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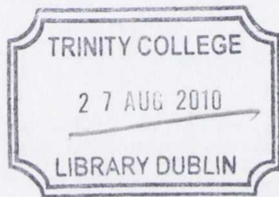
Investigation of the regulation of Bcl-2 family members in apoptosis and cancer

Clare Sheridan

Thesis submitted to Trinity College Dublin for the degree of Doctor of Philosophy

Thesis Supervisor: Prof. Seamus J. Martin

Molecular Cell Biology Laboratory
Department of Genetics
University of Dublin
Trinity College
Dublin 2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SUMMARY

Apoptosis is a highly controlled mechanism of cell suicide that is activated in response to cellular stress and damage. The Bcl-2 family of proteins play a key role in the regulation of this process. Opposing subsets of the Bcl-2 family either promote or inhibit apoptosis, and interaction between rival family members determines whether cell death will proceed. One subgroup, named the BH3-only proteins, are activated in response to pro-apoptotic stimuli and promote the death-inducing function of the Bax-related subgroup. The third subgroup of this family possess anti-apoptotic properties and antagonise the pro-apoptotic members of the Bcl-2 family through sequestration of these proteins.

To ensure that entry to apoptosis is tightly co-ordinated, members of the Bcl-2 family are transcriptionally and post-translationally modified by a myriad of other signalling molecules. However, deregulation of these proteins occurs under some pathological circumstances, such as cancer, contributing to the maintenance and progression of the disease. Therefore, elucidation of the mechanisms used to modulate Bcl-2 family members is important for understanding apoptosis progression under natural conditions and also in disease contexts. In this thesis, we sought to investigate the regulation of Bcl-2 related proteins by anti-apoptotic molecules and pro-apoptotic stimuli. We also explored some of the events driven by Bcl-2 family members during apoptosis, in order to more comprehensively understand the molecular steps that favour the completion of apoptosis.

CHAPTER III

Many cancers, including melanoma skin tumors, display resistance to pro-apoptotic stimuli. This pro-survival characteristic may be mediated through a number of mechanisms including inactivation of important pro-apoptotic molecules. In melanoma, an oncogenic kinase B-Raf^{V600E}, promotes cellular growth and maintenance of tumors. We investigated the effects of B-Raf^{V600E} expression on apoptosis induced by chemotherapeutic drugs, and found that this kinase abrogated cell death initiated by these agents. Further examination of the

mechanism employed by B-Raf^{V600E} to escape apoptosis revealed that B-Raf^{V600E} targeted the BH3-only proteins Bim and Bad for inactivation. This oncogene promoted ERK-dependent phosphorylation of both Bim and Bad, which inhibited their ability to drive cell death. B-Raf^{V600E} also enhanced the expression of the anti-apoptotic Bcl-2 family member Mcl-1. Thus, oncogenic B-Raf augments pro-survival signalling in cells via regulation of a number of Bcl-2 family members. Importantly, ablation of B-Raf^{V600E} levels in melanoma cells sensitised these cancer cells to chemotherapy-induced apoptosis, suggesting that inactivation of this kinase may increase the efficacy of chemotherapeutic treatment in melanoma.

CHAPTER IV

Chemotherapeutic drugs are important components of anti-cancer regimes directed against human malignancies. These agents inhibit crucial cellular processes, leading to cell injury/dysfunction and activation of the cell death machinery downstream. In chapter IV, we explored the molecular mechanism responsible for apoptosis mediated by the DNA-damaging agent cisplatin. We report that cisplatin potently upregulated the BH3-only protein Noxa on a transcriptional level. Interestingly, induction of Noxa was dependent on the kinase ERK, which is more frequently associated with pro-survival signalling. Inhibition of ERK, or ablation of Noxa expression, dramatically attenuated cisplatin-induced apoptosis, demonstrating that these proteins are instrumental effectors of the anti-tumor action of cisplatin. Furthermore, related platinum agents carboplatin and oxaliplatin similarly induced apoptosis through augmentation of Noxa expression. While Noxa levels were increased in response to platinum drugs, Mcl-1 levels were reduced, which further enhanced the initiation of apoptosis. Thus, platinum agents promote cell death through augmentation of the function of pro-apoptotic members of the Bcl-2 family, which then drive Bax/Bak-mediated apoptosis.

CHAPTER V

The pro-apoptotic Bcl-2 family members Bax and Bak, when activated, are localised to mitochondria. From this location, Bax and Bak promote cell death

through the formation of pores in mitochondrial outer membranes facilitating the release of cytochrome *c*, which activates caspases downstream. Mitochondria also become dramatically fragmented during apoptosis. However, the role of fragmentation in cytochrome *c* release is ambiguous. In chapter V, we examined the effect of modulating mitochondrial fission on apoptosis. We observed that mitochondrial fragmentation was not required for Bax/Bak-induced cytochrome *c* release or apoptosis. We report that Bax and Bak promoted mitochondrial fission. However, co-expression of Bax and anti-apoptotic Bcl-2 family members revealed that this event could be uncoupled from the pro-apoptotic function of Bax. Thus, Bax and Bak trigger mitochondrial remodelling separately from their role as mediators of cytochrome *c* release.

ABBREVIATIONS

A1	Bcl-2 related protein-A1
AIDS	Acquired immune deficiency syndrome
AMPK	Adenosine monophosphate-activated protein kinase
AP1	Activator protein 1
Apaf-1	Apoptotic protease activating factor 1
ARF	Alternative reading frame
ASK1	Apoptosis signal-regulating kinase 1
ATF	Activating transcription factor
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3 related
Bad	Bcl-2 antagonist of cell death
Bak	Bcl-2 antagonist killer 1
Bax	Bcl-2-associated x protein
Bcl-2	B-cell lymphoma 2
Bcl-b	Bcl-2 related gene b
Bcl-w	Bcl-2 related gene w
Bcl-xL	Bcl-2 related gene x-long isoform
bFGF	basic Fibroblast growth factor
BH	Bcl-2 homology domain
Bid	BH3-interacting domain death agonist
Bik	Bcl-2 interacting killer
Bim	Bcl-2 interacting protein Bim
Bim_{EL}	Bim-extra long
Bim_{L}	Bim-long
Bim_{S}	Bim-short
Bmf	Bcl-2 modifying factor
Bok	Bcl-2 related ovarian killer
BRCA1	Breast cancer gene 1
BSA	Bovine serum albumin
CAD	Caspase activated Dnase
Caspase	Cysteinyl aspartate-specific protease
CDK	Cyclin-dependent kinase
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CED	Cell death defective
CML	Chronic myelogenous leukemia
CREB	cAMP response element binding protein
DCI	Dichloroisocoumarin
DFG	Aspartic acid (D), Phenylalanine (P), Glycine (G) motif
DLC1	Dyenin light chain 1
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Dream	Dre antagonist modulator
Drp1	Dynamin-related protein 1
EGF	Epidermal growth factor

EGFR	Epidermal growth factor receptor
Egl-1	Egg laying defective 1
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated protein kinase
Ets	E twenty six
FACS	Fluorescence-activated cell sorter
FADD	Fas-associating protein with death domain
FCS	Fetal calf serum
FGF	Fibroblast growth factor
Fis1	Fission 1
FOXO3a	Forkhead Box03a
FRAP	Fluorescence recovery after photobleaching
Fzo1	Fuzzy onion 1
GDAP1	Ganglioside-induced differentiation-associated protein 1
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage-colony stimulating factor
Grb2	Growth factor receptor-bound protein 2
GSK3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
HDM2	Human double minute 2 homolog
HEK	Human embryonic kidney cells
Hif-1α	Hypoxia-inducible factor 1-alpha subunit
HL-60	Human leukemia cells-60
HMGB1	High mobility group box 1
Hrk	Harakiri
Hsc70	Heat shock cognate protein 70 kDa
ICAD	Inhibitor of Caspase activated DNase
IL	Interleukin
INK4	Inhibitor of cyclin-dependent kinase 4
IP	Immunoprecipitation
JAK	Janus kinase
JNK	c-jun kinase
LB	Luria-Bertani broth
MAPK	Mitogen activated protein kinase
Mcl-1	Myeloid cell leukemia 1
MEK	MAPK/ERK kinase
Mfn	Mitofusin
Mgm1	Mitochondrial genome maintenance protein 1
MITF	Microthalmia-associated transcription factor
MNK	MAPK-interacting kinase 1
MOMP	Mitochondrial outer membrane permeabilisation
MTP18	Mitochondrial protein, 18kDa
NfkB	Nuclear factor kappa-light-chain enhancer of activated B cells
Noxa	latin for 'damage'
Opa1	Optic atrophy 1

PEST	Proline (P), glutamic acid (E), serine (S), Threonine (T) rich sequence
PAGE	Polyacrylamide gel
PAK1	p21-activated kinase 1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PIP	Phosphatidylinositol phosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PMA	Phorbol myristic acid
PMSF	Phenylmethylsulphonyl fluoride
PSMA	Prostate specific membrane antigen
PTEN	Phosphatase and tensin homolog
Puma	p53-upregulated modulator of apoptosis
Ras	Rat sarcoma viral oncogene homolog
Rb	Retinoblastoma protein
RFP	Red fluorescent protein
RNA	Ribonucleic acid
ROCK-1	Rho-associated coiled-coil-containing protein kinase 1
RSK	Ribosomal protein S6 kinase
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
siRNA	Small interfering Ribonucleic acid
shRNA	Short hairpin Ribonucleic acid
Smac	Second mitochondrial-derived activator of caspase
Sos	Son of sevenless
STAT	Signal transducer and activator of transcription
TBST	Tris buffered saline with tween 20
TNFα	Tumor necrosis factor alpha
TPCK	Tosyl-L-phenylalanine-chloromethyl-ketone
TRADD	TNF receptor 1-associated death domain protein
TRAIL	TNF-related apoptosis-inducing ligand
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
WNT	Wingless type MMTV integration site family
ZVAD.fmk	Benzylocarbonyl-valine(V) alanine(A) aspartic acid(D)-(OMe)-fluoromethylketone

PUBLICATIONS

Brumatti G[1], **Sheridan C[1]**, Elgendy M, Brunet M and Martin SJ (2009). An ERK-dependent pathway to Noxa expression regulates apoptosis by platinum-based chemotherapeutic drugs. Submitted
[1]These authors contributed equally to this work.

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CHAPTER I

INTRODUCTION

1.1 APOPTOSIS

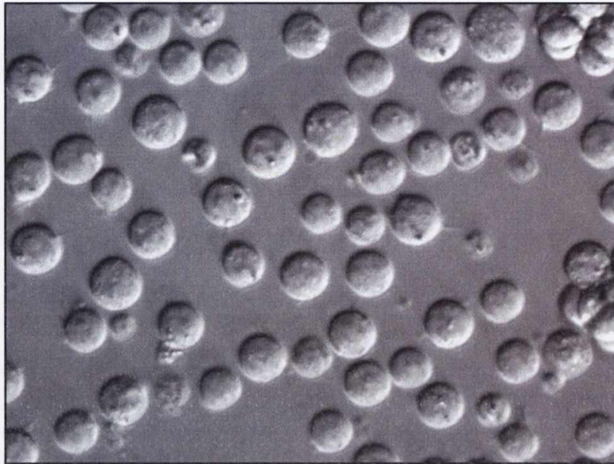
Apoptosis is a form of programmed cellular death that is crucial for efficient development and also maintains cellular homeostasis in adults. During development, apoptosis is involved in processes such as the shaping of bones and digits, involution of tubes and elimination of superfluous cells in the nervous system (Meier *et al.*, 2000). In adults, apoptosis functions to remove old and damaged cells and provides space for proliferating healthy cells. This form of cell death may also be activated in cells that are carrying potentially cancerous mutations or those infected with pathogens. As this process is so important for maintaining a healthy balance of cells in the body, deregulation of apoptosis may contribute to the development of disorders such as neurodegeneration, AIDS or cancer.

1.1.1 *Early advances in apoptosis research*

Apoptosis was first defined by Currie and colleagues in 1972 (Kerr *et al.*, 1972). This seminal study described many of the characteristic features displayed by apoptotic cells, including cytoplasmic condensation and the formation of small apoptotic bodies that are ingested by neighbouring phagocytes (Figure 1.1). Subsequent reports have demonstrated that phagocytes recognise a lipid, phosphatidyserine, which is externalised to the outer membrane of cells during apoptosis and this targets apoptotic cells for engulfment (Fadok *et al.*, 1992; Martin *et al.*, 1995). The controlled demolition and efficient removal of apoptotic cells, ensures the surrounding environment is unaffected by the apoptotic process. In contrast, another form of cell death, necrosis, occurs under conditions of acute injury, where cells rapidly swell and rupture releasing all of their intracellular contents. This alerts the immune system to the possibility of infection and triggers an immune response.

Apoptosis is an evolutionarily conserved process that occurs in mammals, insects and nematodes. Exploration of programmed cell death in the worm, *Caenorhabditis elegans*, resulted in the identification of a series of genes, denoted the *ced* (cell death defective) genes, that define the molecular route to apoptosis (Sulston and

Healthy



Apoptotic

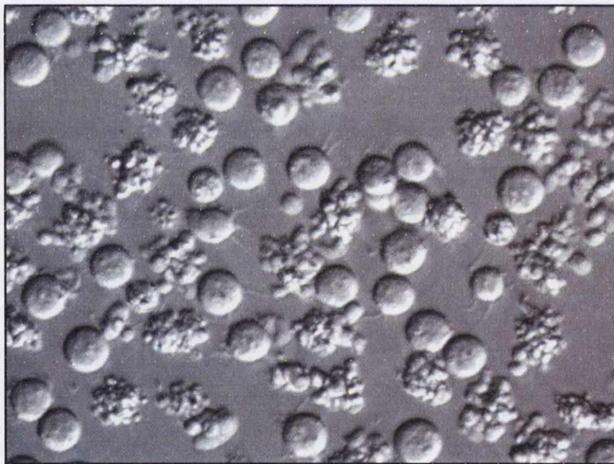


Fig 1.1
Morphology of apoptotic cells
Human HL-60 cells were incubated in the absence or presence of the pro-apoptotic drug Daunorubicin. Dying cells display typical features of apoptosis including membrane blebbing. *In vivo*, small apoptotic bodies break off and are ingested by neighbouring phagocytes.

Horvitz 1977, Ellis and Horvitz, 1986). These elegant studies provided the blueprint from which many mammalian cell death regulators were identified. Subsequent characterisation of these genes revealed that an enzyme, CED-3, was responsible for executing cell death in the worm through proteolysis of a cohort of substrates (Xue *et al.*, 1996; Taylor *et al.*, 2007). This enzyme is activated following binding to an adapter protein CED-4 (Chinnaiyan *et al.*, 1997). However, CED-4 is usually sequestered by CED-9 and is only released when another protein, EGL-1 is upregulated and disrupts this interaction (Spector *et al.*, 1997; Conradt *et al.*, 1998). While the pathway to apoptosis is not identical between C. *elegans* and mammals, the same key players are involved. Thus, the caspase family of enzymes which share homology with CED-3 are important mediators of apoptosis in humans, while Bcl-2 family members, the homologues of CED-9 and EGL-1, play a central regulatory role in the apoptotic process (Yuan *et al.*, 1993; Hengartner *et al.*, 1994).

1.1.2 *Caspases*

In mammals, apoptosis is a highly regulated process with many proteins involved. Cell death stimuli as diverse as UV irradiation, cellular starvation, and pathogenic infection, all activate a common set of effector molecules, although the upstream initiating events may differ. There are two main pathways leading to apoptosis, the intrinsic and extrinsic pathways and both result in the activation of a family of cysteine proteases, the caspases, which are responsible for the execution of apoptosis (Figure 1.2). This family contains 11 members divided into two groups based on their role in apoptosis (caspase 2, 3, 6, 7, 8, 9, 10) or inflammation (caspase 1, 4, 5, 12). Those involved in apoptosis are further divided into initiator (caspase 2, 8, 9, 10) and executioner (caspase 3, 6, 7) caspases (Nicholson and Thornberry, 1997). The initiator caspases contain additional interaction domains, which facilitate binding of adapter proteins and activation of these enzymes (Logue and Martin, 2008). Once activated, the initiator caspases proteolyse and activate executioner caspases, which subsequently cleave a large number of cellular proteins resulting in the demise of the cell (Luthi and Martin, 2007). Important substrates include an inhibitor of the dnase CAD (ICAD), and ROCK-1. Caspase-

mediated cleavage and inactivation of ICAD leads to the characteristic DNA fragmentation observed during apoptosis (Liu *et al.*, 1997). Alternatively, proteolysis of ROCK 1 by caspases activates this kinase, leading to phosphorylation of cytoskeletal proteins and plasma membrane blebbing of apoptotic cells (Sebbagh *et al.*, 2001)

1.1.3 *The Intrinsic Pathway to apoptosis*

The intrinsic pathway is activated in response to cell stress or damage and is regulated by Bcl-2 family members at mitochondria (Figure 1.2). This family is comprised of three subgroups; the anti-apoptotic Bcl-2 family members, the pro-apoptotic BH3-only proteins, and the pro-apoptotic Bax sub-family. BH3-only proteins couple external cell death signals to mitochondria where the interplay of various members of the Bcl-2 family determines the fate of the cell (Labi *et al.*, 2006). Following cellular stress, BH3-only proteins are upregulated and promote Bax and Bak activation through interaction with anti-apoptotic Bcl-2 family members. Once activated, Bax and Bak oligomerise and form a pore in the mitochondrial outer membrane facilitating the release of cytochrome *c*. Cytosolic cytochrome *c* is then incorporated into the apoptosome, a protein complex, which also contains the adapter protein Apaf-1 and the initiator caspase-9 (Hill *et al.*, 2004). Activation of caspase-9 by auto-proteolysis within the apoptosome leads to activation of the downstream executioner caspases-3 and –7. These latter caspases then proteolyse numerous substrates leading to the molecular and morphological changes associated with apoptosis (Slee *et al.*, 1999; Luthi and Martin, 2007). Release of cytochrome *c* from mitochondria is a defining step during the apoptotic process, as cells will die due to mitochondrial dysfunction even if downstream caspases are inhibited (Colell *et al.*, 2007).

1.1.4 *The Extrinsic Pathway to apoptosis*

The extrinsic pathway to apoptosis is utilised by surveillance cells of the immune system to eliminate infected target cells. This pathway is initiated when ligands (TNFα, Fas ligand, TRAIL) expressed on the cell surface of immune cells bind to

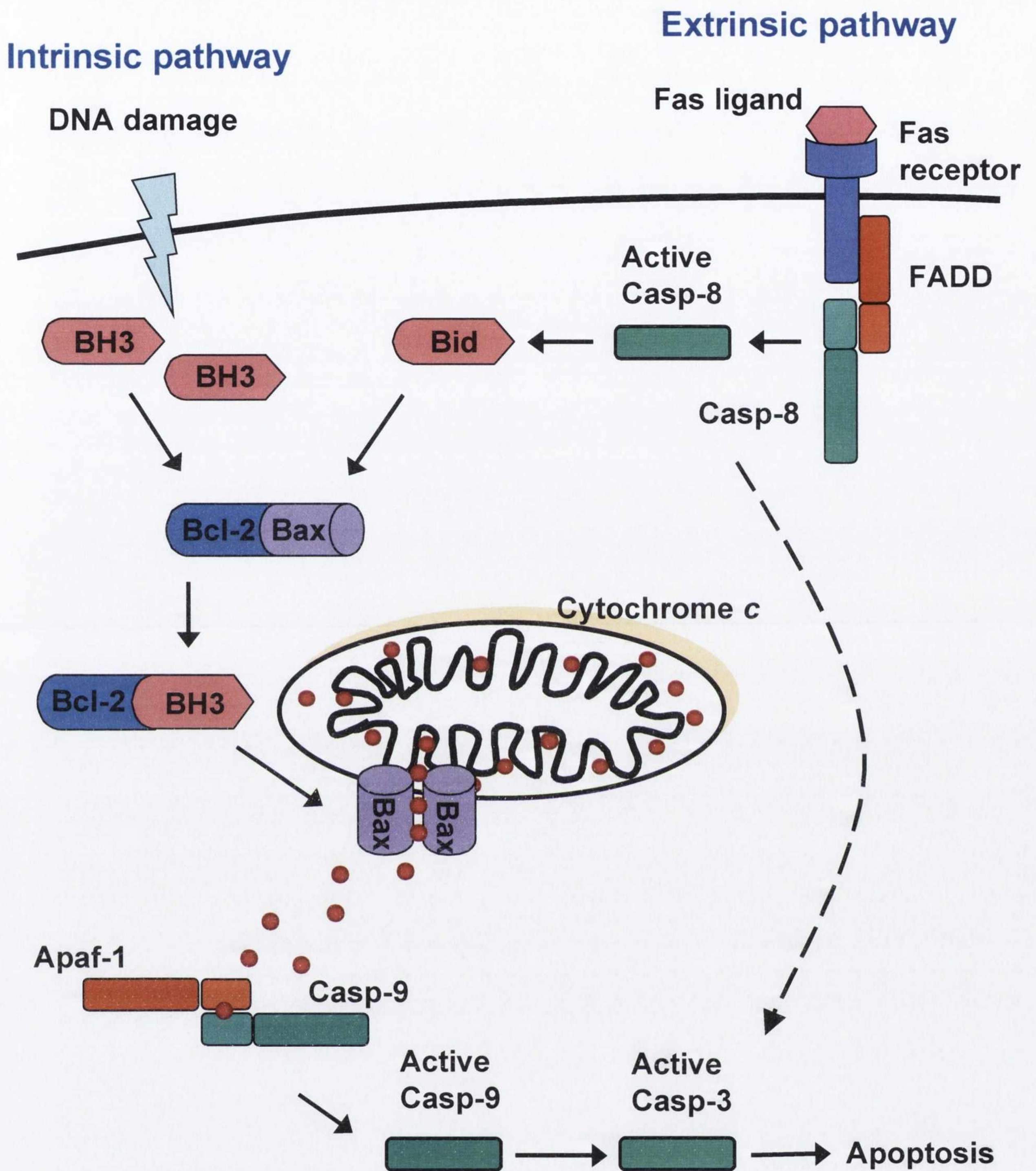


Figure 1.2

Pathways to Apoptosis

In the intrinsic pathway to apoptosis, BH3-only proteins are upregulated in response to cell stress and bind to Bcl-2 proteins releasing Bax and Bak. This results in cytochrome *c* release and caspase-9 activation, followed by caspase-3 activation and cell death. In the extrinsic pathway to apoptosis, Fas ligand binds to the Fas receptor leading to recruitment and activation of caspase-8. Caspase-8 cleaves the BH3-only Bid and/or caspase-3 to induce cell death.

their respective receptors (TNFR, Fas) on target cells. Interactions between these molecules triggers the formation of a death receptor complex, which is comprised of the death receptor, adaptor proteins such as TRADD or FADD, and the initiator caspase-8 (Figure 1.2) (Muzio *et al.*, 1996). Activation of caspase-8 occurs through auto-proteolysis and this enzyme, in turn, cleaves and activates the effector caspase-3 leading to cell death. Caspase-8 may also activate the intrinsic pathway to apoptosis through cleavage and activation of the BH3-only protein Bid (Li *et al.*, 1998).

1.1.5 *The Bcl-2 family*

As outlined above, the Bcl-2 family play a critical role in the regulation of the intrinsic pathway to apoptosis and may also contribute to the extrinsic pathway to death. As the work presented in this thesis focuses on the role of Bcl-2 family members in cell death regulation, these molecules are discussed in greater detail in subsequent sections. Briefly, Bcl-2 family members are grouped based on their function, which is reflected by their protein domain structure (Figure 1.3). Anti-apoptotic Bcl-2 family members contain four conserved Bcl-2 homology (BH) domains, the BH1, BH2, BH3 and BH4 domains. These survival proteins antagonise cell death by binding to and inhibiting both sets of pro-apoptotic proteins (Chipuk and Green, 2008). BH3-only proteins share a single conserved BH3 domain, which is crucial for their ability to bind anti-apoptotic Bcl-2 family members and induce apoptosis (Huang and Strasser, 2000). Bax and Bak contain three conserved Bcl-2 homology domains, BH1, BH2 and BH3 domains, which permits binding to anti-apoptotic members, but also oligomerisation into multimers. This ability to oligomerise facilitates the formation of pores in mitochondrial outer membranes and the release of cytochrome *c* leading to apoptosis (Kuwana and Newmeyer, 2003).

1.2 BH3-ONLY PROTEINS

All eight BH3-only proteins; Bim, Bid, Puma, Bad, Noxa, Bik, Bmf and Hrk, when overexpressed, are potent inducers of apoptosis. These proteins act as sensors of cell stress, as they are induced or modified in response to apoptotic stimuli, and

they are the primordial initiators of apoptosis. These molecules promote cell death principally via binding to anti-apoptotic Bcl-2 related proteins and liberating sequestered Bax, Bak or activator BH3-only proteins. Alternatively, certain BH3-only proteins, Bid, Bim and possibly Puma may directly interact with and activate Bax or Bak to promote cytochrome *c* release (Letai *et al.*, 2002; Kuwana et al, 2005; Kim *et al.*, 2006).

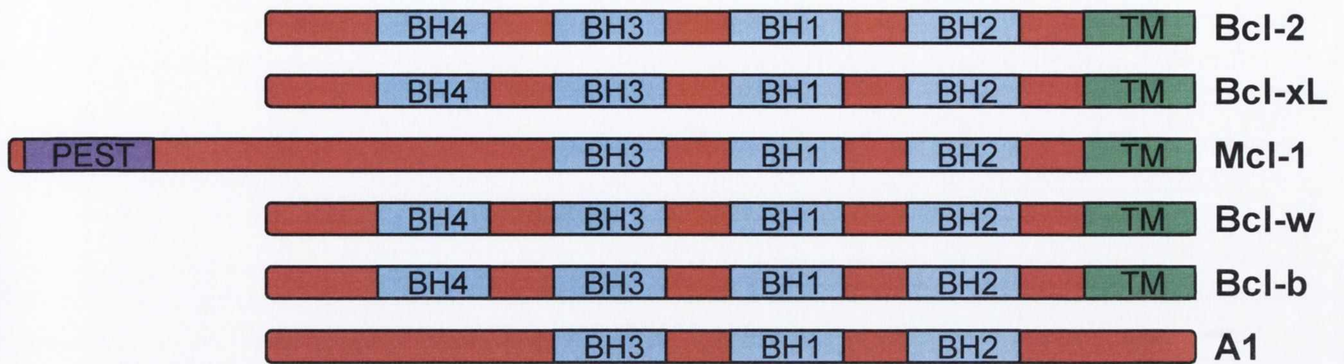
1.2.1 *Regulation of BH3-only proteins*

Although all BH3-only proteins share a homologous BH3 domain, the remainder of their protein sequence is highly divergent. Thus their regulation, expression, and ability to interact with various Bcl-2 family members differs dramatically. Many of the BH3-only proteins are regulated at a transcriptional level. For example, Noxa and Puma are upregulated by p53, while the *hrk* gene is silenced by the transcriptional repressor Dream (Nakano and Vousden, 2001; Oda *et al.*, 2000; Sanz *et al.*, 2001). Bid, Bim and Bad are expressed within healthy cells and are modified in an alternative manner. Bid is cleaved and myristolyated in response to death receptor- and cytotoxic granule-induced apoptosis by caspase-8 and Granzyme B respectively. Cleavage produces a truncated form of Bid that efficiently inserts into mitochondrial membranes and activates Bax (Li *et al.*, 1998; Barry *et al.*, 2000; Zha *et al.*, 2000). Bid may also be cleaved by executioner caspases downstream of mitochondria to drive a feedback loop that amplifies the apoptotic signal (Slee *et al.*, 2000). Cellular Bim and Bad are negatively regulated by phosphorylation and removal of this post-translational modification activates these proteins (Ley *et al.*, 2003; Zha *et al.*, 1996). Finally, Bmf is transcriptionally upregulated by certain apoptotic stimuli and may be activated following histone hyperacetylation of the *bmf* promoter region (Zhang *et al.*, 2006; Schmeizle *et al.*, 2007).

1.2.2 *BH3-only proteins and cell death*

Each cell death stimulus activates a unique cohort of BH3-only proteins and this brings further levels of complexity and regulation to the apoptotic process. Knock-

Anti-apoptotic Bcl-2 family members



Pro-apoptotic Bcl-2 family members

BH3-only proteins

Bax sub-family

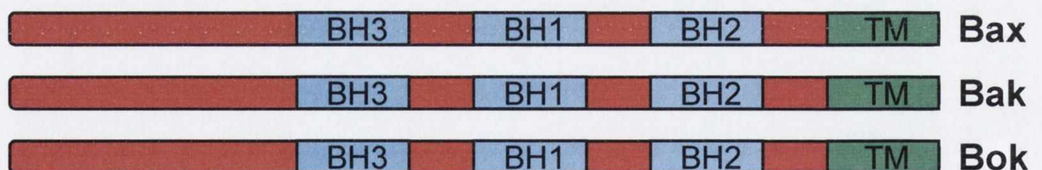


Figure 1.3

Bcl-2 family members

The Bcl-2 family is comprised of anti-apoptotic members and two sub-sets of pro-apoptotic members. The Bcl-2 homology (BH) domains (shown in blue) and the transmembrane domain (shown in green) are important for interactions between family members and integration into mitochondrial membranes respectively. In particular, the BH1, BH2 and BH3 domains of anti-apoptotic Bcl-2 family members form a hydrophobic pocket that is bound by the BH3 domain of pro-apoptotic Bcl-2 family members.

out studies have demonstrated that Puma and Noxa play an important role in DNA damage-induced apoptosis, while mice lacking Bid are refractory to death receptor induced apoptosis (Villunger *et al.*, 2003; Yin *et al.*, 1999). Removal of the *hrk* gene in mice inhibits neuronal apoptosis in response to NGF withdrawl, while Bmf is involved in cell death triggered by histone deacetylase inhibition (Coultas *et al.*, 2007; Labi *et al.*, 2008). Bik knock-out mice showed no impairment in apoptosis (Coultas *et al.*, 2004). However, loss of both Bim and Bik revealed greater apoptotic defects than either single knock-out, indicating that specific cohorts of BH3-only proteins may be redundant for particular functions (Coultas *et al.*, 2005). In addition to these *in vivo* studies, numerous *in vitro* studies have linked certain BH3-only proteins with apoptotic stimuli. For example, Puma plays a role in ER stress-induced apoptosis, Bik promotes apoptosis triggered by proteasome inhibition and Bid is an important mediator of Granzyme B-induced cell death (Reimertz *et al.*, 2003; Zhu *et al.*, 2005; Sutton *et al.*, 2000).

1.2.3 *Interaction with anti-apoptotic Bcl-2 family members*

The ability of BH3-only proteins to interact with Bcl-2 family members varies, and this impacts on their potency as cell death initiators (Figure 1.4). The general consensus from interaction studies is that Bim, Puma and Bid interact with high affinity to most anti-apoptotic Bcl-2 family members (Chen *et al.*, 2005a; Certo *et al.*, 2006). In contrast, Bik, Bmf and Hrk show intermediate binding affinities with a smaller range of anti-apoptotic members, while Bad and Noxa are the least promiscuous. These later two molecules work most efficiently together as Bad binds Bcl-xL, Bcl-2 and Bcl–w, while Noxa binds Mcl-1 and A1. The variations in binding affinities appear to be influenced by particular amino acids within the BH3 domain, as mutation of residues within the Noxa BH3 domain to the corresponding residues found within the Bim BH3 domain enhanced interactions between Noxa and Bcl-xL (Chen *et al.*, 2005a). While these studies are useful for gaining an overall impression of the interactions between pro- and anti-apoptotic Bcl-2 family members, the initial observations were carried out using BH3 peptides and thus may not reflect physiological interactions between full-length proteins in cells.

Furthermore, the abundance, cellular localisation, and post-translational modifications of individual family members is likely to influence interactions within cells.

1.2.4 *Bim*

As the emphasis of chapter III and IV of this thesis lies with the BH3-only proteins Bim, Bad and Noxa, these proteins are described in greater detail below. Bim is an important member of the BH3-only family that was identified in 1998 (O'Connor *et al.*, 1998). Three isoforms Bim_{EL}, Bim_{L}, and Bim_{S} have been described, all of which possess the ability to bind Bcl-2 and induce apoptosis. Bim_{EL} is the most abundant isoform, however Bim_{S} is the most potent of the three. Bim is a particularly powerful cell death inducer as it has the capability to bind efficiently to all anti-apoptotic members of the Bcl-2 family, and also the ability to activate Bax (Chen *et al.*, 2005a; Kuwana *et al.*, 2005). Knock-out studies have revealed that Bim plays an important role in hematopoietic function, as it is responsible for the elimination of autoreactive T lymphocytes and mice lacking the *bim* gene develop autoimmune kidney disease (Bouillet *et al.*, 1999; Bouillet *et al.*, 2002). This BH3-only protein is also involved in the disposal of excess immune cells following an immune response, as Bim-deficient mice accumulate plasma cells throughout adult life (Hildeman *et al.*, 2002; Bouillet *et al.*, 1999; Hughes *et al.*, 2008). *In vitro*, a role for this protein in apoptosis-induced by growth factor withdrawl, anoikis, microtubule disrupters, TRAIL, glucocorticoids, histone deacetylase inhibiors, and ER stress has been reported (Shinjyo *et al.*, 2001; Reginato *et al.*, 2003; Li *et al.*, 2005; Corazza *et al.*, 2006; Abrams *et al.*, 2004; Zhao *et al.*, 2005; Puthalakath *et al.*, 2007). These studies indicate that Bim is a generally important BH3-only protein whose expression influences cell death in multiple contexts. As a mediator of apoptosis, Bim also has a tumor suppressing function and removal of a single *bim* allele accelerated myc-induced B-cell leukemia (Egle *et al.*, 2004).

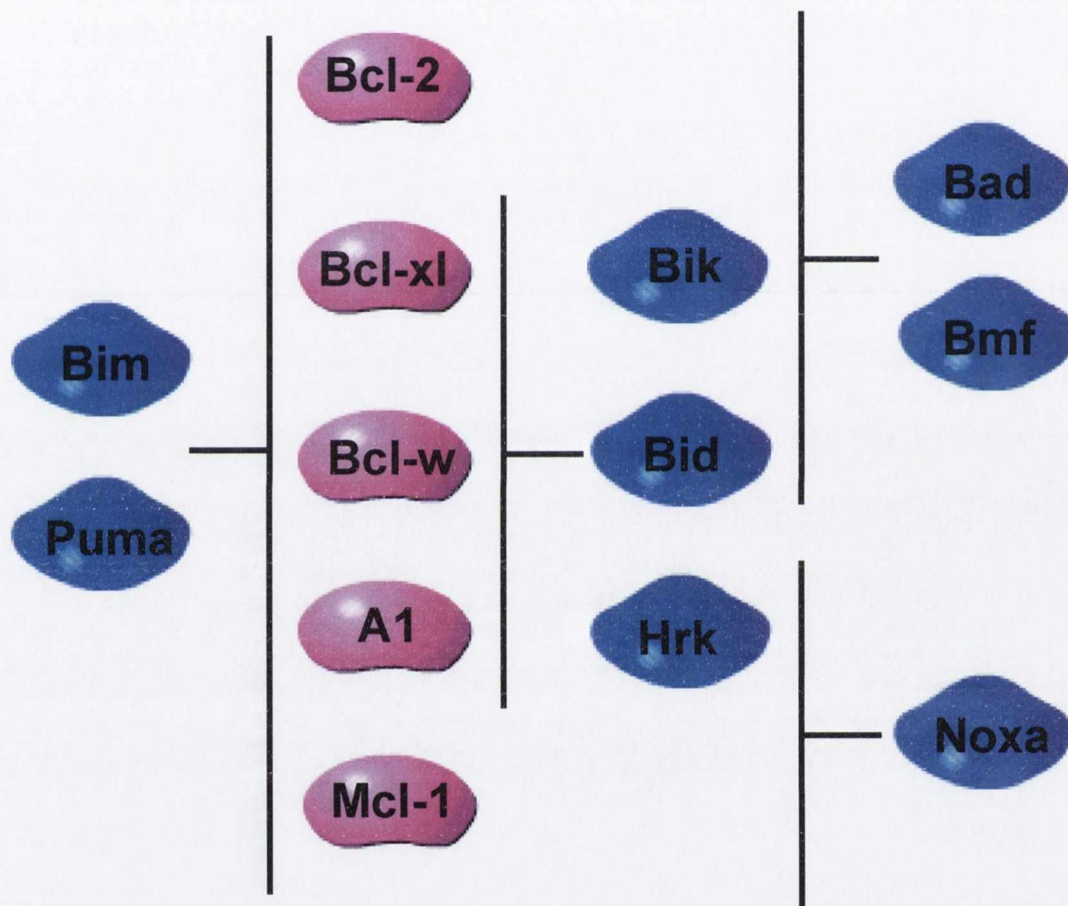


Figure 1.4

Interactions between Bcl-2 family members

Binding of BH3-only proteins with anti-apoptotic Bcl-2 family members is divergent. Bim and Puma can bind all anti-apoptoic proteins. In contrast, Bad binds only Bcl-2, Bcl-xL and Bcl-w, while Noxa binds Mcl-1 and A1. Interactions shown are as described by Chen *et al.*, 2005a.

1.2.5 *Bim regulation*

Bim is heavily regulated at both the transcriptional and post-translational level. Thus different apoptotic stimuli may increase Bim protein expression or enhance the ability of Bim to bind to Bcl-2 family members in different ways. Initial studies describing Bim regulation revealed that cellular Bim is bound to dyenin light chain 1 (DLC1) of the dyenin motor complex and consequently is sequestered to microtubules in healthy cells (Puthalakath *et al.*, 1999). Later studies demonstrated that phosphorylation of Bim by JNK within the DLC1 binding motif, in response to environmental stresses such as UV irradiation, disrupted interactions between Bim and DLC1 and promoted apoptosis (Lei and Davis, 2003). Microtubule disruptors may also interfere with Bim-DLC1 interaction leading to Bim-dependent apoptosis. On the transcriptional level, Bim expression is induced in response cytokine deprivation by the transcription factor FOX03a (Dijkers *et al.*, 2000). This transcription factor is constitutively inactivated in healthy cells via Protein kinase B (PKB) mediated phosphorylation, which prevents its entry into the nucleus. Cytokine removal leads to PKB/AKT downregulation and liberation of FOXO3a resulting in Bim upregulation.

Bim is also regulated at the post-translational level via phosphorylation by ERK. This constitutive phosphorylation event leads to the ubiquitination and degradation of Bim by the proteasome (Weston *et al.*, 2003; Ley *et al.*, 2003; O' Reilly *et al.*, 2009). Dephosphorylation of Bim in response to ERK inactivation during cytokine withdrawl results in increased Bim protein levels within the cell, enhanced interaction between Bim and Bax, and thus apoptosis (Harada *et al.*, 2004). Phosphorylation of Bim by AKT and association with 14-3-3 proteins has also been reported (Qi *et al.*, 2006). In addition, reduction of the RNA binding ability of Hsc70 by cytokines has been described and this hinders Hsc70-mediated stabilisation of Bim mRNA (Matsui *et al.*, 2007).

1.2.6 *Bad*

Bad was the first BH3-only protein identified and largely because of this, extensive studies have been carried out on this protein (Yang *et al.*, 1995). Initial reports revealed that Bad is constitutively phosphorylated in proliferating cells by survival kinases within a 14-3-3 binding site. Phosphorylation of Bad facilitates binding to 14-3-3 and consequently, Bad is sequestered in the cytosol away from mitochondria (Zha *et al.*, 1996). Serum withdrawl leads to downregulation of the kinases responsible for Bad phosphorylation, resulting in the release of Bad from inhibitory constraints, enhanced interaction with Bcl-xL and Bcl-2 and consequently cell death induction. Subsequent studies demonstrated that Bad may be phosphorylated on different residues by PKA, AKT, and an ERK regulated kinase called RSK (Harada *et al.*, 1999; Del Peso *et al.*, 1997; Bonni *et al.*, 1999; Scheid *et al.*, 1999). Bad-deficient mice displayed similar sensitivity as wild type mice to most cell death stimuli with the exception of EGF withdrawl-induced apoptosis. Thus, other BH3-only proteins appear to functionally replace Bad in these mice. However, Bad-deficient mice developed diffuse large B cell lymphomas in later life demonstrating a role for this protein in suppressing tumorigenesis (Ranger *et al.*, 2003).

As the first BH3-only protein to be identified, Bad was considered to play a crucial role in cell death progression. However, more recent studies have revealed that other BH3-only proteins such as Puma and Bim are more potent cell death inducers and are activated in response to a greater number of stimuli. The pro-apoptotic potential of Bad may be hampered by a reduced ability to interact with Mcl-1 and A1. Thus, although Bad can bind Bcl-2 and Bcl-xL and release sequestered Bax or Bak, the remaining Bcl-2 family members may recapture this liberated Bax and Bak and prevent cell death induction.

1.2.7 *Noxa*

Noxa was identified in a screen for p53 target genes that were upregulated in response to x-ray irradiation (Oda *et al.*, 2000). Subsequent knockout studies demonstrated that Noxa plays a significant role in DNA damage-associated

apoptosis, although this role is shared with the fellow p53 target Puma (Shibue *et al.*, 2003; Villunger *et al.*, 2003; Naik *et al.*, 2007). Noxa has also been implicated in apoptosis induced by other cellular stresses such as hypoxia, ER stress, viral infection and T cell apoptosis (Kim *et al.*, 2004; Li *et al.*, 2006; Wang *et al.*, 2009; Sun and Leaman, 2005; Lallemand *et al.*, 2007; Alves *et al.*, 2006; Yamashita *et al.*, 2008). In particular, a cohort of reports have demonstrated that Noxa plays a critical role in apoptosis triggered by proteasome inhibition (Perez-Galan *et al.*, 2005; Qin *et al.*, 2005; Fernandez *et al.*, 2005; Jullig *et al.*, 2006; Nikiforov *et al.*, 2007). Induction of Noxa under these conditions may involve concurrent stabilisation of Noxa protein and transcriptional upregulation of Noxa (Qin *et al.*, 2005; Fernandez *et al.*, 2005).

Regulation of Noxa appears to occur primarily on a transcriptional level. This protein is rapidly upregulated in response to a number of cellular stresses, however the transcription factors responsible vary depending on the stimulus. For example, p53 is important for Noxa upregulation in response to DNA damage, while Hif-1α regulates Noxa expression during hypoxia (Oda *et al.*, 2000; Schuler *et al.*, 2003; Kim *et al.*, 2004). In addition, Myc has been implicated in Noxa induction following proteasome inhibition and CREB may drive Noxa upregulation during viral infection (Nikiforov *et al.*, 2007; Nawrocki *et al.*, 2008; Lallemand *et al.*, 2007). Finally, upregulation of Noxa during ER stress is dependent on the transcription factors ATF3 and ATF4 (Wang *et al.*, 2009). Thus, the upstream pathways that are activated in response to different cellular stresses are important for determining whether Noxa expression is induced, and through which transcription factor this occurs. Noxa may also be transcriptionally repressed in healthy proliferating cells and removal of this negative regulation may enhance Noxa induction during apoptosis (Yamashita *et al.*, 2008, Wang *et al.*, 2009).

Although Noxa is not a potent inducer of apoptosis when overexpressed, and it cannot bind to a large repertoire of anti-apoptotic Bcl-2 family members, it nevertheless appears to be an important BH3-only protein for impeding the anti-

apoptotic function of Mcl-1 (Kim *et al.*, 2004; Fribley *et al.*, 2006; Chen *et al.*, 2005a; Certo *et al.*, 2006). Therefore, Noxa becomes a critical mediator of apoptosis under conditions where cells show a dependency on Mcl-1 expression (Alves *et al.*, 2006; Qin *et al.*, 2006; Mei *et al.*, 2007). Furthermore, there is some evidence to suggest that differential regulation of Noxa in malignant versus non-cancerous cells may enhance tumor selective treatment with chemotherapeutic agents (Nikiforov *et al.*, 2007).

1.3 ANTI-APOPTOTIC BCL-2 FAMILY MEMBERS

1.3.1 *Overview*

Anti-apoptotic Bcl-2 related proteins suppress apoptosis through blocking cytochrome *c* release initiated by diverse death stimuli. The Bcl-2 family contains six anti-apoptotic members; Bcl-2, Bcl-xL, Mcl-1, Bcl-w, Bcl-b and A1, whose ability to interact with pro-apoptotic members of the family, mode of regulation and tissue distribution vary considerably. This diversity between family members ensures that apoptosis does not occur by chance, but rather by intentional and specific inhibition of multiple anti-apoptotic Bcl-2 family members. Similar to BH3-only proteins, anti-apoptotic Bcl-2 family members may also be post-translationally modified through phosphorylation or cleavage (Bassik *et al.*, 2004, Cheng *et al.*, 1997; Weng *et al.*, 2005). However, it appears that their abundance and ability to interact with a repertoire of pro-apoptotic molecules are the most influential determinants of their anti-apoptotic potential. Interaction with BH3-only proteins is facilitated by a hydrophobic pocket formed by the BH1-BH3 domains of anti-apoptotic proteins, which creates a docking site for the BH3 domain in pro-apoptotic Bcl-2 family members, resulting in their sequestration in healthy cells (Petros *et al.*, 2000; Liu *et al.*, 2003).

The importance of anti-apoptotic Bcl-2 related proteins in the regulation of controlled cell death, is exemplified in the phenotypes of knock-out mice. Removal of the *bcl-xL* and *mcl-1* genes in mice is embryonically lethal due to excessive apoptosis, firmly establishing the crucial role of these proteins in development (Motoyama *et*

al., 1995; Rinkenberger *et al.*, 2000). Bcl-2 deficient mice survive until birth, however, many display increased apoptosis in the lymphoid system, develop polycystic kidney disease and die at an early age (Veis *et al.*, 1993). Loss of Al-a leads to spontaneous neutophil apoptosis (Hamasaki *et al.*, 1998). However, Bcl-w deficient mice are healthy, although males display impaired spermatogenesis, demonstrating that some anti-apoptotic Bcl-2 family members have more restricted roles in apoptosis regulation (Print *et al.*, 1998).

1.3.2 *Mcl-1*

Mcl-1 is of particular interest as expression of this anti-apoptotic Bcl-2 family member is modulated by the apoptosis regulators described in this thesis. Mcl-1 is a highly regulated member of the Bcl-2 family. In contrast to the stable expression of Bcl-2 and Bcl-xL, Mcl-1 is a rapidly turned-over protein that rarely achieves high levels of expression. This protein contains a PEST sequence, which targets it for ubiquitination and proteasome-mediated degradation on a continuous basis (Rogers *et al.*, 1986). Further analysis of Mcl-1 deterioration led to the identification of an E3 ubiquitin ligase, Mule, that specifically interacts with and polyubiquitinates Mcl-1, catalysing its degradation (Zhong *et al.*, 2005). Thus, to combat this event in proliferating cells, Mcl-1 is transcriptionally upregulated in response to many growth factors and cytokines such as EGF, IL-6, IL-15 and GM-CSF (Leu *et al.*, 2000; Puthier *et al.*, 1999; Kuo *et al.*, 2001; Huntington *et al.*, 2007; Epling-Burnette *et al.*, 2001). The PI3K and JAK/STAT pathways provide the link between many of these cytokines and Mcl-1 upregulation (Puthier *et al.*, 1999; Kuo *et al.*, 2001; Epling-Burnette *et al.*, 2001). PI3K further enhances Mcl-1 stability through inhibitory phosphorylation of GSK3 (Maurer *et al.*, 2006). This prevents GSK3-mediated phosphorylation of Mcl-1, which promotes Mcl-1 ubiquitination and degradation. In addition, Mcl-1 is regulated by the ERK pathway, through both transcriptional upregulation, and phosphorylation which increases protein stability (Townsend *et al.*, 1999; Domina *et al.*, 2004).

Ablation of Mcl-1 sensitises cells to numerous apoptotic stimuli including anoikis, DNA damage, TRAIL and bacterial infection, which demonstrates the potent anti-apoptotic signal produced by this molecule (Woods *et al.*, 2007; Nijhawan *et al.*, 2003; Taniai *et al.*, 2004; Rajalingam *et al.*, 2008). Conditional knockout of Mcl-1 demonstrated that it is also crucial for survival of B lymphocytes, T lymphocytes and hematopoietic stem cells (Opferman *et al.*, 2003; Opferman *et al.*, 2005). Furthermore, expression of Mcl-1 is enhanced in many cancers indicating that this molecule promotes tumor development and may provide resistance against chemotherapeutic agents (Kaufmann *et al.*, 1998; Thallinger *et al.*, 2003; Wuilleme-Toumi *et al.*, 2005; Nagata *et al.*, 2009). Thus, while Mcl-1 contributes valuable survival signals in healthy proliferating cells, deregulation of Mcl-1 expression can block cell death in damaged or cancerous cells where apoptosis is warranted.

1.4 BAX SUBFAMILY

1.4.1 *Role of Bax and Bak in mitochondrial-associated apoptosis*

Bax and Bak are the Bcl-2 family members responsible for cytochrome *c* release. As a result, the pathway to apoptosis is preceded by a battle between anti-apoptotic Bcl-2 family members and BH3-only proteins over these influential cell death inducers. Bax and Bak are crucial for intrinsic apoptosis progression. While mice lacking either one of these genes may still succumb to cell death, mice lacking both genes are refractory to all forms of apoptosis utilising the mitochondrial pathway (Wei *et al.*, 2001; Ruiz-Vela *et al.*, 2005). As these proteins are essential for developmental apoptosis, the majority of Bax/Bak double knockout mice die perinatally. Those that survive display webbed paws and excessive neuronal and hematopoietic cells due to impaired apoptosis (Lindsten *et al.*, 2000). A third member of this subfamily, Bok may also contribute to mitochondrially driven apoptosis, however, it appears to be significantly less important than Bax and Bak (Hsu *et al.*, 1997).

1.4.2 *Bax/Bak-mediated mitochondrial outer membrane permeabilisation*

Bax and Bak promote apoptosis through mitochondrial outer membrane permeabilisation (MOMP), allowing the release of pro-apoptogenic factors from the mitochondrial intermembrane space such as smac and cytochrome *c*. Although Bax and Bak ultimately achieve the same goal, these proteins possess unique properties. In healthy cells, Bax is a cytosolic protein that exists in an inactive form. Under these conditions, the soluble form of Bax is maintained by sequesteration of the C-terminal region into a hydrophobic cleft formed by BH1 and BH2 domains (Suzuki *et al.*, 2000). Binding of particular BH3-only proteins, such as Bid, to the N-terminal domain of Bax promotes the unfolding of the protein and insertion of the C-terminal region into mitochondrial outer membranes (Desagher *et al.*, 1999; Cartron *et al.*, 2004; Gavathiotis *et al.*, 2008). In contrast, Bak is mitochondrially localised and is held in check by the anti-apoptotic Bcl-2 family members. Bax and Bak are further activated within mitochondrial membranes during apoptosis, triggering dimerisation dependent on the BH1 and BH3 domains, followed by oligomerisation into multimers (George *et al.*, 2007; Dewson *et al.*, 2008). These oligomerised forms of Bax and Bak can form pores in mitochondrial outer membranes by a mechanism that is still undefined, but may depend on the central region of the protein (α-helices 5 and 6) that resembles bacterial pore-forming proteins (Saito *et al.*, 2000; Korsmeyer *et al.*, 2000). These two pro-apoptotic Bcl-2 family members are not only essential, but also sufficient, for this critical step in the apoptotic process, as incubation of Bax and Bak with isolated mitochondria or reconstituted liposomes triggers cytochrome *c* release (Eskes *et al.*, 1998; Kuwana *et al.*, 2002).

While the position of Bax and Bak as mediators of cytochrome *c* release is unequivocal, the regulation and activation of these proteins by other Bcl-2 family members remains under dispute. Currently there are two models proposed for the activation of Bax and Bak, the direct activation model and the displacement model. The first model suggests that Bax and Bak are stimulated by 'direct activator' BH3-only proteins, Bid and Bim, while 'de-repressor' BH3-only proteins promote the release of Bid and Bim from the constraints of anti-apoptotic Bcl-2 family members

(Wei *et al.*, 2000; Letai *et al.,* 2002; Kuwana *et al.*, 2005; Gavathiotis *et al.*, 2008; Merino *et al.*, 2009). The second model suggests that Bax and Bak are already primed for death and liberation of these proteins from anti-apoptotic Bcl-2 family members by any BH3-only protein is sufficient to promote MOMP (Willis *et al.*, 2005; Willis *et al.*, 2007). A combination of both scenarios is likely to be true depending on the availability of each family member and the nature of the apoptotic stimulus present (Figure 1.5). Possibly, in untransformed cells the direct activation model may prevail. However, due to stresses accumulated during transformation, deregulated tumor cells may contain active Bax and Bak, which are sequestered by anti-apoptotic Bcl-2 family members. This latter scenario would facilitate the displacement route to cell death. Furthermore, although high levels of liberated Bax and Bak can induce spontaneous activation, this event may be more efficient in the presence of direct activator BH3-only proteins. In addition, binding of other cellular proteins such as p53 to these pore-forming Bcl-2 family members may also impact on their function and interactions with fellow Bcl-2 family members (Chipuk *et al.*, 2004). There have also been conflicting reports regarding the ability of individual anti-apoptotic Bcl-2 family members to bind Bax and Bak (Willis *et al.*, 2005; Zhai *et al.*, 2008). Thus, many questions have yet to be addressed in this area, as the exact events that occur at mitochondria, the specific interactions between various members of this family, and the manner in which Bax and Bak pores are formed remains elusive.

1.5 MITOCHONDRIAL DYNAMICS

1.5.1 *Mitochondria*

As mitochondria play such a crucial role in the progression of apoptosis, understanding their function in healthy cells contributes to unravelling their role in cell death. Mitochondria are highly dynamic organelles that are constantly extending and dividing to form a network that spans the entire length of the cell (Detmer and Chan, 2007). These organelles are the energy source of the cell, providing ATP that powers everyday cellular processes, and mitochondria also contribute to calcium signalling and metabolism (McBride 2006). Hence it is

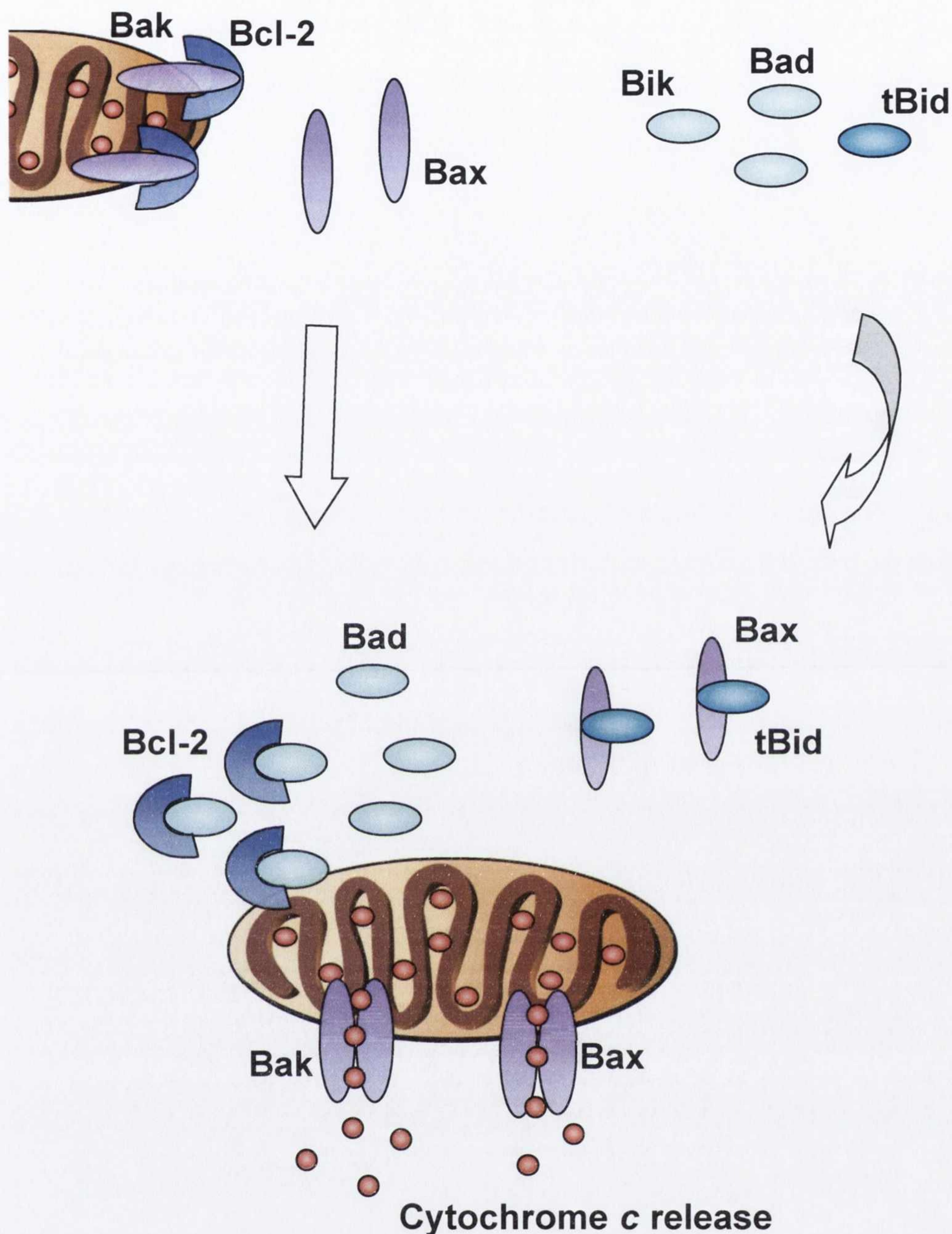


Figure 1.5

Activation of Bax and Bak by BH3-only proteins

In healthy cells, Bak is localised to mitochondria and is held in check by ant-apoptotic Bcl-2 family members, while Bax is found in the cytosol. During cell death, BH3-only proteins are upregulated, or activated in response to pro-apoptotic stimuli. Some BH3-only proteins bind to anti-apoptotic Bcl-2 family members releasing sequestered Bax and Bak. Others BH3-only proteins also directly activate Bax and Bak, leading to mitochondrial outer membrane permeabilisation and cytochrome *c* release

important that the network can stretch into all crevices and protrusions of a cell. The dynamic nature of mitochondria is due to two concurrent processes, mitochondrial fission (division of one mitochondrion) and fusion (merging of two mitochondria) (Figure 1.6) (Chan, 2006a; Cerveny *et al.*, 2007). Mitochondrial fission and fusion are crucial for maintaining the overall mitochondrial morphology, for rapid repair of damaged mitochondria and for sharing of DNA or proteins between mitochondria (Chan, 2006b). Thus, proteins that play an influential role in these processes are important for the maintenance of healthy mitochondria. In addition to mitochondrial remodelling via fission and fusion, mitochondria are also transported within the cell along cytoskeletal tracks (Frederick and Shaw, 2007). Studies using neuronal cells have demonstrated that mitochondria translocate towards axonal areas with high metabolic demands, such as synaptic sites and this mobility enhances the distribution of energy throughout the cell (Hollenbeck and Saxon, 2005).

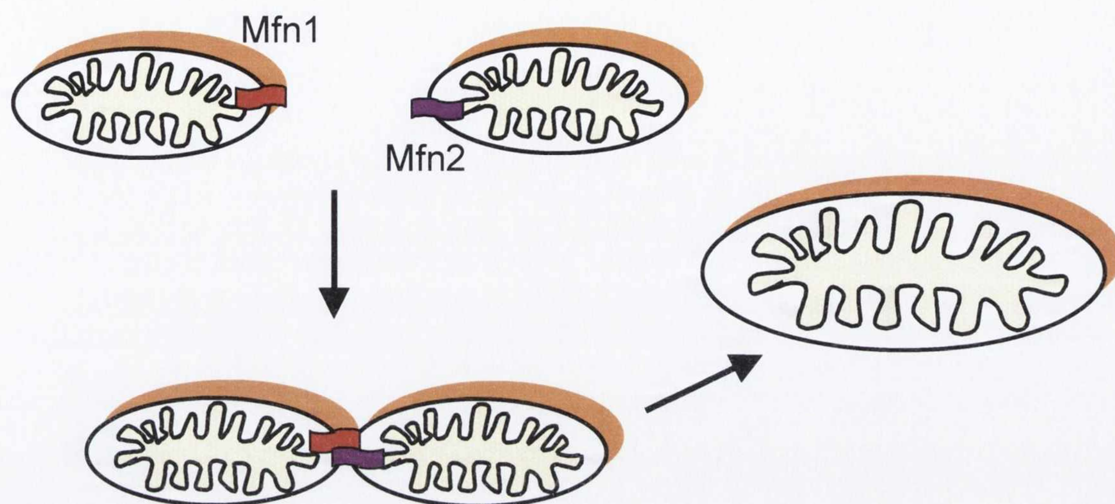
1.5.1 *Mitochondrial fusion*

Mitochondrial fusion involves the tethering of two adjacent mitochondria together followed by merging, or fusion, of the inner and outer mitochondrial membranes. This facilitates the exchange of materials between fused mitochondria and aids the repair of defective mitochondria. Mitochondrial fusion is extremely important as cells with impaired fusion display reduced cell growth, decreased mitochondrial membrane potential and defective respiration (Chen *et al.*, 2005b). Studies in *D. melanogaster* and S. cerevisiae have identified Fzo1 and Mgm1 as the major players in mitochondrial fusion in these organisms (Okamoto *et al.*, 2005; Griffin *et al.*, 2006). The mammalian homologues of Fzo1 are Mitofusin 1 and Mitofusin 2 (Mfn1 and Mfn2), two large GTPases that are localised on mitochondrial outer membranes (Santel *et al.*, 2003; Eura *et al.*, 2003). C-terminal coiled-coil domains facilitate homo- or heterodimeric interactions between these two proteins on adjacent mitochondria. This tethers two mitochondria together, promoting GTPase dependent fusion of mitochondrial outer membranes (Koshiba *et al.*, 2004; Santel *et al.*, 2001; Santel *et al.*, 2003). While mitofusions are important for fusion of the outer

mitochondrial membrane, Opa1, the mammalian homologue of Mgm1, is crucial for the fusion of inner mitochondrial membranes. Opa1 is a dynamin-related protein situated on the mitochondrial inner membrane, and in a similar manner to Mfn1/Mfn2 knockout, ablation of this protein inhibits mitochondrial fusion (Olichon *et al.*, 2002; Chen *et al.*, 2003). Evidence also suggests that Opa1 has an important role to play in maintaining the structure of mitochondrial cristae. These are pockets formed by folds in the inner mitochondrial membrane and loss of Opa1 results in disorganisation of cristae and widening of cristae junctions (Arnoult *et al.*, 2005a; Olichon *et al.*, 2003; Frezza *et al.*, 2006; Yamaguchi *et al.*, 2008). The importance of mitochondrial fusion is further underlined by the identification of human diseases such as Charcot-Marie-Tooth neuropathy type 2A and Dominant Optic Atrophy which are caused by mutations in Mfn2 and Opa1 respectively (Zuchner *et al.*, 2004; Verhoeven *et al.*, 2006; Delettre *et al.*, 2000).

1.5.2 *Mitochondrial fission*

Mitochondrial fission depends largely on the dynamin related protein Drp1, which is localised predominantly in the cytosol and must be recruited to mitochondria for fission to occur (Smirnova *et al.*, 1998; Smirnova *et al.*, 2001). Translocation of Drp1 in yeast is facilitated by its receptor Fis1, which is tethered to the mitochondrial outer membrane. Human Fis1 may also be responsible for recruiting Drp1 to mitochondria in mammals (James *et al.*, 2003). However, translocation of Drp1 still occurred in cells where Fis1 levels were ablated indicating that other receptors may also exist (Lee *et al.*, 2004). Current evidence suggests that Drp1 promotes fission by tethering to mitochondria at specific positions known as fission sites. Drp1 then forms multimeric spirals around mitochondria, constricting mitochondrial tubules and promoting mitochondrial fission (Smirnova *et al.*, 2001). Ablation of Drp1 with siRNA demonstrated the crucial role of this protein in mediating mitochondrial fission as these cells contain elongated, fused mitochondria (Smirnova *et al.*, 2001; Lee *et al.*, 2004). Other proteins have been implicated in mitochondrial fission in humans such as MPT18, Endophilin B1 and GDAP1 and these may aid Drp1-



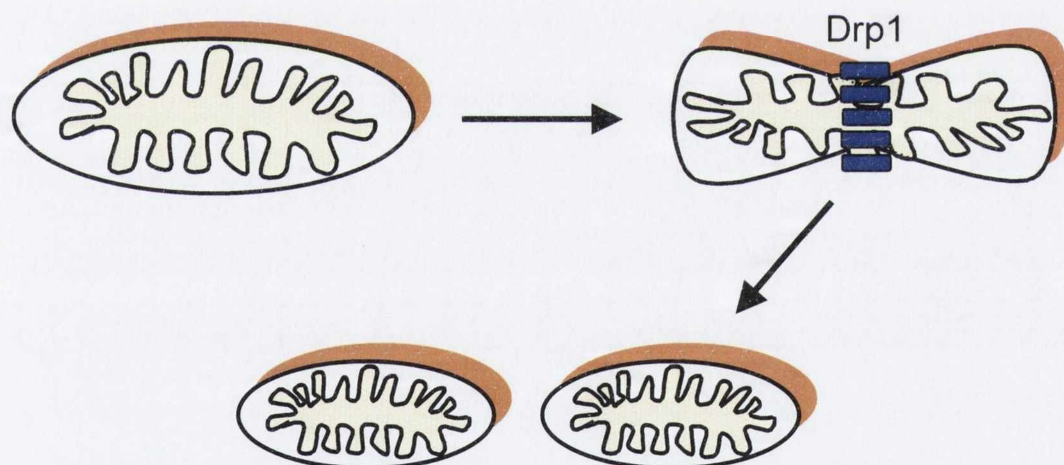


Figure 1.6

Mitochondrial fission and fusion dynamics

Mitochondrial fusion involves the tethering of two mitochondria together through Mfn interactions, followed by GTPase-dependent fusion of the inner and outer mitochondrial membranes. Mitochondrial fission occurs when Drp1, recruited from the cytosol, forms spirals around mitochondria at fission sites. This increases the proximity of mitochondrial membranes and is followed by mitochondrial fragmentation into two smaller mitochondria.

dependent mitochondrial fragmentation (Tondera *et al.*, 2004; Karbowski *et al.*, 2004; Niemann *et al.*, 2005).

1.5.3 *Mitochondrial fission during apoptosis*

As previously mentioned, the release of cytochrome *c* and other pro-apoptotic factors from mitochondria is a crucial step in the apoptotic process. Within a similar time frame, mitochondria fragment from filamentous tubules into numerous small round particles (Frank *et al.*, 2001; Gao *et al.*, 2001; Lee *et al.*, 2004). These fragmented mitochondria often collapse from an extended network into a clustered perinuclear cellular localisation and show reduced motility (De Vos *et al.*, 1998). Drp1 appears to be responsible for this fragmented phenotype as studies have demonstrated that ablation of Drp1 reduces mitochondrial fragmentation during apoptosis (Frank *et al.*, 2001; Karbowski *et al.*, 2002; Estaquier *et al.*, 2007; Sugioka *et al.*, 2004), while overexpression of dominant negative Drp1 prevents apoptosis-induced mitochondrial fragmentation (Frank *et al.*, 2001, Arnoult *et al.*, 2005b). In addition, increased recruitment of Drp1 from the cytosol to mitochondrial sission sites during apoptosis has been demonstrated (Frank *et al.*, 2001, Cassidy-Stone *et al.*, 2008). However, the function of mitochondrial fragmentation during apoptosis is unclear and whether this event contributes to cytochrome *c* release is uncertain. Some studies have proposed that mitochondrial fission facilitates the release of cytochrome *c* (Frank *et al.*, 2001; Lee *et al.*, 2004). Others reports have demonstrated that fragmentation occurs after MOMP and suggest that mitochondrial fission occurs due to the loss of inner mitochondrial proteins (Arnoult *et al.*, 2005a; Estaquier and Arnoult, 2007). Furthermore, the events or proteins that trigger mitochondrial fission during apoptosis have not been identified. Thus the relationship between mitochondrial fragmentation and apoptosis has yet to be clearly defined.

1.6 CANCER

1.6.1 *Cancer development and progression*

Cancer is a potent killer in developed countries including Ireland, superseded only by cardiovascular disease. There are many different types of cancer and tumors may be found in all tissues of the body. Development of cancer occurs over a number of years and is believed to be the culmination of acquiring a number of individual cancerous mutations, on separate occasions, eventually producing a malignant disease (Figure 1.7).

Cancers are defined by a number of characteristics. Firstly, all tumors display reduced dependency on external growth signals. Non-malignant cells receive signals for proliferation through growth factors secreted by neighbouring cells. However tumor cells circumvent this requirement by producing their own growth factors, overexpressing growth factor receptors on their cell membranes or by amplifying the internal proliferation signal through oncogenic mutation of key components of cell signalling pathways (Normanno *et al.*, 2006; Schubbert *et al.*, 2007). Cancer cells also evade anti-proliferative signals, and promote the growth of blood vessels carrying oxygen and nutrients to the tumor in a process named angiogenesis. To achieve the latter aim, many tumor cells enhance the production of angiogenic factors such as VEGF and bFGF (Bhat *et al.*, 2008). Normal cells have a limited replicative potential that is governed by the length of their telomeres. These telomeres are stretches of 6 bp repeats that protect the end of chromosomes and each time a cell divides the telomere length is reduced, eventually leading to chromosomal disarray and cell death (Harley *et al.*, 1990). Tumor cells prevent this telomere degradation, mainly through expression of the enzyme telomerase, providing them with limitless replicative potential that facilitates proliferation long past their original life span (Harley, 2008). While tumors arise in a particular tissue, many cancers develop an ability to metastasise to other sites in the body away from the initial tumor site. Metastasis is responsible for the majority of cancer-related deaths as the multiple tumors distributed throughout the body become untreatable (Leber and Efferth, 2009). This characteristic is usually acquired at a late stage of

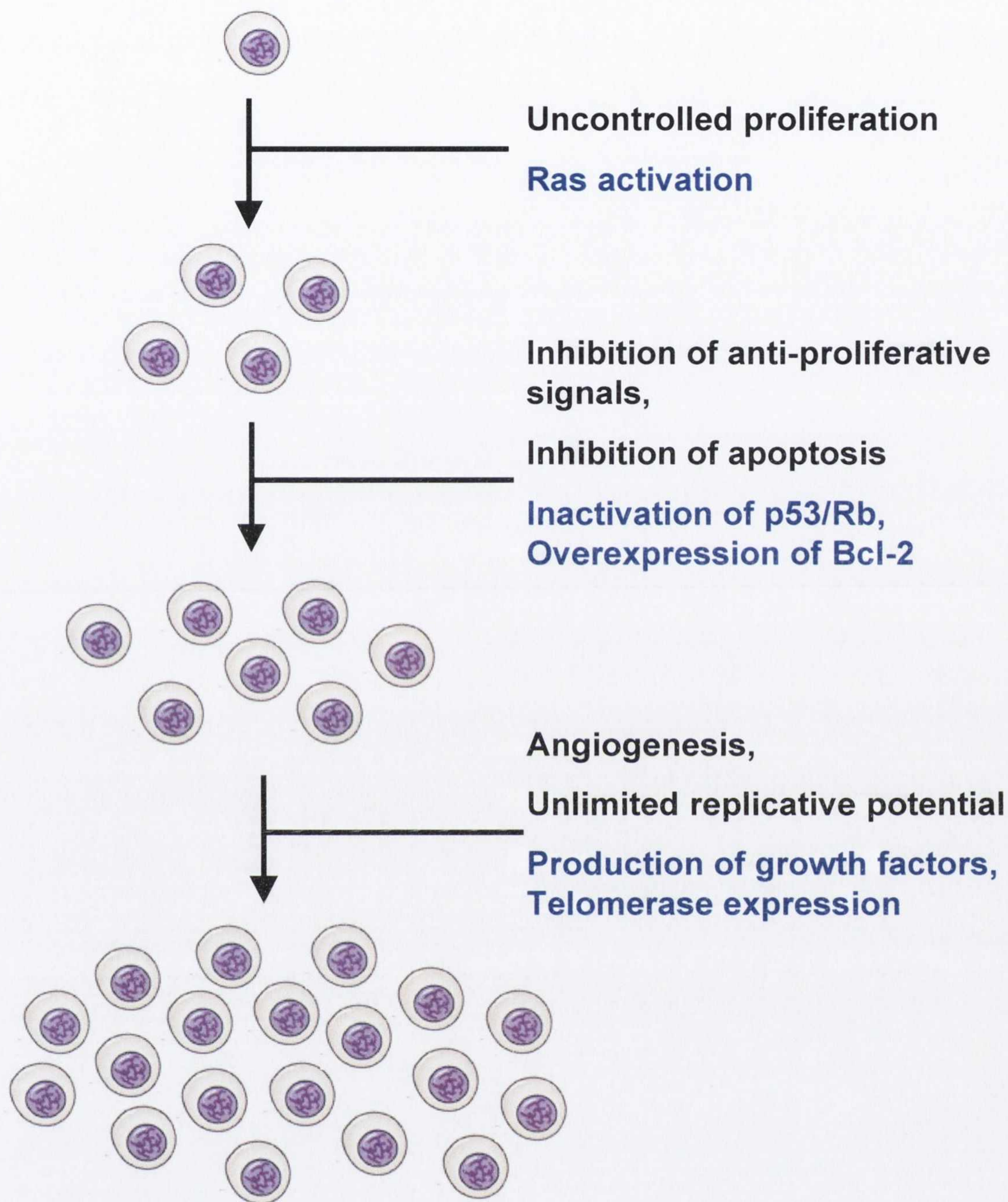


Figure 1.7

Transformation of cells during oncogenesis

Development of cancer is a multistep process. Established tumorigenic cells acquire various characteristics at different points in the transformation process. Some of these traits are described in black, while the events that facilitate tumor development are described in blue.

cancer development, and the cells involved carry numerous mutations that allow them to circumvent natural surveillance systems that target tumor cells (Chiang *et al.*, 2008).

Finally, all cancer cells demonstrate evasion of apoptosis. In untransformed cells, apoptosis acts a surveyor that monitors the cells external and internal environment and purges defective cells where necessary. Numerous scenarios encountered by cancer cells such as cell starvation due to uncontrolled tumor growth, DNA damage leading to cancerous mutations, detachment of a cancer cell from its neighbouring cells, and hyperactivation of intracellular pathways switched on by oncogenes should all conclude with the termination of the cell. However, cancer cells evolve mechanisms to avoid apoptosis and continue proliferating in their defective state (Kasibhatla and Tseng, 2003). This can be achieved through enhancing survival pathways within the cells such as the PI3K pathway, mutation or downregulation of death receptors such as Fas, or modulation of Bcl-2 family members (Zhao and Vogt, 2008; French and Tschopp, 2002; Kirkin *et al.*, 2004; Karst *et al.*, 2006).

1.6.2 *Cancer oncogenes and tumor suppressors*

As cancer cells develop they produce many different molecules that promote their existence, and they recruit various untransformed neighbouring cells to protect and provide for the growing tumor. However, cancer cells originate due to mutations that occur in crucial cell-associated proteins, causing oncogene activation or removal of tumor suppressors.

Ras is one of the most well defined oncogenes whose activation results in the enhancement of two important cell signalling pathways, the MAPK pathway and the PI3K pathway (Karnoub and Weinberg, 2008). Hyperactivation of these pathways promotes growth factor independent proliferation and survival against apoptosis. Mutation of Ras occurs frequently, in approximately twenty percent of cancers, and many other tumors exhibit alternative activating mutations in the same pathways (Downward, 2003). The transcription factor Myc is another established oncogene

that upregulates genes associated with cell cycle progression (Herold *et al.*, 2009). Activation of this oncogene commonly occurs due to gene translocation or amplification rather than direct mutation (Dalla-Favera *et al.*, 1983; Meyer and Penn, 2008). Viral infection may also contribute to cellular transformation, through the expression of viral oncogenes that deregulate existing cell signalling molecules (Boccardo and Villa, 2007). For example, the human papillomavirus E6 and E7 oncoproteins bind to p53 and Rb, inhibiting their functions. Other oncogenes include tyrosine kinase receptors, proteins involved in the WNT pathway, and transcription factors c-fos and c-jun, all of which promote the enlargement of a tumor.

Conversely, inactivation or silencing of the p53 tumor suppressor protein also encourages advancement of tumors, and this has been detected in more than 60 percent of cancers (Bourdon, 2007). This protein plays a crucial role in sensing cellular stress, particularly DNA damage, and induces cell cycle arrest to allow repair of DNA, or apoptosis where appropriate. Thus, inhibition of p53 facilitates the accumulation of DNA mutations that are beneficial to cancers (Sherr and McCormick, 2002). The retinoblastoma protein (Rb) also plays a crucial role in halting cell cycle progression when the nutrients or space for replication are in short supply. This protein functions via binding to the E2F transcription factor, and through this interaction prevents the upregulation of proteins that regulate cell cycle entry (Burkhart and Sage, 2008). In cancer cells Rb appears to be inactivated by a number of different mechanisms, including gene deletion of the upstream regulator $p16^{INK4A}$ and mutation of the *rb* gene itself (Kim and Sharpless, 2006). Additional tumor supressors include PTEN which inhibits the PI3K pathway, a regulator of Ras called neurofibromin and BRCA proteins that are involved in DNA damage repair.

1.6.3 *Cancer treatments and chemotherapy*

A range of different cancer treatments have been developed to combat the uncontrolled proliferation of human tumors. These therapies include surgical removal of tumors, radiation, immunotherapy and chemotherapeutic treatment.

Chemotherapy involves the eradication of cancer cells using toxic drugs, most of which induce apoptosis. These drugs disrupt ongoing cellular processes or damage cellular components in an irreversible way. For example, taxanes prevent microtubule disassembly thereby preventing cell division, topoisomerase inhibitors affect the topology of DNA by inhibiting DNA supercoiling, and alkylating agents crosslink DNA preventing replication and transcription (Wilson and Jordan, 2004; Giles and Sharma 2005; Siddik et al., 2003). TRAIL is another widely used cancer therapeutic that activates the extrinsic pathway to apoptosis through interaction with TRAIL death receptors. TRAIL is an ideal anti-cancer agent as it targets tumor cells preferentially over non-cancerous cells. However, this agent lacks potency and thus is most effective in combination with other chemotherapeutic drugs (Johnstone *et al.*, 2008).

While development of chemotherapy has been a major advance in the treatment of cancer, and these drugs will continue to be an instrumental form of cancer therapy in the future, the side-effects accompanying treatment are often severe. Most chemotherapeutic drugs target rapidly dividing cells by inhibiting cellular division in some manner. Unfortunately non-malignant cells that also divide frequently such as hair follicles and cells in the digestive tract are also targeted, causing hair loss and nausea. In addition tumor cells often acquire resistance to chemotherapeutic drugs such that relapsed tumors are refractory to further chemotherapeutic treatment. Therefore, developing therapies aim to more specifically target tumor cells and the oncogenic mutations that produced them. Imatinub, a tyrosine kinase inhibitor that targets the bcr-abl kinase amplified in chronic myelogenous leukemia (CML) patients, is the most successful example of targeted therapy to date (Druker and Lydon, 2000).

1.6.4 *Bcl-2 family members and cancer*

Bcl-2 was first identified in 1985 as a chromosomal translocation in B cell lymphomas. In these cells, the *bcl-2* gene becomes coupled with the immunoglobulin heavy chain locus, leading to Bcl-2 overexpression and tumor

formation (Tsujimoto *et al.*, 1985). This was the earliest indication of the oncogenic potential of anti-apoptotic Bcl-2 family members and has been followed by association of these proteins with various cancers (Kirkin *et al.*, 2004, Droin and Green, 2004). Although high expression of these genes alone does not typically induce tumor formation, anti-apoptotic members of this family are frequently overexpressed in human cancers and play an important role in preventing apoptosis in cells carrying other harmful mutations. In this regard, an elegant study by Vaux and colleagues demonstrated that Bcl-2 overexpression aided c-Myc induced transformation of pre-B cells, while mice harbouring a Bcl-xL transgene more readily develop chemically-initiated squamous cell carcinomas (Vaux *et al.*, 1988, Pena *et al.*, 1998). Increased expression of anti-apoptotic Bcl-2 related proteins may also be responsible for the resistance of many cancers to apoptosis induced by chemotherapeutic drugs. Consequently, BH3 mimetics such as ABT-737 are being developed as anti-cancer agents to overcome cellular anti-apoptotic Bcl-2 family members (Konopleva *et al.*, 2006, Lessene *et al.*, 2008). In some cancers, down-regulation or inactivation of BH3-only proteins has also been described, such as reduced expression of Bik in renal cell carcinomas, demonstrating that BH3-only proteins may have tumor suppressing abilities (Sturm *et al.*, 2006). Consistent with this theory, Bid and Bad deficient mice develop tumors as they age, while removal of Bim accelerates myc driven tumorogenesis (Zinkel *et al.*, 2003; Ranger *et al.*, 2003; Egle *et al.*, 2004). Furthermore, loss of Bmf accelerates thymic lymphoma development induced by γ-irradiation (Labi *et al.*, 2008). Thus, enhancing the functions of anti-apoptotic Bcl-2 family members or inhibition of pro-apoptotic BH3-only proteins can dramatically augment the oncogenic potential of cells.

1.7 MELANOMA

Melanoma is the most severe form of skin cancer which, once established, is extremely refractory to chemotherapeutic treatment. These tumors develop when benign nevi, or moles, progress to a malignant state, and if detected at an early stage can be cured by surgical removal. However, patients harbouring metastatic melanoma have a poor survival rate of less than one year (Gray-Schophfer *et al.*,

2007). Melanoma is initiated following exposure of skin to UV light and it is believed that intermittent, intensive exposure to sun may be responsible for development of the disease. The incidence of melanoma is on the rise worldwide and fair-skinned Caucasian populations are more susceptible with the highest rate of incidence occurring in Australia.

1.7.1 *Melanoma-associated mutations*

A number of different mutations characterise melanomas. Deletion or mutation of the chromosomal region 9p21, encoding the *cdkn2a* gene, frequently occurs in melanoma (Fitzgerald *et al.*, 1996). This gene encodes two distinct proteins through alternative use of exon 1α or 1β together with exon 2. The first variant encodes p16^{INK4A}, which negatively controls cell cycle progression via inhibition of cyclin-dependent kinases (CDKs) that hyperphosphorylate the retinoblastoma protein (Rb). Phosphorylation of Rb blocks interaction with the E2F transcription factor, thus facilitating the upregulation of genes necessary for cell cycle entry by E2F. The second variant encoded at this locus is p14^{ARF}, which binds to and inhibits HDM2, thus preventing ubiquitination and degradation of p53 (Pomerantz *et al.*, 1998).

Elevated activity of AKT/PKB has been detected in 43-67 % of melanomas resulting in increased proliferation and survival (Stahl *et al.*, 2004). This is facilitated by mutation or gene silencing of PTEN in the majority of these cases (Mirmohammadsadegh *et al.*, 2006). PTEN is a dual lipid and protein phosphatase that negatively regulates AKT by converting PIP_3, which is essential for PI3K mediated activation of AKT, to PIP_2. Ras is also mutated in 20 % of melanomas, most frequently a Q61R mutation, which enhances it's function and leads to augmentation of both the AKT and MAPK pathways. Finally, micropthalmia-associated transcription factor (MITF), a transcription factor involved in survival and differentiation of melanocytes, is amplified in 10-20% of melanomas (Levy *et al.*, 2006).

1.7.2 *B-RAF and melanoma*

In 2002, Davies and colleagues described the mutation of B-Raf in ~70% of malignant melanomas. B-Raf is an important kinase in the MAPK signalling pathway that is described in greater detail below. A number of different B-Raf mutations have been identified, however the most frequent mutations occur at residue 600 where substitution of a valine residue to a glutamic acid renders this kinase up to 500 fold more active (Davies *et al.*, 2002). B-Raf normally resides in an inactive state due to hydrophobic interactions between the P-loop in the regulatory N region and the Asp-Phe-Gly (DFG) motif in the activation segment (Figure 1.8). Phosphorylation of the activating residues Threonine 599 and Serine 602 destabilises this interaction producing an active kinase (Zhang and Guan, 2000). In a similar fashion, mutation of valine 600 to a negatively charged aspartic acid disrupts the auto-inhibitory interactions between these regions resulting in a constitutively active kinase (Wan *et al.*, 2004). Although B-Raf^{V600E} mutations occur most frequently in melanoma they have also been identified in ~40% of papillary thyroid cancers and ~10% of colorectal cancers (Tuveson *et al.*, 2003).

The B-Raf^{V600E} mutation is oncogenic and expression in melanocytes results in phorbol myristic acid (PMA)-independent growth, colony formation and development of tumors when injected into nude mice (Wellbrock *et al.*, 2004a, Hoeflich *et al.*, 2006). Mutation of this kinase appears to be an early event in tumorigenesis as B-Raf^{V600E} is often detected in benign nevi (Pollock *et al.*, 2003). Typically, overexpression of B-Raf^{V600E} alone is not sufficient for tumor formation, as B-Raf^{V600E}–induced senescence blocks cell division (Michaloglou *et al.*, 2005; Wajapeyee *et al.*, 2008). Thus, senescent nevi harbouring B-RAF^{V600E} are transformed by additional genetic lesions, which inactivate p16^{INK4A} or enhance AKT activity (Dankort *et al.*, 2007; Cheung *et al.*, 2008; Goel *et al.*, 2009). B-Raf^{V600E} appears to plays an integral role in melanoma maintenance as inactivation or downregulation of this kinase resulted in tumor regression in mouse models of melanoma (Karasarides *et al.*, 2004; Sumimoto *et al.*, 2004; Sharma *et al.*, 2005; Hoeflich *et al.*, 2006). Furthermore, the tumorigenic activity of B-Raf^{V600E} is

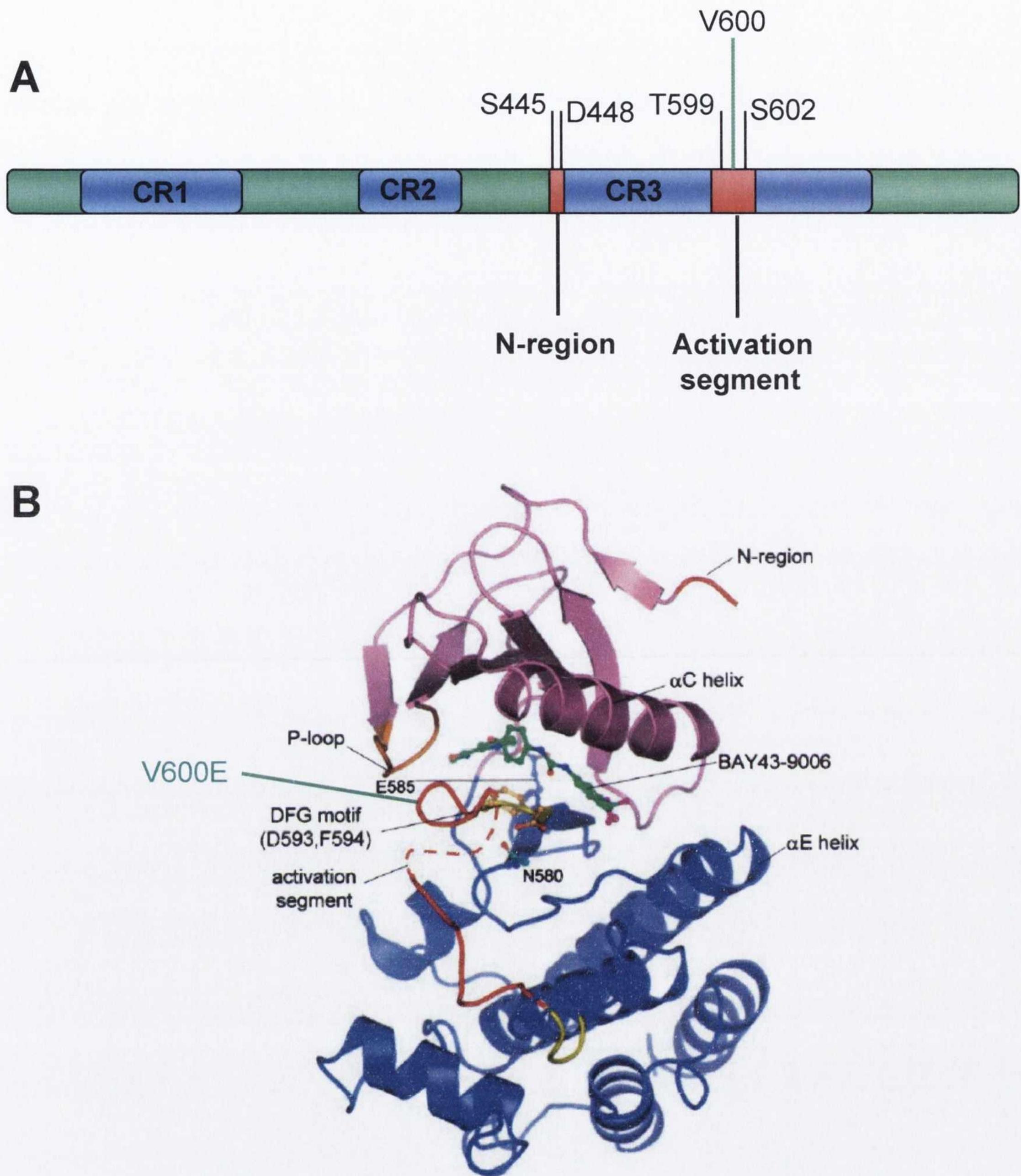


Figure 1.8

Structure of B-Raf

Schematic of the primary structure of B-Raf (A). Regions conserved with other Raf isoforms are shown in blue, the regulatory N-region and activation segment are shown in red and important residues for B-Raf activation are indicated. Ribbon diagram of the B-Raf kinase domain complexed with the Raf inhibitor BAY43-9006. Interactions between the P-loop of the N-region and the DFG motif in the activation segment inhibit kinase activity. Mutation of residue 600 from valine to aspartic acid disrupts this interaction producing a protein confirmation that promotes activity. Structure is from Wan *et al.*, 2004.

dependent on the MAPK pathway as inhibition of MAPKs similarly caused tumor regression in mice injected with melanoma cells harbouring the B-Raf^{V600E} mutation (Collisson *et al.*, 2003; Solit *et al.*, 2005).

1.8 THE MAPK PATHWAY

1.8.1 *Introduction*

The MAPK pathway plays an important role in the growth and division of healthy cells (Figure 1.9). MAPK signalling is activated in response to heterotrimeric G proteins and growth factors such as EGF and FGF (Gutkind, 1998). These growth factors bind to their respective cell surface receptors, and trigger receptor activation, which occurs through auto-phosphorylation of tyrosine residues. Receptor phosphorylation facilitates binding of the adaptor protein Grb2 through its SH2 domain, and subsequent recruitment of the guanine nucleotide exchange factor, Sos through a SH3 domain on the latter molecule (Egan *et al.*, 1993). Activated Sos converts recruited Ras from a GDP bound state to an active GTP bound state (Buday and Downward, 1993).

Active Ras then promotes cell division through activation of two key proliferation pathways within the cell, the AKT pathway and the MAPK pathway. Recruitment of PI3K to RAS results in its activation and subsequent phosphorylation of the survival kinase AKT (Rodriguez-Viciana *et al.*, 1994). In a similar manner, the serine/threonine kinases A-, B- and C-Raf are recruited to the plasma membrane via interaction with active Ras, where they are activated by phosphorylation on various residues (Warne *et al.*, 1993, Moodie *et al.*, 1993). Phosphorylation of S338 by p21-activated kinase (PAK) and Y340 by Src in the regulatory N-region disrupts auto-inhibitory interactions of the C-Raf kinase while phosphorylation of T491 and S494 in the activation loop increases kinase activity (King *et al.*, 1998; Marais *et al.*, 1995; Chong *et al.*, 2001). Raf kinases subsequently phosphorylate the tyrosine/threoinine kinase MEK, which in turn phosphorylates the downstream serine/threoinine kinase, MAPK (also known as ERK) (Kyriakis *et al.*, 1992).

1.8.2 *MAPK targets*

ERK phosphorylates multiple targets including kinases such as RSK, MNK and MSK, which subsequently modulate a myriad of proteins through phosphorylation (Roux and Blenis, 2004). ERK also targets many transcription factors such as c-myc, ATF2 and Ets transcription factors for activation. In particular, ERK phosphorylates Elk-1, which enhances the transactivation function of this transcription factor and leads to c-fos upregulation (Gille *et al.*, 1995). During sustained MAPK activation, cellular c-fos is further phosphorylated and stabilised by ERK, facilitating the formation of the AP-1 transcription complex between c-fos and c-jun (Murphy *et al.*, 2002). This complex is responsible for the upregulation of cyclin D, which activates cyclin-dependent kinases necessary for G1-S phase transition of the cell cycle (Shen *et al.*, 2008; Weber *et al.*, 1997; Xiong *et al.*, 1992). In addition, ERK modulates upstream kinases, MEK and Raf, in a negative feedback loop and also affects cellular morphology through phosphorylation of cytoskeletal proteins (Yoon and Seger, 2006). Thus, via modification of numerous proteins, either directly or indirectly, ERK influences divergent molecular processes including proliferation, differentiation and survival.

1.8.3 *MAPK Pathway and apoptosis*

Although the primary function of the MAPK pathway is to drive cell division, there have been a number of studies linking this pathway to apoptosis resistance. Removal of the *c-raf* gene in mice resulted in embryonic lethality due to fetal liver apoptosis, and increased apoptosis of hematopoietic and fibroblast cells (Mikula *et al.*, 2001). Specific removal of C-Raf from cardiac muscle led to cardiomyocyte apoptosis in an apoptosis signal-regulating kinase-1 (ASK1) dependent manner (Yamaguchi *et al.*, 2004). However, the survival function of C-Raf appears to be MAPK-independent as ERK signalling is unaffected in these mice due to the presence of A- and B-Raf. Subsequently, interactions between C-Raf and Rok-α have been described, leading to reduced clustering and membrane expression of the death receptor Fas, and thus inhibition of liver apoptosis (Piazzolla *et al.*, 2005). B-Raf deficient mice display increased apoptosis in the endothelium further

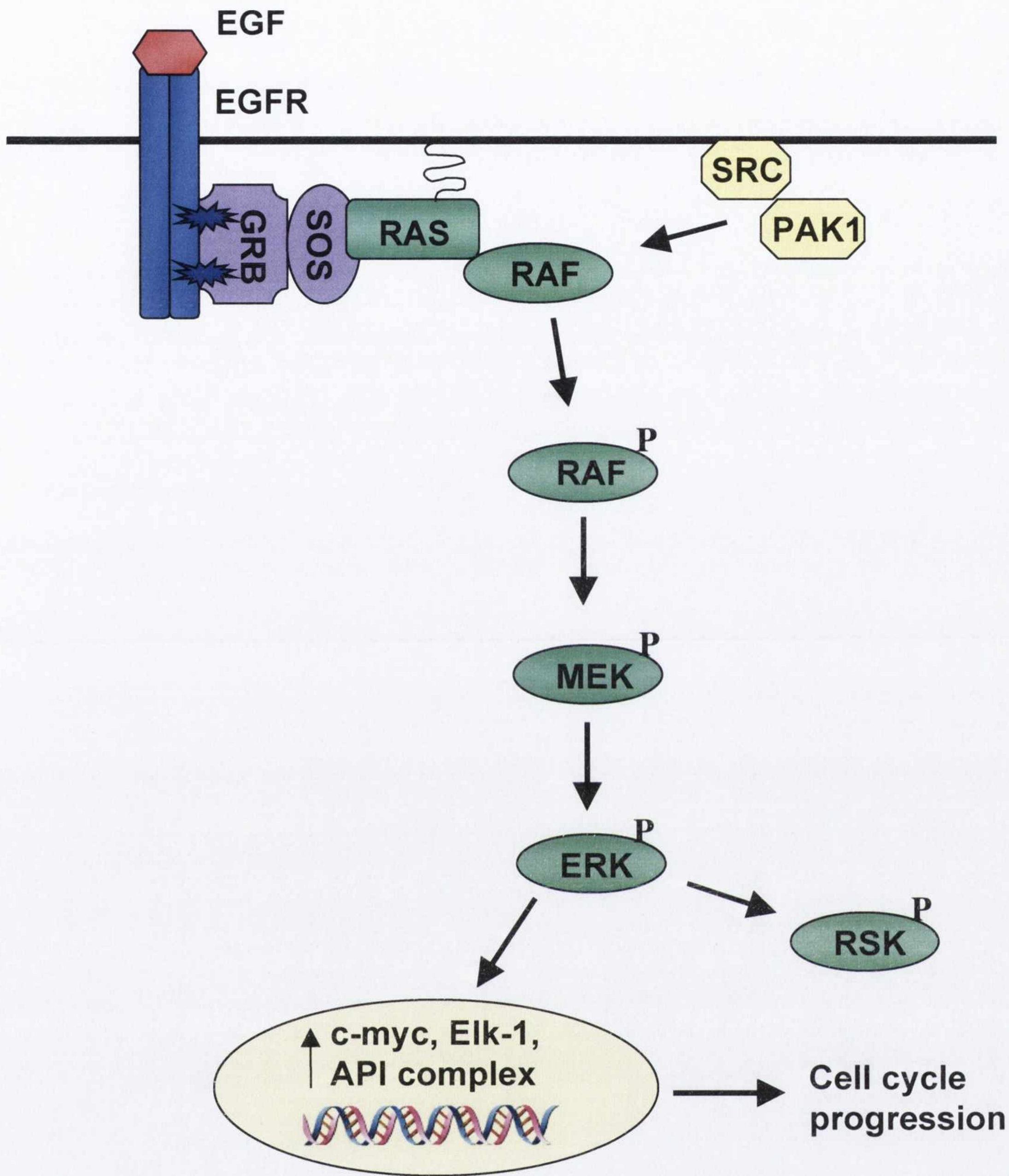


Figure 1.9

The MAPK Pathway

The MAPK pathway is activated in response to EGF binding to the EGF Receptor. This results in autophosphorylation of the receptor which facilitates GRB2 binding, followed by SOS binding. SOS then activates RAS, which is prenylated and attached to the plasma membrane. RAS recruits RAF to the plasma membrane where it is activated following phosphorylation by other kinases. RAF then activates MEK, which activates ERK by phosphorylation. ERK phosphorylates a number of substrates including Elk-1 and c-myc resulting in upregulation of genes necessary for cell cycle progression.

implicating these kinases in apoptosis prevention (Wojnowski *et al.*, 1997). The MAPK pathway is most commonly associated with survival signalling in the presence of growth factors and apoptosis-induced by cytokine withdrawl occurs following MAPK inactivation (Xia *et al.*, 1995; O' Reilly *et al.*, 2009). MAPKs have also been linked to inhibition of Fas and radiation-induced apoptosis, suggesting that these kinases transmit an anti-apoptotic signal in healthy cells (Holmstrom *et al.*, 2000; Shonai *et al.*, 2002).

1.8 THESIS AIMS

Bcl-2 family members are crucial regulators of apoptosis whose actions truly affect the fate of a cell. As these proteins control the release of cytochrome *c* from mitochondria, an event that marks the demise of a cell, modulation of their function may have defining consequences on cellular outcome. Cancer development and apoptosis are inextricably linked as evasion of apoptosis is essential for tumor formation. Conversely, induction of apoptosis plays a prominent role in cancer eradication. Bcl-2 family members are key targets of both cancer-promoting oncogenes and chemotherapeutic agents. The aims of this thesis were to explore the regulation of Bcl-2 family members by both pro- and anti-apoptotic factors, and to gain a more comprehensive understanding of the cellular events governing Bax/Bak-induced cytochrome *c* release.

Malignant melanomas are acutely resistant to chemotherapy-induced apoptosis, such that the survival rate of patients with the malignant form of this disease has not improved in over forty years. The mechanism of melanoma resistance to chemotherapy remains elusive, however, the identification of B-Raf^{V600E} as an oncogene in melanomas has provided a possible rationale. Since its discovery, the role of this oncogene in melanoma tumor progression and proliferation has been established (Wellbrock *et al.*, 2004a). However, the role of the B-Raf kinase in tumor survival and resistance to apoptosis has yet to be determined. In chapter III, we wished to establish whether B-Raf^{V600E} could protect cells against chemotherapeutic drug-induced cell death and unravel the molecular modifications responsible for this. As the Bcl-2 family are critical for cell death induction, the search for apoptosis-related molecules modified by B-Raf^{V600E} expression concentrated on these molecules.

Chemotherapeutic drugs induce apoptosis in target cells through the activation or upregulation of BH3-only proteins. Various subsets of BH3-only proteins are activated depending on the initial cellular stress caused by chemotherapeutic agents, and they provide the link between the toxic insult and cell death induction.

While investigating the effects of widely used chemotherapeutic drugs on the expression of BH3-only proteins, we observed that cisplatin, a DNA damaging agent, promoted Noxa upregulation. As experiments described in chapter III demonstrated that ERK modulates Noxa expression, the goal of chapter IV was to investigate whether ERK was involved in cisplatin-mediated Noxa induction and whether these events were essential for cisplatin-induced apoptosis.

The role of Bax, Bak and mitochondria in the progression of apoptosis is unequivocal. However the composition of pores created by Bax and Bak and the ideal membrane environment for pore formation remain undefined. In particular, whether the morphology of the mitochondrial network impacts on pore formation has been debated. While mitochondrial fission has been observed during apoptosis, the importance of this event with regard to cytochrome *c* release is ambiguous. Both these events occur within a similar timeframe. However, whether fission creates the ideal membrane curvature for Bax/Bak oligomerisation and membrane permeabilisation, or indeed whether fission occurs as a consequence of cytochrome c release is unknown. The final chapter of this thesis examines whether modulation of mitochondrial fission and fusion dynamics impacts on cytochrome *c* release or apoptosis.

CHAPTER II

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 *General reagents*

UO126, PD98059 and ERK inhibitor peptide II were purchased from Cell Signalling. Actinomycin D, Daunorubicin, Cycloheximide, Cisplatin, Oxaliplatin and Hoechst were purchased from Sigma. LY294002, DCI, TPCK, MG132 and Carboplatin were purchased from Calbiochem. Genejuice and Oligofectamine were purchased from Novagen. Mitotracker.CMXRos was purchased from Molecular Probes and DNA-related reagents were purchased from NEB Biosciences. Cell culture materials were purchased from Gibco. Unless otherwise stated all other chemicals and reagents were purchased from sigma.

2.1.2 *Antibodies*

Antibodies were purchased from the following suppliers: α-Bcl-2 mAb, α-Mcl-1 mAb, α-Bcl-xL pAb, α-BID pAb, α-C-RAF mAb, α-Caspase-3 mAb, α-p53 mAb, α-Hif-1α mAb, α-ATF4 mAb, and α-Cytochrome *c* from BD Biosciences; α-ERK pAb, α-P-ERK pAb, α-BAD pAb, α-P-BAD mAb and α-cleaved Caspase-3 pAb from Cell Signalling; α-Bim pAb, α-Bcl-w pAb and α-NOXA mAb from calbiochem; α-Bik pAb, α-Bax pAb, α-B-RAF mAb and α-p73 pAb from Santa Cruz Biotechnology; α-His mAb, α-Myc mAb and α-HA mAb from Roche; α-Flag mAb from Sigma; α-Puma pAb from Prosci; α-Bak pAb from Upstate Biotechnology; α-Actin mAb from MP Biomedicals and α-Smac pAb was generated for our laboratory. Secondary antibodies for immunoblotting, α-mouse, α-rabbit and α-goat were purchased from Jackson ImmunoResearch Laboratories INC. Secondary antibodies for immunofluorescence, α-mouse Alexa Fluor488, α-mouse Alexa Fluor405, α-mouse Texas Red and α-rabbit Texas Red were obtained from Molecular Probes (UK).

2.1.3 *siRNA oligos*

All siRNA were purchased from Ambion Inc. Sequences of siRNA are as follows:

B-Raf sense 5'-GUGGCAUCGUGAUGUGGCA -3'

C-Raf sense 5'-UAGUUCAGCAGUUUGGCUA-3'

Bim sense: 5'-GCACCCAUGAGUUGUGACA -3'

Bad sense: 5'-GUACUUCCCUCAGGCCUAU-3'
Noxa#1 sense: 5'-GGUGCACGUUUCAUCAAUU -3'
Noxa#2 sense: 5'-CCGGACAUAACUGUGGUUC -3'
Noxa#3 sense: 5'-GCUGUGAUAACGUGAAACC -3'
Control sense: 5'-AAGAGAACAGCACCCGAGGAC-3'

2.2 DNA PURIFICATION

2.2.1 *Preparation of competent bacteria*

To prepare E. coli DH5α competent bacteria, a colony from a LB agar plate previously streaked with DH5α cells was used to inoculate a 3 ml starter culture and grown overnight at 37°C, 280 rpm. Up to 3 ml of starter culture was added to 50 ml of SOB (2% bacto tryptone, 0.5% bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ pH 7) to obtain an OD_{600} of ~0.1. The bacterial culture was grown to an OD_{600} of 0.4 at 25°C, 280 rpm. Bacterial cells were chilled on ice for 10 minutes, prior to centrifugation at 2,500 g for 10 minutes at 4°C. The bacterial pellet was resuspended gently in 20 ml of sterile Inoue Transformation Buffer (10 mM Pipes pH 6.7, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl) and incubated on ice for 10 minutes. The bacterial cells were repelleted at 2,500 g for 10 minutes at 4°C before resuspension in 4 ml Inoue Transformation Buffer. Finally, 280 μl of DMSO was added and the bacterial suspension was incubated on ice for 10 minutes before swirling the suspension to mix, and aliquoting into sterile vials. Competent bacteria were stored at –70°C.

2.2.2 *Transformation of competent bacteria*

Transformations were carried out using E. coli DH5α competent bacteria. Source plasmids for transformation were obtained from plasmid midi-preps or freshly ligated DNA plasmids during cloning. Using plasmid midi-preps, 1 μl of plasmid prep was added to 50 μl of DH5α cells and incubated on ice for 30 minutes to allow the plasmid to coat the cells. The bacterial cells were then heat shocked for 40 seconds at 42°C to facilitate entry of the plasmid into the cells, followed by incubation on ice for 2 minutes. Then 450 μl of LB was added to the bacterial cells

and they were incubated at 37°C, 220 rpm for 1 hour to allow expression of the antibiotic resistance gene. Following this step, 50 μl of bacterial culture was plated on LB agar plates containing ampicillin (100 μg/ml) and incubated at 37°C overnight. A negative control, using an equivalent volume of TE instead of plasmid was also set up to ensure that no contamination of bacteria with plasmid had occurred.

2.2.3 *Plasmid DNA preparation*

For mini plasmid preparations, 1 ml of a bacterial starter culture was centrifuged at top speed for 1 minute. The bacterial pellet was resuspended in 100 μl of P1 Buffer (50 mM Tris pH 8, 10 mM EDTA), followed by 200 μl of P2 Buffer (0.2 M NaOH, 1% SDS) to lyse the cells. The suspension was inverted gently to mix, and incubated on ice for 1 minute prior to addition of 200 μl of P3 Buffer (3 M KAc pH 5.5) to precipitate genomic DNA and other cellular material. This solution was inverted to mix and incubated on ice for 3 minutes. The suspension was then centrifuged at top speed for 15 minutes to pellet out unwanted proteins and DNA. To precipitate the plasmid DNA, the supernatant was placed in a clean eppendorf, 350 μl of isopropanol was added and the solution was incubated at room temperature for 20 minutes. Plasmid DNA was pelleted at 15,000 g for 10 minutes, the supernatant was removed and 1 ml of 70 % ice cold ethanol was added to wash the pellet and remove residual isopropanol. Following centrifugation and removal of the supernatant, the pellet was air-dried for 10 minutes and then resuspended in 10 μl of dH_20 and subsequently 10 μl of TE. Plasmid preps were run on a 0.6 % agarose gel alongside a 1 kb DNA ladder to ensure that plasmids were intact and running at the expected size.

Plasmid midi preps were prepared using Qiagen Tip-100 columns as per manufacturers instructions. DNA was quantified on a spectrophotometer by obtaining absorbancy readings at 260 nm. DNA was sterilised using Spin-X tubes (Sigma) prior to use in tissue culture transfections.

2.3 GENE CLONING

2.3.1 *PCR amplification of DNA*

Primers were designed to amplify the complete coding region of BH3-only genes and Bcl-2 family genes. These primers incorporated restriction enzymes sites, typically BamH1 and Xho1, on either side of the gene to facilitate cloning into the pCDNA3 vector. In some cases the sequence encoding a Flag tag was also incorporated into the forward primer, to facilitate detection of proteins expressed by Western Immunoblotting and Immunostaining.

PCR reactions were typically carried out on a 100 μl scale. BH3-only genes were amplified from a Jurkat cDNA library (500 ng per reaction) while Bcl-2 related genes were amplified from plasmid templates (100 ng per reaction). Each reaction contained: template as described above, 10X PCR Buffer, 200 μM of each dNTP, 100 nM of forward and reverse primers and 2.5 Units (0.5 μl) of Taq DNA polymerase. The reaction was brought to a final volume of 100 μl using DNAse free dH_20. A control reaction containing all the components except DNA template was set up to ensure that no DNA contamination had occurred.

The PCR was carried out as follows:

Initial denaturation		94°C	3 min
30 cycles x	denaturation	94°C	1 min
	annealing	55-65°C	1 min
	extension	72°C	1 min
Final extension		72°C	10 min

PCR products were run on a 1.5 % agarose gel alongside a 100 bp DNA ladder, to facilitate analysis of PCR product size, presence of contaminating non-specific PCR products and estimation of DNA yields.

2.3.2 *Genecleaning of DNA from solution or agarose gel*

PCR products were genecleaned from solution by addition of 3 volumes of 6 M NaI, followed by addition of 20 μl of silica beads to the PCR solution. This suspension

was rotated for 15 minutes at room temperature to allow binding of the DNA to the silica. The DNA-bound silica was then pelleted at top speed for 10 seconds, the supernatant was removed and the pellet was resuspended in 500 μl of DNA Wash Buffer (10 mM Tris pH 7, 50 mM NaCl, 2.5 mM EDTA, 50 % EtoH) to remove any remaining contaminants. The silica was repelleted and the wash step was repeated twice more. Finally, the DNA pellet was air-dried for 10 minutes, followed by addition of 20 μl of DNAse free dH_20 to elute the DNA. A 1 μl sample of this was run on a 1.5 % agarose gel for estimation of DNA yields. For genecleaning from agarose gels, the band of interest was excised from the agarose gel under UV illumination and weighed. Three times the volume of the slice of 6 M NaI was added (300 μl for 100 μg agarose) and heated at 65°C to melt the agarose. 20 μl silica was then added per 2 μg DNA and the solution was treated as described above.

2.3.3 *Restriction enzyme digests*

Restriction enzyme digests were carried out on a 20 μl investigative scale or a 50 μl preparative scale. Following genecleaning of PCR products, the DNA was digested in a double digest reaction using the appropriate New England Biolabs (NEB) buffer as recommended by the manufacturers. These reactions typically contained 10X digestion buffer, 10X BSA, 2 μg of vector or 500 ng of PCR product, 1 μl of each digestive enzyme and DNAse free dH_20. Controls containing no enzymes or each enzyme alone with plasmid vectors were also set up. All digestions were incubated overnight at 37°C. A sample of each digestion was run on a 1.5 % agarose gel to ensure that the reaction was successful before proceeding to the next step in the cloning proceedure.

2.3.4 *Ligation of digested DNA*

Following DNA digestion, both the digested vector and insert were run on agarose gels and isolated bands were excised and genecleaned as described above. Genecleaned vector and insert were then ligated together using a DNA ligase enzyme. Ligations typically contained 200 ng vector and twice the molar amount of insert DNA (~ 40 ng of a 500 bp insert when cloning into 5.4 kb pCDNA3). To

separate re-annealed DNA, the DNA was first incubated at 42°C for 5 minutes, followed by addition of I μl of 10X ligase buffer and 0.5 μl (0.2 Units) of T_4 Ligase. The ligation reaction was carried out at 16°C overnight.

Competent bacteria were then transformed with ligated plasmid. Bacterial cells were treated as described in section 2.2.2, except 3 μl of each ligation was added to 200 μl of DH5α cells and 800 μl of LB was added following heat shock. When plating the transformation solution, 250 μl of bacterial cells were plated on LB agar plates containing ampicillin at 100 μg/ml. To screen for positive DNA clones, at least 20 colonies were used to inoculate 3 ml mini cultures. Mini plasmid preparations were prepared and analysed on a 0.6 % agarose gel, to identify plasmids that were larger than the empty vector. These plasmid clones were then digested with restriction enzymes and run on a 1.5 % agarose gel to confirm the presence of an insert of the correct size, followed by sequencing to ensure that the gene was intact and contained no mutations.

2.4 PROTEIN ANALYSIS

2.4.1 *SDS-PAGE gels*

For protein analysis, cell lysates were prepared using SDS-PAGE Buffer (2 % SDS, 50 mM Tris pH 6.8, 10 % Glycerol, 2.5 % β-mercaptoethanol, 0.1 % Bromophenol Blue) or IP Lysis Buffer (150 mM NaCl, 50 mM Tris pH 8, 1 % NP-40, I mM PMSF, 10 μg/ml leupeptin, 1 μg/ml aprotinin). Cells were incubated in lysis buffer for 10 minutes and 5X SDS-PAGE Buffer was added those incubated with IP lysis buffer prior to SDS-PAGE gel electrophoresis. Samples were boiled at 90°C for 7 minutes and then electrophoresed on sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE). Gels were prepared as described by Sambrook and Maniatis (Sambrook *et al.*, 1989). Typically, gels containing 12 % acrylamide were used and samples were run at 55 V through the stacking gel and 75 V through the separation gel in SDS-PAGE Running Buffer (25 mM Tris pH 8.3, 250 mM Glycine, 0.1 % SDS).

2.4.2 *Western Immunoblotting*

Protein samples were electrophoresed on SDS-PAGE gels, and then transferred onto nitrocellulose blots at 35 mA overnight in Transfer Buffer (39 mM Glycine, 48 mM Tris pH 8.3, 0.037 % SDS, 20 % Methanol). Blots were incubated in blocking solution (TBST + 5 % dried milk) for 1 hour to reduce non-specific binding of antibodies. Blots were then incubated in 5 ml of blocking solution containing primary antibody (~1:1000) for 2 hours, or alternatively overnight. To remove unbound primary antibody, blots were washed 3 times in TBST (10 mM Tris pH 8, 150 mM NaCl, 0.05 % Tween) on a shaker for 10 minutes each time. Blots were then incubated with anti-mouse or anti-rabbit secondary antibodies diluted 1:1000 for 1 hour. Blots were washed again in TBST and processed using chemiluminescence solutions, SuperSignal West Pico Peroxide and Luminol/Enhancer solutions (Pierce). Protein expression was analysed by exposing blots to autoradiography film.

2.5 MAMMALIAN CELL CULTURE

2.5.1 *Routine cell culture*

HeLa, HeLa-cytochrome *c*-GFP and HL-60 cells were cultured in RPMI supplemented with 5 % Fetal Calf Serum (FCS) and L-glutamine (2 mM). HEK 293T cells were grown in DMEM containing 10% FCS/ 2 mM L-glutamine. Melanoma cell lines SK-MEL-1, SK-MEL-3 and SK-MEL-30 were purchased from DSMZ and SK-MEL-2, SK-MEL-28 AND SK-MEL-31 were purchased from LGC promochem. All melanoma cell lines were cultured in RPMI supplemented with 10% FCS/ 2 mM L-glutamine, except SK-Mel-31, which was cultured in DMEM supplemented with 10% FCS/ 2 mM L-glutamine. Cells were incubated at 37°C, 5 % CO_2 and were passaged every 2-3 days. Suspension cells were split 1:5 in fresh medium to facilitate continued growth. Adherent cells were passaged through a process of trypsinisation and replating. This involved removal of old medium from adherent cells, incubation of cells in 0.05 % Trypsin-EDTA for 10 minutes, followed by addition of fresh medium to neutralise the trypsin and replating 1:10 in fresh medium onto new plates.

2.5.2 ***Transfection of mammalian cells-Genejuice method***

HeLa cells were plated at 1 x 10^5 cells/well on 6 well plates or 1 x 10^6 cells/10 cm plate and incubated for 24 hours to allow cells to adhere to the plate. For transfection, 100 μl of RPMI containing 2 μl of genejuice was added dropwise to DNA mixes. Genejuice/DNA complexes were incubated for 20 minutes at room temperature and then added dropwise onto cells. Genes of interest were co-transfected with 50-150 ng of pAAV-GFP, pEFmitoGFP or pDsRedmito reporter plasmids to identify transfected cells. Cells were incubated for 24 hours to facilitate protein expression and then harvested for protein analysis or treated pro-apoptotic drugs for cell death assays.

2.5.3 ***Transfection of mammalian cells-Calcium Phosphate method***

HEK 293T cells were plated at 2 x 10^6 cells/10 cm plate, 24 hours prior to transfection. Typically, 5 μg of plasmid and 500 ng pAAV-GFP reporter plasmid were used per reaction, brought to a final volume of 200 μl with dH_20. 50 μl of $CaCl_2$ (2.5 mM) was added to the DNA and this mixture was slowly added dropwise to 250 μl of 2X HBS buffer (280 mM NaCl, 10 mM KCL, 1.5 mM Na_2HPO_4, 12 mM Dextrose, 50 mM Hepes pH 7.1). Complexes were allowed to form for 30 minutes and then added dropwise onto cells. Genes were expressed for 24 hours and cells were harvested for analysis of protein expression via Western Immunoblotting.

2.5.4 ***Transfection of mammalian cells-Oligofectamine method***

Oligofectamine was used to transfect siRNA into adherent cells. HeLa cells were plated at 1 x 10^5 cells/well on 6 well plates and incubated for 24 hours. For transfection, 90 μl of RPM1 was added to siRNA (typically 200 nM of siRNA was used), followed by 20 μl of RPMI containing 4 μl of oligofectamine. This mixture was incubated for 20 minutes at room temperature to allow complexes to form and then added dropwise onto cells. Prior to transfection, culture medium was removed and cells were washed with RPMI to remove traces of FCS. RPMI without FCS was then added to cells for the transfection period. 6 hours later, complete medium was

added to cells and they were incubated for 48-72 hours to facilitate ablation of target genes. Cells were then harvested and protein expression was analysed or alternatively cells were treated with pro-apoptotic drugs for cell death assays.

2.5.4 *Transfection of mammalian cells-Amaxa method*

The Amaxa transfection system was used to transfect siRNA into suspension cells. For each transfection, 1×10^6 SK-MEL-1 cells were centrifuged and resuspended in 100 μl of transfection solution (5 mM KCl, 15 mM $MgCl_2$, 20 mM Hepes, 150 mM Na_2HPO_4 pH 7.2). This suspension was added to siRNA (2 μM) in a cuvette and electroporated using Amaxa program T-020. Cells were diluted in 10 ml of medium, plated at 2×10^6 cells/well and incubated for 24-96 hours.

2.6 CELL CULTURE-RELATED ASSAYS

2.6.1 *Melanoma Growth Assay*

Melanoma cells were plated at 1×10^5 cells/10 cm plate. 24 hours later, cells were treated with UO126 (20 μM), LY294002 (20 μM) or left untreated. Medium containing fresh inhibitors was replenished every 48 hours. Cell growth was monitored over 7 days by photographic analysis.

2.6.2 *Cell survival Assay*

HeLa cells were plated at 2×10^4 cells/well in 6 well plates. 24 hours later, cells were treated with UO126 (20 μM) for 1 h, followed by cisplatin (25 μM) treatment for 3 hours. Medium containing cisplatin and UO126 was removed, replaced with fresh medium and cells were incubated for a further 7 days. Cells were then washed with PBS and incubated in 0.5 % crystal violet viability stain for 1 hour. Cells were washed again and images of plates were taken. Alternatively, 5×10^4 cells/well were transfected with siRNA for 48 hours, and then treated with cisplatin for 3 hours. Following incubation at 37°C for a further 7 days, HeLa cells were stained as described above.

2.6.3 *Apoptosis Assays*

HeLa cells were plated at 2 x 10^5 cells/well in 6-well plates. After 24 hours, cells were treated with a range of pro-apoptotic drugs for a further 12-24 hours. Cell death was assessed based on the morphology of cells, including cytoplasmic condensation, membrane blebbing and detachment from the plate. Cell lysates were then prepared to assess protein expression in dying cells. In some experiments, HeLa cells were plated at 1 x 10^5 cells/well in 6-well plates. Cells were then transfected with various plasmid combinations along with a pAAV-GFP reporter plasmid using genejuice. This allowed us to investigate the effect of overexpression of certain proteins on apoptosis induced by pro-apoptotic drugs. After 24 hours, cells were treated with cytotoxic agents for 12-24 hours and GFP-expressing cells were scored for apoptotic morphology. Apoptosis was also induced by transfection of HeLa cells with pro-apoptotic Bcl-2 family members. Cell death in the absence or presence of other proteins was enumerated in GFP-positive cells 24 hours later. A minimum of 300 cells were counted per treatment for all apoptosis assays. Each assay was performed a minimum of three times.

2.6.4 *Flow cytometric analysis of apoptotic cells*

To detect and quantify apoptosis via flow cytometry, HL-60 cells were plated at 1 x 10^6 cells/well in 6-well plates and treated with pro-apoptotic drugs 24 hours later. After 8 h, cells were centrifuged at 250 g and washed in PBS (NaCl 8 g/L, KCl 0.2 g/L, Na_2HPO_4 1.44 g/L, KH_2PO_4 0.24 g/L pH 7.2). Cells were fixed in 70 % ethanol for 1 hour, centrifuged at 250 g for 5 minutes and resuspended in PBS containing RNase A for 30 minutes. Cells were then centrifuged at 250 g for 5 minutes and resuspended in PBS containing Propidium Iodide (20 μg/ml), which enters permeabilised cells and binds to DNA, for 20 minutes. Cell fluorescence was then measured on a flow cytometer (FACSCalibur; Becton Dickinson, CA) using CellQuest software.

2.7 MICROSCOPIC ANALYSIS OF CELLS

2.7.1 *Assessment of mitochondrial morphology in HeLa cells*

In order to examine the mitochondrial phenotype of HeLa cells under various conditions, cells were plated at 2 x 10^5 cells/well on coverslips in 6-well plates. Cells were transfected with plasmid combinations, together with a mitochondrially targeted-GFP or -RFP reporter plasmid. Where Bax or Bak were transfected, cells were treated with zVAD-fmk (50 µM, BACHEM) to avoid loss of cells due to apoptosis. After 24 hours, medium was removed and cells were washed in 3 x 2 mls PBS. Cells were then fixed in PBS containing 3.7 % Paraformaldehyde for 30 minutes. Cells were washed in 3 x 2 mls PBS, on a shaker for 10 minutes each time. This was followed by nuclear staining using Hoechst (20 µM) for 10 minutes and a final wash in PBS. Coverslips were mounted onto slides with 5 µl of Slow Fade (Molecular probes), and cells were analysed using confocal microscopy.

2.7.2 *Immunostaining*

For immunostaining, HeLa cells plated at 2 x 10^5 cells/well were grown on coverslips for 24 hours, followed by transfection with various plasmid combinations in the presence of Z-VAD.fmk where necessary. After 24 hours, cells were washed in 3 x 2 mls PBS and fixed with PBS containing 3.7 % Paraformaldehyde for 30 min. Wash steps in PBS (3 x 2 mls PBS shaking for 10 minutes) were followed by permeabilization with 0.15 % Triton-X100 for 15 minutes. Cells were incubated in PBS containing 2 % BSA to reduce non specific binding of antibodies for 30 minutes. Primary antibodies were used at 1:100 for 1 hour at room temperature, followed by washes in 3 x 2 mls PBS containing 2 % BSA. Secondary antibodies were used at 1:1000 for 45 min at room temperature. Final washings were carried out with PBS followed by incubation with Hoechst (20 µM) for 10 min. Coverslips were mounted onto slides with 5 µl of Slow Fade (Molecular probes), and cells were analysed using confocal microscopy.

2.7.3 *Light, UV and Confocal Microscopy*

To assess cellular growth and apoptosis under various conditions, images were taken using an inverted microscope (Olympus IX71) with a 20X objective lens. Images were acquired using Analysis image acquisition software and processed using Adobe Indesign. To enumerate apoptosis in GFP-positive cells, these cells were visualised on the inverted microscope under UV light. To examine the mitochondrial morphology of cells under various conditions, and also to assess cellular localisation of certain proteins, HeLa were grown on coverslips and mounted onto slides as described above. Cells were then observed on a laser scanning confocal microscope (Olympus FV1000) with a 60X objective lens, using a 488 nm Argon laser (green fluorescence), a 543 nm HeNe laser (red fluorescence) and a 405 nm LD laser (Hoechst). Confocal images were acquired with Fluoview 1000 V.1 application software and images were processed with Adobe Indesign.

2.7.4 *FRAP Analysis*

For FRAP analysis, HeLa cells were plated on glass-bottomed tissue culture dishes (MatTek Corporation). After 24h, cells were transfected with various plasmid combinations. Following 24 h of expression, cells were stained with Mitotracker CMXRos (50 nM) by incubation in tissue culture medium for 1 h at 37°C, followed by several changes of medium to remove excess Mitotracker. FRAP assessments were carried out at room temperature using Flouroview 1000 application software (PAPP protocol processor). Mitochondrial fluorescence in a specified area was bleached to approximately 50% of the initial signal using a brief pulse of high intensity laser illumination, followed by acquisition of images every 5 seconds for a duration of 140 seconds. Fluorescence recovery in the target area was graphed using KaleidaGraph software.

CHAPTER III

ONCOGENIC B-RAF^{V600E} INHIBITS APOPTOSIS AND TARGETS THE BH3-ONLY PROTEINS BIM AND BAD FOR INACTIVATION

3.1 INTRODUCTION

Malignant melanoma is a highly aggressive form of skin cancer. Although it is not the most commonly occurring form, it is responsible for the highest number of skin cancer related deaths (Rager *et al.*, 2005). Melanomas are initiated following exposure to ultraviolet radiation, which may lead to uncontrolled growth of melanocytes, the pigment producing cells of the skin (Miller and Mihm, 2006). This form of cancer is extremely refractory to chemotherapeutic treatment and frequently metastasises to other sites in the body. As a result, there is a poor survival rate for patients with the advanced form of this disease, and to date, the most effective treatment involves early detection and surgical removal of the tumor (Thompson *et al.*, 2005). The molecular mechanism of chemotherapy resistance in melanomas has not yet been clearly defined. Enhanced activity of AKT and decreased levels of the tumor suppressors $p16^{INK4A}$ and $p14^{ARF}$ in melanoma cells have been reported and may increase resistance to therapy (Stahl *et al.*, 2004; Fitzgerald *et al.*, 1996). More recently, hyperactivation of the MAPK pathway in melanomas, through activating mutations in the upstream kinase B-Raf, has been described (Davies *et al.*, 2002). This may be one of the mechanisms employed by malignant melanomas to evade chemotherapeutic treatment.

Oncogenic B-Raf, B-Raf^{V600E}, is important for melanoma growth and tumor maintenance (Dong *et al.*, 2003, Hoeflich *et al.*, 2006). While the primordial function of this kinase is to enhance proliferation, it promotes tumor development through a number of other mechanisms. B-Raf^{V600E} has been shown to inhibit the AMP-dependent protein kinase (AMPK) pathway, which regulates cell growth during conditions of energy stress (Zheng *et al.*, 2009). B-Raf^{V600E} also enhances angiogenesis and tumor metastasis (Sharma *et al.*, 2005; Sharma *et al.*, 2006a, Liang *et al.*, 2007). However, the role of oncogenic B-Raf in the notorious resistance of malignant melanoma to chemotherapeutic drug treatment has yet to be explored. Preliminary results have indicated that ablation of B-Raf^{V600E} in some melanoma cell lines leads to spontaneous cell death, which suggests that B-Raf^{V600E} may possess an anti-apoptotic function (Hingorani *et al.*, 2003; Karasarides

et al., 2004). However, an extensive investigation into the ability of B-Raf^{V600E} to inhibit apoptosis, or the molecular mechanism of this putative function has not been described.

BH3-only proteins of the Bcl-2 family are major sensors of cellular stress and injury, and are activated under a wide range of conditions such as nutrient deprivation, DNA damage, death receptor stimulation and ER stress (Brunelle and Letai, 2009). BH3-only proteins act by promoting mitochondrial cytochrome *c* release and apoptosis. To prevent this occurring in healthy cells, many molecules that promote proliferation simultaneously target BH3-only proteins for inactivation (Eberle and Hossini, 2008). For example, AKT blocks Bim transcription through phosphorylation and inhibition of the transcription factor FOXO3a (Dijkers *et al.*, 2000). Conversely, other pro-survival molecules upregulate anti-apoptotic Bcl-2 family members as observed with the upregulation of Bcl-xL by NfκB (Bui *et al.*, 2001). In cancerous tumors that display uncontrolled proliferation, many pro-survival pathways are constitutively activated. This can lead to suppression of BH3-only proteins under conditions where cell death is desirable and can also contribute to chemotherapeutic resistance.

In this study we set out to investigate whether hyperactivation of the MAPK pathway, due to B-Raf^{V600E} expression, protects melanoma cells from apoptosis and whether expression of this kinase leads to modification of any of the BH3-only proteins that are crucial for cell death induction by many chemotherapeutic drugs as well as other stresses.

3.2 RESULTS

3.2.1 *MAPK signalling is important for melanoma proliferation*

The role of the MAPK pathway in cell proliferation is well established and this pathway is activated in response to a number of growth factors including EGF, FGF and PDGF (Ramos, 2008). Most human melanomas display augmented MAPK activity due to activating mutations of Ras, or its downstream target B-Raf, which

implies that this signalling pathway is important for the maintenance of melanomas (Cohen *et al.*, 2002). We first wished to explore the role of MAPK signalling in melanoma cell proliferation in a panel of six melanoma cell lines containing B-Raf^{V600E} or Ras^{Q61R} mutations. Cells were treated with the MEK inhibitor U0126 to block MAPK signalling, or an inhibitor of the PI3K pathway LY294002 for comparison. Cell proliferation was monitored over a period of 7 days and medium containing inhibitors was replenished every 48 h to ensure continued inhibition of the signalling pathways. Strikingly, inhibition of the ERK/MAPK pathway resulted in a dramatic reduction of melanoma cell growth, demonstrating the important role of this pathway in promoting melanoma proliferation (Figure 3.1). UO126 treatment quickly led to growth arrest of melanoma cells that eventually succumbed to cell death. The PI3K inhibitor also reduced cell growth, albeit to a lesser extent than MEK inhibition (Figure 3.1). Overall, these results demonstrated that melanoma cells depend on MAPK signalling for long-term survival.

3.2.2 *B-Raf^{V600E} protects against chemotherapeutic drug-induced apoptosis*

Although B-Raf^{V600E} was found to be essential for melanoma proliferation (Wellbrock *et al.*, 2004a, Hoeflich *et al.*, 2006, Figure 3.1), we also wished to explore the possibility that this oncogene could suppress apoptosis. In order to investigate the potential role of B-Raf^{V600E} in cellular resistance to apoptosis, we transiently transfected HeLa cells with the wild type or constitutively active mutant form of B-Raf prior to treatment with chemotherapeutic drugs. Immunoblot analysis revealed that overexpression of both wild type and oncogenic B-Raf enhanced ERK phosphorylation, indicative of activation (Figure 3.2 A). However, ERK activation mediated by B-Raf^{V600E} was significantly greater. Strikingly, cells expressing B-Raf^{V600E} exhibited a marked decrease in apoptosis in response to a number of chemotherapeutic drugs (Figure 3.2 B). As the pro-apoptotic drugs utilised display different modes of action, including inhibition of transcription and inhibition of serine proteases, the anti-apoptotic actions of B-Raf^{V600E} appear to be universal rather than specific to a particular cellular stress. These results demonstrates that the B-

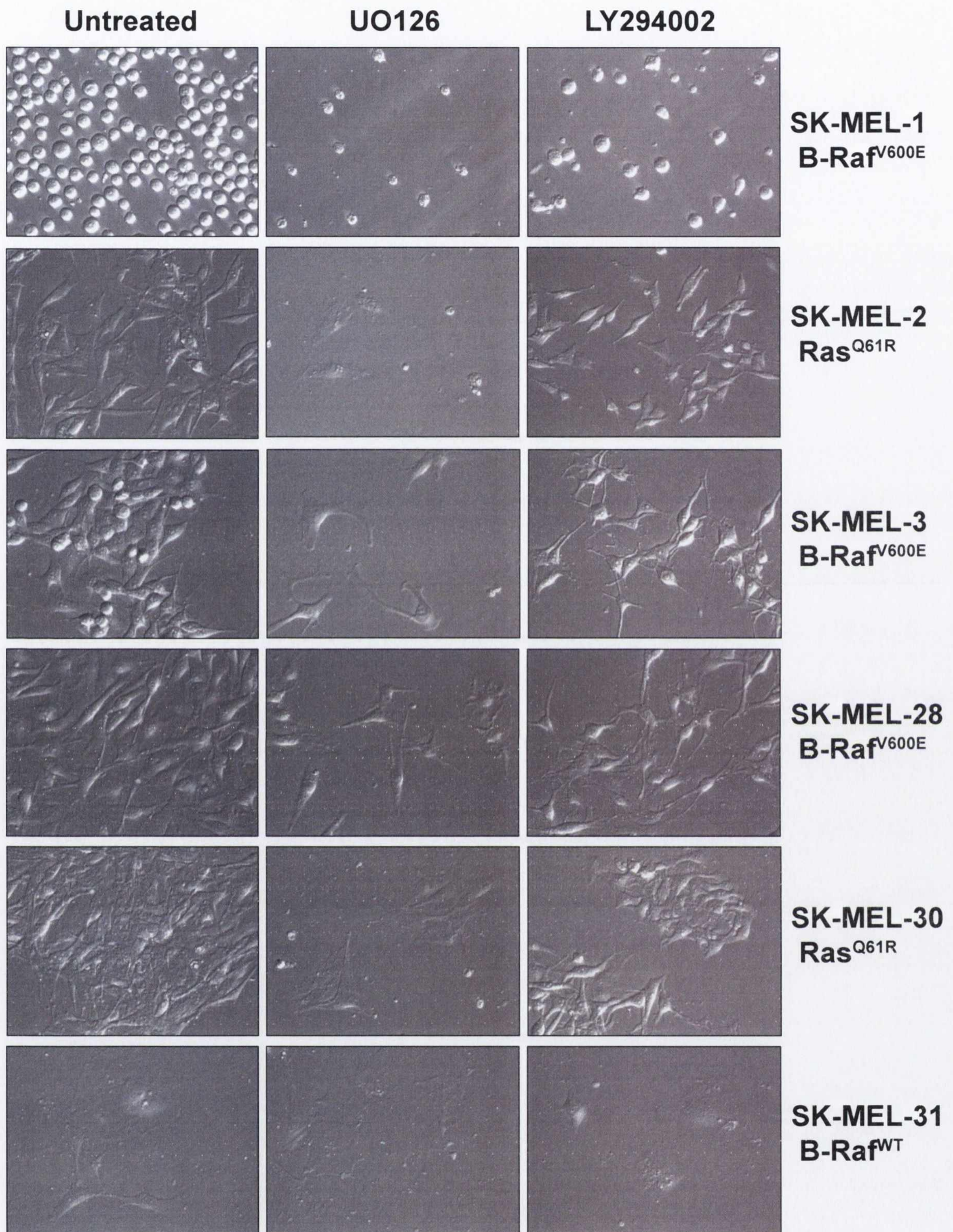
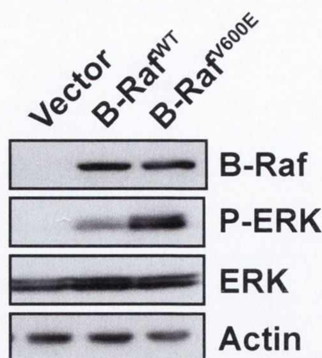


Figure 3.1

MAPK signalling is important for melanoma cell growth

Melanoma cells were plated at 10^5 cells/10 cm dish and treated with UO126 (20 μM) or LY294002 (20 μM) for 7 days. Medium was replenished every two days. Cell growth was assessed by visual analysis of pictures taken on a light microscope with 200X magnification.

A



B

Untreated

Actinomycin D

Daunorubicin

DCI

Apoptosis (%)

80 60 40 20 0

Vector B-Raf^{WT} B-Raf^{V600E} Bcl-xL

Figure 3.2

B-Raf^{V600E} protects against chemotherapeutic drug-induced apoptosis

HeLa cells were plated at 10^5 cells/well on 6-well plates. 24 h later, cells were transfected with 500 ng of the plasmids indicated, together with 50 ng of pAAV-GFP reporter plasmid. After 24 h transfection, cells were lysed in SDS PAGE buffer and protein expression was analysed by Western Immunoblot with the indicated antibodies (A). Transfected cells were treated with Actinomycin D (10 μM), Daunorubicin (10 μM) or DCI (50 μM) for a further 12 h and apoptosis was assessed based on the morphology of GFP-positive cells (B). Results represent triplicate counts from representative experiments with error bars representing standard error of the mean (SEM).

Raf oncogene is not only required for proliferation but also protects cells against apoptosis-inducing agents.

3.2.3 *B-RAF^{V600E} expression leads to modification of the BH3-only proteins Bim and Bad*

BH3-only proteins are important mediators of apoptosis occurring through the mitochondrial pathway. In response to cell stress, these proteins are activated either by transcriptional upregulation or by post-translational modification. Activated BH3-only proteins provoke cytochrome *c* release from mitochondria, which ensures the timely death of cells via the apoptosome pathway. Conversely, downregulation of BH3-only proteins by oncogenes can prevent the elimination of tumorigenic cells. Thus, in order to explore the molecular mechanism of B-Raf^{V600E}-mediated suppression of apoptosis, we asked whether expression of this kinase could modulate BH3-only proteins in any way. To address this question, Hela or HEK 293T cells were transfected with B-Raf^{WT} and B-Raf^{V600E}, cells were harvested and lysates were probed with a panel of antibodies against BH3-only proteins. Strikingly, phosphorylation (indicated by a mobility shift on SDS-PAGE gels) and destabilisation of the BH3-only protein Bim in the presence of oncogenic B-Raf was clearly evident, while phosphorylation of Bad on residue Serine75 was also dramatically increased (Figure 3.3 A, B). Examination of the expression of anti-apoptotic members of the Bcl-2 family revealed that Mcl-1 levels were increased in the presence of oncogenic but not wild type B-Raf. Phosphorylation of the pro-apoptotic proteins Bim and Bad, together with the upregulation of anti-apoptotic Mcl-1, strongly suggests this kinase may regulate the activity of several Bcl-2 family members to inhibit apoptosis. Surprisingly, B-Raf^{V600E} expression also resulted in Noxa upregulation. This may act as a defensive mechanism to detect deregulated MAPK activity and prevent oncogenic transformation of cells.

Titration of B-Raf^{WT} and B-Raf^{V600E} plasmids in HeLa cells revealed that while phosphorylation of Bim and Bad was evident at the highest concentrations of B-Raf^{WT} used, these modifications occurred at all concentrations of B-Raf^{V600E} (Figure

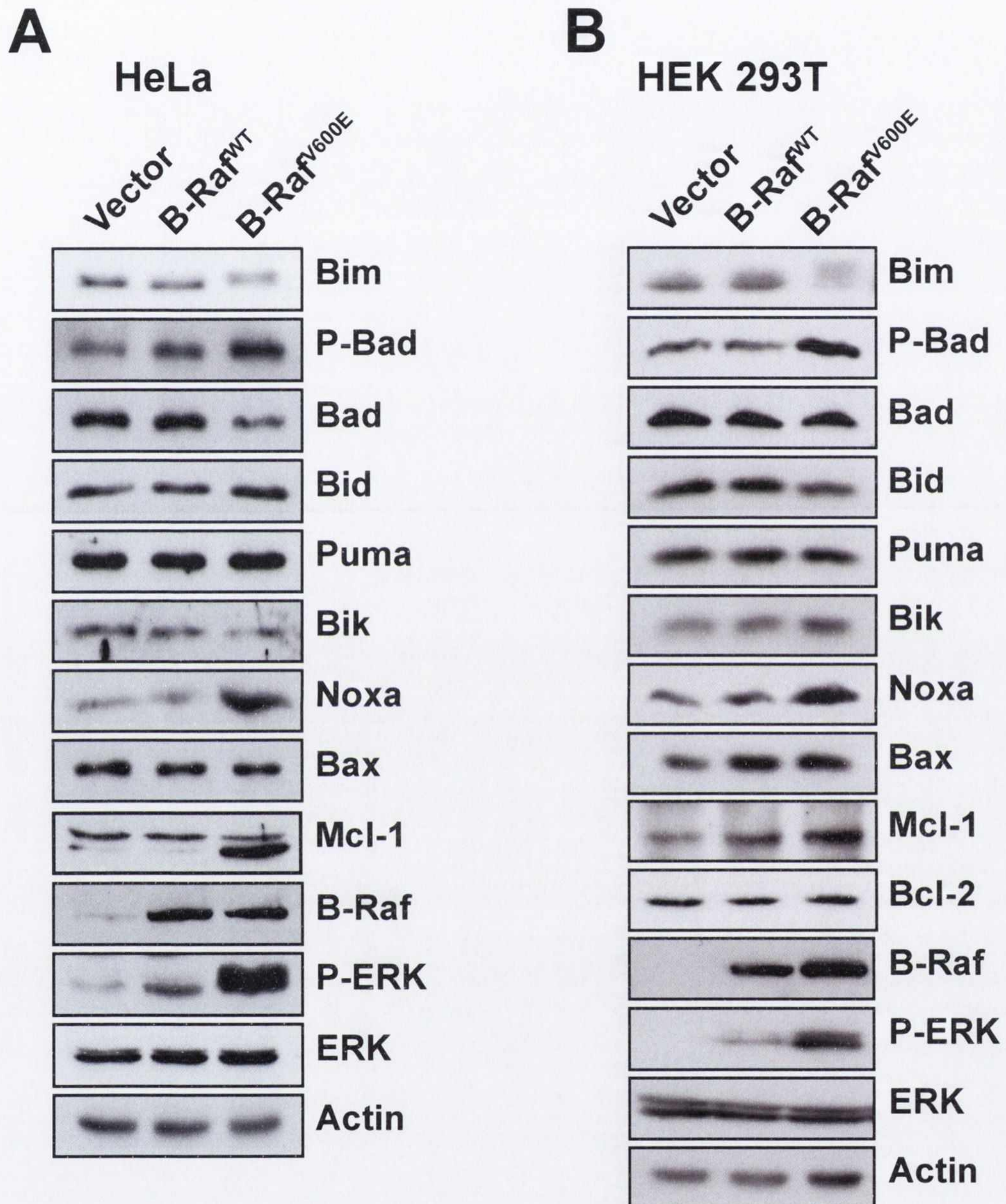
3.4). Of note, phosphorylation and activation of ERK peaked at the highest concentration of B-Raf^{WT} and titrated down at lower concentrations, but B-Raf^{V600E} induced robust phosphorylation of ERK at all concentrations, demonstrating that even low concentrations of B-RAF^{V600E} can hyperactivate ERK.

3.2.4 *Modification of Bim and Bad is ERK-dependent*

As phosphorylation of Bim and Bad correlated with the activation of ERK, we examined whether these modifications were ERK-dependent. HeLa cells were transiently transfected with both forms of B-Raf in the absence or presence of the MEK inhibitor UO126. Inhibition of MEK prevented ERK phosphorylation and activation in the presence of both B-Raf^{WT} or B-Raf^{V600E} and also prevented the phosphorylation of Bim and Bad (Figure 3.5). These results clearly demonstrate an ERK-dependent mechanism of Bim and Bad phosphorylation in response to B-Raf^{V600E} expression.

3.2.5 *Bim is phosphorylated on residues S55 and S65 in response to B-RAF^{V600E} expression*

Previous reports have shown that the serine/threoinine kinase ERK phosphorylates Bim on serine 55, serine 65 and serine 100 (Harada *et al.*, 2004). Using Bim mutants where these target residues have been converted from serine to alanine, we demonstrated that serine 55, serine 65 and to a lesser extent serine 100 are the residues phosphorylated in response to B-Raf^{V600E} expression (Figure 3.6 A and B). Furthermore, a triple mutant with all three serine residues mutated to alanine was not only resistant to phosphorylation in the presence of B-Raf^{V600E}, but was also significantly stabilised indicating that phosphorylation of Bim at these sites leads to its degradation (Figure 3.6 B). Indeed, incubation of B-Raf^{WT} and B-Raf^{V600E} transfected cells with the proteasome inhibitor MG132 blocked B-Raf^{V600E} –mediated degradation of Bim (Figure 3.6 C). Importantly, Bim accumulated in its phosphoylated form when MG132 was present suggesting that phosphoylation of Bim targets it for degradation.



<u>Figure 3.3</u>

B-RAF^{V600E} expression leads to modification of Bim and Bad

HeLa (A) or HEK 293T (B) cells were plated at 10^6 cells/10 cm dish and transfected with 5 μg of each plasmid 24 h later. Following 24 h of expression, cells were lysed in SDS-PAGE buffer and protein expression was analysed by Western Immunoblotting with the indicated antibodies.

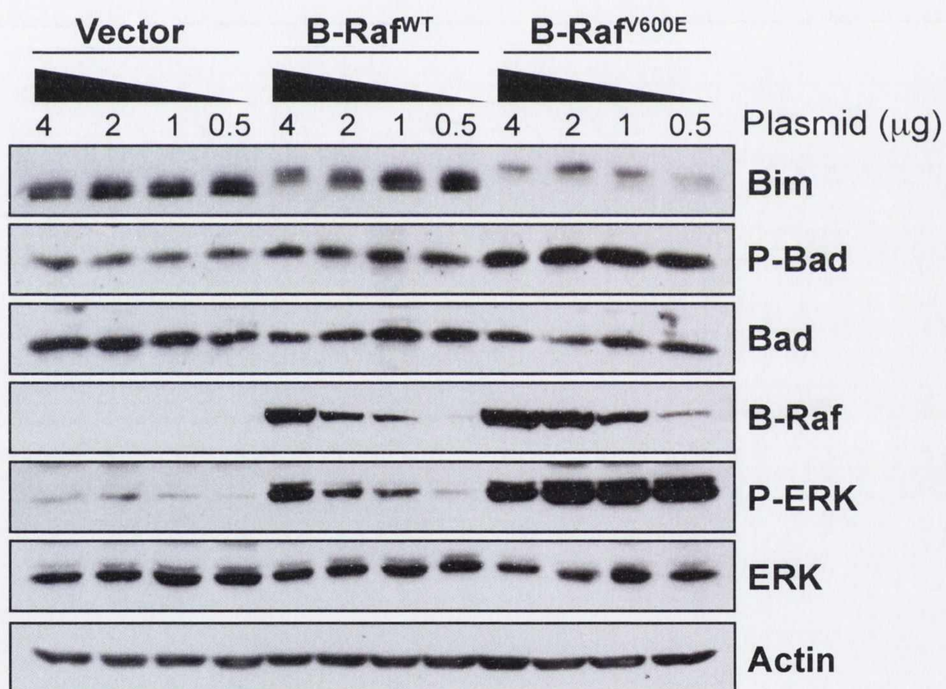


Figure 3.4

Titration of B-Raf^{WT} and B-Raf^{V600E} in HeLa cells

HeLa cells were plated at 5 x 10^5 cells/6 cm dish and transfected with indicated amounts of each plasmid 24 h later. Following 24 h of expression, cells were lysed in SDS-PAGE buffer and protein expression was analysed by Western Immunoblotting with the indicated antibodies.

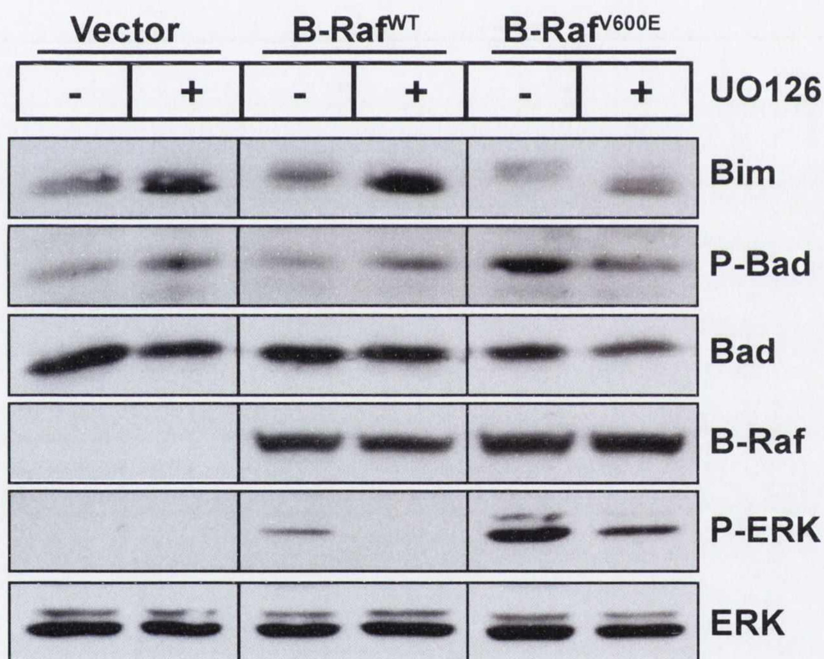
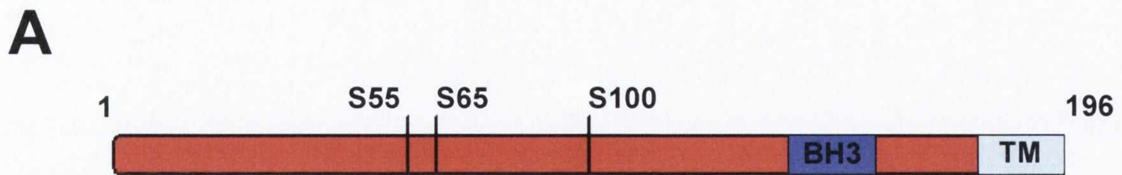
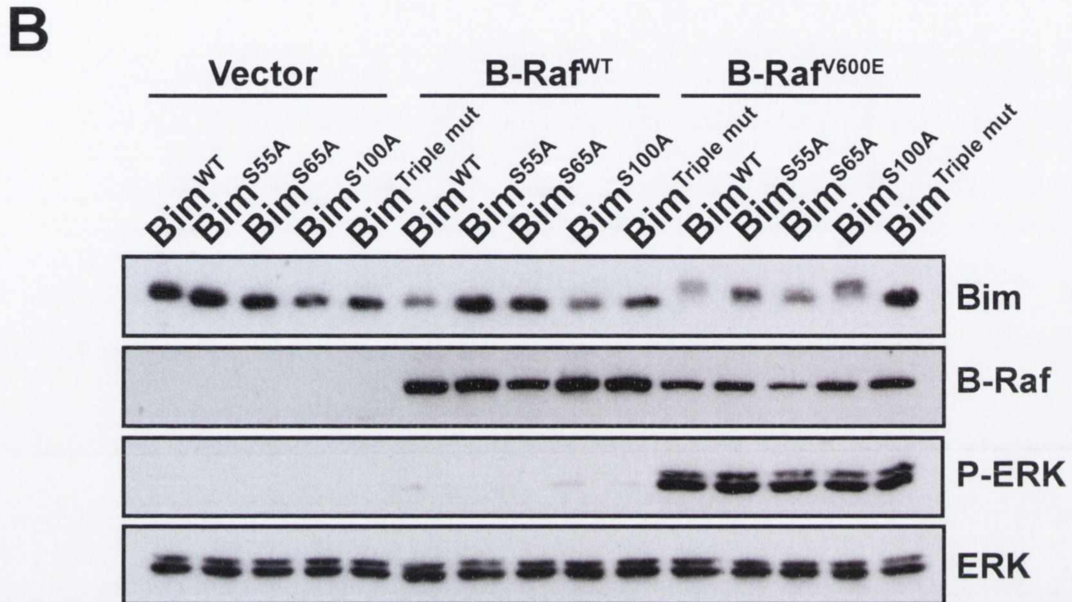


Figure 3.5

B-Raf^{V600E}-mediated modification of Bim and Bad is ERK-dependent

HeLa cells were plated at 5 x 10^5 cells/6 cm dish. 24 h later, cells were transfected with 1 μg of the plasmids indicated, followed by treatment with UO126 (20 μM) 1 h later. Following 24 h of expression, cells were lysed in SDS-PAGE buffer and protein expression was analysed by Western Immunoblotting with the indicated antibodies.





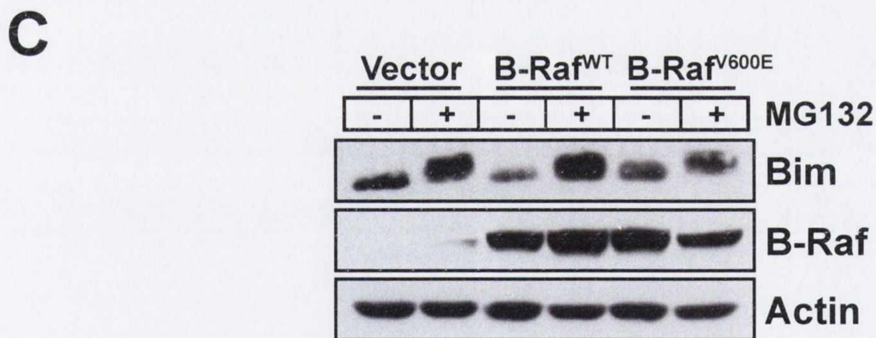


Figure 3.6

Bim is phosphorylated on residues serine 55 and serine 65

Schematic of Bim protein highlighting putative phosphorylation residues, BH3 domain and transmembrane domain (A). HeLa cells were plated at 2 x 10^5 cells/well on 6-well plates and transfected with 1 μg of B-RAF plasmids together with 200 ng of the Bim plasmids indicated. Following 24 h of expression, cells were lysed in SDS-PAGE buffer and protein expression was analysed by Western Immunoblotting with the indicated antibodies (B). HEK 293T cells were transfected with 750 ng of pcDNA3 vector, or B-Raf plasmids for 12 h, followed by treatment with the proteasome inhibitor MG132 (20 μM) for a further 10 h. Lysates were prepared and probed for the indicated proteins (C).

3.2.6 *B-Raf^{V600E} reduces Bim- and Bad-induced apoptosis*

To investigate whether B-Raf^{V600E}–mediated phosphorylation of Bim and Bad modulated their pro-apoptotic potential, HeLa cells were co-transfected with plasmids encoding these BH3-only proteins in conjunction with B-Raf^{WT} or B-Raf^{V600E} plasmids. While Bim and Bad overexpression efficiently induced apoptosis, coexpression of B-Raf^{V600E} suppressed cell death induced by both of these proteins (Figure 3.7 A and B). In contrast, apoptosis mediated by Bid and Noxa overexpression was unaffected by B-Raf^{V600E} (Figure 3.7 C and D). These results demonstrate that B-Raf-mediated phosphorylation of Bim and Bad can interfere with their ability to induce apoptosis.

3.2.7 *Bim and Bad are constitutively phosphorylated in melanoma cells*

As phosphorylation of Bim and Bad can be enhanced by ERK/MAPK we next wished to explore whether melanoma cell lines displaying elevated ERK activity also contained constitutively phosphorylated Bim and Bad. A panel of six melanoma cell lines harbouring B-Raf^{V600E} or Ras^{Q61R} mutations were treated with the MEK inhibitor UO126, or the PI3K inhibitor LY294002 for comparison, and the expression of BH3-only proteins in cell lysates was analysed. Remarkably, treatment with the MEK inhibitor led to dephosphorylation of Bim and a dramatic increase in Bim protein levels in each of the six cell lines examined (Figure 3.8). In addition, using a phosphospecific antibody, dephosphorylation of Bad was also evident in the presence of UO126 in all melanoma cell lines (Figure 3.8). These results suggest that melanoma cells may elicit protection from cell death by raising their apoptotic threshold through constitutive ERK-dependent phosphorylation and inactivation of the BH3-only proteins Bim and Bad. In contrast, other BH3-only proteins such as Bid and Puma were not modified in the presence of ERK pathway inhibitors, while expression the BH3-only proteins Bmf and Hrk could not be detected in these cells. Inhibition of PI3K showed no effect on Bim and Bad, strongly suggesting that inactivation of these proteins occurs specifically through the ERK/MAPK pathway.

Examination of the expression of other Bcl-2 family members revealed few alterations in response to MEK inhibition (Figure 3.9). Interestingly, although Mcl-1 protein levels were increased in response to B-Raf^{V600E} expression in HeLa cells, MEK inhibition did not alter Mcl-1 levels in many of melanoma cell lines examined. This suggests that these cells also use other mechanisms to upregulate Mcl-1.

3.2.8 *Ablation of B-RAF expression in melanoma cells leads to cell death induction*

To more specifically target the B-Raf pathway in melanoma cells, we used siRNA to reduce B-Raf levels in SK-MEL-1 cells harbouring the B-Raf^{V600E} mutation. Ablation of B-Raf^{V600E} expression resulted in spontaneous apoptosis 48-96 hours after treatment with siRNA (Figure 3.10 A and B). Conversely, reduction of the sister kinase C-Raf had no effect on cell death (Figure 3.10 A). Furthermore, cells treated with B-Raf siRNA also exhibited reduced proliferation rates (Figure 3.10 B). These results suggest that SK-MEL-1 cells have developed a dependency on the oncogenic form of B-Raf for survival as well as proliferation.

Examination of lysates from cells treated with B-Raf siRNA revealed that ablation of B-Raf^{V600E}, but not C-Raf, resulted in reduced ERK phosphorylation, indicating that B-Raf^{V600E} is the primary kinase responsible for ERK activation in these cells (Figure 3.11 A). Conversely, ablation of C-Raf and not B-Raf in SK-MEL-2 cells containing a Ras^{Q61R} mutation blocked ERK activation (Figure 3.11 B). This demonstrates that both kinases are important activators of ERK and their role in particular melanoma cells depends on the driving oncogenic signal. In addition, ablation of B-Raf led to dephosphorylation of Bim and Bad, consistant with the previous results observed with the ERK pathway inhibitor UO126 (Figure 3.11 A).

3.2.9 *Ablation of B-Raf expression in melanoma cells increases sensitivity to chemotherapeutic drug-induced apoptosis*

To further investigate B-Raf mediated survival in melanoma cells, we treated SK-MEL-1 cells with siRNA for 72 h, followed by exposure to chemotherapeutic drugs.

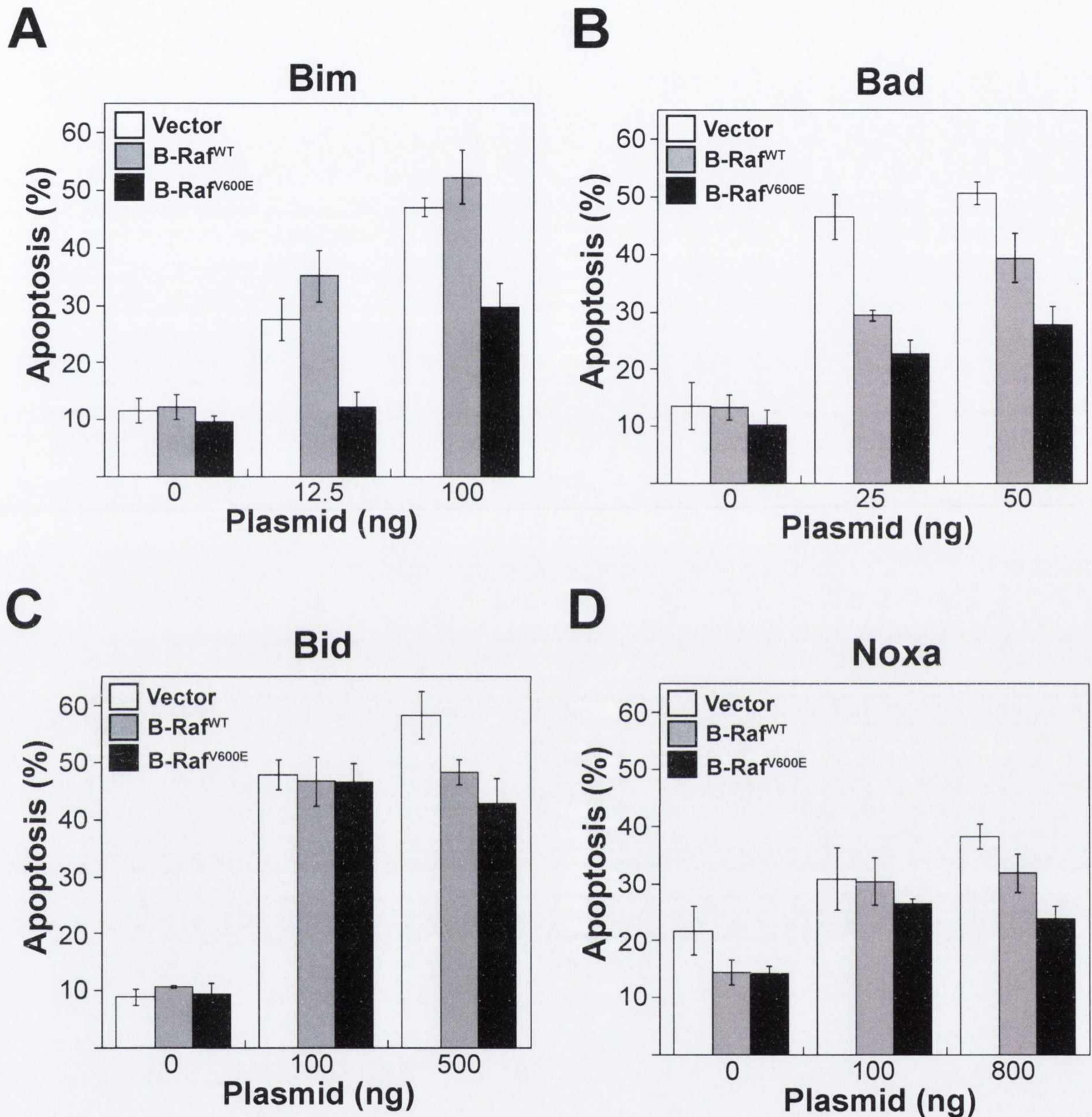


Figure 3.7

B-Raf^{V600E} protects against Bim- and Bad-induced apoptosis

HeLa cells were plated at 10^5 cells/well on 6-well plates. 24 h later, they were transfected with 500 ng of B-Raf plasmids together indicated amounts of BH3-only plasmids and 50 ng of pAAV-GFP reporter plasmid. The effect of B-Raf^{V600E} on Bim (A), Bad (B), Bid (C) and Noxa (D) induced apoptosis was assessed based on the morphology of GFP-positive cells. Results represent triplicate counts from representative experiments with error bars representing SEM.

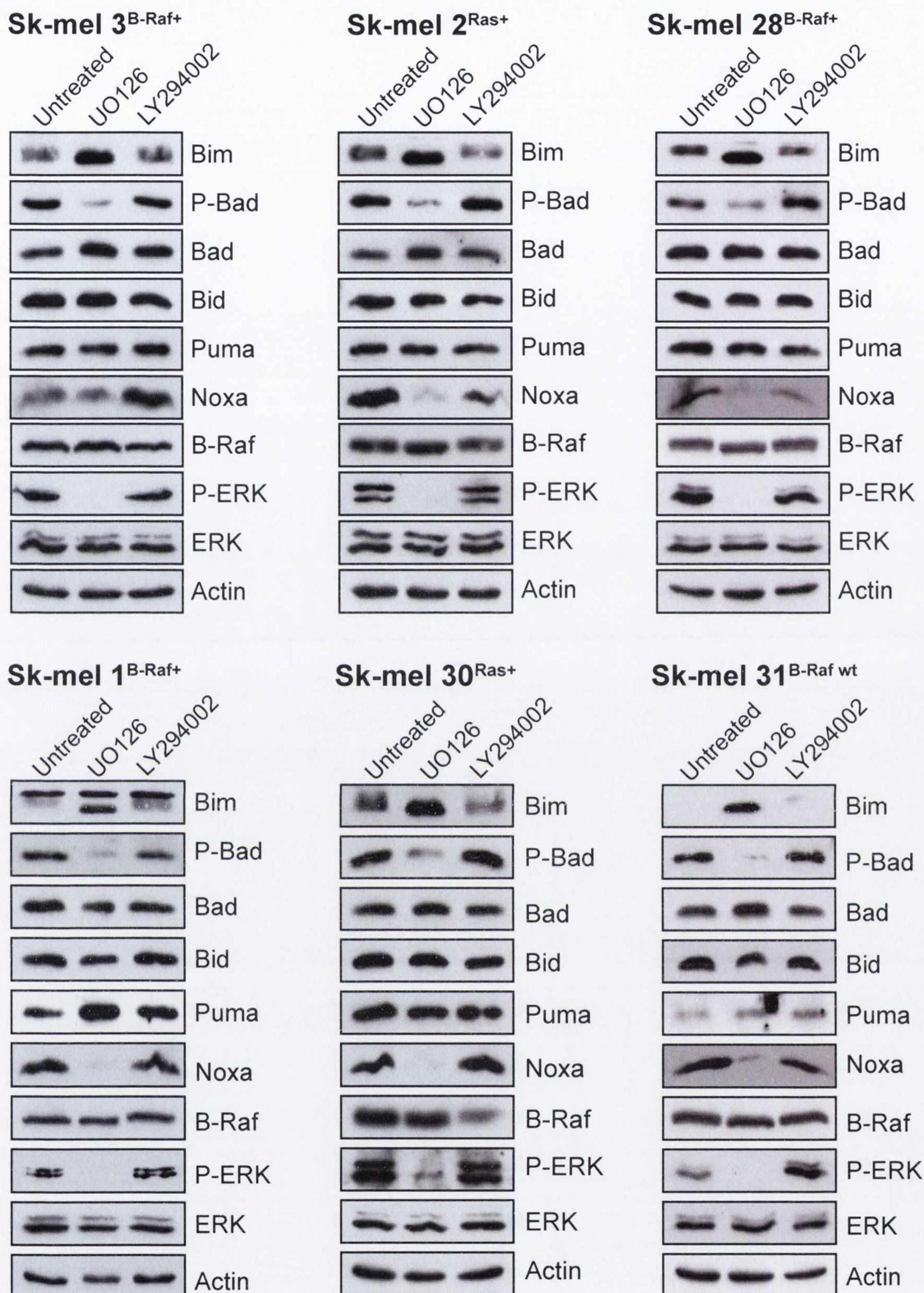


Figure 3.8

Bim and Bad are constitutively phosphorylated in melanoma cells

Melanoma cells were plated at 10^6 cells/10 cm dish. 24 h later, cells were treated with the MEK inhibitor, UO126 (20 μM) or the PI3K inhibitor, LY294002 (20 μM). Following 12 h of treatment, cells were lysed in SDS-PAGE buffer and protein expression was analysed by Western Immunoblotting with the indicated antibodies.

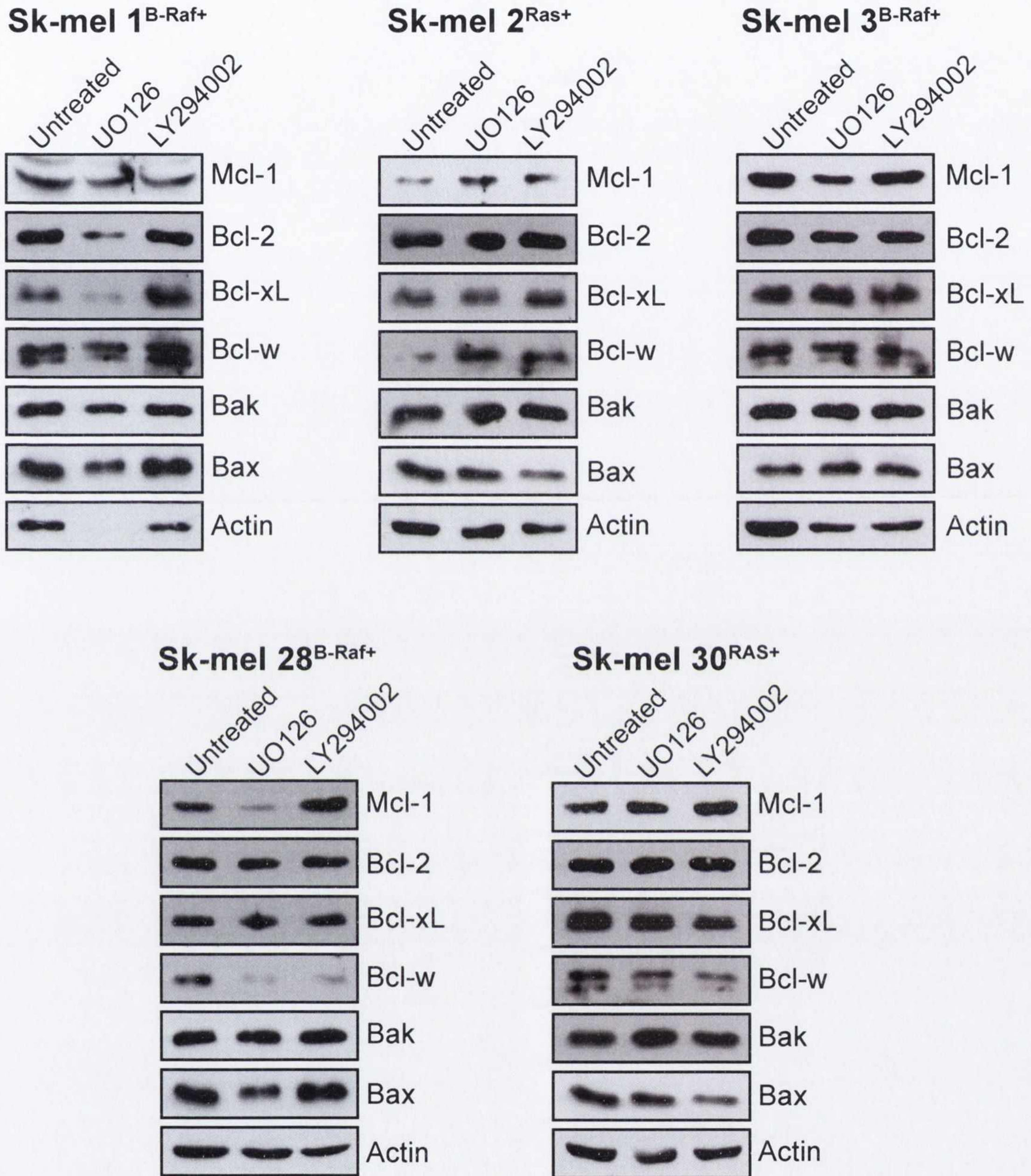
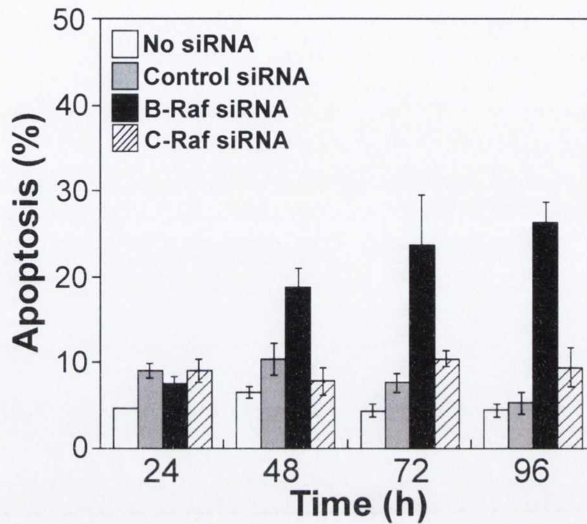


Figure 3.9

Modification of Bcl-2 family members by the MEK inhibitor UO126

Melanoma cells were plated at 10^6 cells/10 cm dish and 24 h later, cells were treated with the MEK inhibitor, UO126 (20 μM) or the PI3K inhibitor, LY294002 (20 μM). Following 12 h of treatment, cells were lysed in SDS-PAGE buffer and protein expression was analysed by Western Immunoblotting with the indicated antibodies.

A



B

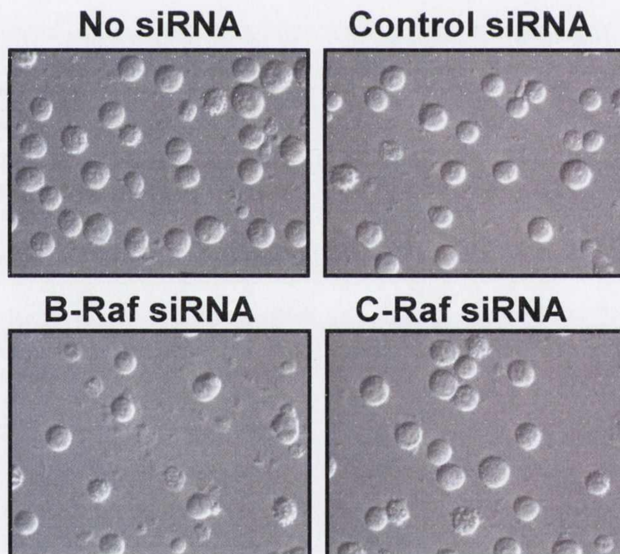
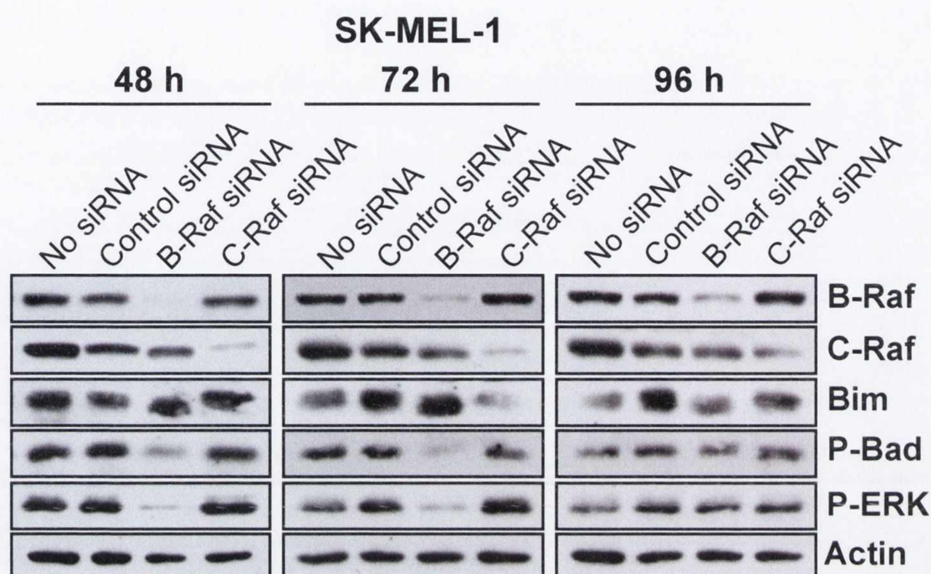


Figure 3.10

Ablation of B-Raf^{V600E} in melanoma cells leads to apoptosis

10^6 SK-MEL-1 cells were treated with 2 μM siRNA using the Amaxa Nucleofection system (sol V and program T-020) and incubated at 37°C for the indicated timepoints. Apoptosis was enumerated based on morphology (A). Results represent triplicate counts from a representative experiment with error bars representing SEM. Cellular growth was assessed by visual analysis of pictures taken on a light microscope with 200X magnification at 72 h (B).



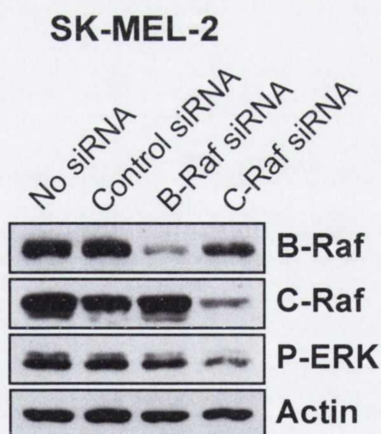


Figure 3.11

Ablation of B-Raf^{V600E} in melanoma cells prevents ERK activation

10^6 SK-MEL-1 cells (B-Raf^{V600E}) were treated with 2 μM siRNA using the Amaxa Nucleofection system (sol V and program T-020) and incubated at 37°C for the indicated timepoints. Cells were lysed in SDS-PAGE buffer and protein expression was analysed by Western Immunoblotting with the indicated antibodies (A). SK-MEL-2 (Ras^{V12G}) were treated with 200nM siRNA using oligofectamine for 72 h. Cells were lysed in SDS-PAGE buffer and protein expression was analysed by Western Immunoblotting with the indicated antibodies (B).

Ablation of B-Raf, but not C-Raf, sensitised these cells to apoptosis induced by a range of chemotherapeutic drugs (Figure 3.12 A). Interestingly, Bim was dephosphorylated and stabilised in the presence of B-Raf siRNA, while Bad was also dephosphorylated (Figure 3.12 B). This suggests that melanoma cells display increased resistance to apoptosis, at least in part due to hyperactivation of the B-Raf kinase and its effects on Bad and Bim.

3.2.10 *Ablation of Bim and Bad does not provide cells with the same apoptosis resistance afforded by B-RAF^{V600E} expression*

While B-Raf^{V600E} clearly modifies the BH3-only proteins Bim and Bad and disrupts their ability to promote apoptosis, we wished to examine whether inactivation of these proteins was sufficient to mediate B-Raf^{V600E}–associated protection against cell death. As a means of mimicking Bim and Bad inactivation, we transfected HeLa cells with siRNAs directed against these BH3-only proteins, alone or in combination, and then treated them with chemotherapeutic drugs. Ablation of Bim or Bad individually had little effect on pro-apoptotic drug-induced apoptosis (Figure 3.13 A). Combinational ablation of both offered greater resistance against drug-induced cell death, however this resistance was weaker than the protection provided by B-Raf^{V600E} expression (Figure 3.13 A, Figure 3.2 B). Furthermore, B-Raf^{V600E} provided additional resistance against apoptosis in cells where expression of the three BH3-only proteins modified by B-Raf^{V600E} was ablated (Figure 3.13 B). These results suggest that B-Raf^{V600E} targets multiple apoptosis regulators including Bim and Bad to increase cell survival in the presence of pro-apoptotic drugs. Of note, although Bim and Bad were not completely responsible for B-Raf^{V600E}–mediated apoptosis resistance, inactivation of these proteins modifies the apoptotic theshold of cells in favour of cell survival. Thus phosphorylation of these proteins could enhance melanoma progression.

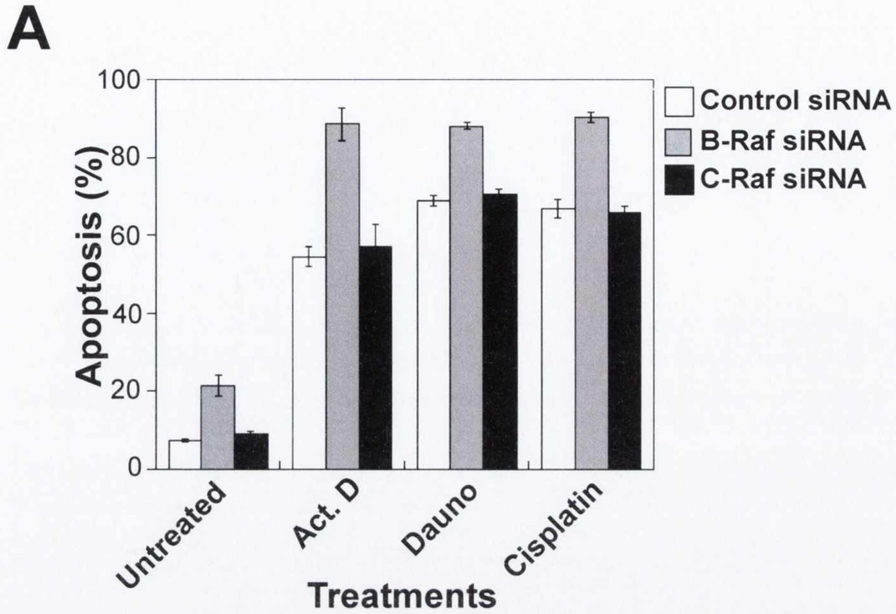
3.2.11 *B-RAF^{V600E} inhibits Bax- and Bak-induced apoptosis*

To further investigate B-Raf^{V600E}-mediated resistance against apoptosis we tested the ability of this kinase to protect against Bax- and Bak- induced programmed cell

death. Strikingly, B-Raf^{V600E} conferred protection against Bax and Bak induced apoptosis (Figure 3.14 A-C), suggesting that B-Raf may also exert effects on the cell death machinery downstream of the BH3-only proteins. Of note, previous experiments revealed that overexpression of B-Raf^{V600E} led to an increase in Mcl-1 levels in HeLa cells (Figure 3.3 A) and this may play a role in the protection provided by B-Raf^{V600E} against Bax- and Bak- induced cell death. Alternatively, B-Raf mediated inactivation of endogenous Bim may inhibit Bax and Bak activation, thus preventing apoptosis induced by these molecules.

3.2.12 *B-Raf^{V600E} inhibits Bax translocation to mitochondria*

Bax activation results in its translocation from the cytosol to mitochondria where it oligomerises and forms pores in mitochondrial outer membranes facilitating cytochrome *c* release. As B-Raf^{V600E} protected cells from Bax-induced apoptosis, we next asked whether B-Raf^{V600E} overexpression could modulate Bax translocation to mitochondria. To address this question, HeLa cells were transfected with GFP-tagged Bax in the presence or absence of B-Raf^{WT} and B-Raf^{V600E}. The cellular localisation of GFP-tagged Bax was then examined using confocal microscopy. Results revealed that B-Raf^{V600E} dramatically reduced Bax translocation to mitochondria, with many cells exhibiting a diffuse pattern of Bax-associated fluorescence throughout the cell rather than the punctate, mitochondrial-associated Bax fluorescence seen in control cells (Figure 3.15 A, B). This suggests that B-Raf also protects against apoptosis, in part, by inhibiting Bax translocation to mitochondria where it is responsible for propagating death signals by promoting the release of cytochrome *c* into the cytosol.



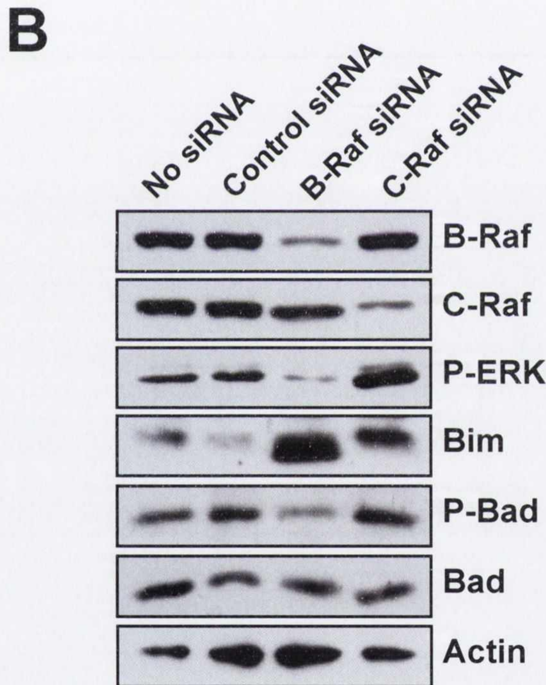
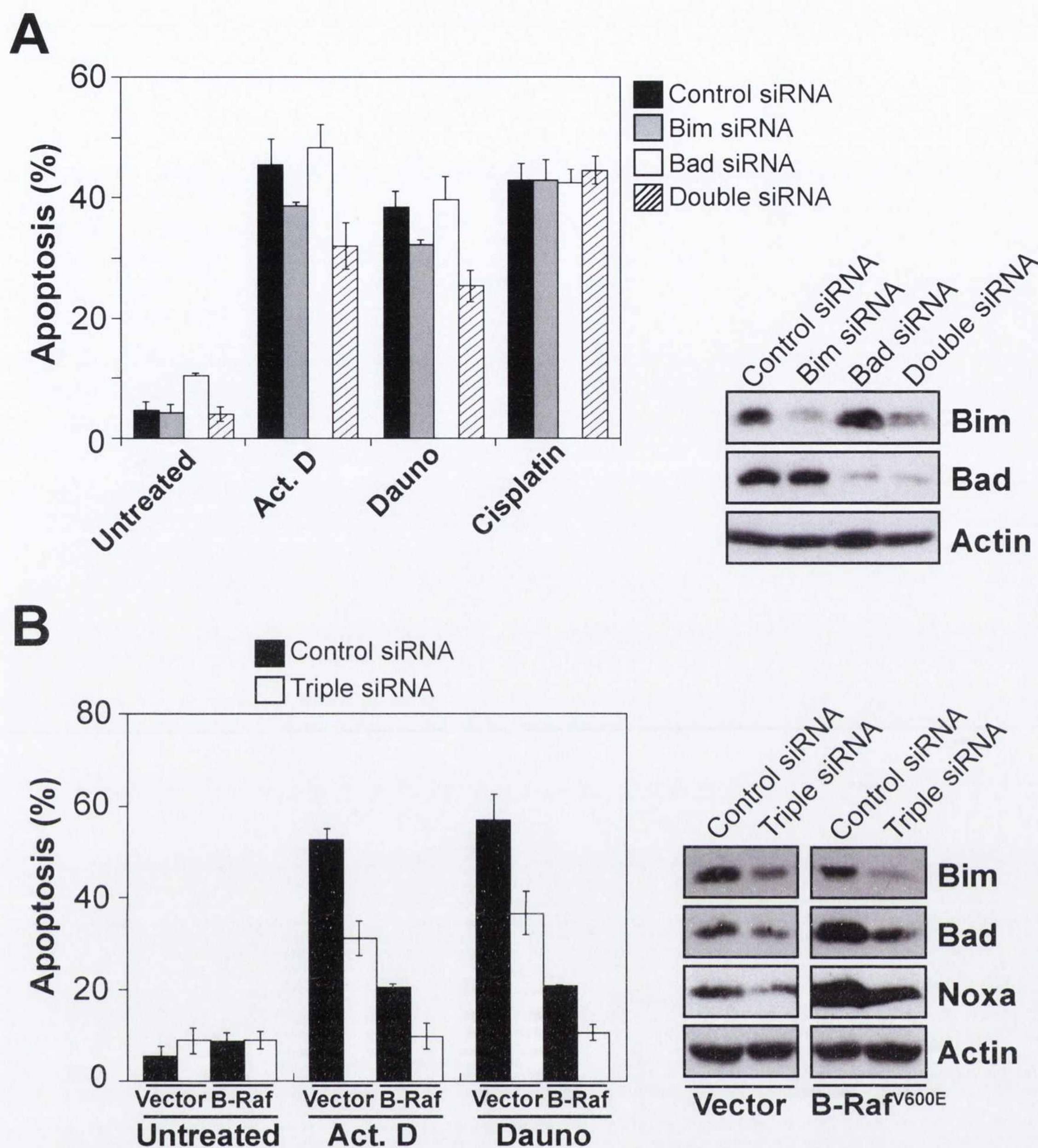


Figure 3.12

Ablation of B-Raf^{V600E} sensitises melanoma cells to apoptosis

10^6 SK-MEL-1 cells were treated with 2 μM siRNA using the Amaxa Nucleofection system (sol V and program T-020) and incubated at 37°C for 72 h, followed by treatment with Actinomycin D (10 μM), Daunorubicin (10 μM) or Cisplatin (300 μM) for 24 h. Cell death was enumerated based on morphology (A). Results represent triplicate counts from a representative experiment with error bars representing SEM. Cells were lysed in SDS-PAGE buffer and protein expression was analysed by Western Immunoblotting with the indicated antibodies (B).



<u>Figure 3.13</u>

SiRNA-mediated silencing of Bad and Bim expression does not replicate the apoptosis-inhibitory effects of B-Raf^{V600E}

HeLa cells were plated at 10^5cells/well and 24 h later were treated with 100 nM of control siRNA, Bim siRNA, Bad siRNA or a combination of Bim and Bad siRNA. After 48 h, cells were treated with actinomycin D (0.5 μM), daunorubicin (5 μM) or cisplatin (75 μM) and apoptosis was enumerated 12 h later. Lysates of untreated cells were prepared and probed for the indicated proteins to validate knockdown (A). HeLa cells were treated with 300 nM of control siRNA or a combination of Bim siRNA, Bad siRNA and Noxa siRNA (100 nM of each). After 24 h, cells were transfected with 750 ng of vector, or B-Raf^{V600E} plasmids, along with 50 ng of pAAV-GFP reporter plasmid. After a futher 24 h, cells were treated with actinomycin D (0.5 μM) or daunorubicin (5 μM) and apoptosis in GFP-positive cells was enumerated 12 h later (B). Lysates of untreated cells were prepared and probed for the indicated proteins to validate knock-down. Results represent triplicate counts from representative experiments with error bars representing SEM.

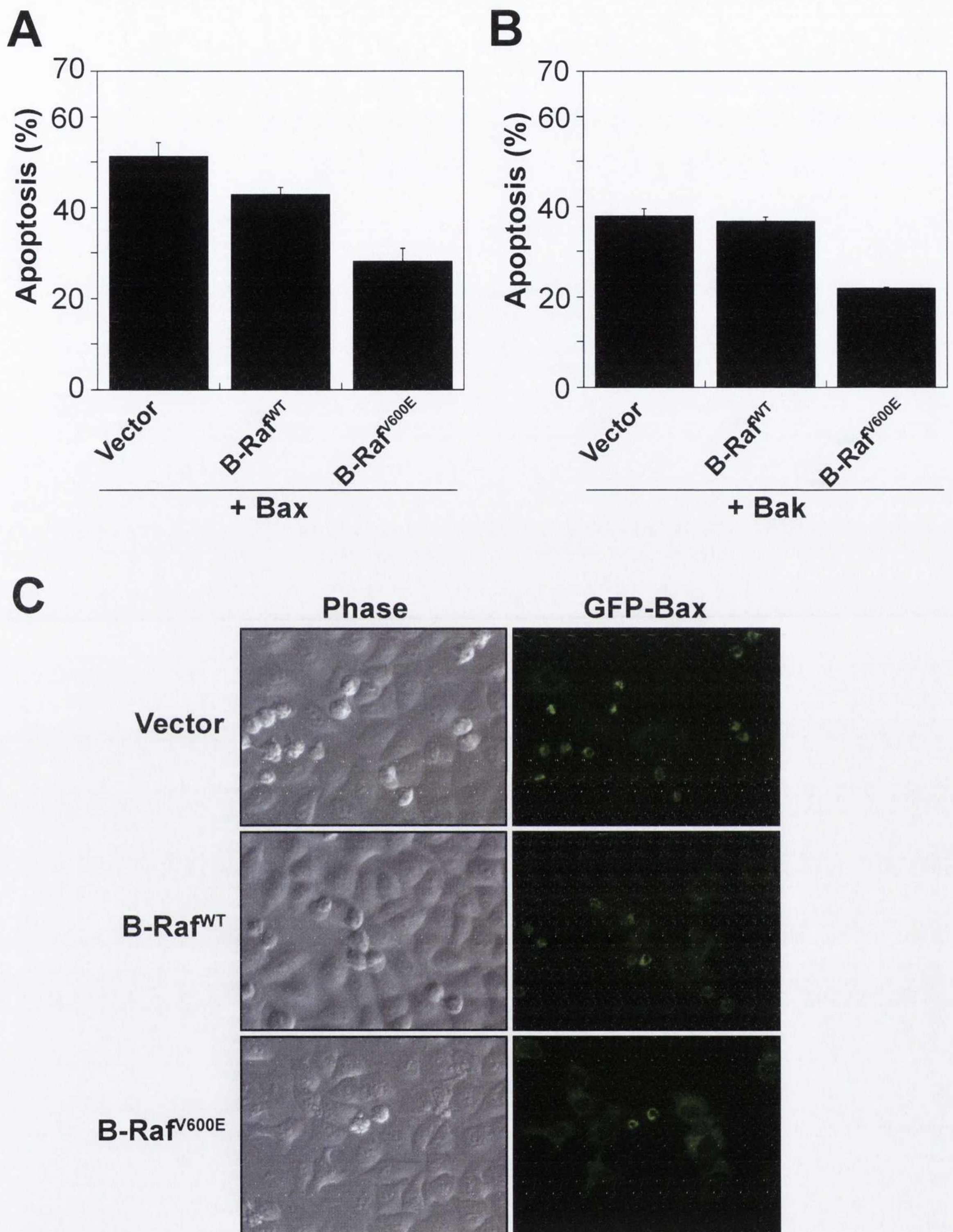
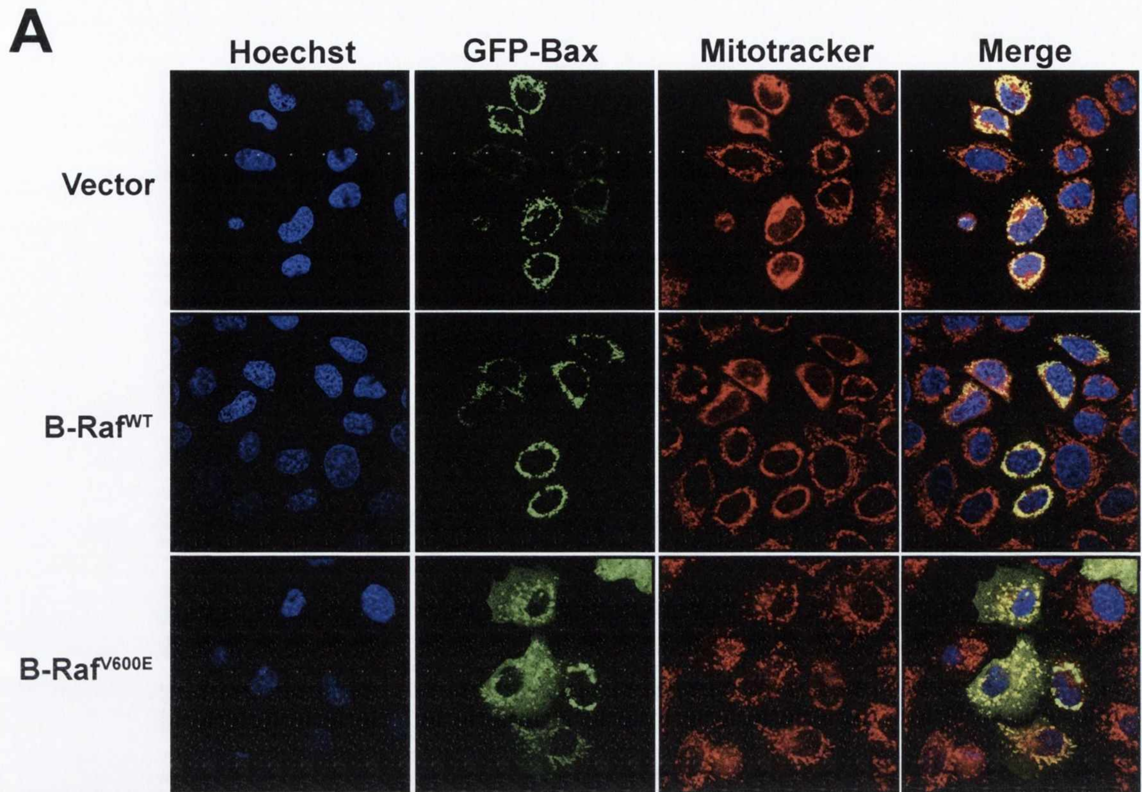


Figure 3.14

B-RAF^{V600E} protects against Bax- and Bak-induced apoptosis

HeLa cells were plated at 10^5 cells/well on 6-well plates. 24 h later, cells were transfected with 500 ng of B-RAF plasmids together with 30 ng of Bak or GFP tagged Bax plasmid. The effect of B-RAF^{V600E}on Bax (A) and Bak (B) induced apoptosis was assessed based on the morphology of GFP-positive cells, and by visual analysis of pictures taken on a light microscope (C). Results represent triplicate counts from representative experiments with error bars representing SEM.



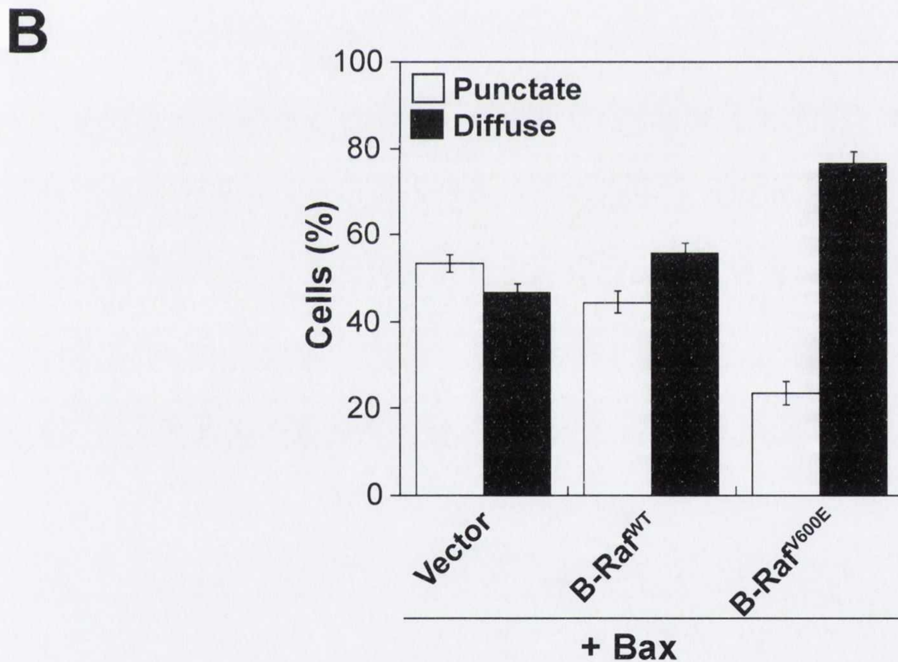


Figure 3.15

B-Raf^{V600E} inhibits Bax translocation to mitochondria

HeLa cells were plated at 10^5 cells/well on coverslips in 6-well plates. 24 h later, cells were transfected with 500 ng of B-Raf plasmids together with 30 ng of GFP-tagged Bax plasmid. Cells were fixed using 3% paraformaldehyde, stained with Hoechst stain (10 μM) and mounted onto slides. The effect of B-Raf^{V600E} on Bax localisation was assessed by visual analysis of pictures taken on a confocal microscope with 400X magnification (A), and also by enumerating the number of cells displaying diffuse verus punctate Bax expression (B). Results represent triplicate counts from a representative experiment with error bars representing SEM.

3.3 DISCUSSION

3.3.1 *Mutation of B-RAF in melanoma*

In 2002, a breakthrough study revealed the presence of mutated B-RAF in 66% of melanomas. A number of different mutations were described but one mutation, B-Raf^{V600E}, was found in 80% of cases (Davies *et al.*, 2002). This mutation may be favoured as it involves only one nucleotide change, T1796A and it produces a kinase that is ~500 fold more active, due to disruption of inhibitory interactions between the regulatory N-region and the activation segment (Wan *et al.*, 2004). The majority of the other mutations identified, such as G468A, also disrupted this interaction, however most of these had lower kinase activity than B-Raf^{V600E}. The cause of the T1796A mutation remains unclear. Although melanoma is a disease induced by ultraviolet radiation, mutation of B-Raf does not correlate with the typical C-T mutation triggered by ultraviolet rays. Furthermore, B-Raf^{V600E} mutations have also been identified in thyroid and colorectal cancers that are unaffected by sun exposure.

B-Raf has two sister kinases A-Raf and C-Raf that share a similar structure and function. However these kinases are rarely mutated in human cancers. Analysis of the activation of Raf kinases revealed that B-Raf is already primed for activation by the existence of a constitutively phosphorylated residue S445 and an acidic residue D448 in positions that require phosphorylation for activation in A- and C-Raf (Welbrock *et al.*, 2004b). Thus, mutation of the residue in C-Raf that corresponds to Valine 600 in B-Raf does not produce a hyperactive kinase (Emuss *et al.*, 2005). However, cells harbouring activating mutations in Ras are dependent on C-Raf for MAPK signalling (Dumaz *et al.*, 2006; our observations). Therefore, although C-Raf is not an oncogene itself, it is a critical mediator of oncogenic signalling promoted by other mutated proteins.

3.3.2 *B-RAF^{V600E} protects against chemotherapeutic drug-induced apoptosis*

Since the identification of the B-Raf^{V600E} mutation, numerous studies have demonstrated the oncogenic potential of this kinase and its involvement in

melanoma progression (Dong *et al.*, 2003; Collisson *et al.*, 2003; Karasarides *et al.*, 2004, Wellbrock *et al.*, 2004a, Hoeflich *et al.*, 2006). Other studies have implicated this kinase in various tumor-promoting functions such as vascular development and melanoma immune evasion (Sharma *et al.*, 2005, Sumimoto *et al.*, 2006). Although a number of oncogenic lesions are required to create melanocytic tumors, once these cancers are established they become highly dependent on B-Raf^{V600E} signalling for continued survival. Through ablation of B-Raf in melanoma, or inhibition of the downstream kinase MEK, a number of reports have showed that interruption of B-Raf signalling in melanoma led to growth arrest, apoptosis and tumor regression (Hingorani *et al.*, 2003; Collisson *et al.*, 2003; Karasarides *et al.*, 2004; Sumimoto *et al.*, 2004; Hoeflich *et al.*, 2006).

Here we examined the ability of oncogenic B-Raf to suppress apoptosis. We observed that B-Raf^{V600E} protected against cell death induced by a range of chemotherapeutic drugs, suggesting that this kinase may contribute to the chemoresistance displayed by malignant melanoma. Conversely, we found that ablation of B-Raf^{V600E} in SK-MEL-1 cells increased sensitivity to chemotherapeutic drug-induced apoptosis. We also observed the dependency of SK-MEL-1 cells on B-Raf^{V600E} function, as ablation of B-Raf induced spontaneous apoptosis in the absence of pro-apoptotic signalling. These results reveal a dual role for B-Raf^{V600E} in melanoma cell growth and survival, and demonstrate the therapeutic potential of targeting this kinase in melanoma treatment. Importantly, our observations show that B-Raf^{V600E} propagates an active anti-apoptoic signal in melanoma cells. Therefore, disruption of MAPK signalling may be necessary to provide the optimum environment for chemotherapeutic drug action.

3.3.3 *B-Raf^{V600E} expression results in modification of the BH3-only proteins Bim and Bad*

Exploration of the molecular mechanism of B-RAF^{V600E}-mediated resistance to apoptosis revealed that the BH3-only proteins Bim and Bad are modified in the presence of B-Raf^{V600E} (Figure 3.16). These proteins are phosphorylated in an

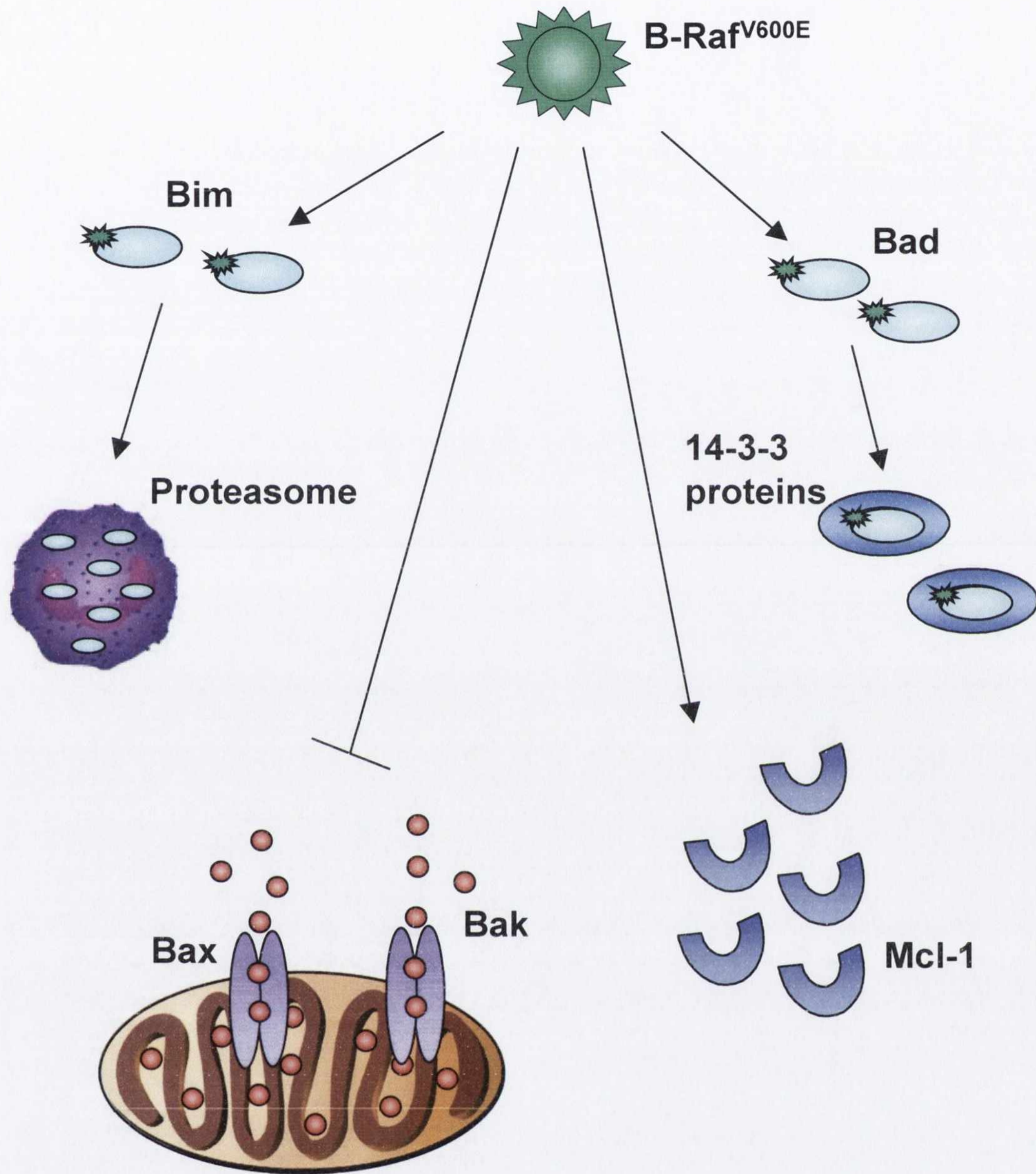


Figure 3.16

Modulation of Bcl-2 family members by B-Raf^{V600E}

B-Raf^{V600E} enhances phosphorylation of the BH3-only protein Bim, resulting in proteasome-mediated degradation of this protein. B-Raf^{V600E} also promotes the phosphorylation of Bad resulting in sequesteration by 14-3-3 proteins. Mcl-1 is upregulated in response to B-Raf^{V600E} expression, while Bax- and Bak-induced apoptosis is inhibited this kinase. Thus, B-Raf^{V600E} targets a number of Bcl-2 family members, to modify the apoptotic threshold in cells in favor of survival.

ERK-dependent manner, and these post-translational modifications reduce the pro-apoptotic potential of Bim and Bad. We have demonstrated that phosphorylation of Bim prompted proteasome-mediated degradation of this protein, thus reducing the availability of Bim within the cell. Through this mechanism B-Raf^{V600E} efficiently removes the pro-apoptotic threat posed by Bim. Other studies have indicated that phosphorylation of Bim on these residues disrupts interactions between Bim and Bax, and also interactions between Bim and anti-apoptotic Bcl-2 family members Bcl-xL and Mcl-1 (Harada *et al.*, 2004; Ewings *et al.*, 2007). Thus prior to degradation, phosphorylated Bim appears to be unable to promote Bax/Bak activation and cytochrome *c* release.

We also observed that B-Raf^{V600E} promoted phosphorylation of Bad and reduced the ability of Bad to initiate apoptosis. This effect may be mediated by RSK, a kinase activated by ERK, that is known to phosphorylate Bad (Bonni *et al.*, 1999; Eisenmann *et al.*, 2003). Phosphorylation leads to enhanced interaction of Bad with 14-3-3 proteins, sequesteration of Bad in the cytosol, and consequently, reduced interaction with anti-apoptotic Bcl-2 family members at mitochondria (Zha *et al.*, 1996). Similar to Bim, phosphorylation of Bad competently disrupts its role as a mediator of cell death.

Surprisingly, we also observed Noxa upregulation in the presence of B-Raf^{V600E}. Under these conditions, Noxa may act as a tumor suppressor, eliminating cells that display sustained hyperactivation of ERK. However, our panel of melanoma cells displayed high constitutive Noxa expression, suggesting that these cells have acquired resistance to Noxa-induced cell death, possibly by simultaneous upregulation of the anti-apoptotic molecule Mcl-1.

3.3.4 *Bim and Bad are constitutively inactivated in human melanomas*

Our results suggest that B-Raf^{V600E} raises the apoptotic threshold in melanoma cells via inactivation of the BH3-only proteins Bim and Bad. Inhibition of ERK signalling in six different melanoma cell lines resulted in dephosphorylation and dramatic

stabilisation of Bim, while Bad was also dephosphorylated. Furthermore, ablation of B-Raf^{V600E} in SK-MEL-1 cells led to dephosphorylation of Bim and Bad and sensitisation of these cells to chemotherapeutic drugs.

Inactivation of Bim and Bad is advantageous to melanoma cells in a number of ways; both of these proteins have been implicated in growth factor withdrawal-induced apoptosis and the rapid proliferation rates of malignant melanoma may result in local growth factor shortage (Zha *et al.*, 1996; Bouillet *et al.*, 1999; Shinjyo *et al.*, 2001). Thus, inactivation of Bim and Bad may prevent the elimination of excessively proliferating cancer cells. Furthermore, Bim and Bad have been linked with detachment-induced cell death, or anoikis (Reginato *et al.*, 2003, Marani *et al.*, 2004; Idogawa *et al.*, 2003). Therefore downregulation of these proteins may be important for metastasising melanoma cells that are detaching from the primary tumor. Consistent with this, inhibition of MAPK and PI3K signalling in melanoma cells increases susceptibility to anoikis (Boisvert-Adamo and Aplin, 2006). In addition, Bim is involved in cell death orchestrated by some natural pro-apoptotic molecules, such as TRAIL, and thus inactivation of Bim would provide melanoma cells with resistance against this endogenous cytotoxic molecule (Han *et al.*, 2006; Corazza *et al.*, 2006).

Mouse models have demonstrated an important role for Bim and Bad in tumor suppression. *Bad*$^{-/-}$ mice develop B cell lymphomas with age, while inactivation of a single allele of *Bim* accelerates myc-induced leukemias (Ranger *et al.*, 2003, Egle *et al.*, 2004). These findings underline the importance of the BH3-only proteins in preventing tumor formation and ensuring elimination of transformed cells. Thus B-Raf^{V600E}-mediated inactivation of Bim and Bad may be important for melanoma development.

Since completion of this study, a number of other reports have similarly described targeting of Bim and Bad by oncogenic B-Raf (Boisvert-Adamo and Aplin, 2008; Cartlidge *et al.*, 2008; Cragg et al., 2008; Wickenden et al., 2008; Golstein *et al.*,

2009). These reports have verified our findings and implicate inactivation of Bim and Bad by the MAPK pathway as an important means of evading anoikis and promoting growth factor-independent proliferation.

3.3.5 *B-RAF^{V600E} protects against Bax- and Bak-induced apoptosis*

Surprisingly, while investigating B-Raf^{V600E}-mediated suppression of apoptosis, we observed that this oncogene could protect against Bax- and Bak-induced cell death. Examination of Bax and Bak expression in the presence of B-Raf^{V600E} did not reveal any changes in protein levels or phosphorylation status, however the antibodies used may not have been able to detect post-translational modifications of these proteins. Bax contains one putative ERK phosphorylation site at serine 87 while Bak does not contain any typical ERK phosphorylation motifs, suggesting that B-Raf^{V600E} may inhibit cell death induced by these pro-apoptotic molecules via indirect means. Analysis of Bax cellular localisation revealed that B-Raf^{V600E} also dramatically reduced Bax translocation to mitochondria. While the mechanism utilised by B-Raf^{V600E} to target Bax and Bak remains unclear, it may occur due to B-Raf^{V600E}-mediated inhibition of endogenous Bim, thus preventing direct activation of Bax by Bim. Alternatively, Mcl-1 upregulation by B-Raf^{V600E} may result in sequestration of Bax and Bak and inhibition of cytochrome c release.

3.3.6 *Therapeutic significance of B-RAF-mediated resistance to apoptosis*

We have demonstrated that B-Raf^{V600E} protects cells against apoptosis and that this is mediated, in part, through phosphorylation and inactivation of the BH3-only proteins Bim and Bad. In addition, ablation of B-Raf^{V600E} in SK-MEL-1 cells sensitised these cells to chemotherapeutic drug-induced apoptosis, demonstrating the potential of targeting B-Raf^{V600E} in melanoma treatment. Moreover, siRNA-mediated ablation of B-Raf^{V600E}, but not C-Raf, resulted in spontaneous apoptosis of these cells demonstrating that melanoma cells become more dependent on the B-Raf oncogene. Consistent with our observations, recent studies have described "oncogene addiction" of cancer cells, where tumor cells with deregulated signalling pathways, such as the MAPK pathway, become dependent on the oncogene for

continued survival (Weinstein, 2002; Sharma *et al.*, 2006b). This proposal was validated by Rosen and colleagues who reported increased sensitivity of melanoma cells harbouring the B-Raf^{V600E} mutation to MEK inhibition, in comparison to wild type melanocytes (Solit *et al.*, 2005). Thus targeting MAPK signalling will provide a mechanism to specifically attack cancer cells while leaving non-malignant cells unscathed.

Preliminary studies using mouse models of melanoma have demonstrated reduced tumor development and regression of existing tumors, when treated with the MEK inhibitor C1 1040 or shRNA targeted against B-Raf^{V600E}, indicating that B-Raf^{V600E} is a promising target for melanoma treatment (Collison *et al.*, 2003; Hoeflich *et al.*, 2006). B-Raf inhibitors such as Sorafenib are in clinical trials for the treatment of various cancers. However, this molecule inhibits multiple kinases and anti-tumorigenic effects seen to date appear to be dependent on angiogenesis inhibition rather than MAPK inhibition. In the future, the use of more specific inhibitors such as PLX4720 will establish whether targeting B-Raf^{V600E} enhances chemotherapeutic action in melanomas (Tsai *et al.*, 2008). Given the importance of the MAPK pathway in melanoma cells harbouring Ras, B-Raf and growth factor receptor mutations, perhaps MEK is the most ideal central target for inhibition. A number of MEK inhibitors are currently in clinical trials for evaluation as novel cancer therapies (LoRusso *et al.*, 2005; Wang *et al.*, 2007). Thus targeting the MAPK pathway in melanoma cells containing oncogenic B-Raf, together with conventional chemotherapy, may be an important means of increasing the efficacy of chemotherapeutic drugs and improving survival rates of patients with malignant melanoma.

CHAPTER IV

CISPLATIN INDUCES APOPTOSIS THROUGH ERK-DEPENDENT UPREGULATION OF THE BH3-ONLY PROTEIN NOXA

4.1 INTRODUCTION

Cisplatin is a widely used chemotherapeutic drug that was first identified in the 1960s, and following clinical trails, was approved for treatment of tumors in 1978 (Muggia, 2009). Since then it has been very effective in the treatment of cancers, in particular testicular and ovarian cancers. Cisplatin acts primarily through binding to DNA, leading to the formation of platinum-DNA adducts that prevent DNA replication and transcription (Jamieson and Lippard, 1999). These adducts are recognised by sensors of DNA damage such as HMGB1 and ATR which transduce DNA damage signals to other cellular proteins, ultimately resulting in the induction of apoptosis (Wang and Lippard, 2005).

The initial stages of cisplatin-induced chemotherapeutic action have been extensively analysed. Cisplatin appears to be imported into cells through copper transporters, and proceeds to the nucleus where the platinum element forms covalent bonds with the N7 position in purine bases (Ishida *et al.*, 2002; Wing *et al.*, 1984). This produces intrastand crosslinks in DNA that prevent DNA polymerase-mediated replication (Harder *et al.*, 1976). However, the later events connecting DNA damage to apoptosis initiation are less well defined. The tumor suppressor protein p53 was initially proposed as an important link, due to its well-defined role in cellular DNA damage response (Perego *et al.*, 1996; Gallagher *et al.*, 1997). Following DNA damage, p53 transcriptionally upregulates p21 leading to cell cycle arrest, facilitating repair by DNA repair enzymes (Riley *et al.*, 2008). Alternatively, upon catastrophic DNA injury, p53 upregulates Puma, Noxa and Bax leading to apoptosis. However, a number of reports have since showed that cells were still susceptible to cisplatin-mediated apoptosis in the absence of p53 (Petit *et al.*, 2003; Jiang *et al.*, 2009). Thus, while p53 may be involved in cisplatin cytotoxicity, this protein is not crucial, and other cell signalling molecules contribute to cisplatin-induced cell death.

Many classical chemotherapeutic agents reduce tumor volume by inducing apoptosis in tumor cells. While these agents trigger apoptosis through diverse

mechanisms, their actions are likely to converge on the Bcl-2 family. It is now well established that apoptosis induced by these drugs occurs through the intrinsic apoptotic pathway. Therefore, cell death almost certainly occurs through activation/upregulation of pro-apoptotic Bcl-2 family members or degradation/sequesteration of anti-apoptotic members. However, as the understanding of apoptosis regulation has lagged behind the identification of chemotherapeutic drugs as anti-tumor agents, the molecular mechanism by which some of these anti-cancer drugs induce apoptosis is still unknown. BH3-only proteins often provide the essential link between drug-induced cellular injury and apoptosis. For example, upregulation of Noxa is responsible for apoptosis induced by camptothecin, while Bim plays a crucial role in apoptosis caused by microtubule disruptors (Mei *et al.*, 2007; Li *et al.*, 2005).

In this chapter, we wished to search for novel modifications of BH3-only proteins by chemotherapeutic drugs. We observed Noxa upregulation in response to cisplatin treatment. Thus, this chapter aimed to explore the mechanism of Noxa upregulation and determine what role Noxa plays in cisplatin-mediated cell death.

4.2 RESULTS

4.2.1 *Modification of BH3-only proteins in response to chemotherapeutic drugs*

Many chemotherapeutic drugs induce cell death through the mitochondrial pathway, however the repitoire of BH3-only proteins activated by each drugs is likely to be different. To search for novel modifications of BH3-only proteins in response to drug treatment, we exposed HeLa cells to a panel of chemotherapeutic drugs that kill through divergent modes of action. For example, actinomycin D inhibits RNA synthesis, cisplatin induces crosslinking of DNA and MG132 inhibits proteasome-mediated degradation of proteins. Following treatment with pro-apoptotic drugs, cells displayed morphological features typical of apoptosis such as cell rounding, detachment from the plate and plasma membrane blebbing (Figure 4.1 A). Cell death was enumerated on the basis of these morphological features (Figure 4.1 A

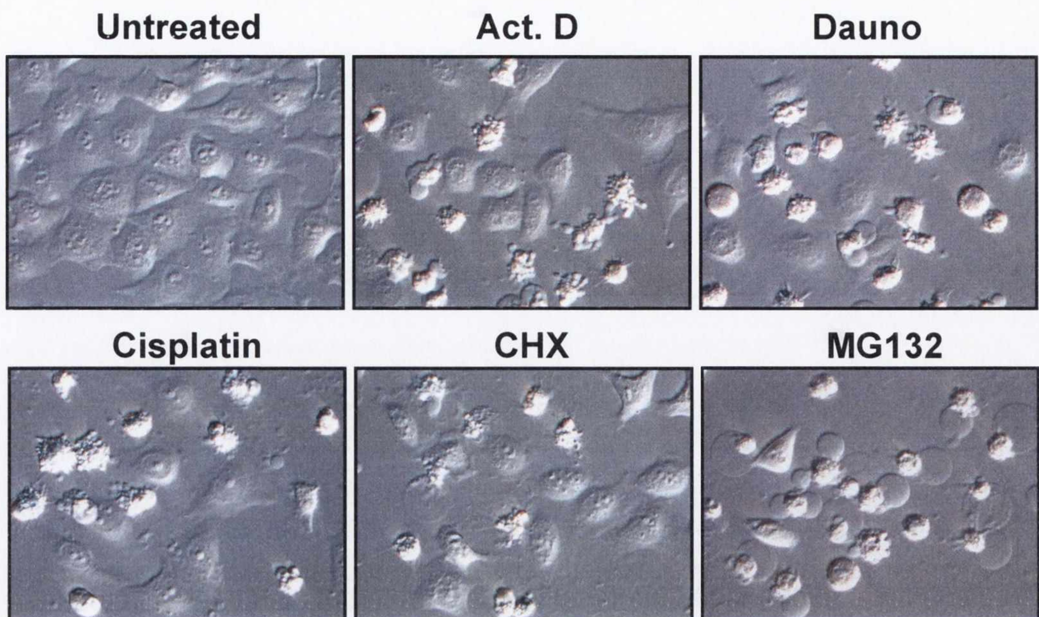
and B). Lysates were then prepared and protein expression of Bcl-2 family members was assessed (Figure 4.1 C). Strikingly, while levels of many of the BH3-only proteins were unchanged or decreased in response to pro-apoptotic drug treament, Noxa was upregulated following cisplatin treatment. As previously described, Noxa was also upregulated by the proteasome inhibitor MG132 (Qin *et al.*, 2005; Fernandez *et al.*, 2005, Perez Galan *et al.*, 2006). However unlike MG132, which also upregulates Noxa's anti-apoptotic interaction partner Mcl-1, cisplatin-induced apoptosis was associated with Mcl-1 downregulation, indicating that these two drugs act through different mechanisms (Figure 4.1 C).

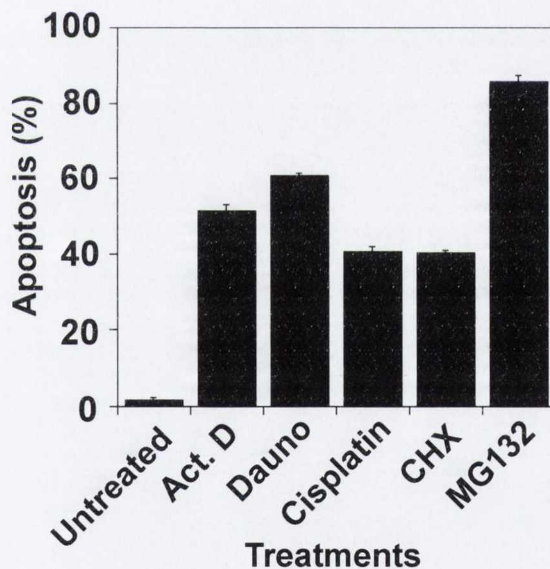
4.2.2 *Noxa and ERK are modulated by cisplatin treatment*

To further examine cisplatin-mediated Noxa upregulation, we treated HeLa cells with cisplatin and prepared lysates at various timepoints. As Figure 4.2 A shows, Noxa is rapidly upregulated within 5 hours of cisplatin treatment and Noxa levels continue to increase over the time period examined. Of note, Noxa induction occurred much earlier than caspase-3 activation, indicating that this is an upstream initiating event during cisplatin-induced apoptosis (Figure 4.2 A). Furthermore, Noxa was upregulated over a range of cisplatin concentrations (Figure 4.2 B). As described in the previous chapter, hyperactivation of ERK by the oncogene B-RAF^{V600E} led to an increase in Noxa expression. Thus we questioned whether ERK may be involved in cisplatin-induced Noxa upregulation. To examine this, we first determined whether ERK was activated during cisplatin treatment. Using a phospho-specific antibody, we observed ERK activation in response to cisplatin treatment in a time- and dose-dependent manner (Figure 4.2 A and B).

4.2.3 *Cisplatin-induced apoptosis and Noxa upregulation are ERK-dependent*

Activation of ERK in response to cisplatin has been previously described (Wang *et al.*, 2000; Schweyer *et al.*, 2004; Kim *et al.*, 2005; Hayakawa *et al.*, 1999; Persons *et al.*, 1999). However whether ERK acts as a pro-apoptotic effector of cisplatin-mediated death, or an anti-apoptotic molecule responsible for cisplatin resistance is under dispute. To investigate the role of ERK in cisplatin-mediated augmentation of





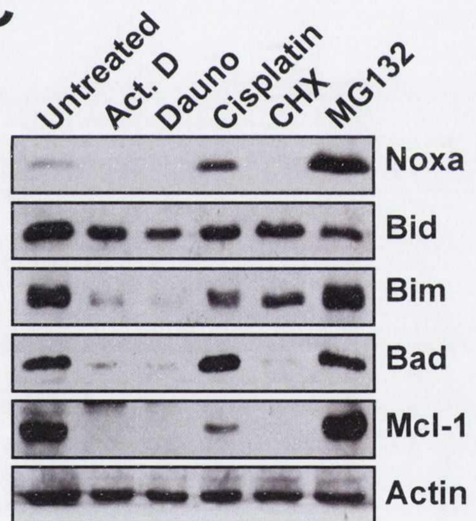


Figure 4.1

Noxa is upregulated in response to cisplatin treatment

HeLa cells were plated at 2 x 10^5 cells/well on 6-well plates. 24 h later, cells were treated with Actinomycin D (Act. D 1 μM), Daunorubicin (Dauno 5 μM), Cisplatin (50 μM) Cyclohexamide (CHX 200 μM) or MG132 (10 μM) for 12-24 h and apoptosis was assessed based on the morphology of cells (A and B). Results represent triplicate counts of a representative experiment with error bars representing SEM. Lysates were prepared and probed for the indicated Bcl-2 family members (C).

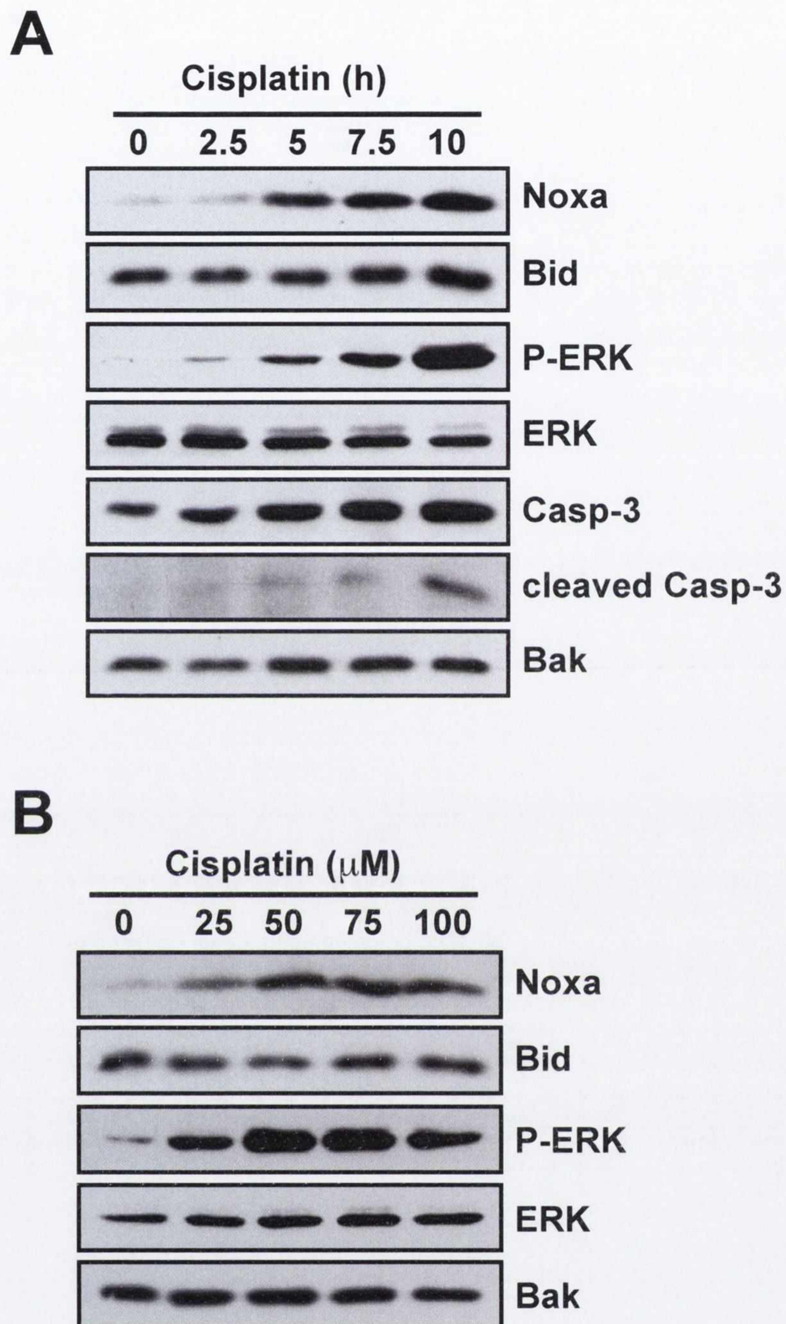


Figure 4.2

Noxa and ERK are modulated by cisplatin treatment

HeLa cells were plated at 5 x 10^5 cells/dish on 6 cm dishes. 24 h later, cells were treated with Cisplatin (50 μM) and lysates were prepared at the indicated timepoints. Protein expression of the indicated proteins was assessed using Western Immunoblotting (A). HeLa cells were plated at 2 x 10^5 cells/well on a 6 well plate. 24 h later, cells were treated with the indicated concentrations of Cisplatin for 8 h. Lysates were prepared and protein expression of the indicated proteins was assessed using Western Immunoblotting (B).

Noxa and apoptosis in HeLa cells, we used a MEK inhibitor that abrogates ERK signalling. Inhibition of MEK by UO126 dramatically attenuated cisplatin-induced apoptosis (Figure 4.3 A and B). Importantly, UO126 provided long-term protection against this drug with many more cells surviving cisplatin treatment over a seven day period (Figure 4.3 C). Strikingly, inhibition of ERK activity completely prevented Noxa upregulation by cisplatin demonstrating that Noxa induction is ERK-dependent in this context (Figure 4.3 D).

4.2.4 *Induction of Noxa expression by cisplatin is p53-independent*

Noxa was originally identified as a target gene of the p53 transcription factor that is activated in response to DNA damage (Oda *et al.*, 2001; Vogelstein *et al.*, 2000). As cisplatin is an alkylating agent that crosslinks DNA, thereby provoking DNA damage, we asked whether p53 is involved in the enhancement of Noxa expression in response to this drug. To address this question we used p53 null HL-60 cells (Wolf *et al.*, 1985). Treatment of HL-60 cells with cisplatin resulted in apoptosis, demonstrating that p53 is not a critical determinant of cisplatin-mediated cell death (Figure 4.4 A). Furthermore, Noxa upregulation in response to cisplatin was still evident in HL-60 cells, demonstrating that this event is not dependent on p53 (Figure 4.4 B). In addition, augmentation of Noxa expression in response to MG132 was also p53-independent (Figure 4.4 B).

4.2.5 *Cisplatin-induced apoptosis and Noxa upregulation are ERK-dependent in HL-60 cells*

To determine whether the pro-apoptotic role of ERK in cisplatin-induced apoptosis was a widespread phenomenon, we treated HL-60 cells with cisplatin in the absence or presence of the MEK inhibitor UO126 and apoptosis was assessed (Figure 4.5 A and B). MEK inhibition dramatically reduced cisplatin-mediated apoptosis in these cells, but not cell death induced by daunorubicin or MG132. This demonstrates that the role of ERK in promoting apoptosis is specific to cisplatin and is conserved across various cell types. Cell death was also assessed by DNA fragmentation analysis (Figure 4.6 A) and again, MEK inhibition was found to

protect cells from cisplatin-induced apoptosis. To determine whether ERK was responsible for Noxa upregulation by this stimulus in HL-60 cells, extracts from cells treated as described above were examined for Noxa expression. Upregulation of Noxa following cisplatin treatment was inhibited by UO126, demonstrating the importance of ERK for cisplatin-mediated Noxa induction (Figure 4.6 B).

To further confirm the role of ERK in cisplatin-induced cell death, we treated HL-60 cells with a panel of ERK pathway inhibitors including PD98059, another MEK inhibitor, and an ERK inhibitory peptide. These latter inhibitors similarly prevented cisplatin-induced apoptosis (Figure 4.7 A). They also potently blocked Noxa induction and caspase-3 cleavage following cisplatin treatment, firmly establishing the influential role played by ERK during cisplatin-mediated apoptosis (Figure 4.7 B).

4.2.6 *Noxa is upregulated in response to Carboplatin and Oxaliplatin treatment*

Cisplatin is one of the first discovered and most used platinum compounds for chemotherapy. However since its development over 40 years ago, other cisplatin analogues have been designed with the aim of producing more effective chemotherapeutic drugs with reduced side effects. While cisplatin has been very successful in the treatment of ovarian and prostate cancers, its widespread use for many different tumor types has been hampered by reduced cytotoxicity against some cancers, and harmful side effects such as nephrotoxicity and neurotoxicity. The development of carboplatin which produces less side effects, and oxaliplatin which is effective on a larger repertoire of cancers have addressed these issues to some extent. We wished to investigate whether ERK activation and Noxa upregulation occurred only with cisplatin or whether these effects were associated with other platinum compounds. To address this question we titrated carboplatin and oxaliplatin onto HeLa cells. Treatment of HeLa cells with these platinum agents led to an induction of apoptosis (Figure 4.8 A and C). Oxaliplatin was similar in potency to cisplatin, however carboplatin was significantly less cytotoxic.

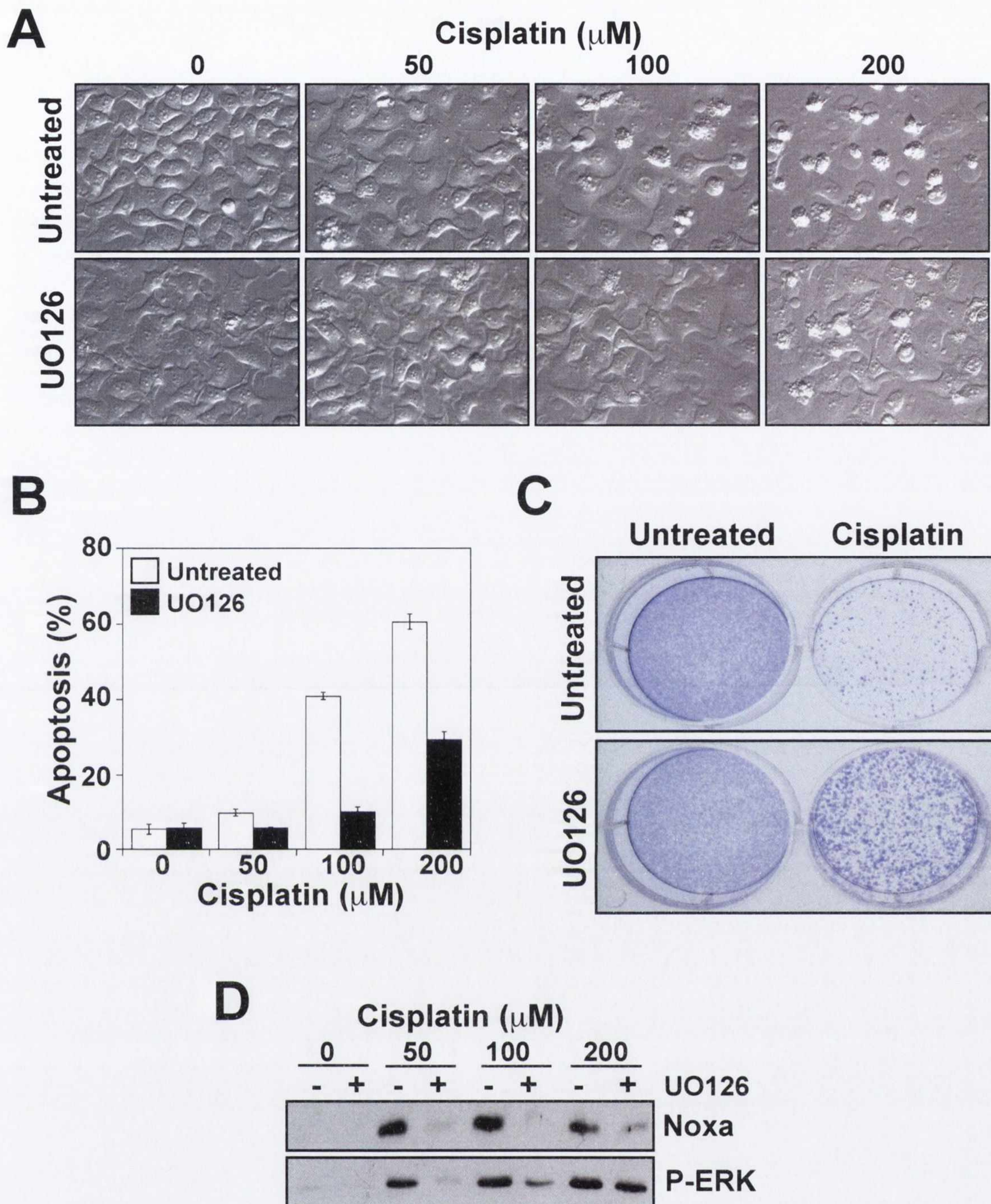
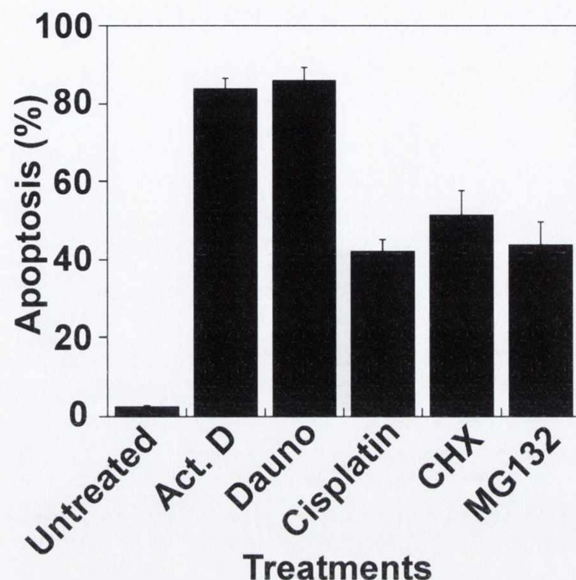
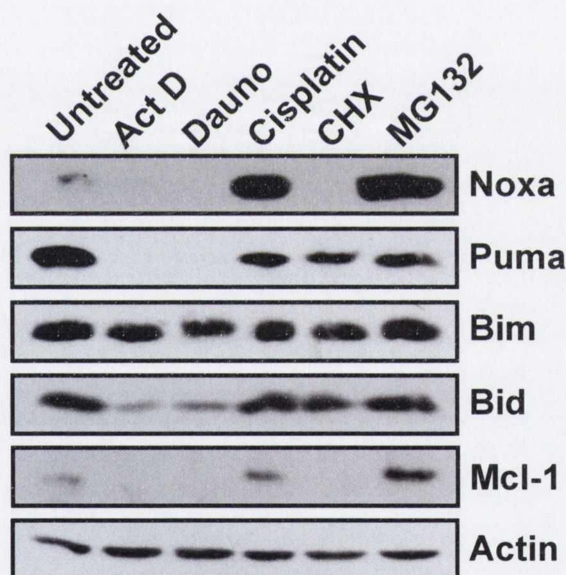


Figure 4.3

Cisplatin-induced apoptosis and Noxa upregulation are ERK-dependent

HeLa cells were plated at 2 x 10^5 cells/well on 6 well plates. 24 h later, cells were treated with Cisplatin in the absence or presence of UO126 (20 μM) and and apoptosis was assessed based on the morphology of cells (A and B). Results represent triplicate counts of a representative experiment with error bars representing SEM. HeLa cells were plated at 2 x 10^4 cells/well on a 6 well plate. 24 h later, cells were pretreated with UO126 (20 μM) for 1 h, followed by Cisplatin treatment (25 μM) for 3 h. Cells were incubated at 37°C for 7 days and then stained with 0.5 % Crystal Violet viability stain (C). Lysates from cells treated as described in (A) were prepared and protein expression of the indicated proteins was assessed using Western Immunoblotting (D).

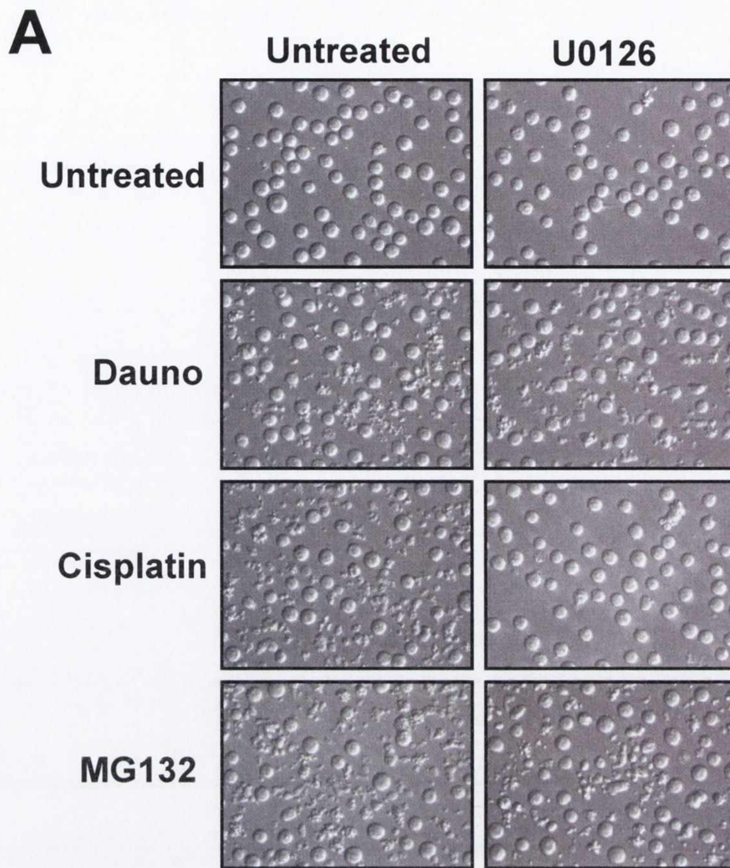


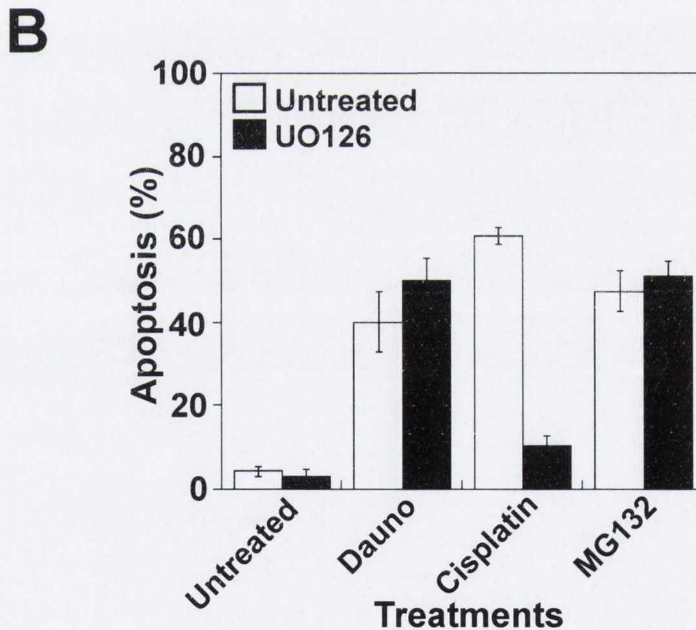


<u>Figure 4.4</u>

Upregulation of Noxa in response to cisplatin is p53-independent

HL-60 cells (p53 null cells) were plated at 10^6 cells/well on 6-well plates. 24 h later, cells were treated with Actinomycin D (Act. D 5 μM), Daunorubicin (Dauno 5 μM), Cisplatin (100 μM) Cycloheximide (CHX 200 μM) or MG132 (5 μM) for 5-9 h and apoptosis was assessed based on the morphology of cells (A). Results represent triplicate counts of a representative experiment with error bars representing SEM. Lysates of treated cells were prepared and protein expression was analysed by Western Immunoblotting with the indicated antibodies (B).





<u>Figure 4.5</u>

Cisplatin-induced apoptosis is ERK-dependent in HL-60 cells

HL-60 cells were plated at 10^6 cells/well on 6-well plates. 24 h later, they were treated with Daunorubicin (Dauno 1 μM), Cisplatin (150 μM) or MG132 (3 μM) in the absence or presence of UO126 (20 μM). Apoptosis was assessed by visual analysis of pictures taken on a light microscope 8 h later (A). Apoptosis was enumerated based on morphology (B). Results represent triplicate counts of a representative experiment with error bars representing SEM.

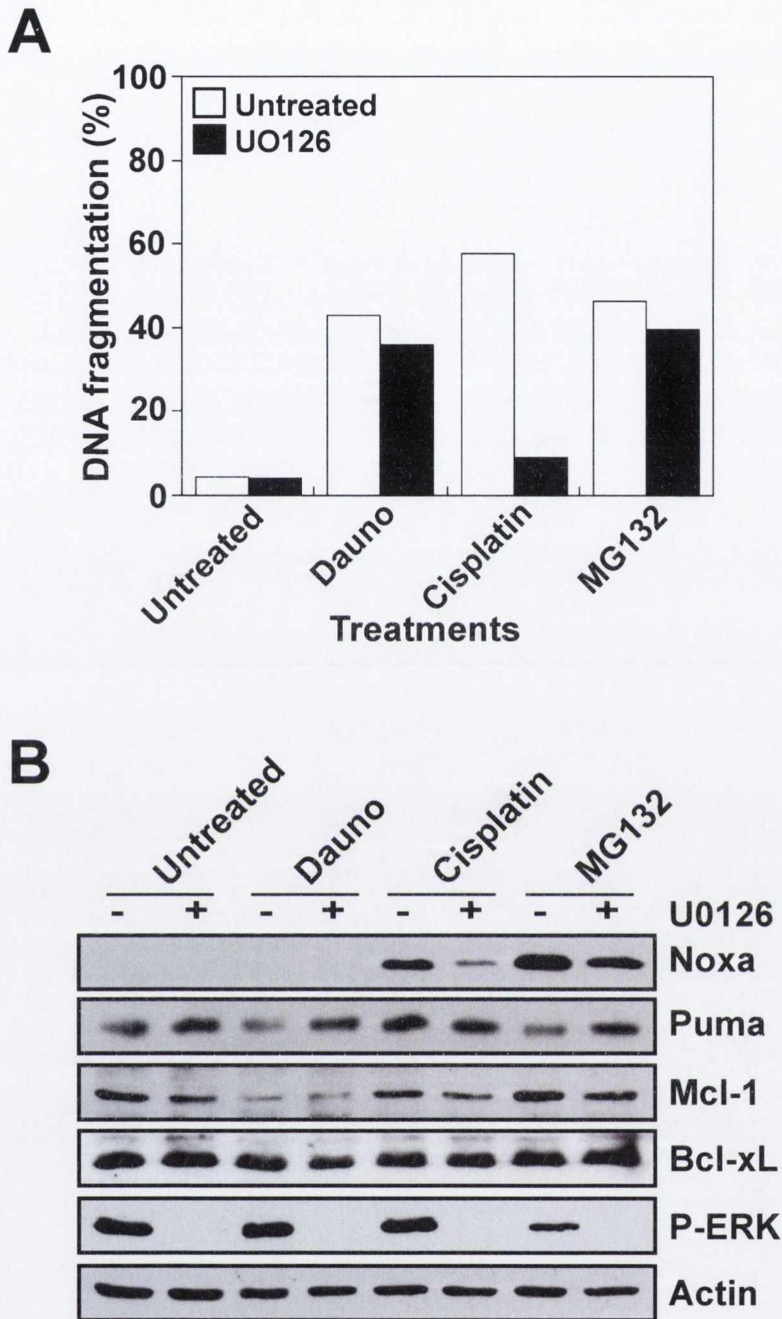


Figure 4.6

Cisplatin-induced apoptosis and Noxa upregulation in HL-60 cells are ERK-dependent

HL-60 cells were plated at 10^6 cells/well on 6-well plates. 24 h later, they were treated with Daunorubicin (Dauno 1 μM), Cisplatin (150 μM) or MG132 (3 μM) in the absence or presence of UO126 (20 μM). Apoptosis was enumerated 8 h later by FACS analysis using Propidium Iodide stain (A). Cells were lysed in SDS-PAGE buffer and protein expression was analysed by Western Immuno-blotting with the indicated antibodies (B).

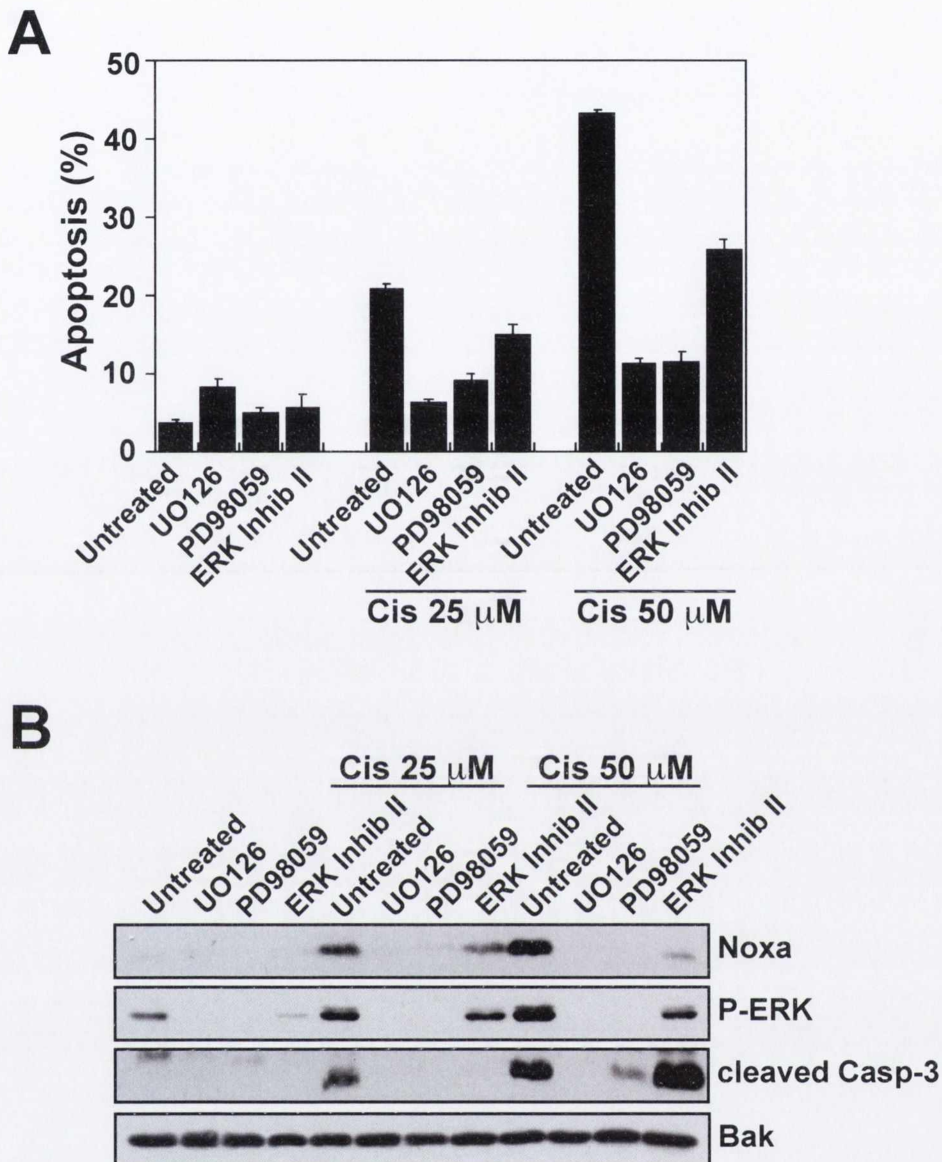


Figure 4.7

Cisplatin-induced apoptosis and Noxa upregulation are inhibited by a number of ERK pathway inhibitors

HL-60 cells were plated at 10^6 cells/well on 6-well plates. 24 h later, cells were treated with Cisplatin in the absence or presence of UO126 (20 μM), PD98059 (50 μM), or ERK Inhibitor peptide II (10 μM). Apoptosis was enumerated based on morphology 12 h later (A). Results represent triplicate counts of a representative experiment with error bars representing SEM. Cells were lysed in SDS-PAGE buffer and protein expression was analysed by Western Immunoblotting with the indicated antibodies (B).

Importantly, Noxa was upregulated over a wide concentration range of both drugs and ERK activation correlated with this induction (Figure 4.8 B and D).

Furthermore, MEK inhibition reduced carboplatin and oxaliplatin-mediated apoptosis and also blocked upregulation of Noxa in response to these drugs (Figure 4.9 A and B; data kindly provided by Dr. Gabriella Brumatti). These results demonstrate that ERK-dependent Noxa upregulation is a conserved event during platinum-mediated apoptosis and indicate that Noxa is likely to play an important role in cell death induced by these compounds.

4.2.7 *Oncogenic Ras and B-Raf induce Noxa expression*

To assess Noxa induction under other conditions of ERK activation, we overexpressed oncogenic Ras^{V12G} and $B\text{-}Raf^{V600E}$ in HeLa cells. Significantly, both oncogenes, which potently induced ERK activation, also increased Noxa expression (Figure 4.10 A). In contrast, dominant negative Ras^{N17S} and $B\text{-}Raf^{WT}$ which failed to activate ERK had no effect on Noxa levels. When these oncogenes were expressed in the presence of UO126, Noxa upregulation no longer occurred demonstrating that ERK was responsible for this effect (Figure 4.10 B). Interestingly, in contrast to cisplatin treatment, Ras^{V12G} and $B\text{-}Raf^{V600E}$ also upregulate anti-apoptotic Mcl-1 in an ERK-dependent manner, which may prevent Noxa-induced apoptosis under these conditions (Figure 4.10 A and B).

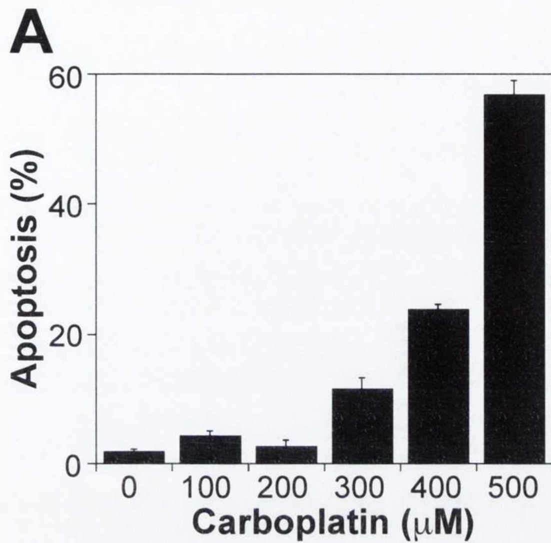
4.2.8 *Noxa is transcriptionally upregulated by cisplatin*

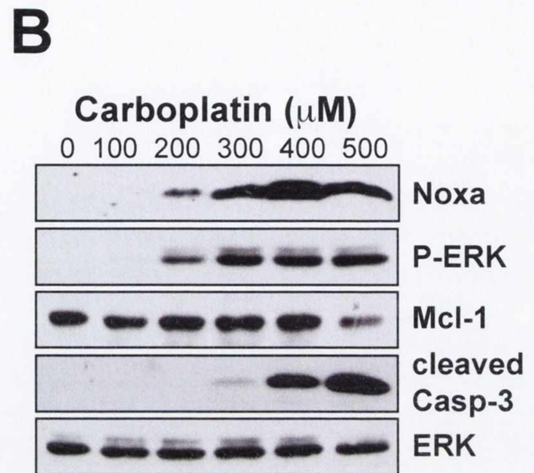
As a BH3-only protein, Noxa is expressed at low levels in healthy dividing cells and cellular Noxa is usual bound and sequestered by anti-apoptotic Bcl-2 family members such as Mcl-1 and A1 (Chen *et al.*, 2005a; Alves et al., 2006). In response to certain death stimuli, Noxa levels are increased allowing this BH3-only protein to overcome the anti-apoptotic Bcl-2 related proteins and promote Bax/Bak-dependent apoptosis (Kim *et al.*, 2004; Fernandez *et al.*, 2005). This can occur through two mechanisms; stabilisation of existing Noxa protein within the cell or *de novo* synthesis of Noxa. To ask whether Noxa is transcriptionally upregulated in

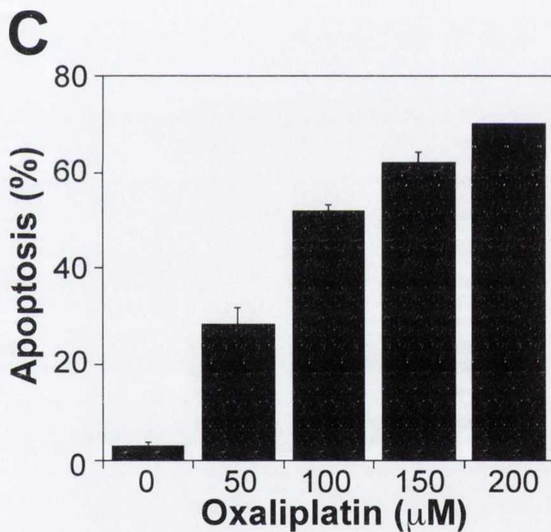
response to cisplatin we treated HeLa cells with the translational inhibitor cycloheximide. As Figure 4.11 A shows, inhibition of new protein synthesis completely blocked Noxa induction by cisplatin. Interestingly, inhibition of Noxa upregulation by cycloheximide also led to a reduction in cisplatin-mediated apoptosis (Figure 4.11 B). While Noxa upregulation was transcriptional in nature, it still remained possible that cisplatin treatment also stabilised Noxa protein. To address this, we treated HeLa cells with cisplatin for 6 hours and then carried out a timecourse of cycloheximide treatment (to block new protein synthesis) to examine the rate of degradation of Noxa in the absence or presence of cisplatin. As Figure 4.11 C shows, cellular Noxa was degraded rapidly with a half-life of less than two hours and disappearance of Noxa occurred at a similar rate in the presence of cisplatin. Thus cisplatin enhances cellular Noxa levels by promoting new protein synthesis and not by preventing Noxa degradation.

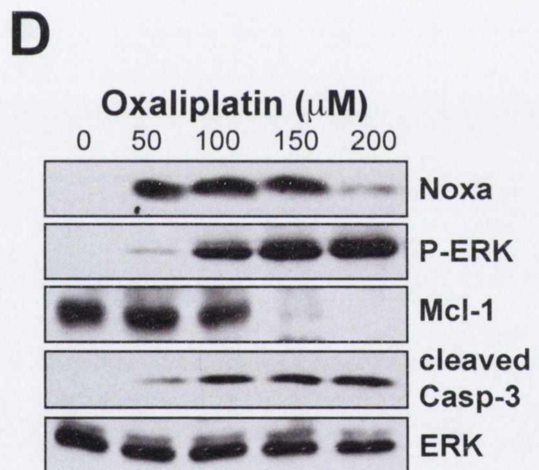
4.2.9 *Known modulators of Noxa expression are not responsible for Noxa upregulation by cisplatin*

Noxa is a p53 target gene and initial studies on Noxa-induced apoptosis attributed transcriptional upregulation of Noxa to p53 (Schuler *et al.*, 2003; Terui *et al.*, 2003; Yakovlev *et al.*, 2004). However, subsequent reports have described p53-independent upregulation of Noxa. A p53-related protein, p73, has also been shown to upregulate Noxa (Lau *et al.*, 2008). Furthermore, this transcription factor has previously been implicated in cisplatin-mediated apoptosis, indicating that p73 may be candidate regulator of Noxa expression during cisplatin treatment (Gong *et al.*, 1999, Shimodaira *et al.*, 2003). Other candidate transcription factors include Hif-1α, which upregulates Noxa during hypoxic cell death, or ATF4 that upregulates Noxa in response to ER-stress (Kim *et al.*, 2004; Wang *et al.*, 2009). Both of these transcription factors are regulated by ERK under certain conditions (Richard *et al.*, 1999; Wang *et al.*, 2004; Thiaville *et al.*, 2008). To explore whether any of these transcription factors are responsible for cisplatin-mediated Noxa induction, we ablated their expression in HeLa cells with siRNA and then treated these cells with cisplatin. Silencing of these transcription factors had no effect on cisplatin-induced





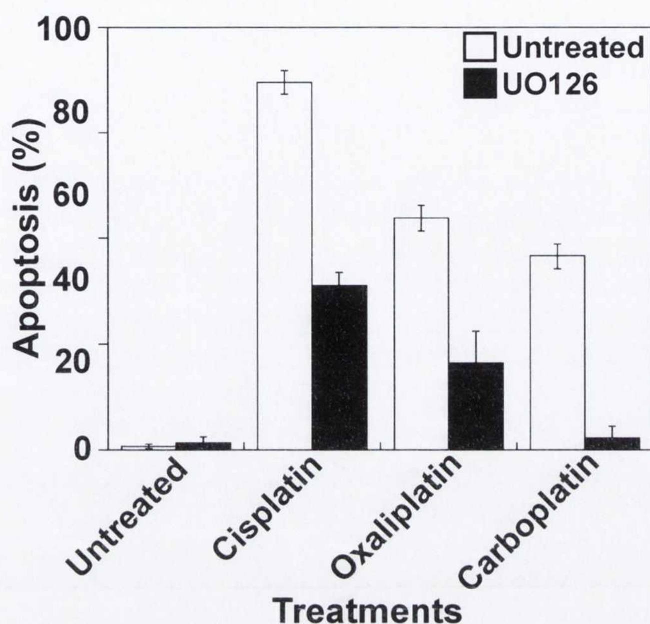




<u>Figure 4.8</u>

Noxa is upregulated in response to Carboplatin and Oxaliplatin treatment

HeLa cells were plated at 2 x 10^5 cells/well on 6-well plates. 24 h later, cells were treated with the indicated amounts of Carboplatin for 24 h and apoptosis was assessed based on the morphology of cells (A). Lysates were prepared and probed for the indicated proteins (B). HeLa cells were plated at 2 x 10^5 cells/well on 6-well plates. 24 h later, cells were treated with the indicated amounts of Oxaliplatin for 18 h and apoptosis was assessed based on the morphology of cells (C). Lysates were prepared and probed for the indicated proteins (D). Graphed results represent triplicate counts of representative experiments with error bars representing SEM.



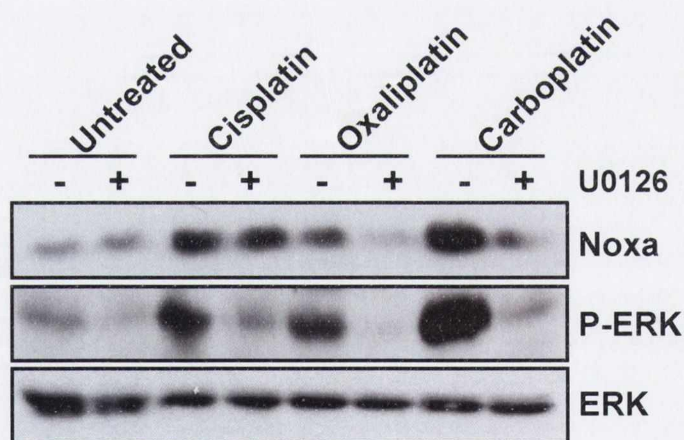


Figure 4.9

Carboplatin- and Oxaliplatin-induced apoptosis and Noxa upregulation are ERK-dependent

HeLa cells were plated at 2 x 10^5 cells/well on 6 well plates. 24 h later, cells were treated with Cisplatin (40 μM), Carboplatin (400 μM) and Oxaliplatin (100 μM) in the absence or presence of UO126 (20 μM). Apoptosis was enumerated based on morphology of cells (A). Results represent triplicate counts of a representative experiment with error bars representing SEM. Lysates from cells treated as described in (A) were prepared and protein expression of the indicated proteins was assessed using Western Immunoblotting (B). Data were kindly provided by Dr. Gabriella Brumatti.

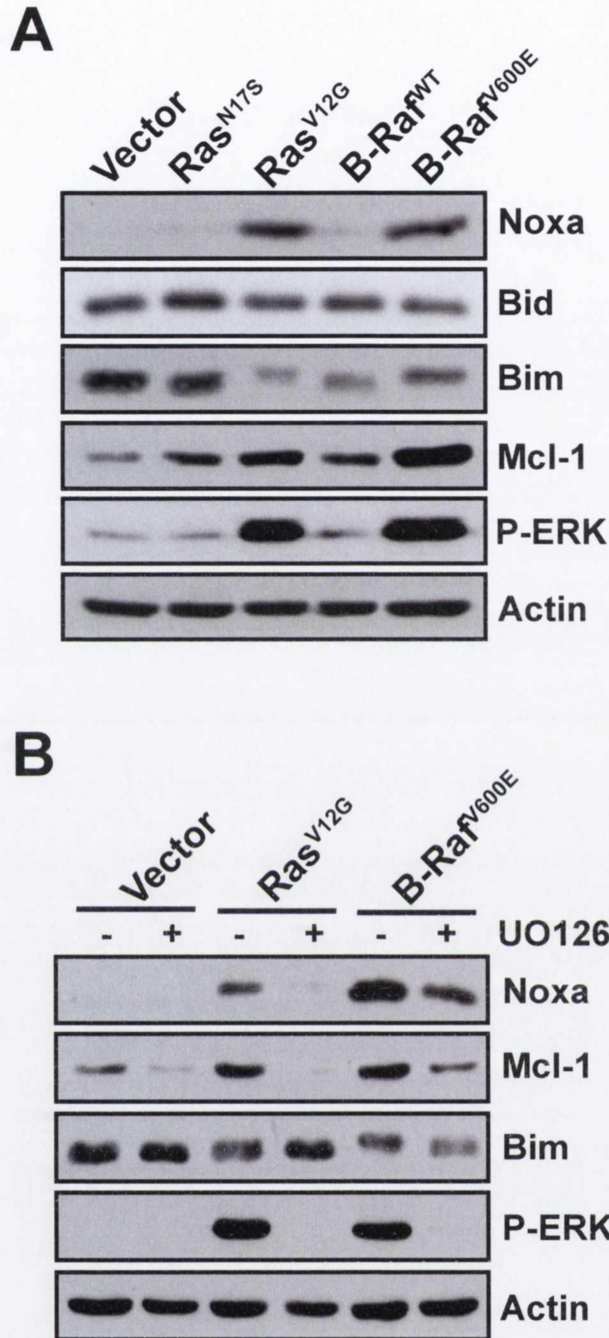


Figure 4.10

Oncogenic Ras and B-Raf upregulate Noxa in an ERK-dependent manner
HeLa cells were plated at 2 x 10^5 cells/well on 6 well plates. 24 h later, cells were transfected with Ras and B-Raf plasmids (1 μg). After 24 h, lysates were prepared and protein expression of the indicated proteins was assessed using Western Immunoblotting (A). HeLa cells were plated at 2 x 10^5 cells/well on 6 well plates. 24 h later, cells were transfected with Ras and B-Raf plasmids (1 μg) in the absence or presence of UO126 (20 μM). After 24 h, lysates were prepared and protein expression of the indicated proteins was assessed using Western Immunoblotting (A).

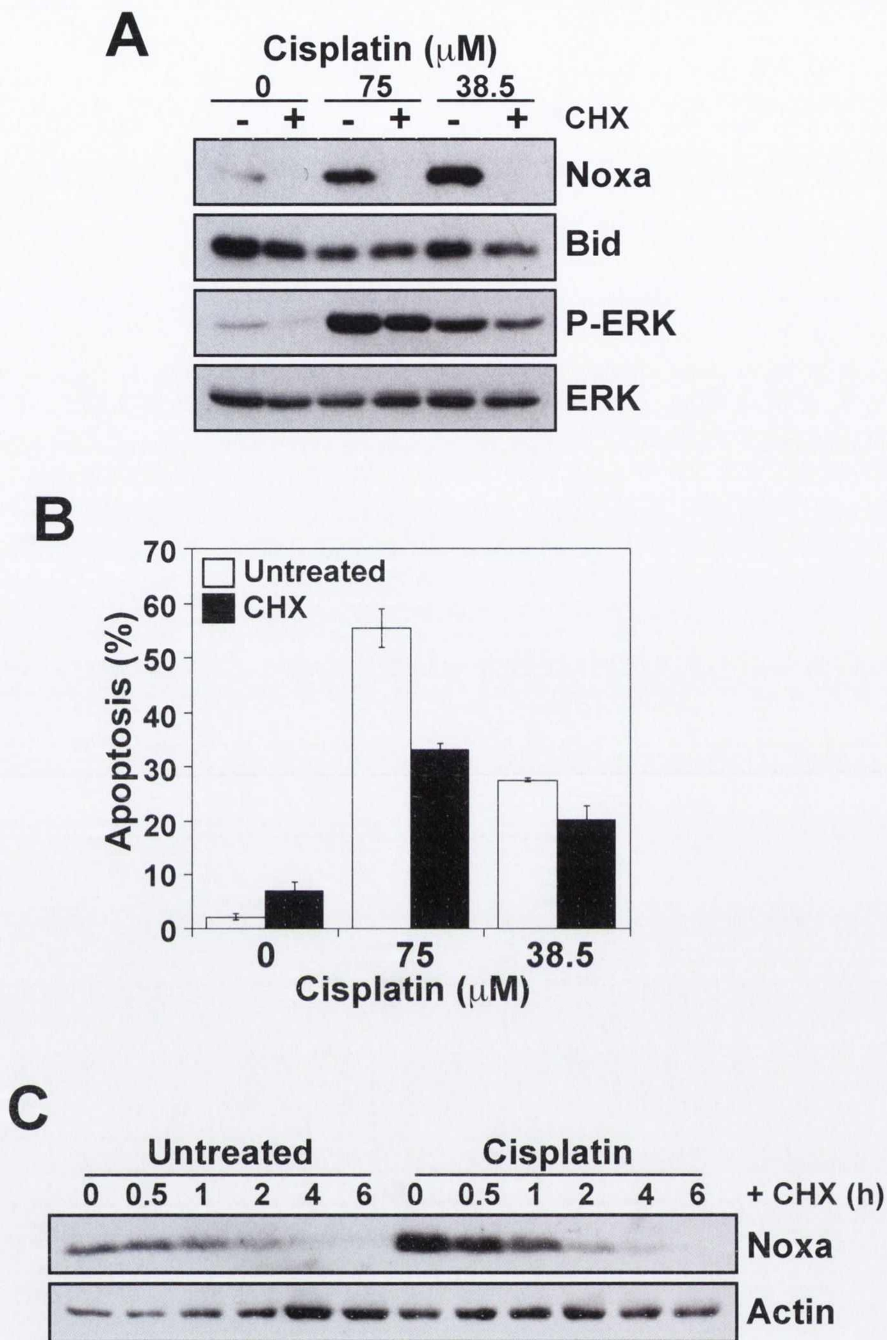


Figure 4.11

Noxa is transcriptionally upregulated by cisplatin

HeLa cells were plated at 2 x 10^5 cells/well on 6 well plates. 24 h later, cells were treated with Cisplatin in the absence or presence of the translational inhibitor Cycloheximide (CHX 10 μg/ml) for 12 h. Lysates were prepared and protein expression of the indicated proteins was assessed using Western Immunoblotting (A). HeLa cells were treated as described in (A) and apoptosis was enumerated based on the morphology of cells (B). Results represent triplicate counts of a representative experiment with error bars representing SEM. HeLa cells were plated at 5 x 10^5 cells/dish on 6 cm dishes. 24 h later, cells were treated with cisplatin (100 μM) for 6 h and then were treated Cycloheximide (CHX 10 μg/ml) for the indicated times. Lysates were prepared and protein expression of Noxa was assessed using Western Immunoblotting (C).

apoptosis or Noxa upregulation (Figure 4.12 A and B). As all of the siRNA utilised efficiently ablated expression of their target genes (Figure 4.12 C), these results demonstrate that p53, p73, Hif-1α and ATF4 are not involved in cisplatin-mediated Noxa regulation. Thus the transcription factor/s responsible are more likely to be ERK-regulated transcription factors that have not previously been linked to Noxa induction.

4.2.10 *Noxa is a critical mediator of cisplatin-induced apoptosis*

To determine the role of Noxa in cisplatin-induced cell death we ablated Noxa expression using siRNA. As controls we also ablated the expression of two other BH3-only proteins Bim and Bad. Reduction of Noxa levels had a dramatic effect on cisplatin-mediated death, while silencing of Bim and Bad had little effect (Figure 4.13 A and B). Noxa ablation also prevented carboplatin and oxaliplatin-induced apoptosis (data not shown). Significantly, Noxa ablation considerably enhanced cell survival following cisplatin treatment over a 7-day period (Figure 4.13 C). This demonstrates that Noxa is an important upstream mediator of cisplatin-induced apoptosis and removal of this protein provides more than a transient reprieve from cisplatin treatment. By preventing Noxa-mediated apoptosis, these cells can continue to proliferate.

Noxa induces apoptosis by binding to anti-apoptotic Bcl-2 family members, thus promoting the release and activation of Bax and Bak. These pro-apoptotic proteins then oligomerise and form pores in mitochondrial outer membranes resulting in cytochrome *c* release. To explore the role of Bax and Bak in cisplatin-induced apoptosis we ablated their expression, and for comparison we also ablated the expression of BH3-only proteins Noxa, Bim and Bad, using shRNA directed against these transcripts followed by treatment with cisplatin. Again, in contrast to Bim and Bad knockdown, Noxa ablation had a profound effect on cisplatin-mediated death (Figure 4.14 A and B). In addition, silencing of Bax and Bak also reduced apoptosis with Bak being the more important player of the pair. Double knockdown of Bax and Bak almost completely prevented cisplatin-induced cell death, demonstrating

that cisplatin causes death primarily through mitochondrial cytochrome *c* release and apoptosis rather than other forms of cell death.

4.2.11 *Mcl-1 modulates cisplatin-mediated apoptosis*

Noxa has been shown to bind to the anti-apoptotic Bcl-2 family members Mcl-1 and A1 (Chen *et al.*, 2005a; Alves *et al.*, 2006). However, A1 has a very restricted pattern of expression making Mcl-1 the most likely inhibitor of Noxa in tumor cells (Choi *et al.*, 1995). To examine the effect of Mcl-1 on cisplatin, we overexpressed Mcl-1 in HeLa cells and treated them with a range of concentrations of cisplatin. Mcl-1 dramatically inhibited cisplatin-induced apoptosis even at the highest concentrations used (Figure 4.15 A). We also ablated Mcl-1 levels in HeLa cells followed by treatment with cisplatin and found that reduction of Mcl-1 profoundly enhanced cisplatin-induced cell death (Figure 4.15 B and C). Thus modulating the balance of Noxa and Mcl-1 in cells is a critical determinant of cisplatin-mediated death. Therefore, reduction of Mcl-1 levels in response to cisplatin (Figure 4.1 C), carboplatin and oxaliplatin (Figure 4.8 B and D) may also be an important step in ensuring that apoptosis occurs in response to these compounds.

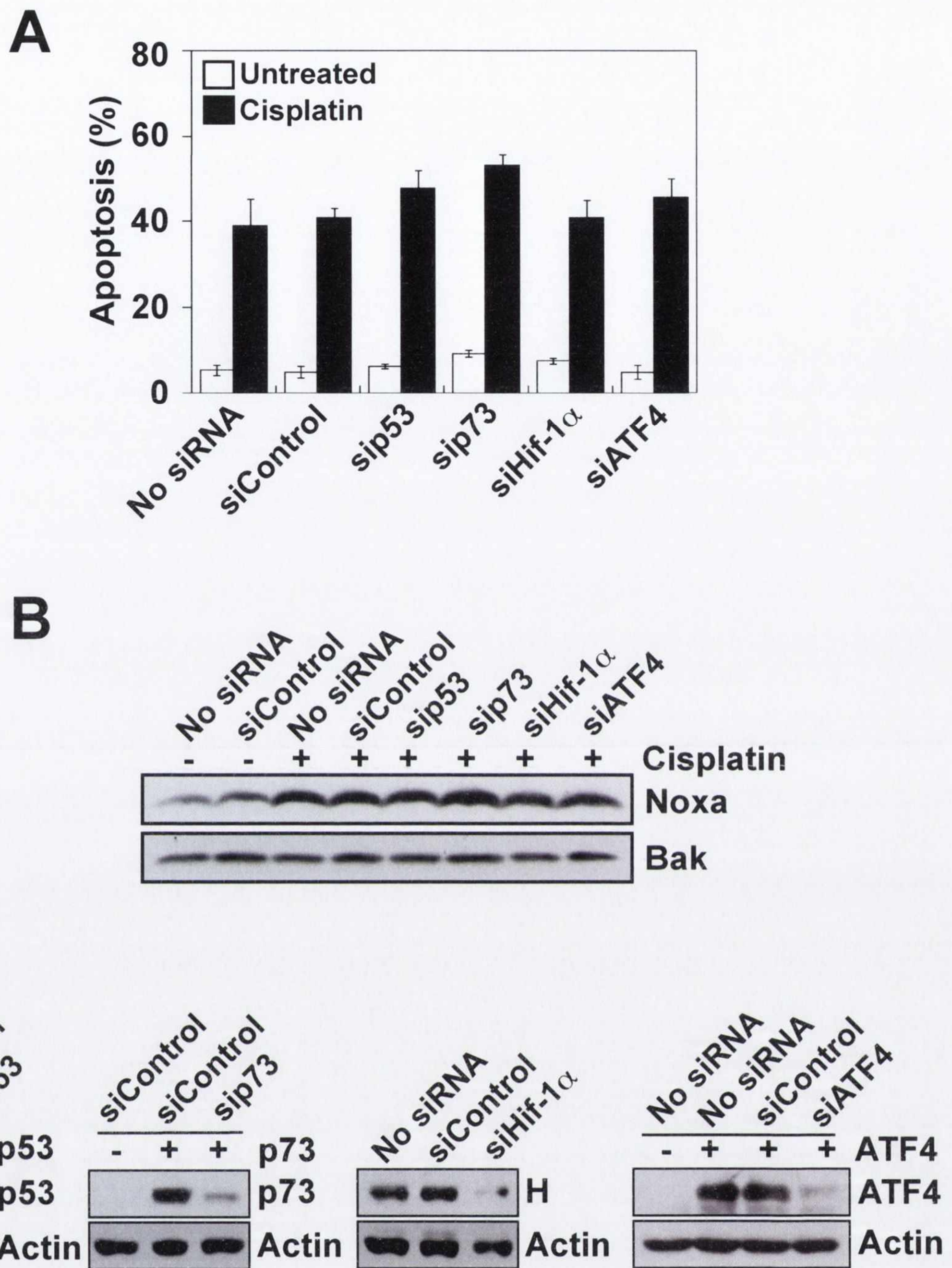


Figure 4.12

Known modulators of Noxa expression are not responsible for Noxa upregulation by cisplatin

HeLa cells were plated at 1 x 10^5 cells/well on 6 well plates. 24 h later, cells were transfected with control siRNA (150 nM) or siRNA against p53, p73, Hif-1α or ATF4. After 48 h, cells were treated with Cisplatin (50 μM) for 12 and apoptosis was enumerated based on morphology of cells (A). Results represent triplicate counts of a representative experiment with error bars representing SEM. Cells were treated as described in (A), lysates were prepared and protein expression of Noxa was assessed using Western Immunoblotting (B). Confirmation of siRNA efficiency, HeLa cells were transfected with p53, p73 or ATF4 together with their respective siRNA. After 48 h lysates were prepared and protein expression of the indicated proteins was assessed using Western Immunoblotting (A).

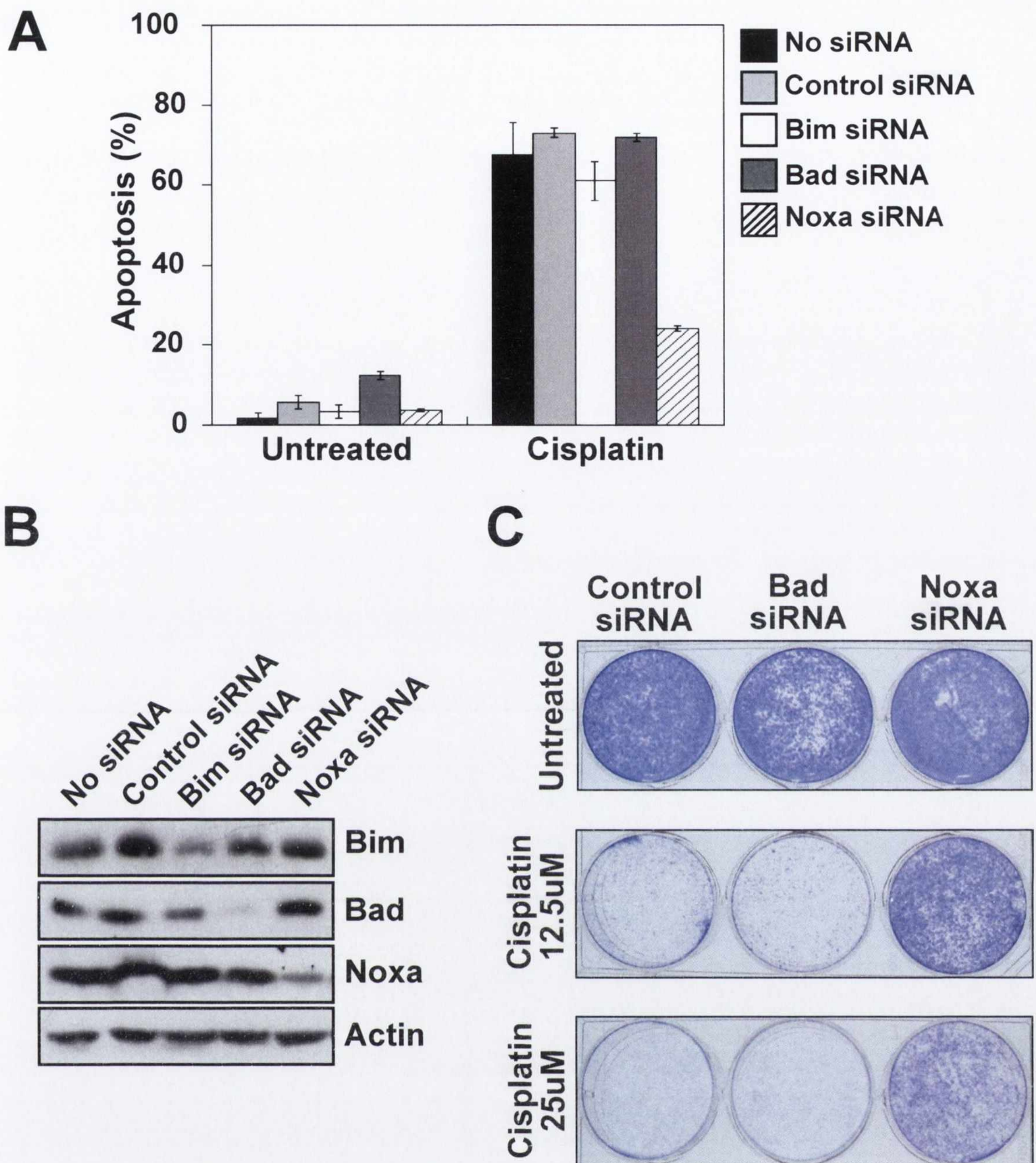
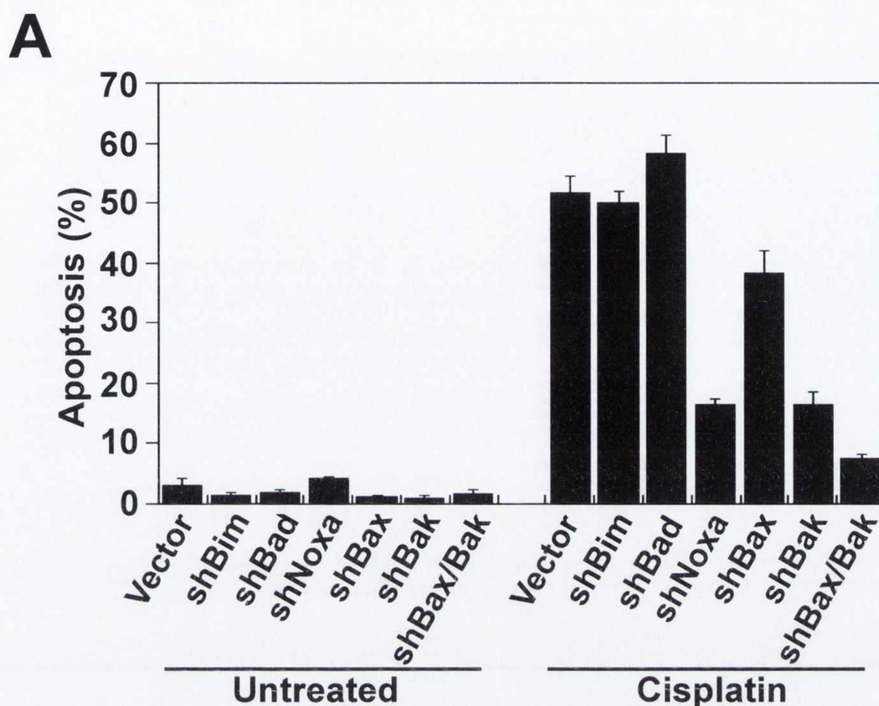


Figure 4.13

Noxa is important for Cisplatin-induced apoptosis

HeLa cells were plated at 1 x 10^5 cells/well on 6 well plates. 24 h later, cells were transfected with control siRNA (200nM) or siRNA against the pro-apoptotic proteins described. After 48 h, cells were treated with Cisplatin (50 μM) for 12 h and apoptosis was enumerated based on morphology of cells (A). Results represent triplicate counts of a representative experiment with error bars representing SEM. Lysates were prepared and protein expression of the indicated proteins was assessed using Western Immunoblotting (B). HeLa cells were plated at 5 x 10^4 cells/well on 6 well plates. 24 h later, cells were transfected with control siRNA (200 nM) or siRNA against Noxa and Bad. After 48 h, cells were treated with Cisplatin for 3 h. Cells were incubated at 37°C for 7 days and then stained with 0.5 % Crystal Violet viability stain (C).



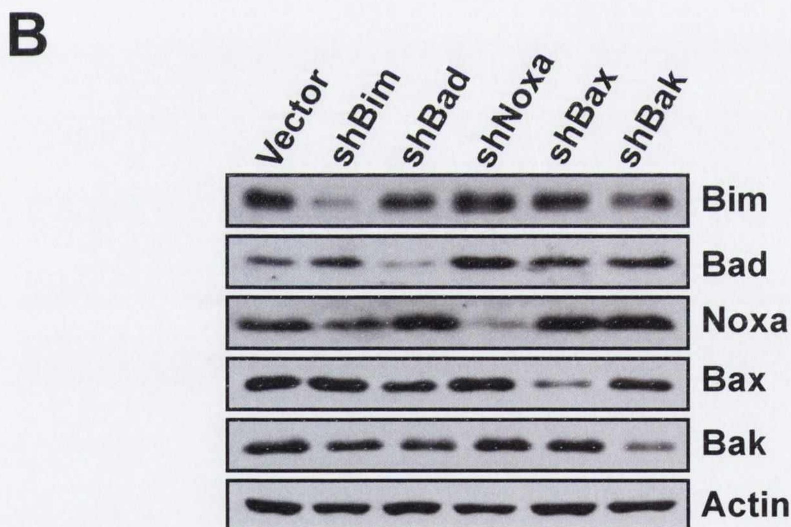


Figure 4.14

Noxa, Bax and Bak are important for Cisplatin-induced apoptosis

HeLa cells were plated at 1 x 10^5 cells/well on 6 well plates. 24 h later, cells were transfected with empty vector, or shRNA (600ng) directed against the pro-apoptotic proteins described. After 48 h, cells were treated with Cisplatin (50 μM) for 12 h and apoptosis was enumerated based on the morphology of cells (A). Results represent triplicate counts of a representative experiment with error bars representing SEM. Lysates were prepared and protein expression of the indicated proteins was assessed using Western Immunoblotting (B).

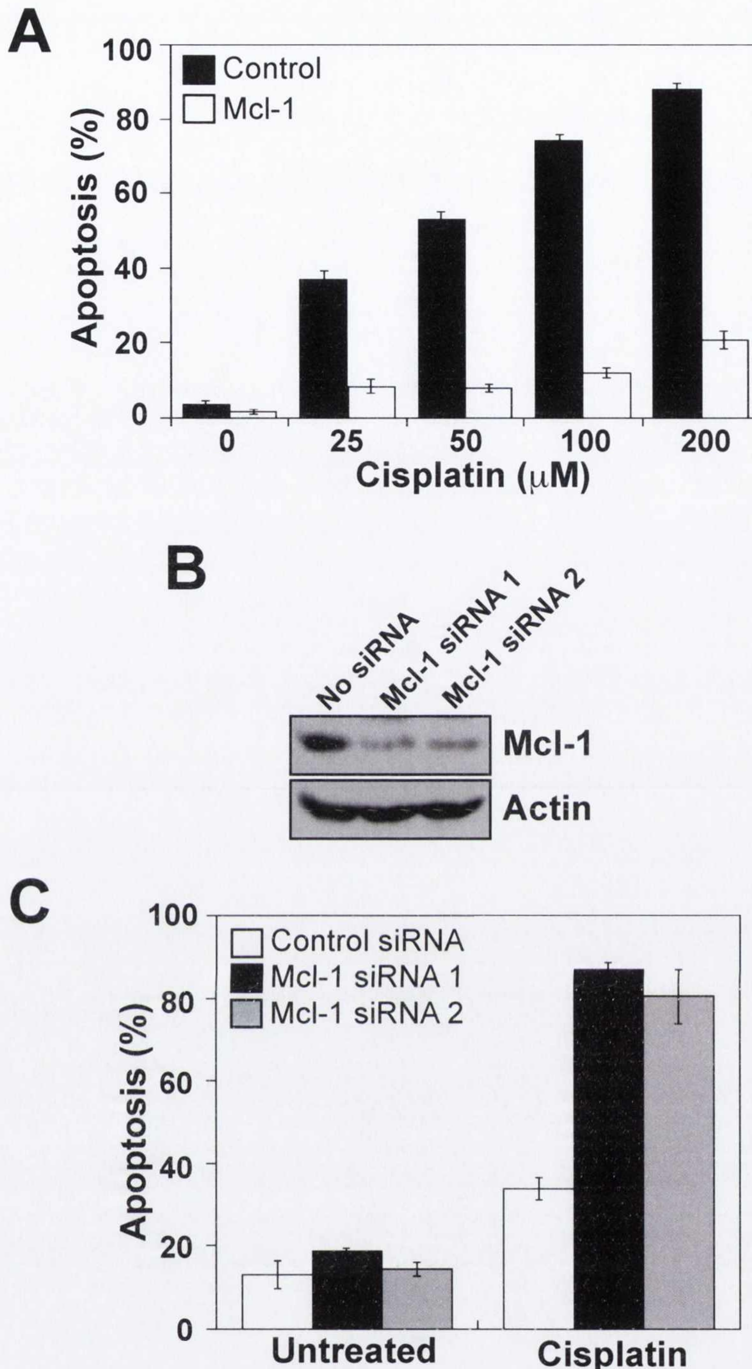


Figure 4.15

Mcl-1 is a potent inhibitor of cisplatin-induced apoptosis

HeLa cells were plated at 1 x 10^5 cells/well on 6 well plates. 24 h later, cells were transfected with empty vector, or Mcl-1 (200 ng). After 24 h, cells were treated with the indicated concentrations of Cisplatin for 12 and apoptosis was enumerated based on the morphology of cells (A). HeLa cells were plated at 1 x 10^5 cells/well on 6 well plates. 24 h later, cells were transfected with control siRNA or siRNA against Mcl-1 (200 nM). After 48 h, lysates were prepared and probed for Mcl-1 using Western Immunoblotting (B). HeLa cells transfected as described in (B) were treated with Cisplatin (50 μM) for 12 h and apoptosis was enumerated based on morphology(C). Graphed results represent triplicate counts of representative experiments with error bars representing SEM.

4.3 DISCUSSION

4.3.1 *Cisplatin in the treatment of cancers*

Cisplatin has been utilised as a chemotherapeutic agent for many years. It is most effective against testicular cancer where treatment provides eighty percent remission in early stage disease (Kollmannsberger *et al.*, 2006). Platninum compounds have also been effective in the treatment of ovarian, non-small-cell lung cancer and head and neck cancers (Muggia *et al.*, 2009; Cosaert *et al.*, 2002; Hao *et al.*, 2006). Development of oxaliplatin widened the scope of cancers efficiently targeted by platinum agents and this compound is used in therapy against colorectal cancers (Kim and Erlichman, 2007). However the efficacy of these cytotoxic agents is hampered by tumor resistance. Therefore it is important to gain a clear understanding of the exact mechanism of action of platinum drugs in order to establish the main causes of resistance.

4.3.2 *Upregulation of Noxa is important for cisplatin-induced apoptosis*

Here we explored cisplatin-induced apoptosis with particular attention to Bcl-2 family members. We observed dramatic upregulation of the BH3-only protein Noxa in response to cisplatin. In contrast, other BH3-only proteins were unaffected. Increased Noxa expression was transcriptional in nature, similar to previous reports describing Noxa regulation by other pro-apoptotic stimuli (Oda *et al.*, 2000; Sun and Leaman, 2005, Qin *et al.*, 2005). Surprisingly, cisplatin-induced Noxa expression was driven by the ERK pathway, rather than p53-mediated transcription. Importantly, Noxa upregulation is a critical step in cisplatin-induced apoptosis, as ablation of Noxa considerably inhibited cisplatin-mediated cytotoxicity. Noxa ablation also prevented carboplatin and oxaliplatin-mediated cell death, indicating that these platinum agents act through a conserved mechanism. Our results provide a crucial link between cisplatin-induced response to cellular damage and activation of pro-apoptotic BH3-only proteins, leading to cytochrome *c* release and apoptosis.

In addition to Noxa upregulation, we also observed concurrent decreases in Mcl-1 levels during cisplatin treatment. This event probably ensures that Noxa overcomes the anti-apoptotic signal provided by Mcl-1, facilitating Bax/Bak activation and apoptosis. Mcl-1 is a highly labile protein that is continually degraded by the proteasome. Thus, inhibition of Mcl-1 transcription caused by cisplatin may result in rapid depletion of cellular Mcl-1 levels. Alternatively, cisplatin may actively promote Mcl-1 removal through enhanced ubiquitination and degradation of this pro-survival protein. Our results establish that these two Bcl-2 family members are important determinants of cisplatin's effectiveness as a pro-apoptotic drug (Figure 4.16). Therefore the abundance of Noxa and Mcl-1 in target cells is likely to impact significantly on the cytotoxic action of platinum agents.

4.3.3 *Upregulation of Noxa by cisplatin is ERK-dependent*

Interestingly, we found that Noxa upregulation by cisplatin is ERK-dependent. Exploration of the role of ERK in cisplatin-mediated apoptosis revealed that ERK plays a novel pro-apoptotic function during cell death induced by platinum agents. Classically ERK has been associated with cell cycle progression and proliferation in response to growth factors and cytokines. ERK has also been associated with inhibition of apoptosis, as extensively shown in the previous chapter. Indeed cytokine withdrawl-induced cell death occurs as a result of Bim accumulation in cells where ERK signalling has been switched off (Weston *et al.*, 2003; O' Reilly *et al.*, 2009). In contrast to these observations we have demonstrated that, under certain circumstances, ERK hyperactivation can be harnessed for the production of a pro-apoptotic protein and can result in a cell death endpoint. Thus prolonged or intense activation of ERK may act as a signal of cell stress, thereby promoting the elimination of cells that are damaged beyond repair.

In addition to cisplatin-mediated Noxa upregulation, we also observed Noxa upregulation in response to oncogene-induced ERK activation. This may have an important role in protecting cells against tumor formation. Thus, activating mutations of the upstream regulators of ERK, such as Ras, B-Raf and EGFR, may

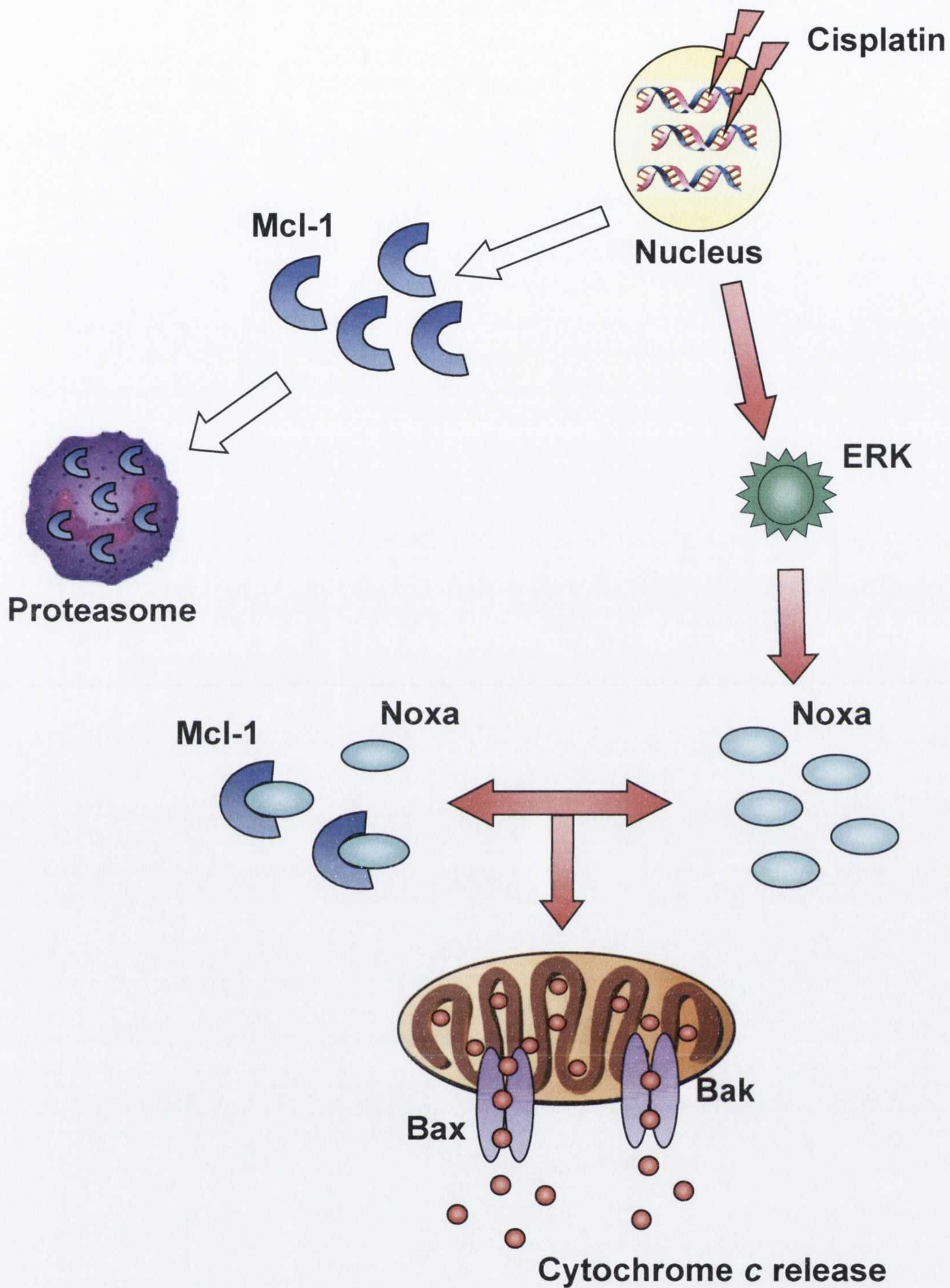


Figure 4.16

Cisplatin-induced apoptosis

Cisplatin is a DNA alkylating agent that crosslinks DNA, inhibiting transcription and replication. Cisplatin treatment of cells leads to ERK activation, and consequently, upregulation of the BH3-only protein Noxa. Noxa then promotes Bax/Bak-mediated cytochrome *c* release through inhibitory interactions with anti-apoptotic Mcl-1. Cisplatin also appears to promote degradation of Mcl-1 by the proteasome, thereby enhancing the apoptotic potential of Noxa.

lead to overexpression of Noxa and apoptosis. In some cases, nascent cancer cells find alternative routes to overcome Noxa overexpression, possibly by enhancing the expression of anti-apoptotic Bcl-2 family members such as Mcl-1. By restraining the death-inducing effects of Noxa, the latter event may facilitate uncontrolled cell division and tumor development. Interestingly, Noxa was first described as a phorbol myristic acid (PMA) responsive gene and PMA is a well known activator of ERK (Hijikata *et al.*, 1990; Nel *et al.*, 1990). Thus ERK may be also responsible for Noxa upregulation by PMA.

Activation of the other MAPKs, p38 MAPK and JNK, in response to cisplatin, has also been described and we also observed the activation of these kinases by cisplatin in HeLa cells (data not shown; Mansouri *et al.*, 2003; Losa *et al.*, 2003; Sanchez-Perez *et al.*, 1998). While our observations indicate that ERK may be the most important MAPK for cisplatin-mediated apoptosis and Noxa upregulation, it is possible that p38 MAPK and JNK may also enhance cell death through activation of other apoptosis-promoting molecules or by enhancing Noxa induction by ERK. Indeed these three MAPKs activate complementary transcription factors such as Fos, Jun and ATFs, and thus activation of all three kinases may lead to prolonged activity of transcription factors on the Noxa promoter and increased Noxa upregulation (Shaulian and Karin, 2001). Similarly, while p53 does not appear to play a role in Noxa upregulation downstream of ERK, activation of p53 by cisplatin may also induce Noxa expression, thereby providing a stronger apoptotic response.

4.3.4 *ERK activation by cisplatin*

Although a number of studies have observed ERK activation in response to cisplatin, the mechanism employed has remained elusive. Ras and Raf proteins are the most well established activators of ERK within the cell. However expression of a dominant negative form of Ras, Ras^{N17S}, had no impact on cisplatin-induced ERK activation or apoptosis in HeLa cells (our observations). A number of reports have described PKC activation by cisplatin and have proposed that PKC is an important mediator of cisplatin-induced death (Basu *et al.*, 2001; Nowak, 2002; Urso

et al., 2005). As PKC is known to activate ERK in response to certain mitogens, this kinase may provide the link between cisplatin-mediated DNA damage and ERK phosphorylation (Nel *et al.*, 1990). Interestingly, a previous study by Eldering and colleagues observed PKC-dependent Noxa expression following CD3 stimulation of T cells (Alves *et al.*, 2006). Of note, although cisplatin is understood to trigger cell death through a DNA damage-related mechanism, cisplatin also binds to numerous cellular proteins. Therefore activation of PKC and ERK may occur independently of DNA damage.

4.3.5 *Implications of Noxa upregulation by platinum agents*

Noxa is an important shared effector of platinum drugs and the extent of Noxa induction in various tumors will likely influence the anti-tumorigenic action of these agents. Cisplatin-resistance, either intrinsic or acquired, has impacted on the types of cancers treated with cisplatin and also the effectiveness of cisplatin as a second line chemotherapeutic agent. This resistance may occur through a number of mechanisms, including impairment of intracellular drug accumulation, drug inactivation by thiol-containing molecules, increased DNA damage repair or inhibition of apoptosis (Siddik, 2003). As Noxa is the final target of platinum compounds prior to cytochrome *c* release, upregulation of this protein could be used as a prognostic biomarker to predict the outcome of chemotherapeutic treatment with these drugs.

Another impairment to cisplatin treatment is toxicity to non-cancerous tissues, in particular nephrotoxicity and neurotoxicity. These side-effects are particularly undesirable in patients that become refractory to cisplatin treatment. Pre-assessment of Noxa upregulation in response to cisplatin, in individual patients, may determine whether this treatment will be effective. Those that fail to show Noxa induction are less likely to respond to platinum treatment and therefore may benefit from treatment with an alternative chemotherapeutic agent.

4.3.6 *Future of cisplatin chemotherapy*

Usually, chemotherapeutic drugs are not utilised as single agents, but rather in combination with other cytotoxic drugs to improve anti-tumorigenic activity. Cisplatin has been successfully used together with other pro-apoptotic drugs such as paclitaxel and histone deacetylase inhibitors. Given the central role of Noxa in platinum-mediated apoptosis, effective combinations may include other enhancers of Noxa expression such as bortezomib. Bortezomib is a proteasome inhibitor that dramatically upregulates Noxa and promotes Noxa-dependent cell death (Qin *et al.*, 2005; Fernandez *et al.*, 2005). Conversely, treatment of tumors with cisplatin, together with an inhibitor of Mcl-1 should enhance the pro-apoptotic function of Noxa. For this purpose, a BH3-mimetic obatoclax, which antagonises Mcl-1 through inhibitory interactions, may augment the cytotoxic action of platinum agents (Nguyen *et al.*, 2007). Both obatoclax and bortezomib have been utilised in clinical trials and bortizimab has already been approved for the treatment of some cancers (Schimmer *et al.*, 2008; Mateos and San Miguel, 2007). Alternatively, as Noxa targets anti-apoptotic Mcl-1, but not Bcl-2 or Bcl-xL, treatment with cisplatin and Bid activators such as TRAIL may produce a more comprehensive pro-apoptotic signal. Thus, unravelling the molecular mechanism of platinum-induced apoptosis is likely to impact on the choice of chemotherapeutic combinations utilised in the future.

Recent focus in platinum-related research has concentrated on developing methods to specifically target platinum drugs to tumor sites. Examples of this include platinum encapsulated nanoparticles that are targeted to prostate cells using prostate specific membrane antigen (PSMA), and co-polymer-linked platinum compounds that are released only in the acidic environment provided by tumors (Dhar *et al.*, 2008; Rice *et al.*, 2006). Achieving this aim will be extremely beneficial as it will facilitate the use of much higher drug concentrations and reduce off-target effects. Furthermore, new platinum compounds are continuously being investigated for enhanced anti-tumorigenic activity when compared with cisplatin. Picoplatin is the most successful to date, as this molecule is more resistant to glutathione-related inactivation and has a broader range of susceptible tumors (Kelland, 2007; Shah

and Dizon, 2009). Thus platinum-based therapy remains an integral part of chemotherapeutic action against human cancers and the identification of Noxa as a contributor to platinum-mediated apoptosis will hopefully impact on numerous anti-cancer regimes.

CHAPTER V

BAX- AND BAK-INDUCED MITOCHONDRIAL FRAGMENTATION CAN BE UNCOUPLED FROM CYTOCHROME *C* RELEASE

5.1 INTRODUCTION

In healthy cells, mitochondria are important organelles responsible for providing the bulk of cellular energy needs through the generation of ATP. Cytochrome *c*, a molecule found within mitochondria is one of the critical components of the electron transport chain of reactions that culminates in the production of ATP (Ow *et al.*, 2008). However, under conditions of extensive cellular stress or injury, mitochondria play an alternate role in promoting cell death progression through the release of cytochrome *c* from the mitochondrial intermembrane space. Cytosolic cytochrome *c* then binds the adapter protein APAF-1 triggering a conformational change, which permits recruitment of caspase-9 into a complex called the apoptosome (Adrain *et al.*, 2006). Activation of caspase-9 within the apoptosome is followed by caspase-3 and -7 activation and apoptosis (Logue and Martin, 2008). Release of cytochrome *c* from mitochondria is a critical step in the cell death process, as cells will die due to impairment of mitochondrial function even if downstream caspases are inhibited.

As the release of cytochrome *c* from mitochondria is a defining event in the fate of a cell, this event is highly regulated by multiple Bcl-2 family members. Augmentation of pro-apoptotic BH3-only proteins in response to cellular stress/damage ensures that these proteins are able to overcome the anti-apoptotic Bcl-2 family members (Labi *et al.*, 2006). This facilitates the release of the pro-apoptotic duo, Bax and Bak, from the constraints of anti-apoptotic Bcl-2 related proteins. Bax and Bak then oligomerise into multimers and form a pore in the mitochondrial outer membrane, which facilitates the release of cytochrome *c* and downstream caspase activation (Leber *et al.*, 2007, Chipuk *et al.*, 2008). Exactly how these proteins cause mitochondrial outer membrane permeabilisation (MOMP) is still unclear, however the composition and topology of mitochondrial membranes are likely to impact on this event.

Mitochondria do not exist as individual organelles within the cell, but rather are continuously fusing and dividing to form a network that covers the entire cell.

Remodelling of the mitochondrial network is facilitated by two sets of mitochondrial-associated proteins. Mitochondrial fusion proteins such as Mfn1, Mfn2 and Opa1 are GTPases that promote tethering of two adjacent mitochondria followed by GTP-dependent fusion of mitochondrial outer and inner membranes (Griffin *et al.*, 2006). Conversely mitochondrial fission proteins, Drp1 and Fis1, enhance mitochondrial tubule constriction and division of one large mitochondrion into two smaller mitochondria (Chan, 2006a).

During apoptosis, concurrently with cytochrome *c* release, mitochondria are dramatically reorganised from long filamentous tubules into small punctate spheres (Frank *et al.*, 2001; Gao *et al.*, 2001; Lee *et al.*, 2004). This is a Drp1 dependent event as ablation of Drp1 reduces apoptosis-associated mitochondrial fragmentation and it has been proposed that increased recruitment of Drp1 to mitochondrial sission sites during apoptosis triggers this fragmentation (Frank et al., 2001; Karbowski *et al.*, 2002; Estaquier *et al.*, 2007; Sugioka *et al.*, 2004; Cassidy-Stone *et al.*, 2008). However, to date it remains unclear whether this mitochondrial fragmentation facilitates cytochrome *c* release or is merely an accompanying event. Initial studies suggested that inhibition of Drp1 mediated mitochondrial fission prevented cytochrome *c* release (Frank *et al.*, 2001; Breckenridge *et al.*, 2003; Lee *et al.*, 2004; Germain *et al.*, 2005; Neuspiel *et al.*, 2005). However, other groups have demonstrated that while Drp1 ablation partially delayed cytochrome *c* release, apoptosis proceeded without hinderance (Parone *et al.*, 2006; Estaquier *et al.*, 2007). Similarly, modulation of mitochondrial fusion proteins in various studies showed opposing effects on cytochrome c release and apoptosis (Neuspiel *et al.*, 2005; Delivani *et al.*, 2006; Arnoult *et al.*, 2005).

Thus the aims of this chapter were to explore BH3-only protein-induced mitochondrial fission and to determine whether mitochondrial fission plays a role in mitochondrial outer membrane permeabilisation, cytochrome *c* release and apoptosis.

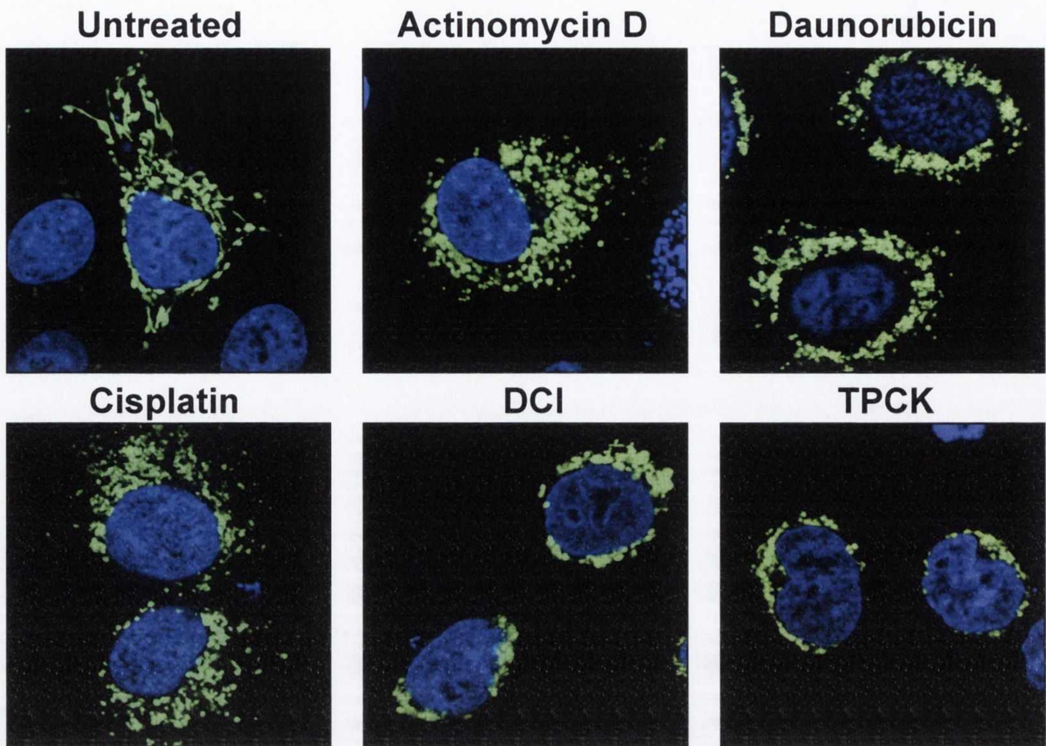
5.2 RESULTS

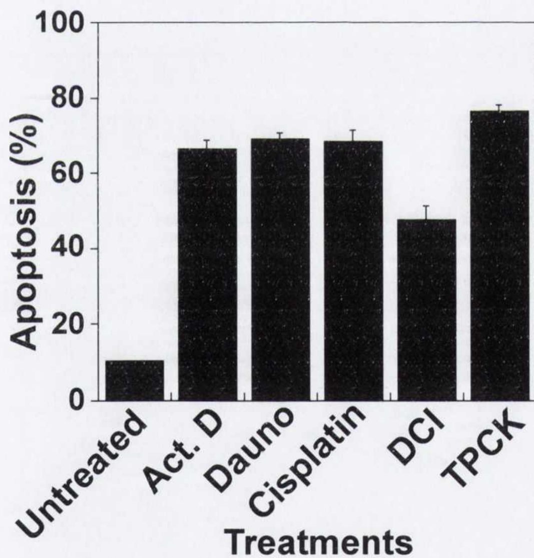
5.2.1 ***Mitochondrial fragmentation is a conserved feature of apoptosis***

To confirm previous reports describing mitochondrial fission during cell death, and to explore whether fission is a conserved feature of apoptosis, we assessed the mitochondrial morphology of HeLa cells treated with pro-apoptotic drugs. While untreated cells contained long filamentous mitochondria connected together to form a network, cells undergoing apoptosis displayed a dramatically fragmented mitochondrial morphology comprised of many individual spherical mitochondria (Figure 5.1 A). In addition, the mitochondrial network collapsed into a perinuclear localisation from the extended network seen in untreated cells. Enumeration of mitochondrial fission in response to cytotoxic drug treatment revealed that mitochondrial fragmentation and apoptosis occurred to a similar extent (Figure 5.1 B and C). Indeed, the level of mitochondrial fragmentation exceeded cell death indicating that this is occurs at an early stage of apoptosis, before the typical morphological features of dying cells are visible.

5.2.2 ***Mitochondrial morphology in cells overexpressing mitochondrial fission and fusion proteins***

Mitochondria are dynamic organelles that constantly fuse and divide to form a network throughout the cell. The rate of mitochondrial fission and fusion in cells determines the overall morphology of the mitochondrial network. Important fusion regulators include Mfn1, Mfn2 and Opa1, while Drp1 and Fis1 regulate mitochondrial fission. To examine the effect of these proteins on mitochondrial morphology we overexpressed each protein in HeLa cells. Cells were immunostained to confirm expression 24 hours later and mitochondrial morphology was visualised using DsRedmito, a mitochondrially-targeted red fluorescent protein (Figure 5.2 A). The percentage of cells displaying normal, fragmented, fused or elongated mitochondria was enumerated (Figure 5.2 B). As expected, overexpression of Mfn1 and Mfn2 produced highly fused mitochondria. These mitochondria displayed a perinuclear localisation and were no longer extended throughout the cell. Inhibition of Drp-1 mediated fission using a dominant negative





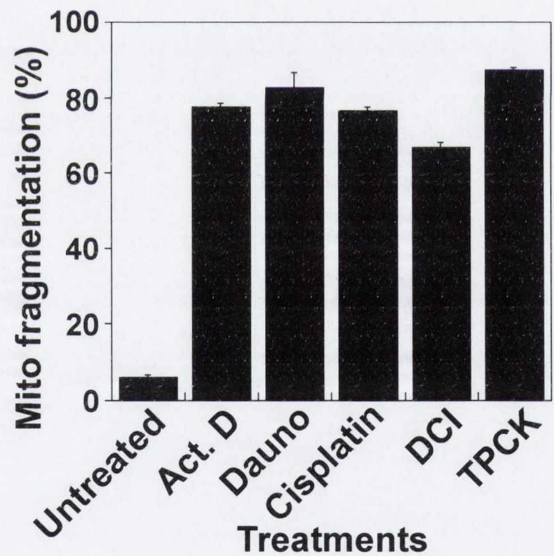
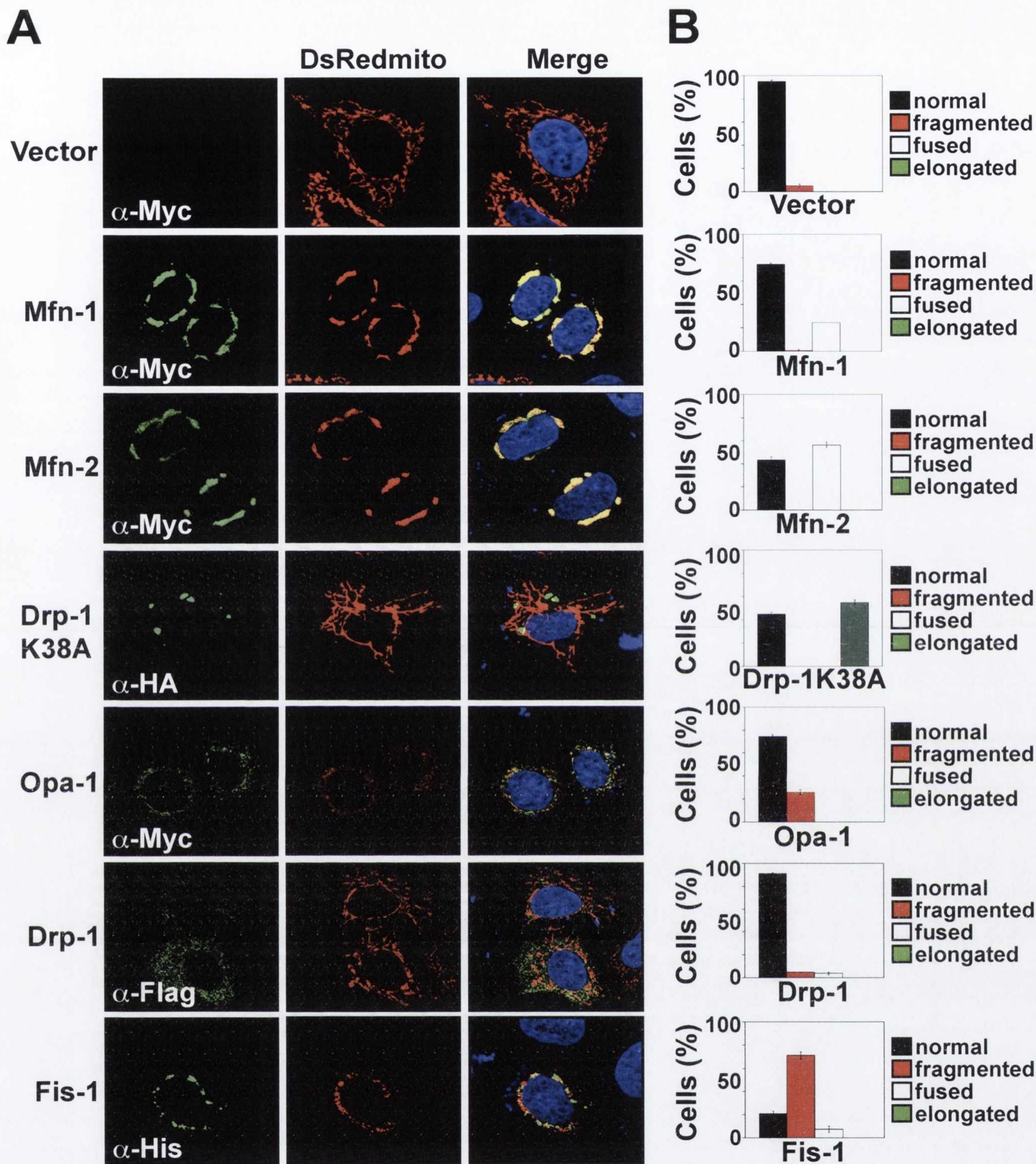


Figure 5.1

Mitochondrial fragmentation is a conserved feature of apoptosis

HeLa cells were plated at 2 x 10^5 cells/well on coverslips in 6-well plates. 24 h later, cells were transfected with pEF-mitoGFP (150 ng) and following 24 h of expression, cells were treated with Actinomycin D (1 μM), Daunorubicin (5 μM), Cisplatin (50 μM), DCI (75 μM), or TPCK (50 μM) for 18 h. Mitochondrial morphology was assessed by visual analysis of pictures taken by confocal microscopy with 600X magnification (A). Apoptosis (B) and mitochondrial fragmentation (C) were assessed in GFP-positive cells based on morphology. Results represent triplicate counts of representative experiments with error bars representing SEM.



<u>Figure 5.2</u>

Mitochondrial morphology in cells expressing mitochondrial fission and fusion proteins

HeLa cells were plated at 2 x 10^5 cells/well on coverslips in 6-well plates. 24 h later, cells were transfected with pDsRed-mito (100 ng) together with plasmids containing mitochondrial fission and fusion genes (800 ng). Following 24 h of expression, cells were fixed and immunostained with the indicated antibodies. Mitochondrial morphology was assessed using confocal microscopy (A) and enumerated in RFP-positive cells (B). Graphed results represent triplicate counts of a representative experiment with error bars representing SEM.

mutant, Drp1 K38A, produced elongated mitochondria, rather than the fused mitochondrial phenotype observed with Mfn overexpression. Surprisingly, Opa1, which is thought to be responsible for mitochondrial inner membrane fusion, did not produce a fused phenotype in HeLa cells, but rather induced some mitochondrial fragmentation. This may be due to a dominant-negative effect of Opa1 overexpression on the function of endogenous Opa1. Overexpression of the mitochondrial fission regulator Drp1 did not affect mitochondrial morphology, indicating that the absence of other rate-limiting fission proteins prevented mitochondrial fragmentation. In contrast, Fis-1 induced mitochondrial fission in almost all cells transfected.

5.2.3 *Mitochondrial fission and fusion regulators do not modulate apoptosis*

While mitochondrial fragmentation has been observed during apoptosis triggered by many different cellular stresses (Figure 5.1), the role of fragmentation in the progression of apoptosis has been hotly debated (Parone *et al.*, 2006; Arnoult, 2007; Cheung *et al.*, 2007; Suen *et al.*, 2008). Previous reports analysing the role of various fission and fusion regulators in cell death have produced opposing results leaving this question still unanswered (Frank *et al.*, 2001; Lee *et al.*, 2004; Parone *et al.*, 2006; Delivani *et al.*, 2006). To investigate the impact of fission and fusion proteins on cell death regulation, we overexpressed these proteins in HeLa cells and treated them with a titration of the pro-apoptotic drug, actinomycin D. With all drug concentrations used, overexpression of the fusion proteins Mfn1, Mfn2 and Opa1, or the fission inhibitor Drp1 K38A, did not delay or prevent apoptosis to any degree (Figure 5.3 A-C). Conversely, overexpression of Fis1 which causes mitochondrial fragmentation did not enhance apoptosis.

While overexpression of these proteins had no effect on apoptosis, it remained possible that they affected the rate of cytochrome *c* release from mitochondria in some manner. To assess this, we overexpressed mitochondrial fission and fusion proteins in HeLa cells, followed by actinomycin D treatment and then immunostained cells for cytochrome *c*. Enumeration of cells displaying cytosolic

cytochrome *c* revealed that mitochondrial fission and fusion regulators did not dramatically alter cytochrome *c* release in response to pro-apoptotic drug treatment (Figure 5.4 A-C). Fis1, which induced low levels of cytochrome *c* release and apoptosis when overexpressed alone, slightly enhanced actinomycin D mediated cytochrome *c* release. However, this was not reflected by increased apoptosis (Figure 5.4 A, Figure 5.3 A). Similarly Drp1 K38A dominant negative mutant partially reduced cytochrome *c* release following actinomycin D treatment but offered no protection against apoptosis (Figure 5.4 C, Figure 5.3 C). Furthermore, mitochondrial fission and fusion proteins had no effect on apoptosis or cytochrome *c* release induced by other apoptosis initiators such as TPCK and DCI drug treatment or Bim overexpression (data not shown).

5.2.4 *Cytochrome c release can occur in the absence of mitochondrial fragmentation*

While it was clear that overexpression of proteins promoting mitochondrial fusion did not alter the levels of cell death in the bulk population when treated with pro-apoptotic drugs, we wished to look more closely at the effect of mitochondrial fission/fusion on apoptosis. Two questions emerged from the previous experiments; (1) Do cells containing highly fused/elongated mitochondrial networks preferentially prevent cytochrome *c* release? and (2) Does pro-apoptotic drug treatment overcome the fused/elongated phenotypes displayed by Mfn1, Mfn2 and Drp1 K38A overexpressing cells? To address these questions we overexpressed fusion proteins in HeLa cells together with a mitochondrially-targeted green fluorescent protein, mitoGFP. We then treated such cells with actinomycin D and immunostained for cytochrome *c*. This allowed us to compare mitochondrial morphology and cytochrome c release within the same cells. Strikingly, we found that cytochrome *c* release still occurred in response to actinomycin D from highly fused or elongated mitochondria (Figure 5.5 A). Furthermore, while cells treated with actinomycin D had reduced the levels of fused and elongated mitochondria, there were still many fused mitochondria that showed cytochrome *c* release (Figure 5.5 B and C). Thus although cytochrome *c* release is usually accompanied by

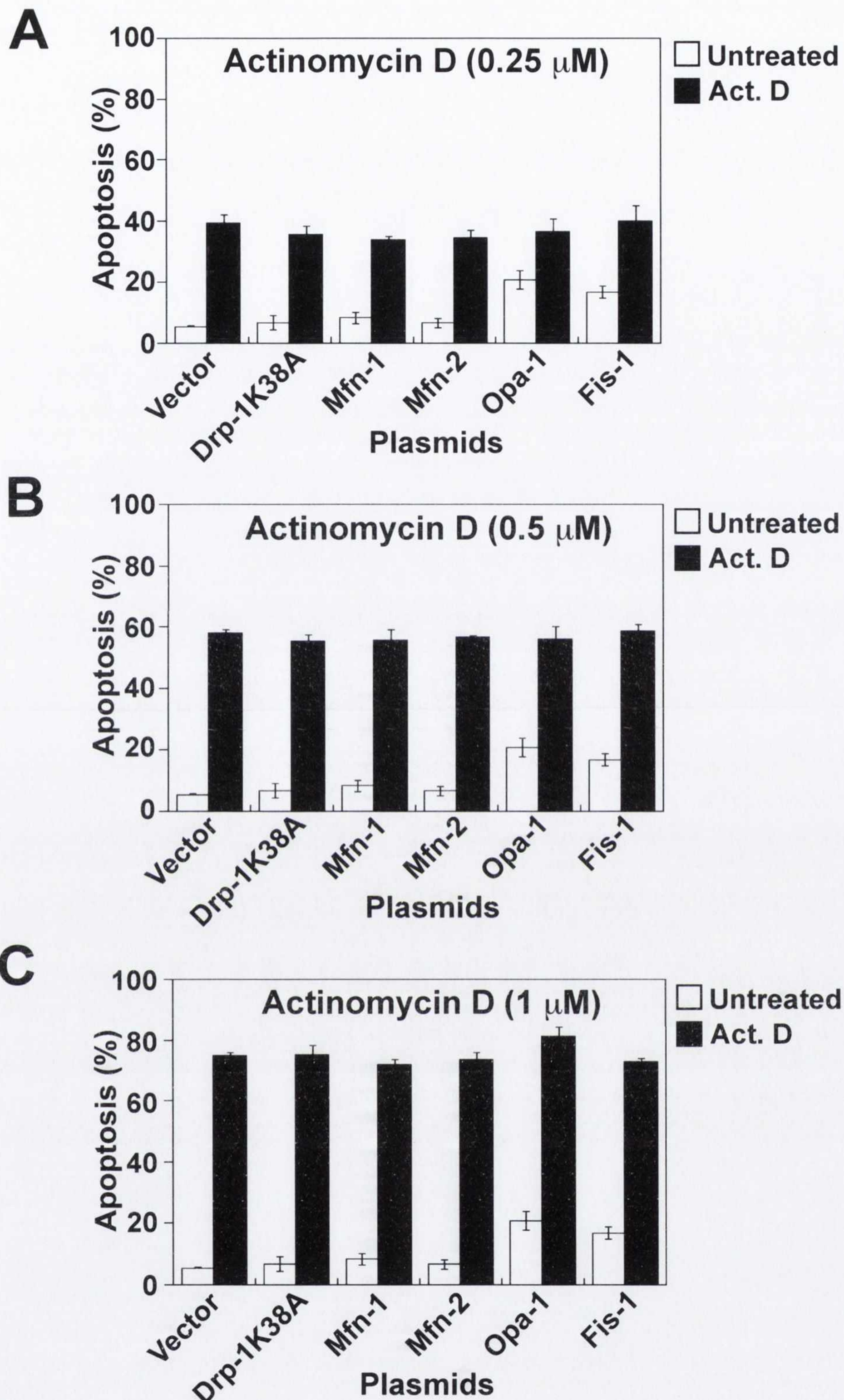


Figure 5.3

Mitochondrial fission and fusion regulators do not modulate apoptosis

HeLa cells were plated at 1 x 10^5 cells/well on 6-well plates. 24 h later, cells were transfected with a GFP-reporter plasmid (50 ng) together with plasmids containing mitochondrial fission and fusion genes (800 ng). Following 24 h of expression, cells were treated with Actinomycin D; 0.25 μM (A), 0.5 μM (B), or 1 μM (C) for 12 h. Apoptosis was assessed in GFP-positive cells based on morphology. Results represent triplicate counts of a representative experiment with error bars representing SEM.

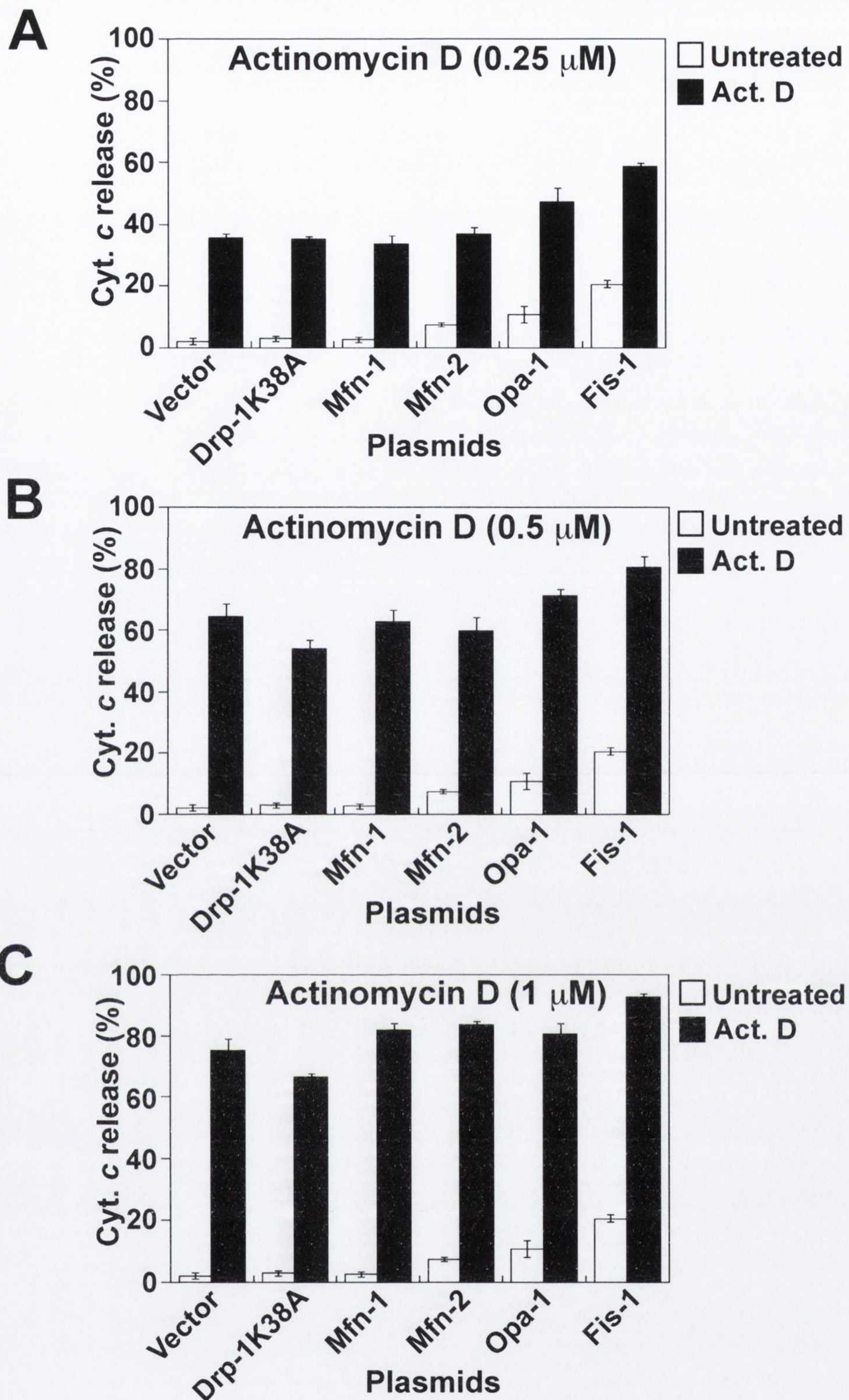
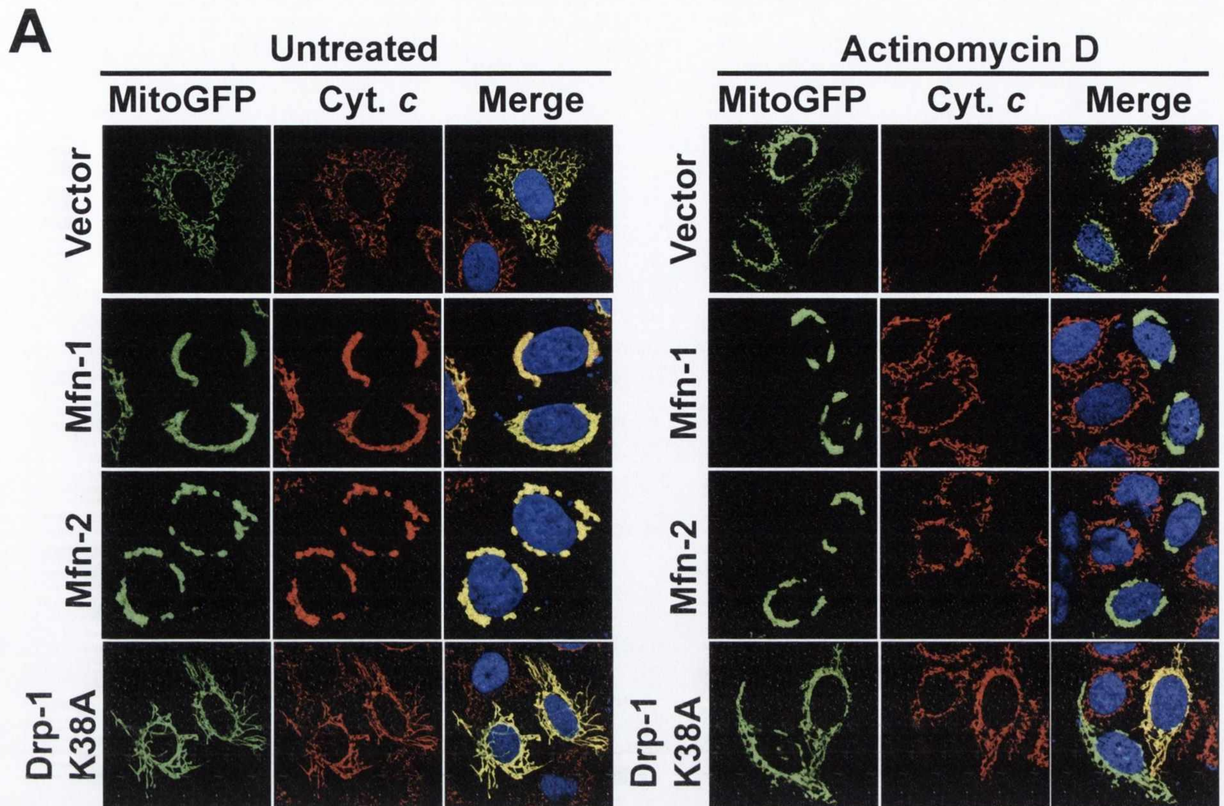
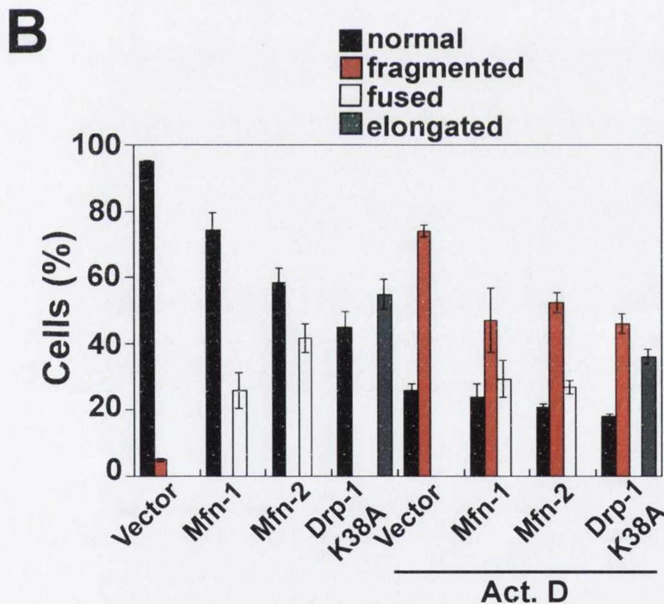


Figure 5.4

Mitochondrial fission and fusion regulators do not modulate cytochrome *c* release from mitochondria during apoptosis

HeLa cells were plated at 2 x 10^5 cells/well on coverslips in 6-well plates. 24 h later, cells were transfected with a pEF-mitoGFP (150 ng) together with plasmids containing mitochondrial fission and fusion genes (800 ng). Following 24 h of expression, cells were treated with Actinomycin D; 0.25 μM (A), 0.5 μM (B), or 1 μM (C) in the presence of Z-VAD.fmk (50 μM) to prevent apoptosis. 12 h later, cells were then fixed, immunostained for cytochrome *c* and release of cytochrome *c* from mitochondria in GFP-positive cells was enumerated. Results represent triplicate counts of representative experiments with error bars representing SEM.





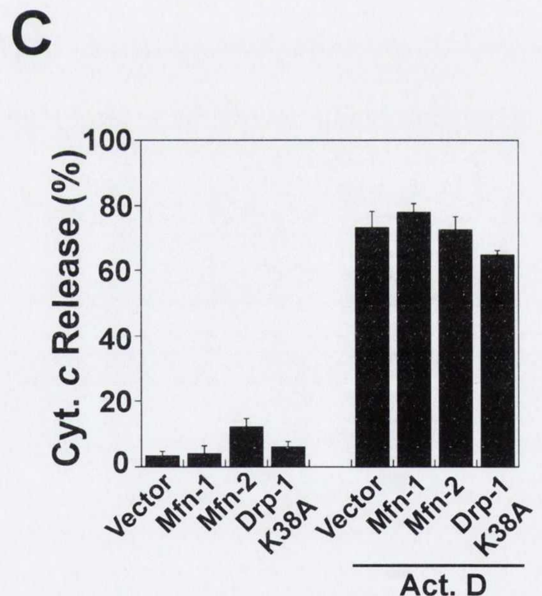


Figure 5.5

Cytochrome *c* release occurs in the absence of fragmentation

HeLa cells were plated at 2 x 10^5 cells/well on coverslips in 6-well plates. 24 h later, cells were transfected with a pEF-mitoGFP (150 ng) together with plasmids containing mitochondrial fission and fusion genes (800 ng). Following 24 h of expression, cells were treated with Actinomycin D (0.5 μM) and Z-VAD.fmk (50 μM) for 12 h. Cells were then fixed, immunostained for cytochrome *c* and mitochondria were visualised using confocal microscopy (A). Mitochondrial morphology (B) and cytochrome *c* release (C) in GFP-positive cells were enumerated. Results represent triplicate counts of representative experiments with error bars representing SEM.

mitochondrial fragmentation, this event is not required for release of this protein from the mitochondrial intermembrane space.

5.2.5 *Mitochondrial fusion regulators do not modulate Smac release from mitochondria during apoptosis*

In healthy cells, cytochrome *c* resides in the mitochondrial intermembrane space. However it is further sequestered within this compartment into pockets formed by folds in the inner mitochondrial membrane, called cristae (Ow *et al.*, 2008). Opa1 is found as oligomers at mitochondrial cristae junctions and this protein is important for maintaining cristae structure and regulating cristae junction opening (Olichon *et al.*, 2003; Arnoult *et al.*, 2005a; Frezza *et al.*, 2006). Previous reports have suggested that while inhibition of mitochondrial fission may not prevent Bax/Bak-mediated pore formation in mitochondrial outer membranes, cytochrome *c* release may be preferentially blocked in comparison to other intermembrane space proteins due to its localisation within mitochondrial cristae (Parone et al., 2006; Estaquier and Arnoult, 2007). To investigate whether fusion proteins differentially affected cytochrome *c* release, and release of other mitochondrial intermembrane proteins, we compared the release of cytochrome *c* and smac from mitochondria in cells treated with actinomycin D. As Figure 5.6 A shows, release of cytochrome *c* and smac occurred to a similar extent in cells treated with actinomycin D. Release of both these proteins was largely unaffected by Mfn2 or Drp1 K38A overexpression and the associated mitochondrial fusion/elongation induced by these proteins (Figure 5.6 B and C).

5.2.6 *Bax- and Bak-induced mitochondrial fragmentation can be uncoupled from cytochrome c release – Bcl-xL-mediated uncoupling*

From the previous experiments it was clear that mitochondrial fragmentation is a feature of apoptosis, however this event does not play a critical role in the progression of cell death. We wished to explore apoptosis-associated mitochondrial fission in greater detail. Bax overexpression induces mitochondrial fission and apoptosis, providing a useful model of cell death-associated mitochondrial

fragmentation (Delivani *et al.*, 2006). As anti-apoptotic Bcl-2 family members competently inhibit Bax-mediated apoptosis, we wondered whether they could also block Bax/Bak-mediated mitochondrial fragmentation.

To address this question we expressed Bax and Bak either alone, or in combination with Bcl-xL, in HeLa cells together with mitochondrially targeted RFP. The poly-caspase inhibitor, Z-VAD.fmk, was added to prevent caspase-dependent cell detachment. After 24 hours, cells were fixed and immunostained for cytochrome *c* to allow comparison of mitochondrial morphology and localisation of cytochrome *c*. As expected, Bax and Bak expression led to mitochondrial fission and cytochrome *c* release from mitochondria (Figure 5.7). Surprisingly, when Bcl-xL was co-expressed with Bax and Bak, cytochrome *c* release was inhibited but mitochondrial fragmentation still occurred (Figure 5.7). Enumeration of apoptosis and cytochrome *c* release in transfected cells revealed that while Bax and Bak potently induced cytochrome *c* release and apoptosis within 24 hours, co-expression of Bcl-xL efficiently blocked these events (Figure 5.8 A and B). In contrast, when mitochondrial morphology was assessed, we observed that Bax and Bak both induced high levels of mitochondrial fission when expressed alone and also when expressed together with Bcl-xL (Figure 5.8 C).

We further compared Bax-induced mitochondrial fragmentation in the absence and presence of Bcl-xL using fluorescence recovery after photobleaching (FRAP). With this method, cells expressing various proteins were incubated with a mitochondrial stain (mitotracker) for one hour. A selected portion of the mitochondrial network was then subjected to a brief pulse of high intensity laser illumination, and the recovery of fluorescence in this area was assessed over a two minute period. Bleached mitochondria that are undergoing constant fission and fusion will regain fluorescence through fusion with neighbouring fluorescent mitochondria. However fragmented mitochondria will continue to display weak fluorescence as they remain separated from the rest of the mitochondrial network. We expressed Drp1 K38A, Bax and Bcl-xL in HeLa cells and determined the ability of mitochondria to recover

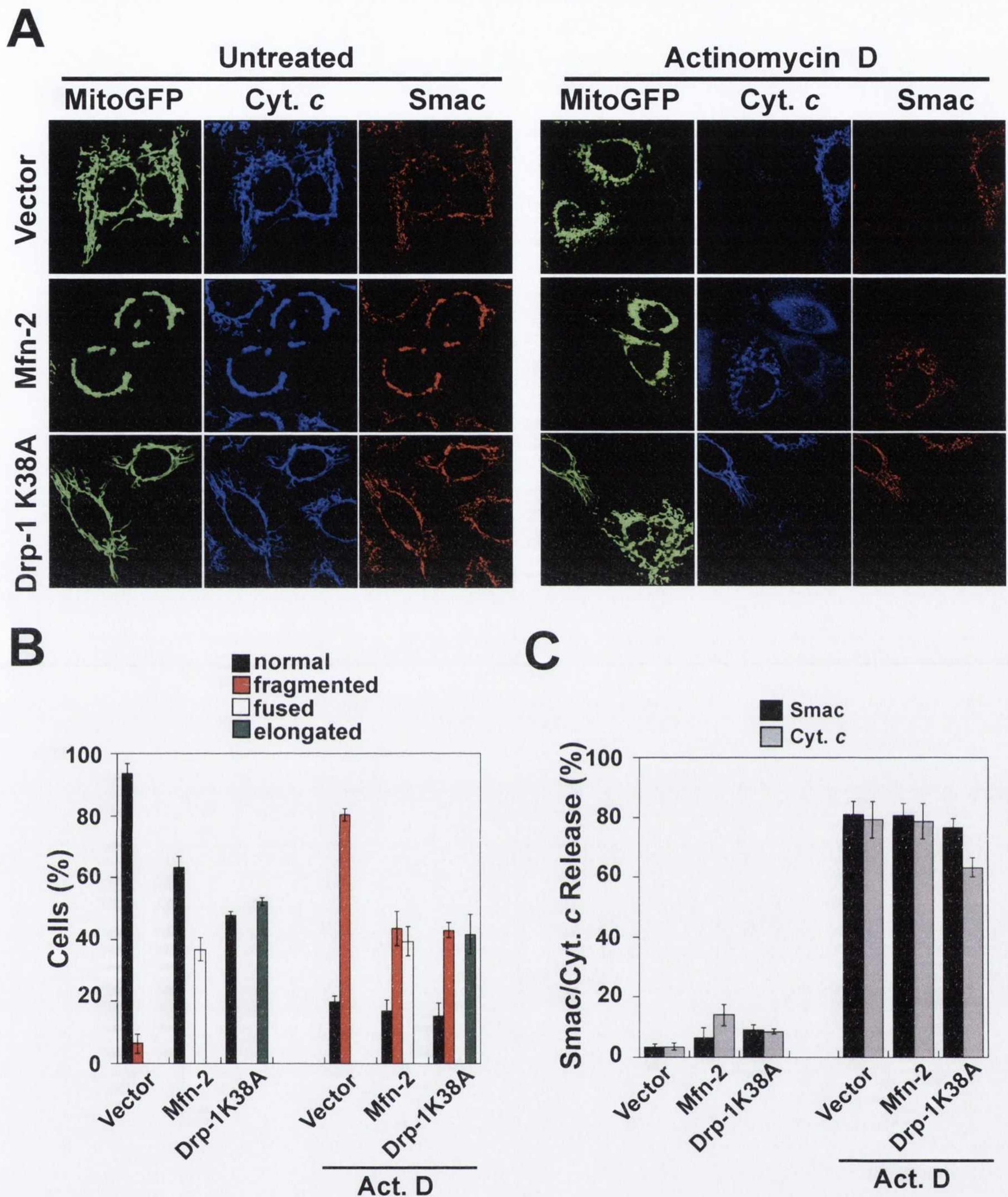


Figure 5.6

Mitochondrial fusion regulators do not modulate Smac release from mitochondria during apoptosis

HeLa cells were plated at 2 x 10^5 cells/well on coverslips in 6-well plates. 24 h later, cells were transfected with a pEF-mitoGFP (150 ng) together with plasmids containing mitochondrial fusion genes (800 ng). Following 24 h of expression, cells were treated with Actinomycin D (0.5 μM) and Z-VAD.fmk (50 μM) for 12 h. Cells were then fixed, immunostained for cytochrome *c* and smac, and mitochondria were visualised using confocal microscopy (A). Mitochondrial morphology (B) and cytochrome *c*/smac release (C) in GFP-positive cells were enumerated. Results represent triplicate counts of representative experiments with error bars representing SEM.

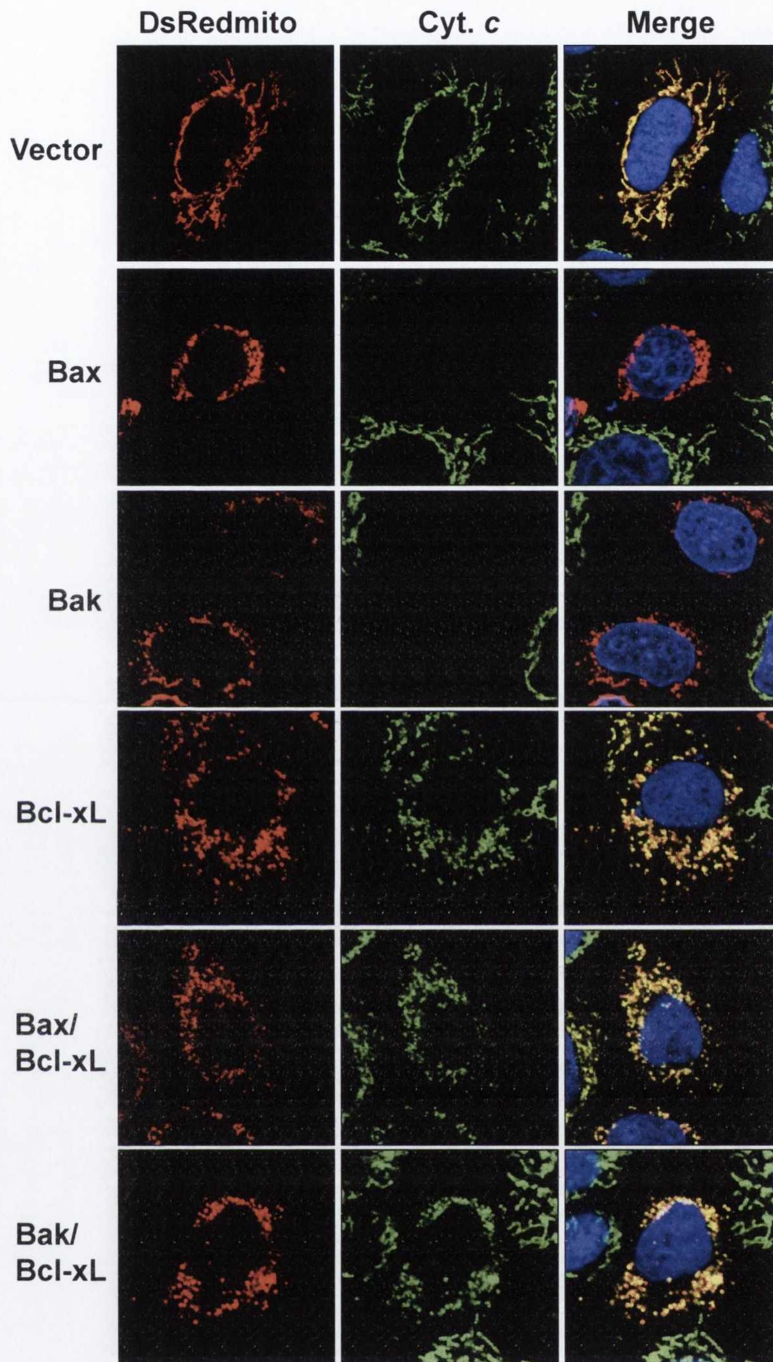


Figure 5.7

Bax and Bak induce mitochondrial fragmentation independently of cytochrome *c* release - separation by Bcl-xL

HeLa cells were plated at 2 x 10^5 cells/well on coverslips in 6-well plates. 24 h later, cells were transfected with a pDsRedmito (100 ng) together with Bax or Bak plasmids (200 ng) and Bcl-xL plasmid (800ng) in the presence of Z-VAD.fmk (50 μM) to prevent apoptosis. Following 24 h of expression, cells were fixed, immunostained for cytochrome *c* and mitochondria were visualised using confocal microscopy.

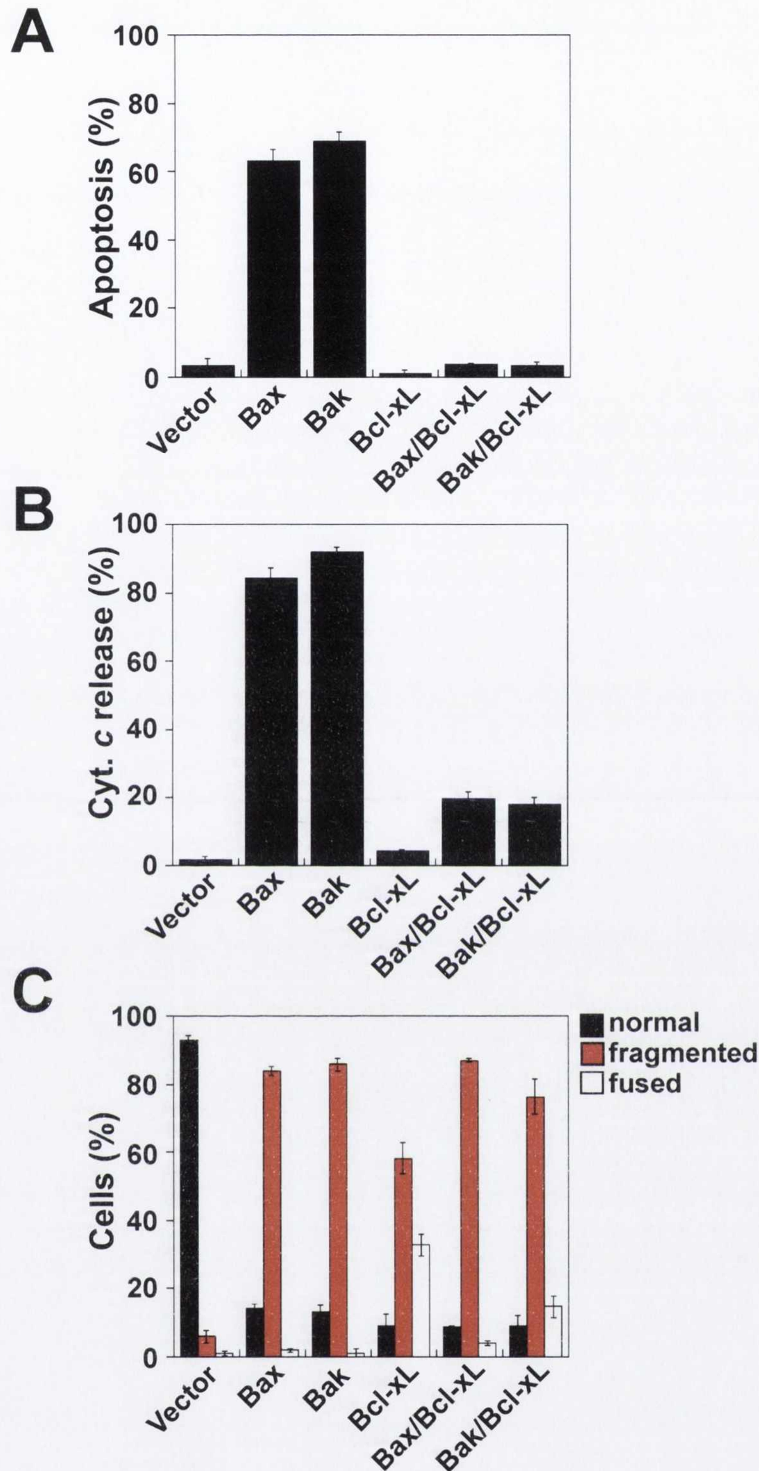
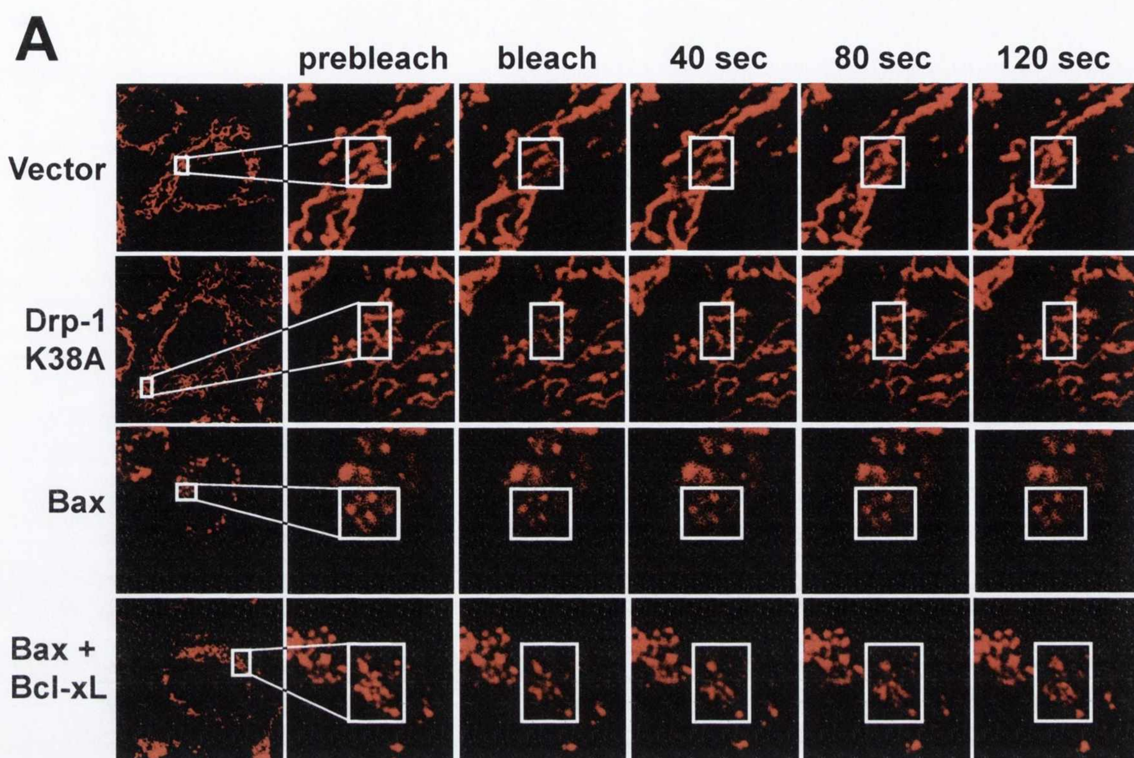


Figure 5.8

Bax and Bak induce mitochondrial fragmentation independently of cytochrome *c* release - separation by Bcl-xL

HeLa cells were plated at 2 x 10^5 cells/well on 6-well plates. 24 h later, cells were transfected with a pDsRed reporter plasmid (100 ng), together with Bax or Bak plasmids (200 ng) and Bcl-xL plasmid (800ng), in the presence of Z-VAD.fmk (50 μM) to prevent apoptosis where necessary. Following 24 h of expression, apoptosis was assessed in transfected cells based on morphology (A). Alternatively, cells were fixed, immunostained for cytochrome *c* and cytochrome *c* release (B) or mitochondrial morphology (C) in RFP-positive cells was enumerated. Results represent triplicate counts of representative experiments with error bars representing SEM.



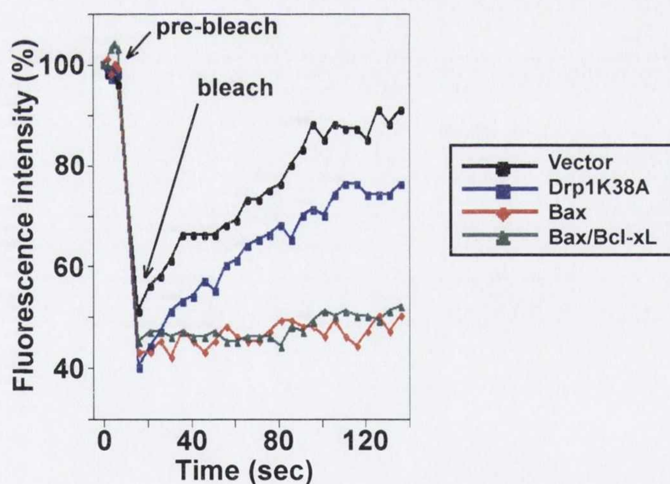


Figure 5.9

FRAP analysis of Bax-induced mitochondrial fragmentation

HeLa cells were plated at 2 x 10^5 cells/dish on glass-bottomed tissue culture dishes. 24 h later, cells were transfected with a pEF-mitoGFP (150 ng) together with Bax plasmid (200 ng) and Bcl-xL or Drp1 K38A plasmids (800ng) in the presence of Z-VAD.fmk (50 μM) to prevent apoptosis. Following 24 h of expression, cells were incubated in Mitotracker CMXRos (50 nM) for 1 h. GFP-positive mitochondria were bleached using a brief pulse of high intensity laser illumination. Images of bleached mitochondria at the indicated timepoints were taken (A) and flourescence recovery over time was measured (B).

fluorescence after laser illumination. We observed that mitochondrial-associated fluorescence from vector and Drp1 K38A transfected cells was recovered by more than 50% after photobleaching. However, fluorescence in mitochondria from Bax alone and Bax/Bcl-xL expressing cells did not increase following photobleaching, demonstrating that mitochondrial networks in the latter treatments are fragmented (Figure 5.9 A and B).

5.2.7 *Bax- and Bak-induced mitochondrial fragmentation can be uncoupled from cytochrome c release – Mcl-1-mediated uncoupling*

Co-expression of Bax and Bcl-xL revealed that Bax-induced mitochondrial fission can be separated from cytochrome c release. However, as Bcl-xL expression alone causes mitochondrial fission in some cells, it remained possible that the fission observed in Bax/Bcl-xL expressing cells was due to Bcl-xL-mediated effects on mitochondria rather than Bax (Figure 5.8 C). To exclude this, we co-expressed Bax and Bak with Mcl-1, which has no effect on mitochondrial dynamics when expressed alone. As described previously, Bax and Bak induced mitochondrial fragmentation and cytochrome *c* release. However, when Bax or Bak were co-expressed with Mcl-1, cytochrome *c* release was inhibited but mitochondrial fragmentation was still evident (Figure 5.10). Cell counts revealed that Mcl-1 potently antagonised the apoptosis-promoting function of Bax and Bak, however the effect of these proteins on mitochondrial morphology was unchanged (Figure 5.11 A-C). These results again demonstrate that Bax and Bak promote mitochondrial fragmentation independently of cytochrome c release.

Using FRAP analysis, we observed that vector and Mcl-1 transfected cells contained filamentous mitochondria that could recover fluorescence after photobleaching. However, mitochondria from cells expressing Bax or Bak, either alone or in combination with Mcl-1, were fragmented and no longer fused with neighbouring mitochondria. Thus, these cells retained the same fluorescence intensity for the duration of FRAP analysis (Figure 5.12 A and B). Furthermore, the average length of individual mitochondria in cells transfected with vector or Mcl-1

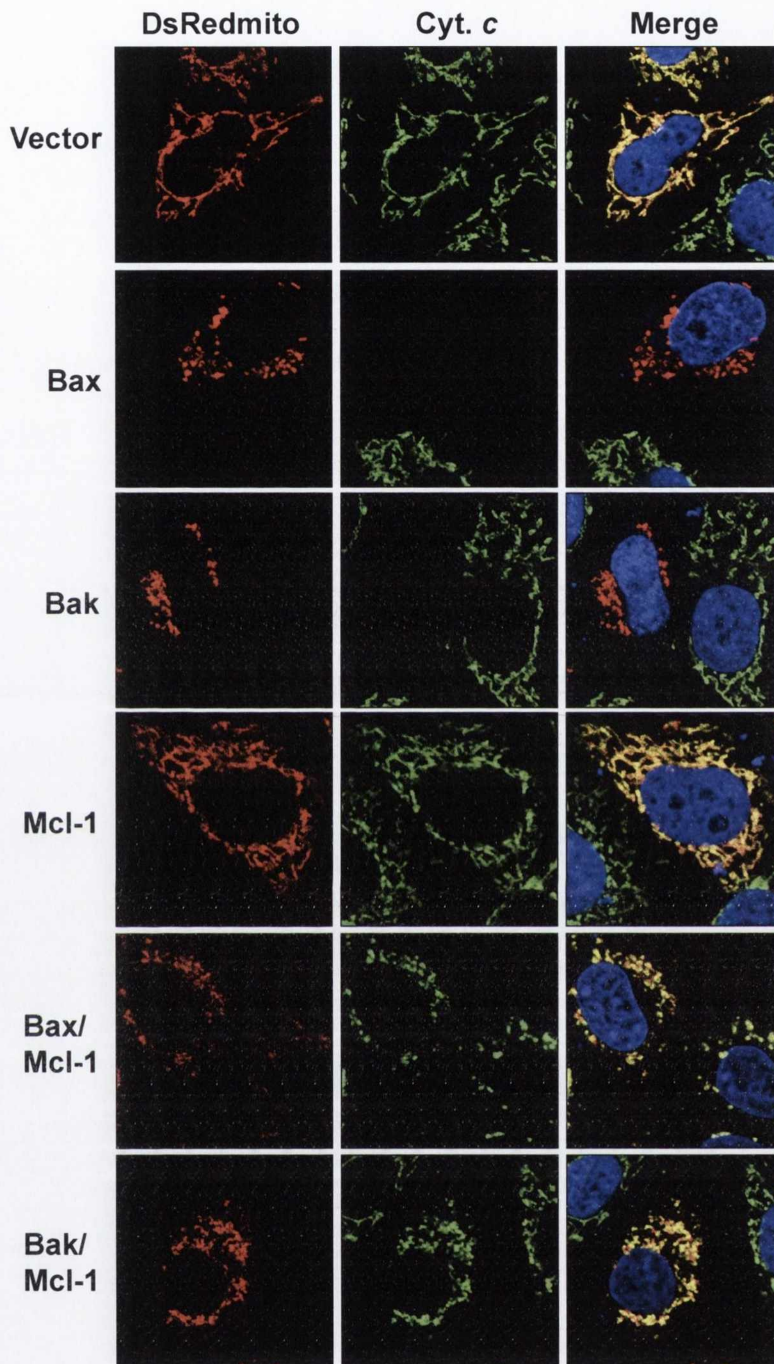
was up to 5 times longer than mitochondria in Bax- and Bax/Mcl-1-expressing cells, demonstrating the fragmented nature of mitochondria in cells expressing the latter proteins (Figure 5.12 C).

5.2.8 *BH3-only proteins induce mitochondrial fragmentation independently of cytochrome c* release

To ensure that the aforementioned results were not caused by overexpression of Bax and Bak, we decided to activate endogenous Bax and Bak using BH3-only proteins. Similar to observations described above, we found that Bcl-xL and Mcl-1 prevented both cytochrome *c* release and apoptosis induced by the BH3-only proteins Bid, Bim and Puma (Figure 5.13 A-C) (data kindly provided by Dr. Petrina Delivani). However, mitochondrial fragmentation induced by these BH3-only proteins was still evident in the presence of anti-apoptotic Bcl-2 family members (Figure 5.13 D) (data kindly provided by Dr. Petrina Delivani). These results further support our proposal that Bax and Bak promote mitochondrial fission separately from cytochrome *c* release. Therefore, the mitochondrial fission seen during apoptosis may occur due to Bax/Bak-mediated perturbation of mitochondrial dynamics, independently of their death promoting roles.

5.2.9 *Fragmented mitochondria in Bax-transfected cells display reduced motility*

In addition to mitochondrial remodelling via fission and fusion, mitochondria are also transported within the cell along cytoskeletal tracks (Frederick and Shaw, 2007). Previous studies have indicated that fragmented mitochondria display reduced motility (Chen *et al.*, 2003). To assess whether Bax-induced mitochondrial fission also resulted in reduced mitochondrial motility, we transfected HeLa cells with Bax, Bcl-xL and Mcl-1 and observed mitochondrial movement over an extended period. We found that vector and Mcl-1 transfected mitochondria were highly motile, displaying branching and directed movement (Figure 5.14 A and E). In contrast, fragmented mitochondria in Bax-transfected cells were more static with little directional movement of individual mitochondria visible (Figure 5.14 B-D). Bcl-xL-



<u>Figure 5.10</u>

Bax and Bak induce mitochondrial fragmentation independently of cytochrome *c* release - separation by Mcl-1

HeLa cells were plated at 2 x 10^5 cells/well on coverslips in 6-well plates. 24 h later, cells were transfected with pDsRed-mito (100 ng) together with Bax or Bak plasmids (200 ng) and Mcl-1 plasmid (800ng) in the presence of Z-VAD.fmk (50 μM) to prevent apoptosis. Following 24 h of expression, cells were fixed, immunostained for cytochrome *c* and mitochondria were visualised using confocal microscopy.

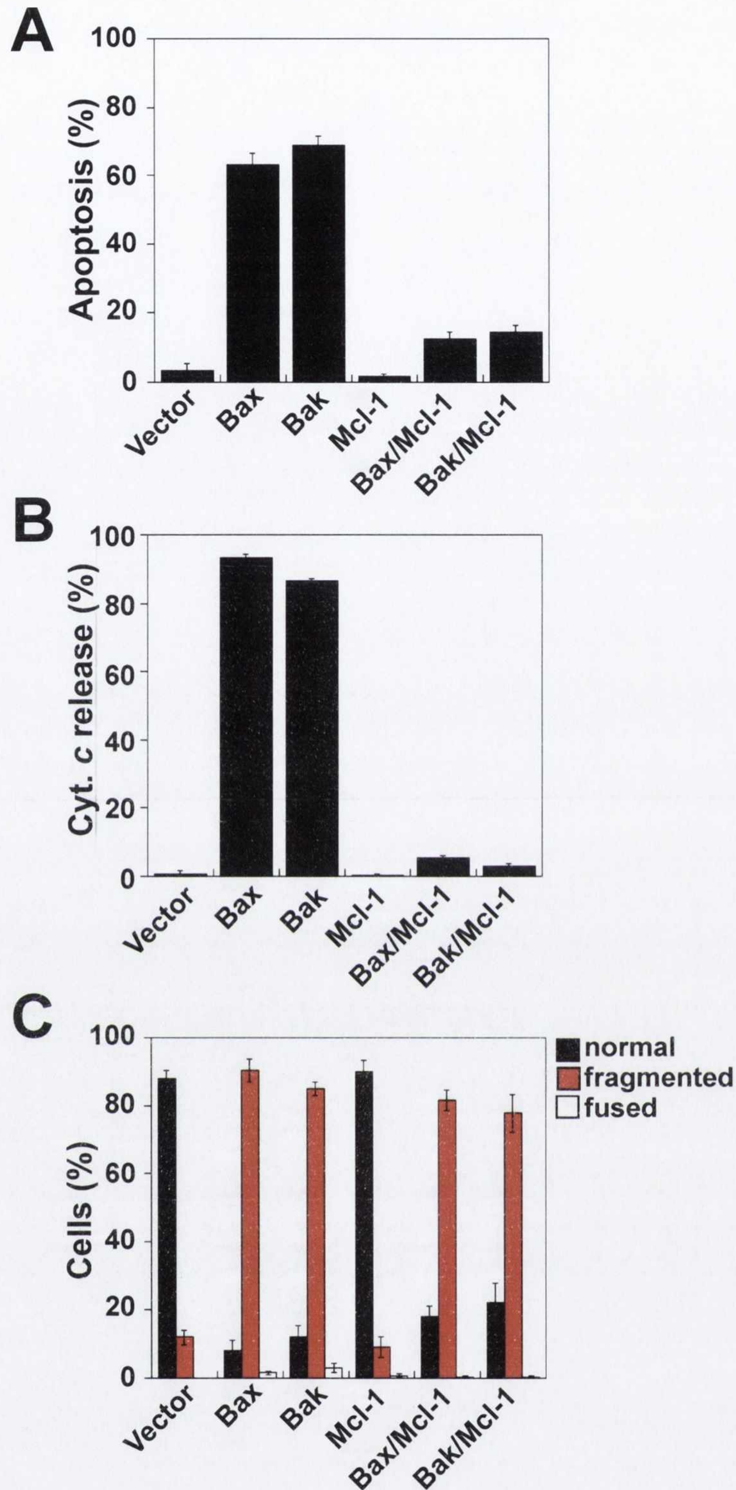


Figure 5.11

Bax and Bak induce mitochondrial fragmentation independently of cytochrome *c* release - separation by Mcl-1

HeLa cells were plated at 2 x 10^5 cells/well on 6-well plates. 24 h later, cells were transfected with a pDsRed reporter plasmid (100 ng), together with Bax or Bak plasmids (200 ng) and Mcl-1 plasmid (800ng), in the presence of Z-VAD.fmk (50 μM) to prevent apoptosis where necessary. Following 24 h of expression, apoptosis was assessed in transfected cells based on morphology (A). Alternatively, cells were fixed, immunostained for cytochrome *c* and cytochrome *c* release (B) or mitochondrial morphology (C) in RFP-positive cells was enumerated. Results represent triplicate counts of representative experiments with error bars representing SEM.

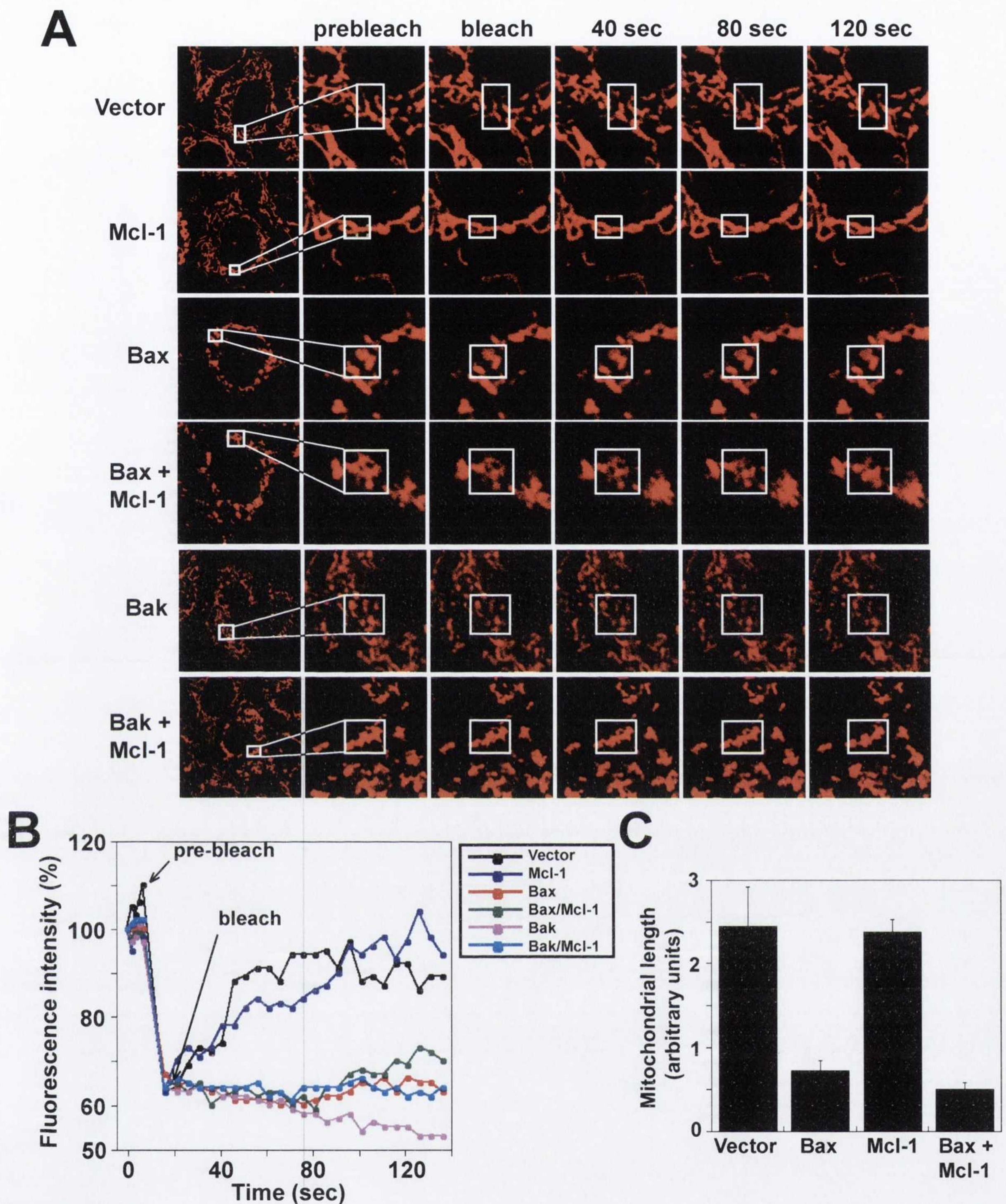
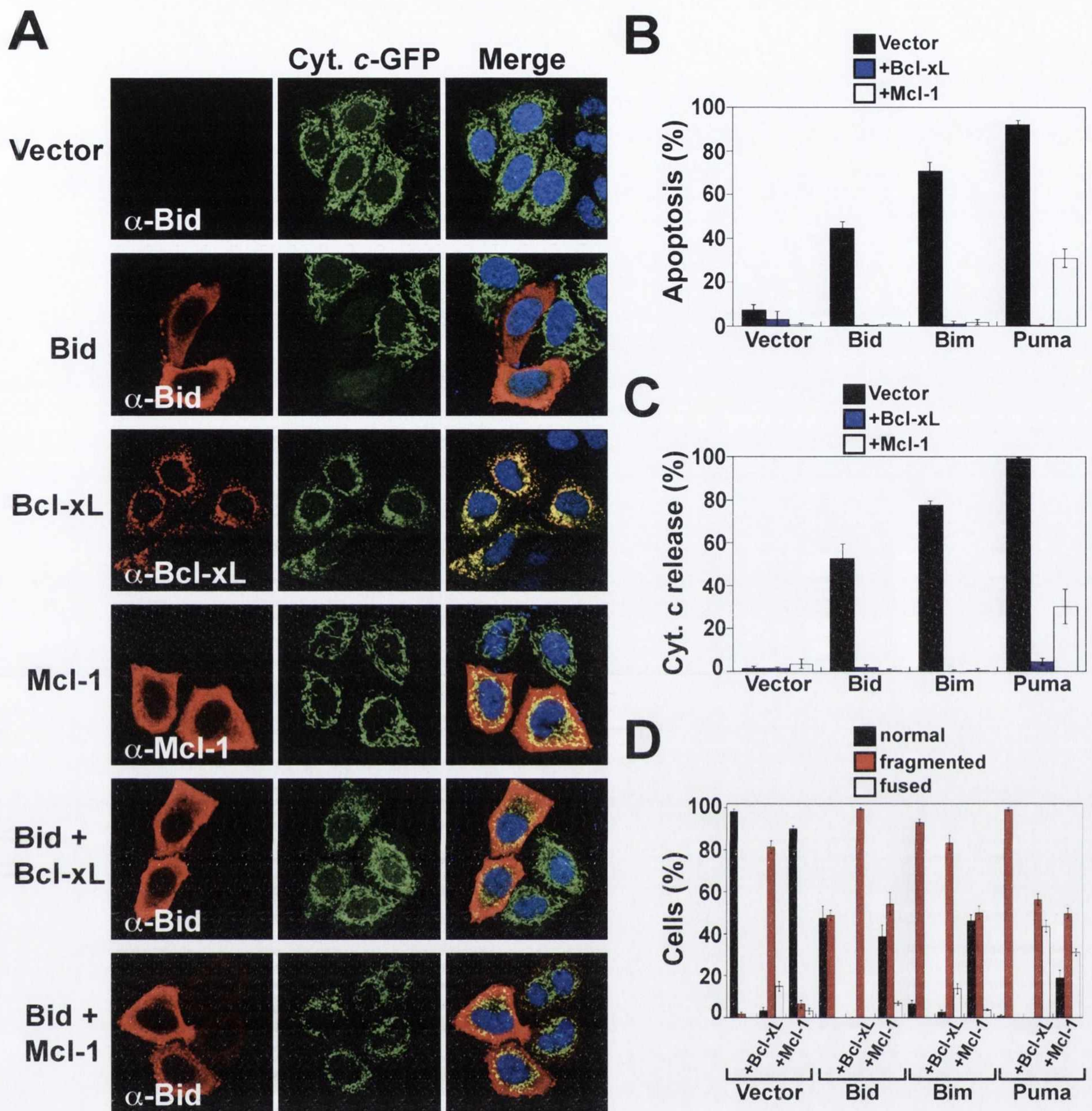


Figure 5.12

FRAP analysis of Bax- and Bak-induced mitochondrial fragmentation

HeLa cells were plated at 2 x 10^5 cells/dish on glass-bottomed tissue culture dishes. 24 h later, cells were transfected with a pEF-mitoGFP (150 ng) together with Bax or Bak plasmids (200 ng) and Mcl-1 plasmid (800ng) in the presence of Z-VAD.fmk (50 μM) to prevent apoptosis. Following 24 h of expression, cells were incubated in Mitotracker CMXRos (50 nM) for 1 h. GFP-positive mitochondria were bleached using a brief pulse of high intensity laser illumination. Images of bleached mitochondria at the indicated timepoints were taken (A) and flourescence recovery over time was measured (B). Relative mitochondrial lengths in HeLa cells after transfection with 150 ng of mitoGFP reporter along with the indicated plasmids were calculated using ImageJ (NIH) software (C).



<u>Figure 5.13</u>

BH3-only proteins induce mitochondrial fragmentation independently of cytochrome *c* release

HeLa cyt. *c*-GFP cells were plated at 2 x 10^5 cells/well on coverslips in 6-well plates. 24 h later, cells were transfected with Bid (200 ng), Bcl-xL (400 ng) or Mcl-1 (800 ng) in the presence of Z-VAD.fmk to prevent apoptosis. Following 24 h of expression, cells were fixed, immunostained for the indicated proteins and mitochondria were visualised using confocal microscopy (A). Cells were transfected with BH3-only plasmids (200 ng) and Bcl-xL (400 ng) or Mcl-1 (800 ng) together with a GFP-reporter plasmid (100 ng). Apoptosis was enumerated 24 h later (B). Alternatively, cells were fixed, immunostained for cytochrome *c* and cytochrome *c* release (B) or mitochondrial morphology (C) in GFP-positive cells was enumerated. Graphed results represent triplicate counts of representative experiments with error bars representing SEM. Data were kindly provided by Dr. Petrina Delivani.

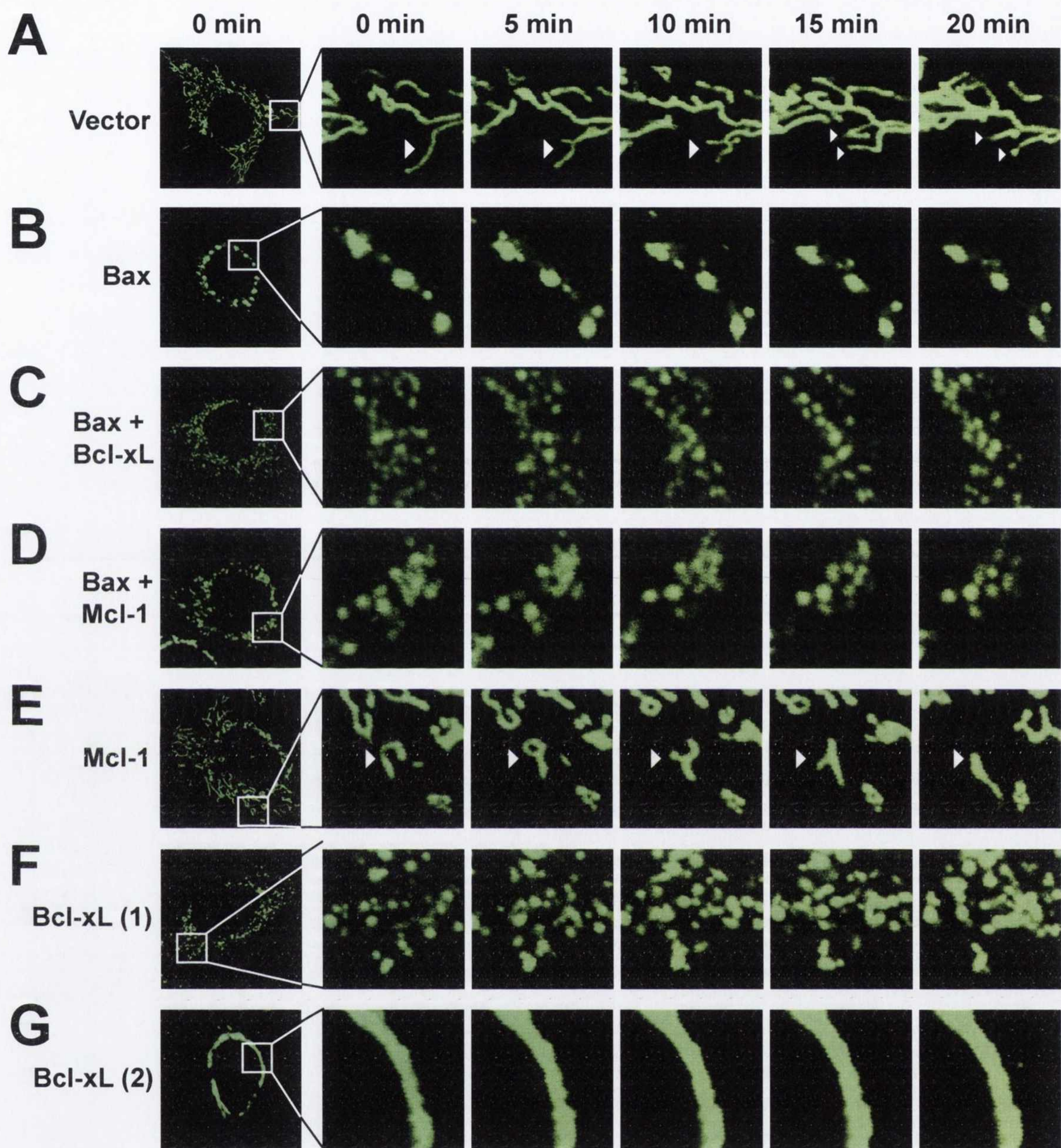


Figure 5.14

Fragmented mitochondria show reduced motility

HeLa cells were plated at 2 x 10^5 cells/dish on glass-bottomed tissue culture dishes. 24 h later, cells were transfected with a pEF-mitoGFP (150 ng) together with vector (1000 ng) (A), Bax (200 ng) (B), Bax (200 ng) and Bcl-xL (800ng) (C), Bax (200 ng) and Mcl-1 (800ng) (D), Mcl-1 (E) or Bcl-xL (F and G). Images of mitochondria taken at the indicated timepoints are shown (A-G).

transfected cells displaying either fragmented or fused mitochondria also displayed poor motility (Figure 5.14 F and G). These results indicate that perturbing the mitochondrial network into a highly fragmented or fused state may interfere with interactions between mitochondrial proteins and proteins associated with tethering mitochondria to the cytoskeletal network such as Miro and Milton. Thus mitochondria are no longer transported throughout the cell and this may result in the peri-nuclear localisation of mitochondria observed in apoptotic cells.

5.2.10 *Effects of Bcl-xL and Mcl-1 on steady-state mitochondrial dynamics*

In addition to Bax/Bak-induced mitochondrial fragmentation, we also observed that the anti-apoptotic protein Bcl-xL altered mitochondrial morphology. To explore this further, we transfected HeLa cells with increasing amounts of Bcl-xL and Mcl-1 plasmids. We found that Bcl-xL induced both mitochondrial fission and fusion, however fusion required greater amounts of Bcl-xL plasmid (Figure 5.15 A). In contrast, Mcl-1 had no effect on mitochondrial morphology even at the highest concentrations utilised. To ask whether Bax or Bak were necessary for Bcl-xL-mediated mitochondrial fission or fusion, we transfected HeLa cells with Bcl-xL and shRNA against Bax and/or Bak. We found that ablation of Bax and Bak only slightly reduced Bcl-xL-mediated mitochondrial remodelling, indicating that these proteins are not downstream effectors of Bcl-xL (Figure 5.15 B and C). However, Mcl-1 promoted mitochondrial fission in the presence of Bax and Bak shRNA, indicating that the overall balance of these proteins in the cell may affect the ability of anti-apoptotic Bcl-2 family members to modulate mitochondrial fission and fusion dynamics.

5.3 DISCUSSION

5.3.1 *Fission and fusion proteins do not regulate cell death*

Mitochondrial fragmentation is a conserved event that occurs during all forms of intrinsic apoptosis. While previous reports have proposed that Drp1-mediated mitochondrial fission is important for cytochrome *c* release, we have clearly demonstrated that regulators of fission and fusion do not impact on apoptosis. A number of studies have blocked Drp-1-mediated fission during apoptosis, using siRNA or a dominant negative inhibitor, and found that cytochrome c release and apoptosis were reduced (Frank *et al.*, 2001; Breckenridge *et al.*, 2003; Lee *et al.*, 2004; Germain *et al.*, 2005). However, we observed that prevention of mitochondrial fragmentation during cell death, through enforced fusion or inhibition of Drp1-mediated fission, had no effect on cytochrome *c* release or apoptosis in response to pro-apoptotic stimuli. Conversely, enhancing mitochondrial fission through Fis1 overexpression, did not significantly enhance cytochrome *c* release or apoptosis. While overexpression of Drp1 K38A and Fis1 slightly altered cytochrome *c* release, these proteins had no effect on apoptosis. Thus, in comparison to the powerful protection afforded by anti-apoptotic Bcl-2 family members, we found the mitochondrial fission and fusion regulators are very weak modulators of cell death. Our results indicate that mitochondrial fission does not have any role to play in MOMP and that fission accompanies rather than drives cytochrome *c* release.

5.3.2 *Mitochondrial fragmentation does not directly facilitate cytochrome c release*

Previously, overexpression of the mitochondrial fission protein Fis-1 has been shown to induce apoptosis, leading to the suggestion that mitochondrial fission is important for apoptosis (James *et al.*, 2003; Yu *et al.*, 2005a, Alirol *et al.*, 2006). However, Fis-1 mediated cell death is inhibited by Bcl-xL overexpression, and is Bax/Bak-dependent, demonstrating that apoptosis occurs due to extensive mitochondrial dysfunction rather than fission-induced mitochondrial permeabilisation (Yu *et al.*, 2005a; Alirol *et al.*, 2006). Similar results have been demonstrated with Opa1 ablation, which results in mitochondrial fragmentation and apoptosis. This is

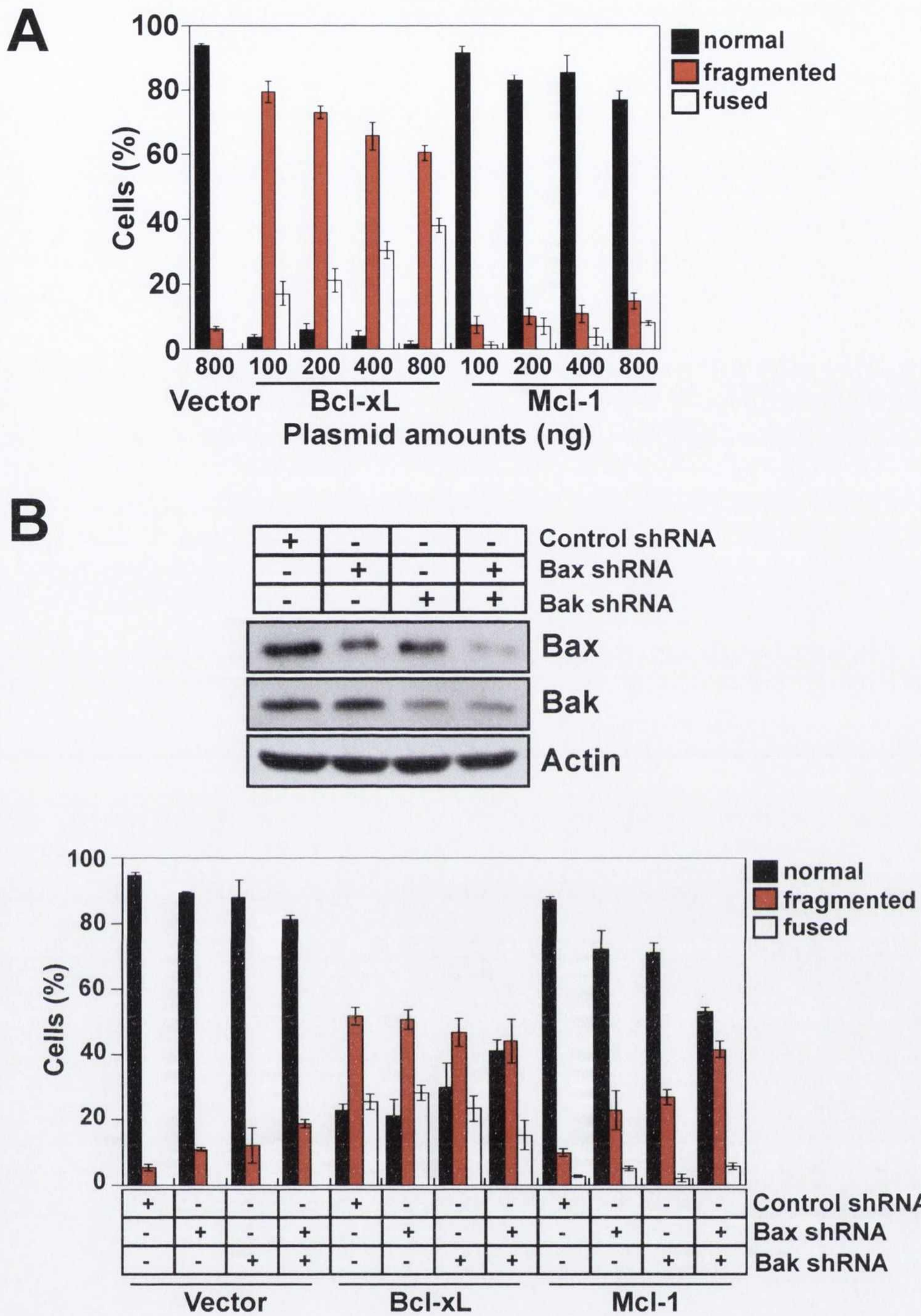


Figure 5.15

Effects of Bcl-xL and Mcl-1 on steady-state mitochondrial network dynamics

HeLa cells were plated at 2 x 10^5 cells/well on coverslips in 6-well plates. 24 h later, cells were transfected with pEF-mitoGFP (150 ng) together with the indicated amounts of Bcl-xL and Mcl-1 plasmids. The mitochondrial morphology of GFP-positive cells was enumerated 24 h later (A). HeLa cells were transfected with plasmids containing shRNA against Bax and Bak (600 ng) for 48 h. Lysates were prepared and Bax and Bak expresion levels were asessed by Western Immunoblotting (B). HeLa cells were transfected with a combination of Bax and Bak shRNA plasmids (600 ng) and Bcl-xL and Mcl-1 plasmids (800ng) together with pEF-mitoGFP (150 ng). After 48 h, the mitochondrial morphology of GFP-positive cells was enumerated (C). Graphed results represent triplicate counts of representative experiments with error bars representing SEM.

also inhibited by Bcl-2, again indicating that death is triggered by the stress of Opa1 loss, rather than fragmentation-induced cytochrome *c* release (Olichon *et al.*, 2003; Lee *et al.*, 2004). Furthermore, many groups have observed dramatically fragmented mitochondria in healthy cells, indicating that mitochondrial fission alone does not necessarily result in cytochrome *c* release and cell death (Chen *et al.*, 2003; Szabadkai *et al.*, 2004, De Vos *et al.*, 2005; Delivani *et al.*, 2006; Karbowski *et al.*, 2006; Taguchi *et al.*, 2007; Norris *et al.*, 2008).

5.3.3 *Mitochondrial fragmentation is not required for cytochrome c release*

We observed cytochrome *c* release from highly fused or elongated mitochondria in cells stimulated with pro-apoptotic drugs, demonstrating that mitochondrial fragmentation is not required for Bax/Bak-mediated pore formation. These results are supported by a previous study in our laboratory, which demonstrated that while the Bcl-2 homologue CED-9 promoted mitochondrial fusion when overexpressed, this protein provided no protection against cytotoxic drug-induced apoptosis (Delivani *et al.*, 2006). Thus, although it is clear that mitochondrial fragmentation is a widespread phenomenon during apoptosis, our results show that this event is not crucial to the progression of programmed cell death. The defining event prior to cytochrome *c* release from mitochondria is the formation of Bax/Bak pores, and to date, there is no evidence to suggest that fission or fusion of mitochondria impacts on pore formation. Therefore, apoptosis is likely to proceed regardless of the mitochondrial phenotype.

5.3.4 *Separation of Bax- and Bak-induced mitochondrial fragmentation and cytochrome c release*

We have shown here that Bax/Bak-mediated mitochondrial fission can be uncoupled from their ability to promote cytochrome *c* release through co-expression of anti-apoptotic Bcl-2 family members. While Bcl-xL and Mcl-1 efficiently block Bax-induced cytochrome *c* release and apoptosis, these proteins have no effect on Bax-induced mitochondrial fragmentation. These results provide yet another example of mitochondrial fission without cytochrome *c* release, and indicate that

Bax independently promotes mitochondrial disintegration and MOMP. Our observations suggest that mitochondrial fragmentation occurs prior to cytochrome *c* release and may be triggered by Bax/Bak-dependent disruption or enhancement of the function of mitochondrial fission and fusion regulators. Thus, although fragmentation accompanies cytochrome *c* release, the separation of these two events during Bax-induced apoptosis demonstrates that they are not inter-dependent steps.

5.3.5 *Mitochondrial fragmentation and apoptosis in model organisms*

Interestingly, mitochondrial fragmentation during apoptosis has also been reported in other organisms such as *S. cerevisae, C. elegans* and *D. melanogaster* (Jagasia *et al.*, 2005; Goyal *et al.*, 2007). A number of studies have addressed the issue of whether mitochondrial fission is important for programmed cell death in these organisms (Fannjiang *et al.*, 2004; Jagasia *et al.*, 2005; Breckenridge *et al.*, 2008; Goyal *et al.*, 2007). Ablation of Dnm1 in yeast and Drp1 in flies enhanced survival following pro-apoptotic stimuli, while overexpression of dominant negative Drp1 reduced developmental cell death in nematodes. However, as mitochondrial cytochrome *c* is not involved in the apoptotic process in these organisms, the impact of mitochondrial fragmentation on cell death is unclear (Dorstyn *et al.*, 2004; Oberst *et al.*, 2008). Indeed a more recent study by Xue and colleagues comprehensively showed that loss of function mutations in fission and fusion genes in *C. elegans* had no effect on apoptotic cell death (Breckenridge *et al.*, 2008). Instead they found that Drp1 and Fis1 promote the elimination of mitochondria in apoptotic cells, indicating that these proteins play a role in the execution phase of apoptosis rather than the initiation phase.

Thus, mitochondrial fragmentation occurring during apoptosis may promote mitochondrial disassembly, enhancing engulfment by neighbouring phagocytes, rather than playing an instrumental role in the initiating events of apoptosis. There is some evidence that caspases target mitochondria during apoptosis, resulting in loss of mitochondrial inner membrane potential and cleavage of proteins important

for mitochondrial function (Dinsdale *et al.*, 1999; Sun *et al.*, 2007; Ricci *et al.*, 2004; Loucks *et al.*, 2009). Therefore, mitochondrial fragmentation may be an early event in the demolition of mitochondria that is concluded by caspases in the later stages of apoptosis.

5.3.6 *Modulation of mitochondrial dynamics by Bcl-2 family members*

While the function of Bcl-2 family members in cell death regulation is well understood, a new housekeeping role for Bcl-2-related proteins in modulating mitochondrial dynamics is emerging. The first indications of this secondary role for Bcl-2 proteins came from a study expressing the *C. elegans* anti-apoptotic Bcl-2 family member, CED-9, in mammalian cells. Strikingly, while overexpression of CED-9 was unable to prevent Bax-induced cytochrome *c* release and apoptosis, this molecule induced dramatic remodelling of the mitochondrial network from long filamentous tubules distributed throughout the cell, to fused mitochondria clustered around the nucleus (Delivani *et al.*, 2006). Here we show that Bcl-xL, the mammalian homologue of CED-9, promotes both mitochondrial fission and mitochondrial fusion when overexpressed. These results suggest that in healthy cells, Bcl-xL regulates mitochondrial fission and fusion dynamics separately from it's anti-apoptotic function.

We also observed that other anti-apoptotic Bcl-2 family members such as Bcl-2, Bcl-w and Bcl-b affected the mitochondrial morphology of cells when overexpressed (data not shown). In contrast, Mcl-1 and A1 do not appear participate in this secondary function. The cytosolic localisation of the latter proteins may affect their ability to regulate mitochondrial status, or the absence of a defined Bcl-2 homology 4 domain (BH4 domain) in these proteins may prevent interactions with other mitochondrial fission and fusion regulators. Bax and Bak ablation did not dramatically alter Bcl-xL-induced mitochondrial phenotypes, indicating that Bcl-xL acts on mitochondria independently of these pro-apoptotic molecules. However ablation of Bax and Bak enhanced Mcl-1-induced fragmentation, suggesting that

these molecules may affect the availability of anti-apoptotic Bcl-2 family members for interactions with other cellular proteins.

Recently, studies utilising neuronal cells have also described a role for Bcl-2 family members in the regulation of mitochondrial dynamics. While Bcl-w deficiency in the brain produces no apoptotic defects, mitochondria in purkinje cells are elongated and these cells have abnormal synapses and dendrites, possibly due to impaired mitochondrial fission (Liu *et al.*, 2008). Expression of Bcl-xL in neurons results in increased mitochondrial fission, fusion and overall mitochondrial biomass, while conditional knockout of Bcl-xL in cortical neurons resulted in a fragmented phenotype (Berman *et al.*, 2008). Expression of Bcl-xL has also been linked with elevated numbers of neuronal synapses due to enhanced mitochondrial localisation at synaptic sites (Li *et al.*, 2008). In addition, pro-apoptotic Bax and Bak have also been linked to regulation of mitochondrial morphology. Double knockout of Bax and Bak in mouse embryonic stem cells gives rise to fragmented mitochondria, indicating that these proteins have a role to play in mitochondrial fusion (Karbowski and Youle, 2006). However, we have observed that overexpression of Bax and Bak similarly induces mitochondrial fission. Thus changes in the ratios of Bax and Bak versus anti-apoptotic Bcl-2 family members or other fission and fusion mediators may determine mitochondrial morphology.

5.3.7 *How do Bcl-2 family members regulate mitochondrial dynamics?*

As none of these proteins contain a GTPase domain found in the well-defined fission and fusion regulators, it is unlikely that Bcl-2 family members act directly on mitochondria. Instead they may act as adaptors or facilitators of mitochondrial fission and fusion. The localisation of many Bcl-2 family members such as Bcl-2, Bcl-xL and Bak on mitochondrial outer membranes places them in an ideal location for modulating the activity and interactions of other fusion and fission mediators. Consistent with this theory, a number of interactions between Bcl-2-related proteins and fission and fusion proteins have been documented. Bcl-xL binds to both Mfn2 and Drp1 in different cell types, which may facilitate the fission and fusion

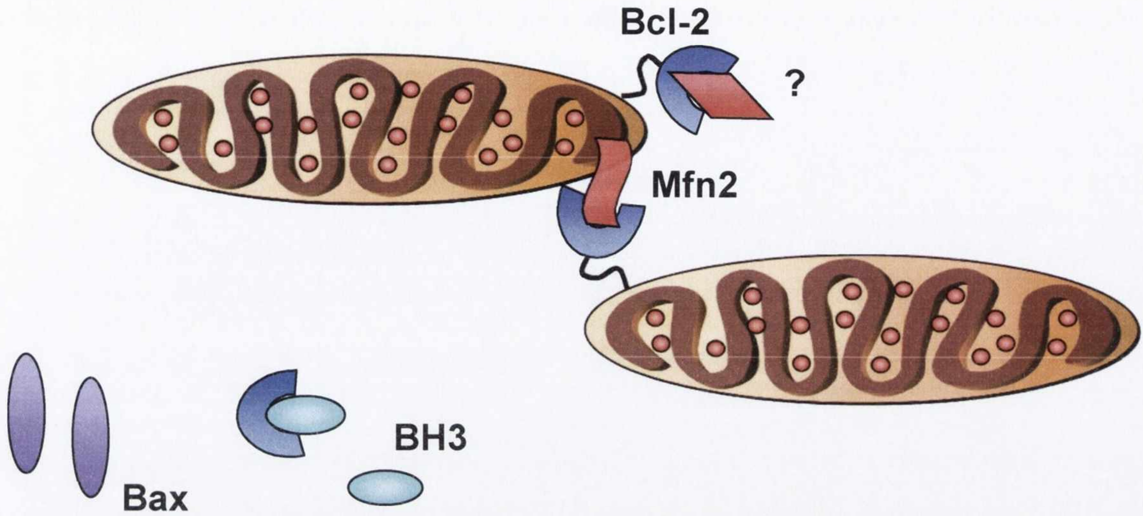
phenotypes seen with overexpression of Bcl-xL (Delivani *et al.*, 2006; Li *et al.*, 2008; Berman *et al.*, 2008). Bak has been shown to interact with Mfn1 and Mfn2, while Bax promotes the activity of Mfn2 (Brooks *et al.*, 2007; Karbowski *et al.*, 2006). In addition Bcl-2 family members may facilitate binding of mitochondria to the cytoskeleton, thus affecting mitochondrial distribution in the cell. Unfortunately, examination of the role of these proteins, particularly Bax and Bak, in the modulation of mitochondrial morphology is hampered by their apoptosis-related properties. Hence, separating the apoptotic functions of the pro- and anti-apoptotic Bcl-2 family members from their ability to modulate mitochondrial morphology will be the key to dissecting this secondary role.

While the mechanism employed by Bcl-2 family members to regulate mitochondrial fission and fusion dynamics is still ambiguous, it appears that this is an important, conserved function of these proteins. *C. elegans* CED-9 also modulates mitochondrial dynamics indicating that this may be an ancient function of the Bcl-2 family (Delivani et al., 2006; Tan *et al.*, 2008; Rolland *et al.*, 2009). Although loss of function mutants in CED-9 revealed no dramatic alterations in mitochondrial morphology, overexpression of CED-9 in *C. elegans* muscle cells resulted in highly interconnected mitochondria, while loss of function mutants enhanced Drp-1 mediated fragmentation (Breckenridge *et al.*, 2009; Tan *et al.*, 2008). This argues that while CED-9 does not directly mediate mitochondrial fission or fusion, it may modulate the activity of other fission and fusion regulators. A role for CED-9 in regulating mitochondrial dynamics provides a possible explanation for the localisation of CED-9 on mitochondrial outer membranes in *C. elegans,* where no role for cytochrome *c* or mitochondria in apoptotic cell death is known. This may also be an important function for Drosophila Bcl-2 family members who play a limited role in Drosophilia apoptosis (Sevrioukov *et al.*, 2007; Galindo *et al.*, 2008).

5.3.8 *Impact of Bcl-2 family members on apoptosis-associated mitochondrial fragmentation*

We have shown that anti-apoptotic Bcl-2 family members may perturb mitochondria in the absence of any cell death phenotype, hinting that these proteins may have a mitochondrial remodelling role in healthy cells that is separate to their role in protecting against apoptosis (Delivani *et al.*, 2006; Liu *et al.*, 2008; Berman *et al.*, 2008). During cell death, increased interactions between anti-apoptotic Bcl-2 related proteins and pro-apoptotic proteins, may disrupt their ability to regulate mitochondrial fusion, culminating in the fragmented mitochondrial phenotype associated with apoptosis (Figure 5.16). We observed mitochondrial fragmentation in response to overexpression of Bax-related molecules and BH3-only proteins, indicating that both sets of proteins may be able to antagonise anti-apoptotic Bcl-2-mediated mitochondrial remodelling. Interestingly, co-expression of *C. elegans* BH3-only protein EGL-1 with CED-9 similarly led to mitochondrial fission, suggesting fragmentation of mitochondria during *C. elegans* apoptosis may result from inactivation of CED-9-promoted mitochondrial fusion (Jagasia *et al.*, 2005; Delivani *et al.*, 2006). In the future, examination of the interplay between Bcl-2 related proteins and members of the fission and fusion machinery may enhance our understanding of the role of fission and fusion modulators in the apoptotic process. Thus, dissecting the part played by Bcl-2 family members in the regulation of mitochondrial morphology in healthy cells may be the key to deciphering what triggers mitochondrial fission during cell death.

Healthy cells



Apoptotic cells

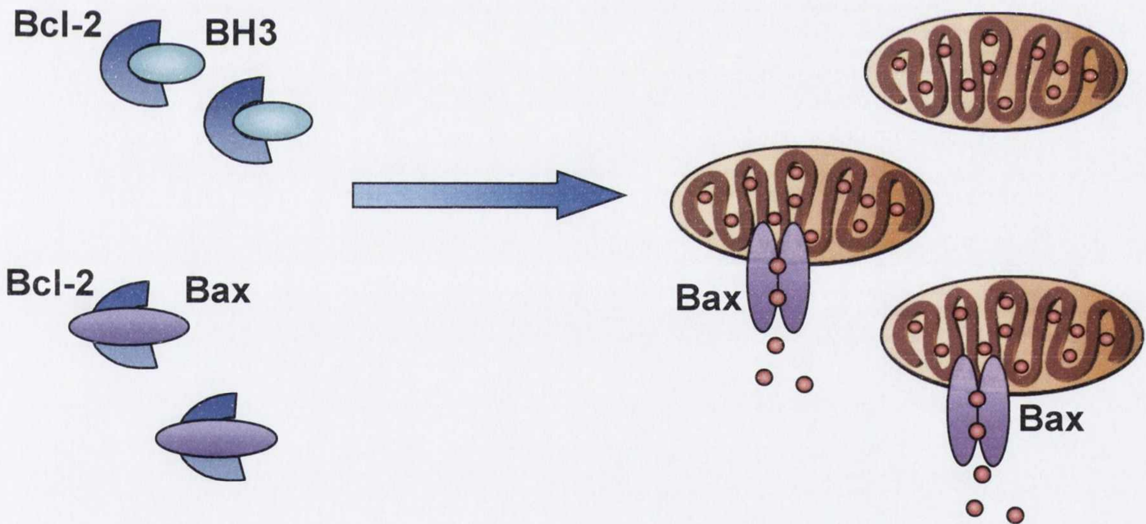


Figure 5.16

Proposed model for mitochondrial fragmentation during apoptosis

In healthy cells, anti-apoptotic Bcl-2 family members hold pro-apoptotic members in check and also contribute to mitochondrial fission and fusion dynamics. In apoptotic cells, anti-apoptotic Bcl-2 family members are out-titrated by pro-apoptotic Bcl-2 family members, and are no longer available to promote mitochondrial fusion, thus contributing to the fragmented phenotype observed in dying cells.

CHAPTER VI

GENERAL DISCUSSION

6.1 INTRODUCTION

The Bcl-2 family are crucial regulators of programmed cell death in response to numerous stimuli. These proteins are involved in the initiation phase rather than the execution phase of apoptosis, and therefore are important determinants of cellular fate. Significantly, Bax/Bak-mediated MOMP signals the demise of the cell, as regardless of downstream events, mitochondrial function cannot be recovered in most cell types. The importance of Bcl-2 family members in promoting the appropriate execution of apoptosis has been exemplified in knockout mice. Bax/Bak double knockout mice die before birth due to impaired apoptosis during development, while deletion of Bim causes accumulation of lymphoid cells (Lindsten *et al.*, 2000; Bouillet *et al.*, 1999). In addition, knockout of the BH3-only proteins Bid, Puma, Noxa and Bim diminishes apoptotic responses to certain stimuli (Yin *et al.*, 1999; Villunger *et al.*, 2003; Puthalakath *et al.*, 2007). Conversely, Bcl-xL knockout mice die during embryogenesis due to excessive apoptosis and disruption of the *bcl-2*, *bcl-w* and *a1* genes leads to elevated apoptosis in certain tissues (Motoyama *et al.*, 1995; Veis *et al.*, 1993, Print *et al.*, 1998; Hamasaki *et al.*, 1998).

Although knockout studies have demonstrated critical roles for certain Bcl-2 family members in response to particular pro-apoptotic stimuli, the ability of many knockout mice to survive to adulthood demonstrates the redundant nature of these proteins (Russell *et al.*, 2002; Coultas *et al.*, 2004). It has become increasingly clear that varying subsets of Bcl-2 family members are important arbiters of cell fate in response to different stimuli and in different tissues. Furthermore, particular Bcl-2 family members appear to be more universally influential in cell death regulation, while others have more specific roles. Therefore, understanding the regulation of these proteins in response to both pro-survival and pro-apoptotic signalling is important for gaining a complete comprehension of the role of individual Bcl-2 family members in apoptotic signalling. In addition, unravelling the events driven by Bax and Bak at mitochondria will aid elucidation of which other cellular proteins influence the progression of apoptosis.

6.2 ROLE OF BCL-2 FAMILY MEMBERS IN CANCER

As Bcl-2 family members are prominent determinants of cell survival, deregulated expression of these proteins can lead to pathological consequences. Aberrant expression of Bcl-2 family members has been linked to autoimmunity, AIDS and tumor formation (Bouillet *et al.,* 2002; Petrovas *et al.*, 2004; Kirkin *et al.*, 2004). In particular, anti-apoptotic Bcl-2 related proteins may be instrumental participants in cancer development, as shown by the enhancement of myc-driven oncogenesis by overexpression of Bcl-2 and Bcl-xL (Vaux *et al.*, 1988, Finch *et al.*, 2006). Inactivation of Bim similarly promotes myc-induced transformation of cells, suggesting that modulation of either sub-groups of the Bcl-2 family may advance tumorigenesis (Egle *et al.*, 2004).

Bcl-2 family members play a key role in cancer for two main reasons. Firstly, in order for tumors to develop, they need to acquire resistance against apoptosis to survive the stressful conditions created by excessive cellular growth. Modulation of Bcl-2 family members can provide cancer cells with this resistance. Secondly, many cancer treatments such as chemotherapy or irradiation act through induction of apoptosis in tumor cells. Thus, deregulation of Bcl-2 family members will have an impact on the effectiveness of anti-cancer treatment. In this thesis we have assessed the mechanism employed by oncogenic B-Raf to increase apoptotic resistance in melanoma cells. We also examined the importance of BH3-only protein upregulation in apoptosis induced by the chemotherapeutic drug cisplatin. These observations, discussed in greater detail below, have expanded our understanding of the role of Bcl-2 family members in driving oncogenesis and contrarily in promoting the demise of tumors during chemotherapeutic drug treatment.

6.3 ONCOGENE-MEDIATED MODULATION OF BCL-2 FAMILY MEMBERS

Many oncogenes promote excessive proliferation of cells in a manner that facilitates tumor formation (Polakis, 2007; Meyer *et al.*, 2008). However, there is a growing understanding of the accompanying ability of some oncogenes to promote cell

survival by regulating the expression of apoptosis-associated molecules (Karnoub *et al.*, 2008).

We have observed that overexpression of oncogenic B-Raf^{V600E} confers resistance against apoptosis. Conversely, ablation of B-Raf^{V600E} expression sensitised melanoma cells to chemotherapeutic drug-induced apoptosis. Thus B-Raf^{V600E} may promote melanoma development by facilitating growth factor-independent proliferation and also by suppression of apoptosis triggered during the transformation process. Examination of the mechanism of apoptotic resistance utilised by oncogenic B-Raf, revealed that this kinase targets a number of Bcl-2 family members through the ERK pathway. B-Raf^{V600E}-dependent phosphorylation of Bim and Bad led to degradation of the former and sequesteration of the latter. Furthermore, co-expression of B-Raf^{V600E} with these BH3-only proteins efficiently blocked apoptosis triggered by either molecule. B-Raf^{V600E} also enhanced Mcl-1 expression. Thus, from our results it is clear that B-Raf^{V600E} does not target one specific protein that is responsible for its anti-apoptotic function. Instead this kinase modulates a number of apoptosis regulators, which modifies the apoptotic threshold of cells in favour of survival, and provides resistance against diverse stimuli.

Surprisingly, B-Raf^{V600E} also increased expression of the BH3-only protein Noxa, which may counterbalance the anti-apoptotic role to this oncogene. As Noxa and Mcl-1 are interaction partners, the effects of upregulating both of these proteins remains unclear. Perhaps the strength and duration of MAPK signalling, or co-regulation of these proteins by accompanying cellular pathways, determines which of these molecules predominate. However, positive regulation of Noxa expression by oncogenic B-Raf^{V600E} suggests that Noxa upregulation may be intended to curb excessive proliferation of cells displaying hyperactivation of ERK and in doing so may act as a 'failsafe' against tumor development.

6.4 TARGETING ONCOGENES IN CANCER TREATMENT

Our results show that B-Raf^{V600E} promotes chemoresistance through modulation of Bcl-2 family members, suggesting that targeting this oncogene, or downstream effectors, may combat melanoma-associated resistance to cytotoxic drugs. We found that all melanoma cell lines used in our study contained elevated ERK activity, either through activating mutations in Ras, B-Raf or via other mechanisms. This suggests that ERK activation is crucial for melanoma maintenance, and thus targeting the MAPK pathway should augment chemotherapeutic action against melanoma. Initial studies using MEK inhibitors in mouse models of melanoma have demonstrated that blocking this pathway reduces metastasis and causes regression of melanocytic tumors (Collison *et al.*, 2003; Sharma *et al.*, 2006). Furthermore, phase 1 clinical trials have been carried out using three different MEK inhibitors CI-1040, PD0325901 and AZD6244 in recent years (LoRusso *et al.*, 2005; Wang *et al.*, 2007; Adjei *et al.*, 2008). While all of these trials indicated that MEK inhibitors display tolerable toxicity to healthy tissue, trials with two of the compounds have been discontinued due to insufficient anti-tumor activity as single agents (Rinehart *et al.*, 2004). However, phase 1 clinical trials were carried out on patients with advanced carcinomas and did not include combination therapy with other chemotherapeutic drugs. Therefore, these studies do not exclude a role for MEK inhibition in combination with other cytotoxic drugs. More recently, sensitisation of cells to apoptosis induced by DNA methyltransferase inhibitors and ionising radiation by the MEK inhibitor AZD6244 has been demonstrated, suggesting that MEK inhibition has potential for future cancer therapies (Nishioka *et al.*, 2009; Chung *et al.*, 2009).

Sorafenib has been the most widely used B-Raf inhibitor for cancer treatments to date. Pre-clinical studies using this inhibitor showed promise and subsequent clinical trials combining sorafenib with cytotoxic drugs have been carried out with encouraging results, particularily in renal carcinomas (Wilhelm *et al.*, 2008). However, sorafenib is not a selective Raf inhibitor but rather a multi-kinase inhibitor that affects all Raf isoforms as well as VEGFR, c-KIT and other kinases (Wilhelm *et*

al., 2004). Therefore, the exact anti-tumorigenic mechanism of action of sorafenib is unclear, but appears to involve inhibition of ERK activity, reduced angiogenesis and increased apoptosis through Mcl-1 downregulation (Murphy *et al.*, 2006; Liu *et al.*, 2006; Yu *et al.*, 2005b). The effect of sorafenib on melanoma has yet to be fully deciphered, although some benefits have been recorded (McDermott *et al.*, 2008). However, the development of more selective inhibitors of B-Raf will more clearly elucidate a possible role for B-Raf inhibition in melanoma cancer treatment (Tsai *et al.*, 2008). B-Raf^{V600E} mutations have also been detected in other cancers such as colorectal, ovarian and thyroid cancers (Yuen *et al.*, 2002; Ueda *et al.*, 2007; Kimura *et al.*, 2003). Thus targeting this kinase may enhance anti-cancer treatment of a number of tumor types.

6.5 ROLE OF NOXA IN CISPLATIN CHEMOTHERAPY

Despite their use for many decades, the molecular mechanism of action of many chemotherapeutic drugs remains ill-defined. Currently, the toxic insult imposed by most of these drugs is known, however the events connecting cellular injury and apoptosis are less well elucidated. Understanding the precise mode of action of chemotherapeutic drugs is important for deciphering why certain patients and types of cancers respond better to a particular anti-cancer treatment than others.

While exploring the link between cisplatin-induced DNA damage and activation of pro-apoptotic Bcl-2 family members, we observed a dramatic increase in the BH3-only protein Noxa. Further investigation revealed that Noxa upregulation was ERK dependent and this event was integral to cisplatin-induced apoptosis. Significantly, Noxa was also upregulated during carboplatin- and oxaliplatin-mediated apoptosis. Our observations show that Noxa is a common target of platinum based therapeutics, transducing the apoptotic signal from DNA-damage to Bax/Bak-dependent cytochrome *c* release. Thus, the ability of platinum agents to induce Noxa within patient tumor cells is likely to impact on their effectiveness as chemotherapeutic agents.

Given the important role of Noxa in platinum-induced apoptosis, combination therapies with drugs that complement the function of this BH3-only protein may improve cisplatin-induced cytotoxicity. For example, treatment of tumors with cisplatin, in combination with drugs that target Mcl-1, such as obatoclax, may enhance the pro-apoptotic potential of Noxa and consequently the outcome of chemotherapy (Nguyen *et al.*, 2007). Furthermore, we have observed that melanoma cells display high levels of Noxa, therefore these tumors may be more susceptible to cisplatin-based therapy. Indeed, other researchers have identified the potential of Bortezomib, which also upregulates Noxa, in the specific treatment of melanoma while exhibiting little toxicity against melanocytes (Fernandez *et al.*, 2005; Qin *et al.*, 2005; Nikiforov *et al.*, 2007).

We have demonstrated the novel upregulation of Noxa by ERK pathway activators. Classically, the ERK pathway is linked to survival signalling and cell cycle progression (Roux and Blenis, 2004). However, we show that under certain circumstances ERK can promote upregulation of Noxa with cell death consequences. Our results provide an example of the complexity of signalling pathways within the cell, where rather than executing one specific function, molecules instead promote different endpoints depending on the upstream activating signals. ERK kinases have multiple targets and can affect the regulation of numerous genes, and thus through different combinations, may orchestrate many different signalling endpoints (Shaul and Seger, 2007). Of interest, Noxa appears to be upregulated under conditions of potent and sustained activation of ERK, indicating that Noxa induction may require prior activation of a number of ERK targets. Our observations suggest that Noxa upregulation in response to persistent ERK activation is a signal that the cell is under duress and upregulation of this BH3-only protein beyond a certain threshold leads to elimination of the cell.

6.6 TARGETED THERAPY DIRECTED AGAINST ANTI-APOPTOTIC BCL-2 FAMILY MEMBERS

In recent years, development of cancer therapies is shifting from empirically identified cytotoxic drugs to targeted therapies against particular cancer-promoting proteins. As anti-apoptotic proteins drive both progression of cancer and tumor chemoresistance they are ideal drug targets. Recently, BH3-mimetics have been developed to inactivate the function of anti-apoptotic Bcl-2 family members. BH3-mimetics are small cell permeable molecules that bind with high affinity to the hydrophobic groove formed by BH1-3 domains in anti-apoptotic Bcl-2 family members, thus releasing endogenous pro-apoptotic molecules (Oltersdorf *et al.*, 2005). One of these drugs. ABT-737, which inhibits Bcl-2, Bcl-xL and Bcl-w proteins, has shown apoptotic synergy with other chemotherapeutic drugs against cancer cells (Trudel *et al.*, 2007; Kang *et al.*, 2007; Whitecross *et al.*, 2009). Interestingly, ABT-737 has also been shown to inhibit autoimmunity by enhancing cell death of autoreactive thymocytes, demonstrating that BH3-mimetics have therapeutic potential outside of cancer (Bardwell *et al.*, 2009). Other BH3-mimetics, such as obatoclax and TW-37, that target the entire repertoire of anti-apoptotic Bcl-2 family members may be even more effective treatments for cancers, while the development of safe mechanisms of introducing siRNA into tumors may provide an additional means of targeting these proteins (Zhai *et al.*, 2006; Konopleva *et al.*, 2006).

6.7 BAX/BAK-MEDIATED MITOCHONDRIAL OUTER MEMBRANE PERMEABILISATION

Bax and Bak form pores in mitochondrial outer membranes, facilitating release of cytochrome *c*, downstream caspase activation and subsequently apoptosis. However the exact structure of these pores and the ideal molecular environment for the formation of Bax/Bak pores is unknown. Previous experiments have indicated that Bax and Bak oligomerise into multimers that may form a channel in mitochondrial outer membranes (George *et al.*, 2007; Dewson *et al.*, 2008). It appears that an activation step is required to produce a protein conformation that

favours oligomerisation and BH3-only proteins facilitate this activation (Wei *et al.*, 2000; Catron *et al.*, 2004; Lovell *et al.*, 2008). Elegant experiments using reconstituted liposomes demonstrated that other mitochondrial proteins are not necessary for permeabilisation, at least in liposomes (Kuwana *et al.*, 2002). However, cardiolipin was required suggesting that mitochondrial outer membrane lipids may be important for creating the ideal environment for Bax/Bak oligomerisation and pore formation.

6.8 ROLE OF MITOCHONDRIAL FRAGMENTATION IN MOMP

In recent years, a debate has unfolded around the idea that mitochondrial membrane remodelling is necessary to produce a conformation that favours Bax/Bak-induced mitochondrial membrane permeabilisation. We explored this question through modulation of mitochondrial fission and fusion during apoptosis and found that Bax and Bak promoted cytochrome *c* release regardless of mitochondrial morphology. In contrast to previous reports, we found that fission and fusion regulators, such as Drp1 and Mfn1/2, did not modulate cytochrome *c* release or apoptosis to any significant degree (Frank *et al.*, 2001; Lee *et al.*, 2004; Sugioka *et al.*, 2004). Importantly, we observed through co-expression with anti-apoptotic Bcl-2 family members, that Bax promotes mitochondrial fragmentation separately from cytochrome *c* release. Thus, mitochondrial fission accompanies, but is not necessary for, release of cytochrome *c* and this phenomenon may occur due to disruption of a mitochondrial remodelling role of anti-apoptotic Bcl-2 family members during apoptosis.

6.9 NON-APOPTOTIC ROLES FOR BCL-2 FAMILY MEMBERS

Through intensive research over the past 20 years, the function of Bcl-2 family members in controlling cell death has been progressively delineated. More recently non-apoptotic functions of Bcl-2-related proteins have emerged. In chapter V we have demonstrated that expression of Bcl-xL in HeLa cells leads to perturbation of the mitochondrial network. We observed that Bcl-xL promoted mitochondrial fission and fusion in a concentration-dependent manner. Remodelling of the mitochondrial

network was also seen with other anti-apoptotic Bcl-2 family members such as Bcl-w and Bcl-2. These observations indicate that mitochondrially localised Bcl-2-related proteins may contribute to mitochondrial dynamics in healthy cells, possibly as binding proteins or scaffolding molecules that facilitate fission and fusion mediated by other proteins. In support of our observations, a number of other studies have linked Bcl-2 family members to the regulation of mitochondrial fission and fusion dynamics and interactions between the anti-apoptotic proteins and mediators of mitochondrial fusion have been detected (Karbowski *et al.*, 2006; Li *et al.*, 2008; Bermann *et al.*, 2008).

Bcl-2 family members have also been associated with other cellular processes in the absence of cell death. Reed and colleagues demonstrated that anti-apoptotic Bcl-2 family members could regulate inflammatory caspase activation through interaction with the adapter protein NALP1 (Bruey *et al.*, 2007; Faustin *et al.*, 2009). This is reminiscent of the role played by the *C. elegans* Bcl-2 homologue CED-9, which directly interacts with the adapter molecule CED-4 during *C. elegans* programmed cell death. Bcl-2 related proteins have also been connected with cell cycle control, where Bcl-2 delays cell cycle re-entry of quiescent cells, possibly through enhanced expression of p27 and subsequent inhibition of cyclin dependent kinases that drive cell cycle progression (0' Reilly *et al.*, 1996; Vairo *et al.*, 2000). Furthermore, Bcl-2 and Bcl-xL inhibit autophagy through sequestration of Beclin-1, a key autophagy mediator (Liang *et al.*, 1999; Pattingre *et al.*, 2005; Oberstein *et al.*, 2007). Thus, Bcl-2 family members are occupied with numerous non-apoptotic functions in healthy cells prior to the epic battle that unfolds during the demise of a cell.

APPENDIX

Table 1: Plasmids used for work undertaken in this thesis

Plasmid name	Cloned by/Received from
pCDNA3	Invitrogen
pEFmB-Raf^{WT}	Laboratory of Prof. Richard Marais
pEFmB-Raf^{V600E}	Laboratory of Prof. Richard Marais
pCDNA3-Flag Bcl-xL	Clare Sheridan (Martin Lab)
pCDNA3-HA BimWT	Laboratory of Dr. Hisashi Harada
pCDNA3-HA BimS55A	Laboratory of Dr. Hisashi Harada
pCDNA3-HA BimS65A	Laboratory of Dr. Hisashi Harada
pCDNA3-HA BimS100A	Laboratory of Dr. Hisashi Harada
pCDNA3-HA BimS55A,S65A,S100A	Laboratory of Dr. Hisashi Harada
pCDNA3-Bim	Clare Sheridan (Martin Lab)
pCDNA3-Bad	Clare Sheridan (Martin Lab)
pCDNA3-Bid	Clare Sheridan (Martin Lab)
pCDNA3-Noxa	Clare Sheridan (Martin Lab)
pCDNA3-Bax	Laboratory of Prof. Douglas Green
pCDNA3-Bak	Laboratory of Prof. Richard Youle
pEGFP-C3 Bax	Laboratory of Prof. Christoph Borner
pEF-Ras^{N17S}	Laboratory of Prof. Douglas Green
pCDNA3-Ras^{V12G}	Laboratory of Prof. Julian Downward
pMKO1-Bim shRNA	Addgene
pMKO1-Bad shRNA	Addgene
pRetroSuper-GFP	Laboratory of Dr. Eric Eldering
pRetroSuper-Noxa	Laboratory of Dr. Eric Eldering
pFUGW-Bax shRNA	Laboratory of Dr. Maria Soengas
pFUGW-Bak shRNA	Laboratory of Dr. Maria Soengas
pCDNA3-Flag Mcl-1	Clare Sheridan (Martin Lab)
pCB6-myc Mfn1	Laboratory of Prof. Luca Scorrano
pCB6-myc Mfn2	Laboratory of Prof. Luca Scorrano
pCDNA3-HA Drp1K38A	Laboratory of Prof. Luca Scorrano
pCMV-myc Opa1	Laboratory of Prof. Luca Scorrano
pCDNA3-Flag Drp1	Dr. Patrick Duriez (Martin Lab)
pCI-His Fis1	Laboratory of Prof. Jean Claude Martinou
pEF-mycMitoGFP	Invitrogen
pDsRed2-mito	Laboratory of Dr. Damien Arnoult
pCDNA3-Puma	Dr. Patrick Duriez (Martin Lab)

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