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HEAT SHOCK-INDUCED APOPTOSIS

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HEAT SHOCK-INDUCED APOPTOSIS

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Dedication

To my parents, Wanda Polewacz Mahajan and Jaswant Rai Mahajan

Thank you for giving me unconditional love always. You nurtured in me the courage to pursue my dreams, even if it was beyond my limitations and seemed too difficult, you helped me to never give in, never give up, smile and forgive always. You taught me with your kind and loving actions what no school could teach. You believed me when I faced doubt. You are my solid rock and source of inspiration always. I can't be thankful enough. This dissertation is for you with all my heart.

"Somewhere, something incredible is waiting to be known".

(Carl Sagan)

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HEAT SHOCK-INDUCED APOPTOSIS

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Apoptosis is a conserved program of cell death that promotes organism homeostasis in all stages of life. Two main pathways activate caspases, which are cysteinyl-aspartate proteases that execute apoptosis. The extrinsic pathway is initiated by cell surface death receptors, while the intrinsic pathway is initiated by intracellular signals that cause permeabilization of the outer mitochondrial membrane (MOMP). The Bcl-2 protein family regulates MOMP, which causes the release of several pro-apoptotic proteins (such as cytochrome c, Smac) into the cytosol. Bcl-2 proteins share homology in up to four “BH” domains and are subdivided into three subgroups. Pro-apoptotic Bax and Bak catalyze pore formation in the mitochondria, while anti-apoptotic members (Bcl-2, Mcl-1) inhibit MOMP. The third subgroup, termed BH3-only, promotes MOMP by either antagonizing Bcl-2 proteins or by directly activating Bax/Bak, and initiate

apoptosis in response to various stressors, including heat shock (HS). Hyperthermia or acute HS reportedly induces apoptosis through caspase-2-mediated cleavage of BID, engaging the intrinsic pathway. However, additional evidence suggests that this pathway could represent an amplification loop. Thus we hypothesized that during HS, another BH3-only protein such as BIM, that does not require cleavage, could engage MOMP. Herein, we report that BIM mediates an alternative HS-induced apoptosis pathway. Cells lacking BIM are resistant to HS and exhibit better short and long-term survival than either *Bid*$^{-/-}$ or *Bax*$^{-/-}$*Bak*$^{-/-}$. Moreover, caspase-2 induces apoptosis in *Bim*$^{-/-}$ but not *Bid*$^{-/-}$ cells, implying that caspase-2 kills exclusively through BID. Interestingly, *Bim*$^{-/-}$ and Bax$^{-/-}$*Bak*$^{-/-}$ cells are entirely resistant to MOMP, but the *Bax*$^{-/-}$*Bak*$^{-/-}$ cells still undergo caspase-3 activation and remain partially sensitive to HS, indicating that BIM triggers caspase-3 activation upstream of mitochondria. Thus, BIM plays an important role in HS-induced apoptosis. Hyperthermia has clinical applications for the treatment of solid tumors. Unfortunately, a practical limitation is the development of thermotolerance, which confers resistance not only to subsequent HS but also to radiotherapy and chemotherapy. Therefore, a better understanding of the molecular mechanisms involved both in heat-induced apoptosis and thermotolerance could lead to new therapeutic interventions. Here we also show evidence for a putative role for the stress kinase JNK signaling pathway in the regulation of thermotolerance.

Table of Contents

List of Figures

List of Abbreviations

ABT-737	Abott laboratories inhibitor of Bcl-2, Bcl-xL, Bcl-w
AIF	Apoptosis-inducing factor
APAF-1	Apoptotic protease-activating factor-1
AP20187	FKBP ligand (homodimerizer)
ASK1	Apoptosis signal-regulating kinase 1
ATP	Adenosine triphosphate
BAD	Bcl-2 associated death promoter
BAK	Bcl-2 antagonist / killer
BAX	Bcl-2-associated X protein
BCL-2	B-cell leukemia/lymphoma-2
BCL-x_L	B-cell lymphoma extra-large
BFL1/A1	Bcl-2 related protein A1
BH	Bcl-2 homology
BH3	Bcl-2 homology domain 3
BID	BH3 interacting domain death agonist
BiFC	Bi-molecular fluorescence complementation
BIK	Bcl-2-interacting killer
BIM	Bcl-2-interacting mediator of cell death
BIR	Baculovirus IAP repeat
BMF	Bcl-2-modifying factor
BRAF	v-raf murine sarcoma viral oncogene homolog B
b-VAD-fmk	Biotinylated-valine-alanine-aspartate-fluoromethylketone
CARD	Caspase activation and recruitment domain
Caspases	Cysteinyl aspartate-specific proteases
CED	Cell death abnormal
c-FLIP	Cellular-FLICE-like inhibitory protein
CH11	Agonistic human CD95 antibody
cIAP1/2	Cellular inhibitor of apoptosis-1/2
c-neu	*v-erb-B2* erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog
cyt. c	Cytochrome c
dATP	Deoxyadenosine triphosphate
DD	Death domain
DDR	DNA damage response
DED	Death effector domain
DISC	Death-inducing signaling complex
DKO	Double knockout

DLC-1	Dynein light chain -1
DMSO	Dimethyl sulfoxide
ER	Endoplasmic reticulum
ERK	Extracellular regulated kinase
Eμ-myc mice	Mice bearing *c-myc* oncogene under control of the immunoglobulin heavy chain (Igh) enhancer
FADD	Fas-associated protein with death domain
FITC	Fluorescein isothiocyanate
FKBP	FK506-binding protein
FOXO3a	Forkhead box O3a
GFP	Green fluorescent protein
HSF1	Heat shock factor -1
HSP	Heat shock protein
HSP70	Heat shock protein 70
HSR	Heat shock response
HRAS	Harvey rat sarcoma viral oncogene homolog
HRK	Harakiri
IAP	Inhibitor of apoptosis
IBM	IAP-binding motif
ICAD	Inhibitor of caspase-activated deoxyribonuclease
IMM	Inner mitochondrial membrane
IMS	Intermembrane space
JNK	c-Jun N-terminal kinase
JIN8	JNK inhibitor 8
LC8	Dynein light chain subunit 8
MAPK	Mitogen-activated protein kinase
MAP2K or MKK	MAPK kinase
MAP3K or MKKK	MAPK kinase kinase
MAPKAP2	MAPK-activated protein kinase 2
MCL-1	Myeloid cell leukemia-1
MEF	Mouse embryonic fibroblast
MEK1/2	MAPK/ERK kinase 1/2
MLK	Mixed-lineage kinase
MMTV/*c-neu* mice	Mice overexpressing the *c-neu* oncogene driven by the mouse mammary tumor virus promoter
MOMP	Mitochondrial outer membrane permeabilization
MULE	Mcl-1 ubiquitin ligase E3
NF-κB	Nuclear factor kappa (κ) B
NIK	NF-κb inducing kinase
N-terminal	Amino-terminal

OMM	Outer mitochondrial membrane
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PI	Propidium iodide
PIDD	p53-induced protein with a death domain
PS	Phosphatidylserine
PUMA	p53 up-regulated modulator of apoptosis
p38	p38 MAPK
q-VD-OPh	Carboxy-terminal valine and aspartate phenoxy conjugate
RAS	Rat sarcoma protein
RAF	Rapidly accelerated fibrosarcoma
RAIDD	RIP-associated ICH1/CED3-homologous protein with death domain
RFP	Red fluorescent protein
RIP	Receptor interacting protein
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
RNA	Ribonucleic acid
RNAi	RNA interference
SAHB	Stabilized alpha helix from Bcl-2 domains
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
shRNA	Small-hairpin RNA
SMAC/DIABLO	Second mitochondria-derived activator of caspases / direct IAP binding protein with low pI
TAK1	Transforming growth factor -beta activated kinase 1
tBID	Truncated bid
TBS	Tris-buffered saline solution
TKO	Triple knockout
TMRE	Tetramethylrhodamine ethyl ester
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRADD	TNFR1-associated death domain
TRAF	TNF-receptor associated factor
TRAIL	TNF-related apoptosis-inducing ligand
T43	Pre-conditioning treatment at 43°C for 30 minutes
UV	Ultraviolet radiation
VC	Vector control
VDAC	Voltage-dependent anion channel
WT	Wild type

XIAP	X-linked IAP
zVAD-FMK	z-valine-alanine-aspartate-fluoromethylketone
$\Delta\Psi m$	Mitochondrial membrane potential
17-AAG	17-allylamino-17-demethoxygeldanamycin
17-DMAG	17-dimethylaminoethylamino-17-demethoxygeldanamycin
5z-oxo	5z-7-oxozeaenol

Chapter 1. Literature Review

1.1. Apoptosis

Apoptosis is an evolutionarily conserved form of cell death, which obeys a genetically encoded program and displays characteristics distinguishable from other forms of cell death. The term apoptosis comes from the Greek terminology for “falling off”, as leaves falling off from trees (1). This cell deletion program regulates development and homeostasis relying on a cascade of signaling events and proteolysis. At center-stage is a family of cysteine proteases, termed *caspases*, that cleave their substrates after aspartic acid residues, resulting in the orderly execution and destruction of the cell (2-5).

Caspases are expressed as inactive zymogens, which must undergo conformational changes and/or processing for full activation. Initiator caspases contain long prodomains, which facilitate recruitment by adaptor proteins and caspase activation (6). Executioner caspases are cleaved and activated by initiator caspases, and once active, they cleave a specific set of substrates that result in the recognition and engulfment of cell corpses by phagocytic cells (7). The typical morphology of an apoptotic cell includes nuclear fragmentation,

membrane blebbing, chromatin condensation, cell shrinkage and formation of apoptotic bodies.

Work in the nematode *C. elegans* has defined a simple and elegant paradigm for controlling cell death during development. In *C. elegans*, apoptosis begins with the transcriptional upregulation of *egl-1,* which encodes a BH3-only protein that antagonizes CED-9, a Bcl-2 homolog. CED-9 precludes apoptosis by sequestering CED-4, which is an adaptor for caspase activation. Binding of Egl-1 to CED-9 activates apoptosis by releasing CED-4 from CED-9 to promote the activation of the caspase CED-3 (8).

Despite the homology of proteins in nematodes and mammals, there are major mechanistic differences in the execution of the apoptotic program. In vertebrate organisms there are two major ways of engaging apoptosis via either the *intrinsic* pathway or the *extrinsic* pathway (Fig. 1.1) (7, 9, 10). In mammals, BCL-2 proteins do not promote direct inhibition of the mammalian CED-4 homolog, Apaf-1. Instead, both positive and negative actions from BCL-2 family members control mitochondrial integrity. The intrinsic pathway is initiated in response to cellular stressors that are sensed by BCL2 proteins (11, 12). Induction of mitochondrial outer membrane permeabilization (MOMP) allows the release of various pro-apoptotic factors such as cytochrome c, Smac, endonuclease G, and AIF (7, 12, 13).

Endonuclease G and AIF are thought to contribute to the nuclear

morphology seen in apoptosis, however their requirement for apoptosis is still a matter of debate (13-15). In contrast, the pro-apoptotic roles of cytochrome c and Smac are well established. Once in the cytosol, cytochrome c binds to Apaf-1 in the presence of dATP to cause Apaf-1 oligomerization followed by recruitment of pro-caspase-9, thereby forming the *apoptosome* (16-18). At this macromolecular complex, caspase-9 is activated to subsequently activate caspases-3 and -7. However, the presence of X-linked inhibitor of apoptosis protein (XIAP) inhibits caspase-9 activity and the processing and activity of caspase-3. Smac is an IAP antagonist and its release from mitochondria allows it to relieve the inhibition of caspases -9 and -3 by XIAP to promote apoptosis (17-20).

In the extrinsic pathway, death receptors located in the cell membrane are engaged by their cognate ligands, causing conformational changes that lead to recruitment of intracellular adaptor proteins (21, 22). The intracellular domain of the death receptor interacts with adaptor proteins via death domains (DD) present in both proteins. For example, during Fas-induced signaling, the adaptor protein FADD (Fas-associated death domain) brings procaspase-8 molecules into the death-inducing-signaling complex (DISC) to become fully activated (21-24). Caspase-8 cleavage of caspase-3 normally results in cell death, however, in a subset of cell types, caspase-8 can cleave BID to engage the intrinsic pathway and create an amplification loop that augments the activation of caspase-3 (25-27).

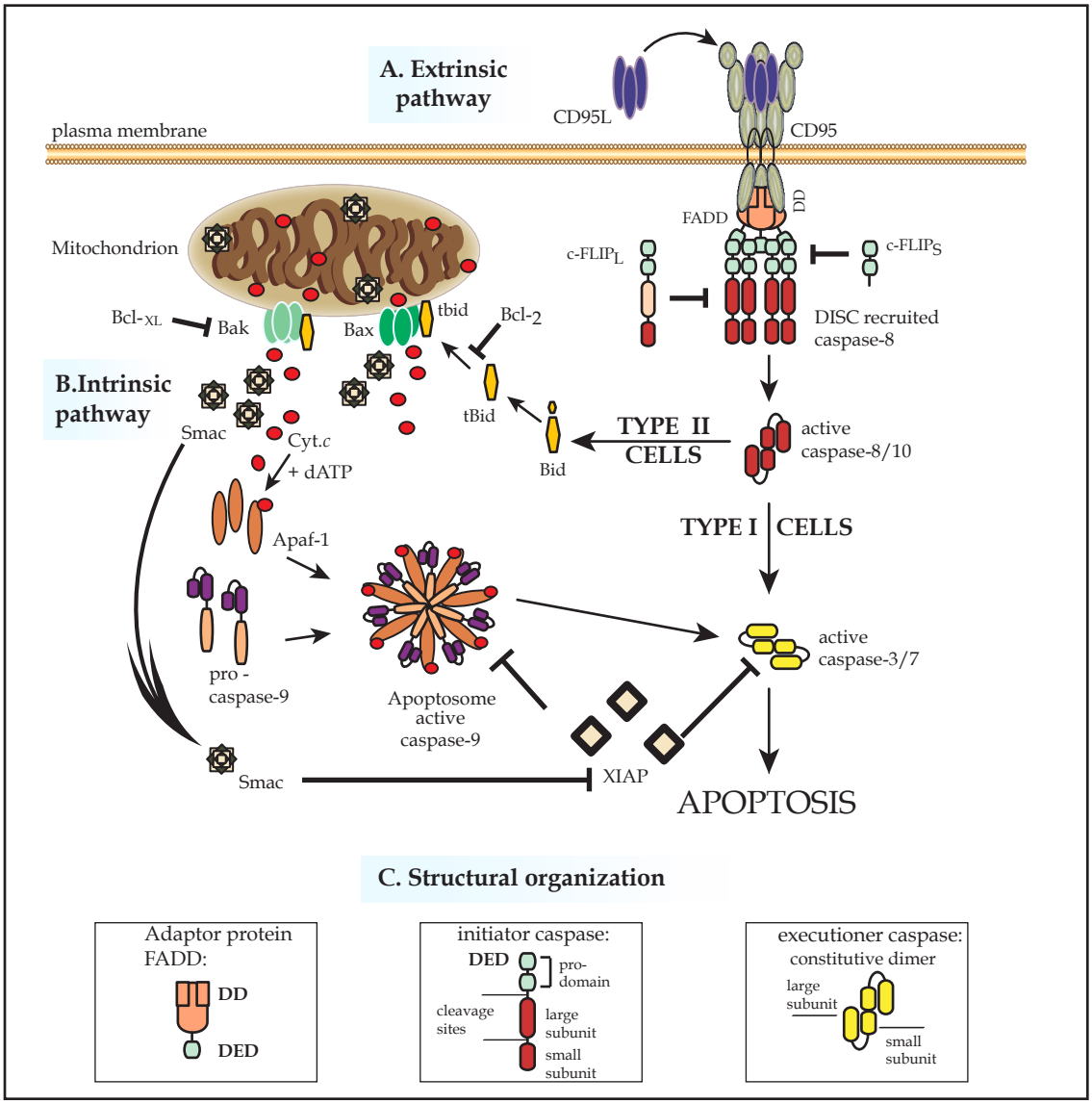


Figure 1.1. Apoptotic pathways. (A). Death-inducing signaling complex (DISC) formation upon extrinsinc pathway engagement by CD95L (Fas ligand) involves binding to its receptor CD95 (Fas), which leads to caspase-8 activation. **(B).** When the intrinsic pathway is initiated by tBid the mitochondria are permeabilized, culminating in caspase-9 activation. The released cytochrome *c* (Cyt.*c*) and Smac respectively contribute to apoptosome formation and XIAP inhibition. Both pathways lead to activation of executioner caspases-3 and -7. Type I cells robustly activate caspase-8; type II cells require amplification via the intrinsic pathway. Thus Bcl-2 anti-apoptotic proteins can block extrinsic apoptosis only in type II cells. **(C).** Structural organization of the adaptor protein FADD, initiator and executioner caspases. *(Modified from : Walczak et al., 2013; Malladi et al., 2009; Bao & Shi, 2007).*

Historical perspective

In 1972, Kerr, Currie and Wyllie coined the term apoptosis and proposed a *consensus* of its defining characteristics. In their original publication, the authors described apoptotic bodies as ultrastructurally well-preserved, membrane-enclosed subcellular fragments, later engulfed and digested by neighboring cells (28). More importantly, the authors recognized the broad biological implications of apoptosis in regulating animal cell populations, as it allowed for extensive elimination of cells in the absence of an inflammatory process (28). The authors also speculated that tumor growth could exploit defects in apoptosis (28, 29). Years later, evasion of cell death was recognized as one of the 10 hallmarks of cancer (30).

Wyllie's article published in Nature in 1980 reported the activation of an endogenous endonuclease during glucocorticoid-induced cell death and provided the first biochemical marker of apoptosis: the formation of a DNA ladder (28, 29, 31-33). By the mid 1990's caspases were recognized as the main executioners of apoptosis and their proteolytic cleavage of key intracellular substrates provided the field with additional apoptotic markers that advanced our understanding of the biochemistry of apoptosis (5, 34-37).

In 2002, H. Robert Horvitz, Sydney Brenner and John E. Sulston shared the Nobel Prize in Physiology or Medicine for their pioneering contributions to deciphering the basic aspects governing developmental cell death using the

nematode *C. elegans* as a model organism. The realization that loss of apoptosis produced “undead” cells led cancer biologists to dissect the molecular mechanisms of apoptosis (31). As a result of this effort, in 1986 Yoshide Tsujimoto and Michael Cleary independently discovered the Bcl-2 gene. B-cell follicular lymphomas exhibit the t(14;18) chromosomal translocation breakpoint, which results in the repositioning of the promoter and enhancer regions of the immunoglobulin heavy chain on chromosome 14 and excessive transcriptional expression of the Bcl-2 gene (38-42). What was most surprising about Bcl-2 discovery is that, unlike the previously discovered oncogenes Ras and Myc, its overexpression did not accelerate cell proliferation rates (43-46). Instead, BCL-2 was shown to prevent cell death caused by unfavorable conditions, such as growth factor deprivation and treatments with ethanol, methotrexate and heat shock. Moreover, Bcl-2 could be targeted to render leukemia cells susceptible to apoptosis (43, 47-51).

The discovery of BCL-2 had a profound impact in understanding how cancers arise, since later it became clear that enhancing cell proliferation, for example by overexpression of an oncogene such as c-Myc, alone, was not sufficient for tumor promotion, and in fact it activated apoptosis (52). However, when a second event, such as excessive BCL-2 levels, was acquired to block apoptosis, efficient tumorigenesis ensued (45, 53). Thus, apoptosis serves to eliminate carcinogenic cells that arise in tissues, and cellular defects that block

apoptosis cooperate in tumor development. These findings led to an outburst of research focused on understanding how BCL-2 and its homologs regulate apoptosis, and on the role of apoptosis deregulation in oncogenesis (10, 54-56).

By the late 1990's apoptosis was the paradigm for programed cell death, in direct opposition to necrosis, which results in the chaotic demise of the cell. Exposure to overwhelming toxic stimulus causes necrosis, characterized by swelling, loss of plasma membrane integrity and ATP depletion (57). Due to the dynamic nature of scientific knowledge, this dichotomous paradigm changed over the last 15 years, and several forms of programmed cell death including necroptosis and autophagic cell death, have now been described (58). For example, necroptosis is a form of cell death that is dependent on the activity of the receptor-interacting protein kinases-1 and -3 (RIP1/3), and occurs when the extrinsic pathway is initiated in the presence of caspase inhibitors or in cells defective in caspase-8 activity (6, 15, 58-60). A further appreciation of the underlying biology of the different modes of programmed cell death will allow for genetic and/or pharmacological manipulation in the future to treat diseases (6, 58).

1.2. Caspases: function and regulation

1.2.1 Structural features, activation and regulatory mechanisms of caspases

Caspases are conserved throughout evolution, being present in invertebrates such as *C. elegans* (CED-3) and insects (Dronc, Drice, DCP-1, etc. in *D. melanogaster*) (61). There are several members of the caspase family in mammals. Humans express 11 of all 14 mammalian caspases identified so far, with the most well characterized initiator caspases being caspases-8, -9 and -10, and executioners being caspase-3, caspase-7 and caspase-6 (61-64). Besides the caspases dedicated to apoptotic function, other caspases have been shown to play important physiological roles in the immune system and inflammatory responses (65).

Caspases proteolytically process their specific targets, activating or inactivating them, depending on the substrate (66). Caspases recognize a minimal 4-aminoacid sequence in their substrates, designated P4-P3-P2-P1 (e.g. D-E-V-D). Cleavage occurs after the c-terminal P1 residue, which is normally an aspartate residue (67). The presence of a glutamate residue on P3 seems to be the preferred choice for all caspases studied so far (3, 67). P4 residue composition varies and contributes to substrate specificity among the different groups of caspases. The binding pocket in caspases is formed by four active site

loops designated S4-S3-S2-S1, respectively, which confers the substrate specificity (67).

The structural organization of a caspase consists of an amino-terminal prodomain, and a carboxy-terminal protease domain critical for proteolytic activity, that is subdivided into a large (~p20) subunit that contains the key catalytic cysteine residue, and a small (~p10) subunit (68). Caspase structure and function are intimately connected, and they are classified based on their position on the apoptotic cascade, where *initiator* caspases act upstream of *effector* caspases (2-5, 61, 67).

Zymogens of initiator caspases-8, -9 and -10 exist as monomers. Their long N-terminal prodomains contain adaptor modules such as the CARD (Caspase-recruitment domain) for caspases-2 and -9, and the DED (death effector domain) for caspases-8 and -10. These conserved moieties are also present in adaptor proteins, which mediate caspase recruitment into activation complexes. Such homotipic interactions bring initiator caspases into close proximity, causing them to dimerize (69-71). The adaptor protein FADD recruits caspase-8 into the Death-Inducing-Signaling-Complex or DISC via their DEDs (23, 24, 72). In contrast, CARD-CARD interactions mediate the Apaf-1 recruitment of caspase-9 into the apoptosome, and the RAIDD interaction with caspase-2 in the PIDDosome (17, 73-76).

Effector or executioner caspases, such as caspases-3 and -7, have much

shorter N-terminal prodomains, comprised of only 20-30 amino acid residues (61). They are present as pre-formed dimers that remain in a latent state until cleavage by initiator caspases at specific aspartate residues on the linker segment that separates large and small subunits (5, 61, 77, 78). Once cleaved, effector caspases undergo conformational changes that re-order the catalytic and the substrate-binding residues (61, 64, 79).

IAP proteins in caspase regulation

Inhibitor of apoptosis (IAP) proteins were first identified in baculoviruses as they potently blocked apoptosis of infected insect host cells (80). IAPs are characterized by the presence of at least one baculovirus IAP repeat (BIR), and some of them contain a RING domain that confers E3 ubiquitin ligase function (81). Later on, several IAPs were discovered in vertebrates, and a subset of them was shown to block caspase activity. Among them, XIAP is the best-characterized member of the IAP family in vertebrates and it directly binds to caspases.

Caspase-9 contains a tetrapeptide sequence on the N-terminal portion of its small subunit that is a conserved IAP binding motif (IBM) through which XIAP binding occurs (19, 82, 83). The BIR3 domain of XIAP can bind to caspase-9 already recruited and processed within the apoptosome, where it prevents

caspase-9 dimerization (17, 19, 82, 83). In contrast, XIAP can inhibit caspases-3 and -7 through interactions of the linker region, between BIR1 and BIR2 on XIAP, with the active site of the caspases (61, 79, 84).

Thus, when XIAP levels are excessive, in order for caspases to kill the cell, XIAP inhibition must be overcome. IAP antagonists are a family of proteins specialized in regulating IAP proteins. Smac and Omi/HtrA2 are two members of this family that reside in the inner mitochondrial space in healthy cells, and which are released into the cytosol following the onset of MOMP. By antagonizing XIAP, Smac contributes to caspase activation and amplifies the initial death signal (20).

1.2.2 The extrinsic pathway - activation of caspase-8

The extrinsic pathway is initiated by plasma membrane receptors, which belong to the TNF superfamily of death receptors. CD95/Fas, TRAIL-R1/DR4 and TRAIL-R2/DR5 are activated by binding of their cognate ligands, CD95L/FasL and TRAIL, respectively (85-93). These ligands are part of a superfamily of cytokines secreted by cytotoxic T cells, where tumor necrosis factor-alpha (TNF-α) is the founding member (21, 94). Thus, the extrinsic pathway is of utmost importance for the immune system, as natural killer cells require it to induce apoptosis and eliminate infected or cancerous target cells. Not surprisingly, cancer cells and some viruses overexpress anti-apoptotic

proteins, such as FLIP, as an evasion mechanism (21, 95).

CD95L and TRAIL induce apoptosis by a similar conserved mechanism (Fig. 1.1). For instance, binding of CD95L/FasL to its death receptor CD95/Fas results in conformational changes that promote the recruitment of FADD to the receptor's intracellular death domains (DDs) (24). The death effector domains (DEDs) of FADD form homotipic interactions with the DEDs on the prodomains of pro-caspase-8 and -10. This complex is known as the DISC where caspases-8 and -10 undergo activation by dimerization (96).

The extrinsic pathway can be regulated by c-FLIP(L) and c-FLIP(s), which are structurally similar to caspase-8, containing the same DEDS but lacking a functional active site. Thus, c-FLIP is recruited to the DISC (Fig.1.1) and can inhibit the activation of caspase-8 by competing with caspase-8 for binding to FADD within the DISC (95, 97-100). Ultimately, a combination of DISC-activated caspase-8 activity (influenced by c-FLIP levels), as well as the XIAP/caspase-3 ratio, determines the fate of the cell and results in the categorization of cells as type I or type II (22, 25, 54, 101, 102).

Depending on the cell type, activation of caspase-8 leads to different downstream events. *Type I cells* have low c-FLIP and XIAP levels, thus in type I cells, activation of caspase-8 results in massive caspase-3/7 activation and cell death (22). However, *type II cells* can contain higher c-FLIP and/or XIAP levels and require cleavage of BID into tBID to engage the intrinsic pathway (discussed

in more detail below) for amplification of the death signal (22). In particular, tBID induces MOMP, causing the release of cytochrome c release and Smac, and both further amplify caspase activation. Therefore, the crucial step for type II cells is the cleavage of BID by caspase-8, followed by its translocation to mitochondria, which explains why in type II cells overexpression of intrinsic pathway inhibitors such as BCL-2 or BCL-x_L can block cell death initiated by death receptors (101, 103).

1.2.3 The intrinsic pathway of apoptosis

1.2.3.1 BCL-2 proteins

The BCL-2 family of proteins, which control mitochondrial integrity, tightly regulates the *intrinsic apoptosis pathway*. The mitochondrion is the cellular organelle responsible for the generation of most ATP. Yet paradoxically, such a vital organelle harbors several pro-apoptotic factors, namely cytochrome c, Smac, AIF, Omi/HtrA2 and endonuclease G (12). Therefore, the initiation of the caspase cascade in this pathway relies on MOMP to release pro-apoptotic factors from the mitochondria into the cytosol (12).

The BCL-2 family of proteins is critical to the initiation of the intrinsic apoptosis pathway, with members that either inhibit or promote cell death in response to stress (46). BCL-2 proteins share homology in up to 4 domains termed BCL-2 homology (BH) domains (12, 104, 105). According to their role in apoptosis and the presence of BH domains, BCL-2 proteins can be classified as anti-apoptotic multi-domain proteins (BCL-2, MCL-1, etc.), multi-domain pro-apoptotic proteins (BAX and BAK), and BH3-only proteins, which share similarity only in the BH3 domain (BID, BIM, PUMA, etc.) (Fig. 1.2) (106).

BCL-2 protein function involves heterodimerization among different subgroup members, which can be between anti-apoptotic members with pro-apoptotic members (e.g. BCL-2-BIM or BCL-2-BAX), or between pro-apoptotic

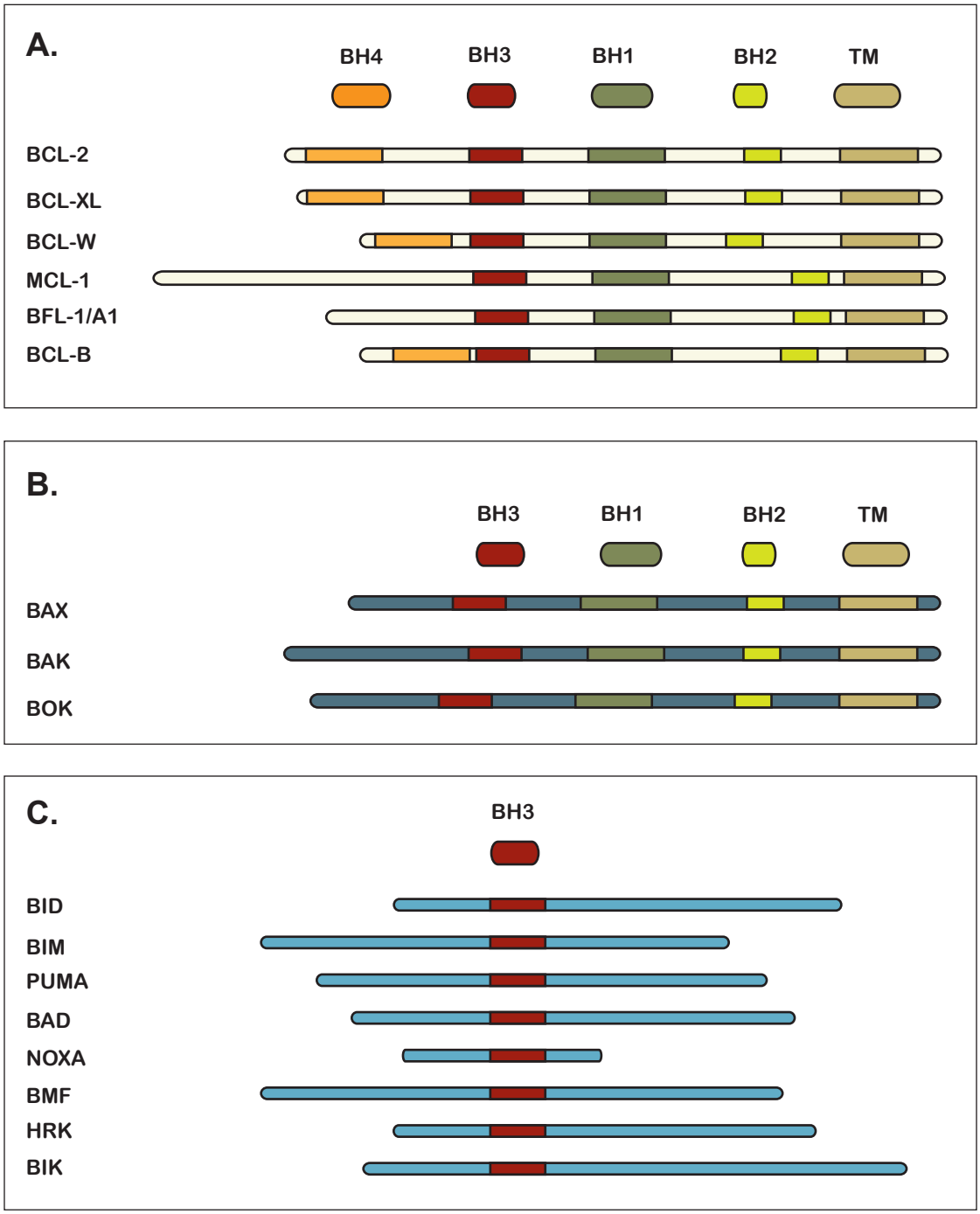


Figure 1.2. Structural representation of Bcl-2 family members. Bcl-2 proteins can share up to four Bcl-2 Homology (BH) domains whose presence subdivide them into **(A)** multi-domain pro-survival proteins; **(B)** multi-domain pro-apoptotic proteins, and **(C)** BH3-only pro-apoptotic proteins. (*Adapted from: Hardwick & Youle, 2009).*

members (e.g. BID-BAX or BAX-BAK) (106-108). Arguably, any BCL-2 family member heterodimerization occur *via* the BH3 domain of one BCL-2 family member with a hydrophobic pocket of the heterodimer partner, although additional binding sites have been identified for certain family members (109-112). Importantly, this BH3:groove interaction takes place when pro-survival BCL-2-like proteins bind to and inhibit BAX and BAK, as well as when they antagonize the various BH3-only members (107, 108).

The BH3 domain is a 9 to 16 amino acid-long region, which contains a conserved L-XXX-GD motif, in which X can be any amino acid (12, 113). In the multi-domain proteins, a hydrophobic groove formed by helices 2, 3, and 4 in BH1, BH2 and BH3 domains, and constitute the major site for BH3 domain binding (114). Interestingly, the groove residues of BAX remain buried until an apoptotic stimulus promotes conformational changes that allow accommodation of a BH3 domain in the groove (111, 114). Due to its transient nature, characterization of BH3-only protein interaction with BAX/BAK has been challenging, but points to involvement of additional N-terminal regions on BAX/BAK besides the hydrophobic groove region described above. Further insight on these interactions and the consequence for MOMP are discussed below.

Anti-apoptotic BCL-2 proteins protect the integrity of mitochondrial outer membranes by directly inhibiting pro-apoptotic members of the family. They

achieve that by directly binding to and sequestering them in an inactive state (115-118). BCL-2, BCL-x_L, MCL-1 and BCL-w are the most well studied members of the anti-apoptotic class and are frequently found overexpressed in cancers, contributing to apoptosis evasion (9, 119, 120). The various anti-apoptotic BCL-2 family members differentially suppress BH3 proteins, BAX and BAK (Fig. 1.3). For instance, while BAX can be inhibited by all of the anti-apoptotic proteins, BAK inhibition is restricted to MCL-1, BCL-x_L and A1 (Fig. 1.3) (121, 122). In contrast, the pro-apoptotic members compromise the integrity of mitochondria either directly or indirectly. The multi-domains BAX and BAK do so by catalyzing pore formation during MOMP, once they are activated by BH3-only proteins (7, 121).

1.2.3.2 BH3-only proteins

BH3-only proteins sense stressful stimuli and, in response, connect initiating apoptotic signals to MOMP. They control pore formation by directly activating BAX and BAK (BIM, BID, PUMA), indirectly by antagonizing the anti-apoptotic members (BAD, NOXA, BMF), or function through a combination of both mechanisms (Fig. 1.4) (118, 121, 123, 124). Since they are potent inducers of apoptosis, BH3-only proteins are subject to tight control (125).

Deletion of BH3-only genes in mammals has been linked to several deleterious phenotypes, and loss of function mutations or genetic deletions have been reported in several cancers (11, 126-128). The BH3-only proteins comprise

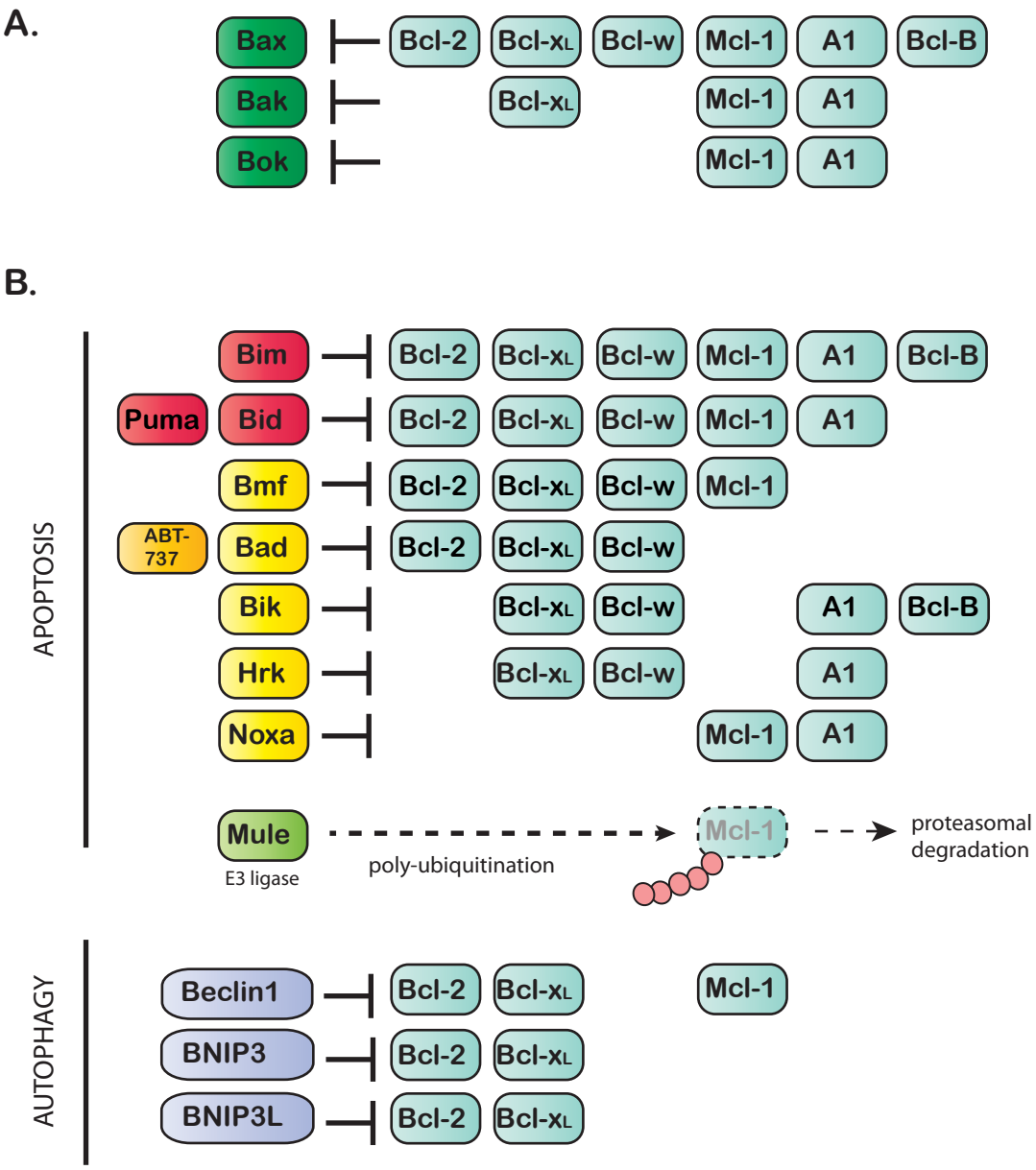


Figure 1.3. Functional interactions among the various sub-classes of Bcl-2 family members. (A). Bax can be inhibited by all anti-apoptotic proteins, while Bak is inhibited by the Bcl-xL, Mcl-1 and A1 subgroup. **(B).** Bim is the universal anti-apoptotic antagonist, while other BH3-only proteins confer specialized antagonism. (*Adapted from: Rautureau et al., 2012; Shamas-Din et al., 2013; Stankiewicz et al., 2009*).

the largest class of BCL-2 family members, comprising 9 mammalian proteins implicated (directly) in apoptosis control (Fig. 1.2) (129). BIM, BID and PUMA can interact with most if not all anti-apoptotic family, as well as with BAX and BAK (Fig. 1.3) (121). Other BH3-only are specialized in antagonizing subsets of pro-survival members: NOXA exclusively antagonizes MCL-1 while BAD can antagonize BCL-2, BCL-w and BCL-x_L, but not Mcl1 (Fig. 1.3) (121). Apart from those that play a role in MOMP, Mule is a BH3-only HECT domain-containing E3 ligase reported to regulate MCL-1 levels (130, 131), which can also be regulated in a ubiquitynation-independent manner (132). Beclin-1, BNIP3 and BNIP3-like (BNIP3L) have been instead implicated in regulation of autophagy (11, 133).

BID

BID (BH3 interacting domain death agonist) is one of the best-characterized BH3-only proteins. It was first identified in 1996 on a cDNA library screen and found capable of binding both recombinant BCL-2 and BAX (134). BID was found to be a BCL-2 antagonist, capable of promoting caspase-dependent cell death, and its BH3 domain was required for its interaction with both BCL-2 and BAX (134).

Since BID activation depends on its proteolytic cleavage (26, 103, 135-140), BID is primarily implicated as a mediator of death receptor signaling on type II cells and as an important player in the activation of caspase amplification loops (102, 141). Importantly, BID is capable of antagonizing all anti-apoptotic

proteins, except for BCL-B, as well as engaging directly BAX and BAK (Fig. 1.3) (121). In the context of this study, it is important to note that BID has been implicated in heat shock-induced apoptosis and this mechanism will be discussed later in detail in the section 1.3 devoted to heat shock-induced apoptosis (142).

BIM

Bim was independently cloned by O'Connor and colleagues (1998) in a yeast two-hybrid screen for BCL-2 interactors and by Hsu and colleagues (1998) in an MCL-1 yeast two-hybrid screen, where it was termed Bod (BCL-2-related ovarian death agonist) (143, 144). Mutation analysis has shown that both the BH3 domain of BIM and its C-terminal transmembrane region were required for killing (144). Later studies have implicated BIM in apoptosis initiated by several apoptotic stimuli such as cytokine withdrawal, DNA damaging agents (gamma-irradiation, doxorubicin, UV), ER stress, cytoskeletal damage and *anoikis*, which is caused by loss of cell adhesion and/or integrin signaling. *Anoikis* is an important cell death mechanism, especially in epithelial tissues as it prevents detached cells from colonizing elsewhere (127, 145-147).

Bim knockout mice display a striking phenotype of lymphoid and myeloid cell hyperplasia, noticeable splenomegaly, lymphadenopathy and systemic *lupus erythematosus*. Indeed two-thirds of the embryos are not viable, with lethality

occurring prior to day E9.5 for unknown reasons (148). Additionally, loss of Bim can accelerate lymphomagenesis in the *Eμ-Myc* tumor model, suggesting a role for Bim as a tumor suppressor (149, 150). Interestingly, loss of Bim can rescue the fatal polycystic kidney disease, premature aging, lymphopenia, and runting caused by loss of Bcl-2, suggesting that BIM initiates apoptosis in various tissues, and modulation of its BH3-only activity/levels by can be a way to control apoptosis (151).

The Bim gene can give rise to several BIM isoforms due to alternative splicing mechanisms (105, 144, 152-154). All three major splice variants of BIM (BIM_{EL}, BIM_L and BIM_S) are potent killers, and stable cell line expression has been achieved only when Bcl-2 or a functional homolog is co-expressed at high levels (113, 144). Even though the BH3 domain of BIM is essential for its apoptotic activity, a BIM version lacking the BH3 was reported to still promote cell toxicity suppressing clonogenicity of L929 cells, apparently by an unknown non-apoptotic cell death mechanism (145). In spite of its hydrophobic C-terminus/membrane localization sequence, upon overexpression BIM is reported to be cytosolic (144, 145). Additional isoforms of BIM have been discovered and their expression pattern, splicing mechanisms, and apoptotic activity vary according to the tissues in which they are found (152-155). For example, BIM-gamma is a splice variant of BIM that is preferentially expressed in prostate cancer cells but not in normal healthy prostate cells (155).

The regions present in BIM_{EL} and BIM_L, but lacking in BIM_S, attenuate their apoptotic potential, making BIM_S the most potent version of BIM (144). Such regions were later shown to contain domains involved in the regulation of the stability, intracellular localization and pro-apoptotic activity of BIM (145, 156, 157). Interestingly, the isoforms that are commonly expressed in most cell lines and normal tissues are BIM_{EL} and BIM_L (144, 158), but whether that reflects a consequence of their regulatory mechanisms or a consequence of preferences in Bim gene splicing is unclear (105).

Yeast two hybrid studies, combined with immunoprecipitation analysis, have demonstrated that both BIM_L and BIM_{EL} bind to light chain-8 (LC8), a component of the dynein light chain-1 (DLC1) microtubule-associated dynein motor complex (145). LC8 binding to BIM is proposed as a regulatory mechanism to sequester BIM_L in the dynein motor complex, decreasing its apoptotic activity until further dissociation, which could be triggered by intrinsic pro-apoptotic stimuli such as cytokine withdrawal, gamma-irradiation, doxorubicin and UV, but not with TNF plus cycloheximide, an extrinsic apoptotic treatment (145, 159).

This BIM-LC8 interaction was hypothesized to be modulated by JNK phosphorylation in response to UV treatment, since JNK phosphorylates a threonine residue (T112) within the DLC1 – binding domain (DLB) of BIM (Fig. 4.1D) (156, 157). Phosphorylation of Thr112 by JNK was required for BIM-induced apoptosis following glucocorticoid treatment and caused increased binding of BIM with BCL-2 (157). Furthermore, cells from mutant Bim^{T112A} knock-

in mice display defects in negative selection of thymocytes, supporting an important role for JNK-mediated phosphorylation of BIM in lymphocyte apoptosis *in vivo* (157).

Although this model relies on BIM being associated with microtubules, there seems to be a discrepancy since in some cell types, such as T cells, BIM is reportedly in constitutive associated with mitochondria (160). Additionally, while the phosphorylation by JNK has been demonstrated to distinctly affect BIM function both *in vitro* and *in vivo*, and with overexpression studies, it is still unclear if JNK phosphorylation-mediated loss of binding to DLC1 recapitulates with endogenous proteins *in vivo* (127, 156, 157). Therefore, the mechanisms regulating BIM protein availability and ultimately its apoptotic activity remain unclear and could vary depending upon the cell type and the stimulus (157).

BIM is also phosphorylated on additional residues by other members of the MAPK family such as extracellular signal-regulated kinase (ERK) and p38 (161). These post-translational modifications reportedly interfere with the ability of BIM to interact with binding partners (156, 162, 163), or regulate BIM protein stability (161, 164, 165). BIM has been shown to be ubiquitinated on Lys3 and Lys108 following ERK phosphorylation on Ser55, Ser65 and Ser73 on mouse BIM_{EL} (corresponding to Ser59/69/77 in human BIM_{EL}) with its subsequent degradation by the 26S proteasome (166).

In order to further understand the physiological role of those phosphor-sites *in vivo*, Hübner and coworkers (2008) generated knock-in mice expressing

Bim mutants on all three serines (Bim3SA), and the mice were viable, suggesting that alternative regulatory mechanisms for BIM turnover exist (157). However the Bim^{3SA} cells were protected from serum-induced BIM phosphorylation and degradation, consistent with previous reports (164, 165). It is important to note that the same serine residues may also be targets for JNK and p38 in response to stress (167-169). While most literature points to ERK-dependent phosphorylation of BIM as a pro-survival mechanism regulating protein stability, JNK and p38 have been associated with an increase in BIM pro-apoptotic activity (164, 165, 167-169).

In addition to post-translational regulation, Bim levels are also controlled at the transcriptional level. The Bim promoter responds to transcriptional activation by E2F1 (170), FOXO3a (171, 172), CHOP-C/EBP-alpha, RelA (173), glucocorticoid receptor (174), and the AP-1 family member c-Jun (175, 176). Conversely, Bim can be transcriptionally repressed by non-canonical NF-κB signaling (177); for example, in *traf2* null MEFs, p100 is constitutively processed to p52 (178), indicating that the non-canonical pathway is constitutively active, and we find BIM protein levels are severely decreased that in those cells, most likely *via* transcriptional downregulation (data not shown).

BIM holds the exclusive capacity to tightly bind and antagonize all 6 anti-apoptotic members of the BCL-2, acting as a universal pro-survival BCL-2 protein antagonist (Fig. 1.3) (179). In addition, evidence points to BIM as a direct activator of BAX and BAK (111, 180-182). With the exception of binding to BCL-

B, both of these properties are largely shared by BID. Although BIM engagement of BAX is not essential for its pro-apoptotic activity in certain tissues, it is required for its full apoptotic regulation to achieve homeostasis in the whole animal, since suppression of pro-survival BCL-2 relatives by itself is not sufficient (183).

1.2.3.3 BAX and BAK

The smallest class in the family, with only 3 members identified to date, is the multi-domain, pore-forming, pro-apoptotic family members BAX, BAK and BOK. Bax and Bak display significant functional overlap, compensating for one another *in vivo* (104). However, a striking phenotype results from the combined deletion of both Bax and Bak. The Bax/Bak double-knockout mice exhibit gross defects in developmental apoptosis, such that 90% of the animals are nonviable, and the 10% that survive display splenomegaly, lymphadenopathy and interdigital webbing. Analysis of the cells derived from the surviving animals indicate resistance to a vast array of toxic stimuli, further implicating BAX and BAK at the core of the mitochondrial apoptotic program (12, 54, 56, 104).

It is important to note that several other organs, such as liver, lungs, and heart, developed normally in Bax/Bak double-knockout mice, suggesting that the intrinsic apoptosis pathway was not essential for their development. Perhaps in these organs the extrinsic pathway predominates, or other forms of programed cell death occur. Alternatively, the poorly characterized protein BOK could be

substituting for Bax and Bak in these tissues, facilitating MOMP and activation of the intrinsic pathway. BCL-2-related-ovarian-killer (Bok) was isolated from a rat ovarian cDNA library and shown to interact specifically with MCL-1 and A1 (Fig. 1.3) (121, 184). Despite an anticipated role for BOK in reproductive tissue homeostasis, as suggested by Bok's expression pattern (184), the recently generated Bok null mice have no apparent developmental defects (185). Therefore, generation of the Bax/Bak/Bok triple knockout could be instrumental in uncovering a physiological role for BOK.

Structurally speaking, both BAX and BAK are globular proteins consisting of nine helices, where the C-terminal α9 functions as a transmembrane domain that anchors BAX and BAK in the mitochondrial outer membrane (MOM) (186, 187). In BAX, this α9 is kept sequestered by the hydrophobic groove, so that in healthy cells, most BAX is cytosolic or loosely attached to the MOM (186-189), while in contrast, BAK is constitutively integrated in the MOM (190, 191). Both BAX and BAK can also be found in small amounts in the ER, but the physiological role of this intracellular localization is unclear (192, 193). During apoptosis, BAX translocate to mitochondria where it inserts into the MOM, and both BAX and BAK undergo a complex set of conformational changes that characterize their activation step (109, 189).

A crucial step in BAX and BAK activation is the exposure of their BH3 domain, which allows their oligomerization (194, 195). BAX and BAK are activated directly by the tBID/BIM/PUMA subset of BH3-only proteins (Fig. 1.3).

There are two proposed binding sites on BAX, the canonical hydrophobic groove, and the α1/α6 rear pocket situated on the opposite side of the BAX molecule (111). The C-terminus of BAX blocks entry to the groove and how exactly it gets displaced is unclear, most likely by the binding of a direct activator BH3-only protein (109, 154, 196). Binding of the BH3 of BIM or tBID to the groove causes exposure of the BAX BH3 domain (located in α2). The subsequent conformational changes cause the displacement of the BH3-only protein and allow for BAX BH3 to bind the groove of an adjacent BAX or BAK molecule (111, 197), causing a chain reaction of oligomerization.

Mitochondrial outer membrane permeabilization – MOMP

Initial insights into the biology of BCL-2 function and regulation came from electrophysiological studies showing that BCL-2, BCL-x_L and BAX can form pores with ion channel characteristics in liposomes, and BCL-2 antagonized the BAX-mediated channel formation observed in these studies (198-201). Crystallographic studies revealed that BCL-2, BCL-x_L and BAX share common structural features. BCL-x_L was the first BCL-2 family member to have its crystal structure solved. The arrangement of alpha-helices in BCL-x_L shows impressive similarity with pore-forming bacterial toxins (202), and akin to such toxins, BAX undergoes profound conformational changes upon its association with lipids of the MOM (203).

Although initially these similarities suggested a common mechanism, there are significant structural differences between bacterial pore-forming toxins and BCL-2 proteins, implying distinct mechanism of pore formation. Currently, the pores that modulate MOMP are thought to be constituted of homo or hetero-oligomers of BAX or BAK, through which apoptogenic factors are released into the cytosol (7). Alternatively, BAX and BAK could catalyze the formation of channels comprised of other MOM integral proteins (such as VDAC) or even constituted by lipids (7).

Even though there is controversy as to the exact mechanism of BAX/BAK pore formation, there is a consensus that initiating events must culminate in induction of conformational changes in BAX/BAK that consist in their activation step prior to their oligomerization. It has been proposed that BH3-only proteins can cause BAX/BAK activation, either by directly binding to them, or by de-repressing BAX/BAK from anti-apoptotic BCL-2 proteins (110, 120, 204, 205). Either way, that promotes the tridimensional reordering of BAX molecules so that they are prone to membrane insertion and oligomerization. Studies with stabilized alpha helix from BCL-2 domains or SAHB peptides modeled on BIM and BID BH3 domains showed measurable high-affinity binding to BAX in the low nanomolar range. The interaction was sequence-dependent and specific to these BH3 domains, as binding interface point mutants, as well as a SAHB modeled on the BH3 domain of BAD, which is a sensitizer BH3-only protein, failed to bind

BAX (206).

Gavathiotis and coworkers (2008) identified an additional BAX binding site for BIM, using a modified BIM SAHB and NMR analyses, located on the opposite side of the canonical BH3-binding groove of BAX (111). Residues from α1 and α6 constitute the novel binding interface on the N-terminal surface of BAX. The authors proposed that the engagement of this new site by select BH3 domains functions as an “on switch” that induces direct BAX activation (111). Besides direct activation by BH3-only proteins (110, 120, 204, 205), physical and chemical agents such as pH variation, H_2O_2 and heat shock, as well as post-translational modifications, such as proteolytic cleavage, and phosphorylation, were found to trigger BAX conformational changes (207-211). Additionally, various non-ionic detergents were shown to promote the exposure of an N-terminal epitope found between amino acid residues 12-24 in BAX, which stays buried in most healthy adult cells, and that can be recognized by the monoclonal antibody 6A7 (212, 213).

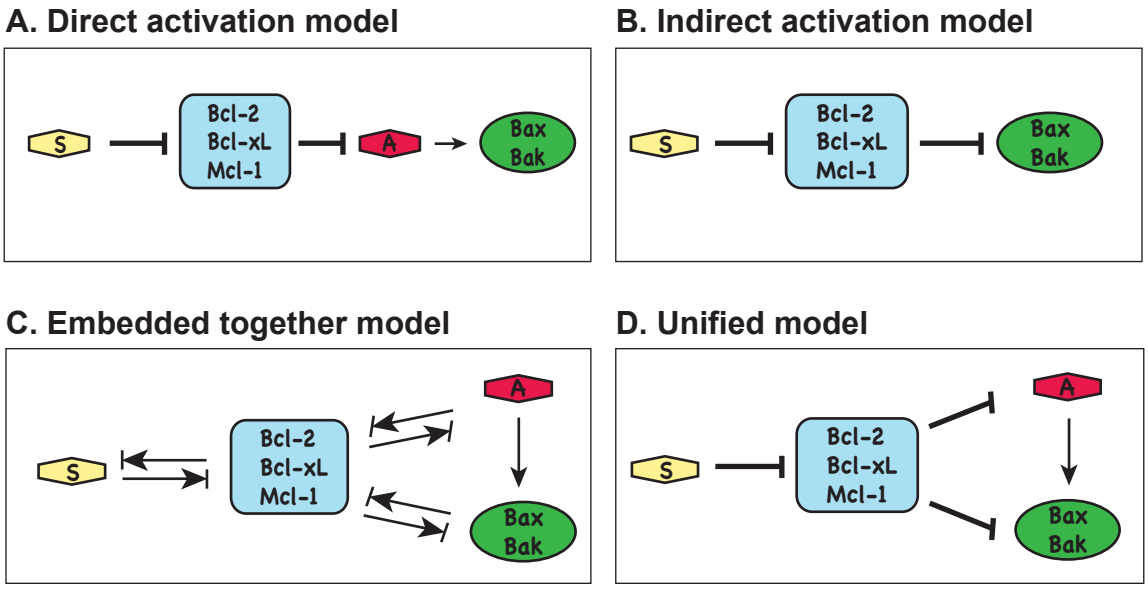
One of the most relevant findings, in the context of heat shock-induced apoptosis, was shown by Pagliari and colleagues (2005). They demonstrated that heat *per se* can promote BAX and BAK to undergo the conformational changes necessary for activation, without the participation of other cellular proteins (208). However, cytosolic factors (such as BCL-x_L, MCL-1) must be overcome in order for MOMP to ensue, as suggested by the delayed onset of

MOMP in cells compared to cell-free extracts (208, 214).

MOMP regulation models

There are currently a few models that attempt to explain the process of MOMP regulation by BCL-2 proteins (Fig. 1.4). According to the *direct activation model* (Fig. 1.4A), a member of the direct activator class of BH3-only proteins must directly engage BAX/BAK to promote the activating conformational changes, independent of any other cellular factors (197, 204, 215). First insights, pointing out to the direct activation of BAX by a BH3 domain, came from the identification of tBID as a BAX and BCL-2 binding protein (134). Additionally, tBID point-mutants were identified, which were still capable of binding BAX and causing apoptosis, even though they lost their ability to bind BCL-2 (134).

The activation-associated conformational changes upon the direct binding of a stapled BID BH3 helix to BAX (206), as well as the observation that BH3 peptides derived from either tBID or BIM could also induce BAX to form pores and permeabilize liposomes in a cell-free system (216-218), further support the "direct activation model". However, the difficulty in detecting the binding of full length BIM, BID or tBID to BAX and BAK (118), argues against it. Nevertheless, more recently, the interactions of BH3 domains with BAX and BAK were found to be fast and transient, suggesting a "hit-and-run" binding mechanism (197).



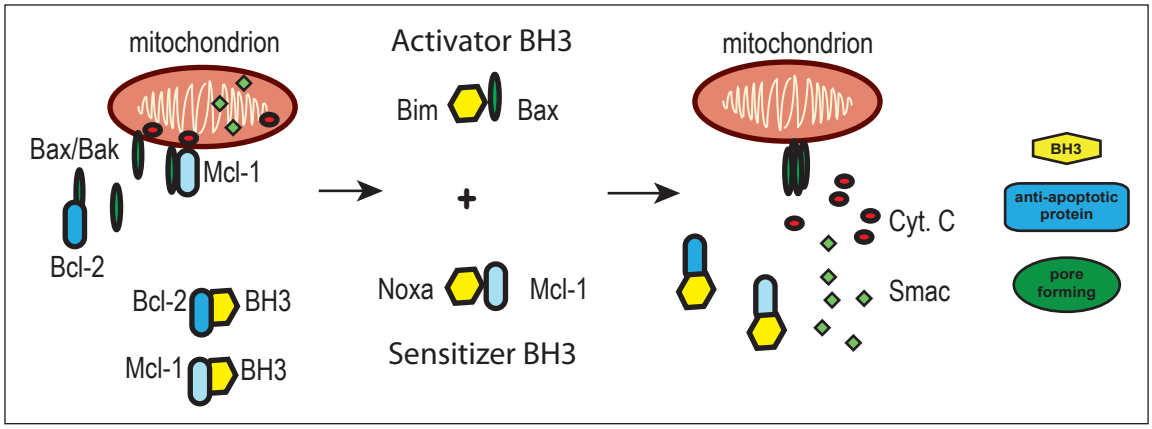


Figure 1.4. Proposed mechanism of action of BH3-only proteins in MOMP induction. (A). *Direct activation model*: initiated when BH3-only sensitizers neutralize the antagonic action of anti-apoptotic proteins primarily in blocking BH3-only activators, which then bind to Bax / Bak promoting their conformational change and activation. **(B).** *Indirect activation model*: all BH3-only proteins are considered as sensitizers in this proposed mechanism of action due to their function simply as derepressors of the inhibition exerted by anti-apoptotic Bcl-2 onto Bax/Bak. **(C).** *Embedded together model*: this model is based on (A) with additional emphasis on the requirement of the mitochondrial membrane in mediating the interactions among the various Bcl-2 proteins involved. **(D).** *Unified model*: in contrast to (C) and (A), the unified model emphasizes the function of BH3-only sensitizers as the main initiators by their de-repression of either BH3-only activators or of Bax/Bak. **(E).** *Overview of MOMP*: diagram of the events culminating in MOMP according to the unified model. *S*, sensitizer; *A*, activator. *(Modified from: Shamas-Din et al., 2013).*

If the “direct activation model” holds true, then deficiency of direct activator BH3s should prevent BAX conformational changes, translocation to mitochondria, oligomerization, MOMP, and cell death in response to intrinsic pathway death stimuli. Cells from Bim/Bid double knock out mice, however, were shown to be sensitive to several apoptotic stimuli, questioning the *in vivo* meaning of the direct activation model (118). PUMA (but not its BH3 peptide) was reported to function as a direct activator (120, 191, 204, 209, 219). Therefore, Ren and coworkers (2010) generated a *Bim*$^{-/-}$*Bid*$^{-/-}$*Puma*$^{-/-}$ (TKO) mouse, which was embryonically lethal, with a phenotype that was strikingly similar to the *Bax*$^{-/-}$ *Bak*$^{-/-}$ DKO mice. Together, these findings suggest that BIM, BID and PUMA act as BH3-only direct activators (220).

In the *indirect activation model* (Fig. 1.4A), BAX is held in check by BCL-2 pro-survival family members, which are displaced by BH3-only proteins. In this scenario, the simple displacement of pro-survival BCL-2-like proteins should ensure BAX activation (115-118). If this holds true, loss of anti-apoptotic members could cause BAX and BAK activation even in the absence of all direct activator BH3-only proteins. However, the profound phenotype exhibited by the *Bim*$^{-/-}$*Bid*$^{-/-}$*Puma*$^{-/-}$ (TKO) mouse described above would strongly argue against this model (220). In contrast, the fact that physical and chemical agents (e.g. detergents and heat shock) can make cells more susceptible to MOMP suggests that direct activation by a BH3-only protein may not be the only way to cause

activating conformational changes in BAX/BAK (208, 212, 213).

The *embedded together model* (Fig. 1.4C) puts emphasis on the role that mitochondrial membranes provide to the process of MOMP. It was shown by Kuwana and coworkers that the mitochondrial membrane lipids provide a favorable biochemical milieu for the cooperation of tBID and BAX to form pores in the outer mitochondrial membrane (215, 221). The “embedded together model” claims that the important functional interactions between BCL-2 proteins occur only in membranes: upon membrane binding those proteins assume appropriate conformations that are conducive and necessary for their interactions to occur (222, 223). In essence, pore formation is the result of sequential recruitment of membrane-bound activated BAX/BAK dimers, which then interact with one another to create a channel of increasing size that allows for cytochrome c and other factors to exit the inner mitochondrial space (7, 215, 224, 225).

Finally, the *unified model* (Fig. 1.4D) incorporates characteristics of all the above-mentioned models and subdivides the inhibition of BCL-2 anti-apoptotic proteins into 2 modes: (A) blockade of activator BH3-only proteins and (B) blockade of BAX/BAK. These 2 modes of inhibition occur simultaneously in cells and display differential efficiencies of suppression. In this model, the mode “A” of inhibition is easily overcome by BH3 sensitizers, which release the activators to act on inhibition mode “B” and promote MOMP. This model is supported by an in vivo study of mice expressing Bim BH3-replacement mutants, where the BH3 of

BIM was swapped for the BH3 domains of BAD, NOXA or PUMA (183). The acquired specificity of prosurvival BCL-2 protein binding accompanied by the loss of binding to BAX in these mutants showed that *in vivo* both functions are required for homeostasis (183).

In summary, the mechanisms controlling mitochondrial integrity by BCL-2 proteins are complex and still not entirely understood. It is possible that variability inherent to cell types and the molecules in question can contribute to the challenge of better describing how this process is regulated. Nevertheless, MOMP is considered "**the point of no return"** in commitment to apoptosis, as it provokes the diffusion into the cytosol of numerous proteins normally sequestered in the intermembrane space (7). Once in the cytosol, their combined action results in the activation of caspases and execution of apoptosis. Ultimately, mitochondria not only "retain-and-release" pro-apoptotic molecules when needed, they also function as a platform where apoptotic signals converge and are integrated to result in MOMP by members of the BCL-2 family (121).

1.2.4 The intrinsic pathway: activation of initiator caspase-9

One of the most relevant consequences of MOMP is the release of the release of Cytochrome c, which is an essential cofactor for the assembly of the caspase-9 activating complex, the **apoptosome** (17, 226). This heptameric complex composed of Apaf-1, cytochrome c, dATP and pro-caspase-9, where

Apaf-1 assists caspase-9 activation via dATP hydrolysis, is assembled when cytochrome c binds to monomers of Apaf-1 causing conformational changes that favor its oligomerization (17, 226). The apoptosome-dependent activation of caspase-9 is essential for the activation of neuronal and fibroblast cell-death processes, as Apaf-1 deficiency causes defects in brain development (227).

The elegant study by Liu and coworkers in 1996 using a biochemical fractionation approach led to the purification and identification of cytochrome c as one of the protein factors that triggered the activation of caspases in the presence of dATP in a cell-free system (35). Cytochrome c is essential for mitochondrial respiration: it associates loosely to the surface of the inner mitochondrial membrane where it accepts electrons from Complex III and diffuses to transfer them to cytochrome c oxidase on Complex IV in the respiratory chain (228, 229). Liu and coworkers demonstrated that HeLa cells undergoing apoptosis released cytochrome c into the cytosol following staurosporine treatment (35).

The pro-apoptotic activity of cytochrome c was later shown to be linked to its ability to bind to Apaf-1 and promote its oligomerization (230, 231). Apaf-1 contains a series of thirteen C-terminal WD40- repeats that provide a binding interface for cytochrome c (230, 232). Additionally, Apaf-1 contains a nucleotide and oligomerization domain (NOD) where a molecule of dADP stays bound and its thought to keep Apaf-1 in an inactive conformation (233). Binding of

cytochrome c induces a cycle of dATP exchange for dADP mediated by hydrolysis of recruited dATP into dADP, powering the assembly of the apoptosome (234, 235). Caspase-9 and Apaf-1 bind to each other via their N-terminal caspase recruitment domains (CARD) in a cytochrome c and dATP-dependent manner (17, 236). Depletion of caspase-9 as well as activate site mutants of caspase-9 suppressed the activation of caspase-3, placing caspase-9 upstream of caspase-3 in the mitochondrial apoptotic pathway (17, 236).

Recent studies brought insight into how are the regulation mechanisms of apoptosome formation and activity. The study by Chandra and coworkers showed that apoptosome formation could be inhibited by high levels of nucleotides, due to their interference with cytochrome c binding to Apaf-1 (237). This model also predicts a safeguard mechanism to prevent unwanted apoptosome activation in case of an “accidental” leak of cytochrome c during favorable conditions for cell survival (237).

Another aspect of apoptosome regulation was elucidated by our laboratory (238). This study favored the activation of caspase-9 by the apoptosome in a highly dynamic process termed “CARD-displacement” model, wherein procaspase-9 has a higher affinity for the complex than the processed enzyme. Consequently, it cycles through the complex until procaspase-9 is depleted. According to this model, the apoptosome functions as a “molecular timer”, in which the intracellular concentration of procaspase-9 dictates the overall duration

of the timer, while the rate of procaspase-9 autoprocessing determines the speed of the process. Autoproteolytic cleavage of procaspase-9, and neither its recruitment to the apoptosome, or its dimerization, activates the timer (238).

1.2.5 Caspase-2

Caspase-2 does not fit neatly into the classical division of labor of initiator and effector caspases, and for this reason has been called the "*Cinderella* caspase" (63). The function of caspase-2 is enigmatic and an active field of investigation. Caspase-2 was one of the first genes discovered with homology to *C. elegans* CED-3 (239, 240). Studies with caspase-2 null mice revealed a modest phenotype of overabundant ovarian germ cells in female mice (241). Most tissues in caspase-2 null mice developed normally, and showed comparable cell death levels in response to various cytotoxic stimuli to wild-type mice (241, 242). Interestingly, caspase-2 knockout mice show premature ageing (243, 244), and caspase-2 was recently shown to be responsive to metabolic cues in Xenopus (245, 246).

Additionally, there is evidence that caspase-2 can function as a tumor suppressor protein (247). *Some* tumors exhibit reduced expression of caspase-2 (248-253). Even though caspase-2 deficient mice do not develop tumors spontaneously, caspase-2 can protect cells from transformation (247, 254, 255), and when crossed onto an oncogenic background, such as Eμ-*Myc* transgenic

mice, loss of caspase-2 accelerates lymphomagenesis (254). Additionally, loss of caspase-2 synergized with the mammary tumor virus (*MMTV*) model of breast carcinoma to promote the formation of mammary tumors in multiparous mice, further implicating a tumor-suppressor role for caspase-2 (256).

Caspase-2 is the only caspase constitutively localized in the cell nucleus, actively imported via a leader sequence in its prodomain (257, 258), but the biological relevance of this localization is unclear. Interestingly, several non-apoptotic functions attributed to caspase-2 to date, such as regulation of cell proliferation, DNA damage response (DDR), and genomic stability, could benefit from its nuclear localization. Additionally, caspase-2 was implicated on mitotic catastrophe - an important mechanism to eliminate aneuploid cells resulting from abnormal mitoses. Altogether, these caspase-2 nuclear functions could contribute to its putative role as a tumor suppressor (254, 256, 259-261).

Structurally speaking, caspase-2 resembles a classical initiator caspase, containing a long pro-domain with a caspase activation and recruitment domain (CARD). Similar to other initiator caspases, caspase-2 is recruited via its CARD pro-domain to an activating complex (262). In response to DNA damage, caspase-2 is recruited to a large molecular complex (76). Later this complex was shown to contain PIDD (p53-induced protein with a death domain) and the CARD-containing protein RAIDD (RIP-associated ICH-1/CED-3 homologous protein with a death domain). RAIDD recruits caspase-2 via CARD-CARD

homotipic interactions to the PIDDosome, where caspase-2 activation occurs by proximity-induced dimerization followed by auto-processing between its small and large subunits (76, 262, 263). A fully mature enzyme is achieved after further processing removes the N-terminal CARD (264). Several studies place caspase-2 upstream of mitochondria in some stress responses, where BID is a caspase-2 substrate and similarly to a caspase-8-dependent pathway, Bid deficiency protects from caspase-2-induced cell death (142, 265, 266).

Even though structurally and mechanistically these studies suggest that caspase-2 functions as an apical caspase, it is still unclear how caspase-2 induces apoptosis, particularly since unlike caspase-8 and -9, caspase-2 does not directly cleave any other mammalian caspase except for its own precursor, and possibly caspase-7 (267, 268). In fact, rather than as an initiator, caspase-2 has been proposed to function as part of an amplification loop by the observation that it can be efficiently cleaved by caspase-3 in various experimental settings (269-272), and has been shown to be activated independently of the PIDDosome (273).

Nevertheless, caspase-2 null mouse embryonic fibroblasts (MEFs) showed partial protection to heat-shock (discussed in more detail on section 1.3) (274). Additional evidence point that caspase-2 caters to a subset of stimuli that include DNA damage, mitotic catastrophe and nutrient depletion (265) (242, 246, 274-276). Together, these studies suggest that caspase-2 can participate in cell

death activation in a cell-type or stimulus-dependent manner, but additional apoptosis pathways are in place conferring redundancy to caspase-2 (264, 272, 277).

1.3. Heat shock-induced apoptosis

Heat shock is one example of an environmental stress that causes apoptosis. Both the intrinsic and extrinsic pathways have been shown to be involved in heat shock-induced apoptosis (278-280). Hyperthermia or heat shock was also shown to promote apoptosis in tumors, contributing to tumor shrinkage and remission after high fevers (281-284). These early reports of fever-induced tumor remission fostered the interest of the biomedical scientific community in the use of heat shock alone or in combination with other chemotherapies for the treatment of solid tumors (284). Therefore, several past and ongoing research efforts have focused on better understanding the basic signaling pathways that govern cellular responses to heat shock, in particular those involved in heat-induced apoptosis (280, 285-287).

As a physical toxicant, heat produces a complex array of effects in the cell (284, 285, 288). The intrinsic thermolability of individual cell component

influences the capacity to withstand elevated temperatures (285). The damage caused by heat positively correlates with thermolability, as well as with the intensity of the heat shock (i.e. the heat shock dose), which results from the combination of the time and the temperature during exposure (285). Several factors that can influence these variables contribute to the complexity of responses displayed by cells.

1.3.1. Cellular responses to heat shock

Organisms have adapted to live in temperatures from 0-113°C, however, for most organisms, heat represents a significant challenge for life. A small increase in temperature can cause protein unfolding and entanglement resulting in nonspecific aggregation and a widespread imbalance in protein homeostasis. Therefore, the heat shock response is an ancient and fairly widespread homeostatic mechanism (284).

The deleterious effects of heat to the cellular organization go beyond the proteome. Changes in plasma membrane fluidity are one of the primary effects of hyperthermia, causing disorganization of membrane lipids and altering permeability (285, 288). Particularly to eukaryotes, the cytoskeleton can be severely damaged by heat. Mild heat stress leads to the reorganization of actin filaments into stress fibers. Severe heat stress however causes the collapse of intermediary, actin and tubulin networks, disruption of the intracellular transport

processes and fragmentation of the Golgi and endoplasmic reticulum network (285, 289, 290). Mitochondrial oxidative phosphorylation is uncoupled, resulting in a dramatic drop in ATP levels during heat stress (284). In the nucleus, RNA splicing is strongly impaired by heat shock. Large granular aggregates of ribosomal proteins and incorrectly processed RNAs form visible deposits in the nucleus, and in the cytosol, stress granules composed of stalled translational RNA-protein complexes are formed and correlate with a global shutdown of protein translation (284, 291).

Combined, these effects can cause different cellular responses in a dose-dependent manner. If the intensity of the heat stress is non-lethal, cells can temporarily acquire tolerance to more severe and otherwise fatal stressors - a phenomenon known as *thermotolerance* (284). This rise in tolerance correlates with the upregulation of so-called heat shock proteins (HSPs) that provide protection to not only additional heat stress but also to several other insults, including ionizing radiation, nutrient withdrawal, UV exposure, polyglutamine repeat expansion, TNF treatment, and chemotherapeutic agents (292-295).

Heat shock factor-1 (HSF-1) is a specialized transcription factor that promotes the fast and transient upregulation of HSPs such as HSP70 and HSP90 when denatured proteins accumulate in the cell (296, 297). These molecular chaperones assist with the refolding of damaged proteins into their native conformations as an attempt to repair the damage. The protective function

of HSPs, as well as other aspects of thermotolerance will be further discussed in chapter 4.

However, when the intensity of the heat causes damage beyond the repair capacity of cells, they may chose to undergo cell cycle arrest or to activate apoptosis to eliminate the injured cells (280). Since apoptosis is a mechanism to delete cells harboring detrimental lesions prone to cancer initiation, it is possible that failure to eliminate such heat-damaged cells could be tumorigenic. From an immunological standpoint, fevers are an intrinsic component of the inflammatory responses to pathogens (298, 299). Fever may contribute to elimination of infected cells by inducing apoptosis, as an alternative to *pyroptosis*, which is a form of programmed cell death activated in infected cells, in response to pathogens (300).

Heat shock can induce both apoptosis and necrosis in a dose-dependent manner in cultured cells and *in vivo* (281-283). However, it is unclear how heat shock causes apoptosis at the molecular level. Previous studies have implicated the extrinsic pathway in heat shock-induced apoptosis (278-280); however these studies were focused on understanding the combined effects of heat shock on death receptor and/or chemotherapy-induced cell death. Although clinically relevant, the complexity of those experimental approaches makes it difficult to tease out the molecular pathways specific to heat shock. Thus, our studies have focused on the mechanism of heat shock alone in inducing apoptosis, resulting in

a model for heat shock-induced apoptosis (discussed below) (142, 208, 301-304).

1.3.2. Current model for heat shock-induced apoptosis

According to the current model, acute exposure to heat, like other environmental stresses, leads to apoptosis by engaging the intrinsic pathway. In 2000, Mosser and coworkers reported that heat shock caused MOMP and activation of caspase-3 (301). However, the initiation events involved in heat shock are distinct from other types of stress, as it implicates the upstream activation of caspase-2 (142, 274, 303). Tu and coworkers (2005) found that heat, in particular, caused the early activation and "*in situ*" trapping of activate caspase-2, but not of initiator caspases-8 or -9, with a biotinylated pan-caspase inhibitor (b-VAD-fmk) (274). Caspase-2 was still labeled even when BCL-2 or BCL-x_L were overexpressed and prevented cell death, placing caspase-2 upstream of the mitochondria (274).

Moreover, splenocytes derived from caspase-2 null mice were resistant to heat shock (43°C/1 h), and so were splenocytes deficient in the caspase-2 adaptor RAIDD (76, 274). The authors reported that heat shock induced PIDD expression, and they speculate that PIDD and RAIDD may both participate in the recruitment and activation of caspase-2 into the PIDDosome, in a similar way to DNA damage (76, 274). Based on their findings, Tu and coworkers (2005)

conclude that heat shock initiates apoptosis by activating caspase-2 in a RAIDD-dependent manner, upstream of the mitochondria (274). However, it is unclear why did caspase-2 and RAIDD null cells not provide better protection against heat shock, if indeed caspase-2 is the essential apical protease. Conversely, it suggests that other caspase(s) must play a redundant role, or alternatively, that caspase-2 could be part of an amplification loop in cells, rather than the true initiator caspase.

Moreover, in the current model, heat *per se* primes BAX and BAK for directly activation by BID (142, 208). Pagliari and coworkers (2005) showed that *in vitro,* heat shock promoted the exposure of N-terminus that stays buried when BAX is inactive, mirroring the action of a direct activator BH3-only protein (208). Importantly, Bax/Bak double knock out (DKO) MEFs were resistant to heat-induced cytochrome c release and apoptosis. However, in cells, the observed onset of MOMP was longer than the observed *in vitro* with pure mitochondria (208). Furthermore, the addition of recombinant BCL-x_L or untreated cytosol prevented MOMP *in vitro* implicating the existence of cytosolic factors that need to be antagonized in order to allow MOMP to take place (208).

Indeed, addition of BH3-only BID or PUMA peptide reverted the protective effect conferred by the cytosolic extract. Together, the authors suggested a model where heat-activated multi-domain BAX and BAK proteins are inhibited by cytosolic anti-apoptotic proteins, such as BCL-x_L, until BH3-only proteins

participate as de-repressors unleashing BAX/BAK to accomplish cytochrome c release from the mitochondria (208). The BH3-only protein BID could be one of the cytosolic factors predicted by Pagliari (2005) to enhance the effect of heat on BAX/BAK in order to accomplish MOMP, and Bonzon and coworkers (2006) later demonstrated that caspase-2-mediated MOMP requires BID. A previous study by Li (1998) found that BID is a caspase-2 substrate, and since BID engages the mitochondrial pathway once it is converted into tBID by caspase-8 cleavage, Bonzon and colleagues hypothesized that caspase-2 could function similar to caspase-8 in the context of heat shock (26, 137, 142, 267).

In agreement, caspase-2 dependent killing was shown to be preventable by BCL-2 overexpression (267). Caspase-2 could not induce MOMP in the absence of BID on a cell-free approach, and when BID was present, BCL-x_L could block the caspase-2-dependent MOMP (142, 266). Caspase-2 cleaves BID at the same site as caspase-8, in a RAIDD-dependent manner (26, 137, 142, 267, 274). Mutation of either the BID cleavage site, or the catalytic cysteine (C320) in caspase-2 protected from cell death upon direct overexpression of caspase-2 (142). Bid null cells were also resistant to heat shock (43°C and 44°C for 1h), but the degree of resistance decreased with time, with the remaining cell death being potentially BID- and caspase-independent necrosis (142).

Previous work from our laboratory failed to find convincing evidence supporting a role for caspase-2, RAIDD and BID in the initiation of apoptosis by

heat shock (302). The study by Milleron and Bratton reported that heat shock could activate a pathway where an apical protease triggers the intrinsic pathway. Intriguingly, even though the putative apical protease was efficiently inhibited by z-VAD-fmk, *all* well-established initiator caspases (caspases-2, -8, -9, -10, -4, -12) were ruled out by the careful utilization of pharmacological inhibitors used in combination with gene-deficient Jurkat T cells and/or MEFs (302).

In contrast with Tu (2005) and Bonzon (2006), Milleron & Bratton (2006) report that primary MEFs deficient in caspase-2 were equally sensitive to hyperthermia as their wild-type counterparts (142, 274, 302); additionally, neither inhibition with the caspase-2 inhibitor, z-VDVAD-fmk, nor depletion of caspase-2 by RNA interference on caspase-8 deficient Jurkat T cells conferred protection to heat-induced MOMP, caspase-3 activation or cell death (302). Using gel filtration studies, Milleron & Bratton (2006) detected oligomerization of PIDD following heat shock. However, neither RAIDD nor caspase-2 were detected in the same complex, indicating that heat shock did not induce the formation of the previously reported PIDDosome (76, 302). However, the pan-caspase inhibitor z-VAD-fmk did block cell death after attenuating MOMP, in agreement with the existence of an apical protease upstream of mitochondria (302).

Shelton and co-workers (2010) sought to solve some of the aforementioned discrepancies. They reported that, similar to Milleron & Bratton's study (2006), a broad caspase inhibitor - Q-VD-OPh - blocked all caspase

processing and cell death. Also, similar to Tu and coworkers (2005), they affinity-labeled caspase-2, but they also captured caspase-8 and caspase-9 in the same assay. Also in contrast to previous studies (142, 274, 302), Shelton and colleagues found only caspase-9 to be essential for cell death, as deficiency of caspase-8, caspase-2, RAIDD, or BID conferred either no protection or partial protection in Jurkat T cells exposed to heat. However, this conclusion was largely based on observations upon RNAi-mediated depletion of Apaf-1, but not upon deficiency in caspase-9 *per se*, in contrast to the studies by Milleron & Bratton where they examined caspase-9 null cells and concluded it was not essential for heat shock-induced apoptosis (302, 304).

Strikingly, the authors find that silencing of Apaf-1 suppressed activation of caspases -3 and -9, truncation of BID and cell death. The cleavage of caspase-2 was also greatly diminished in the Apaf-1 knockdown clones, placing it together with BID as part of an amplification loop that may take place downstream of MOMP (304). This finding does not rule out the possibility that APAF-1 could be playing an apoptosome-independent role in response to heat shock. Recent reports suggest physiological roles for APAF-1, which in principle, are unrelated to its apoptotic function (305, 306). In that context, loss of caspase-9 would not have an effect in spite of the involvement of APAF-1.

The study by Bouchier-Hayes and coworkers (2009) further implicate caspase-2 in heat-shock apoptosis. Based on the premise that dimerization of

initiator caspases, due to their recruitment to an activation platform, constitutes the authentic read-out for initiator caspase activation (66), they implemented a Bimolecular Fluorescence Complementation (BiFC) approach to monitor caspase-2 dimerization and activation (307-309).

The authors show that heat shock, but not Fas or TNF, strongly induced a BiFC signal in the cytosol and not in the nucleus, an often-implicated site of caspase-2 activation (303). Caspase-2 BiFC was RAIDD-dependent, as RAIDD null MEFs failed to show BiFC following heat shock, and caspase-2 CARD mutants that could not bind RAIDD, also failed to exhibit a BiFC signal (303). Even thought the authors validate the caspase-2 BiFC upon enforced expression of the PIDDosome components PIDD and RAIDD, in the absence of heat shock, in this study they did not show direct evidence for the participation or requirement of PIDD as part of the cytosolic caspase-2 complexes formed during heat shock (303). Overexpression of BCL-x_L did not prevent the onset of BiFC after heat shock, even though it protected the HeLa cells from apoptosis, consistent with previous reports that placed the activation of caspase-2 upstream of MOMP (142, 274). Using time-lapse microscopy, the authors found there was a *delay* in the onset of MOMP of about 6-10 hours after the observed BiFC, implicating additional regulatory steps upstream of MOMP, consistent with the study of Pagliari (208, 303).

Heat shock activation of caspase-2 does not require transcriptional

upregulation of additional pro-apoptotic factors, since cycloheximide pre-treatment does not impact heat shock-induced BiFC (303). However, HSF1 null MEFs were more sensitive to induction of BiFC and heat shock-induced apoptosis, suggesting that upregulation of HSPs may prevent caspase-2 activation. Accordingly, silencing of Hsp90 or its pharmacological inhibition with 17-DMAG enhanced BiFC signal (303). It is not entirely surprising that inhibition of chaperone activity might increase the fluorescence of a complex, formed after treatment by an insult that is known to denature proteins and cause protein aggregation. Even though the authors carefully examined that heat shock did not induce the BiFC of Venus *per se*, it is unclear if they ruled out that heat shock does not cause BiFC of additional initiator caspases-8 and -9, which could pointing to the BiFC signal resulting from non-specific heat shock-induced aggregation of proteins that could be more prone to aggregation due to their recruitment domains.

A study by Stankiewicz and coworkers (2009) later identified MCL-1 as an important anti-apoptotic factor, upstream of mitochondria, in preventing MOMP following heat shock (214). According to their findings, in order to undergo apoptosis following heat shock, cells must destroy MCL-1 via ubiquitin-mediated proteasomal degradation, which may be due to the interaction of MCL-1 with its E3-ligase Mule, a non-classical BH3-only protein (214).

BIM and NOXA modulated the MCL-1-MULE interaction, where heat-

induced NOXA displaced MCL-1 from BIM, allowing MULE to bind MCL-1, and HSP70 promoted the stabilization of MCL-1 by preventing MULE binding. This finding is interesting, because it potentially explains the delayed onset of MOMP reported previously (208, 214). Anti-apoptotic proteins such as MCL-1 present in the cytosol probably bind to and hold BAX in check until BH3-only proteins such as tBID or others can displace them from BAX/BAK allowing oligomerization and pore formation (208, 214).

All together, the studies mentioned above have both congruent and conflicting conclusions as to how heat shock triggers the onset of apoptosis (Fig. 1.5). Several studies implicated caspase-2-mediated cleavage of BID to induce MOMP, where tBID binds to BAX/BAK already primed by heat-induced conformational changes (142, 208, 274, 303). In that scenario, degradation of MCL-1 could collaborate with the activation of tBID, which could further de-repress BAX/BAK from other anti-apoptotic family members such as BCL-2 and BCL-x_L (Fig.1.3) (121). However failure to detect the purported caspase-2 activating complexes as well as lack of stronger protection from the knockout cell lines, puts into question some of the aspects of this model, suggesting that alternative mechanisms are involved in the initiation of apoptosis in response to heat.

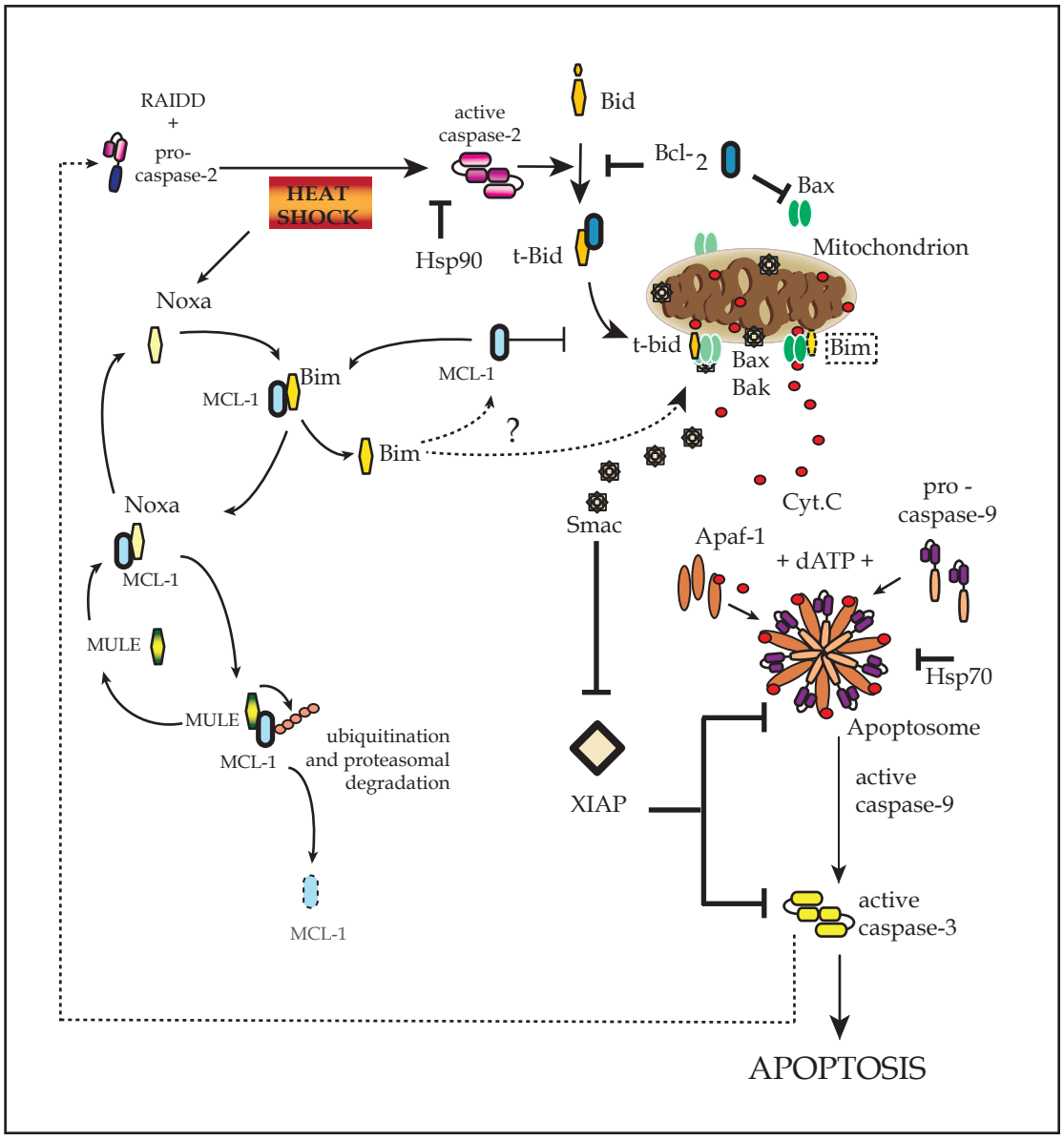


Figure 1.5. Current model for heat shock-induced apoptosis. Heat activates caspase-2 in a RAIDD-dependent manner. Caspase-2 cleaves BID to promote MOMP. Heat directly activates BAX and BAK, promoting Bax translocation to the mitochondria and oligomerization. Heat also causes MCL-1 to change binding partners, and when bound to MULE, it gets ubiquitinated and degraded by the proteasome. Loss of Mcl-1 sensitizes cells to undergo MOMP, and potential role for Bim is especulated. *Adapted from Milleron & Bratton, 2007; Bouchier-Hayes & Green, 2012; Park et al., 2007; Stankiewicz et al., 2009)*

1.4. Study Significance

Hyperthermia or heat shock has gained considerable interest from the biomedical community for its application in cancer therapy (284). Cancer poses a formidable challenge, and in spite of the significant improvement in cancer therapy over the past 4 decades, cancer is the leading cause of death worldwide according to the World Health Organization data in 2008. This global cancer epidemic poses an overwhelming burden to the health care system and to society, urging for new and improved therapeutic approaches.

Hyperthermia is an attractive adjuvant therapy for its targeted tumor toxicity, and solid tumors are the most clinically tractable for hyperthermia interventions (284, 288, 310, 311). Recently developed technologies provide precise control and quantification of delivery when treating tumors with heat (312, 313). Additionally, results from clinical trials indicate the effectiveness of applying hyperthermia in combination with radiotherapy and chemotherapy treatments of various tumors, including superficial cutaneous tumors, breast cancer, head and neck tumors, cervical carcinoma and lymph node metastases (311-313).

The early observation that fevers could contribute to cancer remission was later demonstrated to be in great part due to apoptosis. However, the biological and physiological effects of heat shock in tumors and in normal tissues remain poorly described. The essential steps initiating and regulating apoptosis in

response to heat shock are just beginning to be understood. Furthermore, recurrent heat shock treatments can cause resistance to subsequent heat shock or radio / chemotherapy, undermining therapeutic success (314, 315).

There are promising examples on how basic research on components of the heat shock response fostered the development of small molecule inhibitors to HSP90. Two HSP90 inhibitors, 17-AAG, and its more water-soluble derivative 17-DMAG, are currently in phase I/II clinical trials (316, 317). Recently, the small molecule HSP70 inhibitor VER-155008 was shown to potentiate apoptosis by 17-AAG in HCT116 colon carcinoma cells (318). These examples illustrate that a new generation of pharmacological agents for cancer therapy maybe on its way.

Therefore, a better understanding of the molecular mechanisms involved in both heat-induced apoptosis and thermotolerance could lead to new therapies. For instance, drugs could be designed to exploit the vulnerability of tumors to heat shock, to increase targeted tumor toxicity, or to simulate heat shock effects. Additionally, drugs aimed at circumventing thermotolerance pathways would benefit not only the use of hyperthermia, but also radiotherapy and chemotherapy. In the present study, we sought to achieve a better understanding of the cellular responses to heat shock, with a primary focus on the apoptotic signaling events involving BCL-2 proteins.

1.5. Study Hypotheses And Aims

The literature is controversial regarding the current model of heat shock-induced apoptosis. While some studies implicate caspase-2 as the apical protease that cleaves BID to induce MOMP and cause cell death (142, 274, 303), others have failed to reach the same conclusions (302, 304). Some of the discrepancies are the failure to isolate caspase-2-activating complexes and the modest degree of protection observed in cells lacking caspase-2. Additionally, evidence suggests that caspase-2 is activated downstream of MOMP following heat shock in a caspase-3-dependent manner, and thus is unlikely to be the apical caspase. Moreover, active caspase-2 does not efficiently cleave any other mammalian caspase, except for its own precursor, and it can weakly process and activate the BH3-only protein BID (142, 267). Together, these pieces of evidence propose that caspase-2 participates in an amplification loop (271, 302, 304).

There is no consensus on the putative role of other apical caspases in initiating heat shock-induced apoptosis. Caspases-8 and -9 have either been simultaneously activated by heat (304) or ruled out, along with caspase-12 (302). Thus the puzzle remains, since the role of caspase-2 in activating BID is uncertain, and BID requires cleavage by a caspase or another protease in order to become active. Therefore, we reasoned that if BID is to participate in heat shock-induced apoptosis, heat shock must be activating a protease upstream of

BID, and that protease must need BID to convey the death signal to mitochondria.

The study by Pagliari and colleagues (2005) showed that even though heat shock directly activates BAX and BAK, cytosolic factors must be overcome prior to full permeabilization of the mitochondria. Their study also showed that antagonism of BCL-x_L by tBID aided the onset of MOMP in cell lysates, implicating the need for BH3-only protein to overcome prosurvival BCL-2 proteins. Milleron & Bratton (2006) found that overexpression of BCL-2 blocked MOMP, caspase-3 activation and cell death, strengthening the importance of MOMP in the execution of heat shock apoptosis. Additionally, Stankiewicz and colleagues (2009) implicated MCL-1 in heat shock-induced apoptosis. Since both MCL-1 and BCL-2 can also be antagonized by tBID, it is possible that heat shock utilizes BID to promote MOMP. Even though the activation step of BID is unclear, we reasoned that if BID were required, then loss of Bid should protect cells from heat shock.

Therefore we decided to investigate how Bid null MEFs would respond to heat shock. To our surprise, Bid null MEFs showed only partial protection, in striking contrast to the report of Bonzon (2006), and the reasons for the discrepancy remain unknown. This finding suggested that BID was not is part of an upstream signal, but an initiation cascade, but more likely served to amplify the death signal. Thus, we reasoned that heat shock could be utilizing another

BH3-only protein to initiate the intrinsic pathway.

Of particular interest was the BH3-only protein BIM, which unlike BID, does not require proteolysis in order to be activated. However, similar to BID, BIM is capable of antagonizing all pro-survival BCL-2 family members, and also of directly activating BAX and BAK (179). We hypothesized that BIM might sense the stress of heat shock and respond by promoting MOMP, caspase-3 activation, and cell death. If so, loss of Bim should protect cells from undergoing these apoptotic events. ***Therefore, our first aim was to investigate if the BH3-only protein BIM is required for heat shock-induced MOMP, caspase-3 activation, and cell death.***

Since BIM has been shown to promote MOMP by directly activating BAX and BAK, and/or by antagonizing pro-survival BCL-2-like proteins (12, 121), we hypothesize that loss of Bax/Bak should make cells resistant to heat shock. In contrast, loss or pharmacological inhibition of anti-apoptotic BCL-2 proteins should make cells more sensitive to heat shock-induced MOMP, caspase-3 activation, and cell death. Moreover, MCL-1 was recently implicated in heat shock-induced apoptosis (214). ***Therefore, our second aim was to investigate the roles that additional family members such as BAX, BAK, and MCL-1 in heat shock-induced apoptosis.***

In chapter 3, we show evidence that heat shock requires BIM to cause apoptosis. Thus, we were interested in further understanding how heat shock

activates BIM to kill the cells. BIM is kept inactive by sequestration to the microtubule network via interaction with LC8 (145). The proposed mechanism for BIM activation is its release from the microtubule network, due to phosphorylation by stress-activated kinases such as JNK or p38 (145, 156, 157). Release of BIM is accompanied by its translocation to the mitochondria, where BIM can promote MOMP (144, 145, 157). Thus, we also hypothesized that heat-activated kinases might cause BIM phosphorylation to regulate its pro-apoptotic activity. In this context, we predicted that inhibition of BIM phosphorylation would prevent heat shock-induced MOMP and cell death. ***Therefore, our third aim was to examine whether heat shock causes post-translational modifications on BIM, such as phosphorylation, in order to activate BIM-induced MOMP and apoptosis. If so, we want to determine the kinase phosphorylating BIM, and whether BIM phosphorylation is required for its apoptotic activity.***

Chapter 2. Materials and Methods

2.1. Antibodies and reagents

The following antibodies were purchased from Cell Signaling Technology (Danvers, MA): BAX (CS 2772); BAK (CS 3814); BID (CS 2002), total caspase-3 (CS 9665); cleaved caspase-3 (CS 9662); β-actin (CS 4970); cytochrome c (CS 11940); PARP (CS 9542); cleaved PARP (CS 9544); TAK1 (CS 4505); phospho-TAK1 (CS 4531); BIM (CS 2933); phospho-BIM Ser69 (CS 4581; corresponds to Ser65 in mouse BIM_{EL}); P-JNK (CS 4668); JNK (CS 9252); phospho-c-Jun (Ser63) (CS 2361); phospho-c-Jun (Ser73) (CS 9164); GFP (CS 2956); phospho-HSP27 (2406); phospho-MAPKAP2 (CS 3041) total ERK (CS 9102); phospho-ERK (CS 9101), phospho-p38 (CS 9211). Other antibodies used were as follows: BIM (cat. no. ADI-AAP-330), and inducible HSP70 (cat. no. ADI-SPA-810) were from Enzo Life Sci., Farmingdale, NY); mouse specific anti-MCL-1 (cat. no. 600-401-394S) was from Rockland Immunochemicals, Inc. (Gilbertsville, PA); caspase-2 (cat. no. MAB3501, Chemicon® / EMD Millipore, Billerica, MA); and RFP/mCherry antibody (cat. no. PM005) was from MBL International (Woburn, MA).

The BH3 mimetic ABT-737 (cat. No. S1002) and the p38 inhibitor SB203580 (cat. No. S1076) were purchased from Selleckchem (Houston, TX).

The TAK1 inhibitor 5Z-7-oxozeaenol (Cat. No. NP-009245) was purchased from AnalytiCon Discovery, GmbH (Postdam, Germany). The AP20187 homodimerizer (cat. no. 635060) was purchased from Clontech (Mountainview, CA). TMRE was from Enzo Life Sci. (Farmingdale, NY). Digitonin (D141) was from Sigma (St. Louis, MO). Fetal bovine serum was obtained from Atlanta Biologicals (Lawrenceville, GA), and DMEM and RPMI were purchased from Corning Cellgro (Manassas, VA). The MEK1/2 inhibitor UO126 (cat. no. CS 9903) was from Cell Signaling Technology (Danvers, MA). The JNK inhibitor SP600125 (cat. no. 1496) was from Tocris Bioscience (Bristol, United Kingdom). The JNK inhibitor JIN8 was kindly provided by Dr. Kevin Dalby (University of Texas at Austin, Austin, TX) (319).

2.2. Plasmids

The following sense strand sequence was used to silence *Bim*: 5′-GACCGAGAAGGUAGACAAUUGdTdT-3′ (320) and was cloned into the pSuper/Neo vector. Jurkat cells were then electroporated with 10 μg of either empty vector or pSuper/*shBim*, and after 24 h, live cells were separated on a Ficoll density gradient by centrifugation. Stable clones were then selected in G418 (1 mg/mL), and knockdown levels determined by western blotting. A previously published pSuper/*shBid* clone was similarly constructed (304), and plasmids for GFP-Noxa and GFP-Bad have been previously described (321). To

generate the N-terminal fusion of the dimerization domain of human FK506 binding protein (FKBP1A) with CARD-deleted caspase-2 (*i.e.* pFKBP-Δpro-caspase-2) (322), the *Bam*HI and *Eco*RI sites in the human caspase-2 sequence were first mutated by site-directed mutagenesis using the following primers:

C2_BHI_US: 5'- GAACCACGCAGGGTCCCCTGGGTGCG-3';

C2_BHI_DS: 5'-CGCACCCAGGGGA CCCTGCGTGGTTC-3';

C2_ECORI_US: 5'-CTCCTGGCACAGAGTTCCACCGGTG CA-3';

C2_ ECORI_DS: 5'-TGCACCGGTGGAACTCTGTGCCAGGAG-3'.

The Δpro-caspase-2 sequence (from amino acid 170) was the amplified using the following primers:

BglII_C2LS_US

5'-AGAAGATCTGGTCCTGTCTGCCTTCAGGTG-3';

EcoNot_C2SS_DS

5'-GAAGAATTCGCGGCCGCTCATGTGGGAGGGTGTCCTGG-3'.

The PCR products were then digested with *Bam*HI/*Eco*RI and cloned into pMSCV- FKBP-IRES-GFP (322). The accuracy of all constructs was confirmed by sequencing.

2.3. Cell culture and transfections

MEFs were grown in DMEM supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville, GA), 1% penicillin–streptomycin (100 units/mL), and

2 mM glutamine. Jurkat cells were maintained in RMPI 1640 supplemented with 10% FBS, 1% penicillin–streptomycin, and 1 mM glutamine. Cells were maintained at 37°C in humidified air containing 5% CO_2 and were routinely passaged every 2 days. *Bim*$^{-/-}$ and *Bid*$^{-/-}$ MEFs were a kind gift from Dr. David C. S. Huang (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia). *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs were a kind gift from Dr. Craig B. Thompson (Memorial Sloan–Kettering Cancer Center, NY). Finally, *Mcl-1*$^{-/-}$ MEFs were a kind gift from Dr. Joseph T. Opferman (St. Jude Children's Research Hospital, Memphis, TN).

For the generation of MEFs expressing inducible FKBP-Δpro-caspase-2, GP2-293 cells were transfected with 6 μg each of the pAmphotropic receptor and pFKBP/Δpro-caspase2 plasmids using Lipofectamine®2000, according to the manufacturer's recommendations (Invitrogen). After 48 h transfection, the viral supernatant was mixed with polybrene (7 μg/mL) and exposed to MEFs (30% confluent) for 4 h. After infection, cells were expanded for 3 d and then cell-sorted for GFP-positive cells on a BD FACS- ARIA. The isolated cell pools were then analyzed by immunoblotting for the expression of FKBP-Δpro-caspase-2 fusion and GFP. For the dimerization experiments, 150 x 10^3 cells/well were seeded into 12-well plates and 18 h later the AP20187 homodimerizer (100 nM) was added. The uptake of propidium iodide (PI) was

then quantified 48 h later by flow cytometry.

2.4. Heat shock treatments

MEFs or Jurkat T cells were plated at 0.3 – 0.5 x 10^6 cells/well in 6-well plates 20 h prior to heat shock. Exposures were done in a tissue culture incubator at 44°C with 5% CO_2 for various periods of time, after which the cells were returned to a 37°C incubator for "recovery". Samples were collected for analyses at various time points post-heat shock. To examine long-term survival, cells were prepared and treated as above, except that fresh media was added to the cells after 24 h and the plates were cultured for an additional 48 h at 37°C. At 72 h post-heat shock, the cells were fixed with 70% ethanol for 10 min, stained with crystal violet for 45 min, washed with tap water, and allowed to air dry prior to image analysis (323).

For the thermotolerance studies, MEFs were incubated at 43°C for 30 minutes, returned to the 37°C incubator for a 4 h recovery period, after which they were exposed to lethal heat shock at 44°C for 1.5 h. Samples were collected for cell death analysis at 24 h or for long term survival at 72 h after the lethal heat shock.

2.5. Cell death and ∆ψm assays

Jurkat T cells or trypsinized MEFs (1 x 10^6) were pelleted at 400 x *g* for 4 min, washed with PBS, and resuspended in 1 mL of Annexin-V binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Cells were then incubated with 100 ng/mL Annexin-V-FITC for 8 min, and propidium iodide (PI) was added just prior to flow cytometric analysis. Recombinant Annexin-V was expressed and purified in-house, labeled with FITC, and dialyzed to remove unconjugated FITC. Cell populations, labeled with FITC and/or PI, were analyzed by flow cytometer (Beckman- Coulter, Fullerton, CA, USA).

Similarly, to assess the loss of mitochondrial membrane potential *(∆ψm*), suspensions containing 1x10^6 MEFs or Jurkat T cells were incubated at 37°C for 20 minutes in pre-warmed (37°C) media containing 100 nM TMRE. Cells were washed twice with PBS and analyzed by flow cytometry.

2.6. Cytochrome c release assay

Sixteen hours (16 h) post treatment, 5 x 10^6 MEFs were trypsinized, washed twice in PBS, and permeabilized in MOMP lysis buffer (20 mM HEPES, pH 7.4, 250 mM sucrose, 1 mM EDTA, 75 mM KCl, 2.5 mM $MgCl_2$) containing 0.05% digitonin (always added fresh) on ice for 5 minutes. The cells were then centrifuged at 15,000 x *g* for 10 min at 4°C to collect the “cytosolic fractions”.

The pellets were lysed in RIPA buffer, as described above, to obtain the "mitochondrial fractions". Jurkat cells were similarly permeabilized utilizing 0.02% digitonin. The protein concentration of cytosolic and mitochondrial fractions were measured by the Bradford assay and resolved by SDS-PAGE.

2.7. Western blotting

Cell pellets were lysed in RIPA lysis buffer on ice for 20 minutes, spun at 18,000 x *g* for 10 minutes at 4°C, and the supernatants normalized for protein concentration by the Bradford assay. Equal amounts of protein were resolved by SDS-PAGE, transferred onto nitrocellulose membranes, and blocked in 0.1% Tween-TBS with 5% milk prior to incubation with primary antibodies (1:1,000-2,000) overnight.

Chapter 3. BH3-only protein BIM mediates alternative pathways to heat shock-induced apoptosis

3.1. Introduction

Apoptosis is an evolutionarily conserved programmed form of cell death that involves the activation of caspases (cysteine proteases) (324). These proteases are typically activated in response to stimulation of cell-surface death receptors, such as Fas/CD95, or in response to stressful stimuli, such as oncogene activation, DNA damage, growth factor withdrawal, ER stress, etc. (226). In the latter instances, stress activates the so-called *intrinsic apoptosis pathway*, which generally involves the activation of pro-apoptotic BCL-2 family members. BH3-only proteins, such as BID, BIM, PUMA, BAD, and NOXA, serve as cellular sentinels that are activated in response to distinct types of stress. These BH3-only proteins subsequently activate the multidomain proapoptotic family members, BAX and BAK, which are often restrained by the antiapoptotic BCL-2 family members, BCL-2, BCL-x_L, and/or MCL-1 (11, 12, 116). How BH3-only family members activate BAX and BAK remains controversial, but BID, BIM, and PUMA are thought to directly activate BAX and BAK, whereas BAD, NOXA, and other BH3-only family members indirectly contribute to their activation

through neutralization of the antiapoptotic family members (111, 117, 118, 122, 183, 191, 204, 220).

Once activated, BAX inserts into the outer mitochondrial membrane (OMM), and both BAX and BAK oligomerize into pores that permeabilize the membrane and facilitate the release of intermembrane space proteins, such as cytochrome c (cyt c), into the cytoplasm. Cyt c then binds to apoptotic protease-activating factor 1 (Apaf-1) and triggers a dATP/ATP-dependent conformational change in Apaf-1 that results in its oligomerization into a heptameric caspase-activating complex, known as the Apaf-1 apoptosome (16). Finally, the apoptosome sequentially recruits and activates the initiator caspase-9 and the effector caspase-3, the latter of which targets >800 cellular substrates for proteolytic cleavage. Thus, cells utilize various BH3-only family members to integrate a variety of cellular stressors, all of which induce mitochondrial outer membrane permeabilization (MOMP), apoptosome assembly, caspase activation, and cell death (226).

BID is unique among the BH3-only family members in that it is activated through caspase cleavage, most notably by caspase-8, which allows death receptors to engage the intrinsic pathway. Interestingly, caspase-2 has also been shown to engage the intrinsic pathway through cleavage of BID, upstream of mitochondria, particularly when overexpressed (76, 142, 265, 267, 270, 274, 275, 325). However, characterizing the genuine role of caspase-2 in stress-induced apoptosis *per se* has been challenging. Numerous studies have

suggested that caspase-2 is either critical for DNA damaged-induced apoptosis or irrelevant to this response (326). The caspase-2 knockout mice develop normally, aside from an increase in oocytes, but when crossed with the Eμ-myc or MMTV/c-neu mouse models, they develop significantly more lymphomas and mammary tumors, respectively, indicating a role for caspase-2 as a tumor suppressor (241, 254, 256).

Recently, caspase-2 has been shown to play an important role in cell death induced by microtubule disruption and heat shock (142, 242, 274, 303). Indeed, Green and colleagues have shown that caspase-2 forms a complex with its adapter protein RAIDD, early following heat shock, and can be trapped with a biotinylated version of the poly-caspase inhibitor zVAD-fmk (274, 303). They also find that BID and BAX/BAK-deficient cells are resistant to caspase-2 and heat shock-induced apoptosis (142). In the present study, we provide evidence that BIM mediates heat shock-induced apoptosis and that the caspase-2-BID pathway likely functions as either a parallel pathway in some cell-types or as part of an important amplification loop to enhance cell death, particularly at lower temperatures or decreased exposures.

3.2. Results

3.2.1 Bim is essential for heat shock-induced cell death

Heat shock reportedly induces apoptosis through the canonical intrinsic pathway in which caspase-2 is first activated and in turn cleaves BID to initiate BAX/BAK-dependent MOMP (142, 274, 303). However, in the course of our studies, we uncovered a critical role for the BH3-only protein BIM. Loss of BIM afforded near complete protection from cell death following a 1-1.5 h exposure to heat shock at 44°C, whereas BID-deficient cells were only partially protected following a 1 h treatment (Fig. 3.1A and C). Consistent with these cell death measurements, only *Bim*$^{-/-}$ cells avoided MOMP (as determined by cytochrome c release), loss of mitochondrial inner membrane potential ($\Delta\psi m$), caspase-3 activation, and PARP cleavage (Fig. 3.1B and D). Importantly, other forms of stress, including DNA damage (etoposide) and ER stress (tunicamycin), readily induced apoptosis in *Bim*$^{-/-}$ MEFs (Fig. 3.1F). Thus, collectively, the data indicated that BIM played a specific and apparently dominant role in regulating heat shock-induced apoptosis.

Finally, even though BIM appeared to be critical for short-term protection against heat shock, we questioned whether loss of BIM could provide long-term protection. Therefore, we heat-shocked wild type, *Bim*$^{-/-}$, and *Bid*$^{-/-}$ MEFs for 1-1.5 h and monitored their death/growth up to 72 h. As

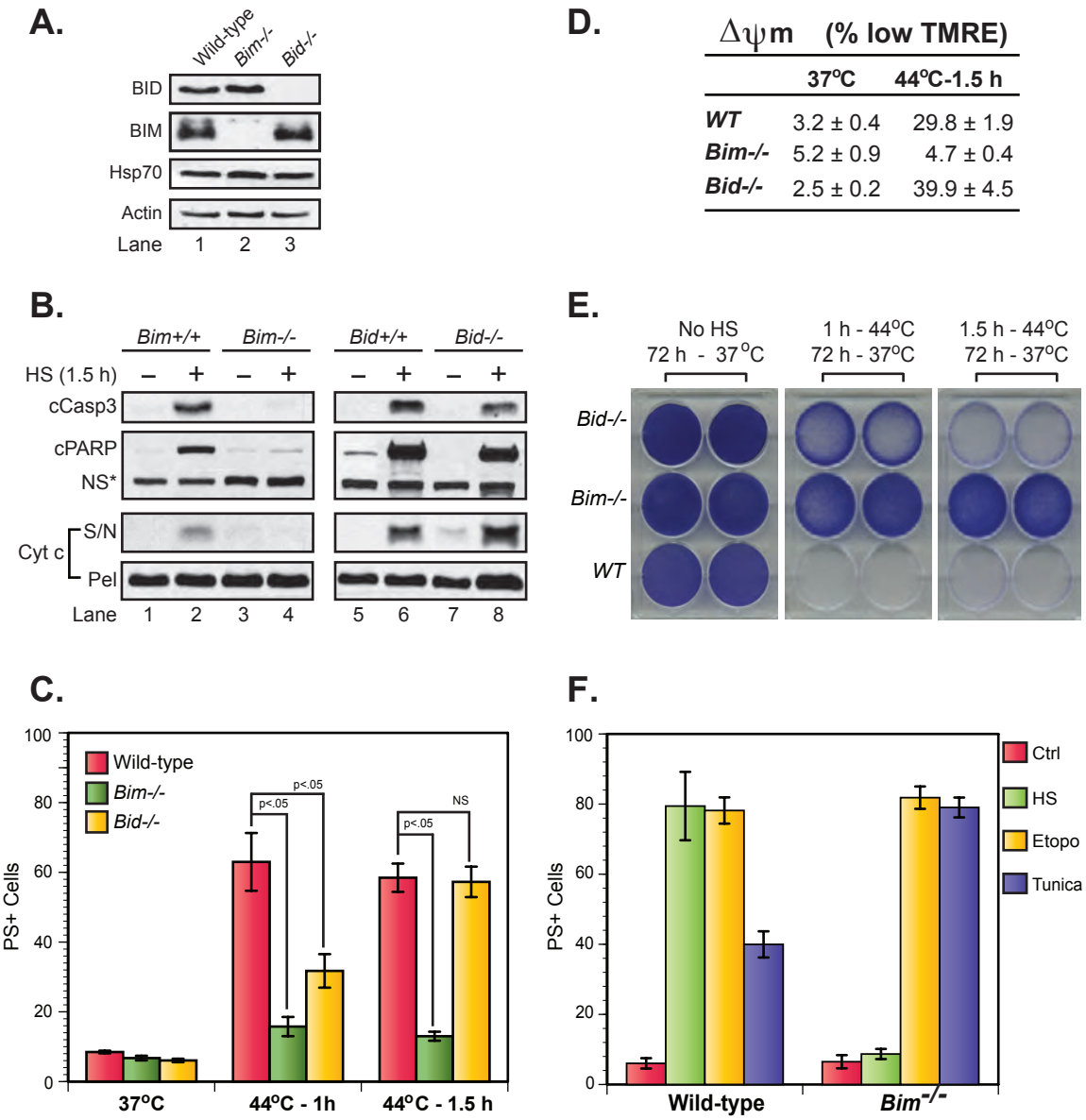
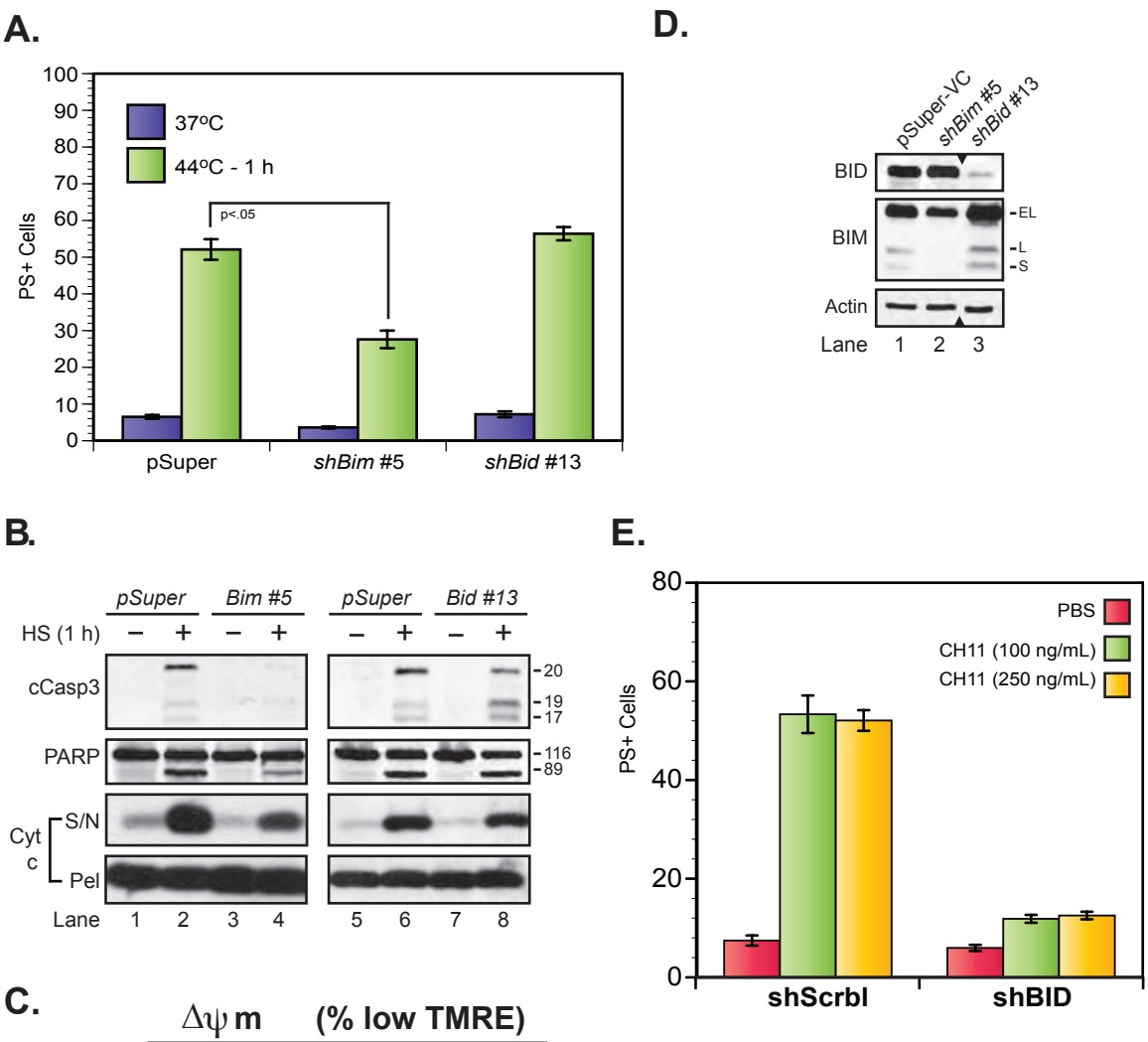


Figure 3.1. BIM is critical for heat shock-induced apoptosis. (**A-D).** *Wild-type, Bim*$^{-/-}$, and *Bid*$^{-/-}$ MEFs (*panel A*) were exposed to heat shock (44°C for 1-1.5 h) in a humidified incubator (5% CO_2 - 95% air). The cells were then transferred to a 37°C incubator and later collected for MOMP (16 h, *panel B*), caspase-3 activation, PARP cleavage (24 h, *panel B*), *Δψm* (24 h, *panel D*), and cell death measurements (24 h, *panel C*). **(E).** *Wild-type, Bim*$^{-/-}$, and *Bid*$^{-/-}$ MEFs were exposed to heat shock as described above, but were left in culture afterwards for 72 h, after which the plates were stained with crystal violet. **(F).** Wild-type and *Bim*$^{-/-}$ MEFs were exposed to heat shock as described above, or treated with etoposide (10 μM) or tunicamycin (1 μM), and subsequently assayed for cell death at 24 h.

shown in Fig. 3.1E, *Bid*$^{-/-}$ cells were partially protected following a 1 h heat shock, but only *Bim*$^{-/-}$ cells were protected from the more extreme 1.5 h exposure.

Previous efforts to generate stable BIM-expressing cell lines have been unsuccessful (113, 144), and despite repeated attempts, we too were unable to stably reintroduce *Bim* into the *Bim*$^{-/-}$ MEFs. Therefore, to confirm BIM's role in heat shock-induced killing, we generated a stable human Jurkat cell line expressing a short-hairpin RNA to *Bim*. The Jurkat T leukemia cell line was chosen since previous studies had been performed on them (274, 302), and also a clone where Bid was stably knockdown had been previously described and was suitable for comparisons with Bim stable knockdown clones (304). RNA interference resulted in complete loss of expression for the BIM_L and BIM_S isoforms, but only partially depleted (~50%) the BIM_{EL} isoform (Fig. 3.2D, lane 2). The BID-deficient clone expressed slightly higher levels of all three BIM isoforms (Fig. 3.2D, lane 3), and as expected, it was resistant to Fas-induced apoptosis (Fig. 3.2E). Using an optimal exposure for Jurkat cells (44°C for 1h, determined by dose-response experiments, data not shown), we observed that only the BIM-depleted cells exhibited resistance to cell death (Fig. 3.2A), which correlated with the extent of total BIM knockdown, as well as the degree of MOMP, $\Delta\psi m$, caspase-3 activation, and PARP cleavage (Fig. 3.2B and 3.2C). These results were consistent with our findings in MEFs, further supporting the



C.

$\Delta\psi$ m	(% low TMRE)	
	37°C	**44°C-1 h**
VC	8.2 ± 1.2	52.9 ± 4.2
Bim #5	7.2 ± 0.9	26.5 ± 1.2
Bid #13	8.3 ± 0.9	60.4 ± 1.6

Figure 3.2. BIM silencing protects Jurkat T cells from heat shock-induced apoptosis. (A-D). Human Jurkat T cells, stably depleted of BIM or BID by RNAi (*panel D*; note: black triangles denote crop marks), were exposed to heat shock (44°C for 1 h) in a humidified incubator (5% CO_2 - 95% air). As before, the cells were then transferred to a 37°C incubator and later collected for MOMP, caspase-3 activation, PARP cleavage (24 h, *panel B*), *Δψm* (24 h, *panel C*), and cell death measurements (24 h, *panel A*). **(E).** Jurkat T cells stably, expressing a scrambled or Bid shRNA, were exposed to agonistic human CD95 antibody (CH11; 100-250 ng/mL) for 4 h and subsequently assayed for cell death by Annexin V-PI staining and flow cytometry. cPARP, cleaved PARP; cCasp3, cleaved/active caspase-3; S/N, supernatant; Pel, mitochondrial pellet.

importance of BIM in heat shock-apoptosis.

3.2.2 Caspase-2 induces cell death independently of BIM

Since heat shock activates the apical caspase-2 in the canonical cell death pathway, we questioned whether caspase-2 might mediate cell death *via* BIM, as has been shown for BID (142, 267). In order to selectively activate caspase-2, we generated an FKBP-Δpro-caspase-2 construct, similar to that previously reported for caspase-9 (31), in which the prodomain of caspase-2 was replaced with a modified FKBP protein that can be induced to dimerize upon exposure to the chemical ligand AP20187 (Fig. 3.3A and B). Since dimerization is thought to mediate the activation of initiator caspases, including caspase-2, we retrovirally-expressed FKBP-Δpro-caspase-2 in wild-type, *Bim*$^{-/-}$, and *Bid*$^{-/-}$ MEFs and sorted the cells using a GFP marker.

Following exposure to AP20187, we observed activation/processing of caspase-2 in each of the isolated cell pools (Fig. 3.3D), but not in cells expressing FKBP alone (Fig. 3.3C). To confirm the cell death was caspase-2-dependent we generated wild-type MEFs expressing a catalytic site caspase-2 mutant (FKBP-Δpro-caspase-2/C320G), which did not die upon addition of AP20187 (Fig. 3.3F). As previously reported (142), the *Bid*$^{-/-}$ cells were resistant to caspase-3 activation and cell death (Fig. 3.3C and D). By contrast, both the wild-type and *Bim*$^{-/-}$ cells displayed BID cleavage,

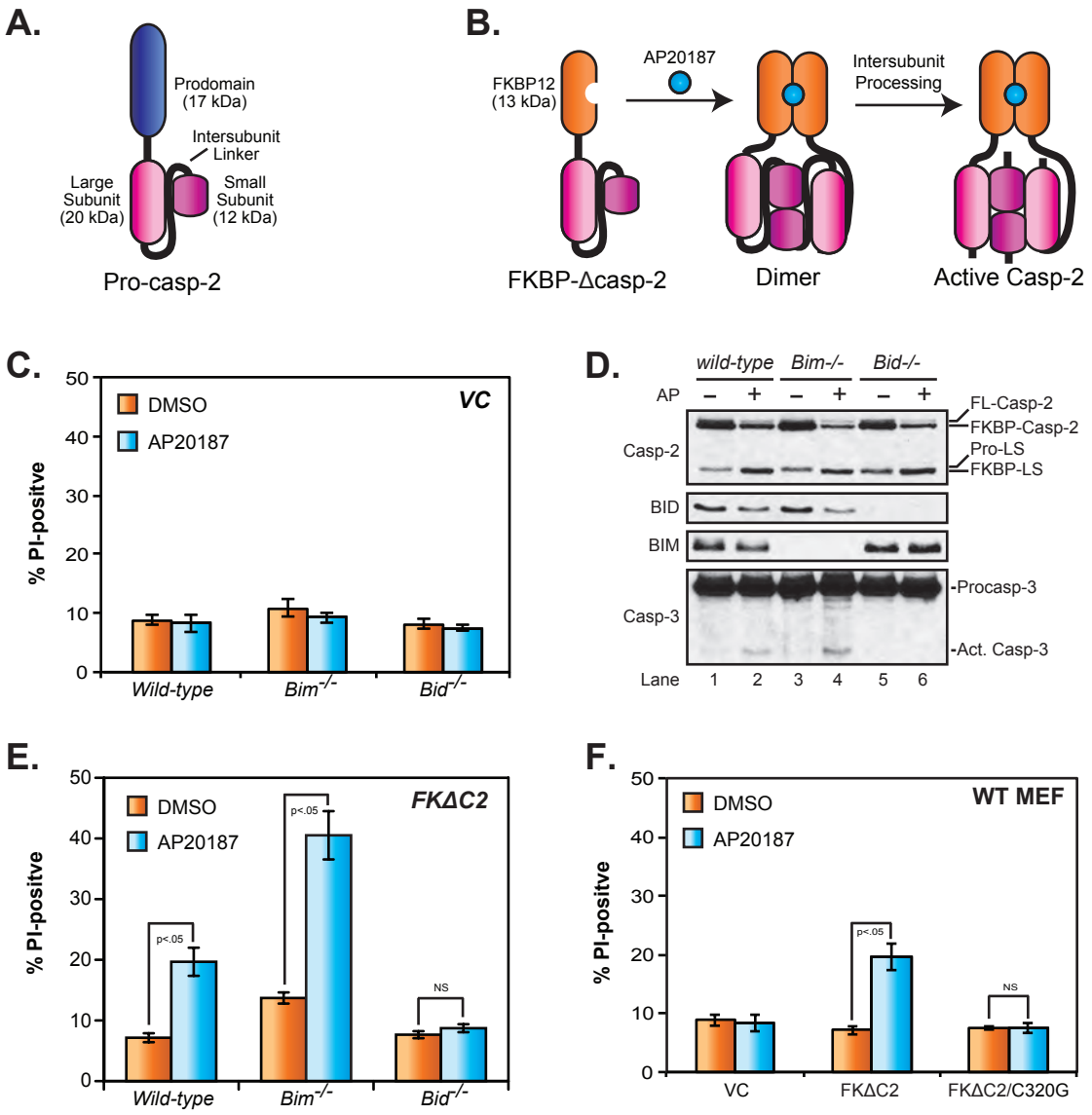


Figure 3.3. Caspase-2 induces apoptosis in a BID-dependent but BIM-independent manner. (A and B). Cartoons depicting wild-type caspase-2 and the FKBP-Δprocaspase-2 fusion, in which the prodomain of caspase-2 is replaced with an FKBP protein that dimerizes upon the addition of AP20187. **(C-F).** Wild-type, *Bim*$^{-/-}$, and *Bid*$^{-/-}$ MEFs were infected with retroviruses either expressing FKBP only (VC, *panel C*) or the FKBP-Δpro-caspase-2 fusion protein (FKΔC2, *panels D-F*); as a negative control in wild type MEFs, a catalytic site caspase-2 mutant FKBP fusion protein was introduced (FKΔC2/C320G, *panel F*). The cells were then sorted by flow cytometry for GFP (marker)-positive cells, and the cell pools incubated in the presence or absence of AP20187 for 48 h, after which they were assayed for cell death via PI uptake (*panels C, E and F*), as well as activation of caspases-2 and -3 and BID cleavage where relevant (*panel D*).

caspase-3 activation, and cell death, indicating that BIM is not essential for caspase-2-mediated cell death.

3.2.3 Heat shock induces cell death through BAX/BAK-dependent and independent pathways

Since BIM played a critical role in heat shock-induced cell death, we expected that it was likely to induce MOMP and cell death through its activation of the multidomain pro- apoptotic BCL-2 family members, BAX and/or BAK. To our surprise, however, we observed only ~50% protection upon BAX and BAK deficiency, i.e. half of the cells still died regardless of BAX/BAK expression (Fig. 3.4A).

Since this finding was quite unexpected, we tested the *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFS with several stimuli dependent on the intrinsic pathway for killing. There was no apparent issue with the *Bax*$^{-/-}$*Bak*$^{-/-}$ cells, as they remained entirely resistant to UV-induced MOMP, $\Delta\psi m$, caspase-3 activation and apoptosis (Fig. 3.5A and B). Remarkably, the *Bax*$^{-/-}$*Bak*$^{-/-}$ cells failed to undergo MOMP or $\Delta\psi m$ following heat shock, but nevertheless activated caspase-3 and cleaved PARP, albeit to a lesser extent (Fig. 3.4B, lanes 2 and 4; 3.4D). Despite the unexpected caspase activation and cell death in approximately half of the *Bax*$^{-/-}$*Bak*$^{-/-}$ cells, those that were alive at 24 h remained viable and populated the culture dish by 72 h (Fig. 3.4C). The *Bax*$^{-/-}$*Bak*$^{-/-}$ cells also showed protection to etoposide and

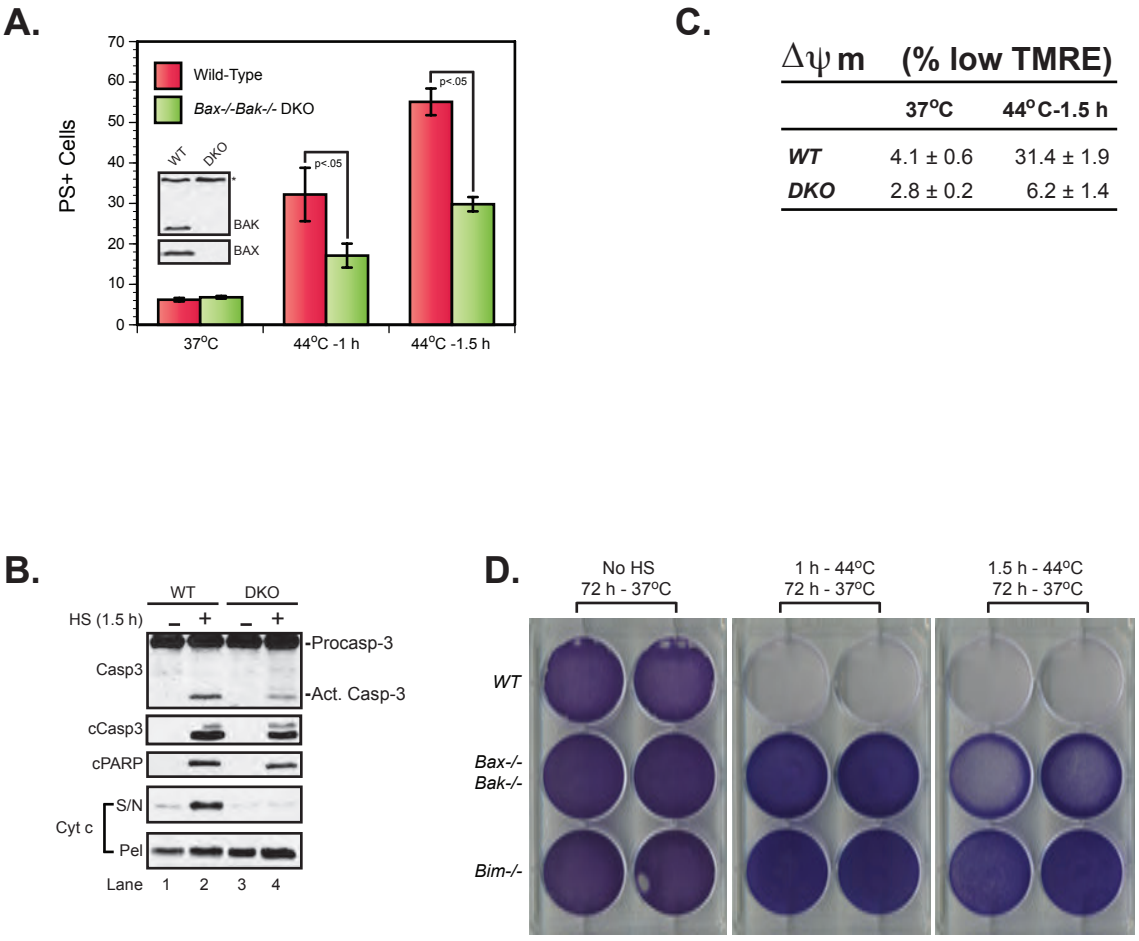


Figure 3.4. *Bax*$^{-/-}$*Bak*$^{-/-}$ cells resist heat shock-induced MOMP and *Δψm*, but still undergo caspase-3 activation and cell death. Wild-type and *Bax*$^{-/-}$*Bak*$^{-/-}$ DKO cells were exposed to **(A-D)** heat shock (44°C for 1-1.5 h) in a humidified incubator (5% CO_2 - 95% air). The cells were then transferred to a 37°C incubator and later collected for MOMP (16 h, *panel B*), caspase-3 activation, PARP cleavage (24 h, *panel B*), *Δψm* (24 h, *panel C*), and cell death measurements (24 h, *panel A*). **(D)** and *Bax*$^{-/-}$*Bak*$^{-/-}$ DKO MEFs were exposed to heat shock as described above, but were left in culture afterwards for 72 h, after which the plates were stained with crystal violet. cPARP, cleaved PARP; cCasp3, cleaved/active caspase-3; S/N, supernatant; Pel, mitochondrial pellet.

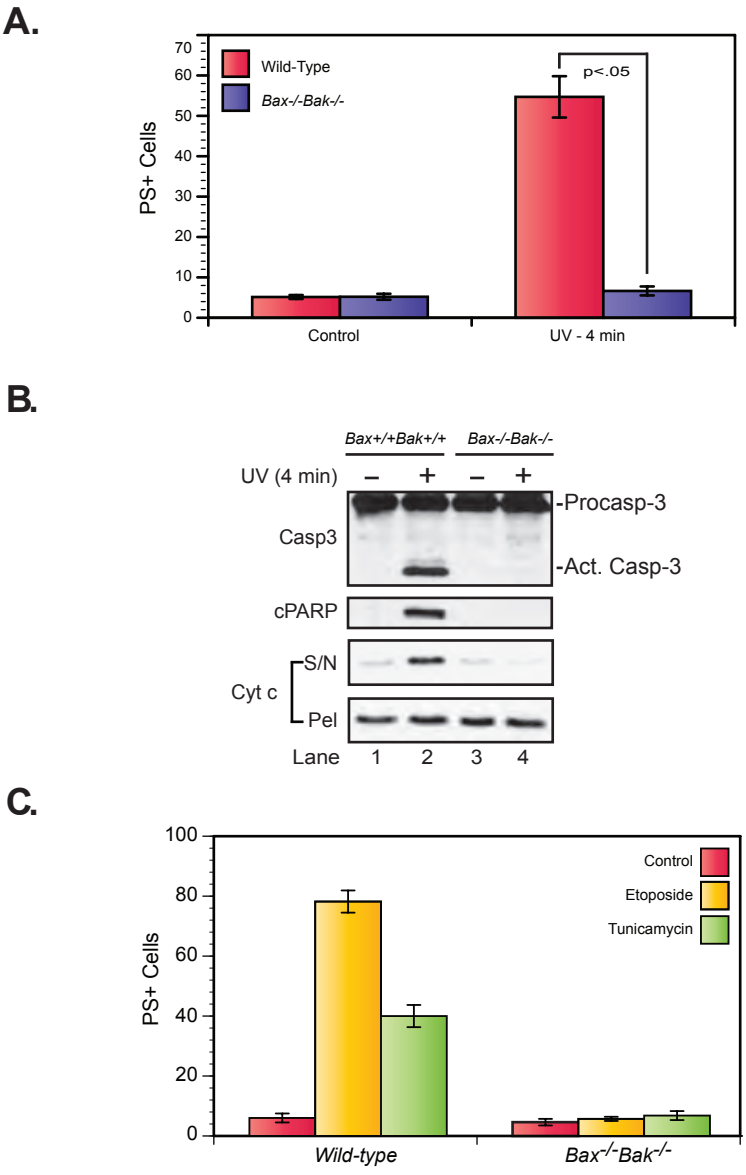


Figure 3.5. *Bax*$^{-/-}$*Bak*$^{-/-}$ cells resist cell death by stressful stimuli other than heat shock. Wild-type and *Bax*$^{-/-}$*Bak*$^{-/-}$ DKO cells were exposed to **(A and B)** UV irradiation (4 min) on a transilluminator. The cells were then transferred to a 37°C incubator and later collected for MOMP (16 h, *panel B*), caspase-3 activation, PARP cleavage (24 h, *panel B*) and cell death measurements (24 h, *panel A*). In **(C)**, wild-type and *Bax*$^{-/-}$*Bak*$^{-/-}$ DKO cells were treated with etoposide (10μM) or tunicamycin (1 μM), and subsequently assayed for cell death at 24 h. cPARP, cleaved PARP; cCasp3, cleaved/active caspase-3; S/N, supernatant; Pel, mitochondrial pellet.

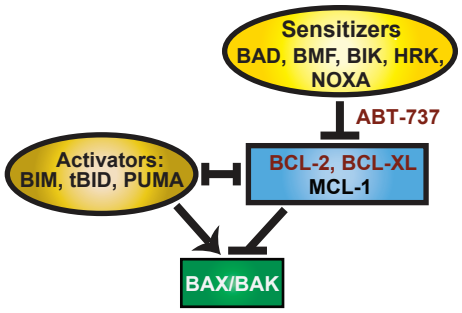
tunicamycin (Fig. 3.5C). Together, the data suggested that the phenotype of partial resistance to heat shock was specific.

3.2.4 Loss of MCL-1 or antagonism of BCL-2/ BCL-x_L sensitizes cells to heat shock-induced apoptosis

Previous studies indicate that BIM, BID, and PUMA function as *direct activators* of BAX and BAK, whereas the remaining BH3-only family members function as *sensitizers*, *i.e.* they cannot directly activate BAX and BAK, but they can neutralize the antiapoptotic family members (BCL-2, BCL-x_L, MCL-1) that inhibit direct activators (Fig. 3.6A, and in more detail, chapter1, Fig. 1.3) (220). Mosser and colleagues have recently reported that MCL-1 plays an important role in suppressing heat shock-induced apoptosis (214). Consistent with their results, loss of MCL-1 sensitized cells to heat shock-induced MOMP, $\Delta\psi m$, caspase-3 activation, PARP cleavage, and cell death (Fig. 3.6B-D).

Additionally, the BCL-2/BCL-x_L antagonist, ABT-737, also sensitized MEFs to heat shock-induced cell death (Fig. 3.7A-D). Since ABT-737 functions as a sensitizer and BIM is critical for heat shock-induced killing, the simplest explanation is that ABT-737 most likely liberated BIM from BCL-2 or BCL-x_L, which in turn activated and/or further de-repressed BAX and/or BAK. This interpretation is also consistent with our previous data in which BCL-2 overexpression protected BAX-deficient Jurkat T cells from heat shock-induced

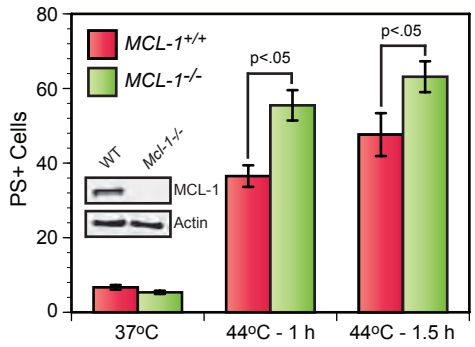
A.



C.

	$\Delta\psi$m (% low TMRE)		
	37°C	44°C-1 h	44°C-1.5 h
Mcl-1$^{+/+}$	5.0 ± 0.9	26.1 ± 2.3	33.7 ± 6.2
Mcl-1$^{-/-}$	7.5 ± 0.9	31.1 ± 3.5	45.3 ± 4.7

B.



D.

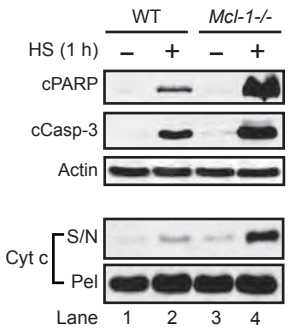


Figure 3.6. Loss of MCL-1 sensitizes cells to heat shock-induced apoptosis. (A). Cartoon illustrating the interactions of BH3-only proteins with multidomain proapoptotic (BAX and BAK) and antiapoptotic (MCL-1, BCL-2, and BCLXL) BCL-2 family members. BH3-only proteins can function as BAX/BAK activators (BIM, tBID, PUMA), or as sensitizers (NOXA, BAD, BMF, BIK, and HRK) that displace activators from antiapoptotic family members. **(B-D).** Wild-type and *Mcl-1*$^{-/-}$ MEFs (*panel B*, inset) were exposed to heat shock (44°C for 1-1.5 h) in a humidified incubator (5% CO_2 - 95% air). The cells were then transferred to a 37°C incubator and later collected for MOMP (16 h, *panel D*), caspase-3 activation, PARP cleavage (24 h, *panel D*), *Δψm* (24 h, *panel C*), and cell death measurements (24 h, *panel B*).

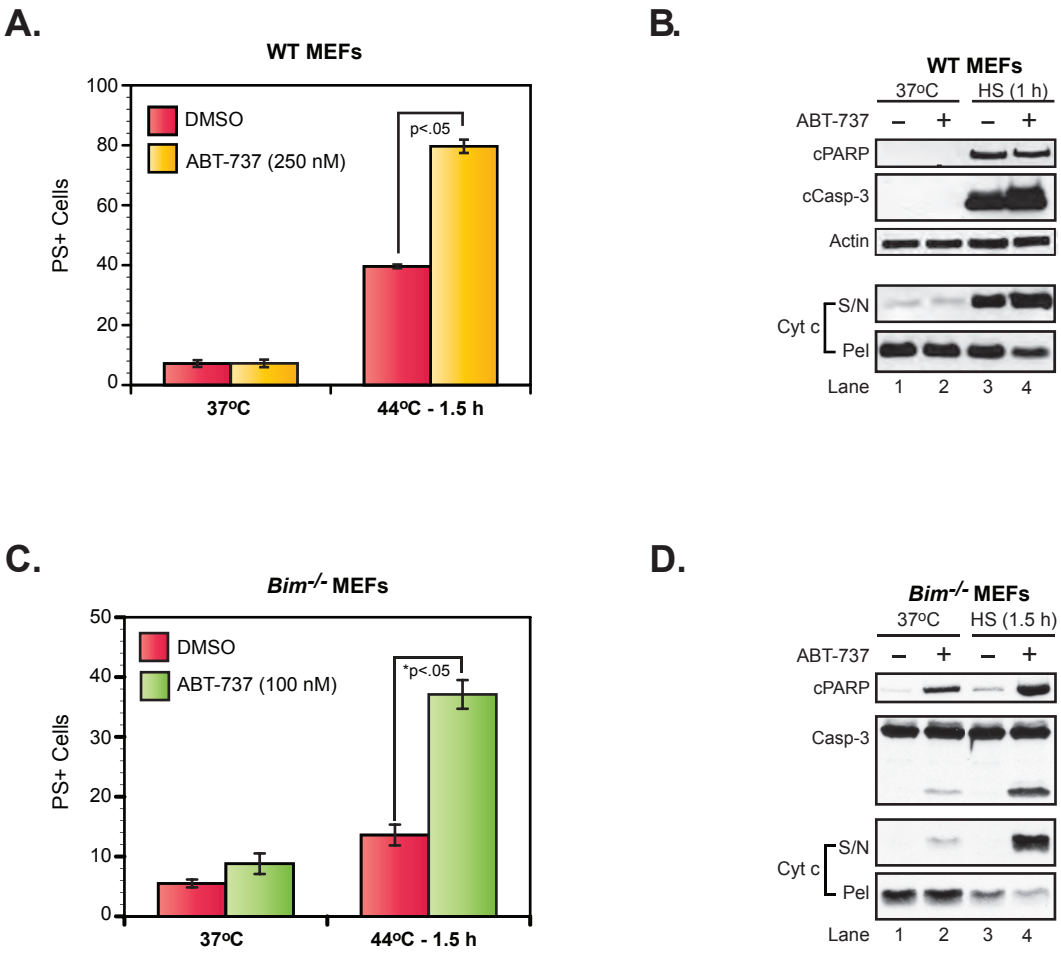


Figure 3.7. Inhibition of BCL-2/BCL-XL with the BH3-mimetic ABT-737 potentiates heat shock- induced apoptosis. (A - D). Wild-type and *Bim*$^{-/-}$ MEFs were preincubated with DMSO (control) or ABT-737 (100 - 250 nM) for 1 h and subsequently exposed to heat shock (44°C for 1.5 h) in a humidified incubator (5% CO_2 95% air). The cells were then transferred to a 37°C incubator and later collected for MOMP (16 h, *panels B and D*) , caspase-3 activation, PARP cleavage (24 h, *panels B and D*) and cell death measurements (24 h, *panels A and C*). cPARP, cleaved PARP; cCasp3, cleaved/active caspase-3; S/N, supernatant; Pel, mitochondrial pellet.

apoptosis, even though BCL-2 does not inhibit BAK (118, 302).

To further investigate the relative roles of MCL-1, BCL-2, and BCL-x_L in heat shock resistance, we transfected HeLa cells with NOXA (MCL-1-specific antagonist) or BAD (BCL-2/ BCL-x_L-specific antagonist). Surprisingly, while ectopic expression of NOXA sensitized HeLa cells to heat shock-induced apoptosis, expression of BAD (or treatment with ABT-737) afforded only a modest increase in cell death (Fig. 3.8A and B). Thus, the cellular context (*i.e.* cell-type, relative expression of BCL-2 family members, etc.) appears to dictate the overall importance of MCL-1, BCL-2 and BCL-x_L in suppressing BIM-induced apoptosis following heat shock.

3.3. Discussion

The ability to adapt and survive heat shock is fundamentally important for cellular life. Heat shock induces various cellular disturbances, including defects in DNA repair, cell cycle arrest, and cytoskeletal damage (284). Hundreds of studies have characterized survival pathways initiated by heat shock, including the upregulation of heat shock proteins that function as chaperones in the repair of misfolded proteins (327). In some cases, however,

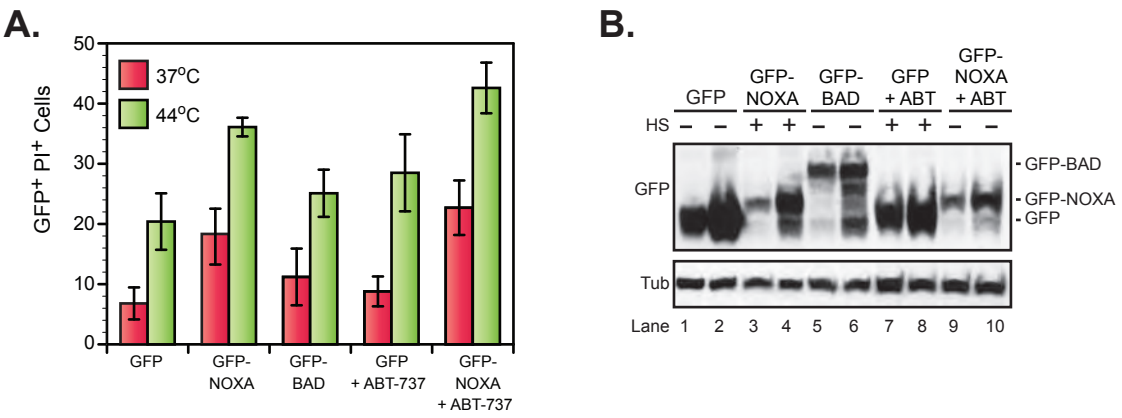


Figure 3.8. HeLa cells can be sensitized to heat shock by Noxa. (A and B). HeLa cervical carcinoma cells were transfected with vector control-GFP, GFP-NOXA, or GFP-BAD (*panel B*) and pretreated with or without ABT-737 (100 nM). Transfected cells were then exposed to heat shock and assayed for cell death (GFP^+/PI^+ cells) by flow cytometry (*panel A*).

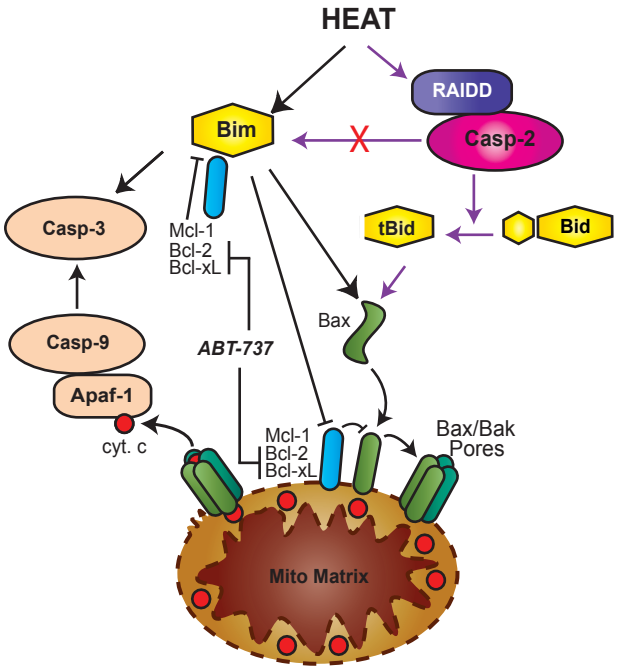


Figure 3.9. Model of BIM-induced cell death following heat shock. In the canonical pathway, heat shock induces formation of a RAIDD-caspase-2 complex that activates caspase-2. Following cleavage by caspase-2, truncated BID (tBID) then stimulates BAX/BAK-dependent MOMP, cytochrome c release, and formation of the Apaf-1/caspase-9 apoptosome complex, which in turn activates the effector caspase-3. In our hands, however, loss of BID or inhibition of the apoptosome provides only modest shortterm protection at lower exposures (1 h, 44°C), whereas loss of BIM profoundly inhibits cell death and facilitates long-term protection. In the context of heat shock, BIM induces MOMP and *Δψm* in a BAX/BAK-dependent manner, but BIM can still induce caspase-3 activation in the absence of detectable mitochondrial injury (i.e. in *Bax*$^{-/-}$*Bak*$^{-/-}$ cells). Thus, BIM appears to mediate alternative (and perhaps dominant) pathways to heat shock-induced apoptosis through activation of both BAX/BAK-dependent and independent pathways.

cells are unable to overcome the damage induced by heat shock and instead initiate a cell death program (280, 301).

There is significant interest in better understanding the underlying mechanisms that mediate heat shock-induced apoptosis, since heat shock preferentially targets malignant cells and cancers for reasons that remain unclear (283, 310, 312). Most data suggest that heat shock kills cells through activation of the intrinsic apoptosis pathway. Several recent papers from Green and colleagues indicate that heat shock stimulates selective formation of a cytoplasmic RAIDD-caspase-2 complex, which in turn activates caspase-2 and cleaves BID (142, 274, 303). tBID then stimulates MOMP, cytochrome c release, and formation of the Apaf-1-caspase-9 apoptosome (Fig. 3.9) (142, 303). Green and colleagues also provide evidence that heat itself induces conformational changes in BAX and/or BAK that render them more susceptible to activation by exogenously added recombinant tBID (208). However, this does not exclude the possibility that another BH3-only protein such as BIM might replace tBID.

Additional reports suggest this canonical pathway may not fully account for the cell death induced by heat shock. In some instances, caspase-2 and BID are not essential for cell death or serve as an amplification loop to promote MOMP and caspase activation downstream of the apoptosome (302, 304). In the current studies, we have discovered that another BH3-only family member, BIM, plays a significant role in mediating cell death, independently of

the caspase-2-BID pathway. By assessing the survival of *Bim*$^{-/-}$, *Bid*$^{-/-}$ and *Bax*$^{-/-}$*Bak*$^{-/-}$cells at different exposure levels, we found that loss of BID afforded some early protection to “low-dose” heat shock (44°C/1 h), but failed to provide short or long-term protection following a “high-dose” exposure. By comparison, loss of BIM afforded significant protection at both doses, even exceeding the protection observed in *Bax*$^{-/-}$*Bak*$^{-/-}$ cells. Indeed, unlike *Bim*$^{-/-}$ cells, Bax/Bak-deficient cells underwent reduced but significant caspase-3 activation, PARP cleavage, and cell death, which suggested that heat shock could induce apoptosis through BAX/BAK-dependent and independent pathways. How heat shock induces BAX/BAK-independent activation of caspase-3, in the absence of MOMP, is unclear but is currently under investigation in our laboratory.

Given that *Bim*$^{-/-}$ cells were more resistant to heat shock than *Bid*$^{-/-}$ cells, it is tempting to conclude that the BIM pathway is the dominant pathway to apoptosis, where the caspase-2-BID pathway represents an amplification loop. However, we remain skeptical of this interpretation, given that caspase-2 associates with RAIDD in cells following heat shock (303) and that adapter proteins typically interact only with apical caspases to initiate a caspase cascade (328). Thus, in our view, the caspase-2-BID pathway likely represents an alternative pathway that is most active at lower temperatures or shorter exposures.

Since active caspase-2 does not require BIM in order to kill cells, the BIM

and caspase-2-BID pathways appear to function independently of one another, although we cannot formally rule out the possibility that BIM impacts, in some way, formation of the RAIDD-caspase-2 complex. Is interesting that in the nematode *C. elegans*, though there is no mitochondrial involvement, there is regulation by BCL-2 proteins. In *C. elegans*, the BCL-2 homolog CED-9 directly interferes with oligomerization of the Apaf-1 homolog CED-4 to block activation of the caspase CED-3. The BH3-only protein Egl-1 regulates apoptosis by displacing CED-9 from CED-4. From an evolutionary standpoint, it is interesting to consider that Bim might similarly regulate the activating complex of caspase-2, which is the most archaic of all human caspases. Such a pathway could represent a “vestigial” pathway that could be activated by an ancient stress such as heat shock.

Even though there are discrepancies between the canonical model for heat shock-apoptosis and our findings, there are aspects in which we find agreement. A role for Bim is consistent with the requirement of BH3-only proteins reported by Green and colleagues (2005). According to their study, de-repression of prosurvival BCL-2 family proteins was the step that, until overcome, delayed the onset of BAX/BAK-mediated MOMP. Moreover, this “de-repression” scenario potentially explains why ABT-737, not being a direct activator BH3-mimetic, still sensitizes cells in the absence of Bim (Fig. 3.6C and D).

Mosser and colleagues (2009) proposed a model where NOXA displaces

MCL-1 from BIM, to bind Mule (214). This BH3-only and E3-ligase protein causes the ubiquitynation and proteasomal degradation of MCL-1. The authors argued that the loss of Mcl-1 was the sufficient de-repression to allow MOMP to occur, since heat shock had already caused the direct activation of BAX/BAK. However, the authors did not pursue the role of BIM, once displaced from MCL-1 by NOXA. We speculate that heat shock may be promoting the upregulation of sensitizer BH3 proteins such as NOXA and BAD, which can antagonize subsets of BCL-2 proteins (Fig. 1.3 on chapter 1) to cause an effect similar to the one achieved by ABT-737.

Finally, it is unknown how BIM is activated following heat shock. It is worth noting that heat shock disrupts intermediary, actin, and tubulin networks (327), and BIM, which associates with the LC8 chain of the dynein motor complex, is liberated in response to cytoskeletal damage (145). Moreover, heat shock is a strong activator of c-Jun N-terminal kinases (JNKs) (329), and BIM_{EL} contains a JNK phosphorylation site at Thr112, which disrupts its association with LC8 (156). Therefore, the mechanism by which heat shock promotes the activation of BIM is currently under investigation in our laboratory, and some of the preliminary findings are presented on chapter 4.

Chapter 4. Role of MAPKs in heat-induced apoptosis and thermotolerance

4.1 Introduction

Heat shock is an ancient stress and cells have devised adaptive mechanisms to either repair damaged components or to undergo cell death if insults are irreparable (280). Heat alters membrane fluidity, disrupts the cytoskeleton and causes massive protein denaturation leading to aggregation and loss of essential housekeeping functions (288, 289, 315, 330). In response, cells mount the heat shock response (HSR), through which a group of conserved transcription factors termed heat shock factors (HSFs) upregulate molecular chaperones better known as heat shock proteins (HSPs), such as HSP70 and HSP90, to care for the proteotoxic damage (314, 331). In healthy cells, HSF1 is kept inactive through sequestration in a complex with HSP90 and HSP70. Heat and other stresses can cause the accumulation of misfolded / denatured proteins, increasing the demand for chaperones and recruiting HSP70/90 away from the complex. This, in turn, frees HSF1 to translocate to the nucleus and promote the transcriptional upregulation of HSPs (332, 333).

Heat shock proteins are the best-studied class of molecular chaperones upregulated by proteotoxic stress. The prototypical member of the class is

HSP70, which contains a 15-kDa C-terminal peptide-binding domain and an N-terminal 44kDa ATPase domain. When ATP is hydrolyzed it induces conformational changes in HSP70 that allow it to recognize and bind to the exposed hydrophobic regions of unfolded proteins and aid them in recovering their native conformation, thus preventing protein aggregation and contributing to the restoration of normal cellular function (284, 297, 301).

Besides the HSR, a specialized group of protein kinases, termed mitogen-activated-protein-kinases (MAPKs) and stress-activated protein kinases (SAPKs), can be activated by the cell in response to inflammatory cytokines, growth factors and environmental stressors such as osmotic shock, ER stress, DNA damage, reactive oxygen species (ROS), and hyperthermia (334, 335). These kinases include the c-Jun-N-terminal kinase (JNK1/2/3), p38 and extracellular signal-regulated kinase (ERK) (Fig. 4.1A). Typically, a hierarchical signaling cascade activates MAPKs, where the stimulus causes the activation of the MAP3K (the upstream most kinase in the cascade), which in turn phosphorylates a MAP2K, responsible for the direct phosphorylation and activation of the MAPK that will execute the cellular response (Fig.4.1A).

The stress-activated protein kinase JNK has been previously implicated in causing MOMP by favoring BAX translocation from the cytosol into the mitochondrial outer membrane (336, 337) and promoting apoptosis in response to several stressful stimuli, including heat shock (338, 339). As previously

discussed in chapter 1, JNK can induce apoptosis by transcriptionally upregulating Bim in neurons (167, 175) or by phosphorylating BIM (T112, see Fig. 4.1D) in response to ER stress and UV, leading to BIM protein stabilization and translocation to the mitochondria (145, 157). In contrast, ERK has been implicated in pro-survival signaling, where phosphorylation of BIMEL on Ser55/65/73 by ERK, under favorable growth conditions, can target it for proteasomal degradation (157, 164, 340). Finally, the importance of p38 for BIM regulation is less well characterized and has been restricted to a role in responding to arsenite and glucocorticoid-induced apoptosis. Studies indicate that p38 regulates BIM directly by activating it *via* phosphorylation on Ser65 and also transcriptionally through phosphorylation and activation of the transcription factor FOXO3a (168, 341, 342).

HSP70 has been shown to prevent apoptosis by inhibiting MOMP (301, 339, 343-348), and caspase activation via the disruption of apoptosome formation and activity (301, 349-353), although the latter has been questioned (354). HSP70 can also interfere with pro-apoptotic JNK signaling (301, 329, 355-360). Previous studies have shown that HSP70 can prevent the activation of pro-apoptotic JNK in response to heat shock and thereby prevent cell death as a novel thermotolerance mechanism of HSP70 (355, 357). Pro-apoptotic JNK activation has been linked to permeabilization of mitochondria to initiate the intrinsic apoptotic pathway (338, 361), and HSP70 was shown to prevent the

translocation of BAX due to its JNK-inhibitory effects (337).

In chapter 3 we reported the identification of the BH3-only protein BIM as a critical regulator of heat shock-induced cell death. Since the mechanism by which heat shock activates BIM is unclear, we hypothesized that MAPKs might regulate the pro-apoptotic activity of BIM in this context. Here we report that heat shock-activated ERK, but not JNK, phosphorylates BIM. Since the MEK1/2 inhibitor UO126 prevents BIM phosphorylation but does not protect cells from heat shock, we speculate that BIM phosphorylation is part of a thermoprotective stress response. Interestingly, when studying the impact of MAPK inhibitors on heat shock-induced cell death and thermotolerance, we observed that the novel JNK inhibitor JIN8 prevents cells from acquiring thermotolerance. In addition, we discovered that the MAP3K TAK1 was required for JNK activation, and that cells deficient in TAK1 were intrinsically thermotolerant, consistent with a pro-apoptotic role for JNK. Interestingly, JNK activation follows a dichotomous pattern of transient activation by mild heat shock *versus* robust and sustained activation with exposure to lethal heat shock. Thus, our preliminary findings suggest a dual role for JNK in response to heat doses, with an unanticipated function in promoting cellular thermoresistance.

4.2 Results

4.2.1 Heat shock activates MAPKs and phosphorylates BIM

Previous studies have demonstrated that heat shock causes the activation of ERK, JNK and p38 kinases (335). These kinases have pleiotropic effects, performing central roles in signaling cell adaptive responses to stress by affecting gene transcription and target protein function. They can act to promote either pro-survival responses or promote apoptosis in certain cellular contexts (Fig. 4.1A).

We found that a 44°C for 1.5 h treatment caused the phosphorylation and activation of p38, ERK and JNKs (Fig. 4.1B), and that BIM_{EL} electrophoretic mobility was delayed after heat shock, potentially due to phosphorylation event(s) (Fig. 4.1C). There are several previously identified phosphorylation sites in BIM_{EL}, with serine 65 being a common site for ERK, JNK and p38 kinases, while the threonine 112 that lies within the LC8-binding domain seems exclusive to JNK (Fig. 4.1D) (156, 157, 168). Using a commercially available antibody raised against the phospho-Ser65 epitope, we confirmed that BIM phosphorylation after heat shock occurs on at least this residue (Fig. 4.1E), though it does not exclude the possibility that other residues are also phosphorylated.

A.

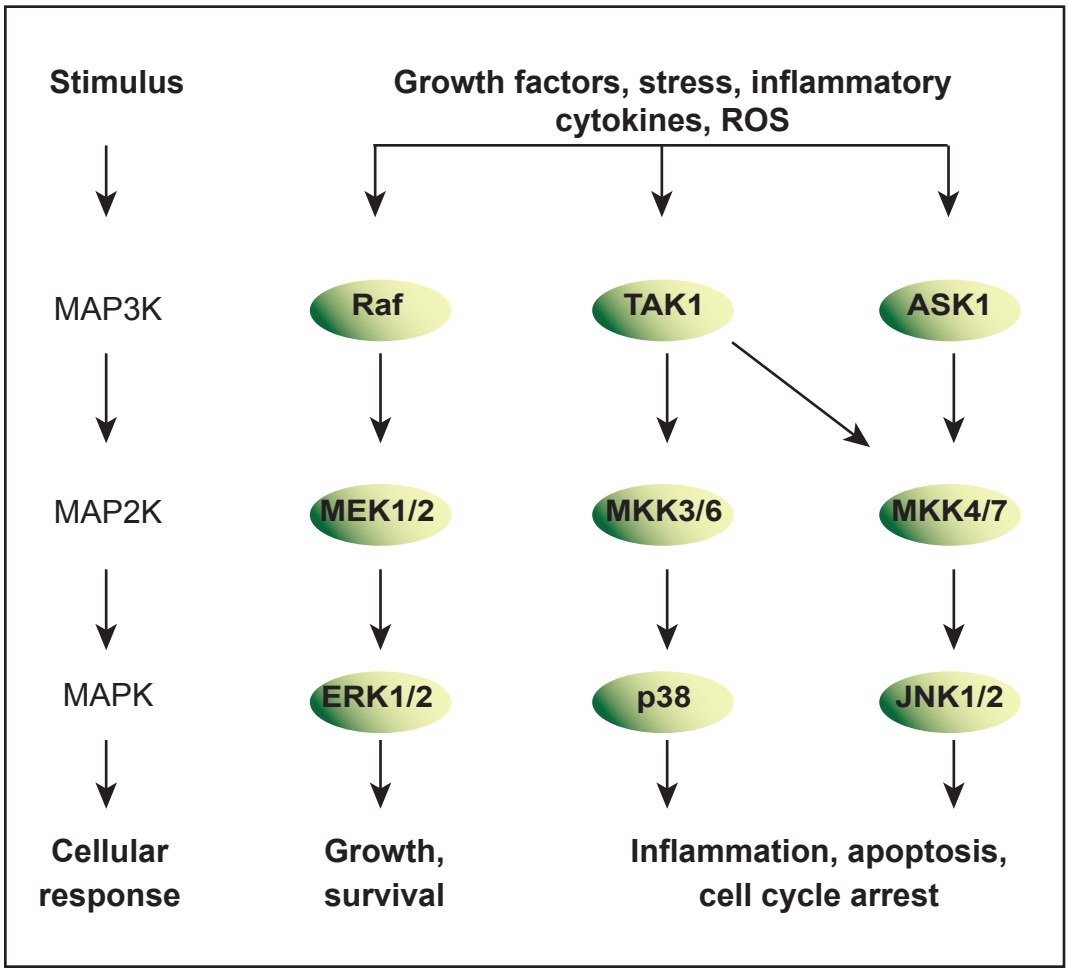


Figure 4.1. Heat shock promotes the activation of ERK, p38 and JNK, and the phosphorylation of BIM on Ser65. (A). Scheme of general hierarchic organization of MAPK signaling modules *(Adapted from Davis, 2000; Kyriakis & Avruch, 2001; Cell Signaling Techology, Inc.).*

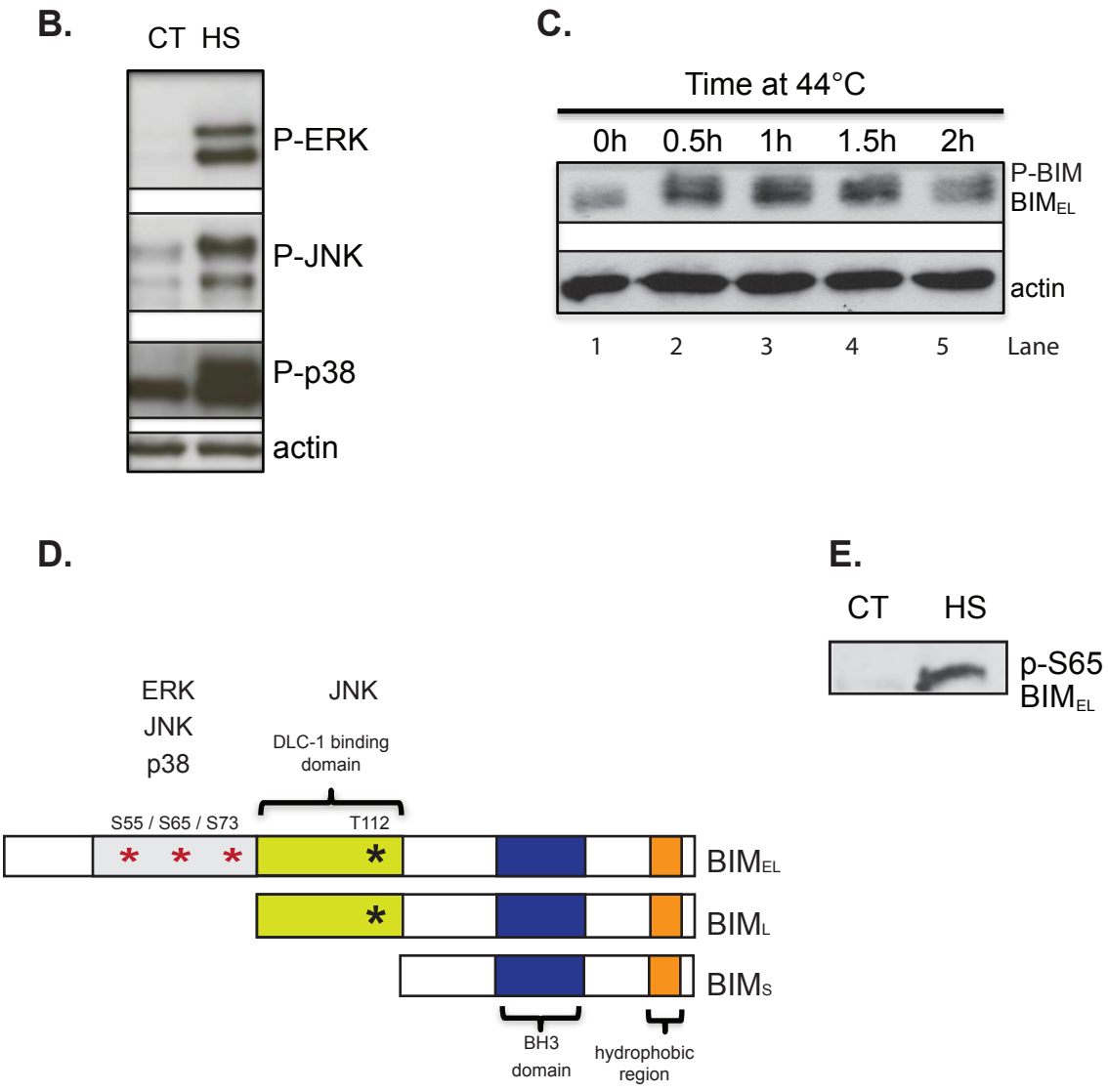


Figure 4.1. (Cont.) Heat shock promotes the activation of ERK, p38 and JNK, and the phosporylation of BIM on Ser65. (B). MEFs were exposed to 44°C for 1.5 h and recovered at 37°C for 1 h prior to collection for western blot analysis. **(C).** MEFs were treated as in (B) but collected after the indicated time points. **(D).** Schematic representation of Bim isoforms generated by alternative splicing with the respective phosphorylation sites present in BimEL and BimL (adapted from O'Connor *et al*., 1998; Puthalakath *et al*., 1999; Lei & Davis, 2003; Hubner *et al*., 2008). **(E).** Using a phospho-specific antibody raised against the phospho-Ser65 of Bim we confirmed that residue is target of phosphorylation after heat shock.

4.2.2 ERK phosphorylates BIM after heat shock

We sought to further identify the heat-activated BIM kinase(s). Since all 3 MAPKs that were activated by heat can target Ser65, we studied the effect of pharmacological inhibitors of their signaling pathways. We found that inhibition of MEK1/2 by UO126 disrupted the phosphorylation of BIM (Fig. 4.2A, lane 5). In contrast, the JNK inhibitor SP600125 did not prevent BIM phosphorylation (Fig. 4.2A, lane 6), but in turn it lowered BIM levels. To a lesser extent, inhibition of p38 by SB203580 also decreased BIM phosphorylation (Fig. 4.2B, lane 6). It is unclear why SB203580 increased the total level of phospho-p38, however it did decrease p38 activity as indicated by a decrease on phospho-MAPKAP2 signal (Fig. 4.2B, lane 6). When testing the effect of SB203580 and UO126 in heat shock-apoptosis, we did not find any significant difference between the treated and the control groups (Fig. 4.2C and 4.2D), suggesting that they may not be essential for apoptosis at the heat shock dose we tested. Preliminary experiments with SP600125 were not interpretable since prolonged incubation (24h) with this inhibitor at this dose (50 μM) caused toxicity at 37°C, requiring further optimization (data not shown). Since ERK phosphorylation of BIM has been linked to its degradation as part of a pro-survival response (157, 163), we postulated that, perhaps in the context of heat, phosphorylation is not regulating BIM activation, but rather could be part of a cell survival mechanism promoted by MAPKs.

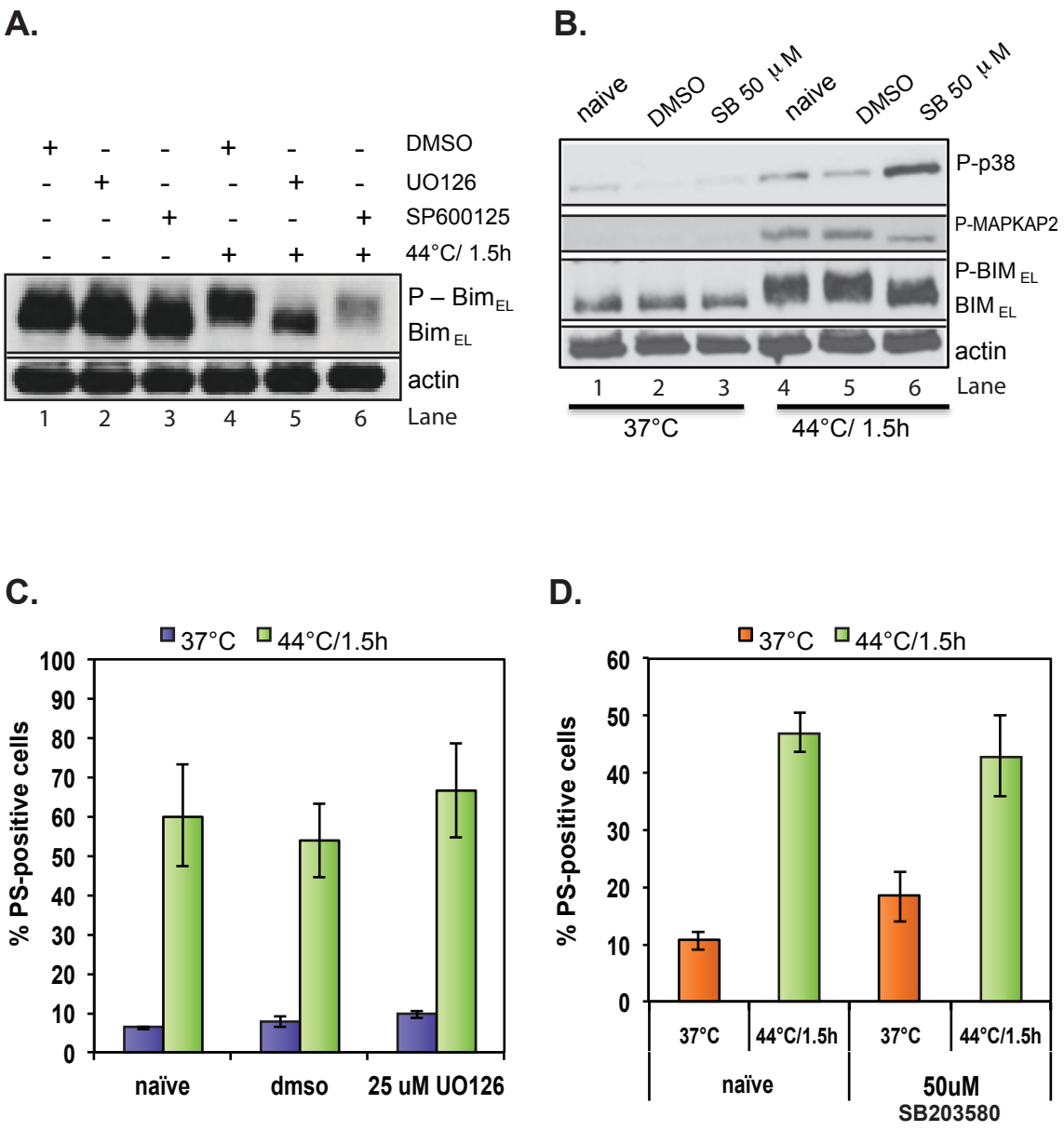


Figure 4.2. Effects of various inhibitors on heat shock-induced MAPK activation and BIM phosphorylation. (A-B). MEFs were pre-incubated with the following inhibitors: ERK inhibitor UO126 (25 μM); JNK inhibitor SP600125 (50 μM), or in (B) p38 inhibitor SB203580 (50 μM), subjected to HS and collected for western blot analysis. **(C,D).** MEFs were treated as in (A-B) and collected for annexin-V-FITC/PI staining and flow cytometry after 24 h.

4.2.3 Pre-conditioning leads to thermotolerance and causes differential phosphorylation of JNK compared to lethal heat shock

Several studies have previously shown that exposure to a mild heat shock makes cells transiently resistant to a subsequent otherwise lethal heat shock. This phenomenon is commonly referred to as thermotolerance (284, 285). We established a standard operating procedure to make wild type MEFs thermotolerant in our laboratory (Fig. 4.3), similar to those reported previously for MEFs (362, 363). Pre-conditioning by a mild heat shock involved exposure at 43°C for 30 minutes (henceforth referred to as “T43”), followed by recovery at 37°C for 4 h. Cells were then exposed to lethal heat shock (44°C for 1.5 h) (Fig. 4.3). At 24 h the T43 group displayed significant protection (Fig. 4.3A), which was followed up to 72 h (Fig. 4.3B), when cells completely repopulated the wells.

Careful observation of the kinetics of stress response activation following the two heat shock doses being used in our studies confirm previous reports (284, 364) that mild heat shock promptly induces HSP70 expression (Fig. 4.3C), whereas this response was inhibited following a lethal dose of heat (Fig. 4.3D). Regarding MAPK activation, we observed that T43 causes a transient JNK activation during the first 3 hours following the insult (Fig. 4.3C), whereas lethal heat shock promotes a sustained JNK activation that starts with the initial heat shock and continues through 24 h, when we normally quantify the number of apoptotic cells (Fig. 4.3D).

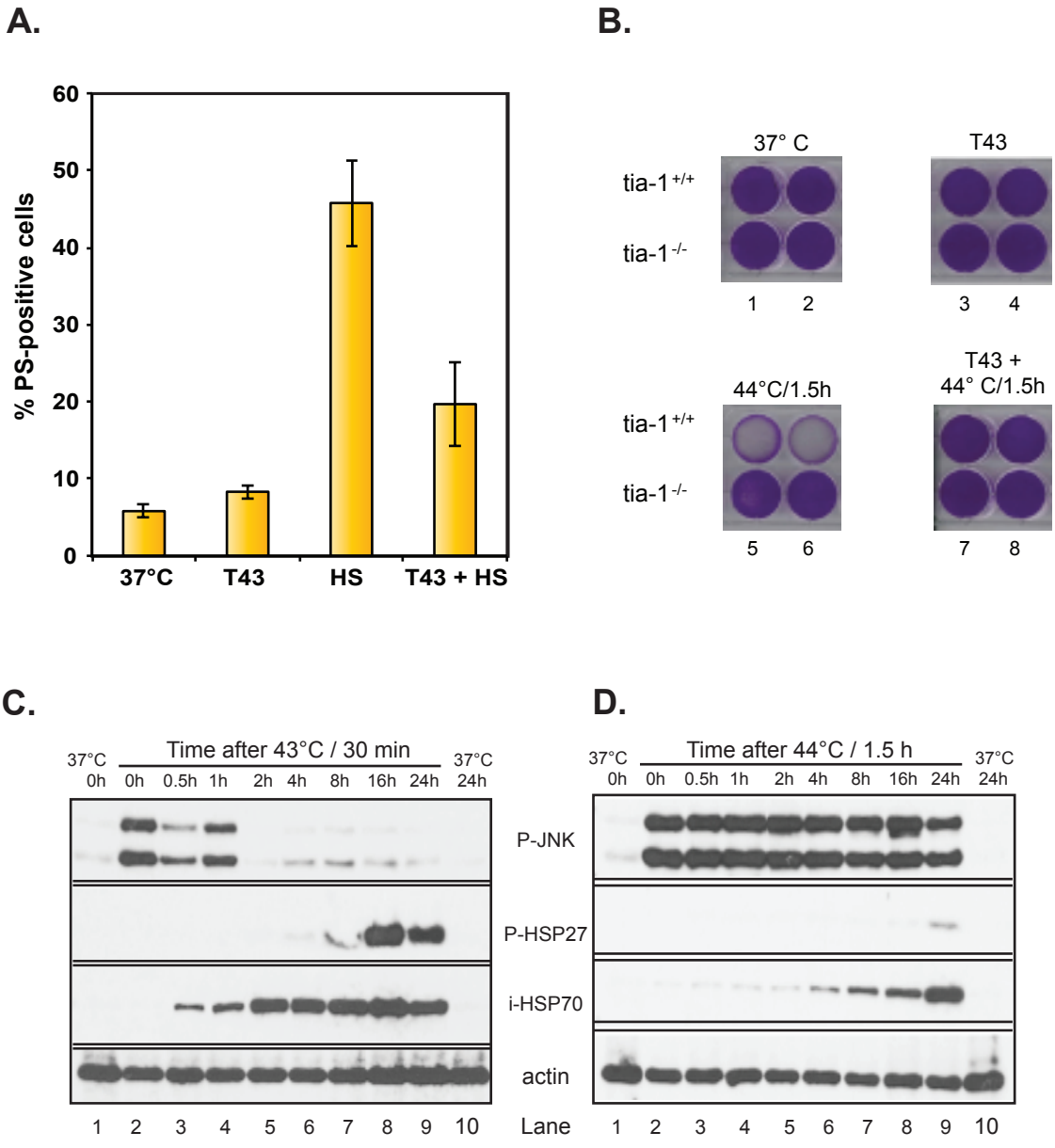


Figure 4.3. MEFs become thermotolerant when exposed to mild heat shock. (**A-B).** Cells where pre-incubated at 43°C for 30 minutes, allowed to recover at 37°C for 4 h, then subjected to a lethal HS (44°C/1.5 h), and analysed for apoptosis at 24 h (*panel A*), and for long-term survival by crystal-violet staining at 72 h (*panel B*). **(C-D).** Cells were treated with indicated doses of heat and collected at several time points for western blot analysis.

4.2.4 Inhibition of JNK does not impact heat shock-induced apoptosis but prevents thermotolerance

We obtained a novel small-molecule JNK inhibitor, JIN8, as a generous gift from Dr. Kelvin Dalby as per collaboration between our research groups (319). Sustained (and not transient) JNK activity has been proposed to contribute to cell death (365), therefore, we investigated the effect of JIN8 in heat shock-apoptosis. JIN8 can efficiently block the phosphorylation of Ser73 in c-Jun, a known substrate of JNK and common read-out of its kinase activity, in response to heat shock (Fig. 4.4A, lanes 7-8). JIN-8 also suppressed basal levels of phospho-c-Jun (Fig. 4.4A, lanes 3-4); it is interesting to note that the pattern of phosphorylation of c-Jun suggests that there is/are additional protein kinase(s) that could be targeting this phosphorylation site and which are not being inhibited by JIN-8.

We confirmed the effect of JIN-8 in blocking phosphorylation of another residue in c-Jun using a phosphor-specific antibody against Ser63 (data not shown). JIN-8 did not prevent BIM phosphorylation (Fig. 4.4A, lanes 5-8), confirming the result observed with SP600125 (Fig. 4.2A, lane 6). We found that pre-treatment of MEFs with JIN8 did not have any impact in heat shock-induced apoptosis (Fig. 4.4B), but surprisingly, JIN8 blocked acquisition of thermotolerance (Fig. 4.4C). Together, these preliminary findings suggest the existence of a pro-survival role for JNK following mild heat shock.

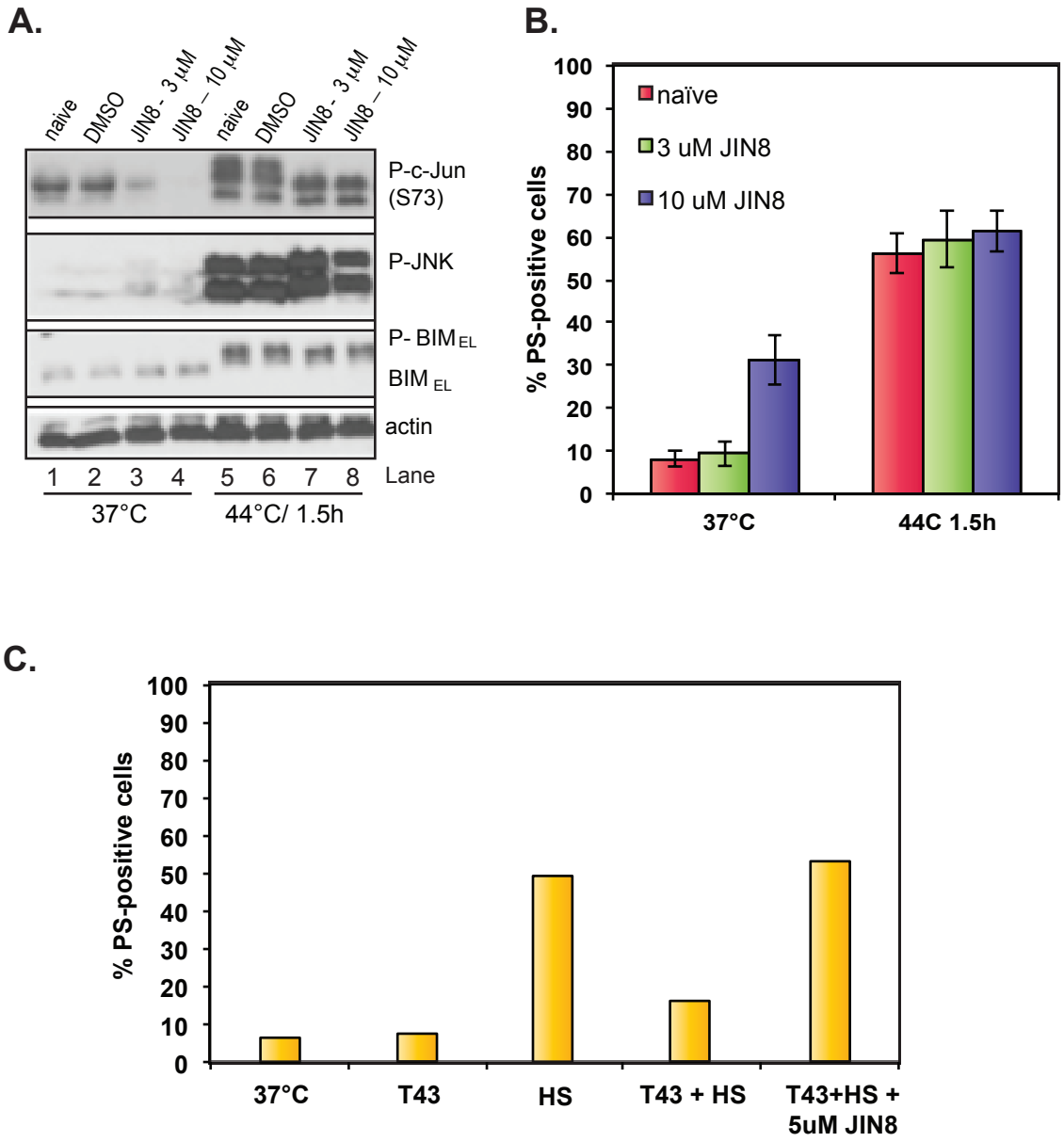


Figure 4.4. Effects of JNK inhibition on signaling, cell death, and thermotolerance. (A-B). Cells were pre-incubated with the novel JNK inhibitor JIN8 for 3 h and heat shocked at 44°C for 1.5 h, collected for western blot at h after HS or for apoptosis analysis at 24 h (mean of 3 independent experiments +/- SEM). **(C).** Effect of JIN8 on thermotolerance: cells were pre-incubated at 43°C prior to lethal HS where indicated by T43; the graph shows the mean of 2 independent experiments.

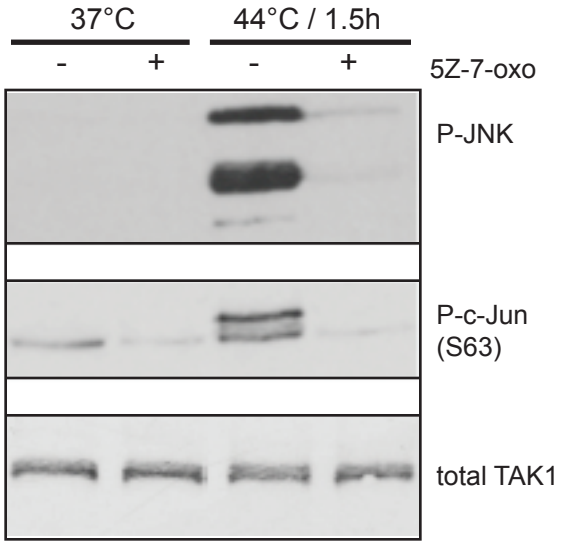
4.2.5 The MAP3K TAK1 is required to activate JNK in response to heat shock

While investigating the role of MAPKs in the stress response to heat, we sought to identify the upstream MAP3K kinases leading to the activation of JNK and other MAPKs. By pre-treating cells with the resorcyclic lactone (5Z)-7-oxozeaenol (5Z-7-oxo), a specific TAK1 inhibitor, we found that it severely impaired the activation of JNK following heat shock (Fig. 4.5A, lane 4). Similar to the above-mentioned MAPK inhibitors, 5Z-7-oxo pretreatment did not contribute nor prevent heat shock-induced cell death (Fig. 4.5B). Since 5Z-7-oxo prevented JNK activation in response to heat shock, and JNK inhibition by JIN8 prevented thermotolerance, we are currently investigating whether 5Z-7-oxo can similarly block thermotolerance by preventing JNK activation in that scenario.

4.2.6 TAK1-deficient MEFs fail to activate JNK and are protected from heat shock

In addition to investigating the role of TAK1 with the pharmacological inhibitor 5Z-7-oxo, we sought to verify the response of *tak1*$^{-/-}$ MEFs to heat shock. When exposed to lethal heat shock, we found that *tak1*$^{-/-}$ MEFs were resistant to apoptosis (Fig. 4.6A). The *tak1*$^{-/-}$ MEFs at 24 h exhibited decreased sustained JNK activation and failed to activate caspase-3 and cleave PARP, compared to their wild-type MEFs (Fig. 4.6B, lanes 3 and 7). Analysis of the early

A.



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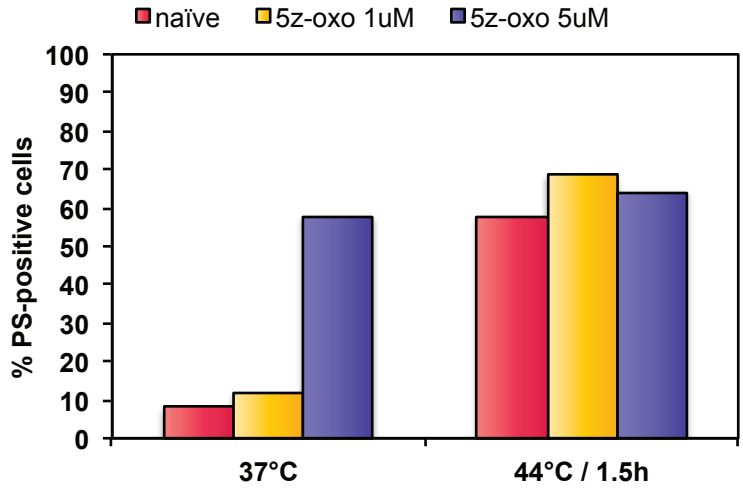


Figure 4.5. The TAK1 inhibitor 5Z-7-oxozeaenol blocks JNK activation following lethal heat shock. (A). Cells were pre-incubated for 1 h with the inhibitor prior to exposure to 44°C for 1.5 h, and collected for western blot analysis 1 h after the heat shock. (**B).** Cells were pre-incubated with the inhibitor prior to lethal HS. Cells were labeled with annexin-V-FITC/PI and analysed by flow cytometry. Obs.: results shown are the mean of 2 independent experiments.

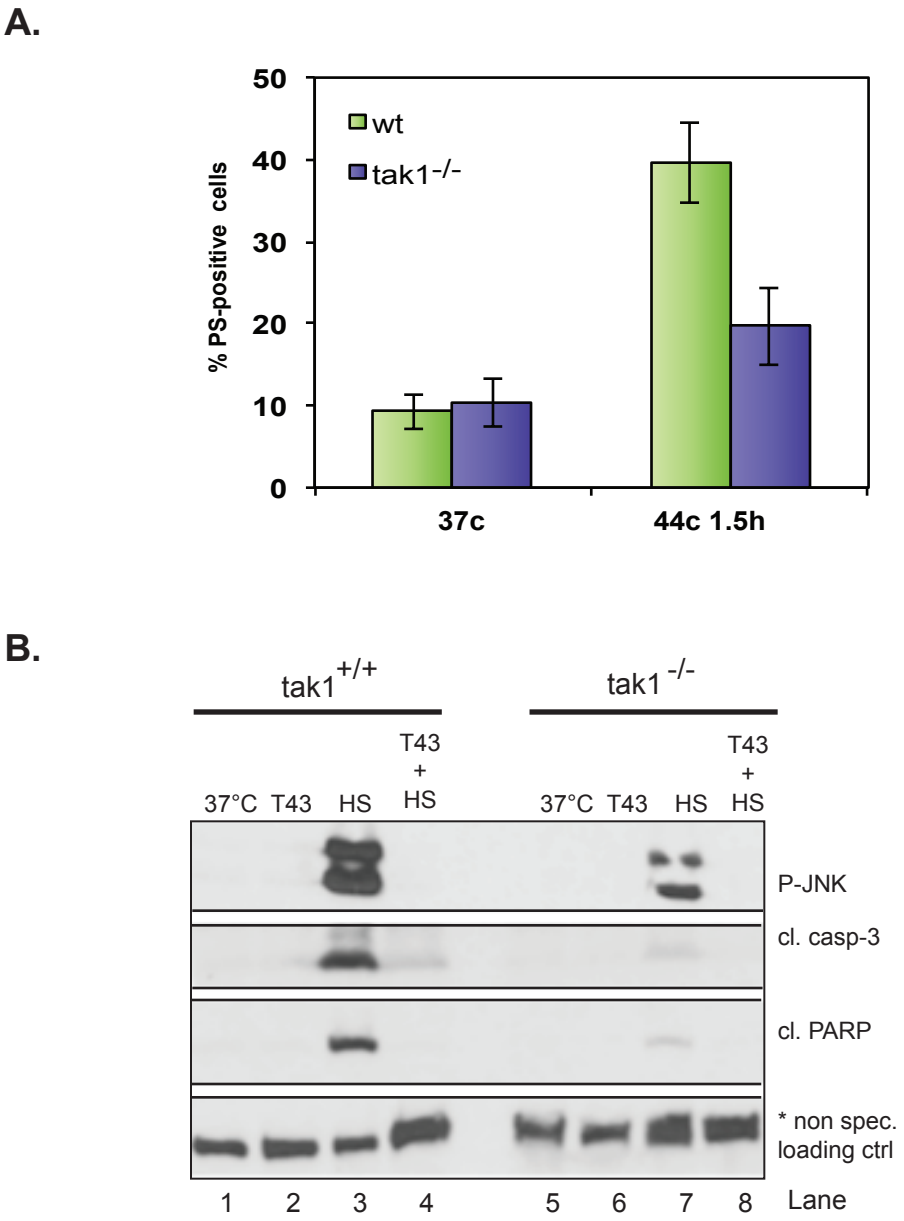
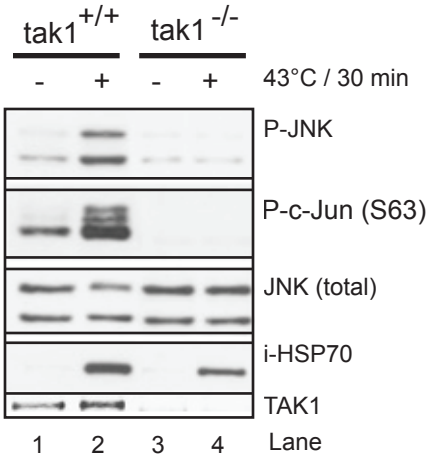


Figure 4.6. TAK1 null MEFs are protected from heat shock-induced apoptosis, and have diminished JNK activation following heat shock. (A). TAK1 null MEFs were exposed to lethal heat shock, and after 24 h, cells were labeled with annexin-V-FITC/PI and analysed by flow cytometry. **(B).** Cells were exposed to lethal heat shock as in (A), with or without pre-incubation at 43°C prior to lethal heat shock, where indicated by T43, and collected for western blot analysis 24 h after the heat shock.

C.



D.

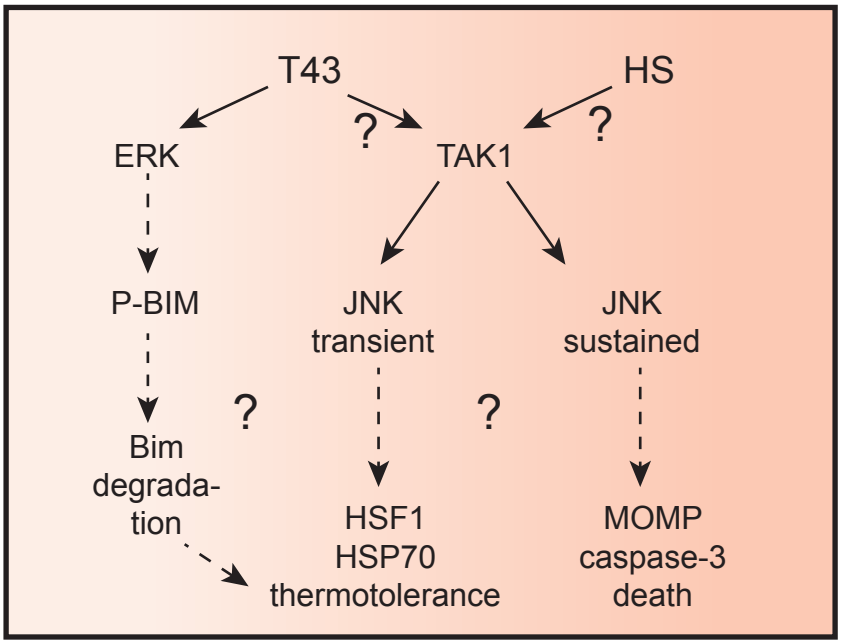


Figure 4.6. (Cont.) TAK1 null MEFs are protected from heat shock-induced apoptosis, and have diminished JNK activation following heat shock. (C). TAK1 null MEFs were pre-incubated at 43°C for 30 minutes, and collected for western blot analysis 1 h after recovery at 37°C. **(D).** Speculative model of MAPK-mediated pathways for apoptosis and thermotolerance after HS.

response of *tak1*$^{-/-}$ MEFs to T43 show that they don't activate JNK after the pre-conditioning heat dose while their matching wild type control cells do (Fig. 4.6C). Together, our findings implicate TAK1 in the regulation of JNK in response to heat shock. The exact mechanism of how heat shock activates TAK1 to activate JNK, as well as the biological consequences of lack of TAK1, or its pharmacological inhibition, are currently being investigated in our laboratory.

4.3 Discussion

In order to maintain homeostasis cells resort to the activation of evolutionarily conserved stress responses, such as the HSR, when faced with stimuli that damage the proteome. Two major signaling responses, the stress-activated MAPK pathways and the heat shock response, promote the upregulation of important cell survival molecules that safeguard cells when injury occurs (284, 332, 335, 358). Heat shock proteins have been extensively studied in part due to their roles in cancer and associated to resistance to chemotherapeutic agents, radiotherapy and poor prognostics (315). Currently there are intense research efforts into the discovery of small-molecule HSF1, HSP70 and HSP90 inhibitors for cancer treatment (284, 315, 316, 318). Therefore, a better understanding of the basic molecular events regulating the

stress response and how it confers a multi-resistance phenotype, such as the development of thermotolerance, will be instrumental for the discovery of novel therapeutic approaches.

In this preliminary study we observed important differences in the kinetics of signaling events following a mild heat stress versus a lethal heat dose: (A) HSP70 induction occurs promptly after mild heat shock but is severely impaired after lethal heat; (B) JNK activation is transient with mild heat, but robust and sustained with lethal heat. Also, we may have uncovered two potentially novel thermotolerance mechanisms: (I) heat-activation of ERK to modulate the pro-apoptotic BH3-only protein BIM, and (II) transient JNK activation as a pro-survival signaling pathway.

The rapid induction of HSP70 with T43 (Fig. 4.3D) occurs presumably due to de-sequestration of HSF1 by HSP70/HSP90, as they leave the trimeric complexes with HSF1 in order to attend the accumulated pool of denatured proteins (284, 331). It is unclear why lethal heat shock delays the upregulation of HSP70 induction, and since HSP70 has anti-apoptotic properties, preventing its induction could function as a pro-apoptotic mechanism (332).

BIM phosphorylation can impact cell fate differentially, depending on the cellular context (157, 163, 167). In our study we found that BIM is a target of ERK phosphorylation after heat shock, but the biological function of the post-translational modification is still unclear. In response to favorable growth

conditions, cells destroy BIM in an ERK-dependent manner to prevent cells from dying. Thus, we hypothesize that when facing heat stress cells activate both pro-survival and apoptotic signaling pathways simultaneously, and it is the balance of these pathways that determines cell fate (Fig. 4.6D). In this scenario, BIM phosphorylation by ERK may constitute an attempt by the cell to survive while repairing the damage caused by the elevated temperature. If true, one would predict that Bim phospho-mutants for the ERK target sites would tend to accumulate in the cell and could hinder the development of thermotolerance. Dr. Roger Davis has kindly provided us with Bim knockin MEFs where the 3-serine residues (S55/65/73) have been substituted for alanine (Bim3SA), and we are currently pursuing those experiments.

Our studies with pharmacological inhibitors of MAPKs showed no significant impact on heat shock induced apoptosis (Fig. 4.2 and Fig. 4.4). This was somewhat disappointing since we predicted that UO126 could sensitize cells to heat shock by promoting accumulation of BIM. However these studies were preliminary and additional optimization to evaluate the efficacy of UO126 in increasing BIM levels in MEFs may depend on longer pre-incubation with the inhibitor (these experiments were done with 3h pre-incubations). Additionally, there are other classes of more potent and specific MEK1/2 inhibitors, such as AZD6244 or PD0325901 (366), which could also be utilized to verify this possibility. Since JNK activation is robust following lethal heat shock, we

predicted that treatment with a JNK inhibitor would protect cells from apoptosis. We did not observe any significant effect in that context. Thus, we shifted our focus to the effect of inhibitors on thermotolerance. To our surprise, we found that inhibition of JNK with JIN8 prevented cells from becoming thermotolerant (Fig. 4.4C). The fact that a JNK inhibitor could prevent thermotolerance supports the notion that JNK activation in response to mild heat is an adaptive, pro-survival response.

This finding, though preliminary, is in striking contrast with the literature that implicates suppression of JNK signaling as a mechanism to undergo thermotolerance (337, 355), in part due to an inhibitory effect of HSP70 on JNK. Indeed, JNK activity has been linked to induction of MOMP and apoptosis (336-338). Thermotolerance prevents caspase-3 activation (Fig. 4.6B, lane 4) and relies on HSP70 function (355, 367), which has been proposed to interfere directly with core components of the apoptotic pathway (301, 343, 345). Therefore, collectively, one would have expected that inhibition of JNK should protect cells from lethal heat shock.

The observation that *tak1*$^{-/-}$ MEFs have less JNK activation following heat shock and were protected from heat shock-induced apoptosis is consistent with a pro-apoptotic role for JNK. It is possible that the protection observed in *tak1*$^{-/-}$ MEFs occurred because these cells failed to maintain a sustained JNK activity following the stress. Alternatively, TAK1 may signal through a JNK-independent

pathway to induce apoptosis. Several studies show a strong correlation of sustained JNK activation with apoptosis (365, 368-370). A recent study proposed a role for TAK1 in thermotolerance, but the authors did not implicate JNK activation in their proposed mechanism (371). Additionally, it is unclear how heat shock promotes the activation of TAK1. TAK1 has been implicated in several signaling pathways, including pro-survival TNFR1 signaling following TNF-α engagement of the receptor, culminating in the activation of MAPKs and NF-κB (22, 372-376). However our preliminary studies with *tnfr1*$^{-/-}$ MEFs failed to implicate TNFR1 in heat shock-induced apoptosis (data not shown). Therefore, our observations await studies with the reintroduction of wild-type TAK1, as well studies on the role of known TAK1 activator/adaptor proteins, to further establish a role for this MAP3K in heat stress responses. Additionally, kinetic studies comparing TAK1 wild type and null MEFs could be fundamental to understanding how TAK1 regulates JNK activation after heat shock.

It is unclear at the moment how JNK could be driving thermotolerance. We are tempted to speculate that an alternative interpretation exists to explain the JNK-HSP70 connection. *Perhaps JNK controls thermotolerance by affecting the function of HSF1*? Previous studies have shown that the affinity of HSF1 for the complex with HSP70 and HSP90 could be modulated through post-translational modifications on HSF1, such as phosphorylation (377-379). However, there is extremely scarce and contradictory evidence in the literature implicating a role for

JNK in the regulation of HSF1 and the HSR. A study by Dai and coworkers mostly based on overexpression approaches, proposes that JNK-mediated phosphorylation of HSF1 inhibits its HSP70 inducing function (378). Conversely, a study by Park & Liu (2001) reports the exact opposite. Even though the authors suggest that JNK can play a role in thermotolerance, their approach is questionable since they use the p38 inhibitor SB203580 at incredibly high concentrations to inhibit JNK. They show that both JNK and p38 are inhibited (100uM), but at this dose they are likely affecting other enzymes in the cell (359, 380). Therefore, a rigorous investigation of the role of JNK in regulating HSF1-mediated responses by a panel of carefully titrated inhibitors, aided by studies with genetic knockouts / knockdowns in cells, could elucidate a novel role for JNK in promoting thermotolerance.

Needless to say, caution should be used in interpreting results obtained with pharmacological kinase inhibitors. Several reports alert to the potential non-specific or “off-target” effects promoted by widely used inhibitors, such as SP600125 or SB203580 (319, 381, 382). Use of “inactive” chemical structural analogs can be useful to help with interpretation of results, as well as the implementation of complimentary approaches such as use of knockout / knock downs by RNAi, as well as overexpression of dominant-negative mutants of the MAPKs involved in the signaling pathways. Nevertheless, our preliminary findings point to a JNK-mediated pathway for cellular thermotolerance, which if

true and universal, could add another layer of complexity into how JNKs and HSP70 cooperate to promote cellular resistance to heat stress and potentially have clinical significance. Acquisition of thermotolerance has been linked to resistance not only to hyperthermia, but also to other modalities of cancer treatment such as radiotherapy and chemotherapy (315, 383). We would like to further investigate, using *xenograft* models, the effects of JIN8 on the response of tumors to hyperthermia alone or in combination with chemotherapy or radiotherapy. It is exciting to imagine that JIN8 might overcome the refractory response of cells to radio/chemotherapy treatments. This novel class of JNK inhibitors (319) could serve as a new generation of chemotherapeutic agents, quickly escalating to clinical trials in the future for the treatment of solid tumors.

Chapter 5. Conclusions

Drastic changes in temperatures, as during hyperthermia or heat shock, represents one of the most ancient challenges that organisms had to adapt to in order to survive. As a result, evolutionarily conserved mechanisms exist in order to assist cells in repairing the damage to cellular components, mainly the proteome, and also protecting the cell against subsequent insults (already discussed in detail in chapters 1 and 4). For instance, the heat shock response utilizes the transcription factor HSF1 to trigger the upregulation of HSPs such as HSP70 and HSP90 (314, 333). These molecular chaperones facilitate “damage control” by refolding denatured proteins, thereby preventing aggregation and rendering cells thermotolerant (284). However, when the damage is irreparable, the cellular suicide program can be activated to eliminate cells that now pose potential harm to the organism (280).

Apoptosis induction involving the intrinsic pathway is essential for the proper elimination of cells damaged by several stressors, such as DNA damaging agents, glucocorticoids, ER stress, and acute heat shock (12, 146, 148, 208, 384-386). Several studies have uncovered an important role for caspase-2 and BID in the induction of MOMP and apoptosis following heat shock (142, 274, 303). However, other studies have challenged this model, since they

did not find convincing evidence that would definitively implicate the same molecular players (302, 304). Additional proteins such as BAX, BAK, JNK, HSP70, and MCL-1 have also been implicated in the regulation of MOMP in heat shock-induced apoptosis (208, 337, 351, 352, 355, 357, 387, 388).

One of the main discrepancies is the modest level of protection observed in cells lacking either caspase-2 or its adaptor protein RAIDD, or the BH3-only BID, proposed to be the direct substrate for heat-activated caspase-2 (274, 302-304). BID requires proteolytic cleavage in order to efficiently engage the intrinsic pathway and antagonize the pro-survival family members and/or directly activate BAX/BAK (26, 103, 135-140, 267, 389). Compared to other initiator caspases, caspase-2 substrate preference is limited, raising skepticism towards its physiological function as an apical protease in a caspase-activating cascade (267-272, 302, 304). Since attempts to implicate additional initiator caspases-8, -9, and -12 were unsuccessful (or conflicting), the heat-activated, z-VAD-inhibitable apical protease remains elusive (302, 304).

Our most important finding reported in this study is that heat shock requires BIM in order to undergo MOMP, caspase-3 activation and cell death. Absence of BIM not only protected cells short term, but allowed repopulation of the dishes after 72 h (Fig. 3.1). Moreover, silencing of Bim in Jurkat T leukemia cells conferred resistance to the cells, correlating with Bim expression levels (Fig. 3.2). In contrast, cells lacking Bid showed only partial protection to lower doses of

heat shock, which decreased with longer exposure times, and was completely lost long-term. We confirmed previous reports that caspase-2 does require BID to kill cells, at least in the context of overexpression (Fig. 3.3) (142, 267). Therefore, together, our data suggest that BIM plays a dominant role in heat shock-induced apoptosis and that BID may be involved in an amplification loop that is critical primarily at lower doses.

In contrast, and to our surprise, *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs were partially protected from heat shock, and despite a complete failure to undergo MOMP (Fig. 3.4). *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs still had significant caspase-3 activation, albeit less than their WT counterparts (Fig. 3.4). This finding suggests that heat shock can trigger caspase-3 activation independent or upstream of MOMP. In agreement with Pagliari (2005), we also find that *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs are entirely resistant to heat shock induced MOMP. However, we find that these MEFs remain somewhat sensitive to heat shock-induced death, implying the existence of a Bax/Bak-independent pathway. Whether this alternative caspase-3 activation mechanism relies on BIM is unknown, and based on the literature, unlikely, since BIM is thought to act through BAX and BAK (in mitochondria or even in other organelles such as lysosomes) (124, 146, 390, 391).

Nevertheless, it is tempting to speculate that BIM could engage other pathways, parallel to the canonical mitochondrial pathway, where BIM might alter the permeability of other organelles such as the ER, Golgi or lysosomes (146,

391-393). BCL-2 family members can form pores in liposomes that behave like channels (discussed in chapter 1) BIM is an intrinsically disordered protein, which remains unstructured in the absence of a binding partner, but that undergoes several conformational rearrangements upon heterodimerization with pro-survival family members (394). Heat *per se* can cause conformational changes that activate BAX and BAK (208). Thus, one could speculate that heat shock may convert BIM into an “independent killer”, perhaps by causing conformational changes in BIM, directly or via post-translational modifications. These tridimensional rearrangements could free BIM from LC8 constraint and allow it to mediate pore-formation directly or through interactions with other proteins or lipids in the outer membranes of organelles other than mitochondria.

Mcl-1 deficient cells exhibited enhanced sensitivity to heat shock compared to their wild-type counterparts, suggesting that, as previously shown by Stankiewicz (2009), MCL-1 is an important player in preventing apoptosis in response to heat shock. However, this does not exclude the importance of other pro-survival BCL-2 proteins in preventing MOMP when cells are faced with heat shock, as suggested by the sensitization observed with ABT-737, a BH3-mimetic that imitates the action of the sensitizer BH3-only protein BAD (395-399). Interestingly, MCL-1 suppression seemed to cause a more prominent role in HeLa cells, suggesting that cell type differences could favor a particular pro-survival member of the BCL-2 family. ABT-737 has been shown to synergize with

MEK and BRAF inhibitors (400, 401). Thus, BH3-mimetics in combination with hyperthermia would represent a potential angle to be exploited therapeutically.

Nevertheless, Bim plays a prominent role in cancer development. Bim is downregulated in several tumors through various mechanisms, including epigenetic promoter silencing, genetic deletion, enhanced proteasomal degradation, and micro-RNA mediated suppression (128, 402-407). Bim is a tumor suppressor and is deleted in mantle cell lymphoma and contributes to lymphomagenesis (402). Additionally, Bim downregulation in diffuse large B cell lymphomas and Burkitt Lymphomas is due to promoter hypermethylation, which is associated with poor prognosis and early relapse (128, 405).

Oncogenic mutations in RAS or BRAF can lead to constitutive MEK1/2-ERK1/2 signaling (406). These tumors are RAS or BRAF-“addicted” and rely on the de-regulated ERK signaling pathway, not only for the increased cell proliferation, but also for the decrease in BIM levels (163, 164, 406, 408-413). As mentioned in chapter 1, ERK controls BIM turnover, and pharmacological inhibitors of components of the ERK signaling pathways are in clinical trials. Indeed, the first BRAF inhibitor, vemurafenib, has been approved for clinical use (406).

Importantly, BRAF inhibitors have been shown to restore BIM levels in some cell types, contributing to the response to these drugs (366, 406) (412, 414). Even though we did not observe an increase in cell death in MEFs upon

pre-treatment with UO126 (Fig 4.2), it is likely that cells that are “addicted” to the ERK pathway will be more responsive to such inhibitors. Additionally, UO126 is a first generation pan-MEK inhibitor, which also inhibits MEK5, and thus could lead to non-specific effects. It is also less potent than newer generation inhibitors, such as AZD6244 or PD0325901 (366). Inhibitors targeting the mutant $BRAF^{600E}$ could also be promising as they are known to synergize with growth factor deprivation to induce Bim expression levels (366). Therefore, it will be interesting to investigate if inhibitors that modulate ERK signaling pathways can increase BIM levels and synergize with hyperthermia in eliminating melanoma and colorectal cancer cells in *xenograft* mouse models, with hopes of being translated to the clinic.

Similarly, other agents that could lead to BIM upregulation would be worth investigating as potential candidates for combination therapies. The ER stressor thapsigargin was shown to induce Bim expression in several cell types (146). Additionally, proteasomal inhibitors are an attractive and relatively new generation of chemotherapeutics with tumor-selective properties (415, 416). Bortezomib (also known as Velcade or PS-341) is a reversible inhibitor or the 20S catalytic core of the proteasome, and as the first-in-class proteasomal inhibitor, has been approved by the FDA to treat multiple myeloma (417, 418). This proteasome inhibitor causes BIM to accumulate in H-RAS-expressing tumors and sensitizes them to taxanes, such as paclitaxel (203). Even though

this could explain the synergy observed in some cases between Bortezomib and paclitaxel, only minor enhancement was seen in a phase II trial for Bortezomib alone or combined with docetaxel.

Additionally, BIM has been implicated in *anoikis*, and resistance to *anoikis* is an important step in establishing metastases, where cancer cells must be capable of surviving once detached from their substrate (419-424). One of the cellular components most affected by heat shock is the cytoskeleton. In cell culture the effect of heat shock is visible soon after the exposure, as many cells round up and detach from the dish. BIM is known to stay in intimate association with the microtubule network in many healthy cells (145). Therefore, one could speculate that drugs such as proteasomal inhibitors, which sensitize cells to cytoskeletal disruptors, would be good candidates for combination therapy with heat shock.

Finally, genetic screening of tumors has already been used to predict the responsiveness of tumor subtypes to a particular chemotherapy regimen (425-429). For example, the ratio of MCL-1 or BCL-2 to BAX is a relatively accurate prognostic indicator for response of CLL patients to chemotherapy (430-432), and expression of estrogen receptor in breast cancers predicts their responsiveness to hormone therapy (433-436). Therefore, knowledge of the expression levels of molecules implicated in heat shock-induced apoptosis could help forecast the responsiveness of a tumor to hyperthermia. We predict that

high BIM to MCL-1/BCL-2/BCL-x_L ratios should increased sensitivity to hyperthermia, and chemotherapy agents that can increase Bim expression levels and/or decrease the levels of pro-survival BCL-2 proteins should synergize with heat shock.

In chapter 4, we sought to study the mechanism by which heat shock activates BIM. Although we found that BIM is phosphorylated early by ERK, this post-translational modification is most likely activated as part of an attempt by the cells to survive the insult. Thus, currently we don't know how heat shock activates BIM to promote MOMP and cell death. However, while studying the role of MAPKs previously implicated in BIM regulation, we found preliminary data showing that TAK1 is the MAP3K involved in the activation of JNK following heat shock. Depending upon the intensity of heat shock, JNK activation displayed two profiles: JNK was transiently activated following mild-heat shock, whereas robust and sustained JNK activity was observed following lethal heat shock. Several reports correlate the transient activation of JNK with pro-survival responses by cells, while the later sustained activation seems to signify the engagement of a pro-apoptotic response (365, 368-370, 437-445).

Our preliminary findings, in agreement with previous literature, could suggest that transient *versus* sustained JNK can have quite distinct biological consequences. Indeed, we found that JNK inhibition in the context of a mild heat shock could prevent the acquisition of thermotolerance. Understanding how

thermotolerance occurs and how MAPKs regulate the HSR could be helpful in overcoming resistance to other types of treatments that have a common underlying mechanism of resistance.

Alternatively, BIM sequestration to microtubules is thought to isolate it from mitochondria, until pro-apoptotic JNK signaling promotes its dissociation from LC8 (145, 156, 157). We found that BIM is phosphorylated immediately after heat shock by ERK; however, we found no evidence for JNK-dependent phosphorylation of BIM. JNK may phosphorylate BIM at later time points, more proximal to the onset of MOMP. Thus, while the initial phosphorylation events are ERK-mediated and most likely induce BIM turnover, likely as a pro-survival mechanism, it is possible that sustained JNK signaling leads to a second wave of BIM phosphorylation that could be associated with its dissociation from LC8, translocation to mitochondria, MOMP and cell death.

For reasons that remain unknown, cancer cells are preferentially more susceptible to heat shock than most healthy tissues. Oncogenic stress may prime tumor cells to heat shock through upregulation of BCL-2 family members, or heat shock and the tumor *milieu*, may confer conformational changes that bypasses canonical activation steps induced in response to other stresses, as seen with BAX and BAK (208). The anti-tumor effect of heat was first observed when infection-induced fevers were found to correlate with tumor shrinkage in some cancer patients (280-284). Although it is clear that excessive heat (e.g. due

to high fever, malignant hyperthermia, or whole body hyperthermia) can have deleterious effects to organisms (446, 447), the idea that fever or heat could be beneficial to cancer patients has been resurrected in the last 25 years (284, 285, 298, 299). Current technological advances are improving the delivery means, making heat shock available as a fairly non-invasive therapy, which may be useful in shrinking otherwise inoperable tumors (448-451). The revived interest in the clinical applications of heat shock makes this a fervent area of investigation, with the promise of improving already existing therapies, and/or creating new approaches to treat cancer (280, 284-287).

In summary, our finding that BIM is required for heat shock-induced apoptosis is relevant, since it has improved our understanding of heat shock-induced apoptosis and uncovered the existence of an alternative pathway to death. This aspect could be exploited for the rational design of therapies that make use of drug combinations that upregulate BIM levels and sensitize cells to hyperthermia, with the aim of improving therapeutic outcomes in the clinic. Further insight into the mechanism(s) by which heat shock activates BIM could prove instrumental in achieving this goal.

The discoveries that have culminated in significant advances in biomedical and pharmaceutical sciences, in particular in the last century, have had a profound impact in increasing human life expectancy and in improving quality of life (452). The importance of translational research is unquestionable. However, it

is impossible to make significant progress in translational research without thoroughly designed, and carefully executed basic research. Therefore, to conclude this study, I'd like to quote one of my favorite insights from Marie Curie:

> *"We must not forget that when radium was discovered no one knew that it would prove useful in hospitals. The work was one of pure science. And this is a proof that scientific work must not be considered from the point of view of the direct usefulness of it. It must be done for itself, for the beauty of science, and then there is always the chance that a scientific discovery may become like the radium a benefit for humanity."* (Marie Curie, Lecture at Vassar College, May 14, 1921)

Appendix. Comparison of various selected relevant studies on heat shock-induced apoptosis.

Study	Main findings	Experimental details
Mosser *et al*., 2000	Heat shock caused MOMP to activate caspase-3; HSP70 overexpression protected from MOMP, caspase-3 activation and cell death.	PEER human acute lymphoblastic leukemia cells ($1x10^6$ cells/mL) were heat shocked at 43°C for 1 h in a circulating water bath, in media containing 20 mM HEPES, pH 7.4; after heat shock, cell suspensions were diluted to $0.5x10^6$ cells/mL with fresh medium at 37°C; 9 h after the heat shock, cell death was measured by Annexin V staining.
Pagliari *et al*., 2005	Heat shock directly activated BAX/BAK; Bax/Bak DKO MEFs were protected from heat shock-induced MOMP and cell death; BCL-x_L prevented the onset of MOMP and cell death.	Liposomes and purified mitochondria were heated in a thermocycler at 43°C for various periods of time; MEFs and Jurkat T cells had their media exchanged for pre-heated media at 43°C and were incubated for 1 h at 43°C in a water bath; when returned to 37°C the media was not exchanged.
Tu *et al*., 2005	Heat shock-activated caspase-2 was identified as the apical caspase in a biotinylated caspase inhibitor (bio-VAD-fmk) pull-down; *caspase-2*$^{-/-}$ and *raidd*$^{-/-}$ MEFs showed partial protection to HS.	Jurkat T cells, *caspase-2*$^{-/-}$ and *raidd*$^{-/-}$ MEFs were maintained in 7% CO_2 humidified incubator in DMEM with antibiotics and 10% FBS; splenocytes were obtained freshly from animals and activated with TPA and ionomycin prior to heat shock (43°C for 1 h).
Stankiewicz *et al*., 2005	HSP70 blocked JNK pro-apoptotic signaling and BAX translocation to mitochondria in response to heat shock.	PEER human acute lymphoblastic leukemia cells ($1x10^6$ cells/mL) were heat shocked at 43°C for 1 h in a circulating water bath; cell death was evaluated at 6 h after the heat shock.
Bonzon *et al*., 2006	Bid deficiency protected cells from HS and from active caspase-2; caspase-2-induced MOMP required BID cleavage by caspase-2.	Study utilized a cell-free system based on *Xenopus laevis* egg-purified mitochondria, as well as transformed *bid*$^{-/-}$ MEFs (44°C for 1 h), grown in IMDM supplemented with β-mercaptoethanol; no further details on the heat shock conditions.
Milleron & Bratton, 2006	Heat shock activated an unknown apical protease; MEFs deficient in caspase-1, -2, -9, and -12, and Jurkat T cells deficient in caspase-8 and/or depleted of caspase-2, were not protected from HS-induced caspase-3 activation and cell death; BCL-2 overexpression protected Jurkat T cells from HS-induced MOMP, caspase-3 activation and cell death.	Jurkat T cells and various transformed knockout MEFs as well as *caspase-2*$^{-/-}$ primary MEFs were heat shocked at 44°C and 45°C for 1-2 h in a humidified incubator (5% CO_2); cell death analysis was measured at 4h, 8h and 24 h after the heat shock by Annexin V-FITC/PI staining; MOMP was evaluated by cyt. *c* release and loss of TMRE staining by flow cytometry; caspase activity was evaluated by western blot analysis and DEVDase activity.
Stankiewicz *et al*., 2009	Heat shock-mediated MCL-1 degradation led to MOMP; HSP70 can prevent MCL-1 degradation.	PEER human acute lymphoblastic leukemia cells were treated in log-phase at 43°C for 1 h, in a circulating water bath.
Bouchier-Hayes *et al*., 2009	Caspase-2 was activated by induced proximity in response to heat shock; FKBP-caspase-2 fusion-induced cell death was prevented by BCL-x_L overexpression (in HeLa); HSF1 deficiency sensitized cells to heat shock.	MEFs were maintained with DMEM supplemented with sodium pyruvate and β-mercaptoethanol. Various HS conditions: HeLa cells (45°C for 1 h); *raidd*$^{-/-}$ MEFs (44°C for 1 h); *hsf1*$^{-/-}$ MEFs (42°C or 43°C/1 h). Media was replaced with pre-heated media prior to transfer to heat shock incubator; when returned to 37°C the media was not exchanged.
Shelton *et al*., 2010	Apaf-1 deficiency protected from HS; loss of raidd, bid, caspase-8, and -2 did not protect cells from HS; BCL-2 and BCL-x_L overexpression protected Jurkat T cells from HS-induced cell death.	Jurkat T cells were incubated at 44°C for 1 h in a humidified incubator (5% CO_2); cell death was analyzed at 6 h after heat shock; caspase proteolytic processing was evaluated by western blot analysis; MOMP was evaluated by cyt. *c* release and $\Delta\psi m$ by flow cytometry with $DiIC_1(5)$.

Obs.: MEFs = mouse embryonic fibroblasts; TPA = tetradecanoylphorbol acetate.

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