



# **The regulation of ER stress-induced cell death by HSPB1 (HSP27)**

A thesis submitted to the National University of Ireland, Galway in fulfilment of the  
requirement for the degree of

Doctor of Philosophy

by

Donna Kennedy

Apoptosis Research Centre, Discipline of Biochemistry, School of Natural Sciences,  
National University of Ireland, Galway, Ireland

Thesis Supervisor: **Prof. Afshin Samali**

Co-supervisor: **Dr Karen Doyle**

November

2013

## Table of Contents

<b>Acknowledgments .....</b>	<b>vi</b>
<b>Abstract.....</b>	<b>vii</b>
<b>Abbreviations .....</b>	<b>vii</b>
<b>1. Chapter 1 .....</b>	<b>1</b>
<b>1. Introduction .....</b>	<b>1</b>
<b>1.1 Programmed cell death .....</b>	<b>2</b>
<b>1.2 Introduction to apoptosis.....</b>	<b>2</b>
<b>1.3 Classification of caspases .....</b>	<b>3</b>
<b>1.4 Activation of caspases .....</b>	<b>4</b>
<b>1.5 The extrinsic pathway.....</b>	<b>5</b>
<b>1.6 The intrinsic pathway .....</b>	<b>7</b>
<b>1.7 The BCL-2 family .....</b>	<b>7</b>
<b>1.8 BAX/BAK activation .....</b>	<b>11</b>
<b>1.9 Cellular Stress .....</b>	<b>14</b>
1.9.1 Proteostasis.....	14
<b>1.10 The ER and the unfolded protein response (UPR) .....</b>	<b>15</b>
1.10.1 Stress sensing by the UPR .....	17
1.10.2 Initiation phase .....	18
1.10.3 Adaptive phase of the UPR: Damage control .....	22
1.10.4 Adaptive phase of the UPR: Induction of transcription factors.....	24

1.10.5	Decision time.....	25
1.10.6	Execution phase of ER stress.....	25
<b>1.11</b>	<b>The heat shock response (HSR).....</b>	<b>29</b>
1.11.1	Stress sensing by the HSR .....	30
1.11.2	Mediators of the HSR.....	32
1.11.3	HSPA (HSP70) family .....	33
1.11.4	HSPH (HSP110).....	34
1.11.5	HSPC (HSP90) .....	34
1.11.6	DNAJ (HSP40) .....	34
<b>1.12</b>	<b>Small heat shock family- members – HSPB.....</b>	<b>35</b>
1.12.1	Functions of HSPB1 .....	36
<b>1.13</b>	<b>Aims and Rationale.....</b>	<b>41</b>
<b>2.</b>	<b>Chapter 2.....</b>	<b>56</b>
<b>2.</b>	<b>Materials and Methods .....</b>	<b>56</b>
<b>2.1</b>	<b>Cell lines and cell culture .....</b>	<b>57</b>
2.1.1	PC12 Cell Culture.....	57
2.1.2	HeLa cell culture.....	58
2.1.3	MEF cell culture.....	58
<b>2.2</b>	<b>Drug treatments .....</b>	<b>60</b>
2.2.2	Heat shock preconditioning.....	61
<b>2.3</b>	<b>Transformation of plasmids.....</b>	<b>61</b>
<b>2.4</b>	<b>Transfections.....</b>	<b>63</b>
2.4.1	DNA transfections .....	63

2.4.2	siRNA transfections .....	63
<b>2.5</b>	<b>Flow Cytometry .....</b>	<b>64</b>
2.5.1	Harvesting cells for flow cytometry .....	64
2.5.2	Sub-G1 analysis of DNA fragmentation .....	64
2.5.3	Annexin V staining.....	65
2.5.4	Measurement of $\Delta\Psi_m$ by TMRE staining .....	65
<b>2.6</b>	<b>Analysis of type II caspase activity .....</b>	<b>66</b>
<b>2.7</b>	<b>mRNA expression analysis .....</b>	<b>67</b>
2.7.1	RNA preparation .....	67
2.7.2	Reverse transcription (RT)-PCR.....	67
2.7.3	Real time Quantitative RT-PCR .....	68
<b>2.8</b>	<b><i>In vivo</i> Chaperone Assay .....</b>	<b>69</b>
<b>2.9</b>	<b>Luciferase reporter Assays.....</b>	<b>70</b>
<b>2.10</b>	<b>Cell Viability Assay .....</b>	<b>71</b>
<b>2.11</b>	<b>Protein expression analysis.....</b>	<b>71</b>
2.11.1	Western Blotting .....	71
2.11.2	Immunostaining .....	74
2.11.3	Assessment of Protein Half-Life .....	74
2.11.4	Assessment of oligomeric status following ER stress .....	74
<b>2.12</b>	<b>Statistical analysis .....</b>	<b>75</b>
<b>3.</b>	<b>Chapter 3.....</b>	<b>76</b>
<b>3.</b>	<b>Cross-talk between different cellular stress response pathways: heat shock preconditioning attenuates ER stress induced cell death.....</b>	<b>76</b>

<b>3.1</b>	<b>Introduction .....</b>	<b>77</b>
<b>3.2</b>	<b>Results .....</b>	<b>80</b>
3.2.1	TG caused induction of HSPs in HeLa cells.....	80
3.2.2	ER stress leads to induction of HSPs .....	81
3.2.3	HSF1 is a functional component of the UPR .....	85
3.2.4	ER stress alters the oligomeric state of HSPB1 .....	88
3.2.5	HSPB1 cells have increased chaperoning capacity in response to ER stress 91	
3.2.6	Induction of HSPs in HeLa and PC12 cell lines following thermal preconditioning.....	93
3.2.7	Heat shock preconditioning activates the UPR.....	95
3.2.8	Heat shock preconditioning activates an adaptive UPR response .....	96
3.2.9	Heat shock preconditioning protects against ER stress-induced apoptosis.....	97
3.2.10	Heat shock preconditioning attenuated caspase activation in HeLa cells 99	
3.2.11	Heat shock preconditioning reduces expression of pro- apoptotic BCL-2 family members .....	102
3.2.12	Heat shock preconditioning reduced ER stress-induced BIM expression 104	
3.2.13	Generation of cell lines overexpressing HSPs .....	106
3.2.14	Over expression of HSPB1 reduces ER stress-induced BIM. ....	107
<b>3.3</b>	<b>Discussion .....</b>	<b>109</b>
<b>4</b>	<b>Chapter 4.....</b>	<b>115</b>
<b>4.</b>	<b>Regulation of ER stress-induced apoptosis by HSPB1 .....</b>	<b>115</b>
<b>4.1</b>	<b>Introduction .....</b>	<b>116</b>

<b>4.2</b>	<b>Results</b> .....	<b>121</b>
4.2.1	HSPB1 over-expression increases cell viability in response to ER stress 121	
4.2.2	HSPB1 over-expression attenuates ER stress induced cell death.....	122
4.2.3	HSPB1 over-expression reduces ER stress induced apoptosis.....	123
4.2.4	HSPB1 mediated protection occurs upstream of the mitochondria .	124
4.2.5	HSPB1 reduces caspase activation following ER stress .....	126
4.2.6	BIM is involved in ER stress-induced apoptosis.....	128
4.2.7	Knock down of BIM attenuates ER stress induced apoptosis.....	129
4.2.8	HSPB1 mediated protection against ER stress is dependent on regulation of BIM .....	130
4.2.9	HSPB1 regulates BIM via a post transcriptional mechanism .....	131
4.2.10	Regulation of BIM by HSPB1 is not due to microRNA activity .....	133
4.2.11	HSPB1 over-expression alters stress kinase signalling.....	135
4.2.12	Phospho-ERK regulated BIM during ER stress .....	137
4.2.13	HSPB1 mediated protection against ER stress induced apoptosis is phospho-ERK dependent .....	139
4.2.14	HSPB1 over-expressing cells have increased levels of phospho-BIM	141
4.2.15	HSPB1 mediated regulation of BIM is partially due to regulation of phospho-ERK.....	142
4.2.16	HSPB1 decreases the protein half-life of BIM.....	144
4.2.17	HSPB1 regulates BIM via proteasomal degradation .....	146
<b>4.3</b>	<b>Discussion</b> .....	<b>148</b>
<b>5</b>	<b>Chapter 5</b> .....	<b>155</b>
	<b>Functional assessment of neuropathy associated HSPB1 mutants</b> .....	<b>155</b>
<b>5.1</b>	<b>Introduction</b> .....	<b>156</b>

<b>5.2</b>	<b>Results .....</b>	<b>158</b>
5.2.1	Generation of mutant HSPB1 over-expressing cell lines .....	158
5.2.2	Mutant HSPB1 expressing cells differentiate upon addition of NGF. ....	160
5.2.3	HSPB1 mutants are more sensitive to ER stress-induced apoptosis. ....	161
5.2.4	HSPB1 mutants fail to protect against loss of $\Delta\Psi_m$ following TG ....	162
	.....	164
5.2.5	HSPB1 mutants have increased caspase-3 cleavage following TG ....	165
5.2.6	HSPB1 mutants have an altered UPR response .....	166
5.2.7	HSPB1 mutants have higher levels of BIM than WT HSPB1.....	168
<b>5.3</b>	<b>Discussion .....</b>	<b>170</b>
<b>6.</b>	<b>Chapter 6.....</b>	<b>175</b>
6.1	Discussion .....	175
<b>7.</b>	<b>Chapter 7.....</b>	<b>190</b>
7.1	References .....	190

## Acknowledgments

The experimental work is completed, the thesis is written and now the time has come for me to thank all the people who have helped me get this far.

I would first and foremost like to thank my supervisor Professor Afshin Samali for his support, guidance and patience. It has been a pleasure working with you. I am extremely grateful to you for giving me the opportunity to work in your lab and for making me into a biochemist!

I would also like to thank Dr Karen Doyle for her help with my thesis.

Many thanks to Professor Vincent Timmerman and Professor Peter Vandenabeele for the opportunity to work in VIB Antwerp and VIB Ghent. It was a valuable experience.

I know in years to come I will remember my time as a PhD student fondly and that is mainly due to the amazing friends I have made over the last four years. Your support both emotionally and scientifically has been invaluable. In particular I especially want to acknowledge the 'winesday crew'. You celebrated the good results with me and help me problem solve the rest. I hope in our future careers we will have the opportunity to work together again.

To my besties Louise, Emma, Tara and Ciara. Not one of you has a clue what I actually do and ye are scattered across the world but ye have supported me nevertheless. Finally I wish to thank my parents, Carmel and Raymond for encouraging my academic pursuits and supporting me in my endeavours. I love guys.

What doesn't kill you makes you stronger, in life and cell biology 😊



## Abstract

Endoplasmic reticulum (ER) stress is associated with several human pathologies including neurodegenerative disorders. ER stress results in accumulation of unfolded proteins which activates the unfolded protein response (UPR). Heat shock proteins (HSP) are stress-inducible cyto-protective proteins which mediate the heat shock response (HSR). We investigated whether heat shock (HS) preconditioning can block ER stress-induced apoptosis. Treatment of cells with ER stress-inducing drugs caused cell death. Heat shock preconditioning prior to ER stress treatment significantly reduced cell death and reduced levels of the pro-apoptotic protein, BIM. Overexpression of HSPB1 could mimic the effect of HS on expression levels of BIM. We conclude that HS is protective against ER stress-induced cell death, partly through HSPB1-mediated reduction BIM.

HSPB1 acts as a suppressor of cell death however its role in ER stress-induced apoptosis has not been elucidated. Using PC12s overexpressing HSPB1 we investigated the molecular mechanism by which HSPB1 protects against ER stress-induced apoptosis and regulates the expression of BIM. Overexpression of HSPB1 reduced ER stress-induced apoptosis. Examination of signalling pathways critical to the regulation of BIM identified HSPB1 as a regulator of the proteasomal degradation of BIM. This was in part due to HSPB1 enhancing phosphor-ERK levels. Moreover knockdown of BIM effectively inhibited the protective effects of HSPB1 highlighting the importance of HPSB1 mediated regulation of BIM in protection against ER stress.

Mutations in HSPB1 are associated with Charcot Marie Tooth (CMT) disease, a peripheral neuropathy. We found that cells which overexpress mutant HSPB1 are more sensitive to ER stress-induced apoptosis and have high levels of BIM.

Collectively we have identified a novel function for HSPB1 as a regulator of ER stress-induced apoptosis, a function which is lost in HSPB1 CMT associated mutations.

**Abbreviations**

ADP	Adenosine diphosphate
ACD	Alpha-crystallin domain
AIF	Apoptosis-inducing factor
ALLN	N-[N-(N-Acetyl-L-leucyl)-L-leucyl]-L-norleucine
APAF-1	Apoptotic peptidase activating factor 1
ARE	Antioxidant response element
ASK1	Apoptosis signal-regulation kinase 1
ATF3	Activating transcription factor 3
ATF4	Activated transcription factor 4
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
BAD	BCL-2-antagonist of cell death
BAK	BCL-2 homologous antagonist/killer
BAX	BCL-2 associated protein X
BCA	Bicinchoninic acid
BCL-2	B-cell lymphoma 2
BCL-xL	B-cell lymphoma 2- extra large
BH	BCL2-homology
BID	BH3-interacting domain death agonist
BIM	BCL-2-interacting mediator of cell death
BOK	BCL-2-related ovarian killer
BOP	BH3 only protein
BSA	Bovine serum albumin
Bzip	basic-leucine zipper
Ca <sup>+2</sup>	Calcium

CaCl <sub>2</sub>	Calcium Chloride
CAD	Caspase-activated DNase
CARD	Caspase recruitment domain
Caspases	Cysteine-dependent aspartate-directed proteases
cDNA	complimentary DNA
c-FLIP	Cellular FLICE inhibitory protein
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHOP	C/EBP-homologous protein
ciAP	Cellular inhibitors of apoptosis
CO	Carbon monoxide
CSR	Cell stress responses
dATP	Deoxyadenosine triphosphate
DAPI	4',6-diamidino-2-phenylindole
DD	Death domain
DED	Death effector domain
DEPC	Diethyl pyrocarbonate
DEVDase	N-Acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin
DISC	Death inducing signalling complex
DNA	Deoxyribonucleic acid
dNTPS	Deoxynucleotide triphosphates
DMEM	Dulbecco's Modified Eagle Medium
DTT	Dithiothreitol
E.coli	Escherichia coli
EDEM1	ER degradation enhancer, mannosidase alpha-like 1
EDTA	Ethylenediaminetetraacetic acid

eIF2 $\alpha$	Eukaryotic translation initiation factor 2 $\alpha$
ER	Endoplasmic Reticulum
ERAD	ER-associated degradation
ERdj4/ HSP40	Heat shock protein 40
ERK	Extracellular signal-regulated kinase
ERO-1	ER oxidoreductin 1
ERP72	Endoplasmic reticulum resident protein 72
ERSE I/ II	ER Stress response Element I/ II
FBS	Foetal Bovine Serum
FITC	Fluorescein isothiocyanate
<i>g</i>	Gravitational acceleration
g	Grams
GADD34	Growth Arrest/DNA Damage Inducible 34
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLS	Golgi localization signals
h	Hours
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HS	Heat shock
HSE	Heat shock element
HRI	Heme-regulated eIF2 $\alpha$
HSF1	Heat shock factor 1
HSF2	Heat shock factor 2
HSPs	Heat shock proteins
HSPA5/GRP78	Heat shock protein A5

HSPA1	Heat shock protein 72
HSPB1	Heat shock protein 27
HSR	Heat shock response
ICAD	Inhibitor of caspase-activated DNase
IDP	Intrinsically disordered proteins
IKK	Inhibitor of NFkB kinase
IRE1	Inositol requiring enzyme-1
JNK	c-JUN terminal kinase
KEAP1	Kelch-like ECH-associated protein
LB broth	Luria-Bertani broth
M	Molar
MAMs	Mitochondria-associated ER membranes
MCL-1	Myeloid cell leukemia sequence 1
MEF	Mouse embryonic fibroblast
miRNA	microRNA
mL	millilitre(s)
$\mu$ L	microlitre(s)
$\mu$ M	micromole(s)
MOMP	Mitochondrial outer membrane permeabilization
MOPs	3-(N-morpholino)propanesulfonic acid
MPP <sup>+</sup>	1-methyl-4-phenylpyridinium
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazonium bromide
NaCl	Sodium Chloride
NaF	Sodium Flouride
Na <sub>3</sub> VO <sub>4</sub>	Sodium orthovanadate

NaDeox	Sodium Deoxycholate
NF-Y	Nuclear transcription factor Y
nM	nanomole(s)
NP-40	nonyl phenoxyethoxyethanol
NRF2	Nuclear factor (erythroid-derived 2)-like 2
p58(IPK)	p58 inhibitor of protein kinase
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate Buffered saline
PCR	Polymerase chain reaction
PDI	Protein disulphide isomerases
PERK	Double-stranded RNA-activated protein kinase (PKR)-like kinase
PKR	Protein kinase R
PLL	Poly-L-lysine
PMSF	Phenylmethanesulfonyl fluoride
PN	Proteostasis network
PI	Propidium Iodide
PUMA	p53 up-regulated modulator of apoptosis
PS	Phosphatidylserine
RIPA Buffer	Radio-Immunoprecipitation Assay Buffer
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RPM	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
ROS	Reactive oxygen species

SDS	Sodium dodecyl sulfate
S1	Site-1 protease
S2	Site-2 protease
Sec	Second(s)
SERCA	Sarco/Endoplasmic Reticulum Ca <sup>2+</sup> -ATPase
siRNA	small interfering RNA
Smac/DIABLO	Second mitochondria-derived activator of caspases (Smac)/direct IAP binding protein with low Pi
SV40	Simian vacuolating virus 40
TG	Thapsigargin
TM	Tunicamycin
TNF	Tumour necrosis factor
TMRE	Tetramethylrhodamine, ethyl ester
TRAF2	TNF-receptor associated factor 2
TP	Thermal Preconditioning
UPR	Unfolded protein response
UPRE	Unfolded Protein Response Element
UT	Untreated
UTR	Untranslated region
WT	Wild type
XBP1	X-box binding protein
XBP1s	X-box binding protein spliced
XIAP	X-linked inhibitor of apoptosis protein
$\Delta\psi_m$	Mitochondrial Membrane Potential

## **Chapter 1**

### **Introduction**



## 1.1 Programmed cell death

The concept of a regulated form of cell death was first proposed in 1965 (Lockshin, 1964). In the past five decades the associated research field has grown considerably and there are now 13 acknowledged biochemically-diverse forms of programmed cell death including anoikis, apoptosis, autophagic death, cornification, entosis, mitotic catastrophe, necroptosis, parthanatos, pyroptosis and ferroptosis (Dixon et al., 2012, Galluzzi et al., 2012). The different modalities can be difficult to distinguish and in addition it is proposed that in certain cases, a continuum of cell death processes occurs simultaneously (Galluzzi et al., 2009).

## 1.2 Introduction to apoptosis

Apoptosis is necessary for embryonic development and morphogenesis which is exemplified by the developmental defects in mice that lack key components of the apoptotic pathway (Degterev A, 2003). In the adult organism, apoptosis enables homeostatic control of cell death and removal of damaged, aged cells or superfluous cells in a manner that escapes unwanted activation of the immune system (Taylor et al., 2008). Dysregulation of apoptosis is associated with a number of pathological processes; resistance to apoptosis can cause autoimmune disease, dysregulated wound healing, and cancer (Wang and Kaufman, 2012). Excessive apoptosis is linked with inflammatory diseases and neurodegenerative diseases such as Alzheimer's disease, Huntington's disease, Amyotrophic Lateral Sclerosis (ALS) and Parkinson's disease (Doyle et al., 2011). Given its over-arching roles in human health, deciphering the precise mechanism of apoptosis is paramount to the design of novel therapeutic targets.

Apoptosis is a tightly regulated, genetically encoded form of programmed cell death. Morphologically, apoptosis is characterized by condensation of chromatin

(pyknosis), cytoplasmic shrinkage, membrane blebbing, nuclear fragmentation (karyorrhexis) and formation of apoptotic bodies (Elmore, 2007, Galluzzi et al., 2012). The biochemical features of apoptosis include mitochondrial outer membrane permeabilization (MOMP), loss of mitochondrial membrane potential ( $\Delta\psi_m$ ), release of intra-mitochondrial space proteins, respiratory chain inhibition, caspase activation, DNA fragmentation and plasma membrane alterations which cause the cells to be recognised by phagocytes and ultimately engulfed. Studies in *C. elegans* provided the first insight into the genetic components of programmed cell death (Yuan et al., 1993). In this organism, 131 of 1090 cells undergo PCD by a process that is genetically mediated by CED-3 CED-4, CED-9 and EGL1 (Lettre and Hengartner, 2006). Apoptosis is highly conserved; however, higher eukaryotes have more sophisticated and fine-tuned regulatory mechanisms.

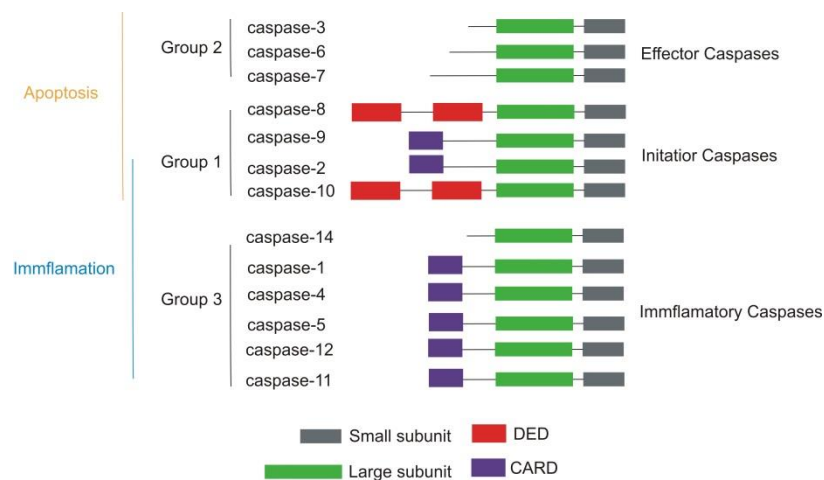
### 1.3 Classification of caspases

Caspases (cysteine dependent aspartate specific proteases) are a family of 14 proteases which are the orthologues of CED-3. The enzymatic properties of caspases are governed by a dominant specificity for protein substrates containing Asp and by the use of a Cys side chain in the caspase active site that is necessary for catalyzing peptide bond cleavage.

Caspases can be loosely classified based on their main role in apoptosis (caspases - 3, -6, -7, -8, -9) or primary role in inflammation (caspases -1, -4, -5, -11(murine) and -12). It is difficult to classify caspase-2, caspase-10 and caspase-14 using this system as they have clear roles in both cellular processes. This dualistic (apoptotic versus inflammatory) approach to caspase classification is overly simplistic as most apoptotic caspases have at least one additional non-apoptotic function. For example, caspase-3 is necessary for diverse functions such as erythroid maturation, thrombopoiesis and osteoblast differentiation (Galluzzi et al., 2008). Similarly the

'inflammatory' caspase, caspase-14, has a role in protection against UVB radiation (Denecker et al., 2008). Inflammatory caspases are also capable of inducing a form of cell death termed pyroptosis which is associated with massive activation of inflammatory cells (Strowig et al., 2012).

Within the apoptotic group of caspases, the terms apical/initiator pertains to caspases -2, -8, -9 and -10 which initiate the apoptotic cascade while effector/executioner caspases (caspases -3, -6, -7) describe caspases that carry out execution of the cell (Lettre and Hengartner, 2006). Structurally, initiator and executioner caspases are differentiated by their pro-domain length with the former possessing a long pro-domain while the latter contains a short pro-domain (Kumar, 2006). The pro-domains of initiator caspases contain homotypic protein interaction motifs which facilitate protein-protein interactions, such as a caspase activation and recruitment domain (CARD) in caspase-9 and caspase-2 and Death Effector Domains (DED) domains in the case of caspase-8 and caspase-10 (See Fig 1.1 for details).



**Fig.1.1 Classification of caspases.** Caspases can be functionally subdivided into Effector caspases, Initiator caspases and inflammatory caspases. Effector caspases have a short pro-domain while initiator have a long pro-domain. Death Effector domains (DED) are depicted in red. Caspase activation and recruitment domains (CARD) are depicted in purple. Small subunits are in grey and large subunits are in green.

## 1.4 Activation of caspases

All caspases are synthesized as inactive zymogens consisting of a pro-domain long or short, one large domain (~20 kDa) and one small domain (~10 kDa). Activation increases the catalytic activity of caspases by several orders of magnitude (Earnshaw et al., 1999). Initiator caspases are present in the cell as monomers with dimerization resulting in inter-chain autocatalytic cleavage and activation. Within the cell, caspase dimerization is facilitated by protein platforms or activating complexes, which provide a focal point to concentrate caspases, leading to proximity-induced activation and autocleavage (Shi, 2004). In the case of caspase-9 this complex is termed the apoptosome. The caspase 8/10 activating complex is called the Death Inducing Signalling Complex (DISC) (Sessler et al., 2012). Inflammatory caspases also require an activating complex namely, the PIDDosome in the case of caspase-2, and the inflammasome in the case of caspases -1, -4 and -5.

The repertoire of initiator caspase substrates is limited to the executioner caspases and BH3 interacting-domain death agonist (BID) in the case of caspase-8. Executioner caspases are present as dimers in non-apoptotic cells. Proteolytic cleavage by initiator caspases between the long and short subunits leads to reorganization of the molecule favouring formation of an active catalytic site (McIlwain et al., 2013). This sequential model of activation serves to prevent inappropriate activation of executioner caspases. In contrast to the initiator caspases, executioner caspases have a wide range of targets enabling the characteristic demolition of the cell that occurs during apoptosis (Shi, 2004, Taylor et al., 2008, Pop and Salvesen, 2009).

## 1.5 The extrinsic pathway

Apoptosis is classified as an intrinsic or extrinsic process wherein the former is activated by cell stressors emanating from within the cell, while the latter is

activated by extracellular stressors. The intrinsic pathway activates caspases via the apoptosome, while the extrinsic pathway utilizes the DISC to induce caspase activity (Mace and Riedl, 2010). Ultimately both pathways converge at the level of activation of executioner caspases (Tait and Green, 2010). Activation of the extrinsic apoptosis pathway is initiated at cell surface death receptors which are members of the tumour necrosis factor (TNF) receptor gene family. Death receptors are characterized by the presence of an 80 amino acid cytoplasmic sequence which encodes a death domain (DD) (McIlwain et al., 2013). Mammals express 8 DD-containing receptors namely Tumor Necrosis factor Receptor 1 (TNFR1), Fas Receptor (FasR), Death Receptor 3 (DR3), Death Receptor 4 (DR4), Death Receptor 5 (DR5), Death Receptor 6 (DR6) and p75 neurotrophin receptor (p75<sup>NTR</sup>). Death receptors are activated cognate ligands of the Tumor Necrosis Factor (TNF) family of cytokines or of the neurotrophins including Nerve Growth Factor (NGF), Brain Derived Neurotrophic Factor (BDNF), Neurotrophin 3 (NT-3) and Neurotrophin 4 (NT-4) in the case of p75<sup>NTR</sup> (Sessler et al., 2012). The DD of the receptor is crucial for transmitting the death signal into the cell. Ligands bind to death receptors via the DD, which leads to receptor trimerization and activation (Li et al., 2013). This stimulates recruitment of adaptor proteins thereby providing a platform that enables binding of signal transduction proteins (Galluzzi et al., 2012). The outcome for the cell depends on which adaptor proteins bind and culminates in either apoptosis via activation of caspase-8 and or caspase-10, or alternatively adaptive responses are mediated by NF- $\kappa$ B and MAPK (Guicciardi and Gores, 2009). Recruitment of Fas Associated Death Domain (FADD) leads to formation of the Death Inducing Signalling Complex (DISC), a caspase-8 activating complex. Activated caspase-8 proteolytically processes and activates executioner caspases -3 and-7 (Sessler et al., 2012). Cells in which sufficient levels of caspase -8 are activated to lead to activation of executioner caspases are termed type one cells. In some cell types, termed type II cell activation of caspase-8 is insufficient to cause direct activation of caspase-3. In this instance the intrinsic apoptotic pathway is engaged via caspase-8 mediated cleavage of BID to tBID. This acts to amplify the apoptotic

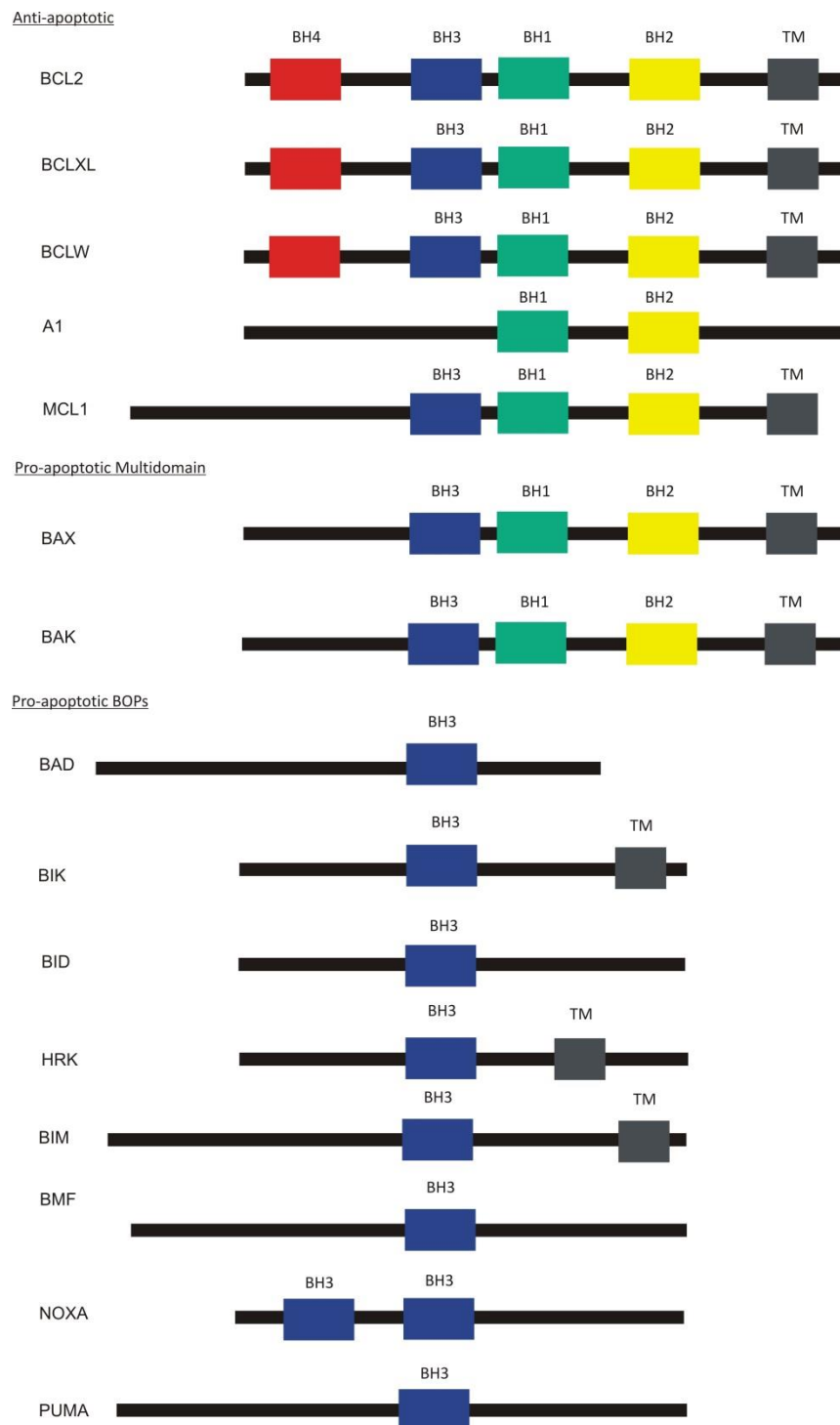
signal (Ozoren and El-Deiry, 2002). Such activation occurs in such as hepatocytes and pancreatic  $\beta$  cells.

## 1.6 The intrinsic pathway

The intrinsic apoptosis pathway, or mitochondrial pathway is activated by withdrawal of pro-survival factors or an increase in pro-apoptotic factors. This leads to mitochondrial outer membrane permeabilization (MOMP) and release of apoptogenic factors from the mitochondrial inter membrane space and the matrix such as cytochrome *c*, Second mitochondrial-derived activator of caspases (SMAC), also known as Direct IAP binding protein with low pI (Diablo), and Omi stress regulated endoprotease/ high temperature requirement protein A2 (HTRA2) (Cecconi et al., 2010). This is a crucial event for driving initiator caspase activation and subsequent apoptosis. SMAC and HTRA2 negate the caspase inhibitory effect of XIAPs (x-linked inhibitors of apoptosis) (Tait and Green, 2010). Cytochrome *c*, in conjunction with Apoptotic protease activating factor 1 (Apaf-1), pro-caspase-9, and the cofactor dATP/ATP, form the apoptosome (Olson and Kornbluth, 2001, Szegezdi et al., 2009). Apaf-1, which is the CED-4 homologue, is the core component of the apoptosome. Apaf-1's CARD domain facilitates interaction with capsase-9 while the WD-40 repeats mediate interaction with cytochrome *c* and a nucleotide binding domain enables interaction with ATP. The ultrastructure of the apoptosome has been resolved revealing a wheel shaped complex in which the CARD and nucleotide binding domains form the centre of the wheel with the WD-40 repeats forming the spokes (Acehan et al., 2002). Specifically, the apoptosome increases the catalytic activity of caspase-9 resulting in activation of downstream effector caspases including caspases-3, -7 and -6.

## 1.7 The BCL-2 family

Death signalling events at the mitochondria and subsequent release of apoptogenic factors are arbitrated by a family of proteins called the BCL-2 family. In mammals there are 15 core BCL-2 proteins with varying degrees of structural similarity in short amino acid motifs called BH (BCL-2 homology) domains designated BH1-4. Family members are classified as pro-apoptotic and include the multi domain members BAX and BAX (containing BH1, 2, and 3) and the BH3 only proteins (BOP) BAD, BIM, BIK, BID, PUMA, BMF, HRK and NOXA whose sequence homology to the other family members is restricted to the BH3 domain. BIM, BAD and BMF are intrinsically disordered proteins. Anti-apoptotic BCL-2 (aaBCL-2) members, BCL-X<sub>L</sub> and MCL-1, BCL-w, BCL-2A1 and BCL-B generally have all 4 BH domains with the exception of MCL1. aaBCL-2 proteins are the orthologous of CED-9 and BOPs are orthologues of EGL-1. Many of the BCL2 proteins including BCL-2, BCL-X<sub>L</sub> and MCL-1, BCL-w, BCL-B, BAX, BAK, BOK, HRK and BIK also express a transmembrane domain (See Fig. 1.2). The dynamic interplay between these proteins regulates MOMP. MOMP is necessary for caspase activation and apoptosis in response to most apoptotic stimuli in vertebrates. MOMP is considered a critical step in the apoptotic cascade and a 'point of no return' for cells, as cells will eventually die even in the absence of caspase activity (Tait and Green, 2010).



**Fig. 1.2 The BCL2 family.** The BCL2 family is subdivided into anti-apoptotic members, multi-domain apoptotic members and BH3 only proteins. The transmembrane domain (TM) is depicted in grey, the BH2 domain is depicted in yellow, BH3 is depicted in blue and BH4 is depicted in red.



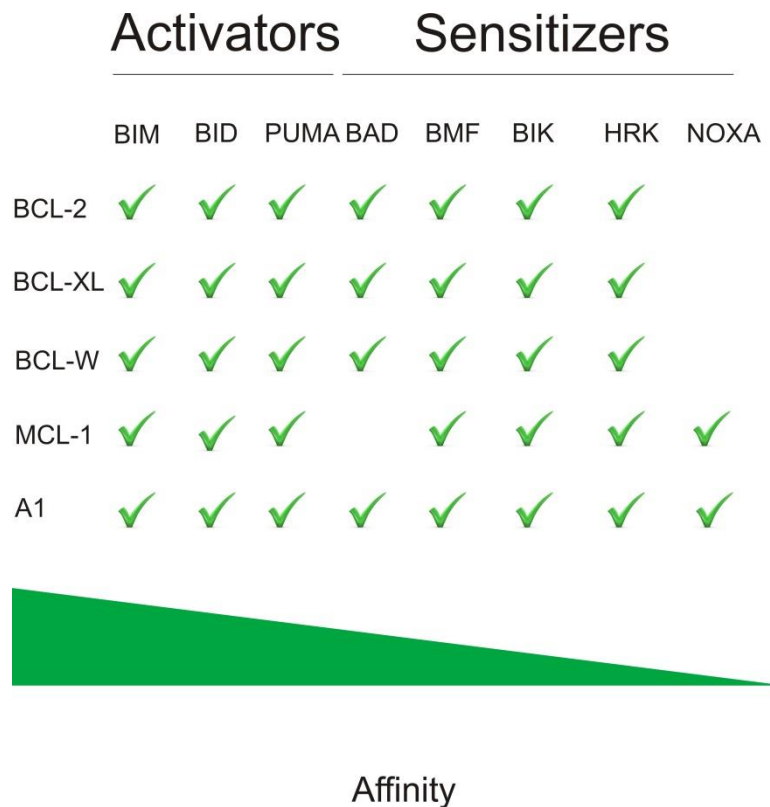
BCL-2 proteins exist in an inactive conformation in viable cells often in the form of heterodimers of anti-apoptotic and pro-apoptotic proteins. Depending on the specific death signals and the particular cell type, transcriptional and post-transcriptional modifications alter the dynamic balance between anti-apoptotic and pro-apoptotic members leading to MOMP (Bouillet and Strasser, 2002). BAX and BAK are the direct mediators of MOMP via formation of a pore in the mitochondrial membrane which leads to activation of the apoptosome. The requirement of BAX and BAK for formation of MOMP is demonstrated by a lack of MOMP in BAX<sup>-/-</sup> BAK<sup>-/-</sup> cells (Wei et al., 2001). While it is clear that BAX and BAK are necessary for MOMP the precise mechanism by which they are activated is unclear. One of the key questions is how are IMS components of the mitochondria are released. Potential mechanisms include a BAX/BAK forming channel called mitochondrial-apoptosis inducing channel (MAC), modulation of existing mitochondrial channels such as the mitochondrial permeability transition pore complex via BAX/BAK (PTPC) or modulation of voltage gated anion channels by BAX/BAK (Tait and Green, 2010).

BAX/BAK activation and therefore MOMP, is regulated by the aaBCL2 proteins and BOPs. Anti-apoptotic proteins inhibit MOMP, whereas members of the BH3-only subfamily induce MOMP (Youle and Strasser, 2008). In unstressed cells BAX is a soluble cytosolic protein due to its transmembrane domain being buried within the globular structure of the protein. A small fraction of the total BAX pool is loosely attached to the mitochondria and ER (Zhong et al., 2003) Cell stress leads to dramatic changes in the cellular location and structural conformation of BAX. Specifically, cell stress leads to interaction of BAX with activator BOPs BIM, PUMA or tBID which results a conformational alteration leading to exposure of an N-terminal mitochondrial targeting epitope, which is normally buried in inactive BAX (Gavathiotis et al., 2010). The structural alteration is also thought to facilitate insertion of BAX into the mitochondria. Unlike BAX, BAK is constitutively expressed

at the mitochondria where it is bound to VDAC2, MCL1 and BCL-XL, with a small proportion residing at the ER (Zong et al., 2003).

## 1.8 BAX/BAK activation

The precise mode of BAX and BAK activation is still not fully elucidated and is a contentious issue in the apoptosis field (Bouillet and Strasser, 2002). A major issue of debate in the field is whether BH3-only proteins bind directly to BAX and BAK (termed the direct activation model) or if they act by displacing anti-apoptotic proteins from BAX and BAK thereby neutralizing their inhibitory effect on BAK and BAX (termed the indirect activation or displacement model (Leber et al., 2007) . The direct activation model classifies BH3-only proteins as activators or sensitizers. In this model, BIM, tBID and PUMA are classed as activators as they have the ability to bind directly to BAX and BAK causing their activation (Marani et al., 2002, Zhang et al., 2009, Shamas-Din et al., 2013, Vela et al., 2013). These proteins can also potentially bind all five anti-apoptotic BCL-2 proteins, as proposed in the indirect activation model. The second class of BH3- only proteins, which includes BAD, BMF, BIK, HRK, and NOXA, act as sensitizers that cause the release of BH3 only proteins. BOPs are bound to anti-apoptotic proteins for which they have high affinity (see figure 1.3 for binding affinity (Leber et al., 2007, Ku et al., 2011).



**Fig. 1.3 Interactions between members of the BCL2 family.** BIM, BID, and PUMA acts as direct activators while BAD, BMF, BIK, HRK and NOXA act as sensitizers. Some BOPs can interact with all 5 aaBLC2 proteins while some such as BAD and NOXA display specificity. Direct activators such as BIM, BID and PUMA directly bind to BAX/BAK leading to activation. Sensitizers such as BAD, BMF, HRK, BIK and NOXA promote BAX/BAK activation by sequestering anti-apoptotic proteins away from BAX/BAK.

The displacement model was proposed due initial lack of evidence showing direct interaction between BOPs and BAX/BAK. This model proposes that BAX and BAK are constitutively active and their activity is suppressed by binding of anti-apoptotic BCL-2 proteins. It is proposed that BH3-only proteins, once activated, displace the anti-apoptotic BCL-2 proteins leading to activation of BAX and BAK. Therefore the displacement model dictates that anti-apoptotic proteins negate the action of proapoptotic proteins by direct interaction (Chipuk et al., 2010). It is difficult to resolve how membrane bound BCL-2 proteins could interact with cytosolic BAX. However It has been proposed that once at the mitochondria, BCL-2 proteins may act as

dominant negative regulators of BAX by competing for membrane insertion (Renault et al., 2013). One caveat of these models is that they assign a passive role for the membrane in activation of BAX and BAX. In fact BH3-only proteins only recruit and activate anti-apoptotic proteins by changing their conformation at the membrane. Leber et al (2007) proposed the 'embedded together model' in which it is suggested that BCL-2 proteins insert into the membrane where they change conformation leading to alterations in function (Leber et al., 2007). Recruitment of cytoplasmic anti-apoptotic BCL-2 to the membrane by all pro-apoptotic BCL-2 proteins has been proposed where the anti-apoptotic proteins inhibit activator BH3-only proteins. Sensitizer BH3-only proteins could displace anti-apoptotic BCL-2 proteins from BH3-only proteins and BAX and BAK leading to activation of BAX/BAK (Shamas-Din et al., 2010). BAX and BAK form homo-oligomers during apoptosis, a phenomenon that is necessary for apoptosis. Oligomerization is facilitated by structural alterations which occur during activation (Westphal et al., 2011).

## 1.9 Cellular Stress

Organisms commonly encounter a wide variety of environmental insults. These insults have deleterious effects on critical bio-molecules such as proteins, lipids and DNA resulting in modifications that inhibit optimal functioning. To maintain cellular homeostasis certain programs, termed cellular stress response (CSR), are initiated (Kültz, 2003). CSRs include the heat-shock response (HSR), the Endoplasmic Reticulum Unfolded Protein Response (UPR<sup>ER</sup>), the Mitochondrial Unfolded Protein Response (UPR<sup>MT</sup>) and autophagy (Fulda et al., 2010), to name but a few (Fulda et al., 2010). A cell's overriding response to cell stress is to initiate protective mechanisms to restore homeostasis. However in certain situations this is an insurmountable task and cell death ensues.

### 1.9.1 Proteostasis

Cell stress responses are considered cardinal components of the proteostasis network (PN), the guardian of protein homeostasis. The PN network is responsible for maintenance of proteostasis throughout the life of a protein, encompassing processes such as transcription, translation, protein localization, folding and degradation (Cohen and Dillin, 2008). This requires a complex interconnected network of molecular chaperones and co-factors, protein degradation systems and signalling pathways all of which which act in concert to protect the proteome. Organisms have a proteome in excess of 10,000 different protein species, which is dictated largely by the genome. Proteins are synthesized as linear chains of amino acids whose primary structure must be folded to yield the final functionally active protein. Protein folding occurs in crowded cellular environments subjecting this process to numerous opportunities for error (Hartl et al., 2011). Molecular chaperones help prevent inappropriate interactions within and between non-native

polypeptides, enhance the efficiency of *de novo* protein folding and promote the refolding of proteins that have become mis-folded as a result of cellular stress (Muchowski and Wacker, 2005). The dynamic nature of proteins dictates a constant need for molecular chaperones to prevent abnormal interactions which could have potentially toxic effects for the cell. A cohesive understanding of how cells regulate proteome stability is lacking and remains a fundamental and urgent question (Hutt et al., 2009).

The importance of the quality control mechanism mediated by the PN is demonstrated by the many examples of disease resulting from loss of proteostasis (Kultz, 2005, Fulda, 2010). Neurodegenerative diseases are characterized by accumulation of mis-folded and/or aggregated protein and are commonly late-onset illnesses. Mis-folded proteins are detrimental to the cell due to the toxic effects of the mutated proteins and through alteration of other protein functions through unwanted interactions. It has been suggested that the decline in the function of CSRs and proteostasis is a contributing factor to age-associated neurodegenerative diseases (Ben-Zvi et al., 2009, Douglas and Dillin, 2010). Indeed the dogma of ageing as a passive deterioration of cell viability is being challenged by studies showing a clear genetic involvement in the ageing process (Blanka, 2011). In particular CSRs have been implicated in the ageing process, and manipulation of CSRs can extend lifespan and improve responses to cell stress (Taylor and Dillin, 2013). Loss of stress responses is associated with induction of apoptosis under stress conditions (Sreedhar et al., 1999)

### 1.10 The ER and the unfolded protein response (UPR)

The UPR is a signalling pathway emanating from the endoplasmic reticulum (ER), a double membrane bound organelle consisting of a network of sacs and tubules called cisternae. Physiologically the ER has a number of functions with particular

emphasis on protein quality control. It is the site of synthesis of membrane and secretory proteins (Palade, 1975) with approximately 30% of all cellular proteins entering the ER for posttranslational modification (Truettner et al., 2009). The ER takes part in the folding of proteins into their native conformation and in post-translational modification of proteins, including N-linked protein glycosylation and disulphide bridge formation. It is responsible for the transport of newly synthesized proteins to the cis-golgi apparatus where further post-translational modifications occur (Teasdale and Jackson, 1996, Ellgaard and Helenius, 2003). Finally it acts as a quality control centre for proteins, targeting mis-folded proteins for degradation in a process termed ER-associated protein degradation (ERAD)(Jentsch, 1993). ERAD will be discussed later in the text. ERAD, along with autophagy, are responsible for the removal of mis-folded or unassembled proteins from the protein folding machinery and targeting them for degradation (Ravikumar et al., 2002, Meusser et al., 2005). The lumen of the ER is an oxidizing environment, which is required for proper protein folding and formation of disulphide bonds (Frand et al., 2000). In addition, the ER is a major internal calcium store (Schröder, 2008). Sarco/Endoplasmic Reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA) pumps are active transporters of  $\text{Ca}^{2+}$  working against a concentration gradient, pumping  $\text{Ca}^{2+}$  into the ER and resulting in  $\text{Ca}^{2+}$  levels in the lower millimolar range. The high concentration of  $\text{Ca}^{2+}$  within the ER is required for the activity of  $\text{Ca}^{2+}$ -dependent molecular chaperones and foldases that aid in protein folding e.g calreticulin and calnexin (Nigam et al., 1994).

In recent years our understanding of the ER and its functions has greatly expanded. Where we once thought of the ER as an independent organelle, it is now apparent that the labyrinth of ER encompasses important functional contact points with other cytoplasmic organelles, including the mitochondria, Golgi, nuclear envelope, lysosomes, endosomes, peroxisomes and plasma membrane (Rowland and Voeltz, 2012). The cells numerous CSR pathways mentioned in 1.9 acts in concert to restore cellular homeostasis. The UPR co-operates with various stress pathways including autophagy to regulate cell fate (Deegan et al., 2013).

Cellular conditions such as nutrient deprivation, alterations in glycosylation, disruption of calcium homeostasis or protein mutations that produce aberrant proteins, impede ER proteostasis leading to a state of ER stress (Gorman et al., 2012b). The cells initial and rapid response to ER stress involves the activation of a set of pro-survival signalling pathways termed the Unfolded Protein Response (UPR). This coordinated biochemical response to ER stress allows cells to deal with ER stress – however, if the stress is prolonged or excessive, apoptosis ensues (Doyle et al., 2011). Numerous pathological conditions are associated with ER stress including cancer and neurodegenerative diseases (Healy et al., 2009, Doyle et al., 2011).

#### **1.10.1 Stress sensing by the UPR**

In mammals, the three major ER stress sensors are IRE1 (inositol requiring 1; ERN1, endoplasmic reticulum-to-nucleus signalling 1), PERK [double-stranded RNA-activated protein kinase (PKR)-like ER kinase; PEK, pancreatic eukaryotic initiation factor 2 $\alpha$  kinase; EIF2AK3] and ATF6 (activating transcription factor 6) (Ron and Walter, 2007). IRE1 and PERK are type I transmembrane proteins with protein kinase activity, whereas ATF6 is a type II transmembrane protein encoding a transcription factor (Schroder and Kaufman, 2005). The ER-luminal domain of PERK, IRE1 and ATF6 interacts with the ER chaperone and HSPA1 homologue, HSPA5 (glucose-regulated protein 78). However, upon accumulation of unfolded proteins, HSPA5 dissociates from these molecules, leading to their activation (Schroder and Kaufman, 2005). Activation of PERK, IRE1, and ATF6 initiates a network of intracellular signalling pathways during the UPR (Doyle et al., 2011, Gorman et al., 2012a).

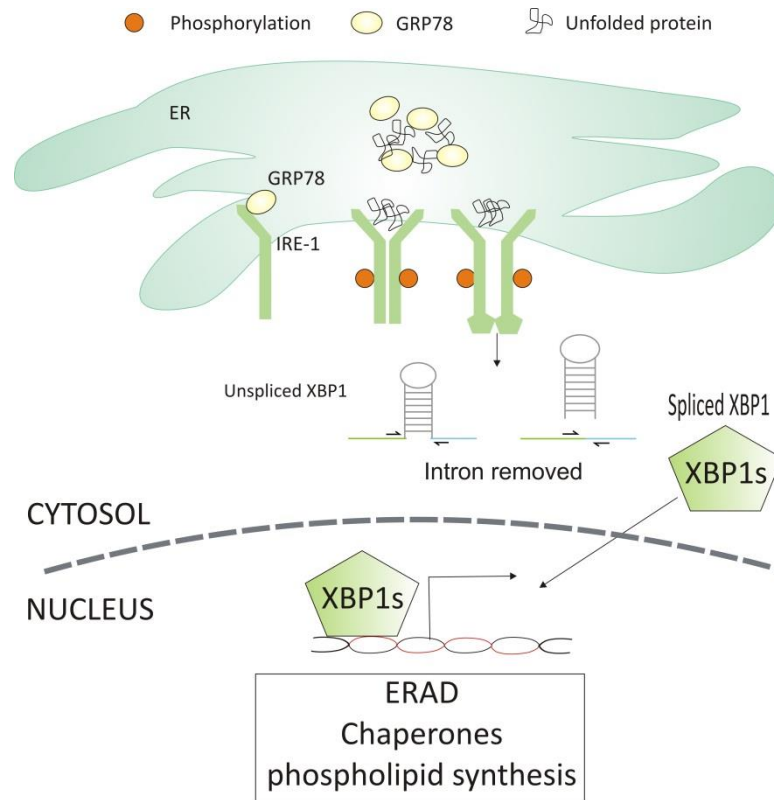
The ER's response to cell stress can be divided into three stages. Firstly the stress inducing stimuli are sensed by the UPR pathway (Initializing phase). The second stage sees protective mechanisms initiated in response to the stress (Adaptive



phase), and finally, if the stress is unresolved cell death occurs (Execution phase) (Szegezdi et al., 2006).

### 1.10.2 Initiation phase

IRE1 exists in two highly conserved isoforms, IRE1 $\alpha$  and IRE1 $\beta$ . IRE1 $\alpha$  is expressed ubiquitously, whereas the expression of IRE1 $\beta$  is limited to respiratory and digestive epithelial cells (Zhang et al., 2011b). IRE1 $\alpha$  knockout mice are embryonically lethal while IRE1 $\beta$ <sup>-/-</sup> mice are viable (Iwawaki et al., 2009, Tsuru et al., 2013). The cytoplasmic domain of IRE1 contains a serine/threonine kinase domain, the purpose of which is to activate downstream targets including a C-terminal endoribonuclease domain (Korennykh and Walter, 2012). Activation of the endoribonuclease domain requires binding of ATP or ADP in the active site of the kinase. The mechanism of action of IRE1 has been the subject of much debate (Korennykh and Walter, 2012). Originally it was thought that ER stress leads to dissociation of HSPA5 from IRE1 resulting in autophosphorylation of IRE1 $\alpha$  and activation of its RNase activity. Subsequent studies have found that IRE1 can be activated in response to stress independent of HSPA5 (Pincus et al., 2010). The sensor domain of IRE1 has a peptide binding domain similar to the peptide-binding groove of major histocompatibility complex I (MHCI). Gardner and Walter (2012) demonstrated that the sensor domain of yeast IRE1 can bind hydrophobic peptides, which leads to oligomerization of IRE1 and subsequent activation of its kinase and ribonuclease (RNase) domains. Therefore, the primary activator of IRE1 appears to be direct interaction with unfolded proteins, with HSPA5 fine-tuning the response. It is thought the role of HSPA5 is to deactivate IRE1 by maintaining sufficient ER stress sensing monomers of IRE1 (Korennykh and Walter, 2012). In this way the ER luminal domain of IRE1 can act as a stress sensor (Korennykh and Walter, 2012). See Fig. 1.4 for details

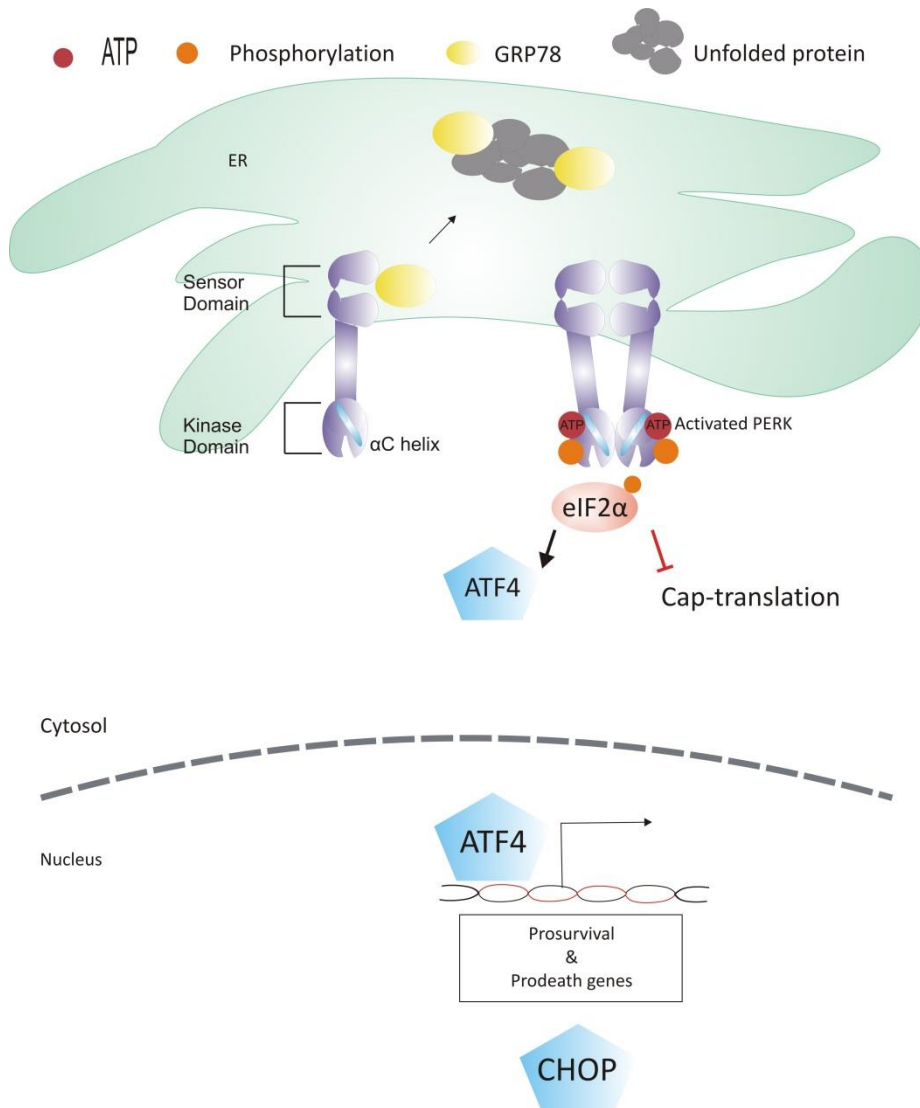


**Fig. 1.5 Activation of the IRE1 pathway.** Activation of IRE1 leads to non-conventional splicing of XBP1 and synthesis of ERAD transcripts, Chaperone and phospholipid synthesis genes.

PERK is an ER-associated transmembrane serine/threonine protein kinase. It belongs to a family of eIF2 $\alpha$  kinases which includes PKR (protein kinase double-stranded RNA-dependent), GCN2 (general control non-derepressible-2), and HRI (heme-regulated inhibitor) (Donnelly et al., 2013). PERK knockout mice are viable and are born at normal Mendelian rate's however, they develop diabetes at an early age, a phenotype that is recapitulated in humans with Wolcott-Rallison syndrome, known also to be caused by mutations in PERK (Lin et al., 2008).

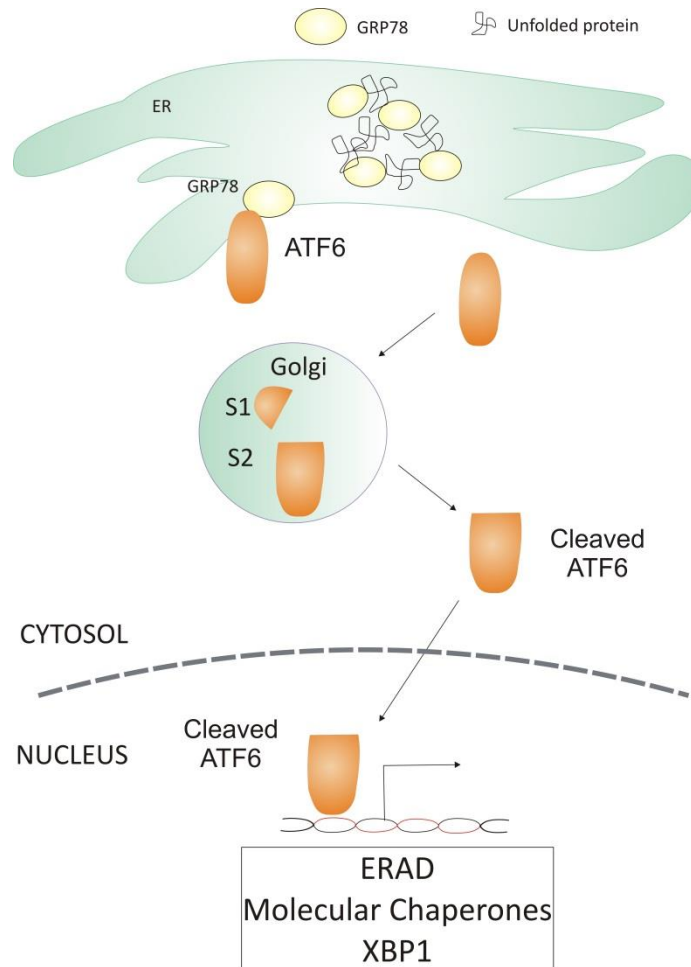
Upon accumulation of unfolded proteins in the ER lumen HSPA5 dissociates from the sensor domain of PERK leading to its dimerization. This produces a back-to-back interface which facilitates trans-autophosphorylation on residues Thr980 and Tyr615 of PERK. Phosphorylation stabilizes the activation loop and causes a structural change in the alpha helix  $\alpha$ C, priming PERK for ATP hydrolysis and docking

of its substrate eukaryotic translation initiation factor 2 alpha (eIF2 $\alpha$ ) (see Figure 1.5) (Korennykh and Walter, 2012, Donnelly et al., 2013). The PERK sensor domain is not well studied and it is unknown if it can bind misfolded proteins. However, given the high degree of sequence homology with the sensor domain of Ire1 (47%), it is plausible that PERK is also activated by direct interaction with mis-folded proteins (Korennykh and Walter, 2012).



**Fig. 1.5. Activation of the PERK pathway by ER stress.** Activation of the PERK pathway causes general protein translation arrest via eIF2 $\alpha$

In mammals, there are two alleles of ATF6, ATF6 $\alpha$  (90 kDa) and ATF6 $\beta$  (110 kDa) and both are synthesized in all cell types as ER transmembrane proteins. A number of other tissue-specific bZIP transcription factors that localize to the ER have been identified including OASIS, CREBH, LUMAN/CREB3, CREB4 and BBF2H7. The precise function of these proteins is unclear but they may serve to tailor the UPR to the needs of specific cell types (Asada et al., 2011). In unstressed cells, ATF6 is localized at the ER membrane and is bound to HSPA5 (Haze et al., 1999). Dissociation from HSPA5 exposes two golgi localizing sequences (GLS) in ATF6 leading to its translocation to the golgi apparatus via COP II vesicles. It is unknown if unfolded proteins directly activate ATF6, or if possibly HSPA5 dissociation permits full activation of ATF6 (Haze et al., 1999). In line with this, removal of the HSPA5 binding site on ATF6 with retention of the GLS leads to constitutive translocation of ATF6 to the golgi, suggesting that HSPA5 is the primary modulator of ATF6 activation. At the golgi, ATF6 undergoes proteolytic processing to yield an active transcription factor. This modification is mediated by site-1 protease and site-2 protease which cleave ATF6 in the luminal domain. The processed forms of ATF6 $\alpha$  and ATF6 $\beta$  translocate to the nucleus and bind to the ATF/cAMP response element and to the ER stress responsive element (ERSE) to activate target genes (Asada et al., 2011). See Fig. 1.6 for details.



**Fig. 1.6. Activation of the ATF6 pathway by ER stress.** ER stress causes dissociation of HSPA5 from AFT6 facilitating its transport to the Golgi Apparatus where it undergoes sequential cleavage by S1 and S2 proteases. This generates an active transcription factor which enters the nucleus and increases expression of ERAD and molecular chaperone genes as well as XBP1.

### 1.10.3 Adaptive phase of the UPR: Damage control

Multiple mechanisms are activated by the UPR with the aim of reducing the load of nascent proteins, these include eIF2 $\alpha$  phosphorylation, co-translational degradation of ER located proteins, autophagy, ER-associated degradation (ERAD) and ER expansion.

PERK-mediated phosphorylation of eIF2 $\alpha$  on Ser51 blocks the binding of Met-tRNA to the ribosome thereby reducing the recognition of the AUG initiation codon. This results in a general translational arrest (Harding et al., 1999). The pro-survival effects of blocking translation are exemplified by Salubrinal, a selective inhibitor of eIF2 $\alpha$  dephosphorylation. Salubrinal has been shown to be protective in ER stress-induced apoptosis in cardiomyocytes (Chun-Lei Liu, 2012) and A $\beta$  induced ER stress leading to apoptosis in neuronal cells (Yuan et al., 2012). An additional mechanism to reduce the load of nascent proteins at the ER is co-translational degradation of ER localized proteins (Oyadomari et al., 2006). These mechanisms provide protection over the short term but are not compatible with cell function over the long-term and are therefore reversible processes. ER expansion acts to dilute the concentration of mis-folded proteins and is dependent on phospholipid synthesis. This may also assist in protein refolding as protein folding is more efficient at low concentration due to a reduction in the propensity to aggregate (Schuck et al., 2009).

The UPR engages two degradation pathways to remove mis-folded proteins namely autophagy and ERAD. These pathways serve as quality control measures by ensuring proper and safe removal of mis-folded proteins, thus preventing illicit protein interactions. Proteins are selected for degradation via the ERAD pathway by ER resident proteins including HERP, EDEM, and HSPA1 family of proteins. Targets exit the ER via retro-translocations created by SEC61 with Derlin protein also contributing (Willer et al., 2008). The driving force for elimination of ERAD targets from the ER seems to be ubiquitination, which ensures that, once in the cytosol, ERAD targets are rapidly degraded (Brodsky, 2012). The ERAD pathway feeds into the ubiquitin-proteasome pathway. Autophagy provides adaptive responses during ER stress where it serves to eliminate misfolded proteins and damaged organelles (Deegan et al., 2013). The combined action of these processes mediates a reduction in the load of nascent proteins arriving at the ER and the removal of mis-folded proteins, giving the cells a window of opportunity to resolve the cellular stress.

#### 1.10.4 Adaptive phase of the UPR: Induction of transcription factors

One of the most salient features of the UPR is the induction of a cohort of transcription factors. Although phosphorylation of eIF2 $\alpha$  inhibits general translation initiation, it paradoxically increases translation of cap-independent mRNA such as activating transcription factor 4 (ATF4) (Lu et al., 2004), activating transcription factor 5 (ATF5) and activating transcription factor 3 (ATF3) due to preferential processing of mRNAs with internal ribosomal entry sites (IRES). ATF4 leads to induction of Growth Arrest/DNA Damage Inducible 34 (GADD34) which results in de-phosphorylation of eIF2 $\alpha$  via protein phosphatase 1 thereby relieving the translational block. ATF4 also induces genes involved in folding, redox balance, amino acid metabolism and autophagy. ATF4 helps cells adapt to cell stress and provides pro-survival signals. However, under conditions of chronic cell stress, ATF4 is associated with induction of apoptosis. In addition to eIF2 $\alpha$ , the bZIP Cap 'n' Collar transcription factor, nuclear respiratory factor 2 (NRF2), is also a substrate of PERK (Cullinan and Diehl, 2006). NRF2 is retained in the cytoplasm through its association with the microtubule-associated protein KEAP1 (Kelch-like Echinoid-associated protein 1) (Cullinan and Diehl, 2006). Upon ER stress, PERK-mediated phosphorylation of NRF2 promotes its dissociation from KEAP1, leading to the nuclear accumulation of NRF2 (Cullinan and Diehl, 2006). NRF2 binds to the antioxidant response element (ARE) to activate transcription of genes encoding detoxifying enzymes.

Studies with ATF6<sup>-/-</sup> cells have recently shown that ATF6 is responsible for transcriptional induction of a cohort of ER proteins which include chaperones such as HSPA5 and HSPC4 (GRP94), folding enzymes like protein disulphide isomerase, and ER-associated degradation (ERAD) components (Wu et al., 2007a). ATF6 is also responsible for induction of XBP1, which is a target of IRE1-mediated endoribonuclease activity (Calfon et al., 2002). Activated IRE1 excises a 26-

nucleotide sequence from XBP1 mRNA. IRE1-mediated XBP1 mRNA splicing causes a shift in the reading frame, such that spliced XBP1 (XBP1s) mRNA encoding a 376 amino acid protein is produced. XBP1s possesses a potent transcriptional transactivation domain in its C-terminal region (Calfon et al., 2002). XBP1 targets include the ERAD genes HERP and EDEM as well as the HSP40 family member p58<sup>IPK</sup> which acts as a cofactor for HSPA5. XBP1s also increases expression of genes involved in phospholipid synthesis, which is required for the expansion of the ER during stress conditions (Sriburi et al., 2007).

#### **1.10.5 Decision time**

The decisive switch that determines the change from an adaptive ER stress response to a maladaptive one remains enigmatic. The amplitude and kinetics of the stress response appear to determine cell fate. In a graded fashion, low doses of ER stress inducers only increase expression of pro-survival genes. Additionally it was found that specific oligomeric configurations of IRE1 have divergent signalling outputs. Such switches in signalling may utilize additional components that may act to fine tune the stress response. Such modulators have been termed the UPRosome and include HSPs that can modulate UPR signalling, such as HSPA1 mediated regulation of IRE1, or stabilize the UPR sensors as is the case for HSPC1 (Marcu et al., 2002, Gupta et al., 2010). Additional regulator mechanisms include post-translational modification of the UPR sensors, for example de-phosphorylation of IRE1 by PP2A, and ribosylation of PERK and IRE1 by PARP16, which acts to enhance their downstream signalling outputs (Jwa and Chang, 2012). Finally recent studies have highlighted the key role of microRNAs in modulating cell fate in response to cell stress (Gupta et al., 2012, Cawley et al., 2013).

#### **1.10.6 Execution phase of ER stress**



Overall, it is thought that ER stress-induced apoptosis occurs via the intrinsic pathway and it is well documented that ER stress leads to activation of executioner caspases (Morishima et al., 2002, Szegezdi et al., 2008). To this end, caspase-3 knock out MEFs are resistant to ER stress-induced cell death, but the identity of the initiator caspase involved in ER stress induced apoptosis is less clear. Identification of the ER resident caspase-12 which is cleaved in response to ER stress saw a period of intense research into its potential role in the regulation of ER stress-induced apoptosis. However, conflicting reports emerged on the response of caspase-12 knockout MEFs with some stating it has no role, while others have reported it as the caspase responsible for ER stress-induced cell death (Kalai et al., 2003). The relevance of caspase-12 as the regulator of ER stress-induced apoptosis is called into question due to the fact the several inactivating mutations occur in human caspase-12 producing truncated proteins (Fischer et al., 2002). However, it may still mediate ER stress-induced apoptosis in other species where caspase-12 is present and functional, e.g., rat and mouse models. Caspase-4 has high homology to caspase-12 and its expression and cleavage is increased during ER stress. It has therefore been suggested that caspase-4 may perform the function of caspase-12 in human cells. More recently, caspase-2 has been linked to ER stress. It was shown that caspase-2 is activated during ER stress leading to cleavage of BID (Upton et al., 2008). Inhibition of caspase-2 confers resistance to ER stress-induced apoptosis. Interestingly, direct modulation of caspase-2 by the UPR has been demonstrated. The IRE1-RIDD pathway causes degradation of microRNAs that normally act to suppress caspase-2 (Upton et al., 2012).

CHOP, also known as growth arrest and DNA damage-inducible gene 153 (GADD153), is a member of the C/EBP family that heterodimerizes with other members of the C/EBP transcription factor family. This 29 kDa factor is expressed at low levels in unstressed cells but is strongly induced in response to numerous cellular stressors including UV light, genotoxic agents, glucose deprivation and amino acid starvation and ER stress (Zinszner et al., 1998b, Maytin et al., 2001). (Reimertz et al., 2003b). The CHOP promoter contains at least two ERSE motifs.

CHOP is induced as early as 4 hours following Thapsigargin (TG) treatment and remains elevated throughout the UPR response (Reimertz et al., 2003b).

CHOP can be induced by all three arms of the UPR, however the induction of CHOP by ER stress is almost fully attenuated in PERK null cells and in eIF2aS51A cells which express a non-phosphorylatable form of eIF2a. These results suggest that the PERK/eIF2a pathway is the primary mediator of CHOP induction. However, the ATF6 and Ire1/XBP-1 signalling pathways are still required for optimal induction of CHOP during ER stress (Harding et al., 2000).

CHOP is composed of two known functional domains, an N-terminal transcriptional activation domain and a C-terminal basic-leucine zipper (bZIP). Deletion of domain fragments has established that the apoptosis inducing effects are due to the bZIP domain. However, the amino-terminal region contains phosphorylation sites which serve as regulatory regions for CHOP activity; Ser<sup>79</sup> and Ser<sup>82</sup> of CHOP are phosphorylated by the p38/MAP kinase family following cellular stress leading to enhanced transcriptional activation (Wang and Ron, 1996). Paradoxically, the PERK/eIF2 $\alpha$ /ATF4 pathway transiently suppresses CHOP mRNA via microRNA mediated repression (Chitnis et al., 2012). This effect likely reflects initial attempts by the PERK arm to allow cells to recover from the stressor. It has been shown that CIAPs functions as an E3 ubiquitin ligase promoting ubiquitination and degradation of CHOP in  $\beta$ -cell survival following ER stress (Qi and Xia, 2012). Therefore, different cell types may have evolved additional mechanisms to deal with CHOP and ER stress.

It has been shown that mouse embryonic fibroblasts derived from CHOP<sup>-/-</sup> animals exhibited significantly less cell death when challenged with ER-stress inducing agents compared to wild type (Zinszner et al., 1998b). In contrast to the defined mechanism of CHOP induction, the downstream mechanisms of CHOP-induced cell death are less well defined. CHOP's pro-apoptotic effects are linked to modulation of BCL2 family members and enhanced production of reactive oxygen species (ROS) (McCullough et al., 2001). Caspase-11 has been reported to act downstream of

CHOP to induce cell death by activating death effector caspases -1 and -3 (Fradejas et al., 2009). CHOP can bind to the promoter region of pro-apoptotic BIM increasing its expression as well as transcriptionally down-regulating BCL-2 and in this way it induces cell death (Puthalakath et al., 2007). Expression of CHOP is not sufficient to bring about ER stress-induced apoptosis as CHOP-deficient mice are only partially resistant to ER stress-dependent cell death (Zinszner et al., 1998a).

Additional pro-death inducing signals emanating from the PERK arm include p53-mediated induction of BH3 only proteins (BOPs). By activating NF $\kappa$ B, PERK leads to transcriptional induction of p53. This potent tumor suppressor enters the nucleus where it drives expression of PUMA and NOXA. P53 knockout mice are partially resistant to ER stress, and fail to induce PUMA and NOXA causing attenuated apoptosis (Li et al., 2006, Lin et al., 2012).

It has been demonstrated that differential oligomerization of IRE1 has divergent effects on cell fate (Bouchecareilh et al., 2011). The best characterized target of IRE1 is XBP1 however IRE1 is also capable of inducing cell death via activation of regulated IRE1 dependent decay (RIDD) and JNK. RIDD is a conserved pathway that mediates degradation of mRNAs. Still relatively new to the ER stress field, the list of RIDD substrates is rapidly growing but is known to include genes involved in lipogenesis and lipoprotein metabolism (So et al., 2012) and in particular ER localized mRNAs (Gaddam et al., 2013).

JNK belongs to the superfamily of mitogen activated protein kinases, which includes ERK and P38. The pro apoptotic inducing abilities of JNK have been demonstrated in response to a variety of cellular stressors including NGF withdrawal, TNF $\alpha$  and staurosporine. This activity is linked to the intrinsic pathway as JNK fails to induce apoptosis in BAX/BAK knock out cells. Activated IRE1 can bind to tumor necrosis factor (TNF)-receptor-associated factor 2 (TRAF2), an adaptor protein that promotes activation of c-JUN terminal kinase (JNK) through apoptosis signal-regulating kinase 1 (ASK1) (Urano et al., 2000). This process seems to be regulated

by calpains as MEFS lacking calpains fail to induce JNK following ER stress and are subsequently protected from ER stress induced apoptosis (Tan et al., 2006).

The primary means by which JNK induces apoptosis is via regulation of the BCL2 family, as JNK can phosphorylate BCL2 and BCLXL thereby inhibiting their anti-apoptotic ability. Secondly JNK can cause phosphorylation of a conserved phosphorylation site on BIML and BMF (S65) causing their release from dynin and myosin motor complexes (Lei and Davis, 2003). JNK is also capable of phosphorylating BIMEL on S65 which enhances its pro-apoptotic capacity (Putcha et al., 2003). Furthermore JNK can enhance translocation of BAX to the mitochondria (Tsuruta et al., 2004).

It is apparent from all of the above that the precise mechanism of ER stress induced cell death and the regulation of such mechanism remains to be determined.

### 1.11 The heat shock response (HSR)

The heat shock response (HSR) is one of the most ancient and evolutionarily conserved cytoprotective mechanisms found in nature (Ritossa, 1996). Following exposure to environmental insults cells dramatically increase the production of a group of highly conserved proteins that lead are collectively known as Heat Shock Proteins (HSPs). As their name suggests, HSPs were originally found to be up-regulated after exposure to elevated temperatures (Lindquist, 1986, Morimoto, 1998). For all living organisms, even minor increases in temperature above the optimum represent a severe stress. This is predominantly due to the deleterious effects of excess temperature on protein leading to protein unfolding and aggregation. In fact many of the morphological and phenotypic effects of excess temperature are due to deregulation of protein homeostasis (Richter et al., 2010). It has been suggested that the heat shock response is not a response to excessive temperature per se, rather it is an adaptive response to unfolded protein

accumulation (Richter et al., 2010). The heat shock response is induced by a wide variety of stressors that are associated with accumulation of mis-folded proteins including ischemia, hypoxia (Kinouchi et al., 1993), oxidative stress (Gomer et al., 1996), increased intracellular calcium and energy depletion (Mosser et al., 1990), heavy metals (Bauman et al., 1993), and infection (Nagasawa et al., 1992). Expression of heat shock proteins is strongly induced in response to proteasomal inhibition (Bush et al., 1997). In addition, diseases such as cancer and neurodegenerative diseases are associated with aberrant expression of heat shock proteins (Westerheide and Morimoto, 2005). This supports the hypothesis that the heat shock response is initiated in response to mis-folded proteins. Severe heat stress leads to irreversible changes and cell death. However, a mild heat stress can provide protection against a later thermal challenge in a phenomenon known as 'thermo- tolerance' (Lindquist, 1986, Morimoto, 1998, Richter et al., 2010). In general, preconditioning with mild stressors can protect against a later, more severe insult (Leak et al., 2006). Interestingly, "crossprotection" is also possible: HSPs induced by one type of stress provide protection against an array of subsequent stressors (Lindquist, 1986, Morimoto, 1998, Richter et al., 2010).

The heat shock response can be divided into three stages. Firstly the stress inducing stimulus is sensed by the CSR pathway (Initializing phase). The second stage sees protective mechanisms initiated to response to the stress (Adaptive phase). Finally, if the stress is unresolved cell death occurs (Execution phase).

### **1.11.1 Stress sensing by the HSR**

The heat shock response is sensed and activated by a family of heat shock factors (HSF). Invertebrates express just one HSF, HSF1, while mammals express four HSF proteins (HSF1, 2, 3, 4). HSF1 is the major stress responsive transcription factor in mammalian cells. In support of this is the observation that HSF1 knockout MEFs are

unable to induce HSP70 in response to thermal stress, while HSF2 knockout MEF cells induce HSP70 normally.

HSF1 is constitutively expressed in an inactive form in the cytoplasm of unstressed cells. Three models have been proposed to explain the activation of HSF1 by cellular stress. The finding that HSPs interact with HSF1 led to the theory that in response to cell stress, HSPs dissociate from HSF1 to restore proteostasis thereby freeing up HSF1 and allowing for its activation. In a negative feedback mechanism, the increased expression of HSPs will eventually accumulate to a level at which sufficient HSPs are expressed so that complexes with inactivated HSF1 can be reformed. Alternatively, HSF1 is reported to exhibit auto-inhibitory ability via interaction of discrete domains thereby preventing oligomerization. Data demonstrate that HSF1 can directly 'sense' cell stress as determined by *in vitro* assays for HSF1 activation/oligomerization, following challenges such as heat shock, treatment with H<sub>2</sub>O<sub>2</sub>, acidic pH and increased intracellular calcium levels (Björk and Sistonen, 2010).

Expression of HSP genes is dependent on the presence of short nucleotide sequences known as heat shock elements (HSEs) located upstream in the promoter region of heat shock responsive genes. HSEs serve as binding sites for HSFs. Interestingly, HSF1 binding per se does not lead to induction of HS targets. HSFs are able to act as both activators and repressors in a targeted gene-dependent manner. In some instances binding of HSF1 will only lead to activation of a target when co-activators are present. For example the co-factor Strap forms a complex on the promoter of Hsp70 with HSF1 and p300, leading to increased acetylation of the target gene. This complex was shown to be necessary for optimal induction of Hsp70. Studies have shown that cofactors operate with HSF1 in stress-specific conditions to induce HSPs. Induction of the HSF target gene CUP1, by glucose starvation in yeast, is dependent on the Snf1 kinase, while CUP1 induction by heat shock occurs in a HSF1 dependent Snf1 kinase independent manner (Hahn and

Thiele, 2004). This stressor specific modulation explains how components of the HSR are utilized by different stress pathways to increase HSPs.

### 1.11.2 Mediators of the HSR

The heat shock response leads to an increase in approx. 100 genes with diverse functions, including molecular chaperones (HSPs), components of the ubiquitination pathway, RNA- and DNA-modifying enzymes, metabolic and detoxifying enzymes, transcription factors or kinases, proteins involved in intracellular transport and membrane-modulating proteins (Richter et al., 2010, Velichko et al., 2013).

HSPs, are divided into five groups based on molecular weight. HSPA (HSP70, HSPH (HSP110), HSPC (HSP90), DNAJ (HSP40) and HSPB (small HSP) (Kampinga et al., 2009). HSPs and their close constitutively expressed relatives function as “molecular chaperones”, proteins that guard against “illicit or promiscuous interactions” between other proteins (Craig et al., 1993; Benjamin and McMillan, 1998). These chaperones protect the proteome from the dangers of mis-folding and aggregation by facilitating protein folding, trafficking, complex assembly, and ubiquitination, as well as proteasomal degradation (Balch et al., 2008). This protection is achieved via a number of ways including through *de novo* protein folding, re-folding of mis-folded proteins and oligomer assembly (Hartl et al., 2011).

### 1.11.3 HSPA (HSP70) family

The human genome encodes 13 HSPA family members.

HSP70 family members display a high degree of sequence and domain homology. Additional characteristics include (i) a conserved ATPase domain at the N-terminus; (ii) a middle region with protease sensitive sites; (iii) a flexible C-terminal peptide substrate binding domain (SBD) (iv) a G/P-rich C-terminal region containing an EEVD-motif that enables the proteins to bind co-chaperones and other HSPs.

In cooperation with other chaperones, the HSPA family carries out numerous house keeping functions many of which rely on the chaperoning function of HSPA proteins. Examples include prevention of protein aggregation, refolding of misfolded or aggregated proteins and folding and translocation of newly translated polypeptides. In addition, the HSPA family coordinates trafficking of proteins between cellular compartments (Daugaard et al., 2007) These chaperones participate in all of these processes through their ability to recognize nonnative conformations of proteins. Alternation between the ATP state with low affinity for clients and the high affinity ADP state is tightly regulated by several classes of associated proteins, or co-chaperones. If the protein cannot be refolded then degradation is favoured. HSPA1 enhances degradation via its co-chaperone CHIP (C-terminus of HSPA1 interacting protein) which is an ubiquitin ligase. When interacting with HSPA1, CHIP ubiquitinates target proteins leading to proteasomal degradation (Turturici et al., 2011).



#### **1.11.4 HSPH (HSP110)**

The human genome encodes 4 HSPH members (HSPH1-4). This family is highly homologous to the HSPA family with the HSPH members expressing a longer linker region and like the HSPA family, HSPH proteins prevent protein aggregation. HSPH proteins primarily act as nucleotide exchange factors for the HSPA family but they have also been shown to be able to refold mis-folded luciferase in the absence of HSPA proteins (Kampinga et al., 2009) .

#### **1.11.5 HSPC (HSP90)**

This family is made up of five members (HSC1-5). In humans the most prominent members of this abundant family are HSPC2 (HSP90 $\alpha$ ) and HSPC3 (HSP90 $\beta$ ) The two isoforms share a high degree of homology (86%) and many studies do not differentiate between the two isoforms because for a long time they have been considered as having the same function in the cells. However, this notion is now being challenged and the two isoforms have some unique functions. The HSPC family functions as part of a multichaperone complex via association with a variety of co-chaperones and client proteins that rely on the complex for maturation and stability. The primary role of HSPC1 is in maintaining the conformation of its client proteins many of which are tyrosine kinases such as BRAF, steroid hormone receptors or signalling proteins such as the transcription factors p53. HSPC1 has also been shown to modulate the stability of the UPR sensor IRE1 (Marcu 2002).

#### **1.11.6 DNAJ (HSP40)**

DNAJ is a family of HSPA1 co-chaperones. The ATPase activity of the HSPA1 family is critical for their activity and is a process that is regulated by DNAJ proteins.

Therefore DNAJ proteins are important for determining activity of HSPA proteins and stabilizing interactions with client proteins (Kampinga et al., 2009).

### 1.12 Small heat shock family- members – HSPB

The sHSPB group is a family of molecular chaperones consisting of ten members which can be classified into two functional categories: Class I sHSPs (HSPB1/ HSP27, CRYAB/HSPB5, HSPB6/ HSP20, and HSPB8/ H11) which are ubiquitously expressed, and the Class II sHSPs (HSPB2, HSPB3, HSPB7/ cvHsp, CRYAA/ HSPB4, HSPB9, and ODF1/HSPB10), which display tissue-restricted patterns of expression (Taylor and Benjamin, 2005). sHSPs are the most widely expressed HSPs however they are highly diverse in sequence, size, client protein specificity and function (Vos et al., 2008a). The tissue-specific expression of sHSPs again points to functional diversity. In addition, mutations in the various sHSPs leads to diverse pathologies. However sHSPs do share certain characteristic features; (i) a conserved  $\alpha$ -crystallin domain (ACD), (ii) a small molecular mass of 12–43 kDa, (iii) formation of large oligomers and (iv) a dynamic quaternary structure and ATP independent chaperone activity. sHSPs are classed as holdases, which are molecular chaperones that bind to client proteins thereby keeping them in a state that is competent for either refolding by ATP dependent HSPs such as HSPA1 or degradation. (Haslbeck et al., 2005, Parcellier et al., 2006). The  $\alpha$ -crystallin domain is a ~90 residue sequence located in the C-terminal of sHSPs. This region is a highly structured  $\beta$ -pleated sheet with several contact points allowing oligomer formation and stabilization (Kim et al., 1998).

In contrast to other regions of sHSP, the ACD is highly structured and is a hot spot for many disease-causing mutations (Baranova et al., 2011). The N-terminal of sHSPs has low sequence homology, with the exception of a highly conserved proline-phenylalanine-rich region containing one or two WD/EPF hydrophobic

motifs (WDPF) (Theriault et al., 2004). The N-terminus also possesses many regulatory post-translational modification sites, which regulate quaternary structure. It appears that although the  $\alpha$ -crystallin domain is important in oligomeric-complex formation, the N-terminal residues before the  $\alpha$ -crystallin domain are necessary not only for complex formation but also for chaperone activity (Theriault et al., 2004). This is due to the role of phosphorylation in the regulation of cellular location and activity of sHSPs. Depending on the oligomeric state sHSPs have different client proteins and binding partners, and cell stressors lead to rapid phosphorylation of sHSPs causing de-oligomerization. Therefore, it has been suggested that the phosphorylation status and structural organization of sHSPs acts as an intracellular stress sensor leading to initiation of adaptive strategies (Arrigo, 2013). sHSPs are capable of homo-oligomerization and hetro-oligomerization giving an infinite number of conformational states with potentially diverse functions. The effects of the chimeric oligomers formed by sHSPs is only beginning to be studied and it is speculated that hetro-oligomers have different client protein profiles to homo-oligomers (Arrigo, 2013). Thus far only a handful of studies have investigated the formation of hetro-complexes formed by WT and mutant HSPBs. It seems that presence of mutated HSPBs alters oligomer size (Arrigo, 2013, Nefedova et al., 2013)

In addition to being constitutively expressed, some members, namely HSPB1, HSPB5, HSPB6 and HSPB8 are stress inducible (Bartelt-Kirbach and Golenhofen, 2013). HSPB11 is a controversial member of the HSPB family. It was identified in 2007 as novel heat-inducible HSP with anti-apoptotic properties (Bellyei et al., 2007). However, sequence and structural analysis showed it does not have an ACD domain which is the defining feature of the HSPB family (Guido et al., 2010).

### **1.12.1 Functions of HSPB1**

The HSPB1 family have diverse functions including inhibition of apoptosis and regulation of cytoskeleton dynamics. HSPB1 specifically inhibits activation of caspases by interacting with cytosolic cytochrome *c* and preventing apoptosome formation (Bruey et al., 2000b, Concannon CG, 2001). Moreover HSPB1 can also prevent release of cytochrome *c* from the mitochondria. This effect is not due to the ability of HSPB1 to retain cytochrome *c* in mitochondria, rather HSPB1 has a stabilizing effect on the mitochondria via binding to F-actin preventing cytoskeletal disruption, Bid intracellular redistribution and cytochrome *c* release (Paul et al., 2002b). A proportion of cytosolic cytochrome *c* has been shown to interact with HSPB1 thereby reducing efficacy of apoptosome formation (Bruey et al., 2000b). Therefore HSPB1 can act both up stream and downstream of the mitochondria to modulate cytochrome *c*. HSPB1 can also directly associate with caspase-3. This protein-protein interaction was demonstrated *in vivo* and with purified proteins. Interaction of HSPB1 and the amino-terminal prodomain of caspase-3 inhibits the second proteolytic cleavage necessary for caspase-3 activation (Voss et al., 2007). HSPB5 has also been shown to inhibit the autocatalytic maturation of caspase-3 (Kamradt et al., 2002).

HSPB1 was reported to inhibit conformational BAX activation, oligomerization and translocation to the mitochondria following metabolic stress. No direct interaction was found between HSPB1 and BAX, rather HSPB1 promotes interaction of AKT, a pro-survival kinase with BAX. Specifically, HSPB1 prevents AKT inactivation by promoting PI3-Kinase activity, an upstream activator of AKT (Havasi et al., 2008b). HSPB1 and AKT are reported to co-exist in a signal complex with p38 MAPK, MK2 which phosphorylates AKT on Ser-473 (Rane et al., 2003). HSPB1 regulates AKT activation and promotes cell survival by scaffolding MK2 to the AKT signal complex (Wu et al., 2007b)

In instances when mis-folded proteins cannot be refolded, proteasomal degradation of the potentially toxic protein occurs. Proteins are flagged for proteasomal degradation in a step wise fashion via the ubiquitin pathway. Ubiquitin

is a highly expressed cytosolic protein which is classed as the smallest HSP (8 kDa). Ubiquitin is considered a HSP because it contains an HSE in the promoter region and its primary function is to target proteins for removal via the ubiquitin-proteasome pathway. The importance of ubiquitination in cell stress and proteostasis is demonstrated by the fact that increased ubiquitin-dependent degradation can replace the essential requirement for HSP induction (Friant et al., 2003).

The proteasome is the main intracellular proteolytic machinery in higher eukaryotic cells. It is found in both the nucleus and cytoplasm. It consists of a multi-subunit complex formed by a 20S proteolytic core and two 19S regulatory subunit complex which are involved in degradation of ubiquitin-tagged proteins and also in some cases non-ubiquitinated substrates (Jariel-Encontre et al., 2008)

Ubiquitination is a multi-step enzymatic pathway involving three classes of enzymes, E1 or ubiquitin activating enzyme, E2 or ubiquitin conjugating enzyme, and E3 or ubiquitin protein ligase (Fang and Weissman, 2004). This process leads to degradation of the tagged protein by the 26S proteasome complex with release of free and reusable ubiquitin catalyzed by ubiquitin-recycling enzymes (Fang and Weissman, 2004). (Fang and Weissman, 2004). E1 forms a thiol-ester bond between its active site cysteine and the carboxyl-terminal glycine of ubiquitin. The activated ubiquitin on E1 is subsequently transferred to the active site cysteine of an E2 by transesterification. E3 binds ubiquitin-charged E2 and substrate and facilitates formation of an isopeptide linkage between the carboxyl-terminal glycine of ubiquitin and an internal lysine residue on the substrate, or an ubiquitin already attached to the protein (Fang and Weissman, 2004). Substrate specificity is largely determined by the E3 ligase (Fang and Weissman, 2004). All known E3s identified so far contain either a HECT (homologous to E6-associated protein C-terminus) domain or RING (really interesting new gene)-finger motifs. Multi-ubiquitin chains are assembled via an isopeptide linkage between the lysine residue of the previous ubiquitin and the C-terminal glycine residue of the next ubiquitin. Different multi-

ubiquitin chains can be formed, depending on the lysine residues used for ubiquitin–ubiquitin linkage. Linkage type and chain length seem to influence the fate of the conjugate. Chains of four or more ubiquitin moieties linked via Lys48 of ubiquitin, promote proteasomal degradation of the substrates. By contrast, Lys63-linked multi-ubiquitin chains, and also mono-ubiquitin, seem to have non-proteolytic functions, such as signal transduction. It was recently shown that efficient multi-ubiquitination of some substrates requires additional conjugation factors. Such factors act as a scaffold to dock E3 ligases with their substrate or to catalyse an extension of the multi-ubiquitin chain in collaboration with E1, E2, and E3 and have been termed E4 enzymes. Therefore E4 ligases can be defined as an E3-like enzyme that cooperate with a substrate-specific ubiquitination machinery to increase the efficacy.

HSPB1 has been shown to enhance proteasome activity (Parcellier et al., 2003, Lanneau et al., 2010). HSPB1 specifically binds to ubiquitin and preferentially interacts with poly-ubiquitinated species increasing their degradation in response to stress (Parcellier et al., 2003, Lanneau et al., 2010). Interestingly HSPB1 was found to specifically enhance degradation of proteins. Expression of HSPB1 leads to enhanced degradation of p27<sup>KIP1</sup> and I $\kappa$ B $\alpha$  while having no effect on other cell cycle proteins such as cyclin E, cyclin D1 cyclin A or  $\beta$ -catenin (Parcellier et al., 2003, Lanneau et al., 2010). HSPB1 can target mutant proteins such as cystic fibrosis transmembrane conductance regulator and mutant HSPB5 for degradation (Ahner et al., 2012). Contradictory to this is the finding that HSPB1 protects the eukaryotic initiation factor eIF2E from ubiquitination and subsequent proteasomal degradation (Andrieu et al., 2010) and knockdown of HSPB1 induces degradation of several client proteins (Gibert et al., 2012). Therefore HSPB1 appears to exert substrate-specific effects on proteasomal degradation and protein stability.

### **1.12.2 Small heat shock proteins and peripheral neuropathies**

Neurodegenerative diseases are characterized by a wide range of cognitive, behavioral and/or motor alterations, which develop as a consequence of the dysfunction and death of specific neuronal populations. Motor neuron diseases (MNDs) are a subclass of neurodegenerative diseases which effects motor neurons leading to dysfunction or death of motor neurons resulting in muscle weakness and atrophy. The unique morphology of motor neurons have been implicated as a sensitizing factor. Motor neurons are highly polarized with long axonal processes and are dependent on efficient proteostasis. For this reason motor neurons are thought to be particularly sensitive to several types of insults that are normally better managed in other neuronal or non neuronal cell types. In particular, motor neurons are highly sensitive to accumulation of mutant proteins.

Overexpression of HSPs had been shown to be protective in numerous cell culture and animal models of neurodegeneration. Conversely mutations in five members of the HSPB family, namely HSPB1, HSPB3, HSPB4, HSPB5 and HSPB8 are associated with the development of neurological disorders and muscular disorders suggesting a key role for these proteins in motor neuron functions and/or survival. In particular, mutations of HSPB1, HSPB3 and HSPB8 have been directly associated with MNDs. HSPB4 and HSPB5, have been associated with the development of congenital cataracts characterized also by neurological disorders and/or with desmin-related myopathy and cardiomyopathy. (Carra *et al* 2012)

### 1.13 Aims and Rationale

Numerous similarities exist between the HSR and the UPR; (i) both are characterized by transcriptional induction of molecular chaperones, (ii) both are induced by a variety of stimuli which lead to protein mis-folding and (iii) the outcome of the cellular stress response depends on the severity and duration of the stressor. Based on these observations from the literature to date, we hypothesized that crosstalk existed between the HSR and the UPR. This hypothesis was investigated using well established inducers of the HSR and the UPR namely thermal preconditioning and ER stress inducing compounds Thapsigargin (TG) and Tunicamycin.

Initially cells mount a pro-survival response and cells can be rendered tolerant to stress by a preconditioning stimulus. Cross-tolerance can be induced wherein one stimulus provided protection against a subsequent toxic insult. We hypothesised that thermal preconditioning induces tolerance to ER stress. Therefore, we investigated if thermal preconditioning can protect cells against ER stress induced apoptosis.

Very little is known on the role of HSPB1 and ER stress. Given its potent anti-apoptotic abilities we investigated the role of HSPB1 in ER stress-induced apoptosis and the mechanism by which it protects cells during cell stress. Data from Chapter 3 suggested HSPB1 could regulate BIM. Therefore in chapter 4 we investigated if expression of HSPB1 alone protects from ER stress induced cell death. We also sought to determine the mechanism by which HSPB1 regulates BIM.

HSPB1 protects against a number of cytotoxic insults and cellular stressors and in neurodegenerative disease models. Additionally, mutations in HSPB1 cause Charcot Marie Tooth Disease (Peripheral Neuropathy). Based on the results of Chapter 4 we hypothesised that HSPB1 mutants would be more sensitive to ER stress and



therefore investigated the response of neuropathy associated HSPB1 mutations to ER stress induced apoptosis and the effect of HSPB1 mutations on expression of BIM.

## **Chapter 2**

### **Materials and Methods**

## 2.1 Cell lines and cell culture

### 2.1.1 PC12 Cell Culture

PC12 cells, a rat pheochromocytoma cell line, were obtained from European Collection of Cell Cultures (ECACC). These cells are a neuronal-like cell line and are widely used to study neuronal cell signalling and cell stress (Gorman et al., 2005). They are a semi adherent cell line, therefore, cells were cultured on poly-L-lysine (PLL) coated flasks to aid attachment of cells. To PLL coat flasks, a 0.001% w/v PLL solution made up in autoclaved ddH<sub>2</sub>O was added to culture flasks for ~3 h prior to seeding. PLL was removed and flasks were washed three times with autoclaved water. PC12s were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, D6429) supplemented with 10% heat inactivated horse serum (Sigma-Aldrich, H1138), 5% foetal bovine serum (FBS) (Sigma-Aldrich, F2442), and 50 U/ml penicillian and 50 µg/mL Streptomycin (Pen/Strep, Sigma-Aldrich, P0781 ) in a 5% CO<sub>2</sub> humidified incubator at 37°C.

#### 2.1.1.1 Generation of HSPB1 overexpressing cell lines

Neo (Control), WT-HSPB1, and mutant HSPB1 expressing cell lines were generated as follows. Wildtype PC12s were seeded as previously described on PLL coated 6 well plates. 24 h later cells were transfected with 0.75 µg of pCDNA-control plasmid (Neo), pCDNA WT-HSPB1, pCDNA-HSPB1S135F, pCDNA-HSPB1R127W, pCDNA-HSPB1R136W, pCDNA-HSPB1t151I as described in section 2.3.1. 24 h after transfection plasmid expressing cells were selecting using 800 ng of G418 thereby enabling selection of stably overexpressing cells.

Cells were maintained in T75 flasks (Greiner CELLSTAR, Cruinn). When the cells had grown to approximately 80% confluency, they were 'trypsinised' using 3 mL of 2X Trypsin/ EDTA (Sigma-Aldrich, T4174) diluted in Hank's balanced salt solution (Sigma-Aldrich H9394) at 37°C for 5 min. Trypsin was inactivated by adding 7 mL of fresh medium. Cells were then centrifuged at 1500 rpm for 5 min at room temperature(Thermo Scientific Sorvall ST16 Centrifuge). The supernatant was

removed and cells were re-suspended in 10 mL of fresh media. For experiments PC12 cells were seeded at 200,000 cells/ml.

### **2.1.2 HeLa cell culture**

HeLa cells are a glandular cervical cancer line and are a well established model to study heat shock proteins. Cells were maintained in T75 flasks (See table 1.1). When the cells had grown to approximately ~ 80% confluency, they were 'trypsinised' using 3 mL of 2X Trypsin/ EDTA in HANKS balanced salt solution at 37°C for 5 min. Trypsin was inactivated by adding 7 mL of medium. Cells were then centrifuged at 1,500 rpm for 5 min. The supernatant was removed and cells were re-suspended in 10 mL of fresh media supplemented with 10% FBS, and 1% penicillin/streptomycin. For experiments HeLa cells were seeded at 30,000/mL.

### **2.1.3 MEF cell culture**

Mouse Embryonic Fibroblasts (MEFs) are a Simian vacuolating virus 40 (SV40) immortalized cell line. MEF cells are a fibroblast cell line. Wild type and HSF1<sup>-/-</sup> MEFs were maintained in T75 flasks (See table 2.1). When the cells had grown to approximately ~ 80% confluency, they were 'trypsinised' using 3mls of 2X Trypsin/ EDTA in HANKS balanced salt solution at 37°C for 5 min. Trypsin was inactivated by adding 7 mL of medium. Cells were then centrifuged at 1,500 rpm for 5 min. The supernatant was removed and cells were re-suspended in 10 mL of fresh media supplemented with 10% FBS, and 1% penicillin/streptomycin. For experiments MEF cells were seeded at 125,000 cells/mL.

Table 2.1 Cell culture flasks

<b>Plate/Flask</b>	<b>96 well plate</b>	<b>24 well plate</b>	<b>6 well plate</b>	<b>T25</b>	<b>T75</b>	<b>T175</b>
<b>Size</b>						
<b>Surface area</b>	<b>0.3 cm<sup>2</sup></b>	<b>2 cm<sup>2</sup></b>	<b>10cm<sup>2</sup></b>	<b>25 cm<sup>2</sup></b>	<b>75 cm<sup>2</sup></b>	<b>175 cm<sup>2</sup></b>
<b>Volume of Media added</b>	100 µL	500 µL	2 mls	5 mls	10 mls	30 mls

Table 1.1 Cell culture flasks

## 2.2 Drug treatments

Two pharmacological inducers of ER stress were used over the course of this study, Thapsigargin (TG) and Tunicamycin (TM).

### 2.2.1.1 *Thapsigargin*

TG is a well-established inducer of ER stress. TG induces ER stress by selectively inhibiting the  $\text{Ca}^{2+}$ -ATPase responsible for  $\text{Ca}^{2+}$  accumulation by the ER. Mobilization of ER-sequestered  $\text{Ca}^{2+}$  to the cytosol and to the extracellular fluid subsequently ensues. Depletion of ER  $\text{Ca}^{2+}$  perturbs optimal functioning of ER-resident chaperones and the increase in cytosolic  $\text{Ca}^{2+}$  leads to apoptosis. TG (Sigma-Aldrich, T9033) was re-suspended to a stock concentration of 4 mM/ml in DMSO. PC12 and MEFs were treated with 0.25  $\mu\text{M}$  TG unless otherwise stated. HeLa cells were treated with 1.5  $\mu\text{M}$  TG (Samali et al, 2009).

### 2.2.1.2 *Tunicamycin*

Tunicamycin (TM) inhibits N-glycosylation in eukaryotes by blocking the transfer of GlcNAc-1-P from UDP-GlcNAc to dolichyl-P. Proteins that are not glycosylated remain trapped in the ER leading to accumulation of mis-folded proteins within the ER lumen. TM (Sigma-Aldrich T 7765) was re-suspended to a stock concentration of 2 mg/ml in DMSO and stored at  $-80\text{ }^{\circ}\text{C}$ . PC12 cells were treated with 2  $\mu\text{g}$  TM. HeLa cells were treated with 1.5  $\mu\text{g}$  TM (Samali et al, 2009).

### 2.2.1.3 *Control treated cells*

TG and TM were made up in DMSO therefore untreated control cells were generated by treating cells with DMSO made up in a similar manor as drugs were made. e.g PC12 were treated with 0.25  $\mu\text{M}$  TG. TG was diluted 1:20 in medium and cells were then treated with appropriate volume of drug to generate final required concentration. For untreated controls DMSO was also first diluted 1:20 in medium and the same volume as was used to treat cells was added to untreated control.

### 2.2.2 Heat shock preconditioning

Cell culture media was replaced with DMEM buffered with 10  $\mu$ M HEPES. 1M stock of HEPES at pH 7.4 was made up in water, aliquoted and stored at -20 °C. 10  $\mu$ l of 1M stock was added per mL of culture media, i.e 50  $\mu$ L to 5 mL in a T25 flask (See table 1.1 for details). It is added to the media to provide extra buffering capacity as cells are outside of the CO<sub>2</sub> incubator for an extended period of time. The culture flask lids were replaced with non-vented lids and were sealed by wrapping parafilm to prevent water from the water bath entering the flasks. Flasks were then placed in a swirling water bath at 42°C for 1 h after which culture media was replaced with 37°C normal culture medium (HEPES free). Cells were allowed to recover at 37°C for 6 h unless otherwise stated.

### 2.3 Transformation of plasmids

Transformation is a technique used to introduce foreign DNA in the form of a plasmid into competent bacterial cells. For the generation of all plasmids, DH5 $\alpha$  E. coli cells were used (Generated in Afshin Samali's Lab, BRB, NUIG). 1 to 5  $\mu$ L of plasmid DNA (usually 10pg to 100ng) was added to 20-50 $\mu$ L of competent cells in an eppendorph tube. Plasmid DNA was combined with the cells and incubated on ice for 30 min. The cold temperature aids the permeabilisation of the cells to ready them for taking up DNA. The mixture was then heat shocked at 42°C for 40 sec. Cells were placed on ice for 2 min and combined with 480  $\mu$ l of Luria-Bertani (LB) Broth. Cells were allowed to recover and to grow at 37°C for 1 h. The cells were spread on LB agar plates containing either 50  $\mu$ g/mL Kanamycin or Ampicillin (Sigma-Aldrich) and grown overnight at 37°C. The next day, bacteria from a single colony, were selected and inoculated into fresh LB broth plus antibiotic. Cells were incubated for approx. 8 h at 37°C with vigorous shaking (approx. 300 rpm). The resulting culture was centrifuged at 5000 g for 20 min to pellet the bacterial cells

containing the plasmid of interest. Plasmid extraction was carried out using Qiagen maxi-prep kit as per manufacturer's guidelines (Quiagen, Cat. No. 12163).



## 2.4 Transfections

### 2.4.1 DNA transfections

For the transfection of PC12 cells, Lipofectamine (Fermentas) was used. It is a cationic polymer-based transfection reagent that forms positively charged complexes with DNA to allow efficient delivery of DNA into cells. 2mL of cells were seeded at 200,000 cells/mL in a 6 well plate. 24 h after seeding the culture media was replaced with DMEM with FBS minus pen/strep and then transfected with the plasmid of interest in a 1:2 ratio of DNA to Lipofectamine or 0.75 µg DNA: 1.5 µL Lipofectamine for a 6 well plate. The DNA-Lipofectamine complex was mixed by gently pipetting and incubated at room temperature for 20 min. The complex was added drop by drop to the cells and mixed gently by swirling the culture plate. The plate was incubated for 5 h at 37°C after which time the media was changed. 24 h later, the cells were used for experiments.

### 2.4.2 siRNA transfections

Darmacon siGENOME siRNA against BIM (ref no. M093533-01-0005) and a non targeting control (D-001206-13-05) were obtained from Fisher Scientific.(Dublin, Ireland) siRNAs were resuspended in Molecular grade water (Sigma-Aldrich, S4502) to a stock concentration of 40 µM and were stored at -80 °C.

For siRNA transfection of PC12 cells Lipofectamine (Fermentas) was used. 500 µl of cells at 200,000 cells/mL were seeded in PLL coated 24 well plate. 24 h later media was replaced with 450 µl DMEM with FBS minus pen/strep. Culture were then transfected with siRNA of interest in a 1:2 ratio of DNA to Lipofectamine or 1 µL of 10 µM siRNA to 2 µL Lipofectamine in 47 µL of serum-free medium. Therefore the final concentration of siRNA was 20 nM siRNA/ well. The DNA-Lipofectamine complex was mixed by gentle pipetting and incubated at room temperature for 20 min. The complex mixture was added dropwise to the cells and the resulting mixture was gently mixed by swirling the plate. The plate was then incubated for 5 h at 37°C. The medium was changed and 24 h later cells were used for experiments.

## 2.5 Flow Cytometry

### 2.5.1 Harvesting cells for flow cytometry

To harvest cells, culture medium was removed and placed in the labelled ependorfs. Cells were washed with 500  $\mu$ L of Hank's Balanced salt solution. Hanks solution was removed and 500  $\mu$ L of 1x trypsin was added to each well. Cells were put at 37°C for 5-7 min to detach cells from culture plate. Trypsinized cells were then recombined with media in ependorfs. This ensures all cells both floating 'dead' cells and attached 'live' cells were collected. Cells were allowed to recover from trypsinization procedure for 15 mins at 37°C. After this time the cells were prepared according to the specific flow cytometry assay.

### 2.5.2 Sub-G1 analysis of DNA fragmentation

Caspase activated endonucleases ndonucleases degrade DNA in small fragments of about 180 bp in the latter stages of apoptosis. Consequently, large numbers of small fragments of DNA accumulate in the cell. If cells are fixed in ethanol and subsequently rehydrated, apoptotic cells can be observed as a fractional (sub-diploid) DNA or 'sub-G1' peak in a DNA histogram. Organic solvents such as ethanol remove lipids and dehydrate the cells, while precipitating the proteins on the cellular architecture and are suitable for subsequent analysis of nucleic acids.

Cells were seeded in duplicate in a 6 well plate and treated as previously described in section 2.1. Cells were harvested as previously detailed in section 2.4.1. Cells were centrifuged at 0.3 *g* for 5 min. Cells were washed in 1 x phosphate buffered saline (PBS) and re-suspended in 150  $\mu$ L of PBS. Cells were fixed by adding 350  $\mu$ L of ice-cold 100 % ethanol drop-wise, while gently vortexing. Cells were placed on ice for a minimum of 1 h for efficient fixation. Fixed cells can be stored at -20 for a maximum of 2 weeks or be analysed immediately. Once fixed, cells were stained with 20  $\mu$ g/mL propidium Iodide (PI) for 30 min in the dark on ice. Cells were then analysed using a FACSCanto flow cytometer (BD Biosciences) with a total of 10,000 events acquired per sample. Analysis was carried out with Cyflogic software.

### 2.5.3 Annexin V staining

During apoptosis, phosphatidyl serine (PS) residues, which are normally located on the internal surface of the plasma membrane, redistribute to the external surface. PS binds the protein, Annexin V, and the change can be observed by incubating unfixed cells with fluorescein isothiocyanate (FITC) labelled Annexin V which is generated in our laboratory (Afshin Samali Lab, BRB, NUIG).

Cells were seeded in duplicate in a 24 well plate and treated as previously described in section 2.1. Following treatment, cells were harvested as previously detailed in section 2.4.1 and collected by centrifugation at 0.3 *g* for 5 min. The supernatant was removed and cells were washed with 1 mL of ice cold 1X PBS. Cells were centrifuged again, the supernatant was removed and cells were re-suspended in 50  $\mu$ L binding buffer (10 nM HEPES, pH 7.5, 140 mM NaCl, 2.5 mM CaCl<sub>2</sub>) containing 1  $\mu$ L of home-made FITC labelled Annexin V and incubated in the dark on ice for 15 min. Cells were analyzed on a FACSCanto flow cytometer with a total of 10,000 events acquired per sample. Analysis was carried out using Cyflogic software.

### 2.5.4 Measurement of $\Delta\Psi_m$ by TMRE staining

Loss of mitochondrial membrane potential,  $\Delta\Psi_m$ , occurs during apoptosis and this can be measured by flow cytometry. Tetramethylrhodamine ethyl ester (TMRE) is a cell permeable, positively-charged, red-orange dye that readily accumulates in active mitochondria due to their relative negative charge. Depolarized or inactive mitochondria have decreased  $\Delta\Psi_m$  and fail to sequester TMRE. Carbonyl cyanide *m*-chlorophenyl hydrozone (CCCP) is a ionophore un-coupler of oxidative phosphorylation and therefore destroys  $\Delta\Psi_m$ .

Cells were seeded in duplicate in a 24 well plate and treated as previously described in section 2.1. To generate a positive control, cells were treated with 40  $\mu$ M CCCP for 3 h prior to harvesting. After treatment, floating cells were collected and the remaining cells were 'trypsinized' and combined with floating cells in

ependorfs. Cells were allowed to recover at 37°C for 15 min, incubated with 100 nM TMRE at room temperature for 30 min in the dark and analysed by flow cytometry in the PE (FL-2, 564-601 nm) channel using a FACScanto flow cytometer. Analysis was carried out using Cyflogic software.

## 2.6 Analysis of type II caspase activity

DEVDase assay allows for detection of type II caspase activity due to specific cleavage of a fluorogenic substrate (N-Acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin or Ac-DEVD-AMC) by these caspases. During the assay, activated type II caspases cleave the substrate between DEVD and AMC, generating highly fluorescent AMC that can be detected using a fluorescence reader. Cleavage of the substrate only occurs in lysates of apoptotic cells; therefore, the amount of AMC produced is proportional to the number of apoptotic cells in the sample.

Cells were seeded in a t25 flask and treated as previously detailed in section 2.1 (see table 1.1). Cells were scraped with a cell scraper, harvested and pelleted by centrifugation at 1,500 RPM. After washing in PBS, the cell pellets were re-suspended in 50  $\mu$ L of ice cold PBS and 25  $\mu$ L aliquot from each sample was transferred to duplicate wells of a microtiter plate and frozen at -80 °C. To initiate the reaction, add 50  $\mu$ L of 50  $\mu$ M of the caspase substrate carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-methyl-coumarin (DEVD-AMC, Peptide Institute Inc.) in assay buffer (100 mM HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS, 5 mM dithiothreitol (DTT) and 0.0001% Igepal-630, pH 7.25) was added to cell lysates. Liberated free AMC was measured by a Wallac Victor 1420 Multilabel counter (Perkin Elmer Life Sciences) using 355 nm excitation and 460 nm emission wavelengths at 37 °C at 60 sec intervals for 25 cycles. After the measurement Bradford assay was performed on the lysates to determine protein concentrations. Briefly, 2  $\mu$ L of lysate was added to 200  $\mu$ L of Bradford reagent in a 96 well plate in triplicate. The plate was measured immediately at 595 nm. The data were analyzed by linear regression and enzyme activity was expressed as nm of AMC released  $\times$  min<sup>-1</sup> $\times$ mg<sup>-1</sup> total cellular

protein. Statistical analysis was carried out on duplicate measurements of two independent repeats.

## 2.7 mRNA expression analysis

### 2.7.1 RNA preparation

For RNA analysis experiments cells were seeded and treated as detailed in 2.1 in a t25 flasks (see table 1.1). Cells were scraped with a cell scraper, collected and centrifuged at 1500 rpm for 5 min. The pellet was then re-suspended in 500  $\mu$ L (Sigma-Aldrich, #9424) of TRI reagent. To separate RNA from DNA and protein, 100  $\mu$ L of molecular grade chloroform (Sigma-Aldrich #496189) was add, mixed well and incubated for 2 min at room temperature. Samples were centrifuged at 12,000  $g$  for 15 min. The upper clear phase was carefully pipetted into freshly autoclaved 1.5 mL tubes, with special care taken not to disturb the interface. To precipitate the RNA, 250  $\mu$ L of isopropanol was added to each sample and incubated at  $-20^{\circ}\text{C}$  overnight. Samples were then centrifuged at 12,000  $g$  for 10 min and the RNA pellet was washed in 75 % Ethanol. All of the ethanol was carefully removed and the pellet was air dried to allow all traces of ethanol to evaporate. The RNA was re-suspended in 25  $\mu$ L of sigma water and stored at  $-80^{\circ}\text{C}$  until ready to use. RNA quality and quantity was determined by spectrophotometry using a Nanodrop reader.

### 2.7.2 Reverse transcription (RT)-PCR

To synthesise cDNA, 2  $\mu$ g of RNA was subjected to DNase treatment with 1  $\mu$ L DNase (Invitrogen) and 1  $\mu$ L 10X Buffer (Invitrogen) added per reaction and incubated for 15 min at room temperature. The DNase was inactivated using 1  $\mu$ L of 25 mM EDTA and incubated at  $65^{\circ}\text{C}$  for 8 min. DNase treatment is conducted to ensure there is no contaminating genomic DNA in final cDNA preparation.

The RNA was then reverse transcribed into cDNA using the Superscript II first strand RT-PCR system and oligo Dts (Invitrogen, No. 18418-012). To each reaction 1  $\mu$ L of a

1 $\mu$ g/ $\mu$ L oligo DTs solution was added to each sample, which was mixed well, and heated at 65°C for 2 min, and then 42°C for 2 min. First strand mastermix consisting of molecular grade water, first strand buffer, 5 mM DTT, 5 mM dNTPs and 1  $\mu$ L of superscript II enzyme was added to each sample and mixed well. The samples were kept at 42°C for 50 min followed by 75°C for 10 min.

Conventional PCR for GAPDH was carried out using 2  $\mu$ l of cDNA to check cDNA quality with 3  $\mu$ L of forward and reverse primer, 4.5  $\mu$ L of sigma water and 12.5  $\mu$ L of 2x Go Taq mastermix (Promega) to a final volume of 25  $\mu$ L. Primers to amplify GAPDH:

GAPDH Fwd 5' ACCACAGTCCATGCCATC 3' and

GAPDH Rev 5' TCCACCCTGTTGCTG 3'.

PCR cycle conditions: 94°C for 3 min, 94°C for 30 sec, 55°C for 30 sec (annealing temp), 72°C for 30 sec, 72°C for 7 min, 4°C for  $\infty$ . Repeat steps 2-4 for 25 cycles.

Anneling temperature was calculated as the primer TM- 3°C and was confirmed by temperature gradient PCR to determine optimal annealing temperature.

### **2.7.3 Real time Quantitative RT-PCR**

Real time QRT PCR is a simple and yet quantitative way of determining differences in gene expression between samples. Taqman chemistry was used for this study which allows for the avoidance of non-specific amplification during PCR and does not require primer optimization steps. PCR using Taqman chemistry, also referred to as the 5' nuclease assay, enlists the participation of forward and reverse primers designed to amplify the target of interest and a MGB (Minor groove binder) probe which hybridizes to the target, between the two primers. It is this probe that allows for Real time detection of product amplification. The probe has a fluorescent reporter dye attached to its 5' end and the MGB and Non Fluorescent Quencher (NFQ) dye tagged at its 3' end. As DNA polymerase amplifies the target it begins to cleave the probe separating the fluorescent dye from the NFQ allowing release of a fluorescent signal detected by the qRT-PCR machine. For qRT-PCR, cDNA is synthesised and diluted to a concentration of 40 ng per 10  $\mu$ L reaction (See section

2.6.2); it is then combined with 5  $\mu\text{L}$  Brilliant III Ultra-Fast QPCR Master Mix (Agilent), 0.5  $\mu\text{L}$  of 20X Taqman gene expression assays (Applied Biosystems and Integrated Dna Technology), 0.15  $\mu\text{L}$  of diluted reference dye and 1.85  $\mu\text{L}$  of sigma water per reaction and accurately dispensed to a fast optical MicroAmp 96-well plate (Applied Biosystems) in triplicate. The PCR is run for 40 cycles on an Applied Biosystems fast 7500 machine using the following cycling conditions: Hold at 95  $^{\circ}\text{C}$  for 3 min, then 40 cycles at 95  $^{\circ}\text{C}$  for 12 sec, and 60  $^{\circ}\text{C}$  for 30 sec.

## 2.8 *In vivo* Chaperone Assay

Double stable PC12 cell lines expressing luciferase and HSPB1 or Neo (See section 2.1.1 for additional info) were seeded in triplicates as previously described in section 2.1 in three different 24-well plates (control-not heat shocked plate, heat shock recovery plate, heat shock no recovery plate). After 24 h the medium from each well in the three plates was replaced by 500  $\mu\text{L}$  of fresh DMEM supplemented with 20 mM MOPS and 40  $\mu\text{g}/\text{mL}$  cycloheximide. Cells were put back for 30 min in the 37  $^{\circ}\text{C}$  incubator to ensure complete inhibition of translation by cycloheximide. Subsequently, two plates were heat-shocked for 30 min at 44 $^{\circ}\text{C}$  in a swirling water bath while one was kept in the 37 $^{\circ}\text{C}$  incubator as a non-heat-shocked control. After the heat shock, one of the plates was allowed to recover at 37 $^{\circ}\text{C}$  for 180 min (Heat shock recovery plate). The second plate was used a heat shock no recovery plate therefore immediately after heat shock medium was removed, and cells were frozen at  $-80^{\circ}\text{C}$ . After the 180 min recovery time, medium was removed from the third plate, and cells were frozen at  $-80^{\circ}\text{C}$ . Luciferase activity was measured using the Promega Dual Glo reporter assay system (Promega E1960) following the manufacturer's instructions as detailed below. The luciferase activity was expressed as a ratio of the luminescence between the heat-shocked conditions and the non-heat-shocked control. Statistical significance was determined using a two-way ANOVA and Tukey Post-hoc analysis using GraphPad Prism Software

## 2.9 Luciferase reporter Assays

Luciferase is a commonly used reporter enzyme which allows the study of gene expression, promoter region activity, intracellular signalling and protein folding. The Promega Dual Glo reporter system (E1960) allows simultaneous measurement of Luciferase and another bioluminescent molecular, Renilla, within the same sample. This allows normalization of Luciferase activity, which is driven by alterations in gene expression, to Renilla which reflects baseline activity. This highly sensitive assay is based on conversion of the chemical energy of luciferin oxidation through electron transition from the product molecule oxyluciferin. Firefly luciferase catalyzes luciferin oxidation using ATP-Mg<sup>+2</sup> as a co-substrate. This reaction produces light proportional to the activity of luciferase.

To carry out expression analysis using the luciferase reporter assay, cells were seeded and treated in a 24 well plate as detailed in section 2.1. For chaperoning assay cells used stably overexpressed luciferase and therefore did not need to be transfected prior to treatments (Section 2.7) For analysis of BIM 3'UTR cells were seeded and 24 h later transfected as outlined in section 2.3.1 with psiCHECK control plasmid or a BIM 3'UTR plasmid. To harvest samples for luciferase assays cell culture media was removed and cells were washed with 500  $\mu$ L 1X PBS. Cells were lysed in 1X passive lysis buffer (PLB) which is supplied as a 5X stock in the promega kit. 100  $\mu$ L volume of PLB was added to cells in each well of 24 well plate. Lysis was carried out by leaving plates on a rocker for 30 min. 20  $\mu$ L aliquote of lysate was transferred by pipetting to to white microwell 96 well plates. White plates provide maximum reflection and minimum auto-fluoresence and autoluminescence. A 100  $\mu$ L volume of Luciferase Assay reagent was added to each wells and luminescence was measured for 10 sec, at a 2 sec inter sample delay. Results were recorded and 100  $\mu$ L of Stop and Glo was added to terminate Luciferase activity and measure Renilla activity.



## 2.10 Cell Viability Assay

The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazonium bromide) assay is based on the principle that viable cells have active mitochondria. The mitochondrial activity of cells can be assayed by monitoring the conversion of MTT to formazan crystals which are solubilised by addition of SDS and dimethyl formamide. Therefore a decrease in the cell viability is inversely proportional to the formazan concentration which can be measured as OD on a plate reader.

For MTT analysis 100  $\mu$ L of cells at the appropriate density (See section 2.1) (200,000 cells/ml for PC12s i.e 20,000 cells/ well in a 96 well plate) were seeded in triplicate. Wells were set up for untreated, blanks and each treatment condition. Outer wells of the plate were not seeded with cells but with Hank's balanced salt solution. After 24 h cells were treated by adding in 2  $\mu$ L of drug at an appropriate concentration to yield final desired concentration (see section 2.1). For example 2  $\mu$ L of 4 mM TG was diluted in 640  $\mu$ L of DMEM to generate an experiment stock of 12.5 mM. Cells were treated by adding 2  $\mu$ L of experiment stock to 100  $\mu$ L of culture media. Following treatment, 10  $\mu$ L of MTT (5 mg/ml stock dissolved in Hank's stored at 4°C until required ) was added to the wells and incubated at 37°C for 3 h. The reaction was stopped by adding 100  $\mu$ L of stop mix solution containing 20% w/v SDS in 40% dimethyl formamide. Plates were placed on an orbital shaker over night to allow the formazan precipitate to dissolve. The colour intensity is measured at 550 nm and the percentage cell viability is calculated using the untreated samples as the reference which is set 100% viability and expressing the viability of treated samples relative to the control.

## 2.11 Protein expression analysis

### 2.11.1 Western Blotting

Western blotting is a well-established technique used in research to separate, identify and assess protein expression. Proteins from a wide variety of biological samples can be separated based on molecular weight through gel electrophoresis. These protein are then transferred to a membrane producing a band for each protein. The membrane is then incubated with antibodies specific to the protein of interest.

#### *2.11.1.1 Preparation of samples for Western Blotting*

Cells were seeded in a 6 well plate and treated as previously described in section 2.1. Following treatment, cells were scraped, collected in appropriately sized tubes and centrifuged at 5000 g for 5 min. The supernatant were removed and 1 mL of PBS was added to each pellet and the mixture centrifuged again. PBS was removed and cell pellets were frozen at -80°C. Time course samples were treated at the same time and harvested at the appropriate time point. All samples were lysed at the same time using in RIPA buffer (50 mM Tris-HCl, pH 8.8, containing 150 mM NaCl, 0.5 % NaDeox, 0.1% SDS, 1% NP-40). Samples harvested from a 6 well plate were lysed in 46 µL of RIPA buffer containing a cocktail of protease inhibitors ( PMSF 1 µM, Pepstatin 1 µg/ml, Leupeptin 10 µM, Aprotinin 2.5 µg/ml and ALLN, 250 µM) and phosphatase inhibitors ( 10 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>). Samples were missed by vortexing and left on ice to lyse for 30 min. Protein concentration was determined by BCA assay (Pierce #23227). Briefly 2 µL of protein lysate was added in triplicate to a clear flat bottomed 96 well assay plate. A standard curve was generated using a 1 mg/mL bovine serum albumin (BSA) stock. 200 µL of BCA assay reagent was added to each well and the plate was incubated at 37°C for 30 min. Absorbance was measured at 590 nM. 10-20 µg of protein was prepared as required for denaturing SDS-PAGE (Section 2.10.1.2) by adding the appropriate volume of 5x Laemmli's buffer (60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) and incubating for 5 min at 96°C.

### 2.11.1.2 SDS-PAGE gel electrophoresis

Proteins were separated on 12% SDS- polyacrylamide gel. The proteins were transferred onto a nitrocellulose membrane with CAPS transfer buffer. Membranes were blocked with 5% non-fat milk for 1 h at room temperature. Membranes were probed with specific antibodies for Actin (Sigma, A2066), AKT (Cell Signalling Technologies #9272), Caspase-3 (Cell Signaling Technologies, #9662) Cleaved Caspase-3 (Cell Signalling Technologies 9664), Caspase-9 (Cell Signalling Technologies #9058) CHOP (Santa Cruz, sc793), eIF2 $\alpha$  (Cell Signalling Technologies, #9722), ERK (Cell Signalling Technologies #4696), HSPB1 (Enzo, ADI-SPA-801-F), Human specific HSPB1 (Enzo, ADI-SPA-803.-F), HSPA1 (Stressgen, SPA811), HspA5 (Stressgen, SPA826), ), HSF1 (Cell Signalling Technologies # 4356), JNK (Cell Signalling Technologies #9258), Mcl-1 (Cell Signalling Technologies #4572), NOXA (Calbiochem OP-180), PERK (Cell Signalling Technologies #3192), PUMA (Cell Signalling Technologies #4976), Phospho-AKT(Cell Signalling Technologies #9271), Phospho-BIM (Cell Signalling Technologies #4581) Phospho-ERK (Cell Signalling Technologies #9101), Phospho-JNK (Cell Signalling Technologies #9255), XBP1 (Bioledgend, 619501). Goat secondary antibodies conjugated to horseradish peroxidase were from Jackson Labs.

### 2.11.1.3 Densitometric analysis

Quantative analysis of western blotting results was carried out using densitometric analysis with ImageJ software. Expression was normalized to the loading control (ACTIN) and expressed relative to untreated sample.

### **2.11.2 Immunostaining**

Cytocentrifuge preparations of PC12 cells were fixed on slides (FischerBrand, 12-550-003) in 3.7% formaldehyde for 5 min at room temperature and then permeabilised with 0.2% Triton X-100 for a further 5 min at room temperature. Free antibody binding sites were blocked for 1 h at room temperature with PBS containing 5% goat serum and 1% BSA followed by 1 h incubation with the primary antibody (rabbit anti-HSP27, diluted 1:1000 with 1% BSA in PBS). Excess antibody was removed by 3×5 min washes in PBS. Then the slides were incubated with goat anti-rabbit IgG-Alexa-546 conjugate (diluted 1:200 in 1% BSA in PBS) for 1 h at room temperature. Unbound antibodies were removed by 3×5 min washes in PBS before mounting the slides with Vectashield containing the nuclear stain DAPI. Cells were visualized on IX51 Olympus microscope

### **2.11.3 Assessment of Protein Half-Life**

PC12 cells were seeded at 200,000 (2 mls) in PLL coated 6 well plates. 24 h later cells were treated with TG for 24 h after which time media was removed and replaced with fresh media containing 10  $\mu$ M cyclohexamide (CHX) for 0.5-3 h. At this time TG control and UT cells were harvested. Cyclohexamide treated cells were harvested after 0.5,1,2 and 3 h. Cells were harvested and analysed by western blot as previously described in section 2.11. Quantative analysis of protein expression was carried out using densitometric analysis normalized to loading control (ACTIN) (See section 2.11.1.3). Data was fit to a monoexponential decay curve using Microsoft excel where TG treated samples were set to 100%.

### **2.11.4 Assessment of oligomeric status following ER stress**

Cells were seeded and treated in a t25 as previously detailed in section 2.1. 4 separate flasks were set up, untreated, TG treated, TM treated and heat shocked. 24hrs later 2 flasks were treated with ER stress-inducing agents TG and TM or DMSO control as previous detailed for 12 h. As a positive control, cells were heat

shocked at 42°C for 1 h and allowed to recover for 2 h in fresh media. Cells were harvested by scraping cells with a cell scraper into media, collecting and centrifuging at 1500 rpm for 5 min, washed 1x in 5 mL of PSB. The supernatant was removed and cells were washed again in 2 mL of PSB. Cells were lysed in 100 µL of lysis buffer (25 mM HEPES, pH 7.4, 3.3% glycerol, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF) and snap frozen with liquid nitrogen and thawed at 37°C. Lysates were centrifuged at 14,000 rpm for 2 min and supernatants was transferred to new tubes. Lysates were chemically cross-linked by incubating with 1 volume of 0.1% glutaraldehyde for 40 minutes at 30 degrees. The cross-linking reaction was stopped by adding one volume of stopping buffer ( 1M Tris-HCl containing 10% SDS and 10 mM EDTA). Bromophenol blue was added to sample and equal volumes of lysate were run on 12 % SDS-polyacrylamide gel (see section 2.11.1) . Crossed-linked HSPB1 species were detected by immunoblotting with antibody to HSPB1 (Lambert et al., 1999).

## 2.12 Statistical analysis

One-way ANOVA followed by Tukey–Kramer multiple comparisons test were used for protein expression data (Densitometry) analysis for Fig 3.2.1, 3.2.2. Two-way ANOVA followed by Tukey–Kramer multiple comparisons test were used for protein expression data (Densitometry) analysis for Fig 3.2.3, 3.2.4, 3.2.5 flow cytometry data analysis. Two-way ANOVA followed by the Tukey–Kramer multiple comparisons test was used to determine significance in chaperoning assay experiments. For all tests, significance was set at  $p < 0.05$ .

## **Chapter 3**

**Cross-talk between different cellular stress response pathways: heat shock preconditioning attenuates ER stress induced cell death**

### 3.1 Introduction

Disruption to cellular homeostasis causes cellular stress, which, if unresolved leads to cell death. Maintaining cellular homeostasis is achieved by cell stress responses including the HSR and the UPR (Kultz, 2005, Fulda, 2010). Both stress response pathways are characterized by transcriptional induction of molecular chaperones which aim to restore cellular homeostasis and prevent proteotoxic stress (Hutt et al., 2009). Molecular chaperones are crucial for maintenance of proteostasis as they facilitate protein folding, prevent aggregation, as well as enhance ubiquitination and proteasomal degradation (Balch et al., 2008).

Heat shock proteins are induced by a wide variety of noxious stimuli, all of which are associated with the accumulation of misfolded proteins, these include ischemia/hypoxia (Kinouchi et al., 1993), oxidative stress (Gomer et al., 1996), energy depletion (Mosser et al., 1990), heavy metals (Bauman et al., 1993), and response to proteasomal inhibition (Bush et al., 1997). This has led to the proposal that the HSR is primarily a cellular response to the accumulation of unfolded proteins. In addition to enhancing expression of HSP, cell stress also leads to post-translational alterations that modify cellular location and activity of HSPs (Lavoie et al., 1995, Keiichi Ishihara, 2003, Rafiee et al., 2003, Mattoo et al., 2013).

As mentioned in Chapter 1, in mammalian cells accumulation of unfolded proteins in the ER leads to activation of PERK, IRE1 and initiates a transcriptional program, the UPR, which enables cells to cope with cell stress (Gorman et al., 2012a). The outcome of the cell's response to ER stress depends on the severity and duration of the stress. Initially cells mount a pro-survival response; however, if the effects of the stressor cannot be resolved, cell death ensues. Stress-induced cell death is primarily executed via the intrinsic apoptotic pathway leading to mitochondrial outer membrane permeabilization (MOMP), dissipation of mitochondrial-innermembrane potential ( $\Delta\Psi_m$ ) and release of pro-apoptotic factors, including cytochrome c (Samali, 2001, Gupta et al., 2009). This leads to activation of caspase-

9 and caspase-3. The BCL-2 family proteins are the key regulators of MOMP and therefore represent critical regulators of cell fate (Kultz, 2005, Fulda, 2010). BCL-2 homology domain 3 (BH3)-only proteins such as BIM and NOXA have been shown to be central mediators of ER stress-induced apoptosis in a number of cell lines, and RNAi-mediated knockdown of either protein shows significant protection against ER stress-induced cell death (Szegezdi et al., 2008, Zhang et al., 2012). NOXA has been shown to be critically involved in heat-induced apoptosis (Stankiewicz et al., 2009). Cells can be protected against heat-induced apoptosis via modulation of apoptotic events upstream of the mitochondria by HSPA1 overexpression (Stankiewicz et al., 2005). HSPB1 has also been shown to modulate events upstream of the mitochondria (Samali, 2001) by promoting the phosphorylation and inactivation of the BH3-only protein BAD (Zoubeidi et al., 2013). A complex relationship exists between the UPR and the HSR. Both pathways are responses to mis-folded proteins, and studies in yeast have shown that the HSR can partially compensate for a defective UPR in IRE1 mutant cells (Liu and Chang, 2008). Genomic analysis of HSR target genes revealed a 25% overlap with the UPR response genes (Liu and Chang, 2008). However, depending on the severity and duration of the thermal stress imposed, the HSR can either activate or suppress the UPR. This is a characteristic feature of hormesis, a term first coined in the 1940s (Mao and Franke, 2013). Hormesis is an adaptive response of cells and organisms to a moderate stress resulting in protection against a subsequent stressor. A classic example of hormesis is thermotolerance, or thermal preconditioning (TP), whereby cells subjected to a mild bout of thermal stress are subsequently protected against a more severe insult (Henle et al., 1979). This is also true for pathological stressors, for example a brief period of ischemia can mediate protection against a subsequent long term ischemic insult (Hausenloy and Yellon, 2011). Interestingly cells exposed to a stress stimulus can also develop cross-tolerance to a different stress stimulus (Lindquist, 1986, Cheng et al., 2011). For example TP can protect PC12 cells from the neurotoxin MPP<sup>+</sup> (Quigney et al., 2003). The protective effects of TP are not restricted to cell culture models. TP has been shown to be beneficial in preventing thrombosis (Li et



al., 2012) and ischemia/reperfusion-induced lung injury (McCormick et al., 2003) . The protection afforded by thermal preconditioning is linked to expression levels of HSPs as the survival advantage is abrogated by inhibitors of HSPs such as triptolide (Shu-Fen Hsu, 2013). We have also shown that overexpression of HSPA1 can protect cells against ER stress-induced cell death (Gupta et al., 2010). Given the apparent overlap between the HSR and the UPR, we sought to characterize the potential crosstalk between the two pathways and the effect of such a phenomenon on cell fate.

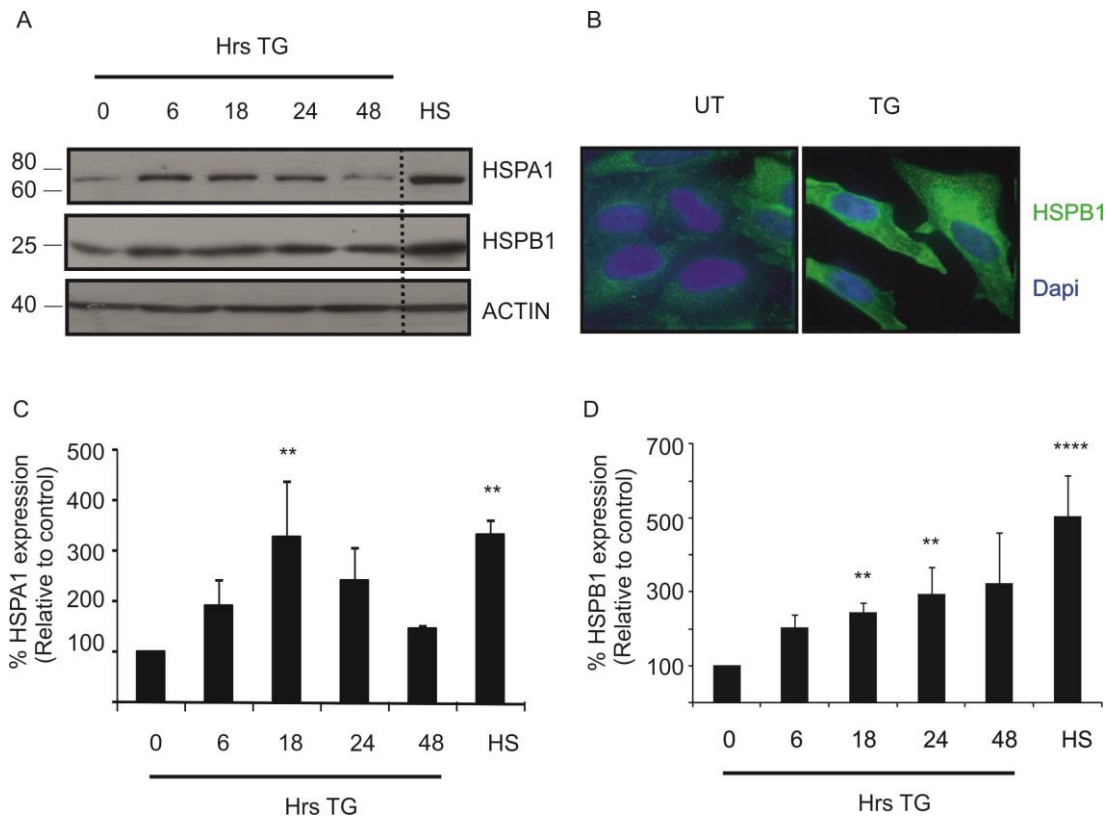
Previous work from our lab reported that a mild heat shock (HS) preconditioning protects cells against variety of cytotoxic agents that otherwise induce apoptosis. We therefore, investigated whether HS preconditioning can block ER stress-induced apoptosis. Cells were heat-shocked for 1 h at 42°C. This led to a rapid induction of HSPA1 (Hsp70) HSPB1 (HSP27). Levels of these proteins remained elevated for at least 24 h post-HS. Treatment of cells with ER stress inducing chemicals, thapsigargin (TG) and tunicamycin (TM) resulted in cell death. HS pre-treatment, for 6 h prior to addition of TG/TM, significantly the induction of cell death. The inhibition of apoptosis was associated with reduced levels of the BH3-only protein, BIM. Overexpression of HSPB1, but not HSPA1, could mimic the effect of HS on expression levels of BIM. We conclude that HS is protective against ER stress-induced cell death, partly through HSPB1-mediated reduction BIM. Furthermore we demonstrated that ER stress inducing agents could cause induction of the HSR as exemplified by induction of HSPs. Activation of the HSR by ER stress provides pro-survival signalling as in the absence of HSF1 cells are more sensitive to ER stress-induced apoptosis. These findings also provide further evidence for a cross talk between stress response pathways.

## 3.2 Results

### 3.2.1 TG caused induction of HSPs in HeLa cells

Given that ER stress is a potent inducer of mis-folded proteins we tested whether HSPs are induced by classical ER stress inducing agents TG (See section 2.1.4.1 for details). Treatment of HeLa cells, a human cervical cancer cell line, with 1.5  $\mu$ M of the ER stress inducing agent, thapsigargin (TG), caused an gradual increase in the expression of HSPA1 which peaked at 18 h and returned to base line levels by 48 h (Fig. 3.2.1 A). HSPB1 was also induced by TG treatment in HeLa cells (Figure 3.2.1A). Cells which were heat shocked at 42°C for 1 h, followed by 24 h recovery at 37°C, were used as a positive control for HSP expression.

Induction of HSPB1 by TG was further confirmed by immunofluorescence analysis of untreated cells, or cells treated with 1.5  $\mu$ M TG for 24 h which causes approx. 20% cell death at 24 h (Fig. 3.2.1B). Induction of HSPA1 and HSPB1 was determined by Western blotting and quantified by densitometry using ImageJ software. It was found that TG treatment caused a transient increase in HSPA1 expression which reached a maximal expression level of 3.3-fold  $\pm$  0.6 at 18 h, which returned to basal expression levels by 48 h (Fig. 3.2.1C). The increase in HSPA1 expression at 18 h was comparable to cells that were heat shocked. HSPB1 induction following TG treatment was also increased by 2.2  $\pm$  0.3 fold after 24 h; however, 48 h after treatment there was no significant difference between untreated and treated cells. Heat shock treatment caused a 5.0  $\pm$  1.1 fold increase in HSPB1 expression and a 3.3  $\pm$  0.1 fold increase in HSPA1 (Fig. 3.2.1D). Therefore TG causes a transient increase in HSPs to a level which is comparable to heat shock.

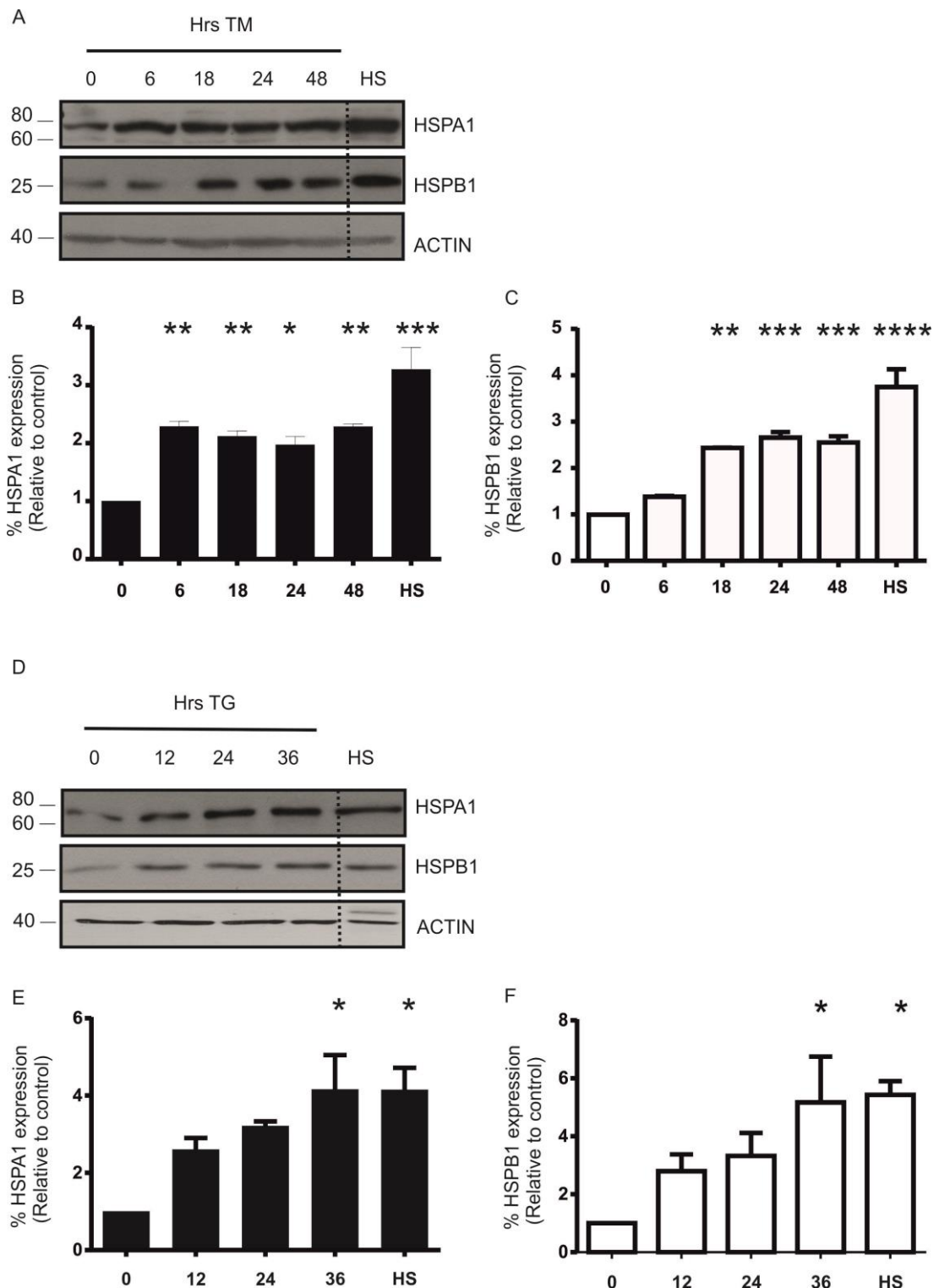


**Fig. 3.2.1 TG induces HSP expression in HeLa cells.** (A) HeLa cells were treated for 0, 6, 12, 24, 48 h with 1.5  $\mu$ M TG. To generate a positive control, HeLa cells were heat shocked for 1 h at 42°C followed by 24 h recovery at 37°C. (B) Cytocentrifuge preparations of untreated HeLa cells or cells treated with 1.5  $\mu$ M TG for 24 h were prepared. Samples were fixed with 3.7% formaldehyde and permeabilized with Triton X followed by staining with labelled anti-HSPB1 antibody and DAPI and visualized by fluorescent microscopy (C) Densitometric analysis of HSPA1 induction by TG in HeLa cells expressed relative to untreated cells. (D) Densitometric analysis of HSPB1 induction by TG in HeLa cells expressed relative to untreated. Values shown are representative of three independent repeats\* P <0.05, \*\* P<0.01, \*\*\* P< 0.001, \*\*\*\* P< 0.0001 One-way ANOVA followed by Tukey's post hoc analysis versus untreated cells.

### 3.2.2 ER stress leads to induction of HSPs

To determine if HSP induction is a specific response to TG-induced ER stress, HeLa cells were also treated with Tunicamycin (TM). TM induces ER stress by inhibiting protein N-glycosylation, causing accumulation of mis-folded proteins within the ER.

Treatment of HeLa cells with 1.5  $\mu$ M TG caused a potent induction of both HSPA1 and HSPB1, indicating induction of HSPs is a general response to ER stress (Fig. 3.2.2A). To determine if HSP induction following ER stress was a cell type-specific response, a rat pheochromocytoma cell line, PC12 cells, were treated with an optimized dose of 0.25  $\mu$ M TG for 0-36 h (See Fig.4.2.3). This dosage typically causes 20% cell death at 24 h and therefore is comparable to the level of cell death induced by 1.5  $\mu$ M TG in HeLa cells at 24 h. TG treatment caused a sustained increase in HSP expression that was comparable to levels in heat shocked control cells as seen in Fig. 3.2.2B. This demonstrates that the induction of HSPs occurs in multiple cell line following ER stress.

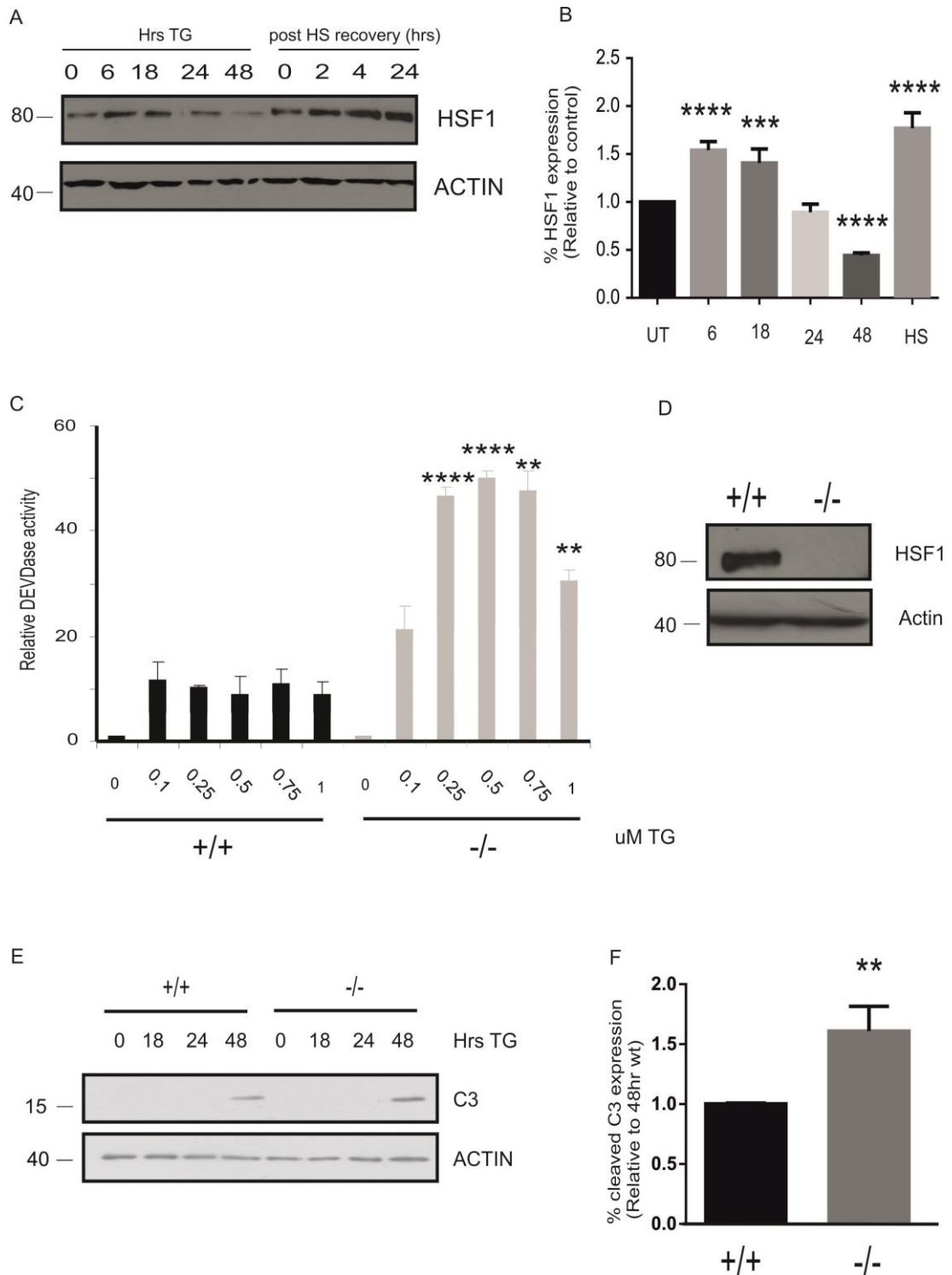


**Fig. 3.2.2 ER stress induces HSPs.** (A) HeLa cells were treated for 0, 6, 12, 24 or 48 h with 1.5  $\mu$ M TM. Expression of HSPA1 and HSPB1 was determined by Western blotting. ACTIN was used as a loading control. To generate a positive control, HeLa cells were heat shocked for 1 h at 42°C followed by 24 h recovery at 42°C. (B) Densitometric analysis of HSPA1 induction following TM treatment in HeLa cells expressed relative to untreated cells.

**(C)** Densitometric analysis of HSPB1 induction following TM treatment in HeLa cells expressed relative to untreated cells. **(D)** PC12 cells were treated with 0.25  $\mu$ M TG for 0, 12, 24 and 36 h. Expression of HSPA1 and HSPB1 was determined by Western blotting and ACTIN was used as a loading control. Heat shocked cells were used as a positive control for HSP induction. **(E)** Densitometric analysis of HSPA1 induction following TG treatment in PC12 cells expressed relative to untreated cells. **(F)** Densitometric analysis of HSPB1 induction following TG treatment in PC12 cells expressed relative to untreated cells. Values shown are representative of three independent repeats. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$  One-way ANOVA followed by Tukey's post hoc analysis versus untreated cells.

### 3.2.3 HSF1 is a functional component of the UPR

To further delineate the role of the HSR during ER stress, we analysed HSF1 induction. Expression of HSPs is primarily governed by the transcription factor, HSF1. We hypothesized that HSF1 would be activated in response to ER stress and thus cause the observed increase in expression of HSPs. We assessed expression levels of HSF1 following ER stress in HeLa cells. We observed a transient induction of HSF1 that reached a peak expression at 6 h of TG treatment, after which levels decline. Induction of HSF1 by TG was much less than that observed following HS recovery. Interestingly, this expression pattern paralleled the expression of HSPA1 following TG treatment (Fig. 3.2.1C). To characterize the role of HSF1 in ER stress we obtained HSF<sup>-/-</sup> and wild type Mouse Embryonic Fibroblasts (MEFs). We assessed the sensitivity of WT and HSF<sup>-/-</sup> MEFs to TG treatments by DEVDase assay. Cells were treated with 0.1, 0.25, 0.5, 0.75, or 1  $\mu$ M TG. HSF<sup>-/-</sup> MEFs were significantly more sensitive to TG at all doses. In agreement with results from the DEVDase assay, HSF<sup>-/-</sup> MEFs had more caspase-3 cleavage following ER stress. We could only detect caspase cleavage by Western blot at 48 h while the DEVDase assay could detect caspase activity at 24 h. This is due to the different sensitivities of the assays. The DEVDase assay detects caspases activity with higher sensitivity than Western blotting. These data suggest that HSF1 plays a protective role in ER stress.



**Fig. 3.2.3 HSF1 is a functional component of the UPR. (A)** HeLa cells were treated for 0, 6, 12, 24 or 48 h with 1.5  $\mu$ M TM or heat-shocked and allowed to recover for 0, 2, 4, or 24 h as a positive control. Expression of HSF1 was assessed by Western blotting. ACTIN was used as a loading control. **(B)** Densitometric analysis of HSF1 expression in HeLa cells following TG treatment expressed relative to untreated control. Values shown are



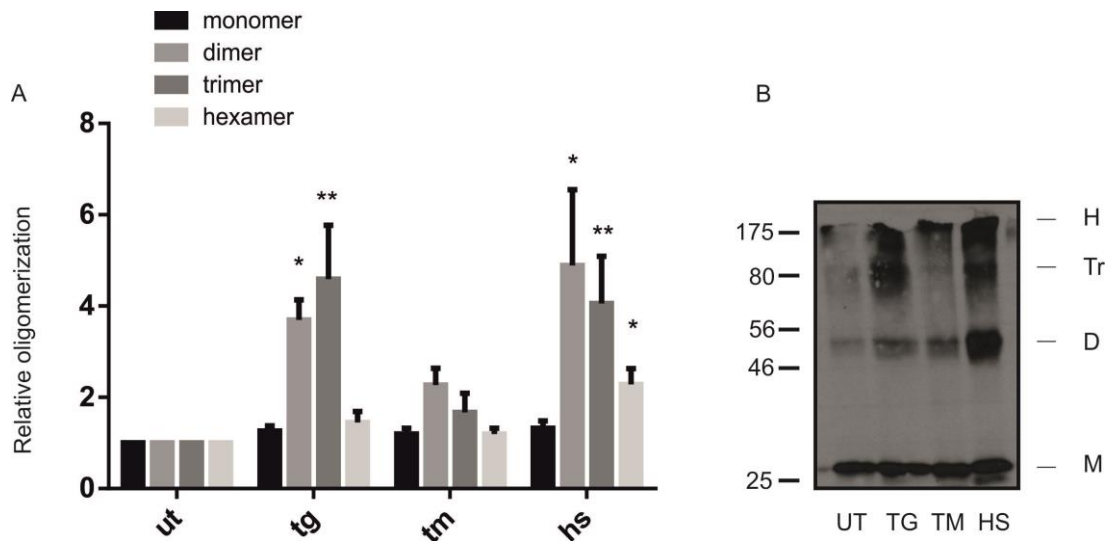
representative of three independent P <0.05, \*\* P<0.01, \*\*\* P< 0.001 One-way ANOVA followed by Tukey's post hoc analysis versus untreated. **(C)** MEFs were treated with 0.1, 0.25, 0.5, 0.75 or 1  $\mu$ M TG for 24 h. DEVD-AMC cleavage activity was measured in whole cell extracts. Values shown are representative of two independent repeats performed in triplicates P <0.05, \*\* P<0.01, \*\*\* P< 0.001 Two-way ANOVA followed by Tukey's post hoc analysis versus wildtype. **(D)** Western Blot analysis of HSF1 expression in +/+ (wildtype) and -/- (knockout) MEFs. **(E)** MEFs were treated with 0.25  $\mu$ G TG for 0, 18, 24, 48 h. Caspase-3 cleavage was assessed by Western blotting using an anti-caspase-3 antibody. ACTIN was used as a loading control. **(F)** Densitometric analysis of cleaved caspase-3 expression in +/+ and -/- HSF1 MEFs expressed relative to 48 h TG treated +/+ cells. Values shown are representative of three independent P <0.05, \*\* P<0.01, \*\*\* P< 0.001 Student t-tests.

### 3.2.4 ER stress alters the oligomeric state of HSPB1

Oligomerization is an essential property HSPs and governs its chaperoning activity. HSPB1 exists in a dynamic equilibrium of oligomeric states with small oligomers reported to mediate the chaperoning activity. Increased molecular chaperoning activity of HSPB1 correlates with the formation of small oligomers, with the HSPB1 dimer being the most active species (Hayes et al., 2009b).

To assess the nature of the HSPB1 oligomerization following ER stress, HSPB1 overexpressing PC12 cells were treated with 0.25  $\mu$ M TG, 2  $\mu$ G TM or HS for 1 h as a positive control. Cells were harvested and lysates were incubated in a protein cross-linking buffer containing 0.5 % glutaraldehyde. Chemical crosslinking offers a direct method to assess protein-protein interactions by generating covalent bonds between interacting proteins. Lysates were crosslinked for 30 min after which time the reaction was stopped with stopping buffer as described in (Lambert et al., 1999). Non-denatured lysates were run on SDS-PAGE and immunoblotting for HSPB1 was carried out. In approach the interaction of HSPB1 can be assessed. A monomer of HSPB1 has a molecular weight of 27 kDa. On crosslinking higher molecular weight bands were observed at  $\sim$ 27 kDa, 54 kDa, 80 kDa and 175 kDa. Such a distribution of sizes in multiples of approximately 27 kDa is indicative of the formation of homotypic dimers, trimers and hexamers of HSPB1 (Fig. 3.2.4B). Densitometric analysis was carried out to assess differences. Data was normalized to UT for each oligomeric state. It was found that revealed that relative to untreated samples TG stress caused a statically significant increase in abundance of dimers and trimers (Fig. 3.2.3A). Heat shock also caused a statically significant increase in abundance of dimers and trimers (Fig. 3.2.3A). Although not statically significant a similar trend was observed in TM treated samples. This suggests in addition to inducing HSPs, ER stress also causes alterations in the oligomeric state of HSPB1. The changes were comparable with HS controls. In particular small oligomers (dimers and trimmers) were increased. As the dimeric form of HSPB1 is

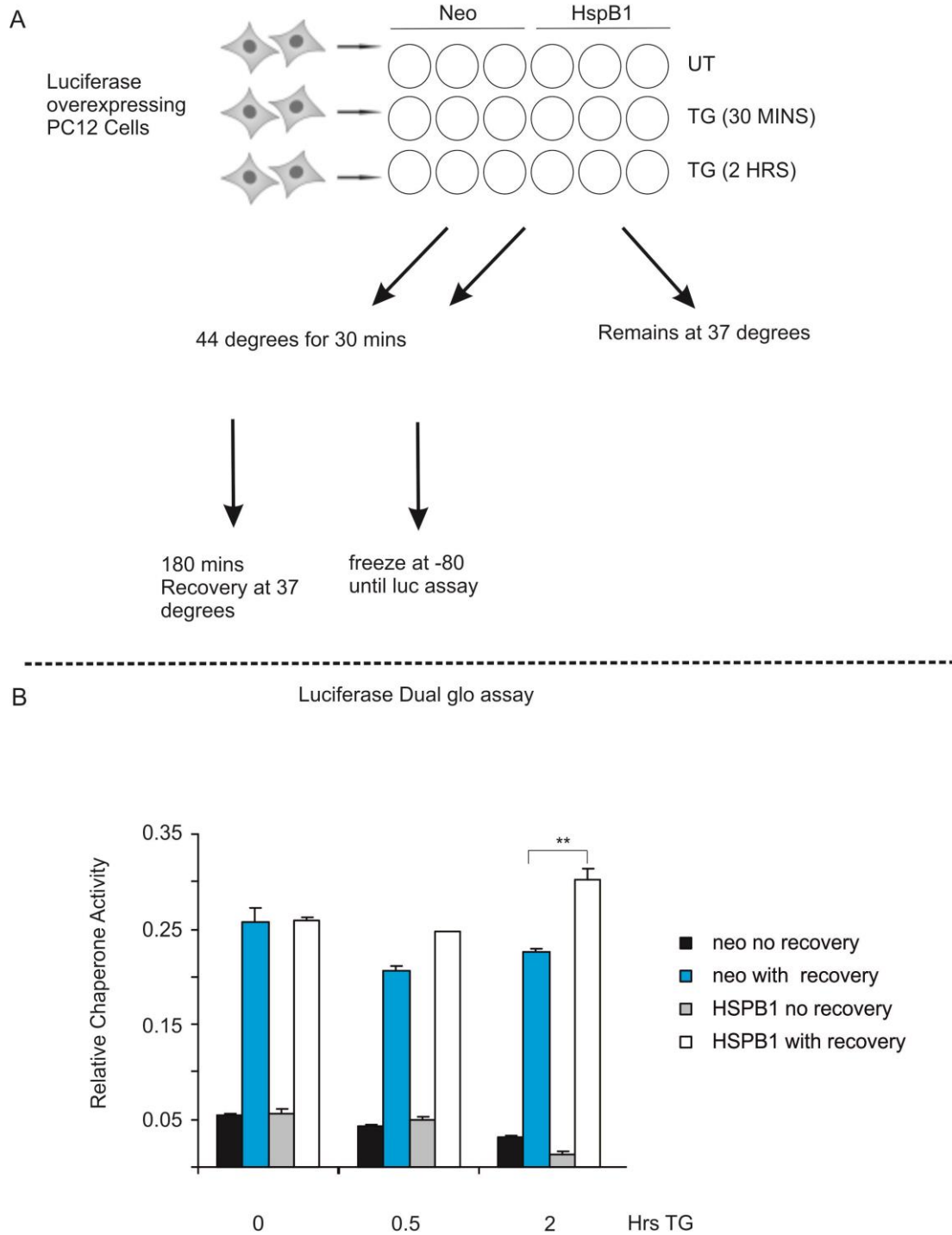
reported to be responsible for the chaperoning effects of HSPB1 this suggests that in response to ER stress the chaperoning activity of HSPB1 is activated.



**Fig. 3.2.4 ER stress alters the oligomeric state of HSPB1.** (A) Representative densitometric analysis of HSPB1 oligomerization following ER stress in glutaraldehyde cross-linked samples. (B) Western Blot analysis of HSPB1 oligomerization following ER stress. UT, TG, TM or HS lysates were incubated with 0.05% glutaraldehyde. Cross-linked proteins were separated by SDS-polyacrylamide gel electrophoresis, and HSPB1 was detected by immunoblot. N=2. . P <0.05, \*\* P<0.01, \*\*\* P< 0.0001 Two-way ANOVA followed by Tukey post hoc analysis

### 3.2.5 HSPB1 cells have increased chaperoning capacity in response to ER stress

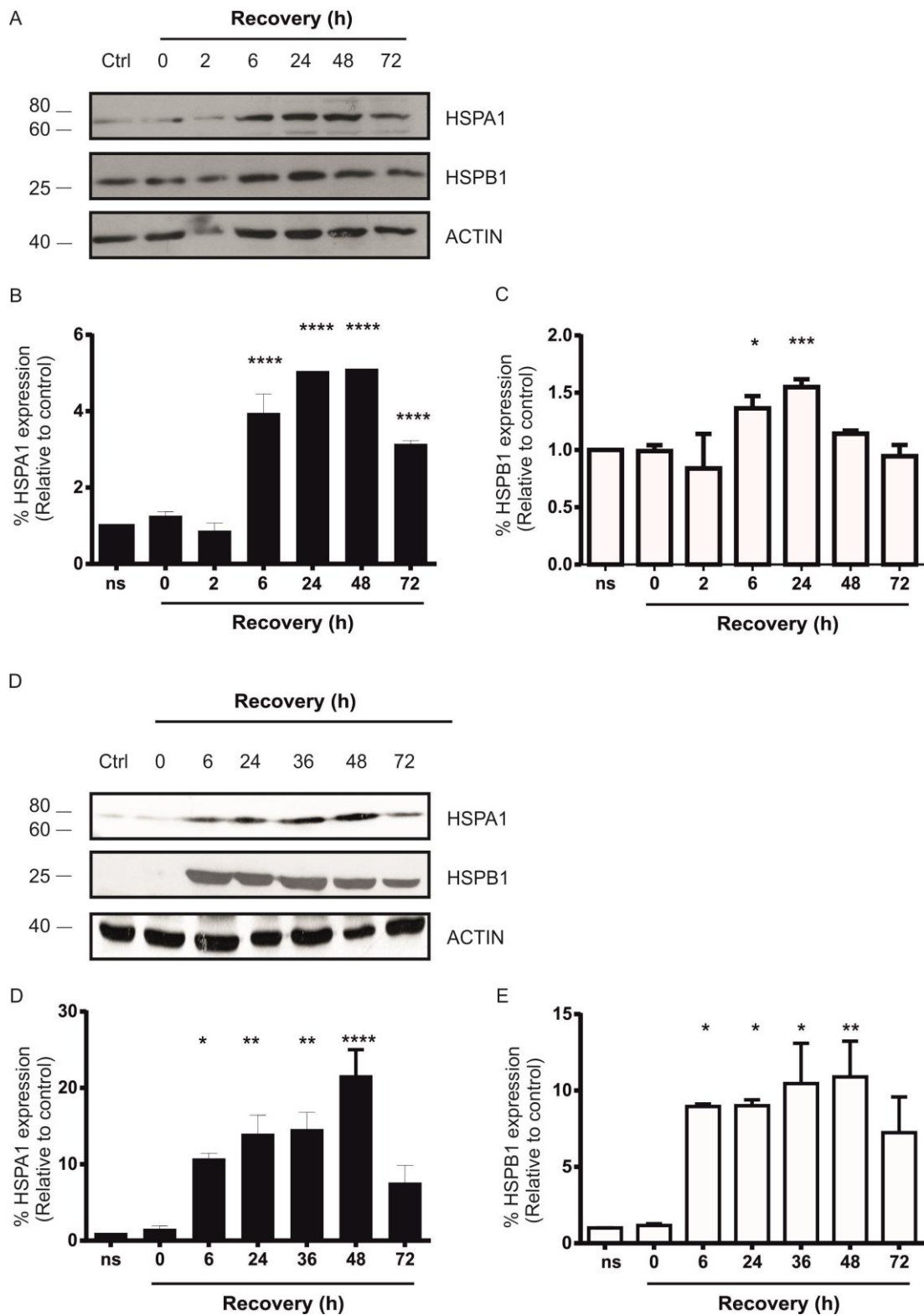
To test whether the increase in HSPB1 dimers during ER stress conditions was associated with an increase in chaperoning activity of HSPB1, an *in vivo* refolding assay was performed. Briefly, this assay measured the refolding of heat-denatured luciferase. Luciferase is a luminescent protein; however, denatured luciferase is not luminescent. Denatured luciferase can be refolded if chaperones within the cell are active resulting in a restoration of luminescence. Using this assay PC12 cells which over-express a Neo vector plus luciferase or HSPB1 plus luciferase were generated (See section 2.1 for details on generation of cell lines). Briefly cells were treated with TG for 0.5 or 2 h after which 20 mM MOPS and 40  $\mu$ M cyclohexamide were added for 30 min. Subsequently two plates of cells per treatment were heat shocked at 44°C (to denature the luciferase) while the other remained at 37 °C (not heat-shocked or control). After heat shock, one plate was harvested and stored at -80°C while the other plate was allowed to recover for 180 min at 37°C. Chaperoning activity was determined as the ratio of luciferase luminescence in heat-shocked: non heat-shocked cells. We can see that the degree of Luciferase denaturation was similar in Neo and HSPB1 cells. Neo and HSPB1 cells recover Luciferase activity to a similar degree in untreated conditions. This suggests that no difference in chaperoning activity occurs between Neo and HSPB1 cells under untreated conditions. Fig. 3.2.5 shows that ER stress caused no significant change in the ability of Neo cells to refold Luciferase. In contrast HSPB1 overexpressing cells had increased chaperoning activity in response to ER stress. Differences were apparent between Neo and HSPB1 cells after 2 h of treatment. These data suggest that, in response to ER stress, the oligomeric state of HSPB1 maybe altered and the chaperoning capacity of HSPB1 is increased.



**Fig. 3.2.5 HSPB1 increases chaperoning capacity of cells.** Chaperoning activity during ER stress was assessed in Neo and HSPB1 cells following treatment with 0.25  $\mu$ g TG for 0.5 or 2 h. Chaperoning activity was determined by the Dual Glo Luciferase Assay. Values shown are means of 4 independent repeats performed in triplicate  $\pm$  SEM.  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  Two-way Anova followed by Tukey's post hoc analysis versus Neo cells

### **3.2.6 Induction of HSPs in HeLa and PC12 cell lines following thermal preconditioning**

To investigate the role of the HSP induction following ER stress we set up a thermal preconditioning (TP) experiment. As previously mentioned, preconditioning is a well-established phenomenon in which a mild stressor can protect against subsequent cytotoxic insults (Gerner et al., 1976). Cross-protection is also possible whereby HSPs induced by one type of stress provide protection against a subsequent stressor (Cheng et al., 2011). We exposed cells to a non-lethal thermal stress by placing cells in a water bath at 42°C for 1 h (Gorman et al., 2005). Cells were allowed to recover for 6-72 h and HSPA1 and HSPB1 expression was assessed (Fig. 3.2.6A, B). The maximal level of HSPs was induced by 6 h of recovery and remained elevated for 48 h. The 6 h time point was selected as the optimal recovery time for subsequent thermal preconditioning experiments as HSPs were significantly increased in both cell lines at this time point.



**Fig. 3.2.6 Induction of HSPs in HeLa and PC12 cell lines following TP.** (A) HeLa cells were heat-shocked in HEPES buffered media for 1 h at 42 °C after which time the culture media was replaced and cells were allowed to recover at 37 °C for 0-72 h. Induction of HSP



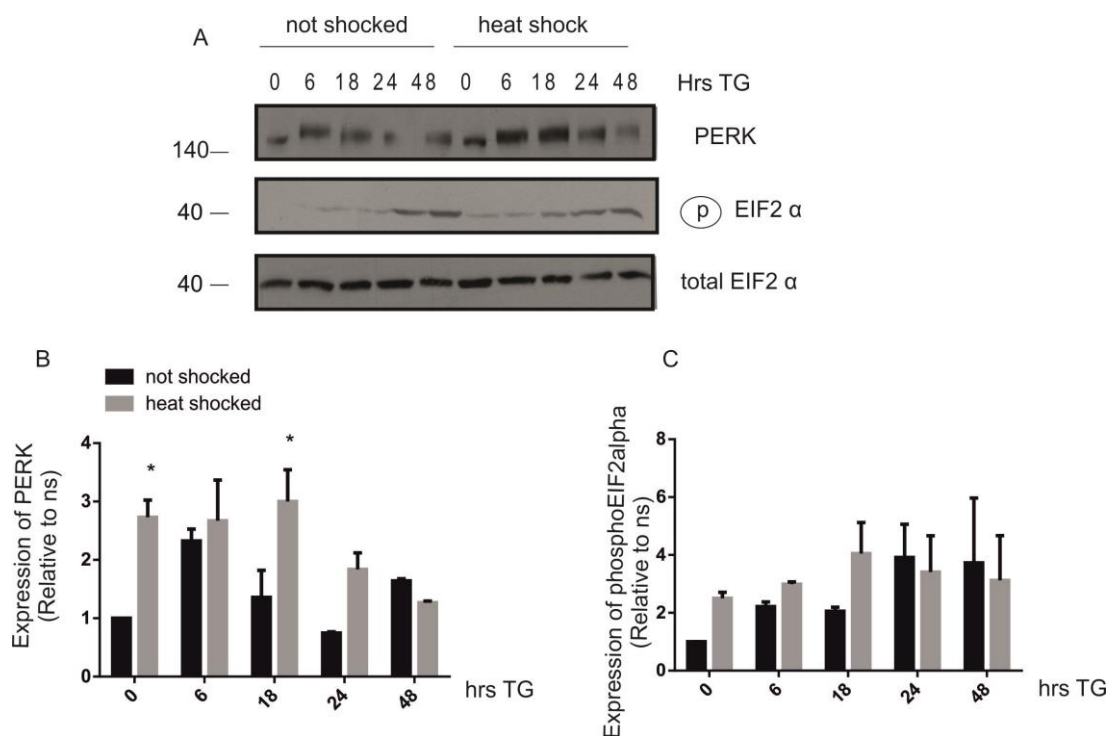
was determined by Western Blotting using specific antibodies for HSPA1 and HSPB1. ACTIN was used as a loading control. **(B)** Densitometric analysis of HSPA1 induction following heat shock in HeLa cells expressed relative to not shocked cells (ns). **(C)** Densitometric analysis of HSPB1 induction following heat shock in HeLa cells expressed relative to not shocked cells (ns). **(D)** PC12 cells were heat shocked in HEPES buffered media for 1 h at 42 °C after which time the culture media was replaced and cells were allowed to recover at 37 °C for 0-72 h. Induction of HSP was determined by Western Blotting using specific antibodies for HSPA1 and HSPB1. ACTIN was used as a loading control. **(E)** Densitometric analysis of HSPA1 induction following heat shock in PC12 cells expressed relative to not shocked cells (ns). **(F)** Densitometric analysis of HSPB1 induction following heat shock in PC12 cells expressed relative to not shocked cells. Values shown for all densitometric analysis are representative of three independent repeats. \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001, \*\*\*\* P < 0.0001 One-way ANOVA followed by Tukey's post hoc analysis versus not shocked cells.

### 3.2.7 Heat shock preconditioning activates the UPR

We then investigated how heat shock preconditioning effected UPR signalling. Using the optimized preconditioning conditions in HeLa cells, we assessed expression of key UPR markers by Western blotting following TG treatment.

We first assessed the effect of heat shock preconditioning on activation of the PERK arm of the UPR. ER stress causes oligomerization of PERK and its autophosphorylation. Therefore activation of PERK can be assessed by Western blotting by examining the reduction in mobility or upshift in the band due to phosphorylation. Consistent with activation of PERK by ER stress there was an up shift in PERK with TG treatments (Fig. 3.2.7A). The expression of PERK was also increased following treatment (Fig. 3.2.7B) Heat shocked cells had significantly increased expression of PERK at basal levels and after 18 h of TG treatment (Fig. 3.2.7B). The downstream consequence of PERK activation is EIF2 $\alpha$  phosphorylation. Using antibodies specific for the phosphorylated form of EIF2 $\alpha$  we found that heat shocked cells appeared to have higher pEIF2 $\alpha$  at basal levels and following 18 h TG treatment while total EIF2 $\alpha$  remained unchanged however changes in EIF2 $\alpha$  were

not spastically significant (Fig. 3.2.7C). These data indicate heat shock preconditioning activates the UPR.

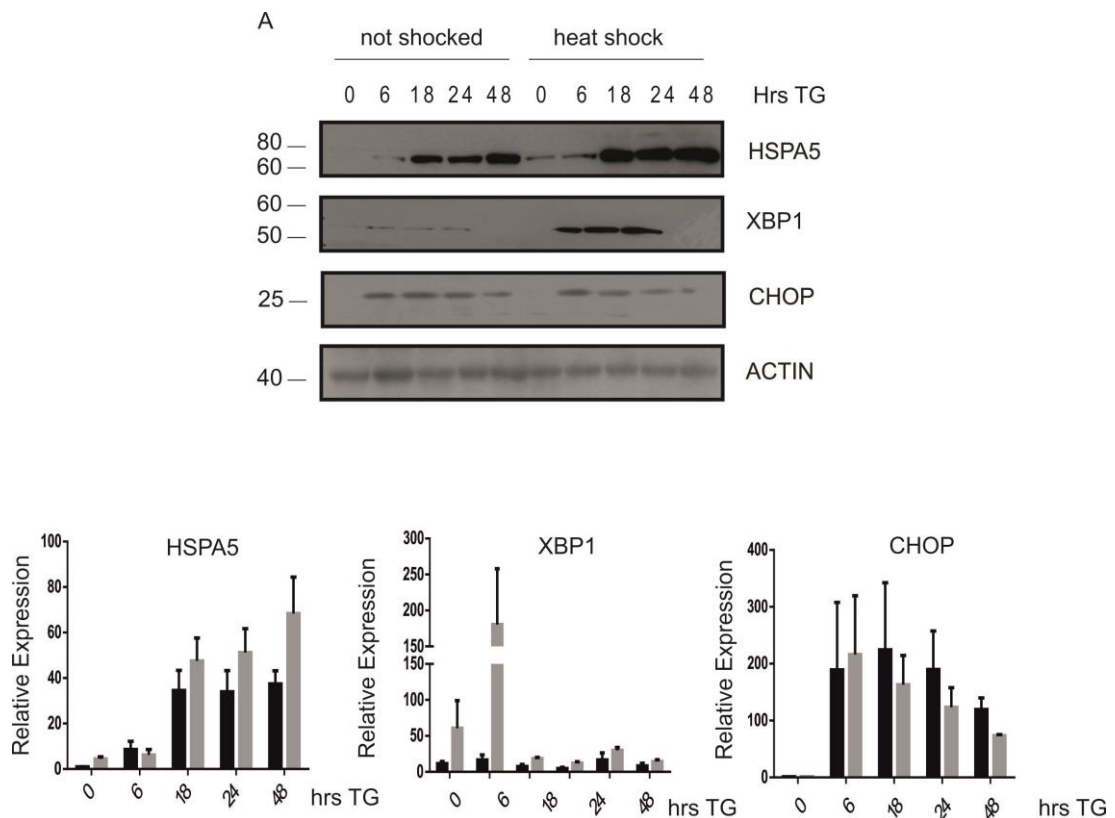


**Fig. 3.2.7 Heat shock preconditioning activates the UPR.** (A) HeLa cells were not shocked or heat shocked and left to recover for 6 h after which they were treated with 1.5  $\mu$ M TG for 0-48 h. Samples were prepared for SDS-PAGE gels and probed using anti-PERK, anti EIF2 $\alpha$  and total EIF2 $\alpha$  antibodies. Data are representative of three independent repeats. (B) Densitometric analysis of PERK expression in TG treated HeLa cells relative to not shocked untreated. (C) Densitometric analysis of phospho-EIF2-alpha expression in TG treated HeLa cells relative to not shocked untreated. Values shown are representative of three independent repeats.\* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001, \*\*\*\* P < 0.0001 Two-way ANOVA followed by Tukey's post hoc analysis versus not shocked cells.

### 3.2.8 Heat shock preconditioning activates an adaptive UPR response

We next assessed expression of downstream targets of the UPR including pro-survival factors such as XBP1s and HSPA5 as well as the pro-death inducing transcription factor, CHOP. As can be seen in Fig. 3.2.8 heat shock preconditioning caused an increase in the basal levels of HSPA5 which was further increased with

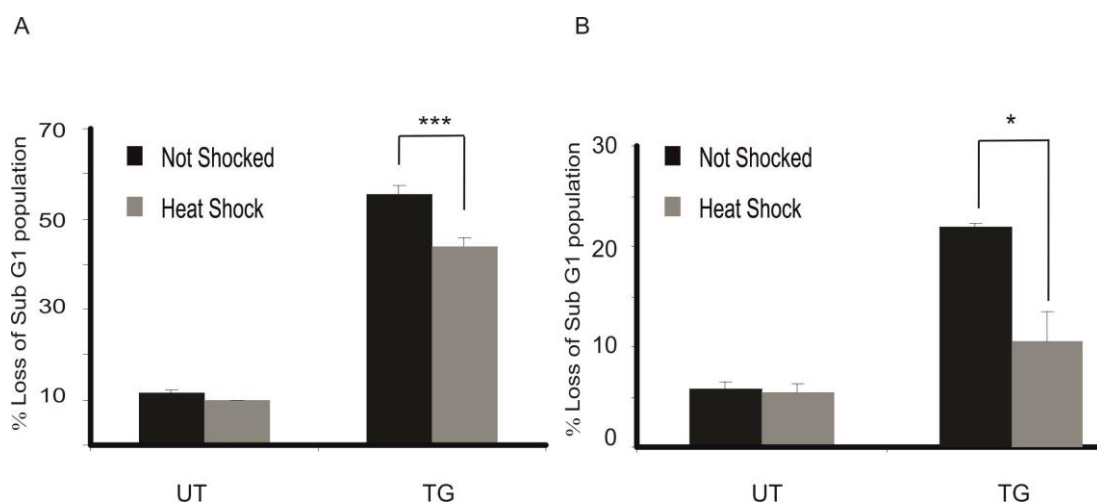
TG treatment. We have previously reported on the role of HSPA1 in enhancing XBP1s. In agreement with this we see that heat shock preconditioning causes more potent induction of XBP1 in heat-shocked cells compared to non-shocked cells. Expression analysis of the pro-apoptotic marker CHOP revealed no difference between non-shocked and heat shocked cells. Collectively these findings suggest that heat shock preconditioning activates an adaptive UPR response.



**Fig 3.2.8 Heat shock preconditioning activates the UPR. (A)** HeLa cells were not shocked or heat-shocked and left to recover for 6 h after which they were treated with 1.5  $\mu$ M TG for 0-48 h. Samples were prepared for SDS-PAGE gels and probed using anti-HSPA5, anti-XBP1s and CHOP antibodies. ACTIN was used as a loading control. All densitometric analysis is representative of representative of three independent repeats\* P <0.05, \*\* P<0.01, \*\*\* P< 0.001, \*\*\*\* P< 0.0001 Two-way ANOVA followed by Tukey's post hoc analysis versus untreated cells.

### 3.2.9 Heat shock preconditioning protects against ER stress-induced apoptosis

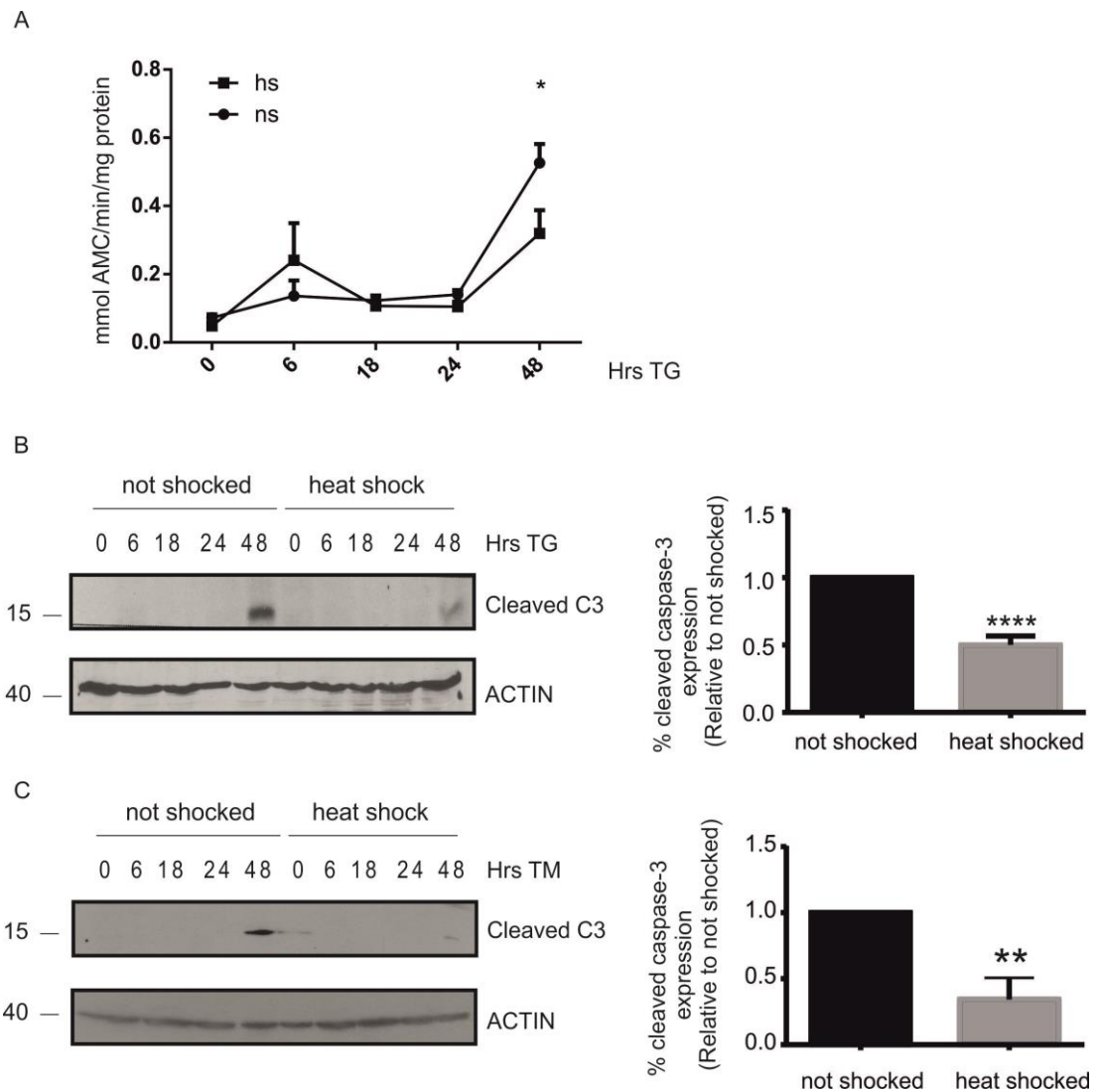
We have shown that heat shock preconditioning can augment expression of PERK phospho-EIF2 $\alpha$  and XBP1, while levels of CHOP remained similar to non shocked cells. However, in order to determine whether heat shock pre-conditioning can protect cells against ER stress-induced apoptosis, cells were heat shocked at 42°C for 1 h and allowed to recover at 37°C for 6 h to allow induction of HSPs. Following recovery, cells were treated with TG for the indicated times. Cell death was determined by Sub-G1 analysis using flow cytometry (See section 2.4.2 for further details). As demonstrated in Fig. 3.2.9C, heat shock preconditioning led to a 10% reduction in the number of HeLa cells with sub-G1 DNA content indicating reduced cell death (Fig. 3.2.9A). Similar results were obtained with PC12 cells where heat shock preconditioning also reduced cell death by 10% (Fig 3.2.9B).



**Fig. 3.2.9 Heat shock preconditioning protects against ER stress-induced apoptosis.** **(A)** HeLa cells were not shocked or heat shocked and left to recover for 6 h after which they were treated with 1.5  $\mu$ M TG for 48 h. Cells were fixed in 70% ethanol and Sub-G1 analysis was determined by PI staining on PE-A channel (FacsCalibur flow cytometer) **(B)** PC12 cells were not shocked or heat-shocked and left to recover for 6 h after which they were treated with 0.25  $\mu$ M TG for 36 h. Cells were fixed in 70% ethanol and Sub-G1 analysis was determined by PI staining on PE-A channel (FACSCanto flow cytometer). Values shown are representative of three independent repeats. \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001, \*\*\*\* P < 0.0001 Two-way ANOVA followed by Tukey's post hoc analysis versus not shocked cells.

### **3.2.10 Heat shock preconditioning attenuated caspase activation in HeLa cells**

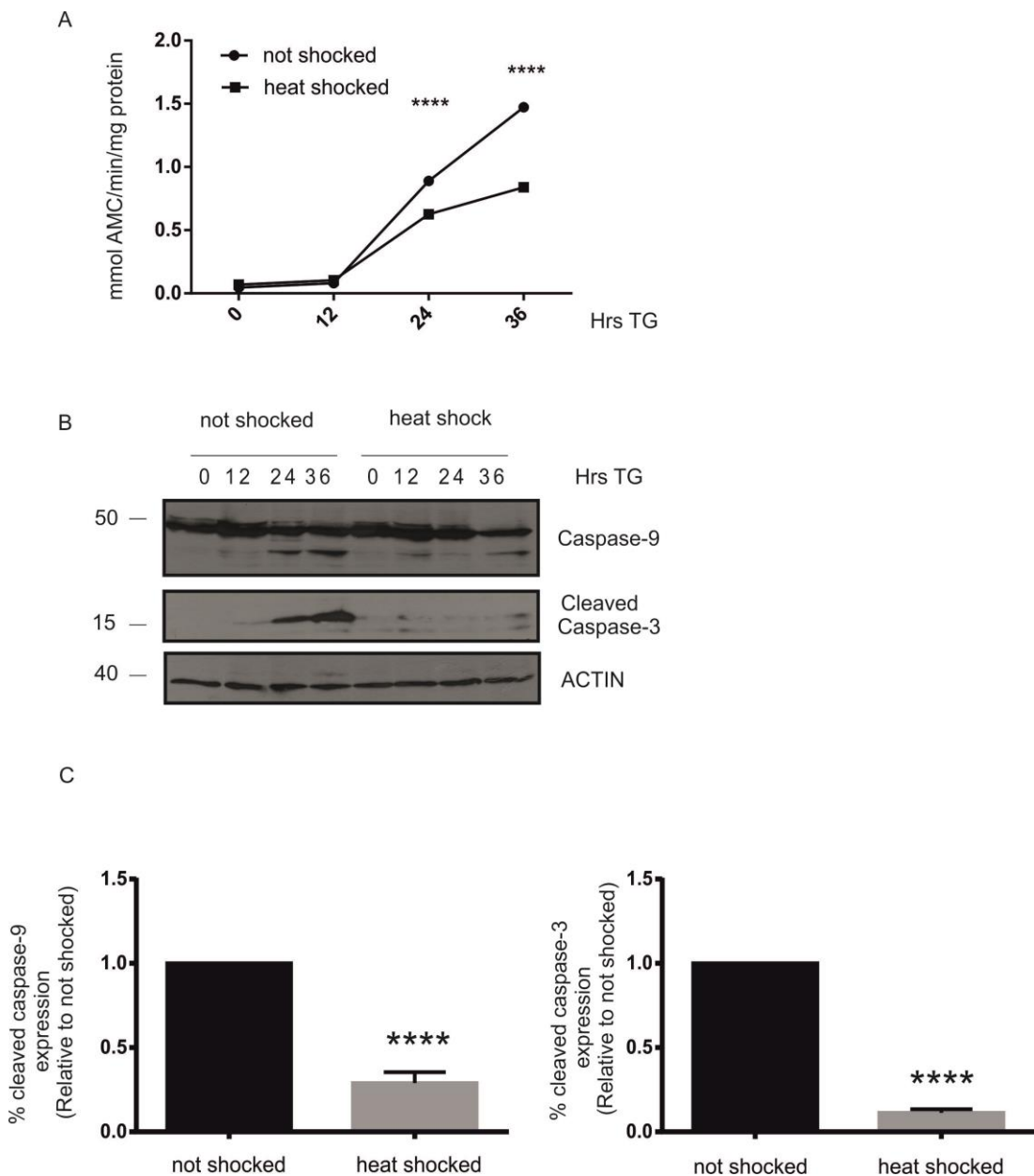
ER stress-induced apoptosis occurs via the intrinsic pathway and is caspase-dependent. We therefore tested if heat shock preconditioning could reduce caspase activity following ER stress. ER stress-induced caspase activity was determined by DEVDase assay. Caspase activity was reduced by 40% in preconditioned HeLa cells 48 h after treatment with 1.5  $\mu$ M TG (Fig. 3.2.10 A). In agreement with reduced caspase activity, preconditioned cells had reduced expression of the active form of caspase-3, the p17 cleaved fragment, following the 48 h TG treatment (Fig. 3.2.10 B). The effect of heat shock preconditioning on caspase activation was not specific to TG treatments as similar results were obtained in HeLa cells treated with 1.5  $\mu$ g of TM (Fig. 3.2.10C).



**Fig. 3.2.10 Heat shock preconditioning attenuated caspase activation in HeLa cells.** **(A)** Non shocked and heat-shocked HeLa cells were treated for 0-48 h with TG and DEVD-AMC cleavage activity was measured in whole cell extracts. Values shown are representative of two independent repeats performed in triplicates  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  Two-way ANOVA followed by Tukey's post hoc analysis versus non-shocked. **(B)** Time course study of TG-induced caspase-3 cleavage in non shocked and heat-shocked HeLa cells detected by Western blotting. Densitometric analysis was used to determine caspase-3 cleavage relative to non-shocked cells. Values shown are representative of three independent repeats. \*\*\*\*  $P < 0.0001$  Student's  $t$  test versus non-shocked. **(C)** Time course study of TM-induced caspase-3 cleavage in not shocked and heat-shocked HeLa was detected by Western blotting. ACTIN was used as a loading control. Densitometric analysis was used to determine caspase-3 cleavage relative to non-shocked cells. Values shown are representative of three independent repeats. \*\*\*\*  $P < 0.0001$  Student's  $t$ -test versus non-shocked.

### 3.2.11 Heat shock preconditioning attenuated caspase activation in PC12 cells

To ensure the protective effects of heat shock preconditioning were not unique to HeLa cells we performed similar experiments in the PC12 model. As can be seen in Fig. 3.2.11 A, heat shock preconditioned cells had 30% less caspase activity at 24 h and a 50 % reduction in caspase activity at 36 h.



**Fig 3.2.11 Heat shock preconditioning attenuates caspase activation in PC12 cells. (A)**

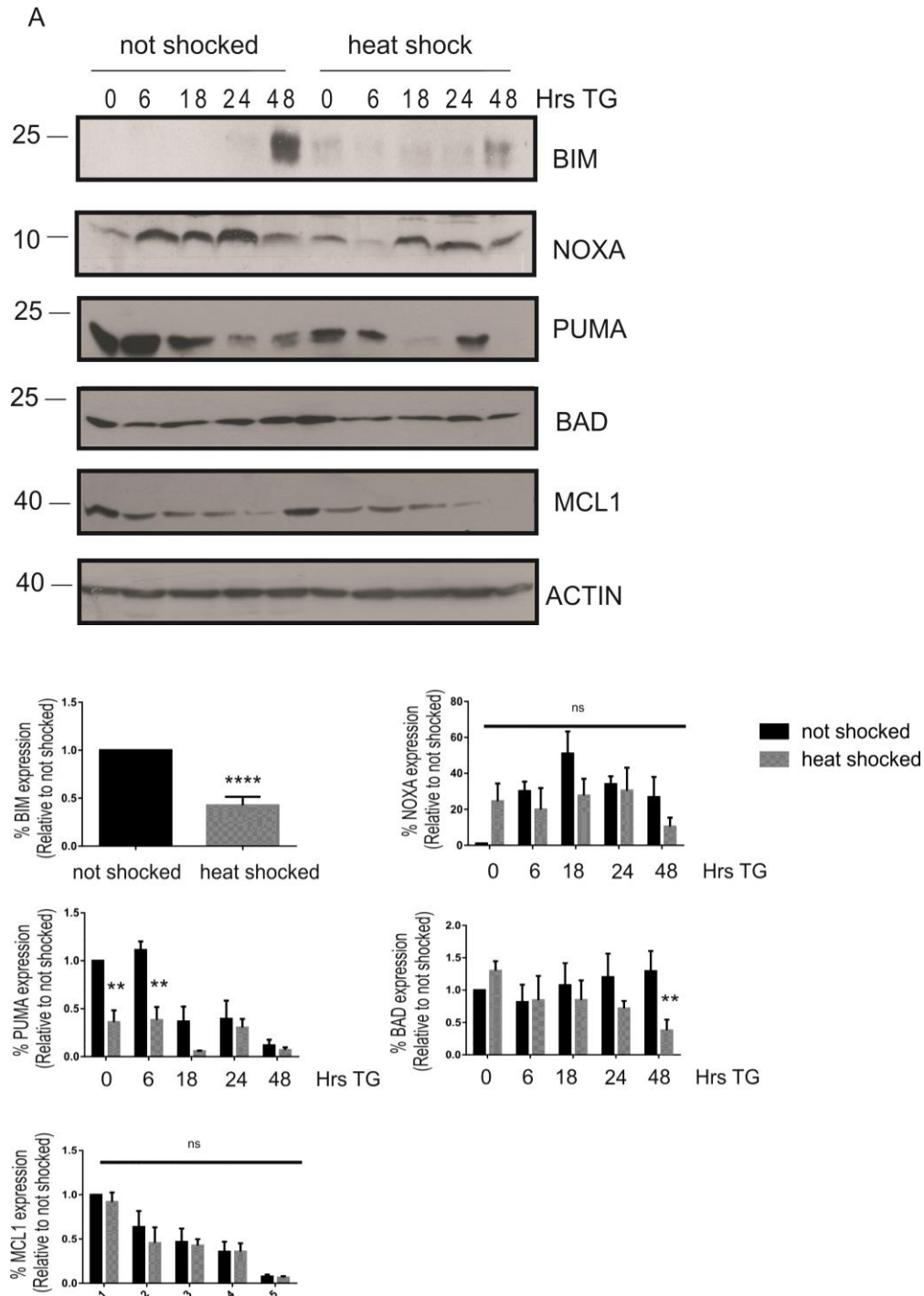
Time course study of TG-induced caspase activity as measured by DEVD-AMC cleavage was measured in whole cell extracts in not shocked and heat-shocked PC12 cells. Values shown are representative of two independent repeats performed in triplicates  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$  Two-way ANOVA followed by Tukey's post hoc analysis versus not shocked **(B)** Time course study of TG-induced caspase-3 and caspase-9 cleavage in not shocked and heat-shocked cells. ACTIN was used as a loading control. **(C)** Densitometric analysis of caspase-9 cleavage expressed relative to 36 hr not shocked. **(D)** Densitometric analysis of caspase-3 cleavage expressed relative to 36 hr not shocked. Values shown in C and D are representative of three independent repeats \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$ . Student's *t*-test versus 36 hr not shocked.

**3.2.12 Heat shock preconditioning reduces expression of pro- apoptotic BCL-2 family members**

We have previously demonstrated that ER stress-induced apoptosis involves activation/induction of BH3-only proteins with subsequent MOMP (Gupta et al., 2009). Previous studies in this group have also demonstrated that HS preconditioning inhibits HS-induced apoptosis at the level of the mitochondria and prior to BAX activation (Samali, 2001). This prompted us to investigate whether HS preconditioning affected expression levels of BCL-2 family members. To this end we carried out Western blot profiling of the BCL-2 proteins BIM, NOXA, PUMA, BAD and MCL1.

TG treatment caused an increase in expression of the pro-apoptotic protein BIM at 48 h. However, HS preconditioning prevented BIM expression (Fig. 3.2.12A). NOXA was also increased following TG treatment and induction was similar in not shocked and heat shocked cells (Fig. 3.2.12B). Unexpectedly, TG treatment caused a reduction in PUMA expression and HS preconditioning further attenuated this. We saw a subtle reduction in the levels of BAD following TG treatment. Heat shock had lower expression levels of BAD Consistent with previous reports we saw down-regulation of MCL1 following ER stress however, this was unaffected by heat shock preconditioning.

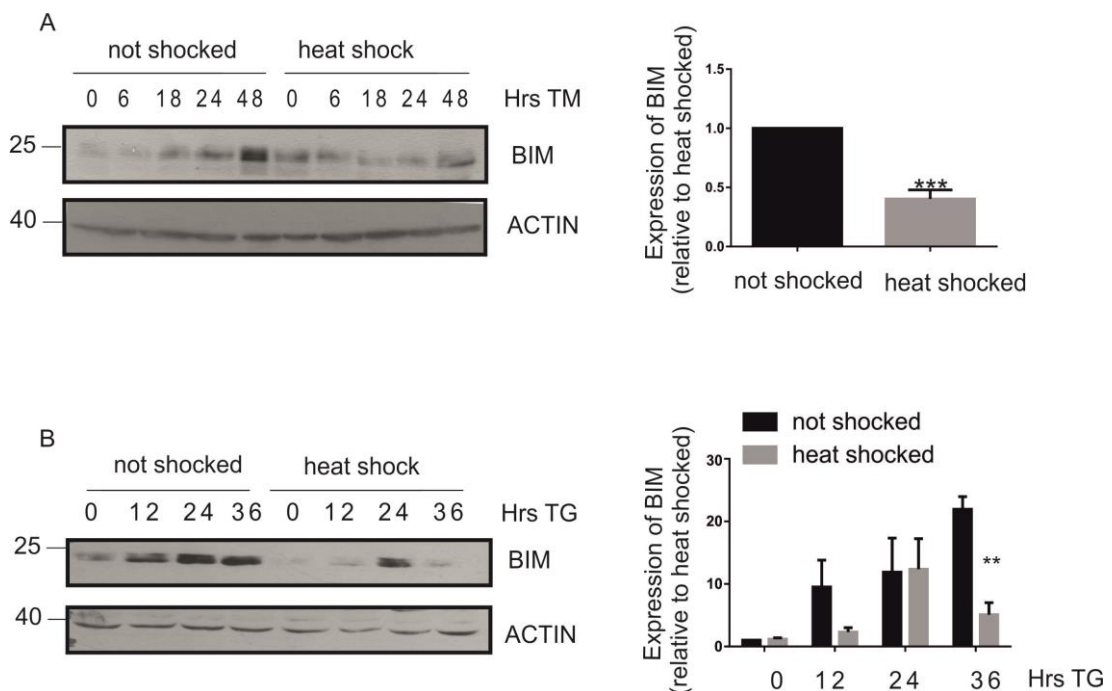




**Fig. 3.2.12 Heat shock preconditioning reduces expression of pro-apoptotic BCL-2 family members.** HeLa cells were not shocked or heat-shocked and left to recover for 6 h after which they were treated with 1.5  $\mu$ M TG for 0-48 h. Samples were prepared for SDS-PAGE gels and probed using anti-BIM, anti-NOXA, anti-PUMA, anti-BAD, anti-MCL1 antibodies. ACTIN was used as a loading control. Densitometric analysis of BCL-2 family proteins expressed relative to not shocked. Values shown are representative of three independent repeats \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$  Two-way ANOVA followed by Tukeys post hoc analysis.

### 3.2.13 Heat shock preconditioning reduced ER stress-induced BIM expression

We decided to further investigate the effect of heat shock preconditioning on BIM expression given the crucial role of BIM in ER stress induced apoptosis (Puthalakath et al., 2007) We treated HeLa cells with TG and found that heat shock preconditioned cells had significantly reduced expression of BIM (Fig. 3.2.13 A). This demonstrates that activation of the HSR caused inhibition of ER stress induced BIM expression. We saw similar results in PC12 cells indicating that regulation of BIM by the HSR is a general and not a cell type specific response (Fig. 3.2.13 B).

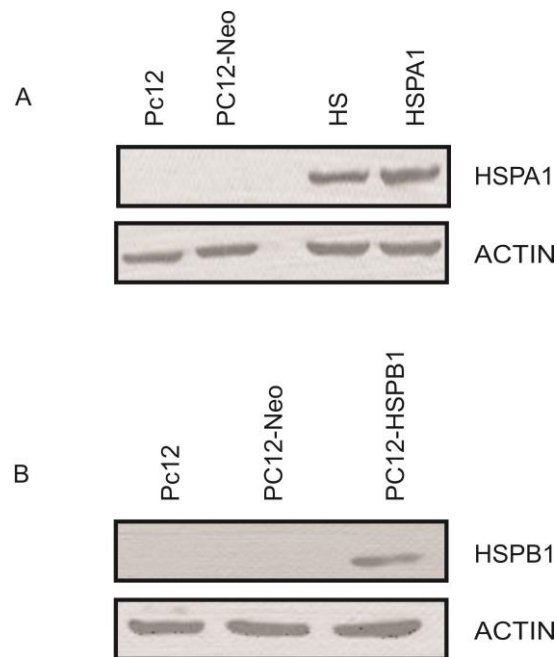


**Fig. 3.2.13 Heat shock preconditioning reduces expression of pro-apoptotic BCL-2 family members.**(A) HeLa cells were not shocked or heat-shocked and left to recover for 6 h after which they were treated with 1.5  $\mu$ M TG for 0-48 h. Samples were prepared for SDS-PAGE gels and probed using anti-BIM, ACTIN was used as a loading control. Densitometric and statistical analysis was carried out on BIM expression in TG treated HeLa cells. (B) PC12 cells were not shocked or heat shocked and left to recover for 6 h after which they were treated with 0.25  $\mu$ M TG for 0-36 h. Samples were prepared for SDS-PAGE gels and probed using anti-BIM, ACTIN was used as a loading control. Densitometric analysis of BIM expressed relative to not shocked untreated. Values shown are representative of three

independent repeats \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$  Two-way ANOVA followed by Tukeys post hoc analysis versus not shocked.

### **3.2.14 Generation of cell lines overexpressing HSPs**

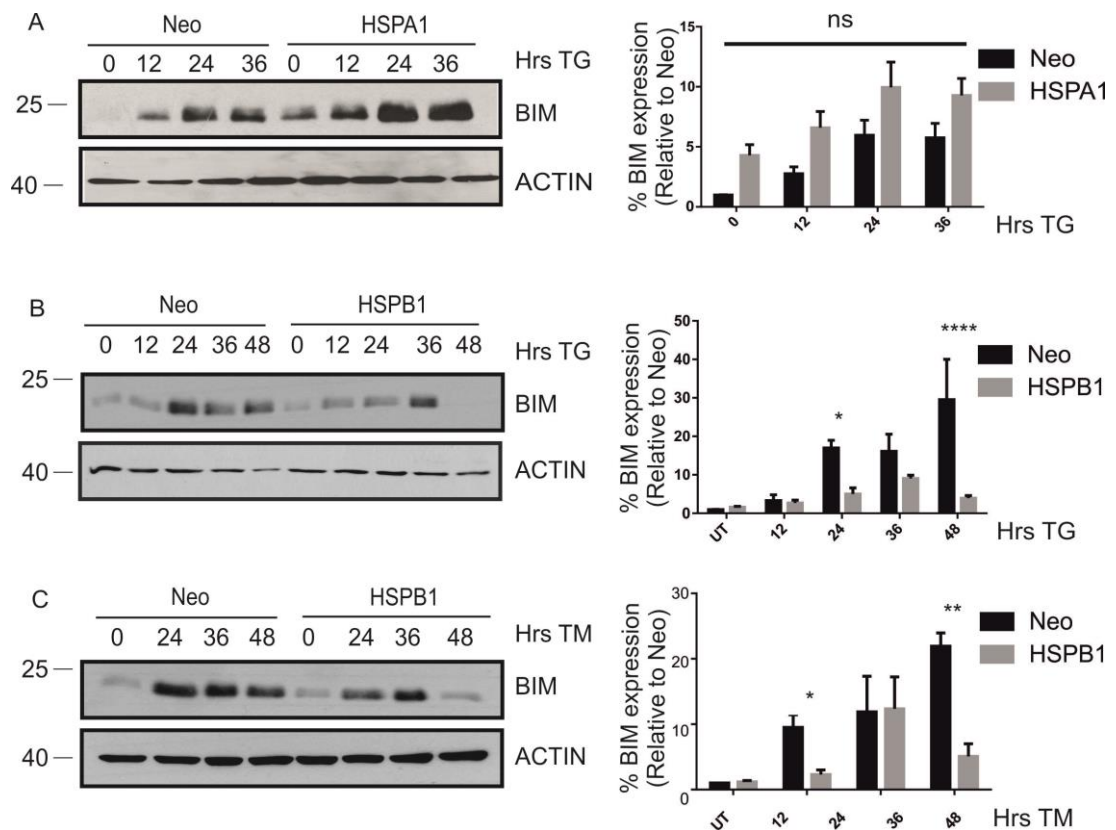
To investigate which HSP was responsible for regulation of BIM following heat shock preconditioning we set up a HSP over-expression system. The rat pheochromocytoma cell line, PC12, was initially selected to address this question as PC12 cells express very low levels of endogenous HSPs (Quigney et al., 2003). In addition these cells induce BIM expression following ER stress and would therefore provide a suitable model system to decipher, which HSP is leading to a reduction in BIM following heat shock preconditioning. HSPA1 and HSPB1 are the most potently induced heat shock proteins following HS preconditioning, therefore these two HSPs were selected for further study. Cells stably overexpressing either HSPA1, HSPB1 or a control empty vector (Neo) were generated by transient transfection of pcDNA-HSPA1, pcDNA-HSPB1, or a control vector pcDNA. Cells that contained the overexpression plasmids were then selected for using the antibiotic resistant marker in the plasmid, G418, this allowed generation of a stable pool of colonies. Cells were validated for overexpression of HSPs by Western blotting (Fig 3.2.14).



**Fig 3.2.14. Generation of stably over expressing HSPA1 and HSPB1 cells. (A)** Protein extracts from Neo and HSPA1 cells were analyzed by SDS-PAGE followed by Western blotting using a specific anti-HSPA1 antibody. **(B)** Protein extracts from Neo and HSPB1 cells were analyzed by SDS-PAGE followed by Western blotting using a specific anti-HSPB1 antibody. ACTIN was used as a loading control.

### 3.2.15 Over expression of HSPB1 reduces ER stress-induced BIM.

A time course study of Neo, HSPA1 and HSPB1 PC12 cells was carried out with TG using the previously optimized drug concentration of 0.25  $\mu\text{M}$ . Samples were then probed for expression levels of BIM. As can be seen in Fig. 3.2.15 TG treatment lead to a time dependant increase in BIM expression, however, over-expression of HSPA1 did not reduce BIM expression (Fig. 3.2.15 A). In contrast, PC12 cells overexpressing HSPB1 were found to have reduced levels of BIM expression following TG treatment (Fig. 3.2.15 B). This was not a stimulus specific effect as HSPB1 cells treated with 2  $\mu\text{g}$  of TM also expressed lower levels of BIM. This indicates that HSPB1 may have a role in the reduction of BIM expression following ER stress.



**Fig 3.2.15 Overexpression of HSPB1 reduces ER stress induced BIM. (A)** Neo and HSPA1 PC12 cells were treated with TG and protein extracts were assessed for levels of BIM. Actin was used as a loading control. Densitometric and statistical analysis was carried out on TG treated Neo and HSPA1 cells **(B)** Protein extracts from TG treated Neo and HSPB1 cells were analysed by SDS-PAGE followed by Western blotting using a specific BIM antibody. ACTIN was used as a loading control. Densitometric and statistical analysis was carried out on TG treated Neo and HSPB1 cells **(C)** Protein extracts from TM treated Neo and HSPB1 cells were analysed by SDS-PAGE followed by Western blotting using a specific BIM antibody. ACTIN was used as a loading control. Densitometric and statistical analysis was carried out on TM treated Neo and HSPB1 cells. Values shown are representative of three independent repeats. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$  Two-way ANOVA followed by Tukey's post hoc analysis versus Neo

### 3.3 Discussion

The HSR and the UPR are induced by common stimuli including mis-folded protein accumulation, proteasome inhibition, oxidative stress, and infection. We propose that co-activation of the stress pathways occurs in response to noxious stimuli in order to help cells cope. The UPR is a biphasic stress response pathway in which protective mechanisms are initially employed to help cells survive; however, if these measures fail, apoptosis ensues. We have shown that ER stress inducing drugs cause induction of both HSPA1 and HSPB1 in PC12 and HeLa cell lines. We observed a transient increase in HSPs following ER stress treatment. Of note, the decline of HSPs levels, which occurred at 48 h, coincided with initiation of cell death (Fig. 3.2.1 and Fig 3.2.2). Potentially the reduction of HSPs in the latter stages of the UPR is one of the elusive switching mechanisms in the transition from an adaptive to a maladaptive UPR response. Induction of HSPs was found to occur in both the human cell line (HeLas) and rat cell line (PC12s) and in response to different ER stress inducing agents (Fig. 3.2.1 and Fig.3.2.2). This suggests that induction of HSPs is general adaptive feature of the UPR and may act to help cells deal with ER stress and prevent ER stress-induced apoptosis.

The increase in expression of HSPs was paralleled by a transient increase in expression of HSF1 the main transcription factor involved in the classical HSR. Consistent with HSF1 and heat shock proteins having a role in ER stress we see that HSF1<sup>-/-</sup> MEFs are more sensitive to ER stress-induced apoptosis. This demonstrates the functional significance of co-activation of the HSR and the UPR. HSF1 mediated activation of the HSR is an integral component of the proteostasis network. It is therefore logical that loss of this network would cause activation of the UPR due to the build-up of mis-folded proteins. Our data shows that in the absence of HSF1 cells are more sensitive to ER stress-induced caspase activation (Fig. 3.2.3). These data suggest that UPR mediated activation of the HSR enhances the cells capacity to prevent ER stress induced apoptosis.

Inhibition of HSF1 has previously been shown to enhance apoptosis in response to a proteasome inhibitor, PS-341 (Tay et al., 2012). Co-operation between the HSR and the UPR is further exemplified by the observation that loss of vitamin B12 causes ER stress due to impaired activation of HSF1 (Ghemrawi et al., 2013). This suggests that the HSR is involved in setting the basal threshold to ER stress. It also demonstrates the redundancy of the HSR and the UPR as loss of one leads to a compensatory activation of the other, a feature that has previously been noted in HSF1 knockout MEFs (Liu and Chang, 2008). This again highlights the potential crosstalk of these two key proteostasis pathways.

We were interested in further delineating the role of the HSPs in ER stress given the fact that HSPA1 and HSPB1 were induced in HeLa and PC12 cell lines in response to ER stress. HSPB1 acts as an ATP-independent molecular chaperone and has previously been shown to protect against apoptosis, however its role in ER stress has not been addressed.

Post translational modifications of HSPs adds an additional layer of regulation to the HSR, a measure that is particularly important in stress conditions (Hatayama et al., 1994, Rafiee et al., 2003). The dynamic organization of HSP oligomers appears to be a crucial feature governing HSP activity (Bruey et al., 2000a). It has been demonstrated that in response to different stress stimuli HSPB1 alters its oligomerization status which maybe enable differential client protein interactions (Paul et al., 2012). In this way HSPB1 can mount a tailored response to the imposed stress. We therefore investigated the regulation of HSPB1 functionality under ER stress conditions. HSPB1 exists in an equilibrium of oligomers in unstressed cells with cellular stress leading to rapid phosphorylation on residues S15, S78, and S82 by mitogen-activated protein kinase–activated protein kinase 2 and 3 (MK2, MK3). This causes disassembly of large oligomers into small oligomers.

There is an as yet incomplete understanding of the nature of HSPB1 oligomeric state required for optimal chaperoning capacity. Rogalla et al (1999) used in vitro



refolding of citrate synthase to assess chaperoning activity. Comparing light scattering ability of aggregates in the presence of HSPB1, or the triple mutant HSPB1 -S15D,S78D,S82D (phospho-mimic mutant), they found that the triple mutant had significantly decreased ability to act as a molecular chaperone, to suppress thermal denaturation and facilitate refolding (Rogalla et al., 1999). These data suggest that un-phosphorylated HSPB1 acts as a molecular chaperone. These findings contradict a recent finding by Hayes et al (2009) who performed a comparative analysis of HSPB1, triple phosphorylated by MK2 kinase, and the HspB1-S15D, S78D, S82D triple mutant. It was shown that the widely used triple mutant was only a partial and qualitative mimic for phosphorylated HSPB1 (Hayes et al., 2009a). MK2 phosphorylated HSPB1 was smaller in size than the mutant and showed enhanced protection against aggregation compared to the mutant (Hayes et al., 2009a). Therefore phosphorylated HSPB1 was suggested to be the chaperone competent form of HSPB1 (Hayes et al., 2009a). Current opinion is that stress induced phosphorylation and subsequent de-oligomerization of HSPB1 provides a transient increase in chaperoning capacity so that unfolded protein may be refolded by the HSPA1 machinery. Failure of this mechanism is purported to lead to formation of large oligomers which sequester misfolded proteins and prevent aberrant potentially cytotoxic protein interactions (Bryantsev, 2007).

In agreement with current literature, which suggests that small oligomers of HSPB1 mediate chaperoning activity, we observed both increased formation of HSPB1 dimers and increased chaperoning activity in HSPB1 over-expressing cells in response to ER stress.

Our data suggest that HSPB1 is present in a chaperoning 'inactive' state in unstressed cells as no difference was observed between Neo and HSPB1 cells with regards to chaperoning capacity in untreated conditions. In contrast, treatment with the ER stress inducer, TG, causes an increase in chaperoning activity of HSPB1 (Fig.3.2.3). It is also uncertain as to which phosphorylation state and hence which oligomeric state of HSPB1, prevents apoptosis. In the case of ischemic injury,

phosphorylated HSPB1 is necessary to provide protection (Stetler *et al.* 2009). In a recent study, Gibert *et al.* (2013) generated peptide aptamers which specifically interact with HSPB1 (Gibert *et al.*, 2013). Characterization of the biochemical properties of the effect of aptamers on the oligomeric state of HSPB1 revealed the aptamers resulted in a redistribution of HSPB1 to large oligomers and a concomitant reduction in phosphorylation of S78 (Gibert *et al.*, 2013). Transfection of these aptamers into HeLa cells caused increased sensitivity to staurosporine-induced apoptosis compared to the control (Gibert *et al.*, 2013). These data suggest that phosphorylated HSPB1 is necessary to protect from staurosporine-induced apoptosis and highlights the importance of alterations in HSPB1 oligomerization and its resultant effect on cell fate. Conversely, non-phosphorylated HSPB1 was reported to protect cells from cisplatin and etoposide. The apparent discrepancies may reflect stimulus specific alterations in HSPB1 to allow cells to fine tune stress response to the stress imposed. Indeed Paul *et al.* 2010 demonstrated the stimulus specific HSPB1 dynamics (Paul *et al.*, 2012). They observed that etoposide led to formation of large oligomers. In accordance with Bruey *et al.* 2000, the large oligomers are necessary for protection against etoposide (Bruey *et al.*, 2000b). Therefore it seems that different stimuli cause discrete changes in the structural organization of HSPB1 producing a stressor-specific survival strategy. With this in mind it is plausible that ER stress induces formation of small oligomers of HSPB1, thereby increasing its chaperoning capacity and aiding in the attenuation of ER-stress induced apoptosis. In agreement with this hypothesis it has been reported that ER stress causes phosphorylation of HSPB1 (Ito *et al.*, 2005). In the case of HSPA1 its chaperoning property is essential for mediating protection against apoptosis (Mosser *et al.*, 2000). This was demonstrated by assessment of heat induced cell death in cells expressing WT HSPA1 or an ATPase deletion mutant, an EEVD deletion mutant or a mutated EEVD construct, all of which which all reduce the chaperoning ability of HSPA1. It was found that only WT HSPA1 could protect against heat-induced cell death thereby emphasizing the prosurvival role of HSPs chaperoning activity.

Our data pointed to co-activation, co-operation and cross modulation of the HSR and UPR. We sought to better clarify the role of the HSR in modulating ER stress by setting up a thermal preconditioning model. Previous reports have established that the UPR is activated following thermal stress however the effect of thermal preconditioning on cell fate has not been explored. In this work thermal preconditioning induced HSPA1 and HSPB1 expression in HeLa and PC12 cells (Fig 3.2.6). Co-activation of the HSR and the UPR was further exemplified in the increased expression of pro-survival UPR markers HSPA5 and XBP1s in preconditioned cells (Fig 3.2.8). This suggests that preconditioning leads to mild ER stress which causes activation of a pro-survival UPR response in a manner reminiscent of hormesis. Specific activation of pro-survival aspects of the UPR has previously been demonstrated in cells treated with very low concentrations of ER stress inducers TG or TM (Rutkowski et al., 2006, Mendes et al., 2009). As such, thermal preconditioning primes cells for a subsequent ER stress insult. We hypothesized that thermal preconditioning could therefore confer a survival advantage to cells in ER stress conditions. The pro-survival effects of thermal preconditioning in response to apoptosis inducing agents has previously been reported however the exact mechanisms are not well understood (Cheng et al., 2011). Our data clearly demonstrate that thermal preconditioning can protect against ER stress-induced apoptosis (Fig.3.2.9, Fig.3.2.10, Fig.3.2.11). ER stress-induced apoptosis is mediated via the mitochondrial/intrinsic pathway (Gupta et al., 2009). HSPs, in particular HSPA1 and HSPB1 have been linked to interference with the intrinsic pathway via inhibition of the release of cytochrome c and subsequent caspase activation (Concannon CG, 2001). We too saw reduced caspase activation and activity in preconditioned cells.

The role of the BCL-2 family as critical regulators of events at the mitochondria and therefore cell fate is well established (Bouillet and Strasser, 2002, Stankiewicz et al., 2009, Zhang et al., 2012) . Previous reports have demonstrated the ability of HSPB1

to regulate BAD via the AKT pathway (Zoubeidi et al., 2010). HSPA1 has also been shown to regulate NOXA. Additionally cross-tolerance induced by thermal preconditioning of SHSY5Y cells has been shown to reduce BAX activation in response to staurosporine (Cheng et al., 2011). This suggested to us that the cytoprotective effects of TP on ER stress-induced apoptosis could be due to regulation of the BCL-2 family. We have shown that preconditioned cells have lower levels of the key ER stress-associated BH3-only protein BIM. To date, no reports have linked HSPs to the regulation of BIM, and given its crucial role in ER stress induced apoptosis we were interested in identifying the HSP capable of regulating BIM expression. We found that cells which over-express HSPB1 had reduced expression of BIM in response to ER stress and we propose that thermal preconditioning modulates BIM, in part, via increased expression of HSPB1. As HSPs were induced by ER stress in our hands, we propose a role for HSPs in the ER stress response, modulation of cell fate and in particular we propose a HSPB1-BIM pathway in the adaptive phase of the UPR. To test the aforementioned hypothesis we further explored the role of HSPB1 in ER stress-induced apoptosis in PC12s cell which overexpress human HSPB1 or a control vector. Using this model system we also investigated the mechanism by which HSPB1 regulates BIM expression as details in chapter 4.

## **Chapter 4**

### **Regulation of ER stress-induced apoptosis by HSPB1**

## 4.1 Introduction

HSPB1 is the best characterized and most studied member of the small HSP family. HSPB1 has been shown to mediate protection from diverse cell stressors including heat shock (Samali, 2001), oxidative stress (Rogalla et al., 1999), metabolic stress (Havasi et al., 2008a), 6-hydroxydopamine (Gorman et al., 2005) staurosporine (Paul et al., 2002a), and DNA damage (Kanagasabai et al., 2013). The commonality between these stressors is activation of the mitochondrial apoptotic pathway.

In Chapter 3, we demonstrated that HSPB1 could reduce expression of the pro-apoptotic BOP, BIM during conditions of ER stress. We therefore investigated whether overexpression of HSPB1 could protect cell from ER stress. In this chapter, we speculate that HSPB1 mediated regulation of BIM would afford HSPB1 cells protection during ER stress and seek to identify how HSPB1 regulates expression of BIM.

BIM is a key pro-apoptotic member of BCL-2 family of protein, the expression of which is necessary for apoptosis in diverse cell types including hematopoietic, epithelial, neuronal, and germ cells, both in vitro and in a pathological setting within the whole animal (Puthalakath et al., 2007, Wakeyama et al., 2007). BIM is induced by numerous stimuli including ER stress (Puthalakath et al., 2007), NGF withdrawal (Whitfield et al., 2001), and by BCL-2-inhibitable stress stimuli, including cytokine deprivation or treatment with ionomycin or taxol (Bouillet et al., 1999). BIM has been shown to be important in neuronal cell death (Kudo et al., 2012, Sanphui and Biswas, 2013). Depletion of BIM reduces both developmental cell death and trophic factor withdrawal-induced apoptosis in neurons (Putcha et al., 2001). Additionally depletion of BIM reduces ER stress-induced apoptosis indicating it is a player in UPR-mediated cell death (Puthalakath et al., 2007, Szegezdi et al., 2008, Gupta et al., 2012).

Alternative splicing results in several BIM mRNA splice variants encoding different BIM isoforms. The major splice variants are BIM<sub>EL</sub>, BIM<sub>L</sub> and BIM<sub>S</sub>. Other less well studied BIM isoforms have been identified, namely BIM $\alpha$ 1, BIM $\beta$ 1, BIM $\beta$ 2, BIM $\alpha$ 2, BIM $\beta$ 3, BIM $\beta$ 4 (Marani et al., 2002). These isoforms show variable expression among normal tissues at least in humans, suggesting a tissue-specific transcriptional regulation of BIM. Of these lesser studied isoforms, only BIM $\alpha$ 1 and  $\alpha$ 2 contain a BH3 domain, however their biological significance remains to be fully defined (U et al., 2001, Marani et al., 2002). The other isoforms which lack the BH3 domain may function as decoys of pro-apoptotic BIM splice variants, thereby representing a novel regulatory mechanism for BIM (Adachi et al., 2004). With regard to ER stress BIM<sub>EL</sub> is the primary isoform induced at protein level (Puthalakath et al., 2007, Szegezdi et al., 2008). BIM<sub>EL</sub>, like BIM<sub>L</sub> is bound and sequestered to the cytoskeleton microtubular motor complex via a dynein light chain binding domain (DLC), a domain that is absent from BIMs and is regarded as the reason for the apoptotic potency of BIMs (Adachi et al., 2004)

BIM can be regulated by a variety of transcriptional, post-transcriptional and post-translational modifications. The regulation of BIM is highly complex and varies with cellular context (Ley et al., 2005). Examination of the proximal BIM promoter reveals binding sites for FoxO, MYB, C-JUN and ERG-1, and CHOP-C/EBP $\alpha$  (Biswas et al., 2007). BIM can be transcriptionally regulated by the pro-survival UPR gene, XBP1. Modulation of XBP1 expression by siRNA knock down was shown to increase BIM at mRNA level (Kurata et al., 2011), although it was not investigated whether a binding site for XBP1 is located in the BIM promoter.

The post-transcriptional regulation of gene expression is an important control mechanism particularly in the case of cellular stress when cells need to respond to change rapidly. BIM is regulated by a number of microRNAs, which are small non coding RNAs which bind to the 3'UTR of target genes and repress translation of mRNA into protein. MicroRNAs that regulate BIM include mir-24 (Qian et al. 2011), and the ER stress regulated clusters miR-17-92 (Molitoris et al., 2011) and miR-106B-25 (Gupta et al., 2012).

An important regulator of BIM during cell stress conditions involves post-translational modification by phosphorylation. BIM can be phosphorylated by ERK, JNK, P38, ATK and PKA (Ley et al., 2003, Luciano et al., 2003, Putcha et al., 2003, Qi et al., 2006, Moujalled et al., 2011). Phosphorylation may increase or decrease the apoptotic potential of BIM, alter its interaction with anti-apoptotic BCL-2 family proteins, or alter BIM protein stability (Hübner et al., 2008).

BIM<sub>EL</sub> is reported to have five phosphorylation sites, three of which are due to phospho-ERK signalling, S55, S65 ( S69 in humans) and S73 (Hübner et al., 2008). Phosphorylation of these sites is thought to occur in a hierarchical fashion where phosphorylation of S65 drives phosphorylation of the other residues. The consequence of ERK mediated phosphorylation of BIM on S65 is enhanced proteasomal degradation (Ley et al., 2003). Additionally in response to IL-3, phosphorylation by ERK decreases interaction of BAX with BIM, thereby reducing apoptosis (Harada et al., 2004).

Data also indicate that, in neuronal cells, JNK may phosphorylate BIM on S65 (Putcha et al., 2003, Ley et al., 2004). The mechanism of how phosphorylation of BIM by JNK can enhance apoptotic ability, while phosphorylation by ERK increases proteasomal degradation is unclear. In vitro both ERK and JNK can phosphorylate BIM<sub>EL</sub>, however, ERK is more effective (Ley et al., 2005). It is possible that S65 may undergo JNK mediated phosphorylation in the absence of ERK signalling. Additional sites phosphorylated by JNK include S100, S112, S114, the consequence of which is stabilization of BIM leading to increased apoptosis (Geissler et al., 2013).

The identity of the E3 ligase responsible for polyubiquitination of BIM has been controversial. Reported E3 ligases for BIM include  $\beta$ TrCP an F box protein (Dehan et al., 2009), and CBL (Akiyama et al., 2003), a ring finger protein, however, both of these findings have proven difficult to reproduce (Wiggins et al., 2007, Wiggins et al., 2011). Other reported ligases include Rack1, CIS, DRG1 and TRIM2.

Interestingly it has been shown that BIM can undergo ERK-driven degradation in an ubiquitin independent manner (Wiggins et al., 2011). BIM is an intrinsically



disordered protein (IDP) which does not have a well-defined tertiary structure and commonly undergoes structural transition upon binding to cognate partners. IDPs often undergo degradation by uncapped 20S proteasome without poly-ubiquitination (Wiggins et al., 2011). BIM<sub>EL</sub> has been shown to undergo ubiquitin independent proteasomal degradation (Wiggins et al., 2011).

In relation to ER stress, BIM has been shown to be transcriptionally regulated by CHOP (Puthalakath et al., 2007). Post-transcriptionally, ER stress suppresses microRNAs of the miR-17-92 family and miR-106-92 family, leading to increased mRNA stability and increased BIM protein expression. Post-translational modifications of BIM have also been reported during ER stress, due to dephosphorylation of BIM by PP2A (Puthalakath et al., 2007). In melanoma cell lines this was found to be an indirect effect, via PP2A-mediated dephosphorylation of ERK, leading to stabilization of BIM and increased apoptosis (Tay et al., 2012).

Results from Chapter 3 demonstrated the pro-survival effects of TP in ER stress. We also identified HSPB1 as a novel regulator of BIM expression. The aim of this study was to delineate the role of HSPB1 in ER stress-induced apoptosis and to examine the mechanisms by which HSPB1 can regulate BIM accumulation in the cells following ER stress. PC12 cells which expressed a control vector (Neo) or HSPB1 were used to investigate the protective effects of HSPB1 during ER stress-induced apoptosis. Using an siRNA knock down approach we demonstrated that regulation of BIM is key to the protective effects afforded by HSPB1 overexpression. Molecular analysis of the pathways which regulate BIM was carried out to determine how HSPB1 regulates BIM.

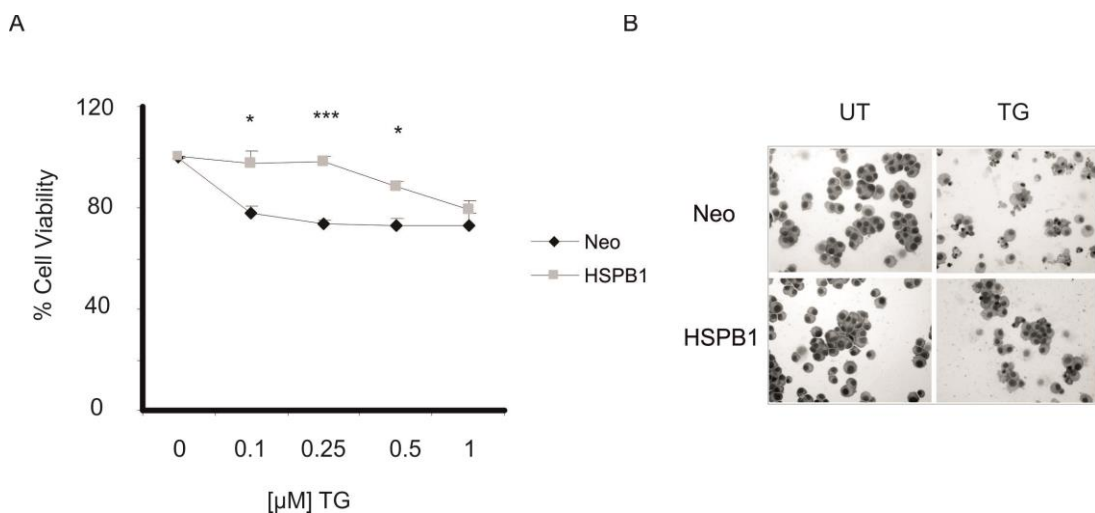
Although HSPs including HSPA1, HSPA5, and HSPB5 have previously been linked to regulation of ER stress-induced cell death, nothing is currently known about the role of HSPB1. Our previous results revealed induction and modulation of HSPB1 by TG suggesting it may have a role in regulation of cell death induced by ER stress. The findings reported in this Chapter demonstrate that HSPB1 is a novel regulator

of ER stress-induced apoptosis and has uncovered HSPB1 as a stimulator of BIM degradation.

## 4.2 Results

### 4.2.1 HSPB1 over-expression increases cell viability in response to ER stress

Results from Chapter-3 found that induction of HSP by heat shock preconditioning could attenuate ER stress-induced apoptosis. Heat shock preconditioning induces numerous HSPs. We therefore investigated whether over-expression of the small HSP, HSPB1, could provide protection against ER stress-induced cell death. Following 24 h treatment with TG, cell viability in Neo and HSPB1 cells was determined by MTT assay. As can be seen in Fig 4.2.1 A, HSPB1 over-expressing cells showed significantly enhanced survival at a range of TG concentrations of 0.1, 0.25 and 0.5  $\mu$ M. Cell morphology was assessed by making cytopsin preparations of cells followed by hemotoxylin-eosin staining. Neo cells treated with TG displayed characteristic features of apoptosis including cell shrinkage, nuclear condensation and membrane blebbing. Cells over-expressing HSPB1 displayed less apoptotic features (4.2.1 B)

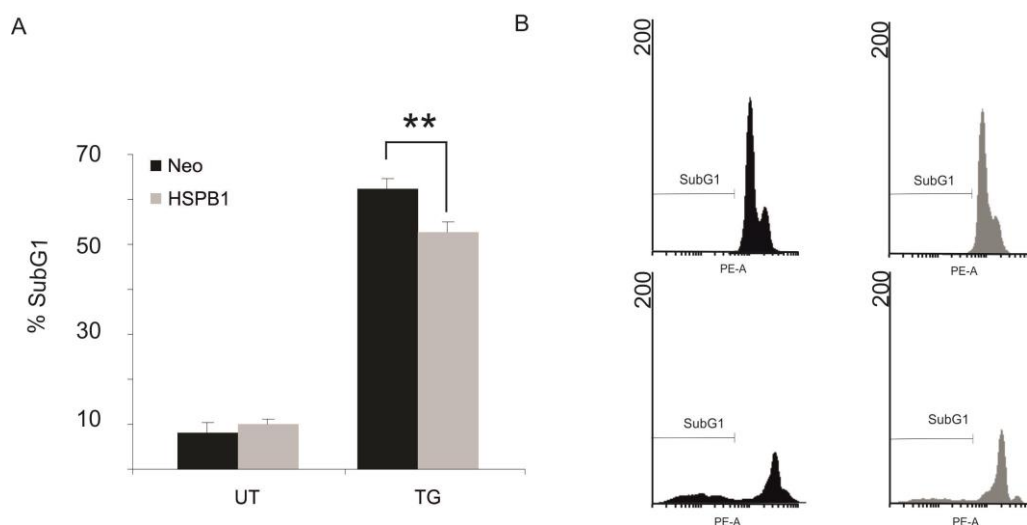


**Figure 4.2.1 HSPB1 over-expression increased cell viability in response to ER stress** (A) Neo and HSPB1 PC12 cells were treated with 0, 0.1, 0.25 0.5, 1  $\mu$ M TG for 24 h followed by MTT assessment of cell viability. Values shown are representative of three independent repeats. \* P <0.05, \*\* P<0.01, \*\*\* P< 0.001, \*\*\*\* P< 0.0001 Two- way ANOVA followed by Tukey's post hoc analysis versus Neo

Neo versus HSPB1 cells. **(B)** The morphology of the untreated and cells treated with 0.25  $\mu\text{M}$  TG for 48 h was examined by making cytocentrifuge preparations of cells followed by staining with haematoxylin and eosin dyes

#### 4.2.2 HSPB1 over-expression attenuates ER stress induced cell death.

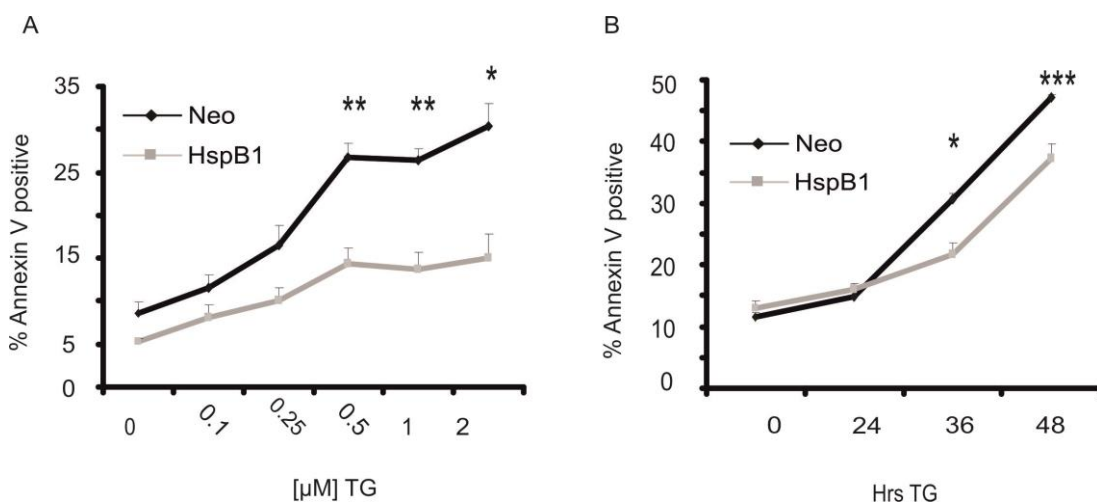
To confirm that the reduction in cell viability was due to apoptosis, we carried out a panel of apoptosis assays. Cells that are undergoing apoptosis have fragmented DNA due to the action of nucleases which can be measure by Subg1 analysis (See section 2.4.2 for details). Cell cycle profiles were assessed for Neo and HSPB1 cells following 36 h of 0.25  $\mu\text{M}$  TG treatments. It was found that HSPB1 cells have a significant reduction in the % of cells with a sub-G1 population (10% reduction). Fig 4.1.2 B shows representative histograms.



**Fig 4.2.2 HSPB1 over-expression attenuates DNA fragmentation following ER stress** **(A)** Neo and HSPB1 PC12 cells were treated with 0.25  $\mu\text{M}$  TG for 36 h. Cells were fixed in 70 % ethanol and Sub-G1 analysis was determined by PI staining on PE-A channel (FacsCanto flow cytometer). Values shown are representative of three independent repeats.\* P <0.05, \*\* P<0.01, \*\*\* P< 0.001, \*\*\*\* P< 0.0001 Two-way ANOVA followed by Tukey's post hoc analysis versus Neo. **(B)** Representative histograms of Neo (Black) and HSPB1 (Grey) cells treated with 0.25  $\mu\text{M}$  TG.for 36 h.

### 4.2.3 HSPB1 over-expression reduces ER stress induced apoptosis.

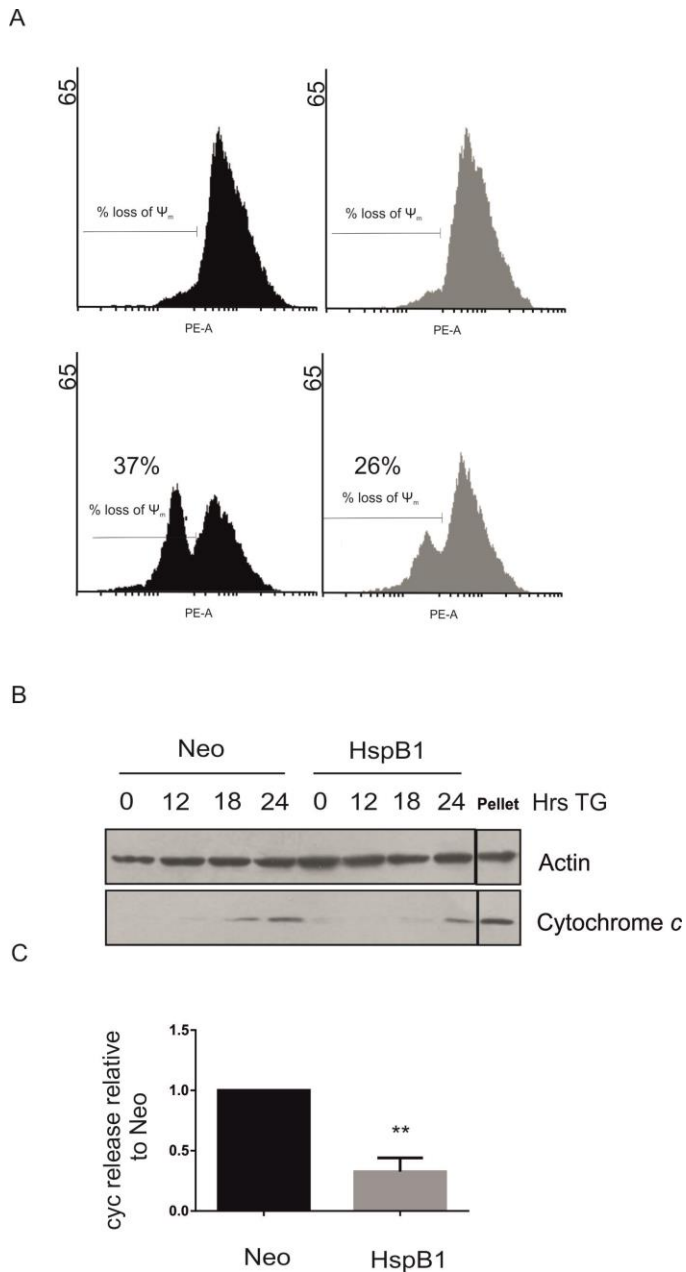
To verify the protective effects of HSPB1 on ER stress induced by TG treatment, annexin V staining was carried out (See section 2.4.3 for details). Annexin V binds to phosphatidylserine (PS) which is exposed on the surface of apoptotic cells (Galluzzi et al., 2009). HSPB1 over expressing cells showed reduced annexin V binding in both a dose independent and time independent manner (Figure 4.2.3 A,B). Significant protection against cell death was seen in HSPB1 cells following treatment with 0.5  $\mu\text{M}$ , 1  $\mu\text{M}$  and 2  $\mu\text{M}$  TG at 24 hrs (approx. 50% less annexin V positive cells in HSPB1 cells than in the neo). HSPB1 over-expression caused a significant 10% reduction in apoptosis at 36 h and 48 h of TG treatment. Collectively, these data demonstrate the protective effect of HSPB1 in ER stress-induced apoptosis.



**Figure 4.2.3 HSPB1 over-expression reduces ER stress induced apoptosis. (A)** Neo and HSPB1 cells were treated with 0.1 0.25 0.5, 1 or 2.0  $\mu\text{M}$  TG for 24 h. The % cell death was estimated by flow cytometry based measurement of Annexin V. **(B)** Time course study of Neo and HSPB1 cells treated with 0.25  $\mu\text{M}$  TG for 0-48 h. The % cell death was estimated by flow cytometry based measurement of Annexin V. Values shown are representative of three independent repeats.\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$  Two-way ANOVA followed by Tukey's post hoc analysis versus Neo.

#### **4.2.4 HSPB1 mediated protection occurs upstream of the mitochondria**

HSPB1 can positively modulate cell fate by regulating events upstream and downstream of the mitochondria. We investigated how HSPB1 regulated apoptotic events during ER stress. Apoptosis induced by ER stress occurs via the mitochondrial pathway; therefore, we investigated the intrinsic pathway in Neo and HSPB1 cells. We assessed alterations in mitochondrial membrane potential using tetramethyl rhodamine ethyl ester (TMRE) (See section 2.4.4 for details). A greater proportion of HSPB1 cells retained mitochondrial membrane potential compared to Neo cells (Figure 4.2.4 A). Consistent with HSPB1 protecting cells from ER stress-induced apoptosis upstream of the mitochondria, release of cytochrome c was reduced in HSPB1 over expressing cells (Figure 4.2.4 B).



**Figure 4.2.4 HSPB1 mediated protection occurs upstream of the mitochondria. (A)**

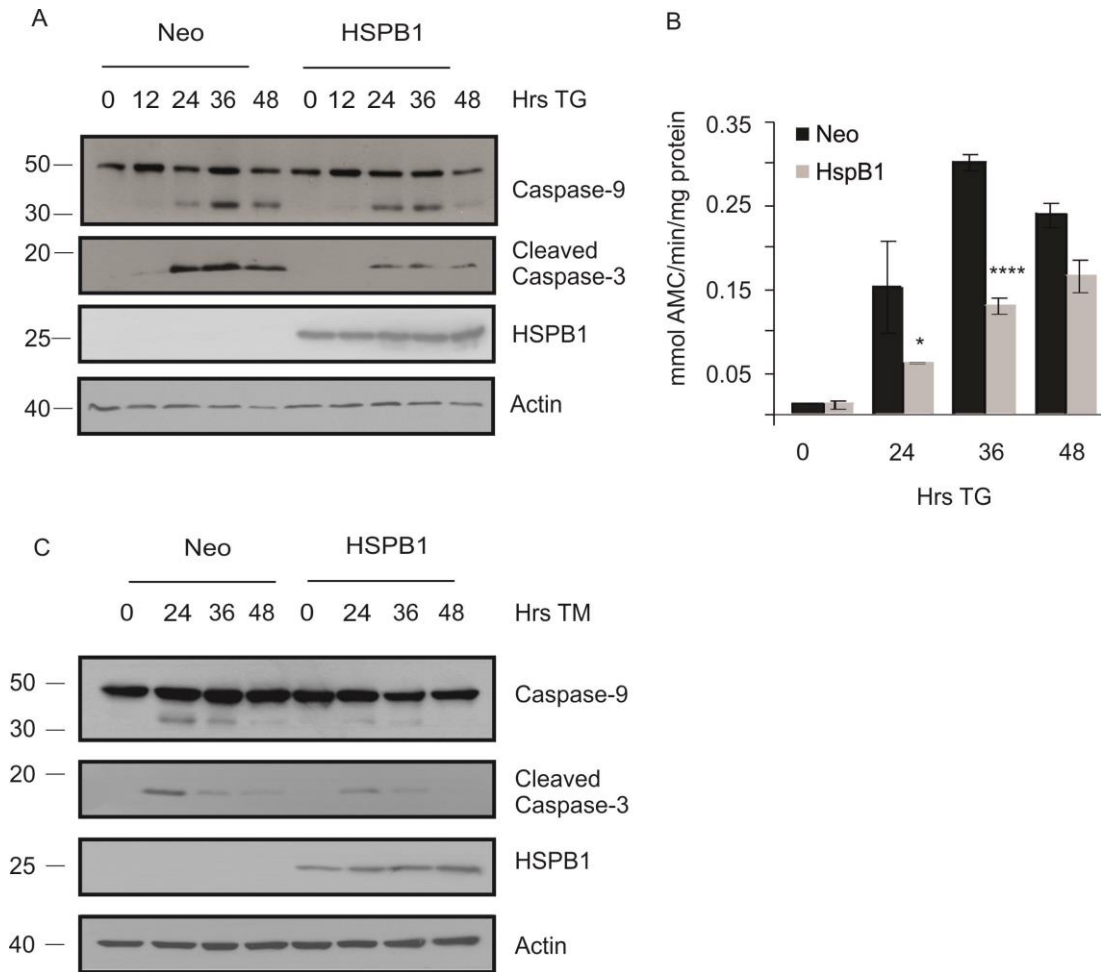
Changes in  $\Delta\Psi_m$  in Neo and HSPB1 cells treated with 0.25  $\mu\text{M}$  TG-treated cells were determined by Flow cytometry based measurement of TMRE at 48 h **(B)** Release of cytochrome *c* into cytosol was detected by Western blot analysis of cytosolic fraction obtained by Digitonin permeabilization of cells treated with TG at indicated time points. ACTIN was used as a loading control. Data is representative of three independent repeats.**(C)** Densitometric analysis of cytochrome *c* release was carried out and statically significant assessed by students t-test versus Neo. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$

#### 4.2.5 HSPB1 reduces caspase activation following ER stress

Apoptosis is defined as a caspase-dependent form of programmed cell death. We therefore tested caspase activation in Neo and HSPB1 cells following ER stress. Caspase activation can be monitored by Western blot analysis and probing for caspase cleavage products (See section 2.10. for details).

Following TG treatment, caspase-9 undergoes cleavage, producing a ~35 kDa fragment. This process was reduced in HSPB1 over-expressing cells. Similarly caspase-3 is cleaved to the p17 fragment following 24, 34, and 48 h of TG treatment. This was significantly attenuated in HSPB1 over-expressing cells. As can be seen in Fig 4.2.5A, HSPB1 cells have reduced caspase-9 and caspase-3 cleavage. Activity of type II caspases can be determined by using a fluorescently labelled peptide substrate, DEVD-AMC. In agreement with Western blot data, HSPB1 cells have lower levels of caspase enzymatic activity (Fig 4.2.5 B). The modulatory effect of HSPB1 on caspase activation is not specific to TG treatments as similar results were obtained in cells treated with TM (Fig 4.2.5C).

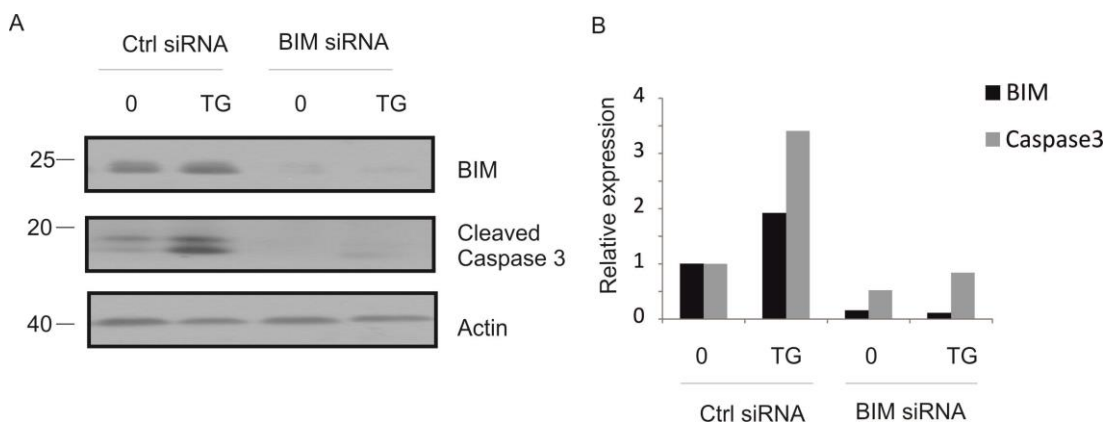




**Fig 4.2.5 HSPB1 reduces caspase activation following ER stress. (A)** Neo and HSPB1 PC12 cells were treated with 0.25  $\mu$ M TG for 0,12, 24,36 and 48 h. Caspase cleavage was assessed by Western blotting using anticaspase-9 and anti-caspase-3 antibodies. ACTIN was used as loading control **(B)** Caspase activation was measured by DEVDase activity of whole cell lysate at indicated time points after TG treatment. **(C)** Neo and HSPB1 PC12 cells were treated with 2  $\mu$ g TM for 0, 24, 36 and 48 h. Caspase cleavage was assessed by Western blotting using anticaspase-9 and anti-caspase-3 antibodies. ACTIN was used as loading control Data is representative of three independent repeats.

#### 4.2.6 BIM is involved in ER stress-induced apoptosis

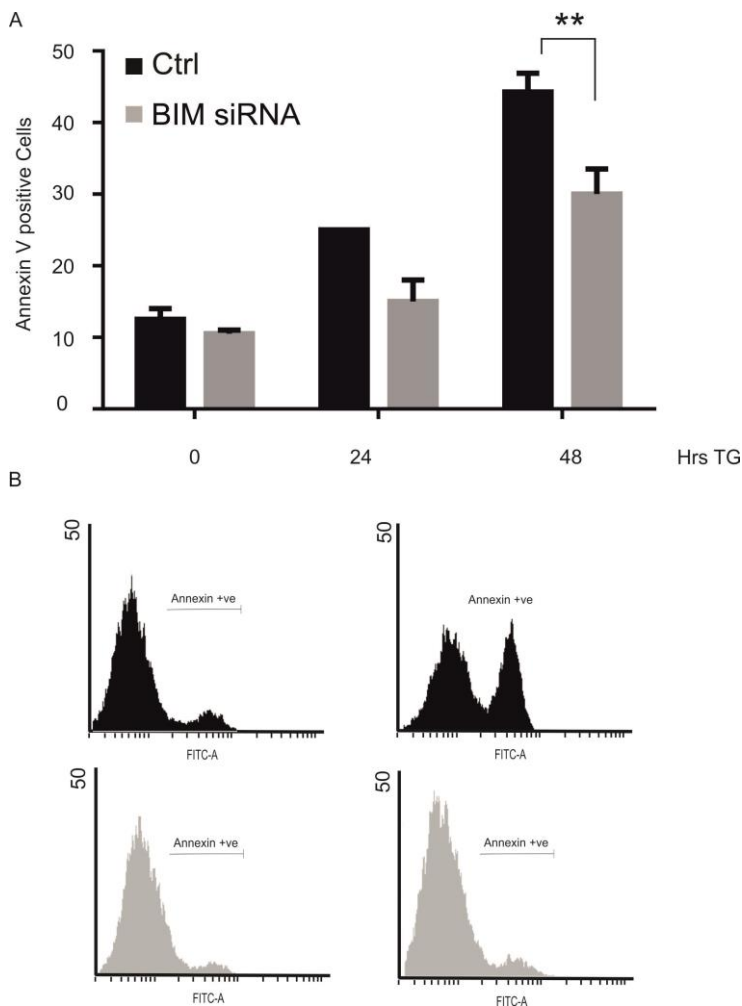
Previous reports have demonstrated the involvement of BIM in ER stress-induced apoptosis (Puthalakath et al., 2007, Gupta et al., 2012, Zhang et al., 2012). We wanted to assess the role for BIM in ER stress-induced apoptosis in PC12s treated with TG. Cells were transfected with 20 nM BIM siRNA or a non-targeting control siRNA using lipofectamine for 5 h (See section 2.3.2 for details) 24 h after transfection, cells were treated with TG for 0, 24 or 48 h. Knockdown efficiency was determined by Western blotting of untreated cells or cells treated with 0.25  $\mu$ M TG for 0 or 48 hrs. Fig 4.2.6 demonstrates that 20 nM of siRNA effectively knocked down BIM expression. In agreement with BIMs role in ER stress induced apoptosis we observed reduced caspase-3 cleavage in cells treated with BIM siRNA (Fig 4.2.6). This suggests that BIM is important for initiating mitochondrial apoptosis in response to TG in PC12 cells.



**Fig 4.2.6 Involvement of Bim in ER stress induced apoptosis. (A)** 24 h after siRNA transfection PC12 cells were untreated or treated with 0.25  $\mu$ M TG for 48 h. BIM and Caspase-3 cleavage were assessed by Western blotting using anti-caspase-3 and anti-BIM antibodies. ACTIN was used as loading control. Data is representative of two independent repeats. **(B)** Densitometric analysis of BIM and caspase3 expression in control and BIM siRNA treated cells.

#### 4.2.7 Knock down of BIM attenuates ER stress induced apoptosis

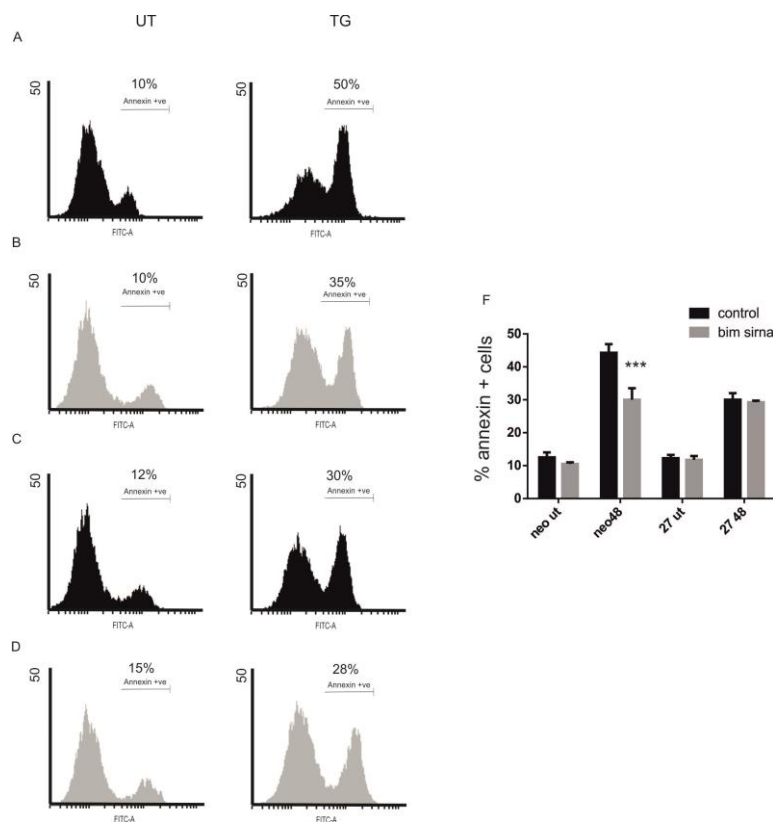
We next determined what % of cell death was due to increased expression of BIM following TG treatment. Cell death was determined by annexin V staining and flow cytometry. As can be seen in Fig 4.2.7 A knock down of BIM significantly reduced ER stress induced apoptosis following 48 h treatment with TG. Fig 4.2.6 B shows representative histograms.



**Fig 4.2.7 Knock down of BIM attenuates ER stress-induced apoptosis (A)** 24 h after siRNA transfection PC12 cells were treated with or 0.25  $\mu$ M TG for 0, 24, 48 h. The % cell death was estimated by flow cytometry based measurement of Annexin V. Values shown are representative of three independent repeats. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$  Two-way ANOVA followed by Tukey's post hoc analysis versus Neo **(B)** Representative Histograms from (A).

#### **4.2.8 HSPB1 mediated protection against ER stress is dependent on regulation of BIM**

Given that HSPB1 is a potent regulator of apoptotic events downstream of the mitochondria including activation of caspases (Bruey *et al* 2000, Concannon *et al* 2001), we wanted to test the significance of HSPB1 mediated regulation of BIM. The protective effects observed in HSPB1 over-expressing cells may be due to previously reported downstream regulation of caspase-3 and cytochrome *c* release mechanism. Alternatively, HSPB1 mediated regulation of BIM may be the upstream event which ultimately leads to reduced caspase activation. To test this hypothesis we transfected Neo and HSPB1 cells with control siRNA or a BIM targeting siRNA. 24 hpost-transfection cells were treated with TG. Cell death was determined by annexin V staining. As observed in previous experiments, HSPB1 over-expression led to a 15% protection against ER stress in control siRNA treated cells compared to Neo control cells (fig 4.2.8B vs A). When BIM was knocked out with BIM targeting siRNA, ER stress induced cell death was reduced by 20% of Neo cells (Fig 4.2.8 C vs A). There was a 2% difference in cell death between Neo and HSPB1 cells when BIM was knock down (Fig 4.2.8 D vs C). This data demonstrates that ~85% of the protective effects of HSPB1 overexpression are due to regulation of BIM (Fig 4.2.8 B vrs B).



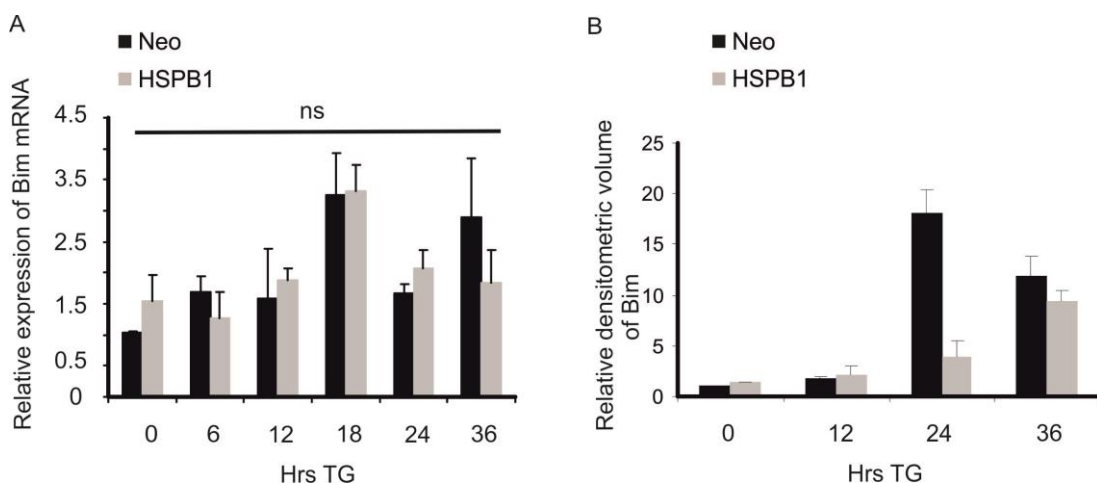
**Figure 4.2.8 HSPB1 protects against ER stress via regulation of BIM.** (A) Neo cells were transfected with ctrl siRNA and followed by treatment with TG for 48 h. (B) HSPB1 cells were transfected with ctrl siRNA and followed by treatment with TG for 48 h. (C) Neo cells were transfected with BIM siRNA and followed by treatment with TG for 48 h. (D) HSPB1 cells were transfected with BIM siRNA and followed by treatment with TG for 48 h. The % cell death was estimated by flow cytometry based measurement of Annexin V. (E) Values shown are representative of three independent repeats. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$  Two-way ANOVA followed by Tukey's post hoc analysis

#### 4.2.9 HSPB1 regulates BIM via a post transcriptional mechanism

Results from Figures 4.2.1-4.2.5 show that HSPB1 over-expression can protect against ER stress induced apoptosis. Figures 4.2.6 and 4.2.7 demonstrate that BIM is a key mediator of ER stress induced apoptosis. Importantly, we demonstrated the protective effects mediated by HSPB1 over-expression are dependent on regulation

of BIM (Fig 4.2.8). Subsequent work sought to identify the mechanism by which HSPB1 can regulate BIM expression.

BIM is subject to multiple modes of regulation at transcriptional, post transcriptional and posttranslational levels. To this end, we tested if HSPB1 over-expression was causing a reduction in BIM mRNA, and subsequently reducing BIM at the protein level. qRT-PCR analysis of BIM mRNA was carried out in Neo and HSPB1 PC12s following TG treatment for 0, 6, 12, 18, 24, or 36 h. As can be seen in Fig 4.2.9A TG-induced a modest maximal (approx. 3.5-fold) increase in BIM mRNA. Crucially, no difference was observed between Neo and HSPB1 cells. This modest increase in BIM mRNA fails to correlate with an approx. 18-fold increase in BIM at protein level (Fig 4.2.9 B). These data suggest that post-transcriptional regulation of BIM is the key regulator of expression during ER stress and HSPB1-mediated reduction in BIM protein levels may be due to post-transcriptional regulation.

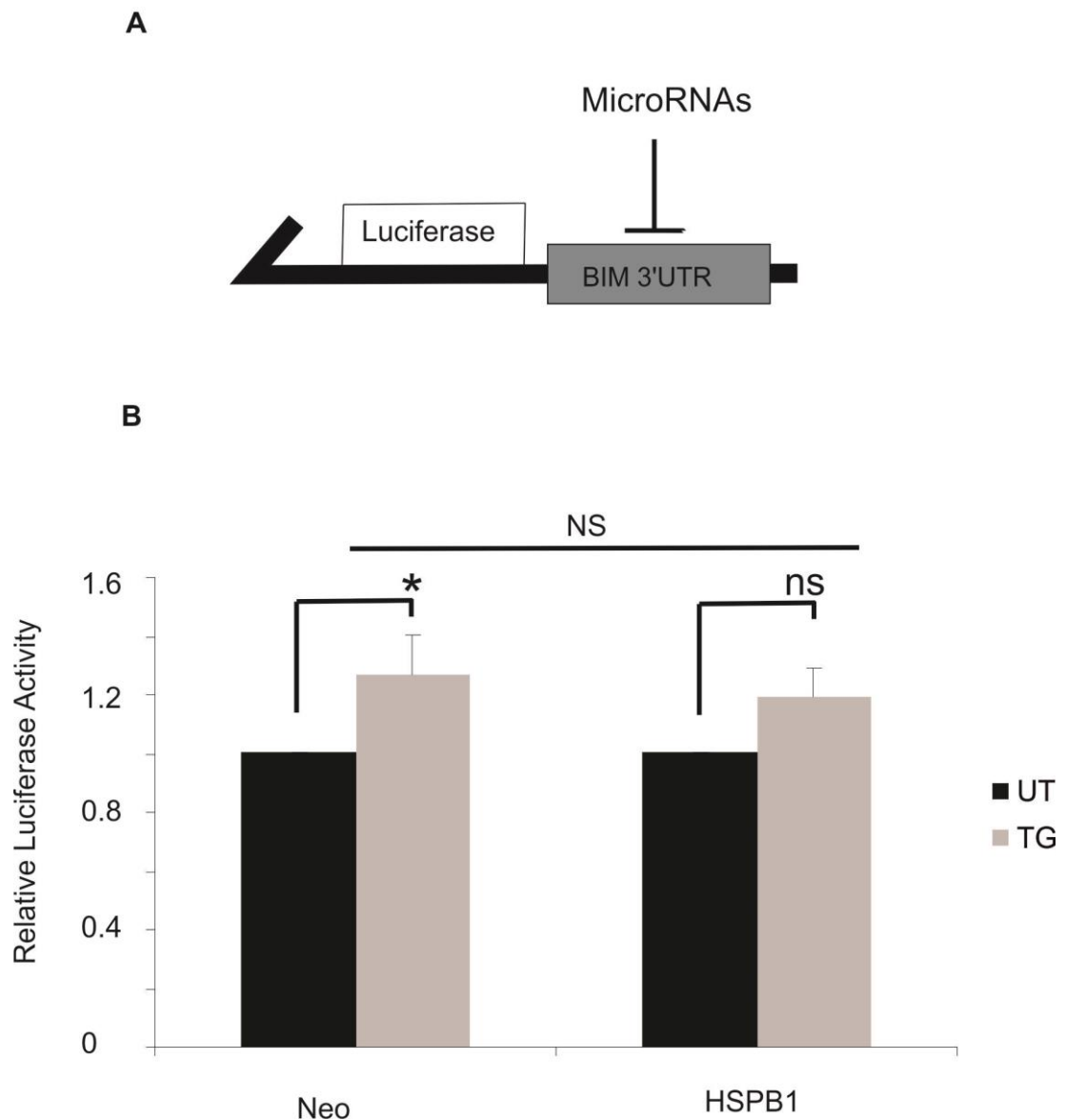


**Fig 4.2.9 HSPB1 regulates BIM via a post-transcriptional mechanism. (A)** Expression levels of BIM mRNA levels in Neo and HSPB1 cells following 0.25  $\mu$ M TG for 0-36 h was quantified by real-time RT-PCR, normalizing against GAPDH. **(B)** Densitometric analysis of BIM expression following Western Blotting was determined using Image J software. Values shown are representative of three independent repeats non significant (Neo versus HSPB1) as determined by Two-way ANOVA followed by Tukey's post hoc analysis versus Neo

#### 4.2.10 Regulation of BIM by HSPB1 is not due to microRNA activity

BIM is a target of two paralogue microRNA clusters, miR-17-92 and miR-106B-25 leading to translational repression (Gupta et al., 2012). Both of these microRNA clusters are down regulated during ER stress resulting in an increase in BIM (Gupta et al 2012). Therefore we sought to determine if microRNA-mediated repression was responsible for reduced induction of BIM in HSPB1 cells.

To determine if regulation occurs through direct binding of members of the miR-17-92 cluster to BIM 3'-UTR, a BIM 3'-UTR reporter plasmid was used which contains the conserved binding sites (for members of miR17-92) in the psiCHECK2 vector, downstream of the *renilla* luciferase gene (see schematic Fig. 4.2.10 A). We have also found that this construct is responsive to the 106B-25 cluster and therefore is an ideal tool to assess activity of miR17-92 AND miR106B clusters at the BIM 3'UTR. Neo and HSPB1 cells were transfected with either a control psiCHECK2 plasmid or the BIM 3'-UTR plasmid. 24 h later cells were treated with TG for a further 24 h (See section 2.3.1 for details). Luciferase activity was determined with the DualGlo Luciferase assay kit (See section 2.8 for details). Results were normalized to control vector. In agreement with previous findings, it was observed that TG caused an increase in luciferase activity of the reporter construct in Neo cells indicative of a reduction in microRNA activity. No difference was seen between the activity of the BIM 3'UTR as determined by luciferase activity in HSPB1 cells compared to Neo cells. Therefore these data suggests that the differential expression of BIM in HSPB1 cells is not due to microRNA-mediated repression.



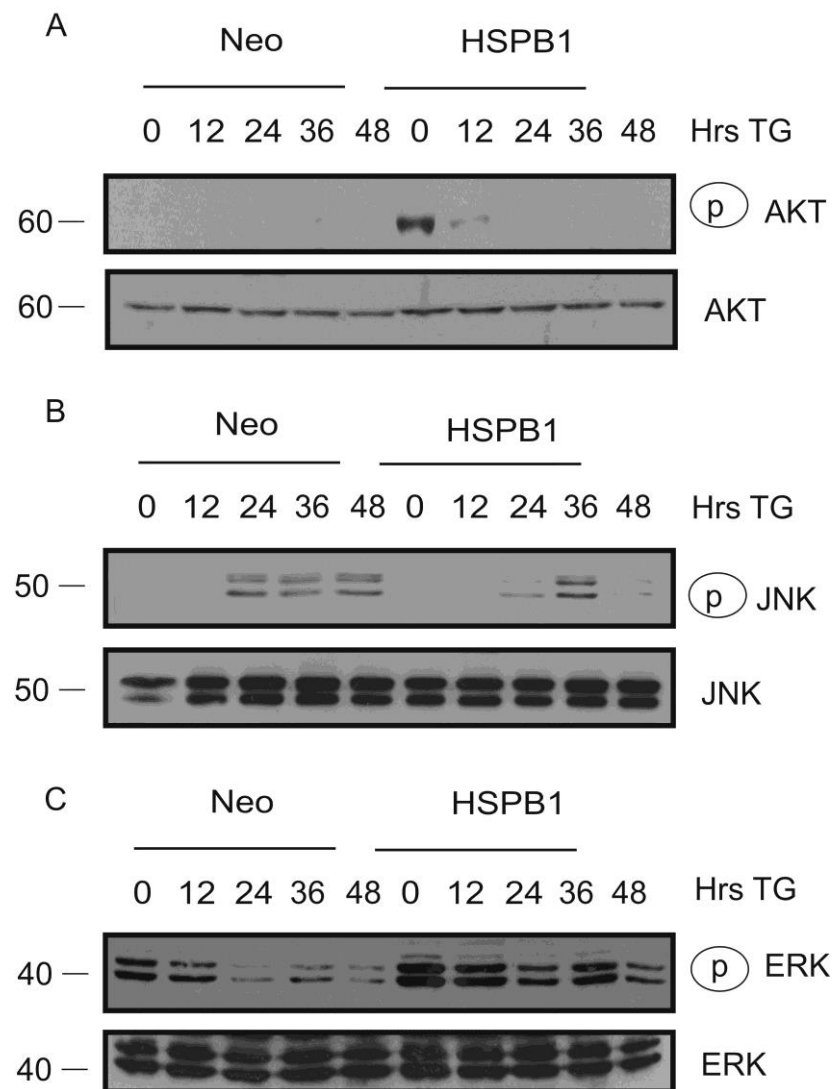
**Fig 4.2.10 Regulation of BIM 3'UTR.** (A) Schematic representation of the BIM 3'UTR reporter construct (B) PC12 cells were transfected with BIM 3'UTR reporter or control plasmid. 24 h post-transfection cells were left untreated (UT) or treated with 0.25  $\mu$ M TG for 24 h. Luciferase activity was assessed using DualGlo assay system and normalized to Luciferase activity (Renilla/Firefly) and expression relative to control the plasmid is shown. Values shown are representative of three independent repeats. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$  Two-way ANOVA followed by Tukey's post hoc analysis



#### 4.2.11 HSPB1 over-expression alters stress kinase signalling

Previous results in this chapter suggest HSPB1 regulates BIM expression via a post-translational mechanism. Stress kinases are important regulators of BIM, acting to regulate its activity and expression via post-translational modifications (Ley et al., 2004). We carried out analysis of AKT, JNK and ERK during ER stress as these kinases have been previously shown to regulate BIM.

Using antibodies which specifically detect the phosphorylated form of AKT we found that HSPB1 cells express higher basal levels of phospho-AKT. We could not detect AKT in Neo cells. TG treatment caused a reduction in levels of phospho-AKT relative to total AKT in HSPB1 cells. We used an antibody which detects levels of phosphorylated p46 and p54 JNK isoforms dually phosphorylated at both threonine 183 and tyrosine 185. This antibody does not recognize unphosphorylated JNK. JNK was found to undergo phosphorylation during ER stress as indicated by an increase in the levels of phospho-JNK relative to total JNK. HSPB1 cells had earlier attenuation of phospho-JNK and a delayed activation of phospho-JNK. To study activation of ERK, we used an antibody which detects endogenous levels of p44 and p42 MAP Kinase isoforms phosphorylated either individually or dually at Thr202 and Tyr204 of Erk1 (Thr185 and Tyr187 of Erk2). Expression of phospho-ERK was down-regulated by TG treatment in a time dependant manner. HSPB1 over-expressing cells had higher basal levels of phospho-ERK and the decline following TG treatment was significantly less than for Neo cells, suggesting phospho-ERK is stabilized in HSPB1 cells.

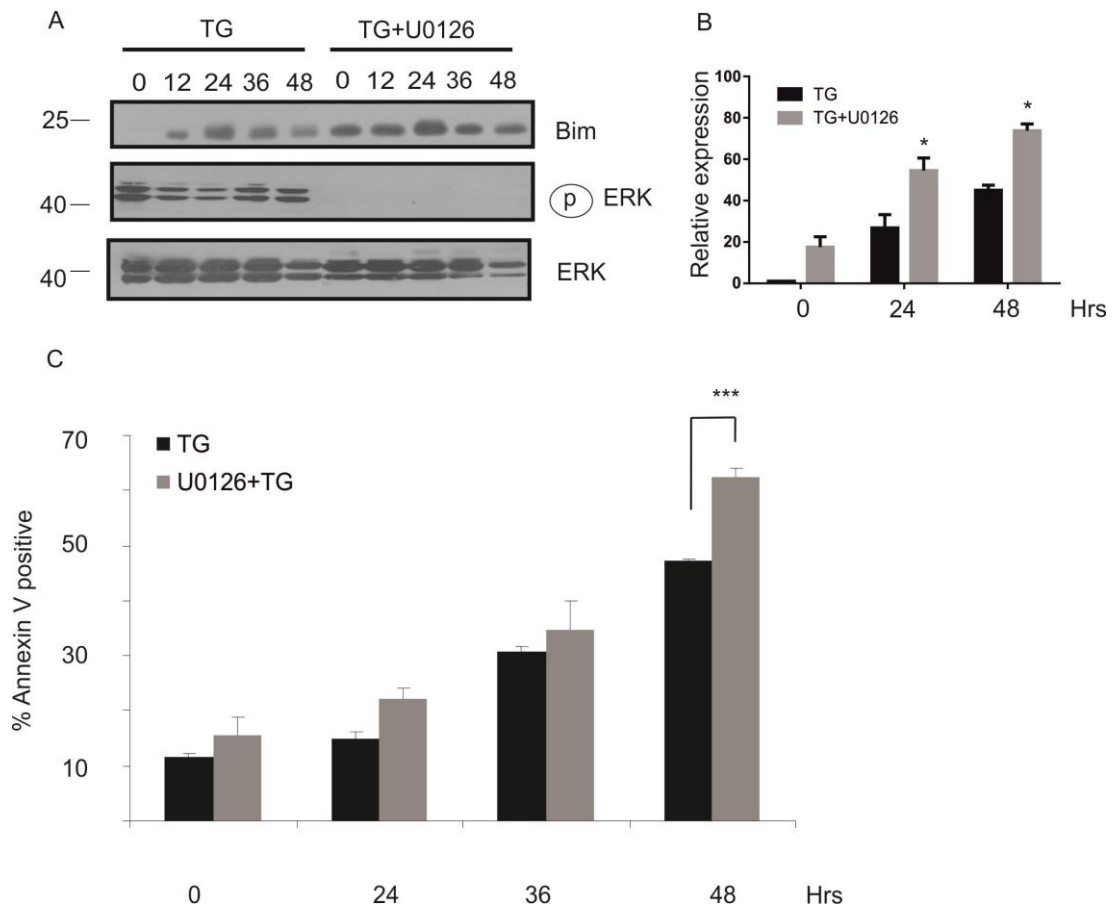


**Figure 4.2.11 HSPB1 modulates the PI3K/AKT and MEK/ERK and JNK pathways. (A)** Western Blot analysis of total and Phospho-AKT in Neo and HspB1 PC12s following 0.25  $\mu$ M TG. **(B)** Western Blot analysis of total and Phospho-JNK in Neo and HspB1 PC12s following 0.25  $\mu$ M TG. **(C)** Western Blot analysis of total and Phospho-ERK in Neo and HspB1 PC12s following 0.25  $\mu$ M TG. Data is representative of at least three independent repeats.

#### 4.2.12 Phospho-ERK regulated BIM during ER stress

We noted a parallel inverse relationship between expression of BIM and phospho-ERK. Significant differences in expression level of BIM occur at 24 h in Neo and HSPB1 cells (Fig 4.2.9 B). Similarly we see prominent differences in expression levels of phospho-ERK at 24 h between Neo and HSPB1 cells (Fig 4.2.9C). This suggested to us a casual relationship between phospho-ERK signalling and BIM expression.

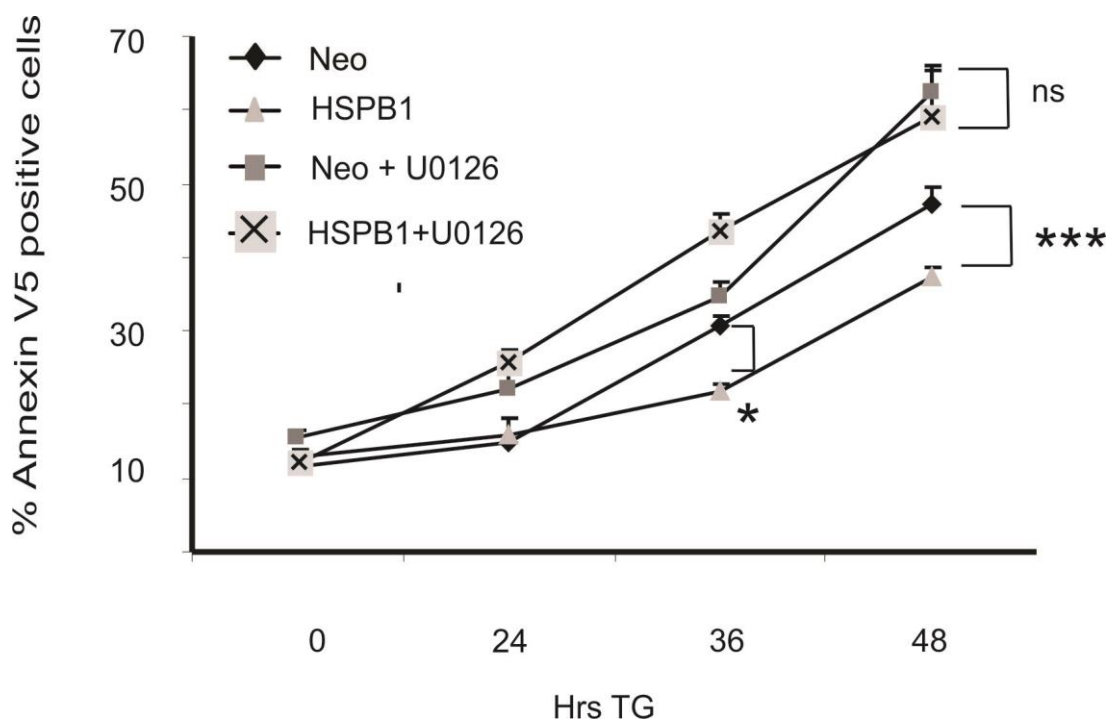
To delineate the role of phospho-ERK signalling during ER stress we inhibited phospho-ERK with a MEK1 inhibitor, U0126. ERK is the only known target of MEK1 therefore U0126 is widely used as a tool to inhibit phospho-ERK (Roberts and Der, 2007). PC12s were treated with 10  $\mu$ M of a 1:1000 stock of U0126 every 12 h or with DMSO as a control. Top-up treatments were necessary due to the short half life of U0126 in culture. U0126 caused abrogation of phospho-ERK while levels of total ERK remained unchanged (Fig 4.2.12 A). Inhibition of phospho-ERK activity was paralleled by increased levels of BIM (Fig 4.2.12 A). These results suggest that ERK may be involved in the regulation of BIM expression during ER stress. We next investigated the effect of phospho-ERK on cell fate during ER stress. Cells were treated with TG or with TG in the presence of absence of U0126. Cell death was measured by annexin V staining. No toxicity was observed in cells treated with U0126 alone as can be seen at the 0 hour treatment in U0126 treated cells (Fig. 4.2.12B). However, U0126 led to an increase in cell death at 48 h (Fig 4.2.12B). No significant difference in cell death was observed at earlier time points. These data suggest that loss of phospho-ERK signalling contributes to cell death.



**Figure 4.2.12 Phospho-ERK can regulate BIM during ER stress. (A)** Western Blot analysis of total and Phospho-ERK and total and Phospho-BIM in Neo PC12s following 0.25  $\mu$ M TG or TG in combination with 10  $\mu$ M U0126 for 0, 24, 36 or 48 h. Samples were harvested and prepared for SDS-PAGE. Protein expression was determined by western blotting using anti BIM and caspase-3 antibodies. **(B)** Annexin V determination of apoptosis following TG treatment in presence or absence of ERK inhibitor U0126. Values shown are means of three independent repeats  $\pm$  SEM. Un-paired Students *t*-test \*  $P < 0.05$ , \*\*  $P < 0.01$  \*\*\*  $P < 0.0001$  TG vrs TG+U0126.

#### 4.2.13 HSPB1 mediated protection against ER stress induced apoptosis is phospho-ERK dependent

We wanted to test if increased phospho-ERK signalling was responsible for the protective effects observed in HSPB1 cells. We reasoned that HSPB1 would not be able to protect against ER stress in the absence of ERK. To test this hypothesis we treated Neo and HSPB1 PC12 cells with TG alone or TG plus the MEK inhibitor U0126. The previously optimized concentrations of 10  $\mu$ M U01236 every 12 h and 0.25  $\mu$ m TG were used. The U0126 0 hour sample was treated with U0126 for the duration of the experiment, i.e., 48 h and therefore represents maximal toxicity induced by U0126 alone. Cells were treated for 0, 24, 36, or 48 h. Results are shown in Fig 4.2.13. TG treated HSPB1 cells had reduced apoptosis compared to Neo cells. However in the presence of U0126 HSPB1 could no longer protect cells from ER stress, indicating that HSPB1 mediated protection against ER stress is dependent on regulation of phospho-ERK.

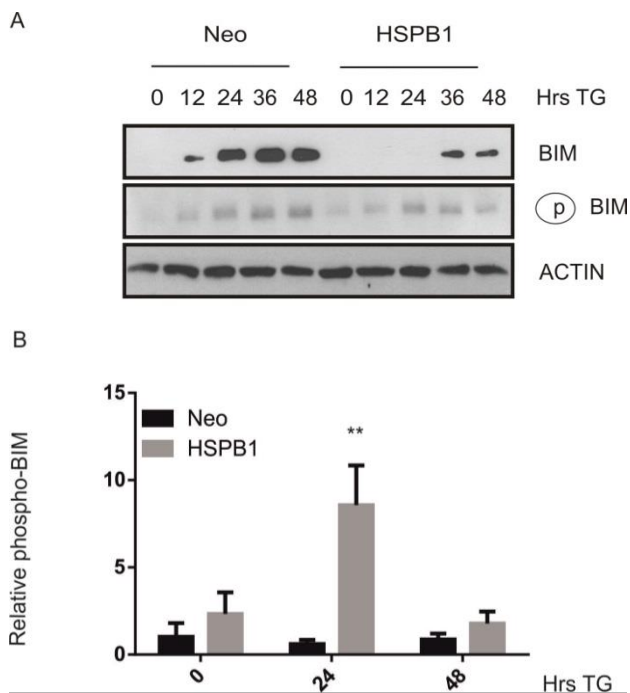


**Figure 4.2.13 HSPB1 mediated protection against ER stress induced apoptosis is ERK dependant. (A)** Annexin V determination of apoptosis following TG treatment in presence or absence of ERK inhibitor U0126 over time Values shown are representative of three

independent repeats.\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$  Two-way ANOVA followed by Tukey's post hoc analysis versus Neo

#### 4.2.14 HSPB1 over-expressing cells have increased levels of phospho-BIM

Increased levels of phospho-ERK in HSPB1 cells could lead to increased phosphorylation of BIM on S65. To test this we used a BIM antibody which was specific for BIM which has been phosphorylated on S65. We found that a small proportion of BIM was phosphorylated on S65 in Neo cells in response to TG treatment. HSPB1 cells had similar levels of phospho-BIM to Neo cells. When taking into account the lower levels of total BIM in HSPB1 cells, there is a higher ratio of phospho BIM to total BIM in HSPB1 cells compared to Neo cells. This analysis was carried out by densitometry. Fig 4.2.14 shows one representative experiment of three separate repeats. Therefore a higher percentage of BIM is phosphorylated in HSPB1 cells



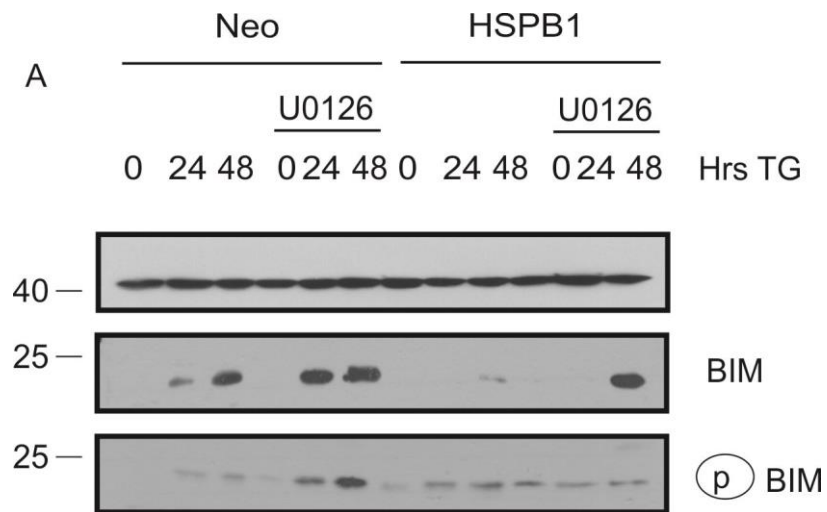
**Fig. 4.1.14. HSPB1 cells have higher levels of S65 phospho-BIM.** **(A)** Western Blot analysis of total and Phospho-BIM Neo and HSPB1 PC12 cells following 0.25  $\mu$ M TG for 0, 12, 24, 36 or 48 h. Samples were harvested and prepared for SDS-PAGE. **(B)** Densitometric analysis of relative phospho-BIM levels in Neo and HSPB1 cells following 0.25  $\mu$ M TG for 0, 24 and 48 hrs. Data is representative of three independent repeats  $\pm$  SEM.\* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001, \*\*\*\* P < 0.0001 Two-way ANOVA followed by Tukey's post hoc analysis versus Neo

#### **4.2.15 HSPB1 mediated regulation of BIM is partially due to regulation of phospho-ERK**

We next investigated the effect of loss of phospho-ERK signalling on BIM in expression in Neo and HSPB1 cells. We hypothesised that loss of phospho-ERK would lead to similar expression levels of BIM in Neo and HSPB1 cells. Cells were treated for 0, 24 or 48 h with TG alone or TG U0126. In agreement with previous results TG caused a loss of phospho-ERK. Treatment of cells with U0126 efficiently reduced basal levels of phospho-ERK while expression levels of total ERK remained unchanged. BIM was induced in a time dependant manner in Neo cells and addition of U0126 led to earlier induction of BIM. HSPB1 cells had reduced levels of BIM following TG treatment compared to Neo cells. Inhibition of phospho-ERK in HSPB1 cells caused massive accumulating of BIM however only at 48 hours of treatment.

Treatment of HSPB1 cells with U0126 causes a reduction in levels of phospho-BIM on S65. Unexpectedly inhibition of phospho-ERK was paralleled by an increase in the levels of phospho-BIM in Neo cells. Therefore an alternative kinase must be phosphorylating BIM in the absence of ERK signalling in Neo cells.





**Fig 4.2.15 Regulation of Phospho-BIM (A)** Neo and HSPB1 PC12s were treated for 0, 24, 48 h with 0.25  $\mu$ M TG alone or TG plus 10  $\mu$ M U0126. Samples were prepared for SDS-PAGE and expression of ERK,phospho-ERK, BIM and phosphor-BIM were determined by Western Blot. Data is representative of four independent repeats.

#### 4.2.16 HSPB1 decreases the protein half-life of BIM

Given the well-established role of phospho-ERK in enhancing degradation of BIM via the proteasome and the proteasome enhancing capabilities of HSPB1, we hypothesised that HSPB1 was acting as an E4 ligase to enhance BIM degradation. To test this hypothesis, we assessed protein half-life of BIM in Neo and HSPB1 over-expressing cells using cycloheximide which interferes with the translocation step in protein synthesis, thus blocking translation elongation. This makes it a useful tool to study protein stability/half-life.

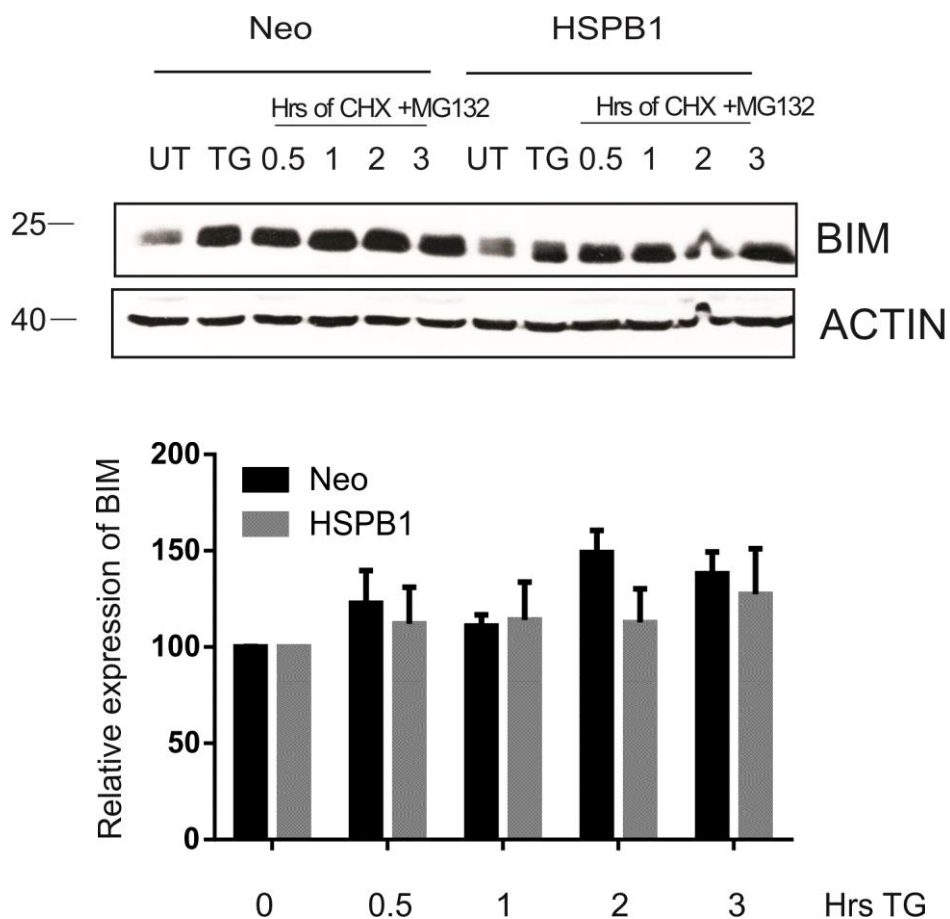
Neo and HSPB1 cells were pre-treated with TG for 24 h. After 24 hours untreated control cells and TG treated positive control cells were harvested and frozen. The remaining samples were treated with fresh media containing 10  $\mu$ M cycloheximide (CHX) for 30 min, 1 h, 2 h or 3 h after which time cells were harvested and all samples were processed according to the Western blot methods (See section 2.10 and 2.10.3 for details)

The cycloheximide time course experiment showed that in HSPB1 over expressing cells, BIM had a shorter half-life compared to Neo cells (Fig 4.2.16A). In HSPB1 overexpressing cells BIM levels had returned to that of untreated conditions by 30 min while in Neo cells BIM levels show a slow decline but even by 3 h BIM expression was still elevated (Fig 4.2.16 A). Protein half-life can be calculated by fitting densitometric analysis to a mono exponential decay curve. BIM half-life was calculated by setting TG treated BIM levels ( $T=0$ ) in Neo and HspB1 cells as 100 % and expressing cycloheximide treated samples (0.5,1,2,3 h) relative to  $t=0$ . In this way the reduced expression of BIM in HSPB1 cells is accounted for in calculating protein half-life. As can be seen in (Fig 4.2.16 B) in HSPB1 over expressing cells BIM has a half-life of less than 2 h while in Neo cells the half-life is  $\sim$  13 h. Therefore BIM degradation is enhanced in the presence of HSPB1.



#### 4.2.17 HSPB1 regulates BIM via proteasomal degradation

To show that HSPB1 is enhancing proteasomal degradation of BIM we pre-treated cells with TG for 24 h after which time the media was replaced with media containing CHX (10  $\mu$ M) plus proteasome inhibitor MG132 (20  $\mu$ M). This was required to ensure BIM was not being degraded via an alternative degradation pathway such as the autophagy pathway which has previously been shown to contribute to the degradation of BIM in an ERK independent manner. Figure 4.2.17 shows that proteasomal inhibition blocks HSPB1 mediated degradation of BIM.



**Fig 4.2.17 HSPB1 causes proteasomal degradation of BIM.** PC12 cells were pre-treated with TG for 24 h to induce BIM, followed by 0.5, 1, 2, 3 h of CHX and MG132 treatment. Samples were harvested and BIM expression was determined by Western Blotting using anti-BIM antibodies. ACTIN was used as a loading control. Densitometric analysis of BIM

expression was carried out expressed relative to 0 hours of CHX treatment. Values shown are representative of three independent repeats no significance Two-way ANOVA followed by Tukey's post hoc analysis versus Neo

### 4.3 Discussion

The ER stress pathway is an important physiological stress response, which is also implicated in patho-physiological situations. The signalling pathways which lead to ER stress induced cell death are not completely understood (Gorman et al., 2012a). It is evident that understanding the molecular switches that govern change from an adaptive to a maladaptive response is an important area of research.

As previously mentioned, HSPB1 provides a potent pro-survival signal in response to diverse apoptotic stimuli. In recent years HSPB1 has garnered attention as a possible therapeutic target due to the pro-survival effect mediated by its expression, especially in neuronal cells (Wagstaff et al., 1999, Zourlidou et al., 2004, Patel et al., 2005, Stetler et al., 2008a, Stetler, 2009, 2011). These studies emphasise the protection afforded by HSPB1 in response to neurodegenerative disease. ER stress and deregulated UPR signalling is a feature of many neurodegenerative diseases including Alzheimer's Disease (Katayama et al., 2001, Hoozemans et al., 2009, Ho et al., 2012), Parkinson's Disease (Holtz and O'Malley, 2003) and ALS (Farg et al., 2012) (Atkin et al., 2013). Several authors have reported that cell death occurring in neurodegenerative diseases is associated with the ER and ER stress. Therefore, modulation of the UPR is a potential treatment aimed at a common feature of neurodegenerative diseases regardless of aetiology.

This work highlights the importance of HSPB1 in mediating protection against ER stress induced cell death. Moreover it identifies a novel function of HSPB1 as a regulator of degradation of BIM.

HSPB1 has been shown to be phosphorylated in response to ER stress (Ito et al., 2005). However the functional relevance of HSPB1 and its effect on ER stress induced apoptosis has, until now, not previously been studied to the authors' best knowledge. We demonstrate that HSPB1 over expression could protect cells from ER stress induced apoptosis. This was evident from increased cell viability, reduced DNA fragmentation, reduced exposure of phosphatidylserine, reduced caspase

cleavage and reduced caspase enzymatic activity, as well as reduced loss of mitochondrial membrane potential and release of cytochrome *c* from the mitochondria. The latter result, release of cytochrome *c* from the mitochondria, indicates that the protective effects of HSPB1 are a result of modulation of events up-stream of the mitochondria.

Previous studies have highlighted the protective effects of HSPB1 downstream of the mitochondria through regulating the release of cytochrome *c* and reducing activation of caspase-3. Direct interaction occurs between HSPB1 and caspase-3 reducing the enzymatic activity of caspase-3 due to a reduction in proteolytic cleavage necessary for caspase-3 activation (Voss et al., 2007).

Arrigo (2002) demonstrated the HSPB1 exerts its regulation over cytochrome *c* release from the mitochondria via stabilization of F-actin in the cytoskeleton. This inhibits BID redistribution to the mitochondria and subsequent activation of the intrinsic pathway. Of interest is that stabilization of the cytoskeleton could only partially explain the protective effects of HSPB1 upstream of the mitochondria; in the presence of an F-actin stabilizer, phalloidin, HSPB1 was still able to enhance cell survival (Paul et al., 2002b). Therefore, additional, novel regulatory mechanisms must exist that explain how HSPB1 can regulate the intrinsic pathway upstream of the mitochondria.

An obvious node of intervention of mitochondrial apoptosis would be regulation of the BCL-2 family. HSPB1 has previously been shown to reduce BAX activation (Havasi et al., 2008b) and cause BAD inactivation (Zoubeidi et al., 2010). In the present study, we have demonstrated that both heat shock preconditioning and over-expression of HSPB1 leads to a reduction in expression of the pro-apoptotic BH3 only protein BIM. We were able to show that 85% of the protective effects of HSPB1 over-expression was due to regulation of BIM.

The importance of BIM in ER stress-induced apoptosis has previously been demonstrated (Puthalakath et al., 2007, Gupta et al., 2012, Zhang et al., 2012). The authors typically observed 50% less cell death in BIM knock down cells compared to

control cells. In the present study, we see a 30% difference in cell death between control siRNA-treated cells and BIM siRNA-treated cells. One difference between this study and Gupta et al (2012), which also used PC12 cells, is that different methods were used to knock down BIM, i.e., transient transfection of siRNA (present work) versus stably over-expressing BIM shRNA in PC12 cells (Gupta et al, 2012). Nevertheless, BIM seems to play a role in ER stress induced apoptosis, albeit a fractional one, as cells are not completely resistant to ER stress-induced cell death in the absence of BIM. It may be that several other BH3-only proteins cooperate to induce ER stress-induced cell death. Alternatively, it is possible that in the absence of BIM, other BH3 only proteins take over as the key mediator of BAX/BAK activation. It would therefore be insightful to investigate the compensatory effect the BCL2 family by knocking down multiple BH3 only proteins at once. This may cause a more striking phenotype with regards protection against apoptosis.

HSPB1 has been reported to protect cells via a number of ways. Therefore we wanted to investigate the significance of HSPB1 regulation of BIM on cell fate. We saw that HSPB1 over-expressing cells had 30% less cell death relative to Neo control cells. In the absence of BIM, HSPB1 could only afford a modest protection of 7% relative to Neo BIM siRNA following TG treatment. These data highlight the physiological importance of HSPB1 mediated regulation of BIM for the protective effects seen by HSPB1 over-expression.

We next investigated the mechanism by which HSPB1 may regulate BIM. The mRNA expression levels of BIM can be increased in response to cell stress; however, this differs greatly between cell types (Reimertz et al., 2003b) meaning that there is cell type specific regulation of BIM. We saw only a three-fold increase in BIM mRNA levels following TG treatment and no difference was observed between Neo and HSPB1 cells. This is in agreement with reports which saw only modest (Puthalakath et al., 2007) or no induction of BIM mRNA following TG treatment (Reimertz et al., 2003a). The induction of BIM mRNA observed by Puthalakath et al (2007) was due to direct binding of CHOP to the promoter of BIM. We observed no difference in expression levels of CHOP between Neo and HSPB1 cells (data not shown), again



highlighting the potential role of post-transcriptional mechanisms in the regulation of BIM in our system. Of note, Szegezdi *et al* (2008) did see an increase in BIM mRNA expression in PC12 cells following TG treatment. However, the dosage of TG used was six times higher than the dosage used in our study (Szegezdi *et al.*, 2008). This suggests that, depending on the amplitude of the stressor imposed, BIM can be differentially regulated.

MicroRNAs are emerging as critical determinants of cell fate during cell stress conditions (Gupta *et al.*, 2012, Upton *et al.*, 2012, Cawley *et al.*, 2013). Previous studies from our group have established a role for two microRNA families in the regulation of BIM during ER stress, namely the 106b and 17-92 clusters. We questioned whether differential expression of BIM in Neo and HSPB1 cells was due to increased expression of BIM targeting microRNAs in HSPB1 overexpressing cells. We addressed this question using a reporter of activity at the BIM 3'UTR, the site where microRNAs exert their effect. In line with previous reports, we saw an increase in activity at the BIM 3'UTR but no difference between Neo and HSPB1 cells, therefore ruling out a role for HSPB1. This result was confirmed by qRT-PCR analysis of specific microRNAs and of the host genes for the 106b and 17-92 clusters. No difference in expression was observed, thereby ruling out HSPB1 modulation of BIM targeting microRNAs as the regulator of BIM protein expression in our model.

Collectively our data suggests a role for post-translational modification of HSPB1. Such regulation is important in stress situations when cells require rapid alteration in protein expression or function (Thomas and Lieberman, 2013). Given the well-documented regulation of BIM by stress kinases, we hypothesised that HSPB1 modulation of BIM was due to altered phosphorylation of BIM. This hypothesis was supported by literature which has previously identified HSPB1 in the regulation of AKT (Havasti *et al* 2008) and JNK (Stetler *et al* 2008).

Havasti *et al* (2008) demonstrated that HSPB1 could prevent BAX activation via modulation of AKT. HSPB1 has also been shown to cause inactivation of BAD via AKT. By phosphorylating BAD, AKT precludes its binding to the anti-apoptotic BCL2

family members leading to increased cell survival (Havasi et al., 2008b). Therefore regulation of stress kinases may provide an explanation as to how HSPB1 regulates events upstream of the mitochondria. Szegezdi *et al* (2008) identified a role for the PI3K/AKT pathway in regulating TG-induced apoptosis. It was found that inhibition of AKT with LY294002 led to an increase in TG-induced BIM expression. We were unable to observe phospho-AKT in Neo cells at basal levels or in response to ER stress. While HSPB1 over-expressing cells had higher levels of AKT at basal levels, phospho-AKT was undetectable following TG treatment. This suggested a lack of relevance of AKT in TG-induced BIM expression in our model. A study by Stetler *et al* also observed increased expression of phospho-AKT in cortical neurons treated with HSPB1 adenovirus. In their OGD inhibition of the upstream kinase of AKT, PI3K failed to affect the protective properties of HSPB1 against OGD or bleomycin (Stetler et al., 2008a). In contrast MEF cells which exhibit high levels of phospho-AKT at basal levels, treatment with ER stressors causes significant reduction in phospho-AKT signalling (Qin et al., 2010). Phospho-AKT is linked with transcriptional and post-transcriptional regulation of BIM (Qi et al., 2006, Zhu et al., 2008). Phospho-AKT has also been reported to phosphorylate BIM on S87 leading to a reduction in its apoptotic capacity. We see a difference in expression of BIM protein levels between Neo and HSPB1. Therefore, as AKT only affects apoptotic ability, and not expression it could not be responsible for the post-transcriptional difference observed between Neo and HSPB1 cells with respect to BIM expression. Phospho-AKT has also been shown to regulate BIM transcriptionally via FoxO3a (Sunters et al., 2003). However, we saw no difference in mRNA expression of BIM between Neo and HSPB1 cells. Therefore, we reason that AKT is not responsible for reduced levels of BIM in HSPB1 cells. Despite this, the higher basal levels of phospho-AKT may mediate a survival advantage to the HSPB1 cells in a BIM independent manner. We would need to inhibit AKT and assess cell death following TG treatment to confirm this observation.

JNK has been shown to be important for ER stress-induced cell death. JNK is activated in response to ER stress via the IRE1 pathway. HSPB1 has been linked to regulation of JNK activity (Stetler et al., 2008a), as has HSPA1 (Mosser et al., 2000).

Regulation of JNK by HSPA1 has been demonstrated to regulate BIM UV induced apoptosis. In the presence of JNK, BIM<sub>L</sub> translocates to the mitochondria to initiate cell death. HSPA1 impedes this process (Li et al., 2010).

The physiological importance of HSPB1 mediated JNK regulation was shown by Stetler *et al* (2008). They investigated the mechanism by which HSPB1 overexpression protected against ischemic brain injury that they pinpointed to regulation of the ASK1-JNK pathway. ASK1 is an upstream kinase that regulates JNK. Via a physical interaction with ASK1, HSPB1 acts as a dominant negative in the ASK1-JNK pathway. Of interest this study assessed the potential of HSPB1 to regulate the BH3 family by treating control and HSPB1 overexpressing cells with a BH3 peptide. This peptide contains residues 57-72 of the BH3 only domain and can induce cell death by interacting with and antagonizing anti-apoptotic members of the BCL-2 family of proteins. It was found that HSPB1 overexpression was unable to protect cells against BH3 peptide induced cell death. Based on these results, Stetler *et al* (2008) ruled out a role for HSPB1 in the regulation of BIM. Critically, this peptide lacks the BIM degron, the minimal sequence required for degradation of BIM which consists of an ERK docking site, an ERK phosphorylation site and a lysine residue (Wiggins et al., 2010). Therefore the study by Stetler *et al* 2008 shows that HSPB1 cannot regulate a non-degradable BIM.

We saw that HSPB1 over-expressing cells had reduced levels of phospho-JNK. In addition we saw that ER stress caused a reduction in levels of ERK, however levels of phospho-ERK are maintained at high levels in HSPB1 over-expressing cells. Both JNK and ERK have been implicated in the proteasomal degradation of BIM. In the case of ERK this is well established and occurs due to S65 phosphorylation of BIM causing increased proteasomal degradation (Ley et al., 2003, Luciano et al., 2003, Ley et al., 2004). To date, only one report has demonstrated that JNK can influence degradation of BIM. It was show that in neuronal cells, JNK also can phosphorylate

S65 thereby inhibiting its proteasomal degradation (Putcha et al., 2003). It is perplexing how phosphorylation of the same site can have opposite effect on protein turnover. A possible explanation for this may be provided by the observation that while both JNK and ERK can bind to BIM, ERK does so with much greater affinity (Ley et al., 2004). Therefore in non-stressed cells when phospho-ERK levels are high, BIM is rapidly turned over by proteasomal degradation. When cell stress occurs and phospho-ERK levels decline, this provides JNK with an opportunity to interact with BIM and promote its stability. Our data would suggest such a model. We see that at the times when phospho-ERK is most down regulated, p-JNK expression is increased. Therefore the cells seem to have synchronized alterations in stress kinase expression that can regulate BIM stability. Our data on BIM phosphorylation during ER stress also supports this hypothesis. We see that in the absence of phospho-ERK signalling S65 of BIM undergoes phosphorylation. In this instance ERK cannot be mediating the increase in phosphor-BIM so an additional kinase must be responsible.

We saw that inhibition of phospho-ERK led to similar expression levels of BIM in Neo and HSPB1 cells at 48 h. We have excluded the possibility that the effects of U0126 on cell death and BIM are due to DMSO toxicity as the levels of cell death at 0 hours of TG were comparable with previous experiments in which cells were treated with a single vehicle dose of DMSO.

At 24 hours HSPB1 could still reduce BIM indicating that HSPB1 mediated regulation of ERK can only partially account for its effect on BIM. Clearly additional mechanisms occur which facilitate HSPB1 regulation of BIM. Certainly our data show that the half-life of BIM is reduced from 7 h to 1.7 h in the presence of HSPB1. Phospho-JNK can suppress proteasomal degradation of BIM. HSPB1 cells express reduced levels of phospho-JNK and thereby reducing the block imposed by phospho-JNK on BIM degradation. Therefore a dual regulation of stress may be what leads to HSPB1 cells expressing lower levels of BIM. We postulate that HSPB1 acts as an E4 ligase scaffolding ERK and BIM together facilitating proteasomal degradation. This process is further aided by reduced expression of JNK.

## **Chapter 5**

### **Functional assessment of neuropathy associated HSPB1 mutants**

## 5.1 Introduction

Motor neuron diseases are a heterogeneous group of progressive neurodegenerative disorders affecting the central and peripheral nervous systems. Conditions can be classified as sporadic or hereditary. The causes of sporadic MNDs are not known, but environmental, toxic, viral, or genetic factors have been implicated (Kanning et al., 2010)). Sporadic MNDs include progressive bulbar palsy, primary lateral sclerosis and amyotrophic lateral sclerosis (ALS), which is the most common MND. Inheritable MNDs account for 10% of cases and include neurofibromatosis, Friedreich's ataxia, Huntington's Disease and Charcot-Marie-Tooth disease (CMT) (Carra et al., 2012). With an estimated prevalence of 1 in 2,500, CMT is one of the most common inherited neurological disorders (d'Ydewalle et al., 2012). It is characterized by progressive degeneration of peripheral nerves which leads to weakness and atrophy of distal muscles in lower limbs, feet and hands with eventual difficulty in ambulation and fine motor tasks (Ackerley et al., 2006). Current treatments of CMT are symptomatic treatments only, such as pain relief and surgery to correct foot and hand deformities. No therapy has been approved to slow disease progression (d'Ydewalle et al., 2012).

CMT is subdivided into two classifications. CMT1 is the demyelinating form that is associated with abnormalities in myelination by Schwann cells in the periphery. CMT2, is the axonal, nondemyelinating form, in which the abnormality develops in the axons of peripheral neurons (Suter and Scherer, 2003). CMT can be transmitted in an autosomal dominant, autosomal recessive or X-linked dominant manner (Zuchner and Vance, 2006). Subtypes are further classified on the basis of the genetic mutation involved. Mutations in HSPB1 are a cause of Charcot-Marie-Tooth disease type 2F (CMT2F) and cause distal hereditary motor neuropathy (dHMN)(Dierick et al., 2005, Ackerley et al., 2006, Dierick et al., 2007, Zhai et al., 2007, James et al., 2008). Interestingly, mutations in other small heat shock proteins such as, HSPB3 and HSPB8 are also a cause of dHMN, perhaps highlighting

the importance of small HSPs in neuronal survival (Irobi et al., 2010, Kolb et al., 2010).

Having established the protective role of WT HSPB1 in ER stress induced apoptosis (chapter 4) we undertook a study to investigate the effect of mutant HSPB1 in ER stress and ER stress-induced cell death. We chose our well characterized neuronal-like model system PC12 cells to investigate this question.

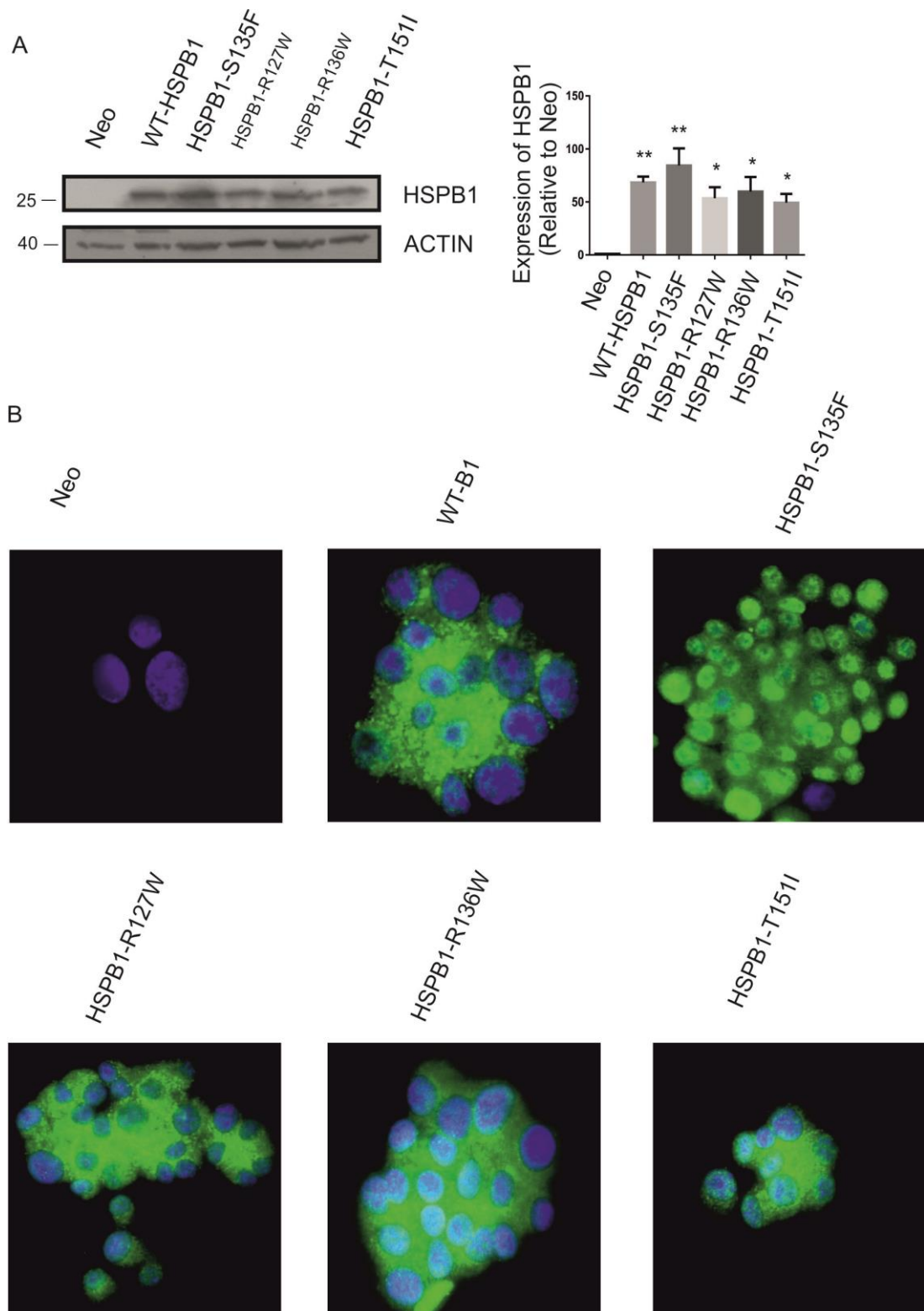
## 5.2 Results

### 5.2.1 Generation of mutant HSPB1 over-expressing cell lines

Given the protection afforded by HSPB1 over-expression in ER stress induced apoptosis we were interested in investigating the effect of mutant HSPB1 overexpression in cells under ER stress. To this end we generated stable cell lines that over-expressed a Neo control plasmid, wildtype HSPB1 or S135F, R127W, R136W or T151I in PC12 cells(See section 2.1.1.1 for details). These mutants were chosen because they occur in the alpha-crystallin domain of HSPB1 which has been identified as a 'hot spot' in relation to HSP associated neuropathies. Cells were validated for over expression of HSPs by Western Blotting using an antibody specific for human HSPB1 (Fig 5.2.1 A). We can see that Neo cells express no HSPB1 while HSPB1 was expressed to a similar level in WT HSPB1, S135F, R127W, R136W and T151I, thereby allowing for comparison of function between the cell lines.

We were interested to see if the mutant showed a normal diffuse cytoplasmic staining as is expected for HSPB1. Such a pattern of expression is evident in WT HSPB1 and the mutant cell lines, R127W, R136W. However, S135F shows a predominantly nuclear localization with T151I also displaying higher nuclear expression than WT HSPB1.





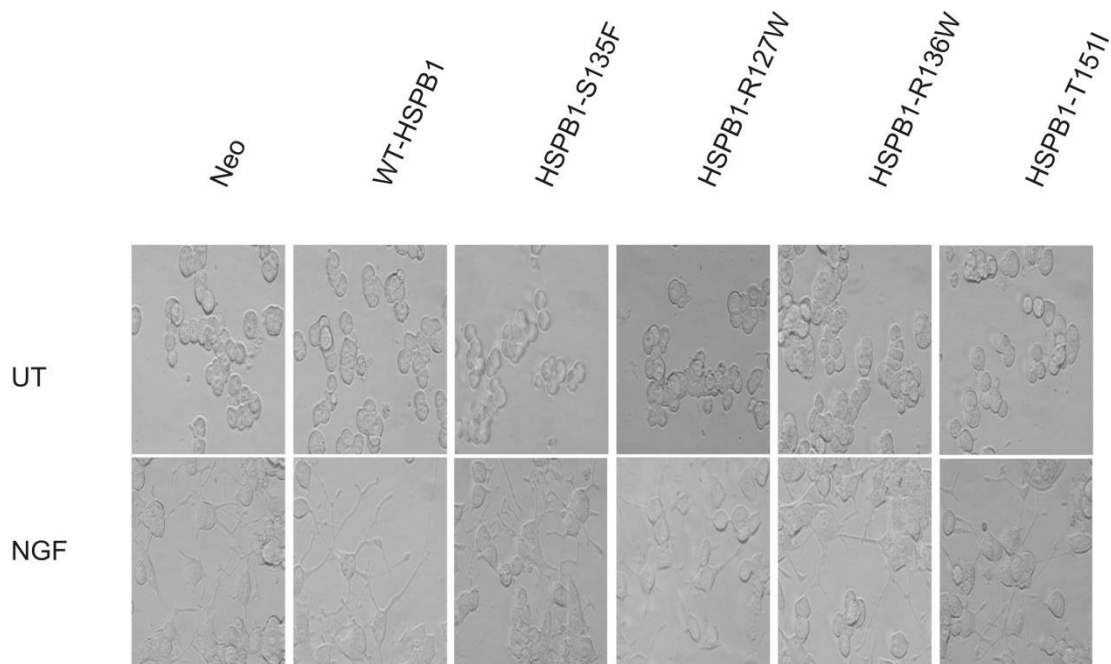
**Fig 5.2.1 Generation of mutant HSPB1 over-expressing cell lines. (A)** Protein extracts from in Neo, WT HSPB1, S135F, R127W, R136W and T151I PC12s were analyzed by SDS-

PAGE followed by Western blotting using a specific anti-HSPB1 antibody. Densitometric analysis of HSPB1 expression in cells . \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$  One-way ANOVA followed by Tukey's post hoc analysis versus Neo cells. **(B)** Cytocentrifuge preparations of Neo, WT HSPB1, S135F, R127W, R136W and T151I PC12s were stained with anti-HSPB1 and DAPI. Cells were visualized by fluorescent microscopy with 60x oil magnification.

### **5.2.2 Mutant HSPB1 expressing cells differentiate upon addition of NGF**

PC12 cells are neuronal-like cells which can be stimulated to extend neurites and differentiate into a sympathetic neuron-like phenotype by the addition of Nerve Growth Factor (NGF) (Drubin et al., 1985). We were interested in investigating the functionality of mutant HSPB1. We therefore asked if the mutant expressing cells would differentiate normally and form neurites. To test this we added 100 ng of NGF to PC12 cells for 7 days after which time cells were imaged. NGF was added only at the beginning of the experiment. Results are depicted in Fig 5.2.2.

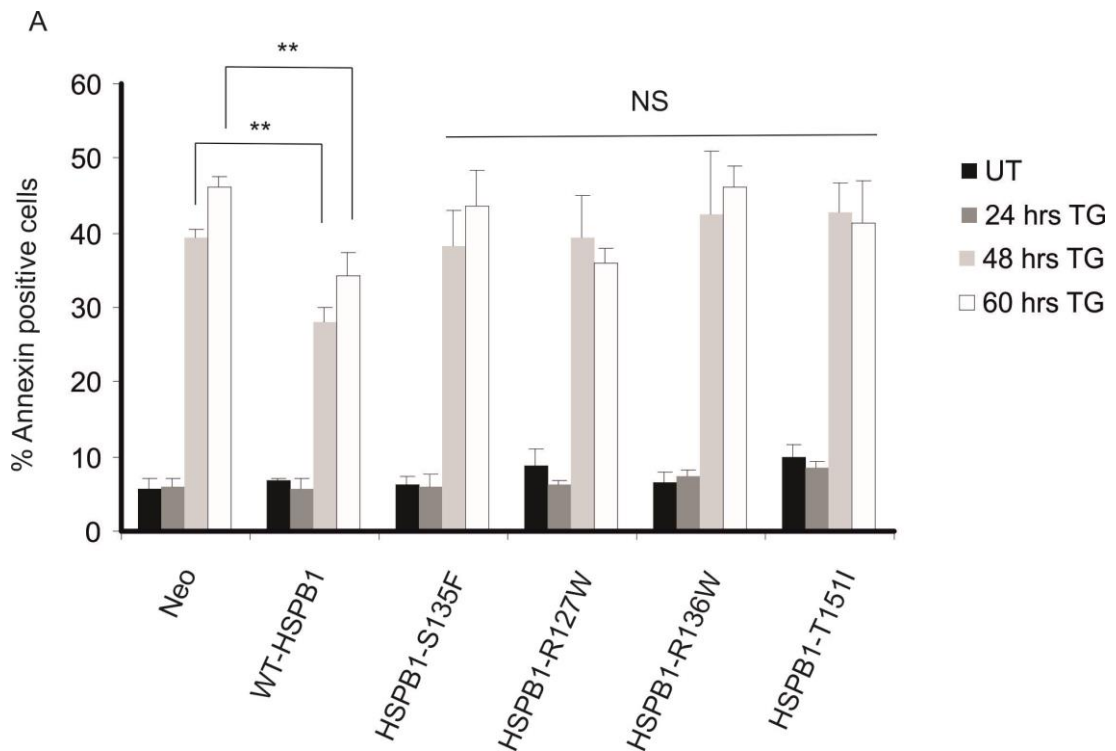
Undifferentiated PC12 cells are spherical in appearance and all six cell lines had a similar morphology in untreated conditions. The addition of NGF caused formation of neurities in all cell lines. We observed no difference in the degree of differentiation as indicated by neurite outgrowth and neurite branching between the cell lines. Therefore data indicated that mutant HPSB1 expressing cells differentiate like wild-type cells.



**Fig 5.2.2 Mutant HSPB1 expressing cells differentiate upon addition of NGF.** Neo, wildtype HSPB1, S135F, R127W, R136W and T151I were seeded at 15,000 cells per well of a 24 well plate. 24 hours later 100 ng/ml of mouse NGF was added for 7 days or cells were left untreated. Cells were visualized by light microscopy at 40x magnification. N=2

### 5.2.3 HSPB1 mutants are more sensitive to ER stress-induced apoptosis

We next investigated if mutant HSPB1 cells were capable of protecting cells against ER stress in a similar manner to wild type HSPB1. Neo, wildtype HSPB1, S135F, R127W, R136W and T151I cells were treated with 0.25  $\mu$ M TG for 0, 24, 48 or 60 hours. There was no significant increase in cell death at 24 hours of TG treatment in Neo and HSPB1 cells. Similar results were obtained for levels of cell death at 24 hours in the mutant cell lines. At 48 and 60 hours of TG treatment, WT HSPB1 over-expressing cells showed attenuated ER stress induced apoptosis compared to Neo cells. No significant difference in levels of cell death was observed between Neo and S135F, R127W, R136W or T151I cells. Therefore mutant HSPB1 cells cannot protect against ER stress induced apoptosis.



**Fig 5.2.3 HSPB1 over-expressing mutants are more sensitive to ER stress induced apoptosis. (A)** Time course study of Neo, wildtype HSPB1, S135F, R127W, R136W and T151I cells treated with 0.25  $\mu$ M TG for 0-60 hrs. The % cell death was estimated by flow cytometry based measurement of Annexin V. Values shown are representative of three independent repeats. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$  Two-way ANOVA followed by Tukey's post hoc analysis versus Neo cells.

#### 5.2.4 HSPB1 mutants fail to protect against loss of $\Delta\Psi_m$ following TG

Mitochondrial outer membrane permeabilization (MOMP) and loss of  $\Delta\Psi_m$  are critical events in ER stress induced apoptosis, and are governed by the BCL2 family. MOMP initiates the activation of caspases due to release of cytochrome c. We have previously shown that wildtype HSPB1 protects cells from ER stress upstream of the mitochondria (Chapter 4). We were therefore interested in how mutant HSPB1 cells regulated mitochondrial dynamics by flow cytometry using TMRE (See section 2.4.4 for details). Neo, wildtype HSPB1, S135F, R127W, R136W and T151I cells were treated with 0.25  $\mu$ M TG for 0, 24 or 48 hours. As a positive control for mitochondrial depolarisation, cells were treated with 40  $\mu$ M carbonyl cyanide 3-chlorophenylhydrazone (CCCP) for 3 hrs. Following treatments, cells were

incubated with 100 nM TMRE for 30 minutes in the dark. The loss of  $\Delta\Psi_m$  was detectable as a loss of TMRE signal, as determined by flow cytometry.

WT-HSPB1 cells were protected from loss of mitochondrial membrane potential following ER stress compared to Neo cells. However, neither S135F, R127W, R136W or T151I could reduce ER stress-induced loss of mitochondrial membrane potential.

Collectively data from Figures 5.2.3 and 5.2.4 demonstrate that HSPB1 mutants are more sensitive to ER stress-induced cell death. While WT HSPB1 can protect against MOMP to inhibit ER stress-induced apoptosis, mutants fail to do so and this may result in increased cell death.

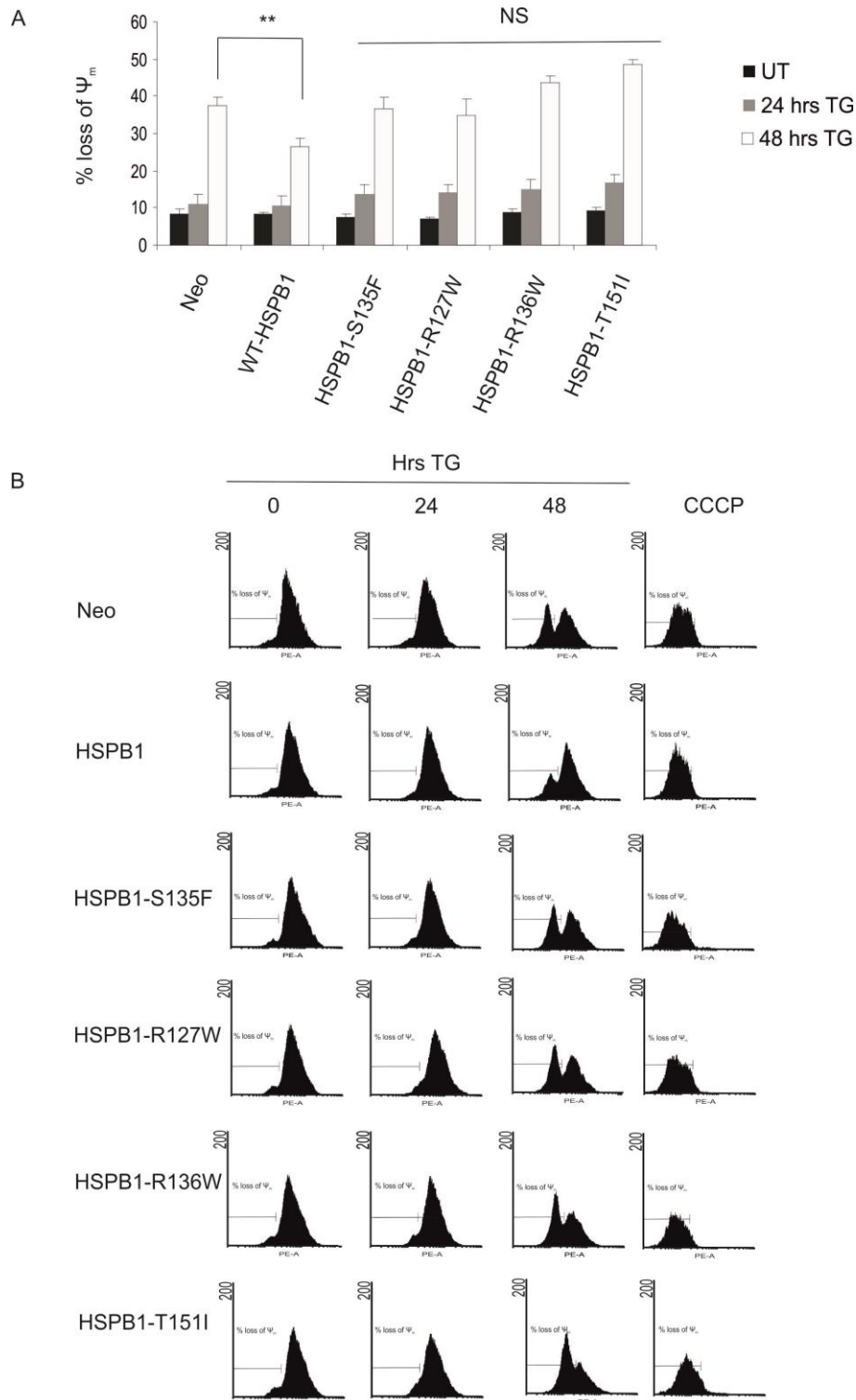
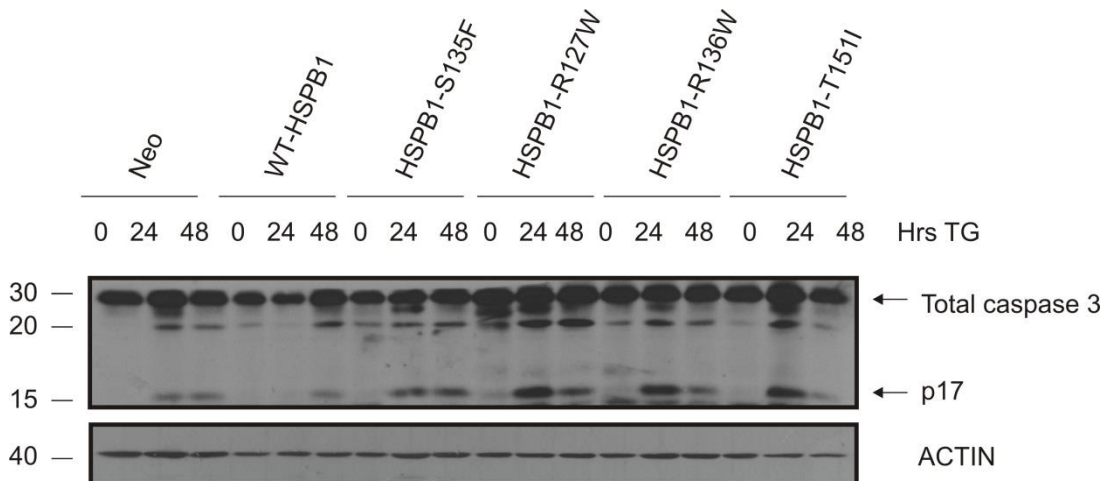


Fig. 5.2.4 HSPB1 mutants fail to protect against loss of mitochondrial membrane potential following ER stress. **(A)** Time course study of Neo, wildtype HSPB1, S135F, R127W, R136W and T151I cells treated with 0.25  $\mu$ M TG for 0-48 hrs. Loss of  $\Delta\Psi_m$  was determined by Flow cytometry based measurement of TMRE. CCCP treated cells were used as a positive control **(B)** Representative histograms of data shown in **(A)** Values shown are

representative of three independent repeats. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$  Two-way ANOVA followed by Tukey's post hoc analysis versus Neo cells.

### 5.2.5 HSPB1 mutants have increased caspase-3 cleavage following TG

To further characterize the effect of HSPB1 mutants in response to ER stress-induced apoptosis, we assessed caspase-3 cleavage in Neo, WT- HSPB1, S135F, R127W, R136W and T151I cells in untreated cells and cells given TG treatment for 24 or 48 hours. Caspase-3 exists as a 30 kDa protein in unstressed cells. Upon exposure to apoptotic stimuli it is cleaved into 3 fragments namely, p19, p17 and p12. Our antibody is unable to detect the p12 fragment. We observed cleavage of caspase-3 to a p19 and p17 fragment in all cell lines. In response to TG, caspase cleavage-3 was reduced in HSPB1 cells. A higher degree of caspase-3 cleavage was observed in cells expressing S135F, R127W and T151I. We did not observe any p17 expression in untreated conditions.



**Fig. 5.2.5 Mutant HSPB1 cells have increased caspase-9 cleavage in response to ER stress.** Activation of caspase-3 was assessed in Neo, WT-HSPB1, S135F, R127W, R136W and T151I. Cells were treated with 0.25  $\mu\text{M}$  TG for 0-48 hrs. Samples were harvested and

prepared for SDS-PAGE followed by probing with anti-caspase-3 antibody. Data is representative of four independent repeats.

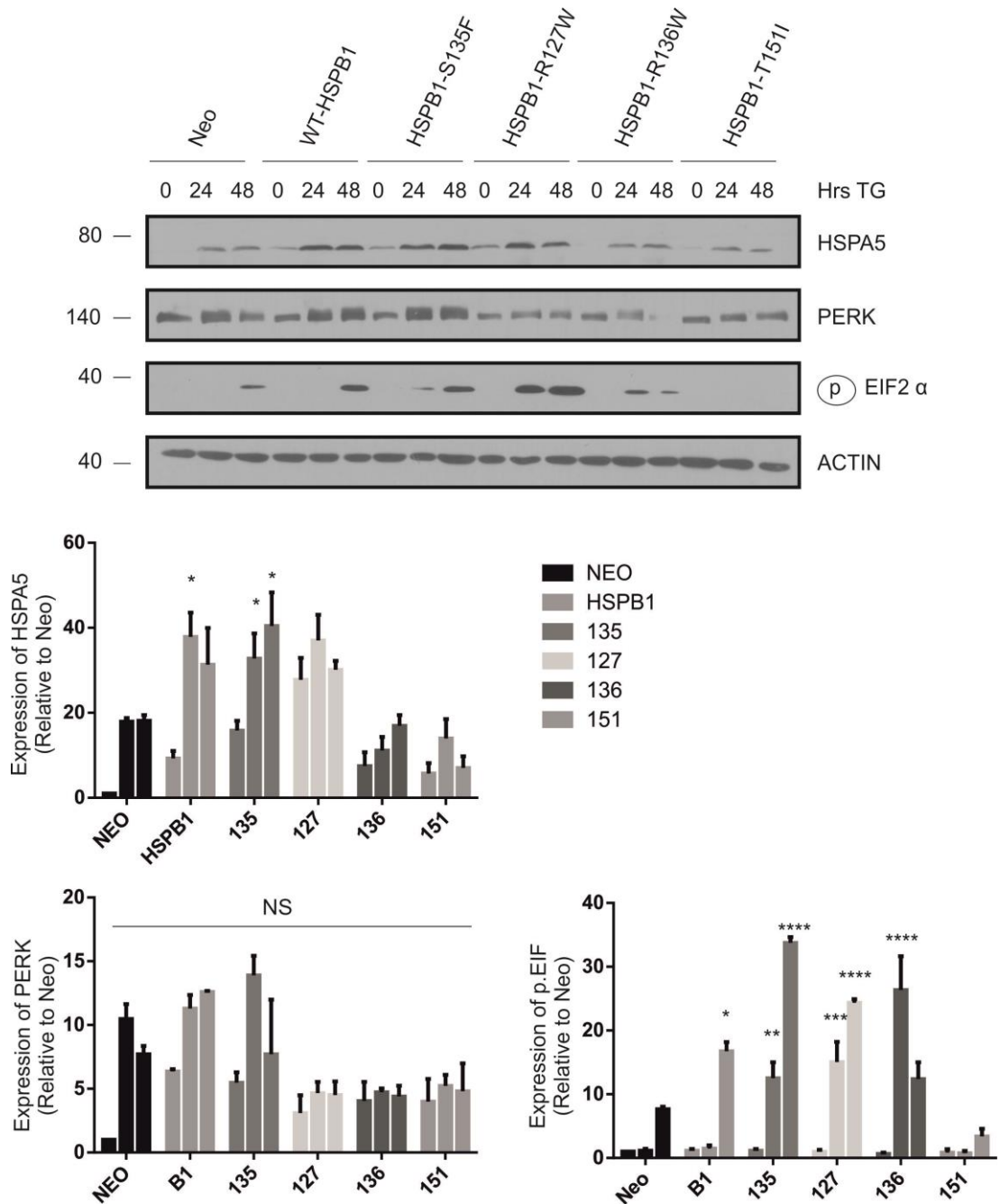
### 5.2.6 HSPB1 mutants have an altered UPR response

We next investigated the UPR response of Neo, wildtype HSPB1, S135F, R127W, R136W and T151I cells. Cells were treated with 0.25  $\mu$ M TG for 0-48 hrs. We assessed expression of some of the key UPR markers including HSPA5, PERK, and phosphor-EIF2 $\alpha$ . We saw induction of HSPA5 in all cells upon TG treatment (Fig 5.2.6). WT- HSPB1 cells had higher expression of HSPA5 than Neo cells. S135F and R127W expressing cells had similar levels of HSPA1 to WT- HSPB1. Cells expressing mutants R136W and T151I had expression levels of HSPA1 similar to Neo cells.

PERK is one of the UPR sensors and autophosphorylates upon activation. This can be seen as a retarded mobility, or upshift on SDS-PAGE gels. We saw an upshift in PERK in Neo, WT-HSPB1 and S135F cells following 24 and 48 hrs of TG treatment. In contrast no upshift in PERK was seen in R127W, R136W AND T151I cells (Fig 5.2.6). Expression levels of PERK remains the same as untreated conditions in these cells possibly indicating a failure to activate the UPR.

We also assessed expression of phosphor-EIF2 $\alpha$  in Neo, WT- HSPB1, S135F, R127W, R136W and T151I cells. WT- HSPB1 expressing cells had higher levels of phospho-EIF2 $\alpha$  than Neo cells. Mutants S135F had earlier phosphorylation of EIF2 $\alpha$  than WT-HSPB1. Mutant R136W also displayed earlier phosphorylation of EIF2 $\alpha$  however levels were lower at 48 hrs than WT-HSPB1, with levels more comparable to that of Neo cells. Cells expressing mutant R127W had significantly higher levels of phosphorylation of EIF2 $\alpha$  than WT- HSPB1. Cells expressing mutant T151I failed to induce phosphorylation of EIF2 $\alpha$  in response to TG treatment.

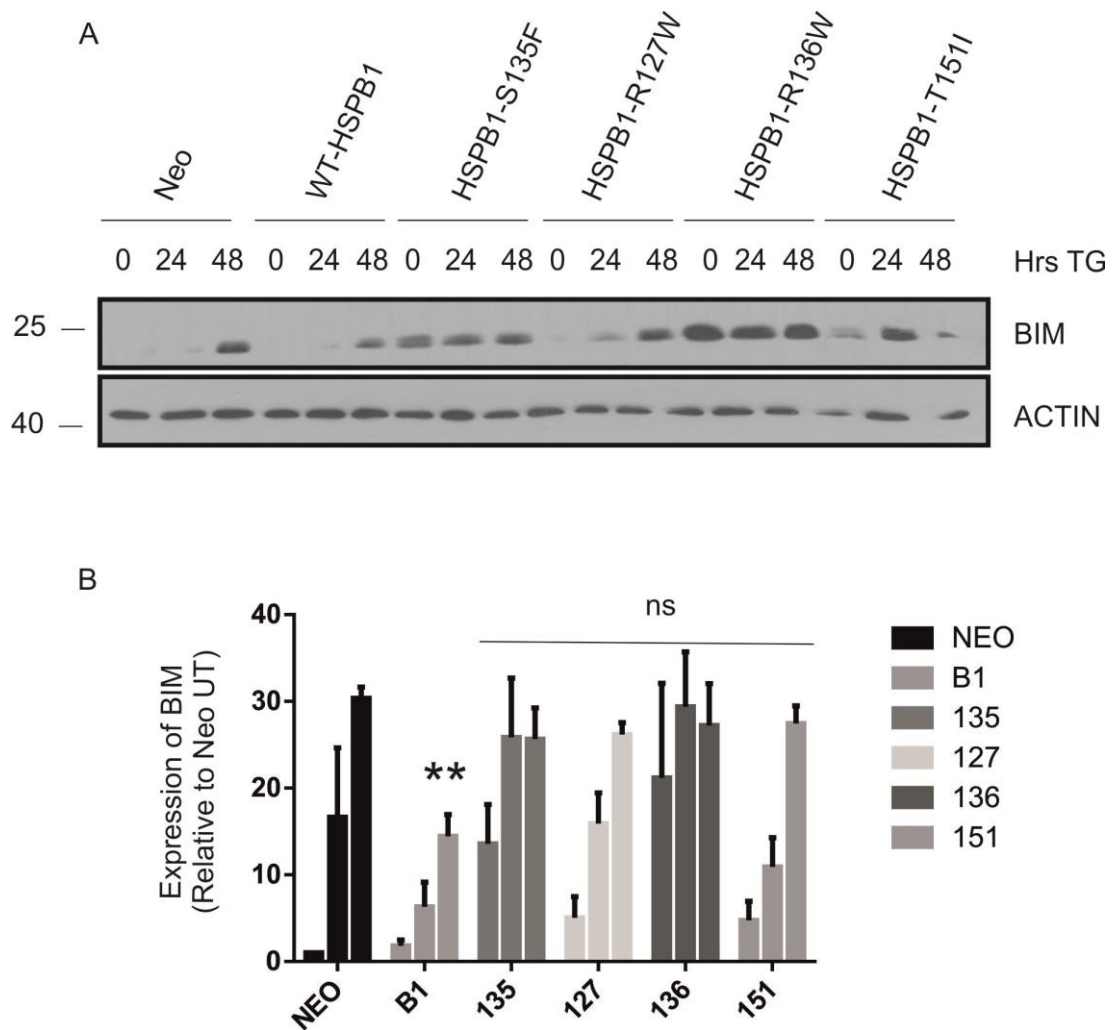




**Fig. 5.2.6 Mutant HSPB1 cells have an altered UPR response.** Neo, WT-HSPB1, S135F, R127W, R136W and T151I cells were treated with 0.25  $\mu$ M TG for 0-48 hrs. Samples were harvested and prepared for SDS-PAGE followed by probing with anti-HSPA5, PERK, Phospho- EIF2 $\alpha$ . ACTIN was used as a loading control. Data is representative of four independent repeats. Densitometric analysis of HSPB1 expression in cells . \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001, \*\*\*\* P < 0.0001 Two-way ANOVA followed by Tukey's post hoc analysis versus Neo untreated cells.

### 5.2.7 HSPB1 mutants have higher levels of BIM than WT HSPB1

Our previous data demonstrated a novel function of HSPB1 in regulation of BIM during ER stress (Chapter 4). We questioned whether mutated forms of HSPB1 could have lost the potential to regulate BIM and therefore have become more sensitive to ER stress. We took our Neo, WT- HSPB1, S135F, R127W, R136W and T151I cells and treated them with 0.25  $\mu$ M TG for 0, 24, 48 hours. In agreement with previous results, the WT-HSPB1 cells had reduced levels of BIM compared to Neo cells. In comparison to WT-HSPB1 cells mutant S135F had high basal levels of BIM and higher levels of BIM following treatment. R127W had no BIM in untreated conditions but showed earlier induction of BIM and expressed higher levels of BIM at 48 hours than WT-HSPB1 and Neo cells. R136W had very high levels of BIM in untreated conditions and these remained elevated throughout treatments. T151I had slightly higher levels of BIM at untreated conditions and higher levels at 24 and 48 hours (Fig 5.2.7).



**Fig 5.2.7 HSPB1 mutants have higher levels of BIM.** Neo, WT-HSPB1, S135F, R127W, R136W and T151I cells were treated with 0.25  $\mu$ M TG for 0-48 hrs. Samples were harvested and prepared for SDS-PAGE followed by probing with anti-BIM. ACTIN was used as a loading control. Data is representative of four independent repeats. Densitometric analysis of HSPB1 expression in cells . \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$  Two-way ANOVA followed by Tukey's post hoc analysis versus Neo untreated cells.

### 5.3 Discussion

The human genome encodes 10 classical HSPB's (Garrido et al., 2012). Although it remains elusive as to how such ubiquitously expressed proteins specifically lead to neuronal disturbances, given the cytoprotective role of HSPBs it is not surprising that mutation of small heat shock proteins correlates with neurological and muscular disorders. Mutations in eight family members namely HSPB1, HSPB2, HSPB3, HSPB4, HSPB5, HSPB6, HSPB7 and HSPB8 are associated with human diseases, many of which occur in the alpha-crystallin domain (Carra et al., 2012). In particular HSPB1 and HSPB8 have been linked to dHMN and CMT (Evgrafov et al., 2004, Irobi et al., 2010) Neurons are particularly sensitive to accumulation of misfolded proteins, a phenomenon which results from cell stress. HSPs are the first line of defence against proteotoxic stress and this may, at least in part, explain the neuronal specific sensitivity to sHSP mutations.

Much of the research on the pathological mechanism of HSPB1 mutants has focused on interaction of mutants with neurofilament light (NFL) and medium chains (NFM). Neurons are hypersensitive to disturbances and this has been suggested to account for the neurodegenerative phenotype of HSPB1 mutations (Dierick et al., 2005, Almeida-Souza et al., 2010). Mitochondrial disturbances have been reported in a model of CMT2E, an event which precedes neurofilament (NF) disruptions (Tradewell et al., 2009).

Twenty different mutations in HSPB1 have been identified with diverse biochemical features. R127W, R136W and S135F have increased monomerization and preferentially interact with tubulin to induce hyperphosphorylation of NFs and reduce anterograde transport of NFs (Almeida-Souza et al., 2010). To date there have been no studies to the authors best knowledge, on the response of mutant HSPB1 cells to cell stress. Given the role of ER stress in progressive neurodegenerative diseases and our finding on the protective role of HSPB1 in ER

stress, we were interested in characterizing the response of mutant HSPB1 overexpressing cell to ER stress.

We generated stable mutant HSPB1 expressing PC12 cells. All the mutants expressed to a similar level as wildtype protein overexpressing cells. Morphologically the cells were similar to WT-HSPB1. Immunofluorescence analysis revealed that WT HSPB1 R127W, R136W and T151I displayed a diffuse primarily cytoplasmic expression profile. This is in agreement with previous publications which show a cytoplasmic expression profile for HSPB1 with a concentrated expression in the perinuclear region (Gorman et al., 2005, Paul et al., 2012). One mutant, S135F showed altered cellular distribution in PC12 cells and to a much lesser extent T151L also. Immunofluorescence assessment revealed a predominantly nuclear expression for S135F. Microinjection of S135F cDNA into motor neurons also displayed a diffusely distributed expression profile predominantly in the perikarya (supplementary data (Zhai et al., 2007)). How this mutant results in nuclear accumulation is unknown at the present time.

Numerous reports have shown that transient overexpression of mutant HSPB1 as well as mutant HSPB8 leads to formation of intracellular aggregates within the cell (Ackerley et al., 2006, James et al., 2008). We too observed formation of aggregates upon transient transfection of R127W, T151I and R136W in PC12s (data not shown). However none of the mutants formed aggregates in stably overexpressing cells (Fig. 5.2.1 B). Formation of aggregates may be due to protein overload of the cells induced by transient transfection. It can be seen that even wildtype HSPB1 will form aggregates when transiently overexpressed due to massive overexpression of the protein (Ackerley et al., 2006). Similarly transient overexpression of wild type HSPB8 leads to formation of aggregates in 17% of COS cells (Fontaine et al., 2006). Additionally, other studies have reported reduced cell viability by transient transfection of mutant HSPs (Evgrafov et al., 2004). We observed no difference in cell death between Neo, WT HSPB1, R127W, R136W and T151I in untreated conditions (Fig. 5.2.3 and Fig 5.2.4). In agreement with these results we saw no

caspase-3 cleavage in untreated conditions in any of the mutant expressing cells (Fig 5.2.5). Therefore our stably transfected model enables the study of the long term effects of mutant HSPB1 expression in cells and represents a more physiologically relevant model to investigate the consequence of HSPB1 mutations.

Our previous work (Chapter 4) demonstrated the importance of the HSPB1-ERK-BIM axis in conditions of ER stress. Here we demonstrate that mutant forms of HSPB1 fail to regulate BIM. Current understanding of the pathophysiology of HSPB1 mutants S135F, R127W and R136W is that they show increased chaperoning activity and increased interaction with client proteins, as determined by tandem affinity purification of HSPB1 and HSPB1 mutants in 293Ts (Almeida-Souza et al., 2010). However, mutants T151I and P182L do not have altered chaperoning activity or interaction with client proteins (Almeida-Souza et al., 2010). Our data show that all mutants tested showed increased sensitivity to ER stress and higher expression levels of BIM. One could postulate that the regulation of BIM by HSPB1 is independent of the chaperoning activity, as mutants which have higher chaperoning activity still cannot reduce levels of BIM. Alternatively mutations in HSPB1 may alter client specificity such that mutants show increased interaction with certain clients leading to a consequent reduction in interaction with other client proteins. Additionally, regulation of BIM by HSPB1 may be a multi-step process requiring different functional states of HSPB1

Heat shock stress was found to cause a cyclic alteration in HSPB1 oligomerization (Almeida-Souza et al., 2010). Thirty min after heat shock, a significant proportion of HSPB1 existed in a monomeric state, which returned to a dimeric state by 60 min, and was fully restored to a dimeric state by 24 hours (Almeida-Souza et al., 2010). In contrast, the mutant S135F and R127W cells maintained heat induced monomeric form (Almeida-Souza et al., 2010). Although the HSPB1 mutations R127W and S135F are present predominantly as monomers, they still form oligomers. SEC analysis by Souza *et al* (2010) revealed that S135F and R127W mutants had a slightly increased propensity to be in oligomeric state. However, in

contrast to the WT-HSPB1 oligomer formed by mutants S135F and R127W were primarily made up of monomeric HSPB1 and not dimeric HSPB1.(Almeida-Souza et al., 2010). It is possible that the oligomer of monomers may not be functional in the same way as an oligomer of dimers would so do. Souza *et al* (2010) demonstrated that phosphorylation of HSPB1 was dispensible for the dimer to monomer transition. Therefore we propose that the altered oligomeric state of mutants S135F, R127W and R136W may result in an inability to degrade HSPB1.

We observed an altered UPR response in the mutant HSPB1 cells, particularly in R136W and T151I. Of note, while mutant R136W did have higher chaperoning activity than wildtype HSPB1, it was significantly lower than the other mutants S135W and R127W. We see that R136W and T151I generally had reduced activation of the UPR response as demonstrated by reduced PERK upshift and therefore phosphorylation of PERK as well as reduced HSPA5 expression and EIF2 $\alpha$  phosphorylation. HSP regulation of EIF2 $\alpha$  has previously been noted where overexpression of HSPB8 causes increased EIF2 $\alpha$  phosphorylation resulting in increased autophagy, a prosurvival degradation pathway (Carra et al., 2009b). We have not explored regulation of autophagy in HSPB1 cells but potentially this pathway aids in the increased survival of HSPB1 cells in response to ER stress. Impaired induction of EIF2 $\alpha$  in mutant T151I and R136W may exacerbate ER stress induced cell death. Alternatively, or in addition, the impaired activation of EIF2 $\alpha$  in the UPR in R136W and T151I could lead to a loss of the damage controlling action of reduced translation typically induced by ER stress. Therefore T151I and R136W cells could experience an high burden of protein folding in the ER in response to cell stress. A lack of induction of UPR associated chaperones such as HSPA5 would exacerbate this condition leading to ER stress induced cell death.

It is interesting that the four mutants, which only differ in one amino acid substitution, vary so much in their regulation of the UPR. This may be explained by the differential structural make up of oligomers formed by S135F, R127W versus R136W and T151I, in which the latter show higher propensity to monomer based

oligomers(Almeida-Souza et al., 2010). It would be interesting to do a detailed study of oligomeric state following TG treatment in Neo, WT-HSPB1 and mutant cell lines and test if a correlation exists with oligomeric state and regulation of UPR components.

Suppression of UPR signalling by a neurodegenerative disease associated protein mutation has previously been reported for Presenilin 1 (PS1), a protein associated with Alzheimer's Disease (Katayama et al., 2001). It has been found that PS1 causes reduction in HSPA5 expression and PERK signalling (Katayama et al., 2001). More recently ER stress induced cell death and activation of the intrinsic apoptotic pathway has been implicated in the pathogenesis of AD (Kudo et al., 2012). Taken together with our data, this highlights the role of disease causing protein mutations and regulation of ER stress in neurodegenerative diseases.



## Chapter 6

### 6.1 Discussion

*Proteostasis; the link between aging and neurodegeneration?*

The proteostasis network includes the HSR and the UPR and permits survival in response to cellular stress. The aetiology of neurodegenerative diseases is diverse, yet one commonality in susceptibility is advanced age, typically emerging in the fifth decade of life. It has in fact been suggested the level level of activity of the proteostasis network governs the cells capacity to cope with cell stress and that age-dependent onset of many neurodegenerative diseases is due to a decline in the proteostasis pathway leading to accumulation of toxic and mis-folded proteins (Douglas and Dillin, 2010). In dividing cells, damaged proteins can be sequestered in the mother cell, thereby preventing transmission a potentially lethal protein to the daughter cell. In post-mitotic cells such as neurons this is not possible and the proteostasis pathway is key for cell survival (Douglas and Dillin, 2010). Principally this involves molecular chaperones and members of the heat shock family of proteins (Cohen and Dillin, 2008). The neuronal-specific effect of disease-causing protein mutations may also be explained by proteostasis network differences between cell types. Protein mutations and, in particular, mutations in molecular chaperones are associated with neuronal-specific degeneration (Irobi et al., 2010) Autophagic clearance of mutant superoxide dismutase (mSOD) was found to be higher in muscle cells than motor neuron cells (Crippa, 2013). Therefore investigation into regulation of proteostasis networks in neuronal cells will be crucial for understanding mechanism of neurodegeneration.

*Cross talk between stress response pathways*

We have demonstrated co-activation and co-operation of two key components of the proteostasis network, the UPR and the HSR. Co-activation of stress pathways is not a novel concept. The UPR has been shown to induce and regulate the autophagy pathway at multiple levels (Deegan et al., 2013).

Similarly, crosstalk exists between the HSR and autophagy, where the HSR suppresses activation of autophagy. Loss of HSF1 led to a compensatory increase in autophagy (Dokladny et al., 2013). The aforementioned studies demonstrate the interplay and cross-regulation of stress response pathways in the cell. Additionally it suggests a multi-tier system in which one stress pathway is dominant in certain conditions, but loss of the dominant pathway can result in activation of an alternative pathway. Our data supports such a model. We see that the HSR is induced by ER stress as exemplified by increased HSPs and HSF1, however, not to the same extent as seen with classical heat shock. In this instance, the UPR is the dominant pathway, while the HSR serves to assist the UPR in coping with ER stress. The compensatory nature of the stress responses is further exemplified by the study by Liu and Chang (2008) who used a yeast model system which lacked a UPR response due to deletion of IRE1. By over-expressing HSF1 they could relieve some of the defects seen in the UPR-deficient cells (Liu and Chang, 2008).

The induction of HSPs that we observed following ER stress may be due to HSF1. This could be investigated by determining expression of HSP in HSF1<sup>-/-</sup> and wildtype MEFs following ER stress. If HSF1 is responsible for HSP induction following ER stress then there would be no induction in HSF1<sup>-/-</sup> MEFs. An alternative hypothesis is that the UPR is leading to activation of HSP during ER stress. The HSPB1 promoter contains binding sites for ATF3 and ATF5 which are PERK-dependent transcription factors (Nakagomi et al., 2003, Wang et al., 2007). This could be assessed by treating cell with TG alone or in combination with GSK2656157, a PERK inhibitor (Atkins et al., 2013). A different approach would be to investigate HSP expression in PERK<sup>-/-</sup> ATF6<sup>-/-</sup> and IER1<sup>-/-</sup> MEFs and corresponding wildtypes. This would facilitate detailed study of the individual arms of the UPR for induction of HSPs. Of note, other studies have reported no increase in expression of HSPA1 in response to ER stressors in myeloma cells (Davenport et al., 2007).

We see that HSF1<sup>-/-</sup> MEFs are more sensitive to ER stress-induced apoptosis. Similarly inhibition of HSPC1 via 17-AAG led to induction of ER stress and cell death

in myeloma cells. Cell death was in part due to ER stress (Davenport et al., 2007). This again highlights the importance of the HSR and HSPs in survival of cells following ER stress. It has recently been demonstrated in yeast cells that over expression of a constitutively active HSF1 caused increased resistance to ER stress (Hou et al., 2013). To the authors knowledge, this study is the first to characterize the role of activation of the HSR on cell fate in mammalian cells. Previous reports have indicated activation of the HSR by ER stress however none have shown the functional consequence of the HSR in modulating ER stress induced apoptosis. Our data demonstrate that activation of the HSR by thermal preconditioning protected cells from subsequent ER stress-induced cell death.

We were able to investigate in further details the regulation of HSPs by ER stress. We observed alterations in HSPB1 oligomerization consistent with an increase in chaperoning activity which was further demonstrated by an *in vivo* chaperoning assay. The observed alterations in HSPB1 oligomerization in response to ER stress again suggest cross modulation of stress response pathways whereby a component of one stress pathway is modified and recruited into a different stress response. We hypothesize that the altered oligomerization of HSPB1 observed in response to ER stress could cause increased cellular protection and this supports investigation into strategies to promote formation of small HSPB1 oligomers as a cytoprotective measure against ER stress induced cell death.

Future work will be required to characterize the role of phosphorylation of HSPB1 in ER stress. One strategy to address this question would be transfection of phosphor-mimicking and non-phosphorylatable HSPB1 into PC12 cells which express very low levels of endogenous HSPs and therefore results would not be complicated by expression of wild type proteins. However, results would have to be interpreted carefully given that the phosphor-mimic would only be a partial representative of *in vivo* phosphorylated HSPB1. An alternative approach could be to block HSPB1 phosphorylation with inhibitors.

### *Modulation of the UPR by HSPs*

Our data highlight the functional significance of crosstalk between the HSR and the UPR. Using both a thermal preconditioning model and over-expression system we demonstrated the protective effects of HSPs in ER stress conditions. Thermal preconditioning is an example of mild acute stress, such transient stressors lead to activation of pro-survival pathways. In contrast, chronic stress such as that observed in neurodegenerative diseases is a persistent stress which cumulates over the course of a number of years. Clearly a mechanism must be in place to enable survival of cells in spite of persistent cell stress (Rutkowski and Kaufman, 2007). Disease-causing protein mutations such as those caused by mSOD, are tolerated for years without any apparent cell death (Douglas and Dillin, 2010).

Long term stress may cause partial suppression or modification of the UPR sensors in order to promote cell survival (Rutkowski and Kaufman, 2007). Full activation of the UPR leads to engagement of apoptotic pathways, a response which is mediated by all three arms of the UPR. Therefore regulation of the UPR sensors or their downstream signalling pathway must occur in order to cope with chronic stress. An elegant example of this is the divergent outcome on cell fate that depends on the oligomeric state of IRE1. Regulation of the UPR pathway and ER stress by heat shock proteins may be part of the regulatory system that facilitates long term survival during chronic stress. HSPA5 is a HSP70 family member and acts to regulate activation of the UPR sensors IRE1, ATF6 and PERK. Like other molecular chaperones HSPA5 has a protective role in the cells. Knock-down of HSPA5 with siRNA potentiates apoptosis in PC12 cells in response to NGF withdrawal or staurosporine treatment (Yu et al., 1999). The mechanism of regulation of the UPR sensors is emerging as a more complex situation than initially thought. In the case of IRE1, HSPA5 is thought to deactivate IRE1 by maintaining sufficient ER stress

sensing monomers of IRE1 (Korennykh and Walter, 2012). This may be a mechanism by which cells adapt to chronic cell stress.

It has been shown that over-expression of HSPA1 in PC12 cells can protect against ER stress induced apoptosis via modulation of IRE1 mediated XBP1 splicing (Gupta et al., 2010). The HSP40 family member p58<sup>ipk</sup> is an XBP1 target. The function of p58<sup>ipk</sup> is to deactivate PERK and restore protein translation, an event which is required for cells to recover from ER stress (Yan et al., 2002). It has also been proposed that p58<sup>ipk</sup> acts in conjunction with HSPA1 to regulate co-translational degradation of ER localized proteins which helps reduce build-up of polypeptides at the ER during ER stress (Oyadomari et al., 2006). HSPC1 has been shown to be involved in stabilization of PERK and IRE1 as HSPC1 inhibitors reduced protein half-life of both PERK and IRE1. Additional roles for HSPs in the modulation of the UPR response include Cdc37-Hsp90 which is thought to inhibit IRE1 autophosphorylation by binding to the kinase domain - dimer interface, thereby suppressing activation of IRE1 (Ota and Wang, 2011).

The role of sHSPs in the regulation of ER stress induced apoptosis is not well defined. To date the only sHSP linked to regulation of ER stress-induced apoptosis is HSPB5 ( $\alpha\beta$ -crystallin). It was found that both ER stress and HSPB5 were activated in response to myocardial infarction (MI) (Mitra et al., 2013). Additionally, knockdown of HSPB1 led to increased cytochrome *c* release and caspase activation in response to MI. Although this study highlights a role for HSPB5 in MI, it does not clearly address the regulation of ER stress-induced apoptosis by HSPB5 (Mitra et al., 2013).

A more detailed investigation was carried out by Dou *et al* 2012 where they showed that deficiency of HSPB5 caused increased sensitivity of retinal cells to ER stress. This was attributed to increased BAX activation and increased caspase 3 activation. However the upstream regulation of this effect was not identified. To date, no studies, to the author's knowledge, have investigated the role of HSPB1 in ER stress induced cell death. We have shown that HSPB1 is protective against ER-stress. We

have further detailed the mechanism of this protection and have established a role for ERK-driven proteasomal degradation of BIM via HSPB1. Given our observation that HSPB1 is induced by ER stress it is tempting therefore to speculate that induction of HSPB1 is a feature of the pro-survival UPR response and the return of expression and chaperoning activity to basal levels may represent a threshold stage in the switch to a cell death- inducing UPR response.

We have demonstrated cross talk between two components of the proteostasis pathway however it is likely that others stress response pathways are modulated by HSPB1s in stress situations. Expanded polyglutamine repeats, which are a feature of Huntington's Disease and spinocerebellar ataxia, lead to induction of ER stress-induced apoptosis (Kouroku et al., 2006). As a protective mechanism, autophagy is induced via the PERK-EIF2 $\alpha$  pathway, inhibition of which leads to increased aggregate formation (Kouroku et al., 2006). Interestingly, the HSPs have also been implicated in this pathway with respect to autophagy clearance of ALS-associated mutant superoxide dismutase, mSOD. HSPB8 has independently shown to increase autophagy via EIF2 $\alpha$  (Carra, 2009, Carra et al., 2009a) as well as aiding in the clearance of mSOD (Crippa et al., 2010). Currently it is unknown if HSPB1 can regulate autophagy but it is plausible given the similarities in functional properties of HSPB1 and HSPB8.

The aforementioned studies highlight the stress modulating and prosurvival effects of HSPs. It is apparent therefore that HSPs are an integral part of stress response pathways. Preconditioning and hormesis show a high degree of protective plasticity. Preconditioning with ER stress agents was found to protect against UV, H<sub>2</sub>O<sub>2</sub> and mutation induced retinal degeneration, and thermal preconditioning can protect against diverse stimuli. This suggests that therapeutic strategies which act to augment one stress response pathway may be beneficial in cases where another stress response pathway has been de-regulated or mutated. Therefore chemical modulators of the HSR may be beneficial under the conditions where the UPR is dysregulated or pathogenic. Work from our laboratory would suggest that such

interventions could support activation of a pro-survival UPR response via the combined action of HSPB1 and HSPA1, as well as attenuating ER stress induced apoptosis. In addition increased expression of HSPB1 would attenuate cell death via regulation of BIM.

Considerable progress has now been made towards a detailed understanding of the signalling pathways of the ER stress response, with a view to developing UPR targeted therapeutics. Chaperones which reduce phosphorylation of PERK and IRE1 were shown to improve glucose tolerance and insulin sensitivity in insulin-resistant obese mice (Özcan et al., 2006). Furthermore, treatment with salburinal, a small-molecule inhibitor of eIF2 $\alpha$  de-phosphorylation, results in sustained eIF2 $\alpha$  phosphorylation, and protects cells from ER stress and viral infection (Boyce et al., 2005). What is apparent from the literature is that while ER stress is a common feature of neurodegenerative diseases, UPR signalling can vary greatly. For example PS1 mutants inhibit IRE1 signalling (Katayama et al., 1999), whereas in Parkinsons Disease (PD) there is increased activation of the IRE1-JNK pathway. Therefore detailed analysis of UPR signalling would be required for each disease state and possibly each mutation. Our study of the UPR response in HSPB1 mutants further exemplified the differences in UPR signalling mediated by proteins that differ by only one amino acid substitution. Potentially, mechanisms that increase activity of the HSR and/or individual HSPs, could be a viable option for defective UPR signalling associated diseases. The defective IRE1 signalling in PS1 mutants could be restored by overexpression of HSPA5 indicating the protective effects of HSPs in ER stress (Katayama et al., 1999). We demonstrate that the HSR can modify the UPR and improve survival in ER stress conditions. Two compounds which boost HSF1-activity, GGA and BGP-15, seem to specifically boost HSF1 activity in conditions of proteotoxic stress and have been found to be protective in models of disease states (Tanaka and Mizushima, 2009, Gombos et al., 2011, Zhang et al., 2011a).



*HSPB1 as general regulator of cell death*

It has been noted that neuroprotective strategies which act downstream of the mitochondria may only serve to delay, rather than prevent, cell death. The proteolytic avalanche that is the caspase cascade means that cellular destruction is imminent once initiator caspases become activated. A more prudent neuroprotective intervention would be to preserve the integrity of the mitochondria, thus minimizing caspase activation. We have demonstrated the ability of HSPB1 to protect cells up stream of the mitochondrial pathway.

The protective effects of thermal preconditioning and HSPs are not limited to regulation of apoptotic cell death (Wagstaff et al., 1999). Heat stroke-induced neurodegeneration is associated with necrotic cell death (Kourtis et al., 2012). Preconditioning *C. elegans* at mildly elevated temperatures protects animals from heat-induced necrosis. This protective effect of preconditioning was mediated by the sHSP, HSP16.1 (Kourtis et al., 2012). HSPB1 can also regulate the extrinsic apoptotic pathway. DAXX acts as an adapter of Fas that can recruit and activate apoptosis signal-regulated kinase 1 (Ask1), a mitogen-activated protein kinase kinase kinase. Activation of Ask1 can then lead to the activation of JNK. HSPB1, when in its dimeric form, has been shown to interact with DAXX preventing its association with Fas and Ask1 (Charette et al., 2000). DAXX is primarily a nuclear protein; however, co-expression with ASK1 leads to redistribution to the cytoplasm (Charette and Landry, 2000). Heat stroke-induced neurodegeneration is associated with necrotic cell death (Kourtis et al., 2012).

HSPC1 has been recently linked to the regulation of necroptosis, an RIP1-dependant form of regulated necrosis (Yan et al., 2013). When the intrinsic apoptotic pathway is blocked, cell death can switch modalities and execute elimination of the cells via an alternative mechanism. This can occur when caspases are blocked or the pro-survival BCL-2 proteins are overexpressed. It was found that inhibition of HSPBC caused cell death in BCL-2 overexpressing cells via necroptosis (Yan et al., 2013). To date, no studies have been carried out on the effect of sHSPs on necroptosis, but

potentially sHSPs may act as general modulators of programmed cell death modalities.

*Identification of a novel stress response axis; HSPB1-BIM*

In addition to highlighting the role of HSPB1 in ER stress, we have identified a novel function for HSPB1, specifically in the regulation of the pro-apoptotic protein BIM. We have shown that ER stress leads to induction and alteration of HSPB1 which alters the expression levels of BIM kinases. We were also able to demonstrate the physiological relevance of the novel HSPB1-BIM pathway. It can be seen that HSPB1 mutants, which have high levels of BIM, are more sensitive to ER stress induced cell death. Numerous studies have now highlighted the role of BIM in neuronal cell death, in particular in disease states. Work carried out in our lab has also revealed that HSPB8 can reduce BIM expression. However, the mechanism by which this occurs is currently unknown. HSPB8 mutants are also associated with CMT and distal hereditary motor neuropathies (dHMN). Potentially BIM is a common pathogenic target in CMT and dHMN. Mutations in the myelin sheath protein P0, cause CMT1B. The mutant P0 is retained in the ER causing activation of the UPR response (Pennuto et al., 2008). Ablation of CHOP expression reversed motor function defects in mouse models (Pennuto et al., 2008). These authors did not investigate the intrinsic apoptosis pathway in their model but as CHOP is a transcriptional regulator of BIM, the protective effects of CHOP may be a result of reduced BIM expression. BIM is also involved in neuronal cell death in ALS models (Suzuki et al., 2011, Soo et al., 2012, Matus et al., 2013). HSPB1 has been shown to afford protection in transgenic mouse models of mSOD ALS (Sharp et al., 2008). Given the importance of BIM in ALS associated degeneration, we speculate that protection afforded by HSPB1 in ALS models may, in part, be accounted for by HSPB1-mediated regulation of BIM.

A study of the anti-apoptotic effects of HSPA4 ( $\alpha$ -crystallin) demonstrated that overexpression in CHO cells could reduce staurosporine and etoposide induced apoptosis (Pasupuleti et al., 2010). The authors also demonstrated that HSPA4 could protect cells from cell death induced by overexpression of BIM (Pasupuleti et al., 2010). However, it is unlikely that HSPB4 was involved in the regulation of BIM expression, as BIM was expressed equally in empty vector control cells and HSPB4 cells (Pasupuleti et al., 2010). Therefore while both HSPB1 and HSPB4 can protect from BIM-induced apoptosis, the mechanisms appears to differ.

Activation of the ER stress pathway is a salient feature of neurodegenerative diseases (Doyle et al., 2011). Our data show that induction of HSPs provides partial protection against ER stress-induced apoptosis. In light of this, induction or activation of HSPs may be a potential therapeutic intervention in neurodegenerative diseases in which ER stress plays a pathophysiological role.

In addition to being important for ER stress induced apoptosis, BIM is recognised as an important modulator of neurodegenerative disease associated cell death. Two recent publications have demonstrated the role of BIM in neuronal cell death induced by Beta-Amyloid ( $A\beta$ ) (Kudo et al., 2012, Sanphui and Biswas, 2013). Both studies demonstrated induction of BIM in response to  $A\beta$ . Sanphui and Biswas (2013) showed that  $A\beta$  caused a reduction in AKT and other kinases, which regulate FOXO2A, a transcriptional regulator of BIM. Therefore a model can be suggested in which  $A\beta$  causes increased BIM expression leading to activation of the intrinsic pathway. We and others have shown that HSPB1 can modulate AKT. We additionally demonstrate that HSPB1 can reduce BIM levels by proteasomal degradation. Therefore increasing expression and/or activity of HSPB1 may be a therapeutically exploitable avenue in AD, as HSPB1 would act at multiple levels of the cell death pathway and inhibit both transcriptional induction of BIM, as well as clearing protein levels via the proteasome.

Kudo et al (2012) demonstrated the role of the BAX/BAK-dependant apoptotic pathway in A $\beta$  induced cell death. It was shown that A $\beta$  caused a 4-fold increase in PIP1 uptake and this was reduced to 1.5 fold in BAX knockout cells. That detectable levels of cell death still occurred in the absence of an intact mitochondrial pathway indicates that multiple cell death modalities occur simultaneously in response to A $\beta$ . As a modulator of multiple cell death modalities, HSPB1 holds therapeutic promise.

Data from our thermal preconditioning model in HeLa cells revealed reduced expression of PUMA and NOXA in response to preconditioning. We were able to ascertain that HSPB1 is the HSP capable of regulating BIM expression. However, it is plausible that HSPB1-mediated protection against ER stress is via regulation of multiple BH3-only proteins. Alternatively the reduction in PUMA and/or NOXA may be due to modulation by another HSP. For our study we chose to investigate HSPA1 and HSPB1 as they are the most widely expressed and most potently induced HSPs in response to thermal stress. Alternative candidates for HSP regulation of BH3-only proteins include HSPA1, HSPB5, HSPB6, HSPA5 and HSPA6 (Vos et al., 2008b, Bartelt-Kirbach and Golenhofen, 2013). It was demonstrated that HSBP1 was not heat inducible in HeLa cells (Chowdary et al., 2004). Therefore, it is unlikely that HSPB8 is accounting for regulation of BH3-only proteins in our thermal preconditioning HeLa model as HSPB8 is not induced by heat shock in HeLa cells. The ideal model to address this would be siRNA knock down of stress-inducible HSPs in HeLa cells followed by western blot profiling for the BCL-2 family under ER stress conditions. The benefit of such a system is minimal genetic manipulation and therefore adaptation of the cells.

We have demonstrated that HSPB1 is capable of regulating BIM, in part, via regulation of phospho-ERK. In our thermal preconditioning model we also observed a reduction in the expression levels of NOXA and PUMA. Other recent studies have shown that loss of phospho-ERK signalling via Vemurafenib treatment

increased expression PUMA and NOXA (Beck et al., 2013). This suggests that phospho-ERK is involved in inhibition of BIM, PUMA and NOXA. Potentially the reduced expression levels of BIM, PUMA and NOXA in thermal preconditioned cells is due to HSPB1 regulation of phospho-ERK. To study the requirement for phospho-ERK in the protective effects and BH3-only regulating effects of thermal preconditioning, we would need to chemically inhibit ERK using U0126 or genetically inhibit phospho-ERK with siRNA. If thermal preconditioning could still protect cells and reduce levels of BH3 only proteins in the absence of phospho-ERK this would mean that ERK does not have a role in our thermal preconditioning model. Alternatively, if the protection of thermal preconditioning is abrogated in the absence of phospho-ERK this would suggest it is necessary for protecting cells via thermal preconditioning. A role for phospho-ERK in heat shock enhanced NGF outgrowth has already been shown (Read et al., 2008). This suggests that phospho-ERK is activated by heat shock.

JNK too is reported as a regulator of the BCL2 family specifically BIM<sub>EL</sub>, BIM<sub>L</sub> and BMF. We could only detect a faint band for BMF at 6 h in HeLa cells following treatment. Given the fact that cell death occurs after 24 h, it is unlikely BMF plays a role in TG induced cell death in HeLa cells. Expression of phospho-JNK in the thermal preconditioned cells was not investigated however it may be reduced compared to non shocked cells due to the fact that both HSPA1 and HSPB1 regulate JNK. It would be interesting to investigate the contribution of altered phospho-JNK signalling in thermally preconditioned cells by inhibiting or knocking down JNK. We think that the regulation of BIM via HSPB1 is due to dual regulation of both ERK and JNK; a similar situation may be occurring in the thermal precondition model. Although thermal preconditioning models have been used against various insults, the exact molecular mechanism by which preconditioning protects cells is not well defined. Certainly HSPs are required but which aspect of HSP functioning is required and which signalling pathways are involved remains to be determined.

*HSPB1 regulation of IDP*

Previous studies have demonstrated the role of HSPB1 in regulation of proteasome activity and degradation of certain clients. Expression of HSPB1 leads to enhanced degradation of p27<sup>KIP1</sup> and I $\kappa$ B $\alpha$ . Like BIM, p27<sup>KIP1</sup> (Dunker and Uversky, 2008) and I $\kappa$ B $\alpha$  (Mathes et al., 2010) are intrinsically disordered proteins IDP. IDP can be partially or entirely unstructured when in solution by themselves and only fold when in complex with partner proteins. Unstructured regions mean that IDP have larger surface areas for protein interaction, and post-translational regulation of such regions facilitate biodiversity of these proteins (Babu et al., 2011). Given their unusual properties, IDP must be carefully regulated in the cell in order to prevent unwanted interactions. This is certainly true for BIM which has complex regulatory mechanisms in place at transcriptional, post-transcriptional and post-translational levels. It has been observed that overexpression or mutation of IDP such as ataxin-1, a-synuclein and huntingtin are often associated with neurodegenerative diseases (Babu et al., 2011). It has been estimated that 30 % of eukaryotic cell proteomes consist of IDPs (Dunker et al., 2001). A characteristic feature of IDP is ubiquitin-independent degradation by the proteasome. Ubiquitin usually flags proteins for degradation. How are IDP degraded in a regulated fashion in the absence of a clear signalling molecular such as ubiquitin? Potentially HSPB1 interaction with IDP facilitates degradation and over rides the requirement for ubiquitin. This may be important in stress response pathways where many proteins are being ubiquitinated for signal transduction and degradation. Ubiquitin-independent degradation pathways would free up ubiquitin for the aforementioned processes. It has been shown that in addition to reduced proteostasis aged cells have reduced capacity to maintain metastable proteins such as IDPs (Ben-Zvi et al., 2009). The loss of molecular chaperones, as a result of proteostasis collapse, may account for this effect.

Our future work will focus on further delineating the regulation of BIM by HSPB1. HSPB1 can increase ubiquitination of client proteins; however BIM can be degraded

independent of ubiquitin. We will therefore assess levels of ubiquitinated BIM in Neo and HSPB1 cells. To investigate this we will transfect Neo and HSPB1 cells with HA-ubiquitin. After immuno-precipitation pull down of ubiquitinated proteins has been performed, expression levels of BIM will be determined by Western blotting.

Thus far, we have focused on the role of ERK in HSPB- mediated regulation of BIM. However, our data with U0126 clearly demonstrate that other mechanism are at play due to the fact that HSPB1 cells still have significantly less BIM even when the ERK pathway is inhibited. Analysis of phospho-BIM revealed increased expression following ERK inhibition. We postulate that JNK signalling is responsible for this effect and will attempt to delineate the contribution of JNK in the protection and reduction of BIM seen in HSPB1 cells. Further characterization of the mutant HSPB1 cell lines will be performed, specifically focusing on investigating ERK and JNK signalling.

## Chapter 7

### 7.1 References

- Acehan D, Jiang X, Morgan DG, Heuser JE, Wang X, Akey CW (2002) Three-Dimensional Structure of the Apoptosome: Implications for Assembly, Procaspase-9 Binding, and Activation. *Molecular Cell* 9:423-432.
- Ackerley S, James PA, Kalli A, French S, Davies KE, Talbot K (2006) A mutation in the small heat-shock protein HSPB1 leading to distal hereditary motor neuropathy disrupts neurofilament assembly and the axonal transport of specific cellular cargoes. *Human Molecular Genetics* 15:347-354.
- Adachi M, Zhao X, Imai K (2004) Nomenclature of dynein light chain-linked BH3-only protein Bim isoforms. *Cell Death and Differentiation* 12:192-193.
- Ahner A, Gong X, Schmidt BZ, Peters KW, Rabeh WM, Thibodeau PH, Lukacs GL, Frizzell RA (2012) Small heat shock proteins target mutant cystic fibrosis transmembrane conductance regulator for degradation via a small ubiquitin-like modifying dependent pathway. *Molecular Biology of the Cell* 24:74-84.
- Akiyama T, Bouillet P, Miyazaki T, Kadono Y, Chikuda H, Chung U, Fukuda A, Hikita A, Seto H, Okada T, Inaba T, Sanjay A, Baron R, Kawaguchi H, Oda H, Nakamura K, Strasser A, Tanaka S (2003) Regulation of osteoclast apoptosis by ubiquitylation of proapoptotic BH3-only Bcl-2 family member Bim. *EMBO J* 22:6653-6664.
- Almeida-Souza L, Goethals S, de Winter V, Dierick I, Gallardo R, Van Durme J, Irobi J, Gettemans J, Rousseau F, Schymkowitz J, Timmerman V, Janssens S (2010) Increased Monomerization of Mutant HSPB1 Leads to Protein Hyperactivity in Charcot-Marie-Tooth Neuropathy. *Journal of Biological Chemistry* 285:12778-12786.



- Andrieu C, Taieb D, Baylot V, Ettinger S, Soubeyran P, De-Thonel A, Nelson C, Garrido C, So A, Fazli L, Bladou F, Gleave M, Iovanna JL, Rocchi P (2010) Heat shock protein 27 confers resistance to androgen ablation and chemotherapy in prostate cancer cells through eIF4E. *Oncogene* 29:1883-1896.
- Arrigo A-P (2013) Human small heat shock proteins: Protein interactomes of homo- and hetero-oligomeric complexes: An update. *FEBS letters* 587:1959-1969.
- Asada R, Kanemoto S, Kondo S, Saito A, Imaizumi K (2011) The signalling from endoplasmic reticulum-resident bZIP transcription factors involved in diverse cellular physiology. *Journal of Biochemistry* 149:507-518.
- Atkin JD, Farg MA, Soo KY, Walker AK, Halloran M, Turner BJ, Nagley P, Horne MK (2013) Mutant SOD1 inhibits ER-Golgi transport in amyotrophic lateral sclerosis. *Journal of Neurochemistry* . 129(1):190-204
- Atkins C, Liu Q, Minthorn E, Zhang S-Y, Figueroa DJ, Moss K, Stanley TB, Sanders B, Goetz A, Gaul N, Choudhry AE, Alsaïd H, Jucker BM, Axten JM, Kumar R (2013) Characterization of a Novel PERK Kinase Inhibitor with Antitumor and Antiangiogenic Activity. *Cancer Research* 73:1993-2002.
- Babu MM, van der Lee R, de Groot NS, Gsponer J (2011) Intrinsically disordered proteins: regulation and disease. *Current Opinion in Structural Biology* 21:432-440.
- Baranova EV, Weeks SD, Beelen S, Bukach OV, Gusev NB, Strelkov SV (2011) Three-Dimensional Structure of  $\alpha$ -Crystallin Domain Dimers of Human Small Heat Shock Proteins HSPB1 and HSPB6. *Journal of Molecular Biology* 411:110-122.
- Bartelt-Kirbach B, Golenhofen N (2013) Reaction of small heat-shock proteins to different kinds of cellular stress in cultured rat hippocampal neurons. *Cell Stress and Chaperones* 1-9.

- Bauman JW, Liu J, Klaassen CD (1993) Production of Metallothionein and Heat-Shock Proteins in Response to Metals. *Toxicological Sciences* 21:15-22.
- Beck D, Niessner H, Smalley KSM, Flaherty K, Paraiso KHT, Busch C, Sinnberg T, Vasseur S, Iovanna JL, Driessen S, Stork B, Wesselborg S, Schaller M, Biedermann T, Bauer J, Lasithiotakis K, Weide B, Eberle J, Schittek B, Schadendorf D, Garbe C, Kulms D, Meier F (2013) Vemurafenib Potently Induces Endoplasmic Reticulum Stress-Mediated Apoptosis in BRAFV600E Melanoma Cells. *Science Signalling*. 29;6(260):ra7.
- Bellyei S, Szigeti A, Boronkai A, Pozsgai E, Gomori E, Meleg B, Janaky T, Bognar Z, Hocsak E, Sumegi B, Gallyas F, Jr. (2007) Inhibition of cell death by a novel 16.2 kD heat shock protein predominantly via Hsp90 mediated lipid rafts stabilization and Akt activation pathway. *Apoptosis* 12:97-112.
- Ben-Zvi A, Miller EA, Morimoto RI (2009) Collapse of proteostasis represents an early molecular event in *Caenorhabditis elegans* aging. *Proceedings of the National Academy of Sciences* 106:14914-14919.
- Biswas SC, Shi Y, Sproul A, Greene LA (2007) Pro-apoptotic Bim Induction in Response to Nerve Growth Factor Deprivation Requires Simultaneous Activation of Three Different Death Signaling Pathways. *Journal of Biological Chemistry* 282:29368-29374.
- Björk JK, Sistonen L (2010) Regulation of the members of the mammalian heat shock factor family. *FEBS Journal* 277:4126-4139.
- Blanka R (2011) A grand challenge for genetics of aging: adding healthy years to our lives. *Frontiers in Genetics* 2.
- Bouchecareilh M, Higa A, Fribourg S, Moenner M, Chevet E (2011) Peptides derived from the bifunctional kinase/RNase enzyme IRE1 modulate IRE1 activity and protect cells from endoplasmic reticulum stress. *The FASEB Journal* 25:3115-3129.

- Bouillet P, Metcalf D, Huang DCS, Tarlinton DM, Kay T, Kantgen F, Adams JM, Strasser A (1999) Proapoptotic Bcl-2 Relative Bim Required for Certain Apoptotic Responses, Leukocyte Homeostasis, and to Preclude Autoimmunity. *Science* 286:1735-1738.
- Bouillet P, Strasser A (2002) BH3-only proteins - evolutionarily conserved proapoptotic Bcl-2 family members essential for initiating programmed cell death. *Journal of Cell Science* 115:1567-1574.
- Boyce M, Bryant KF, Jousse C, Long K, Harding HP, Scheuner D, Kaufman RJ, Ma D, Coen DM, Ron D, Yuan J (2005) A Selective Inhibitor of eIF2 $\alpha$  Dephosphorylation Protects Cells from ER Stress. *Science* 307:935-939.
- Brodsky Jeffreyal (2012) Cleaning Up: ER-Associated Degradation to the Rescue. *Cell* 151:1163-1167.
- Bruey J-M, Ducasse C, Bonniaud P, Ravagnan L, Susin SA, Diaz-Latoud C, Gurbuxani S, Arrigo A-P, Kroemer G, Solary E, Garrido C (2000a) Differential regulation of HSP27 oligomerization in tumor cells grown in vitro and in vivo. *Oncogene* 19:4855-4863.
- Bruey J-M, Ducasse C, Bonniaud P, Ravagnan L, Susin SA, Diaz-Latoud C, Gurbuxani S, Arrigo A-P, Kroemer G, Solary E, Garrido C (2000b) Hsp27 negatively regulates cell death by interacting with cytochrome c. *Nature Cell Biology* 2:645-652.
- Bryantsev AL, Kurchashove S Y, Golyshev S A, Polyakov V Y, Wunderink H F, Budagova K R, Kakakov A E, Kampinga H H. (2007) Regulation of stress-induced intracellular sorting and chaperone function of Hsp27 (HspB1) in mammalian cells. *Biochemical Journal* 407:407-417.
- Bush KT, Goldberg AL, Nigam SK (1997) Proteasome Inhibition Leads to a Heat-shock Response, Induction of Endoplasmic Reticulum Chaperones, and Thermotolerance. *Journal of Biological Chemistry* 272:9086-9092.

- Calton M, Zeng H, Urano F, Till JH, Hubbard SR, Harding HP, Clark SG, Ron D (2002) IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature* 415:92-96.
- Carra S (2009) The stress-inducible HspB8-Bag3 complex induces the eIF2 $\alpha$  kinase pathway: Implications for protein quality control and viral factory degradation? *Autophagy* 5:428-429.
- Carra S, Brunsting JF, Lambert H, Landry J, Kampinga HH (2009a) HspB8 Participates in Protein Quality Control by a Non-chaperone-like Mechanism That Requires eIF2 $\alpha$  Phosphorylation. *Journal of Biological Chemistry* 284:5523-5532.
- Carra S, Brunsting JF, Lambert H, Landry J, Kampinga HH (2009b) HspB8 Participates in Protein Quality Control by a Non-chaperone-like Mechanism That Requires eIF2 $\alpha$  Phosphorylation. *Journal of Biological Chemistry* 284:5523-5532.
- Carra S, Crippa V, Rusmini P, Boncoraglio A, Minoia M, Giorgetti E, Kampinga HH, Poletti A (2012) Alteration of protein folding and degradation in motor neuron diseases: Implications and protective functions of small heat shock proteins. *Progress in Neurobiology* 97:83-100.
- Cawley K, Logue SE, Gorman AM, Zeng Q, Patterson J, Gupta S, Samali A (2013) Disruption of microRNA Biogenesis Confers Resistance to ER Stress-Induced Cell Death Upstream of the Mitochondrion. *PLoS ONE* 8:e73870.
- Cecconi F, D'Amelio M, Ferraro E, Fuoco C, Strappazon F (2010) Apoptosome Structure and Regulation. In: *Apoptosome*, pp 27-39: Springer Netherlands.
- Charette SJ, Landry J (2000) The Interaction of HSP27 with Dax Identifies a Potential Regulatory Role of HSP27 in Fas-Induced Apoptosis. *Annals of the New York Academy of Sciences* 926:126-131.
- Charette SJ, Lavoie JN, Lambert H, Landry J (2000) Inhibition of Daxx-Mediated Apoptosis by Heat Shock Protein 27. *Molecular Cell Biology* 20:7602-7612.

- Cheng L, Smith DJ, Anderson RL, Nagley P (2011) Modulation of Cellular Hsp72 Levels in Undifferentiated and Neuron-Like SH-SY5Y Cells Determines Resistance to Staurosporine-Induced Apoptosis. *PLoS ONE* 6:e24473.
- Chipuk JE, Moldoveanu T, Llambi F, Parsons MJ, Green DR (2010) The BCL-2 Family Reunion. *Molecular Cell* 37:299-310.
- Chitnis NS, Pytel D, Bobrovnikova-Marjon E, Pant D, Zheng H, Maas NL, Frederick B, Kushner Jake A, Chodosh Lewis A, Koumenis C, Fuchs Serge Y, Diehl JA (2012) miR-211 Is a Prosurvival MicroRNA that Regulates chop Expression in a PERK-Dependent Manner. *Molecular Cell*. 9;48(3):353-64.
- Chowdary TK, Raman B, Ramakrishna T, Rao CM (2004) Mammalian Hsp22 is a heat-inducible small heat-shock protein with chaperone-like activity. *Biochemical Journal* 381:379-387. 48(3):353-64
- Chun-Lei Liu XL, Guo-Liang Hu, Rui-Jun Li, Yun-Yun He, Wu Zhong, Song Li, Kun-Lun He, and Li-Li Wang (2012) Salubrinal protects against tunicamycin and hypoxia induced cardiomyocyte apoptosis via the PERK-eIF2 $\alpha$  signaling pathway. *Journal Geriatric Cardiol.ogy*
- Cohen E, Dillin A (2008) The insulin paradox: aging, proteotoxicity and neurodegeneration. *Nature Reviews Neuroscience* 9:759-767.
- Concannon CG S, Samali A. (2001) Hsp27 inhibits cytochrome c-mediated caspase activation by sequestering both pro-caspase-3 and cytochrome c. *Gene Expression* 9:195-201.
- Crippa V, Carra S, Rusmini P, Sau D, Bolzoni E, Bendotti C, De Biasi S, Poletti A (2010) A role of small heat shock protein B8 (HspB8) in the autophagic removal of misfolded proteins responsible for neurodegenerative diseases. *Autophagy* 6:958-960.

- Crippa V, Valeria Crippa, Mariarita Galbiati, Alessandra Boncoraglio, Paola Rusmini, Elisa Onesto, Elisa Giorgetti, Riccardo Cristofani, Arianna Zito and Angela Poletti (2013) Motoneuronal and muscle-selective removal of ALS-related misfolded proteins. *Biochemical Society Transactions* 41.
- Cullinan SB, Diehl JA (2006) Coordination of ER and oxidative stress signaling: the PERK/Nrf2 signaling pathway. *International Journal Biochemical & Cell Biology* 38:317-332.
- d'Ydewalle C, Benoy V, Van Den Bosch L (2012) Charcot-Marie-Tooth disease: Emerging mechanisms and therapies. *The International Journal of Biochemistry & Cell Biology* 44:1299-1304.
- Daugaard M, Rohde M, Jäättelä M (2007) The heat shock protein 70 family: Highly homologous proteins with overlapping and distinct functions. *FEBS Letters* 581:3702-3710.
- Davenport EL, Moore HE, Dunlop AS, Sharp SY, Workman P, Morgan GJ, Davies FE (2007) Heat shock protein inhibition is associated with activation of the unfolded protein response pathway in myeloma plasma cells. *Blood* 110:2641-2649.
- Deegan S, Saveljeva S, Gorman A, Samali A (2013) Stress-induced self-cannibalism: on the regulation of autophagy by endoplasmic reticulum stress. *Cellular and Molecular Life Sciences* 70:2425-2441.
- Degterev A BMAJ (2003) A decade of caspases. *Oncogene* 22:8543–8567
- Dehan E, Bassermann F, Guardavaccaro D, Vasiliver-Shamis G, Cohen M, Lowes KN, Dustin M, Huang DCS, Taunton J, Pagano M (2009) [beta]TrCP- and Rsk1/2-Mediated Degradation of BimEL Inhibits Apoptosis. *Molecular Cell* 33:109-116.
- Denecker G, Ovaere P, Vandenabeele P, Declercq W (2008) Caspase-14 reveals its secrets. *The Journal of Cell Biology* 180:451-458.

- Dierick I, Irobi J, De Jonghe P, Timmerman V (2005) Small heat shock proteins in inherited peripheral neuropathies. *Annals of Medicine* 37:413-422.
- Dierick I, Irobi J, Janssens S, Theuns J, Lemmens R, Jacobs A, Corsmit E, Hersmus N, Van Den Bosch L, Robberecht W, De Jonghe P, Van Broeckhoven C, Timmerman V (2007) Genetic variant in the HSPB1 promoter region impairs the HSP27 stress response. *Human Mutation* 28:830-830.
- Dixon Scott J, Lemberg Kathryn M, Lamprecht Michael R, Skouta R, Zaitsev Eleina M, Gleason Caroline E, Patel Darpan N, Bauer Andras J, Cantley Alexandra M, Yang Wan S, Morrison B, Stockwell Brent R (2012) Ferroptosis: An Iron-Dependent Form of Nonapoptotic Cell Death. *Cell* 149:1060-1072.
- Dokladny K, Zuhl MN, Mandell M, Bhattacharya D, Schneider S, Deretic V, Moseley PL (2013) Regulatory coordination between two major intracellular homeostatic systems: heat shock response and autophagy. *Journal of Biological Chemistry*.
- Donnelly N, Gorman A, Gupta S, Samali A (2013) The eIF2 $\alpha$  kinases: their structures and functions. *Cellular and Molecular Life Sciences* 1-19.
- Douglas PM, Dillin A (2010) Protein homeostasis and aging in neurodegeneration. *The Journal of Cell Biology* 190:719-729.
- Doyle KM, Kennedy D, Gorman AM, Gupta S, Healy SJM, Samali A (2011) Unfolded proteins and Endoplasmic Reticulum stress in neurodegenerative disorders. *Journal of Cellular and Molecular Medicine*.
- Drubin DG, Feinstein SC, Shooter EM, Kirschner MW (1985) Nerve growth factor-induced neurite outgrowth in PC12 cells involves the coordinate induction of microtubule assembly and assembly-promoting factors. *The Journal of Cell Biology* 101:1799-1807.
- Dunker AK, Lawson JD, Brown CJ, Williams RM, Romero P, Oh JS, Oldfield CJ, Campen AM, Ratliff CM, Hipps KW, Ausio J, Nissen MS, Reeves R, Kang C,

- Kissinger CR, Bailey RW, Griswold MD, Chiu W, Garner EC, Obradovic Z (2001) Intrinsically disordered protein. *Journal of Molecular Graphics and Modelling* 19:26-59.
- Dunker AK, Uversky VN (2008) Signal transduction via unstructured protein conduits. *Nature Chemical Biology* 4:229-230.
- Earnshaw WC, Martins LM, Kaufmann SH (1999) Mammalian Caspases: Structure, Activation, Substrates, and Functions During Apoptosis. *Annual Review of Biochemistry* 68:383-424.
- Ellgaard L, Helenius A (2003) Quality control in the endoplasmic reticulum. *Nat Rev Molecular Cell Biology* 4:181-191.
- Elmore S (2007) Apoptosis: A Review of Programmed Cell Death. *Toxicologic Pathology* 35:495-516.
- Evgrafov OV, Mersyanova I, Irobi J, Van Den Bosch L, Dierick I, Leung CL, Schagina O, Verpoorten N, Van Impe K, Fedotov V, Dadali E, Auer-Grumbach M, Windpassinger C, Wagner K, Mitrovic Z, Hilton-Jones D, Talbot K, Martin J-J, Vasserman N, Tverskaya S, Polyakov A, Liem RKH, Gettemans J, Robberecht W, De Jonghe P, Timmerman V (2004) Mutant small heat-shock protein 27 causes axonal Charcot-Marie-Tooth disease and distal hereditary motor neuropathy. *Nature Genetics* 36:602-606.
- Fang S, Weissman AM (2004) Ubiquitin-proteasome system. *Cellular and Molecular Life Sciences* 61:1546-1561.
- Farg MA, Soo KY, Walker AK, Pham H, Orian J, Horne MK, Warrach ST, Williams KL, Blair IP, Atkin JD (2012) Mutant FUS induces endoplasmic reticulum stress in amyotrophic lateral sclerosis and interacts with protein disulfide-isomerase. *Neurobiology of Aging* 33:2855-2868.



- Fischer H, Koenig U, Eckhart L, Tschachler E (2002) Human caspase 12 has acquired deleterious mutations. *Biochemical and Biophysical Research Communications* 293:722-726.
- Fontaine J-M, Sun X, Hoppe AD, Simon S, Vicart P, Welsh MJ, Benndorf R (2006) Abnormal small heat shock protein interactions involving neuropathy-associated HSP22 (HSPB8) mutants. *The FASEB Journal* 20:2168-2170.
- Fradejas N, Pastor MD, Burgos M, Beyaert R, Tranque P, Calvo S (2009) Caspase-11 mediates ischemia-induced astrocyte death: Involvement of endoplasmic reticulum stress and C/EBP homologous protein. *Journal of Neuroscience Research* 9999:
- Frand AR, Cuozzo JW, Kaiser CA (2000) Pathways for protein disulphide bond formation. *Trends in Cell Biology* 10:203-210.
- Friant S, Meier KD, Riezman H (2003) Increased ubiquitin-dependent degradation can replace the essential requirement for heat shock protein induction. *EMBO J* 22:3783-3791.
- Fulda S, Gorman AM, Hori O, Samali A (2010) Cellular Stress Responses: Cell Survival and Cell Death. *International Journal of Cell Biology* 2010.
- Fulda SG, AM. Hori, O. and Samali, A. (2010) Cellular Stress Responses: Cell Survival and Cell Death. *International Journal of Cell Biology* 2010:23.
- Gaddam D, Stevens N, Hollien J (2013) Comparison of mRNA localization and regulation during endoplasmic reticulum stress in *Drosophila* cells. *Molecular Biology of the Cell* 24:14-20.
- Galluzzi L, Aaronson SA, Abrams J, Alnemri ES, Andrews DW, Baehrecke EH, Bazan NG, Blagosklonny MV, Blomgren K, Borner C, Bredesen DE, Brenner C, Castedo M, Cidlowski JA, Ciechanover A, Cohen GM, De Laurenzi V, De Maria R, Deshmukh M, Dynlacht BD, El-Deiry WS, Flavell RA, Fulda S,

- Garrido C, Golstein P, Gougeon ML, Green DR, Gronemeyer H, Hajnoczky G, Hardwick JM, Hengartner MO, Ichijo H, Jaattela M, Kepp O, Kimchi A, Klionsky DJ, Knight RA, Kornbluth S, Kumar S, Levine B, Lipton SA, Lugli E, Madeo F, Malorni W, Marine JC, Martin SJ, Medema JP, Mehlen P, Melino G, Moll UM, Morselli E, Nagata S, Nicholson DW, Nicotera P, Nunez G, Oren M, Penninger J, Pervaiz S, Peter ME, Piacentini M, Prehn JHM, Puthalakath H, Rabinovich GA, Rizzuto R, Rodrigues CMP, Rubinsztein DC, Rudel T, Scorrano L, Simon HU, Steller H, Tschoop J, Tsujimoto Y, Vandenabeele P, Vitale I, Vousden KH, Youle RJ, Yuan J, Zhivotovsky B, Kroemer G (2009) Guidelines for the use and interpretation of assays for monitoring cell death in higher eukaryotes. *Cell Death and Differentiation* 16:1093-1107.
- Galluzzi L, Joza N, Tasdemir E, Maiuri MC, Hengartner M, Abrams JM, Tavernarakis N, Penninger J, Madeo F, Kroemer G (2008) No death without life: vital functions of apoptotic effectors. *Cell Death and Differentiation* 15:1113-1123.
- Galluzzi L, Vitale I, Abrams JM, Alnemri ES, Bachrecke EH, Blagosklonny MV, Dawson TM, Dawson VL, El-Deiry WS, Fulda S, Gottlieb E, Green DR, Hengartner MO, Kepp O, Knight RA, Kumar S, Lipton SA, Lu X, Madeo F, Malorni W, Mehlen P, Nunez G, Peter ME, Piacentini M, Rubinsztein DC, Shi Y, Simon HU, Vandenabeele P, White E, Yuan J, Zhivotovsky B, Melino G, Kroemer G (2012) Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. *Cell Death and Differentiation* 19:107-120.
- Garrido C, Paul C, Seigneuric R, Kampinga HH (2012) The small heat shock proteins family: The long forgotten chaperones. *The International Journal of Biochemistry & Cell Biology* 44:1588-1592.
- Gavathiotis E, Reyna DE, Davis ML, Bird GH, Walensky LD (2010) BH3-Triggered Structural Reorganization Drives the Activation of Proapoptotic BAX. *Molecular Cell* 40:481-492.

- Geissler A, Haun F, Frank DO, Wieland K, Simon MM, Idzko M, Davis RJ, Maurer U, Borner C (2013) Apoptosis induced by the fungal pathogen gliotoxin requires a triple phosphorylation of Bim by JNK. *Cell Death Differ* 20:1317-1329.
- Gerner EW, Boone R, Connor WG, Hicks JA, Boone MLM (1976) A Transient Thermotolerant Survival Response Produced by Single Thermal Doses in HeLa Cells. *Cancer Research* 36:1035-1040.
- Ghemrawi R, Pooya S, Lorentz S, Gauchotte G, Arnold C, Gueant JL, Battaglia-Hsu SF (2013) Decreased vitamin B12 availability induces ER stress through impaired SIRT1-deacetylation of HSF1. *Cell Death Dis* 4:e553.
- Gibert B, Eckel B, Fasquelle L, Moulin M, Bouhallier F, Gonin V, Mellier G, Simon S, Kretz-Remy C, Arrigo A-P, Diaz-Latoud C (2012) Knock Down of Heat Shock Protein 27 (HspB1) Induces Degradation of Several Putative Client Proteins. *PLoS ONE* 7:e29719.
- Gibert B, Simon S, Dimitrova V, Diaz-Latoud C, Arrigo A-P (2013) Peptide aptamers: tools to negatively or positively modulate HSPB1(27) function. *Philosophical Transactions of the Royal Society B: Biological Sciences* 368.
- Gombos I, Crul T, Piotto S, GÜngör B, Török Z, Balogh G, Péter M, Slotte JP, Campana F, Pilbat A-M, Hunya Á, Tóth N, Literati-Nagy Z, Vigh L, Jr., Glatz A, Brameshuber M, Schütz GJ, Hevener A, Febbraio MA, Horváth I, Vigh L (2011) Membrane-Lipid Therapy in Operation: The HSP Co-Inducer BGP-15 Activates Stress Signal Transduction Pathways by Remodeling Plasma Membrane Rafts. *PLoS ONE* 6:e28818.
- Gomer CJ, Ryter SW, Ferrario A, Rucker N, Wong S, Fisher AMR (1996) Photodynamic Therapy-mediated Oxidative Stress Can Induce Expression of Heat Shock Proteins. *Cancer Research* 56:2355-2360.

- Gorman AM, Healy SJM, Jager R, Samali A (2012a) Stress management at the ER: Regulators of ER stress-induced apoptosis. *Pharmacology & Therapeutics* 134:306-316.
- Gorman AM, Healy SJM, Jager R, Samali A (2012b) Stress management at the ER: Regulators of ER stress-induced apoptosis. *Pharmacology & Therapeutics* 134:306-316.
- Gorman AM, Szegezdi E, Quigney DJ, Samali A (2005) Hsp27 inhibits 6-hydroxydopamine-induced cytochrome c release and apoptosis in PC12 cells. *Biochemical and Biophysical Research Communications* 327:801-810.
- Guicciardi ME, Gores GJ (2009) Life and death by death receptors. *The FASEB Journal* 23:1625-1637.
- Guido K, Boelens W, Jong W (2010) Why proteins without an alpha-crystallin domain should not be included in the human small heat shock protein family HSPB. *Cell Stress and Chaperones* 15:457-461.
- Gupta S, Cuffe L, Szegezdi E, Logue SE, Neary C, Healy S, Samali A (2009) Mechanisms of ER Stress-Mediated Mitochondrial Membrane Permeabilization. *International Journal of Cell Biology* 2010.
- Gupta S, Deepti A, Deegan S, Lisbona F, Hetz C, Samali A (2010) HSP72 Protects Cells from ER Stress-induced Apoptosis via Enhancement of IRE1-XBP1 Signaling through a Physical Interaction. *PLoS Biol* 8:e1000410.
- Gupta S, Read DE, Deepti A, Cawley K, Gupta A, Oommen D, Verfaillie T, Matus S, Smith MA, Mott JL, Agostinis P, Hetz C, Samali A (2012) Perk-dependent repression of miR-106b-25 cluster is required for ER stress-induced apoptosis. *Cell Death and Disease* 3:e333.

- Hahn J-S, Thiele DJ (2004) Activation of the *Saccharomyces cerevisiae* Heat Shock Transcription Factor Under Glucose Starvation Conditions by Snf1 Protein Kinase. *Journal of Biological Chemistry* 279:5169-5176.
- Harada H, Quearry B, Ruiz-Vela A, Korsmeyer SJ (2004) Survival factor-induced extracellular signal-regulated kinase phosphorylates BIM, inhibiting its association with BAX and proapoptotic activity. *Proceedings of the National Academy of Sciences of the United States of America* 101:15313-15317.
- Harding HP, Novoa I, Zhang Y, Zeng H, Wek R, Schapira M, Ron D (2000) Regulated Translation Initiation Controls Stress-Induced Gene Expression in Mammalian Cells. *Molecular Cell* 6:1099-1108.
- Harding HP, Zhang Y, Ron D (1999) Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. *Nature* 397:271-274.
- Hartl FU, Bracher A, Hayer-Hartl M (2011) Molecular chaperones in protein folding and proteostasis. *Nature* 475:324-332.
- Haslbeck M, Franzmann T, Weinfurter D, Buchner J (2005) Some like it hot: the structure and function of small heat-shock proteins. *Nature Structural Molecular Biology* 12:842-846.
- Hatayama T, Nishiyama E, Yasuda K (1994) Cellular Localization of High-Molecular-Mass Heat Shock Proteins in Murine Cells. *Biochemical and Biophysical Research Communications* 200:1367-1373.
- Hausenloy DJ, Yellon DM (2011) The therapeutic potential of ischemic conditioning: an update. *Nature Reviews Cardiology* 8:619-629.
- Havasi A, Li Z, Wang Z, Martin JL, Botla V, Ruchalski K, Schwartz JH, Borkan SC (2008a) Hsp27 Inhibits Bax Activation and Apoptosis via a Phosphatidylinositol 3-Kinase-dependent Mechanism. *Journal of Biological Chemistry* 283:12305-12313.

- Havasi A, Li Z, Wang Z, Martin JL, Botla V, Ruchalski K, Schwartz JH, Borkan SC (2008b) Hsp27 Inhibits Bax Activation and Apoptosis via a Phosphatidylinositol 3-Kinase-dependent Mechanism. *Journal of Biological Chemistry* 283:12305-12313.
- Hayes D, Napoli V, Mazurkie A, Stafford WF, Graceffa P (2009a) HSP27: phosphorylation dependence of its multimeric size and molecular chaperone function. *Journal of Biological Chemistry*.
- Hayes D, Napoli V, Mazurkie A, Stafford WF, Graceffa P (2009b) Phosphorylation Dependence of Hsp27 Multimeric Size and Molecular Chaperone Function. *Journal of Biological Chemistry* 284:18801-18807.
- Haze K, Yoshida H, Yanagi H, Yura T, Mori K (1999) Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. *Mol Biol Cell* 10:3787-3799.
- Healy SJM, Gorman AM, Mousavi-Shafaei P, Gupta S, Samali A (2009) Targeting the endoplasmic reticulum-stress response as an anticancer strategy. *European Journal of Pharmacology* 625:234-246.
- Henle KJ, Bitner AF, Dethlefsen LA (1979) Induction of Thermotolerance by Multiple Heat Fractions in Chinese Hamster Ovary Cells. *Cancer Research* 39:2486-2491.
- Ho Y-S, Yang X, Lau JC-F, Hung CH-L, Wuwongse S, Zhang Q, Wang J, Baum L, So K-F, Chang RC-C (2012) Endoplasmic Reticulum Stress Induces Tau Pathology and Forms a Vicious Cycle: Implication in Alzheimer's Disease Pathogenesis. *Journal of Alzheimer's Disease* 28:839-854.
- Holtz WA, O'Malley KL (2003) Parkinsonian Mimetics Induce Aspects of Unfolded Protein Response in Death of Dopaminergic Neurons. *Journal of Biological Chemistry* 278:19367-19377.

- Hoozemans JJM, van Haastert ES, Nijholt DAT, Rozemuller AJM, Eikelenboom P, Scheper W (2009) The Unfolded Protein Response Is Activated in Pretangle Neurons in Alzheimer's Disease Hippocampus. *The American journal of pathology* 174:1241-1251.
- Hou J, Tang H, Liu Z, Österlund T, Nielsen J, Petranovic D (2013) Management of the ER Stress by Activation of the Heat Shock Response in Yeast. *FEMS Yeast Research* 10.1111/1567
- Hübner A, Barrett T, Flavell RA, Davis RJ (2008) Multisite Phosphorylation Regulates Bim Stability and Apoptotic Activity. *Molecular Cell* 30:415-425.
- Hutt D, M, Powers ET, Balch WE (2009) The proteostasis boundary in misfolding diseases of membrane traffic. *FEBS Letters* 583:2639-2646.
- Irobi J, Almeida-Souza L, Asselbergh B, De Winter V, Goethals S, Dierick I, Krishnan J, Timmermans J-P, Robberecht W, De Jonghe P, Van Den Bosch L, Janssens S, Timmerman V (2010) Mutant HSPB8 causes motor neuron-specific neurite degeneration. *Human Molecular Genetics* 19:3254-3265.
- Ito H, Iwamoto I, Inaguma Y, Takizawa T, Nagata K-i, Asano T, Kato K (2005) Endoplasmic reticulum stress induces the phosphorylation of small heat shock protein, Hsp27. *Journal of Cellular Biochemistry* 95:932-941.
- Iwawaki T, Akai R, Yamanaka S, Kohno K (2009) Function of IRE1 alpha in the placenta is essential for placental development and embryonic viability. *Proceedings of the National Academy of Sciences* 106:16657-16662.
- James PA, Rankin J, Talbot K (2008) Asymmetrical late onset motor neuropathy associated with a novel mutation in the small heat shock protein HSPB1 (HSP27). *Journal of Neurology, Neurosurgery & Psychiatry* 79:461-463.

- Jariel-Encontre I, Bossis G, Piechaczyk M (2008) Ubiquitin-independent degradation of proteins by the proteasome. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer* 1786:153-177.
- Jean-Marie Bruey CP, Annie Fromentin, Sophie Hilpert1, André-Patrick Arrigo, Eric Solary and Carmen Garrido (2000) Differential regulation of HSP27 oligomerization in tumor cells grown in vitro and in vivo. *Oncogene* 19:4855-4863.
- Jentsch TSS (1993) A protein translocation defect linked to ubiquitin conjugation at the endoplasmic reticulum. *Nature* 365:176 - 179
- Jwa M, Chang P (2012) PARP16 is a tail-anchored endoplasmic reticulum protein required for the PERK- and IRE1 $\hat{\pm}$ -mediated unfolded protein response. *Nature Cell Biology* 14:1223-1230.
- Kalai M, Lamkanfi M, Denecker G, Boogmans M, Lippens S, Meeus A, Declercq W, Vandenaabeele P (2003) Regulation of the expression and processing of caspase-12. *The Journal of Cell Biology* 162:457-467.
- Kampinga H, Hageman J, Vos M, Kubota H, Tanguay R, Bruford E, Cheetham M, Chen B, Hightower L (2009) Guidelines for the nomenclature of the human heat shock proteins. *Cell Stress and Chaperones* 14:105-111.
- Kamradt MC, Chen F, Sam S, Cryns VL (2002) The Small Heat Shock Protein  $\hat{\pm}$ B-crystallin Negatively Regulates Apoptosis during Myogenic Differentiation by Inhibiting Caspase-3 Activation. *Journal of Biological Chemistry* 277:38731-38736.
- Kanagasabai R, Karthikeyan K, Vedam K, Qien W, Zhu Q, Ilangoan G (2013) Hsp27 Protects Adenocarcinoma Cells from UV-Induced Apoptosis by Akt and p21-Dependent Pathways of Survival. *Molecular Cancer Research* 8:1399-1412.



- Kanning KC, Kaplan A, Henderson CE (2010) Motor Neuron Diversity in Development and Disease. *Annual Review of Neuroscience* 33:409-440.
- Katayama T, Imaizumi K, Honda A, Yoneda T, Kudo T, Takeda M, Mori K, Rozmahel R, Fraser P, George-Hyslop PS, Tohyama M (2001) Disturbed Activation of Endoplasmic Reticulum Stress Transducers by Familial Alzheimer's Disease-linked Presenilin-1 Mutations. *Journal of Biological Chemistry* 276:43446-43454.
- Katayama T, Imaizumi K, Sato N, Miyoshi K, Kudo T, Hitomi J, Morihara T, Yoneda T, Gomi F, Mori Y, Nakano Y, Takeda J, Tsuda T, Itoyama Y, Murayama O, Takashima A, St George-Hyslop P, Takeda M, Tohyama M (1999) Presenilin-1 mutations downregulate the signalling pathway of the unfolded-protein response. *Nature Cell Biology* 1:479-485.
- Keiichi Ishihara NY, Takumi Hatayama (2003) Protein kinase CK2 phosphorylates Hsp105 alpha at Ser509 and modulates its function. *Biochemical Journal* 371:917-925.
- Kim KK, Kim R, Kim S-H (1998) Crystal structure of a small heat-shock protein. *Nature* 394:595-599.
- Kinouchi H, Sharp FR, Hill MP, Koistinaho W, Sagar SM, Chan PH (1993) Induction of 70-kDa Heat Shock Protein and hsp70 mRNA Following Transient Focal Cerebral Ischemia in the Rat. *Journal of Cerebral Blood Flow and Metabolism* 13:105-115.
- Kolb SJ, Snyder PJ, Poi EJ, Renard EA, Bartlett A, Gu S, Sutton S, Arnold WD, Freimer ML, Lawson VH, Kissel JT, Prior TW (2010) Mutant small heat shock protein B3 causes motor neuropathy: Utility of a candidate gene approach. *Neurology* 74:502-506.
- Korennykh A, Walter P (2012) Structural Basis of the Unfolded Protein Response. *Annual Review of Cell and Developmental Biology* 28:251-277.

- Kouroku Y, Fujita E, Tanida I, Ueno T, Isoai A, Kumagai H, Ogawa S, Kaufman RJ, Kominami E, Momoi T (2006) ER stress (PERK//eIF2[alpha] phosphorylation) mediates the polyglutamine-induced LC3 conversion, an essential step for autophagy formation. *Cell Death and Differentiation* 14:230-239.
- Kourtis N, Nikolettou V, Tavernarakis N (2012) Small heat-shock proteins protect from heat-stroke-associated neurodegeneration. *Nature* 490:213-218.
- Ku B, Liang C, Jung JU, Oh B-H (2011) Evidence that inhibition of BAX activation by BCL-2 involves its tight and preferential interaction with the BH3 domain of BAX. *Cell Research* 21:627-641.
- Kudo W, Lee HP, Smith MA, Zhu X, Matsuyama S, Lee Hg (2012) Inhibition of Bax protects neuronal cells from oligomeric A[beta] neurotoxicity. *Cell Death and Disease* 3:e309.
- Kultz D (2005) Molecular and Evolutionary Basis of the Cellular Stress Response. *Annual Review of Physiology* 67:225–257.
- Kültz D (2003) Evolution of the cellular stress proteome: from monophyletic origin to ubiquitous function. *Journal of Experimental Biology* 206:3119-3124.
- Kumar S (2006) Caspase function in programmed cell death. *Cell Death and Differentiation* 14:32-43.
- Kurata M, Yamazaki Y, Kanno Y, Ishibashi S, Takahara T, Kitagawa M, Nakamura T (2011) Anti-apoptotic function of Xbp1 as an IL-3 signaling molecule in hematopoietic cells. *Cell Death and Disease* 2:e118.
- Lambert H, Charette SJ, Bernier AF, Guimond A, Landry J (1999) HSP27 Multimerization Mediated by Phosphorylation-sensitive Intermolecular Interactions at the Amino Terminus. *Journal of Biological Chemistry* 274:9378-9385.

- Lanneau D, Wettstein G, Bonniaud P, Garrido C (2010) Heat Shock Proteins: Cell Protection through Protein Triage. *The Scientific World Journal* 10:1543-1552.
- Lavoie JN, Lambert H, Hickey E, Weber LA, Landry J (1995) Modulation of cellular thermoresistance and actin filament stability accompanies phosphorylation-induced changes in the oligomeric structure of heat shock protein 27. *Molecular and Cellular Biology* 15:505-516.
- Leak RK, Liou AKF, Zigmond MJ (2006) Effect of sublethal 6-hydroxydopamine on the response to subsequent oxidative stress in dopaminergic cells: evidence for preconditioning. *Journal of Neurochemistry* 99:1151-1163.
- Leber B, Lin J, Andrews D (2007) Embedded together: The life and death consequences of interaction of the Bcl-2 family with membranes. *Apoptosis* 12:897-911.
- Lei K, Davis RJ (2003) JNK phosphorylation of Bim-related members of the Bcl2 family induces Bax-dependent apoptosis. *Proceedings of the National Academy of Sciences* 100:2432-2437.
- Lettre G, Hengartner MO (2006) Developmental apoptosis in *C. elegans*: a complex CEDnario. *Nature Reviews Molecular Cell Biology* 7:97-108.
- Ley R, Balmanno K, Hadfield K, Weston C, Cook SJ (2003) Activation of the ERK1/2 Signaling Pathway Promotes Phosphorylation and Proteasome-dependent Degradation of the BH3-only Protein, Bim. *Journal of Biological Chemistry* 278:18811-18816.
- Ley R, Ewings KE, Hadfield K, Cook SJ (2005) Regulatory phosphorylation of Bim: sorting out the ERK from the JNK. *Cell Death and Differentiation* 12:1008-1014.
- Ley R, Ewings KE, Hadfield K, Howes E, Balmanno K, Cook SJ (2004) Extracellular Signal-regulated Kinases 1/2 Are Serum-stimulated "BimEL Kinases" That

- Bind to the BH3-only Protein BimEL Causing Its Phosphorylation and Turnover. *Journal of Biological Chemistry* 279:8837-8847.
- Li H, Liu L, Xing D, Chen WR (2010) Inhibition of the JNK/Bim pathway by Hsp70 prevents Bax activation in UV-induced apoptosis. *FEBS Letters* 584:4672-4678.
- Li J, Lee B, Lee AS (2006) Endoplasmic Reticulum Stress-induced Apoptosis: multiple pathways and the activation of P53 up regulated modulator of apoptosis (PUMA) and NOXA by P53 *Journal of Biological Chemistry* 281:7260-7270.
- Li J, Yin Q, Wu H, Frederick WA (2013) Chapter Five - Structural Basis of Signal Transduction in the TNF Receptor Superfamily. In: *Advances in Immunology*, vol. Volume 119, pp 135-153: Academic Press.
- Li P-C, Yang C-C, Hsu S-P, Chien C-T (2012) Repetitive progressive thermal preconditioning hinders thrombosis by reinforcing phosphatidylinositol 3-kinase/Akt-dependent heat-shock protein/endothelial nitric oxide synthase signaling. *Journal of vascular surgery : official publication, the Society for Vascular Surgery [and] International Society for Cardiovascular Surgery, North American Chapter* 56:159-170.
- Lin JH, Walter P, Yen TSB (2008) Endoplasmic Reticulum Stress in Disease Pathogenesis. *Annual Review of Pathology: Mechanisms of Disease* 3:399-425.
- Lin W-C, Chuang Y-C, Chang Y-S, Lai M-D, Teng Y-N, Su I-J, Wang CCC, Lee K-H, Hung J-H (2012) Endoplasmic Reticulum Stress Stimulates p53 Expression through NF-KB Activation. *PLoS ONE* 7:e39120.
- Lindquist S (1986) The Heat-Shock Response. *Annual Review of Biochemistry* 55:1151-1191.
- Liu Y, Chang A (2008) Heat shock response relieves ER stress. *EMBO J* 27:1049-1059.

- Lockshin RA, and Williams, C.M. J (1964) Programmed cell death-II. Endocrine potentiation of the breakdown of the intersegmental muscles of silkmths. . *Journal of Insect Physiology* 10:643-649.
- Lu PD, Harding HP, Ron D (2004) Translation reinitiation at alternative open reading frames regulates gene expression in an integrated stress response. *J Cell Biol* 167:27-33.
- Luciano F, Jacquelin A, Colosetti P, Herrant M, Cagnol S, Pages G, Auberger P (2003) Phosphorylation of Bim-EL by Erk1//2 on serine 69 promotes its degradation via the proteasome pathway and regulates its proapoptotic function. *Oncogene* 22:6785-6793.
- Mace PD, Riedl SJ (2010) Molecular cell death platforms and assemblies. *Current Opinion in Cell Biology* 22:828-836.
- Mao L, Franke J (2013) Hormesis in Aging and Neurodegeneration Prodigy Awaiting Dissection. *International Journal of Molecular Sciences* 14:13109-13128.
- Marani M, Tenev T, Hancock D, Downward J, Lemoine NR (2002) Identification of Novel Isoforms of the BH3 Domain Protein Bim Which Directly Activate Bax To Trigger Apoptosis. *Molecular and Cellular Biology* 22:3577-3589.
- Marcu MG, Doyle M, Bertolotti A, Ron D, Hendershot L, Neckers L (2002) Heat Shock Protein 90 Modulates the Unfolded Protein Response by Stabilizing IRE1 $\alpha$ . *Molecular and Cellular Biology* 22:8506-8513.
- Mathes E, Wang L, Komives E, Ghosh G (2010) Flexible Regions within I $\kappa$ B $\alpha$  Create the Ubiquitin-independent Degradation Signal. *Journal of Biological Chemistry* 285:32927-32936.
- Mattoo RUH, Sharma SK, Priya S, Finka A, Goloubinoff P (2013) Hsp110 Is a Bona Fide Chaperone Using ATP to Unfold Stable Misfolded Polypeptides and

- Reciprocally Collaborate with Hsp70 to Solubilize Protein Aggregates. *Journal of Biological Chemistry* 288:21399-21411.
- Matus S, Lopez E, Valenzuela V, Nassif M, Hetz C (2013) Functional Contribution of the Transcription Factor ATF4 to the Pathogenesis of Amyotrophic Lateral Sclerosis. *PLoS ONE* 8:e66672.
- Maytin EV, Ubeda M, Lin JC, Habener JF (2001) Stress-Inducible Transcription Factor CHOP/gadd153 Induces Apoptosis in Mammalian Cells via p38 Kinase-Dependent and -Independent Mechanisms. *Experimental Cell Research* 267:193-204.
- McCormick PH, Chen G, Tlerney S, Kelly CJ, Bouchier-Hayes DJ (2003) Clinically relevant thermal preconditioning attenuates ischemia-reperfusion injury. *The Journal of Surgical Research* 109:24-30.
- McCullough KD, Martindale JL, Klotz L-O, Aw T-Y, Holbrook NJ (2001) Gadd153 Sensitizes Cells to Endoplasmic Reticulum Stress by Down-Regulating Bcl2 and Perturbing the Cellular Redox State. *Molecular Cell Biology* 21:1249-1259.
- McIlwain DR, Berger T, Mak TW (2013) Caspase Functions in Cell Death and Disease. *Cold Spring Harbor Perspectives in Biology* 5.
- Mendes CS, Levet C, Chatelain G, Dourlen P, Fouillet A, Dichtel-Danjoy M-L, Gambis A, Ryoo HD, Steller H, Mollereau B (2009) ER stress protects from retinal degeneration. *EMBO J* 28:1296-1307.
- Meusser B, Hirsch C, Jarosch E, Sommer T (2005) ERAD: the long road to destruction. *Nature Cell Biology* 7:766-772.
- Mitra A, Basak T, Datta K, Naskar S, Sengupta S, Sarkar S (2013) Role of [alpha]-crystallin B as a regulatory switch in modulating cardiomyocyte apoptosis by mitochondria or endoplasmic reticulum during cardiac hypertrophy and myocardial infarction. *Cell Death and Disease* 4:e582.

- Molitoris JK, McColl KS, Distelhorst CW (2011) Glucocorticoid-Mediated Repression of the Oncogenic microRNA Cluster miR-17-92 Contributes to the Induction of Bim and Initiation of Apoptosis. *Molecular Endocrinology* 25:409-420.
- Morimoto RI (1998) Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes & Development* 12:3788-3796.
- Morishima N, Nakanishi K, Takenouchi H, Shibata T, Yasuhiko Y (2002) An Endoplasmic Reticulum Stress-specific Caspase Cascade in Apoptosis: cytochrome c-independent activation of caspase-9 by caspase-12. *Journal of Biological Chemistry* 277:34287-34294.
- Mosser DD, Caron AW, Bourget L, Meriin AB, Sherman MY, Morimoto RI, Massie B (2000) The Chaperone Function of hsp70 Is Required for Protection against Stress-Induced Apoptosis. *Molecular and Cellular Biology* 20:7146-7159.
- Mosser DD, Kotzbauer PT, Sarge KD, Morimoto RI (1990) In vitro activation of heat shock transcription factor DNA-binding by calcium and biochemical conditions that affect protein conformation. *Proceedings of the National Academy of Sciences* 87:3748-3752.
- Moujalled D, Weston R, Anderton H, Ninnis R, Goel P, Coley A, Huang DCS, Wu L, Strasser A, Puthalakath H (2011) Cyclic-AMP-dependent protein kinase A regulates apoptosis by stabilizing the BH3-only protein Bim. *EMBO Rep* 12:77-83.
- Muchowski PJ, Wacker JL (2005) Modulation of neurodegeneration by molecular chaperones. *Nature Reviews Neuroscience* 6:11-22.
- Nagasawa H, Oka M, Maeda K, Jian-Guo C, Hisaeda H, Ito Y, Good RA, Himeno K (1992) Induction of heat shock protein closely correlates with protection against *Toxoplasma gondii* infection. *Proceedings of the National Academy of Sciences* 89:3155-3158.

- Nakagomi S, Suzuki Y, Namikawa K, Kiryu-Seo S, Kiyama H (2003) Expression of the Activating Transcription Factor 3 Prevents c-Jun N-Terminal Kinase-Induced Neuronal Death by Promoting Heat Shock Protein 27 Expression and Akt Activation. *The Journal of Neuroscience* 23:5187-5196.
- Nefedova VV, Sudnitsyna MV, Strelkov SV, Gusev NB (2013) Structure and properties of G84R and L99M mutants of human small heat shock protein HspB1 correlating with motor neuropathy. *Archives of Biochemistry and Biophysics* 538:16-24.
- Nigam SK, Goldberg AL, Ho S, Rohde MF, Bush KT, Sherman MYu (1994) A set of endoplasmic reticulum proteins possessing properties of molecular chaperones includes Ca(2+)-binding proteins and members of the thioredoxin superfamily. *Journal of Biological Chemistry* 269:1744-1749.
- Olson M, Kornbluth S (2001) Mitochondria in Apoptosis and Human Disease. *Current Molecular Medicine* 1:91-122.
- Ota A, Wang Y (2011) Cdc37/Hsp90-mediated regulation of IRE1 $\alpha$  activity in ER stress response and insulin synthesis in INS-1 cells. *Journal of Biological Chemistry*.
- Oyadomari S, Yun C, Fisher EA, Kreglinger N, Kreibich G, Oyadomari M, Harding HP, Goodman AG, Harant H, Garrison JL, Taunton J, Katze MG, Ron D (2006) Cotranslocational Degradation Protects the Stressed Endoplasmic Reticulum from Protein Overload. *Cell* 126:727-739.
- Özcan U, Yilmaz E, Özcan L, Furuhashi M, Vaillancourt E, Smith RO, Görgün CZ, Hotamisligil GS (2006) Chemical Chaperones Reduce ER Stress and Restore Glucose Homeostasis in a Mouse Model of Type 2 Diabetes. *Science* 313:1137-1140.
- Ozoren N, El-Deiry WS (2002) Defining Characteristics of Types I and II Apoptotic Cells in Response to TRAIL. *Neoplasia* 4:551.



- Palade (1975) Intracellular aspects of the process of protein synthesis. *Science* 189; 347-358.
- Parcellier A, Brunet M, Schmitt E, Col E, Didelot Cl, Hammann A, Nakayama K, Nakayama KI, Khochbin S, Solary E, Garrido C (2006) HSP27 favors ubiquitination and proteasomal degradation of p27Kip1 and helps S-phase re-entry in stressed cells. *The FASEB Journal* 20:1179-1181.
- Parcellier A, Schmitt E, Gurbuxani S, Seigneurin-Berny D, Pance A, Chantome A, Plenchette S, Khochbin S, Solary E, Garrido C (2003) HSP27 Is a Ubiquitin-Binding Protein Involved in I- $\kappa$ B $\alpha$  Proteasomal Degradation. *Molecular Cell Biology* 23:5790-5802.
- Pasupuleti N, Matsuyama S, Voss O, Doseff AI, Song K, Danielpour D, Nagaraj RH (2010) The anti-apoptotic function of human  $\alpha$ A-crystallin is directly related to its chaperone activity. *Cell Death and Disease* 1:e31.
- Patel YJK, Payne Smith MD, de Bellerocche J, Latchman DS (2005) Hsp27 and Hsp70 administered in combination have a potent protective effect against FALS-associated SOD1-mutant-induced cell death in mammalian neuronal cells. *Molecular Brain Research* 134:256-274.
- Paul C, Manero F, Gonin S, Kretz-Remy C, Virost S, Arrigo A-P (2002a) Hsp27 as a Negative Regulator of Cytochrome c Release. *Molecular and Cellular Biology* 22:816-834.
- Paul C, Manero F, Gonin S, Kretz-Remy C, Virost S, Arrigo A-P (2002b) Hsp27 as a Negative Regulator of Cytochrome c Release. *Mol Cell Biol* 22:816-834.
- Paul C, Simon S, Gibert B, Virost S, Manero F, Arrigo A-P (2012) Dynamic processes that reflect anti-apoptotic strategies set up by HspB1 (Hsp27). *Experimental Cell Research* 316:1535-1552.

- Pennuto M, Tinelli E, Malaguti M, Del Carro U, D'Antonio M, Ron D, Quattrini A, Feltri ML, Wrabetz L (2008) Ablation of the UPR-Mediator CHOP Restores Motor Function and Reduces Demyelination in Charcot-Marie-Tooth 1B Mice. *Neuron* 57:393-405.
- Pincus D, Chevalier MW, Aragan Ts, van Anken E, Vidal SE, El-Samad H, Walter P (2010) BiP Binding to the ER-Stress Sensor Ire1 Tunes the Homeostatic Behavior of the Unfolded Protein Response. *PLoS Biol* 8:e1000415.
- Pop C, Salvesen GS (2009) Human Caspases: Activation, Specificity, and Regulation. *Journal of Biological Chemistry* 284:21777-21781.
- Putcha GV, Le S, Frank S, Besirli CG, Clark K, Chu B, Alix S, Youle RJ, LaMarche A, Maroney AC, Johnson Jr EM (2003) JNK-Mediated BIM Phosphorylation Potentiates BAX-Dependent Apoptosis. *Neuron* 38:899-914.
- Putcha GV, Moulder KL, Golden JP, Bouillet P, Adams JA, Strasser A, Johnson Jr EM (2001) Induction of BIM, a Proapoptotic BH3-Only BCL-2 Family Member, Is Critical for Neuronal Apoptosis. *Neuron* 29:615-628.
- Puthalakath H, O'Reilly LA, Gunn P, Lee L, Kelly PN, Huntington ND, Hughes PD, Michalak EM, McKimm-Breschkin J, Motoyama N, Gotoh T, Akira S, Bouillet P, Strasser A (2007) ER Stress Triggers Apoptosis by Activating BH3-Only Protein BIM. *Cell* 129:1337-1349.
- Qi X-J, Wildey GM, Howe PH (2006) Evidence That Ser87 of BimEL Is Phosphorylated by Akt and Regulates BimEL Apoptotic Function. *Journal of Biological Chemistry* 281:813-823.
- Qi Y, Xia P (2012) Cellular Inhibitor of Apoptosis Protein-1 (cIAP1) Plays a Critical Role in Cell Survival under Endoplasmic Reticulum Stress: Promoting ubiquitination and degradation of CHOP. *Journal of Biological Chemistry* 287:32236-32245.

- Qian L, Van Laake LW, Huang Y, Liu S, Wendland MF, Srivastava D miR-24 inhibits apoptosis and represses Bim in mouse cardiomyocytes. *The Journal of Experimental Medicine* 208:549-560.
- Qin L, Wang Z, Tao L, Wang Y (2010) ER stress negatively regulates AKT/TSC/mTOR pathway to enhance autophagy. *Autophagy* 6:239-247.
- Quigley DJ, Gorman AM, Samali A (2003) Heat shock protects PC12 cells against MPP+ toxicity. *Brain Research* 993:133-139.
- Rafiee P, Shi Y, Pritchard KA, Ogawa H, Eis ALW, Komorowski RA, Fitzpatrick CM, Tweddell JS, Litwin SB, Mussatto K, Jaquiss RD, Baker JE (2003) Cellular Redistribution of Inducible Hsp70 Protein in the Human and Rabbit Heart in Response to the Stress of Chronic Hypoxia: role of protein kinases *Journal of Biological Chemistry* 278:43636-43644.
- Rane MJ, Pan Y, Singh S, Powell DW, Wu R, Cummins T, Chen Q, McLeish KR, Klein JB (2003) Heat Shock Protein 27 Controls Apoptosis by Regulating Akt Activation. *Journal of Biological Chemistry* 278:27828-27835.
- Ravikumar B, Duden R, Rubinsztein DC (2002) Aggregate-prone proteins with polyglutamine and polyalanine expansions are degraded by autophagy. *Human Molecular Genetics* 11:1107-1117.
- Read DE, Reed Herbert K, Gorman AM (2008) Heat shock enhances NGF-induced neurite elongation which is not mediated by Hsp25 in PC12 cells. *Brain Research* 1221:14-23.
- Reimertz C, Kagel D, Rami A, Chittenden T, Prehn JHM (2003a) Gene expression during ER stress-induced apoptosis in neurons: induction of the BH3-only protein Bbc3/PUMA and activation of the mitochondrial apoptosis pathway. *The Journal of Cell Biology* 162:587-597.

- Reimertz C, Kagel D, Rami A, Chittenden T, Prehn JHM (2003b) Gene expression during ER stress induced apoptosis in neurons. *The Journal of Cell Biology* 162:587-597.
- Renault TT, Teijido O, Antonsson B, Dejean LM, Manon S (2013) Regulation of Bax mitochondrial localization by Bcl-2 and Bcl-xL: Keep your friends close but your enemies closer. *The International Journal of Biochemistry & Cell Biology* 45:64-67.
- Richter K, Haslbeck M, Buchner J (2010) The Heat Shock Response: Life on the Verge of Death. *Molecular Cell* 40:253-266.
- Ritossa (1996) Discovery of the Heat Shock Response. *Cell stress and chaperones* 1(2):97-98.
- Roberts PJ, Der CJ (2000) Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. *Oncogene* 26:3291-3310.
- Rogalla T, Ehrnsperger M, Preville X, Kotlyarov A, Lutsch G, Ducasse C, Paul C, Wieske M, Arrigo A-P, Buchner J, Gaestel M (1999) Regulation of Hsp27 Oligomerization, Chaperone Function, and Protective Activity against Oxidative Stress/Tumor Necrosis Factor alpha by Phosphorylation. *Journal of Biological Chemistry* 274:18947-18956.
- Ron D, Walter P (2007) Signal integration in the endoplasmic reticulum unfolded protein response. *Nature Reviews Molecular Cell Biology* 8:519-529.
- Rowland AA, Voeltz GK (2012) Endoplasmic reticulum-mitochondria contacts: function of the junction. *Nature Reviews Molecular Cell Biology* 13:607-625.
- Rutkowski DT, Arnold SM, Miller CN, Wu J, Li J, Gunnison KM, Mori K, Sadighi Akha AA, Raden D, Kaufman RJ (2006) Adaptation to ER Stress Is Mediated by Differential Stabilities of Pro-Survival and Pro-Apoptotic mRNAs and Proteins. *PLoS Biol* 4:e374.

- Rutkowski DT, Kaufman RJ (2007) That which does not kill me makes me stronger: adapting to chronic ER stress. *Trends in Biochemical Sciences* 32:469-476.
- Samali AR, J. Peterson, E. Manero, F. van Zeijl, L. Paul, C. Cotgreave, I. Arrigo, A. and Orrenius, S. (2001) Hsp27 protects mitochondria of thermotolerant cells against apoptotic stimuli. *Cell Stress and Chaperones* 6:49-58.
- Sanphui P, Biswas SC (2013) FoxO3a is activated and executes neuron death via Bim in response to [beta]-amyloid. *Cell Death and Disease* 4:e625.
- Schröder M (2008) Endoplasmic reticulum stress responses. *Cellular and Molecular Life Sciences* 65:862-894.
- Schroder M, Kaufman RJ (2005) The mammalian unfolded protein response. *Annual Review of Biochemistry* 74:739-789.
- Schuck S, Prinz WA, Thorn KS, Voss C, Walter P (2009) Membrane expansion alleviates endoplasmic reticulum stress independently of the unfolded protein response. *The Journal of Cell Biology* 187:525-536.
- Sessler T, Healy S, Samali A, Szegezdi E (2012) Structural determinants of DISC function: New insights into death receptor-mediated apoptosis signalling. *Pharmacology & Therapeutics* 140:186-199.
- Shamas-Din A, Bindner S, Zhu W, Zaltsman Y, Campbell C, Gross A, Leber B, Andrews DW, Fradin C (2013) tBid Undergoes Multiple Conformational Changes at the Membrane Required for Bax Activation. *Journal of Biological Chemistry* 288:22111-22127.
- Shamas-Din A, Brahmhatt H, Leber B, Andrews DW (2010) BH3-only proteins: Orchestrators of apoptosis. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 1813:508-520.

- Sharp PS, Akbar MT, Bouri S, Senda A, Joshi K, Chen H-J, Latchman DS, Wells DJ, de Bellerocche J (2008) Protective effects of heat shock protein 27 in a model of ALS occur in the early stages of disease progression. *Neurobiology of Disease* 30:42-55.
- Shi Y (2004) Caspase Activation: Revisiting the Induced Proximity Model. *Cell* 117:855-858.
- Shu-Fen Hsu CM, Wu-Tein Huang, Mao-Tsun Lin, Bor-Chih Cheng (2013) Attenuating heat-induced cellular autophagy, apoptosis and damage in H9c2 cardiomyocytes by pre-inducing HSP70 with heat shock preconditioning. *International Journal of Hyperthermia* 29:239-247.
- So J-S, Hur Kyua Y, Tarrío M, Ruda V, Frank-Kamenetsky M, Fitzgerald K, Koteliansky V, Lichtman Andrew H, Iwawaki T, Glimcher Laurie H, Lee A-H (2012) Silencing of Lipid Metabolism Genes through IRE1-Mediated mRNA Decay Lowers Plasma Lipids in Mice. *Cell Metabolism* 16:487-499.
- Soo KY, Atkin JD, Farg M, Walker AK, Horne MK, Nagley P (2012) Bim Links ER Stress and Apoptosis in Cells Expressing Mutant SOD1 Associated with Amyotrophic Lateral Sclerosis. *PLoS ONE* 7:e35413.
- Sreedhar AS, Pardhasaradhi BVV, Begum Z, Khar A, Srinivas UK (1999) Lack of heat shock response triggers programmed cell death in a rat histiocytic cell line. *FEBS Letters* 456:339-342.
- Sriburi R, Bommasamy H, Buldak GL, Robbins GR, Frank M, Jackowski S, Brewer JW (2007) Coordinate Regulation of Phospholipid Biosynthesis and Secretory Pathway Gene Expression in XBP-1(S)-induced Endoplasmic Reticulum Biogenesis. *Journal of Biological Chemistry* 282:7024-7034.
- Stankiewicz AR, Lachapelle G, Foo CPZ, Radicioni SM, Mosser DD (2005) Hsp70 Inhibits Heat-induced Apoptosis Upstream of Mitochondria by Preventing Bax Translocation. *Journal of Biological Chemistry* 280:38729-38739.

- Stankiewicz R, Livingstone M, Mohseni N, Mosser D (2009) Regulation of heat-induced apoptosis by Mcl-1 degradation and its inhibition by Hsp70. *Cell Death Differ* 16:638-647.
- Stetler RA, Armando P, Signore, Yanqin Gao, Guodong Cao, and Jun Chen (2009) HSP27: Mechanisms of Cellular Protection Against Neuronal Injury. *Current Molecular Medicine* 9:863-872.
- Stetler RA, Cao G, Gao Y, Zhang F, Wang S, Weng Z, Vosler P, Zhang L, Signore A, Graham SH, Chen J (2008a) Hsp27 Protects against Ischemic Brain Injury via Attenuation of a Novel Stress-Response Cascade Upstream of Mitochondrial Cell Death Signaling. *Journal of Neuroscience* 28:13038-13055.
- Stetler RA, Cao G, Gao Y, Zhang F, Wang S, Weng Z, Vosler P, Zhang L, Signore A, Graham SH, Chen J (2008b) Hsp27 Protects against Ischemic Brain Injury via Attenuation of a Novel Stress-Response Cascade Upstream of Mitochondrial Cell Death Signaling. *The Journal of Neuroscience* 28:13038-13055.
- Stetler RA, Yanqin Gao, Lili Zhang, Zhongfang Weng, Feng Zhang, Xiaoming Hu, Suping Wang, Peter Vosler, Guodong Cao, Dandan Sun, Steven H. Graham, and Jun Chen (2011) Phosphorylation of HSP27 by Protein Kinase D Is Essential for Mediating Neuroprotection against Ischemic Neuronal Injury. *Journal Neuroscience* 32:2667-2682.
- Strowig T, Henao-Mejia J, Elinav E, Flavell R (2012) Inflammasomes in health and disease. *Nature* 481:278-286.
- Sunters A, Fernández de Mattos S, Stahl M, Brosens JJ, Zoumpoulidou G, Saunders CA, Coffey PJ, Medema RH, Coombes RC, Lam EW-F (2003) FoxO3a Transcriptional Regulation of Bim Controls Apoptosis in Paclitaxel-treated Breast Cancer Cell Lines. *Journal of Biological Chemistry* 278:49795-49805.
- Suter U, Scherer SS (2003) Disease mechanisms in inherited neuropathies. *Nature Reviews Neuroscience* 4:714-726.

- Suzuki H, Lee K, Matsuoka M (2011) TDP-43-induced Death Is Associated with Altered Regulation of BIM and Bcl-xL and Attenuated by Caspase-mediated TDP-43 Cleavage. *Journal of Biological Chemistry* 286:13171-13183.
- Szegezdi E, Logue SE, Gorman AM, Samali A (2006) Mediators of endoplasmic reticulum stress-induced apoptosis. *EMBO Rep* 7:880-885.
- Szegezdi E, MacDonald DC, Ni Chonghaile T, Gupta S, Samali A (2009) Bcl-2 family on guard at the ER. *American Journal of Cell Physiology* 296:C941-953.
- Szegezdi E, Reed Herbert K, Kavanagh ET, Samali A, Gorman AM (2008) Nerve growth factor blocks thapsigargin-induced apoptosis at the level of the mitochondrion via regulation of BIM. *Journal of Cellular and Molecular Medicine* 12:2482-2496.
- Tait SWG, Green DR (2010) Mitochondria and cell death: outer membrane permeabilization and beyond. *Nature Reviews Molecular Cell Biology* 11:621-632.
- Tan Y, Dourdin N, Wu C, De Veyra T, Elce JS, Greer PA (2006) Ubiquitous Calpains Promote Caspase-12 and JNK Activation during Endoplasmic Reticulum Stress-induced Apoptosis. *Journal of Biological Chemistry* 281:16016-16024.
- Tanaka K-I, Mizushima T (2009) Protective role of HSF1 and HSP70 against gastrointestinal diseases. *International Journal of Hyperthermia* 25:668-676.
- Tay KH, Jin L, Tseng HY, Jiang CC, Ye Y, Thorne RF, Liu T, Guo ST, Verrills NM, Hersey P, Zhang XD (2012) Suppression of PP2A is critical for protection of melanoma cells upon endoplasmic reticulum stress. *Cell Death and Disease* 3:e337.
- Taylor RC, Cullen SP, Martin SJ (2008) Apoptosis: controlled demolition at the cellular level. *Nature Reviews Molecular Cell Biology* 9:231-241.



- Taylor Rebecca C, Dillin A (2013) XBP-1 Is a Cell-Nonautonomous Regulator of Stress Resistance and Longevity. *Cell* 153:1435-1447.
- Taylor RP, Benjamin IJ (2005) Small heat shock proteins: a new classification scheme in mammals. *Journal of Molecular and Cellular Cardiology* 38:433-444.
- Teasdale RD, Jackson MR (1996) Signal mediated sorting of membrane proteins between the endoplasmic reticulum and the golgo apparatus. *Annual Review of Cell and Developmental Biology* 12:27-54.
- Theriault JR, Lambert H, ChÃ¡vez-Zobel AT, Charest G, Lavigne P, Landry J (2004) Essential Role of the NH2-terminal WD/EPF Motif in the Phosphorylation-activated Protective Function of Mammalian Hsp27. *Journal of Biological Chemistry* 279:23463-23471.
- Thomas MP, Lieberman J (2013) Live or let die: posttranscriptional gene regulation in cell stress and cell death. *Immunological Reviews* 253:237-252.
- Tradewell ML, Durham HD, Mushynski WE, Gentil BJ (2009) Mitochondrial and Axonal Abnormalities Precede Disruption of the Neurofilament Network in a Model of Charcot-Marie-Tooth Disease Type 2E and Are Prevented by Heat Shock Proteins in a Mutant-Specific Fashion. *Journal of Neuropathology & Experimental Neurology* 68:642-652  
610.1097/NEN.1090b1013e3181a1095deeb.
- Truettner JS, Hu K, Liu CL, Dietrich WD, Hu B (2009) Subcellular stress response and induction of molecular chaperones and folding proteins after transient global ischemia in rats. *Brain Research* 1249:9-18.
- Tsuru A, Fujimoto N, Takahashi S, Saito M, Nakamura D, Iwano M, Iwawaki T, Kadokura H, Ron D, Kohno K (2013) Negative feedback by IRE1<sup>1</sup> optimizes mucin production in goblet cells. *Proceedings of the National Academy of Sciences* 110:2864-2869.

- Tsuruta F, Sunayama J, Mori Y, Hattori S, Shimizu S, Tsujimoto Y, Yoshioka K, Masuyama N, Gotoh Y (2004) JNK promotes Bax translocation to mitochondria through phosphorylation of 14-3-3 proteins. *EMBO J* 23:1889-1899.
- Turturici G, Sconzo G, Geraci F (2011) Hsp70 and Its Molecular Role in Nervous System Diseases. *Biochemistry Research International* 2011.
- U M, Miyashita T, Shikama Y, Tadokoro K, Yamada M (2001) Molecular cloning and characterization of six novel isoforms of human Bim, a member of the proapoptotic Bcl-2 family. *FEBS Letters* 509:135-141.
- Upton J-P, Austgen K, Nishino M, Coakley KM, Hagen A, Han D, Papa FR, Oakes SA (2008) Caspase-2 Cleavage of BID Is a Critical Apoptotic Signal Downstream of Endoplasmic Reticulum Stress. *Molecular and Cellular Biology* 28:3943-3951.
- Upton J-P, Wang L, Han D, Wang ES, Huskey NE, Lim L, Truitt M, McManus MT, Ruggero D, Goga A, Papa FR, Oakes SA (2012) IRE1 Cleaves Select microRNAs During ER Stress to Derepress Translation of Proapoptotic Caspase-2. *Science* 338:818-822.
- Urano F, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP, Ron D (2000) Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science* 287:664-666.
- Vela L, Gonzalo O, Naval J, Marzo I (2013) Direct Interaction of Bax and Bak Proteins with Bcl-2 Homology Domain 3 (BH3)-only Proteins in Living Cells Revealed by Fluorescence Complementation. *Journal of Biological Chemistry* 288:4935-4946.
- Velichko A, Markova E, Petrova N, Razin S, Kantidze O (2013) Mechanisms of heat shock response in mammals. *Cellular and Molecular Life Sciences* 1-13.

- Vos MJ, Hageman J, Carra S, Kampinga HH (2008a) Structural and Functional Diversities between Members of the Human HSPB, HSPH, HSPA, and DNAJ Chaperone Families. *Biochemistry* 47:7001-7011.
- Vos MJ, Hageman J, Carra S, Kampinga HH (2008b) Structural and Functional Diversities between Members of the Human HSPB, HSPH, HSPA, and DNAJ Chaperone Families†. *Biochemistry* 47:7001-7011.
- Voss OH, Batra S, Kolattukudy SJ, Gonzalez-Mejia ME, Smith JB, Doseff AI (2007) Binding of Caspase-3 Prodomain to Heat Shock Protein 27 Regulates Monocyte Apoptosis by Inhibiting Caspase-3 Proteolytic Activation. *Journal of Biological Chemistry* 282:25088-25099.
- Wagstaff MJD, Collaço-Moraes Y, Smith J, de Belleruche JS, Coffin RS, Latchman DS (1999) Protection of Neuronal Cells from Apoptosis by Hsp27 Delivered with a Herpes Simplex Virus-based Vector. *Journal of Biological Chemistry* 274:5061-5069.
- Wakeyama H, Akiyama T, Kadono Y, Nakamura M, Oshima Y, Nakamura K, Tanaka S (2007) Posttranslational Regulation of Bim by Caspase-3. *Annals of the New York Academy of Sciences* 1116:271-280.
- Wang H, Lin G, Zhang Z (2007) ATF5 promotes cell survival through transcriptional activation of Hsp27 in H9c2 cells. *Cell Biology International* 31:1309-1315.
- Wang S, Kaufman RJ (2012) The impact of the unfolded protein response on human disease. *The Journal of Cell Biology* 197:857-867.
- Wang XZ, Ron D (1996) Stress-induced phosphorylation and activation of the transcription factor CHOP (GADD153) by p38 MAP Kinase. *Science* 1996 May 31;272(5266):1347-9.
- Wei MC, Zong W-X, Cheng EH-Y, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB, Korsmeyer SJ (2001) Proapoptotic BAX

- and BAK: A Requisite Gateway to Mitochondrial Dysfunction and Death. *Science* 292:727-730.
- Westerheide SD, Morimoto RI (2005) Heat Shock Response Modulators as Therapeutic Tools for Diseases of Protein Conformation. *Journal of Biological Chemistry* 280:33097-33100.
- Westphal D, Dewson G, Czabotar PE, Kluck RM (2011) Molecular biology of Bax and Bak activation and action. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 1813:521-531.
- Whitfield J, Neame SJ, Paquet L, Bernard O, Ham J (2001) Dominant-Negative c-Jun Promotes Neuronal Survival by Reducing BIM Expression and Inhibiting Mitochondrial Cytochrome c Release. *Neuron* 29:629-643.
- Wiggins CM, Band H, Cook SJ (2007) c-Cbl is not required for ERK1/2-dependent degradation of BimEL. *Cellular Signalling* 19:2605-2611.
- Wiggins CM, Johnson M, Cook SJ (2010) Refining the minimal sequence required for ERK1/2-dependent poly-ubiquitination and proteasome-dependent turnover of BIM. *Cellular Signalling* 22:801-808.
- Wiggins CM, Tsvetkov P, Johnson M, Joyce CL, Lamb CA, Bryant NJ, Komander D, Shaul Y, Cook SJ (2011) BIMEL, an intrinsically disordered protein, is degraded by 20S proteasomes in the absence of poly-ubiquitylation. *Journal of Cell Science* 124:969-977.
- Willer M, Forte GMA, Stirling CJ (2008) Sec61p Is Required for ERAD-L: genetic dissection of the translocation and ERAD-L functions of Sec61p using novel derivatives of CPY. *Journal of Biological Chemistry* 283:33883-33888.
- Wu J, Rutkowski DT, Dubois M, Swathirajan J, Saunders T, Wang J, Song B, Yau GD, Kaufman RJ (2007a) ATF6alpha optimizes long-term endoplasmic reticulum function to protect cells from chronic stress. *Developmental Cell* 13:351-364.

- Wu R, Kausar H, Johnson P, Montoya-Durango DE, Merchant M, Rane MJ (2007b) Hsp27 Regulates Akt Activation and Polymorphonuclear Leukocyte Apoptosis by Scaffolding MK2 to Akt Signal Complex. *Journal of Biological Chemistry* 282:21598-21608.
- Yan C, Oh JS, Yoo SH, Lee JS, Yoon YG, Oh YJ, Jang MS, Lee SY, Yang J, Lee SH, Kim HY, Yoo YH (2013) The targeted inhibition of mitochondrial Hsp90 overcomes the apoptosis resistance conferred by Bcl-2 in Hep3B cells via necroptosis. *Toxicology and Applied Pharmacology* 266:9-18.
- Yan W, Frank CL, Korth MJ, Sopher BL, Novoa I, Ron D, Katze MG (2002) Control of PERK eIF2 $\gamma$  kinase activity by the endoplasmic reticulum stress-induced molecular chaperone P58IPK. *Proceedings of the National Academy of Sciences* 99:15920-15925.
- Youle RJ, Strasser A (2008) The BCL-2 protein family: opposing activities that mediate cell death. *Nature Reviews Molecular Cell Biology* 9:47-59.
- Yu Z, Luo H, Fu W, Mattson MP (1999) The Endoplasmic Reticulum Stress-Responsive Protein GRP78 Protects Neurons Against Excitotoxicity and Apoptosis: Suppression of Oxidative Stress and Stabilization of Calcium Homeostasis. *Experimental Neurology* 155:302-314.
- Yuan J, Shaham S, Ledoux S, Ellis HM, Horvitz HR (1993) The *C. elegans* cell death gene *ced-3* encodes a protein similar to mammalian interleukin-1 $\beta$ -converting enzyme. *Cell* 75:641-652.
- Yuan T, Luo B-l, Wei T-h, Zhang L, He B-m, Niu R-c (2012) Salubrinal Protects Against Cigarette Smoke Extract-induced HBEpC Apoptosis Likely Via Regulating the Activity of PERK-eIF2 $\gamma$  Signaling Pathway. *Archives of Medical Research* 43:522-529.
- Zhai J, Lin H, Julien J-P, Schlaepfer WW (2007) Disruption of neurofilament network with aggregation of light neurofilament protein: a common pathway leading to

motor neuron degeneration due to Charcot–Marie–Tooth disease-linked mutations in NFL and HSPB1. *Human Molecular Genetics* 16:3103-3116.

Zhang D, Ke L, Mackovicova K, Der Want JJLV, Sibon OCM, Tanguay RM, Morrow G, Henning RH, Kampinga HH, Brundel BJM (2011a) Effects of different small HSPB members on contractile dysfunction and structural changes in a *Drosophila melanogaster* model for Atrial Fibrillation. *Journal of Molecular and Cellular Cardiology* 51:381-389.

Zhang K, Wang S, Malhotra J, Hassler JR, Back SH, Wang G, Chang L, Xu W, Miao H, Leonardi R, Chen YE, Jackowski S, Kaufman RJ (2011b) The unfolded protein response transducer IRE1[alpha] prevents ER stress-induced hepatic steatosis. *EMBO J* 30:1357-1375.

Zhang L, Lopez H, George NM, Liu X, Pang X, Luo X (2012) Selective involvement of BH3-only proteins and differential targets of Noxa in diverse apoptotic pathways. *Cell Death and Differentiation* 18:864-873.

Zhang Y, Xing D, Liu L (2009) PUMA Promotes Bax Translocation by Both Directly Interacting with Bax and by Competitive Binding to Bcl-XL during UV-induced Apoptosis. *Molecular Biology of the Cell* 20:3077-3087.

Zhu S, Evans S, Yan B, Povsic TJ, Tapson V, Goldschmidt-Clermont PJ, Dong C (2008) Transcriptional Regulation of Bim by FOXO3a and Akt Mediates Scleroderma Serum–Induced Apoptosis in Endothelial Progenitor Cells. *Circulation* 118:2156-2165.

Zinszner H, Kuroda M, Wang X, Batchvarova N, Lightfoot RT, Remotti H, Stevens JL, Ron D (1998a) CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. *Genes & Development* 12:982-995.

Zinszner H, Kuroda M, Wang X, Batchvarova N, Lightfoot RT, Remotti H, Stevens JL, Ron D (1998b) CHOP is implicated in programmed cell death in response to

impaired function of the endoplasmic reticulum. *Genes & Development* 12:982-995.

Zong W-X, Li C, Hatzivassiliou G, Lindsten T, Yu Q-C, Yuan J, Thompson CB (2003) Bax and Bak can localize to the endoplasmic reticulum to initiate apoptosis. *The Journal of Cell Biology* 162:59-69.

Zoubeydi A, Zardan A, Wiedmann RM, Locke J, Beraldi E, Fazli L, Gleave ME (2010) Hsp27 Promotes Insulin-Like Growth Factor-I Survival Signaling in Prostate Cancer via p90Rsk-Dependent Phosphorylation and Inactivation of BAD. *Cancer Research* 70:2307-2317.

Zoubeydi A, Zardan A, Wiedmann RM, Locke J, Beraldi E, Fazli L, Gleave ME (2013) Hsp27 Promotes Insulin-Like Growth Factor-I Survival Signaling in Prostate Cancer via p90Rsk-Dependent Phosphorylation and Inactivation of BAD. *Cancer Research* 70:2307-2317.

Zourlidou A, Payne Smith MD, Latchman DS (2004) HSP27 but not HSP70 has a potent protective effect against  $\alpha$ -synuclein-induced cell death in mammalian neuronal cells. *Journal of Neurochemistry* 88:1439-1448.

Zuchner S, Vance JM (2006) Mechanisms of Disease: a molecular genetic update on hereditary axonal neuropathies. *Nature Clinical Practice Neurology* 2:45-53.