Cellular response to stress: role of autophagy and cell death in caspase-9 deficient cells

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

Doctor of Philosophy

by

Karolina Pakos-Zebrucka

Discipline of Biochemistry, School of Natural Science,

National University of Ireland, Galway

Thesis Supervisors: Prof Afshin Samali, Dr Adrienne Gorman

Head of School: Prof Ciaran Morrison

Acknowledgments

*"Look deep into **nature**, and then you will understand everything better"* Albert Einstein

Go raibh maith agat. Thank you.

I would like to express my deepest gratitude to Prof. Afshin Samali for immeasurable support and guidance he has provided throughout this study. His deepest insight into this project led me to the right way. Thank you for your encouragement and inspiration during this PhD. For all your kindness, advise and help. Thank you very much for giving me the opportunity to follow my dreams. This was a fantastic journey.

I would also like to extend my gratitude to Dr Adrienne Gorman who has provided me with the assistance and encouragement. Her expert advice in scientific writing is invaluable. Thank you, Adrienne.

I am especially grateful to Dr Susan Logue for giving me guidance and advice during my research. I am thankful for your help and friendly conversations. Your dedication and interest in science is an inspiration. It was a pleasure to work with you and I had a privilege to learn from you. You have opened the doors to research for me and showed me how to be a scientist.

My sincere appreciation is extended to Dr Shane Deegan. His invaluable suggestions, advice and support have enabled me to complete this project. I have learnt lots from you. It was a pleasure to discuss science with you.

I am extremely thankful to Dr Marion Maurel, Dr Mila Ljujic and Dr Sandra Healy. Marion, we were for a short time together in the lab but we have become friends for life. Your sense of humour made me happy every day, and I am looking forward to celebrate with you with "baby Guinness". Mila it was a pleasure to work with you. Our conversations about life and fashion were an important part of my day. Sandra, you were always close to give advice for science and daily life problems.

Susan, Mila and Sandra "we did it", it was a pleasure to complete the Connemarathon in such a fantastic company!

My special thanks are to Dr Eva Szegezdi for her friendly advice and help throughout this project.

To all my colleagues in the laboratory, thank you. Your contribution was invaluable. It was a pleasure to work with all of you.

I am very grateful to everyone in Apoptosis Research Centre. The opportunity to discuss the scientific issues during Wednesday mornings made my work easier and more enjoyable.

I would like to express my gratitude to all my friends in CÚRAM. Thank you for your warm welcome, support and help. It is a pleasure to be a part of such a fantastic team.

Biochemistry department Michael, Anne, John, Ashla, Robert, Oliver and Martina deserve special recognition for making sure everything is done top notch. Thank you.

I sincerely appreciate the support from my friends in Ireland. My special thanks are to Mike Burke, who always supports me with the best jokes ever. You were extremely helpful during my write up, reminding me about coffee breaks. To my *Anam Cara* William Henry, who showed me the beauty of Ireland, who introduced me to Irish culture, and who always is a friend with the best advice. Willy, we will write the next book together. Iwona Machaj your encouragement and enthusiasm always led me to make the right decisions. Niamh Killeen you were so patient for the last years waiting to meet with me only just for a coffee, and those coffees were very precious for me during this PhD. We always had a good fun.

Special thanks to my whole family and friends in Poland. The support from grandmothers, cousins, aunts and uncles was just amazing. I am so lucky to have you all. It's my dream to spend more time with you now and share my stories from Ireland.

My lovely **Parents,** thank you. Thank you for everything. Thank you for your encouragement, advice and support. Thank you for giving me direction and helping in making the right decisions. Thank you for your patience, during the time when I was so dedicated to this project forgetting about the family events. Thank you for understanding that sometimes in life you need to choose a difficult pathway and follow your dreams. You taught me that there are ideas which require our special attention. Thank you. You are the best.

My dear, **husband** Krzysztof without your support and encouragement this project would be different. You were waiting for me so long but every day you gave me a strength and positive attitude. Your sense of humour together with patience and understanding allowed me to successfully complete this project. It was a privilege that you share this journey with me. Thank you my love, my friend.

To my Parents,

To my Husband,

This work is dedicated to you.

Table of Contents

Abstract

Chronic or unresolved stress can lead to cell death, and induction of apoptosis is crucial when the cellular adaptive mechanisms are not able to resolve persistent stress. Malfunction of the apoptotic process caused by blockade of the mitochondrial apoptotic pathway allows cells to resist stress-inducing agents and to therefore evade cell death. However, if the mitochondrial apoptotic pathway is impaired, certain stress stimuli can activate an alternative cell death pathway. Indeed, in cells in which some of the components of the mitochondrial death pathway, such as caspase-9, are deficient, a delayed mode of cell death associated with proteins involved in autophagy (ATG5) and apoptosis (caspase-8) has been observed upon prolonged stress. This alternate cell death pathway leads to the activation of caspase-8 in an autophagy-dependent mechanism. Caspase-8 acts as an initiator caspase in this model, activating executioner caspases that carry out cell death. Autophagy normally plays a pro-survival role and is involved in maintaining cellular homeostasis. However, in response to various stresses such as genotoxic and ER stress the level of autophagy is greatly increased and autophagy may contribute to cellular demise. Furthermore, while the expression of many autophagy-related genes (ATGs) is regulated downstream of eIF2α-ATF4, the precise underlying mechanisms are not yet fully explained.

The aim of this study was to investigate stress-induced cell death under conditions of caspase-9 deficiency. In this thesis, I demonstrate that various stress stimuli induce cell death and caspase-8 activation. The same stress-inducing agents also activate autophagy and ATF4 through phosphorylation of eIF2α. Moreover, I elucidate a common, alternative cell death pathway activated in response to distinct forms of stress. Finally, I conclude that the integrated stress response (ISR) pathway is not involved in the regulation of autophagy-dependent stress-induced cell death. The work presented in this thesis provides a first comprehensive overview of an alternative cell death pathway and contributes to a better understanding of the mechanisms of cell death particularly in cells that are refractory to apoptosis. The thesis describes cellular responses to various stresses and highlights the importance of the pro-death role of autophagy in this novel death pathway.

List of Publications

Deegan S, Svaeljeva S, Logue SE, Pakos-Zebrucka K, Gupta S, Vandenabeele P, Bertrand MJM, Samali A (2014) Deficiency in the mitochondrial apoptotic pathway reveals the toxic potential of autophagy under ER stress conditions. Autophagy, 2014 Nov 2; 10(11):1921-36

Saveljeva S, Cleary P, Mnich K, Ayo A, Pakos-Zebrucka K, Patterson BJ, Logue SE and Samali A (2016) Endoplasmic reticulum stress-mediated induction of SESTRIN 2 potentiates cell survival. Oncotarget. 2016 Mar 15;7(11):12254-66

Klionsky DJ et al, Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition); Autophagy, 2016 Jan; 12(1):1-222

Pakos-Zebrucka K, Koryga I, Mnich K, Ljujic M, Samali A, Gorman AM. (2016) Integrated Stress Response. EMBO reports (2016) 17, 1374-1395. (KPZ and IK contributed equally to this work)

Pakos-Zebrucka K, Gorman AM, Chintha C, Chevet E, Samali A and Mnich K (2017) Chapter title "EIF2S1". In: *Encyclopedia of Signaling Molecules*, *S. Choi (2nd edition.),* Springer, and DOI 10.1007/978; 101587-1.

Abbreviations

5'UTR	The 5' untranslated region
AARE	Amino Acid Response Element
Akt	Protein kinase B
AMBRA1	Activating molecule in BECN1-regulated autophagy protein 1
AMPK	5'-AMP-activated protein kinase catalytic subunit alpha-1
APAF-1	Apoptotic protease-activating factor 1
ASK1	Apoptosis-signal-regulating-kinase
ASNS	Asparagine synthetase
Asp	Aspartic acid, aspartate
ATP	Adenosine triphosphate
ATF/CREB	Activating transcription factor/cyclic AMP response element binding
ATF	Activating transcription factor

Atg	Autophagy-related
ARE	Antioxidant Response Element
BAD	BCL2-associated agonist of cell death
BAX	BCL-2-associated x protein
BAK	Bcl-2 homologous antagonist/killer
BBC3	BCL2 Binding Component 3
BCL-2	B-cell lymphoma 2
BCL2-A1	Bcl2-related protein A1
BCL-B	Bcl-2-like protein 10
BCL-XL	B-cell lymphoma-extra large
BCL-W	Bcl-2-like protein 2
Becn1	Beclin 1
BH3	BCL-2 homology domain 3
BID	BH3-interacting domain death agonist
BIK	Bcl-2-interacting killer
BIM	BCL-2-interacting mediator of cell death
BMF	BCL-2-modifying factor
BNIP3L	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like
BOK	Bcl-2-related ovarian killer protein
bZIP	basic leucine zipper domain
C/EBPβ	CCAAT/enhancer-binding protein beta
CARE	C/EBP-ATF Response Element
CARD	Caspase activation and recruitment domain
CDS	Coding sequence
C	Celsius
CHOP	C/EBP homologous protein

CReP	Constitutive repressor of eIF2α phosphorylation
DAMPS	Danger-associated molecular patterns
DD	Death domain
DED	Death effector domain
DIABLO	Diablo homolog, mitochondrial
DISC	Death-inducing signaling complex
DSB	Double strand breaks
DR 2, 4, 5, 6	Death receptor 2, 4, 5, 6
dsRNA	double-stranded RNA
eEF2	eukaryotic elongation factor 2
eIF2	Eukaryotic translation initiation factor 2
eIF2B	Eukaryotic translation initiation factor 2B
eIF2α	Eukaryotic translation initiation factor 2 alpha
eIF2β	Eukaryotic translation initiation factor 2 subunit 2
eIF2γ	Eukaryotic translation initiation factor 2 subunit 3
eIF3, 5	Eukaryotic translation initiation factor 3, 5
eIF4E	Eukaryotic translation initiation factor 4E
eIF4F	Eukaryotic translation initiation factor 4F
eIF4G	Eukaryotic translation initiation factor 4 gamma
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum (ER)-associated protein degradation
ERO1α	Endoplasmic oxidoreductin-1-like protein
FasL	FAS Ligand
FADD	FAS-associated death domain protein
Fip200	ULK1-associated protein
FLIP	FLICE-inhibitory protein

Gabarap	Gamma-aminobutyric acid receptor-associated protein, Gabarapl2 Gamma-aminobutyric acid receptor-associated protein-like 2,
GβL	Target of rapamycin complex subunit LST8
GADD34	Growth arrest and DNA damage-inducible protein GADD34
GCN2	General control non-derepressible protein 2
GDP	Guanosine diphosphate
GRP78 (BiP)	78 kDa glucose-regulated protein; Immunoglobulin heavy chain-binding protein
GTP	Guanosine-5'-triphosphate
Gy	gray
h	hour
Hsp 94	Heat shock protein HSP 90-beta
Hsp 78	Heat shock 70 kDa protein 1A
HR	Homologous recombination
HRI	Heme-regulated inhibitor kinase
HRK	Harakiri
HSV	Herpes simplex virus
IAPs	Inhibitor of Apoptosis Proteins
iDISC	Intracellular death-inducing signaling complex
IRE1	Inositol-requiring enzyme 1
IRES	Internal Ribosome Entry Sites
ISR	Integrated Stress Response
ISRIB	Integrated Stress Response inhibitor
JNK	c-Jun N-terminal kinase; Mitogen-activated protein kinase 8
Keap1	Kelch-like ECH-associated protein 1
LAMP1/2	Lysosome-associated membrane glycoprotein 1/2
Map1lc3b (LC3)	Microtubule-associated proteins 1A/1B light chain 3B,

MEF	Mouse embryonic fibroblast
MCL1	Myeloid cell leukemia-1
min	minute
µg	microgram
µl	microliter
ml	milliliter
MLKL	Mixed lineage kinase like protein
MOMP	Mitochondrial Outer Membrane Permeabilization
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin kinase protein
mTORC1	mTOR complex 1
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHEJ	Nonhomologous end-joining
NOXA	Phorbol-12-myristate-13-acetate-induced protein 1
NRF2	Nuclear factor erythroid 2-related factor 2
nM	nanomolar
mM	millimolar
$P58^{IPK}$	DnaJ homolog subfamily C member 3
p53	Cellular tumor antigen p53
p62	Sequestosome-1
PDI	Protein disulfide-isomerase
PERK	PKR-like ER kinase
PI3K	Phosphoinositide 3-kinase
PI3P	Phosphatidylinositol-3-phosphatase
PIC	Pre-initiation complex
PKR	double-stranded RNA-dependent protein kinase

PP1c	Protein phosphatase 1c
Ppp1r15a	Protein phosphatase 1 regulatory subunit 15A; GADD34
PUMA	p53 up-regulated modulator of apoptosis
RAPTOR	Regulatory-associated protein of mTOR
PRAS40	Proline-rich Akt substrate of 40 kDa
REDD1	Regulated in development and DNA damage responses 1
RIDD	Regulated IRE1-dependent decay of mRNA
RIPK1	Receptor-interacting serine/threonine-protein kinase 1
RIPK3	Receptor-interacting serine/threonine-protein kinase 3
ROS	Reactive oxygen species
Rpl7	60S ribosomal protein L7
S1P	Membrane-bound transcription factor site 1 protease
S2P	Membrane-bound transcription factor site 2 protease
SSB	single strand breaks
TRADD	Tumor necrosis factor receptor type 1-associated death domain protein
TNF	Tumor necrosis factor
TLR3,4	Toll-like receptor 3,4
TRAF2	TNF receptor-associated factor 2
TRAIL	TNF-related apoptosis-inducing ligand
TRB3,	Tribbles homolog 3
tRNAs	transfer RNAs
TXNIP	Thioredoxin-interacting protein
ULK	Serine/threonine-protein kinase ULK
ULK1/2	Serine/threonine-protein kinase ULK1/2
uORF	Upstream Open Reading Frame
uORF1, 2, 3	Upstream Open Reading Frame1, 2, 3

UPR	Unfolded protein response
UVRAG	UV radiation resistance-associated gene protein
VEGF	Vascular endothelial growth factor
V	volt
VPS34/15	Vacuolar protein sorting-associated protein 34/15
Wars	Tryptophan--tRNA synthetase
XIAP	X-Linked Inhibitor Of Apoptosis
XBP1	X-box-binding protein 1
z-VAD-fmk	Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone

Chapter 1: Introduction

1.1 Contribution

This work is a part of the literature review: "The Integrated Stress Response" Pakos-Zebrucka K, Koryga I, Mnich K, Ljujic M, Samali A, Gorman AM. EMBO reports (2016) 17, 1374-1395

Sections covered by ISR review are as follows:

1.3; 1.3.1; 1.3.2.1; 1.3.2.2; 1.3.3.1, 1.3.3.2, 1.7

1.2 Cellular Stress

Cellular stress responses are a physiological response to fluctuations in the environment. Stress stimuli such as genotoxic stress, nutrient stress, temperature shock, ionizing radiation, hypoxia, or endoplasmic reticulum (ER) stress continuously disrupt cellular homeostasis. In response to different stresses, cells activate specific strategies to repair the damage or to build tolerance and avoid rapid cell death. Therefore, the cellular stress response constitutes a defense mechanism of cells against internal or external insults. Exposure to prolonged stress may cause irreversible changes in the structure and function of cells and their integral components and can have serious consequences for cellular functionality (Kultz, 2005). Depending on the nature, duration and intensity of stress, cells can induce several stress response pathways, which, although initially promote survival, "can ultimately induce cell death" pathways (Fulda et al., 2010).

1.2.1 ER stress

ER stress is caused by the accumulation of unfolded and misfolded proteins within the ER (Hetz et al., 2015). Environmental stresses that result in changes in cellular energy levels, calcium concentrations, redox status, and protein degradation, cause accumulation and aggregation of unfolded proteins within the ER as they reduce the protein folding capacity of the organelle (Rutkowski et al., 2006, Szegezdi et al., 2006). Disruptions in protein homeostasis induce ER stress and activate the unfolded protein response (UPR), an adaptive signal transduction pathway that promotes a restoration of physiological ER function and proteostasis (Hetz et al., 2015). However, if ER stress is

persistent and overwhelms the capacity of the UPR to restore homeostasis, it can also trigger apoptosis (Logue et al., 2013).

1.2.1.1 UPR signaling

Cells recognize and sense the accumulation of unfolded and misfolded proteins through three ER transmembrane receptors: pancreatic ER kinase (PKR)-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1) (Fig. 1.1) (Hetz et al., 2015, Szegezdi et al., 2006). All three branches of the UPR become activated when there is an imbalance between unfolded proteins, misfolded proteins, and chaperones. Under normal physiological conditions, all three ER transmembrane receptors are bound to the ER chaperone glucose-regulated protein 78 kDa (GRP78). However, during ER stress, GRP78 shows a higher affinity for misfolded or unfolded proteins, and thus dissociates from the all ER receptors resulting in their oligomerization and activation (Walter and Ron, 2011).

PERK

PERK (also known as PEK, EIF2AK3) is a type I transmembrane protein localized in the ER membrane. This kinase consists of an ER luminal sensing domain (N-terminal) which contains chaperone binding sites and is important for dimerization and a cytosolic domain (C-terminal) which harbors Ser/Thr kinase activity (Harding et al., 1999, Liu et al., 2000). Once activated, PERK phosphorylates eukaryotic translation initiation factor 2 alpha (eIF2α) leading to an overall decrease in protein translation, thereby limiting the load of nascent polypeptides in the ER and facilitating the folding of proteins already present in the ER (Bertolotti et al., 2000, Harding et al., 2000a, Harding et al., 1999). Phosphorylation of eIF2α results in the preferential translation of *ATF4* mRNA. ATF4 is a transcription factor which activates a gene expression program including factors involved in protein folding, amino acid metabolism, oxidative stress, and the regulation of apoptosis and autophagy (Kroemer et al., 2010, Rzymski et al., 2009)

Another target of active PERK is nuclear factor (erythroid-derived2)-like two (NRF2), which is involved in cellular redox homeostasis. NRF2, also known as NFE2L2, is a member of the basic leucine zipper (bZIP) class of transcription factors. It regulates the

expression of antioxidants that protect cells against oxidative stress. Under unstressed conditions, NRF2 is bound by the suppressor protein Kelch-like ECH-associated protein 1 (KEAP1) in the cytosol (Cullinan et al., 2003). Under stress conditions when the UPR is triggered, PERK activation leads to phosphorylation of NRF2 on Thr80 resulting in KEAP1 dissociation (Lo et al., 2006). This allows NRF2 to translocate to the nucleus where it acts as a major regulator of the expression of antioxidant genes containing the Antioxidant Response Element (ARE) (Hybertson et al., 2011). Thus, NRF2 activity leads to a reduction of ROS and DNA damage in cells, protecting cells from the harmful effects of oxidative stress (Donnelly et al., 2013). Furthermore, it has also been shown that the PERK/eIF2α/ATF4 pathway is required not only for translational control, but also for activation of another arm of the UPR, ATF6 (Teske et al., 2011).

ATF6

Another ER transmembrane receptor is ATF6. There are two isoforms of ATF6, ATF6α and ATF6β, and both are expressed in all cell types. ATF6 is a type II transmembrane receptor and belongs to the basic leucine zipper transcription factor superfamily. Upon dissociation of GRP78, ATF6 translocates to the Golgi apparatus where it undergoes cleavage by Site 1 (S1P) and Site 2 (S2P) proteases which releases the active fragment of 50 kDa (Haze et al., 1999). Processed ATF6 translocates to the nucleus where it acts as a transcription factor for genes involved in protein folding such as protein disulfide isomerase (PDI), ER chaperones GRP94 and GRP78 as well as XBP1, the latter of which is integral to the IRE1 pathway (Walter and Ron, 2011, Yamamoto et al., 2007).

IRE1

The evolutionarily oldest branch of the UPR is triggered by the activation of IRE1. In mammalian cells there are two isoforms of IRE1, IRE1α and IRE1β. IRE1α is expressed ubiquitously in all cell types, whereas IRE1β is expressed only in epithelial cells (Tirasophon et al., 2000). IRE1 is a type I transmembrane protein containing N-terminal luminal and C-terminal cytoplasmic domains. The N-terminal domain is regulated by GRP78 binding, while the cytoplasmic domain contains both the serine-threonine kinase domain and the endoribonuclease domains, which confer IRE1 with dual enzymatic activity (Hetz et al., 2015). Upon activation, the endoribonuclease domain of IRE1

removes a 26-nucleotide intron from *XBP1* mRNA, which is then translated into a transcription factor known as XBP1s (Calfon et al., 2002, Tirasophon et al., 2000). In the nucleus, XBP1s activates a number of genes including ER chaperones, antioxidant enzymes, $P58^{IPK}$, as well as factors involved in ER-associated protein degradation (ERAD), and lipid biosynthesis (Hetz et al., 2015, van Huizen et al., 2003). $P58^{IPK}$ plays a critical role in the UPR as it is responsible for a negative feedback loop that relieves the eIF2α-dependent translational block. $P58^{IPK}$ achieves this by binding to PERK and inhibiting its ability to phosphorylate eIF2α (van Huizen et al., 2003). Additionally, IRE1 RNase activity is also involved in a mechanism known as regulated IRE1-dependent decay (RIDD). RIDD is implicated in the degradation of ER-localized mRNAs, ribosomal RNAs and microRNAs (Hetz et al., 2015). It has been suggested that IRE1 RNase activity has two outcomes: the first is *XBP1* mRNA splicing, which is reported to be pro-survival in cancer and the second is RIDD, which is pro-apoptotic (Chen and Brandizzi, 2013).

IRE1 also possesses kinase activity. During prolonged ER stress, IRE1 can initiate cell death through the c-Jun N-terminal kinase (JNK) pathway (Logue et al., 2013, Urano et al., 2000). Activation of the IRE1 serine-threonine kinase domain leads to recruitment of the adaptor molecule TNF-receptor-associated factor 2 (TRAF2). Next, association of IRE1 with TRAF2 recruits apoptosis-signal-regulating-kinase (ASK1), which in turn activates JNK leading to phosphorylation of BCL-2 family proteins (Yamamoto et al., 1999). JNK can act either as a suppressor of apoptosis by phosphorylating BCL2, or as an activator of apoptosis by phosphorylating BH3-only proteins (Hetz et al., 2015, Logue et al., 2013). Additionally, JNK, through its phosphorylation of anti-apoptotic BCL-2 proteins and the subsequent release of Beclin 1, can also initiate autophagy to promote adaptation to ER stress (Deegan et al., 2013, Sinha and Levine, 2008). This effect is explained in more detail in the Autophagy subsection.

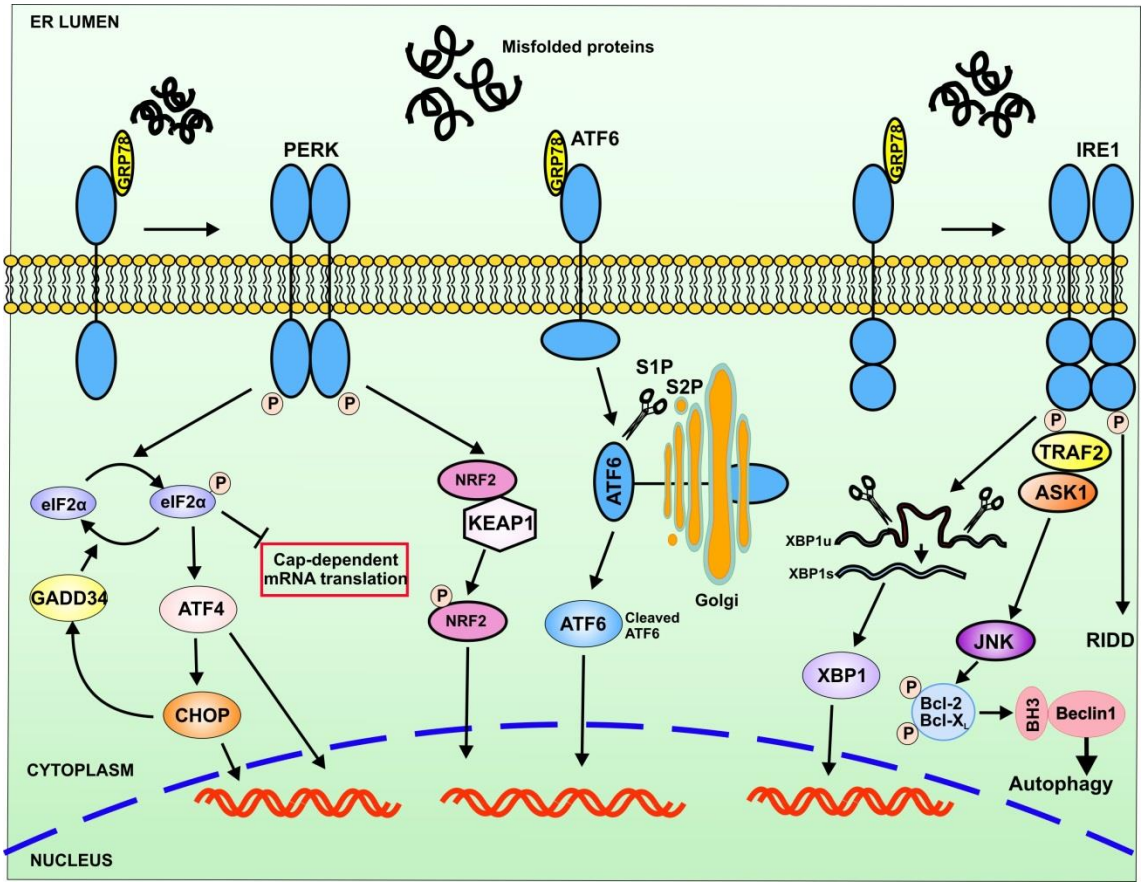


Figure 1.1. Schematic of the classical UPR. Upon ER stress caused by accumulation of unfolded and misfolded proteins within the ER lumen, GRP78 dissociates from PERK, ATF6, and IRE1. PERK dimerizes and autophosphorylates leading to subsequent phosphorylation of eIF2α. This event results in a block in the translation of cap-dependent mRNAs and promotes the preferential translation of *ATF4* mRNA. ATF4 is a bZIP transcription factor which regulates the expression of downstream target CHOP, which in turn controls expression of GADD34. GADD34 is a phosphatase that carries out the dephosphorylation of eIF2α. ATF6 is cleaved in the Golgi by site 1 protease (S1P) and site 2 protease (S2P) to generate active ATF6. The endoribonuclease domain of IRE1 carries out unconventional splicing to generate X-box binding protein 1s (XBP1s), in addition to activation of regulated IRE1-dependent decay (RIDD). All three branches of the UPR generate bZIP transcription factors that regulate the expression of genes involved in cellular adaptation programmes, autophagy and apoptosis. For more detail, see main text. Modified from (Cubillos-Ruiz et al., 2017, Hetz et al., 2015, Deegan et al., 2013).

1.2.2 Genotoxic stress

Genotoxic stress is caused by exposure to toxic agents such as UV irradiation, ionizing radiation, and alkylating agents that cause damage to cellular DNA and thus cause various mutations and potentially neoplastic progression (Garcia-de Teresa et al., 2017, Lavigne et al., 2017, Kultz, 2005). Many commonly used chemotherapeutics such as etoposide, doxorubicin, cisplatin, and 5-fluorouracil have the capacity to covalently modify DNA molecules and thus trigger the activation of an adaptive signaling pathway

known as the DNA damage response (DDR) (Woods and Turchi, 2013). Environmental agents, such as aflatoxins, are also known to modify DNA, and are thus also potent carcinogens (Kultz, 2005). The DNA damage response is a protective signaling pathway mediated by the transcription factor p53, which has the ability to sense DNA damage and regulate the transcription of target genes involved in genomic integrity and the maintenance of DNA stability. Although cells have evolved the ability to repair DNA damage, other toxic agents may compromise these responses, leading to cell death (De Zio et al., 2013). It has been shown that exposure to genotoxic agents such as doxorubicin also activates double-stranded RNA dependent protein kinase (PKR) (Peidis et al., 2011).

Gamma irradiation is a cytotoxic stress that leads to DNA damage and the production of reactive oxygen species (ROS). Exposure to γ-irradiation results in damage to cellular macromolecules, the most well-characterized of which are single-strand breaks (SSB) and double-strand breaks (DSB) to DNA . The destructive effects of γ-rays are also mediated by the generation of ROS, which cause protein oxidation and thus potentiate the overall damage signal (Daly, 2012). DNA damage is sensed by DNA repair programs such as homologous recombination (HR) and nonhomologous end-joining (NHEJ). A failure in these DSB repair programs leads to cell death. Cell death induced by γ-irradiation can be related to cell cycle arrest as most non-transformed irradiated cells are arrested at the G1/S or G2/M checkpoints. In contrast, cancer cells activate cell cycle arrest in the G2 phase (Vucic et al., 2006). Additionally, p53 is known to be essential for induction of apoptosis after exposure to γ-irradiation (Halacli et al., 2013). Radiation therapy is commonly used in the treatment of many types of cancer. Interestingly, it has been shown that combined therapy with both radiation and high temperature treatment can induce apoptosis in a prostate cancer cell line (Li and Franklin, 1998).

1.2.3 Metabolic stress

Metabolic stress is caused by an imbalance in cellular metabolism due to decreased glucose levels (Chaube and Bhat, 2016, Wellen and Thompson, 2010). Two main metabolic pathways: glycolysis and mitochondrial oxidation are responsible for

maintaining homeostasis in cells (Lee and Yoon, 2015). Defects at any stages of these pathways result in imbalances in glucose metabolism and force cells to activate alternative pathways to sustain metabolic processes (Wong et al., 2017). It has been shown that under mild metabolic stress cells activate the PERK/Akt adaptation pathway. However, exposure to sustained metabolic stress promotes extracellular signal-regulated kinase (ERK2)-dependent activation of a GCN2/eIF2α/ATF4 cell death pathway (Shin et al., 2015). Nutrient homeostasis is sensed by specific kinases such as adenosine monophosphate-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) (Wellen and Thompson, 2010). Both of these kinases are sensitive to metabolite deficiency and are involved in induction of autophagy. AMPK is the main sensor of ATP in cells and inhibits growth and proliferation in response to a drop in energy levels. mTOR is directly regulated by amino acid availability and controls translation, proliferation and autophagy (Kim et al., 2011).

1.2.4 Cytoskeletal damage: microtubule polymerization stress

Microtubules together with actin filaments and intermediate filaments are the principal components of the cytoskeleton (Parker et al., 2014). Microtubule damage leads to the destabilization and loss of the cytoskeleton. This damage disrupts not only the intracellular architecture but also impacts many processes that include cell movement, intracellular trafficking, and mitosis. Microtubules are composed of a combination of α-tubulin and β-tubulin isotypes that form heterodimers (Parker et al., 2014). Tubulin heterodimers polymerize to form microtubules. Microtubule stability is regulated by the binding of GTP to β-tubulin resulting in polymerization (Xiao et al., 2006). Microtubules are very sensitive to pharmacological agents and physical stresses such as high and low temperature. Cell stress has a negative impact on microtubule stability resulting in microtubule depolymerization (Xiao et al., 2006). The integrity of the microtubule skeleton is important for growth and proliferation. Microtubules carry out their main cellular function during cell division where they are involved in the separation of chromosomes during mitosis and are an integral component of centrioles. Thus, alterations in microtubule integrity are reported to cause cell cycle arrest, mostly in G1 phase. Compounds that disturb microtubule stability are widely used in cancer treatment due to their ability to disturb mitosis (Parker et al., 2014). For example,

colchicine inhibits microtubule polymerization by binding to both isotypes of tubulin resulting in mitotic arrest. In contrast, Taxol promotes mitotic arrest by inhibiting microtubule depolymerization and thus stabilizing the microtubule polymer. It has also been suggested that malfunctions in the microtubule network are not directly correlated with cancer development but rather affect the cellular response to chemotherapeutics, in this way contributing to chemotherapy resistance and tumor development.

1.3 The Integrated Stress Response

In response to diverse stress stimuli, eukaryotic cells activate a common adaptive pathway, termed the integrated stress response (ISR), to restore cellular homeostasis. The core event in this pathway is the phosphorylation of eIF2α by one of four members of the eIF2α kinase family, which leads to a decrease in global protein synthesis and induction of selected genes, including the transcription factor ATF4, that together promote cellular recovery. The gene expression program activated by the ISR optimizes the cellular response to stress and is dependent on the cellular context, as well as on the nature and intensity of the stress stimuli. Although the ISR is primarily a pro-survival, homeostatic program, exposure to severe stress can drive signaling towards cell death (Pakos-Zebrucka et al., 2016).

1.3.1 ISR Activation-eIF2α kinases

The eIF2α kinases act as early responders to disturbances in cellular homeostasis. There are four members of the family: PKR-like ER kinase (PERK) (Perkins and Barber), double-stranded RNA dependent protein kinase (PKR), heme-regulated eIF2α kinase (HRI) and general control non-derepressible 2 (GCN2) (Fig. 1) (Donnelly et al., 2013, Wek et al., 2006). All four eIF2α kinases share extensive homology in their catalytic kinase domains but possess distinct regulatory domains (Harding et al., 1999, Berlanga et al., 1998, Shi et al., 1998, Chen et al., 1991, Ramirez et al., 1991, Meurs et al., 1990). Each eIF2α kinase dimerizes and autophosphorylates for full activation (Lavoie et al., 2014). However, each kinase responds to distinct environmental and physiological stresses (Fig. 1.2), which reflects their unique regulatory mechanisms (Donnelly et al., 2013).

1.3.2 Components of the ISR

1.3.2.1eIF2α

eIF2α, eIF2β and eIF2γ together form the eIF2 complex and eIF2α is the main regulatory subunit of this complex since it contains both the phosphorylation and RNA binding sites. Under normal conditions, eIF2 plays a key role in the initiation of mRNA translation and recognition of the AUG start codon (Jackson et al., 2010, Pain, 1996). It forms a ternary complex with GTP and Met-tRNAi that binds the 40S ribosome subunit, and together with two small initiation factors, eIF1 and eIF1A, forms the 43S pre-initiation complex (PIC) (Lomakin and Steitz, 2013, Aitken and Lorsch, 2012). The 43S PIC is recruited to the 5'methylguanine cap of mRNA in a process that is facilitated by the eIF4F complex among others. The eIF4F complex consists of the cap-binding protein eIF4E, eIF4G which acts as a scaffold protein, and the RNA helicase eIF4A (Hinnebusch and Lorsch, 2012). The interaction between eIF4G and eIF3 further stabilizes the PIC leading to its migration to the AUG start codon (Lomakin and Steitz, 2013, Aitken and Lorsch, 2012, Hinnebusch, 2011). Upon binding of the Met-tRNAi anticodon and the AUG start codon, eIF1 dissociates from the complex and the GTP on eIF2 is hydrolyzed with the aid of eIF5. This leads to dissociation of the eIF2–GDP complex from the 40S ribosomal complex and its recycling for another round of initiation of mRNA translation. Exchange of GDP for GTP is catalyzed by the guanine nucleotide exchange factor eIF2B and this converts eIF2 back to its active form (Jackson et al., 2010, Pain, 1996).

In response to ISR activation, phosphorylated eIF2α blocks the eIF2B-mediated exchange of GDP for GTP, thereby preventing formation of the 43S PIC. This results in the global attenuation of 5'cap-dependent protein synthesis and concomitant translation of selected mRNAs that contain a short upstream open reading frame (uORF) in their 5' untranslated regions (5'UTR). Examples of these preferentially translated mRNAs include *ATF4, ATF5, CHOP, and GADD34* (Hinnebusch, 2011, Palam et al., 2011, Lee et al., 2009).These mRNAs do not require cap recognition by the eIF4F complex, and their translation relies on a re-initiation mechanism or direct recruitment of ribosomes to internal ribosome entry sites (IRES) (Chan et al., 2013).

Phosphorylation of eIF2α occurs at S51. Homozygous mutation at this site effectively prevents eIF2α phosphorylation permitting normal mRNA translation even under conditions of ER stress or glucose deprivation (Scheuner et al., 2001). Early work showed that mice with homozygous mutation of the eIF2α phosphorylation site die shortly after birth due to prolonged hypoglycemia, demonstrating the importance of translation initiation in glucose metabolism (Scheuner et al., 2001). These data suggest that eIF2α phosphorylation is crucial for sustaining proper physiological function of the liver and pancreas after birth, as eIF2α phosphorylation is required for induction of gluconeogenic enzymes and insulin (Scheuner et al., 2001). Also, it is noteworthy that eIF2α S51A/S51A MEF cells are hypersensitive to ER stress and require supplementation by non-essential amino acids and a reducing agent, which indicates that eIF2α phosphorylation protects against metabolic and oxidative stress (Scheuner et al., 2001). Moreover cells with homozygous eIF2α S51A mutation are unable to induce autophagy upon viral infection, starvation, or ER stress (Kouroku et al., 2007, Talloczy et al., 2002). Thus, the phosphorylation of eIF2α at S51 appears to be crucial for stress-induced autophagy.

1.3.2.2 ATF4

ATF4 is a 39 kDa protein consisting of 351 amino acids (Ameri and Harris, 2008). The expression of stress-responsive ATF4 is regulated transcriptionally, translationally and posttranslationally. Although ATF4 is expressed constitutively at the mRNA level in most non-stressed cells, stimuli such as ER stress, hypoxia, amino acid, and glucose deprivation induce both transcription and translation of ATF4 (Ameri and Harris, 2008).

ATF4 is translationally upregulated in an eIF2α phosphorylation-dependent manner. This reversible phosphorylation results in selective translation of specific mRNAs containing upstream pen reading frames (uORF) in their 5'UTR including *ATF4* mRNA. *ATF4* contains two conserved uORF implicated in translational regulation of *ATF4* (Vattem and Wek, 2004). This mechanism of translational control is highly conserved, and yeast regulate the levels of Gcn4p (a functional homologue of ATF4) using a very similar mechanism (Hinnebusch, 2005, Hinnebusch and Natarajan, 2002). Since yeast do not have PERK, global inhibition of translation is dependent on activation of Gcn2p

(the yeast homologue of GCN2) and eIF2α phosphorylation upon amino acid depletion (Hinnebusch and Natarajan, 2002). This results in preferential synthesis of Gcn4p, a transcription factor which controls the expression of genes involved in amino acid synthesis and transport (Hinnebusch, 2005).

Translational control of yeast *GCN4* requires four uORFs (Mueller and Hinnebusch, 1986). When the ternary complex is abundant, ribosomes initiate scanning at uORF1 and re-initiate at inhibitory downstream uORFs that preclude translation of the *GCN4* coding sequence (CDS). Under amino acid depletion, more time is required for the ribosome to re-initiate translation due to low levels of ternary complex allowing ribosomes to scan through uORF4 and re-initiate at the *GCN4* CDS (Hinnebusch, 1997). Mouse *ATF4* has similar features to *GCN4* (Lu et al., 2004). The mouse gene contains two uORFs (uORF1 and uORF2) that are located 5' to the *ATF4* CDS (Fig. 2), while human *ATF4* mRNA contains three upstream open reading frames (uORF1, uORF2, and uORF3) (Harding et al., 2000a). Under normal cellular conditions, translation of mouse *Atf4* mRNA is initiated at uORF1 that encodes a peptide just three amino acids in length and re-initiates at uORF2. This precludes translation of *Atf4* mRNA because, unlike *GCN4*, the uORF2 (uORF3 in human) sequence overlaps with the *Atf4* CDS in an out-of-frame manner (Ameri and Harris, 2008, Vattem and Wek, 2004, Harding et al., 2000a). In stressed cells, ribosomes scan through uORF2 and re-initiate at the *Atf4* CDS (Vattem and Wek, 2004, Wek et al., 2006).

ATF4 acts as the master transcription factor during the ISR and also has an important role in regulating both normal metabolic and redox processes. ATF4 fulfills a vital function in many tissues and plays a central role in the regulation of obesity, glucose homeostasis, energy expenditure, and neural plasticity. *Atf4* knockout mice have revealed critical roles for ATF4 in embryonic lens formation (Hettmann et al., 2000, Tanaka et al., 1998), fetal-liver hematopoiesis (Masuoka and Townes, 2002), and bone development (Yang et al., 2004). *Atf4* knockout mice are also smaller and leaner (Masuoka and Townes, 2002), suggesting a role for ATF4 in lipid metabolism. They have decreased fat mass caused by a reduction in the volume rather than number of cells, suggesting that deletion of ATF4 increases fat mobilization and suppresses fatty

acid synthesis (Wang et al., 2010). Knock out of *Atf4* in mice has also revealed a physiological role in thermoregulation as these mice exhibit higher core body temperatures in response to cold stress (Wang et al., 2013). ATF4 has also been implicated as a vital mediator of muscle weakness and atrophy in ageing muscles as a reduction in its expression improves muscle quality, strength, and mass in mice (Ebert et al., 2015). Although the role of ATF4 in models of learning and memory formation is somewhat controversial, with both positive and negative effects suggested (Sidrauski et al., 2015, Trinh et al., 2012, Wei et al., 2012, Costa-Mattioli et al., 2005), targeted knockdown of *Atf4* in the mouse hippocampus has indicated that it is necessary for memory formation and neural plasticity (Pasini et al., 2015).

During stressful conditions, elevated translation of *ATF4* mRNA facilitates transcriptional upregulation of stress-responsive genes. ATF4 regulates transcription of its target genes through binding to C/EBP-ATF response element (CARE) sequences that can mediate transcriptional activation in response to various stimuli (Kilberg et al., 2012, Kilberg et al., 2009, Fawcett et al., 1999). During amino acid starvation these sequences function as amino acid response elements (AARE) and ATF4 is the only transcription factor known to bind all known AARE sequences (B'Chir et al., 2013, Averous et al., 2004, Siu et al., 2002). ATF4 can form homo- and heterodimers with several other bZIP transcription factors, including its downstream target CHOP (Ameri and Harris, 2008, Siu et al., 2002) Upon ER stress and amino acid depletion in mouse cells, ATF4 alone, or together with CHOP, preferentially binds to proximal promoter regions of target genes, including *Atf3*, *Ppp1r15a*, *Trib3*, *Wars*, and *Rpl7* (Han et al., 2013). Although it is well established that ATF4 alone regulates the expression of genes involved in amino acid transport and biosynthesis, Kaufman and colleagues showed that, in response to ER stress, ATF4 and CHOP interact to regulate common genes involved in cellular amino acid metabolic processes, mRNA translation, and the unfolded protein response (UPR) (Han et al., 2013).

1.3.2.3 CHOP

C/EBP homologous protein (CHOP), also known as DDIT3/GADD153, belongs to the basic leucine zipper transcription factor superfamily. It is a downstream target of ATF4;

however, its expression is also enhanced by ATF3 (Jiang et al., 2004). It has been shown that CHOP activity can be increased at the posttranslational level by the IRE1-ASK1-p38 MAPK pathway (Kim et al., 2008). CHOP is well known to promote cell death upon exposure to lethal stress by upregulating the pro-apoptotic BCL-2 proteins NOXA and PUMA (Bertolotti et al., 2000) as well as TRB3 (Ohoka et al., 2005). Elevated levels of CHOP are required to trigger apoptosis induced by UV irradiation (Dey et al., 2010). However, CHOP expression alone is not sufficient to execute death and it was suggested that CHOP expression of prolonged duration leads to increased sensitivity to UV irradiation. In addition, CHOP enhances the expression of additional transcription factors which are involved in the cellular stress response. One of CHOP's downstream targets is ATF5 and CHOP is required for its transcription in response to different stress stimuli. CHOP binds to CARE elements in the ATF5 promoter. Together, CHOP and ATF5 enhance pro-death signalling in response to ER stress (Teske et al., 2013).

Together, ATF4 and CHOP bind to specific *cis*-regulatory elements in the promoters of their target genes, leading to the transcriptional upregulation of genes involved in the induction and development of autophagy (B'Chir et al., 2013).

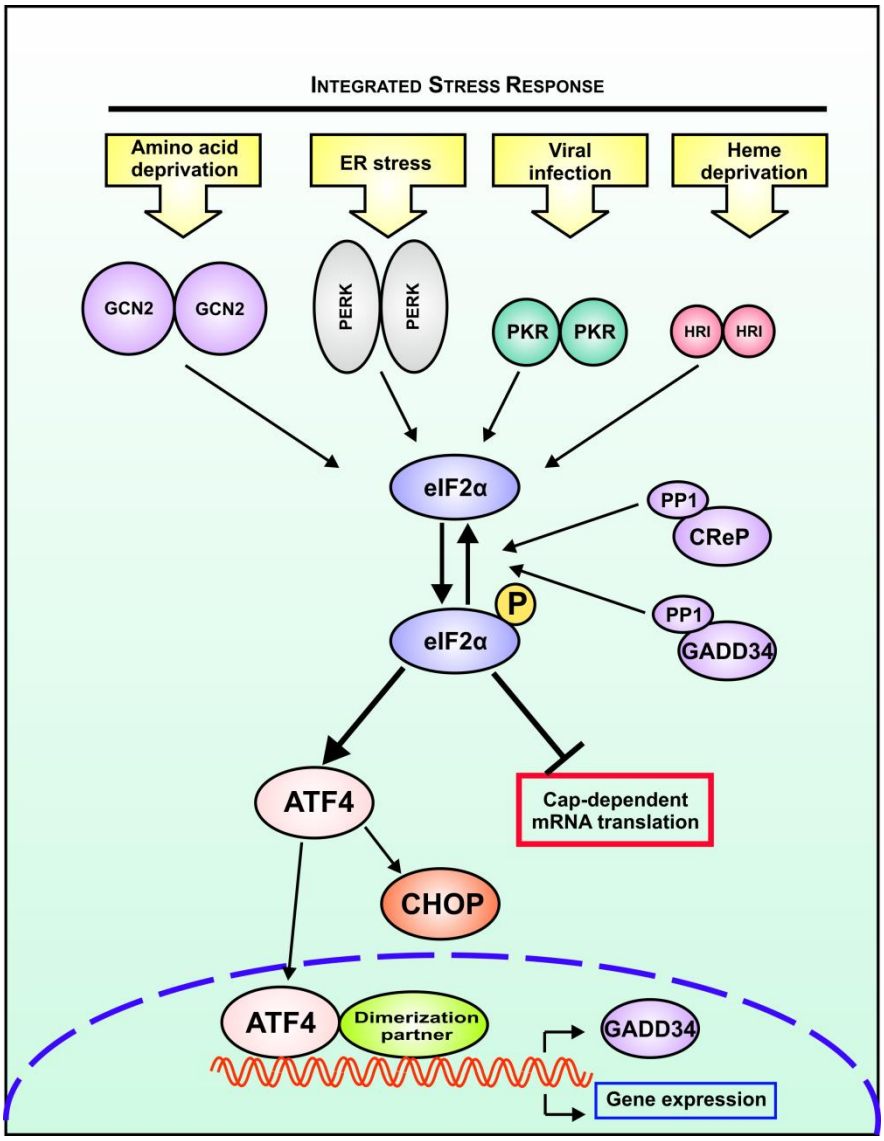


Figure 1.2 Schematic of the Integrated Stress Response signaling in mammals. Nutrient deprivation, ER stress, viral infection and heme deficiency activate a family of eIF2α kinases, GCN2, PERK, PKR, and HRI, that lead to phosphorylation of eIF2α. This event results in a block of cap-dependent translation and promotes the preferential translation of *ATF4* mRNA and other ISR-specific mRNAs. ATF4 is the main effector of the ISR and regulates the expression of CHOP and other genes involved in adaptation and survival. Dephosphorylation of eIF2α is regulated by two phosphatases: CReP which is constitutively expressed and GADD34 which is induced by stress. Arrows denote activation or induction, while blunt lines indicate inhibition. Modified from (Pakos-Zebrucka et al., 2016).

1.3.3 Cellular outcome of the ISR

1.3.3.1 Autophagy regulation by the ISR

Through its activation of autophagy, the ISR can regulate cell survival and cell death pathways. Autophagy promotes the bulk removal and degradation of unfolded proteins or aggregates as well as damaged organelles, while also serving as a means to replenish depleted amino acids for building proteins and to provide energy to a starved cell. Although the precise mechanisms by which phosphorylated eIF2α leads to autophagy are still poorly understood, it is interesting to note that distinct intrinsic and extrinsic stresses that lead to phosphorylation of eIF2α have also been shown to induce autophagy. For example, ER stress leads to increased phosphorylation of eIF2α and subsequent upregulation of the autophagy receptors SQSTM1, NBR1, and BNIP3L in a PERK-dependent manner (Deegan et al., 2015). Pharmacological inhibition of PERK abolished the transcriptional upregulation of these autophagy receptors in mammalian cells (Deegan et al., 2015). In addition, PERK-mediated phosphorylation of eIF2α mediates increased ATG12 and LC3 conversion upon expression of polyQ72 aggregates in C2C5 cells (Kouroku et al., 2007). In fact, the PERK arm of the UPR can regulate all stages of autophagy including induction, vesicle nucleation, phagophore elongation, and maturation (Deegan et al., 2013). Consistent with this, another study showed that ER stress caused by bluetongue virus induces autophagy through the PERK-eIF2α pathway in infected cells (Lv et al., 2015). In that study, both: pharmacological inhibition of PERK activity or genetic silencing of eIF2α were shown to significantly reduce the level of the autophagy marker LC3 (Lv et al., 2015). Furthermore, induction of autophagy during hypoxia is also dependent on PERK-mediated eIF2α phosphorylation, as disruption of PERK signaling or exposure of mutant cells with non-phosphorylatable eIF2α S51A to hypoxia results in decreased levels of MAP1LC3B and ATG5 transcripts (Rouschop et al., 2010). It has also been shown that GCN2-mediated phosphorylation of eIF2α is essential for induction of autophagy upon amino acid starvation in tumor cells (Ye et al., 2010). While GCN2 knockout cells displayed reduced levels of LC3, the eIF2α S51A mutant cells could not induce LC3 processing (Ye et al., 2010). Similarly, S51 of eIF2α has also been demonstrated to be important for amino acid starvation-

induced autophagy in yeast and MEFs (Talloczy et al., 2002). Together, these findings identify eIF2α phosphorylation as the central link between a variety of different stresses and the induction of autophagy.

Downstream of eIF2α phosphorylation, ATF4 has been shown to be required for autophagy induction. However, it has been suggested that processes triggered by eIF2α phosphorylation other than selective translation of *ATF4* mRNA might also be involved in the activation of autophagy (Kroemer et al., 2010). During hypoxia, ER stress, or amino acid deprivation, ATF4 transcriptionally upregulates essential autophagy genes involved in autophagosome biogenesis and function, e.g., *Map1lc3b* and *Atg5* (Deegan et al., 2015, B'Chir et al., 2013, Rzymski et al., 2010). ATF4 can also promote increased expression of regulated in development and DNA damage response 1 (REDD1; also known as Ddit4), which suppresses mTOR complex 1 (mTORC1) activity leading to autophagy induction upon ER stress and starvation (Dennis et al., 2013). ATF4 activation due to amino acid deprivation stimulates the upregulation of several genes involved in autophagy including *Atg3, Atg5, Atg7, Atg10, Atg12, Atg16, Becn1, Gabarap, Gabarapl2, Map1lc3b, and Sqstm1* (B'Chir et al., 2013). By upregulating key autophagy genes, the ISR contributes to increased autophagic flux helping the cell to deal with the stress caused by hypoxia and nutrient deprivation through enhanced recycling of cytoplasmic components and maintenance of cellular biosynthetic capacity and ATP levels, e.g., by supplying amino acids for *de novo* protein synthesis and providing substrates for the tricarboxylic acid cycle, such as amino acids and free fatty acids (Ye et al., 2010, Rzymski et al., 2009). It is worth noting that different autophagy genes can have different levels of dependence on ATF4 and CHOP signaling and that the activation of such genes is determined by the ratio of ATF4 and CHOP proteins that are bound to specific *cis*-regulatory elements, thus allowing subtle tuning of gene expression that can be tailored to meet specific cellular needs (B'Chir et al., 2013). Interestingly, a cytoprotective function of eIF2α phosphorylation against conditions that mimic viral infection or induce ER stress has been reported to occur through the activation of pathways that promote cell survival, such as PI3K and its downstream target Akt/mTOR (Kazemi et al., 2007). In this regard, it is also of interest to consider the effect of proteasome inhibition on survival signaling by the ISR. Proteasome

inhibition, e.g., using bortezomib, leads to activation of the ISR through GCN2 as a result of depletion of amino acids for protein synthesis (Suraweera et al., 2012). In mammalian cells, the depletion of amino acids due to proteasome inhibition also activates autophagy through mTOR in an attempt to restore amino acid levels. Supplementation with critical amino acids that are depleted by proteasome inhibition attenuates eIF2α phosphorylation and suppresses autophagy (Suraweera et al., 2012). Thus, amino acid depletion constitutes a link between autophagy induction and ISR activation to promote the survival of cells.

1.3.3.2 Cell death signaling by the ISR

The ISR encompasses pathways that can lead to the induction of cell death if the adaptive response does not restore homeostasis. These are mainly regulated through the transcriptional activity of ATF4 and some of its downstream targets, particularly CHOP and ATF3. One of the best studied mechanisms of ISR-induced cell death is through ATF4-mediated activation of CHOP. CHOP is a transcription factor that promotes cell death signaling through multiple mechanisms. In several cellular models CHOP has been shown to induce cell death by upregulation of the BH3-only pro-apoptotic BCL-2 family members BCL2L11and BBC3, thus promoting ER stress-induced apoptosis (Galehdar et al., 2010, Puthalakath et al., 2007). CHOP can also contribute to cell death by enhancing expression of one of the death receptors, DR5, that plays a role in induction of apoptosis under ER stress (Zou et al., 2008). In addition, CHOP induces expression of the oxidase ERO1α that disturbs the oxidizing environment of ER (Marciniak et al., 2004). Additionally, CHOP can further regulate gene expression by binding to other ATF/CREB family members, such as ATF4 or ATF3, thus altering their DNA binding specificity (B'Chir et al., 2013). CHOP is also essential for induction of PPP1R15A, which is important for both adaptation and cell death signaling (Marciniak et al., 2004). It is important to note that although there is a well-established role for CHOP in cell death signaling, CHOP expression alone is not sufficient to induce cell death. In fact, CHOP-deficient cells are only partially resistant to ER stress-induced cell death indicating the role of others factors in mediating cell death under these conditions (Young et al., 2016).

ATF4 can also itself promote cell death, for instance, through direct transcriptional activation of the pro-apoptotic BCL-2 family member PMAIP1, which was found to mediate ER stress-induced cell death in neuroectodermal tumor cells (Armstrong et al., 2010). ATF4 can also promote cell death by forming heterodimers with CHOP or with ATF3, both of which are transcriptional targets of ATF4. ATF4-ATF3 heterodimers can increase the transcription of PMAIP1 and thus promote apoptosis through the intrinsic pathway. ATF4 and CHOP together can also induce the pseudokinase TRB3. Under normal conditions TRB3 regulates NF-κB-induced gene expression, while during the cellular response to ER stress it negatively regulates ATF4, thus limiting the transcription of other ATF4-dependent stress responsive genes (Jousse et al., 2007). Moreover, TRB3 can interact with CHOP and downregulate its own induction through a negative feedback loop whereby it represses ATF4-CHOP transactivation (Ohoka et al., 2005). Notably, HEK293 and HeLa cells in which TRB3 was silenced displayed increased resistance to ER stress-induced apoptosis (Ohoka et al., 2005). Additionally, the ISR can also promote cell death through translational and posttranslational regulation of the caspase inhibitor X chromosome linked inhibitor of apoptosis (XIAP) (Hiramatsu et al., 2014). PERK has been shown to downregulate XIAP translation through phosphorylation of eIF2α, while ATF4 promotes XIAP protein degradation through the ubiquitin-proteasome system (Hiramatsu et al., 2014).

During prolonged ER stress, ATF4 induces an amino acid transporter network which leads to mTORC1 activation, inhibition of autophagy, and phosphorylation of multiple factors including eukaryotic elongation factor 2 (eEF2) and, as such, positively regulates translation (Guan et al., 2014). While this series of events could serve to restore cellular homeostasis it can also contribute to cell death execution during chronic ER stress, as constant synthesis of nascent peptides without production of mature proteins causes proteotoxic stress.

Although apoptosis is the main cell death pathway regulated by ISR, it can also trigger other forms of cell death. For example, the ISR can initiate ATF4-dependent necrosis, especially in response to glucose deprivation, as recently reported. Intriguingly, ATF4-

dependent apoptosis can be induced by 2-deoxyglucose in the same cellular model (Leon-Annicchiarico et al., 2015).

1.4 Autophagy

The term *autophagy* was first coined in 1963 by Christian de Duve to describe the process of self-eating (Klionsky, 2008). Autophagy is a portmanteau of two words derived from Greek: "auto" which means oneself and "phagy", to eat. The molecular mechanism of autophagy was initially described in yeast by Yoshinori Ohsumi; in 1993, he discovered 15 genes that are essential for the activation of autophagy in eukaryotic cells. Both de Duve and Ohsumi were awarded the Nobel Prize; de Duve in 1974 for the discovery of the lysosome and in 2016 Yoshinori Ohsumi received the Nobel Prize in Physiology or Medicine "for his discoveries of mechanisms for autophagy".

Macroautophagy (hereafter referred to as autophagy) is an evolutionarily conserved, lysosomal-mediated, catabolic process for the bulk degradation of proteins, organelles and other cellular components (Levine and Klionsky, 2004). Autophagy is characterized by the induction of a small isolation membrane known as a phagophore, which elongates to form a double membrane vacuole known as an autophagosome (Fig. 1.3). The mature autophagosome fuses with a lysosome to create a single membrane vacuole known as an autolysosome, where resident cathepsins degrade the contents (Kroemer et al., 2010). The core autophagy machinery consists of a family of proteins known as the autophagy related protein (ATG) family, and can be regulated by various cellular stresses such as starvation, ER stress, hypoxia, heat shock, or microbial infection (Ding et al., 2007, Yorimitsu et al., 2006). During basal conditions cells utilize autophagy for the turnover of organelles and protein aggregates; however, during conditions of cellular stress autophagy is upregulated to remove damaged cellular components and maintain nutrient and energy homeostasis, implicating autophagy as a pro-survival process during periods of cell stress. In certain cases, however, autophagy can also contribute to cell death (Salazar et al., 2009, Kroemer and Levine, 2008, Levine and Yuan, 2005). Autophagy is involved in many physiological processes, including differentiation and development, cell growth control, aging, nutrient and energy homeostasis, and innate immunity (Levine and Klionsky, 2004). Malfunction of the autophagic process has been suggested

to contribute to developmental abnormalities, neurodegeneration, cancer, aging, heart disease, and inflammation (Galluzzi et al., 2017b, Levine and Kroemer, 2008, Rubinsztein et al., 2007).

1.4.1 Autophagy induction

The induction of canonical autophagy starts with the assembly of the multiprotein ULK complex consisting of ATG13, ATG101, scaffold protein FIP200, and the ULK1/2 kinase (Galluzzi et al., 2017a). The role of ATG13 in this complex is to mediate the interaction between FIP200 and ULK1/2 (Jung et al., 2009). The ULK complex is regulated upstream by the mammalian TOR Ser/Thr kinase (mTOR) which forms two multiprotein complexes, mTORC1 and mTORC2. mTORC1 is involved in autophagy regulation and apart from mTOR, consists of RAPTOR, GβL and PRAS40 (Efeyan and Sabatini, 2010, Kroemer et al., 2010, Hara et al., 2002). Additionally, AMP-activated protein kinase (AMPK) also regulates the ULK complex (Kim et al., 2011). mTORC1 is a key regulator of autophagy. Under basal conditions, mTORC1 binds to and inhibits the ULK complex through direct phosphorylation of ATG13 and ULK. However, during nutrient deprivation AMPK senses ATP depletion and phosphorylates mTOR within mTORC1. This event leads to dissociation of mTORC1 from ULK and subsequent phosphorylation of ULK by AMPK (Kim et al., 2011). However, ULK can also further phosphorylate ATG13 and FIP200 within the complex. It is also suggested that ULK, through its phosphorylation of AMPK, can mediate a negative feedback loop for autophagy induction (Loffler et al., 2011). The released ULK promotes the induction of the isolation membrane also known as a phagophore.

1.4.2 Vesicle nucleation – Beclin 1

Vesicle nucleation is initiated by the multiprotein Class III phosphatidylinositol 3-kinase (PI3K) complex. The main function of this complex is the production of phosphatidylinositol-3 phosphate (PI3P) that is required for vesicle elongation. In mammals two PI3K complexes have been identified, both of which are essential for autophagosome formation. The PI3K complex I consists of Beclin 1 (BECN1), VPS34, VPS15, and ATG14 (Itakura and Mizushima, 2009). The lipid kinase VPS34 plays a crucial role in the accumulation of phosphatidylinositol 3-phosphate (PI3P) molecules

on membranes, while ATG14 is responsible for membrane curvature (Deegan et al., 2013). Therefore, complex I is involved in an early stage of autophagosome formation. The PI3K complex II consists of VPS34, VSP15, and Beclin 1. The other regulatory proteins in this complex are AMBRA1, UVRAG, and endophilin B1/Bax-interacting factor 1 (BIF-1) (Itakura and Mizushima, 2009). Beclin 1 interacts with BIF1 through UVRAG and promotes autophagosome formation (Kang et al., 2011). AMBRA1 is activated by ULK1 and constitutes a scaffold with which Beclin 1 is associated. Both UVRAG and AMBRA are positive regulators of autophagy (Itakura and Mizushima, 2009). It has been shown that Beclin 1 is a BH3-only protein which interacts with anti-apoptotic BCL-2 (Liang et al., 1998) leading to negative regulation of autophagy (Pattingre et al., 2005). Of note, ATG14 in complex I competes with UVRAG in complex II for interaction with Beclin 1 (He and Levine, 2010).

The core of both complexes is constituted by VPS34 and Beclin 1. While VPS34 produces PI3P which is required for further expansion of the autophagosomal membrane and binding of WIPI-1 and WIPI-2, Beclin 1 is important for localization of ATG proteins to the isolation membrane (Kang et al., 2011).

Beclin 1 is also linked to apoptosis. It has been shown that effector caspases cleave Beclin 1 affecting its ability to induce autophagy but promoting apoptosis (Zhu et al., 2010). Moreover, in response to starvation, JNK phosphorylates BCL-2, which in turn dissociates from Beclin-1 leading to autophagy induction (Wei et al., 2008). However, the interaction of ER-localized BCL-2/BCL-X_L with Beclin 1 results in autophagy inhibition (Kang et al., 2011).

1.4.3 Autophagosome elongation

The expansion of autophagosome membranes requires two conjugation systems: the ATG5-ATG12 system and the ATG8 system. These systems are crucial for membrane elongation and completion and are regulated by ubiquitin-like reactions (Kroemer et al., 2010).

1.4.3.1 ATG5-ATG12 conjugation system

The core event in this system is the covalent binding of ATG12 to ATG5. This reaction is mediated by the E1-like enzyme ATG7 and the E2-like enzyme ATG10. Next, the ATG12-ATG5 conjugate forms a complex associating with ATG16. In contrast, ATG16 is bound to ATG5 in a noncovalent manner (Yang and Klionsky, 2010). Incorporation of the ATG5-ATG12-ATG16 complex requires activation of the PI3K complex. Moreover, the ATG5-ATG12-ATG16 complex is in turn required for the second conjugation system and acts as a E3-like enzyme (Deegan et al., 2013).

The expression of ATG5 is regulated by several factors and seems to be stress stimulus- and cell type-specific. Classical inducers of autophagy such as starvation or rapamycin have been shown to maintain low levels of ATG5 while apoptotic stimuli such as etoposide or staurosporine increase the levels of ATG5 thus regulating the overall outcome of autophagy (Tsujimoto and Shimizu, 2005).

1.4.3.2 LC3 conjugation system

There is a single ATG8 conjugation system in yeast, while in mammals the ATG8 family consists of three subfamilies: γ-aminobutyric acid receptor-associated protein (GABARAP), Golgi-associated ATPase enhancer of 16 kDa (GATE-16), and microtubule-associated protein 1 light chain 1 (LC3). Moreover, there are also three variants of LC3, LCA, LC3B, and LC3C; LC3B plays the predominant role with regard to autophagy (Shpilka et al., 2011).

The second system requires the conjugation of LC3 to a lipid. In the first step, the pro-form of LC3 is cleaved by the protease ATG4 to generate LC3-I with an exposed glycine at the C-terminal end. Next, phosphatidylethanolamine (PE) is bound to the exposed glycine of LC3-I in a reaction facilitated by the E1-like enzyme ATG7 and the E2-like enzyme ATG3 to form LC3-II. Recruitment of LC3-II to the autophagosomal membrane requires the ATG5-ATG12-ATG16 complex. The LC3 conjugation system is important for the elongation and closure of autophagic membranes (Deegan et al., 2013, Kroemer et al., 2010).

Lipidated LC3-II is stably associated with autophagosome membranes and is used as a hallmark for detection of autophagy. Similarly, the conversion of LC3-I to LC3-II constitutes a biochemical readout of autophagy induction.

1.4.4 Autophagosome maturation

In the final stage, the outer membrane of the enclosed autophagosome fuses with the lysosomal membrane to form the autophagolysosome. This molecular event requires the involvement of several proteins from different cellular compartments. Autophagosome-lysosome fusion is regulated by RAB proteins, for example Rab7, which bind to LAMP1/2 on the lysosomal membrane. Additionally, the SNARE complex together with ATG9 are also involved in this reaction (Galluzzi et al., 2017a, Deegan et al., 2013). Moreover, the microtubule network is also important in intracellular trafficking of the autophagosome to lysosomes. Following fusion, mature autophagolysosomes digest the cargo of engulfed cytoplasm through the action of lysosomal acid hydrolases including cathepsins.

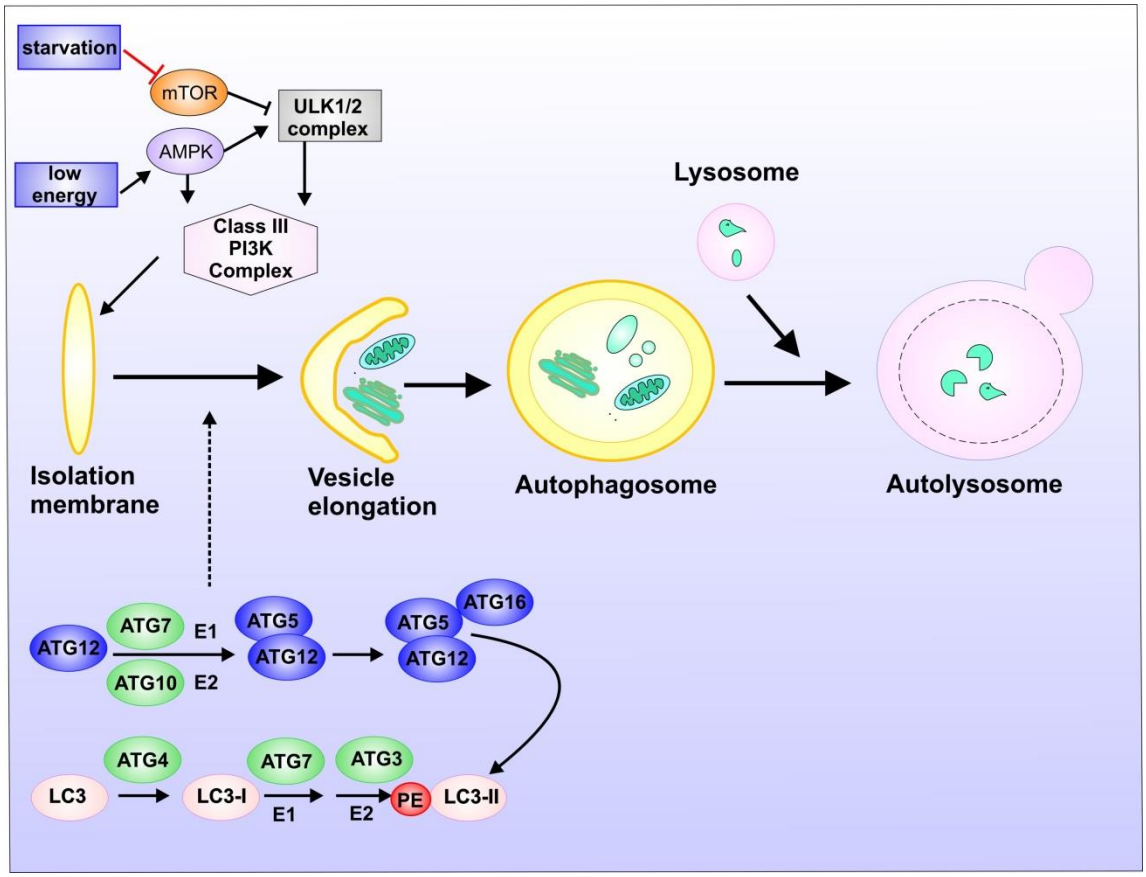


Figure 1.3. Schematic of canonical autophagy in mammals. Autophagy initiation requires mTOR kinase inhibition and AMPK-mediated phosphorylation of the ULK1/2 complex. Following ULK1/2 stimulation, vesicle nucleation involves the activation of the class III PI3K complex. Vesicle elongation requires two ubiquitin-like conjugation systems. One pathway involves the process of covalent binding of ATG12 to ATG5, which is mediated by ATG7 and ATG10. Next, ATG5-ATG12 is non-covalently bound to ATG16 to form a complex necessary for the second conjugation reaction. In the second reaction, LC3 is cleaved by the protease ATG4 to expose the glycine in the C-terminal end and form soluble LC3-I. LC3-I is further processed by two ubiquitin-like enzymes, ATG7 and ATG3, resulting in the phosphatydylethanolamine (PE) conjugation and formation of LC3-II, which is attached to the autophagosome membranes. The mature double-membraned autophagosome fuses with lysosomes to create single-membraned autolysosomes where the degradation processes occur. For more details, see the main text. Arrows denote activation or induction, while blunt lines indicate inhibition. Modified from (Cicchini et al., 2015, Green and Levine, 2014).

1.4.5 Non-canonical autophagy

Autophagy is a highly controlled process orchestrated by the ATG proteins. The core event in this process is the formation of the double-membrane autophagosomes. The ATG proteins control the autophagic process from the induction to the maturation of the autophagosome; therefore, they have been considered as essential components of autophagy. However, it has now been shown that autophagosomes can be formed

independently of some of the core autophagy components in a manner which does not require the involvement of the ATG proteins (Codogno et al., 2011). This alternative mode of autophagosome formation has been termed non-canonical autophagy in order to distinguish it from canonical autophagy in which the full set of ATG proteins is required. However, in accordance with the recommendation from the "autophagy committee" it is suggested to use the expressions independent-/dependent-autophagy only following experimental verification (Galluzzi et al., 2017a). Experiments demonstrating the independence of ULK, Beclin-1, VPS34, ATG5, ATG7 for autophagy in various experimental setting suggest the existence of alternative forms of autophagy (Ma et al., 2015, Wu et al., 2014, Cheong et al., 2011). The existence of alternative autophagy is also supported by an *in vivo* study where this autophagic mode was observed in several embryonic tissues (Nishida et al., 2009).

Nishida and colleagues reported the existence of ATG5/ATG7-independent autophagy that was characterized by the presence of autophagosomes (Nishida et al., 2009). This pathway was not associated with LC3-I to LC3-II conversion but was regulated by the ULK and Beclin 1 complex in response to etoposide. Moreover, the autophagosomal membranes were derived from late endosomes and the *trans*-Golgi. The importance of ATG5/ATG7-independent autophagy was evaluated during erythrocyte differentiation (Nishida et al., 2009). A similar study by the group of Shimizu further supported the idea that ULK1-dependent and ATG5-independent autophagy is an important pathway for the removal of mitochondria from developing reticulocytes (Honda et al., 2014).

There is an increasing body of literature describing Beclin 1-independent autophagy. The first study about the Beclin-1-independent pathway came from the Chu group, who implicated this modality in neuronal cell death (Zhu et al., 2007). Similarly, studies carried out in breast cancer cells in the Codogno laboratory also reported Beclin 1- and VPS34-independent autophagy. The authors showed that resveratrol-induced Beclin 1-independent autophagy acts as a cell death mechanism independently of caspase-3. Moreover, this alternative autophagy was dependent on ATG7 (Scarlatti et al., 2008).

Alternative autophagy displays the same features of the autophagic process as conventional autophagy, and, moreover, the function is also similar and is related to the

bulk degradation of the cytoplasmic content (Dupont et al., 2017). However, it seems that this alternative mode of autophagy is induced only in certain cellular settings and in response to specific stress stimuli.

1.5 Cell death modalities

In accordance with the recommendations of the Nomenclature Committee on Cell Death (NCCD) from 2015, there are two main types of cell death: accidental cell death (ACD) and regulated cell death (RCD). While ACD is induced by exposure to severe physical, chemical, and mechanical stresses that lead to rapid and uncontrolled cell death, RCD is a genetic program which is activated in response to conditions of extracellular and intracellular stress. In general, ACD constitutes a complete mode of cell death that cannot be prevented by pharmacological or genetic intervention. In contrast, RCD can usually be modulated either by pharmacological compounds or genetic approaches, and this mode of cell death is often considered a response to unresolved stress. Programmed cell death (PCD) is a type of RCD which is induced by physiological stress during development. Cell death modalities are defined by morphological and biochemical criteria (Galluzzi et al., 2015).

1.5.1 Apoptosis

The term *apoptosis* was first coined by Kerr, Wyllie, and Currie in 1972 to describe a mechanism of controlled cell death (Kerr et al., 1972). Apoptosis is a natural, physiological form of cell death that eliminates superfluous, irreversibly damaged, and toxic cells from the body without the induction of an immune response, in particular those cells containing pathological mutations. It is a programmed self-destructive process involving characteristic biochemical and morphological events. Apoptosis is accompanied by shrinkage, nuclear chromatin condensation, membrane blebbing, cellular and nuclear fragmentation (karyorrhexis), and creation of apoptotic bodies (Taylor et al., 2008, Horvitz, 1999, Kerr, 2002). Biochemical manifestations of apoptosis include activation of caspase proteases, DNA fragmentation, and exposure of phosphatidylserine on the surface of the plasma membrane (Taylor et al., 2008). However, it has also been reported that caspase activation does not always lead to execution of cell death and that caspases can also be involved in biological processes

other than apoptosis. For example, caspase-3 and caspase-9 are required for thrombopoiesis while caspase-8 was found to be involved in macrophagic maturation (Galluzzi et al., 2008) and in the development of liver cancer through proliferation-associated DNA damage (Boege et al., 2017). Similarly, exposure of phosphatidylserine is not a uniquely apoptotic feature, as it has been shown that this event is also associated with necroptosis (Zargarian et al., 2017). Apoptosis plays an important role in the maintenance of homeostasis; however, dysregulation of the apoptotic pathway can lead to many diseases such as cancer, neurodegeneration, and autoimmunity (Favaloro et al., 2012).

1.5.1.1 Role of the BCL-2 protein family in apoptosis

Apoptosis is regulated by the evolutionarily conserved B-cell lymphoma-2 (BCL-2) protein family. In mammals, there are at least 20 members that are divided into three main groups: (1) anti-apoptotic, (2) pro-apoptotic, and (3) BH3-only proteins based on the presence of BCL-2 homology (BH) domains (Pihan et al., 2017).

The BCL-2 proteins of the anti-apoptotic group have four BH domains and their role is to inhibit apoptosis. The members of this group directly bind to BH3-only proteins to prevent BAX and BAK oligomerization. To this group belong BCL-2, BCL-XL, BCL-W, MCL-1, BCL-B, and BCL-A1 (Szegezdi et al., 2009). The pro-apoptotic group consists of BAX, BAK, and BOK. These proteins have three BH domains and also a transmembrane (TM) domain, and are therefore associated with membranes. BAX and BAK are key regulators of the mitochondrial-mediated apoptotic pathway mediated by mitochondrial outer membrane permeabilization (MOMP). Because MOMP is considered the point of no return of the intrinsic apoptotic pathway, BAX and BAK act as gatekeepers of MOMP (Chipuk et al., 2006). Exposure to stress activates cytoplasmic BAX which subsequently translocates to the mitochondrial membrane where it oligomerizes with BAK leading to release of cytochrome *c*. This event is essential for mitochondria-dependent caspase activation and subsequent cell death (Tait and Green, 2010). Of note, BOK has recently been shown to induce MOMP and trigger apoptosis in the absence of BAX and BAK (Llambi et al., 2016).

The BH-3-only protein family consists of BIM, PUMA, NOXA, BAD, HRK, BIK, BID, BMF and BNIP3, which all only bear the BH3 domain. The role of these proteins is to promote apoptosis (Taylor et al., 2008). Interestingly, although BNIP3 possess the BH3 domain, it is not as potent as other pro-apoptotic members of the BH3-only proteins (Chinnadurai et al., 2008). Therefore, there is a controversy what type of cell death is triggered by BNIP3. It has been shown that BNIP3 is associated with the mitochondria and is involved in necrosis (Vande Velde et al., 2000), autophagy (Azad et al., 2008, Daido et al., 2004) and apoptosis (Burton and Gibson, 2009, Regula et al., 2002). Moreover, BNIP3 was also shown to induce cell death in cells lacking Apaf-1, caspase-9 and caspase-3 (Vande Velde et al., 2000).

Activation of BAX and BAK is regulated by their direct or indirect interaction with other BCL-2 family members. BAX and BAK activity can be directly promoted by BH3-only proteins such as BIM and BID. For example, caspase-8- mediated cleavage of BID results in its activation (Li et al., 1998). Truncated BID (tBID) directly promotes oligomerization of BAX and BAK, the effectors of MOMP. In the other, indirect scenario, BH3-only proteins are required for neutralization of anti-apoptotic proteins that are in protein complexes with BAX and BAK. Thus, BH3-only proteins antagonize anti-apoptotic proteins, while at the same time they directly activate BAX and BAK which effectively trigger MOMP (Chipuk and Green, 2008).

BAX and BAK are crucial for cell death induced by several apoptotic stimuli. It has been shown that Bax/Bak$^{-/-}$ double knock out MEFs are resistant to cell death caused by ER stress, serum deprivation, or radiation (Szegezdi et al., 2009, Wei et al., 2001). Similarly, Bax/Bak$^{-/-}$ mice display numerous abnormalities in neurological and hematopoietic systems, and are therefore resistant to cell death during development and tissue homeostasis (Lindsten et al., 2000). Moreover, it has been also shown that Bax and Bak deficient cells, or cells overexpressing BCL-XL undergo necrotic cell death in response to prolonged ER stress (Ullman et al., 2008).

1.5.1.2 Caspases

Caspases (cysteine aspartic acid-specific proteases) are a family of proteases, which cleave proteins after an aspartate (Asp) residue. Fourteen caspases have been characterized in total in mammals, 11 have been identified in humans, two are unique to mice, and one is found only in bovines (Zhivotovsky, 2003). Table 1.1 presents an overview of caspases. Although caspase activation is a hallmark of apoptosis, not all the caspases are involved in the apoptotic process: caspase-1, caspase-4, caspase-5, and caspase-12 trigger the inflammatory response. The caspases involved in apoptosis are divided into two main groups: the initiator caspases including caspase-2, -8, -9, and -10 and the effector caspases consisting of caspase-3, -6, and -7 (Degterev et al., 2003, Cohen, 1997). The initiator caspases process and activate downstream substrates resulting in activation of effector caspases which include caspase-3, caspase-6, and caspase-7 (Zhivotovsky, 2003, Wang and Lenardo, 2000).

All caspases share similar features such as the presence of the conserved QACXG penta-peptide containing the active cysteine site and the ability to recognize and cleave specific tetrapeptide sequences (Degterev et al., 2003). The domain structure is also similar among caspases, as they all consist of a pro-domain, large, and small domain (Li and Yuan, 2008, Zhivotovsky, 2003). Caspases are constitutively synthetized as inactive pro-enzymes (zymogens). Activation of caspases requires dimerization and autoproteolytic cleavage at a specific aspartic acid residue between the large and small subunits.

The initiator caspases are characterized by the presence of a large pro-domain. So far, two distinct pro-domains have been identified: the caspase activation and recruitment domain (CARD) and the dead effector domain (DED) (Degterev et al., 2003). The effector caspases, on the other hand, contain short pro-domains. Depending on the stress stimuli, activation of effector caspases and subsequent cell death occurs by three main routes: (1) the extrinsic pathway, (2) the intrinsic pathway and (3) granzyme B. The extrinsic and intrinsic pathways are illustrated in Fig. 1.4.

Table 1.1 Characterization of caspases

Caspase	Pro-domain	Function	Function in apoptosis	Organisms	Ref.
Caspase-1	CARD	Inflammation		Human and mouse	(Galluzzi et al., 2016, Degterev et al., 2003, Szegezdi et al., 2003)
Caspase-2	CARD	Apoptosis	Initiator	Human and mouse	
Caspase-3	Short	Apoptosis	Effector	Human and mouse	
Caspase-4	CARD	Inflammation		Human	
Caspase-5	CARD	Inflammation		Human	
Caspase-6	Short	Apoptosis	Effector	Human and mouse	
Caspase-7	Short	Apoptosis	Effector	Human and mouse	
Caspase-8	DED	Apoptosis	Initiator	Human and mouse	
Caspase-9	CARD	Apoptosis	Initiator	Human and mouse	
Caspase-10	DED	Apoptosis	Initiator	Human and mouse	
Caspase-11	CARD	Inflammation		Mouse	
Caspase-12	CARD	Apoptosis	Initiator	Mouse	
Caspase-13	Orthologue of caspase -4			Bovine	
Caspase-14	Short	Differentiation		Human and mouse	

1.5.1.3 Intrinsic pathway

The intrinsic apoptotic pathway, also called the mitochondrial-mediated pathway, is activated in response to intracellular stress signals such as heat, radiation, nutrient deprivation, viral infection, hypoxia, DNA damage, and ER stress. The mitochondrial-mediated apoptotic pathway is tightly controlled by the BCL-2 family proteins. While the pro-apoptotic proteins BAX and BAK induce cell death, the anti-apoptotic BCL-2 and BCL-XL proteins play important roles in inhibiting cell death. Most apoptotic stimuli initiate an imbalance between pro-apoptotic and anti-apoptotic proteins, which determine the decision regarding a cell's fate (Tait and Green, 2013). Once activated, BH3-only proteins promote BAX-BAK oligomerization in the mitochondrial outer membrane resulting in pore formation, which is considered as the point of no return (Tait and Green, 2013). MOMP results in the subsequent release of intramembrane space proteins such as cytochrome *c*, the second mitochondria-derived activator of caspases/direct inhibitor of apoptosis-binding protein with a low isoelectric point (Smac/DIABLO), and a serine protease, the high temperature requirement protein A2/Omi (HtrA2/Omi) into the cytosol (Tait and Green, 2010, Du et al., 2000, Cai et al., 1998). The released cytochrome *c* together with ATP and apoptotic protease-activating factor-1 (Apaf-1) form a caspase activation platform called the apoptosome (Chinnaiyan, 1999). The apoptosome recruits, oligomerizes, and activates pro-caspase-9 through the interaction of its CARD domains with those of Apaf-1. In the intrinsic pathway, caspase-9 is an initiator caspase which leads to proteolytic cleavage and activation of the downstream effector caspases caspase-3 and caspase-7 (Taylor et al., 2008). All components of this pathway including BCL-2 protein, cytochrome *c*, Apaf-1, and caspase-9 are essential for the mitochondrial-mediated cell death pathway. In their absence, cells are resistance to various apoptotic stimuli (Janssen et al., 2007, Wei et al., 2001).

In the intrinsic pathway, caspase activity can be also regulated by other pro-apoptotic proteins that are released from the mitochondria such as Smac/DIABLO and HtrA2/Omi. Indeed, it has been shown that both Smac/DIABLO and HtrA2/Omi indirectly regulate caspase activity though the suppression of members of the Inhibitors of Apoptosis Proteins (IAPs) family (Du et al., 2000). IAPs are characterized by the

presence of the baculovirus IAP repeats (BIR) domains to which Smac/DIABLO and HtrA2/Omi bind with their N-terminal IAP Binding Motif (IBM) resulting in neutralization of IAPs (Suzuki et al., 2004). Although Smac/DIABLO can interact with XIAP, cIAP1 and cIAP2, it has ability to enhance only cIAP1 and cIAP2 ubiquitination resulting in their proteasomal degradation (Yang and Du, 2004). In contrast, Smac/DIABLO binds to the BIR3 domain of XIAP disrupting the interaction of XIAP with caspase-9 and thus enhances caspase-9 activity and apoptosis (Srinivasula et al., 2001). HtrA2/Omi cleaves and degrades the IAP resulting in enhancement of caspase activity and induction of apoptosis (Suzuki et al., 2004). Alternatively, HtrA2/Omi can also regulate caspase activation through cleavage of unidentified target leading to MOMP and subsequent release of cytochrome *c* (Suzuki et al., 2004). Interestingly, while the release of cytochrome c under stress seems to be a general response, the cytochrome c-dependent activation of caspases is observed only in vertebrates (Saelens et al., 2004).

Endonuclease G (Endo G) is a specific nuclease localized in the mitochondrial intermembrane space. In response to apoptotic stimuli and following MOMP, EndoG translocates from the mitochondria to the nucleus, where cleaves genomic DNA independent of caspase activity (Li et al., 2001). Similarly, the release of apoptosis-inducing factor (AIF) during MOMP is crucial for chromatin condensation and DNA fragmentation (Susin et al., 1999). Although, both Endo G and AIF have been proposed to serve as effectors of caspase-independent apoptosis, it has been also shown that downstream caspase activation is required for the release of Endo G and AIF (Arnoult et al., 2003). However, it has been proposed that following MOMP, while there is simultaneous release of mitochondrial intermembrane space proteins such as Smac/DIABLO, HtrA2/Omi and cytochrome c, there is also a selective and slower release of AIF (Munoz-Pinedo et al., 2006).

1.5.1.4 Extrinsic pathway

The extrinsic apoptotic pathway, also called the death receptor pathway, involves the binding of death ligands to transmembrane death receptors. The members of the death receptor family, which include tumor necrosis factor 1 (TNFR1), Fas (also known as

DR2, CD95, APO-1), APO-3 (also known as TRAMP, LARD, DR3, WSL1), TNF-related apoptosis-inducing ligand receptor 1 (TRAIL-R1; also known as APO-2 and DR4), TRAIL-R2 (also known as KILLER, TRICK2 and DR5), ectodysplasin A receptor (EDAR), nerve growth factor receptor (NGFR1), and death receptor 6 (DR6) contain multiple cysteine-rich repeats in the extracellular domain and a death domain (DD) in the cytoplasmic tail (Locksley et al., 2001, Ashkenazi and Dixit, 1998). The presence of the DD is crucial for translating the death signals from the cell surface into downstream events that lead to apoptosis. The receptors are activated by binding to specific members of the TNF ligand family. Upon ligation of the death receptors, cytoplasmic adapter proteins are recruited. In the case of Fas ligand (FasL) and TRAIL that bind to transmembrane death receptor CD95 and TRAIL-R1/TRAIL-R2, respectively, the adapter protein Fas-associated death domain (FADD) is recruited, whereas the binding of TNFα to the TNFR1 receptor results in the recruitment of the adapter protein TRADD together with FADD and RIP (Wajant, 2002, Hsu et al., 1995). FADD, in turn associates with pro-caspase-8 through its death effector domain (DED) leading to the formation of the death-inducing signaling complex (DISC) (Kischkel et al., 1995). Once pro-caspase-8 is recruited to the DISC, it dimerizes and undergoes auto-processing to generate its active form. Next, activated caspase-8 carries out proteolytic cleavage and subsequent activation of the effector caspases caspase-3 and caspase-7. Crosstalk between the extrinsic and intrinsic pathways through the caspase-8-mediated cleavage of pro-apoptotic BID (tBID) to initiate the mitochondrial pathway has also been reported.

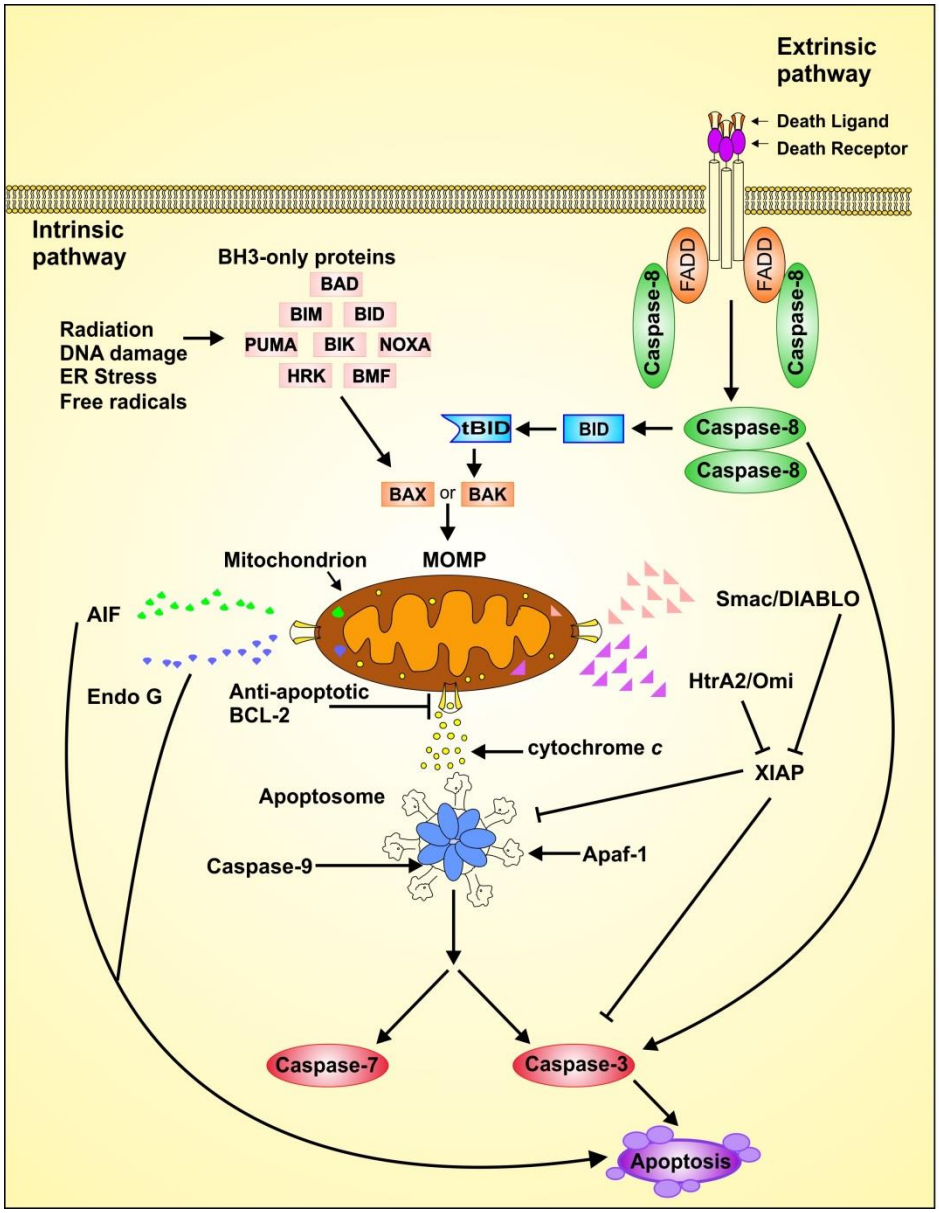


Figure 1.4 Intrinsic and extrinsic apoptosis pathways. The intrinsic pathway is activated in response to intracellular stress stimuli and involves activation of the BH3-only protein family. Once activated, BH3-only proteins trigger MOMP leading to release of cytochrome *c*, which promotes the formation of the apoptosome complex. Active caspase-9 processes the downstream effectors caspase-3 and caspase-7. The extrinsic pathway is activated through ligation of death receptors. This results in recruitment of the adaptor protein FADD and pro-caspase 8. Activated caspase-8, in turn, proteolytically cleaves the downstream effectors caspase-3 and caspase-7. In addition, active caspase-8 also cleaves the BH3-only protein BID. Truncated BID (tBID) triggers MOMP and leading to apoptosome formation and effector caspase activation. Following MOMP, Smac/DIABLO and HtrA2/Omi are released from mitochondria into the cytoplasm where they inhibit X-linked inhibitor of apoptosis (XIAP) leading to caspase activation. Additionally, the release of other mitochondrial proteins into the cytosol such as AIF and Endo G triggers caspase-independent apoptosis. Modified from (Tait and Green, 2010, Taylor et al., 2008, Saelens et al., 2004).

1.5.2 Necrosis and Necroptosis

In 2009, the definition of necrosis was re-evaluated in accordance with the recommendations of the NCCD. Previously, based on morphological criteria, necrosis was defined as Type III cell death. Necrosis is a form of nonapoptotic and accidental of cell death (Fink and Cookson, 2005). Like apoptosis, necrosis can occur during development, but unlike apoptosis necrotic cells display a lack of or limited chromatin condensation and caspase activation. Morphologically, necrosis is characterized by an increase in cell volume (oncosis), swelling of organelles, and early plasma membrane rupture resulting in loss of intracellular content. The process of necrosis leads to ATP depletion, lipid peroxidation, enhanced generation of reactive oxygen species (ROS) and mitochondrial dysfunction, and nuclear changes (Kroemer et al., 2009). Generally, necrotic cell death is accompanied by inflammation due to release of danger-associated molecular patterns (DAMPS) (Taylor et al., 2008). Necrosis is induced by non-physiological stress such as physical or chemical stimuli, for example, heat and osmotic shock, freezing, and thawing (Nicotera et al., 1998). Although well-characterized by its morphological changes, there are still no biochemical markers that can be used to identify necrotic cell death.

Although necrosis was defined as an uncontrolled and unregulated form of cell death, it has recently been shown that necrotic death can also be executed in a controlled fashion (Sosna et al., 2014). Necroptosis is a form of programmed necrosis which is induced by extracellular stimuli such as the TNF family and the Toll-like receptors TLR3, TLR4. Stimulation of the death receptor TNFR1 by TNFα is the most recognized in this regard. In 1988, Laster reported that TNF induced both apoptotic and necrotic cell death (Laster et al., 1988). Necroptosis is defined as a caspase-independent death pathway that depends on the receptor-interacting protein kinase 1 (RIPK1)-RIPK3 complex IIb called the necrosome and which can be inhibited by necrostatin-1 (Kaczmarek et al., 2013, Galluzzi et al., 2012). Necroptosis also requires inhibition of caspase-8 either by pharmacological or genetic intervention. Mixed lineage kinase like protein (MLKL) is a downstream target of RIPK3, which is crucial for necroptosis induction (Sun et al., 2012). Necroptosis is negatively regulated by FADD, caspase-8 and $FLIP_L$ (Kaczmarek

et al., 2013, Cho et al., 2011). As in necrosis, necroptotic cells are morphologically characterized by cellular swelling, organelle swelling and increased membrane permeability. Unlike apoptotic cells, cells undergoing necroptosis do not exhibit chromatin condensation and caspase activation.

1.5.3 Autophagy mediated cell death

Autophagic cell death (ACD) was originally defined by morphological changes characterized by the massive vacuolization of the cytoplasm in the absence of chromatin condensation. This mode of cell death was classified as Type-II programmed cell death. However, in accordance with the NCCD recommendation from 2012 and updated in 2015, autophagic cell death describes a type of cell death that can be inhibited using pharmacological compounds or genetic approaches targeting at least two components of the autophagic machinery (Galluzzi et al., 2015, Galluzzi et al., 2012). In addition, with respect to biochemical changes it has been proposed that autophagic cell death is also associated with the lipidation of LC3 and degradation of p62 (Galluzzi et al., 2015). Importantly, it was suggested that the term 'autophagic cell death' should be replaced by 'death mediated by autophagy'.

Although cell death mediated by autophagy is still a controversial topic, there is increasing evidence supporting a role for autophagy in cell death. However, the molecular mechanisms of this mode of cell death remain unclear. It also remains to be determined whether autophagy and apoptosis act in the same signaling pathway, and, in this way, contribute to stress-induced cell death. It is important to note that the same autophagic genes, such as *ATG5* and *ATG7*, can be involved in both pro-survival and pro-death autophagy. Therefore, characterizing the induction and execution of autophagic cell death is of high interest in order to find new possibilities for the treatment of cancer and other human diseases. Autophagy has been shown to promote physiological cell death of *Drosophila melanogaster* salivary glands. Inhibition of class-I PI3K and the knockdown of specific autophagy genes, for example, *atg3, atg6, agt7 or atg12* reduced cell death *in vivo* (Berry and Baehrecke, 2007). In the cellular setting, autophagy has been shown to contribute to cell death in cells lacking Bax and Bak or

caspases. For example, Yu and colleagues described Atg7- and Beclin 1-dependent nonapoptotic cell death in L929 cells in the presence of z-VAD-fmk, a pancaspase inhibitor (Yu et al., 2004). Similarly, selective cytotoxic stimuli have been shown to induce autophagic death associated with upregulation of Atg5/Atg6 in Bax/Bak$^{-/-}$ MEFs (Tsujimoto and Shimizu, 2005). Recently, autophagosomal membranes were linked to cell death through the formation of the iDISC (Young et al., 2012) that constitutes a platform for the activation of caspase-8 either in apoptotic-competent or -incompetent cells.

1.5.4 Autosis, an alternative cell death modality

Autosis was first coined by Levine and colleagues, as a term comprising two cellular processes: 'auto', autophagy and 'tosis' for death. Autosis is an autophagy-gene dependent, non-apoptotic, and non-necroptotic form of cell death. It is characterized by morphological features such as fragmentation of the ER in early stages and its disappearance in later stages, enhanced cell substrate adhesion, and nuclear membrane changes. Focal perinuclear swelling, nuclear shrinkage and appearance of nuclear-derived round vacuole-like balloons are the most characteristic features of autosis (Liu and Levine, 2015). Like autophagic cell death, autosis is characterized by an increased number of autophagosomes and autolysosomes. Similarly to apoptosis, autosis is accompanied by mild chromatin condensation. Autosis is induced by starvation, the synthetic autophagy-inducing peptides Tat-Beclin 1 and Tat-vFLPI α2, and hypoxia-ischemia (Liu et al., 2013). Autosis is mediated by the Na^+,K^+ATPase ionic pump and can be inhibited by cardiac glycosides that are chemical antagonists of Na^+, K^+ ATPase (Liu et al., 2013). Autosis has been shown to occur in the presence of the pharmacological caspase inhibitor z-VAD and in cells deficient in Bax and Bak. Additionally, application of necrostatin-1 or genetic ablation of Ripk1 and Ripk3 were not able to block autosis (Liu et al., 2013).

1.6 Role of autophagy in cancer

Autophagy is a catabolic process that is involved in the degradation of intracellular proteins and damaged organelles. Autophagy is constitutively active to maintain basal

cellular homeostasis, while during stress conditions it is responsible for sustaining metabolism and energy status. Recycling by autophagy is essential for cellular survival under various stress conditions. Autophagy is a tightly controlled and dynamic process regulated by several ATG proteins (Levine and Klionsky, 2004). Malfunctions in the apoptotic pathway contribute to many diseases such as neurodegenerative conditions, and can also be part of the pathogenesis of cancers (Wong, 2011). The role of autophagy in cancer seems to be context-dependent as autophagy can act either as a tumor suppressor or tumor promoter.

Given that autophagy is a survival pathway with the ability to degrade macromolecules, cancer cells rely on this pathway under hypoxic conditions and nutrient deprivation. In this regard, autophagy promotes tumor growth. It has been shown that pancreatic cancers are characterized by elevated levels of basal autophagy (Yang et al., 2011). Indeed, this study highlighted the crucial role of autophagy in proliferation *in vitro* and *in vivo*. Inhibition of autophagy either by genetic or pharmacological approaches significantly reduced pancreatic cancer growth or resulted in tumor regression and prolonged survival. Melanoma is another example of a cancer with a high level of basal autophagy. It has been shown that enhanced levels of autophagy in aggressive melanoma cells contributes to tumor growth as autophagy protects these cells against metabolic stress. Moreover, inhibition of autophagy resulted in cell death and high levels of autophagy were correlated with survival and resistance to therapy (Xie et al., 2013). Additionally, autophagy suppresses tumor-induced inflammation, thus supporting tumor progression. In this context, autophagy was shown to cooperate with apoptosis to preventing death by necrosis which is associated with inflammation (Degenhardt et al., 2006). Moreover, recently it has been shown that microenvironmental autophagy also supports tumor growth and survival implicating a role for non-autonomous autophagy in malignancy (Katheder et al., 2017).

As autophagy has the capacity to dispose of potentially dangerous cytoplasmic organelles, macromolecules, and pathogens, it has also been implicated in the suppression of malignant transformation. In this context, inhibition of autophagy through genetic ablation of the core autophagy genes *Atg5* and *Atg7* results in cell-type

specific tumorigenesis (Takamura et al., 2011). In contrast, loss of the essential autophagy gene *beclin 1* was shown to initiate tumor formation in multiple organs, including the liver and lungs (Qu et al., 2003).

Furthermore, it has been reported that malignant tumors are associated with defective autophagy. For example, *Beclin 1* which acts as a tumor suppressor is monoallelicaly deleted in 40% to 75% of human breast, ovarian and prostate cancers (Qu et al., 2003). This notion was also supported by the impairment of the progression of the KRAS-driven lung cancers by homozygous deletion of *Atg5* (Rao et al., 2014). Induction of autophagic cell death can also be considered as tumor-suppressive. Indeed, a study by Martin and colleagues showed that deregulation of the oncogene *HRAS* induced a caspase-independent mode of cell death associated with autophagy that could potentially limit oncogenic Ras signals (Elgendy et al., 2011). In human cancer, mutations of the core autophagy genes are not common and are limited to only a few genes such as *ATG7*, *ULK4*, and *FIP200* (Amaravadi et al., 2016).

1.7 Role of the ISR in cancer

Tumor cells are exposed to several cell intrinsic and microenvironmental stresses that activate a variety of adaptive mechanisms to favor cell transformation and promote cancer progression. Cancer cells are constantly challenged by low levels of oxygen, glucose, and other nutrients due to poor vasculature and high metabolic rates leading to oxidative stress, ER stress, and subsequent ISR activation (Bi et al., 2005). Therefore, ISR activation is often enhanced in tumors to promote adaptation to physiological stress and it contributes to the resistance of cancer cells to a variety of stresses in their tumor microenvironment. Recently, it has been shown that ATF4 contributes to mesenchymal tumor metastasis (Dey et al., 2015). Additionally, in the context of cancer biology, the ISR can also be activated by cell-autonomous insults such as the activation of oncogenes like *Ras* and *Myc* (Hart et al., 2012, Denoyelle et al., 2006). Increased activity of the proto-oncogene *Myc* elicits activation of the PERK/eIF2α/ATF4 pathway resulting in cellular transformation and tumorigenesis (Hart et al., 2012), whereas amplified *HRAS* induces the PERK/eIF2α/ATF4 pathway and cellular senescence in a premalignant model of melanoma (Denoyelle et al., 2006). Activation of GCN2 was also reported in

response to prolonged glucose deprivation in human fibrosarcoma and colorectal adenocarcinoma cells (Ye et al., 2010). Although the precise mechanism by which GCN2 is activated by uncharged tRNAs was not defined, it is suggested that cancer cells use amino acids as an alternative source of carbon to produce energy thus indirectly leading to the activation of GCN2 (Ye et al., 2010). UV-irradiated mouse embryonic fibroblast cells also activate GCN2 (Deng et al., 2002).

In tumors, activation of ISR has been described to exert anti-cancer effects. However, a cytoprotective ISR can also be induced in response to anti-cancer therapies and may promote therapy resistance, as is the case in pancreatic ductal carcinoma cells and an orthotropic mouse model subjected to gemcitabine treatment (Palam et al., 2015). Drug-induced enhancement of ISR signaling through eIF2α kinases can be exploited in combined therapies against resistant cancers as it can lead to G1/S checkpoint activation through eIF2α-dependent depletion of cyclin D (Stockwell et al., 2012). Inhibition of ISR signaling through inhibition of the eIF2α kinases also represents a strategy to overcome cancer resistance and to target neurodegenerative diseases. There is an increasing number of eIF2α kinase inhibitors becoming available that attenuate eIF2α phosphorylation. Some examples inhibitors include GSK2606414 and GSK2656157 that target PERK and prevent its autophosphorylation (Axten et al., 2013, Axten et al., 2012). It has been shown that deficiency in either PERK or GCN2 impacts on ATF4 expression and thus results in decreased resistance to hypoxic and nutrient stress leading to reduced tumor cell growth both *in vitro* and *in vivo* (Bobrovnikova-Marjon et al., 2010, Ye et al., 2010). Interestingly, both strategies to suppress the ISR as well as to enhance ISR signaling have been reported to inhibit tumor growth *in vivo*, which reflects the dual role of the ISR in promoting survival and cell death (Chen et al., 2011, Ye et al., 2010). Therefore, pharmacological modulation of the ISR, aiming at inhibition or enhancement of ISR signaling represents a promising therapeutic strategy.

Aims of the thesis

The main aims of this project are to investigate the mode of stress-induced cell death in cells whose mitochondrial apoptotic pathway is compromised, and to examine a novel stress-induced caspase-mediated death pathway dependent on autophagy.

Objectives

1. To investigate if various stresses can induce cell death in cells that cannot undergo apoptosis
2. To investigate the role of stress-induced autophagy in cell death
3. To investigate the role of the ISR in regulation of the alternative cell death pathway in response to various stresses

Chapter 2: Materials and Methods

2.1 Cell Culture

Caspase-9 (C-$9^{-/-}$) and Caspase-$9^{+/+}$ (WT; C-$9^{+/+}$) Mouse Embryonic Fibroblasts (MEFs) cells were obtained from Prof. Tak Mak from University of Toronto, Canada. MEFs were maintained in Dulbecco's modified Eagle medium (DMEM) with 4.5 mg/ml glucose supplemented with 10% (vol/vol) fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml), non-essential amino acids (NEAA), L-glutamine and sodium pyruvate. Lentivirus transduced $Casp9^{-/-}$MEFs expressing either pGIPZ or pLKO vector were selected with addition of 5 μg/ml of puromycin for 72 h and for another two passages in 2 μg/ml of puromycin. After selection cells were left for recovery for 2 passages before experimental work. All reagents used in tissue culture are certified and were purchased from Sigma-Aldrich as per **Table 2.1**.

2.1.1 Subculturing

Cells were cultured at 37°C in a humidified 5% CO_2 atmosphere. They were aseptically passaged 2-3 times weekly. Before subculturing, cells distribution was monitored under microscope. Since MEFs are adherent cells, 1x Trypsin-EDTA was used to detach the cells from flask followed by wash in small volume of Hanks' Balanced Salt Solution. After the cells have been dissociated into a single-cell suspension, they were diluted to the desired concentration and transferred into culture flask with the pre-heated growth medium and incubated at 37°C where they reattached, grew and divided.

2.1.2 Recovery of cryopreserved cells (thawing)

The vial with the cryopreserved C-$9^{-/-}$ MEFs was removed from the liquid nitrogen and thawed by gentle agitation in a 37°C water bath. When the ice crystals were melted the vial was transferred to a laminar flow tissue culture hood. Next the thawed content approx. 1 ml was added dropwise to 9 ml cold complete growth medium and mixed thoroughly by gentle pipetting. The cell suspension was divided into two T25 flasks with total volume of 5 ml and incubated at 37°C. The cultures were examined after 24 h and the medium was exchanged for fresh media. Cells were subcultured 48 h after thawing or as needed.

2.1.3 Cryopreservation (freezing)

Cryopreservation was carried out on the freshly thawed cells with the lowest passage number. Before freezing cell culture was checked for contamination from bacteria or fungi. The freezing medium consisting of 95% FBS and 5% DMSO was prepared in advance. C-9$^{-/-}$ MEFs were detached with 1x Trypsin-EDTA and next collected by gentle centrifugation 1000 x g for 5 min. The supernatant was discarded and cells were resuspended in the freezing medium at a concentration of 1×10^6 viable cells/ml. Immediately 1 ml of cells suspension was added to appropriate labelled vials with the name of the cell line, concentration and the date. The vials were placed into freezing container containing a pre-cooled 100% isopropyl alcohol and next the chamber was transferred to a mechanical freezer at –80°C for at least 24 hours. After 24 hours at –80 °C, one vial was removed for quality control and the cells were restored as per normal cell culture routines outlined above.

Table 2.1 Tissue Culture Reagents

Reagent	Catalogue Number	Company
DMEM-high glucose	D6429	Sigma-Aldrich
FBS	F7524	Sigma-Aldrich
Pen-Strep	P0781	Sigma-Aldrich
L-Glutamine	G7513	Sigma-Aldrich
NEAA	M7145	Sigma-Aldrich
Sodium pyruvate	S8636	Sigma-Aldrich
RPMI-1640	R0883	Sigma-Aldrich
Hanks' Balanced Salt	H9394	Sigma-Aldrich
Trypsin-EDTA Solution	T4174	Sigma-Aldrich
Puromycin	P8833	Sigma-Aldrich

2.2 Treatment

2.2.1 Drug treatment

Cells were seeded at the required density (40,000 cells/well in 12 well plates; 100,000 cells/well in 6 well plates; 2,500,000 cells/T25 flasks) 24 h prior to stress treatment, the medium was then removed and cells were replenished with medium containing required drug concentration. All the drugs were prepared according to the manufacture's instruction and dissolved in the dimethysulfoxide (DMSO). The drugs were aliquoted and stored at -20°C. Inhibitors were added 1 h prior treatment or otherwise as specified. Spautin-1 and PERK inhibitor were replenished every 24 h whereas ISRIB was added every 48 h. All drugs used in the experimental work are summarized in **Table 2.2.**

Table 2.2. Drug treatment

Drug	Catalogue Number	Source/Supplier
Brefeldin A	B6542	Sigma-Aldrich
Chloroquine diphosphate salt	C6628	Sigma-Aldrich
Etoposide	E1383	Sigma-Aldrich
ISRIB	SML0843	Sigma-Aldrich
PERK inhibitor	Compound 44	Amgen
Paclitaxel	T7402	Sigma-Aldrich
Rapamycin	R-500	LC Laboratories
Spautin-1	SML0440	Sigma-Aldrich

2.2.2 Inhibitor's mechanism of action

Brefeldin A (BFA) is a fungal-derived antibiotic, macrocyclic lactone produced by organisms such as *Penicillium brefeldianum*. On the molecular level the mode of action of BFA is implicated in intracellular trafficking. BFA alters the structure and function of Golgi apparatus (GA) and leads to the reduction in the number of secretory cisternae in Golgi stack. Defects in Golgi tabulation and its disassembly cause rapid block in transport of secretory proteins from the ER to the Golgi (Driouich et al., 1993). This

results in accumulation and aggregation of nascent proteins within the ER leading to activation of UPR (Moon et al., 2012).

Chloroquine (CQ) is a widely used anti-malarial drug with other medical applications in relation to amebiasis and rheumatic disease. It is a lysosomotropic agent that impairs lysosomal acidification inside the lysosome or inhibits autophagosome-lysosome fusion, thus resulting in defects in the autophagic flux (Slater, 1993).

Etoposide (Etop) is a podophyllotoxin extracted from the roots of the *Podophyllum peltatum*. It is topoisomerase inhibitors that forms a complex with topoisomerase II and DNA resulting in formation of double-stranded breaks (Montecucco et al., 2015) and subsequent cell death (Nitiss and Wang, 1996).

ISRIB (**I**ntegrated **S**tress **R**esponse **i**nhi**b**itor) acts downstream of all four eIF2α kinases (GCN2, PERK, PKR and HRI) reversing the effects of eIF2α phosphorylation. (Sidrauski et al., 2013). The molecular mechanism of ISRIB is based on either enhancing eIF2B activity or reducing its sensitivity to eIF2 phosphorylation (Sidrauski et al., 2015, Sidrauski et al., 2013).

Paclitaxel (Taxol) was isolated from the bark of the *Taxus brevifolia.* It promotes microtubule polymerization and stabilization. Its mechanism of action is mediated by stabilization of the microtubule polymerization resulting in increased assembly of tubulin (Weaver, 2014, Orr et al., 2003). Additionally, Taxol destroys the microtubule structure and causes mitotic arrest, which subsequently leads to cell death (Xiao et al., 2006).

Rapamycin (Rap) is an immunosuppressive and anti-cancer agent widely used in research to monitor autophagy. The mechanism of rapamycin action is mediated by inhibition of mTOR protein kinase within mTORC1 resulting in induction of autophagy (Law, 2005).

Spautin-1(Specific and potent autophagy inhibitor-1) inhibits two ubiquitin peptidases, USP10 and USP13, which regulate the deubiquitination of Beclin1 in Vps34

complexes. Spautin-1 indirectly leads to Beclin 1 degradation thus inhibits autophagy at the induction stage (Liu et al., 2011).

2.2.3 Gamma irradiation (γ-IR) treatment

Prior to treatment cells were cultured in a T175 flask. On the day of the treatment cells were trypsinized and diluted in fresh medium to final volume of 20 ml. Next the suspension was split into two tubes one for control and the rest for treatment. Cells were exposed to 33 Gy and then collected by gentle centrifugation at 300 x g for 5 min. The supernatant was discarded and pellets were resuspended in fresh growth medium and seeded at required density. Cells were left for recovery and harvested at indicated time.

2.3 Microscopy

Examination of the morphology of the cells in culture was carried out on daily basis. The shape and appearance of the cells were monitored before subculturing, and during the experimental settings. To analyze cytotoxicity and kinetics of morphological changes of cell death induced by stress-stimuli Olympus CKX41 inverted microscope with bright field function was used.

2.3.1 Bright Field Microscopy

C-9$^{-/-}$ MEFs cells, pLKO and *Casp8* shRNA C-9$^{-/-}$ MEFs and pGIPZ and *Atg5* shRNA C-9$^{-/-}$ MEFs were treated with BFA, Etop Taxol and γ-irradiation for the indicated timepoints. Before harvesting images of the cells were taken in bright field using the Zeiss AXIO Vert. A1 inverted microscope at 10x and 20x magnification.

2.3.2 Fluorescent Microscopy

To monitor autophagosome formation, C-9$^{-/-}$ MEFs cells were transfected with a vector expressing a GFP-LC3 fusion protein as described below in Transfection section. After 24 h post-transfection cells were subjected to treatment with either brefeldin A, etoposide and taxol alone or in combination with Spautin-1. At indicated time of treatment cell nucleus was stained using 5 μg/ml 2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole trihydrochloride trihydrate (Hoechst 33342) (Molecular Probes; cat # H-3570) a DNA stain which is excited by ultraviolet light and

emits blue fluorescence at 460 to 490 nm. The cells were visualized using the Operetta High Content Imaging System in GFP green and blue channel at 20x magnification.

2.4 Plasmid Isolation

Plasmid pEGFP-LC3 (Addgene; cat # 24920) was transformed into competent *Escherichia coli* strain DH5α. The competent bacteria were thawed on ice and aliquoted into eppendorf of total volume 50 µl. A 10 µl volume of plasmid was added into competent bacteria and the content was mixed gently by pipette tip. The transformation mixture was left on ice for 30 min incubation. Next eppendorf was placed on heat block at 42°C and bacteria were heat shocked for 30 sec to allow plasmid uptake. Immediately eppendorf was placed back onto ice immediately and left for 2 min recovery. Next 250 µl of LB Broth (no antibiotic) was added directly to the eppendorf and the content was incubated with shaking at 200 rpm for 1 h at 37°C. Following incubation, the total content of transformation mixture was spread onto LB agar plate containing 50 µg/ml of kanamycin. The plate was placed in the incubator upside down for overnight incubation at 37°C. Next day well 3 defined colonies were added to 3 ml of LB Broth in 3 separated tubes containing 50 µg/ml of kanamycin (starter culture) and then placed into orbital shaker with vigorous shaking at 260 rpm for overnight incubation at 37°C. The following day 1 ml of a starter culture from each tube was transferred into 3 separate eppendorf s and centrifuge at 17000 x g for 1 min. The supernatant was removed and bacterial cell pellets were resuspended in 100 µl of P1 buffer (50 mM Tris-HCl pH 8; 10 mM EDTA) by vigorous pipetting. Next bacteria were lysed by addition of 200 µl of P2 buffer (0.2 M NaOH; 1% SDS) and suspensions were mixed gently by inversion. The mixtures were left for 1 min incubation on ice and then 200 µl of P3 buffer (3M KAc pH 5.5) was added to precipitate genomic DNA. Following incubation for 3-5 min on ice the mixtures were pelleted at 17000 x g for 15 min. Supernatants were removed to the new eppendorfs and DNA plasmids were precipitated by addition of 0.7 volume of isopropanol and left for 20 min incubation at room temperature. Next the plasmids were pelleted at microcentrifuge at 17000 x g for 10 min. The supernatants were removed and the pellets were washed in 1 ml of ice-cold 70% Ethanol and centrifuged again. The supernatants were removed and the pellets were left to air dry for 10 min. Following drying the DNA was resuspended in 10 µl sterile H_2O and then 5 min later 10 µl of

sterile TE (10mM Tris, 1 mM EDTA, pH 8; sterile by autoclaving) was added. To visualize the plasmid 2-3 µl of isolated plasmids were run on a 0.6% agarose gel. After visualization the starter culture with the best quality plasmid was chosen to be used going forward. The following day the starter was centrifuged at 5000 x g for 20 min to pellet the bacterial cells containing the plasmid of interest. Plasmid extraction was carried out in accordance with Qiagen mid-prep kit (Qiagen; cat #12145).

2.5 Transfection

2.5.1 Transfection of cells with siRNA

Delivery of siRNAs into C-9$^{-/-}$ MEFs was optimized for cationic –lipid transfection reagent method. Transfection was carried out using DharmaFECT1 transfection reagent (Dharmacon; cat # T-2001-01). C-9$^{-/-}$ MEF were plated at 40,000 cells/well in total volume of 1 ml in 12 well plates 24 h prior to transfection. Next day, 5 µM siRNA solutions for both non-targeting siRNA (Dharmacon; cat # D-001810-10-05) and ATF4 siRNA (Dharmacon; cat # L-042737-01-0005) in 1x siRNA buffer were prepared. Follow by that appropriate amount of siRNA and DharmaFECT1 were diluted with FBS free medium and incubated for 5 min at room temperature. After incubation diluted siRNA was mixed with diluted DharmaFECT1 for a total volume of 200 µl and incubated for 20 min at room temperature. During incubation culture media was removed from wells and cells were washed with antibiotic-free media. Transfection mix was added to 800 µl of antibiotic-free complete medium for a final volume of 1000 µl and a final siRNA concentration of 20 nM of non-targeting siRNA and 20 nM of ATF4 siRNA. Cells were incubated with transfection medium at 37°C in 5% CO_2 for 5 h. The transfection mixture was removed after 5 h and replaced with either fresh growth medium for control samples or medium containing the appropriate drug concentration for testing samples. Next, cells were incubated for desired time and harvested at indicated time point for analysis.

Transfection of siRNA CHOP and Beclin1 into MEFs was carried out as described above. Briefly, either 20 nM or 25 nM CHOP siRNA (Dharmacon; cat # L-062068-00-0005) and 25 nM Becn1 siRNA (Dharmacon; cat # L-055895-00-0005) were mixed with 2.5 µl of DharmaFECT1 transfection reagent in 200 µl of serum free media and

incubated for 20 min at room temperature. Next, the mixture was added drop by drop to 800 µl of antibiotic free media and cells were incubated at 37°C in 5% CO_2 for 5 h. After incubation time the transfection mixture was removed and replaced with normal growth medium and the cells were put back into the incubator for 24 h. The drug treatment was carried out 24 h post transfection and the control samples for both non-targeting siRNA and CHOP siRNA or non-targeting siRNA and Becn1 siRNA were harvested on the time of treatment.

2.5.2 Transfection of cells with plasmid

Transient transfection of GFP-LC3 expression plasmid into C-9$^{-/-}$ MEFs was conducted using the transfection reagent Turbofect (Fermentas; cat # R0531). C-9$^{-/-}$ MEFs were seeded at 40,000 cells/well in total volume of 1 ml in 12 well plates 24 h prior to transfection. Transfection of C-9$^{-/-}$ MEFs cells requires 1:2.5 ratio of DNA to lipid. 1 µg of GFP-LC3 expression plasmid and 2.5 µl of Turbofect transfection reagent were mixed in the same tube containing FBS free medium in total volume of 200 µl. The mixture was incubated for 20 minutes at room temperature to form DNA-lipid complexes. During this time the growth medium was removed from the cells and cells were washed once with antibiotic free medium. After incubation the DNA-lipid mixture was added drop-wise to each well of cells containing 800 µl FBS free medium for a total volume of 1 ml. The medium in each well was swirled gently to mix and then the cells were incubated for 5 h at 37°C. The transfection mixture was removed after indicated time and replaced with growth medium in each well. Cells were incubated at 37°C for 24 h, then the transfection efficiency was determined by fluorescent microscopy and treatment was applied.

2.6 Protein Sample Preparation

2.6.1 Harvesting cells

Cells were seeded in T25 flasks or 6-well and 12-well plates for protein sample preparation. After treatment, cells were harvested at indicated times by scraping into the media. Neither proteases nor phosphatases inhibitors were added to the media. Media and floating cells were transferred into labelled 1.5 ml Eppendorf or 15 ml tube on ice and next centrifuged at 4000x rpm for 5 min at 4°C. After spin down the tubes were

placed on ice, the supernatant was removed and the pellet was washed once with 150 µl of ice-cold 1x PBS pH 7.4 by gently resuspension. Next, cell's suspension was transferred into 1.5 ml Eppendorf and centrifuged at 4000x rpm for 5 min at 4°C. After spin-down the supernatant was removed from the cell pellets and discarded. The pellets were stored in -20°C freezer for up to a week prior to lysis until full time course was collected.

2.6.2 Lysis of cells

All cells from an experiment were lysed together in 1x SDS-PAGE lysis buffer prepared freshly before use from 2x SDS-PAGE lysis buffer. The compositions of the lysis buffers were described in the **Table 2.3** and **Table 2.4.** The volume of the 1x SDS-PAGE lysis buffer was determined based on the cell number. Briefly, the appropriate volume of 1x SDS-PAGE lysis buffer was added directly to pellet on ice. The pellet was immediately re-suspended by pipetting up and down several times and left on ice for 5 min. Next, all samples were boiled at 95°C for 5 min on the heat block. Later, all samples were placed on ice for 2 min. After incubation, the samples were pulse centrifuged at 14000x rpm. Samples could be used immediately for analysis or stored at -20°C. Sonication was not carried out during preparation of protein extracts.

Table 2.3. 2x SDS-PAGE Lysis Buffer

Component	**Final Concentration**
10% SDS solution	4% SDS
1 M Tris HCl pH 6.8	100 mM Tris HCl pH 6.8
Bromophenol Blue	0.1% Bromophenol Blue
Glycerol	20% Glycerol
dH_2O	End Volume to 50 ml H_2O

Above composition is for 50 ml of 2x SDS-PAGE buffer that can be stored at room temperature for up to 3 months. Prior to use 2x SDS-PAGE lysis buffer was diluted 1:2 in total volume of 2 ml.

Table 2.4. 1x SDS-PAGE Lysis Buffer

Ingredient	Volume (µl)
2x SDS-PAGE	950
β-mercaptoethanol	50
dH_2O	1000

2.7 Polyacrylamide Gel Electrophoresis (PAGE)

Appropriate volume of the cell lysates for protein analysis were directly loaded into 12-15% SDS-PAGE gels alongside the color protein molecular weight marker, broad range (11-245 kDa) (NEB; cat # P7712). Briefly, the SDS-PAGE gels were placed in a tank and held vertically between the electrodes chamber during electrophoresis. The composition of the handcast gels is described in **Table 2.5.** The electrode reservoirs were filled with the 1x running buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS) and electrophoresis was carried out at 60 V for 30 min followed by 90 V until the front dye reached the bottom of the gel.

Table 2.5. Composition of the SDS-PAGE gel

Components	Resolving gel		5% Stacking gel (ml)
	12% (ml)	15% (ml)	
H_2O	3.4	2.4	3.4
30% acrylamide mix	4	5	0.85
1.5 M Tris pH 8.8	2.6	2.6	0.65
10% SDS	0.1	0.1	0.05
10% ammonium persulfate (APS)	0.1	0.1	0.05
TEMED	0.004	0.004	0.005
Total volume	10	10	10

2.8 Western blot (Immunoblotting)

2.8.1 Wet (tank) transfer

Briefly, after separation by electrophoresis protein samples were transferred onto nitrocellulose membrane. The SDS-PAGE gel and membrane were sandwiched between sponges and filter papers in the following order: sponge, filter paper, gel, membrane, filter paper, sponge. The sandwich was tightly closed in the cassette and submerged in the cold transfer buffer (10 mM CAPS pH 11, 20% (v/v) Methanol) in the tank which was inserted in a box of ice. The protein samples were transferred onto nitrocellulose membrane for 90 min at 110 V. After the transfer the membrane was stained with Ponceau S to visualize the protein translocation. Next, the membrane was washed several times with PBS-T (0.1% Tween-20 in 1x PBS) to remove the dye. Blocking of non-specific binding was achieved by placing the membrane in 5% non-fat milk in PBS-T for 1 h at room temperature. After blocking, the membrane was incubated with the primary antibody under gentle agitation either for 1 h at room temperature or overnight at 4°C depends on optimal antibody condition. Membrane then was washed three times with PBS-T and further incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1-2 h at room temperature. The list of used antibodies is summarized in the **Table 7.** Next, antibodies were visualized using Western Lighting Plus-ECL substrates (PerkinElmer; cat # NEL 105001EA) and Western blot Luminol Reagent ImmunoCruz (SantaCruz; cat # SC-2048).

2.8.2 Semi-dry transfer

Proteins in the sample were separated by gel electrophoresis and next transferred to nitrocellulose membrane. The SDS-PAGE gel and membrane were sandwiched between filter papers. Four filter papers were soaked in the Anode buffer I (300 mM Tris-HCL pH 10.4, 20% (v/v) Methanol) and placed onto saturated anode plate with the Anode buffer I. Next two filter papers were pre-soaked in the Anode buffer II (25 mM Tris-HCL pH 10.4, 20% (v/v) Methanol) and placed on the top of the four filter papers, removing any air trapped between the added filters. The membrane was equilibrated in Anode buffer II and then carefully placed on the top of the six filter papers. The SDS-PAGE gel was incubated in the Cathode buffer (25 mM Tris-HCL pH 9.4, 10 mM

glycine, 20% (v/v) Methanol) and later gently placed on the top of the membrane. In this step any trapped air was also removed. Next, six filter papers were soaked in the Cathode buffer and then laid one by one on the top of the gel. During adding the filter papers each layer was rolled out to remove the air trapped between the papers. The cathode plate was saturated with the cathode buffer and then carefully placed onto the transfer stack. The protein samples were transferred onto nitrocellulose membrane at a constant current 230 mA for 75 min with limited voltage (25 V) and wattage (10 W). Upon completion of the run the gel and membrane sandwich was disassembled and membrane was incubated in the Ponceau S staining to visualize the protein translocation and check the efficiency of the transfer. Next membrane was distained in PBS-T until background cleared. Later membrane was proceed immunodetection protocol as described above in the 2.7.1 Wet transfer section.

Table 2.6. Antibodies used in Western blot

Antibody	**Host**	**Incubation Condition**	**Catalogue Number**	**Company**
Primary				
ATF4	rabbit	O/N 4°C	11815	CST
Atg5	rabbit	O/N 4°C	12994	CST
Actin	rabbit	1 h RT	A2066	Sigma
Beclin1	rabbit	1 h RT	sc-11427	Santa Cruz
CASP-3	rabbit	O/N 4°C	9662	CST
CASP-8	rabbit	O/N 4°C	4790	CST
CASP-9	mouse	O/N 4°C	9508	CST
CASP-8 cleaved	rabbit	O/N 4°C	8592	CST
CHOP	mouse	O/N 4°C	2895	CST
Phospho-eIF2α	rabbit	O/N 4°C	3398	CST
Total-eIF2α	rabbit	1 h RT	5324	CST
LC3	rabbit	1 h RT	L7543	Sigma
Secondary				

Anti-Mouse IgG (H+L)	goat	1-2 h RT	115-035-003	Jackson
Anti-Rabbit IgG (H+L)	goat	1-2 h RT	111-035-003	Jackson

O/N indicates overnight incubation. RT is referred to room temperature. (H+L) indicates heavy and light chains of gamma immunoglobins.

2.9 Clonogenic assay (Colony forming assay)

pLKO and caspase-8 shRNA C-9$^{-/-}$ MEFs were seeded at two different, low densities (10,000 and 20,000 cells/well) in a 6-well plate 24 prior to treatment. Next day, cells were subjected to treatment with Brefeldin A (0.3 μg/ml), Etoposide (50 μM) and Taxol (1 μM) or exposed to γ-irradiation (33 Gy). After 72 h treatment the media containing drug were removed from the cells and replaced with the fresh growth medium. The fraction of remaining live cells on the plate was allowed to recover and retain the capacity to produce colonies for 7-10 days. Following 7-10 day recovery the media was removed from the cells and the cells were washed with 1x PBS pH 7.4. Next, cells were stained with crystal violet staining solution (0.5%) (20% (v/v) methanol, 0.5% crystal violet) for 10 min at room temperature. Excess stain was removed by washing three times with 1x PBS pH 7.4 and plate was imaged.

2.10 Flow Cytometry Analysis of Cell Viability

Prior to treatment cell were seeded at low density in a 12-well plate. Next day, cells were subjected to the treatment for indicated time. Following treatment, media from cells were transferred to an eppendorf and cells were washed once with Hank's solution and harvested by trypsinization. Washed and trypsinized cells were collected in the same eppendorf and centrifuge at 3000g for 5 min at 4°C. The supernatant was removed and the cells were resuspended in 300 μl of ice-cold 1x PBS pH 7.4. Prior to analysis 4 μl of of propidium iodide (PI) (50 μg/ml) was added to the cells and immediately tested using BD Accuri C6 flow cytometer (Becton Dickinson) serial # 2783. Samples were analysed using C flow software V.1.0.264.15.

2.11 Statistics

The values were reported as mean+ standard error of the mean of three independent experiments. Differences between the treatment groups were assessed using graph pad's. The significance level are *$p<0.05$, **$p<0.01$, ***$p<0.001$ by two-way ANOVA followed by Student's t-test when two-way ANOVA showed significance differences between the groups ($p<0.05$). In multiple comparisons post *hoc* tests the Bonferroni's correction was applied for two-way ANOVA and the Holm-Sidak for Student's t-test.

Chapter 3: Investigation of stress-induced cell death in cells deficient in the intrinsic apoptotic pathway: Role of caspase-8

3.1 Contributions

This work will be a part of a manuscript for a research paper "Stressosome -novel stress inducing complex". Authors: Karolina Pakos-Zebrucka (KPZ), Izabela Glogowska (IG), Katarzyna Mnich, Shane Deegan, Susan Logue, Adrienne Gorman, Afshin Samali

The contribution to the experimental results is as follows:

Treatment optimization– KPZ, IG

Fig. 3.1 A (2x KPZ, 1x IG), B (2x KPZ, 1x IG), C (2x KPZ, 1x IG) , D (1x KPZ, 2x IG)

Fig. 3.5 C (2x KPZ, 1x IG)

Fig. 3.6 C (1x KPZ, 2x IG)

Fig. 3.7 C (1x KPZ, 2x IG)

Fig. 3.8 C (2x KPZ, 1x IG)

3.2 Introduction and Objectives

Cells possess a diverse array of stress response pathways which help them adapt to various stress stimuli. However if the stress is too severe or prolonged, the cell will undergo cell death. Apoptosis and necrosis are the most well-characterised modes of cell death executed in response to cellular stress; however, other forms of cell death have recently been identified. Depending on the model and the stress used, such alternative modes of cell death include autophagy-dependent cell death (Torricelli et al., 2012) and necroptosis (Vandenabeele et al., 2010). The molecular pathways that lead to the activation of these alternative modes of cell death and the exact mechanics and components of the death signalling complexes involved are not clearly defined. Similarly, the cellular responses to different chemotherapeutic agents are not well understood. Although several reports suggest that apoptosis is the cell death mode that is

preferentially induced of by various anticancer drugs, cell death and the molecular mechanisms of its regulation require further clarification. The molecular mechanisms of cell death in apoptosis-resistant cells exposed to BFA, Taxol, γ-irradiation, and etoposide (Etop) remain to be elucidated. How ER stress-inducing agents and other stress stimuli trigger cell death in C-9$^{-/-}$ MEFs is the question under examination in this thesis.

BFA is an antibiotic and a potent inducer of apoptosis in several human cancer cell lines including epithelial ovarian carcinoma, chronic lymphocytic leukemia, multiple myeloma, and follicular lymphoma (Lee et al., 2013, Wlodkowic et al., 2007, Carew et al., 2006, Shao et al., 1996). It has been shown that BFA induces ER stress-mediated apoptosis in Bcl-2-overexpressing follicular lymphoma cells (Wlodkowic et al., 2007). Taxol is a chemotherapeutic agent with broad cytotoxic activity. It is a microtubule-targeting drug which is widely used in the treatment of several resistant cancers such as ovarian, breast, lung, and Kaposi's sarcoma (Weaver, 2014, Liebmann et al., 1993). Apparently, Taxol induces apoptosis via the mitochondrial-mediated pathway as shown in a human breast cancer cell line (Miller et al., 2013). The cytotoxic effect of Taxol is concentration-dependent, and although its application is widely studied the precise mechanism by which Taxol induces cell death is still unclear. Taxol induced Bcl-2 phosphorylation in the PC3 prostate carcinoma cell line which concomitantly with cell death (Haldar et al., 1996). Moreover, in the human colon cancer HT29-D4 cell line Taxol induced apoptosis via caspase-8 activation independently of death receptor ligation (Goncalves et al., 2000). Etop is another commonly used chemotherapeutic agent with broad anticancer activity. It has been reported that Etop induces apoptosis through the mitochondrial-mediated pathway and promotes caspase activation in several cancer cell lines, for instance, pancreatic and neuroblastoma cells (Zhang and Huang, 2013, Day et al., 2009). Although the effect of Etop was already studied in C-9$^{-/-}$MEFs, a detailed mechanism of how Etop triggers cell death has not been reported (Hakem et al., 1998). Of note, in the neuroblastoma cell line SK-N-As, Etop has been shown to trigger apoptosis in a caspase-8-dependent manner (Day et al., 2009). Besides chemotherapeutics, radiotherapy is also currently used as an anticancer strategy. γ-

irradiation was shown to induce cell death via the mitochondrial-mediated pathway in a human lymphoblast cell line (Bishay et al., 2000). Furthermore, it is suggested that γ-irradiation-induced cell death is associated with p53 and subsequent suppression of the anti-apoptotic BCL-2 proteins and induction of pro-apoptotic BAX (Zhou et al., 2003, Bishay et al., 2000, Arai et al., 1996). However, molecular insight into how γ-irradiation triggers apoptosis is lacking especially in the context of cells defective in the mitochondrial-mediated apoptotic pathway.

Activation of the intrinsic apoptotic pathway is commonly observed in cells treated with radiation (Hao et al., 2005) or most chemotherapeutic drugs (Wei et al., 2001, Li et al., 2000), and is characterized by mitochondrial membrane permeabilization, cytochrome *c* release, apoptosome formation, and caspase-9 activation. Given that caspase-9 is an initiator caspase in the intrinsic apoptotic pathway, its function and the mechanisim of its activation has been widely studied. Indeed, it has been shown that deficiency of caspase-9 protects cells from stress-induced cell death in response to apoptotic stimuli such as sorbitol, cisplatin, Etop, Adriamycin, or exposure to UV and γ-irradiation (Hakem et al., 1998, Kuida et al., 1998). Furthermore, depending on cell type and stress stimulus, four different apoptotic pathways have been proposed based on caspase-9 and caspase-3 involvement: (1) caspase-9- and caspase-3-dependent pathway; (2) caspase-9- and caspase-3-independent pathway; (3) caspase-9-dependent and caspase-3-independent pathway; and (4) caspase-9-independent and caspase-3-dependent mode of cell death (Hakem et al., 1998). The existence of the caspase-9-independent and caspase-3-dependent non-canonical pathway also suggests the possibility of other caspases such as caspase-8 being the apical caspase activated in the absence of caspase-9. However, the molecular mechanism of stress-induced cell death in caspase-9-deficient MEFs was not elucidated (Hakem et al., 1998). The requirements for crucial components of the intrinsic pathway have been widely examined using various genetic models. Cells deficient in the pro-apoptotic BCL-2 family proteins BAX/BAK displayed increased resistance to apoptosis when treated with various stress-inducing drugs such as Etop, staurosporine, tunicamycin, or thaspigargin (Shimizu et al., 2004, Wei et al., 2001). Furthermore, the overexpression of anti-apoptotic BCL-2 family proteins has also

been shown to be associated with resistance to proteasome inhibition (Laussmann et al., 2011). Similarly, deficiency in Apaf-1 also prevents apoptosis induced by microtubule polymerisation stress (Janssen et al., 2007) and Etop treatment (Perkins et al., 2000). Although clear inhibition of stress-induced cell death has been shown in these scenarios, recent studies have implied that the same stress stimulus can also lead to cell death in caspase-9- or Bax/Bak-deficient cells (Lindsten and Thompson, 2006, Ekert et al., 2004). Malfunction in mitochondrial-mediated cell death can in this way delay programmed cell death or alternatively may induce an alternate mode of cell death. It is already well established that other forms of cell death such as autophagy-mediated cell death or necroptosis can also be executed in response to severe intracellular stress stimuli (Fulda, 2013, Yuan and Kroemer, 2010, Brown and Attardi, 2005, Brown and Wilson, 2003). The exact mechanism used by cells deficient in caspase-9 to trigger death upon sustained stress is still a matter of debate; however, recent studies have implicated caspase-8 as the apical caspase activated in response to intracellular stress when the intrinsic apoptotic pathway is compromised (Deegan et al., 2014b, Caro-Maldonado et al., 2010). Of note, this caspase-8 activation was independent of death ligands and was induced by nutrient deprivation and exposure to the proteasome inhibitor bortezomib (Laussmann et al., 2011, Caro-Maldonado et al., 2010). Although the precise molecular mechanism is not well defined it seems that caspase-8 might require the involvement of other cellular pathways such as autophagy to execute cell death. Previous studies carried out in our laboratory revealed that caspase-9-deficient cells treated with ER stress-inducing agents were resistant to ER stress-induced cell death when compared to their wild type counterparts; however, prolonged treatment with ER stress did eventually induce cell death which was dependent on caspase activation (Deegan et al., 2014a). Furthermore, subsequent studies demonstrated the existence of alternative cell death pathways in response to unresolved ER stress (Estornes et al., 2014, Deegan et al., 2014b). It has been shown that cells lacking components of the mitochondrial death pathway, such as caspase-9 or BAX and BAK undergo a delayed form of cell death associated with the formation of a novel death inducing-complex which requires components of the apoptosis (caspase-8, FADD) and autophagy (ATG5) machinery (Deegan et al., 2014b). In our laboratory we have termed

this novel death-inducing protein complex the "stressosome". This novel complex constitutes a platform for activation of pro-caspase-8 which has been identified as the initiator caspase in caspase-9-deficient MEFs. Importantly, caspase-8 was required for activation of downstream effector caspases and cell death. Moreover, this caspase-8 activation was found to be dependent on the autophagy machinery (Deegan et al., 2014b). Exposure to sustained ER-stress led to caspase-dependent cell death in both C-$9^{-/-}$MEFs and Bax/$Bak^{-/-}$ MEFs suggesting the engagement of a novel alternative death pathway upon prolonged drug treatment. However, there are still unanswered questions in relation to this pathway. First, we still do not know if this a general response to various stresses or just ER stress. Further, it remains unclear how cells lacking a functional mitochondrial pathway utilize this alternative death pathway when subjected to different types of severe stress. Based on the fact that impairment of the mitochondrial pathway is common in highly resistant cancers, understanding the molecular mechanism of stress-induced cell death in cells defective in intrinsic apoptosis seems to be crucial for the development of more effective therapies. Therefore, in this study C-$9^{-/-}$ MEFs were chosen as a relevant model to investigate cell death mechanisms in cell deficient in the intrinsic apoptotic pathway.

Here, the effect of various stresses on cell death was investigated in C-$9^{-/-}$ MEFs. In particular, the role of caspase-8 was examined in the context of a signaling pathway which was recently discovered to be involved in stress-induced cell death in cells defective in the mitochondrial-mediated pathway.

The aims of this study were to investigate:

1. Whether different cellular stresses can induce cell death in C-$9^{-/-}$ MEFs
2. Whether stress-induced cell death is associated with caspase-8 and caspase-3 activation in C-$9^{-/-}$ MEFs
3. Whether caspase-8 is essential for the execution of cell death in C-$9^{-/-}$ MEFs in response to different stress stimuli which would normally trigger the mitochondrial apoptotic pathway

3.3 Results

3.3.1 C-9$^{-/-}$ MEFs undergo cell death in response to prolonged stress

It has been reported that the mitochondria-mediated apoptosis pathway is activated by diverse stress stimuli including DNA damage, heat shock, UV and γ-irradiation as well as exposure to chemotherapeutic agents (Tait and Green, 2010).

In order to determine if cells with a compromised mitochondrial death pathway remain sensitive to cell death in response to various stresses, both C-9$^{+/+}$ and C-9$^{-/-}$ MEFs were subjected to prolonged treatment with BFA, Etop, Taxol and γ-irradiation. Following these treatments, cells were stained with propidium iodide (PI) and cell death was analyzed by flow cytometry. Interestingly, prolonging the time of exposure to BFA, Etop, or Taxol increased cell death from 10% at 24 h to approximately 30-35% at 72 h in C-9$^{-/-}$ MEFs (Fig. 3.1A-D). However, although exposure to γ-irradiation showed a similar trend, the increase in cytotoxicity was not as evident as with other stresses. The PI uptake rose from 5% at 24 h to about 25% at 72 h post irradiation. These data indicate that C-9$^{-/-}$ MEFs are highly resistant to stress stimuli compared with C-9$^{+/+}$ MEFs which exhibited approximately 75% dead cells within 72 h (Fig. 3.1A-D). Moreover, in all cases C-9$^{+/+}$ MEFs died more rapidly compared with C-9$^{-/-}$ MEFs. The strongest incorporation of PI by C-9$^{+/+}$ MEFs was observed upon Etop treatment. However, further investigations with time match controls are warranted in order to fully understand observed changes. Taken together, C-9$^{-/-}$ MEFs undergo a delayed form of cell death that occurs at slower rate.

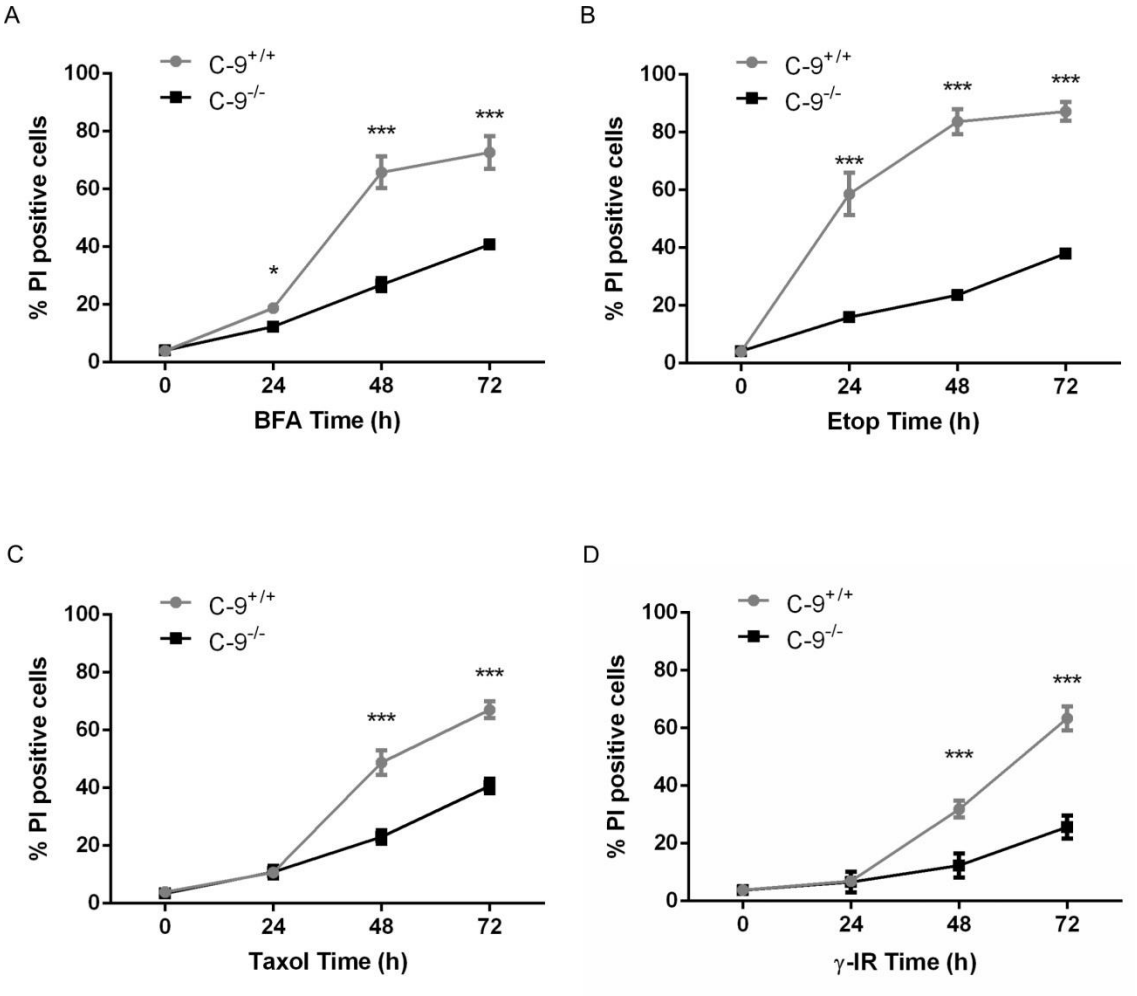


Figure 3.1 **C-9$^{-/-}$ MEFs undergo a delayed cell death in response to various stresses**. C-9$^{+/+}$ and C-9$^{-/-}$ MEFs were treated with (A) 0.3 µg/ml BFA, (B) 50µM Etop, (C) 1µM Taxol and (D) exposed to 33Gy γ-irradiation and left for recovery for indicated time. Cell viability was assessed at the indicated time points using flow cytometry and propidium iodide staining. The values were reported as mean ± SEM of three independent experiments. The significance level are *p<0.05, **p<0.01, ***p<0.001

In order to determine the phenotype of cell death induced by BFA, Etop, Taxol and γ-irradiation in C-9$^{-/-}$ MEFs, the morphological changes were analyzed during prolonged treatment.

At the earliest time point tested, 24 h, C-9$^{-/-}$ MEFs did not display characteristics of cell death changes upon all treatments. However, during the treatments the cells rounded up and lost connection with neighbouring cells. Following, 72 h of BFA, Etop, Taxol and γ-irradiation treatment, C-9$^{-/-}$ MEFs started to display clear morphological changes characteristic of apoptosis such as detachment, and cell shrinkage (Fig. 3.2). However, further investigations with time match controls are warranted in order to fully understand observed changes.

Taken together, the appearance of cell roundness and the abnormal morphology during prolonged treatment with BFA, Etop, Taxol and γ-irradiation was more similar to the morphology of cells dying by apoptosis suggesting that various stresses could trigger apoptotic mode of cell death in C-9$^{-/-}$ MEFs.

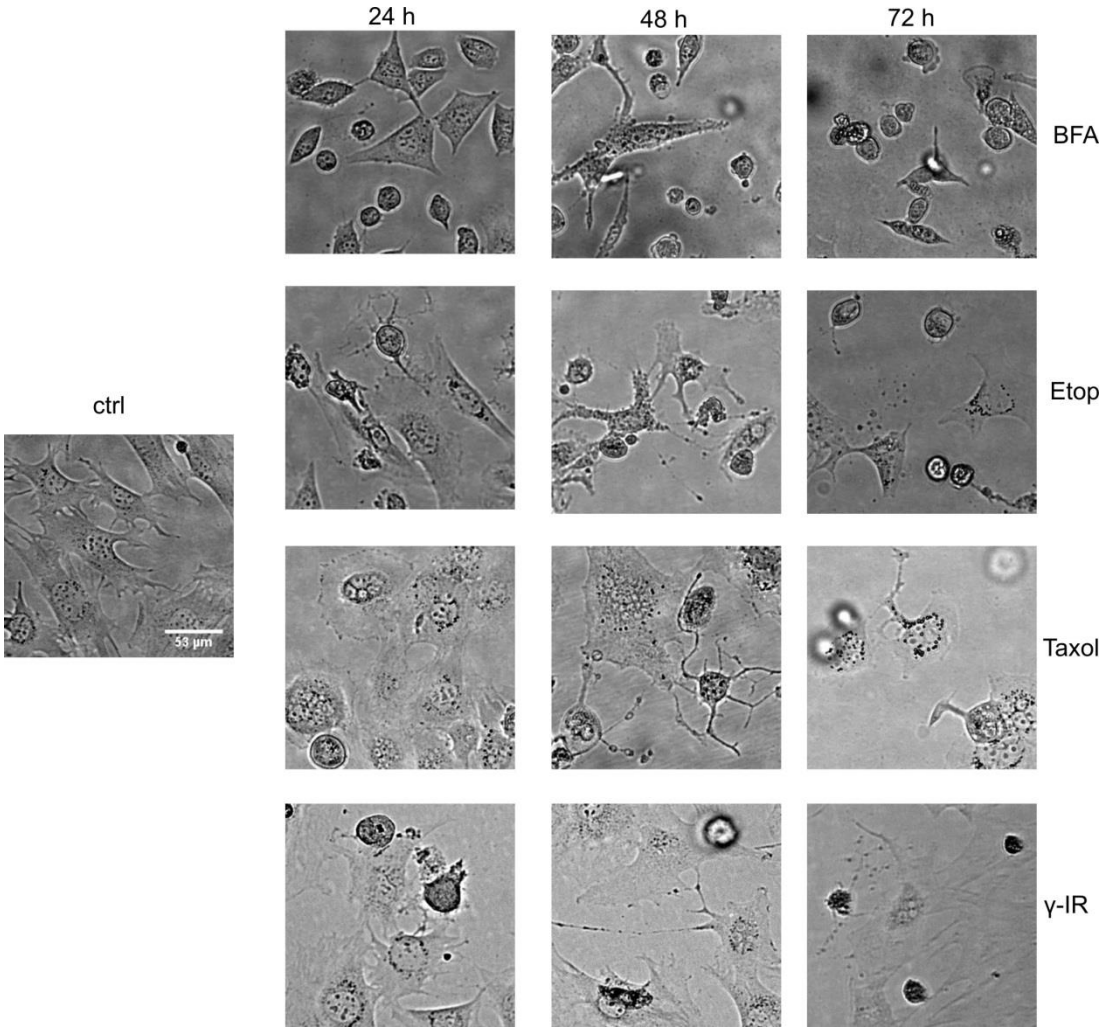


Figure 3.2 **Various treatments lead to cell death in C-9$^{-/-}$ MEFs**. C-9$^{-/-}$ MEFs were treated with 0.3 µg/ml BFA, 50 µM Etop, 1 µM Taxol and exposed to 33 Gy γ-IR and left for recovery for indicated time points and assessed by bright field microscopy; magnification 10X. The results shown are representative of three independent experiments for BFA, Etop and Taxol and two independent experiments for γ-irradiation.

During apoptosis cells display nuclear condensation and DNA fragmentation, which can be detected by staining with Hoechst 33342 and fluorescence microscopy (Purschke et al., 2010). Hoechst 33342 is a blue fluorescent dye that stains the DNA. Therefore to confirm that various types of stress induce apoptosis in C-9$^{-/-}$ MEFs Hoechst 33342 staining was performed. Following prolonged treatment with BFA, Etop or Taxol, apoptotic cells were determined according to the changes in the nuclear morphology including shrinkage, chromatin condensation and fragmentation. Nuclear staining with Hoechst 33342 showed that the control, untreated cells have regular and round nuclei. In contrast, as illustrated in Fig. 3.3, C-9$^{-/-}$ MEFs exhibited numerous cells with fragmented nuclei upon BFA, Etop and Taxol treatment at 72 h of treatment. Interestingly, fragmentation of nuclei and shrinkage was strongly observed also at the early time points upon Taxol treatment. These results, suggest that BFA, Etop and Taxol induce apoptosis in C-9$^{-/-}$ MEFs. The observed nuclei fragmentations also suggest caspase activation. However, further investigations with time match controls are warranted in order to fully understand observed changes.

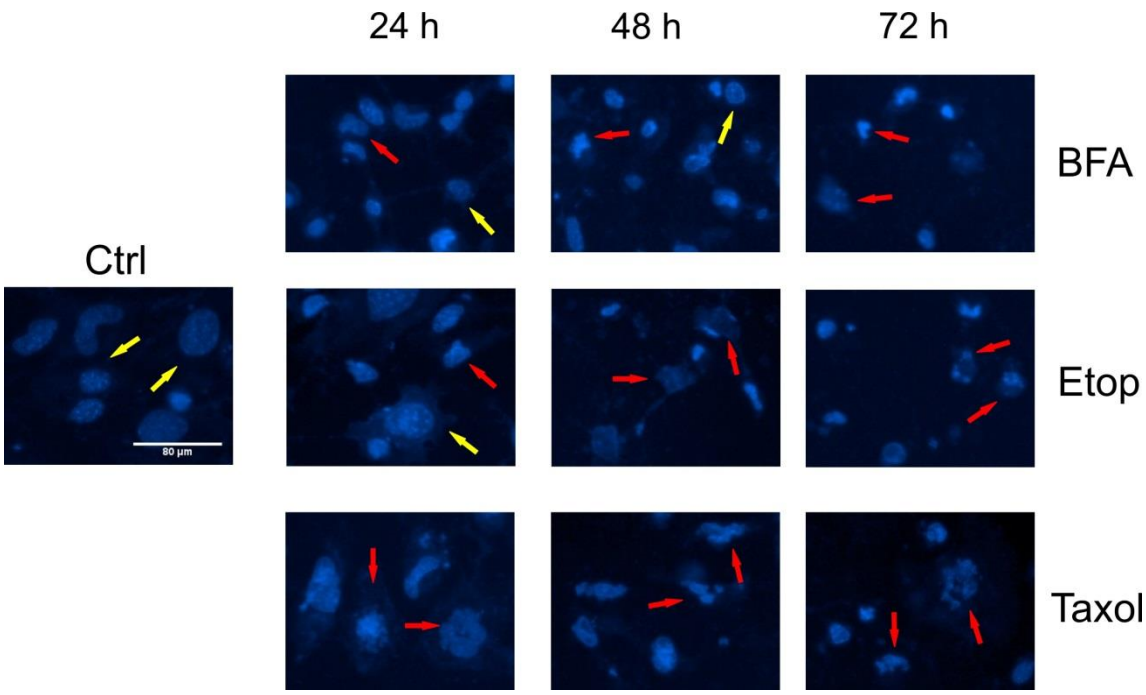


Figure 3.3 **Nuclear morphology of C-9$^{-/-}$ MEFs exposed to prolonged treatment with various stress stimuli**. C-9$^{-/-}$ MEFs were treated with 0.3 µg/ml BFA, 50 µM Etop or 1 µM Taxol for the indicated time points. Cellular DNA was stained with 5 µg/ml Hoechst 33342 for 10 min at room temperature and assessed by fluorescence microscopy; magnification 20X. The results shown are representative of three independent experiments. Yellow arrows indicate healthy nuclei and red arrows depict apoptotic nuclei

3.3.2 Death triggered by diverse cellular stresses in caspase-9$^{-/-}$ MEF cells is associated with caspase-8 and -3 processing

Given that activation of caspases is a hallmark of apoptosis, the processing of caspases was examined in C-9$^{-/-}$ MEFs in response to various types of stress.

As illustrated in Fig. 3.4A-C exposure to sustained treatment with BFA, Etop or Taxol triggered caspase processing in both C-9$^{+/+}$ and C-9$^{-/-}$ MEFs. Although there was more pronounced processing of pro-caspase-8 and caspase-3 in C-9$^{+/+}$ MEFs, there was also a significant processing of both caspases observed at 72 h in C-9$^{-/-}$ MEFs (Fig.3.4). Similarly, there was substantial cleavage of caspase-3 in C-9$^{+/+}$ MEFs compared with C-9$^{-/-}$ MEFs. This suggests that caspase-9 is active and processes caspase-3 in C-9$^{+/+}$ MEFs. In contrast, the observed processing of pro-caspase-8 and caspase-3 in C-9$^{-/-}$ MEFs suggest that various stresses might activate alternate death pathway for caspase processing when the primary route via activation of apoptosome is defective. However, further investigations with time match controls are warranted in order to fully understand observed changes.

Taken together, these data confirm that different types of stresses lead to activation of caspase-8 and caspase-3 in C-9$^{-/-}$ MEFs. Caspase-3 processing occurred in an apoptosome-independent manner but may be associated with caspase-8 activation.

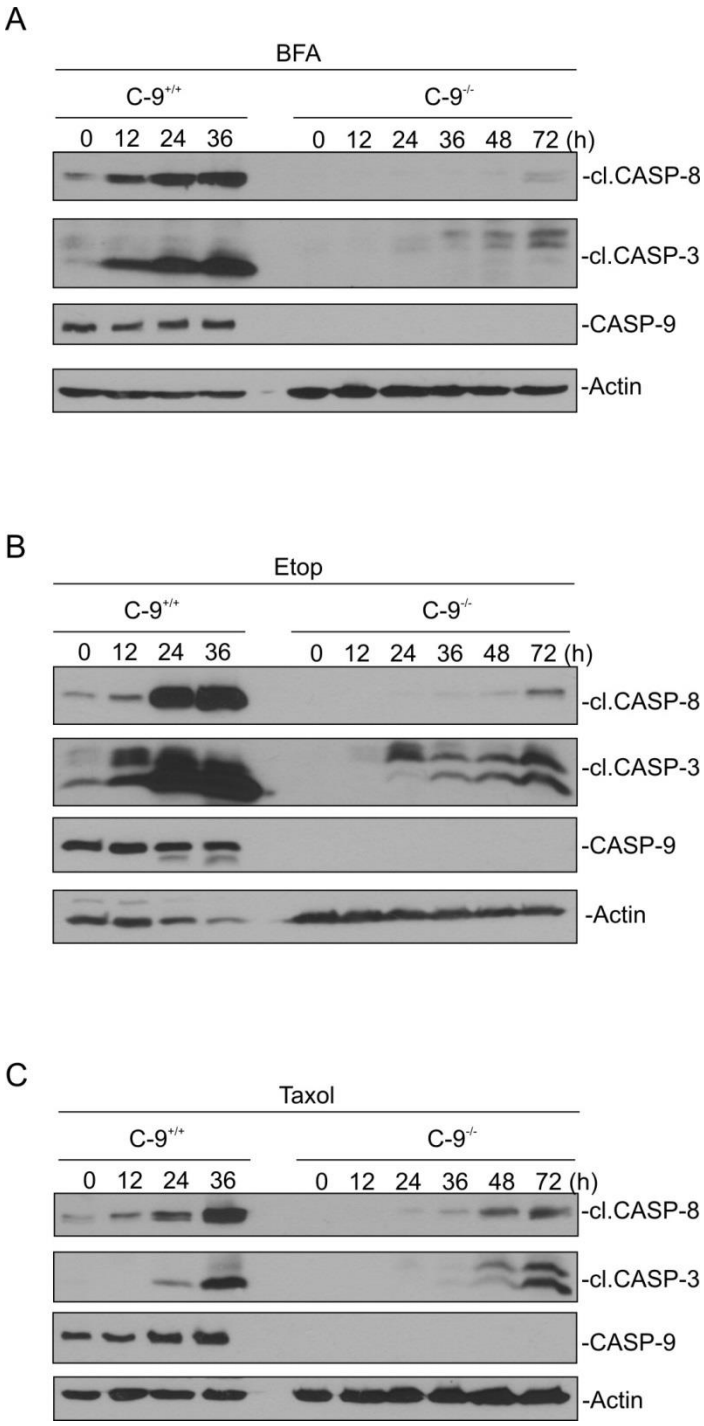


Figure 3.4 **Stress-induced cell death in C-9$^{-/-}$ MEFs is accompanied by caspase-8 and caspase-3 processing.** C-9$^{+/+}$ and C-9$^{-/-}$ MEFs were treated with (A) 0.3 μg/ml BFA, (B) 50 μM Etop and (C) 1 μM Taxol. Protein levels of processed caspase 8 (cl. CASP-8) and caspase-3 (cl. CASP-3), full length caspase-9 (CASP-9) and Actin were assessed at indicated time points by using Western blot. Blots shown are representative of 2 independent experiments.

3.3.3 Knockdown of caspase-8 protects caspase-9$^{-/-}$ MEF from BFA-induced cell death.

To confirm whether the knockdown of caspase-8 would have an effect on ER stress-induced cell death in caspase-9 deficient cells, the stable C-9$^{-/-}$ MEFs transduced with *Casp8* shRNA lentivirus or pLKO empty lentivirus (control transduced cell line), previously generated in our laboratory were used. The morphological changes of BFA-treated C-9$^{-/-}$ MEFs pLKO and C-9$^{-/-}$ MEFs expressing *Casp8* shRNA were first examined. Bright field microscopy images were analyzed at the indicated time points (Fig. 3.5A). Following treatment with BFA, pLKO C-9$^{-/-}$ MEFs died much more rapidly with cell death observed as early as 24 h and almost complete loss of viability at 48 h as illustrated by cell roundness and detachments in Fig. 3.5A. In contrast, *Casp8* shRNA C-9$^{-/-}$ MEFs cells appeared largely refractory to stress as the majority of cells were not affected by long-term treatment. *Casp8* shRNA C-9$^{-/-}$ MEFs started to display morphological signs of cell death at 72h (Fig. 3.5A).

In addition, cell death was examined by PI uptake (Fig. 3.5C). FACS analysis revealed that following treatment with BFA control pLKO C-9$^{-/-}$ MEFs displayed increased cell death over time, reaching around 65% cell death at 72 h. In contrast approximately 25% of cell death at 72 h was observed in *Casp8* shRNA C-9$^{-/-}$ MEFs. This result suggests that knockdown of caspase-8 significantly reduced cell death upon BFA treatment (Fig. 3.5C).

Next, to fully confirm that caspase-8 is the apical caspase in the examined cellular system and is required for activation of effector caspases, Western blot analysis was carried out. Both pLKO C-9$^{-/-}$ and *Casp8* shRNA C-9$^{-/-}$ MEFs were subjected to treatment with BFA for 24 h, 48 h and 72h. Whole cell lysates were harvested at the indicated time points and assessed for caspase-8 and caspase-3 processing, and pro-caspase-8 expression. Western blot analysis revealed complete knockdown of caspase-8, as determined by lack of detection of pro-caspase-8 in the *Casp8* shRNA C-9$^{-/-}$ MEFs, compared with its control pLKO C-9$^{-/-}$ MEF where pro-caspase-8 was observed. As expected, knockdown of caspase-8 resulted in almost complete inhibition of caspase-3

processing confirming that caspase-3 processing occurred in a caspase-8-dependent manner (Fig. 3.5B).

Furthermore, to investigate whether caspase-8 deficiency could provide long term protection against BFA in C-9$^{-/-}$ MEFs, a clonogenic survival assay was carried out after 72h of BFA treatment. As illustrated in Fig. 3.5D the presence of caspase-8 in pLKO C-9$^{-/-}$ significantly reduced formation of colonies compared to the *Casp8* shRNA C-9$^{-/-}$ MEFs. However, loss of caspase-8 enhanced long-term survival and allowed recovery after initial stress. This demonstrates that in C-9$^{-/-}$ MEFs, caspase-8 is important for execution of cell death following treatment with BFA. In summary, these data demonstrate that BFA induced caspase-8 dependent cell death in C-9$^{-/-}$ MEFs. Furthermore, caspase-8 was required for activation of the effector caspase-3.

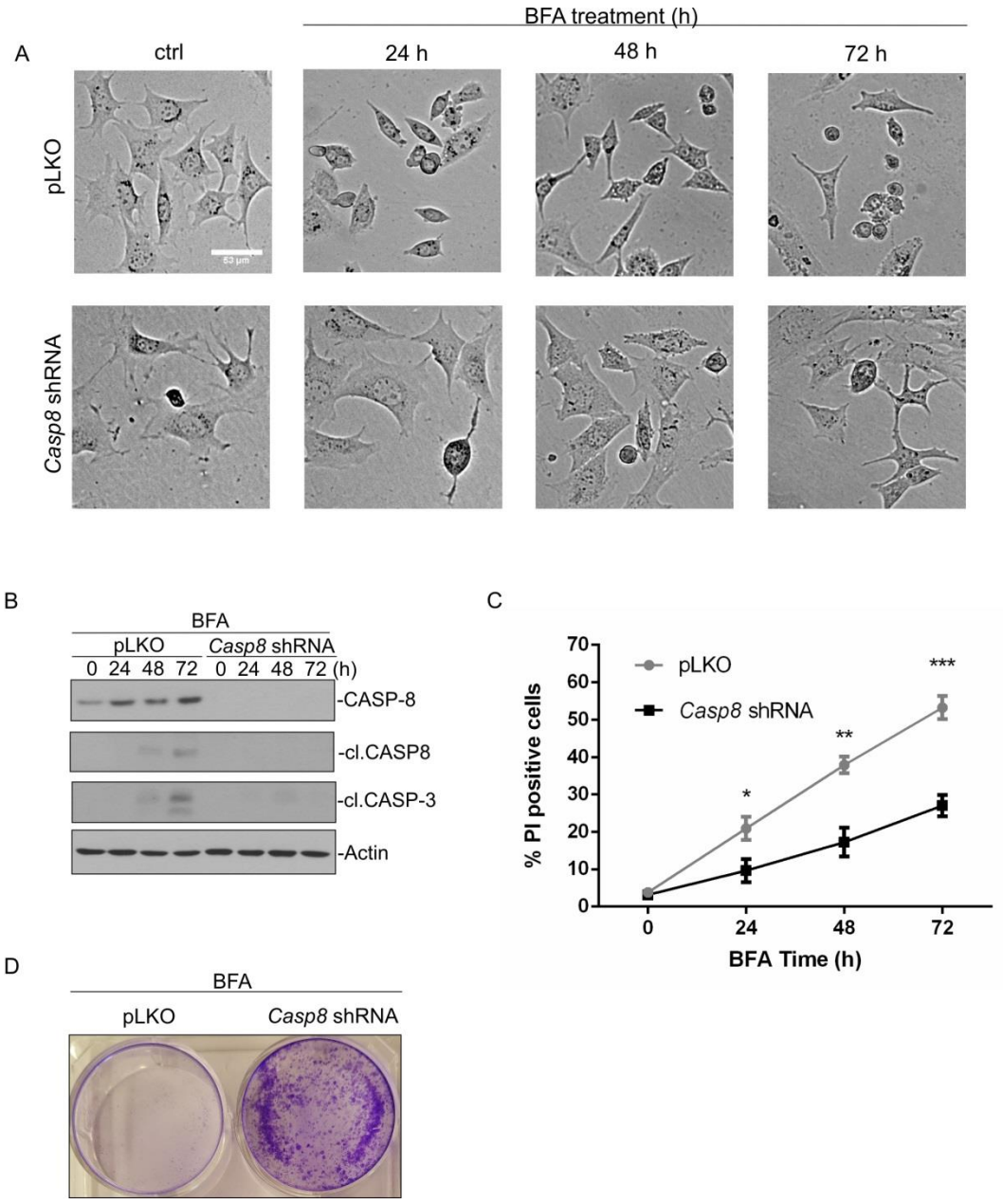


Figure 3.5 **Knockdown of caspase-8 in C-9$^{-/-}$ MEFs protects against prolonged BFA treatment.** (A). pLKO and *Casp8* shRNA C-9$^{-/-}$ MEFs were treated with 0.3 μg/ml BFA for the indicated time points and assessed by bright field microscopy. (B) Whole cell lysates were subjected to Western blot for expression of pro-caspase-8 (CASP-8), cleaved caspase-8 (cl.CASP-8) and caspase-3 (cl.CASP-3) and Actin. (C) Cell viability was assessed at the indicated time points by using flow cytometry and propidium iodide staining. Values represent the mean ± SEM of three independent experiments. The significance levels are*p<0.05, **p<0.01, ***p<0.001. (D) Clonogenic survival of pLKO and *Casp8* shRNA C-9$^{-/-}$ MEFs treated with 0.3 μg/ml BFA for 72 h. After 72 h the treatment was washed off and cells were allowed to form colonies for 7 days. Colonies were stained with crystal violet and picture taken.

3.3.4 Knockdown of caspase-8 reduces processing of caspase-3 and decreases cell death induced by Taxol in caspase-9-/- MEFs

In order to understand the mechanism of cell death induced by Taxol, both pLKO C-9$^{-/-}$ and *Casp8* shRNA C-9$^{-/-}$ MEFs were subjected to prolonged treatment with Taxol. Firstly, the cell death was determined by morphological changes using bright field microscopy. Upon exposure to sustained treatment with Taxol, pLKO control cells displayed cell shrinkage and detachment while *Casp8* shRNA C-9$^{-/-}$ MEFs appeared to be resistant as the majority of cells did not show apoptotic features, suggesting that these cells die in caspase-8 dependent manner. Interestingly, in the absence of caspase-8 cells exhibited increase size and swelling (Fig. 3.6A). Because Taxol affects microtubule network resulting in disruption in cytoskeleton therefore this observation may reflect the mechanism of action of Taxol.

To further determine if the phenotypes observed in *Casp8* shRNA C-9$^{-/-}$ MEFs were due to loss of caspase-8, both pLKO C-9$^{-/-}$ and *Casp8* shRNA C-9$^{-/-}$ MEFs were stained with PI and subjected to FACS analysis. Assessment of cell death showed clear PI uptake in pLKO C-9$^{-/-}$ MEFs following 24 h of Taxol treatment reaching around 45% dead cells within 72 h (Fig. 3.6C). In contrast, there was a significant reduction in PI uptake in *Casp8* shRNA C-9$^{-/-}$ MEFs over the time course, suggesting that caspase-8 is required for Taxol-induced cell death in C-9$^{-/-}$ MEFs.

In the absence of a mitochondria-mediated death pathway it has been shown that caspase-8 is an initiator for caspase cascade in response to intracellular stress (Deegan et al., 2014a, Deegan et al., 2014b, Caro-Maldonado et al., 2010). In order to further specify whether caspase-8 was an apical caspase in Taxol-induced apoptosis in C-9$^{-/-}$ MEFs Western blot analysis was performed. As expected, knockdown of caspase-8 reduced processing of pro-caspase-8 and procaspase-3 (Fig. 3.6B). These results suggest that caspase-8 is an initiator caspase required for activation of downstream effector caspase-3 upon prolonged treatment with Taxol.

In another approach to determine the possible role of the caspase-8 in Taxol-induced cell death in C-9$^{-/-}$ MEFs, the effect of knockdown of caspase-8 on long-term survival

was examined. As illustrated in Fig. 3.6D either presence or absence of caspase-8 did not affect colony formation in Taxol-treated C-9$^{-/-}$ MEFs. There was a slight increase in the number of viable colonies in *Casp8* shRNA C-9$^{-/-}$ MEFs compared with control pLKO C-9$^{-/-}$ MEFs; therefore, it raises the possibility of involvement of caspase-8 in Taxol-induced cell death. Of note, the cytotoxicity of Taxol has been widely studied *in vitro* through clonogenic assay. It has been shown that ability to form colonies varies depending on drug concentration and administration of a higher dose of Taxol resulted in an increase in cell survival (Liebmann et al., 1993). In summary, these data demonstrate that Taxol induces caspase-8 dependent cell death in C-9$^{-/-}$ MEFs. Caspase-8 is required for downstream cleavage of caspase-3 in Taxol treated C-9$^{-/-}$ MEFs.

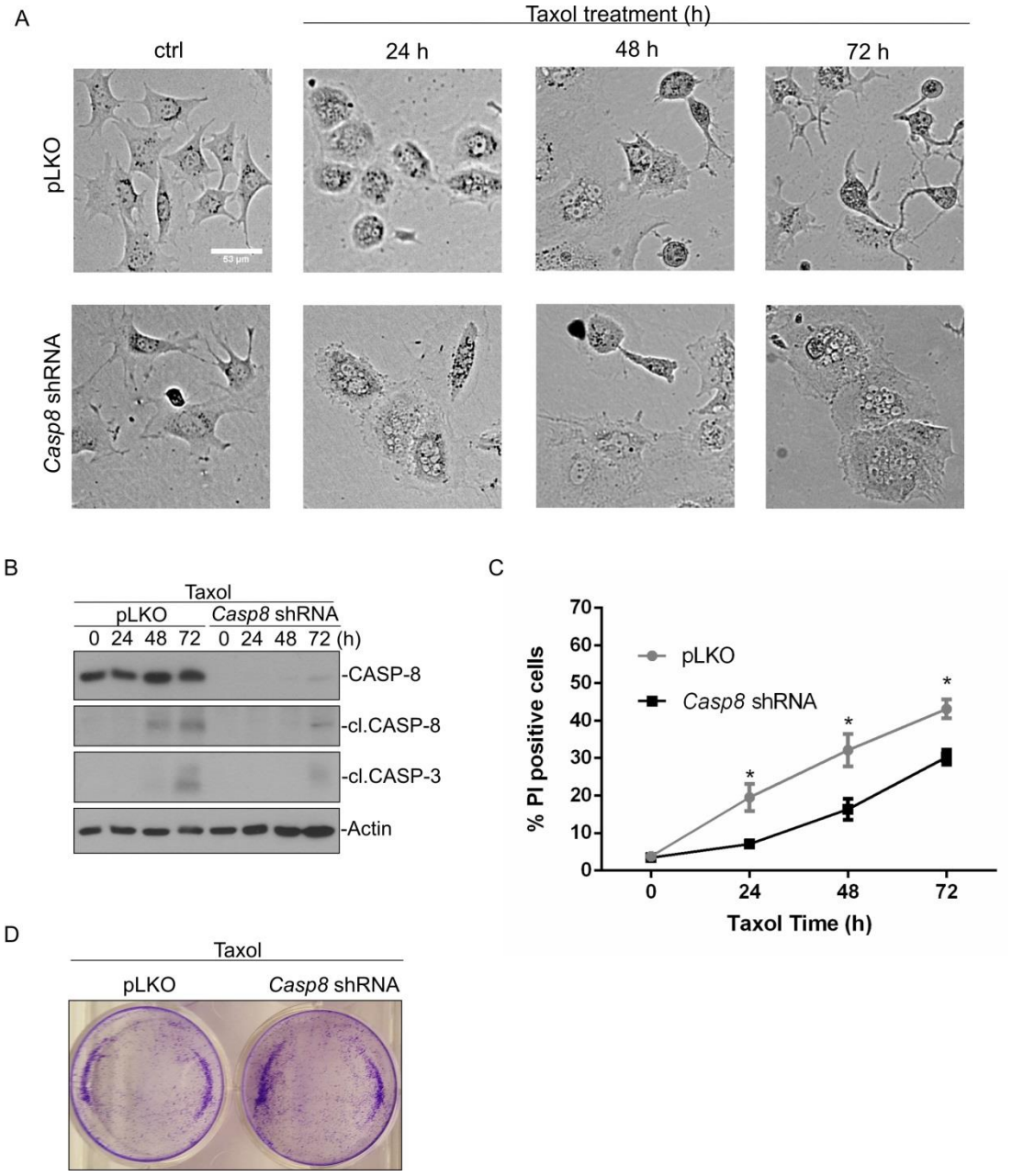


Figure 3.6 **Knockdown of caspase-8 in C-9$^{-/-}$ MEFs protects against prolonged Taxol treatment**. (A). pLKO and *Casp8* shRNA C-9$^{-/-}$ MEFs were treated with 1 μM Taxol for the indicated time points and assessed by bright field microscopy. (B)Whole cell lysates were subjected to Western blot for expression of pro-caspase-8 (CASP-8), cleaved caspase-8 (cl. CASP-8) caspase-3 (cl. CASP-3) and Actin. (C) Cell viability was assessed at the indicated time points by using flow cytometry and propidium iodide staining. Values represent the mean ± SEM of three independent experiments. The significance level is*$p<0.05$. (D) Clonogenic survival of pLKO and *Casp8* shRNA C-9$^{-/-}$ MEFs treated with 1 μM Taxol for 72 h. After 72 h the treatment was washed off and cells were allowed to form colonies for 7 days. Colonies were stained with crystal violet and picture taken.

3.3.5 Knockdown of caspase-8 reduces processing of caspase-3 and decreases cell death induced by γ-irradiation in caspase-9$^{-/-}$ MEFs

To determine whether γ-irradiation induces caspase-8 dependent cell death both pLKO C-9$^{-/-}$ and *Casp8* shRNA C-9$^{-/-}$ MEFs were subjected to γ-irradiation and left for recovery for 24 h, 48 h and 72 h. The morphology of dead pLKO C-9$^{-/-}$ MEFs at 72 h post-treatment displayed changes typical for apoptosis such as: shrinkage, roundness and detachment. In contrast, *Casp8* shRNA C-9$^{-/-}$ MEFs were resistant to γ-irradiation-induced cell death, as exhibited by the normal morphology of the cells (Fig. 3.7A). These data, suggest that cell death induced by γ-irradiation is caspase dependent.

Next, to determine whether caspase-8 is required for processing of downstream executioner caspase-3 in C-9$^{-/-}$ MEFs, Western blot analysis was performed. It was confirmed that knockdown of caspase-8 reduced processing of caspase-3 (Fig. 3.7B). These results demonstrate that activation of effector caspase-3 requires upstream initiator caspase-8. Given that caspases are hallmark of apoptosis, the next aim was to fully verify that observed activation of caspases contribute to cell death. To test whether γ-irradiation-induced cell death is triggered in a caspase-8-dependent manner PI staining was performed on both pLKO C-9$^{-/-}$ MEFs and *Casp8* shRNA C-9$^{-/-}$ MEFs. As expected loss of caspase-8 significantly reduced cell death over time. *Casp8* shRNA C-9$^{-/-}$ MEFs seemed to be protected against γ-irradiation stress. However, although the results were found statistically significant, the overall trend show that both pLKO C-9$^{-/-}$ MEFs and *Casp8* shRNA C-9$^{-/-}$ MEFs slowly die reaching approximately of 25% of dead cells in the presence of caspase-8 and around 15% when caspase-8 was absent (Fig. 3.7C). The observed low level of cell death suggests that γ-irradiation may require the intrinsic apoptotic pathway and subsequent apoptosome formation to efficiently induce cell death.

Additionally, to identify whether knockdown of caspase-8 rescued cells already exposed to γ-irradiation treatment measurement of cell ability for long-term survival was performed. Indeed, irradiation of both pLKO C-9$^{-/-}$ MEFs and *Casp8* shRNA C-9$^{-/-}$ MEFs with 33Gy was able to slightly increase colony formation in *Casp8* shRNA C-9$^{-/-}$

MEFs compare with pLKO C-9$^{-/-}$ MEFs, thus raising the possibility of involvement of caspase-8 in γ-irradiation-induced cell death. Taken together, γ-irradiation induces cell death associated with caspase-8 activation in C-9$^{-/-}$ MEFs. Caspase-8 is required for activation of the executioner caspase-3 and subsequent cell death.

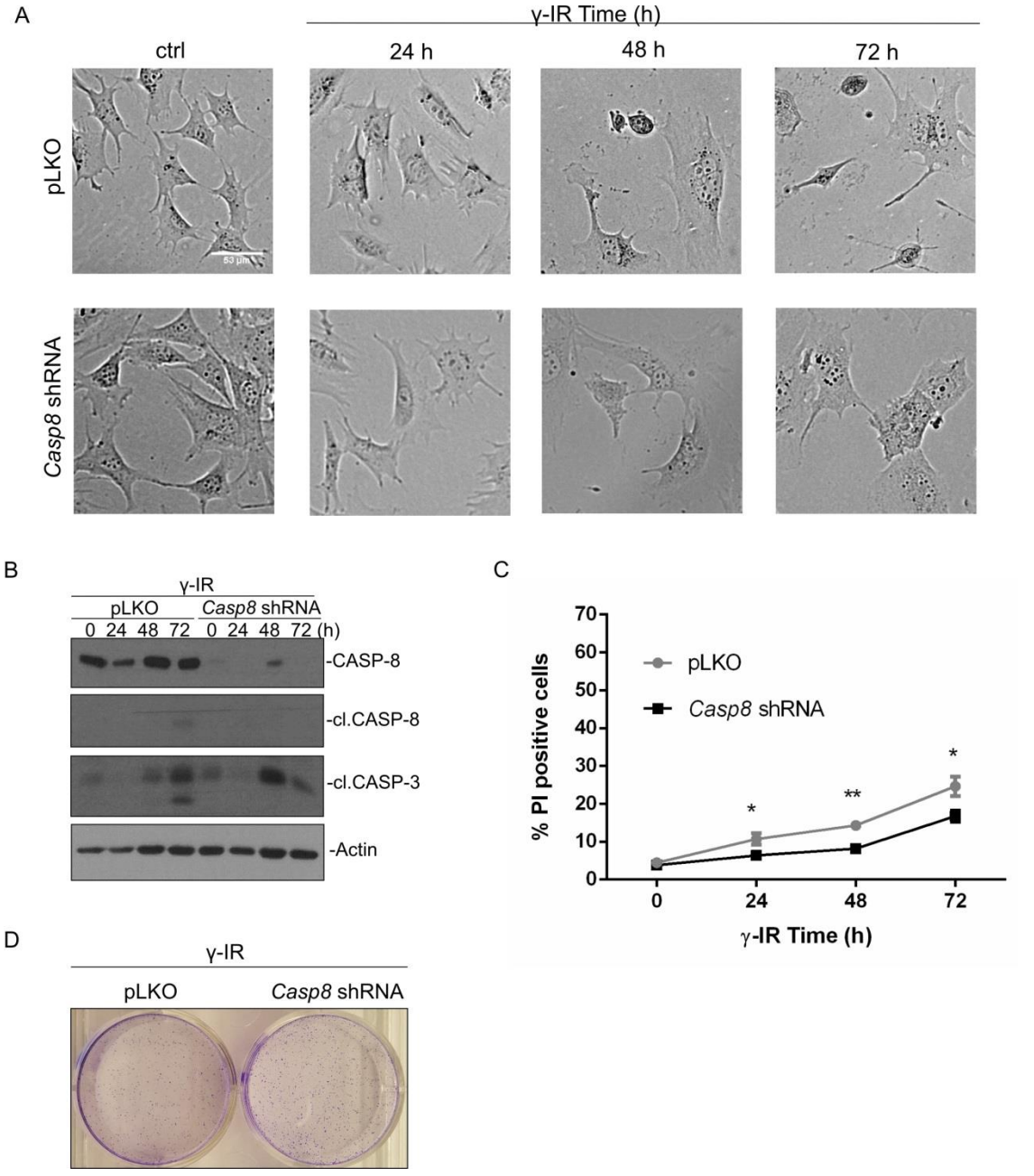


Figure 3.7 **Knockdown of caspase-8 in C-9$^{-/-}$ MEFs protects against prolonged γ-irradiation treatment.** (A). pLKO and *Casp8* shRNA C-9$^{-/-}$ MEFs were treated with 33Gy γ-IR for the indicated time points and assessed by bright field microscopy. (B) Whole cell lysates were subjected to Western blot for expression of pro-caspase-8 (CASP-8), cleaved caspase-8 (cl. CASP-8) caspase-3 (cl. CASP-3) and Actin. (C) Cell viability was assessed at the indicated time points using flow cytometry and propidium iodide staining. Values represent the mean ± SEM of three independent experiments. The significance levels are*p<0.05, **p<0.01. (D) Clonogenic survival of pLKO and *Casp8* shRNA C-9$^{-/-}$ MEFs treated with 33Gy γ-IR for 72 h. 72 h post-treatment media was washed off and cells allowed to form colonies for 7 days. Colonies were stained with crystal violet and picture taken**.**

3.3.6 Knockdown of caspase-8 reduces processing of caspase-3 induced by Etop but does not decrease cell death in caspase-9$^{-/-}$ MEFs

To investigate whether cell death induced by Etop occurs in caspase-8 dependent manner in C-9$^{-/-}$MEFs, control pLKO and *Casp8* shRNA C-9$^{-/-}$ MEFs were subjected to prolonged treatment with Etop.

Bright field microscopy images were analyzed at the indicated time points. As assessed by morphology, prolonged treatment with Etop led to cell death of both pLKO and *Casp8* shRNA C-9$^{-/-}$ MEFs. Moreover, the features of apoptotic cell death were already observed at early stages of treatment with Etop in pLKO, while in *Casp8* shRNA C-9$^{-/-}$ MEFs cell death appeared at 72 h (Fig. 3.8A). Knockdown of caspase-8 did not protect cells against Etop treatment as the majority of cells displayed roundness and detachment similar to the pLKO control. Morphological examination suggests that caspase-8 is not required for cell death induced by prolonged treatment with etoposide in C-9$^{-/-}$ MEFs.

To further determine the role of caspase-8 in cell death both pLKO and *Casp8* shRNA C-9$^{-/-}$ MEFs were stained with PI and subjected to flow cytometry analysis. As illustrated in Fig. 3.8C knockdown of caspase-8 significantly reduced cell death induced by Etop at 24 h and 48 h of treatment, whereas sustained exposure to Etop lead to cell death of both pLKO and *Casp8* shRNA C-9$^{-/-}$ MEFs reaching approximately 35% dead cells at 72 h (Fig. 3.8C). Although analysis of cell death at 72 h was not statistically significant there was still a clear trend that reduction of caspase-8 leads to a loss of potency to protect cells against Etop over prolonged treatment.

Although, these results do not support a role of caspase-8 in Etop-induced cell death, it does not mean that caspase-8 is not involved. Indeed, my previous data show that Etop induce caspase-dependent mode of cell death in C-9$^{-/-}$ MEFs. Therefore to delineate the importance of caspase-8 activation Western blot analysis was performed. As illustrated in Fig. 3.8B complete knockdown of caspase-8 was determined by lack of detection of pro-caspase-8 in the *Casp8* shRNA C-9$^{-/-}$ MEFs, compared to its control pLKO C-9$^{-/-}$ MEF. Moreover, knockdown of caspase-8 resulted in almost complete inhibition of both

pro-caspase-8 and caspase-3 processing indicating that upon Etop treatment caspase-3 processing occurred in a caspase-8-dependent manner.

To determine whether knockdown of caspase-8 directly affected the long-term survival of C-9$^{-/-}$ MEFs exposed to Etop, a clonogenic assay was performed after 72 h of treatment. Since Etop treatment leads to DNA damage its application can inhibit ability of cells to proliferate and division and thus limit clonogenicity. As illustrated in Fig. 3.8B knockdown of caspase-8 did not enhance long-term survival. Moreover, treatment with Etop blocked formation of colonies in both pLKO and *Casp8* shRNA C-9$^{-/-}$ MEFs and did not allow for recovery. This suggests that prolonged treatment with Etop might lead to an irreversible effect that is lethal for C-9$^{-/-}$ MEFs independent on caspase-8.

Taken together, these results show that although caspase-8 is not required for cell death in C-9$^{-/-}$ MEFs upon Etop treatment, it is indeed important for downstream effector caspase activation. Knockdown of caspase-8 in C-9$^{-/-}$ MEFs appears to protect for an initial phase of treatment, however cells eventually die when subject to prolonged exposure to Etop.

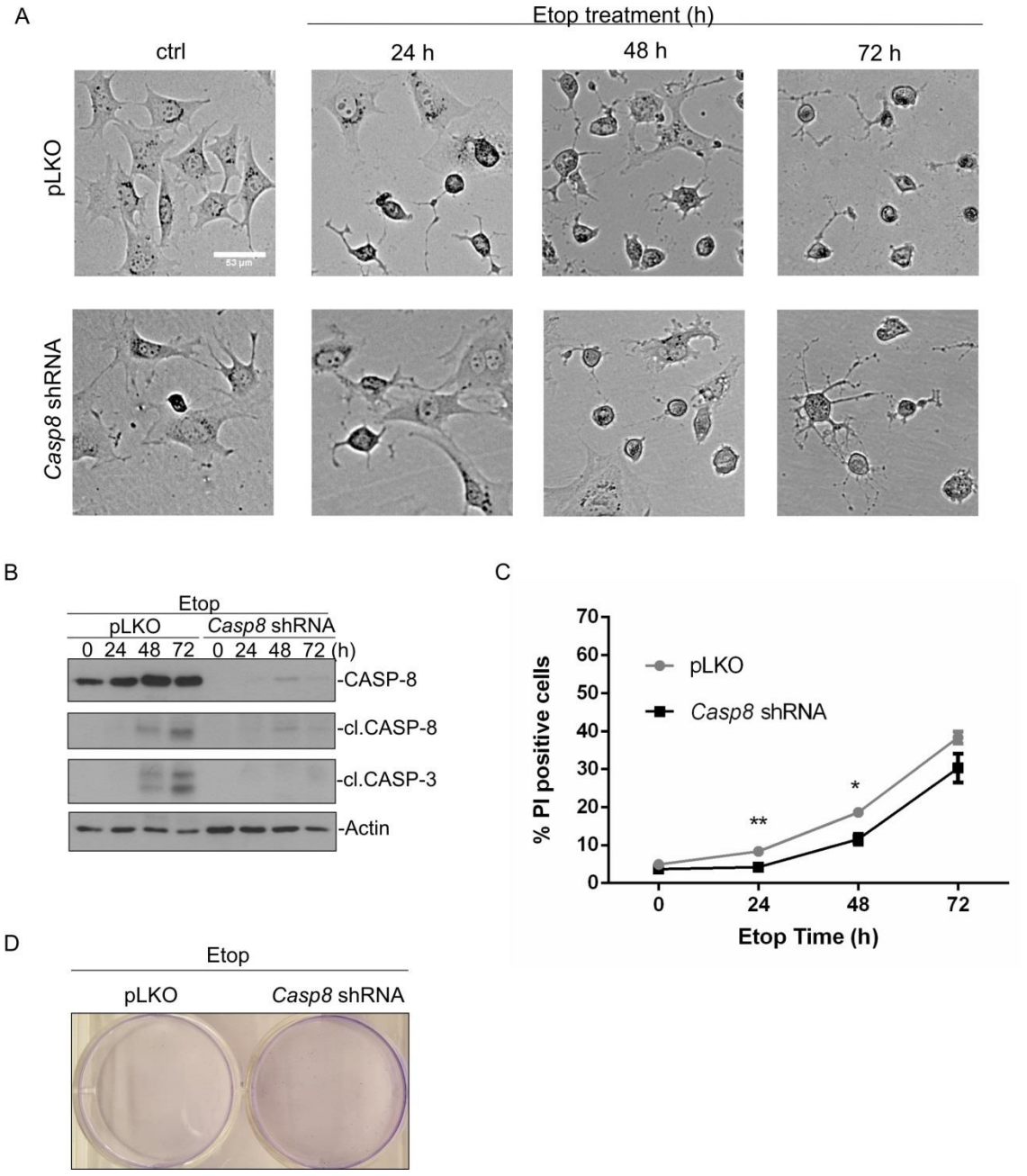


Figure 3.8 **Knockdown of caspase-8 in C-9$^{-/-}$ MEFs cells does not protect against prolonged Etop treatment.** (A) pLKO and *Casp8* shRNA C-9$^{-/-}$ MEFs were treated with 50 μM Etop for the indicated time points and assessed by bright field microscopy. (B) Whole cell lysates were subjected to Western blot for expression of pro-caspase-8 (CASP-8), cleaved caspase-8 (cl. CASP-8) caspase-3 (cl. CASP-3) and Actin. (C) Cell viability was assessed at the indicated time points using flow cytometry and propidium iodide staining. Values represent the mean ± SEM of three independent experiments. The significance levels are*p<0.05, **p<0.01. (D) Clonogenic survival of pLKO and *Casp8* shRNA C-9$^{-/-}$ MEFs treated with 50μM Etop for 72 h. After 72 h the treatment was washed off and cells allowed to form colonies for 7 days. Colonies were stained with crystal violet and picture taken.

3.4 Discussion

Apoptosis is the preferential mode of cell death induced by chemotherapeutics and radiation. It is already well established that mitochondria are essential for the execution of cell death induced by most apoptotic stimuli. However, in many instances, cancer cells develop resistance to conventional therapy like chemotherapeutic drugs and radiotherapy. Indeed, malfunctions in the mitochondrial-mediated pathway are common in tumor cells and are associated with resistance to most apoptotic stimuli (Miyashita and Reed, 1993, Lotem and Sachs, 1993). The ability of cancer cells to proliferate and evade cell death is then a hallmark of cancer. Therefore, an understanding of alternative mechanisms of cell death would facilitate the identification of novel targets that can be therapeutically exploited to overcome drug resistance.

Previous work in our laboratory has demonstrated the existence of a novel and alternative stress-induced cell death pathway that could be exploited for the treatment of highly chemoresistant cancer cells (Deegan et al., 2014b). The study of Deegan *et al*. identified an atypical caspase-8 activation pathway under sustained pharmacological ER stress in cells lacking the principle components of the apoptotic pathway. Using the knockdown of caspase-8 in C-9$^{-/-}$ MEFs it was shown that caspase-8 acts as the initiator caspase which is required for both effector caspase activation and cell death in this system. Generally, activation of caspase-8 is dependent on ligation of death receptors (Ashkenazi and Dixit, 1998). However, in this system blockade of death receptor signalling showed that activation of capsase-8 did not depend on activation of the extrinsic pathway (Deegan et al., 2014b). Additionally, application of the general caspase inhibitor Boc-d-fmk to C-9$^{-/-}$ MEFs treated with the ER stress-inducing agents tunicamycin and thapsigargin resulted in reduced processing of caspase-3 implicating caspase-8 as the apical caspase in this examined cellular system. Despite these intriguing findings, it remains unclear whether this novel pathway is specific to ER stress or represents a more general response that is activated in response to a wide range of different stresses. During treatment, cancer cells are exposed to various types of stresses such as genotoxic stress and ER stress as well as γ-irradiation. Therefore, to identify the molecular mechanisms underlying the alternative cell death pathways

activated in response to various commonly used chemotherapeutics, cells devoid of functional caspase-9 were used in this chapter.

Mak and colleagues demonstrated that cells lacking functional caspase-9 are resistant to a wide range of pro-apoptotic insults, highlighting the importance of caspase-9 in the mitochondrial pathway (Hakem et al., 1998). This is also supported by studies showing that caspase-9-deficient MEFs are protected from Taxol-induced apoptosis (Janssen et al., 2007). Furthermore, cells devoid of caspase-9 were also shown to be resistant to ER-stress, suggesting that caspase-9 is indispensable for stress-induced apoptosis (Deegan et al., 2014a). Several novel pathways have been identified that might explain how cells defective in the intrinsic apoptotic pathway trigger cell death when exposed to various stresses. However, little is known about exposure to chronic stress. Similarly, there is little known about the mode of cell death used by cells lacking a functional mitochondrial death pathway when subjected to stress. Since a variety of cellular stress stimuli lead to disruption of homeostasis and subsequent cell death, elucidating the molecular mechanisms underlying stress-induced cell death in resistant cells is of high importance.

In this study, I first investigated whether various stresses lead to cell death in C-9$^{-/-}$ MEFs. Consistent with a previous study from our laboratory, the ER stress-inducing agent BFA was used in parallel with other stimuli such as Etop, Taxol and γ-irradiation. Although all chosen stimuli have different modes of action, they have all been shown to induce apoptosis in various cellular settings. Of note, in the analysis presented in this chapter, the results were compared with control (untreated) at time zero. The treated samples were harvested at the indicated time and the changes caused by the treatments, either in cell death as determined by PI uptake or expression of proteins of interest as determined by Western blot, were analysed to the start control. However, further investigations with time match controls are warranted in order to fully understand observed changes.

Following the kinetics of cell death induction in isogenic C-9$^{+/+}$ and C-9$^{-/-}$ MEFs confirmed that C-9$^{-/-}$ MEFs displayed reduced cell death when exposed to various stress-

inducing agents as determined by PI uptake. Moreover, it was shown that cells defective in the mitochondrial apoptotic pathway underwent a delayed form of cell death that occurred at slower rate, in reponse to different stress stimuli. C-9$^{-/-}$ MEFs appeared to be protected for the initial phase of treatment; however, they eventually died in response to prolonged exposure to various stresses.

Following prolonged treatment with different stimuli, changes in morphology characteristic of apoptosis were also observed. Therefore, to clarify whether apoptosis is the primary mode of cell death in this system other methods for apoptosis detection such as Hoechst staining were used. The obtained results clearly suggest that BFA, Etop, and Taxol were all capable of inducing chromatin condensation and nuclei fragmentation.

As it remains unclear whether stress-induced cell death in cells defective in the mitochondrial-mediated pathway is caspase-dependent or caspase-independent, caspase processing was analyzed upon prolonged exposure to cellular stress. These data show that the absence of caspase-9 did not prevent caspase-3 processing completely in C-9$^{-/-}$ MEFs. Indeed, there was a significant activation of caspase-3 detected at 48 h and 72 h in response to BFA, Etop and Taxol treatments in C-9$^{-/-}$ MEFs. Caspase-3 is an executioner caspase and thus requires processing by an apical caspase for its full activation. Detection of caspase-8 processing confirmed its role in this system. Indeed, processing of caspase-8 was noted to slowly increase over time reaching a maximum at 72 h in response to BFA, Etop and Taxol treatments in C-9$^{-/-}$ MEFs. Furthermore, through the use of a matched pair of C-9$^{+/+}$ and C-9$^{-/-}$ MEFs, I provided evidence that C-9$^{-/-}$ MEFs suppress the stress-induced activation of caspase-3. At the same time, I detected pronounced processing of caspase-3 in C-9$^{+/+}$ MEFs suggesting that activation of this caspase occurs via the canonical intrinsic pathway. Therefore, based on those findings, it seems that an alternative death pathway is involved in caspase processing and subsequent cell death in response to BFA, Etop and Taxol treatments in C-9$^{-/-}$ MEFs when the primary, canonical mitochondria-mediated pathway is compromised. Together, the presented data showed that C-9$^{-/-}$ MEFs were initially resistant to stress-induced cell death; however, they eventually succumbed to cell death in response to prolonged cellular stress. The delayed mode of cell death observed in C-9$^{-/-}$ MEFs was

associated with caspase processing. Furthermore, these data also suggest that various stresses trigger caspase-8 processing in the absence of caspase-9.

A previous study reported that several anticancer drugs such as daunorubicin, doxorubicin or etoposide led to activation of caspase-8 in the absence of death receptor signaling in Jurkat cells (Wesselborg et al., 1999). The possibility that caspase-8 activation might be triggered by another pathway independent of classical death receptor signaling in response to ER stress was demonstrated by the groups of Vandenabeele and Bertrand (Estornes et al., 2014). This is also in agreement with our previous study which introduced a novel caspase-8 activation pathway independent of death receptor ligation in cells compromised in the mitochondrial-mediated death pathway when exposed to sustained ER stress (Deegan et al., 2014b). Furthermore, Caro-Maldonado and colleagues also previously demonstrated that Bax/Bak-deficient MEFs undergo caspase-8-mediated apoptosis upon glucose deprivation (Caro-Maldonado et al., 2010). Similarly, the study of Laussmann *et al* identified caspase-8 as the apical caspase in Bcl-2-overexpressing human cancer cells that are resistant to proteasome inhibition (Laussmann et al., 2011). Consistent with these reports, my data provided convincing evidence that BFA-, Taxol- and γ-irradiation-mediated cell death relies on caspase-8 activation. Indeed, knockdown of caspase-8 significantly inhibited cell death upon prolonged treatment with all three stimuli and, moreover, it also reduced processing of the downstream effector caspase-3. These results confirm that caspase-3 processing in C-9$^{-/-}$ MEFs is dependent on caspase-8 activation. Interestingly, it was noted that although deficiency of caspase-8 in C-9$^{-/-}$ MEFs protected against cell death upon prolonged stress, it had a different effect on long-term survival in response to various stresses. While upon BFA treatment, clonogenic assays clearly demonstrated a survival advantage of caspase-8-deficient C-9$^{-/-}$ MEFs compare with the control, caspase-8 deficiency did not protect efficiently against Taxol and γ-irradiation. Indeed, although the loss of caspase-8 in C-9$^{-/-}$ MEFs provided long-term protection against Taxol, the same response was observed in the control, however to a slightly lesser extent. Based on this observation, I cannot rule out the possible involvement of caspase-8 in Taxol-mediated cell death. However, as already mentioned in the results section, the effect of Taxol is not only dose-dependent, but may also be cell-type specific. It has been shown

that either caspase-9 deficiency or Apaf-1 deficiency confers long-term stress resistance on MEFs, whereas deficiency of caspase-9 was unable to rescue Jurkat cells upon Taxol treatment (Janssen et al., 2007). Additionally, Taxol, as an inhibitor of microtubule depolymerisation also affects the mitotic spindle, leading to mitotic arrest and subsequent apoptosis (Yvon et al., 1999). Therefore, upon drug removal during long-term survival assays, some cells might escape the mitotic arrest and regain their proliferative capacity resulting in a survival advantage. Furthermore, knockdown of caspase-8 in C-9$^{-/-}$ MEFs protected against Taxol-induced cell death as determined by morphological changes. However, the observed increase in size and swelling of cells may be a consequence of Taxol's known effects on the microtubules, or due to the cells undergoing a different mode of cell death in the absence of caspase-8. This is in agreement with the cell death analysis, as although knockdown of caspase-8 in C-9$^{-/-}$ MEFs significantly protected against prolonged Taxol treatment, caspase-8-deficient C-9$^{-/-}$ MEFs were still dying. Therefore, the observed swelling in the absence of caspases could indicate that damage induced by Taxol was too severe and cells ultimately underwent either necrosis or necroptosis. However, further investigations with time match controls are warranted in order to fully understand observed changes.

Although γ-irradiation does exert anti-proliferative effects, which could potentially result in limited clonogenicity, my results showed that after exposure to that stress cells were able to form colonies ether in the absence or presence of caspase-8 in C-9$^{-/-}$ MEFs. However, similarly to Taxol treatment, there was a slight increase in the number of viable colonies observed in caspase-8-deficient C-9$^{-/-}$ MEFs. Therefore, it is difficult to precisely determine the effect of caspase-8 knockdown on cell survival in response to γ-irradiation. However, the possibility that caspase-8 may be partially involved is demonstrated by Western blot analysis; thus, its role cannot be ruled out by these data. Of note, there are also differences in caspase-3 processing upon γ-irradiation compared with other stresses, observed in caspase-8-deficient C-9$^{-/-}$ MEFs. Caspase-3 is an executioner caspase that is constitutively synthetized as an inactive pro-enyzme (zymogen). Pro-caspase-3 (35 kDa) consists of a pro-domain, large subunit (17 kDa) and small subunit (12 kDa) (Liu et al., 2005). Processing of pro-caspase-3 occurs in a two-step process. The first reaction requires involvement of the initiator caspases such

as caspase-8, -10 ,-9 or -2, leading to generation of an intermediate fragment p19, consisting of large subunit p17 and pro-domain (Kavanagh et al., 2014). The first reaction also leads to the release of the fragment p12 which, together with p19, creates a p19/p12 heterodimer. In the second reaction the processing of p19 to p17 is carried out during autocatalytic processing leading to removal of pro-domain and generation of fully active p17 fragment (Kavanagh et al., 2014, Liu et al., 2005, Martin et al., 1996). Interestingly, upon γ-irradiation, although knockdown of caspase-8 in C-9$^{-/-}$ MEFs reduced processing of caspase-3, the intermediate p19 fragment of caspase-3 was still observed. This could be explained by the possibility that, in the absence of caspase-8, partial processing of pro-caspase-3 occurs due to activity of other caspases such as caspase-2. This could be possible as it has been shown that in response to DNA damage caspase-2 is involved in apoptosis (Bouchier-Hayes, 2010, Giagkousiklidis et al., 2005). Moreover, γ-irradiation activates the intrinsic apoptotic pathway, where apart from cytochrome c, there is also release of other apoptotic factors such as Smac/DIABLO or HtrA2/Omi from mitochondria into the cytosol, which are also involved in caspase-3 activation (Wang and Youle, 2009). Indeed, it has been shown that Smac/DIABLO enhanced processing of pro-caspase-3 in γ-irradiated SH-EP neuroblastoma cells (Giagkousiklidis et al., 2005). Alternatively, processing of pro-caspase-3 to its intermediate fragment can also occur in a granzyme B-dependent manner, as has been shown in Jurkat cells (Martin et al., 1996). However, the lack of appearance of the p17 fragment observed in caspase-8-deficient C-9$^{-/-}$ MEFs upon gamma irradiation could also indicate that the autocatalytic cleavage of immature p19 fragment is blocked, perhaps due to inhibition by IAPs (Roy et al., 1997). Indeed, it has been recently shown that the conversion of the intermediate p19 to p17 fragment of caspase-3 is controlled by the cIAP2 in microglia cells (Kavanagh et al., 2014). Additionally, the activity of processed caspase-3 has been shown to be inhibited by XIAP upon DNA damage (Datta et al., 2000). However, further investigations with time match controls are warranted in order to fully understand observed changes.

The requirement for caspase-8 for Etop-induced cell death in C-9$^{-/-}$ MEFs was also examined. Neither morphological observation nor quantification of cell death through PI uptake revealed any protection in caspase-8-deficient C-9$^{-/-}$ MEFs. However, it is

interesting that knockdown of caspase-8 resulted in clear resistance to cell death over the initial 24 h to 48 h of stress, while prolonged treatment with Etop eventually resulted in cell death. This observation indicates that the duration of exposure had an effect on Etop cytotoxicity. Moreover, the fact that at the initial phase of stress cells were significantly protected raises the possibility that knockdown of caspase-8 may switch Etop-induced apoptosis to another form of cell death. My experiments showed that caspase-8 is required for the activation of downstream effector caspases. Indeed, loss of caspase-8 in C-9$^{-/-}$ MEFs reduced processing of caspase-3. Therefore, these results together with PI analysis clearly suggest that in the absence of caspase-8 another, caspase-independent form of cell death occurs.

However, and interesting to note that the processing of caspase-3 upon Etop differs from γ-irradiation treated caspase-8-deficient C-9$^{-/-}$ MEFs. While in γ-irradiated caspase-8-deficient C-9$^{-/-}$ MEFs the intermediate of caspase-3 p19 was detected, this was not observed in response to Etop. Indeed, the knockdown of caspase-8 in C-9$^{-/-}$ MEFs almost completely reduced caspase-3 processing upon Etop treatment. This can be explained by the fact that, although in response to both treatments caspase-8 is activated and is required for processing of caspase-3, it seems that prolonged treatment with Etop in caspase-8-deficient C-9$^{-/-}$ MEFs might lead to an irreversible effect and the damage inflicted on the cell is too severe, therefore the cells ultimately induce caspase-independent cell death. Moreover, deficiency of caspase-8 was not able to rescue C-9$^{-/-}$ MEFs after Etop as determined by clonogenic survival. These data provide evidence that caspase-8 is not required for Etop-induced cell death. Thus, although Etop drives caspase-8-dependent apoptosis, in the absence of caspase-8 cells can switch to a caspase-independent mode of cell death. It has been already shown that loss of caspase-8 leads to RIP3-dependent necroptosis (Kaiser et al., 2011).

The data presented in this chapter provide new, additional information about the existence of an alternative mode of cell death induced in response to various stresses. The study also clearly reveals the importance of the mitochondrial-mediated pathway for the execution of stress-induced cell death. The results also show that when the intrinsic apoptotic pathway is compromised cells can activate an alternative death

pathway which effectively trigger cell death but with slower kinetics. The data also highlight the involvement of caspase-8 in this alternative, stress-induced caspase activation pathway in response to various stresses which constitutes a compensatory death pathway in cells devoid of functional caspase-9. Additionally, the data here show that, depending on the stress-stimulus, cells can either utilize caspase-8 to execute apoptotic cell death or switch to a non-apoptotic mode of cell death. These results may explain how cells defective in the intrinsic apoptotic pathway execute cell death when subjected to prolonged treatment with different chemotherapeutics, implicating a novel and alternative caspase activation pathway as a potential target for the treatment of highly resistant cancer cells.

In summary, here it has been shown that various apoptotic stimuli induce cell death in caspase-9-deficient cells. A delayed mode of cell death associated with the processing of caspase-8 and caspase-3 was identified in response to different stresses. These data also reveal that caspase-8 acts as the initiator caspase and is required for processing of caspase-3 upon treatment with different stresses (Fig. 3.9). Importantly, the data reveal that the involvement of caspase-8 in the alternative death pathway is stress-stimulus specific. Under conditions of caspase-9 deficiency, exposure to BFA, Taxol, and γ-irradiation triggers caspase-8-dependent cell death, while Etop drives a caspase-8-independent mode of cell death. However, there remain unanswered questions about how is caspase-8 activated in this alternative death pathway. What are the other components of this novel caspase-mediated death pathway?

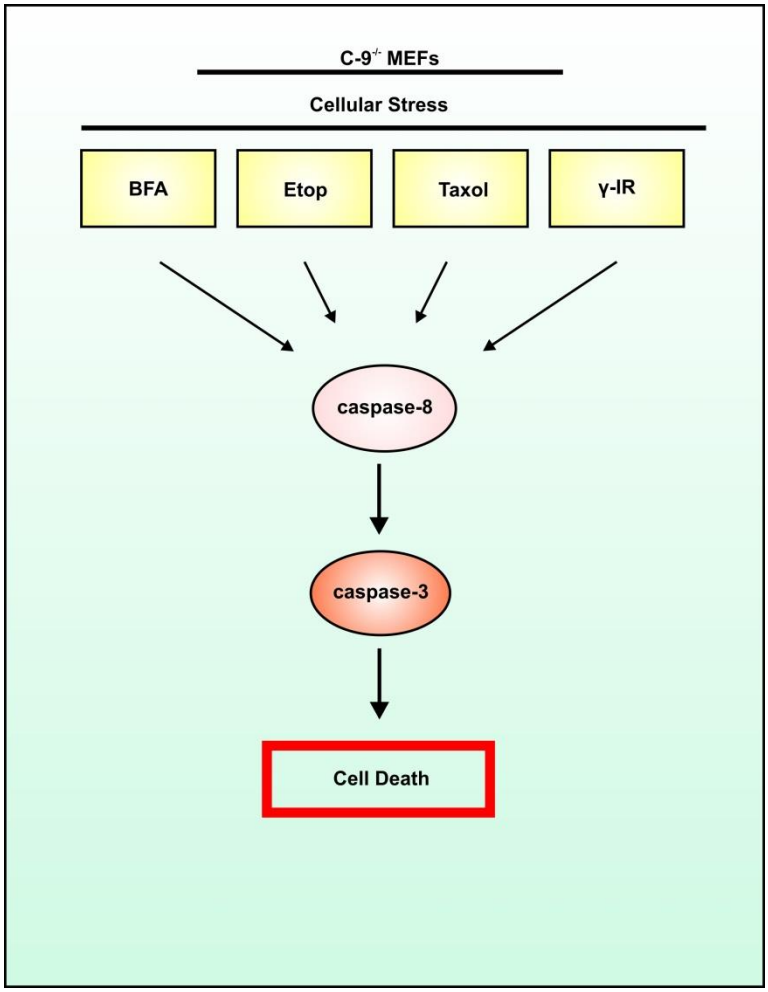


Figure 3.9 **Various stresses induce caspase-8-mediated cell death in C-9$^{-/-}$ MEFs**. A graphic representing a model of the stress-induced caspase activation pathway. Caspase-8 mediates cell death in cells deficient in caspase-9 in response to different stresses.

Chapter 4: Role of stress-induced autophagy on cell death in cells deficient in intrinsic apoptotic pathway in response to various stresses

4.1 Contributions

This work will be a part of a manuscript for a research paper "Stressosome -novel stress inducing complex". Authors: Karolina Pakos-Zebrucka (KPZ), Izabela Glogowska (IG), Katarzyna Mnich, Shane Deegan, Susan Logue, Adrienne Gorman, Afshin Samali

The contributions to the experimental results are as follows:

Fig. 4.4 B (2x KPZ, 1x IG)

Fig. 4.5 B (1x KPZ, 2x IG)

Fig. 4.6 B (1x KPZ, 2x IG)

Fig. 4.7 B (1x KPZ, 2x IG)

4.2 Introduction and Objectives

Autophagy acts as a pro-survival mechanism and is central to adaptation to cellular stresses such as starvation, energy depletion, ER stress, oxidative stress, and hypoxia. Moreover, autophagy is also involved in innate and adaptive immune mechanisms, protecting against pathogens and virus infection (Levine et al., 2011).

Although it plays a multifunctional role in cell survival, autophagy has also been implicated in cell death (Fitzwalter and Thorburn, 2015, Levine and Yuan, 2005). It has been shown that autophagy-mediated cell death occurs in cell defective in the mitochondrial-mediated pathway. Indeed, cells lacking Bax and Bak and thus highly resistant to apoptosis were described to undergo a non-apoptotic cell death associated with autophagy (Ullman et al., 2008, Buytaert et al., 2006, Tsujimoto and Shimizu, 2005). Similarly, human cervical cancer cells overexpressing BCL-2 are resistant to apoptotic stimuli, but do trigger autophagy-dependent cell death (Laussmann et al., 2011). A study from our laboratory also showed that cells lacking either caspase-9 or Bax and Bak are also protected against ER-stress induced apoptosis; however, they eventually succumb to cell death when exposed to prolonged treatment via autophagy-mediated cell death (Deegan et al., 2014b).

Several line of evidence have connected autophagy with apoptosis (Tsujimoto and Shimizu, 2005, Yu et al., 2004). Although apoptosis is the best characterized and understood programmed mode of cell death, it seems that, depending on cellular context and stress-stimuli, other pathways may provide an alternative mode of cell death. Additionally, many apoptotic stimuli such as chemotherapeutics can induce both apoptosis and autophagy. There is still controversy regarding whether cells undergo autophagy-mediated cell death or whether autophagy executes it by itself. Although the morphological appearance of cells undergoing such a form of cell death is characterized by massive vacuolization of the cytoplasm and the lack of chromatin condensation, there is as yet no defined marker for cell death mediated by autophagy (Levine and Yuan, 2005). The involvement of caspases is also questioned, as in some cellular settings a caspase-dependent mode of cell death (Laussmann et al., 2011) is proposed while in others a caspase-independent pathway has been implicated (Ullman et al., 2008, Inbal et al., 2002). Moreover, the inconsistency between *in vitro* and *in vivo* studies also raises questions about the nature of cell death mediated by autophagy.

A previous study by Shimuzu *et al.* explored the involvement of nonapoptotic cell death dependent on autophagy in cells deficient in Bax and Bak (Shimizu et al., 2004). Using pharmacological autophagy inhibition or knockdown of Atg5 or Beclin 1 they were able to rescue cells exposed to different type of stresses. The authors concluded that various stimuli induce Atg5-, Beclin 1-dependent autophagy that is required for non-apoptotic cell death in Bax/Bak-deficient cells (Shimizu et al., 2004). Similarly, the interaction of Beclin 1 with BCL-2 proteins implies a role in cell death (Kang et al., 2011, Wei et al., 2008). Moreover, it has been shown that Beclin 1 is involved in cell death mediated by autophagy in response to various stresses (Valentim et al., 2006, Yu et al., 2004). A recent *in vivo* study by Arakawa et al. proposed a role for Atg5-dependent cell death in Bax/Bak double knockout mice in response to genotoxic stress. The authors showed that Atg5 is necessary for autophagy-dependent death under conditions of apoptosis deficiency. Moreover, application of various types of stress indicated that the observed Atg5-dependent mode of cell death was stimulus-specific (Arakawa et al., 2017). While the first study suggested non-apoptotic cell death with necrotic features, the second study demonstrated that apoptosis was the primary and most common mode of cell

death. However, the mechanism of cell death was not fully elucidated and is still not well understood. Furthermore, there is no clear evidence yet in the literature about the mechanism of autophagy-dependent cell death upon chronic and unresolved stress in cells defective in the intrinsic apoptotic pathway. Although a previous study carried out in our laboratory explained the mechanism partially, it requires further elucidation. Indeed, our study showed that in cells compromised in the mitochondrial apoptotic pathway, autophagy is an important mediator of the caspase-dependent mode of cell death. We showed that ER stress-induced cell death is executed in a manner which is dependent on Atg5 and Atg7, proteins that are associated with autophagosome formation (Deegan et al., 2014b). In this context, we described non-canonical caspase-8 activation that requires the adaptor protein FADD that is associated with Atg5 resulting in the formation of the novel death-inducing protein complex. It is proposed that this novel protein complex, similarly to intracellular death-inducing complex (iDISC), constitutes a platform for the activation of pro-caspase-8 and the subsequent initiation of the apoptotic cascade and execution of cell death (Iurlaro and Munoz-Pinedo, 2016, Young et al., 2012). Thus, the autophagosomal membrane seems to be crucial for stress-induced cell death. Moreover, recently it has been shown that the accumulation of immature autophagosomes can also contribute to caspase-8-dependent cell death in response to nutrient deprivation (Tang et al., 2017). Therefore, the importance of autophagic membranes in caspase-8 activation independent of death receptor ligation implicates autophagy in cell death.

In a previous section of this thesis I showed that C-9$^{-/-}$ MEFs have less apoptotic response to a wide variety of stress stimuli. Therefore, in this chapter, I employed C-9$^{-/-}$ MEFs for further examination, as they should constitute an excellent experimental model system in which to investigate autophagy-dependent cell death. Furthermore, many apoptotic stimuli such as chemotherapeutics can also induce autophagy. Therefore, the response of C-9$^{-/-}$ MEFs to various stresses which were already employed in Chapter 3 was investigated in more detail. As previously explained, the application of these drugs with their diverse mechanisms of action allows for a better understanding of

the mechanisms of cell death in cells compromised in the mitochondrial-mediated pathway.

The results described below highlight the importance of an alternative stress-induced caspase-mediated cell death pathway that is dependent on Atg5, a protein involved in autophagosome formation. This stress-induced cell death was investigated in the context of an alternative caspase-8 activation death pathway that is activated to compensate for deficiency in the canonical intrinsic apoptotic pathway.

The aims of this chapter were to elucidate:

1. Whether various stresses can induce autophagy in C-9$^{-/-}$ MEFs
2. The exact role of Atg5 in stress-induced cell death
3. Whether other components of the autophagic machinery upstream of autophagosome formation are involved in stress-induced autophagy

4.3 Results

4.3.1 Diverse stresses lead to the induction of autophagy in C-9$^{-/-}$ MEFs

It has been shown that different types of cellular stress induce autophagy in cells deficient in the mitochondria-mediated apoptotic pathway (Laussmann et al., 2011, Shimizu et al., 2004). With this in mind, to determine whether various stresses induce macroautophagy in C-9$^{-/-}$ MEFs, both C-9$^{+/+}$ and C-9$^{-/-}$ MEFs were subjected to prolonged treatment with either BFA, Etop, Taxol or γ-irradiation. The changes in the cellular level of the macroautophagy marker LC3-II was analyzed by Western blot. Upon autophagy induction cytoplasmic LC3-I is conjugated to phosphatidylethanolamine to form LC3-II which is recruited to autophagosomal membranes. The treatment of both C-9$^{+/+}$ and C-9$^{-/-}$ MEFs with either BFA, Etop, Taxol or γ-irradiation resulted in an increase in LC3-II levels over time (Fig. 4.1). Interestingly, C-9$^{-/-}$ MEFs displayed high levels of LC3-II at later timepoints (24 < h) in response to all treatments. However further investigation with time match controls is needed in order to fully understand the observed changes.

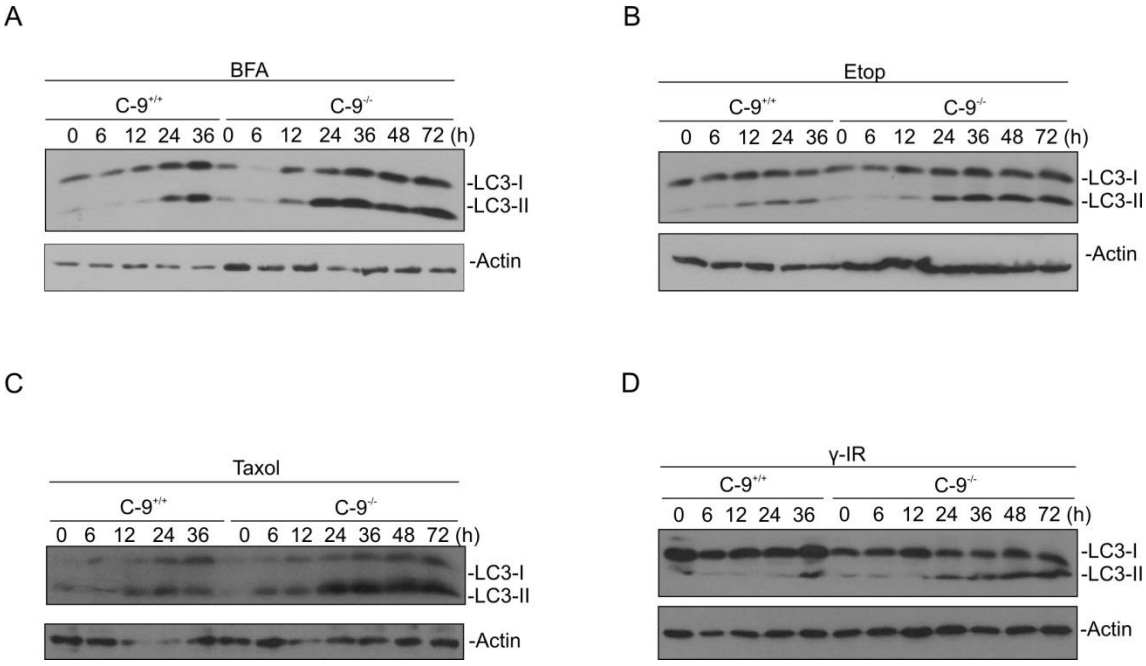
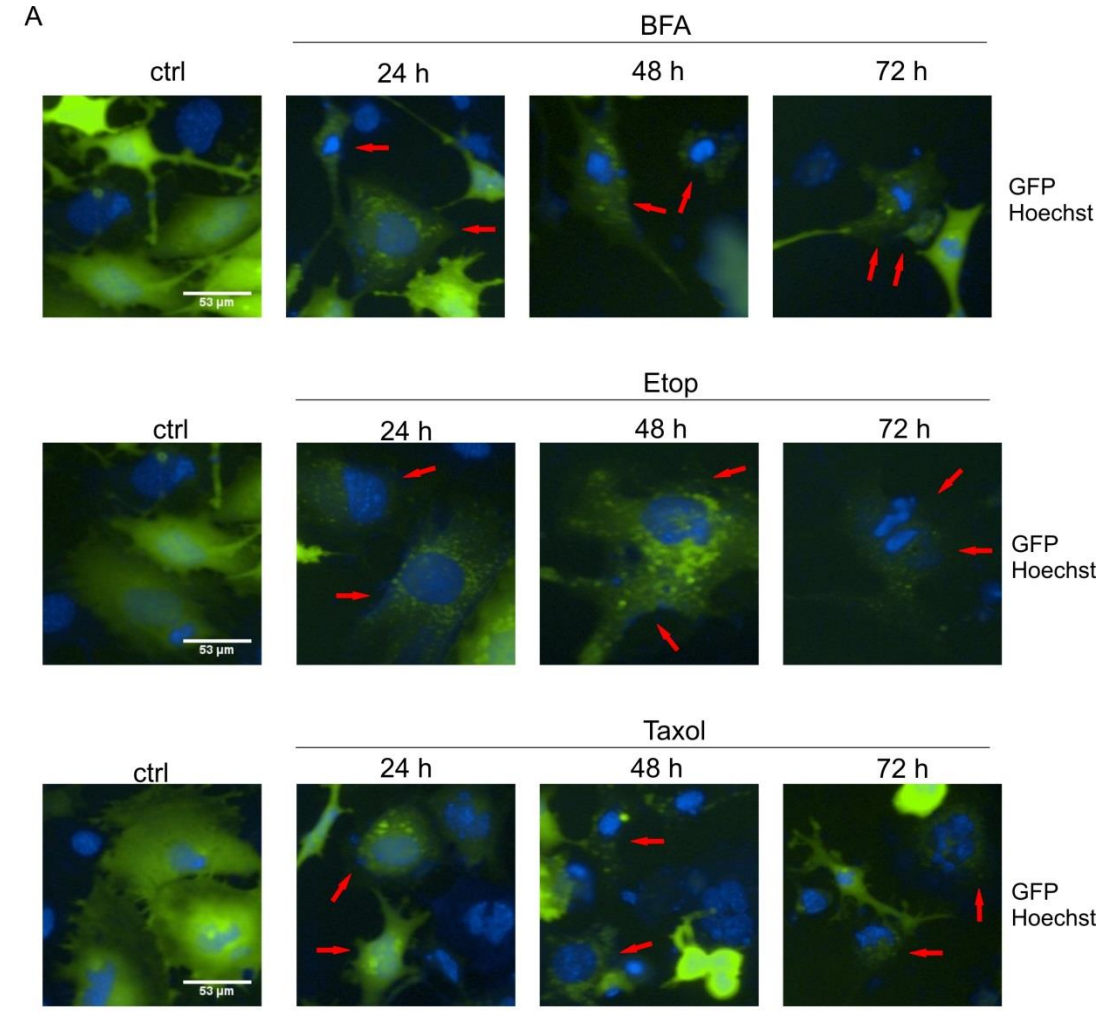


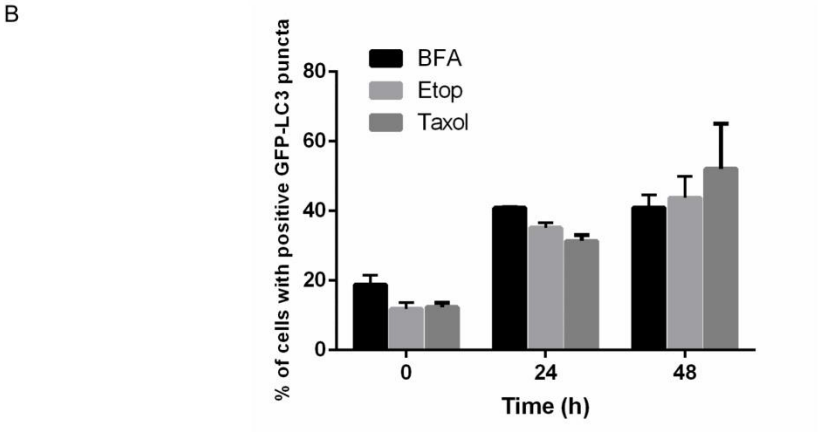
Figure 4.1 **Various stresses induce LC3 I to LC3 II conversion in both C-9$^{+/+}$ and C-9$^{-/-}$ MEFs**. C-9$^{+/+}$ and C-9$^{-/-}$ MEFs were treated with (A) 0.3 μg/ml BFA, (B) 50 μM Etop, (C) 1 μM Taxol and (D) 33Gy γ-IR for the indicated times and whole cell lysates were subjected for Western blot for LC3-I to LC3-II conversion and Actin.

To further confirm that BFA, Etop, Taxol and γ-irradiation induce autophagy in the examined cellular system, C-9$^{-/-}$ MEFs were transfected with a GFP-LC3 expression construct. During autophagy LC3-II associates with the inner and outer autophagosomal membranes. This results in an accumulation of GFP-LC3 puncta in cells, and can be observed using fluorescent microscopy. After prolonged treatment with either BFA, Etop or Taxol a significant increase in the number of cells with punctate fluorescence was observed in C-9$^{-/-}$ MEFs (Fig. 4.2A). A quantitative analysis of GFP-LC3 transfected cells showed gradual increase of total cells displayed positive puncta when treated with BFA, Etop or Taxol for up to 48 h (Fig. 4.2B). These results confirm a strong rise in autophagosome biogenesis.

To further confirm that the observed autophagosome increase in C-9$^{-/-}$ MEFs upon BFA, Etop or Taxol treatment was due to autophagy induction and not impaired autophagosome-lysosome fusion, an autophagic flux experiment with addition of the

lysosomal inhibitor, chloroquine (CQ) was performed. The process of autophagic flux includes the autophagosome formation, fusion with the lysosome, the autophagolysosome formation and the degradation step. Based on the observation that LC3-II is degraded in the autolysosome, thus the level of LC3-II constitutes a hallmark for monitoring functional autophagy process. Increased conversion of cytosolic form of LC3-I to the lipidated, membrane-bound form LC3-II can indicate induction of autophagy or defective autophagosome degradation (Mizushima et al., 2010). Therefore, administration of CQ that blocks degradation of LC3-II results in accumulation of LC3-II. C-$9^{-/-}$MEFs were subjected to treatment with BFA, Etop or Taxol with or without CQ. Cells were harvested at the indicated time points and whole cell lysates were assessed by Western blot for LC3-I to LC3-II conversion. Autophagic flux examination showed that administration of CQ to BFA, Etop or Taxol enhanced the level of LC3-II compared with either CQ or treatment alone in C-$9^{-/-}$ MEFs, with a stronger effect observed at 48 h (Fig. 4.2C). These results confirm that an excessively high level of autophagy is induced by BFA, Etop or Taxol rather than autophagosome degradation. These results confirm that BFA, Etop and Taxol induce functional autophagy in C-$9^{-/-}$ MEFs.


A
BFA
ctrl
24 h
48 h
72 h
GFP
Hoechst
53 µm
Etop
ctrl
24 h
48 h
72 h
GFP
Hoechst
53 µm
Taxol
ctrl
24 h
48 h
72 h
GFP
Hoechst
53 µm


B
% of cells with positive GFP-LC3 puncta
80
60
40
20
0
BFA
Etop
Taxol
0
24
48
Time (h)

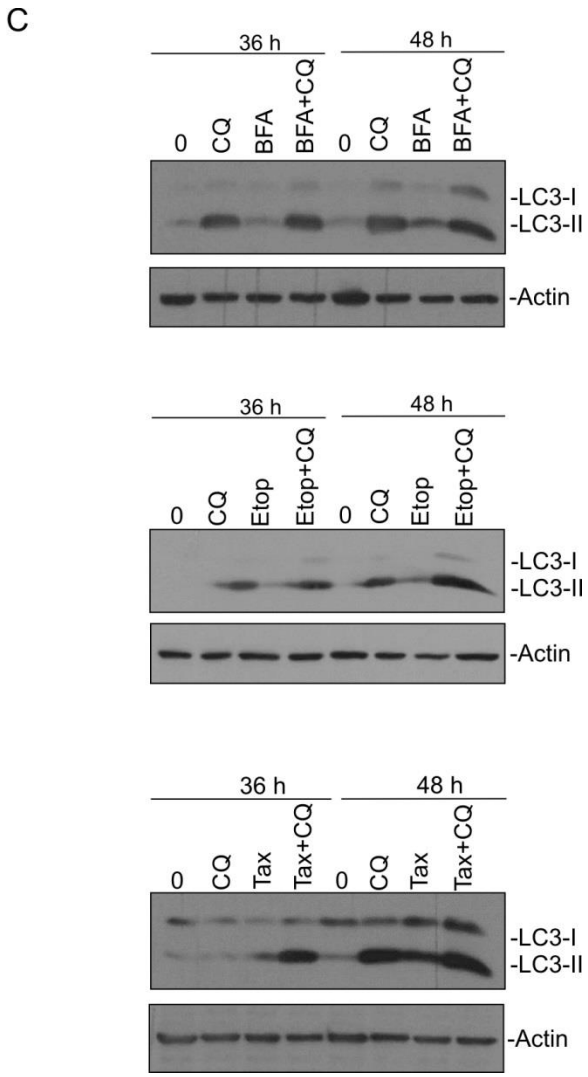


Figure 4.2 **Autophagy is induced by various stresses in C-9$^{-/-}$ MEFs.** (A) Monitoring of autophagosome formation in C-9$^{-/-}$ MEFs. C-9$^{-/-}$ MEFs were transfected with GFP-LC3 plasmid and 24 h post-transfection were treated with 0.3 μg/ml BFA, 50 μM Etop and 1 μM Taxol for the indicated times. Cells were co-stained with 5 μg/ml Hoechst 33342 prior to analysis. The punctation of GFP-LC3 was assessed by fluorescent microscopy at 20X magnification. (B) Three fields of at least 100 cells/field were counted from three independent experiments. (C) Autophagic flux was evaluated in C-9$^{-/-}$ MEFs in response to 0.3 μg/ml BFA, 50 μM Etop and 1 μM Taxol by monitoring LC3-I to LC3-II conversion and Actin was used as a loading control.

4.3.2 Role of ATG5 in stress-induced cell death

As already mentioned in the introduction, the importance of ATG5 in autophagy-dependent cell death has been previously linked to apoptosis and subsequent non-canonical activation of caspase-8. Indeed, autophagosomes were shown to serve as a platform for assembly of a caspase activating complex, where FADD associates with ATG5 and thus recruits caspase-8, leading to downstream caspase activation (Young et al., 2012, Bell et al., 2008).

4.3.2.1 Knockdown of ATG5 reduces stress-induced autophagy in C-9$^{-/-}$ MEFs

To demonstrate that stress-induced cell death in C-9$^{-/-}$ MEFs upon prolonged exposure to various stresses is mediated by autophagy, autophagy was impaired by silencing of *Atg5* gene expression. To perform the experimental procedure previously generated in our laboratory C-9$^{-/-}$ MEFs transduced with A*tg5* shRNA lentivirus or pGIPZ empty lentivirus were used. The functionality of *Atg5* knockdown was confirmed by autophagic flux. Both pGIPZ and *Atg5* shRNA C-9$^{-/-}$ MEFs were treated either with BFA, Etop or Taxol in the presence or absence of CQ for 48 h prior to Western blot for LC3-I to LC3-II conversion. Autophagic flux was lower in cells that were transfected with *Atg5* shRNA as compared with the cells transfected with control pGIPZ. Although conversion of LC3-I to LC3-II was not observed in *Atg5* shRNA C-9$^{-/-}$ MEFs with BFA, Etop or Taxol alone, administration of CQ to BFA, Etop or Taxol-treated *Atg5* shRNA C-9$^{-/-}$ MEFs significantly increased LC3-II levels. As evident it is a lower accumulation of LC3-II in BFA, Etop and Taxol samples co-treated with CQ in *Atg5* shRNA cells compared with pGIPZ control cells (Fig. 4.3). These results confirm that deficiency of Atg5 gene expression reduced stress-induced autophagy triggered by BFA, Etop and Taxol, indicating that various stresses induce ATG5-dependent autophagy in C-9$^{-/-}$ MEFs.

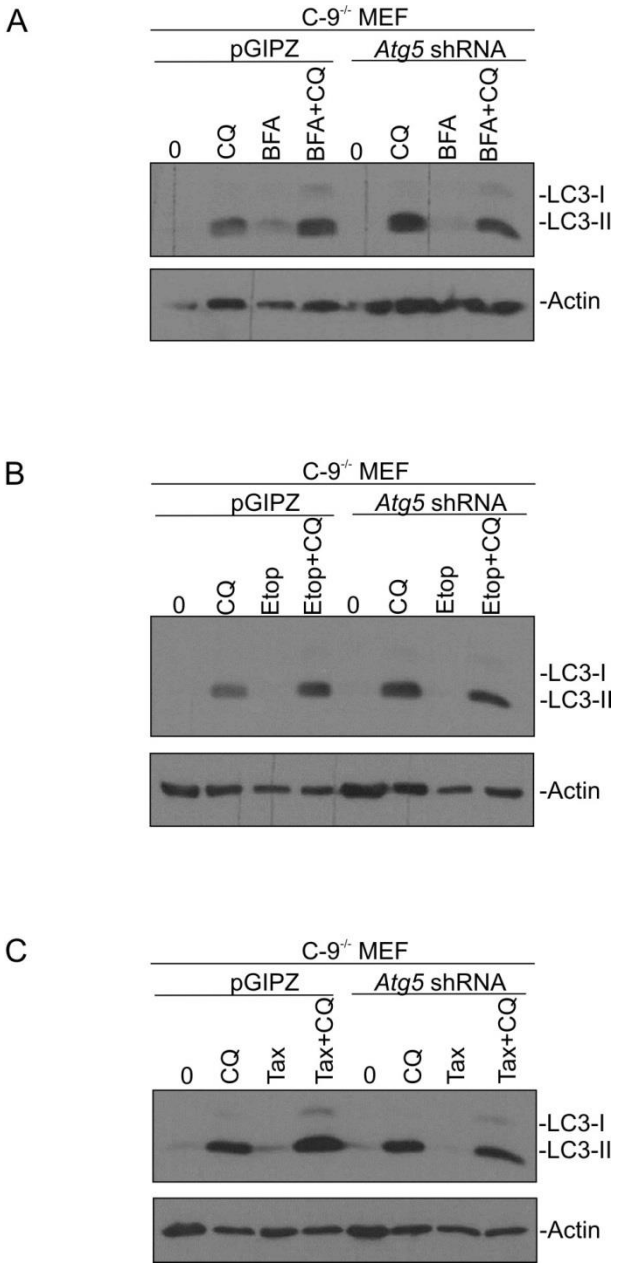


Figure 4.3 **Autophagic flux in C-9$^{-/-}$ MEFs deficient for *Atg5*.** C-9$^{-/-}$ MEFs stably expressing pGIPZ and *Atg5* shRNA were treated with 20 μM chloroquine alone, 0.3 μg/ml BFA, 50 μM Etoposide and 1 μM Taxol alone, or a combination of 20 μM chloroquine and 0.3 μg/ml BFA, 50 μM Etoposide and 1 μM Taxol for the indicated times. Whole cell lysates were subjected to Western blot and analysed for LC3-I to LC3-II conversion and Actin.

4.3.2.2 Knockdown of Atg5 inhibits ER stress stress-induced caspase activation and death in C-9$^{-/-}$ MEFs

To investigate whether ATG5 contributes to ER stress-induced cell death in C-9$^{-/-}$ MEFs, pGIPZ and *Atg5* shRNA C-9$^{-/-}$ MEFs were subjected to prolonged treatment with BFA. Western blot analysis of caspases processing was performed in parallel with cell death analysis using ToPro3 staining. As expected, knockdown of Atg5 greatly decreases processing of pro-caspase-8 and caspase-3 upon prolonged exposure to BFA (Fig. 4.4A). Because, the pronounced activation of both caspases was detected in pGIPZ C-9$^{-/-}$ MEFs, it suggests that ATG5 drives a caspase-dependent mode of cell death.

Furthermore, knockdown of Atg5 in C-9$^{-/-}$ MEFs also correlated with a reduction in cell death as determined by the ToPro3 staining. FACS analysis depicts that following treatment with BFA, pGIPZ shRNA C-9$^{-/-}$ MEFs died much more rapidly with approximately 25% cell death observed as early as 48 h and almost 35% dead cells at 72 h. In contrast, knockdown of Atg5 in C-9$^{-/-}$ MEFs significantly reduced cell death upon BFA treatment (Fig. 4.4B). Interestingly, under the initial stress at 24 h *Atg5* shRNA C-9$^{-/-}$ MEFs were not significantly protected against BFA, suggesting that ATG5 might potentially contributes to sustained stress.

Because *Atg5* shRNA C-9$^{-/-}$ MEFs showed resistance to BFA, the clonogenic assay was performed in order to investigate whether Atg5 deficiency could also provide long-term protection. As illustrated in Fig. 4.4C lack of ATG5 expression resulted in a marked improvement of cell viability compare with the pGIPZ shRNA C-9$^{-/-}$ MEFs. *Atg5* shRNA C-9$^{-/-}$ MEFs were able to recover after initial stress and proliferate. Therefore, these results indicate that BFA-induced cell death is mediated in ATG5-dependent manner. Taken together, the obtained results demonstrate that ATG5 contributes to BFA -induced autophagy-mediated caspase-dependent cell death in C-9$^{-/-}$ MEFs.

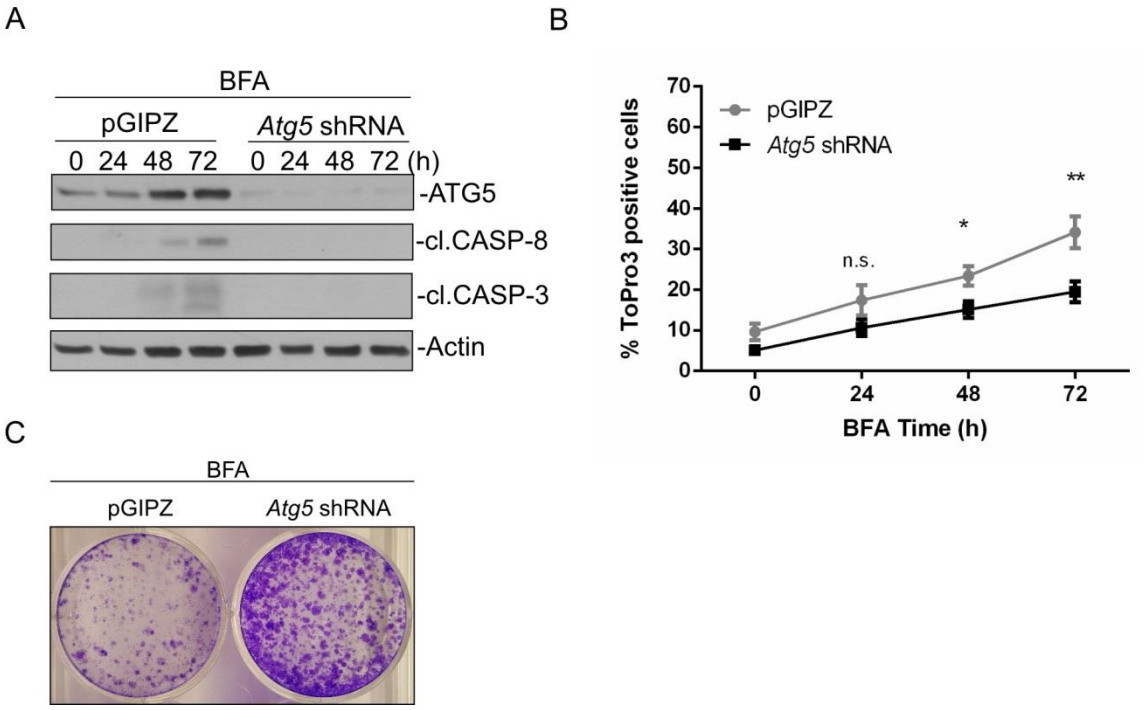


Figure 4.4 **Knockdown of Atg5 reduces cell death upon exposure to sustained treatment with BFA, in C-9$^{-/-}$ MEFs.** (A) pGIPZ and *Atg5* shRNA C-9$^{-/-}$ MEFs were treated with 0.3 µg/ml BFA. Whole cell lysates were subjected to Western blot for expression of ATG5, cleaved caspase-8 (cl.CASP-8), caspase-3 (cl.CASP-3) and Actin. (B) Cell death was assessed at the indicated time points by using flow cytometry and ToPro3 staining. Values represent the mean ± SEM of three independent experiments. The significance level are ** = $p<0.001$ and * = $p<0.05$, n.s. indicates no significant difference. (C) Clonogenic assay of pGIPZ and *Atg5* shRNA C-9$^{-/-}$ MEFs after 72 h of BFA treatment.

4.3.2.3 Knockdown of Atg5 inhibits Etop-induced caspase processing and cell death in C-9$^{-/-}$ MEFs

To investigate whether ATG5 contributes to genotoxic stress-induced cell death, both pGIPZ and *Atg5* shRNA C-9$^{-/-}$ MEFs were subjected to prolonged treatment with Etop. Timecourse analysis revealed that knockdown of Atg5 significantly decreases processing of pro-caspase-8 and caspase-3 compared with pGIPZ where pronounced detection of these proteins was observed even at 24 h (Fig. 4.5A). This suggests that Etop triggers an ATG5-dependent caspase activation mechanism. Moreover, knockdown of Atg5 in C-9$^{-/-}$ MEFs significantly reduced cell death as determined by ToPro3 uptake at 48 h and 72 h (Fig. 4.5B). Indeed, the FACS analysis depicts that following treatment with Etop, pGIPZ shRNA C-9$^{-/-}$ MEFs died faster compared with *Atg5* shRNA C-9$^{-/-}$ MEFs. Similar to BFA treatment, a statistically insignificant lack of protection was observed of *Atg5* shRNA C-9$^{-/-}$ MEFs at the initial stress 24 h of Etop treatment,

suggesting that ATG5 contributes to Etop-induced cell death at the latest stage. Furthermore, to investigate the importance of ATG5-dependent cell death, the clonogenic assay was assessed. The absence of ATG5 did not provide long-term survival and was unable to rescue *Atg5* shRNA C-9$^{-/-}$ after Etop. Interestingly, the same effect was observed in the pGIPZ shRNA C-9$^{-/-}$ MEFs, suggesting that prolonged treatment with Etop inhibits the proliferative ability of cells (Fig. 4.5C). The lack of viable colonies may indicate that the dose of Etop used can lead to irreversible damage and cell cycle arrest which cannot be restored. These results do not support the role of ATG5 in Etop-induced cell death; however it does not mean that ATG5 is not involved in this mode of cell death induced by Etop. Taken together, Etop drives ATG5-dependent caspase activation and cell death in C-9$^{-/-}$ MEFs.

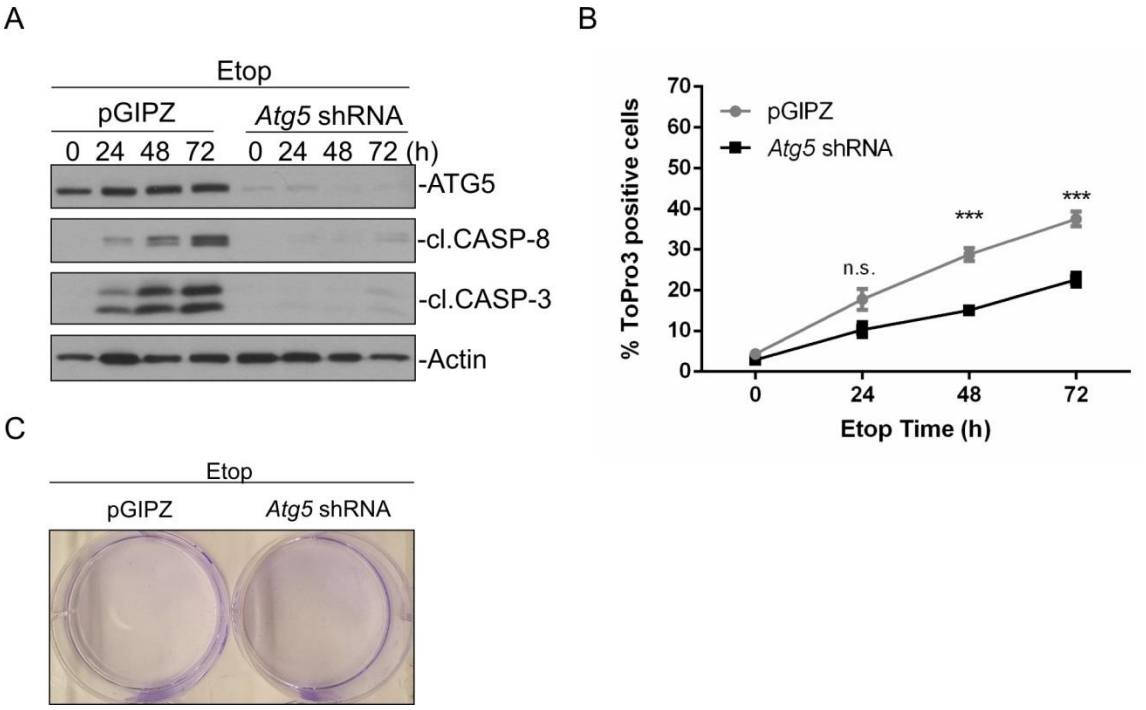


Figure 4.5 **Knockdown of Atg5 reduces cell death upon exposure to sustained treatment with etoposide in C-9$^{-/-}$ MEFs.** (A) pGIPZ and *Atg5* shRNA C-9$^{-/-}$ MEFs were treated with 50 μM Etop. Whole cell lysates were subjected to Western blot for expression of ATG5, cleaved caspase-8 (cl.CASP-8) caspase-3 (cl.CASP-3) and Actin. (B) Cell death was assessed at the indicated time points by using flow cytometry and ToPro3 staining. Values represent the mean ± SEM of three independent experiments. The significance level are *** = p<0.0001, n.s. indicates no significant difference. (C) Clonogenic assay of pGIPZ and Atg5 shRNA C-9$^{-/-}$ MEFs after 72 h of Etop treatment.

4.3.2.4 Knockdown of Atg5 reduces Taxol -induced caspase processing but does not protect against Taxol -induced cell death in C-9$^{-/-}$ MEFs

To determine whether ATG5 contributes to cytoskeletal damage-induced cell death both pGIPZ and *Atg5* shRNA C-9$^{-/-}$ MEFs were subjected to prolonged treatment with Taxol and analysed for expression of processing pro-caspase-8 and caspase-3 by Western blot. As illustrated in Fig. 4.6A, knockdown of Atg5 reduced processing of both caspase-8 and caspase-3 upon prolonged treatment with Taxol, whereas expression levels of these proteins were unaffected in pGIPZ shRNA C-9$^{-/-}$ MEFs.

Furthermore, FACS analysis with ToPro3 staining revealed that *Atg5* shRNA C-9$^{-/-}$ MEFs were not protected against Taxol-induced cell death. Although, the results were statistically insignificant, the trend of the increased cell death in both pGIPZ and *Atg5* shRNA C-9$^{-/-}$ MEFs was very clear. Both cell lines died rapidly reaching maximum of

around 35% dead cells at 72 h (Fig. 4.6B). These data indicate that ATG5 is not required for Taxol-induced cell death.

Because Taxol showed conflicting results, to clarify if ATG5 could protect against sustained stress, clonogenic assay was applied. Although knockdown of Atg5 slightly rescued C-9$^{-/-}$ MEFs after initial stress, it also rescued pGIPZ shRNA C-9$^{-/-}$ MEFs (Fig. 4.6C). These results suggest that ATG5 is not necessary for long-term survival as recovery after Taxol restored reproductive capability of cells.

Taken together, ATG5 is required for Taxol-induced caspase activation, but is not required for cell death. This might suggest that although ATG5 is necessary for caspase-mediated cell death is not sufficient to execute cell death through this mechanism upon Taxol treatment. Most likely, an alternative caspase-independent mode of cell death might be induced in the absence of ATG5.

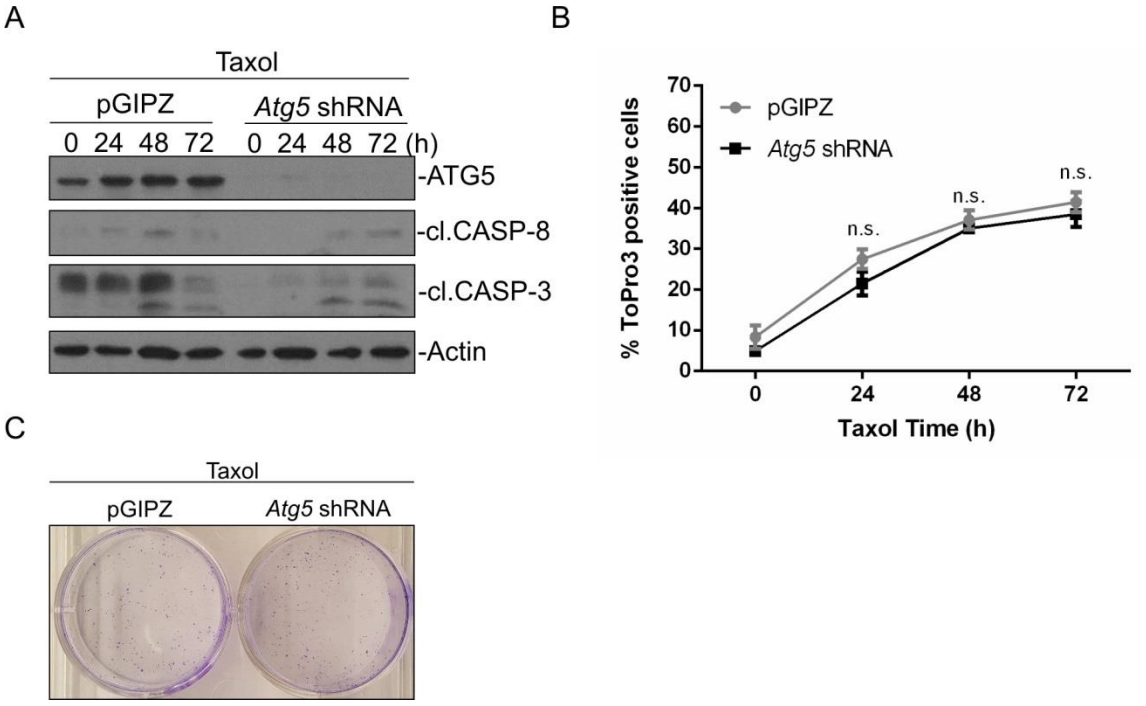


Figure 4.6 **Knockdown of Atg5 in C-9$^{-/-}$ MEFs does not protect against prolonged Taxol treatment. (A)** pGIPZ and *Atg5* shRNA C-9$^{-/-}$ MEFs were treated with 1 μM Taxol for the indicated time points. Whole cell lysates were subjected to Western blot for expression of ATG5, cleaved caspase-8 (cl.CASP-8) caspase-3 (cl.CASP-3) and Actin. (B) Cell death was assessed at the indicated time points by using flow cytometry and ToPro3 staining. Values represent the mean ± SEM of three independent experiments. n.s. indicates no significant difference. (C) Clonogenic assay of pGIPZ and Atg5 shRNA C-9$^{-/-}$ MEFs after 72 h of Taxol treatment.

4.3.2.5 Knockdown of Atg5 reduces γ-irradiation -induced caspase processing but does not protect against γ-irradiation -induced cell death in C-9$^{-/-}$ MEFs

To evaluate whether the alternative caspase activation pathway is a general response activated by various stress, the role of ATG5 in C-9$^{-/-}$ MEFs was investigated in response to γ-irradiation treatment. Both pGIPZ and *ATG5* shRNA C-9$^{-/-}$ MEFs were subjected to prolonged treatment with γ-irradiation and Western blot analysis was performed for pro-caspase-8 and caspase-3 processing followed by FACS analysis of cell death.

Knockdown of Atg5 decreases pro-caspase-8 and caspase-3 processing after treatment with γ-irradiation as illustrated by Fig. 4.7A. Also, similar to previous stresses, pGIPZ shRNA C-9$^{-/-}$ MEFs displayed pronounced cleavage of both caspase-8 and caspase-3 confirming apoptotic mode of cell death induced by γ-irradiation in C-9$^{-/-}$ MEFs. Unlike

BFA and Etop, but like Taxol, knockdown of Atg5 in C-9$^{-/-}$ MEFs did not inhibit γ-irradiation–induced cell death as determined by ToPro3 staining. Although the results were found statistically insignificant, the overall trend of ToPro3 uptake showed a slow increase in cell death of both pGIPZ and *Atg5* shRNA C-9$^{-/-}$ MEFs. Furthermore and interestingly, *Atg5* shRNA C-9$^{-/-}$ MEFs exhibited more cell death compared with pGIPZ shRNA C-9$^{-/-}$ MEFs as presented by Fig. 4.7B.

To this end, the measurement of the cell's ability for long-term survival was applied in order to identify whether knockdown of Atg5 can rescue irradiated C-9$^{-/-}$ MEFs. Again, like Taxol treatment, exposure to γ-irradiation conferred a survival advantage in both pGIPZ and *Atg5* shRNA C-9$^{-/-}$ MEFs and restored a proliferative activity of C-9$^{-/-}$ MEFs independent of ATG5 (Fig. 4.7C). This suggest that ATG5 is not required for cell death induced by γ-irradiation. Taken together, these results indicate that ATG5 is necessary for γ-irradiation-induced caspase activation but it is not required for cell death in C-9$^{-/-}$ MEFs.

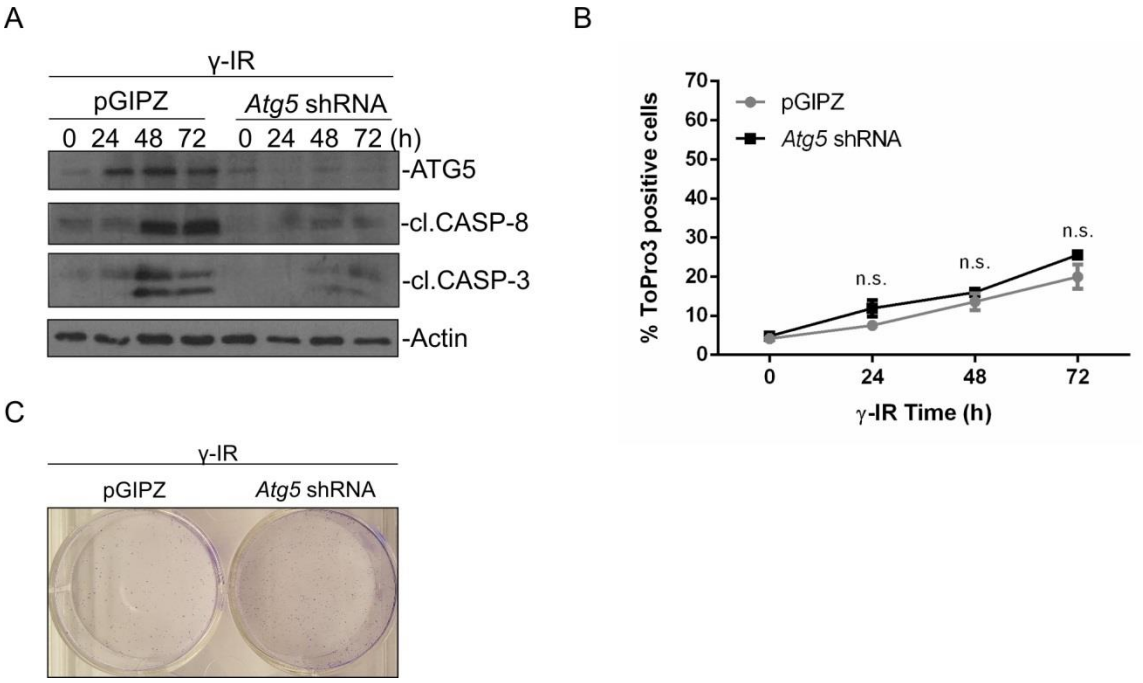


Figure 4.7 **Knockdown of Atg5 in C-9$^{-/-}$ MEFs does not protect against prolonged γ-irradiation treatment.** (A) pGIPZ and *Atg5* shRNA C-9$^{-/-}$ MEFs were treated with 33Gy γ-IR and allowed to recover for the indicated time points. Whole cell lysates were subjected to Western blot for expression of Atg5, cleaved caspase-8 (cl.CASP-8), caspase-3 (cl.CASP-3) and Actin. Cell death was assessed at the indicated time points by using flow cytometry and ToPro3 staining. Values represent the mean ± SEM of three independent experiments. n.s. indicates no significant difference. (C) Clonogenic assay of pGIPZ and *Atg5* shRNA C-9$^{-/-}$ MEFs after 72h of γ-IR exposure.

4.3.3 The impact of autophagy inhibition upstream of autophagosome elongation on stress-induced cell death

To verify that examined alternative stress-induced caspase-activation pathway is not specific to ATG5 but rather reflects involvement of autophagy in general, pharmacological and genetic approaches were used to investigate whether the inhibition of autophagy upstream of autophagosome elongation can rescue C-9$^{-/-}$ MEFs from BFA, Etop and Taxol-induced cell death.

4.3.3.1 Spautin-1 inhibits rapamycin-induced autophagy in caspase-9 deficient MEFs

To determine if spautin-1 is functional, C-9$^{-/-}$ MEFs were treated with rapamycin, a widely used inducer of autophagy, either on its own or in combination with spautin-1. As already explained in Chapter 2: Material and Methods SP-1 specifically inhibits

autophagy through Beclin 1 ubiquitination and its subsequent degradation (Liu et al., 2011).

To demonstrate that rapamycin is a potent inducer of autophagy, C-9$^{-/-}$ MEFs were transiently transfected with a GFP-LC3 expression construct and treated with rapamycin. GFP-LC3 puncta were analysed using fluorescnce microscopy at 0, 24, 48 and 72 h. As illustrated in Fig. 4.8, rapamycin caused a significant accumulation of GFP-LC3 puncta in the C-9$^{-/-}$ MEFs over the timecourse. Furthermore, it was also examined whether prolonged treatment with rapamycin would induce cell death in C-9$^{-/-}$ MEFs. To address this question Hoechst staining was performed. As presented in Fig. 4.8 prolonged exposure to rapamycin in C-9$^{-/-}$ MEFs did not lead to either chromatin condensation or nuclei fragmentation compared to the control. These results indicate that rapamycin does not induce cell death characterized by typical apoptotic features and thus can be used to monitor cells with GFP-LC3 puncta over prolonged timecourse.

Taken together, these findings imply that rapamycin is a potent inducer of autophagy in C-9$^{-/-}$ MEFs and can be used as a positive control for further experiments. Additionally, rapamycin-induced autophagy is not correlated with cell death.

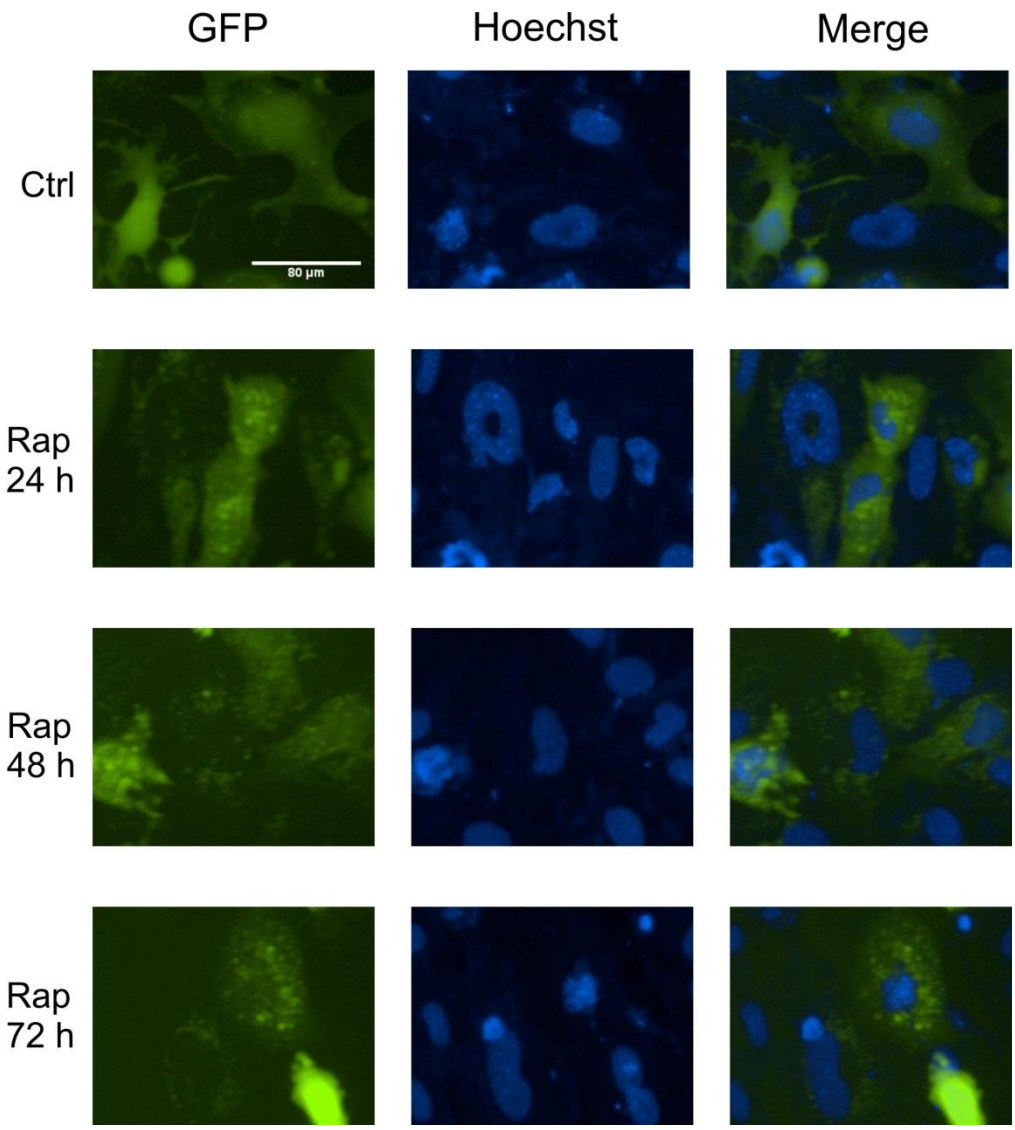
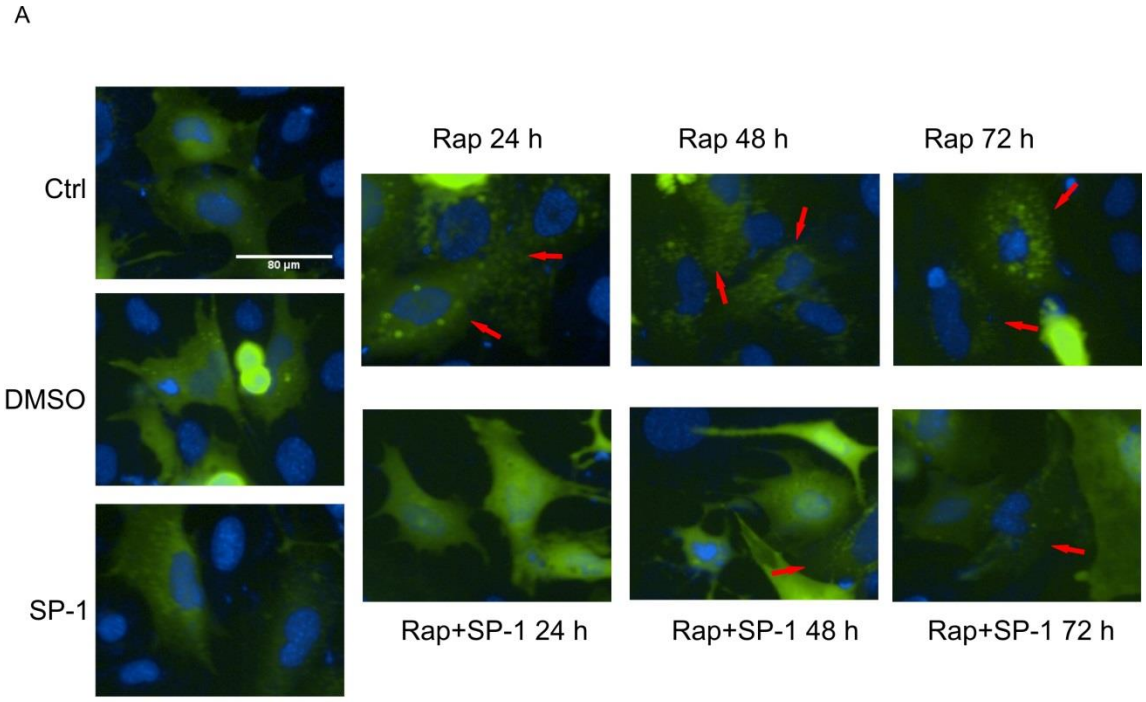


Figure 4.8 **Autophagy is induced by Rapamycin in C-9$^{-/-}$ MEFs.** (A) Monitoring of autophagosome formation in C-9$^{-/-}$ MEFs. C-9$^{-/-}$ MEFs were transfected with GFP-LC3 plasmid and 24 h post-transfection were treated with 400 nM Rap for the indicated times. Cells were co-stained with 5 µg/ml Hoechst 33342 prior to analysis. The punctation of GFP-LC3 was assessed by fluorescent microscopy at 20X magnification.

To examine pharmacological inhibition of stress-induced autophagy upstream of autophagosome elongation, the effects of spautin-1 with rapamycin treatment was investigated. C-9$^{-/-}$ MEFs were transfected with the GFP-LC3 expression construct and subjected to prolonged treatment with rapamycin, either alone or in combination with SP-1. Changes in GFP-LC3 puncta were first examined by fluorescent microscopy.

As expected stimulation of GFP-LC3 transfected cells with rapamycin led to a significant increase in the level of cells with GFP-LC3 compare to vehicle control. A quantitative analysis of GFP-LC3 transfected cells showed around 60% of total cells displayed a positive puncta when treated with rapamycin at 24 h. This level of cells with GFP-LC3 was slight reduced over time reaching around 40% of total cells displayed a positive GFP-LC3 puncta at 72 h. Application of SP-1 reduced the number of cells displaying GFP-LC3 puncta from around 30% at 24 h to 20% at 72 h compared to rapamycin treatment alone (Fig. 4.9A). To avoid misinterpretation of results, due to treatment and its effect on cell morphology caused by cell death, Hoechst staining was performed simultaneously. Therefore, cells with apoptotic features, based on nuclear morphology or cell roundness were excluded from analysis (Klionsky et al., 2016). As illustrated in Fig. 4.9A either rapamycin or spautin-1 did not have an effect on nuclear condensation, thus the analysis was carried out for up to 72 h.

Based on these data, it can be concluded that spautin-1 inhibits autophagy in C-9$^{-/-}$ MEFs. Moreover these results also indicate that spautin-1 has a potency to inhibit autophagy over a prolonged timecourse.



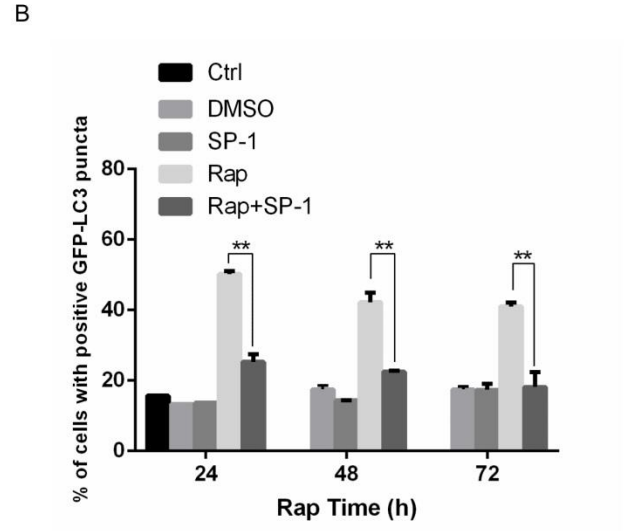
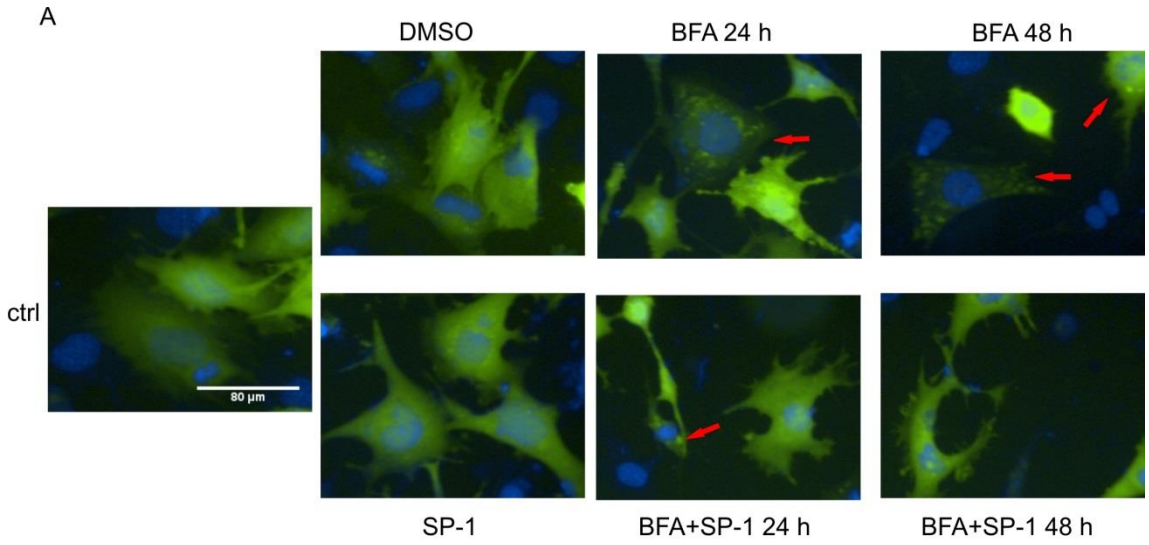


Figure 4.9 **Spautin-1 inhibits Rapamycin-induced autophagy in C-9$^{-/-}$ MEFs.** C-9$^{-/-}$ MEFs transiently transfected with GFP-LC3 were treated with 400 nM Rapamycin ± 10 µM SP-1 and the percentage of GFP-LC3 positive punctate cells counted up to 72 h. (A) Representative fluorescent images are shown at magnification 20x. (B) Three fields of at least 100 cells/field were counted from two independent experiments The significance level is ** = $p<0.001$.

4.3.3.2 Inhibition of autophagy with Spautin-1 slows cell death induced by BFA

To find out whether BFA-induced autophagy is inhibited by spautin-1, C-9$^{-/-}$ MEFs were transiently transfected with GFP-LC3 and treated with BFA with or without SP-1. As predicted, after treatment with BFA a considerable increase in the number of cells with GFP-LC3 puncta over the timecourse was observed compared to the vehicle control (Fig. 4.10A). Indeed, after exposure to BFA the number of cells with punctate GFP-LC3 reached around 40% at 48 h of treatment (Fig. 4.10B). Application of spautin-1 significantly reduced number of cells with GFP-LC3 puncta at 48 h compared to BFA alone as observed by fluorescence microscopy. A quantitative analysis confirms these results indicating that spautin-1 lowered the number of cells displaying GFP-LC3 from around 40% at 24 h to around 15% at 48 h of cotreatment compared to BFA alone. Additionally Hoechst staining was carried out in order to exclude from analysis dying cells. This allowed us to complete analysis up to 48 h.



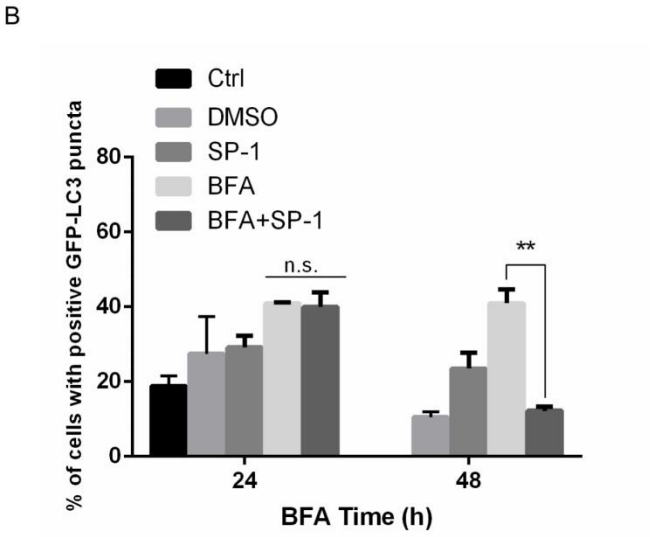


Figure 4.10 **Spautin-1 reduces BFA-induced autophagosome formation at the early time in C-9$^{-/-}$ MEFs.** C-9$^{-/-}$ MEFs transiently transfected with GFP-LC3 were treated with 0.3 µg/ml BFA ± 10 µM SP-1 and the percentage of GFP-LC3 positive punctate cells counted up to 48 h. (A) Representative fluorescent images are shown at magnification 20x. (B) Three fields of at least 100 cells/field were counted from three independent experiments (approx. 1500-2000 cells in total per replicate). The significance level is ** = $p<0.001$ and, n.s. indicates no significant difference.

To further confirm these results, the conversion of LC3-I to LC3-II was also investigated by Western-blot. C-9$^{-/-}$ MEFs were subjected to prolonged treatment with BFA in the presence or absence of spautin-1. Rapamycin was used as a positive control. As illustrated in Fig. 4.11A prolonged pre-incubation with spautin-1 reduced the level of Beclin 1 protein progressively in time-dependent manner. The strongest reduction of Beclin 1 either on basal level or BFA or rapamycin-induced was observed at the latest 72 h time point. Interestingly, the effect of spautin-1 on Beclin 1 was correlated with LC3-II level. Cotreatment with spautin-1 significantly reduced the level of LC3-II compared to BFA alone at 24 h whereas no significant difference was observed at 72 h. In contrast cotreatment of rapamycin with spautin-1 reduced the level of LC3-II over the timecourse (Fig. 4.11A), suggesting that spautin-1 strongly inhibits rapamycin-induced autophagy.

However, the cellular level of LC3-II alone may not correctly indicate the functionality of autophagy (Tanida et al., 2005). Therefore the densitometric ratio between LC3-I and LC3-II (LC3-II/LC3-I) which provides a more accurate measurement of autophagic activity was analysed (Klionsky et al., 2016). A quantitative analysis of Western blot showed that spautin-1 inhibits functional rapamycin-induced autophagy at the latest time point. A statistically insignificant reduction in LC3-II/LC3-I was observed, caused by spautin-1 in BFA–induced autophagy (Fig. 4.11B). Even though Beclin 1 level was decreased in the presence of spautin-1, either with BFA or rapamycin, the effect on functionality of autophagy is the opposite.

Furthermore, these results suggest that spautin-1 has a potency to significantly inhibit autophagy induced by rapamycin in a Beclin 1-dependent manner, whereas it is not efficient at inhibiting BFA-induced autophagy. It is also possible that this might be a Beclin 1-independent event. However, further examination needs to be performed in order to fully validate these data.

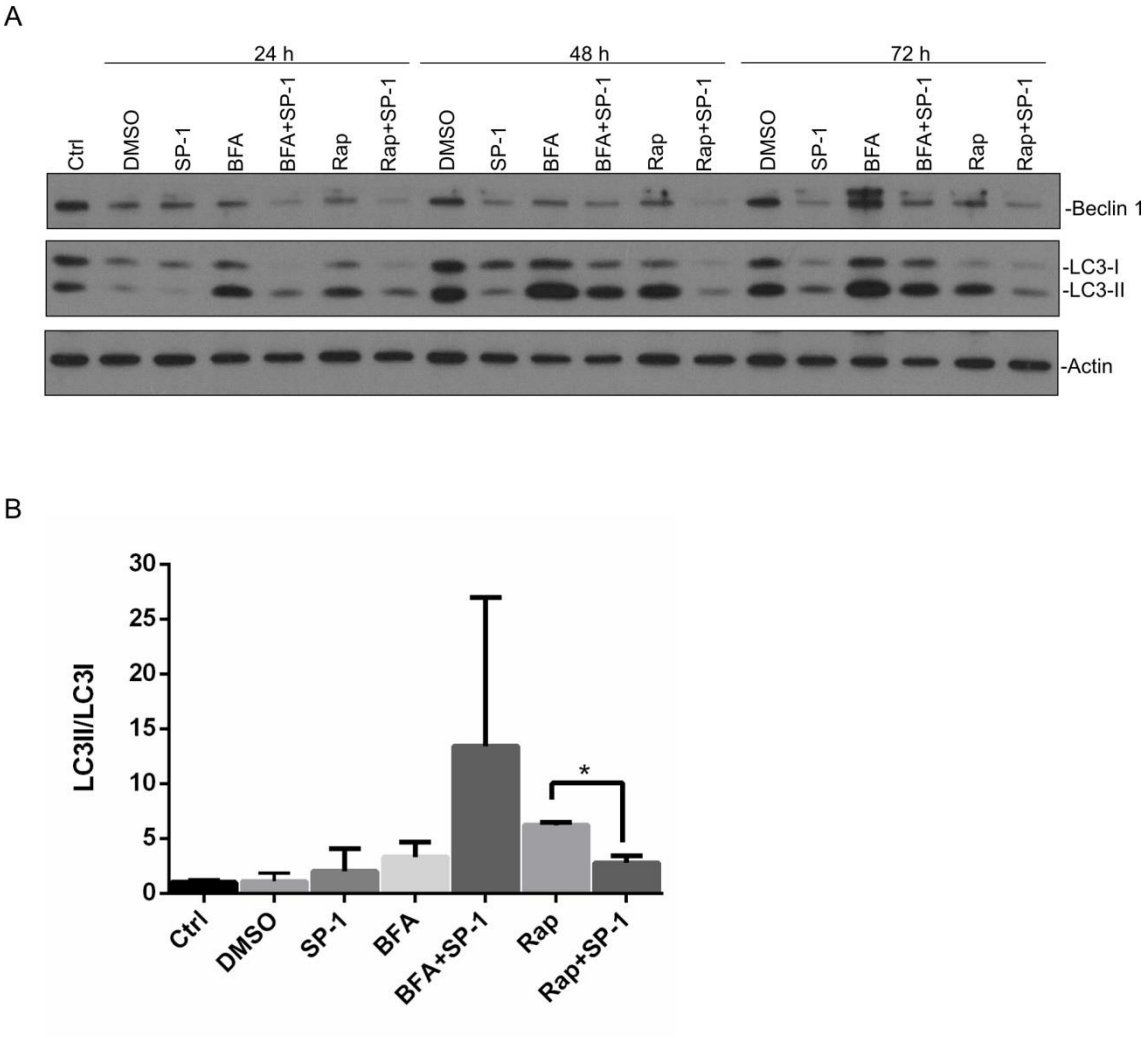


Figure 4.11 **Spautin-1 decreases BFA-induced autophagy at the early time but effectively reduces rapamycin-induced autophagy in C-9$^{-/-}$ MEFs.** C-9$^{-/-}$ MEFs were treated with 0.3 μg/ml BFA alone, 10 μM SP-1 alone , 400 nM Rap alone or a combination of BFA and SP-1 and Rap and SP-1 for up to 72 h. (A) Whole cell lysates were subjected to Western blot for expression of Beclin 1, LC3-I to LC3-II conversion and Actin. (B) Densitometric analysis of BFA, BFA+SP-1, Rap, Rap+SP-1 at 72 h normalized to control. Results are mean $\pm$ SD, * = $p<0.05$.

To this end, to determine the role of autophagy in cell death, Western blot analysis for caspase-8 activation was carried out in parallel with PI staining on C-9$^{-/-}$ MEFs treated with BFA either with or without spautin-1. Cotreatment with spautin-1 had no effect on BFA-induced caspase-8 activation at the latest time points as illustrated in Fig. 4.12A. Interestingly, while enhanced processing of caspase-3 was detectable at 48 h in cotreated samples; there was a slight decrease in caspase-3 processing at 72 h. Moreover, FACS analysis through PI uptake showed that spautin-1 significantly reduced cell death compared to BFA alone at 72 h, whereas it has no significant effect at the earliest time points (Fig. 4.12B). Taken together, spautin-1 inhibits functional BFA-induced autophagy at the early time points. Cell death induced by BFA is slowed down by spautin-1 at the latest stage of stress.

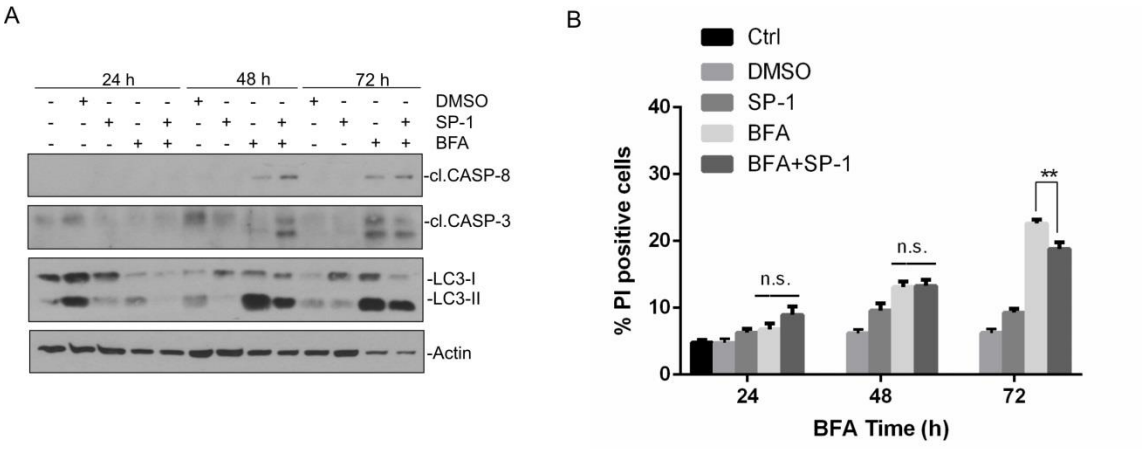


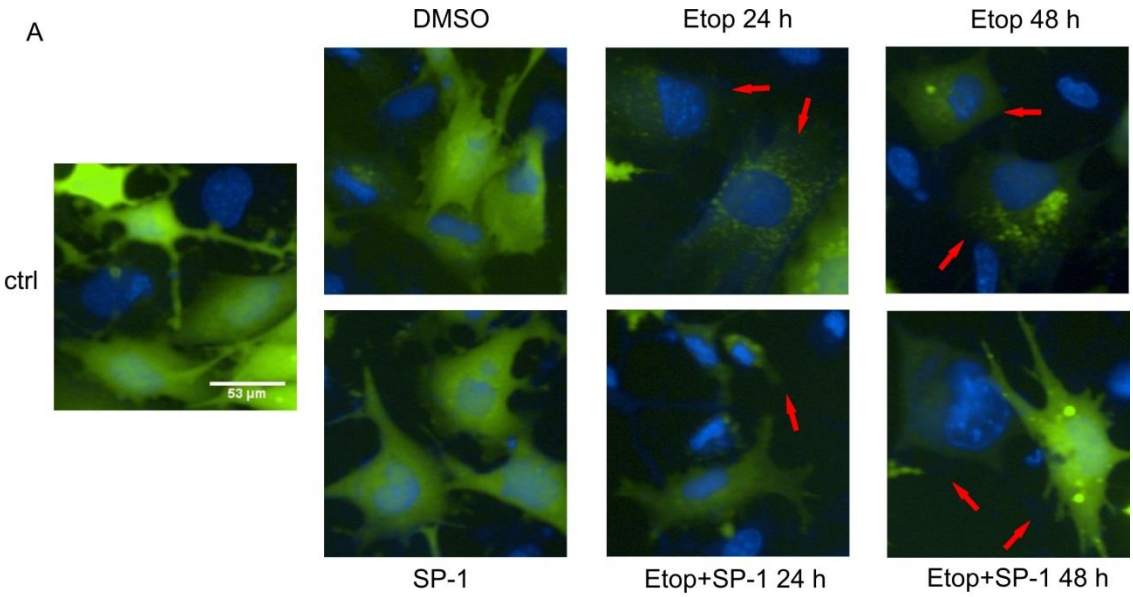
Figure 4.12 **Inhibition of autophagy by Spautin-1 slows cell death induced by BFA in C-9$^{-/-}$ MEFs.** C-9$^{-/-}$ MEFs were treated with 0.3 μg/ml BFA alone, 10 μM SP-1 alone or a combination of BFA and SP-1 for up to 72 h. (A) Whole cell lysates were subjected to Western blot for expression of cleaved caspase-8 (cl.CASP-8) and caspase-3 (cl.CASP-3), LC3-I to LC3-II conversion and Actin. (B) Cell death was assessed at the indicated time points by PI uptake. Values represent the mean ± SEM of three independent experiments. The significance level is ** = $p<0.001$ and n.s. indicates no significant difference.

4.3.3.3 Inhibition of autophagy upstream of ATG5 has not effect on cell death induced by Etop and Taxol

Since an enhanced level of autophagy has been shown to mediate cell death in cells compromised in apoptotic pathway (Deegan et al., 2014b, Shimizu et al., 2004), it was of interest to determine whether inhibition of autophagy upstream of autophagosome formation would reduce stress-induced cell death in C-9$^{-/-}$ MEFs.

Spautin-1 was used to further investigate the role of Etop and Taxol-induced autophagy on cell death. Investigation of GFP-LC3 transiently transfected C-9$^{-/-}$ MEFs cell subjected to prolonged treatment either with Etop or Taxol alone confirmed a significant increase in the number of cells with GFP-LC3 puncta over timecourse compare to the vehicle control as observed by fluorescence microscopy and illustrated in Fig. 4.13A and Fig. 14A respectively. Following Etop treatment addition of spautin-1 significantly reduced level of cells with GFP-LC3 at early time points. Indeed, there were around 40% of cells with GFP-LC3 puncta treated with Etop at 24 h whereas application of spautin-1 reduced the number of GFP-LC3 cell to 20% (Fig. 4.13B). In contrast, a statistically insignificant reduction was found in cells displaying GFP-LC3 punctate following Taxol treatment when cotreated with spautin-1 over timecourse (Fig. 4.14B).

Additionally, Hoechst staining was performed to observe the changes in the nucleus upon both treatments and exclude from analysis cells with apoptotic features. Therefore the quantification of GFP-LC3 puncta was provided only up to 48 h.



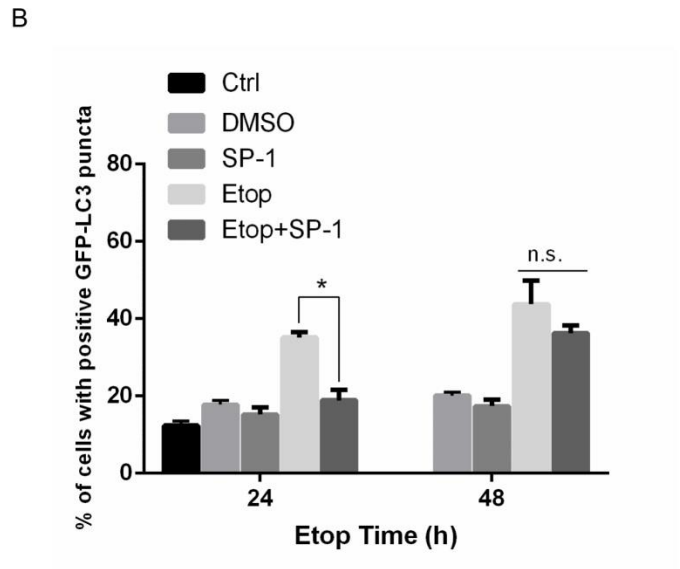
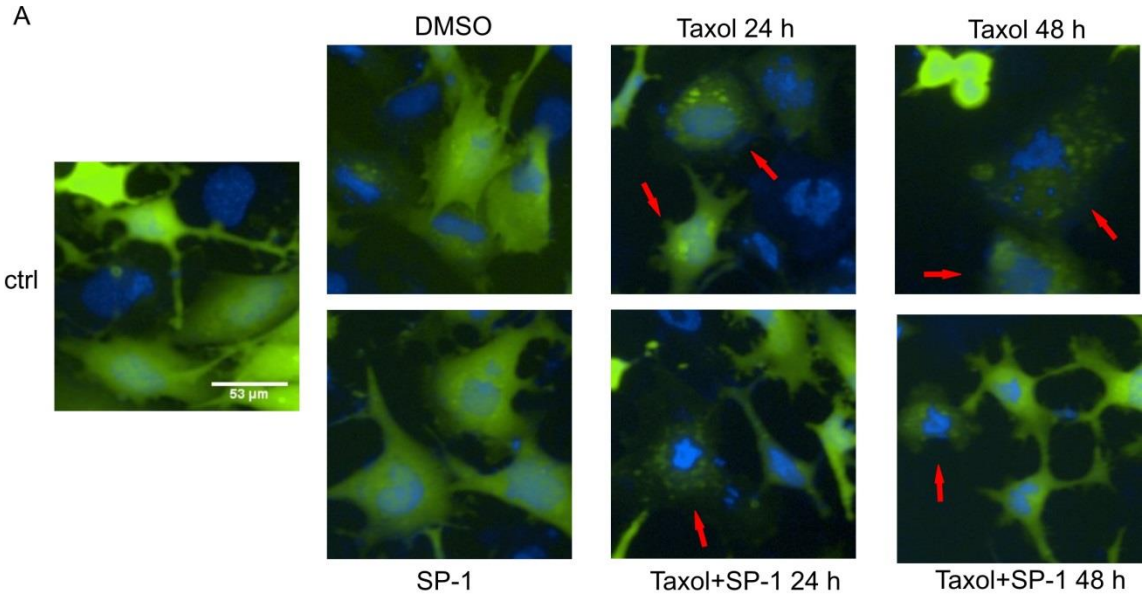


Figure 4.13 **Spautin-1 inhibits Etop-induced autophagosome formation at early timepoints in C-9$^{-/-}$ MEFs.** (A) C-9$^{-/-}$ MEFs transiently transfected with GFP-LC3 were treated with 50 µM Etop ± 10 µM SP-1 and the percentage of GFP-LC3 positive punctate cells counted up to 48 h. Representative fluorescent images are shown at magnification 20x. (B) Three fields of at least 100 cells/field were counted from three independent experiments (approx. 1500-2000 cells in total per replicate). The significance level is * = $p<0.05$, n.s. indicates no significant difference.



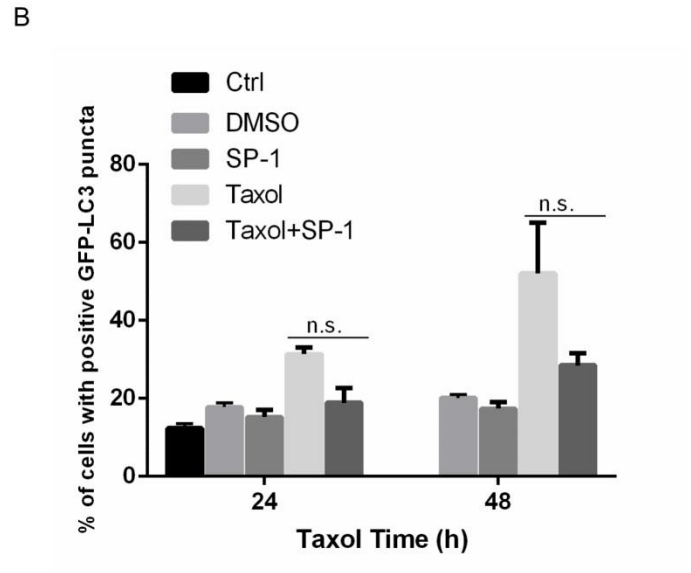


Figure 4.14 **Spautin-1 reduces Taxol-induced autophagosome formation in C-9$^{-/-}$ MEFs.** C-9$^{-/-}$ MEFs transiently transfected with GFP-LC3 were treated with 1 µM Taxol ± 10 µM SP-1 and the percentage of GFP-LC3 positive punctate cells counted up to 72 h. (A) Representative fluorescent images are shown at magnification 20x. (B) Three fields of at least 100 cells/field were counted from three independent experiments (approx. 1500-2000 cells in total per replicate). n.s. indicates no significant difference.

Because spautin-1 inhibited Etop-induced autophagy only at the earliest time, to further confirm these data LC3-I to LC3-II conversion was also monitored by Western blot upon prolonged pre-incubation with spautin-1. Etop elicited an increase in LC3-II level over the time course with a slight decrease when cotreated with spautin-1 only at 48 h. Interestingly, no significant decrease in Beclin 1 level was observed in Etop in the presence of spautin-1, a reduction in Beclin 1 was observed when spautin-1 was added to rapamycin (Fig. 4.15A).

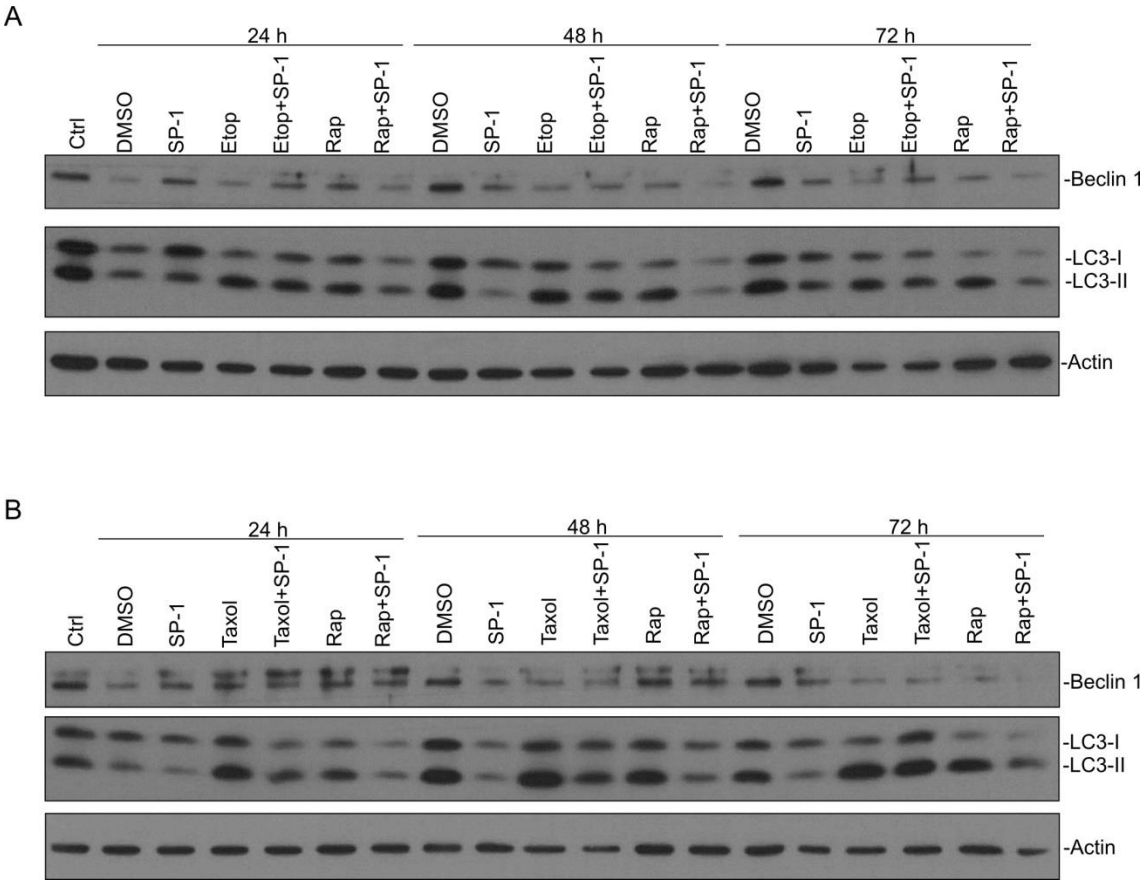


Figure 4.15 **Spautin-1 reduces Etop and Taxol-induced autophagy at the earliest timepoint but effectively reduces rapamycin-induced autophagy in C-9$^{-/-}$ MEFs.** C-9$^{-/-}$ MEFs were treated with (A) 50 μM Etop and (B) 1 μM Taxol alone, 10 μM SP-1 alone , 400 nM Rap alone or a combination of Etop/Taxol and SP-1 and Rap and SP-1 for up to 72 h. Whole cell lysates were subjected to Western blot for expression of Beclin 1, LC3-I to LC3-II conversion and Actin.

Similarly, Western blot analysis showed that cotreatment of Taxol with spautin-1 reduced levels of LC3-II only at early timepoints (Fig. 4.15B). Thus, the effect of spautin-1 on Taxol-induced autophagy could be an early event. Moreover, similar to

BFA and Etop, rapamycin-induced autophagy is strongly inhibited by spautin-1 over the timecourse (Fig. 4.15B).

These results confirm that spautin-1 was not able to reduce significantly LC3-II after prolonged treatment with Etop and Taxol. Taken together, spautin-1 inhibits autophagy induced by Etop and Taxol only at the initial stages of stress and has no effect on prolonged treatment.

Finally, to investigate whether the inhibition of initial stage of autophagy will rescue C-$9^{-/-}$ MEFs from Etop and Taxol-induced cell death, C-$9^{-/-}$ MEFs were subjected to prolonged treatment with Etop and Taxol in the presence and absence of spautin-1 for up to 72 h. Cotreatment with spautin-1 had no effect on Etop-induced caspase-8 activation at the latest time point, as monitored by Western blot (Fig. 4.16A). Similarly, processing of caspase-3 was also detected at 72 h upon Etop and did not change after addition of spautin-1. In contrast, cotreatment of Taxol with spautin-1 showed a slight reduction in both caspase-8 and caspase-3 processing at 48 h and 72 h, as illustrated by Fig. 4.16C. Moreover, addition of spautin-1 did not inhibit either Etop-induced or Taxol-induced cell death as analysed by PI uptake (Fig. 4.16B, D).

Taken together, these results confirm that Etop and Taxol induce formation of autophagosomes. Spautin-1 reduces level of autophagosomes and LC3-II only at the early time points. Inhibition of autophagy upstream of autophagosome elongation slightly decreases pro-caspase-8 and caspase-3 processing upon Taxol. This might be correlated with a reduction in autophagosomal membrane. Overall, spautin-1 had no effect on cell death elicited by Etop and Taxol in C-$9^{-/-}$ MEFs. These findings confirmed that stimulation of autophagy upstream of autophagosome elongation is not involved in Etop and Taxol-induced cell death.

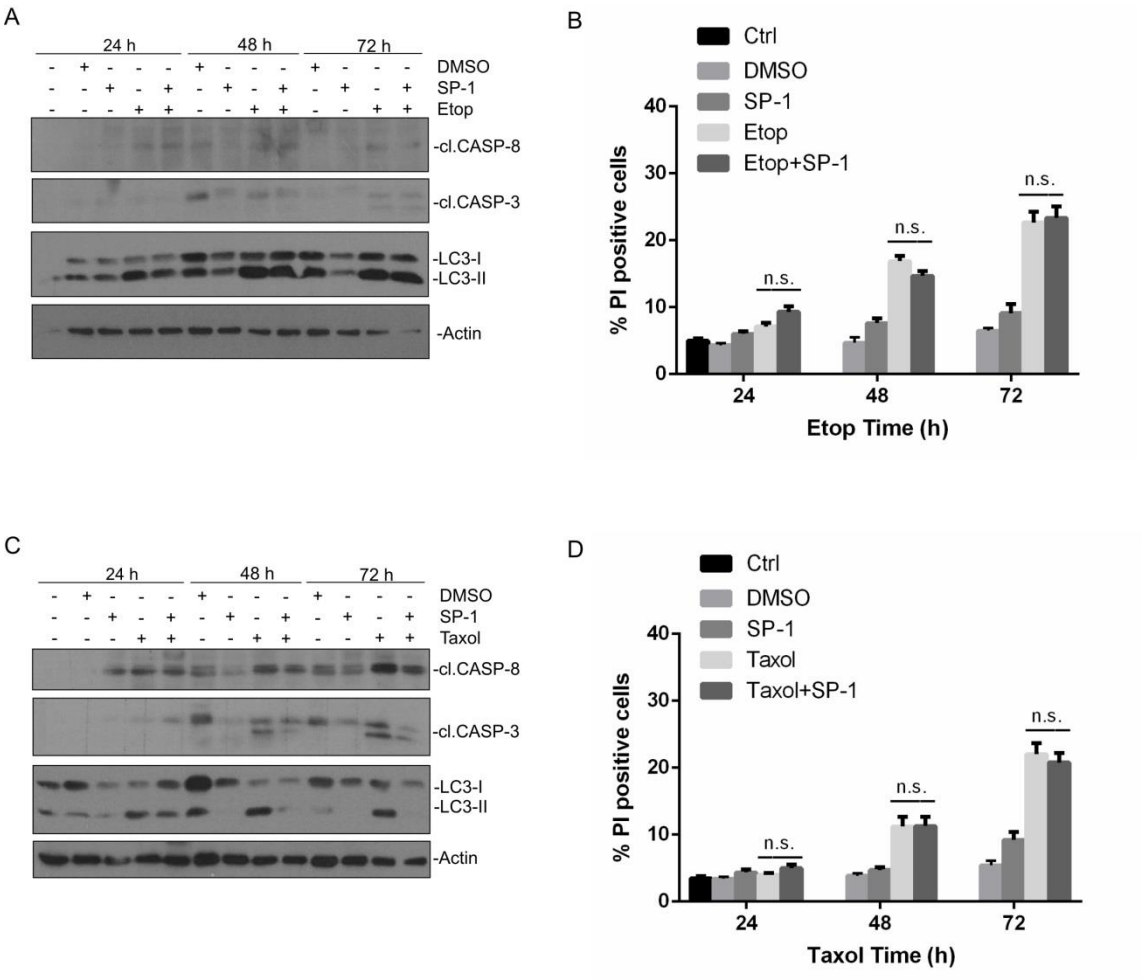


Figure 4.16 **Inhibition of autophagy upstream of autophagosome elongation does not affect cell death induced by Etop and Taxol in C-9$^{-/-}$ MEFs.** C-9$^{-/-}$ MEFs were treated with 50 μM Etop alone, 1 μM Taxol alone, 10 μM SP-1 alone or a combination of Etop and SP-1 , or Taxol and SP-1 for up to 72 h. (A, C) Whole cell lysates were subjected to Western blot for expression of cleaved caspase-8 (cl. CASP-8) and caspase-3(cl.CASP-3), LC3-I to LC3-II conversion and Actin. (B, D) Cell death was assessed at the indicated time points by PI uptake. Values represent the mean ± SEM of three independent experiments. n.s. indicates no significant difference.

4.3.3.4 Validation of Beclin 1 siRNA

To exclude the possibility of a non-specific effect of spautin-1, a genetic approach with siRNA was used to investigate the role of autophagy upon BFA, Etop and Taxol condition. To validate the functionality of *Beclin 1* siRNA changes in the cellular level of LC3-II under basal and stress-induced condition were analyzed. Rapamycin was used as positive control. C-9$^{-/-}$ MEFs were transiently transfected with *Beclin 1* siRNA and subjected to prolonged treatment with rapamycin. Western blot analysis confirmed

Beclin 1 knockdown under basal and rapamycin-induced conditions. Reduction in Beclin 1 was more pronounced under rapamycin treatment compare to vehicle control indicating the differences in the functionality of the autophagy process (Fig. 4.17).

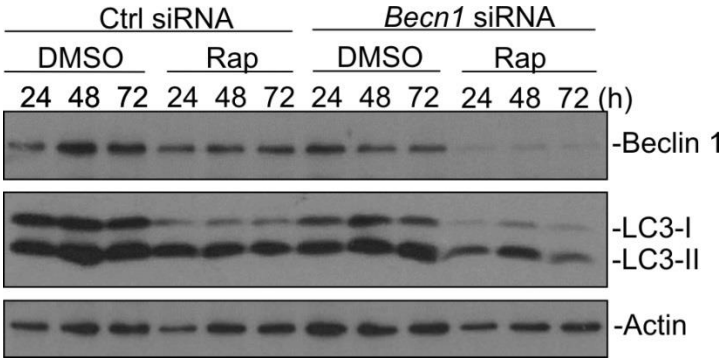


Figure 4.17 **Knockdown of Beclin-1 decreases rapamycin-induced autophagy in C-9$^{-/-}$ MEFs.** C-9$^{-/-}$ MEFs were treated with 400 nM Rapamycin for up to 72 h. Whole cell lysates were subjected to Western blot for expression of Beclin 1, LC3-I to LC3-II conversion and Actin**.**

Because rapamycin is a potent inducer of autophagy its application could activates the autophagic process at early timepoints, leading to a significant turnover of Beclin 1 over time. But because DMSO is not an autophagy inducer, the increased level of LC3-II is a result of accumulation of this protein overtime rather than induction of autophagy. Time course analysis upon rapamycin treatment showed that the level of Beclin 1 is significantly reduced at 72 h. As the half-life of Beclin 1 is around 60 h (Lian et al., 2011), this might explain effectiveness of knockdown at the latest time point. The level of LC3-II was considerably decreased in rapamycin-induced Beclin 1 knockdown. These results confirm that Beclin 1 knockdown was efficient at reducing rapamycin-induced autophagy.

4.3.3.5 Knockdown of Beclin 1 does not inhibit stress-induced autophagy in C-9-/- MEFs

To further confirm whether Beclin 1 is required for stress-induced autophagy Beclin 1 knockdown using siRNA was performed in C-9$^{-/-}$ MEFs. Transiently transfected cells were exposed to prolonged treatment either with BFA, Etop or Taxol and rapamycin was used as a positive control. Cells were harvested at indicated time and subjected for Western blot analysis for conversion of LC3-I to LC3-II.

As expected, a significant decrease in LC3-I to LC3-II level was observed upon treatment with rapamycin with *Beclin 1* siRNA (Fig. 4.18A-C). The strongest reduction in LC3-I to LC3-II conversion was detected at 72 h. As explained previously, this is correlated with the efficient reduction of Beclin 1 level, which half–life is around 60 h. Interestingly, no major difference was observed in the level of LC3-I to LC3-II conversion upon BFA, Etop or Taxol treatment, indicating that the levels of autophagy are not also affected in *Beclin 1* siRNA C-9$^{-/-}$ MEFs. As illustrated in Fig. 4. 18A-C the strongest difference between the levels of LC3-I to LC3-II conversion was observed with BFA compared to the other stresses. Western blot analysis confirmed also knockdown of *Beclin-1*. Although Beclin 1 seems to be efficiently reduced in Etop and Taxol treatments, it is difficult to conclude its efficiency under BFA treatment. But because the knockdown of *Beclin 1* is efficient in rapamycin under all three treatments, these results cannot be ruled out and are valid.

Taken together, these results revealed that reduction of Beclin 1 did not affect BFA, Etop or Taxol-induced autophagy but strongly inhibits autophagy induced by rapamycin. BFA, Etop and Taxol might potentially induce autophagy in Beclin-1-independent manner. However, more examination needs to be conducted to verify this observation and I do not address this possibility within the scope of this work.

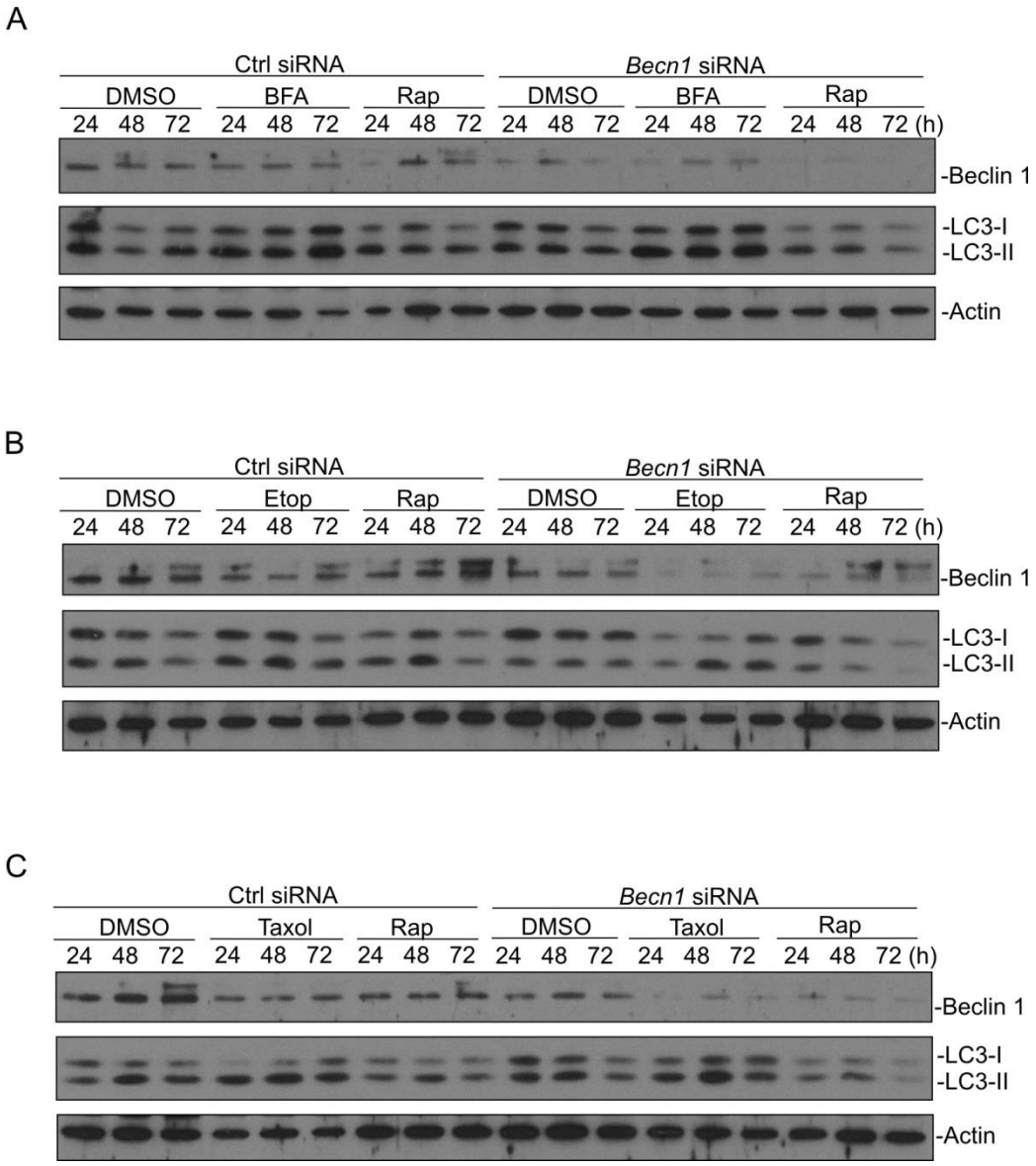


Figure 4.18 **Knockdown of Beclin 1 does not reduce stress-induced autophagy in C-9$^{-/-}$ MEFs**. C-9$^{-/-}$ MEFs were treated with (A) 0.3 μg/ml BFA, (B) 50 μM Etop, (C) 1 μM Taxol and 400 nM Rapamycin for up to 72 h. Whole cell lysates were subjected to Western blot for expression of Beclin1, LC3-I to LC3-II conversion and Actin.

Thus far, the results showed that downregulation of Beclin 1 in C-9$^{-/-}$ MEFs did not decrease LC3-I to LC3II conversion upon BFA, Etop and Taxol treatment. Therefore, it was important to determine whether the level of autophagy would be affected in response to various stresses in *Beclin 1* siRNA C-9$^{-/-}$ MEFs. Autophagic flux was examined in response to BFA, Etop and Taxol, in the presence or absence of CQ. Western blot analysis showed that autophagic flux was not affected in cells that were transfected with *Beclin 1* siRNA as compared with the cells transfected with control siRNA. As illustrated in Fig. 4.19A-C, the same increase in LC3-II level was observed in cells cotreated with BFA, Etop or Taxol and CQ. Because, the obtained results showed that Beclin 1 was not fully reduced it might suggest that the *Beclin 1* knockdown was not efficient enough to inhibit stress-induced autophagy.

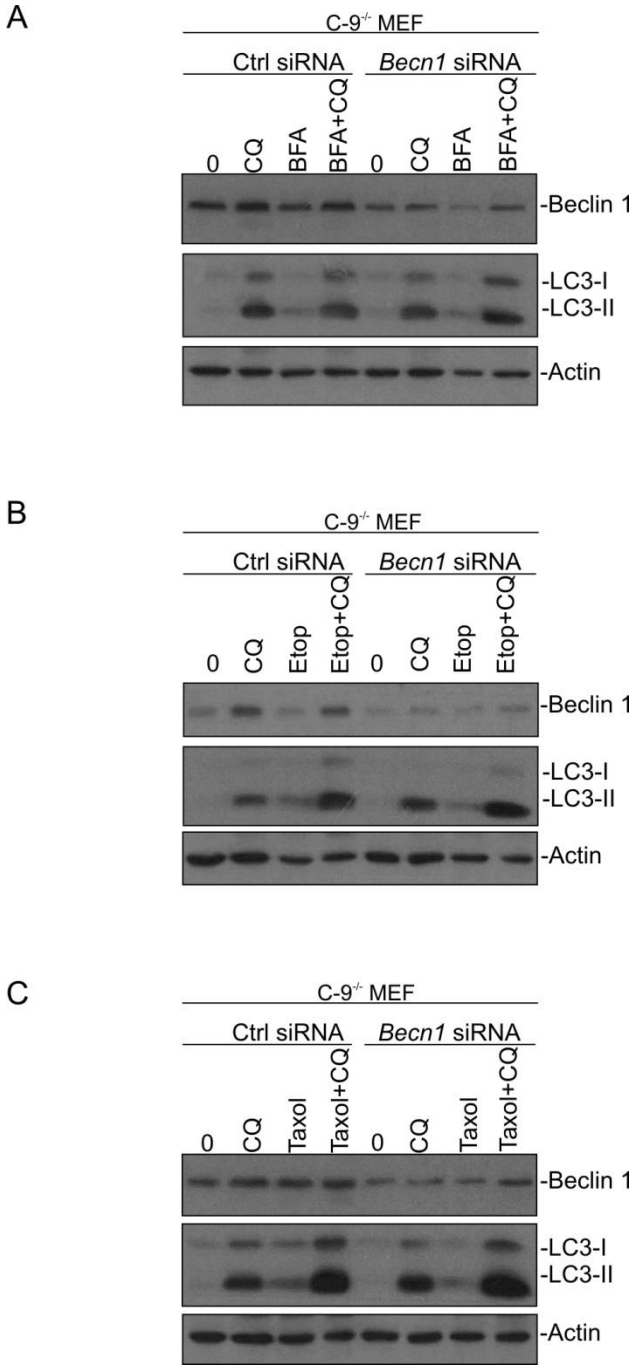


Figure 4.16 **Autophagic flux in C-9$^{-/-}$ MEFs deficient for Beclin-1.** *Ctrl* and *Beclin1* siRNA C-9$^{-/-}$ MEFs were treated with (A) 0.3 μg/ml BFA, (B) 50 μM Etop, (C) 1 μM Taxol alone, 20 μM chloroquine (CQ) alone or a combination of BFA, Etop or Taxol and CQ for 48 h. Whole cell lysates were subjected to Western blot for expression of LC3-I to LC3-II conversion and Actin**.**

4.3.3.6 Knockdown of Becn1 does not inhibit stress-induced caspase activation and cell death in C-9-/- MEFs

To determine the effect of autophagy inhibition upstream of ATG5 on cell fate under diverse stress condition, transient knockdown of Becn1was performed in C-9$^{-/-}$ MEFs.
It was observed that the abrogation of *Beclin 1* gene expression was accompanied with the strongest expression of processed pro-caspase-8 and caspase-3 in the presence of BFA, Etop and Taxol (Fig. 4.20A-C, left panel). Interestingly, upon Etop treatment more pronounced activation of caspase-3 was observed in *Beclin 1* siRNA compare with the control siRNA (Fig. 4.20B left panel). It is important to note that knockdown of Beclin 1 was not efficient in BFA, Etop and Taxol to potently reduce caspase cleavage. But because the same trend was observed in previous experimental settings I cannot rule out these data however more investigation need to be done in order to fully examine role of Beclin 1 in C-9$^{-/-}$ MEFs.

Furthermore, knockdown of *Beclin 1* in C-9$^{-/-}$ MEFs did not affect stress-induced cell death as determined by PI staining. FACS analysis showed that following treatment with either BFA, Etop or Taxol knockdown of *Beclin 1* in C-9$^{-/-}$ MEFs was not able to rescue C-9$^{-/-}$ MEFs (Fig. 4.20 A-C right panel).

These results indicate that inhibition of autophagy upstream of autophagosome elongation does not affect BFA, Etop and Taxol-induced cell death in C-9$^{-/-}$ MEFs.

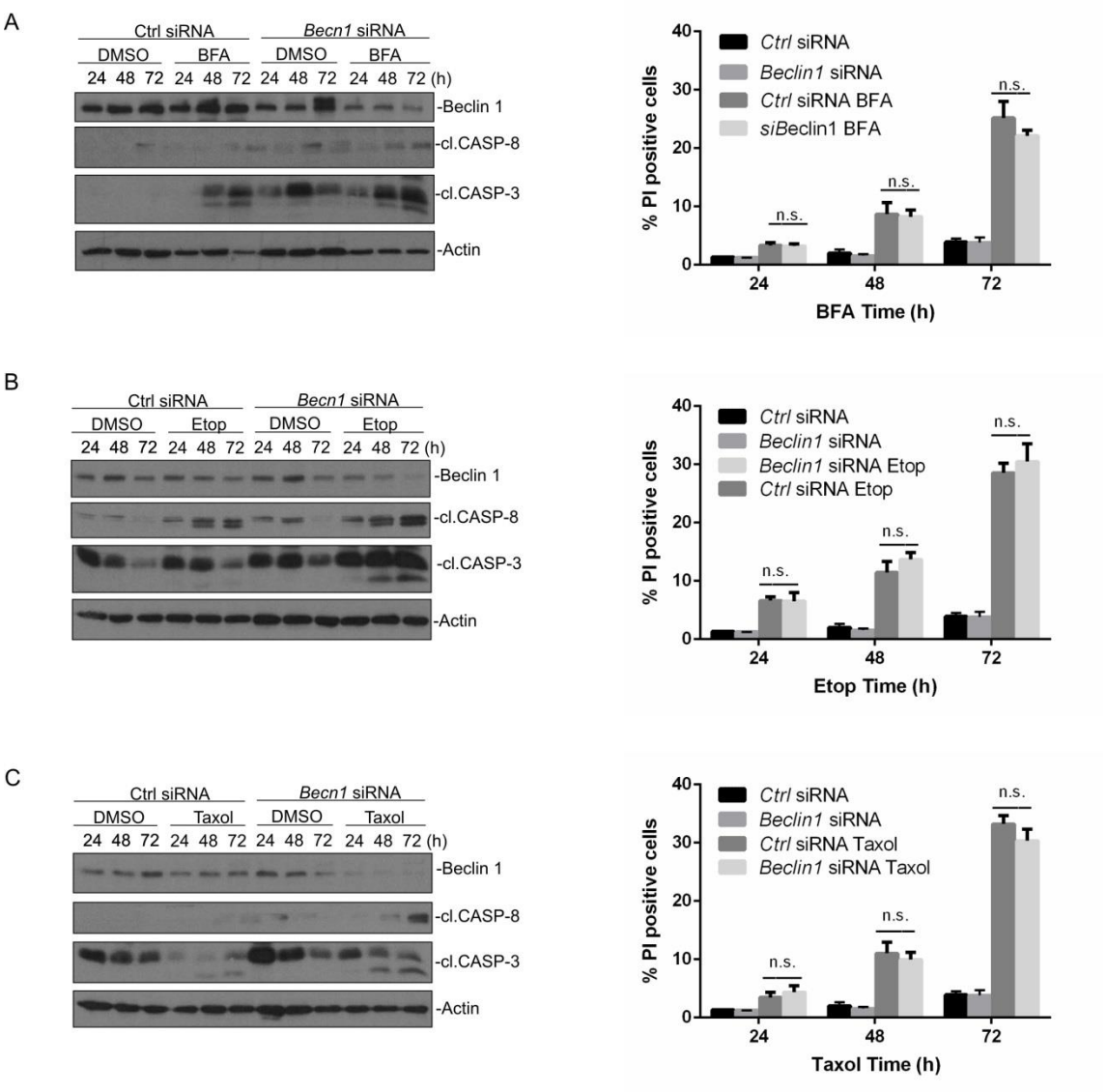


Figure 4.20 **Knockdown of Beclin does not reduce cell death and caspase-8 and caspase-3 processing upon exposure to sustained treatment with BFA, Etop and Taxol in C-9$^{-/-}$ MEFs .** C-9$^{-/-}$ MEFs transfected with *Becn1* or Ctrl siRNA were treated with (A) 0.3 μg/ml BFA, (B) 50 μM Etop, (C) 1 μM Taxol for indicated time after which lysates were subjected to Western blot for Becn1, cleaved caspase-8 (cl.CASP-8) and caspase-3 (cl.CASP-3) and Actin. Values represent the mean ± SEM of three independent experiments. n.s. indicates no significant difference.

4.4 Discussion

Autophagy maintains cellular homeostasis through degradation of cellular components. It also acts as a mechanism that promotes adaptation to a variety of different types of stress by recycling cellular macromolecules and by removing protein aggregates and dysfunctional cytoplasmic organelles (Ding et al., 2007, Yorimitsu et al., 2006, Levine and Klionsky, 2004). However, upon sustained exposure to stress, excessive autophagy has been implicated in the execution of cell death. Autophagy-mediated cell death has been widely described in cells deficient in the intrinsic apoptotic pathway (Tsujimoto and Shimizu, 2005), but the molecular mechanisms of this mode of cell death and how autophagy is linked with the cellular death machinery is still unclear and remains to be fully elucidated.

Given that an alternative caspase-mediated death pathway is activated to compensate for a lack of caspase-9 or BAX and BAK, this raises the possibility that such a pathway might be a general response to cellular stress. Since previous work carried out in our laboratory had focused only on ER stress, in this chapter I evaluated the importance of autophagy in this alternate death pathway in response to additional cell death-promoting stimuli.

The experiments presented in the previous chapter partially characterized how BFA, Etop, Taxol, and γ-irradiation trigger cell death in C-$9^{-/-}$ MEFs. The results showed that the examined stresses activate capase-8; however, not all the stresses were fully reliant on caspase-8 to induce cell death, suggesting that apart from the novel caspase activation pathway examined in this thesis, other alternative pathways are also potentially activated to execute cell death. However, if caspase-8 is the main driver of cell death in the cellular system under investigation, a better understanding of how its activation is regulated is still required. Atg5 contributes to autophagy-mediated cell death through its interaction with FADD (Pyo et al., 2005), and in this way is also involved in the activation of caspase-8 (Deegan et al., 2014b, Young et al., 2012, Bell et al., 2008). Therefore, in this chapter I focused on the role of Atg5 as an essential component of the alternative stress-induced caspase activation pathway.

Following BFA, Etop, and Taxol treatments, functional autophagy was strongly induced and was dependent on ATG5. To detect ATG5 the monoclonal antibody that recognizes both the endogenous level of total ATG5 and ATG5-ATG12 conjugated form was used. Of note, the level of p62 protein which serves as an alternative approach to monitor the autophagic activity, was not performed within the scope of this thesis, however it was previously tested in this cellular system. Since p62 is degraded during functional autophagy, the reduction in p62 level can be detected through Western blot. Indeed, in our preliminary examination it was difficult to conclude the changes in the cellular level of p62 in C-9$^{-/-}$ MEFs. Moreover, application of autophagic flux in Atg5 deficient C-9$^{-/-}$ MEFs did not show convincing results in p62 reduction upon tunicamycin treatment. Furthermore, in addition to LC3 processing, p62 was also used as readout of autophagic activity in rapamycin dose-response optimization. However, transient increase in p62 level in rapamycin dose-dependent manner was observed in C-9$^{-/-}$ MEFs. Therefore, it was difficult to conclude whether p62 could potentially serve as marker of autophagy in C-9$^{-/-}$ MEFs. Considering the fact that the changes in the p62 level can be either cell-type or context specific (Klionsky et al., 2016), raises the possibility that detection of LC3-I to LC3-II conversion and autophagic flux with CQ may be more accurate readout of autophagy in C-9$^{-/-}$ MEFs. Nevertheless, further investigation of p62 is needed in order to fully understand stress-induced autophagy in C-9$^{-/-}$ MEFs.

Although several studies have already demonstrated that various stresses induce autophagy, there is still an incomplete understanding about how autophagy contributes to cell death. A recent *in vivo* study showed that ATG5 is implicated in cellular stress responses such as inflammation, stress-induced senescence and apoptosis (Lin et al., 2014). Moreover, it has been shown that in response to several apoptotic stimuli Atg5 could promote either apoptosis or activate pro-survival autophagy in Bax/Bak-deficient cells (Tsujimoto and Shimizu, 2005). Interestingly, in this study, the authors showed that etoposide and staurosporine upregulate expression of Atg5 leading to cell death. Moreover, the study presented by Kim and colleagues showed that Bax/Bak$^{-/-}$MEFs, although resistant to gamma irradiation, also induce autophagy which was correlated with cell death, suggesting that in the absence of the intrinsic apoptotic pathway, γ-irradiation induces an alternative cell death pathway dependent on autophagy (Kim et

al., 2006). Inhibition of autophagy has also been shown to enhance Taxol-induced caspase-dependent apoptosis in nasopharyngeal carcinoma (Song et al., 2017).

The involvement of ATG5 in stress-induced cell death in C-9$^{-/-}$ MEFs was tested by knockdown of Atg5. Because full activation of caspase-8 requires the formation of a multiprotein complex on membranes, an involvement of Atg5 could mean that autophagosome membranes are necessary for this process. The obtained results demonstrate that Atg5 contributes to stress-induced caspase-dependent cell death in response to all treatments. Knockdown of Atg5 efficiently reduced processing of pro-caspase-8 and caspase-3 upon BFA, Etop, Taxol, and γ-irradiation, suggesting an involvement of the autophagy pathway. It is interesting that BFA which triggers ER stress, Etop and γ-irradiation which induce genotoxic stress, and Taxol which affects microtubule polymerization, all show dependency on Atg5; this suggests that the autophagosomal membrane might be an essential platform for the activation of caspases and subsequent cell death in C-9$^{-/-}$ MEFs in response to these stress conditions. Some recent reports support the idea that Atg5 directly interacts with FADD and pro-caspase-8 to mediate caspase-dependent cell death (Young et al., 2012, Bell et al., 2008). However, in contrast to these studies my experiments show that Atg5 was not required for stress-induced cell death in response to all treatments. Indeed, while BFA- and Etop-induced cell death was dependent on Atg5, cells lacking Atg5 still died in response to Taxol and γ-irradiation, suggesting that another caspase-independent cell death pathway is activated, for instance necrosis or necroptosis. This is an important observation, as so far only a few reports have provided insight into the mechanism of Taxol-induced cell death (Impens et al., 2008, Torres and Horwitz, 1998). Yeng and colleagues suggested that high concentrations of Taxol lead to necrosis due to the accumulation of microtubules and subsequent inhibition of cell cycle in S phase (Yeung et al., 1999). Indeed, it has been shown that when apoptosis is defective, then necrosis takes over as a cell death modality (Golstein and Kroemer, 2005). However, the fact that my previous data showed that the applied dose of Taxol induced caspase-dependent cell death suggests that necroptosis rather than necrosis might execute cell death in this cellular setting. Indeed, when caspases are inhibited due to the loss of Atg5, Taxol can induce

the necroptotic pathway to terminate C-9$^{-/-}$ MEFs. This suggests that necroptosis could possibly constitute a backup not only to canonical apoptosis as already suggested (Long and Ryan, 2012), but, as shown in this study, it may also be a compensatory pathway for novel stress-induced caspase activation pathway in C-9$^{-/-}$ MEFs. However, further investigation is needed in order to confirm this phenomenon.

Consistent with this, deficiency of Atg5 in C-9$^{-/-}$ MEFs was able to restore cellular proliferative ability after the initial stress of Taxol and γ-irradiation, and conferred a survival advantage almost to the same extent as in the control shRNA cells. This might suggest that Atg5 is necessary but not sufficient to execute Taxol and γ-irradiation-induced cell death in C-9$^{-/-}$ MEFs. In contrast, abrogation of Atg5 gene expression did not rescue C-9$^{-/-}$ MEFs in a long-term survival assay upon Etop treatment like control shRNA, but was able to rescue C-9$^{-/-}$ MEFs after BFA treatment. Therefore, these results underline the fact that various stresses possess different modes of action and that BFA is the only stress that allowed cellular recovery in C-9$^{-/-}$ MEFs in the absence of Atg5. A similar effect was described in Chapter 3 where in the absence of caspase-8, BFA restored the proliferative ability of C-9$^{-/-}$ MEFs, confirming that although all the examined stresses activate a novel caspase activation pathway, ER stress induced by BFA seems to depend only on this pathway to execute cell death. Consistent with this, several reports demonstrate that prolonged ER stress promotes cell death through sustained autophagy (Deegan et al., 2014b, Sano and Reed, 2013). Therefore, it seems that compounds that selectively induce ER stress directly trigger a novel caspase activation cell death pathway in highly resistant cells and thus merit consideration as potential alternative therapies. This is a very interesting and important observation that may facilitate a better understanding of the mechanism of action of drugs in the context of the efficient treatment of different tumors.

After examining the involvement of Atg5, I next tested whether other components of the autophagic machinery are involved in stress-induced cell death in C-9$^{-/-}$ MEFs. Using spautin-1, a novel and specific autophagy inhibitor, I looked at the effect of inhibiting the initial steps in the autophagic process upstream of autophagosome elongation. The molecular mechanism by which spautin-1 blocks autophagy is through the inhibition of

two specific ubiquitin peptidases, USP10 and USP13 that regulate the levels of Beclin 1. The original study by Liu and colleagues showed that spautin-1 effectively blocked Etop-induced autophagy in Bax/Bak$^{-/-}$ MEFs (Liu et al., 2011). The efficiency of spautin-1 was validated against rapamycin, which is a classical inducer of mTOR-dependent autophagy (Law, 2005). Both rapamycin and spautin-1 were potent in either promoting or inhibiting autophagy in C-9$^{-/-}$ MEFs, respectively. Indeed, as a consequence of rapamycin treatment I observed an increased proportion of cells positive for GFP-LC3, while the addition of spautin-1 significantly reduced this number. As explained already, accumulation of GFP-LC3 is correlated with an increased number of autophagosomes; therefore, monitoring the number of cells with GFP-LC3 puncta provides an effective approach to monitor induction of autophagy.

Having established the efficacy of spautin-1, I now asked how inhibition of autophagy upstream of autophagosome elongation affected cell death induced by BFA, Etop, and Taxol. Spautin-1 treatment significantly lowered the number of cells with GFP-LC3 at 48 h when applied to BFA-treated cells. However, the increase in LC3-I to LC3-II conversion after prolonged treatment although insignificant, clearly shows that spautin-1 was not able to efficiently inhibit BFA-induced autophagy at the latest time point, whereas it significantly reduced rapamycin-induced autophagy. In comparison, although spautin-1 treatment significantly reduced the number of GFP-LC3-positive cells at the initial stage of Etop treatment it was not able to significantly reduce this number upon Taxol treatment, although a clear decreasing trend was observed. In addition, analysis of LC3-I to LC3-II conversion confirmed that spautin-1 was not able to block accumulation of LC3-II upon prolonged treatment with Etop and Taxol, but robustly inhibited rapamycin induced-autophagy over the same time course. This could suggest that BFA, Etop, and Taxol might be stronger inducers of autophagy than rapamycin or that their cytotoxic effects overwhelmed the capacity of cells to respond to spautin-1. Therefore, even though spautin-1 was replenished every 24 h during the duration of the time course it was observed to lose its potency against BFA, Etop, and Taxol. Thus, following prolonged BFA, Etop, and Taxol treatment, the increase in autophagosomes seems to reach a threshold level above which enhanced autophagy is accompanied with cell death. This suggests that the cell death induced by these drugs strongly depends on

autophagy. As spautin-1 should decrease the levels of Beclin 1, I also looked at the changes in the expression levels of this protein. As expected, spautin-1 significantly reduced the levels of Beclin 1 upon rapamycin treatment. This is not surprising as rapamycin is a classical inducer of autophagy that requires activation of all components of the canonical autophagy pathway, implicating Beclin 1 as an essential part for this pathway. Although I observed that Beclin 1 levels were slightly reduced by the addition of spautin-1 to BFA-treated cells, spautin-1 addition did not bring about a convincing reduction in the levels of Beclin 1 upon Etop or Taxol treatment. Therefore, it is difficult to conclude whether Beclin 1 is involved or not in BFA-, Etop- and Taxol-induced autophagy.

On the basis of previous reports suggesting that autophagy can exhibit pro-death properties especially in cells defective in the apoptotic pathway, and to fully address the role of autophagy in stress-induced cell death, the activity of PI3 kinase complexes was blocked and the amount of cell death over time was measured. The class III phosphatidylinositol 3-kinase complex consists of Beclin 1, VPS34, VPS15, ATG14, and NRFB2 and plays an essential role in autophagosome elongation (Galluzzi et al., 2017a). Because spautin-1 was able to inhibit autophagy induced by BFA, Etop, and Taxol only in the initial, early phase of stress, I did not expect it would protect cells from the effects of prolonged stress. Indeed, spautin-1 was not able to either significantly reduce pro-caspase-8 and caspase-3 processing or cell death as determined by PI uptake in response to prolonged Etop treatment. However, it indeed resulted in lower levels of cell death at the last timepoint of BFA treatment. This can potentially be explained by the observation that in response to BFA treatment, a significant reduction in GFP-LC3 was detected at 48 h only; therefore, the decrease in cell death might be correlated with a reduction in autophagosomal membranes. Similarly, cotreatment with Taxol slightly decreased caspase processing; however, spautin-1 had no effect on Taxol-mediated cell death. The reason for the lack of an effect of spautin-1 on cell death in C-$9^{-/-}$ MEFs upon prolonged treatment with either Etop or Taxol and the only partial effect with BFA is unknown, but from the experimental observations it can be speculated that the amount of autophagosomes formed upon BFA, Etop, and Taxol although reduced by

spautin-1 in response to initial stress, is sufficient for caspase activation and subsequent cell death.

Spautin-1 indirectly promotes Beclin 1 degradation; therefore, a siRNA approach was used in order to avoid the side effects of pharmacological inhibition. Because Beclin 1 is an important molecule in the autophagy process, its loss could potentially impact autophagic degradation. Therefore, I examined LC3-I to LC3-II conversion upon BFA, Etop, and Taxol treatments in the presence and absence of Beclin 1. My experiments showed that Beclin 1 efficiently reduced rapamycin-induced autophagy but did not affect BFA-, Etop- and Taxol-induced autophagy. This suggests that BFA, Etop, and Taxol might potentially induce a non-canonical form of autophagy that is independent of Beclin 1. There are several reports suggesting that this mode of Beclin 1-independent autophagy is also involved in cell death (Sun et al., 2015, Scarlatti et al., 2008). For example, the study of Grishchuk et al. showed that in response to various apoptotic stimuli cortical neurons trigger Beclin 1-independent but ATG5/ATG7-dependent autophagy which is required for both caspase-dependent and -independent pathways of cell death (Grishchuk et al., 2011). However, this exciting observation was not examined within the scope of this thesis, since I focused on the role of autophagy in stress-induced cell death. However, further investigations are warranted in order to fully understand this phenomenon.

Interestingly, knockdown of Beclin 1 was not able to reduce the level of stress-induced autophagy triggered by BFA, Etop, and Taxol. Therefore, it would be surprising if cells deficient in Beclin 1 would be protected against stress-induced cell death. Indeed, knockdown of Beclin 1 although not efficient, did not reduce the processing of pro-caspase-8 and caspase-3 upon BFA, Etop, and Taxol treatments and also did not reduce cell death. Because a pool of siRNAs was used to knockdown Beclin 1, off-target effects can be ruled out. However, knockdown of Beclin 1 may not always have been sufficient to have a functional effect on the conducted experiments. Therefore, to fully investigate the role of Beclin 1, it would be important to perform a stable genetic knockdown with shRNA, as it could provide a more definitive answer as to its role in stress-induced cell death in C-9$^{-/-}$ MEFs. However, it is important to note that my experiments with siRNA-

mediated knockdown of Beclin 1 gave similar results to pharmacological inhibition with spautin-1, except for BFA treatment where spautin-1 slowed down cell death.

Taken together, the data presented in this chapter show that stress-induced cell death associated with caspase processing tends to be dependent on ATG5 but not necessarily on the whole process of autophagy. Although the induction of functional autophagy is crucial for the formation of autophagosomes, inhibition of the initial stage of the autophagy process showed no effect on cell death. The importance of ATG5 in stress-induced cell death in C-9$^{-/-}$ MEFs was clearly confirmed. Thus, these results support the concept of an alternative caspase-activation death pathway, which is dependent on ATG5. However, it is important to mention that in the original study of Deegan *et al.*, the role of Atg7 was also examined (Deegan et al., 2014b). As ATG7 is responsible for the covalent binding of ATG5 and ATG12 and the subsequent formation of the protein complex ATG5-ATG12-ATG16, it is also required for lipidation of LC3 (Yang and Klionsky, 2010). Therefore the role of ATG7 is linked to the elongation step of autophagosome formation. Thus, knockdown of either Atg5 or Atg7 confirmed that the novel death-inducing protein complex was not specific to just Atg5 but rather reflects a more general autophagic response related to autophagosome membranes (Deegan et al., 2014b). For that reason, in this chapter, although I specifically focused on ATG5, I was rather interested in its integral role as part of the autophagosome membrane rather than in the ATG5 molecule itself.

The experiments described in this chapter showed new evidence for the crucial role of ATG5-dependent cell death that is required for caspase-8 activation in C-9$^{-/-}$ MEFs in response to various stresses (Fig. 4.21). These results highlight that various stresses induce autophagy in caspase-9-deficient MEFs. Although ATG5 is required for caspase processing in response to different stresses, the requirements for ATG5 were found to be stress stimulus-specific. While BFA and Etop trigger ATG5-dependent cell death under conditions of caspase-9-deficiency, exposure to Taxol and γ-irradiation was observed to drive an ATG5-independent mode of cell death

This is the first time that a pathway involving ATG5 has been shown to induce cell death in response to various stress in C-9$^{-/-}$ MEFs. Since cell death modalities are

extensively studied in cells lacking the components of the intrinsic apoptotic pathway due to their high resistance to various apoptotic stimuli, this novel caspase activation pathway seems to be an attractive alternative signaling network that could be targeted for therapeutic purposes.

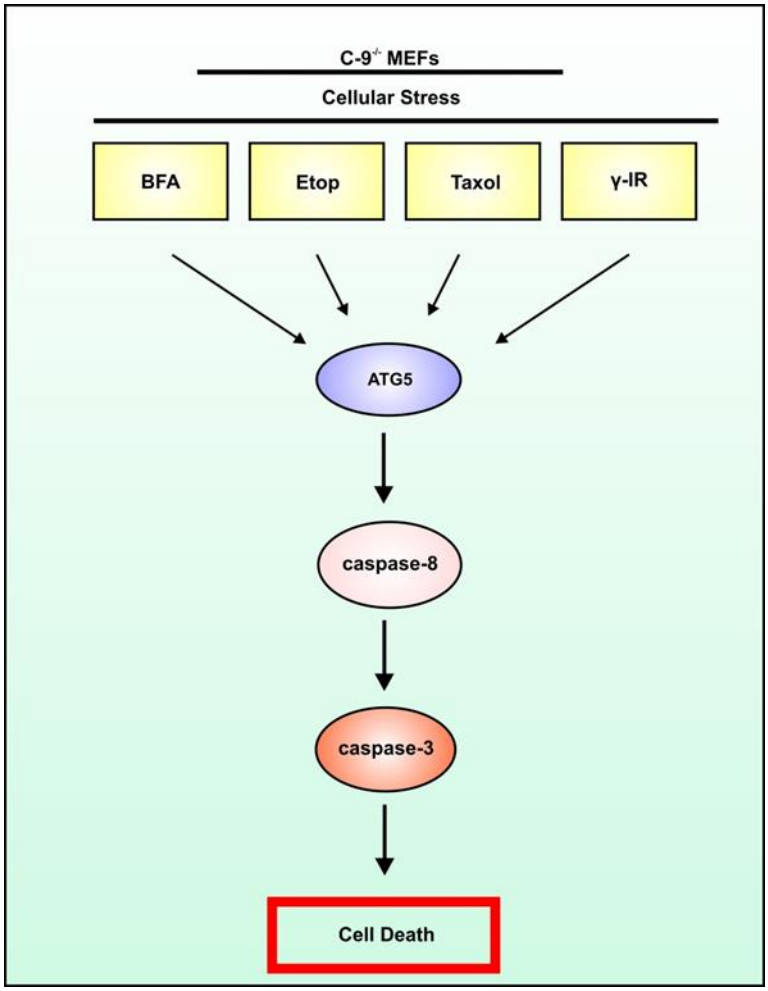


Figure 4.21 **Various stresses induce ATG5-dependent caspase-mediated cell death in C-9$^{-/-}$ MEFs**. The graphic represents a model of the stress-induced caspase activation pathway. ATG5 triggers caspase-8-mediated stress-induced cell death in cells deficient in caspase-9 in response to different stresses.

Chapter 5: Role of ATF4 and CHOP in stress-induced autophagy and cell death in C-9$^{-/-}$ MEFs

5.1 Introduction and Objectives

In response to variety of different stresses, cells activate a common stress response pathway characterized by phosphorylation of eIF2α. In mammals, this molecular event is mediated by four eIF2α kinases: PERK, PKR, GCN2, and HRI and it is an important stimulator of an adaptive cellular program known as the ISR. The role of the ISR is to protect cells from the negative consequences of several unrelated stresses such as metabolic stress, pathogen infection, and ER stress. However, depending on the nature of the stress, its frequency and intensity, it is also reported that the ISR can switch to cell death. Although the mechanisms by which the ISR mediates cell death are not fully clear, several reports have linked ATF4 and CHOP with apoptosis (Pakos-Zebrucka et al., 2016).

ATF4 and CHOP are basic leucine zipper (bZIP) transcription factors that are central to the ISR. Elevated translation of ATF4 during stressful conditions facilitates transcriptional upregulation of stress-responsive genes. In this process, ATF4 can form homo- and heterodimers with several other bZIP transcription factors, including its downstream target CHOP (Ameri and Harris, 2008).

The role of ATF4 is crucial as a mediator in the crosstalk between different stresses and for the induction and regulation of adaptation pathways, autophagy and potentially apoptosis depending on stress duration and intensity (Milani et al., 2009). Studies have shown that the pro-apoptotic effects of eIF2α of phosphorylation are mediated through the induction of ATF4 and CHOP (Jiang and Wek, 2005). It is well established that ATF4 exerts cytoprotective effects in the short-term, whereas prolonged activation leads to a switch to apoptosis (Han et al., 2013). Previous studies have described CHOP as a pro-apoptotic protein that is involved in ER stress-induced cell death (Han et al., 2013, Ohoka et al., 2005), and cells lacking CHOP are protected from stress-induced apoptosis (Marciniak et al., 2004, Zinszner et al., 1998).

Recent studies have connected ATF4 and CHOP with autophagy induction. Stress-inducible eIF2α phosphorylation has been shown to regulate autophagy in response to hypoxia, starvation, and ER stress through transcriptional control of autophagy genes by ATF4 and CHOP (B'Chir et al., 2013, Rouschop et al., 2010). It has been shown that ATF4 and/or CHOP regulate the formation, development, and function of autophagosomes (B'Chir et al., 2013). ATF4 and/or CHOP can bind to the specific *cis*-regulatory elements in the promoters of their target genes. In this context, ATF4 alone has been shown to regulate *Becn1, Map1lc3b, Atg3, Atg12, Atg16l1,* and *Gabarapl2*, while together with CHOP it regulates *Atg7, p62,* and *Nbr1* (B'Chir et al., 2013). In addition, CHOP has been shown to directly regulate *Atg5, Atg10,* and *Gabarap* (B'Chir et al., 2013). Of note, this ATF4 and/or CHOP-dependent transcriptional upregulation of autophagy genes also requires activation of eIF2α kinases confirming that in response to different stresses such as starvation or ER stress complete activation of ISR signalling is essential to maintain autophagy. ATF4 has also been shown to mediate autophagy through direct *Map1lc3b* upregulation under severe hypoxia (Rzymski et al., 2010). Both ATF4-dependent autophagy through LC3 and CHOP-dependent autophagy through Atg5 constitute survival mechanisms. However, intense stress can switch this response to a cytotoxic one (B'Chir et al., 2014).

In the previous chapters, I described the activation of an alternative death pathway in response to various stresses in C-9$^{-/-}$ MEFs. This novel pathway facilitated cell death in an Atg5-dependent manner leading to non-canonical caspase-8 activation. Induction of the alternative stress-induced caspase activation pathway seems to be necessary for execution of cell death in cells lacking functional caspase-9, and which are thus defective in the mitochondrial-mediated cell death pathway. Moreover, the importance of this novel caspase-activation pathway has been shown in the context of highly resistant cells that rely on this signaling mechanism to overcome sustained stress. How this alternative pathway is regulated is still not understood and has not been elucidated. The fact that stress-induced cell death in the examined cellular system is mediated by Atg5, an autophagy component suggests that the ISR could be a potential regulator of this novel caspase activation pathway. Given that the eIF2α-ATF4 pathway is essential for the transcriptional upregulation of a set of autophagy genes in response to various

forms of stress, I hypothesized that the ISR may participate in cell death in C-9$^{-/-}$ MEFs, and that ATF4/CHOP may be at the core of a cell's decision to switch from a survival response to the induction of cell death. However, this interplay remains widely uncharacterized, especially in relation to cell death mediated by autophagy in C-9$^{-/-}$ MEFs. Despite increasing evidence that the eIF2α/ATF4/CHOP pathway is involved in autophagy and cell death, the role of the ISR and its components in C-9$^{-/-}$ MEFs is poorly characterized. Furthermore, it is unclear whether the ISR is required for direct regulation of autophagy in this novel caspase-activation pathway, or whether it potentially regulates other components of this pathway leading to cell death.

The main objectives of the experiments described below were to determine the role of the ISR in Atg5-dependent cell death in caspase-9-deficient cells. In this chapter I investigated:

1. The role of the ISR, specifically ATF4 and CHOP, in stress-induced autophagy in response to different apoptotic stimuli
2. The role of the ISR, specifically ATF4 and CHOP, in stress-induced cell death

These approaches provide strong evidence for a possible involvement of the ISR and these two transcription factors in regulation of the novel caspase activation death pathway in cells deficient in the intrinsic apoptotic pathway.

5.2 Results

5.2.1 Various stresses induce ISR in C-9$^{-/-}$ MEFs

To investigate whether the ISR signalling pathway is induced in response to different stresses, both C-9$^{+/+}$ and C-9$^{-/-}$MEFs were subjected to prolonged treatment with either BFA, Etop, Taxol or γ-irradiation. As already mentioned in Chapter 3, BFA is an ER-stress inducer, Etop and γ-irradiation cause genotoxic stress, while Taxol leads to cytoskeletal damage. The changes in the cellular level of the ISR components were analyzed by Western blot.

The treatment of both C-9$^{+/+}$ and C-9$^{-/-}$ MEFs with either BFA, Etop and Taxol resulted in a time-dependent accumulation of eIF2α phosphorylation. A quantitative analysis of ratio phosphorylated eIF2α to total eIF2α detected by Western blot showed that ISR is induced by various stresses over the time course in both C-9$^{+/+}$ and C-9$^{-/-}$ MEFs (Fig. 5.1 A-D below panels). During ER stress the enhanced level of ATF4 and CHOP is mediated in eIF2α phosphorylation manner. Consistent with this, time course analysis revealed that BFA increases the level of ATF4 and CHOP in C-9$^{+/+}$ and C-9$^{-/-}$ MEFs (Fig. 5.1A). Similarly, prolonged exposure to Etop, Taxol or γ-irradiation led to enhanced induction of ATF4 and CHOP in C-9$^{-/-}$ MEFs (Fig. 5.1 B-D). Interestingly, C-9$^{-/-}$ MEFs displayed high levels of ATF4 and CHOP at later timepoints (24 h <) in response to all treatments compared with C-9$^{+/+}$ MEFs. This could suggest that enhanced ATF4 and CHOP levels at the latest time point might be associated with the delayed mode of cell death observed in C-9$^{-/-}$ MEFs. Importantly, these data also imply that the activation of ISR in C-9$^{+/+}$ MEFs is more transient compared with that in C-9$^{-/-}$ MEFs, where activation of ISR is sustained. However, further investigations with time match controls are warranted in order to fully understand observed changes. Also, further investigation is required to determine the role of ATF4 and CHOP in C-9$^{-/-}$ MEFs. Taken together, these results demonstrate that various stresses induce ISR in C-9$^{-/-}$ MEFs.

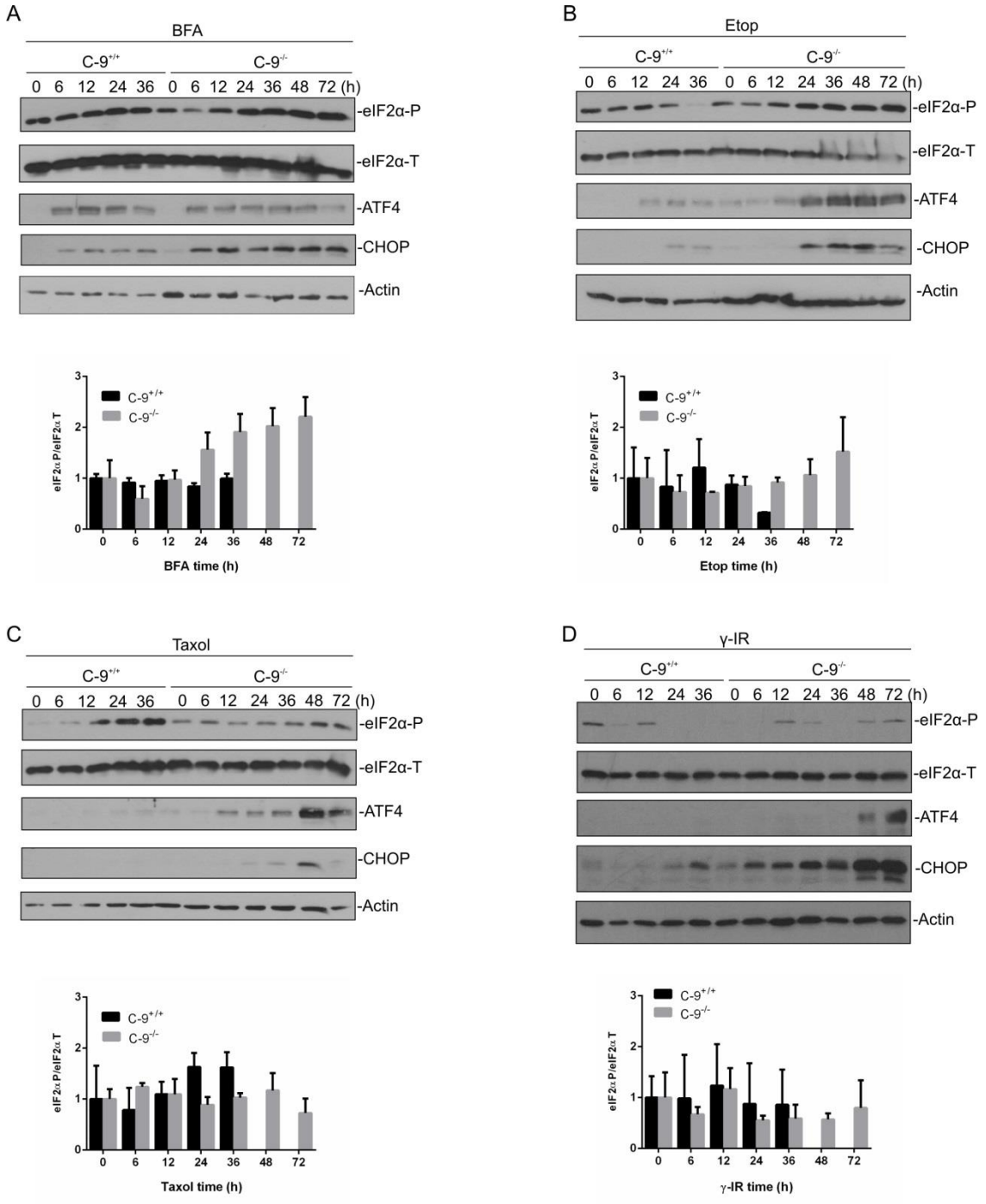


Figure 5.1 **ISR is activated by various stresses in both C-9$^{+/+}$ and C-9$^{-/-}$ MEFs**. C-9$^{+/+}$ and C-9$^{-/-}$ MEFs were treated with (A) 0.3 μg/ml BFA, (B) 50 μM Etop, (C) 1 μM Taxol and (D) 33 Gy γ-IR for the indicated times and whole cell lysates were subjected to Western blot for expression of phosphorylated eIF2α (eIF2α-P), total eIF2α (eIF2α-T), ATF4, CHOP and Actin. Densitometric analysis of eIF2α-P/eIF2α-T of BFA, Etop, Taxol and γ-IR normalized to control. Results are mean + SD of two independent experiments.

5.2.2 Role of ISR and its components in stress-induced autophagy

To investigate whether the inhibition of ISR and its component reduced autophagy induced by BFA, Etop and Taxol in C-9$^{-/-}$ MEFs pharmacological and genetic approaches were used.

To address this question, first C-9$^{-/-}$ MEFs were subjected to prolonged treatment with BFA, Etop and Taxol in the presence or absence of ISRIB. Cells were harvested at indicated time and analyzed by Western blot. As already explained in Chapter 2 Material and Method ISRIB is a potent inhibitor of ISR which acts downstream of eIF2α phosphorylation.

As expected, ISRIB significantly reduced the level of ATF4 upon all three treatments over a prolonged time course. Levels of ATG5 following ISR inhibition were comparable to the levels in those samples that were treated with BFA, Etop or Taxol alone, suggesting that inhibition of ISR has no significant effect on ATG5 level. Additionally, ISRIB did not reduce conversion of LC3-I to LC3-II at any time point as illustrated in Fig. 5.2 A, D and G. The quantitative analysis of Western blots confirmed that inhibition of ISR has no effect either of ATG5 or LC3-II/LC3-I ratio (Fig. 5.2 B, C, E, F, H, I). These results demonstrate that inhibition of ISR signaling by ISRIB results in attenuation of ATF4. Importantly, reduced levels of ATF4 did not affect autophagy markers.

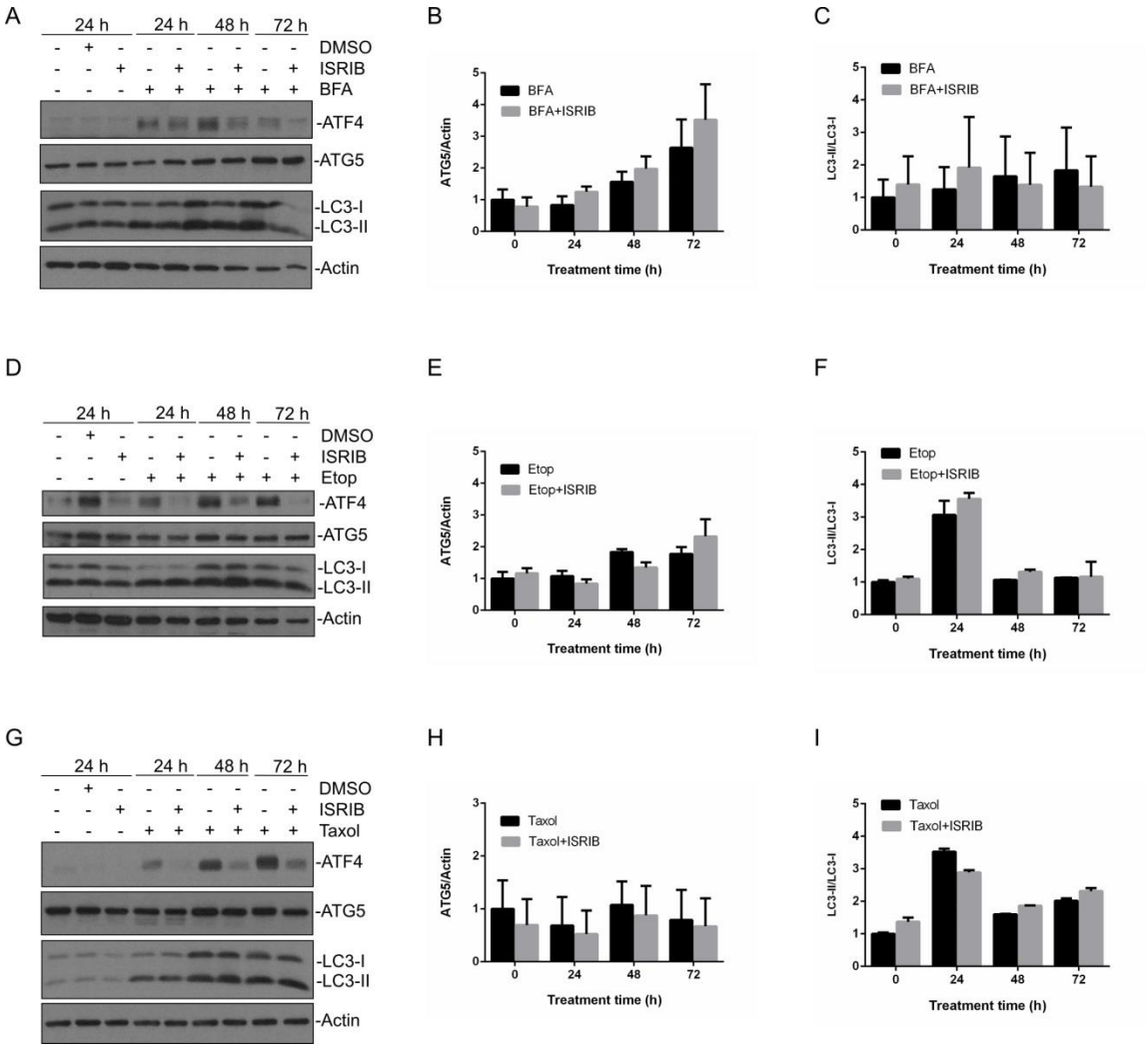


Figure 5.2 **Inhibition of ISR by ISRIB does not reduce autophagy markers upon exposure to sustained treatment with BFA, Etop and Taxol in C-9$^{-/-}$ MEFs.** C-9$^{-/-}$ MEFs were treated with (A) 0.3 μg/ml BFA, (D) 50 μM Etop, (G) 1 μM Taxol for indicated time after which lysates were subjected to Western blot for ATF4, ATG5, LC3-I to LC3-II conversion and Actin. Densitometric analysis of ATG5and LC3-II/LC3-I ratio of cotreted with ISRIB samples BFA (B, C), Etop (E, F), Taxol (H, I) over the time course normalized to control. Results are mean + SD of two separate determinations of Taxol and two independent experiments (BFA, Etop).

Thus far, I have shown that inhibition of ISR did not reduce autophagy markers upon prolonged exposure to different stresses. Therefore, it was of interest whether ISR plays an important role in the induction of autophagy. To test this, an autophagic flux experiment was performed, C-9$^{-/-}$ MEFs were subjected to treatment with BFA, Etop or Taxol, with or without CQ, in the absence or presence of ISRIB.

Cells were harvested at the indicated time points and whole lysates were assessed by Western blot for LC3-I to LC3-II conversion. To confirm functionality of ISRIB the level of ATF4 and CHOP was also monitored.

As predicted, ISRIB treatment decreased both ATF4 and CHOP under BFA, Etop and Taxol treatment. Interestingly, inhibition of the late stage of autophagy by CQ significantly increased level of ATF4; however this was considerably reduced by addition of ISRIB in all three treatments. The autophagic flux examination revealed that although BFA, Etop and Taxol alone cannot significantly induce LC3-II accumulation either with or without ISRIB, administration of CQ to BFA, Etop or Taxol enhanced the level of LC-II compared with either CQ or treatment alone in C-9$^{-/-}$ MEFs. Of note, addition of ISRIB did not affect an increase in LC3 processing with CQ upon BFA and Etop (Fig. 5.3A-D). However, by comparison, ISRIB slightly decreases LC3-I to LC3-II conversion upon Taxol treatment with CQ, suggesting that components of ISR might be involved in Taxol-induced autophagy (Fig. 5.3 E). A quantitative analysis of Western blot showed a statistically insignificant reduction in LC3-II/LC3-I, caused by ISRIB in Taxol-induced autophagy (Fig. 5.3 F), however more investigation need to be done in order to fully examine effect of ISRIB on Taxol-induced autophagy in C-9$^{-/-}$ MEFs.

Taken together, these results demonstrate that ISR and its components are not involved in induction of autophagy caused by BFA and Etop but could be in Taxol-induced autophagy. Considering the fact that ISRIB inhibits signaling downstream of eIF2α phosphorylation without affecting either basal or stress-induced eIF2α phosphorylation this raises the possibility that under Taxol treatment, other mechanisms directed from phosphorylated eIF2α might be involved in autophagy induction.

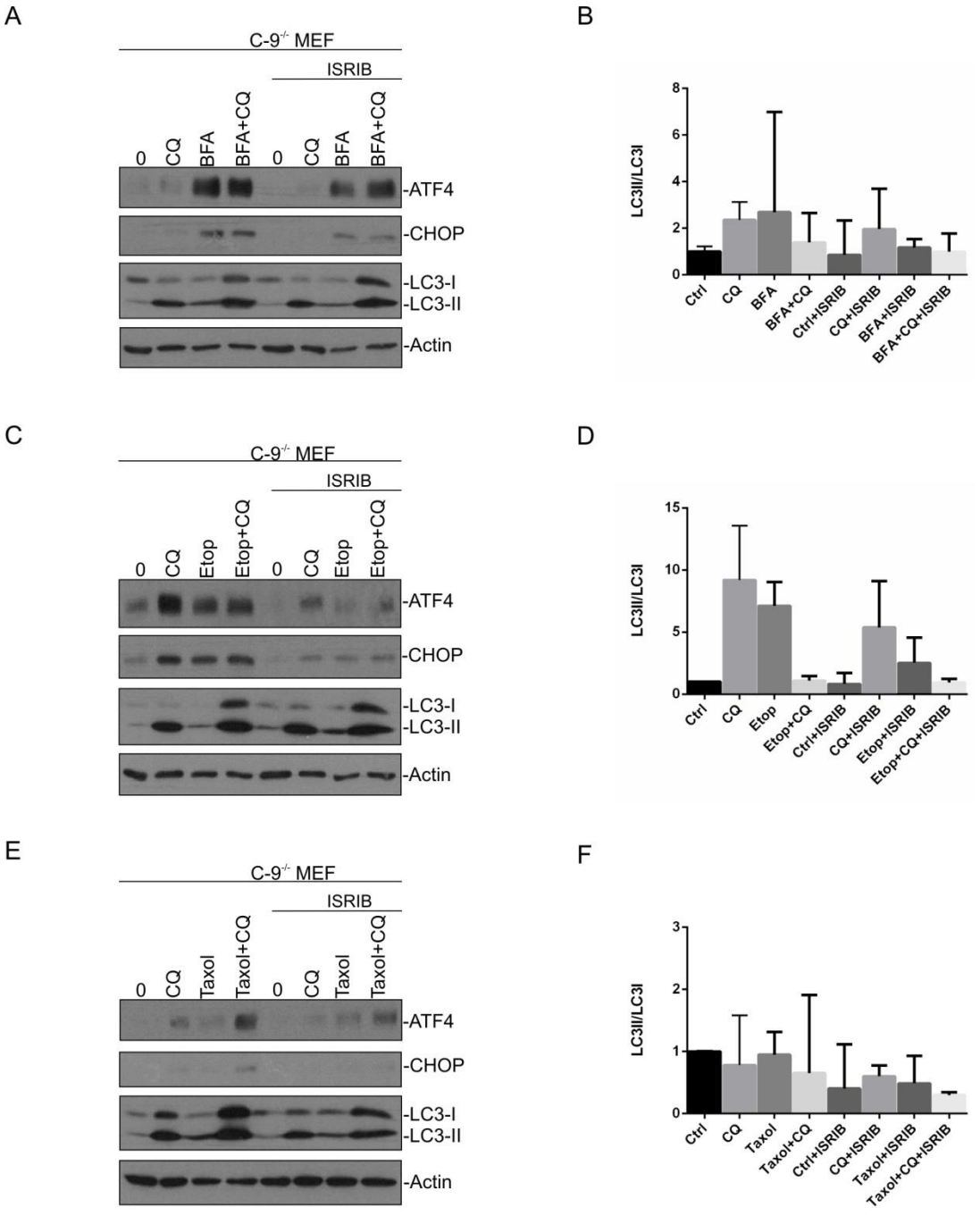


Figure 5.3 **ISR does not regulate stress-induced autophagy**. Autophagic flux in C-9$^{-/-}$ MEFs with inhibited ISR pathway. C-9$^{-/-}$ MEFs were treated with (A) 0.3 μg/ml BFA, (C) 50 μM Etop, (E) 1 μM Taxol alone, 20 μM chloroquine (CQ) alone or a combination of BFA, Etop or Taxol, CQ and ISRIB for 48 h. Whole cell lysates were subjected to Western blot for expression of ATF4, LC3-I to LC3-II conversion and Actin. Densitometric analysis of samples BFA (B), Etop (D), Taxol (F) over the time course normalized to control. Results are mean + SD of two independent experiments.

5.2.3 Autophagy is not dependent on ATF4

To determine whether stress-induced autophagy in C-9$^{-/-}$ MEFs is regulated by ATF4 the knockdown of ATF4 was performed. Both control and *ATF4* siRNA C-9$^{-/-}$ MEFs were subjected to prolonged treatment either with BFA, Etop or Taxol and analysed for detection of autophagy markers by Western blot.

As illustrated in Fig. 5.4 A, C, E, the abrogation of Atf4 gene expression was confirmed by reduced level of ATF4 protein triggered by BFA, Etop and Taxol. Conversely, Atf4 knockdown did not reduce ATG5 levels in any of the three treatments. Similarly, loss of ATF4 did not decrease LC3-I to LC3-II conversion upon BFA, Etop or Taxol treatment. The same, time-dependent increase in LC3 processing was detected either in the presence or absence of ATF4. The quantitative analysis of Western blot further confirmed that knockdown of Atf4 has no functional effect on BFA, Etop and Taxol-induced autophagy (Fig. 5.4 B, D, F). These data show that BFA, Etop and Taxol induce ATF4-independent autophagy, as demonstrated by lack of sensitivity of autophagy markers ATG5 and LC3II.

To further demonstrate the role of ATF4 in stress induced autophagy, the autophagic flux experiment with or without CQ was applied. Western blot analysis confirmed that ATF4 was significantly reduced in Atf4 siRNA samples compared with the control siRNA. Consistent with the previous results, knockdown of Atf4 did not reduce the level of LC3-I to LC3-II conversion compared with non-targeting control siRNA. Moreover, the autophagic flux examination showed enhanced level of LC3-II upon co-treatment of BFA, Etop and Taxol with CQ compared with either CQ alone or drug treatment alone in both control and A*tf4* siRNA (Fig. 5.5). These results confirm that an excessively high level of autophagy is induced by BFA, Etop or Taxol either in the presence or absence of ATF4. As illustrated in Fig. 5.6 C, the specific knockdown of ATF4 did not affect LC3 processing upon Taxol treatment, suggesting that ATF4 is not required for stress-induced autophagy in this examined cellular system. Taken together these data suggest that ATF4 is not essential for regulating autophagy induced by prolonged treatment with BFA, Etop or Taxol in C-9$^{-/-}$ MEFs.

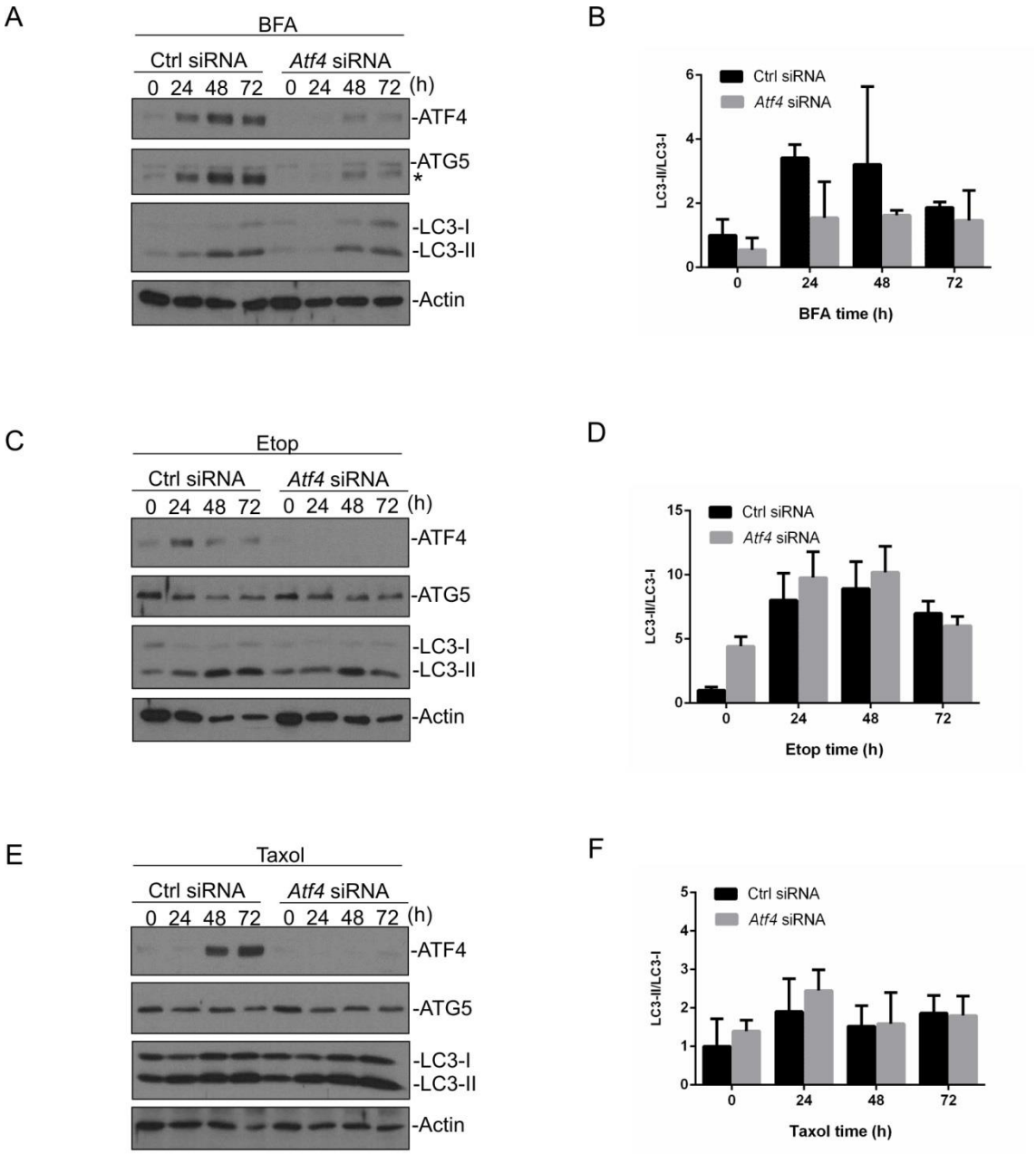


Figure 5.4 **Knockdown of ATF4 does not reduce autophagy markers upon exposure to sustained treatment with BFA, Etop and Taxol in caspase-9 deficient MEFs.** *Ctrl* and *Atf4* siRNA C-9$^{-/-}$ MEFs were treated with (A) 0.3 μg/ml BFA, (C) 50 μM Etop, (E) 1 μM Taxol for the indicated time after which lysates were subjected to Western blot for ATF4, ATG5, LC3-I to LC3-II conversion and Actin. * denotes non-specific band. Densitometric analysis of LC3-II/LC3-I ratio in samples BFA (B), Etop (D), Taxol (F) over the time course normalized to control. Results are mean + SD of two independent experiments.

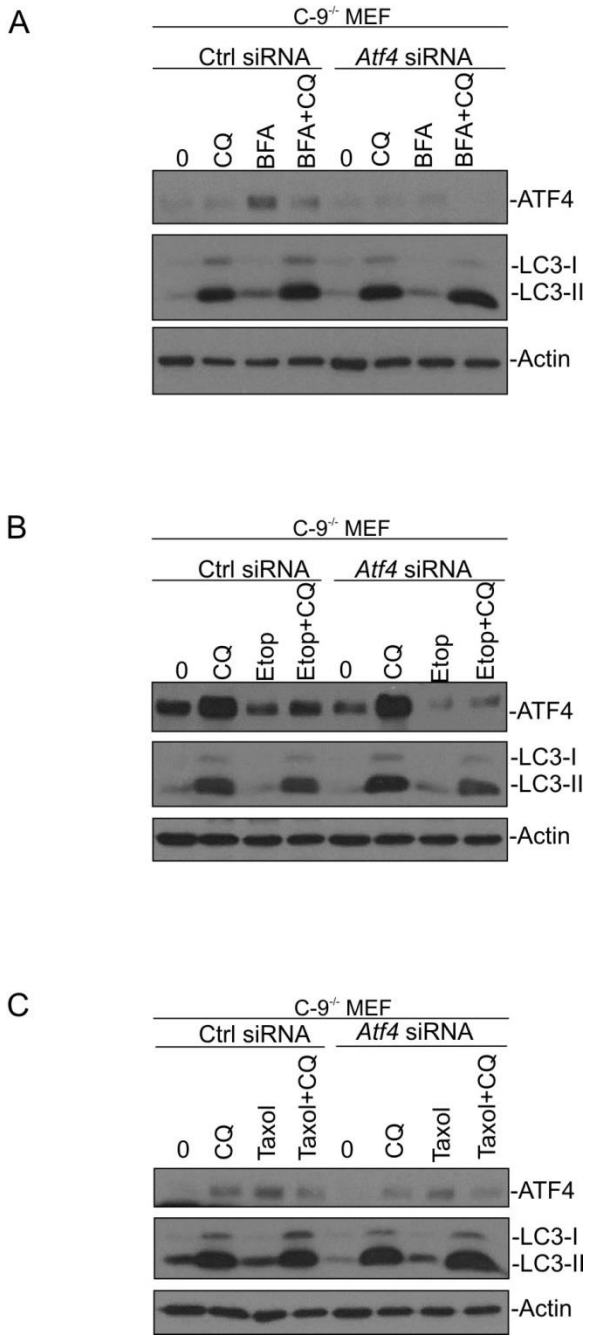


Figure 5.5 **Autophagic flux in C-9$^{-/-}$ MEFs deficient for ATF4.** *Ctrl* and *Atf4* siRNA C-9$^{-/-}$ MEFs were treated with (A) 0.3 μg/ml BFA, (B) 50 μM Etop, (C) 1 μM Taxol alone, 20 μM chloroquine (CQ) alone or a combination of BFA, Etop or Taxol and CQ for 48 h. Whole cell lysates were subjected to Western blot for expression of ATF4, LC3-I to LC3-II conversion and Actin

5.2.4 CHOP-independent autophagy

To assess the role of CHOP in the regulation of stress-induced autophagy, timecourse analysis of BFA, Etop and Taxol treatment in both non-targeting siRNA and *Chop* siRNA C-9$^{-/-}$ MEFs was performed. The knockdown of CHOP was confirmed in all three treatments as illustrated in Fig. 5.6. Later, to evaluate autophagy in the different stress-conditions, the LC3-I to LC3-II conversion and expression of ATG5 were measured. Immunoblot analysis revealed that the level of LC3 protein, which is involved in autophagosome formation and is a target of autophagy itself, is not affected by *Chop* knockdown but increased similarly in time-dependent manner in both control siRNA and *Chop* siRNA upon prolonged BFA, Etop and Taxol treatment (Fig. 5.6). Taken together, these data suggest that CHOP's transcriptional programme does not contribute to LC3 accumulation.

Moreover, the expression of ATG5 protein was not affected by CHOP knockdown during prolonged exposure to all three stresses. Indeed, induced expression of the autophagy protein ATG5 in *Chop* siRNA C-9$^{-/-}$ MEFs was similar to non-targeting siRNA C-9$^{-/-}$ MEFs, indicating that CHOP is not involved in regulation of the ATG5.

Taken together, CHOP is not involved in autophagy regulation in C-9$^{-/-}$ MEFs. Importantly, these results also indicate that ATG5 is regulated independently of CHOP in the system I tested. Overall, these data suggest that CHOP is not required for stress-induced autophagy.

In summary, ISR and its components ATF4 and CHOP are not involved in regulation of stress induced autophagy in C-9$^{-/-}$ MEFs caused by BFA, Etop, and Taxol. Indeed, BFA, Etop and Taxol induced ATF4- and CHOP- independent autophagy in C-9$^{-/-}$ MEFs.

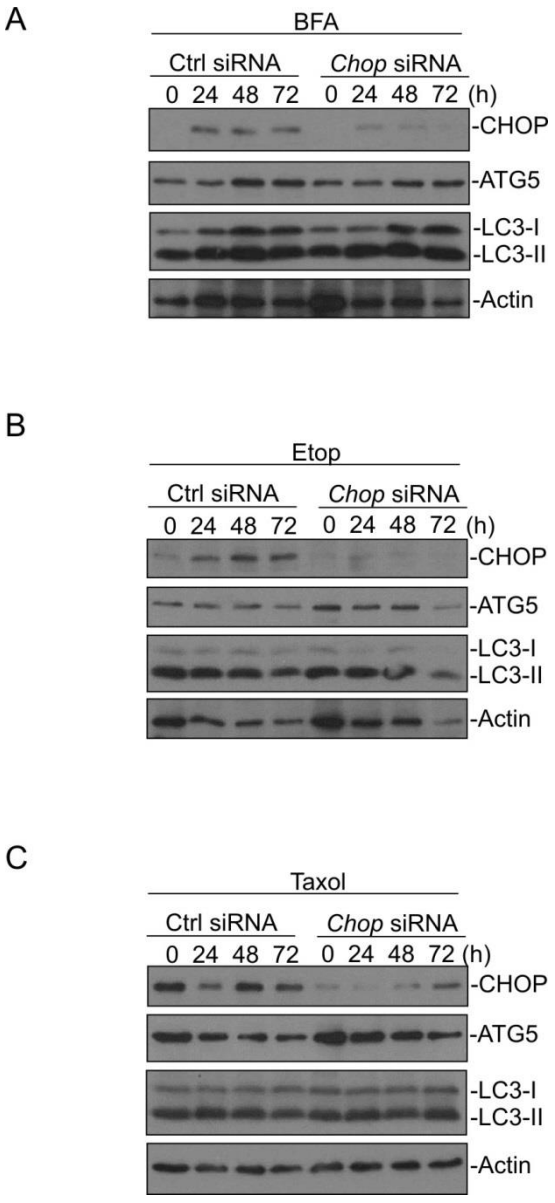


Figure 5.6 **Knockdown of CHOP does not reduce autophgay markers upon exposure to sustained treatment with BFA, Etop and Taxol in C-9$^{-/-}$ MEFs.** *Ctrl* and *Chop* siRNA C-9$^{-/-}$ MEFs were treated with (A) 0.3 μg/ml BFA, (B) 50 μM Etop, (C) 1 μM Taxol for indicated time after which lysates were subjected to Western blot for ATF4, ATG5, LC3-I to LC3-II conversion and Actin.

5.2.5 Inhibition of ISR does not protect against stress-induced cell death in C-9$^{-/-}$ MEFs

Considering the fact that changes in the duration of ATF4 and CHOP activity results in different effects on cellular outcome, as both transient and sustained ISR signalling lead to the various gene expression, could possibly involve ISR and its component in regulation of other, unidentified yet elements of the novel caspase-activation pathway. Therefore, I wished to determine the effect of ISR and its components on stress-induced cell death.

To investigate whether the ISR pathway modulates survival, C-9$^{-/-}$ MEFs cells were incubated for up to 72 h with BFA, Etop and Taxol with or without ISRIB. The Western blot analysis for processing of pro-caspase-8 and caspase-3 was performed together with PI staining on C-9$^{-/-}$ MEFs. First, the functionality of ISRIB was confirmed by reduced level of ATF4 upon all three treatments. It was observed that ISRIB reduced processing of caspase-3 and slightly decreased also procaspase-8 at 48 h upon BFA treatment. However, ISRIB had no effect on BFA-induced pro-caspase-8 and caspase-3 processing as monitored by Western blot at the latest time points (Fig. 5.7 A). Interestingly, enhanced processing of caspase-3 was detectable at 48 h and 72 h in co-treated samples with Etop and ISRIB (Fig. 5.7 B). Similarly, prolonged treatment with Taxol led to processing of both pro-caspase-8 and caspase-3 in C-9$^{-/-}$ MEFs. The addition of ISRIB to Taxol-treated cells was not able to reduce processing of caspase-3 either at 48 h or 72 h; however, there was a slight decrease in pro-caspase-8 processing observed only at 72 h. These results suggest that even though ISRIB was able to reduce the level of ATF4 upon all three treatments, it was not able to inhibit significantly processing of pro-caspase-8 and caspase-3 during a prolonged time course.

Next, to examine whether inhibition of ISR and its component reduces C-9$^{-/-}$ MEFs viability subjected to prolonged treatment with BFA, Etop and Taxol, flow cytometry was used. FACS analysis through PI uptake showed that ISRIB did not affect cell death caused by BFA or Taxol, whereas treatment significantly increased cell death upon Etop treatment (Fig. 5.7 A-C, left panel). Importantly, addition of ISRIB alone did not affect cell viability. These results, suggest that stress-induced cell death resulted from

activation of apoptosis as the processing of the executioner caspases-3 was significantly induced under these three conditions. Moreover, addition of ISRIB enhanced processing of caspase-3 upon Etop treatment suggesting that either loss of ATF4 or restoration of protein translation process upon stress condition enhanced stress-induced cell death.

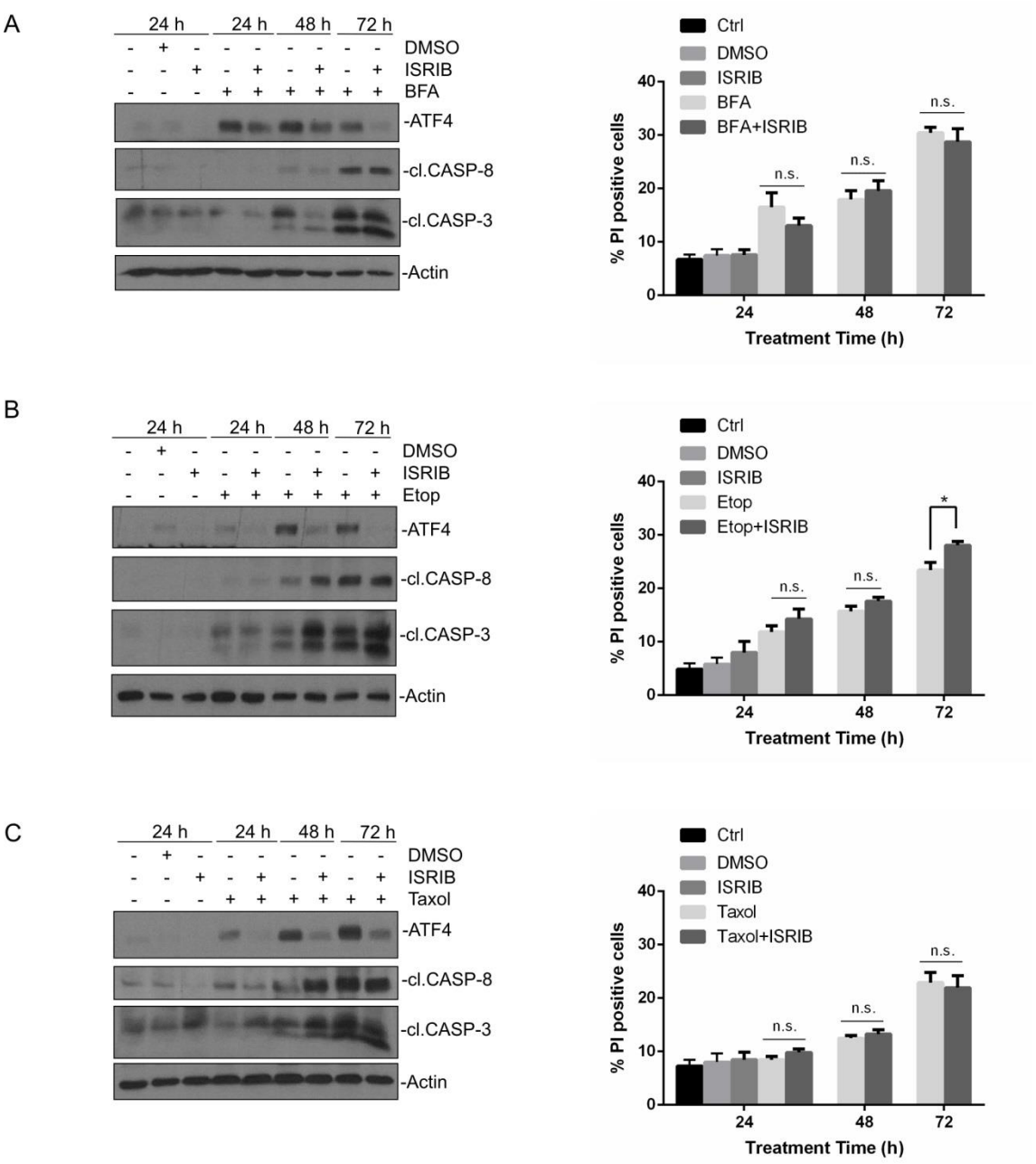


Figure 5.7 **Caspase-8 and caspase-3 cleavage is sustained in stressed C-9$^{-/-}$ MEFs treated with ISRIB.** C-9$^{-/-}$ MEFs were treated with (A) 0.3 μg/ml BFA, (B) 50 μM Etop, (C) 1 μM Taxol for the indicated time points and whole cell lysates were subjected to Western blot for expression of ATF4, cleaved caspase-8 (cl.CASP-8) and caspase-3 (cl.CASP-3) and Actin. Cell death was assessed at the indicated time points by using flow cytometry and propidium iodine staining.Values represent the mean ± SEM of three independent experiments. The significance level is * = $p<0.05$ and n.s. indicates no significant difference.

5.2.6 ATF4 is not required for stress-induced cell death in C-9$^{-/-}$ MEFs

To determine whether stress-induced cell death is regulated by ATF4, the effect of siRNA-mediated knockdown of ATF4 on pro-caspase-8 and caspase-3 processing and cell death was examined in C-9$^{-/-}$ MEFs.

Of note, the activation of caspase-8 and subsequent processing of caspase-3 in this cellular system constitute the readout of functional alternative stress-induced caspase activation pathway. I found that abrogation of ATF4 gene expression did not affect pro-caspase-8 and caspase-3 processing in response to BFA, Etop and Taxol treatment during a prolonged time course (Fig. 5.8 A- C).

Furthermore to investigate whether knockdown of Atf4 reduce BFA, Etop and Taxol-induced cell death in C-9$^{-/-}$ MEFs, FACS analysis was performed. It was observed that loss of ATF4 did not affect stress-induced cell death as determined by PI staining. FACS analysis showed that following treatment with BFA, Etop or Taxol knockdown of *Atf4* in C-9$^{-/-}$ MEFs was not able to rescue C-9$^{-/-}$ MEFs (Fig. 5.8 A-C right panel).

Taken together, these results showed that ATF4 a main component and effector of ISR signalling is not required for cell death induced by BFA, Etop and Taxol. Importantly, these data also imply that novel caspase-activation pathway is regulated independently of ATF4.

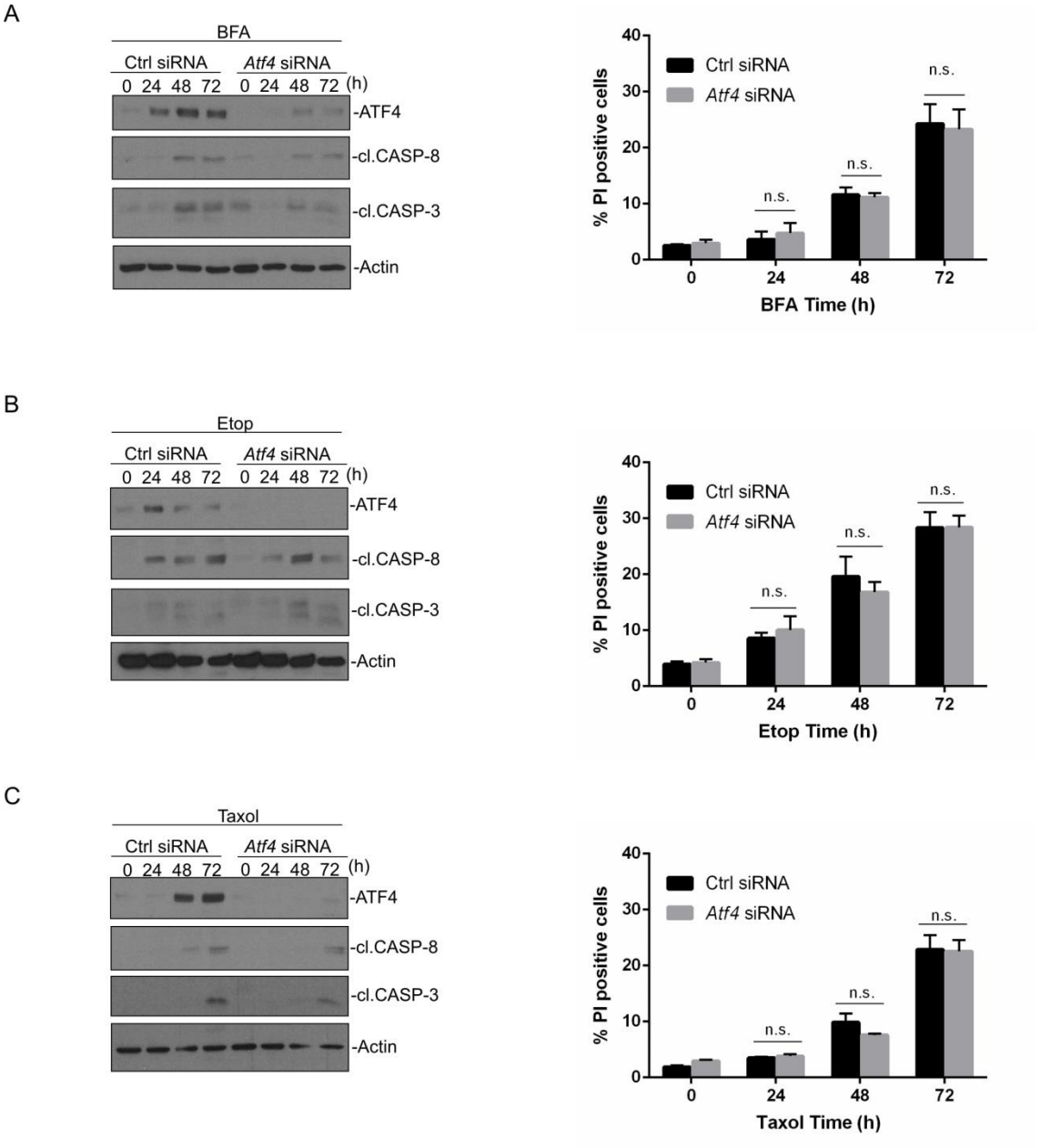


Figure 5.8 **ATF4 is not required for stress-induced cell death in C-9$^{-/-}$ MEFs.** C-9$^{-/-}$ MEFs were transiently transfected with 20 nM Ctrl siRNA and ATF4 siRNA and next treated with (A) 0.3 μg/ml BFA, (B) 50 μM Etop and (C) 1 μM Taxol for the indicated time points and whole cell lysates were subjected to Western blot for expression of ATF4, cleaved caspase-8 (cl.CASP-8) and caspase-3 (cl.CASP-3) and Actin. Cell death was assessed at the indicated time points by using flow cytometry and propidium iodide staining.Values represent the mean ± SEM of three independent experiments. n.s. indicates no significant difference.

5.2.7 Role of CHOP in stress-induced cell death in C-9$^{-/-}$ MEFs

The enhanced level of CHOP observed during prolonged treatment with various stresses in C-9$^{-/-}$ MEFs, may have additional effect on the cellular outcome, especially in context of regulation of the alternative caspase activation pathway and cell death. With this in mind, it was of interest to determine whether CHOP is implicated in cell death in C-9$^{-/-}$ MEFs.

To address this question, the levels of processing of pro-caspase-8 and caspase-3 in both non-targeted siRNA and CHOP siRNA cells exposed to prolonged treatment with BFA, Etop and Taxol were analysed. As already explained, activation of pro-caspase-8 and caspase-3 in this cellular system constitute the readout of functional alternative caspase-mediated cell death pathway. As illustrated in Fig. 5.9 A-C (left panel) processed pro-caspase-8 increased from 24 h of BFA and Etop treatment while in Taxol from 48 h. By, contrast cleaved caspase-3 increased from 48 h upon BFA treatment, and from 24 h upon Etop treatment. Importantly, the maximum amount of processed pro-caspase-8 and caspase-3 was observed at 72 h in both control and *Chop* siRNA in all three treatments (Fig. 5.9 A-C, left panel). Following Taxol treatment, processing of caspase-3 was slightly reduced in *Chop* siRNA cells compared with the non-targeting siRNA.

Furthermore, to investigate the role of CHOP in stress-induced cell death, C-9$^{-/-}$ MEFs were also subjected to flow cytometry analysis. The obtained results showed that knockdown of C*hop* had no effect on cells death as determined by PI uptake in all three treatments. Silencing of CHOP was not able to reduce the percentage of PI positive cells in response to prolonged treatment with BFA, Etop and Taxol (Fig. 5.9 A-C right panel). These results, suggest that CHOP is dispensable for stress-induced cell death in C-9$^{-/-}$ MEFs upon prolonged drug treatment

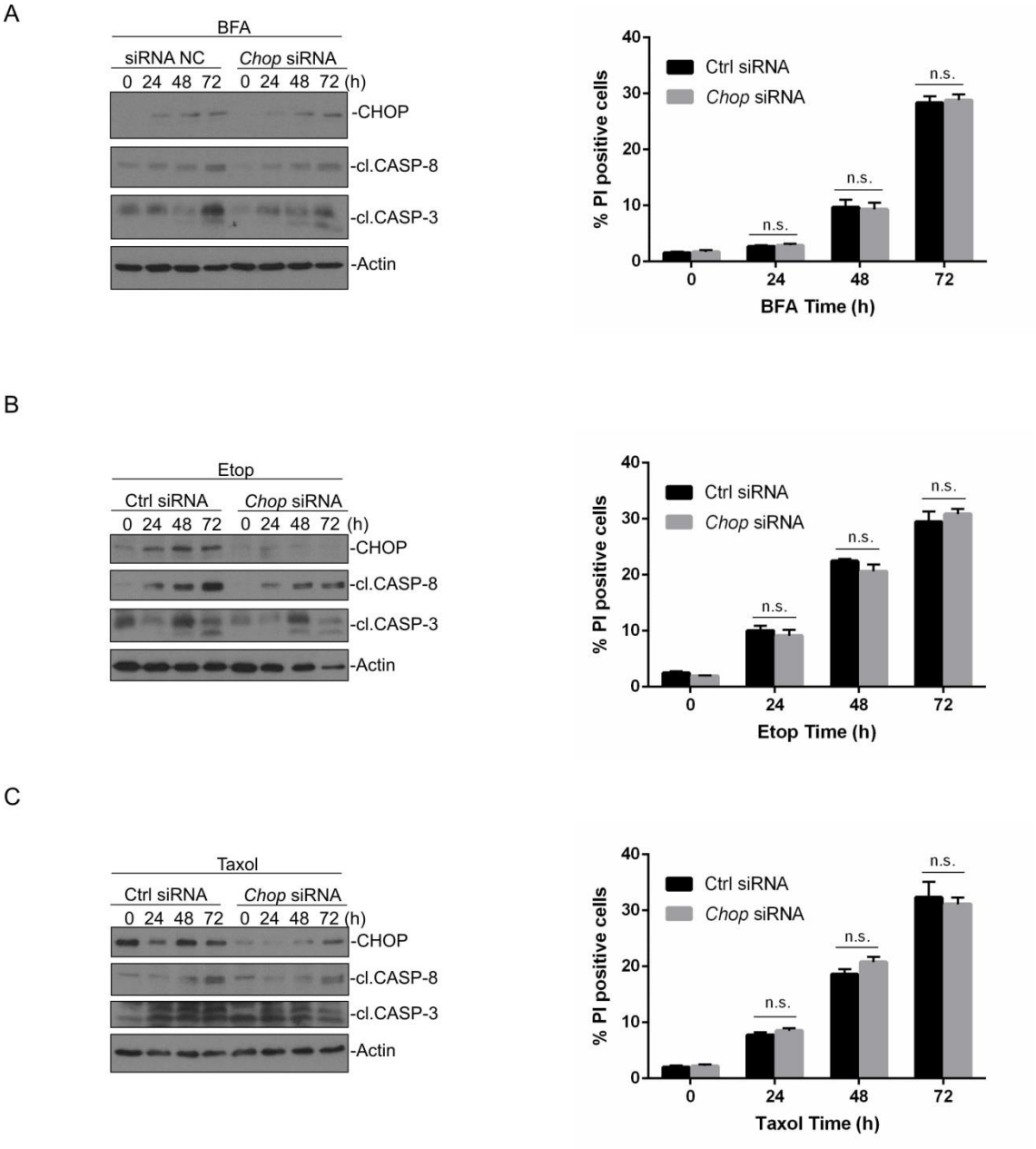


Figure 5.9 **CHOP is not required for stress-induced cell death in C-9$^{-/-}$ MEFs**. Ctrl and *Chop* siRNA C-9$^{-/-}$ MEFs were treated with (A) 0.3 μg/ml BFA,(B) 50 μM Etop, (C) 1 μM Taxol for the indicated time points and whole cell lysates were subjected to Western blot for expression of CHOP, cleaved caspase-8 (cl. CASP-8) and caspase-3 (cl.CASP-3) and Actin. Cell death was assessed at the indicated time points by using flow cytometry and propidium iodine staining.Values represent the mean ± SEM of three independent experiments. n.s. indicates no significant difference.

5.2.8 PERK signaling is involved but not required for ER stress-induced autophagy and caspase processing in C-9$^{-/-}$ MEFs

To block ISR activation under ER stress condition, highly selective and potent PERK inhibitor Amgen, compound 44, that inhibits PERK kinase activity was used (Smith et al., 2015).

To determine the role of PERK-eIF2α-ATF4 pathway in ER stress mediated autophagy, selective inhibition of PERK activity with compound 44 in C-9$^{-/-}$ MEFs subjected to prolonged treatment with BFA was performed. First, it was confirmed that PERK, one of four eIF2α kinases is activated upon BFA treatment, as demonstrated by its phosphorylation observed as an upshift on the SDS-PAGE gel (Fig. 5.10). The results also showed that compound 44 had a good inhibitory effect visualised by as reduced phosphorylation of PERK over prolonged timecourse. During ER stress, PERK phosphorylates eIF2α, thus its increased level along with other downstream targets like ATF4 and CHOP was observed (Fig. 5.10). Interestingly, the level of phosphorylated eIF2α dropped after application of compound 44 at 48 h. However, a statistically insignificant reduction in eIF2α-P/eIF2α-T was observed, caused by compound 44 in BFA treated C-9$^{-/-}$ MEFs (Fig. 5.10 B). Similarly, addition of compound 44 significantly reduced the level of ATF4 during the time course, while CHOP level was reduced only at 24 h and 48 h of cotreatment. Consistently with this, an elevated level of LC3-I to LC3-II conversion was observed, indicating that BFA induced autophagy. However, blockage of PERK by compound 44 was not able to reduce the level of LC3 processing. A quantitative analysis of Western blot confirmed that compound 44 has no functional effect on BFA-induced autophagy (Fig. 5.10 C).

Prolonged and severe pharmacological ER stress can trigger caspase-mediated cell death through a PERK-dependent mechanism (Szegezdi et al., 2006). Therefore, finally, to address the question of whether PERK-eIF2α-ATF4 regulates stress-induced cell death, the processing of pro-caspase-8 and caspase-3 that constitute the readout of an alternative caspase-mediated death pathway was also examined. The obtained results confirm that significantly high level of processed pro-caspase-8 and caspase-3 was detected at the latest time point, and application of compound 44 was not able to reduce this level but rather enhanced it. A quantitative analysis of Western blot further

confirmed that compound 44 enhanced BFA-induced processing of pro-caspase-8 (Fig. 5.10 D). These data suggest that PERK signalling does not contribute to ER stress induced autophagy and is not required for caspase processing over sustained stress.

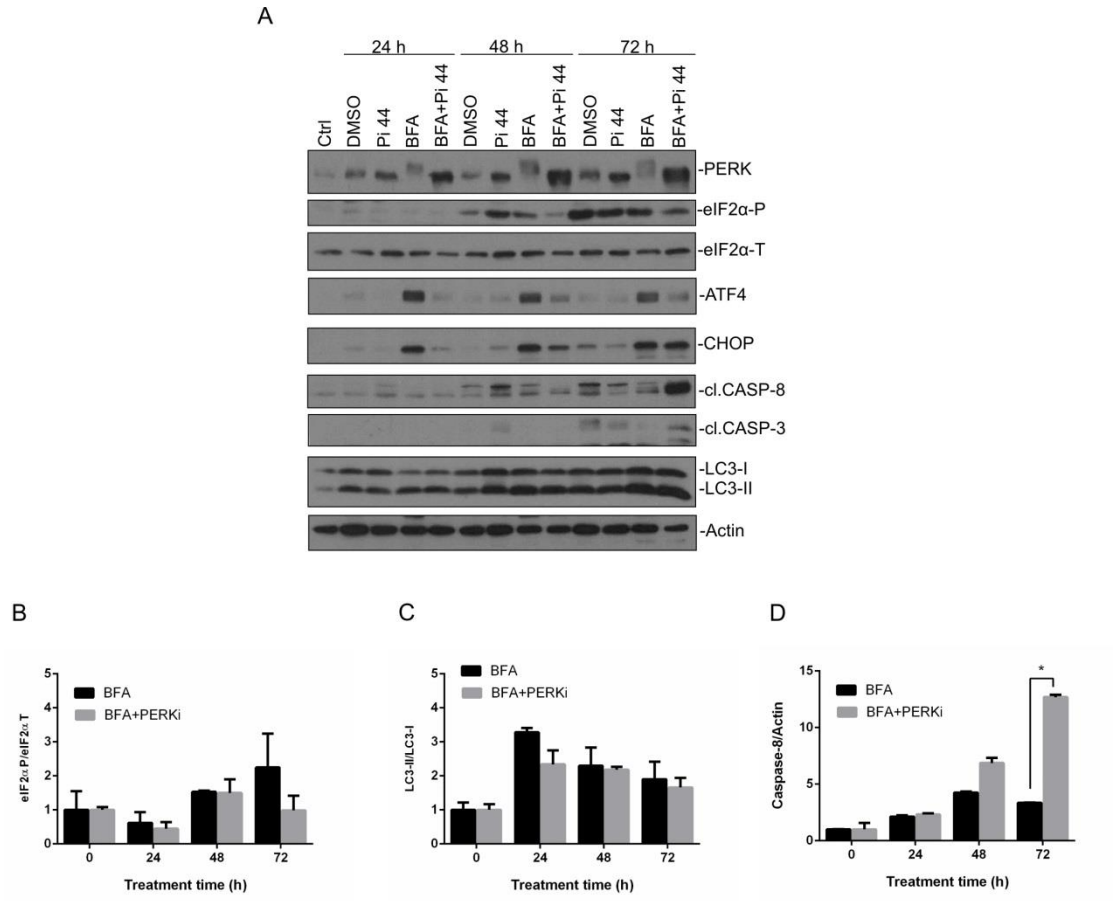


Figure 5.10 **PERK signalling is not required for ER stress-induced autophagy and caspase processing in C-9$^{-/-}$ MEFs**. (A) C-9$^{-/-}$ MEFs were treated with 0.3 μg/ml BFA for the indicated time points and whole cell lysates were subjected to Western blot for expression of PERK, P-eIF2α, T-eIF2α, ATF4, CHOP, cleaved caspase-8 (cl.CASP-8) and caspase-3 (cl.CASP-3) and Actin. Densitometric analysis of (B) eIF2-P/eIF2-T, (C) LC3-II/LC3-I and (D) Caspase-8 h normalized to control. Results are mean + SD, * = $p<0.05$ of two independent experiments.

5.3 Discussion

Apoptosis and autophagy are independent processes; however, in response to various stresses these two processes can be interlinked (Marino et al., 2014). Although autophagy is primarily an adaptive response and apoptosis leads to cellular demise, it has been shown that in response to sustained ER stress autophagy can also favour the induction of apoptosis (Tang et al., 2017, Young et al., 2012). The crosstalk between autophagy and apoptosis can be mediated through the PERK/eIF2α/ATF4 pathway. Central to these responses is the ISR, which is activated by a variety of stimuli. While eIF2α/ATF4 signaling is essential for apoptosis induction through upregulation of CHOP, Noxa, and TRB3 (Teske et al., 2013, Jousse et al., 2007), it is also necessary for autophagy via ATF4 and CHOP in response to various stresses such as hypoxia, starvation, and ER stress (B'Chir et al., 2013, Rzymski et al., 2010). It has been shown that ATF4- and CHOP-dependent transcriptional regulation of autophagy is cytoprotective, implicating ATF4 and CHOP in cell survival. However, there are also reports that have linked stress-induced autophagy to cell death (Kroemer and Levine, 2008, Levine and Yuan, 2005). Although there are many controversies in relation to autophagy-mediated cell death, it is already well established that autophagy can be cytotoxic in cells compromised in the intrinsic apoptotic pathway. Furthermore, the involvement of the ISR in cell death mediated by autophagy is also elusive; however, depending on the duration and nature of the stress both ATF4 and CHOP can promote different cellular outcomes (B'Chir et al., 2014). The functional relationship between ISR signalling, autophagy, and cell death mediated by autophagy is not fully understood and requires clarification. Further, the mechanism by which ATF4 contributes to autophagy initiation is still poorly characterized. However, based on the fact that ATF4 integrates signals from several unrelated stresses, it could be suggested that ATF4 may be the major transcription factor that coordinates autophagy induction as part of the cellular response to stress. Therefore, ATF4 might be a crucial mediator of the crosstalk between different stresses, the transcriptional activation of specific target genes and regulation of autophagy, and in this way facilitate the induction of an alternative stress-induced caspase-mediated death pathway.

Chemotherapeutics can trigger a number of cellular responses and stress signaling pathways. Thus far, I have demonstrated that BFA, Etop, and Taxol induce cell death, autophagy, and the ISR in C-9$^{-/-}$ MEFs. Therefore, I was next interested in how these different pathways are involved in mediating apoptotic cell death. Also, it is important to elucidate how these pathways are regulated to converge on the alternative stress-induced death pathway to initiate caspase-8 activation. The fact that direct and indirect links between the ISR and autophagy, the ISR and cell death, and autophagy and cell death have been demonstrated suggests close connections between these processes. Furthermore, the mechanisms by which the ISR promotes cell death through the induction of the alternative caspase activation pathway remained unknown. The experiments presented below represent the first analysis of the contribution of the ISR and its components to the regulation of the alternative caspase activation pathway under severe stress in C-9$^{-/-}$ MEFs.

The data show that stress-inducible eIF2α phosphorylation promoted enhanced induction of ATF4 and CHOP in C-9$^{-/-}$ MEFs over the course of prolonged drug treatment. Previous results have indicated that in response to various stresses both ATF4 and CHOP regulate the expression of genes involved in the induction of autophagy (B'Chir et al., 2013, Rouschop et al., 2010). The best characterized autophagy genes are *Atg5* and *Lc3*, which have been shown to be upregulated in ATF4- and CHOP-dependent manners, respectively (B'Chir et al., 2013). Using the pharmacological ISRIB inhibitor, which blocks the induction of ATF4 (Sidrauski et al., 2013), I demonstrated that the ISR and its components are not involved in autophagy induced by BFA and Etop. The levels of ATG5 and LC3 did not change after ISRIB application over a period of prolonged treatment with BFA, Etop, and Taxol. However, the reduced conversion of LC3-I to LC3-II observed in autophagic flux experiments upon Taxol treatment in the presence of ISRIB suggested that autophagy induced by Taxol might be regulated by the ISR. Since ISRIB significantly reduced the levels of ATF4 upon all three treatments, this could suggest that ATF4 is involved in Taxol-induced autophagy. However, in contrast to the well-characterized mechanism of autophagy that requires ATF4, my data demonstrate that BFA-, Etop-, and Taxol-induced autophagy is not dependent on ATF4. Direct silencing of ATF4 did not reduce either ATG5 or LC3 levels in response to

prolonged treatment with these drugs. Instead, a time-dependent increase in LC3-I to LC3-II conversion was observed both in control siRNA and *Atf4* siRNA cells, suggesting that BFA, Etop, and Taxol elicit excessively high level of autophagy in C-9$^{-/-}$ MEFs. Determination of autophagic flux further supported and confirmed these observations, implicating ATF4 as a nonessential regulator of autophagy in C-9$^{-/-}$ MEFs. The knockdown of ATF4 was also confirmed in both experiments verifying the effectiveness of ATF4 silencing. However, in autophagic flux experiments, a minimal level of ATF4 was detected in response to Taxol treatment in *Atf4* siRNA-transfected cells compared with cells transfected with control siRNA. This slight difference in the ATF4 levels seems to be within experimental variation, and therefore its significance is hard to determine. Knockdown of ATF4 was achieved using a pooled siRNA approach to minimize off-target effects; however, for further determination of the role of ATF4 in the regulation of the alternative caspase activation pathway a different genetic approach such as a shRNA system could provide more convincing evidence.

Rouschop et al. demonstrated that CHOP is involved in the regulation of autophagy in response to severe hypoxia (Rouschop et al., 2010). These data are in agreement with the recent study of B'Chir et al. that implicated CHOP as an important regulator of starvation-induced autophagy (B'Chir et al., 2014). Thus, based on the existing knowledge, it was of interest to determine the role of CHOP in stress-induced autophagy in C-9$^{-/-}$ MEFs. Transient knockdown of Chop was applied in this examined cellular system. Silencing of Chop did not affect either ATG5 or LC3 levels in response to prolonged treatment with BFA, Etop, and Taxol in C-9$^{-/-}$ MEFs. These results indicate that ATG5 is regulated independently of CHOP in the system under investigation. The same time-dependent increase in LC3-I to LC3-II conversion was observed in both the presence and absence of CHOP. Therefore, although CHOP is upregulated in response BFA, Etop, and Taxol treatments, it seems that CHOP does not contribute to the regulation of autophagy in C-9$^{-/-}$ MEFs.

There thus seems to be a contradiction between what has already been described in the literature and what has been shown in this study. Indeed, Rzymski et al. have shown that an increase in the levels of autophagosomes requires ATF4-dependent LC3 upregulation

under severe hypoxia in a breast cancer cell line (Rzymski et al., 2010). Similarly, a study of Rouschop *et al.* also indicated that increased expression of LC3 was regulated by ATF4 and CHOP under hypoxia in the same cell lines (Rouschop et al., 2010). Since my experiments were performed in cells lacking a functional mitochondrial death pathway, it raises the possibility that the observed differences might be cell-type specific. Also, it is important to note that the studies of Rzymski, Rouschop, and Dey (Dey et al., 2015, Rouschop et al., 2010, Rzymski et al., 2010) all demonstrated the role of ATF4/CHOP-mediated cytoprotective autophagy in cancer cell lines. In the context of the novel caspase activation pathway, autophagy has been shown to exert its pro-death function in cells defective in the mitochondrial apoptotic pathway under sustained ER stress (Deegan et al., 2014b). This is also supported by the results presented in Chapter 4 in this thesis, where knockdown of Atg5 protected C-9$^{-/-}$ MEFs against BFA- and Etop-induced cell death. Therefore, it seems that ATF4 which generally protects cell from the negative consequences of several unrelated stresses and which is also the main effector of the ISR, may not simultaneously regulate the death pathway. However, I have also observed enhanced ATF4 expression in response to prolonged treatment with all stimuli. This suggests that ATF4 may have additional effects on cellular outcome and could be potentially associated with the delayed mode of cell death occurring in C-9$^{-/-}$ MEFs. The pro-death role of ATF4 has been shown in neuroectodermal tumor cells sensitized to ER stress-mediated apoptosis (Armstrong et al., 2010) and upon glucose deprivation where ATF4 drives DR5 and TRAIL receptor-mediated cell death (Iurlaro et al., 2017). The notion that ATF4 triggers cell death is also supported by enhanced expression of its downstream target CHOP which is implicated in cell death upon exposure to lethal stress. Although the mechanism by which ATF4 contributes to cell death is still unknown it is believed that the type of stress might be a deciding factor. While the ISR is primarily a cytoprotective pathway that promotes cell survival, it is suggested that when stress is too severe and overwhelms the capacity of cell to restore homeostasis the ISR can trigger cell death (Pakos-Zebrucka et al., 2016). Furthermore, recent studies have demonstrated that during prolonged nutrient deprivation stress, CHOP, apart from regulating autophagy, can also induce apoptosis (B'Chir et al., 2014). This dual role of CHOP is regulated by the stress condition; CHOP can either participate

in cell survival through transcriptional upregulation of autophagy genes induced by short exposure to stress stimuli, or, alternatively, in response to chronic stress CHOP may promote cell death either by limiting the transcriptional activation of autophagy genes or by regulation of pro-apoptotic factors (B'Chir et al., 2014). Indeed, it has been shown that during ER stress CHOP transcriptionally upregulates pro-apoptotic protein BIM (Puthalakath et al., 2007) and downregulates anti-apoptotic BCL-2 protein (McCullough et al., 2001).

Considering the proposed model for the alternative caspase activation pathway, it was of interest whether ATF4 and CHOP could regulate this pathway through their ability to modulate autophagy. However, although ATF4 and CHOP were shown to be dispensable for stress-induced autophagy their presence during the prolonged treatment suggested that they might still be involved in the cell death triggered in the tested system. Indeed, the ISR, specifically ATF4 and CHOP, could possible regulate other as yet unknown components of the novel ATG5-dependent caspase-mediated pathway and thus control stress-induced cell death in C-9$^{-/-}$ MEFs. However, the obtained results revealed that neither pharmacological inhibition with ISRIB nor genetic silencing of ATF4 and CHOP reduced stress-induced cell death in C-9$^{-/-}$ MEFs. Moreover, the level of processed pro-caspase-8 and caspase-3 was not affected either in ATF4 or CHOP knockdown cells in response to any of the three treatments. Of note, I could not achieve a convincing reduction in CHOP levels in *Chop* siRNA cells after 72 h of BFA treatment. Therefore, it is difficult to conclude whether CHOP reduces caspase processing or not in response to cell death induced by prolonged treatment with BFA. Because silencing of CHOP was efficient at early time points and showed no effect on caspase processing these results cannot be rule out. Interestingly, an increase in caspase-3 processing was observed in cells cotreated with ISRIB and Etop at 48 h and 72 h compared with cells treated with Etop alone. Consistent with this, enhanced cell death was also detected at 72 h as determined by PI uptake. The specific knockdown of either ATF4 or CHOP did not reduce the cell death upon Etop treatment; therefore, inhibition by ISRIB rather suggests that direct mechanisms dependent on phosphorylated eIF2α might increase cell death upon Etop treatment. These results could indicate that eIF2α phosphorylation alone or additional events downstream of eIF2α phosphorylation are

required for Etop-induced cell death. Alternatively, there is also the possibility that the restoration of translation caused by ISRIB application might enhance cell death. Additionally, it is of interest to note that CHOP transcriptional activity has been implicated in regulation of BCL-2 family members during ER stress. Therefore, it would not be surprising that siRNA-mediated CHOP knockdown could lead to suppression of BIM induction, thus reducing stress-induced cell death. Nevertheless, thus far, the role of ATF4 and CHOP in regulation of the alternative caspase activation pathway seems to be nonessential.

The results presented in this thesis showed that BFA, Etop, and Taxol activate the alternative caspase-mediated pathway in C-9$^{-/-}$ MEFs as determined by pro-caspase-8 and caspase-3 processing. However, individual components of the ISR appear to be not required for induction of this novel alternative caspase activation pathway to execute cell death in C-9$^{-/-}$ MEFs. Therefore, to definitively elucidate the ability of the ISR and its components to modulate the alternative caspase activation pathway the novel and specific PERK inhibitor compound 44 was used. PERK is one of four eIF2α kinases that regulate the integrated stress response. Upon ER stress, PERK is activated leading to phosphorylation of eIF2α (Pakos-Zebrucka et al., 2016). In addition, it has been shown that the PERK pathway is also implicated in autophagy induction (Deegan et al., 2015, B'Chir et al., 2013, Kouroku et al., 2007) and cell death (Szegezdi et al., 2006, Harding et al., 2000b). Using compound 44 it was further confirmed that PERK is not involved in the regulation of autophagy in C-9$^{-/-}$ MEFs. It was found that the levels of LC3 processing were not changed in the presence of the PERK inhibitor. This indicates that when cells are under ER stress, LC3 processing can be enhanced independently of PERK activation. Furthermore, chronic stress can reach a threshold level above which C-9$^{-/-}$ MEFs strongly depend on autophagy to induce cell death. Interestingly, these results are in agreement with others showing that autophagy precedes apoptosis (Maiuri et al., 2007). Indeed, my results showed that LC3 induction is followed by caspase activation, confirming that autophagy serves a primarily cytoprotective function; however, stress of pronounced duration and intensity results in apoptosis. The enhanced level of LC3 processing observed upon sustained ER stress also further supports the notion that autophagy contributes to the caspase-dependent mode of cell death. This

latter phenomenon was not reversed by the PERK inhibitor. Inhibition of PERK was not sufficient to significantly inhibit the processing of pro-caspase-8 and caspase-3. Thus, these data further support those from a previous study showing that prolonged treatment with an ER stress-inducing agent led to cell death in C-9$^{-/-}$ MEFs (Deegan et al., 2014a). Moreover, inhibition of PERK enhanced processing of pro-caspase-8, suggesting that cells became more sensitive to ER stress-induced apoptosis. In fact, it has been shown that in PERK-deficient cells activation of Noxa could contribute to increased sensitivity to ER stress (Gupta et al., 2012). Alternatively, this could be explained by the fact that prolonged inhibition of PERK affected the phosphorylation of eIF2α leading to increased protein synthesis and thus higher levels of ER stress (Harding et al., 2000b). Nevertheless, the obtained results confirmed the nonessential role of PERK in ER stress-induced autophagy. Interestingly, these results are also in agreement with the results already presented in this chapter. Indeed, the specific knockdown of ATF4 and CHOP did not reduce stress-induced autophagy even at the early time points. Furthermore, application of ISRIB also did not affect the expression of autophagy genes. The examination of ISR and its components was carried out in response to BFA treatment, Etop and Taxol, and I observed similar effects for all three treatments. Therefore, it seems that complete activation of ISR pathways involving signalling upstream of eIF2α phosphorylation (PERK/eIF2α/ATF4) is also nonessential for maintaining autophagy during ER stress. This observation is in contrast to the study of Kouroku *et al.*, who showed that the PERK/eIF2α phosphorylation pathway was required for autophagy (Kouroku et al., 2007). However, considering the fact that ISRIB inhibits signalling downstream of eIF2α phosphorylation without affecting either basal or stress-induced eIF2α phosphorylation itself, this raises the possibility that under stress, either eIF2α phosphorylation or other signals downstream of phosphorylated eIF2α might be involved in autophagy induction. This notion is in agreement with previous studies that showed that cells expressing a non-phosphorylatable eIF2α mutant were unable to induce autophagy upon ER stress, starvation, and viral infection (Kouroku et al., 2007, Talloczy et al., 2002).

The mechanisms of ISR, ATF4, and CHOP-mediated cell death are likely to be stress stimulus- and context-dependent. Since many signal transduction pathways are activated

in one cell upon drug treatment, it would seem unlikely that the ISR, and specifically ATF4/CHOP, trigger cytotoxic effects. The fact that constitutive activation of the ISR pathway was observed in the cellular system I tested, suggests that this pathway may be associated with drug resistance rather than with regulation of the alternative caspase activation pathway. This is in agreement with previous reports indicating that ATF4 overexpression correlates with resistance to commonly used chemotherapeutics such as cisplatin, doxorubicin, and etoposide (Igarashi et al., 2007, Tanabe et al., 2003). Moreover, it has been shown that gemcitabine resistance was associated with activation of ISR and expression of anti-apoptotic protein such as BCL2-A1 in pancreatic cancer cells (Palam et al., 2015). Interestingly, administration of ISRIB and siRNA-mediated knockdown of ATF4 reduced level of BCL2-A1, suggesting that ISR is involved in drug resistance through regulation of BCL-2 family proteins (Palam et al., 2015). The effect of ISRIB on BCL-2 family expression is not well understood but it is suggested that during stress, ISRIB could restore phospho-eIF2α-dependent translation either through enhancing eIF2B activity or via eIF2α-phosphorylation-mediated stress granule formation (Palam et al., 2015, Sidrauski et al., 2015).

The data presented in this chapter provide evidence that the ISR, ATF4, and CHOP are dispensable for the modulation of autophagy and cell death; thus, a precise characterization of the regulation of the alternative caspase activation pathway as well as its functional role in C-9$^{-/-}$ MEFs is still lacking. Elucidating the regulation of this alternative caspase activation pathway is complicated by the fact that several signaling pathways can simultaneously induce both apoptosis and autophagy. The transcription factor p53 has been implicated in the regulation of both autophagy and apoptosis and therefore seems to be an attractive target for further examination (Maiuri et al., 2007). p53 is the central tumor suppressor activated in response to various stresses such as DNA damage, metabolic stress, and hypoxia (Marino et al., 2014, Maiuri et al., 2007). p53 was first linked to autophagy through activation of its downstream target damage-regulated autophagy modulator DRAM leading to induction of apoptosis (Crighton et al., 2006). mTOR kinase, which constitutes an upstream regulator of autophagy that integrates signals from different stresses such as nutrient and energy deprivation, might also be considered as playing a role in the regulation of the alternative caspase-mediated

death pathway. In this context, mTOR could induce autophagy through the AMPK pathway. Several lines of evidence also suggest that c-Jun-N-terminal kinase (JNK) regulates both apoptosis and autophagy through its capacity to phosphorylate BCL-2 family members resulting in the release of Beclin 1 (Marino et al., 2014).

In this chapter I particularly focused on characterizing a novel role for the ISR in the regulation of the alternative caspase activation pathway. The results show that although various stresses induce the ISR in caspase-9-deficient MEFs, activation of the ISR, and of ATF4 and CHOP, is not required for stress-induced autophagy in C-9$^{-/-}$ MEFs. Furthermore, neither pharmacological inhibition of the ISR nor genetic silencing of ATF4 or CHOP reduced cell death in C-9$^{-/-}$ MEFs. These results can be explained in the context of ATG5-dependent caspase-mediated cell death that constitutes an alternative caspase-8 activation death pathway to compensate for the deficiency of the canonical intrinsic apoptotic pathway. Since ATG5 is involved in cell death execution it appears that the presence of autophagosomes are necessary for caspase processing. Therefore, these findings suggest that ATF4 and CHOP, because they are dispensable for autophagy induction in C-9$^{-/-}$ MEFs, are also not required for regulation of the alternative caspase activation pathway.

This is the first time that a novel caspase activation pathway consisting of ATG5 has been examined in the context of the ISR in response to various stresses in C-9$^{-/-}$ MEFs (Fig. 5.11). A better understanding of the complex regulation of this alternative caspase activation pathway in response to various stress-inducing agents in the context of cancer treatment can contribute to identification of potential new therapeutic targets. It will be important to characterize the molecular mechanisms and functions, but also the regulation of this pathway as it could allow for the development of more effective new strategies to overcome drug resistance. For example, a combined therapy based on drugs that induce autophagy-dependent cell death in apoptosis-resistant cells might represent an effective treatment. Therefore, the results presented in this chapter may be highly significant in relation to the treatment of highly resistant cancers.

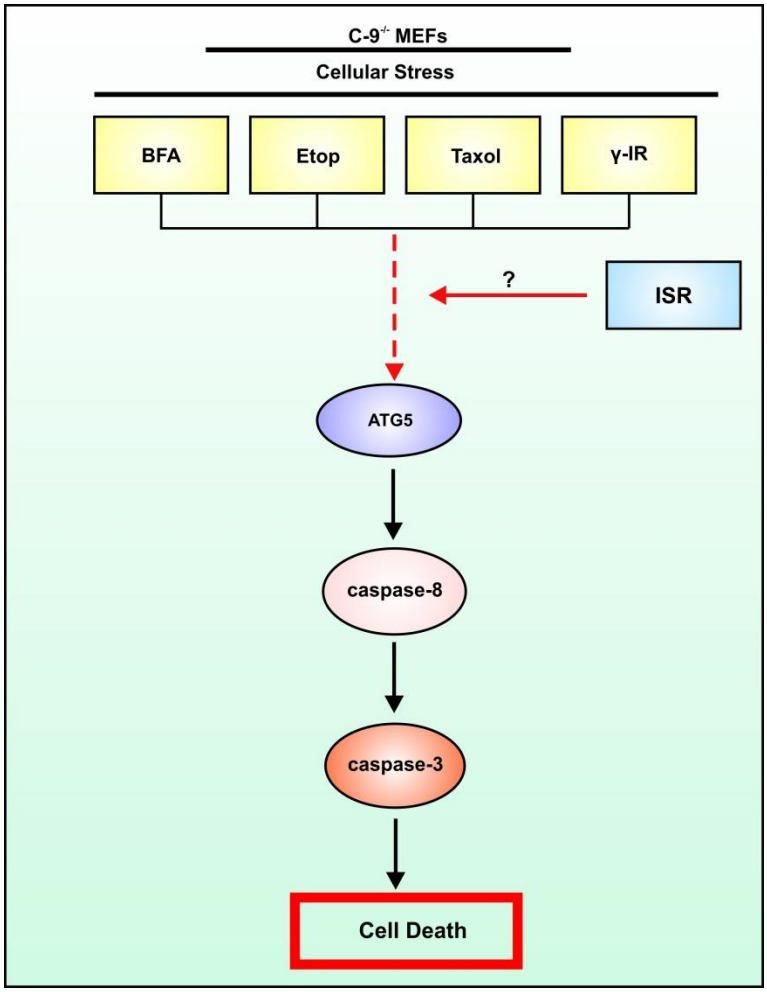


Figure 5.11 **Various stresses induce ATG5-mediated caspase-dependent cell death in C-9$^{-/-}$ MEFs.** The graphic represents a model of the stress-induced caspase activation pathway. ATG5 mediates caspase-8 dependent stress-induced cell death in cells deficient in caspase-9 in response to different stresses. The question mark indicates the possible involvement of the ISR in the regulation of the stress-induced caspase activation pathway.

Chapter 6: General Discussion and Concluding Remarks

In this study I investigated how chemotherapeutic drugs and γ-irradiation, treatments with distinct cellular targets, activate a novel, alternative cell death pathway in resistant cells. The experiments presented in this thesis provide additional evidence and new insights into another death pathway that is activated to compensate for the primary route of cell death in C-9$^{-/-}$ MEFs.

The findings presented in this thesis are of high importance with regard to understanding the molecular mechanisms of cell stress responses in response to chemotherapy. The acquisition of chemoresistance is an impediment to efficient anticancer therapy, and this thesis has revealed a potential method for overcoming this drug resistance.

Cell death is crucial for maintaining homeostasis in multicellular organisms. During development and morphogenesis, cells trigger the physiological process of programmed cell death which is associated with the removal of superfluous and harmful cells (Haanen and Vermes, 1996). However, exposure to pharmacological stress can also induce apoptosis to execute cell death. Defects in apoptosis are linked to the pathogenesis of cancer (Delbridge et al., 2012). Malfunction of the intrinsic apoptotic pathway confers resistance to a variety of stress stimuli and enables evasion of cell death. For example, Bax/Bak double knockout MEFs are resistant to ER stress and DNA damage-inducing agents (Wei et al., 2001, Ruiz-Vela et al., 2005). Abnormalities in BCL-2 expression are frequently found in human cancers and are correlated with the cancer progression and malignancy (Delbridge and Strasser, 2015). Indeed, BAX deficiency contributes to colon cancer development (Rampino et al., 1997), while BCL-2 overexpression in lymphoma cells provides resistance to various stresses (Strasser et al., 1994). Similarly, deficiency in caspase-9 and Apaf-1, crucial components of the mitochondrial apoptotic pathway also protects MEFs against stress-induced cell death (Ruiz-Vela et al., 2005). Downregulation of Apaf-1 contributes to cancer progression and is frequently observed in malignant melanoma (Dai et al., 2004). However, although mutation or loss of caspase-9 is not a common feature in human cancer, it is reported that caspase-9 may possess a tumour suppressor function (Ekert et al., 2004, Marsden et

al., 2002). It is still not known how cells defective in the apoptotic pathway resist the effects of drug treatment. Therefore, it is important to understand in detail the mechanisms of stress-induced cell death underlying the development of cancer.

In the first results chapter (Chapter 3) of this thesis, I focused on the existence of an alternative caspase-activation pathway initiated when the intrinsic apoptotic pathway is compromised. Since cancer cells are resistant to cell death due to malfunctions and defects in their apoptotic pathway, cells devoid of functional caspase-9 were used in this study to understand the molecular mechanisms of alternative cell death pathways activated in response to various stresses. The applications of different chemotherapeutics were shown to activate the alternative caspase-mediated death pathway in the examined cellular system. This is important as in response to drug therapy stress stimuli can trigger many stress responsive pathways (Ricci and Zong, 2006). Moreover, cancer cells can adapt to various cytotoxic drugs contributing to their progression and malignancy (Fodale et al., 2011, Hanahan and Weinberg, 2011). Therefore, the existence of a backup mechanism that executes cell death has important implications for overcoming drug resistance, especially in cells with a dysfunctional apoptotic pathway. Indeed, I show that this alternative death pathway can effectively trigger cell death but with slower kinetics. Even though the mode of cell death that was observed was delayed, the existence of this pathway has the potential to bypass drug resistance. The challenge is to identify the regulators of this stress-induced cell death pathway as it would promote the pharmacological exploitation of novel or already existing stress responses to combat highly-resistant cancers. Furthermore, at the molecular level, it was intriguing to characterize the dynamics of this caspase-8 activation pathway which executed cell death. Depending on the stress stimulus, cells could either utilize caspase-8 to execute apoptotic cell death or switch to a nonapoptotic mode of cell death.

In the second results chapter (Chapter 4), I examined the role of stress-induced autophagy in the context of this novel alternative caspase activation pathway. My study showed that various stresses induced autophagy; however, autophagy as a process was not involved in the execution of stress-induced cell death. Pharmacological and genetic inhibition of the initial stage of autophagy, upstream of autophagosome formation, was

shown to have no effect on the function of the alternative caspase activation pathway. Interestingly, however, my data suggest that various stresses can potentially induce a non-canonical form of autophagy that is independent of Beclin 1 in C-9$^{-/-}$ MEFs. This fascinating observation requires further examination. It was confirmed that ATG5 is required for the optimal functioning of this novel death pathway. In this study, I discovered that although ATG5 is required for caspase processing in response to different stresses, the requirements for ATG5 in cell death are stress stimulus-specific. These findings further substantiate the concept of an alternative caspase activation death pathway which is dependent on ATG5 and provide additional information regarding its functioning. The involvement of autophagy in this alternative pathway underscores the notion that autophagy, which is considered to serve as a backup mechanism to trigger cell death especially in cells defective in the intrinsic apoptotic pathway, would rather contributes to cell death promoting apoptosis than executing it itself. The precise role of autophagy in cancer is still not well understood. Defects in the autophagic process are found in many human cancers. For example, in human breast, ovarian, and prostate cancers the loss of Beclin 1 is frequently observed, while deficiency in Atg5 is correlated with tumorigenesis in a kidney cell line (White and DiPaola, 2009). Importantly, in cancer therapy, interest in the clinical manipulation of the autophagic process is focused on inhibiting autophagy (Levy et al., 2017). This is not surprising as autophagy typically acts as a survival mechanism allowing cancer cells to adapt to several stresses in the tumor microenvironment (Amaravadi et al., 2016, Katheder et al., 2017). The genetic and pharmacological inhibition of autophagy was shown to reduce tumor growth and caused tumor regression (Katheder et al., 2017, Levy et al., 2014). However, in the context of highly resistant cancer cells that are characterized by dysfunction of the intrinsic apoptotic pathway autophagy has an opposite effect. Therefore, understanding the molecular mechanisms underlying cell death pathways that involve autophagy is of high importance for optimizing cancer therapy.

In the third results chapter (Chapter 5), I focused on the regulation of the alternative ATG5-dependent caspase-mediated death pathway. I examined the pro-death role of the ISR and its components and established a novel link between the ISR and the induction of autophagy to promote cell death. In this study, I showed that the novel and alternative

caspase activation pathway is not regulated by ISR components. The role of ATF4 and CHOP was dispensable both for autophagy regulation and for cell death in cells defective in the intrinsic apoptotic pathway. Application of the pharmacological inhibitor ISRIB fully confirmed this observation. In addition, inhibition of the eIF2α kinase PERK one of eIF2α kinase, was shown to have no effect on stress-induced autophagy. With this in mind, I can assume that full activation of the ISR by an upstream kinase is not required for regulation of autophagy in C-9$^{-/-}$ MEFs. Furthermore, inhibition of PERK had no effect on the functioning of the alternative caspase activation pathway. This may also implicate eIF2α phosphorylation which is at the core of the ISR. Whether phosphorylation of eIF2α or as yet unknown downstream targets are essential for the regulation of this novel caspase-activating pathway dependent on ATG5 is a matter for debate and further examination. However, the results presented in this thesis partially answer this question, demonstrating that eIF2α is dispensable for the regulation of the alternative caspase activation pathway. The experiments presented in this chapter were challenging due to the fact that the ISR is generally considered as a pro-survival pathway. In the context of cancer, tumor cells often induce the ISR to adapt to physiological stress conditions in their microenvironment caused by nutrient and oxygen deprivation (Dey et al., 2015, Hart et al., 2012). It is also intriguing that in cancer biology the activity of oncogenes can result in cell-autonomous activation of the ISR including its downstream target ATF4. For example, increased activity of proto-oncogene Myc induces the eIF2α/ATF4 pathway leading to cytoprotective autophagy (Hart et al., 2012). Therefore, inhibition of ISR could represent a strategy to combat malignancies with Myc overexpression (Hart et al., 2012). Cancer cells frequently exhibit higher levels of ATF4, which correlates with their resistance to a wide range of chemotherapeutics (Milani et al., 2009, Igarashi et al., 2007, Bi et al., 2005, Tanabe et al., 2003). Moreover, the chemotherapeutics used in this study simultaneously induced a number of cellular responses such as autophagy, cell death, and the ISR. From the clinical point of view, the activation of a pathway favouring cell survival may limit the efficacy of the applied anticancer strategy. The examined alternative caspase activation pathway is characterized by the interlinking of two molecular pathways (apoptosis and autophagy); therefore, the development of a

combined therapy might be the most effective approach to treat certain types of resistant cancer.

Overall, the data presented in this thesis show that inhibition of the primary route of cell death (apoptosis) can induce another type of cell death with manifestation of autophagic and apoptotic features. The assumption that the alternative caspase activation pathway may constitute a backup mechanism is based on the comprehensive studies carried out in this thesis showing that this pathway is activated by various forms of stress. The existence of this novel death pathway also highlights the importance of the caspases that execute cell death in an ATG5-dependent manner. Inhibition of the initiator caspase-8 prevented the activation of executioner caspases and cell death upon prolonged exposure to BFA, Taxol, and γ-irradiation. However, in response to Etop treatment, although caspase-8 inhibition blocked the downstream caspases, it did not prevent cell death, which was therefore most likely executed through another mechanism such as necrosis or perhaps necroptosis. Similarly, ATG5 was found to be required for caspase activation in response to all the various examined stresses. However, its depletion was not able to protect against cell death caused by Taxol and γ-irradiation, suggesting that additional mechanisms were activated to trigger cell death. Based on the experiments in this thesis, it can be assumed that, BFA which is an inducer of ER stress, is the only stimulus that requires the alternative caspase activation pathway to execute cell death in C-9$^{-/-}$ MEFs. Although the other stresses activate this novel ATG5-dependent caspase-mediated pathway in C-9$^{-/-}$ MEFs, targeted inhibition of either caspase-8 or ATG5 may stimulate alternative pathways for cell death execution. This could suggest that ER stress preferentially utilizes the alternative ATG5-dependent caspase-mediated pathway to trigger cell death under conditions when the apoptotic pathway is compromised. This may also have important therapeutic implications as drugs which selectively induce ER stress could potentially be used to treatment highly resistant cancers.

Altogether, the study of cellular responses to various stresses in C-9$^{-/-}$ MEFs imply the existence of a novel caspase-activation pathway which constitutes a compensatory death pathway when the primary route of cell death is supressed. Comprehensive examination of this novel cell death pathway revealed that different stress responses converge on the

alternative caspase activation pathway to execute cell death. These findings are very interesting in the context of cancer biology. The examination of this alternative caspase activation pathway in the context of commonly used cancer therapies such as chemotherapeutics (Etop, Taxol) and radiotherapy (γ-IR) delivers new insights into the molecular mechanisms of cell death induced by a variety of stress stimuli.

The components and proposed regulators of the alternative ATG5-dependent caspase-mediated pathway are summarized in Table 6.1.

Table 6.1. A summary of the regulation of the alternative caspase activation pathway in response to various stresses

	Effect on	ISR			ATF4			CHOP			Becn-1			ATG5			CASP-8		
BFA	Caspase activation	–			–			–			–			+	+	+	+	+	+
	Cell death	ns			ns			ns			ns			+	+	+	+	+	+
Etop	Caspase activation	–			–			–			–			+	+	+	+		
	Cell death	+			ns			ns			ns			+	+	+	–		
Taxol	Caspase activation	–			–			–			–			+			+	+	+
	Cell death	ns			ns			ns			ns			ns			+	+	+
γ-IR	Caspase activation													+			+	+	+
	Cell death													ns			+	+	+

The results of experiments are expressed in the form of + indicating a positive effect on caspase activation or/and cell death. Increasing numbers of + suggest a strong response. The – symbol has been used to describe a negative effect on caspase activation or/and cell death. n.s. indicates no significant difference.

Chapter 7: Future Directions

The work presented in this thesis has been carried out with a well-established cell line; therefore, it would be important in the future to confirm whether the observations described in this thesis also hold true in a human model of therapy-resistant cancer cells. Since C-9$^{-/-}$ MEFs constitute an appropriate and suitable model to investigate the alternative mode of cell death in resistant cells, I would like in future, in order to exploit the role of this novel ATG5-dependent caspase-mediated pathway for therapeutic purposes, to use a panel of malignant human melanoma cell lines (e.g., MeWo and A-375) that are highly resistant to induction of cell death due to reduced Apaf-1 expression, a protein with a key role in the mitochondrial apoptotic pathway (Su et al., 2009, Soengas et al., 2001).

Many current cancer therapies rely on an induction of autophagy (Levine and Kroemer, 2008, Rubinsztein et al., 2007). Autophagy modulation may therefore be effective as a therapeutic strategy in melanoma. The effect of vemurafenib (drug used in melanoma treatment) on cell death showed a close relationship between ER stress response and autophagy and thus provides a rationale for combination approaches for melanoma treatment (Ma et al., 2014). Recently, the development of a small molecule that triggers ER stress was also shown to induce cell death associated with autophagy and apoptosis in melanoma (Cerezo et al., 2016). Therefore, understanding the role of factors that regulate the novel and alternative caspase activation pathway and cell death is crucial. Furthermore, finding the regulators of the alternative ATG5-dependent caspase-mediated pathway would potentially facilitate the discovery of other components of this novel pathway.

It would be interesting to use a panel of already tested chemotherapeutics to determine if they led to the induction of the alternative ATG5-dependent caspase-mediated pathway in melanoma cells. Considering already established treatments, it would also be of interest to examine the effects of more physiological stresses (glucose deprivation,

hypoxia, or heat shock) as this approach would be more informative with respect to stress in tumor microenvironments as well as to potential treatment. As combined therapy seems to be the most effective strategy for treating highly resistant cells, a combination of chemotherapeutics with radiation or high temperature is attractive. First, I would determine whether any of the melanoma cell lines respond to treatment with drugs (BFA, Etop, Taxol) and physical stress (γ-irradiation, heat shock) by inducting cell death, and then subsequently I would determine the mode of cell death. In this approach, it would be necessary to confirm whether the resulting cell death depended on caspase-8. Furthermore, it would be of interest to determine whether treatment with this well-established panel of stress stimuli activated autophagy and whether the observed cell death is triggered in an ATG5-dependent manner. Finally, it would be important to elucidate whether other components of the autophagy machinery are involved in this novel and alternative caspase-mediated death pathway.

My final goal would be to determine the regulators of this novel and alternative ATG5-dependent caspase-mediated pathway induced in response to these treatments. To clearly establish the role of the stress-inducible ATF4 protein in the ATG5-dependent caspase-mediated pathway, I would use inducible CRISPR/CAS9 or a doxycycline-inducible shRNA system in C-$9^{-/-}$ MEFs. Application of inducible CRISPR/CAS9 will allow for efficient knockout of ATF4 in the examined cellular model. Using the CRISPR/CAS9 knockout system would enable assessment of the functional role of ATF4 in the regulation of the novel and alternative caspase activation pathway at different stages of prolonged treatment.

Future investigation is required to address if the ISR is required for the induction of the alternative ATG5-dependent caspase-mediated pathway. Cells expressing the nonphosphorylatable eIF2α mutant S51A would be most useful for addressing this question. As the experiments described in this thesis were performed in cells lacking functional caspase-9, either eIF2α siRNA or shRNA should be applied first in C-$9^{-/-}$ MEFs and later in the melanoma cell line. It would also be interesting to determine the involvement of the complete ISR signaling pathway (eIF2α kinase/eIF2α/ATF4). To address this question, pharmacological inhibition of PERK, GCN2, PKR, and HRI in

parallel with si/shRNA-mediated knockdown of PERK, GCN2, PKR, and HRI should be applied in C-9$^{-/-}$ MEFs and later in a melanoma cell line.

The regulation of the alternative ATG5-dependent caspase-mediated pathway seems to be linked to autophagy; apart from ATF4 and CHOP, autophagy can also be transcriptionally controlled by p53 which upregulates ATG genes such as ATG7, ATG4, ATG2, ULK1, and UVRAG (Fullgrabe et al., 2014). Besides, p53 is the main tumor suppressor in cells and mutation of this gene has been linked to chemoresistance. Generally, p53 is induced upon DNA damage (Lakin and Jackson, 1999); however, it is also activated in response to various other stresses leading to apoptosis through its regulation of PUMA, Noxa and other BH3-only proteins. The function of p53 is regulated by posttranslational modification (phosphorylation and ubiquitination) and is dependent on its subcellular localization (Fullgrabe et al., 2014). In light of what is currently known, it can be assumed that different stresses induce p53 leading to the activation of a novel ATG5-dependent caspase-mediated pathway through transcriptional regulation of the core autophagy machinery. Thus, p53 degradation should then reduce ATG5-dependent caspase-mediated pathway activation and/or function resulting in a decrease in cell death. Finally, investigation of the role of mTOR in this system as an upstream regulator of autophagy is also crucial. Based on my data, it seems that various stresses induce a mode of autophagy (pro-death) that is different to that induced by rapamycin which was not observed to trigger cell death in the system under investigation. Thus, the rationale for these experiments is linked to the mode of action of the applied stressors. Since rapamycin induces autophagy through inhibition of mTORC1 and results in activation of pro-survival mechanisms, this raises the possibility that various stresses might use other routes to activate autophagy, or alternatively that mTORC1 constitutes a switch between life and death decisions upon autophagy induction.

In the context of melanoma with mutation of the oncogene BRAF, the role of MAPK kinases is also of interest, in particular JNK, as it was already implicated in autophagy control. Indeed, activated JNK leads to phosphorylation of BCL-2 proteins resulting in the dissociation of the BCL-2-Beclin 1 complex and subsequent autophagy activation (Wei et al., 2008). JNK is particularly interesting in light of the fact that it has been

linked to the UPR, specifically IRE1 (Cheng et al., 2014). Since ER stress-inducing agents are widely used in cancer therapy, examination of this pathway in the context of combined therapy would be interesting.

References

AITKEN, C. E. & LORSCH, J. R. 2012. A mechanistic overview of translation initiation in eukaryotes. *Nat Struct Mol Biol,* 19**,** 568-76.

AMARAVADI, R., KIMMELMAN, A. C. & WHITE, E. 2016. Recent insights into the function of autophagy in cancer. *Genes Dev,* 30**,** 1913-30.

AMERI, K. & HARRIS, A. L. 2008. Activating transcription factor 4. *Int J Biochem Cell Biol,* 40**,** 14-21.

ARAI, T., KIDA, Y., HARMON, B. V. & GOBE, G. C. 1996. Comparative alterations in p53 expression and apoptosis in the irradiated rat small and large intestine. *Br J Cancer,* 74**,** 406-12.

ARAKAWA, S., TSUJIOKA, M., YOSHIDA, T., TAJIMA-SAKURAI, H., NISHIDA, Y., MATSUOKA, Y., YOSHINO, I., TSUJIMOTO, Y. & SHIMIZU, S. 2017. Role of Atg5-dependent cell death in the embryonic development of Bax/Bak double-knockout mice. *Cell Death Differ,* 24**,** 1598-1608.

ARMSTRONG, J. L., FLOCKHART, R., VEAL, G. J., LOVAT, P. E. & REDFERN, C. P. 2010. Regulation of endoplasmic reticulum stress-induced cell death by ATF4 in neuroectodermal tumor cells. *J Biol Chem,* 285**,** 6091-100.

ARNOULT, D., GAUME, B., KARBOWSKI, M., SHARPE, J. C., CECCONI, F. & YOULE, R. J. 2003. Mitochondrial release of AIF and EndoG requires caspase activation downstream of Bax/Bak-mediated permeabilization. *EMBO J,* 22**,** 4385-99.

ASHKENAZI, A. & DIXIT, V. M. 1998. Death receptors: signaling and modulation. *Science,* 281**,** 1305-8.

AVEROUS, J., BRUHAT, A., JOUSSE, C., CARRARO, V., THIEL, G. & FAFOURNOUX, P. 2004. Induction of CHOP expression by amino acid limitation requires both ATF4 expression and ATF2 phosphorylation. *J Biol Chem,* 279**,** 5288-97.

AXTEN, J. M., MEDINA, J. R., FENG, Y., SHU, A., ROMERIL, S. P., GRANT, S. W., LI, W. H., HEERDING, D. A., MINTHORN, E., MENCKEN, T., ATKINS, C., LIU, Q., RABINDRAN, S., KUMAR, R., HONG, X., GOETZ, A., STANLEY, T., TAYLOR, J. D., SIGETHY, S. D., TOMBERLIN, G. H., HASSELL, A. M., KAHLER, K. M., SHEWCHUK, L. M. & GAMPE, R. T. 2012. Discovery of 7-methyl-5-(1-{[3-(trifluoromethyl)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-7H-p yrrolo[2,3-d]pyrimidin-4-amine (GSK2606414), a potent and selective first-in-class inhibitor of protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK). *J Med Chem,* 55**,** 7193-207.

AXTEN, J. M., ROMERIL, S. P., SHU, A., RALPH, J., MEDINA, J. R., FENG, Y., LI, W. H., GRANT, S. W., HEERDING, D. A., MINTHORN, E., MENCKEN, T., GAUL, N., GOETZ, A., STANLEY, T., HASSELL, A. M., GAMPE, R. T., ATKINS, C. & KUMAR, R. 2013. Discovery of GSK2656157: An Optimized PERK Inhibitor Selected for Preclinical Development. *ACS Med Chem Lett,* 4**,** 964-8.

AZAD, M. B., CHEN, Y., HENSON, E. S., CIZEAU, J., MCMILLAN-WARD, E., ISRAELS, S. J. & GIBSON, S. B. 2008. Hypoxia induces autophagic cell death in apoptosis-competent cells through a mechanism involving BNIP3. *Autophagy,* 4**,** 195-204.

B'CHIR, W., CHAVEROUX, C., CARRARO, V., AVEROUS, J., MAURIN, A. C., JOUSSE, C., MURANISHI, Y., PARRY, L., FAFOURNOUX, P. & BRUHAT, A. 2014. Dual role for CHOP in

the crosstalk between autophagy and apoptosis to determine cell fate in response to amino acid deprivation. *Cell Signal,* 26**,** 1385-91.

B'CHIR, W., MAURIN, A. C., CARRARO, V., AVEROUS, J., JOUSSE, C., MURANISHI, Y., PARRY, L., STEPIEN, G., FAFOURNOUX, P. & BRUHAT, A. 2013. The eIF2alpha/ATF4 pathway is essential for stress-induced autophagy gene expression. *Nucleic Acids Res,* 41**,** 7683-99.

BELL, B. D., LEVERRIER, S., WEIST, B. M., NEWTON, R. H., ARECHIGA, A. F., LUHRS, K. A., MORRISSETTE, N. S. & WALSH, C. M. 2008. FADD and caspase-8 control the outcome of autophagic signaling in proliferating T cells. *Proc Natl Acad Sci U S A,* 105**,** 16677-82.

BERLANGA, J. J., HERRERO, S. & DE HARO, C. 1998. Characterization of the hemin-sensitive eukaryotic initiation factor 2alpha kinase from mouse nonerythroid cells. *J Biol Chem,* 273**,** 32340-6.

BERRY, D. L. & BAEHRECKE, E. H. 2007. Growth arrest and autophagy are required for salivary gland cell degradation in Drosophila. *Cell,* 131**,** 1137-48.

BERTOLOTTI, A., ZHANG, Y., HENDERSHOT, L. M., HARDING, H. P. & RON, D. 2000. Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nat Cell Biol,* 2**,** 326-32.

BI, M., NACZKI, C., KORITZINSKY, M., FELS, D., BLAIS, J., HU, N., HARDING, H., NOVOA, I., VARIA, M., RALEIGH, J., SCHEUNER, D., KAUFMAN, R. J., BELL, J., RON, D., WOUTERS, B. G. & KOUMENIS, C. 2005. ER stress-regulated translation increases tolerance to extreme hypoxia and promotes tumor growth. *EMBO J,* 24**,** 3470-81.

BISHAY, K., ORY, K., LEBEAU, J., LEVALOIS, C., OLIVIER, M. F. & CHEVILLARD, S. 2000. DNA damage-related gene expression as biomarkers to assess cellular response after gamma irradiation of a human lymphoblastoid cell line. *Oncogene,* 19**,** 916-23.

BOBROVNIKOVA-MARJON, E., GRIGORIADOU, C., PYTEL, D., ZHANG, F., YE, J., KOUMENIS, C., CAVENER, D. & DIEHL, J. A. 2010. PERK promotes cancer cell proliferation and tumor growth by limiting oxidative DNA damage. *Oncogene,* 29**,** 3881-95.

BOEGE, Y., MALEHMIR, M., HEALY, M. E., BETTERMANN, K., LORENTZEN, A., VUCUR, M., AHUJA, A. K., BOHM, F., MERTENS, J. C., SHIMIZU, Y., FRICK, L., REMOUCHAMPS, C., MUTREJA, K., KAHNE, T., SUNDARAVINAYAGAM, D., WOLF, M. J., REHRAUER, H., KOPPE, C., SPEICHER, T., PADRISSA-ALTES, S., MAIRE, R., SCHATTENBERG, J. M., JEONG, J. S., LIU, L., ZWIRNER, S., BOGER, R., HUSER, N., DAVIS, R. J., MULLHAUPT, B., MOCH, H., SCHULZE-BERGKAMEN, H., CLAVIEN, P. A., WERNER, S., BORSIG, L., LUTHER, S. A., JOST, P. J., WEINLICH, R., UNGER, K., BEHRENS, A., HILLERT, L., DILLON, C., DI VIRGILIO, M., WALLACH, D., DEJARDIN, E., ZENDER, L., NAUMANN, M., WALCZAK, H., GREEN, D. R., LOPES, M., LAVRIK, I., LUEDDE, T., HEIKENWALDER, M. & WEBER, A. 2017. A Dual Role of Caspase-8 in Triggering and Sensing Proliferation-Associated DNA Damage, a Key Determinant of Liver Cancer Development. *Cancer Cell,* 32**,** 342-359 e10.

BOUCHIER-HAYES, L. 2010. The role of caspase-2 in stress-induced apoptosis. *J Cell Mol Med,* 14**,** 1212-24.

BROWN, J. M. & ATTARDI, L. D. 2005. The role of apoptosis in cancer development and treatment response. *Nat Rev Cancer,* 5**,** 231-7.

BROWN, J. M. & WILSON, G. 2003. Apoptosis genes and resistance to cancer therapy: what does the experimental and clinical data tell us? *Cancer Biol Ther,* 2**,** 477-90.

BURTON, T. R. & GIBSON, S. B. 2009. The role of Bcl-2 family member BNIP3 in cell death and disease: NIPping at the heels of cell death. *Cell Death Differ,* 16**,** 515-23.

BUYTAERT, E., CALLEWAERT, G., VANDENHEEDE, J. R. & AGOSTINIS, P. 2006. Deficiency in apoptotic effectors Bax and Bak reveals an autophagic cell death pathway initiated by photodamage to the endoplasmic reticulum. *Autophagy,* 2**,** 238-40.

CAI, J., YANG, J. & JONES, D. P. 1998. Mitochondrial control of apoptosis: the role of cytochrome c. *Biochim Biophys Acta,* 1366**,** 139-49.

CALFON, M., ZENG, H., URANO, F., TILL, J. H., HUBBARD, S. R., HARDING, H. P., CLARK, S. G. & RON, D. 2002. IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature,* 415**,** 92-6.

CAREW, J. S., NAWROCKI, S. T., KRUPNIK, Y. V., DUNNER, K., JR., MCCONKEY, D. J., KEATING, M. J. & HUANG, P. 2006. Targeting endoplasmic reticulum protein transport: a novel strategy to kill malignant B cells and overcome fludarabine resistance in CLL. *Blood,* 107**,** 222-31.

CARO-MALDONADO, A., TAIT, S. W., RAMIREZ-PEINADO, S., RICCI, J. E., FABREGAT, I., GREEN, D. R. & MUNOZ-PINEDO, C. 2010. Glucose deprivation induces an atypical form of apoptosis mediated by caspase-8 in Bax-, Bak-deficient cells. *Cell Death Differ,* 17**,** 1335-44.

CEREZO, M., LEHRAIKI, A., MILLET, A., ROUAUD, F., PLAISANT, M., JAUNE, E., BOTTON, T., RONCO, C., ABBE, P., AMDOUNI, H., PASSERON, T., HOFMAN, V., MOGRABI, B., DABERT-GAY, A. S., DEBAYLE, D., ALCOR, D., RABHI, N., ANNICOTTE, J. S., HELIOT, L., GONZALEZ-PISFIL, M., ROBERT, C., MORERA, S., VIGOUROUX, A., GUAL, P., ALI, M. M. U., BERTOLOTTO, C., HOFMAN, P., BALLOTTI, R., BENHIDA, R. & ROCCHI, S. 2016. Compounds Triggering ER Stress Exert Anti-Melanoma Effects and Overcome BRAF Inhibitor Resistance. *Cancer Cell,* 30**,** 183.

CHAN, C. P., KOK, K. H., TANG, H. M., WONG, C. M. & JIN, D. Y. 2013. Internal ribosome entry site-mediated translational regulation of ATF4 splice variant in mammalian unfolded protein response. *Biochim Biophys Acta,* 1833**,** 2165-75.

CHAUBE, B. & BHAT, M. K. 2016. AMPK, a key regulator of metabolic/energy homeostasis and mitochondrial biogenesis in cancer cells. *Cell Death Dis,* 7**,** e2044.

CHEN, J. J., THROOP, M. S., GEHRKE, L., KUO, I., PAL, J. K., BRODSKY, M. & LONDON, I. M. 1991. Cloning of the cDNA of the heme-regulated eukaryotic initiation factor 2 alpha (eIF-2 alpha) kinase of rabbit reticulocytes: homology to yeast GCN2 protein kinase and human double-stranded-RNA-dependent eIF-2 alpha kinase. *Proc Natl Acad Sci U S A,* 88**,** 7729-33.

CHEN, T., OZEL, D., QIAO, Y., HARBINSKI, F., CHEN, L., DENOYELLE, S., HE, X., ZVEREVA, N., SUPKO, J. G., CHOREV, M., HALPERIN, J. A. & AKTAS, B. H. 2011. Chemical genetics identify eIF2alpha kinase heme-regulated inhibitor as an anticancer target. *Nat Chem Biol,* 7**,** 610-6.

CHEN, Y. & BRANDIZZI, F. 2013. IRE1: ER stress sensor and cell fate executor. *Trends Cell Biol,* 23**,** 547-55.

CHENG, X., LIU, H., JIANG, C. C., FANG, L., CHEN, C., ZHANG, X. D. & JIANG, Z. W. 2014. Connecting endoplasmic reticulum stress to autophagy through IRE1/JNK/beclin-1 in breast cancer cells. *Int J Mol Med,* 34**,** 772-81.

CHEONG, H., LINDSTEN, T., WU, J., LU, C. & THOMPSON, C. B. 2011. Ammonia-induced autophagy is independent of ULK1/ULK2 kinases. *Proc Natl Acad Sci U S A,* 108**,** 11121-6.

CHINNADURAI, G., VIJAYALINGAM, S. & GIBSON, S. B. 2008. BNIP3 subfamily BH3-only proteins: mitochondrial stress sensors in normal and pathological functions. *Oncogene,* 27 Suppl 1**,** S114-27.

CHINNAIYAN, A. M. 1999. The apoptosome: heart and soul of the cell death machine. *Neoplasia,* 1**,** 5-15.

CHIPUK, J. E., BOUCHIER-HAYES, L. & GREEN, D. R. 2006. Mitochondrial outer membrane permeabilization during apoptosis: the innocent bystander scenario. *Cell Death Differ,* 13**,** 1396-402.

CHIPUK, J. E. & GREEN, D. R. 2008. How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? *Trends Cell Biol,* 18**,** 157-64.

CHO, Y., MCQUADE, T., ZHANG, H., ZHANG, J. & CHAN, F. K. 2011. RIP1-dependent and independent effects of necrostatin-1 in necrosis and T cell activation. *PLoS One,* 6**,** e23209.

CICCHINI, M., KARANTZA, V. & XIA, B. 2015. Molecular pathways: autophagy in cancer--a matter of timing and context. *Clin Cancer Res,* 21**,** 498-504.

CODOGNO, P., MEHRPOUR, M. & PROIKAS-CEZANNE, T. 2011. Canonical and non-canonical autophagy: variations on a common theme of self-eating? *Nat Rev Mol Cell Biol,* 13**,** 7-12.

COHEN, G. M. 1997. Caspases: the executioners of apoptosis. *Biochem J,* 326 (Pt 1)**,** 1-16.

COSTA-MATTIOLI, M., GOBERT, D., HARDING, H., HERDY, B., AZZI, M., BRUNO, M., BIDINOSTI, M., BEN MAMOU, C., MARCINKIEWICZ, E., YOSHIDA, M., IMATAKA, H., CUELLO, A. C., SEIDAH, N., SOSSIN, W., LACAILLE, J. C., RON, D., NADER, K. & SONENBERG, N. 2005. Translational control of hippocampal synaptic plasticity and memory by the eIF2alpha kinase GCN2. *Nature,* 436**,** 1166-73.

CRIGHTON, D., WILKINSON, S., O'PREY, J., SYED, N., SMITH, P., HARRISON, P. R., GASCO, M., GARRONE, O., CROOK, T. & RYAN, K. M. 2006. DRAM, a p53-induced modulator of autophagy, is critical for apoptosis. *Cell,* 126**,** 121-34.

CUBILLOS-RUIZ, J. R., BETTIGOLE, S. E. & GLIMCHER, L. H. 2017. Tumorigenic and Immunosuppressive Effects of Endoplasmic Reticulum Stress in Cancer. *Cell,* 168**,** 692-706.

CULLINAN, S. B., ZHANG, D., HANNINK, M., ARVISAIS, E., KAUFMAN, R. J. & DIEHL, J. A. 2003. Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival. *Mol Cell Biol,* 23**,** 7198-209.

DAI, D. L., MARTINKA, M., BUSH, J. A. & LI, G. 2004. Reduced Apaf-1 expression in human cutaneous melanomas. *Br J Cancer,* 91**,** 1089-95.

DAIDO, S., KANZAWA, T., YAMAMOTO, A., TAKEUCHI, H., KONDO, Y. & KONDO, S. 2004. Pivotal role of the cell death factor BNIP3 in ceramide-induced autophagic cell death in malignant glioma cells. *Cancer Res,* 64**,** 4286-93.

DALY, M. J. 2012. Death by protein damage in irradiated cells. *DNA Repair (Amst),* 11**,** 12-21.

DATTA, R., OKI, E., ENDO, K., BIEDERMANN, V., REN, J. & KUFE, D. 2000. XIAP regulates DNA damage-induced apoptosis downstream of caspase-9 cleavage. *J Biol Chem,* 275**,** 31733-8.

DAY, T. W., WU, C. H. & SAFA, A. R. 2009. Etoposide induces protein kinase Cdelta- and caspase-3-dependent apoptosis in neuroblastoma cancer cells. *Mol Pharmacol,* 76**,** 632-40.

DE ZIO, D., CIANFANELLI, V. & CECCONI, F. 2013. New insights into the link between DNA damage and apoptosis. *Antioxid Redox Signal,* 19**,** 559-71.

DEEGAN, S., KORYGA, I., GLYNN, S. A., GUPTA, S., GORMAN, A. M. & SAMALI, A. 2015. A close connection between the PERK and IRE arms of the UPR and the transcriptional regulation of autophagy. *Biochem Biophys Res Commun,* 456**,** 305-11.

DEEGAN, S., SAVELJEVA, S., GORMAN, A. M. & SAMALI, A. 2013. Stress-induced self-cannibalism: on the regulation of autophagy by endoplasmic reticulum stress. *Cell Mol Life Sci,* 70**,** 2425-41.

DEEGAN, S., SAVELJEVA, S., GUPTA, S., MACDONALD, D. C. & SAMALI, A. 2014a. ER stress responses in the absence of apoptosome: a comparative study in CASP9 proficient vs deficient mouse embryonic fibroblasts. *Biochem Biophys Res Commun,* 451**,** 367-73.

DEEGAN, S., SAVELJEVA, S., LOGUE, S. E., PAKOS-ZEBRUCKA, K., GUPTA, S., VANDENABEELE, P., BERTRAND, M. J. & SAMALI, A. 2014b. Deficiency in the mitochondrial apoptotic pathway reveals the toxic potential of autophagy under ER stress conditions. *Autophagy,* 10**,** 1921-36.

DEGENHARDT, K., MATHEW, R., BEAUDOIN, B., BRAY, K., ANDERSON, D., CHEN, G., MUKHERJEE, C., SHI, Y., GELINAS, C., FAN, Y., NELSON, D. A., JIN, S. & WHITE, E. 2006. Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. *Cancer Cell,* 10**,** 51-64.

DEGTEREV, A., BOYCE, M. & YUAN, J. 2003. A decade of caspases. *Oncogene,* 22**,** 8543-67.

DELBRIDGE, A. R. & STRASSER, A. 2015. The BCL-2 protein family, BH3-mimetics and cancer therapy. *Cell Death Differ,* 22**,** 1071-80.

DELBRIDGE, A. R., VALENTE, L. J. & STRASSER, A. 2012. The role of the apoptotic machinery in tumor suppression. *Cold Spring Harb Perspect Biol,* 4.

DENG, J., HARDING, H. P., RAUGHT, B., GINGRAS, A. C., BERLANGA, J. J., SCHEUNER, D., KAUFMAN, R. J., RON, D. & SONENBERG, N. 2002. Activation of GCN2 in UV-irradiated cells inhibits translation. *Curr Biol,* 12**,** 1279-86.

DENNIS, M. D., MCGHEE, N. K., JEFFERSON, L. S. & KIMBALL, S. R. 2013. Regulated in DNA damage and development 1 (REDD1) promotes cell survival during serum deprivation by sustaining repression of signaling through the mechanistic target of rapamycin in complex 1 (mTORC1). *Cell Signal,* 25**,** 2709-16.

DENOYELLE, C., ABOU-RJAILY, G., BEZROOKOVE, V., VERHAEGEN, M., JOHNSON, T. M., FULLEN, D. R., POINTER, J. N., GRUBER, S. B., SU, L. D., NIKIFOROV, M. A., KAUFMAN, R. J., BASTIAN, B. C. & SOENGAS, M. S. 2006. Anti-oncogenic role of the endoplasmic reticulum differentially activated by mutations in the MAPK pathway. *Nat Cell Biol,* 8**,** 1053-63.

DEY, S., BAIRD, T. D., ZHOU, D., PALAM, L. R., SPANDAU, D. F. & WEK, R. C. 2010. Both transcriptional regulation and translational control of ATF4 are central to the integrated stress response. *J Biol Chem,* 285**,** 33165-74.

DEY, S., SAYERS, C. M., VERGINADIS, II, LEHMAN, S. L., CHENG, Y., CERNIGLIA, G. J., TUTTLE, S. W., FELDMAN, M. D., ZHANG, P. J., FUCHS, S. Y., DIEHL, J. A. & KOUMENIS, C. 2015. ATF4-dependent induction of heme oxygenase 1 prevents anoikis and promotes metastasis. *J Clin Invest,* 125**,** 2592-608.

DING, W. X., NI, H. M., GAO, W., HOU, Y. F., MELAN, M. A., CHEN, X., STOLZ, D. B., SHAO, Z. M. & YIN, X. M. 2007. Differential effects of endoplasmic reticulum stress-induced autophagy on cell survival. *J Biol Chem,* 282**,** 4702-10.

DONNELLY, N., GORMAN, A. M., GUPTA, S. & SAMALI, A. 2013. The eIF2alpha kinases: their structures and functions. *Cell Mol Life Sci,* 70**,** 3493-511.

DRIOUICH, A., ZHANG, G. F. & STAEHELIN, L. A. 1993. Effect of brefeldin A on the structure of the Golgi apparatus and on the synthesis and secretion of proteins and polysaccharides in sycamore maple (Acer pseudoplatanus) suspension-cultured cells. *Plant Physiol,* 101**,** 1363-73.

DU, C., FANG, M., LI, Y., LI, L. & WANG, X. 2000. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell,* 102**,** 33-42.

DUPONT, N., NASCIMBENI, A. C., MOREL, E. & CODOGNO, P. 2017. Molecular Mechanisms of Noncanonical Autophagy. *Int Rev Cell Mol Biol,* 328**,** 1-23.

EBERT, S. M., DYLE, M. C., BULLARD, S. A., DIERDORFF, J. M., MURRY, D. J., FOX, D. K., BONGERS, K. S., LIRA, V. A., MEYERHOLZ, D. K., TALLEY, J. J. & ADAMS, C. M. 2015. Identification and Small Molecule Inhibition of an Activating Transcription Factor 4 (ATF4)-dependent Pathway to Age-related Skeletal Muscle Weakness and Atrophy. *J Biol Chem,* 290**,** 25497-511.

EFEYAN, A. & SABATINI, D. M. 2010. mTOR and cancer: many loops in one pathway. *Curr Opin Cell Biol,* 22**,** 169-76.

EKERT, P. G., READ, S. H., SILKE, J., MARSDEN, V. S., KAUFMANN, H., HAWKINS, C. J., GERL, R., KUMAR, S. & VAUX, D. L. 2004. Apaf-1 and caspase-9 accelerate apoptosis, but do not determine whether factor-deprived or drug-treated cells die. *J Cell Biol,* 165**,** 835-42.

ELGENDY, M., SHERIDAN, C., BRUMATTI, G. & MARTIN, S. J. 2011. Oncogenic Ras-induced expression of Noxa and Beclin-1 promotes autophagic cell death and limits clonogenic survival. *Mol Cell,* 42**,** 23-35.

ESTORNES, Y., AGUILETA, M. A., DUBUISSON, C., DE KEYSER, J., GOOSSENS, V., KERSSE, K., SAMALI, A., VANDENABEELE, P. & BERTRAND, M. J. 2014. RIPK1 promotes death receptor-independent caspase-8-mediated apoptosis under unresolved ER stress conditions. *Cell Death Dis,* 5**,** e1555.

FAVALORO, B., ALLOCATI, N., GRAZIANO, V., DI ILIO, C. & DE LAURENZI, V. 2012. Role of apoptosis in disease. *Aging (Albany NY),* 4**,** 330-49.

FAWCETT, T. W., MARTINDALE, J. L., GUYTON, K. Z., HAI, T. & HOLBROOK, N. J. 1999. Complexes containing activating transcription factor (ATF)/cAMP-responsive-element-binding protein (CREB) interact with the CCAAT/enhancer-binding protein (C/EBP)-ATF composite site to regulate Gadd153 expression during the stress response. *Biochem J,* 339 (Pt 1)**,** 135-41.

FINK, S. L. & COOKSON, B. T. 2005. Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. *Infect Immun,* 73**,** 1907-16.

FITZWALTER, B. E. & THORBURN, A. 2015. Recent insights into cell death and autophagy. *FEBS J,* 282**,** 4279-88.

FODALE, V., PIEROBON, M., LIOTTA, L. & PETRICOIN, E. 2011. Mechanism of cell adaptation: when and how do cancer cells develop chemoresistance? *Cancer J,* 17**,** 89-95.

FULDA, S. 2013. Alternative cell death pathways and cell metabolism. *Int J Cell Biol,* 2013**,** 463637.

FULDA, S., GORMAN, A. M., HORI, O. & SAMALI, A. 2010. Cellular stress responses: cell survival and cell death. *Int J Cell Biol,* 2010**,** 214074.

FULLGRABE, J., KLIONSKY, D. J. & JOSEPH, B. 2014. The return of the nucleus: transcriptional and epigenetic control of autophagy. *Nat Rev Mol Cell Biol,* 15**,** 65-74.

GALEHDAR, Z., SWAN, P., FUERTH, B., CALLAGHAN, S. M., PARK, D. S. & CREGAN, S. P. 2010. Neuronal apoptosis induced by endoplasmic reticulum stress is regulated by ATF4-CHOP-mediated induction of the Bcl-2 homology 3-only member PUMA. *J Neurosci,* 30**,** 16938-48.

GALLUZZI, L., BAEHRECKE, E. H., BALLABIO, A., BOYA, P., BRAVO-SAN PEDRO, J. M., CECCONI, F., CHOI, A. M., CHU, C. T., CODOGNO, P., COLOMBO, M. I., CUERVO, A. M., DEBNATH, J., DERETIC, V., DIKIC, I., ESKELINEN, E. L., FIMIA, G. M., FULDA, S., GEWIRTZ, D. A., GREEN, D. R., HANSEN, M., HARPER, J. W., JAATTELA, M., JOHANSEN, T., JUHASZ, G., KIMMELMAN, A. C., KRAFT, C., KTISTAKIS, N. T., KUMAR, S., LEVINE, B., LOPEZ-OTIN, C., MADEO, F., MARTENS, S., MARTINEZ, J., MELENDEZ, A., MIZUSHIMA, N., MUNZ, C.,

MURPHY, L. O., PENNINGER, J. M., PIACENTINI, M., REGGIORI, F., RUBINSZTEIN, D. C., RYAN, K. M., SANTAMBROGIO, L., SCORRANO, L., SIMON, A. K., SIMON, H. U., SIMONSEN, A., TAVERNARAKIS, N., TOOZE, S. A., YOSHIMORI, T., YUAN, J., YUE, Z., ZHONG, Q. & KROEMER, G. 2017a. Molecular definitions of autophagy and related processes. *EMBO J,* 36**,** 1811-1836.

GALLUZZI, L., BRAVO-SAN PEDRO, J. M., LEVINE, B., GREEN, D. R. & KROEMER, G. 2017b. Pharmacological modulation of autophagy: therapeutic potential and persisting obstacles. *Nat Rev Drug Discov,* 16**,** 487-511.

GALLUZZI, L., BRAVO-SAN PEDRO, J. M., VITALE, I., AARONSON, S. A., ABRAMS, J. M., ADAM, D., ALNEMRI, E. S., ALTUCCI, L., ANDREWS, D., ANNICCHIARICO-PETRUZZELLI, M., BAEHRECKE, E. H., BAZAN, N. G., BERTRAND, M. J., BIANCHI, K., BLAGOSKLONNY, M. V., BLOMGREN, K., BORNER, C., BREDESEN, D. E., BRENNER, C., CAMPANELLA, M., CANDI, E., CECCONI, F., CHAN, F. K., CHANDEL, N. S., CHENG, E. H., CHIPUK, J. E., CIDLOWSKI, J. A., CIECHANOVER, A., DAWSON, T. M., DAWSON, V. L., DE LAURENZI, V., DE MARIA, R., DEBATIN, K. M., DI DANIELE, N., DIXIT, V. M., DYNLACHT, B. D., EL-DEIRY, W. S., FIMIA, G. M., FLAVELL, R. A., FULDA, S., GARRIDO, C., GOUGEON, M. L., GREEN, D. R., GRONEMEYER, H., HAJNOCZKY, G., HARDWICK, J. M., HENGARTNER, M. O., ICHIJO, H., JOSEPH, B., JOST, P. J., KAUFMANN, T., KEPP, O., KLIONSKY, D. J., KNIGHT, R. A., KUMAR, S., LEMASTERS, J. J., LEVINE, B., LINKERMANN, A., LIPTON, S. A., LOCKSHIN, R. A., LOPEZ-OTIN, C., LUGLI, E., MADEO, F., MALORNI, W., MARINE, J. C., MARTIN, S. J., MARTINOU, J. C., MEDEMA, J. P., MEIER, P., MELINO, S., MIZUSHIMA, N., MOLL, U., MUNOZ-PINEDO, C., NUNEZ, G., OBERST, A., PANARETAKIS, T., PENNINGER, J. M., PETER, M. E., PIACENTINI, M., PINTON, P., PREHN, J. H., PUTHALAKATH, H., RABINOVICH, G. A., RAVICHANDRAN, K. S., RIZZUTO, R., RODRIGUES, C. M., RUBINSZTEIN, D. C., RUDEL, T., SHI, Y., SIMON, H. U., STOCKWELL, B. R., SZABADKAI, G., TAIT, S. W., TANG, H. L., TAVERNARAKIS, N., TSUJIMOTO, Y., VANDEN BERGHE, T., VANDENABEELE, P., VILLUNGER, A., WAGNER, E. F., et al. 2015. Essential versus accessory aspects of cell death: recommendations of the NCCD 2015. *Cell Death Differ,* 22**,** 58-73.

GALLUZZI, L., JOZA, N., TASDEMIR, E., MAIURI, M. C., HENGARTNER, M., ABRAMS, J. M., TAVERNARAKIS, N., PENNINGER, J., MADEO, F. & KROEMER, G. 2008. No death without life: vital functions of apoptotic effectors. *Cell Death Differ,* 15**,** 1113-23.

GALLUZZI, L., LOPEZ-SOTO, A., KUMAR, S. & KROEMER, G. 2016. Caspases Connect Cell-Death Signaling to Organismal Homeostasis. *Immunity,* 44**,** 221-31.

GALLUZZI, L., VITALE, I., ABRAMS, J. M., ALNEMRI, E. S., BAEHRECKE, E. H., BLAGOSKLONNY, M. V., DAWSON, T. M., DAWSON, V. L., EL-DEIRY, W. S., FULDA, S., GOTTLIEB, E., GREEN, D. R., HENGARTNER, M. O., KEPP, O., KNIGHT, R. A., KUMAR, S., LIPTON, S. A., LU, X., MADEO, F., MALORNI, W., MEHLEN, P., NUNEZ, G., PETER, M. E., PIACENTINI, M., RUBINSZTEIN, D. C., SHI, Y., SIMON, H. U., 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 Differ,* 19**,** 107-20.

GARCIA-DE TERESA, B., HERNANDEZ-GOMEZ, M. & FRIAS, S. 2017. DNA Damage as a Driver for Growth Delay: Chromosome Instability Syndromes with Intrauterine Growth Retardation. *Biomed Res Int,* 2017**,** 8193892.

GIAGKOUSIKLIDIS, S., VOGLER, M., WESTHOFF, M. A., KASPERCZYK, H., DEBATIN, K. M. & FULDA, S. 2005. Sensitization for gamma-irradiation-induced apoptosis by second mitochondria-derived activator of caspase. *Cancer Res,* 65**,** 10502-13.

GOLSTEIN, P. & KROEMER, G. 2005. Redundant cell death mechanisms as relics and backups. *Cell Death Differ,* 12 Suppl 2**,** 1490-6.

GONCALVES, A., BRAGUER, D., CARLES, G., ANDRE, N., PREVOT, C. & BRIAND, C. 2000. Caspase-8 activation independent of CD95/CD95-L interaction during paclitaxel-induced apoptosis in human colon cancer cells (HT29-D4). *Biochem Pharmacol,* 60**,** 1579-84.

GREEN, D. R. & LEVINE, B. 2014. To be or not to be? How selective autophagy and cell death govern cell fate. *Cell,* 157**,** 65-75.

GRISHCHUK, Y., GINET, V., TRUTTMANN, A. C., CLARKE, P. G. & PUYAL, J. 2011. Beclin 1-independent autophagy contributes to apoptosis in cortical neurons. *Autophagy,* 7**,** 1115-31.

GUAN, B. J., KROKOWSKI, D., MAJUMDER, M., SCHMOTZER, C. L., KIMBALL, S. R., MERRICK, W. C., KOROMILAS, A. E. & HATZOGLOU, M. 2014. Translational control during endoplasmic reticulum stress beyond phosphorylation of the translation initiation factor eIF2alpha. *J Biol Chem,* 289**,** 12593-611.

GUPTA, S., GIRICZ, Z., NATONI, A., DONNELLY, N., DEEGAN, S., SZEGEZDI, E. & SAMALI, A. 2012. NOXA contributes to the sensitivity of PERK-deficient cells to ER stress. *FEBS Lett,* 586**,** 4023-30.

HAANEN, C. & VERMES, I. 1996. Apoptosis: programmed cell death in fetal development. *Eur J Obstet Gynecol Reprod Biol,* 64**,** 129-33.

HAKEM, R., HAKEM, A., DUNCAN, G. S., HENDERSON, J. T., WOO, M., SOENGAS, M. S., ELIA, A., DE LA POMPA, J. L., KAGI, D., KHOO, W., POTTER, J., YOSHIDA, R., KAUFMAN, S. A., LOWE, S. W., PENNINGER, J. M. & MAK, T. W. 1998. Differential requirement for caspase 9 in apoptotic pathways in vivo. *Cell,* 94**,** 339-52.

HALACLI, S. O., CANPINAR, H., CIMEN, E. & SUNGUROGLU, A. 2013. Effects of gamma irradiation on cell cycle, apoptosis and telomerase activity in p53 wild-type and deficient HCT116 colon cancer cell lines. *Oncol Lett,* 6**,** 807-810.

HALDAR, S., CHINTAPALLI, J. & CROCE, C. M. 1996. Taxol induces bcl-2 phosphorylation and death of prostate cancer cells. *Cancer Res,* 56**,** 1253-5.

HAN, J., BACK, S. H., HUR, J., LIN, Y. H., GILDERSLEEVE, R., SHAN, J., YUAN, C. L., KROKOWSKI, D., WANG, S., HATZOGLOU, M., KILBERG, M. S., SARTOR, M. A. & KAUFMAN, R. J. 2013. ER-stress-induced transcriptional regulation increases protein synthesis leading to cell death. *Nat Cell Biol,* 15**,** 481-90.

HANAHAN, D. & WEINBERG, R. A. 2011. Hallmarks of cancer: the next generation. *Cell,* 144**,** 646-74.

HAO, Z., DUNCAN, G. S., CHANG, C. C., ELIA, A., FANG, M., WAKEHAM, A., OKADA, H., CALZASCIA, T., JANG, Y., YOU-TEN, A., YEH, W. C., OHASHI, P., WANG, X. & MAK, T. W. 2005. Specific ablation of the apoptotic functions of cytochrome C reveals a differential requirement for cytochrome C and Apaf-1 in apoptosis. *Cell,* 121**,** 579-91.

HARA, K., MARUKI, Y., LONG, X., YOSHINO, K., OSHIRO, N., HIDAYAT, S., TOKUNAGA, C., AVRUCH, J. & YONEZAWA, K. 2002. Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. *Cell,* 110**,** 177-89.

HARDING, H. P., NOVOA, I., ZHANG, Y., ZENG, H., WEK, R., SCHAPIRA, M. & RON, D. 2000a. Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Mol Cell,* 6**,** 1099-108.

HARDING, H. P., ZHANG, Y., BERTOLOTTI, A., ZENG, H. & RON, D. 2000b. Perk is essential for translational regulation and cell survival during the unfolded protein response. *Mol Cell,* 5**,** 897-904.

HARDING, H. P., ZHANG, Y. & RON, D. 1999. Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. *Nature,* 397**,** 271-4.
HART, L. S., CUNNINGHAM, J. T., DATTA, T., DEY, S., TAMEIRE, F., LEHMAN, S. L., QIU, B., ZHANG, H., CERNIGLIA, G., BI, M., LI, Y., GAO, Y., LIU, H., LI, C., MAITY, A., THOMAS-TIKHONENKO, A., PERL, A. E., KOONG, A., FUCHS, S. Y., DIEHL, J. A., MILLS, I. G., RUGGERO, D. & KOUMENIS, C. 2012. ER stress-mediated autophagy promotes Myc-dependent transformation and tumor growth. *J Clin Invest,* 122**,** 4621-34.
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**,** 3787-99.
HE, C. & LEVINE, B. 2010. The Beclin 1 interactome. *Curr Opin Cell Biol,* 22**,** 140-9.
HETTMANN, T., BARTON, K. & LEIDEN, J. M. 2000. Microphthalmia due to p53-mediated apoptosis of anterior lens epithelial cells in mice lacking the CREB-2 transcription factor. *Dev Biol,* 222**,** 110-23.
HETZ, C., CHEVET, E. & OAKES, S. A. 2015. Proteostasis control by the unfolded protein response. *Nat Cell Biol,* 17**,** 829-38.
HINNEBUSCH, A. G. 1997. Translational regulation of yeast GCN4. A window on factors that control initiator-trna binding to the ribosome. *J Biol Chem,* 272**,** 21661-4.
HINNEBUSCH, A. G. 2005. Translational regulation of GCN4 and the general amino acid control of yeast. *Annu Rev Microbiol,* 59**,** 407-50.
HINNEBUSCH, A. G. 2011. Molecular mechanism of scanning and start codon selection in eukaryotes. *Microbiol Mol Biol Rev,* 75**,** 434-67, first page of table of contents.
HINNEBUSCH, A. G. & LORSCH, J. R. 2012. The mechanism of eukaryotic translation initiation: new insights and challenges. *Cold Spring Harb Perspect Biol,* 4.
HINNEBUSCH, A. G. & NATARAJAN, K. 2002. Gcn4p, a master regulator of gene expression, is controlled at multiple levels by diverse signals of starvation and stress. *Eukaryot Cell,* 1**,** 22-32.
HIRAMATSU, N., MESSAH, C., HAN, J., LAVAIL, M. M., KAUFMAN, R. J. & LIN, J. H. 2014. Translational and posttranslational regulation of XIAP by eIF2alpha and ATF4 promotes ER stress-induced cell death during the unfolded protein response. *Mol Biol Cell,* 25**,** 1411-20.
HONDA, S., ARAKAWA, S., NISHIDA, Y., YAMAGUCHI, H., ISHII, E. & SHIMIZU, S. 2014. Ulk1-mediated Atg5-independent macroautophagy mediates elimination of mitochondria from embryonic reticulocytes. *Nat Commun,* 5**,** 4004.
HORVITZ, H. R. 1999. Genetic control of programmed cell death in the nematode Caenorhabditis elegans. *Cancer Res,* 59**,** 1701s-1706s.
HSU, H., XIONG, J. & GOEDDEL, D. V. 1995. The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. *Cell,* 81**,** 495-504.
HYBERTSON, B. M., GAO, B., BOSE, S. K. & MCCORD, J. M. 2011. Oxidative stress in health and disease: the therapeutic potential of Nrf2 activation. *Mol Aspects Med,* 32**,** 234-46.
IGARASHI, T., IZUMI, H., UCHIUMI, T., NISHIO, K., ARAO, T., TANABE, M., URAMOTO, H., SUGIO, K., YASUMOTO, K., SASAGURI, Y., WANG, K. Y., OTSUJI, Y. & KOHNO, K. 2007. Clock and ATF4 transcription system regulates drug resistance in human cancer cell lines. *Oncogene,* 26**,** 4749-60.
IMPENS, F., VAN DAMME, P., DEMOL, H., VAN DAMME, J., VANDEKERCKHOVE, J. & GEVAERT, K. 2008. Mechanistic insight into taxol-induced cell death. *Oncogene,* 27**,** 4580-91.

INBAL, B., BIALIK, S., SABANAY, I., SHANI, G. & KIMCHI, A. 2002. DAP kinase and DRP-1 mediate membrane blebbing and the formation of autophagic vesicles during programmed cell death. *J Cell Biol,* 157**,** 455-68.

ITAKURA, E. & MIZUSHIMA, N. 2009. Atg14 and UVRAG: mutually exclusive subunits of mammalian Beclin 1-PI3K complexes. *Autophagy,* 5**,** 534-6.

IURLARO, R. & MUNOZ-PINEDO, C. 2016. Cell death induced by endoplasmic reticulum stress. *FEBS J,* 283**,** 2640-52.

IURLARO, R., PUSCHEL, F., LEON-ANNICCHIARICO, C. L., O'CONNOR, H., MARTIN, S. J., PALOU-GRAMON, D., LUCENDO, E. & MUNOZ-PINEDO, C. 2017. Glucose Deprivation Induces ATF4-Mediated Apoptosis through TRAIL Death Receptors. *Mol Cell Biol,* 37.

JACKSON, R. J., HELLEN, C. U. & PESTOVA, T. V. 2010. The mechanism of eukaryotic translation initiation and principles of its regulation. *Nat Rev Mol Cell Biol,* 11**,** 113-27.

JANSSEN, K., POHLMANN, S., JANICKE, R. U., SCHULZE-OSTHOFF, K. & FISCHER, U. 2007. Apaf-1 and caspase-9 deficiency prevents apoptosis in a Bax-controlled pathway and promotes clonogenic survival during paclitaxel treatment. *Blood,* 110**,** 3662-72.

JIANG, H. Y. & WEK, R. C. 2005. Phosphorylation of the alpha-subunit of the eukaryotic initiation factor-2 (eIF2alpha) reduces protein synthesis and enhances apoptosis in response to proteasome inhibition. *J Biol Chem,* 280**,** 14189-202.

JIANG, H. Y., WEK, S. A., MCGRATH, B. C., LU, D., HAI, T., HARDING, H. P., WANG, X., RON, D., CAVENER, D. R. & WEK, R. C. 2004. Activating transcription factor 3 is integral to the eukaryotic initiation factor 2 kinase stress response. *Mol Cell Biol,* 24**,** 1365-77.

JOUSSE, C., DEVAL, C., MAURIN, A. C., PARRY, L., CHERASSE, Y., CHAVEROUX, C., LEFLOCH, R., LENORMAND, P., BRUHAT, A. & FAFOURNOUX, P. 2007. TRB3 inhibits the transcriptional activation of stress-regulated genes by a negative feedback on the ATF4 pathway. *J Biol Chem,* 282**,** 15851-61.

JUNG, C. H., JUN, C. B., RO, S. H., KIM, Y. M., OTTO, N. M., CAO, J., KUNDU, M. & KIM, D. H. 2009. ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. *Mol Biol Cell,* 20**,** 1992-2003.

KACZMAREK, A., VANDENABEELE, P. & KRYSKO, D. V. 2013. Necroptosis: the release of damage-associated molecular patterns and its physiological relevance. *Immunity,* 38**,** 209-23.

KAISER, W. J., UPTON, J. W., LONG, A. B., LIVINGSTON-ROSANOFF, D., DALEY-BAUER, L. P., HAKEM, R., CASPARY, T. & MOCARSKI, E. S. 2011. RIP3 mediates the embryonic lethality of caspase-8-deficient mice. *Nature,* 471**,** 368-72.

KANG, R., ZEH, H. J., LOTZE, M. T. & TANG, D. 2011. The Beclin 1 network regulates autophagy and apoptosis. *Cell Death Differ,* 18**,** 571-80.

KATHEDER, N. S., KHEZRI, R., O'FARRELL, F., SCHULTZ, S. W., JAIN, A., RAHMAN, M. M., SCHINK, K. O., THEODOSSIOU, T. A., JOHANSEN, T., JUHASZ, G., BILDER, D., BRECH, A., STENMARK, H. & RUSTEN, T. E. 2017. Microenvironmental autophagy promotes tumour growth. *Nature,* 541**,** 417-420.

KAVANAGH, E., RODHE, J., BURGUILLOS, M. A., VENERO, J. L. & JOSEPH, B. 2014. Regulation of caspase-3 processing by cIAP2 controls the switch between pro-inflammatory activation and cell death in microglia. *Cell Death Dis,* 5**,** e1565.

KAZEMI, S., MOUNIR, Z., BALTZIS, D., RAVEN, J. F., WANG, S., KRISHNAMOORTHY, J. L., PLUQUET, O., PELLETIER, J. & KOROMILAS, A. E. 2007. A novel function of eIF2alpha kinases as inducers of the phosphoinositide-3 kinase signaling pathway. *Mol Biol Cell,* 18**,** 3635-44.

KERR, J. F. 2002. History of the events leading to the formulation of the apoptosis concept. *Toxicology,* 181-182**,** 471-4.

KERR, J. F., WYLLIE, A. H. & CURRIE, A. R. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer,* 26**,** 239-57.

KILBERG, M. S., BALASUBRAMANIAN, M., FU, L. & SHAN, J. 2012. The transcription factor network associated with the amino acid response in mammalian cells. *Adv Nutr,* 3**,** 295-306.

KILBERG, M. S., SHAN J FAU - SU, N. & SU, N. 2009. ATF4-dependent transcription mediates signaling of amino acid limitation.

KIM, I., XU, W. & REED, J. C. 2008. Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities. *Nat Rev Drug Discov,* 7**,** 1013-30.

KIM, J., KUNDU, M., VIOLLET, B. & GUAN, K. L. 2011. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat Cell Biol,* 13**,** 132-41.

KIM, K. W., MUTTER, R. W., CAO, C., ALBERT, J. M., FREEMAN, M., HALLAHAN, D. E. & LU, B. 2006. Autophagy for cancer therapy through inhibition of pro-apoptotic proteins and mammalian target of rapamycin signaling. *J Biol Chem,* 281**,** 36883-90.

KISCHKEL, F. C., HELLBARDT, S., BEHRMANN, I., GERMER, M., PAWLITA, M., KRAMMER, P. H. & PETER, M. E. 1995. Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *EMBO J,* 14**,** 5579-88.

KLIONSKY, D. J. 2008. Autophagy revisited: a conversation with Christian de Duve. *Autophagy,* 4**,** 740-3.

KLIONSKY, D. J., ABDELMOHSEN, K., ABE, A., ABEDIN, M. J., ABELIOVICH, H., ACEVEDO AROZENA, A., ADACHI, H., ADAMS, C. M., ADAMS, P. D., ADELI, K., ADHIHETTY, P. J., ADLER, S. G., AGAM, G., AGARWAL, R., AGHI, M. K., AGNELLO, M., AGOSTINIS, P., AGUILAR, P. V., AGUIRRE-GHISO, J., AIROLDI, E. M., AIT-SI-ALI, S., AKEMATSU, T., AKPORIAYE, E. T., AL-RUBEAI, M., ALBAICETA, G. M., ALBANESE, C., ALBANI, D., ALBERT, M. L., ALDUDO, J., ALGUL, H., ALIREZAEI, M., ALLOZA, I., ALMASAN, A., ALMONTE-BECERIL, M., ALNEMRI, E. S., ALONSO, C., ALTAN-BONNET, N., ALTIERI, D. C., ALVAREZ, S., ALVAREZ-ERVITI, L., ALVES, S., AMADORO, G., AMANO, A., AMANTINI, C., AMBROSIO, S., AMELIO, I., AMER, A. O., AMESSOU, M., AMON, A., AN, Z., ANANIA, F. A., ANDERSEN, S. U., ANDLEY, U. P., ANDREADI, C. K., ANDRIEU-ABADIE, N., ANEL, A., ANN, D. K., ANOOPKUMAR-DUKIE, S., ANTONIOLI, M., AOKI, H., APOSTOLOVA, N., AQUILA, S., AQUILANO, K., ARAKI, K., ARAMA, E., ARANDA, A., ARAYA, J., ARCARO, A., ARIAS, E., ARIMOTO, H., ARIOSA, A. R., ARMSTRONG, J. L., ARNOULD, T., ARSOV, I., ASANUMA, K., ASKANAS, V., ASSELIN, E., ATARASHI, R., ATHERTON, S. S., ATKIN, J. D., ATTARDI, L. D., AUBERGER, P., AUBURGER, G., AURELIAN, L., AUTELLI, R., AVAGLIANO, L., AVANTAGGIATI, M. L., AVRAHAMI, L., AWALE, S., AZAD, N., BACHETTI, T., BACKER, J. M., BAE, D. H., BAE, J. S., BAE, O. N., BAE, S. H., BAEHRECKE, E. H., BAEK, S. H., BAGHDIGUIAN, S., BAGNIEWSKA-ZADWORNA, A., et al. 2016. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy,* 12**,** 1-222.

KOUROKU, Y., FUJITA, E., TANIDA, I., UENO, T., ISOAI, A., KUMAGAI, H., OGAWA, S., KAUFMAN, R. J., KOMINAMI, E. & MOMOI, T. 2007. ER stress (PERK/eIF2alpha phosphorylation) mediates the polyglutamine-induced LC3 conversion, an essential step for autophagy formation. *Cell Death Differ,* 14**,** 230-9.

KROEMER, G., GALLUZZI, L., VANDENABEELE, P., ABRAMS, J., ALNEMRI, E. S., BAEHRECKE, E. H., BLAGOSKLONNY, M. V., EL-DEIRY, W. S., GOLSTEIN, P., GREEN, D. R., HENGARTNER, M., KNIGHT, R. A., KUMAR, S., LIPTON, S. A., MALORNI, W., NUNEZ, G., PETER, M. E.,

TSCHOPP, J., YUAN, J., PIACENTINI, M., ZHIVOTOVSKY, B., MELINO, G. & NOMENCLATURE COMMITTEE ON CELL, D. 2009. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell Death Differ,* 16**,** 3-11.

KROEMER, G. & LEVINE, B. 2008. Autophagic cell death: the story of a misnomer. *Nat Rev Mol Cell Biol,* 9**,** 1004-10.

KROEMER, G., MARINO, G. & LEVINE, B. 2010. Autophagy and the integrated stress response. *Mol Cell,* 40**,** 280-93.

KUIDA, K., HAYDAR, T. F., KUAN, C. Y., GU, Y., TAYA, C., KARASUYAMA, H., SU, M. S., RAKIC, P. & FLAVELL, R. A. 1998. Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. *Cell,* 94**,** 325-37.

KULTZ, D. 2005. Molecular and evolutionary basis of the cellular stress response. *Annu Rev Physiol,* 67**,** 225-57.

LAKIN, N. D. & JACKSON, S. P. 1999. Regulation of p53 in response to DNA damage. *Oncogene,* 18**,** 7644-55.

LASTER, S. M., WOOD, J. G. & GOODING, L. R. 1988. Tumor necrosis factor can induce both apoptic and necrotic forms of cell lysis. *J Immunol,* 141**,** 2629-34.

LAUSSMANN, M. A., PASSANTE, E., DUSSMANN, H., RAUEN, J. A., WURSTLE, M. L., DELGADO, M. E., DEVOCELLE, M., PREHN, J. H. & REHM, M. 2011. Proteasome inhibition can induce an autophagy-dependent apical activation of caspase-8. *Cell Death Differ,* 18**,** 1584-97.

LAVIGNE, M. D., KONSTANTOPOULOS, D., NTAKOU-ZAMPLARA, K. Z., LIAKOS, A. & FOUSTERI, M. 2017. Global unleashing of transcription elongation waves in response to genotoxic stress restricts somatic mutation rate. *Nat Commun,* 8**,** 2076.

LAVOIE, H., LI, J. J., THEVAKUMARAN, N., THERRIEN, M. & SICHERI, F. 2014. Dimerization-induced allostery in protein kinase regulation. *Trends Biochem Sci,* 39**,** 475-86.

LAW, B. K. 2005. Rapamycin: an anti-cancer immunosuppressant? *Crit Rev Oncol Hematol,* 56**,** 47-60.

LEE, M. & YOON, J. H. 2015. Metabolic interplay between glycolysis and mitochondrial oxidation: The reverse Warburg effect and its therapeutic implication. *World J Biol Chem,* 6**,** 148-61.

LEE, S. A., KIM, Y. J. & LEE, C. S. 2013. Brefeldin a induces apoptosis by activating the mitochondrial and death receptor pathways and inhibits focal adhesion kinase-mediated cell invasion. *Basic Clin Pharmacol Toxicol,* 113**,** 329-38.

LEE, Y. Y., CEVALLOS, R. C. & JAN, E. 2009. An upstream open reading frame regulates translation of GADD34 during cellular stresses that induce eIF2alpha phosphorylation. *J Biol Chem,* 284**,** 6661-73.

LEON-ANNICCHIARICO, C. L., RAMIREZ-PEINADO, S., DOMINGUEZ-VILLANUEVA, D., GONSBERG, A., LAMPIDIS, T. J. & MUNOZ-PINEDO, C. 2015. ATF4 mediates necrosis induced by glucose deprivation and apoptosis induced by 2-deoxyglucose in the same cells. *FEBS J,* 282**,** 3647-58.

LEVINE, B. & KLIONSKY, D. J. 2004. Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev Cell,* 6**,** 463-77.

LEVINE, B. & KROEMER, G. 2008. Autophagy in the pathogenesis of disease. *Cell,* 132**,** 27-42.

LEVINE, B., MIZUSHIMA, N. & VIRGIN, H. W. 2011. Autophagy in immunity and inflammation. *Nature,* 469**,** 323-35.

LEVINE, B. & YUAN, J. 2005. Autophagy in cell death: an innocent convict? *J Clin Invest,* 115**,** 2679-88.

LEVY, J. M., THOMPSON, J. C., GRIESINGER, A. M., AMANI, V., DONSON, A. M., BIRKS, D. K., MORGAN, M. J., MIRSKY, D. M., HANDLER, M. H., FOREMAN, N. K. & THORBURN, A. 2014. Autophagy inhibition improves chemosensitivity in BRAF(V600E) brain tumors. *Cancer Discov,* 4**,** 773-80.

LEVY, J. M. M., TOWERS, C. G. & THORBURN, A. 2017. Targeting autophagy in cancer. *Nat Rev Cancer,* 17**,** 528-542.

LI, H., ZHU, H., XU, C. J. & YUAN, J. 1998. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell,* 94**,** 491-501.

LI, J. & YUAN, J. 2008. Caspases in apoptosis and beyond. *Oncogene,* 27**,** 6194-206.

LI, K., LI, Y., SHELTON, J. M., RICHARDSON, J. A., SPENCER, E., CHEN, Z. J., WANG, X. & WILLIAMS, R. S. 2000. Cytochrome c deficiency causes embryonic lethality and attenuates stress-induced apoptosis. *Cell,* 101**,** 389-99.

LI, L. Y., LUO, X. & WANG, X. 2001. Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature,* 412**,** 95-9.

LI, W. X. & FRANKLIN, W. A. 1998. Radiation- and heat-induced apoptosis in PC-3 prostate cancer cells. *Radiat Res,* 150**,** 190-4.

LIAN, J., WU, X., HE, F., KARNAK, D., TANG, W., MENG, Y., XIANG, D., JI, M., LAWRENCE, T. S. & XU, L. 2011. A natural BH3 mimetic induces autophagy in apoptosis-resistant prostate cancer via modulating Bcl-2-Beclin1 interaction at endoplasmic reticulum. *Cell Death Differ,* 18**,** 60-71.

LIANG, X. H., KLEEMAN, L. K., JIANG, H. H., GORDON, G., GOLDMAN, J. E., BERRY, G., HERMAN, B. & LEVINE, B. 1998. Protection against fatal Sindbis virus encephalitis by beclin, a novel Bcl-2-interacting protein. *J Virol,* 72**,** 8586-96.

LIEBMANN, J. E., COOK, J. A., LIPSCHULTZ, C., TEAGUE, D., FISHER, J. & MITCHELL, J. B. 1993. Cytotoxic studies of paclitaxel (Taxol) in human tumour cell lines. *Br J Cancer,* 68**,** 1104-9.

LIN, H. H., LIN, S. M., CHUNG, Y., VONDERFECHT, S., CAMDEN, J. M., FLODBY, P., BOROK, Z., LIMESAND, K. H., MIZUSHIMA, N. & ANN, D. K. 2014. Dynamic involvement of ATG5 in cellular stress responses. *Cell Death Dis,* 5**,** e1478.

LINDSTEN, T., ROSS, A. J., KING, A., ZONG, W. X., RATHMELL, J. C., SHIELS, H. A., ULRICH, E., WAYMIRE, K. G., MAHAR, P., FRAUWIRTH, K., CHEN, Y., WEI, M., ENG, V. M., ADELMAN, D. M., SIMON, M. C., MA, A., GOLDEN, J. A., EVAN, G., KORSMEYER, S. J., MACGREGOR, G. R. & THOMPSON, C. B. 2000. The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. *Mol Cell,* 6**,** 1389-99.

LINDSTEN, T. & THOMPSON, C. B. 2006. Cell death in the absence of Bax and Bak. *Cell Death Differ,* 13**,** 1272-6.

LIU, C. Y., SCHRODER, M. & KAUFMAN, R. J. 2000. Ligand-independent dimerization activates the stress response kinases IRE1 and PERK in the lumen of the endoplasmic reticulum. *J Biol Chem,* 275**,** 24881-5.

LIU, H., CHANG, D. W. & YANG, X. 2005. Interdimer processing and linearity of procaspase-3 activation. A unifying mechanism for the activation of initiator and effector caspases. *J Biol Chem,* 280**,** 11578-82.

LIU, J., XIA, H., KIM, M., XU, L., LI, Y., ZHANG, L., CAI, Y., NORBERG, H. V., ZHANG, T., FURUYA, T., JIN, M., ZHU, Z., WANG, H., YU, J., LI, Y., HAO, Y., CHOI, A., KE, H., MA, D. & YUAN, J. 2011. Beclin1 controls the levels of p53 by regulating the deubiquitination activity of USP10 and USP13. *Cell,* 147**,** 223-34.

LIU, Y. & LEVINE, B. 2015. Autosis and autophagic cell death: the dark side of autophagy. *Cell Death Differ,* 22**,** 367-76.

LIU, Y., SHOJI-KAWATA, S., SUMPTER, R. M., JR., WEI, Y., GINET, V., ZHANG, L., POSNER, B., TRAN, K. A., GREEN, D. R., XAVIER, R. J., SHAW, S. Y., CLARKE, P. G., PUYAL, J. & LEVINE, B. 2013. Autosis is a Na+,K+-ATPase-regulated form of cell death triggered by autophagy-inducing peptides, starvation, and hypoxia-ischemia. *Proc Natl Acad Sci U S A,* 110**,** 20364-71.

LLAMBI, F., WANG, Y. M., VICTOR, B., YANG, M., SCHNEIDER, D. M., GINGRAS, S., PARSONS, M. J., ZHENG, J. H., BROWN, S. A., PELLETIER, S., MOLDOVEANU, T., CHEN, T. & GREEN, D. R. 2016. BOK Is a Non-canonical BCL-2 Family Effector of Apoptosis Regulated by ER-Associated Degradation. *Cell,* 165**,** 421-33.

LO, S. C., LI, X., HENZL, M. T., BEAMER, L. J. & HANNINK, M. 2006. Structure of the Keap1:Nrf2 interface provides mechanistic insight into Nrf2 signaling. *EMBO J,* 25**,** 3605-17.

LOCKSLEY, R. M., KILLEEN, N. & LENARDO, M. J. 2001. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell,* 104**,** 487-501.

LOFFLER, A. S., ALERS, S., DIETERLE, A. M., KEPPELER, H., FRANZ-WACHTEL, M., KUNDU, M., CAMPBELL, D. G., WESSELBORG, S., ALESSI, D. R. & STORK, B. 2011. Ulk1-mediated phosphorylation of AMPK constitutes a negative regulatory feedback loop. *Autophagy,* 7**,** 696-706.

LOGUE, S. E., CLEARY, P., SAVELJEVA, S. & SAMALI, A. 2013. New directions in ER stress-induced cell death. *Apoptosis,* 18**,** 537-46.

LOMAKIN, I. B. & STEITZ, T. A. 2013. The initiation of mammalian protein synthesis and mRNA scanning mechanism. *Nature,* 500**,** 307-11.

LONG, J. S. & RYAN, K. M. 2012. New frontiers in promoting tumour cell death: targeting apoptosis, necroptosis and autophagy. *Oncogene,* 31**,** 5045-60.

LOTEM, J. & SACHS, L. 1993. Regulation by bcl-2, c-myc, and p53 of susceptibility to induction of apoptosis by heat shock and cancer chemotherapy compounds in differentiation-competent and -defective myeloid leukemic cells. *Cell Growth Differ,* 4**,** 41-7.

LU, P. D., HARDING, H. P. & RON, D. 2004. Translation reinitiation at alternative open reading frames regulates gene expression in an integrated stress response. *J Cell Biol,* 167**,** 27-33.

LV, S., SUN, E. C., XU, Q. Y., ZHANG, J. K. & WU, D. L. 2015. Endoplasmic reticulum stress-mediated autophagy contributes to bluetongue virus infection via the PERK-eIF2alpha pathway. *Biochem Biophys Res Commun,* 466**,** 406-12.

MA, T., LI, J., XU, Y., YU, C., XU, T., WANG, H., LIU, K., CAO, N., NIE, B. M., ZHU, S. Y., XU, S., LI, K., WEI, W. G., WU, Y., GUAN, K. L. & DING, S. 2015. Atg5-independent autophagy regulates mitochondrial clearance and is essential for iPSC reprogramming. *Nat Cell Biol,* 17**,** 1379-87.

MA, X. H., PIAO, S. F., DEY, S., MCAFEE, Q., KARAKOUSIS, G., VILLANUEVA, J., HART, L. S., LEVI, S., HU, J., ZHANG, G., LAZOVA, R., KLUMP, V., PAWELEK, J. M., XU, X., XU, W., SCHUCHTER, L. M., DAVIES, M. A., HERLYN, M., WINKLER, J., KOUMENIS, C. & AMARAVADI, R. K. 2014. Targeting ER stress-induced autophagy overcomes BRAF inhibitor resistance in melanoma. *J Clin Invest,* 124**,** 1406-17.

MAIURI, M. C., ZALCKVAR, E., KIMCHI, A. & KROEMER, G. 2007. Self-eating and self-killing: crosstalk between autophagy and apoptosis. *Nat Rev Mol Cell Biol,* 8**,** 741-52.

MARCINIAK, S. J., YUN, C. Y., OYADOMARI, S., NOVOA, I., ZHANG, Y., JUNGREIS, R., NAGATA, K., HARDING, H. P. & RON, D. 2004. CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. *Genes Dev,* 18**,** 3066-77.

MARINO, G., NISO-SANTANO, M., BAEHRECKE, E. H. & KROEMER, G. 2014. Self-consumption: the interplay of autophagy and apoptosis. *Nat Rev Mol Cell Biol,* 15**,** 81-94.

MARSDEN, V. S., O'CONNOR, L., O'REILLY, L. A., SILKE, J., METCALF, D., EKERT, P. G., HUANG, D. C., CECCONI, F., KUIDA, K., TOMASELLI, K. J., ROY, S., NICHOLSON, D. W., VAUX, D. L., BOUILLET, P., ADAMS, J. M. & STRASSER, A. 2002. Apoptosis initiated by Bcl-2-regulated caspase activation independently of the cytochrome c/Apaf-1/caspase-9 apoptosome. *Nature,* 419**,** 634-7.

MARTIN, S. J., AMARANTE-MENDES, G. P., SHI, L., CHUANG, T. H., CASIANO, C. A., O'BRIEN, G. A., FITZGERALD, P., TAN, E. M., BOKOCH, G. M., GREENBERG, A. H. & GREEN, D. R. 1996. The cytotoxic cell protease granzyme B initiates apoptosis in a cell-free system by proteolytic processing and activation of the ICE/CED-3 family protease, CPP32, via a novel two-step mechanism. *EMBO J,* 15**,** 2407-16.

MASUOKA, H. C. & TOWNES, T. M. 2002. Targeted disruption of the activating transcription factor 4 gene results in severe fetal anemia in mice. *Blood,* 99**,** 736-45.

MCCULLOUGH, K. D., MARTINDALE, J. L., KLOTZ, L. O., AW, T. Y. & HOLBROOK, N. J. 2001. Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. *Mol Cell Biol,* 21**,** 1249-59.

MEURS, E., CHONG, K., GALABRU, J., THOMAS, N. S., KERR, I. M., WILLIAMS, B. R. & HOVANESSIAN, A. G. 1990. Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon. *Cell,* 62**,** 379-90.

MILANI, M., RZYMSKI, T., MELLOR, H. R., PIKE, L., BOTTINI, A., GENERALI, D. & HARRIS, A. L. 2009. The role of ATF4 stabilization and autophagy in resistance of breast cancer cells treated with Bortezomib. *Cancer Res,* 69**,** 4415-23.

MILLER, A. V., HICKS, M. A., NAKAJIMA, W., RICHARDSON, A. C., WINDLE, J. J. & HARADA, H. 2013. Paclitaxel-induced apoptosis is BAK-dependent, but BAX and BIM-independent in breast tumor. *PLoS One,* 8**,** e60685.

MIYASHITA, T. & REED, J. C. 1993. Bcl-2 oncoprotein blocks chemotherapy-induced apoptosis in a human leukemia cell line. *Blood,* 81**,** 151-7.

MIZUSHIMA, N., YOSHIMORI, T. & LEVINE, B. 2010. Methods in mammalian autophagy research. *Cell,* 140**,** 313-26.

MONTECUCCO, A., ZANETTA, F. & BIAMONTI, G. 2015. Molecular mechanisms of etoposide. *EXCLI J,* 14**,** 95-108.

MOON, J. L., KIM, S. Y., SHIN, S. W. & PARK, J. W. 2012. Regulation of brefeldin A-induced ER stress and apoptosis by mitochondrial NADP(+)-dependent isocitrate dehydrogenase. *Biochem Biophys Res Commun,* 417**,** 760-4.

MUELLER, P. P. & HINNEBUSCH, A. G. 1986. Multiple upstream AUG codons mediate translational control of GCN4. *Cell,* 45**,** 201-7.

MUNOZ-PINEDO, C., GUIO-CARRION, A., GOLDSTEIN, J. C., FITZGERALD, P., NEWMEYER, D. D. & GREEN, D. R. 2006. Different mitochondrial intermembrane space proteins are released during apoptosis in a manner that is coordinately initiated but can vary in duration. *Proc Natl Acad Sci U S A,* 103**,** 11573-8.

NICOTERA, P., LEIST, M. & FERRANDO-MAY, E. 1998. Intracellular ATP, a switch in the decision between apoptosis and necrosis. *Toxicol Lett,* 102-103**,** 139-42.

NISHIDA, Y., ARAKAWA, S., FUJITANI, K., YAMAGUCHI, H., MIZUTA, T., KANASEKI, T., KOMATSU, M., OTSU, K., TSUJIMOTO, Y. & SHIMIZU, S. 2009. Discovery of Atg5/Atg7-independent alternative macroautophagy. *Nature,* 461**,** 654-8.

NITISS, J. L. & WANG, J. C. 1996. Mechanisms of cell killing by drugs that trap covalent complexes between DNA topoisomerases and DNA. *Mol Pharmacol,* 50**,** 1095-102.

OHOKA, N., YOSHII, S., HATTORI, T., ONOZAKI, K. & HAYASHI, H. 2005. TRB3, a novel ER stress-inducible gene, is induced via ATF4-CHOP pathway and is involved in cell death. *EMBO J,* 24**,** 1243-55.

ORR, G. A., VERDIER-PINARD, P., MCDAID, H. & HORWITZ, S. B. 2003. Mechanisms of Taxol resistance related to microtubules. *Oncogene,* 22**,** 7280-95.

PAIN, V. M. 1996. Initiation of protein synthesis in eukaryotic cells. *Eur J Biochem,* 236**,** 747-71.

PAKOS-ZEBRUCKA, K., KORYGA, I., MNICH, K., LJUJIC, M., SAMALI, A. & GORMAN, A. M. 2016. The integrated stress response. *EMBO Rep,* 17**,** 1374-1395.

PALAM, L. R., BAIRD, T. D. & WEK, R. C. 2011. Phosphorylation of eIF2 facilitates ribosomal bypass of an inhibitory upstream ORF to enhance CHOP translation. *J Biol Chem,* 286**,** 10939-49.

PALAM, L. R., GORE, J., CRAVEN, K. E., WILSON, J. L. & KORC, M. 2015. Integrated stress response is critical for gemcitabine resistance in pancreatic ductal adenocarcinoma. *Cell Death Dis,* 6**,** e1913.

PARKER, A. L., KAVALLARIS, M. & MCCARROLL, J. A. 2014. Microtubules and their role in cellular stress in cancer. *Front Oncol,* 4**,** 153.

PASINI, S., CORONA, C., LIU, J., GREENE, L. A. & SHELANSKI, M. L. 2015. Specific downregulation of hippocampal ATF4 reveals a necessary role in synaptic plasticity and memory. *Cell Rep,* 11**,** 183-91.

PATTINGRE, S., TASSA, A., QU, X., GARUTI, R., LIANG, X. H., MIZUSHIMA, N., PACKER, M., SCHNEIDER, M. D. & LEVINE, B. 2005. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell,* 122**,** 927-39.

PEIDIS, P., PAPADAKIS, A. I., MUADDI, H., RICHARD, S. & KOROMILAS, A. E. 2011. Doxorubicin bypasses the cytoprotective effects of eIF2alpha phosphorylation and promotes PKR-mediated cell death. *Cell Death Differ,* 18**,** 145-54.

PERKINS, C. L., FANG, G., KIM, C. N. & BHALLA, K. N. 2000. The role of Apaf-1, caspase-9, and bid proteins in etoposide- or paclitaxel-induced mitochondrial events during apoptosis. *Cancer Res,* 60**,** 1645-53.

PERKINS, D. J. & BARBER, G. N. 2004. Defects in translational regulation mediated by the alpha subunit of eukaryotic initiation factor 2 inhibit antiviral activity and facilitate the malignant transformation of human fibroblasts. *Mol Cell Biol,* 24**,** 2025-40.

PIHAN, P., CARRERAS-SUREDA, A. & HETZ, C. 2017. BCL-2 family: integrating stress responses at the ER to control cell demise. *Cell Death Differ,* 24**,** 1478-1487.

PURSCHKE, M., RUBIO, N., HELD, K. D. & REDMOND, R. W. 2010. Phototoxicity of Hoechst 33342 in time-lapse fluorescence microscopy. *Photochem Photobiol Sci,* 9**,** 1634-9.

PUTHALAKATH, H., O'REILLY, L. A., GUNN, P., LEE, L., KELLY, P. N., HUNTINGTON, N. D., HUGHES, P. D., MICHALAK, E. M., 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**,** 1337-49.

PYO, J. O., JANG, M. H., KWON, Y. K., LEE, H. J., JUN, J. I., WOO, H. N., CHO, D. H., CHOI, B., LEE, H., KIM, J. H., MIZUSHIMA, N., OSHUMI, Y. & JUNG, Y. K. 2005. Essential roles of Atg5 and FADD in autophagic cell death: dissection of autophagic cell death into vacuole formation and cell death. *J Biol Chem,* 280**,** 20722-9.

QU, X., YU, J., BHAGAT, G., FURUYA, N., HIBSHOOSH, H., TROXEL, A., ROSEN, J., ESKELINEN, E. L., MIZUSHIMA, N., OHSUMI, Y., CATTORETTI, G. & LEVINE, B. 2003. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *J Clin Invest,* 112**,** 1809-20.

RAMIREZ, M., WEK, R. C. & HINNEBUSCH, A. G. 1991. Ribosome association of GCN2 protein kinase, a translational activator of the GCN4 gene of Saccharomyces cerevisiae. *Mol Cell Biol,* 11**,** 3027-36.

RAMPINO, N., YAMAMOTO, H., IONOV, Y., LI, Y., SAWAI, H., REED, J. C. & PERUCHO, M. 1997. Somatic frameshift mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype. *Science,* 275**,** 967-9.

RAO, S., TORTOLA, L., PERLOT, T., WIRNSBERGER, G., NOVATCHKOVA, M., NITSCH, R., SYKACEK, P., FRANK, L., SCHRAMEK, D., KOMNENOVIC, V., SIGL, V., AUMAYR, K., SCHMAUSS, G., FELLNER, N., HANDSCHUH, S., GLOSMANN, M., PASIERBEK, P., SCHLEDERER, M., RESCH, G. P., MA, Y., YANG, H., POPPER, H., KENNER, L., KROEMER, G. & PENNINGER, J. M. 2014. A dual role for autophagy in a murine model of lung cancer. *Nat Commun,* 5**,** 3056.

REGULA, K. M., ENS, K. & KIRSHENBAUM, L. A. 2002. Inducible expression of BNIP3 provokes mitochondrial defects and hypoxia-mediated cell death of ventricular myocytes. *Circ Res,* 91**,** 226-31.

RICCI, M. S. & ZONG, W. X. 2006. Chemotherapeutic approaches for targeting cell death pathways. *Oncologist,* 11**,** 342-57.

ROUSCHOP, K. M., VAN DEN BEUCKEN, T., DUBOIS, L., NIESSEN, H., BUSSINK, J., SAVELKOULS, K., KEULERS, T., MUJCIC, H., LANDUYT, W., VONCKEN, J. W., LAMBIN, P., VAN DER KOGEL, A. J., KORITZINSKY, M. & WOUTERS, B. G. 2010. The unfolded protein response protects human tumor cells during hypoxia through regulation of the autophagy genes MAP1LC3B and ATG5. *J Clin Invest,* 120**,** 127-41.

ROY, N., DEVERAUX, Q. L., TAKAHASHI, R., SALVESEN, G. S. & REED, J. C. 1997. The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. *EMBO J,* 16**,** 6914-25.

RUBINSZTEIN, D. C., GESTWICKI, J. E., MURPHY, L. O. & KLIONSKY, D. J. 2007. Potential therapeutic applications of autophagy. *Nat Rev Drug Discov,* 6**,** 304-12.

RUIZ-VELA, A., OPFERMAN, J. T., CHENG, E. H. & KORSMEYER, S. J. 2005. Proapoptotic BAX and BAK control multiple initiator caspases. *EMBO Rep,* 6**,** 379-85.

RUTKOWSKI, D. T., ARNOLD, S. M., MILLER, C. N., WU, J., LI, J., GUNNISON, K. M., MORI, K., SADIGHI AKHA, A. A., RADEN, D. & KAUFMAN, R. J. 2006. Adaptation to ER stress is mediated by differential stabilities of pro-survival and pro-apoptotic mRNAs and proteins. *PLoS Biol,* 4**,** e374.

RZYMSKI, T., MILANI, M., PIKE, L., BUFFA, F., MELLOR, H. R., WINCHESTER, L., PIRES, I., HAMMOND, E., RAGOUSSIS, I. & HARRIS, A. L. 2010. Regulation of autophagy by ATF4 in response to severe hypoxia. *Oncogene,* 29**,** 4424-35.

RZYMSKI, T., MILANI, M., SINGLETON, D. C. & HARRIS, A. L. 2009. Role of ATF4 in regulation of autophagy and resistance to drugs and hypoxia. *Cell Cycle,* 8**,** 3838-47.

SAELENS, X., FESTJENS, N., VANDE WALLE, L., VAN GURP, M., VAN LOO, G. & VANDENABEELE, P. 2004. Toxic proteins released from mitochondria in cell death. *Oncogene,* 23**,** 2861-74.

SALAZAR, M., CARRACEDO, A., SALANUEVA, I. J., HERNANDEZ-TIEDRA, S., LORENTE, M., EGIA, A., VAZQUEZ, P., BLAZQUEZ, C., TORRES, S., GARCIA, S., NOWAK, J., FIMIA, G. M., PIACENTINI, M., CECCONI, F., PANDOLFI, P. P., GONZALEZ-FERIA, L., IOVANNA, J. L., GUZMAN, M., BOYA, P. & VELASCO, G. 2009. Cannabinoid action induces autophagy-mediated cell death through stimulation of ER stress in human glioma cells. *J Clin Invest,* 119**,** 1359-72.

SANO, R. & REED, J. C. 2013. ER stress-induced cell death mechanisms. *Biochim Biophys Acta,* 1833**,** 3460-3470.

SCARLATTI, F., MAFFEI, R., BEAU, I., CODOGNO, P. & GHIDONI, R. 2008. Role of non-canonical Beclin 1-independent autophagy in cell death induced by resveratrol in human breast cancer cells. *Cell Death Differ,* 15**,** 1318-29.

SCHEUNER, D., SONG, B., MCEWEN, E., LIU, C., LAYBUTT, R., GILLESPIE, P., SAUNDERS, T., BONNER-WEIR, S. & KAUFMAN, R. J. 2001. Translational control is required for the unfolded protein response and in vivo glucose homeostasis. *Mol Cell,* 7**,** 1165-76.

SHAO, R. G., SHIMIZU, T. & POMMIER, Y. 1996. Brefeldin A is a potent inducer of apoptosis in human cancer cells independently of p53. *Exp Cell Res,* 227**,** 190-6.

SHI, Y., VATTEM, K. M., SOOD, R., AN, J., LIANG, J., STRAMM, L. & WEK, R. C. 1998. Identification and characterization of pancreatic eukaryotic initiation factor 2 alpha-subunit kinase, PEK, involved in translational control. *Mol Cell Biol,* 18**,** 7499-509.

SHIMIZU, S., KANASEKI, T., MIZUSHIMA, N., MIZUTA, T., ARAKAWA-KOBAYASHI, S., THOMPSON, C. B. & TSUJIMOTO, Y. 2004. Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes. *Nat Cell Biol,* 6**,** 1221-8.

SHIN, S., BUEL, G. R., WOLGAMOTT, L., PLAS, D. R., ASARA, J. M., BLENIS, J. & YOON, S. O. 2015. ERK2 Mediates Metabolic Stress Response to Regulate Cell Fate. *Mol Cell,* 59**,** 382-98.

SHPILKA, T., WEIDBERG, H., PIETROKOVSKI, S. & ELAZAR, Z. 2011. Atg8: an autophagy-related ubiquitin-like protein family. *Genome Biol,* 12**,** 226.

SIDRAUSKI, C., ACOSTA-ALVEAR, D., KHOUTORSKY, A., VEDANTHAM, P., HEARN, B. R., LI, H., GAMACHE, K., GALLAGHER, C. M., ANG, K. K., WILSON, C., OKREGLAK, V., ASHKENAZI, A., HANN, B., NADER, K., ARKIN, M. R., RENSLO, A. R., SONENBERG, N. & WALTER, P. 2013. Pharmacological brake-release of mRNA translation enhances cognitive memory. *Elife,* 2**,** e00498.

SIDRAUSKI, C., MCGEACHY, A. M., INGOLIA, N. T. & WALTER, P. 2015. The small molecule ISRIB reverses the effects of eIF2alpha phosphorylation on translation and stress granule assembly. *Elife,* 4.

SINHA, S. & LEVINE, B. 2008. The autophagy effector Beclin 1: a novel BH3-only protein. *Oncogene,* 27 Suppl 1**,** S137-48.

SIU, F., BAIN, P. J., LEBLANC-CHAFFIN, R., CHEN, H. & KILBERG, M. S. 2002. ATF4 is a mediator of the nutrient-sensing response pathway that activates the human asparagine synthetase gene. *J Biol Chem,* 277**,** 24120-7.

SLATER, A. F. 1993. Chloroquine: mechanism of drug action and resistance in Plasmodium falciparum. *Pharmacol Ther,* 57**,** 203-35.

SMITH, A. L., ANDREWS, K. L., BECKMANN, H., BELLON, S. F., BELTRAN, P. J., BOOKER, S., CHEN, H., CHUNG, Y. A., D'ANGELO, N. D., DAO, J., DELLAMAGGIORE, K. R., JAECKEL, P., KENDALL, R., LABITZKE, K., LONG, A. M., MATERNA-REICHELT, S., MITCHELL, P., NORMAN, M. H., POWERS, D., ROSE, M., SHAFFER, P. L., WU, M. M. & LIPFORD, J. R. 2015. Discovery of 1H-pyrazol-3(2H)-ones as potent and selective inhibitors of protein kinase R-like endoplasmic reticulum kinase (PERK). *J Med Chem,* 58**,** 1426-41.

SOENGAS, M. S., CAPODIECI, P., POLSKY, D., MORA, J., ESTELLER, M., OPITZ-ARAYA, X., MCCOMBIE, R., HERMAN, J. G., GERALD, W. L., LAZEBNIK, Y. A., CORDON-CARDO, C. & LOWE, S. W. 2001. Inactivation of the apoptosis effector Apaf-1 in malignant melanoma. *Nature,* 409**,** 207-11.

SONG, Y., LI, W., PENG, X., XIE, J., LI, H. & TAN, G. 2017. Inhibition of autophagy results in a reversal of taxol resistance in nasopharyngeal carcinoma by enhancing taxol-induced caspase-dependent apoptosis. *Am J Transl Res,* 9**,** 1934-1942.

SOSNA, J., VOIGT, S., MATHIEU, S., LANGE, A., THON, L., DAVARNIA, P., HERDEGEN, T., LINKERMANN, A., RITTGER, A., CHAN, F. K., KABELITZ, D., SCHUTZE, S. & ADAM, D. 2014. TNF-induced necroptosis and PARP-1-mediated necrosis represent distinct routes to programmed necrotic cell death. *Cell Mol Life Sci,* 71**,** 331-48.

SRINIVASULA, S. M., HEGDE, R., SALEH, A., DATTA, P., SHIOZAKI, E., CHAI, J., LEE, R. A., ROBBINS, P. D., FERNANDES-ALNEMRI, T., SHI, Y. & ALNEMRI, E. S. 2001. A conserved XIAP-interaction motif in caspase-9 and Smac/DIABLO regulates caspase activity and apoptosis. *Nature,* 410**,** 112-6.

STOCKWELL, S. R., PLATT, G., BARRIE, S. E., ZOUMPOULIDOU, G., TE POELE, R. H., AHERNE, G. W., WILSON, S. C., SHELDRAKE, P., MCDONALD, E., VENET, M., SOUDY, C., ELUSTONDO, F., RIGOREAU, L., BLAGG, J., WORKMAN, P., GARRETT, M. D. & MITTNACHT, S. 2012. Mechanism-based screen for G1/S checkpoint activators identifies a selective activator of EIF2AK3/PERK signalling. *PLoS One,* 7**,** e28568.

STRASSER, A., HARRIS, A. W., JACKS, T. & CORY, S. 1994. DNA damage can induce apoptosis in proliferating lymphoid cells via p53-independent mechanisms inhibitable by Bcl-2. *Cell,* 79**,** 329-39.

SU, D. M., ZHANG, Q., WANG, X., HE, P., ZHU, Y. J., ZHAO, J., RENNERT, O. M. & SU, Y. A. 2009. Two types of human malignant melanoma cell lines revealed by expression patterns of mitochondrial and survival-apoptosis genes: implications for malignant melanoma therapy. *Mol Cancer Ther,* 8**,** 1292-304.

SUN, L., LIU, N., LIU, S. S., XIA, W. Y., LIU, M. Y., LI, L. F. & GAO, J. X. 2015. Beclin-1-independent autophagy mediates programmed cancer cell death through interplays with endoplasmic reticulum and/or mitochondria in colbat chloride-induced hypoxia. *Am J Cancer Res,* 5**,** 2626-42.

SUN, L., WANG, H., WANG, Z., HE, S., CHEN, S., LIAO, D., WANG, L., YAN, J., LIU, W., LEI, X. & WANG, X. 2012. Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. *Cell,* 148**,** 213-27.

SURAWEERA, A., MUNCH, C., HANSSUM, A. & BERTOLOTTI, A. 2012. Failure of amino acid homeostasis causes cell death following proteasome inhibition. *Mol Cell,* 48**,** 242-53.

SUSIN, S. A., LORENZO, H. K., ZAMZAMI, N., MARZO, I., SNOW, B. E., BROTHERS, G. M., MANGION, J., JACOTOT, E., COSTANTINI, P., LOEFFLER, M., LAROCHETTE, N., GOODLETT, D. R., AEBERSOLD, R., SIDEROVSKI, D. P., PENNINGER, J. M. & KROEMER, G. 1999. Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature,* 397**,** 441-6.

SUZUKI, Y., TAKAHASHI-NIKI, K., AKAGI, T., HASHIKAWA, T. & TAKAHASHI, R. 2004. Mitochondrial protease Omi/HtrA2 enhances caspase activation through multiple pathways. *Cell Death Differ,* 11**,** 208-16.

SZEGEZDI, E., FITZGERALD, U. & SAMALI, A. 2003. Caspase-12 and ER-stress-mediated apoptosis: the story so far. *Ann N Y Acad Sci,* 1010**,** 186-94.

SZEGEZDI, E., LOGUE, S. E., GORMAN, A. M. & SAMALI, A. 2006. Mediators of endoplasmic reticulum stress-induced apoptosis. *EMBO Rep,* 7**,** 880-5.

SZEGEZDI, E., MACDONALD, D. C., NI CHONGHAILE, T., GUPTA, S. & SAMALI, A. 2009. Bcl-2 family on guard at the ER. *Am J Physiol Cell Physiol,* 296**,** C941-53.

TAIT, S. W. & GREEN, D. R. 2010. Mitochondria and cell death: outer membrane permeabilization and beyond. *Nat Rev Mol Cell Biol,* 11**,** 621-32.

TAIT, S. W. & GREEN, D. R. 2013. Mitochondrial regulation of cell death. *Cold Spring Harb Perspect Biol,* 5.

TAKAMURA, A., KOMATSU, M., HARA, T., SAKAMOTO, A., KISHI, C., WAGURI, S., EISHI, Y., HINO, O., TANAKA, K. & MIZUSHIMA, N. 2011. Autophagy-deficient mice develop multiple liver tumors. *Genes Dev,* 25**,** 795-800.

TALLOCZY, Z., JIANG, W., VIRGIN, H. W. T., LEIB, D. A., SCHEUNER, D., KAUFMAN, R. J., ESKELINEN, E. L. & LEVINE, B. 2002. Regulation of starvation- and virus-induced autophagy by the eIF2alpha kinase signaling pathway. *Proc Natl Acad Sci U S A,* 99**,** 190-5.

TANABE, M., IZUMI, H., ISE, T., HIGUCHI, S., YAMORI, T., YASUMOTO, K. & KOHNO, K. 2003. Activating transcription factor 4 increases the cisplatin resistance of human cancer cell lines. *Cancer Res,* 63**,** 8592-5.

TANAKA, T., TSUJIMURA, T., TAKEDA, K., SUGIHARA, A., MAEKAWA, A., TERADA, N., YOSHIDA, N. & AKIRA, S. 1998. Targeted disruption of ATF4 discloses its essential role in the formation of eye lens fibres. *Genes Cells,* 3**,** 801-10.

TANG, Z., TAKAHASHI, Y., CHEN, C., LIU, Y., HE, H., TSOTAKOS, N., SERFASS, J. M., GEBRU, M. T., CHEN, H., YOUNG, M. M. & WANG, H. G. 2017. Atg2A/B deficiency switches cytoprotective autophagy to non-canonical caspase-8 activation and apoptosis. *Cell Death Differ*.

TANIDA, I., MINEMATSU-IKEGUCHI, N., UENO, T. & KOMINAMI, E. 2005. Lysosomal turnover, but not a cellular level, of endogenous LC3 is a marker for autophagy. *Autophagy,* 1**,** 84-91.

TAYLOR, R. C., CULLEN, S. P. & MARTIN, S. J. 2008. Apoptosis: controlled demolition at the cellular level. *Nat Rev Mol Cell Biol,* 9**,** 231-41.

TESKE, B. F., FUSAKIO, M. E., ZHOU, D., SHAN, J., MCCLINTICK, J. N., KILBERG, M. S. & WEK, R. C. 2013. CHOP induces activating transcription factor 5 (ATF5) to trigger apoptosis in response to perturbations in protein homeostasis. *Mol Biol Cell,* 24**,** 2477-90.

TESKE, B. F., WEK, S. A., BUNPO, P., CUNDIFF, J. K., MCCLINTICK, J. N., ANTHONY, T. G. & WEK, R. C. 2011. The eIF2 kinase PERK and the integrated stress response facilitate activation of ATF6 during endoplasmic reticulum stress. *Mol Biol Cell,* 22**,** 4390-405.

TIRASOPHON, W., LEE, K., CALLAGHAN, B., WELIHINDA, A. & KAUFMAN, R. J. 2000. The endoribonuclease activity of mammalian IRE1 autoregulates its mRNA and is required for the unfolded protein response. *Genes Dev,* 14**,** 2725-36.

TORRES, K. & HORWITZ, S. B. 1998. Mechanisms of Taxol-induced cell death are concentration dependent. *Cancer Res,* 58**,** 3620-6.

TORRICELLI, C., SALVADORI, S., VALACCHI, G., SOUCEK, K., SLABAKOVA, E., MUSCETTOLA, M., VOLPI, N. & MAIOLI, E. 2012. Alternative Pathways of Cancer Cell Death by Rottlerin: Apoptosis versus Autophagy. *Evid Based Complement Alternat Med,* 2012**,** 980658.

TRINH, M. A., KAPHZAN, H., WEK, R. C., PIERRE, P., CAVENER, D. R. & KLANN, E. 2012. Brain-specific disruption of the eIF2alpha kinase PERK decreases ATF4 expression and impairs behavioral flexibility. *Cell Rep,* 1**,** 676-88.

TSUJIMOTO, Y. & SHIMIZU, S. 2005. Another way to die: autophagic programmed cell death. *Cell Death Differ,* 12 Suppl 2**,** 1528-34.

ULLMAN, E., FAN, Y., STAWOWCZYK, M., CHEN, H. M., YUE, Z. & ZONG, W. X. 2008. Autophagy promotes necrosis in apoptosis-deficient cells in response to ER stress. *Cell Death Differ,* 15**,** 422-5.

URANO, F., WANG, X., BERTOLOTTI, A., ZHANG, Y., CHUNG, P., HARDING, H. P. & RON, D. 2000. Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science,* 287**,** 664-6.

VALENTIM, L., LAURENCE, K. M., TOWNSEND, P. A., CARROLL, C. J., SOOND, S., SCARABELLI, T. M., KNIGHT, R. A., LATCHMAN, D. S. & STEPHANOU, A. 2006. Urocortin inhibits Beclin1-mediated autophagic cell death in cardiac myocytes exposed to ischaemia/reperfusion injury. *J Mol Cell Cardiol,* 40**,** 846-52.

VAN HUIZEN, R., MARTINDALE, J. L., GOROSPE, M. & HOLBROOK, N. J. 2003. P58IPK, a novel endoplasmic reticulum stress-inducible protein and potential negative regulator of eIF2alpha signaling. *J Biol Chem,* 278**,** 15558-64.

VANDE VELDE, C., CIZEAU, J., DUBIK, D., ALIMONTI, J., BROWN, T., ISRAELS, S., HAKEM, R. & GREENBERG, A. H. 2000. BNIP3 and genetic control of necrosis-like cell death through the mitochondrial permeability transition pore. *Mol Cell Biol,* 20**,** 5454-68.

VANDENABEELE, P., GALLUZZI, L., VANDEN BERGHE, T. & KROEMER, G. 2010. Molecular mechanisms of necroptosis: an ordered cellular explosion. *Nat Rev Mol Cell Biol,* 11**,** 700-14.

VATTEM, K. M. & WEK, R. C. 2004. Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells. *Proc Natl Acad Sci U S A,* 101**,** 11269-74.

VUCIC, V., ISENOVIC, E. R., ADZIC, M., RUZDIJIC, S. & RADOJCIC, M. B. 2006. Effects of gamma-radiation on cell growth, cycle arrest, death, and superoxide dismutase expression by DU 145 human prostate cancer cells. *Braz J Med Biol Res,* 39**,** 227-36.

WAJANT, H. 2002. The Fas signaling pathway: more than a paradigm. *Science,* 296**,** 1635-6.

WALTER, P. & RON, D. 2011. The unfolded protein response: from stress pathway to homeostatic regulation. *Science,* 334**,** 1081-6.

WANG, C., HUANG, Z., DU, Y., CHENG, Y., CHEN, S. & GUO, F. 2010. ATF4 regulates lipid metabolism and thermogenesis. *Cell Res,* 20**,** 174-84.

WANG, C., XIA, T., DU, Y., MENG, Q., LI, H., LIU, B., CHEN, S. & GUO, F. 2013. Effects of ATF4 on PGC1alpha expression in brown adipose tissue and metabolic responses to cold stress. *Metabolism,* 62**,** 282-9.

WANG, C. & YOULE, R. J. 2009. The role of mitochondria in apoptosis*. *Annu Rev Genet,* 43**,** 95-118.

WANG, J. & LENARDO, M. J. 2000. Roles of caspases in apoptosis, development, and cytokine maturation revealed by homozygous gene deficiencies. *J Cell Sci,* 113 (Pt 5)**,** 753-7.

WEAVER, B. A. 2014. How Taxol/paclitaxel kills cancer cells. *Mol Biol Cell,* 25**,** 2677-81.

WEI, M. C., ZONG, W. X., CHENG, E. H., LINDSTEN, T., PANOUTSAKOPOULOU, V., ROSS, A. J., ROTH, K. A., MACGREGOR, G. R., THOMPSON, C. B. & KORSMEYER, S. J. 2001. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science,* 292**,** 727-30.

WEI, W., COELHO, C. M., LI, X., MAREK, R., YAN, S., ANDERSON, S., MEYERS, D., MUKHERJEE, C., SBARDELLA, G., CASTELLANO, S., MILITE, C., ROTILI, D., MAI, A., COLE, P. A., SAH, P., KOBOR, M. S. & BREDY, T. W. 2012. p300/CBP-associated factor selectively regulates the extinction of conditioned fear. *J Neurosci,* 32**,** 11930-41.

WEI, Y., PATTINGRE, S., SINHA, S., BASSIK, M. & LEVINE, B. 2008. JNK1-mediated phosphorylation of Bcl-2 regulates starvation-induced autophagy. *Mol Cell,* 30**,** 678-88.

WEK, R. C., JIANG, H. Y. & ANTHONY, T. G. 2006. Coping with stress: eIF2 kinases and translational control. *Biochem Soc Trans,* 34**,** 7-11.

WELLEN, K. E. & THOMPSON, C. B. 2010. Cellular metabolic stress: considering how cells respond to nutrient excess. *Mol Cell,* 40**,** 323-32.

WESSELBORG, S., ENGELS, I. H., ROSSMANN, E., LOS, M. & SCHULZE-OSTHOFF, K. 1999. Anticancer drugs induce caspase-8/FLICE activation and apoptosis in the absence of CD95 receptor/ligand interaction. *Blood,* 93**,** 3053-63.

WHITE, E. & DIPAOLA, R. S. 2009. The double-edged sword of autophagy modulation in cancer. *Clin Cancer Res,* 15**,** 5308-16.
WLODKOWIC, D., SKOMMER, J. & PELKONEN, J. 2007. Brefeldin A triggers apoptosis associated with mitochondrial breach and enhances HA14-1- and anti-Fas-mediated cell killing in follicular lymphoma cells. *Leuk Res,* 31**,** 1687-700.
WONG, C. C., QIAN, Y. & YU, J. 2017. Interplay between epigenetics and metabolism in oncogenesis: mechanisms and therapeutic approaches. *Oncogene,* 36**,** 3359-3374.
WONG, R. S. 2011. Apoptosis in cancer: from pathogenesis to treatment. *J Exp Clin Cancer Res,* 30**,** 87.
WOODS, D. & TURCHI, J. J. 2013. Chemotherapy induced DNA damage response: convergence of drugs and pathways. *Cancer Biol Ther,* 14**,** 379-89.
WU, C. A., HUANG, D. Y. & LIN, W. W. 2014. Beclin-1-independent autophagy positively regulates internal ribosomal entry site-dependent translation of hypoxia-inducible factor 1alpha under nutrient deprivation. *Oncotarget,* 5**,** 7525-39.
XIAO, H., VERDIER-PINARD, P., FERNANDEZ-FUENTES, N., BURD, B., ANGELETTI, R., FISER, A., HORWITZ, S. B. & ORR, G. A. 2006. Insights into the mechanism of microtubule stabilization by Taxol. *Proc Natl Acad Sci U S A,* 103**,** 10166-73.
XIE, X., WHITE, E. P. & MEHNERT, J. M. 2013. Coordinate autophagy and mTOR pathway inhibition enhances cell death in melanoma. *PLoS One,* 8**,** e55096.
YAMAMOTO, K., ICHIJO, H. & KORSMEYER, S. J. 1999. BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G(2)/M. *Mol Cell Biol,* 19**,** 8469-78.
YAMAMOTO, K., SATO, T., MATSUI, T., SATO, M., OKADA, T., YOSHIDA, H., HARADA, A. & MORI, K. 2007. Transcriptional induction of mammalian ER quality control proteins is mediated by single or combined action of ATF6alpha and XBP1. *Dev Cell,* 13**,** 365-76.
YANG, Q. H. & DU, C. 2004. Smac/DIABLO selectively reduces the levels of c-IAP1 and c-IAP2 but not that of XIAP and livin in HeLa cells. *J Biol Chem,* 279**,** 16963-70.
YANG, S., WANG, X., CONTINO, G., LIESA, M., SAHIN, E., YING, H., BAUSE, A., LI, Y., STOMMEL, J. M., DELL'ANTONIO, G., MAUTNER, J., TONON, G., HAIGIS, M., SHIRIHAI, O. S., DOGLIONI, C., BARDEESY, N. & KIMMELMAN, A. C. 2011. Pancreatic cancers require autophagy for tumor growth. *Genes Dev,* 25**,** 717-29.
YANG, X., MATSUDA, K., BIALEK, P., JACQUOT, S., MASUOKA, H. C., SCHINKE, T., LI, L., BRANCORSINI, S., SASSONE-CORSI, P., TOWNES, T. M., HANAUER, A. & KARSENTY, G. 2004. ATF4 is a substrate of RSK2 and an essential regulator of osteoblast biology; implication for Coffin-Lowry Syndrome. *Cell,* 117**,** 387-98.
YANG, Z. & KLIONSKY, D. J. 2010. Mammalian autophagy: core molecular machinery and signaling regulation. *Curr Opin Cell Biol,* 22**,** 124-31.
YE, J., KUMANOVA, M., HART, L. S., SLOANE, K., ZHANG, H., DE PANIS, D. N., BOBROVNIKOVA-MARJON, E., DIEHL, J. A., RON, D. & KOUMENIS, C. 2010. The GCN2-ATF4 pathway is critical for tumour cell survival and proliferation in response to nutrient deprivation. *EMBO J,* 29**,** 2082-96.
YEUNG, T. K., GERMOND, C., CHEN, X. & WANG, Z. 1999. The mode of action of taxol: apoptosis at low concentration and necrosis at high concentration. *Biochem Biophys Res Commun,* 263**,** 398-404.
YORIMITSU, T., NAIR, U., YANG, Z. & KLIONSKY, D. J. 2006. Endoplasmic reticulum stress triggers autophagy. *J Biol Chem,* 281**,** 30299-304.
YOUNG, M. M., TAKAHASHI, Y., KHAN, O., PARK, S., HORI, T., YUN, J., SHARMA, A. K., AMIN, S., HU, C. D., ZHANG, J., KESTER, M. & WANG, H. G. 2012. Autophagosomal membrane

serves as platform for intracellular death-inducing signaling complex (iDISC)-mediated caspase-8 activation and apoptosis. *J Biol Chem,* 287**,** 12455-68.

YOUNG, S. K., PALAM, L. R., WU, C., SACHS, M. S. & WEK, R. C. 2016. Ribosome Elongation Stall Directs Gene-specific Translation in the Integrated Stress Response. *J Biol Chem,* 291**,** 6546-58.

YU, L., ALVA, A., SU, H., DUTT, P., FREUNDT, E., WELSH, S., BAEHRECKE, E. H. & LENARDO, M. J. 2004. Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8. *Science,* 304**,** 1500-2.

YUAN, J. & KROEMER, G. 2010. Alternative cell death mechanisms in development and beyond. *Genes Dev,* 24**,** 2592-602.

YVON, A. M., WADSWORTH, P. & JORDAN, M. A. 1999. Taxol suppresses dynamics of individual microtubules in living human tumor cells. *Mol Biol Cell,* 10**,** 947-59.

ZARGARIAN, S., SHLOMOVITZ, I., ERLICH, Z., HOURIZADEH, A., OFIR-BIRIN, Y., CROKER, B. A., REGEV-RUDZKI, N., EDRY-BOTZER, L. & GERLIC, M. 2017. Phosphatidylserine externalization, "necroptotic bodies" release, and phagocytosis during necroptosis. *PLoS Biol,* 15**,** e2002711.

ZHANG, S. H. & HUANG, Q. 2013. Etoposide induces apoptosis via the mitochondrial- and caspase-dependent pathways and in non-cancer stem cells in Panc-1 pancreatic cancer cells. *Oncol Rep,* 30**,** 2765-70.

ZHIVOTOVSKY, B. 2003. Caspases: the enzymes of death. *Essays Biochem,* 39**,** 25-40.

ZHOU, L., YUAN, R. & SERGGIO, L. 2003. Molecular mechanisms of irradiation-induced apoptosis. *Front Biosci,* 8**,** d9-19.

ZHU, J. H., HORBINSKI, C., GUO, F., WATKINS, S., UCHIYAMA, Y. & CHU, C. T. 2007. Regulation of autophagy by extracellular signal-regulated protein kinases during 1-methyl-4-phenylpyridinium-induced cell death. *Am J Pathol,* 170**,** 75-86.

ZHU, Y., ZHAO, L., LIU, L., GAO, P., TIAN, W., WANG, X., JIN, H., XU, H. & CHEN, Q. 2010. Beclin 1 cleavage by caspase-3 inactivates autophagy and promotes apoptosis. *Protein Cell,* 1**,** 468-77.

ZINSZNER, H., KURODA, M., WANG, X., BATCHVAROVA, N., LIGHTFOOT, R. T., REMOTTI, H., STEVENS, J. L. & RON, D. 1998. CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. *Genes Dev,* 12**,** 982-95.

ZOU, W., YUE, P., KHURI, F. R. & SUN, S. Y. 2008. Coupling of endoplasmic reticulum stress to CDDO-Me-induced up-regulation of death receptor 5 via a CHOP-dependent mechanism involving JNK activation. *Cancer Res,* 68**,** 7484-92.