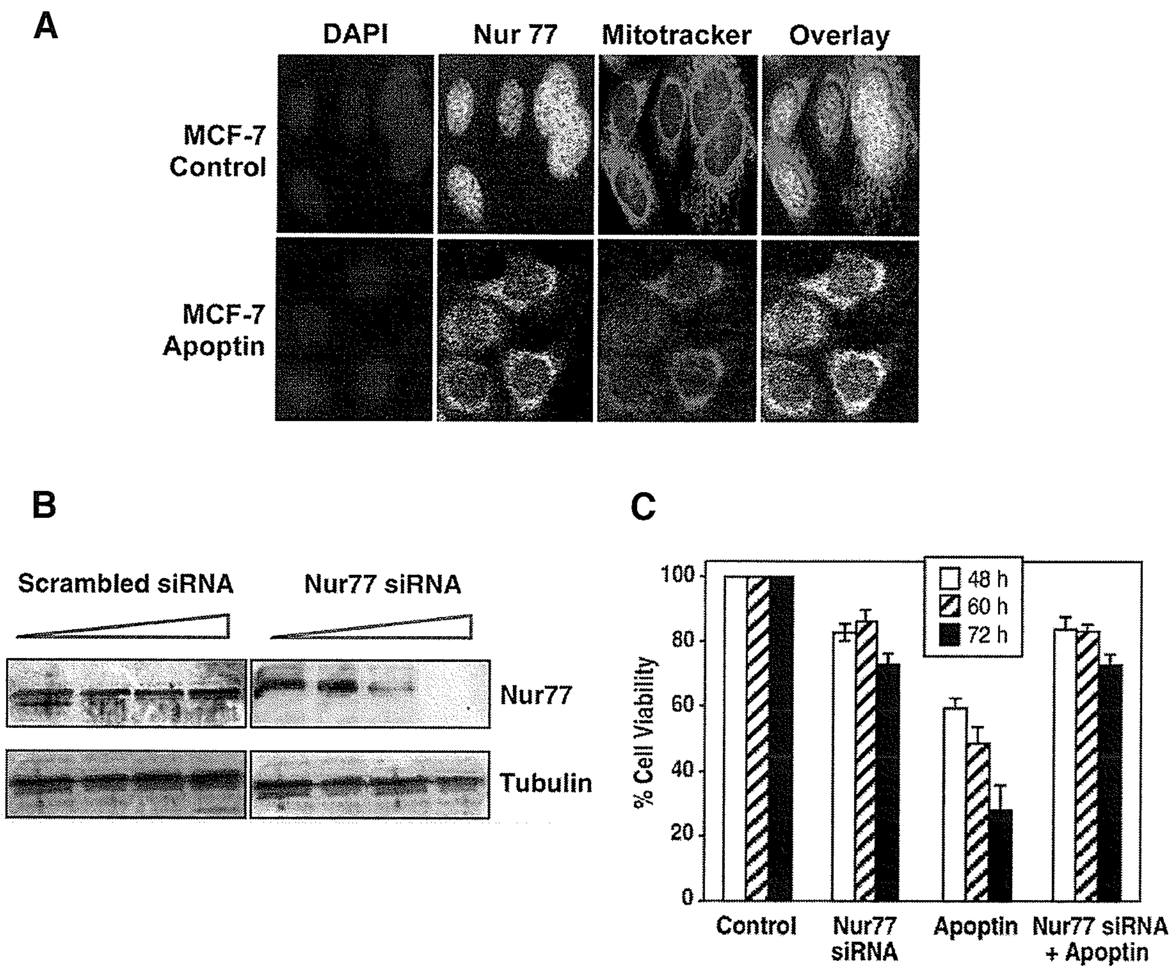
Figure 34: Akt mediated phosphorylation enhances the degradation of p27^{kip1} via proteosomal degradation pathway. (A) The levels of p27^{kip1} in GFP-Apoptin transfected PC-3 cells were followed within 24 hours post transfection by Western blot. Tubulin acted as a loading control. **(B)** PC-3 cells were transfected with Apoptin alone or transfected

after pre-treatment with with PD98059, Wortmannin, or MG-115 and the levels of p27^{kip1} was detected by immunoblotting. Tubulin was used as a loading control **(C)** The effect of selected kinases on p27^{kip1} protein levels, in the presence of Apoptin. p27^{kip1} was detected by immuno-blotting in lysates of PC-3 cells 24 hours after transfection either with GFP, GFP-Apoptin alone or upon co-transfection with PI3-K-DN, PDK1-DN, Akt-DN and PKC-DN. **(D)** PC-3 cells were transfected with either p27^{kip1} siRNA or scrambled (control) siRNA for 36 hours and the p27^{kip1} expression was detected by immuno-blotting. **(E)** PC-3 cells were transfected with GFP (ctrl), GFP-Apoptin (Ap), p27^{kip1}-siRNA alone, or co-transfection with Apoptin. Apoptosis was measured by flow cytometry (Nicoletti) at the indicated time points. The data represents average results from three independent experiments. The error bars shown in the graph represent the standard deviation and the data is statistically significant ($p<0.03$) according to the student's t-test.

3.5.3 Nur77 transmits the Apoptin-induced death signal from nucleus to the mitochondria.

The phosphorylation-dependent nuclear retention of Apoptin occurs only in transformed cells and it plays an important role in Apoptin's toxicity. Yet, ultimately Apoptin activates the mitochondrial pathway. Nur77 and p53 are the best-known candidates for nucleo-cytoplasmic apoptotic signalling (Moll et al., 2006). Also, Akt phosphorylates Nur77 and regulates its function (Pekarsky et al., 2001). Since it has been reported that Apoptin triggered cell death is p53-independent (Zhuang et al., 1995a), we have tested if Nur77, a nuclear orphan receptor of steroid/thyroid receptor family member, plays a role in the Apoptin-induced pathway. Nur77 was predominantly localized in the nucleus, but Nur77 was translocated to cytosol and co-localizes at the mitochondria around 24 h of TAT-Apoptin treatment (Fig. 35A). Furthermore, the knock down of Nur77 expression by specific siRNA but not a scrambled siRNA (Fig. 35B) strongly protected from Apoptin induced cell death (Fig. 35C). Also, cells stably transfected with Nur77 dominant negative vector showed reduced toxicity to Apoptin compared to Nur77 wild type cells indicating the negative effect of Nur77 loss on Apoptin induced cell death (Fig.

35D). We further investigated whether Nur77 translocation is upstream of mitochondrial death pathway activation. As shown in Figure 35E, Nur77 translocation was unaffected by caspase inhibition using zVAD-fmk. The prevention of Nur77 translocation by using a nuclear export inhibitor Leptomycin B (Fig35E) partially blocked the release of cytochrome c from the mitochondria to the cytosol (Fig. 35F), indicating that Nur77 translocation is upstream of the mitochondrial death pathway. Together, these data show that Nur77 is an integral molecule within the Apoptin-triggered cell death pathway that is required for nucleus to mitochondrial signaling.



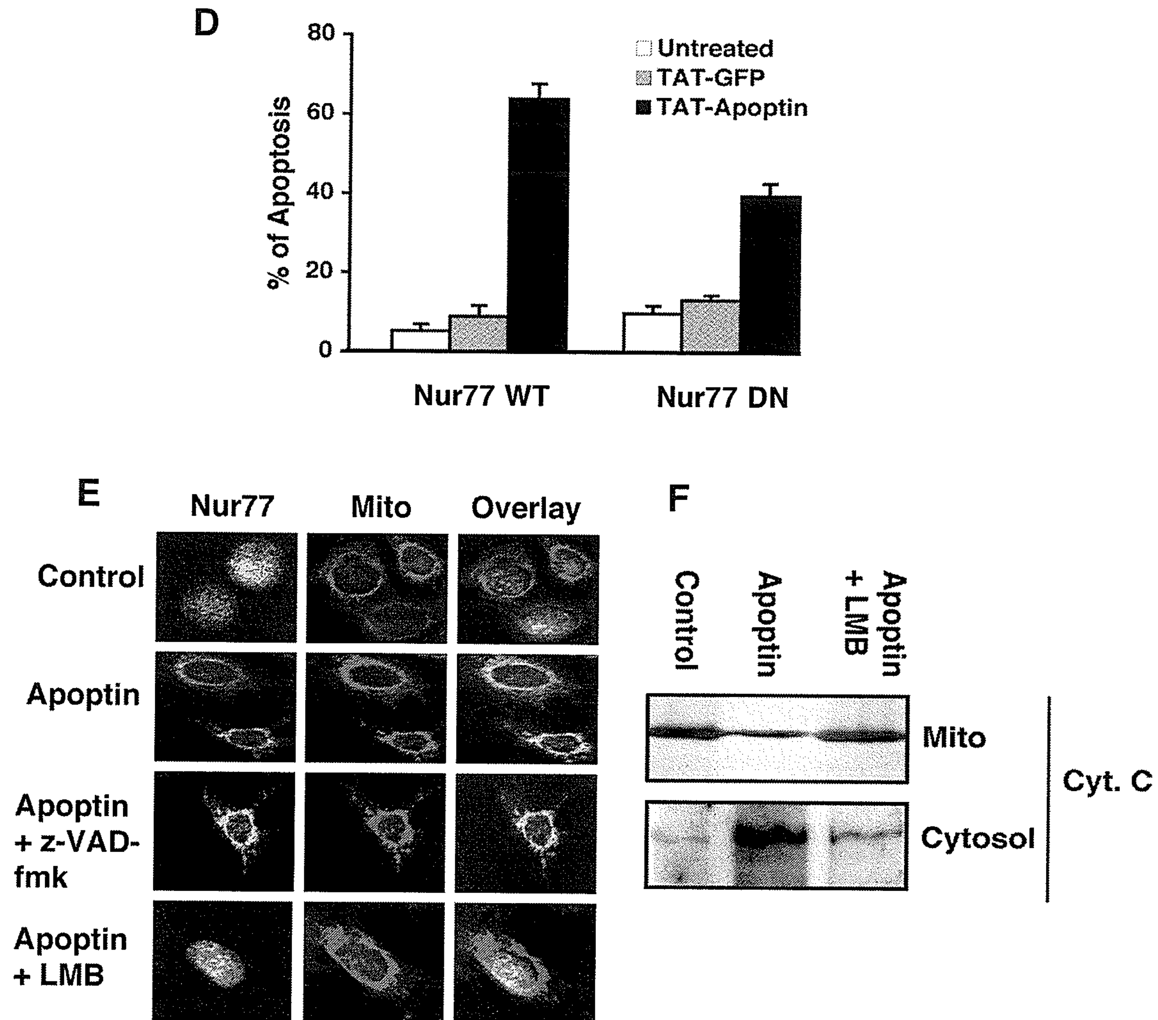


Figure 35: Nur77 transmits the Apoptin-induced death signal from the nucleus to the mitochondria. (A) Confocal microscopy visualization of Nur77 (FITC labelled) in MCF-7 cells either left untreated or treated with TAT-Apoptin for 30 hours. Mitochondria are stained by Mitotracker Red and nuclei with DAPI. **(B)** MCF-7 cells were either transfected with scrambled siRNA or Nur77 targeting siRNA and the level of Nur77 was analysed at 0, 12, 24 or 48 h post transfection. Tubulin was used as a loading control. **(C)** Cell viability of MCF-7 cells treated with TAT-GFP (control), transfected with Nur77 siRNA, or treated with TAT-Apoptin either alone or in the presence of Nur77 siRNA, as determined by MTT assay at the indicated time points. The error bars shown in the graph represent the standard deviation and the data is statistically significant ($p<0.05$) according to the student's t-test. **(D)** Nur77 Wild type (WT) or Nur77 dominant negative cells (DN) were either left untreated, treated with TAT-GFP or TAT-Apoptin and after 48 h the cell death was assayed by the Nicoletti method. The error bars shown in the graph represent the standard deviation and the data is statistically significant ($p<0.04$) according to the student's t-test. **(E)** Confocal microscopy visualization of Nur77 in MCF-7 cells either left untreated,

treated with TAT-Apoptin for 30 h, pretreated with z-VAD-fmk or Leptomycin B (LMB) followed by Apoptin. **(F)** Cells were either left untreated, treated with TAT-Apoptin alone or in combination with LMB and after 30 h, the mitochondrial and cytosolic cytochrome c was detected by western blotting.

3.5.4 PI3-Kinase pathway indirectly regulates Apoptin mediated Nur77 translocation

Previously, the phosphorylation of Nur77 and further regulation of its transcription and nucleo-cytoplasmic translocation was well documented (Moll et al., 2006) Thus, here we tested whether Nur77 gets post-translationally modified in the presence of Apoptin. Nur77 immunoprecipitated from Apoptin treated cells compared to untreated cells showed increased levels of phosphorylation at serine residues but not threonine residues (Fig. 36A). Akt regulates Nur77 activity by phosphorylation, and at the same time we observed Akt activation and its nuclear translocation in Apoptin treated cells (Fig. 21A). We tested whether Nur77 translocation is dependent on PI3-Kinase/Akt activity. The pre-treatment of cells with Wortmannin, a PI3-kinase specific inhibitor reduced the Apoptin-induced translocation of Nur77 to the mitochondria (Fig. 36B). But, the Akt mediated phosphorylation of Nur77 at Serine 351 residue was un-affected either in the presence or absence of Apoptin, though the global serine Nur77 phosphorylation is reduced in the presence of Wortmannin (Fig. 36C). Thus these results indicate that Nur77 phosphorylation and further cytoplasmic translocation in the presence of Apoptin is dependent on the activation of PI3-kinase, but mediated by an unknown downstream intermediate kinase.

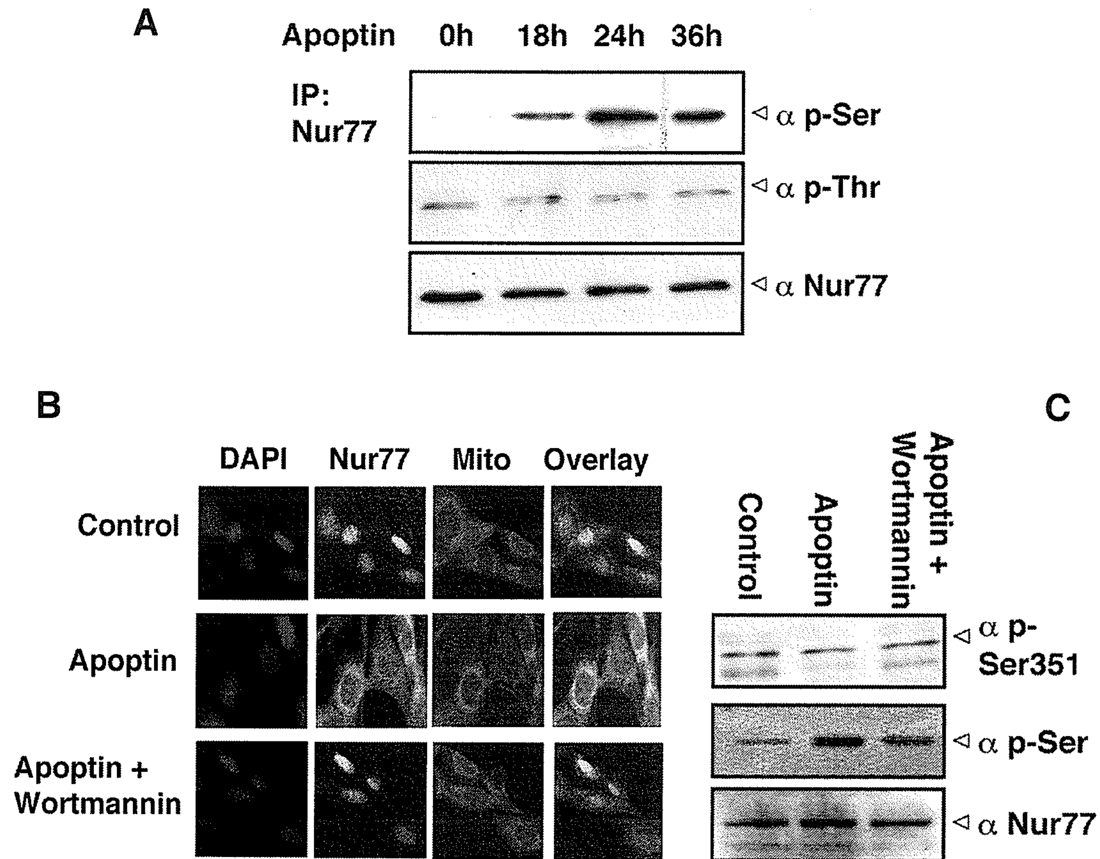


Figure 36: PI3-Kinase pathway indirectly regulates Nur77. (A) MCF-7 cells were treated with Apoptin for the indicated times and the phosphorylation of Nur77 at serine and threonine residues was detected by anti-phospho-serine or anti-phospho-Thr-specific antibodies after immuno-precipitation with anti-Nur77 antibody. **(B)** Confocal imaging analysis of Nur77 localization after immunostaining in either MCF-7 untreated, Apoptin alone or in combination with Wortmannin is shown. **(C)** Cells either untreated, treated with Apoptin alone or pre-treatment with Wortmannin followed by Apoptin were immunoprecipitated with anti-Nur77 antibody. The phosphorylation at serine 351 residue and the total serine residues was detected by immunoblotting with the respective antibodies.

3.5.5 Nur77 interacts with anti-apoptotic Bcl-2 family members

Mitochondrial translocated Nur77 interacts with Bcl-2 and was shown to convert it from an anti-apoptotic molecule to a pro-apoptotic molecule (Lin et al., 2004). Thus to understand the role of the mitochondrial targeted Nur77 during Apoptin induced cell death, we tested the interaction of Nur77 and Bcl-2 family members. Nur77 interacted with Bcl-2 and Bcl-xL (Fig. 37A), the anti-apoptotic Bcl-2 family members, but no interaction was seen with either Bax or Bad, the pro-apoptotic Bcl-2 family members (Other studies in the lab, data not shown). To further test the idea of the conversion of Bcl-2 to a pro-apoptotic molecule and its requirement for Nur77 to induce cell death mediated by Apoptin, we transfected the cells with Bcl-2 siRNA then followed by Apoptin treatment. Interestingly, the knock down of Bcl-2 using siRNA has no deleterious effect on Apoptin's action (Fig. 37B). In fact a slight increase in cell death was observed in the presence of Apoptin and Bcl-2 siRNA compared to Apoptin alone. Taken together, these results indicate that Nur77 interaction with Bcl-2 and Bcl-xL may not be required for their conversion from anti-apoptotic to pro-apoptotic molecules; instead Nur77 may sequester them away from their anti-apoptotic function.

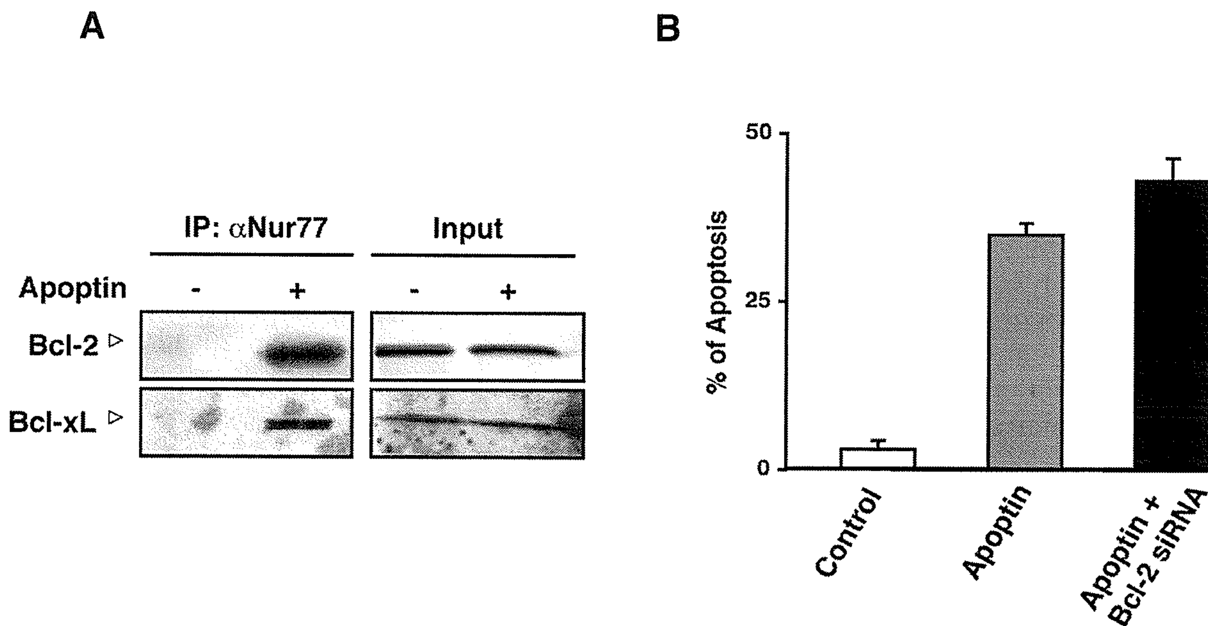


Figure 37: Apoptin interacts with anti-apoptotic Bcl-2 members. (A) MCF-7 cells were either untreated or treated with TAT-Apoptin for 36 h and immunoprecipitation was performed with anti-Nur77 antibody. Bcl-2 and Bcl-xl presence in the complex was detected by immunoblotting with their respective antibodies. **(B)** MCF-7 cells were treated with TAT-Apoptin alone or in combination with the transfected Bcl-2 siRNA for 24 h and the cell death was assessed by PI uptake, followed by flow cytometry.

4. Discussion

4.1 Mechanisms of Apoptin induced apoptosis

Using Apoptin as a model molecule, here we studied the role of different intracellular signalling pathways during apoptosis. Apoptin, a protein derived from chicken anemia virus, induces p53-independent apoptosis upon expression in tumor and transformed cells, but not in normal primary cells (Danen-Van Oorschot et al., 1997; Oro and Jans, 2004). Since, the current available cancer therapies, like chemotherapy and radiation therapy lack specificity in killing cancer cells alone without affecting normal cells, the cancer selective toxicity of Apoptin suggested by both *in vitro* and *in vivo* studies (Pietersen et al., 1999; Poon et al., 2005b) intrigues researchers in developing this as a safe and effective candidate future anti-cancer therapy molecule. Recently, the tumor selective toxicity of Apoptin became controversial as a study from Guelen et al., (Guelen et al., 2004) reported that the normal human embryonal lung fibroblasts and non-transformed MDCK dog-epithelial cells were sensitive to Apoptin induced cell death. In addition, HB4a normal breast epithelial cells expressing a temperature sensitive SV40 large T-antigen were equally killed by Apoptin at both the permissive and non-permissive temperatures. In another study, He et al., (He et al., 2005) reported that Apoptin induced chromatin condensation and apoptosis like phenotype in human bone marrow stromal cells isolated from femurs of a normal 5-month old fetus and normal embryo lung fibroblasts when they were infected with adenovirus containing FLAG-tagged Apoptin. In addition, we observed some toxicity in non-transformed mouse embryonic fibroblasts towards Apoptin induced cell death. Thus, it is possible that the sensitivity seen with the fetal lung cells, embryonic fibroblasts and fetal human marrow stromal cells towards Apoptin might

be partially due to their embryonic stage, as Apoptin is known to be toxic to young but not adult chicken secondary fetal cells. Furthermore, it can not be excluded that the primary cells growing in culture may lack some of the growth factors that are normally available *in vivo* and thus might therefore become sensitive to Apoptin induced cell death. Nonetheless, the reasons for the observed discrepancies in the selective toxicity of Apoptin are currently unclear and need to be further studied in detail using broader normal cell systems and varied cancer cell lines. We have found in our studies that all the other normal cell systems tested were resistant to Apoptin where as all the cancer/transformed cells were sensitive to Apoptin induced cell death.

The mechanism of Apoptin's selective toxicity towards cancer cells is not yet known. The elucidation of the signalling molecules involved in the Apoptin induced cell death pathways may provide novel drug targets resulting in the making of improved anti-tumor therapies and thus can be applied to broader types of cancers. It has been recently proposed that Apoptin kills cancer cells by interfering with the function of the anaphase promoting complex (Teodoro et al., 2004). The interference with the final stages of mitosis certainly causes cellular stress that would most likely activate the mitochondrial death pathway. Since in primary cells Apoptin shows cytoplasmic localization, whereas it accumulates in the nuclei of transformed cells, it is tempting to speculate that the selective nuclear localization in combination with the mitotic interference largely explains the cancer selective effects of Apoptin. Although several studies have attempted to reveal the molecular basis of Apoptin's tumor specific nuclear localization, including the role of c-terminal bipartitite nuclear localization signals (NLS), N-terminal nuclear export signals (NES) (Danen-Van Oorschot et al., 2003) and post-translational modifications like Thr-

108 tumor specific phosphorylation (Rohn et al., 2002), the complete mechanism is not clearly understood. However, Apoptin artificially directed to the nuclei of primary cells by the attachment of nuclear localization signal still remains non-toxic for the primary cells (Danen-Van Oorschot et al., 2003; Guelen et al., 2004; Rohn et al., 2002) thus indicating that the interaction with other molecules or additional modifications in cancer cells may be required for Apoptin's toxicity.

Several anticancer agents use components of the death receptor machinery such as FADD (Mishima et al., 2003), caspase-8 (Seki et al., 2000), CD95 (Fulda et al., 1998) and TRAIL-receptors (Walczak and Krammer, 2000; Wen et al., 2000) to kill cancer cells (Wesselborg and Lauber, 2005). Furthermore, it has been previously reported that Apoptin itself partially co-localizes with FADD in mammalian cells (Guelen et al., 2004). In addition, Apoptin interacting proteins like DEDAF (Danen-van Oorschot et al., 2004) and Hippi (Cheng et al., 2003) were assigned a major role in caspase-8/FADD dependent death pathway. Thus, using an array of cell lines that are either defective- or overexpress components of apoptotic machinery, we have shown that caspase-8 and FADD, both critical DISC-components of pro-apoptotic TNF-R/NGF-R family members, do not play a role in Apoptin induced cell death. A number of anticancer drugs such as Doxorubicin and cisplatin up regulate CD95L, that in turn may contribute to their anticancer activity in an autocrine or paracrine manner (Fulda and Debatin, 2005; Muller et al., 1997). Furthermore, some previous reports indicate that CD95 can still signal atypical apoptotic cell death in the absence of caspase-8 (Holler et al., 2000). Also, some reports have suggested that anti-cancer drugs upregulate CD95L by a ceramide-dependent mechanism, which then subsequently induces CD95-induced apoptosis (Gulbins, 2003). Though, recent studies

reported that Apoptin partially mediates apoptosis by modulating the ceramide signalling in cells (Liu et al., 2006a; Liu et al., 2006b), using an anti-CD95 mAb (APO-1, IgG_1) that inhibits the interaction between CD95 and the CD95L, we have shown that the Apoptin induced cell death does not involve the CD95 receptor triggered by autocrine- or paracrine-delivered CD95L. Together, these data exclude the role of the TNF-R/NGF-R -family death receptors in Apoptin-induced apoptosis.

The mitochondrial/apoptosome death pathway is ultimately activated by a number of anticancer drugs and stress stimuli (Creagh and Martin, 2003; Del Bello et al., 2004). These stimuli frequently cause the loss of mitochondrial membrane potential as well as the release of cytochrome c, AIF and other pro-apoptotic molecules (Hill et al., 2003; Martinou et al., 2000). In the cytosol, cytochrome c oligomerizes with dATP and Apaf-1, which leads to the exposure of Apaf-1 CARD motif, a caspase-9 interaction motif. The recruited caspase-9 is auto-proteolytically activated and propagates the apoptotic process by proteolytic activation of the executor caspases. Apoptin causes the loss of mitochondrial membrane potential in both wild type cells and in clones devoid of functional caspase-8 or FADD. Further, cytochrome c along with AIF is released from the mitochondria which leads to the cleavage of downstream effector caspases like caspase-3, and caspase-7 but not caspase-8. This data shows that Apoptin kills cancer cells by activation of the mitochondrial death pathway and furthermore, it shows that the caspase-8/tBid dependent signal amplification loop is not important for Apoptin-induced death. Further, we have previously shown that the treatment of different tumor cell lines such as, MCF-7 breast cancer cells, Jurkat T cell leukaemia, HeLa cervical carcinoma and D283 medulloblastoma cells with a broad caspase inhibitor zVAD-fmk significantly inhibited Apoptin induced cell

death, indicating that caspases are required for this process of apoptosis (Burek et al., 2006). Since both the MCF-7 parental cell line that lacks caspase-3 and MCF-7/caspase-3 cells were killed by Apoptin, it might be possible that caspase-3 is functionally substituted by other effector caspases such as caspase-6 and caspase-7.

The mitochondrial/intrinsic death pathway is modulated by pro- and anti-apoptotic Bcl-2 family members (Marsden and Strasser, 2003). Moreover, anti-apoptotic Bcl-2 family members, Bcl-2 and Bcl-X_L efficiently protect against Apoptin triggered apoptosis, thus confirming indirectly the involvement of the mitochondrial death pathway. This data is in contrast to previous reports by Noteborn and colleagues (Danen-Van Oorschot et al., 1999a; Danen-Van Oorschot et al., 1999b). They claimed that the anti-apoptotic Bcl-2 does not inhibit, but even facilitates Apoptin-induced apoptosis. This discrepancy regarding the effect of Bcl-2 family members on Apoptin induced cell death may possibly be attributed to different cell lines used in our study. Nevertheless, we have also observed strong inhibition of Apoptin induced cell death in MCF7 breast cancer cell lines stably transfected with either Bcl-2 or with Bcl-X_L, a finding that was confirmed in other parallel studies with different unrelated cell lines (K562, KG1 cell lines) that over express Bcl-2 (Burek et al., 2006). Thus, anti-apoptotic Bcl-2 family members seem to counteract Apoptin triggered death in our experimental systems. Moreover, in our studies Apoptin induced apoptosis was partially compromised in DU145 prostate cancer cells, which carry a frameshift mutation in the Bax gene and do not express Bax protein. Accordingly the expression of Bax in these cells sensitized them towards Apoptin induced cell death. This data corroborates the experimental results reported here that support the involvement of the mitochondrial death pathway in Apoptin induced cell death. In addition, Apoptin

modulates the mitochondrial death pathway by controlling the balance between the pro- and anti-apoptotic Bcl-2 family members. The upregulation of Bad and Bax is at the mRNA level and down regulation of Bcl-2 is at the protein level. Recently, it was shown that Apoptin enhances the NF-κB transcriptional activity (Tavassoli et al., 2005). A preliminary analysis of the Bad promoter indicated an NF-κB binding site at -562/-574 promoter regions and others have shown that NF-κB regulates Bax (Grimm et al., 2005), thus it might be possible that the NF-κB regulates Bad and Bax at the transcriptional level, which needs to be further studied.

It is widely believed that Bcl-2 family proteins regulate the mitochondrial permeability transition pores that mediate the release of cytochrome c and other apoptotic factors. However, Bcl-2 proteins can also induce or suppress caspase independent non-apoptotic cell death (Kane et al., 1995). Moreover, recent reports have also suggested that Bcl-2 can regulate activation of caspases independently of the classical apoptosome mediated apoptotic pathway, arguing that they operate by mechanisms other than Apaf-1 (Marsden et al., 2002). Thus, we tested if the loss of Apaf-1 affects Apoptin induced cell death by comparing Apaf-1 wild type cells and the Apaf-1 null cells. These comparison studies have clearly demonstrated that Apaf-1 is essential for Apoptin induced cell death. In conclusion, Apoptin induces apoptosis independently of the death receptor pathway, but it activates the intrinsic/mitochondrial apoptotic-signalling cascade. At the mitochondrial level, the Apoptin-triggered signal(s) causes the release of various mitochondrial pro-apoptotic molecules that either directly propagate the apoptotic process or activate downstream molecules in the apoptotic signalling pathway.

4.2 Role of PI3-K/Akt and CDK2 pathways during Apoptin's selective toxicity

In an attempt to define the tumor specific toxicity of Apoptin, we identified components of the PI3-K/Akt pathway as Apoptin's interacting partners in the cell. Interestingly, our results show that the PI3-K/Akt signaling pathway, universally accepted to promote cell survival and proliferation, also has a role in promoting cell death upon apoptosis triggering by certain stimuli. Moreover, we demonstrate that the inhibition of PI3-K activation by either pharmacological inhibitors or by genetic methods clearly abrogated the cell death triggered by Apoptin. Interestingly, we have recently observed that expression of Apoptin in yeast (Saccharomyces cerevisiae) did not induce any growth arrest or cell death (Los M & Zuse A, unpublished results). These results indicate the crucial role of PI3-K/Akt pathway components during Apoptin induced cell death in mammalian cells as these yeast strains lack the functional homologues of PI3-K and Akt (Rodriguez-Escudero et al., 2005). Although PI3-K is known to be involved in cell survival, several publications hinted that the active PI3-K might contribute to the triggering of apoptosis under certain conditions (Aki et al., 2001; Aki et al., 2003; Bar et al., 2005; Lu et al., 2006; Nimbalkar et al., 2003; Ono et al., 2004; Shack et al., 2003). Furthermore, the application of PI3-K inhibitors in experimental cancer therapy has so far been only moderately successful (Stein and Waterfield, 2000; Workman, 2004). Here, I provided for the first time, the molecular basis for the role of the PI3-K/Akt pathway in apoptosis induction and propagation (summarized in Fig. 38). The PI3-K/Akt pathway, under normal conditions, targets several substrates either in the cytosol or the nucleus, and promotes cell survival and proliferation. This is achieved by various mechanisms, including the control of the abundance, activity and stability of the cell survival and cell cycle regulatory

components (reviewed in Cantley, 2002; Marte and Downward, 1997). We have observed that activated Akt, if translocated to the nucleus, stimulates rather than inhibits apoptosis induced by some cytotoxic stimuli, including Apoptin and several anticancer drugs. We observed in our experimental system, that "pro-cell death" Akt-activity in the nucleus does not preclude that nuclear Akt may also have cell death-neutral- or even pro-survival activity under a different signaling context. We hypothesize that, for example, in the presence of Apoptin, the activated Akt may target alternate substrates and/or pathways that may lead to the aberrant activation of CDK2, disturbance of the cell cycle progression, and in ultimately, cell death. This suggests that the net-outcome of CDK2-activation could vary depending on the "signaling context", the type of stimuli and the temporal characteristic of signals that they trigger (transient vs constitutive signaling). Interestingly, a recent report suggested that the transient activation of Akt supports cell survival, whereas its chronic activation leads to apoptosis, which clearly supports our hypothesis (van Gorp et al., 2006). In addition, there are well-established examples of such context-dependent dramatic changes in the final outcome of activation of certain signaling pathways. For example, the proto-oncogene c-myc stimulates cell proliferation in the presence of appropriate survival stimuli (including active PI3-K/Akt-pathway) and triggers apoptosis in their absence (Henriksson and Luscher, 1996; Hueber et al., 1997). Several other molecules in the literature have been assigned a dual role in both cell survival and cell death mechanisms, for instance the, NF-κB (Barkett and Gilmore, 1999), molecules of Ras/Map kinase pathway (Brown and Benchimol, 2006; Downward, 2003), Bcl-2 (Cory and Adams, 2002; Subramanian and Chinnadurai, 2003), caspases (Los et al., 2001), and even an orphan nuclear receptor Nur77 (Lin et al., 2004). These molecules are all involved in promoting

either cell proliferation or cell death, depending on the context and the stimulus. This dual capacity ensures that cell growth is restricted to the correct paracrine environment, co-activation of a pro-survival signaling pathway, and/or co-expression of anti-apoptotic molecules, and is thereby strictly controlled by multiple mechanisms (Baudino et al., 2003; Kauffmann-Zeh et al., 1997).

I have demonstrated that the interaction of Apoptin with the p85 regulatory subunit constitutively activates PI3-K. The interaction of different molecules with the SH3 domain of p85 is known to change the conformation and activate the p110 catalytic subunit, thus leading to constitutive PI3-K activation. Interestingly, a detailed analysis of the protein sequence of Apoptin revealed a proline rich motif between amino acids 80 and 90. The Apoptin's amino acid sequence found in this region PKPPSK (amino acids 81-86) constitutes a perfect consensus motif recognised by SH3 domains (PxxPxR/K). Accordingly, Apoptin mutants lacking these residues are severely impaired in inducing apoptosis. Thus, Apoptin by interacting with the SH3 domain of the p85 regulatory subunit activates PI3-K constitutively. SH3 domain (Src homology 3 domain) was first identified as part of the Rous sarcoma oncogene product Src. SH3 domains found in different signalling proteins such as c-Src, Lck, Plc-γ, Grb2, Nck, Abl, Ras GAP, Crk, PI3-Ks, STATs and VAV proteins play a crucial role in intercellular communication and intracellular signal transduction (Pawson, 1995). Each SH3 domain is a small, conserved sequence of about 50-60 amino acids that interacts with proline-rich peptides of approximately 6-10 amino acids (PXXP sites) (Kay et al., 2000). SH3 domains mediate interactions in many key signaling pathways, including epidermal growth factor receptor signalling, cellular localization of cytoplasmic proteins, up-regulation of GTPase activity

of dynamin, membrane trafficking and other cellular signalling events (Bar-Sagi et al., 1993; Gout et al., 1993; Lowenstein et al., 1992). Thus, it is possible that Apoptin might also be mediating its apoptotic process by interacting with other SH3 domain containing proteins in addition to PI3-Kinase. In fact, a preliminary screening in our studies for the Apoptin interacting SH3 domain containing proteins using SH3 domain arrays, revealed several potential candidates like Abl, Vinexin, OSF (osteoclast stimulating factor) and Plc-γ. Further studies have to be performed to confirm whether these potential interactions occur *in vivo* and to establish the functional significances of the Apoptin-SH3 domain protein interactions.

In addition to Apoptin's interaction with the PI3-K, we have also observed the interaction with Akt, though it occurs transiently. As Apoptin interacts with both PI3-kinase and Akt (molecules in the common pathway) in the cell, the formation of Apoptin-PI3-kinase-Akt trimeric complex cannot be excluded and further needs to be investigated. Furthermore, the interaction of Akt with Apoptin enhances the nuclear localization of Akt. Also, there was an increase in the nuclear PI-3 kinase levels, again supporting the idea that they may exist in a trimeric complex. Recent studies have indicated that under certain conditions, activated Akt could be translocated to the nucleus with the help of some cytosolic proteins, as Akt does not have an inherent nuclear localization signal sequence (Kunstle et al., 2002; Pekarsky et al., 2000). Here, we have shown that Akt nuclear translocation occurs early during Apoptin triggered apoptosis. Furthermore, the interaction of an activated Akt with Apoptin is crucial for its nuclear translocation. This was shown by two separate experiments: (*i*) We demonstrated that Akt was translocated to the nucleus only in the presence of those Apoptin mutants which have the ability to activate PI3-K, and

the secondly (*ii*) Akt was observed in the nucleus in the presence of only those Apoptin mutants that have the ability to accumulate in the nucleus. This may indicate that either Apoptin interacts transiently with Akt, and functions as a transporter of Akt, or it contributes to the assembly of a complex that promotes the nuclear transport of Akt. Apoptin thus causes Akt's translocation to the nucleus, where the novel apoptotic Akt targets are potentially located. Furthermore, we tested if the forced nuclear translocation of Akt by transfecting the cells with NLS-Akt will itself induce apoptosis. This was proven not to be the case as NLS-Akt alone was not very effective in inducing cell death, but it potentiates cell death induced by Apoptin. Moreover, the NLS-Akt was potentiated some, but not all, classical cell death stimuli, for example Methotrexate, docetaxel, Doxorubicin, and Cisplatin, but not cell death induced by staurosporine or CD95-triggering. The nuclear Akt may have pro-survival and proliferation-promoting functions under some experimental conditions. For example, Trotman and colleagues reported that the PML-tumor suppressor prevents cancer by dephosphorylating and inactivating Akt inside the nucleus (Trotman et al., 2006). Other researchers have reported in a cell-free experimental system, that phosphorylated-, nuclear, but not cytoplasmic Akt interacts with Ebp1, (an inhibitor of CAD-dependent apoptotic DNA-fragmentation), and enhances its anti-apoptotic action independently of Akt kinase activity (Ahn et al., 2006). Thus, nuclear Akt may contribute towards cell death pathways only in the presence of certain apoptotic stimuli.

The PI3-Kinase activation is not only seen in Apoptin transfected cancer cells but also in primary cells. Though there was a two-fold activation of PI3-kinase in primary cells by Apoptin, the activation was not up to the level of PI3-Kinase activation in cancer cells (4-6 fold). Interestingly, the activation of PI3-Kinase activity in primary cells induces

cellular senescence, a varied response than its activation in cancer cells. Several recent publications indicated in primary cells that if the oncogenic signaling including the PI3-kinase/Akt pathway becomes aberrantly activated, they will not undergo apoptosis but will enter cellular senescence (Braig et al., 2005; Chen et al., 2005; Minamino et al., 2004; Miyauchi et al., 2004). It is proposed that this is one of the early mechanisms that protect cells form entering further steps of carcinogenesis. Interestingly, the process of cellular senescence in response to the oncogenic signaling is dependent on the activation of p53 dependent pathways. Though, it has been shown that p53 is not required for Apoptin induced cell death in cancer cells, the requirement of p53 for the process of Apoptin induced cellular senescence in primary cells cannot be excluded and further has to be studied in detail. Also, though Apoptin activates PI3-kinase/Akt pathway in primary cells, the targets for Akt in the process of senescence may vary as our preliminary results suggested that there is no nuclear accumulation of Akt in primary cells. Also, in primary cells in response to Apoptin we didn't observe any significant activation of CDK2, a downstream target for Akt in cancer cells to induce cell death (data not shown).

There are several reported nuclear targets for Akt, including FOXO3a, Nur77 and $p21^{waf1}$. Here, we report $p27^{kip1}$ is a nuclear target for Akt-phosphorylation and demonstrated the importance of this signaling event in Apoptin induced cell death. $p27^{kip1}$, a cyclin dependent kinase inhibitor and a negative regulator of the cell cycle, plays a role both in proliferation and apoptosis in a stimulus-dependent manner (Sgambato et al., 2000; Toyoshima and Hunter, 1994). $p27^{kip1}$ is downregulated during Apoptin induced cell death and this is dependent on Akt activation. Akt phosphorylates $p27^{kip1}$ at Thr-157 in the nucleus and targets it for proteosomal degradation. Previously, it was shown that $p27^{kip1}$-

phosphorylation at Thr-187 by cyclin dependent kinases triggers the ubiquitination of $p27^{kip1}$ by SCF/Skp2 ubiquitin ligase complexes and promotes its degradation (Tsvetkov et al., 1999). We have tested the levels of phospho-$p27^{kip1}$-Thr-187 during Apoptin treatment and found no change in its level, although CDK2 was activated during Apoptin induced cell death. Also, it was reported that $p27^{kip1}$ phosphorylation at Ser-10, Thr-157 and Thr-198 by the PI3-K/Akt pathway affects the nuclear localization of the protein resulting in the cytoplasmic accumulation via 14-3-3 scaffold protein and thus blocking $p27^{kip1}$ ability to inhibit CDKs (Liang et al., 2002; Sekimoto et al., 2004). However, we didn't observe any change in the nuclear localization of $p27^{kip1}$ during Apoptin-induced cell death. Akt is also known to control the expression of $p27^{kip1}$ at the mRNA level, via the inactivation of a FOXO3a transcription factor (Medema et al., 2000). Though, we have observed the phosphorylation and inhibition of FOXO3a by Akt during Apoptin induced cell death (data not shown), we did not observe any changes in the mRNA levels of the $p27^{kip1}$. Thus taken together, Akt mediated phosphorylation of $p27^{kip1}$ at Thr-157 only affects the lifespan of the protein through the proteosome pathway and thus it relieves downstream effector molecules from $p27^{kip1}$-mediated inhibition. In other studies in our lab, we have also observed the downregulation of $p21^{cip/waf}$ 24 hours after cell death induction by Apoptin, but this occurs independently of Akt activation, as the inhibition of Akt by Wortmannin did not affect $p21^{waf1}$ levels (unpublished data). The downregulation of $p21^{waf1}$ may be the result of its cleavage by caspases (a secondary event, downstream of mitochondrial death pathway activation).

As a consequence of $p27^{kip1}$ downregulation, cyclin A associated CDK2 is constitutively activated by Apoptin. Though CDK2 is a crucial player in the cell cycle

during the progression from G1 to S phase, it is also known to regulate apoptosis. CDK2 inactivation by either pharmacological inhibitors or by siRNA severely impairs Apoptin induced cell death. Furthermore, we demonstrated that cyclin A/CDK2 also regulates Apoptin's nuclear localization by directly phosphorylating the Thr-108 residue. Previously, the phosphorylation at Thr-108 has been reported to be specific for tumor cells and it has been shown to be required for Apoptin induced cell death (Rohn et al., 2002). Interestingly, in these studies, the phosphorylation level of Apoptin was severely impaired by the Apoptin mutant lacking residues 80-90, even if the residues around the phosphorylation site (Thr-108) are intact. The results reported here explain this interesting observation. We have shown that Apoptin interacts with PI3-K via the amino acids 80-90, and this interaction is required for downstream CDK2 activation. In contrast to previous results, the Ala-108 Apoptin mutant is still toxic for cancer cells under our experimental conditions, suggesting that the Thr-108 phosphorylation is a secondary event, and a consequence of CDK2 activation during Apoptin induced cell death, rather than a triggering event directly involved in Apoptin's toxicity. Our results, however, still confirm that the Thr-108 phosphorylation is important for the nuclear localization of Apoptin. We observed that the nuclear localization of Akt and Apoptin occur in parallel and are dependent on the CDK2 mediated Thr-108 phosphorylation and at the same time the interaction of Apoptin with Akt is transient. Based on these observations, we suspect that Akt and Apoptin interact and are carried to the nucleus, where Akt phosphorylates CDK2, which in turn phosphorylates Apoptin at Thr-108 site. This may release the Apoptin-Akt complex by changing the conformation of Apoptin and in the end results in both proteins to be sequestered in the nuclear compartment. Nevertheless, aberrant CDK2 activation is

crucial and absolutely necessary for Apoptin induced cell death. The targets for CDK2 during apoptosis are still unknown. Though one report suggested that p53 is activated downstream of CDK2 (Hakem et al., 1999), we and others, have ruled out the role of p53 in Apoptin-induced cell death. We have however observed that upon cytoplasmic translocation, CDK2 phosphorylates Bcl-2, and targets it for the proteosome-dependent degradation. This, in turn, affects the balance between anti- and pro-apoptotic Bcl-2-family members, in favour of the later ones, and thus promoting apoptosis. On the other hand, CDK2 activation downstream of Akt is important, but may not be the sole mechanism for the pro-apoptotic role of PI3-K/Akt pathway during Apoptin induced cell death. We predict that some additional direct and indirect Akt targets, either in the nucleus or in the cytoplasm, exist that are phosphorylated in the course of Apoptin-triggered cell death.

The nuclear localization of Apoptin in transformed cells and the ultimate activation of the mitochondrial death pathway suggests that cell death activating signals must be sent from the nucleus to mitochondria. Our experiments indicate that the cytoplasmic translocation of Nur77 is critical for Apoptin's toxicity. In addition, we have shown that Apoptin mediated cell death is dependent on Nur77 expression in the cells, as the cells knocked down for Nur77 expression by specific siRNA are resistant to Apoptin induced cell death. Our data corroborates with the recent finding (Lee et al., 2005) that Nur77 in concert with RXRα translocates from nucleus to mitochondria, where it directly activates the mitochondrial death pathway. It is unlikely that the previously proposed scenario of Nur77-mediated conversion of Bcl-2 from the antiapoptotic to proapoptotic molecule (Lin et al., 2004) is among mechanisms contributing to Apoptin's toxicity since our experimental data clearly underlines the protective role of both Bcl-2 and Bcl-X_L against

Apoptin triggered cell death. On the other hand transcriptional activation of proapoptotic genes that are controlled by Nur77 still cannot be ruled out. Nur77 is one of the known Akt targets in the nucleus, but the phosphorylation of Nur77 by Akt promotes the pro-survival mechanism of Nur77 and not the cell death process (Pekarsky et al., 2001). In addition, we did not observe any phosphorylation of Nur77 at the serine-351 site, an Akt consensus site, but Wortmannin inhibited the cytoplasmic translocation of Nur77 during Apoptin induced cell death. Thus it may be possible that some additional kinase pathways downstream of PI3-kinase may be regulating Nur77. Previously, the Trk/Ras/MAP kinase pathway was shown to regulate Nur77 phosphorylation and its nuclear localization. Also, JNK activation regulates the nucleo-cytoplasmic translocation of Nur77. Interestingly, Apoptin was also shown to activate JNK in previous studies (Ben et al., 2005), thus JNK might be one of the possible PI3-K downstream candidates that can phosphorylate Nur77 and may regulate the nucleo-cytoplasmic shuttling. The expression level of Nur77 varies in different cells. Nur77 is highly expressed in a broad range of cancer cells as compared to normal cells (Uemura and Chang, 1998; Wu et al., 1997). Also, it has recently been shown that Nur77 is one of the 17 signature genes associated with the metastasis of primary solid tumors (Ramaswamy et al., 2003). More evidence for the role of Nur77 in cancer came from studies indicating that EWS, a member of 'Nur77' orphan receptor family is involved in chromosomal translocation in human chondrosarcomas and the fusion protein is in fact 250 fold more potent in activating TR3 responsive element mediated transcription (Clark et al., 1996; Labelle et al., 1999). The difference in the expression level and the role of Nur77 (and Nur77 family members) in normal and cancer cells may indicate, in part, the tumor specific toxicity of Apoptin. It remains to be determined whether Nur77 acts here solely as

the signalling molecule, or concomitantly activating the expression of pro-apoptotic genes that cooperate in the activation of apoptotic pathways in cancer cells (Rajpal et al., 2003).

Different components of the PI3-K/Akt pathway are involved in tumorogenesis and are highly active in various types of cancers compared to normal cells. Furthermore, PTEN, a phosphatase that counteracts PI3-K's action, is the second most commonly mutated tumor suppressor gene after p53 (Vivanco and Sawyers, 2002). Both CDK2 and cyclin A are reported to be highly overexpressed in several tumors compared to the normal tissues (Yam et al., 2002). Hyperactivation of the pathways mentioned above leads to a poor clinical prognosis and also contributes to drug resistance during cancer treatment. Thus, Apoptin's targeting of these various pathways may explain its unique tumor specific toxicity. Thus, considering the above observations and rather slow and asynchronous death kinetics triggered by Apoptin, we assume that Apoptin employs several different mechanisms relying on multiple signalling pathways to kill cancer cells specifically (Fig. 38). Thus, targeting these pathways either alone or in combination might lead to the development of new cancer therapies. In my studies, PI3-K/Akt pathways have a dual role in both survival and cell death processes depending on the stimulus. My results establish a link between the survival and cell death pathways during an apoptotic process, suggesting a completely new and innovative strategy for modulating apoptosis in cancer. The prime discovery of this novel mechanism of redirecting survival signaling into death pathways, will likely lead to the development of a novel class of anti-tumor drugs.

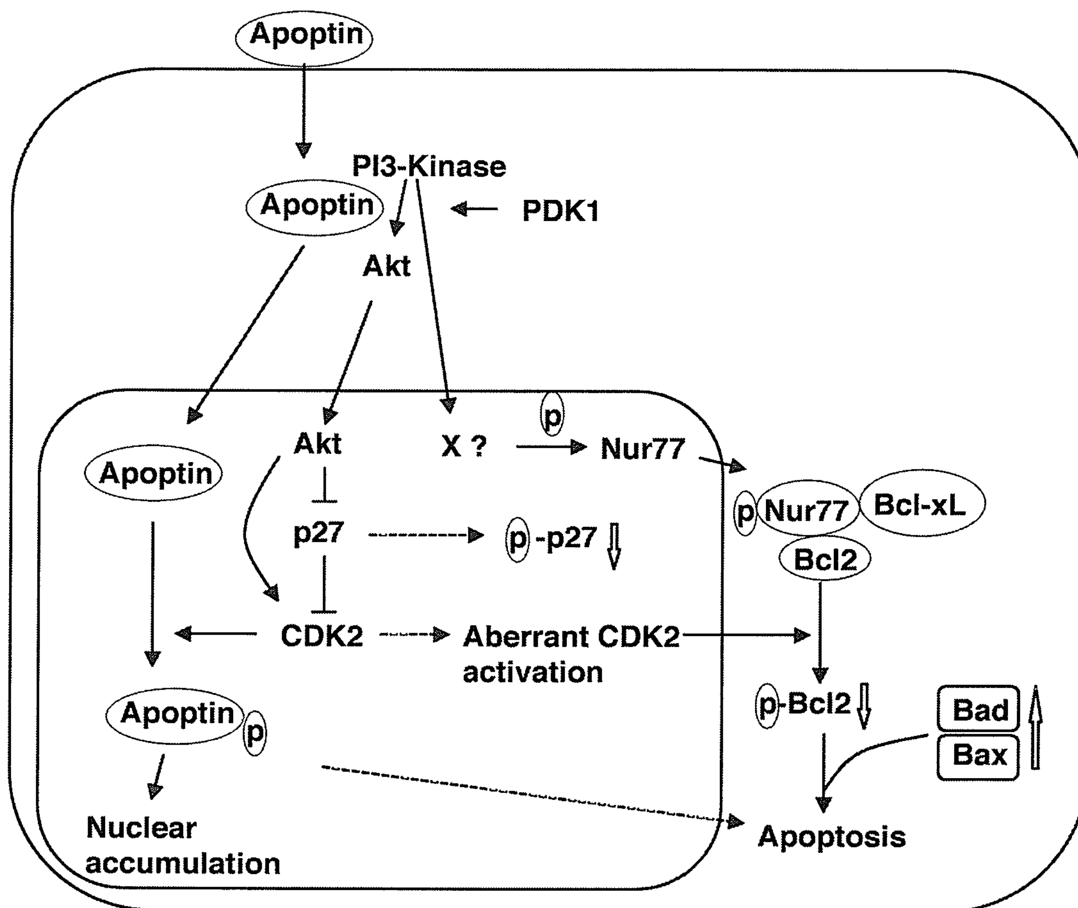


Figure 38: Model for Apoptin activated signaling pathways during cell death. Apoptin interacts with the SH3 domain of PI3-Kinase resulting in its constitutive activation, which leads to PDK1-dependent Akt activation and nuclear translocation of Akt. The nuclear Akt activates CDK2 by both direct and indirect mechanisms. Akt phosphorylates and enhances the proteosome dependent degradation of $p27^{kip1}$, which leads to indirect activation of CDK2. The activated CDK2 phosphorylates, among other substrates, apoptin at Thr-108 site and regulates its nuclear accumulation in cancer cells. On the other hand, Akt directly phosphorylates CDK2 and leads to its cytoplasmic translocation. Subsequently, phosphorylated CDK2 translocates to cytoplasm and phosphorylates Bcl-2. Phosphorylated Bcl-2 is targeted for proteosome-dependent degradation. In addition, PI3-Kinase activates an unknown kinase, which phosphorylates Nur77. The phosphorylated Nur77 translocates to cytoplasm and thus assists in transmitting the nuclear apoptin induced signal to mitochondrial activation. Cytoplasmic Nur77 sequesters anti-apoptotic Bcl-2 and Bcl-xL away from pro-apoptotic members. The Nur77 mediated Bcl-xL sequestering and CDK2 mediated Bcl-2 downregulation in parallel to the upregulation of Bax and Bad shifts the balance between pro-and anti-apoptotic molecules at the mitochondria towards apoptosis.

4.3 Direct link between Akt and CDK2 - A dual role in cell cycle and apoptosis

In addition to the role of Apoptin induced signalling pathways, we also provided the studies linking the cell survival, cell cycle and apoptotic pathways in a physiological context. This was also observed in the presence of other anti-cancer agents and therefore can be used in the modulation of these pathways for effective cancer treatments. Akt targets several proteins during cell cycle progression, in particular G1/S progression (Liang and Slingerland, 2003). In our studies, for the first time, we demonstrate CDK2 as a direct novel target for Akt and its role not only during cell cycle progression, but also during apoptosis. The Akt mediated CDK2 T39 phosphorylation is mainly important for regulating the subcellular distribution of CDK2. CDK2 has a very important role as an S-phase cyclin dependent kinase, in particular during priming of DNA synthesis and its progression (Jiang et al., 1999; Krude et al., 1997; Nougarede et al., 2000). Oscillation of CDK2 activity during G/S phase is required for proper DNA synthesis and prevention of DNA endo-reduplication (Dahmann et al., 1995). Thus, it is possible that CDK2 phosphorylation and transient cytoplasmic relocalization by Akt during late S and G2 phases may be essential for the prevention of DNA endo-reduplication and also mediate proper DNA segregation in the cell. In fact, our preliminary observations indicated that the transfection of CDK2 T39A results in a three-fold increase in the number of 8N and 16N (>4N) DNA containing cells compared to the wild type CDK2 containing cells. Further studies need to be done to confirm that the inhibiton of CDK2 cytoplasmic translocation leads to uncontrolled DNA replication and further genomic instability. In fact, transgenic mice overexpressing cyclin A, the binding partner for CDK2, show higher incidence of genomic instability and cancers due to uncontrolled DNA replication (Girard et al., 1991;

Liao et al., 2001). Although CDK2 T39 phosphorylation enhances its kinase activity by increased cyclin A binding, we did not observe any defect in the kinase activity of the mutant lacking T39 phosphorylation site indicating that T39 phosphorylation is not a regulatory event for the CDK2 kinase activity but serves other purposes. The phosphorylation of CDK2 occurs at a specific stage of the cell cycle (during late S and G2 phases) and results in the relocalization of CDK2, which is required for normal cell cycle progression from S to G2 phase. The experiment demonstrating the entry from S to G2 phase of the cell cycle in the presence of different CDK2 mutants clearly explains the importance of CDK2 phosphorylation, as the CDK2 T39A mutant which is non-phosphorylatable but is still active as a CDK2 kinase delays the entry of cells from S to G2/M phases. It has been previously reported that Akt should only be transiently active during cell cycle as the prolonged activation of Akt resulted in G2/M cell cycle arrest (Alvarez et al., 2001). Here we observed a similar effect as constitutive Akt expression resulted in G2/M cell cycle arrest. This might be partly due to the CDK2 phosphorylation. The expression of Akt-CA might result in constitutive CDK2 cytoplasmic relocalization, and thus CDK2 has no access to the nucleus to make a progression through the G1 phase of the cell cycle. In agreement with the transient Akt activation during cell cycle, we also report here that not only Akt activation is transient but also the nuclear localization of Akt occurs transiently. Akt is nuclear only during late S and G2 phases, thus acting on a specific set of substrates during these phases. In addition to CDK2, Akt might also target other cyclin dependent kinases for phosphorylation as we observed CDK1 phosphorylation by Akt *in vitro* (Unpublished results). Thus, finding additional targets for Akt during different phases of the cell cycle will provide a clearer picture of the role of Akt during cell

cycle progression. Akt has no nuclear localization signal, and therefore it may require additional interaction partners or nuclear targeting proteins for Akt nuclear localization during a particular phase of the cell cycle. Identifying these Akt partner proteins might give us a better understanding of the regulation of this selective nuclear localization.

In addition to the role of Akt/CDK pathway in the progression of cell cycle at S-G2/M transition, we have also demonstrated that it has a very important role during apoptosis induced by some selected anti-cancer drugs in addition to Apoptin. Interestingly, here we report that Akt is activated and translocated to the nucleus constitutively in cells treated with anticancer drugs. The translocation of Akt to the nucleus is required for their cell death inducing activity. Under normal conditions, Akt is transiently activated and is nuclear only during late S and G2 phases of the cell cycle, but is rapidly translocated to the cytoplasm during the cell cycle progression. In contrast, in the presence of anti-cancer drugs, Akt is constitutively active and is constitutively localized in the nucleus irrespective of the phase of the cell cycle, thus contributing to the pro-apoptotic role of Akt, instead of normal cell cycle progression. In addition to the positive role of Akt, the phosphorylation and cytoplasmic localization of CDK2 is also important for cell death inducing activity of methotrexate and docetaxel. In additon, several studies have suggested that CDK2 inactivation is required for G1 and intra-S phase check point activation and apoptosis in response to DNA damage (Huang et al., 2006a; Mailand et al., 2000). Our results indicating that CDK2 re-localization to the cytoplasm from the nucleus in the presence of DNA damaging agents might explain an additional mechanism for DNA damage mediated apoptosis. The downstream targets for cytoplasmic CDK2 during methotrexate and docetaxel induced cell death are not known. Depending on the localization, CDK2 either

promotes cell cycle or cell death. In the presence of Apoptin, we have shown that Bcl-2 is targeted by CDK2 in the cytoplasm. Although, Bcl-2 is phosphorylated and downregulated during docetaxel treatment (Wang et al., 1999b), it remains to be determined if CDK2 mediated phosphorylation targets Bcl-2 in the presence of these anti-cancer drugs. Identification of additional nuclear and cytoplasmic CDK2 targets might provide clues about the differential roles of CDK2 during the cell cycle and apoptosis. Taken together, the differential activation of Akt/CDK2 (transient versus constitutive) and their differential localization (nuclear versus cytoplasmic) might contribute to the varied responses of these pathways either to participate in cell cycle progression or apoptosis (Summarized in Fig 39). Thus, taking advantage of the differential responses of these molecules and designing molecules that target these pathways might contribute to the development of novel anti-cancer therapies.

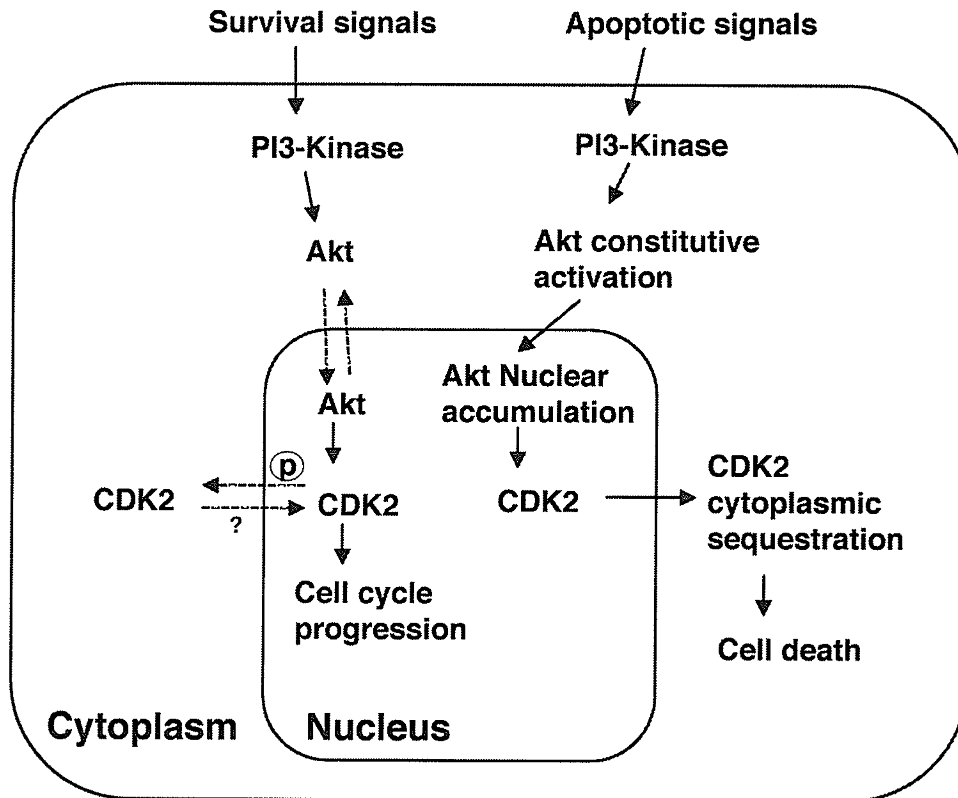


Figure 39: Model for the dual role of Akt/CDK2 pathway in cell cycle progression and cell death. In the presence of survival signals, PI3-Kinase activates Akt and the transiently activated Akt translocates to the nucleus only during late S and G2 phases and shuttles back to the cytoplasm. The transient nuclear Akt phosphorylates CDK2 in the nucleus at late S and G2 phases and leads to the temporary CDK2 re-location to the cytoplasm. When Akt is shuttled back to the cytoplasm, CDK2 rapidly translocates to the nucleus and helps in the cell cycle progression. But in the presence of selected apoptotic signals, PI3-Kinase constitutively activates Akt and leads to the constitutive nuclear Akt accumulation, where it phosphorylates CDK2. The constitutively phosphorylated CDK2 is sequestered in the cytoplasm and directed to the different cytoplasmic substrates, which ultimately leads to cell death.

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