Investigation of IRE1/XBP1s pathway and its potential as a therapeutic target in breast cancer

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Doctor of Philosophy

By

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Communications arising from this work

Research Articles

- **Cleary P[1],** Saveljeva S[1], Logue SE[1], Abiodun A, Patterson JB, Samali A, Endoplasmic Reticulum Stress mediated induction of Sestrin 2 potentiates cell survival, Oncotarget. (manuscript submitted)

- **Cleary P**, Logue SE, McGrath E, Mnich K, Patterson JB, Samali A, IRE1/XBP1s signaling axis drives proliferation of breast cancer cells by regulating production/release of extracellular factors. Target journal; Genes and Development (manuscript in preparation)

Review Articles

- Logue, SE, **Cleary P**, Saveljeva S, Samali A, New Directions in ER stress-induced cell death – Apoptosis. 2013 May;18(5):537-46

- Logue SE, Gorman AM, **Cleary P**, Keogh NA, Samali A (2013) Current Concepts in ER Stress-Induced Apoptosis. J Carcinogene Mutagene S6:002. doi: 10.4172/2161-1149.S6-002

Oral Presentations

- Belspoe Symposium, Leuven, Belgium , June 2013: Delivered a presentation on my PhD work entitled: "Role of XBP1 in Breast Cancer development and resistance to therapy"

- ER Stress Symposium, Laboratory of Cell Death Research & Therapy, Leuven, Belgium , April 2012: Gave a presentation on my work titled: "Role of XBP1 in Breast Cancer development and resistance to therapy"

Poster Presentations

- IACR conference 2014; Presented a poster entitled: Investigation of XBP1s signalling and its potential as a therapeutic target in Breast Cancer, **Patricia Cleary**, Susan E. Logue, Afshin Samali

- IACR conference 2013; Presented a poster entitled: Investigation of XBP1s signalling and its potential as a therapeutic target in Breast Cancer, **Patricia Cleary**, Susan E. Logue, Afshin Samali

Abstract

X-Box binding protein 1 (XBP1) is an integral component of the unfolded protein response (UPR), a pro-survival mechanism, triggered by the onset of endoplasmic reticulum stress. Activation of the endoplasmic reticulum localized stress sensor IRE1α removes a 26 nucleotide intron from XBP1 mRNA generating the active transcription factor XBP1 spliced (XBP1s). Signalling via this arm of the UPR is generally thought to be pro-survival and represents a mechanism through which cells can endure stressful conditions. High levels of XBP1s are linked to breast cancer, with overexpression reported across a range of subtypes where it correlated with a worse patient outcome. In this study routinely used breast cancer cell lines (MCF-7, T47D, SKBR3, MDA-MB231) were found to have basal protein expression of XBP1s. To determine the relevance of basal XBP1s levels a novel chemical inhibitor of IRE1 was employed which specifically inhibits IRE1 ribonuclease activity and therefore block XBP1 splicing. Addition of the IRE1 inhibitor efficiently reduced basal levels of XBP1s in all cell lines tested. Prolonged treatment with the IRE1 inhibitor significantly reduced cell proliferation in breast cancer cells and induced cell death under reduced serum conditions. Interesting, cell death was found to be mediated through modulation of extracellular factors. Moreover, screening of commonly used anti-cancer drugs revealed that IRE1 inhibition could enhance the cytotoxicity of commonly used anti-cancer drugs. In parallel to this study a novel downstream target of IRE1/XBP1s was identified; Sestrin 2. Sestrin 2 knockdown highlighted the pro-survival effects of Sestrin 2 during treatment of breast cancer cells with Methotrexate and Bortezomib, presenting a new therapeutic target in breast cancer treatment.

In summary, this thesis investigates the role of IRE1/XBP1s signalling in breast cancer and demonstrates the clinical potential of IRE1 inhibition.

Abbreviations

ACD	Alpha-crystallin domain
AIF	Apoptosis-inducing factor
APAF-1	Apoptotic peptidase activating factor 1
ARE	Antioxidant response element
ASK1	Apoptosis signal-regulation kinase 1
ATF3	Activating transcription factor 3
ATF4	Activated transcription factor 4
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
BAD	BCL-2-antagonist of cell death
BAK	BCL-2 homologous antagonist/killer
BAX	BCL-2 associated protein X
BCL-2	B-cell lymphoma 2
BCL-xL	B-cell lymphoma 2- extra large
BID	BH3-interacting domain death agonist
BIM	BCL-2-interacting mediator of cell death
BOK	BCL-2-related ovarian killer
BOP	BH3 only protein
B-zip	basic-leucine zipper
CAD	Caspase-activated DNase
CARD	Caspase recruitment domain
CASPASES	Cysteine-dependent aspartate-directed proteases
c-FLIP	Cellular FLICE inhibitory protein
CHOP	C/EBP-homologous protein
cIAP	Cellular inhibitors of apoptosis
CO	Carbon monoxide
CSR	Cell stress responses
DD	Death domain
DED	Death effector domain

DISC	Death inducing signalling complex
DNA	Deoxyribonucleic acid
EDEM1	ER degradation enhancer, mannosidase alpha-like 1
eIF2α	Eukaryotic translation initiation factor 2 α
ER	Endoplasmic Reticulum
ERAD	ER-associated degradation
ERdj4/ HSP40	Heat shock protein 40
ERK	Extracellular signal-regulated kinase
ERO-1	ER oxidoreductin 1
ERP72	Endoplasmic reticulum resident protein 72
ERSE I/ II	ER Stress response Element I/ II
GADD34	Growth Arrest/DNA Damage Inducible 34
HSP	Heat shock protein
HSR	Heat shock responce
ICAD	Inhibitor of caspase-activated DNase
IKK	Inhibitor of NFkB kinase
IRE1	Inositol requiring enzyme-1
JNK	c-JUN terminal kinase
KEAP1	Kelch-like ECH-associated protein
MAM's	Mitochondria-associated ER membranes
MCL-1	Myeloid cell leukemia sequence 1
MEF	Mouse embryonic fibroblast
miRNA	microRNA
MOMP	Mitochondrial outer membrane permeabilization
NF-Y	Nuclear transcription factor Y
NRF2	Nuclear factor (erythroid-derived 2)-like 2
p58(IPK)	p58 inhibitor of protein kinase
PARP	Poly (ADP-ribose) polymerase
PCR	Polymerase chain reaction
PDI	Protein disulphide isomerases
PERK	Double-stranded RNA-activated protein kinase (PKR)-like kinase

PKR	Protein kinase R
PN	Proteostasis network
PUMA	p53 up-regulated modulator of apoptosis
PS	Phosphotidylserine
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
S1	Site-1 protease
S2	Site-2 protease
SERCA	Sarco/Endoplasmic Reticulum Ca^{2+}-ATPase
siRNA	small interfering RNA
Smac/DIABLO	Second mitochondria-derived activator of CASPASES (Smac)/direct IAP binding protein with low pI
TNF	Tumour necrosis factor
TRAF2	TNF-receptor associated factor 2
UPR	Unfolded protein response
UPRE	Unfolded Protein Response Element
XBP1u	X-box binding protein unspliced
XBP1s	X-box binding protein spliced
XIAP	X-linked inhibitor of apoptosis protein
$\Delta\psi_m$	Mitochondrial Membrane Potent

Chapter 1 Introduction

1.1 Endoplasmic Reticulum (ER)

The endoplasmic reticulum (ER) is a membrane bound organelle of eukaryotic cells**,** whose main function is to govern the correct folding and synthesis of secreted and transmembrane proteins. It is the largest organelle in most cell types and typically comprises of a network of tubules, sacs, and flattened cisternae extending from the phospholipid membrane of the nuclear envelope (1, 2). The peripheral phospholipid sheets can associate with ribosomes on the cytosolic face of the ER membrane, termed the rough ER, or they can remain a smooth complex network, termed the smooth ER. The overall morphology and organization of the ER can vary depending on the specific needs and function of the cell; typically the smooth ER governs drug detoxification, fatty acid, steroid biosynthesis and calcium storage, while the majority of protein folding and post translational modifications occurs in the rough ER (1, 3).

The ER is the start site of proteins entering the secretory pathway (4). This pathway begins with the synthesis of proteins at ribosomes which are often associated with the ER outer membrane. A signal sequence at the N terminus of nascent polypeptides is required for their translocation into the ER. This translocation typically occurs via a signal recognition particle (SRP) receptor and Sec61 translocon (5). Once in the ER the polypeptide is folded into its near native structure, chaperones and folding enzymes aid correct folding and shield the polypeptide from interaction with other polypeptides (reviewed in (6)). Unlike cytosolic protein folding, proteins within the ER fold into near native monomers before assembling into oligomers (7). Some membrane bound proteins remain attached to the inner membrane of the ER reducing their movement in order to facilitate oligomer assembly. However, while this may aid protein folding it is not paramount, as a study in 2001 showed that proteins without their transmembrane domain or glycosylphosphatidylinositol anchor (GPI) have a similar folding capacity (8). Transmembrane domains or GPI are crucial for protein association with lipid bilayers. Transmembrane domains which are hydrophobic polypeptides; GPI are

complex gylcolipids covalently attached to over 100 eukaryotic cell surface proteins providing stable association with membranes (9).

A series of post translational modifications such as disulphide bridge formation, glycosylation, signal peptide cleavage, and prolylhydroxylation are necessary for correct protein function. The ER's oxidative environment, alongside Ca^{2+} and ATP dependent molecular chaperones, ensures that correct protein folding and trafficking occurs within the cell (6). Evidence of folding factors that solely work within the ER, and not in other cellular compartments, comes from studies showing that proteins lose their malleability, and become insensitive to unfolding treatments once they exit the ER (10-12). Folding intermediates that fail to fold correctly, or whose folding is delayed can be retrotransolated into the cytosol and ubiquitinated before undergoing proteasomal degradation. Other folding intermediates can persist in the ER, forming protein aggregates. These aggregates can often sequester folding factors and chaperones. Autophagy is often necessary for the removal of such aggregates. Correctly folded proteins can exit the ER via cargo receptors and transport vesicles that transport the cargo to an ER/Golgi intermediate before finally entering the Golgi where final protein folding occurs (6). To facilitate this intricate process, the ER must strictly control its homeostasis to ensure adequate protein folding occurs, as disruption of this process will affect cellular homeostasis. Alteration of ER homeostasis, followed by the build up of protein aggregates, is termed ER stress.

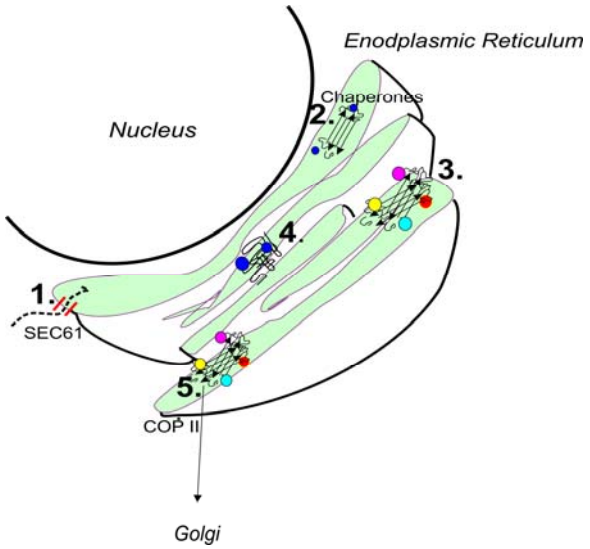


Figure 1.8 Polypeptide pathway through the ER. polypeptide enters the ER via SEC61. **2** Chaperones aid correct folding. **3** Posttranslational modifications are attached.**4** Occasional protein aggregate formation. **5** Fully folded proteins exit to the Golgi

1.1.1 ER Stress

The complexity of the protein folding pathway requires a highly functioning ER and homeostasis to ensure adequate protein folding. Alterations to ER homeostasis which can occur from multiple different sources can affect this folding capacity. Chemical impairment of ER functions through compounds such as glycosylation inhibitors (e.g., Tunicamycin), vesicle transport inhibitors (e.g., Brefeldin A), or compounds which alter luminal Ca^{2+} storage (e.g., Thapsigargin) can decrease the protein folding capacity of the ER and lead to build up of misfolded proteins (13). Loss of ER homeostasis can also be caused by a range of pathophysiological stresses such as hypoxia, oxidative stress, and nutrient deprivation; conditions often associated with tumour microenvironments. These conditions can occur as the tumour's ability to maintain nutrient supply becomes inadequate to sustain growth (14). Several other disease states including Alzheimer's disease, Cystic Fibrosis, and Parkinson's disease are caused by mutations to genes involved in the protein folding pathway, which impair proper folding, resulting in protein aggregation within the ER (15, 16). These perturbations can be morphologically observed by expansion of the ER membrane or by inclusion bodies consisting of aggregated proteins. However, ER stress is not limited to disease states and can also be observed under normal physiological conditions. In highly secretory cell types, such as insulin secreting pancreatic β cells, the ER must adapt to cope with the elevated protein folding requirements. The cell responds to ER stress through removal of terminally misfolded proteins via ER associated degradation (ERAD) and activation of the unfolded protein response (UPR)(17).

1.1.1.1 ER associated degradation

ERAD is the process by which terminally misfolded proteins are removed from the lumen of the ER for degradation in the cytosol. As such, it also plays an important role in cellular adaption to ER stress. There are four main steps in the ERAD pathway namely substrate recognition, retrotranslocation, ubiquitination, and proteasomal degradation (18). Targeting of soluble substrates to ERAD occurs via chaperone

mediated selection with chaperones such as GRP78, HSP40 and PDI homologues linked to substrate aggregation prior to retrotranslocation (19, 20). Another method of substrate selection is coupled to N-linked glycosylation; a post-translational modification added to most secretory and membrane bound proteins. Sequential trimming of sugar residues appears to determine protein fate in the ER. Trimming of glucose residues promotes interactions with chaperones such as calnexin and calreticulin which can aid correct folding of the polypeptide (18). On the other hand, mannose residue trimming occurs following unsuccessful folding, thereby targeting the polypeptide to ERAD by ER resident chaperone-like lectins, such as ER mannosidase and the ER-degradation-enhancing a-mannosidase-like proteins (EDEM)(21).

While we are gaining a clearer image of how ERAD substrates are targeted to the ERAD machinery in mammals, our understanding of retrotranslocation in mammals is still evolving. Retrotranslocation has been reported to occur via SEC61(22) and/or DERLIN family members (23). The mechanical force of this elimination is thought to be facilitated by a ubiquitination process. While ubiquitin ligases are numerous in eukaryotic cells, surprisingly few are ER associated (24). In *S. cerevisiae*, two ER ubiquitin ligases Doa10 and Hrd1 are linked to ERAD function. Interestingly, these are thought to be selective based on the location of the misfolded protein. A terminally misfolded domain within the cytoplasmic face of a protein entering/exiting the ER is targeted and ubiquitinated by Doa10 complex while soluble and membrane misfolded domains is the targets of Hrd1 complex. Once ubiquitinated the protein is dissociated from the ER membrane is via a Cdc48 (p97 in mammals) complex and conveyed to the proteasome by accessory factors such as RAD23p and DSK2p (25, 26).

The importance of ERAD to human physiology is evident from the research that links it to over sixty human diseases (27). While further research may be necessary to fully elucidate this pathway, it is clear that ERAD is a vital component of ER function.

1.2 The Unfolded Protein Response (UPR)

The UPR acts to increase the biosynthetic capacity of the ER and counteracts the stress to maintain cellular homeostasis (28). Glucose response protein 78 (GRP78 or BIP, HSPA5) in homeostatic state is bound to three ER transmembrane proteins: Inositol requiring enzyme 1α (IRE1α), PKR-like ER kinase (PERK) and activating transcription factor 6 (ATF6), can dissociate from them due to its higher affinity for unfolded proteins, leading to the activation of the receptors. GRP78 release induces homo-oligomerisation of IRE1α and transautophosphorylation causing activation of its kinase and endoribonuclease domains (29). Similarly, PERK dimerizes and autophosphorylates leading to activation of its kinase domain (29). ATF6 translocates to the Golgi apparatus where it is cleaved by site 1 and site 2 proteases to become active (30). While the UPR is initially a pro-survival response, relieving protein load and aiding adaption to the stress, prolonged or severe stress can cause a switch in response from pro-survival to pro-death, which is typically mediated through the intrinsic apoptosis pathway (31).

1.2.1 PERK

PERK is a type 1 ER transmembrane protein with a cytoplasmic kinase domain and luminal ER stress sensor. Upon accumulation of unfolded proteins in the ER lumen, GRP78 dissociates from the N-terminus which leads to PERK dimerization while resulting in trans-autophosphorylation of its C-terminal (32). PERK-mediated phosphorylation of the alpha subunit of eukaryotic translation initiation factor 2 alpha (eIF2α) at Ser51 leads to cap dependent translational attenuation (32). Although phosphorylation of eIF2α inhibits general translation initiation, it paradoxically increases non-canonical translation of activating transcription factor 4 (ATF4) through a cap-independent process (33). This block in translation gives the cells an opportunity to remove the unfolded proteins from the system without the addition of newly synthesized proteins worsening the burden. The pro-survival effect of this block is clearly evident in $PERK^{-/-}$ MEFs hypersensitivity to ER stress induced cell death (34).

ATF4 is a transcription factor which binds cAMP response elements leading to transcription of numerous genes involved in acid metabolism, redox balance, protein folding, autophagy and apoptosis (35-37). In addition to eIF2α, PERK can also phosphorylate nuclear respiratory factor 2 (NRF2) (38). NRF2 is retained in the cytoplasm through its association with the microtubule-associated protein Kelch-like Ech-associated protein 1 (KEAP1). Upon ER stress, PERK-mediated phosphorylation of NRF2 promotes its dissociation from KEAP1, leading to the nuclear accumulation of NRF2 (38). NRF2 binds to the antioxidant response element to activate transcription of genes encoding detoxifying enzymes. Upregulation of these genes coupled to block in protein translation should reduce the levels of ER stress, however during prolonged or severe ER stress ATF4 can switch to a pro-apoptotic response through upregulation of CHOP.

CHOP also known as growth arrest and DNA damage-inducible gene 153 (GADD153), which is a member of the C/EBP family that heterodimerizes with other members of the C/EBP transcription factor family. This 29 kDa factor is strongly induced in response to ER stress (39). CHOP is a common point of convergence during UPR with ATF4, XBP1 and ATF6 binding sites found within it promoter sequence highlighting its importance in ER stress (34, 40, 41). It has been shown that mouse embryonic fibroblasts derived from *Chop*$^{-/-}$ animals exhibited significantly less cell death when treated with ER-stress inducing agents compared to wild type (39). Pro-apoptotic effects of CHOP are linked to down-regulation of BCL-2 and enhanced production of reactive oxygen species (ROS) thus shifting the balance to a more favourable apoptotic state (42). BH3 only, namely BIM and PUMA, regulation has also been linked to CHOP activity through interaction with FOXO3a in neuronal cells treated with tunicamycin. Additionally, CHOP is linked to increased BIM expression while transcriptionally down-regulating BCL-2 (43). A study by Puthalakath *et al* also highlighted the importance of BIM to ER stress induced cell death, with BIM knockdown attenuating ER stress induced cell death (44). However, CHOP is not the only factor required for BIM induction, post translational modifications via protein phosphatase 2a (PP2a) are

required to elicit its pro-apoptotic effects in these conditions (44, 45). Another transcriptional target of CHOP is endoplasmic reticulum oxidoreductin 1 (ERO1α) (Fig 1.2) which is reported to hyperoxidise the ER environment and also stimulate calcium release from the ER via activation of inositol triphosphate receptor (IP3R) thus pushing the cell towards cell death (46, 47). CHOP can also induce transcription of GADD34 which in turns allows protein phosphatase 1 to dephosphorylate p-eIF2α thus relieving the inhibition on global protein translation stimulating cell death induction (48).

While CHOP appears to be one of the main factors controlling the switch to ER stress induced cell death much yet remains to be understood as to how this switch occurs or what governs the decision making, it is clear that CHOP plays an important role in ER stress induced cell death. Paradoxically, as mentioned *Perk*$^{-/-}$ cells with decreased CHOP levels, remain sensitive to ER stress-induced apoptosis, an observation linked to regulation of NOXA (49). This indicates redundancy in the system with the ability of CHOP-independent cell death mechanisms to mediate ER stress induced cell death (34).

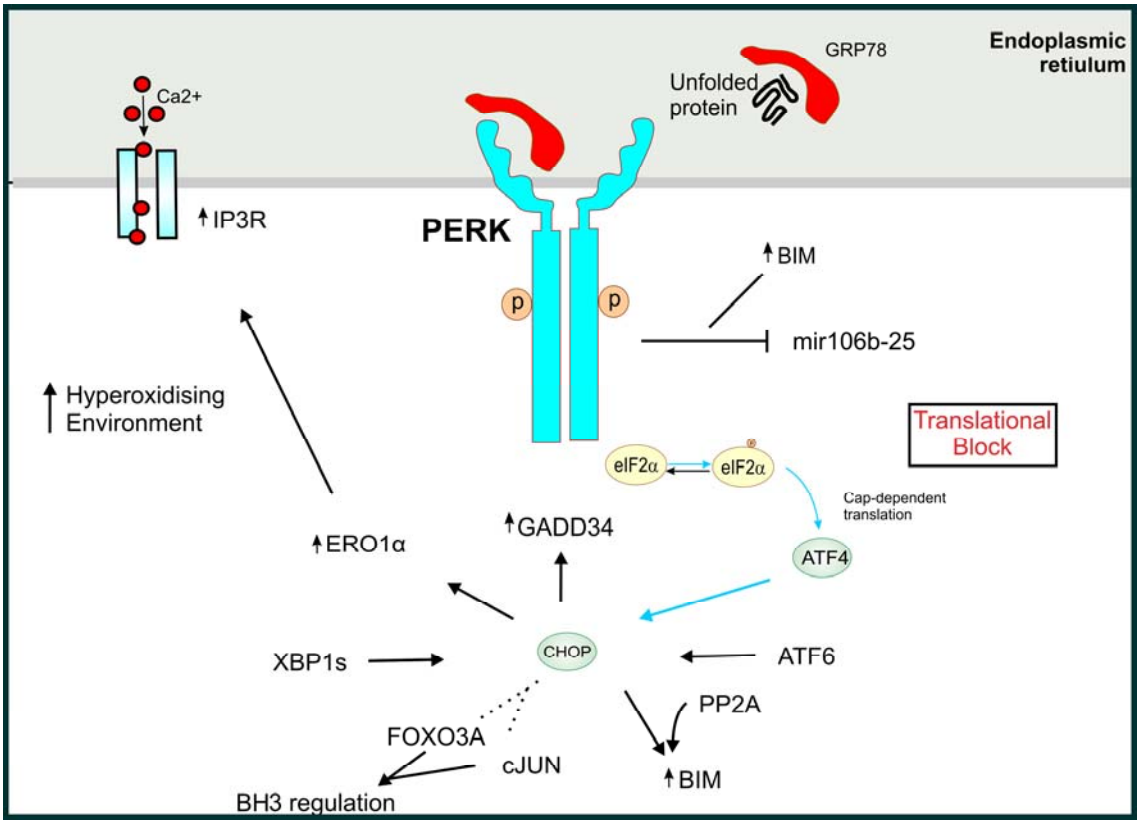


Figure 1.9 PERK Activation. PERK dimerizes and autophosphorylates following Grp78 dissociation. Active PERK mediates its response via phosphorylation of eIF2α leading to translational block and cap independent translation of ATF4. ATF4 induces CHOP which has multiple downstream targets.

1.2.2 ATF6

In mammals, there are two isoforms of ATF6, ATF6α (90 kDa) and ATF6β (110 kDa), both are synthesized in all cell types as ER transmembrane proteins. Owing to an ER targeting hydrophobic sequence ATF6 is tethered to the ER membrane. In unstressed cells, ATF6 is bound to GRP78. In response to ER stress, GRP78 dissociation permits trafficking of ATF6 to the Golgi complex, where ATF6 is sequentially cleaved by two proteases (50). This movement from the ER also depends on the cysteine oxidation status of ATF6, as Nadanaka *et al* showed that only reduced ATF6 monomers are translocated to the Golgi (51). Moreover, protein disulfide isomerase A5 (PDIA5) was

also reported to be necessary for full ATF6 activation during ER stress through disulphide bond rearrangement of ATF6 (52).

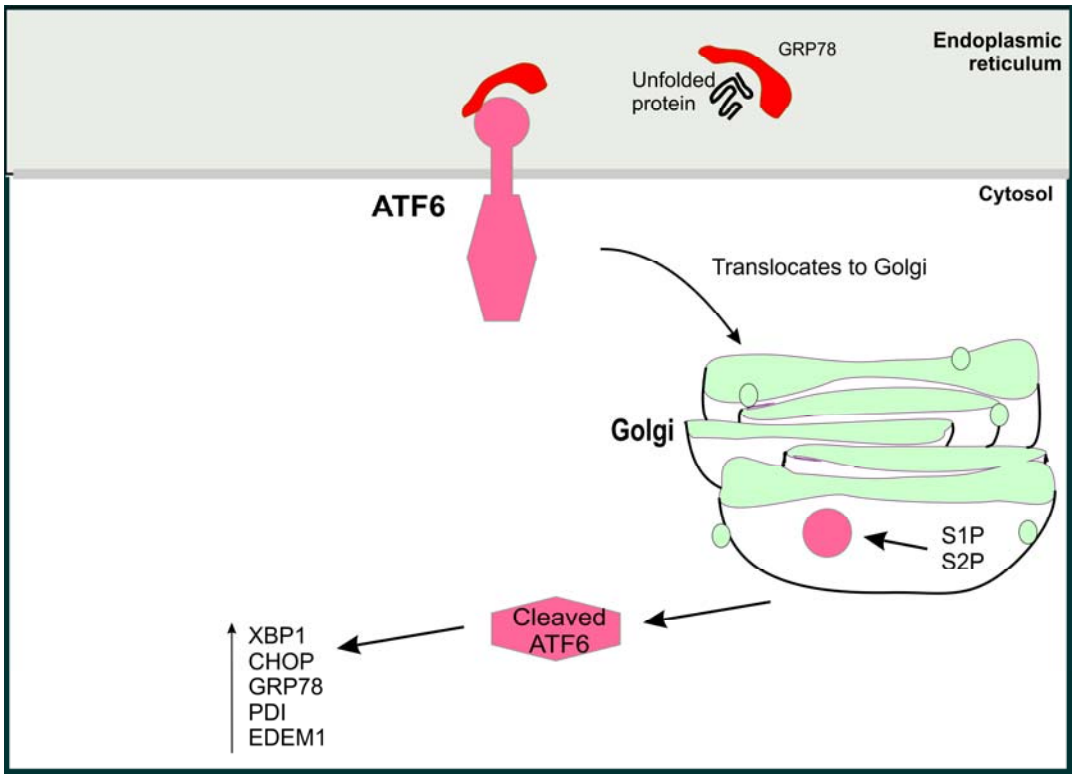


Figure 1.10 ATF6 activation. Following GRP78 dissociation, ATF6 is transported to the Golgi where it is cleaved from its membrane anchor. Little is known about ATF6 regulated pathways but it is involved in the upregulation of UPR associated genes XBP1, CHOP, GRP78, PDI and EDEM1

In the Golgi, site-1 protease cleaves ATF6 in the luminal domain. The N-terminal portion is subsequently cleaved by the site-2 protease as shown in Fig 1.3. Following this processing, ATF6α translocates to the nucleus and binds to the ATF/cAMP response element and ER stress responsive element to activate numerous target genes, many involved in adaption to ER stress. Studies with *Atf6*$^{-/-}$ cells have recently shown that ATF6 is responsible for transcriptional induction of a cohort of ER proteins which

includes chaperones, folding enzymes and ER-associated degradation (ERAD) components (53). ATF6 also increases transcription of XBP1, a target of the IRE1α pathway (54). While predominantly thought to be a pro-survival arm of the UPR, ATF6 has been shown to induce CHOP expression during prolonged ER stress conditions and is linked to downregulation of MCL-1, an anti- apoptotic protein (41, 55). ATF6 remains the most poorly understood UPR arm and more research is needed to fully elucidate its role in ER stress responses.

1.2.3 IRE1

IRE1 (ERN1) is a type I ER transmembrane protein containing a serine/threonine kinase domain and an endoribonuclease domain. IRE1 is conserved from yeast to humans. Two conserved paralogs IRE1α and IRE1β are found in mammals. IRE1α is expressed ubiquitously, whereas the expression of IRE1β is limited to gut epithelial cells (56). GRP78/BIP dissociates from the ER luminal side of IRE1 resulting in conformational changes that leads to IRE1 activation, in yeast these changes can also result after direct binding of unfolded proteins to the luminal domain, a phenomenon not conserved in mammals (57). Crystal analysis of IRE1 in yeast reveals a bilobal structure comprising of a large C terminal lobe and smaller N terminal lobe which is typical for canonical protein kinases (58-60). The luminal domain containing the GRP78 binding domain is tethered via the transmembrane domain to the cytosolic portion which comprised of a linker domain, kinase domain and RNase domain. The RNase domain folds into a kinase extension nuclease (KEN) domain which is structurally uncommon amongst nucleases. During IRE1 activation, the activation domain loop which protrudes from the kinase domain is phosphorylated at residues S841 and T844 leading to complete IRE1 activation (61, 62). Walter's group recently proposed a step-wise activation of IRE1 in yeast, which suggested that the luminal domain but not kinase or RNase domain is indispensable for the oligomerisation of IRE1. Furthermore, they identified the linker region as the targeting and docking site of HAC1, which they postulate can minimize the chance of editing other mRNAs by mistake (62). In the mammalian system XBP1,

the human ortholog of HAC1, is targeted to the ER membrane via a hydrophobic region within the unspliced XBP1 mRNA forming a mRNA- ribosome nascent chain complex (63).

To date XBP1 is considered the main downstream target of IRE1 RNase domain. The activated RNase domain unconventionally splices a 26 nucleotide intron from XBP1 unspliced (XBP1u) mRNA resulting in XBP1 spliced (XBP1s) which is subsequently ligated by HSPC117 (RtcB in worm) (54, 64, 65). This splicing causes a frame shift resulting in the translation of XBP1s protein that now contains a nuclear translocation signal at its C terminus (Fig 1.4). The XBP1u nascent peptide is responsible for directing the mRNA-ribosome nascent chain complex to the ER membrane in order for splicing to occur. It has recently been reported that a conserved peptide module at the C terminus of XBP1u is essential for mRNA translational pausing of XBP1u and is responsible for this targeting effect (66) (The role of XBP1 will be discussed in detail later in the text). While IRE1 RNase activity has been successfully shown to splice XBP1 its activity has recently been linked to the degradation of ER localised mRNA in a process termed IRE1-dependent decay of mRNA (RIDD) (67).

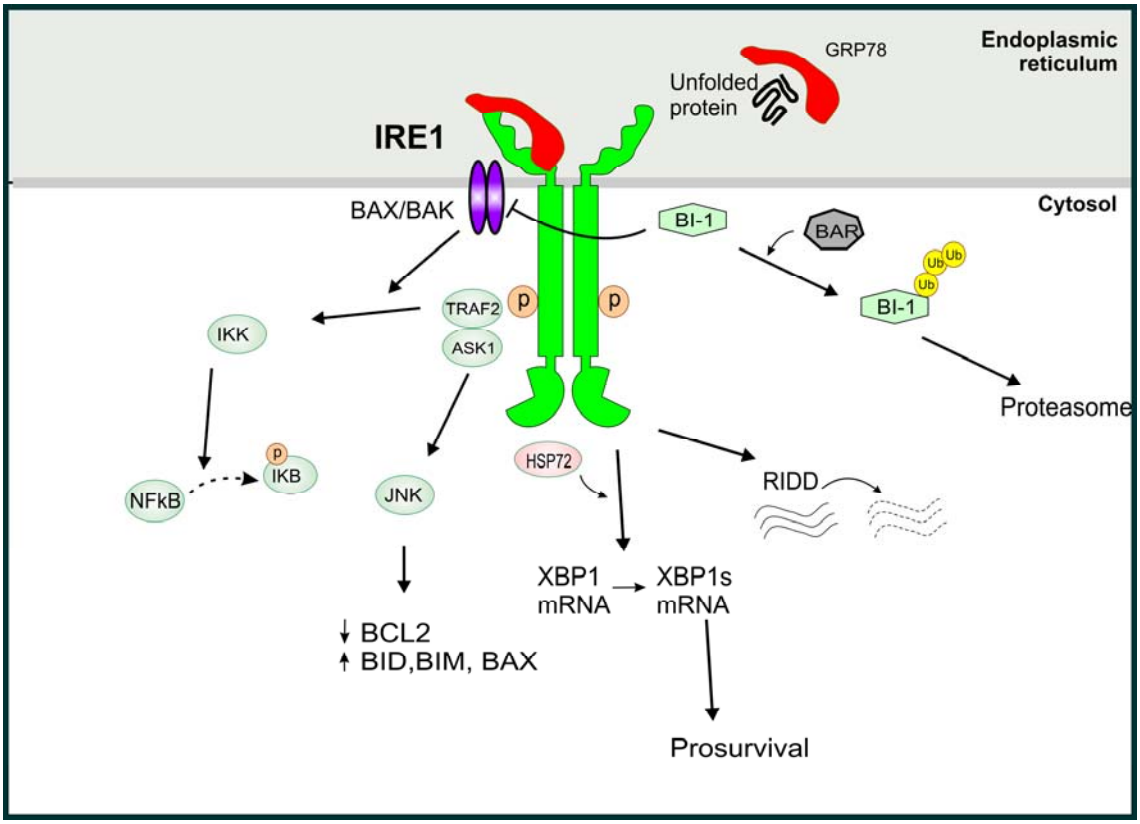


Figure 1.11 IRE1 Activation Following dissociation of Grp78, IRE1α oligomerizes and autophosphorylates facilitating its activation. Active IRE1α induces splicing of XBP1mRNA to XBP1s and also activates JNK via TRAF2 and ASK1. Furthermore, active IRE1a has been linked to downstream NF-κB activation and also RIDD, which can lead to the degradation of pro-survival mRNA.

1.2.3.1 Regulators of IRE1

It is clear that IRE1 plays an important role in adaption to ER stress conditions and while frequently considered a pro-survival arm of the UPR it can also lead to apoptosis via ASK1/JNK activation (see below); therefore regulation of IRE1 activity is important for cell fate. While this regulation can often appear to be cell type specific, our knowledge of the landscape of regulation is still only developing. Here I discuss the possible regulators of IRE1 activity.

Heat shock proteins

HSP90 is reported to bind to the cytoplasmic portion of IRE1 and maintain protein stability. HSP90 inhibition by geldanamycin was able to reduce the protein half-life of IRE1 from >5 h to <1 h (68). Additionally, in 2012 Ota *et al* showed that the kinase specific HSP90 co-chaperone CDC37, can bind to kinase domain of IRE1 and regulate basal suppressing of IRE1 phosphorylation in INS1 cells; a function they report is independent of HSP90's ability to maintain IRE1 stability (69).

HSP72 has also recently been demonstrated to bind to and regulate IRE1a signalling. Our lab reported binding of HSP72 to the cytosolic domain of IRE1α, an interaction which increased the RNase activity of IRE1α resulting in an increase in XBP1 splicing (70)

Self regulation

Randall Kaufmann's group reported that IRE1 can down regulate its own mRNA levels via site specific cleavage in the 5' mRNA (71). Interestingly XBP1 deficient cells exhibit IRE1 RNase hyperactivation, suggesting a negative feedback loop mediated by XBP1smay occur by currently unidentified mechanism (72, 73).

Ubiquitination

Synoviolin is a E3 ubiquitin ligase most associated with its role in ERAD but has also been shown to catalyse the ubiquitination and degradation of IRE1 in synovial fibroblasts (74). A second ERAD associated E3 ubiquitin ligase has also been implemented in IRE1 regulation: Carboxyl terminus of HSC70-interacting protein (CHIP). CHIP was shown to ubiquitinate IRE1 in a K63-linked manner but interestingly was not linked to its degradation, instead CHIP ubiquitintated the kinase domain of IRE1 leading to enhanced ASK1/JNK signalling (75).

Dephosphorylation

Endoplasmic reticulum (ER) membrane targeted protein phosphatase (PP2Ce) was identified as an endogenous IRE1-specific phosphatase that dephosphorylated Ser-724 of IRE1 which lead to down regulated IRE1 activity (76).

RACK1 was reported to interact with IRE1 following ER stress and glucose stimulation in pancreatic β-cells. Following this observation the authors examine formation of IRE1-RACK1-PP2a complex causing dephosphorylation of IRE1 during glucose stimulation. However, the authors observed that during ER stress stimulation PP2a dissociates from RACK1, which they speculate may be to facilitate optimal IRE1 phosphorylation (77).

BCL-2 Family

IRE1 has been functionally linked to BCL-2 family members, work predominantly undertaken by Claudio Hetz and colleagues. A *Science* article published in 2006 reported that *Bax* and *Bak* double knockout mice had reduced XBP1s expression following tunicamycin treatment. They attributed this observation to the ability of BAX and BAK to form a protein complex with the cytosolic domain of IRE1 promoting complete IRE1 activation (78). Later BIM and PUMA, both BH-3 only proteins, were shown to bind to IRE1 and regulate its RNAse activity during conditions of mild ER stress. This involvement may be limited to mild stress as the authors suggest that under more severe conditions BIM and PUMA would switch to their better characterised function and enforce mitochondria mediated apoptosis (79).

Bailly Maitre *et al* observed that *Bax Inhibitor-1* (*BI-1*) null mice were more sensitive to ER stress induced death and had increased IRE1 activity and ATF6 cleavage during ischemic reperfusion (80) and later in models of obesity (81). In 2009, Hetz group explored this observation and showed that BI-1 could directly bind to IRE1 and negatively regulate its RNase activity (82) and also IRE1 mediated JNK activation (83). We now know that BI-1 can itself be regulated during ER stress by bifunctional

apoptosis regulator (BAR) which acts as an ER associated RING type E3 ligase and promotes the proteasomal degradation of BI-1 (84).

ER stress independent activation of IRE1

Poly(ADP-ribose) polymerases 16 (PARP16) overexpression caused a marked increase in globular ER structure. Further analysis in HeLa cells revealed that both PERK and IRE1 required ADP-ribosylation by PARP16 for their activity and this was mediated by increasing the activity of their kinase and endonuclease activity. This paper also demonstrated that PARP16 could activate IRE1 and PERK arms independently from an ER stress stimulus. It was postulated this regulation appeared to work independently from ATF6 due to lack of PARP-16 C-tail binding pocket (85).

A study published in 2010 showed ER stress independent activation of IRE1 during the host immune response. Martinon *et al* reported that TLR2 and TLR4 while inhibiting an overall ER stress response, can selectively trigger IRE1 activation in macrophages. This selective activation required the NADPH oxidase NOX2 and TRAF6. IRE1/XBP1 activity in this situation did not induce a classical downstream ER stress response but rather lead to the upregulation of inflammatory mediators such as TNF , interferon- β and interleukin-6 (IL-6) (86). A recent study reported suggested a feedback loop occurs via IL-6, IRE1 and STAT3. STAT3, which is typically induced by IL-6, binds to the IRE1 cytosolic domain, an interaction they report to be required for full STAT3 phosphorylation. Once phophorylated STAT3 can up regulate a cohort of cytokines including IL-6 (87).

Another example of ER stress independent activation of IRE1 was shown in endothelial cells. Zeng *et al* reported that VEGF signalling through VEGF receptor 2, (also known as kinase insert domain receptor) caused receptor internalization and translocation to the ER and where it interacted directly with the cytoplasmic domain of IRE1 leading to increased XBP1 splicing. This increase in XBP1 splicing was linked to upregulation of endothelial proliferation and angiogenesis (88).

1.2.3.2 IRE1 – JNK activation

The attenuation of IRE1 signalling during prolonged periods of ER stress coupled to the pro-survival effect of XBP1s has suggested that IRE1 is predominantly a pro-survival response (89). However, this is not entirely true as overexpression of IRE1 in HEK293t leads to cell death suggesting that IRE1 may also play a role in apoptotic signalling (90). In fact, Urano and colleagues showed that IRE1 phosphorylation may recruit TRAF2 and lead to JNK activation. JNK has been shown to negatively modulate anti-apoptotic BCL-2/BCL-X_L while conversely enhancing the pro-apoptotic function of BIM and BID (91, 92).

An alternative mode of JNK activation through IRE1 has been proposed through co-localisation of RIP1, TNF Receptor 1 and IRE1 at the endoplasmic membrane which triggers downstream JNK activation (93). JNK activation may be one mechanism by which IRE1 contributes to apoptosis; alternatively studies have suggested that IRE1 activation may lead to TNFα release which acts in an autocrine fashion to induce cell death (94). This pathway can also function through TRAF2 recruitment to IRE1 leading to IKK phosphorylation and subsequent activation. Once activated IKK can in turn phosphorylate IκB causing it to be degraded and release its inhibition of NFκB, thus allowing NFκB activation and upregulation of target genes such as TNFα (94).

1.2.3.3 RIDD

RIDD function was initially identified in *D. Melanogaster,* it has recently been shown to occur in mammalian cells (61, 95). It is reported that RIDD can selectively degrade mRNA encoding secretory proteins thus may initially relieve the load of misfolded proteins within the ER, however prolonged RIDD activity has been linked to an increase in apoptosis (61). It is unknown what might mediate the switch from pro-survival RNase splicing of XBP1 to pro-apoptotic degradation of mRNA for survival proteins. Han *et al* postulates it may be the oligomeric state of IRE1α, as overexpression of an IRE1 kinase derived peptide domain enhances XBP1 activation but conversely

diminishes RIDD and JNK activation (61). As RIDD is a quite a new discovery to the field, its full potential remains to be elucidated.

1.2.4 XBP1

XBP1u is non-conventionally spliced by the IRE1 RNAse domain, which removes a 26 nucleotide sequence resulting in a frameshift and the subsequent translation of a larger XBP1s protein containing a nuclear localisation signal. Structurally XBP1s is a basic leucine zipper protein with motifs including serine/threonine rich region, glutamine rich region, a proline rich region, and an acidic domain.

Acidic domains are regions in which glutamic acid or aspartic acid account for 29% of the amino acids and are a common feature of transcription factors. The acidic domain of XBP1s is located between amino acids 142 and 167. The C-terminal of XBP1s (151-260 amino acids) contains distinct regions: a glutamine rich domain, a serine/threonine rich domain and a glutamine/proline rich domain. This C terminal is the key region required for XBP1s transactivation activity, and contains the nuclear localisation signal (96). The basic region of these proteins (both XBP1u and XBP1s) is similar to many other transcription factors which contain a DNA binding region; however XBP1u is thought to be predominantly an unstable cytoplasmic protein. XBP1s on the other hand is more stable and can bind DNA as a monomer but has also been shown to form a heterodimer with various partners including c-FOS during the transcription of a subset of MHC class II genes (97). Interestingly XBP1s can also dimerise with ATF6α for the transcriptional upregulation of major ERAD components (98).

1.2.4.1 XBP1s Regulation

XBP1 undergoes post translational regulation via acetylation and SUMOylation. SIRT1 (sirtuin1) is a close mammalian homologue to SIR2 (silent information regulator 2), a highly conserved family of histone deactelyases and has been reported to cause

deacetylation of XBP1s. Deacetylation results in a decrease in protein level and repression of transcriptional activity. P300, a histone acetlytransferase, which has already been identified to acetylate ATF2 and ATF4, has been linked to acetylation of XBP1s (99).

SUMOylation has also been identified as a regulatory process of XBP1 activity. SUMO (SENTRIN) is a novel ubiquitin like protein that is catalyzed by activating, conjugating, and ligating enzymes but is removed by a family of SENTRIN/SUMO- specific proteases (100).It is not yet known which enzyme is responsible for XBP1 SUMOylation but a recent paper has shown that de-SUMOylation of XBP1 is by SENP1, and can promote XBP1 transactivation, therefore playing an important role in the expression of XBP1s target genes (100).

1.2.4.2 Function of XBP1

As mentioned XBP1s is predominantly a pro-survival transcription factor with downstream targets aiding protein folding, maturation and degradation thus enabling cell survival under stressful conditions (101). XBP1 is expressed in almost all adult tissues and is essential for embryonic development as knockout studies highlight a fundamental role of XBP1 in cardiac myogenesis, and hepatogenesis which is mediated predominantly via αFP, a possible regulator of hepatocyte growth and three acute phase protein family members (102, 103). Knockout studies further linked the essential role of XBP1 to secretory exocrine gland development, and plasma cell differentiation as XBP1 is the only known transcription factor to be essential for terminal differentiation of B lymphocytes to plasma cells (104, 105). Earlier this year XBP1s was reported to be necessary for eosinophil differentiation (106). This result is intriguing as eosinophils can be linked to tumour burden and survival of memory plasma cells (107, 108). Moreover *Xbp1* knockout mice completely lacked Paneth cells in the intestine and had a diminished level of mucin-secreting goblet cells which resulted in a decrease in the

production of antibacterial peptides, further establishing XBP1's essential role in immunity (72).

XBP1s induces the transcription of various proteins involved in the maintenance of ER homeostasis such as ER chaperones (for example GRP78, ERDJ4, ERDJ5, HEDJ, GRP58 and PDI-P5), ER associated degradation (ERAD) components (EDEM, OS9, HERP, $p58_{IPK}$) components of secretory pathway (SEC23B, SEC24C, SEC61A, SRP54) alongside those involved in glycolsylation, autophagy and transcription factors such as CHOP and XBP1 (101). While located in most adult tissues some downstream targets are activated by XBP1s only in a tissue specific manner (105). Using *Xbp1s* knockout MEFs it was shown that XBP1s alone was specifically essential for expression of DnaJ like accessory proteins Erdj4 and $p58_{IPK}$. DNAJ like proteins regulate HSP70 protein activity via activation of their ATPase activity and by stabilising their interaction with unfolded proteins (109, 110). DNAJ proteins are also linked to regulation of protein transportation and ERAD (111). ERDJ4 is an ER localised type II DNAJ homologue that is positioned as an ER membrane anchor with its J domain facing the ER lumen. From this position it can interact with GRP78 via its J domain and has been shown to increase GRP78 ATPase activity in a concentration dependent manner. ERDJ4 also co-localises with GRP94 in the ER (112).

$p58_{IPk}$ was originally identified as an influenza induced inhibitor of PKR via binding to its kinase domain (113), therefore it is proposed to relieve eIF2α phosphorylation upon ER stress and act in a negative feedback manner to regulate XBP1 that is induced by eIF2α dependent ATF4. $P58_{ipk}$ can also complex with HSP40 with inhibitory consequences that can be relieved upon heat shock (i.e. a form of ER stress). A further link to heat shock response is found with $p58_{IPk}$ ability to interact with HSC70 and regulate its ATPase activity (114).

HEDJ is a human HSP40 chaperone which is found co-localized with calnexin at the endoplasmic reticulum. Its transcription is induced by XBP1s under stressful conditions and mediates haperone-assisted protein folding occurs in the cytosol (115).
RAMP4 is another downstream target of XBP1s transcriptional activity (101). Yamaguchi and colleagues showed that overexpression of RAMP4 during ER stress could limit aggregation and degradation of newly synthesized proteins but also assist in their glycosylation once the stress was removed. RAMP4 was also shown to interact with the molecular chaperone calnexin and subunit of translocon, these findings thus implicates RAMP4s role in membrane protein glycosylation and stability in response to stress (116).

XBP1s can also induce transcription of PDI-P5 which has sequence homology to disulphide isomerases and has substrates specificity to chaperones. EDEM, a type II ER transmembrane protein is linked to ERAD pathway via its ability to facilitate substrate degradation (117). EDEM has been shown to act during the calnexin cycle to remove misfolded glycoproteins, but not correctly folding glycoproteins. Down regulation of this activity prolonged protein folding and delayed ERAD (118). Key players of ER stress such as GRP78 and CHOP are also induced by this transcription factor but studies have shown that these are not solely dependent upon XBP1 but can be also induced by alternative arms of the UPR (101).
At a chromosomal level XBP1 targets have been linked to large scale chromatin remodelling (119), extending the scope of this transcription factor far beyond ER homeostasis alone.

1.2.5 IRE1-XBP1 and Malignancies

XBP1 is linked to numerous malignant conditions here I look at four types of malignancies and what therapeutic potential the regulation of IRE1/XBP1s axis may have.

1.2.5.1 Cancer

Rapid tumour growth generates a distinctive microenvironment conditions which include hypoxia, nutrient deprivation, and oxidative stress; leading to activation of the UPR in various solid tumours (120). The UPR was first suggested to impact oncogenic processes in 2004 (121). Now it is well accepted that the UPR can act as an adaptive mechanism to the microenvironmental changes associated with cancer. In 2007 an analysis of somatic mutations across a range of cancer subtypes identified IRE1 somatic mutations as possible drivers of oncogenic processes (122), later two other groups independently confirmed IRE1 mutations in glioblastoma (123) and hepatocellular carcinomas (124). While the functional effects of these mutations still need to be clarified, the role of IRE1/XBP1 has been linked to numerous other cancer types. For example, XBP1s is reported to be overexpressed in triple negative breast cancer (TNBC) conferring an aggressive phenotype (125). There are indications, that XBP1s may be involved in early stages of tumourigenesis: transformed mouse fibroblasts or human fibrosarcoma cells that are deficient in XBP1 are impaired in their ability to form tumours in SCID mice. This observation is suggested to occur due to the inability of tumours to survive hypoxic conditions in the absence of XBP1s (126). In fact last year XBP1s was shown to be required for a complete hypoxic response through direct binding to HIF1α in TNBC (125).

The role of XBP1 in multiple myeloma has been studied extensively as XBP1 is required for B-cell differentiation into plasma cells and B-cell defence mechanisms (104, 127). Carrasco and colleagues showed that XBP1s overexpression alone in *Eμ-Xbp1s* transgenic mice causes multiple myeloma-like disease (MGUS-MM) with human disease characteristics at molecular, pathological and clinical level (128). Moreover clinical samples displayed elevated XBP1s level when compared to normal plasma cells, suggesting that XBP1 may have a causative role in MM development (128). Recently prostate cancer has been linked to XBP1s as a recent paper identified androgens as selective activators of the IRE/XBP1 arm resulting in a survival and growth advantage. Similarly, in melanoma constitutive activation of IRE1/XBP1 arm

was observed and could attribute a survival advantage (129). It is clear that XBP1s can confer a survival advantage to a range of cancer subtypes and so it is easy to surmise that inhibition of XBP1s may be a therapeutically relevant option across a range of cancer types.

1.2.5.2 Neurodegenerative diseases

Alzheimer's is an incurable neurodegenerative disorder typically characterised by progressive cognitive impairment commonly due to the abnormal accumulation of amyloidβ 1-42 peptide in the brain (Aβ). It is reported that diseased tissues of Alzheimers patients have elevated levels of XBP1 (130). Both drosophila models expressing Aβ and mammalian cells cultured with Aβ oligomers, revealed that XBP1s can elicit a neuroprotective effect. The authors suggest that this protection is mediated via XBP1s down regulation of Ryanodoine Ca^{2+} channels thus reducing the accumulation of free calcium in the system (131).

Parkinsons is a neurodegenerative disorder characterised by loss if dopaminergic neurons in substantia nigra pars compacta of the midbrain. Mitochondria and Ubiquitin/proteasome pathway (UPS) dysfunction have both been linked as possible causes of this condition; one common feature between both is the ability to induce ER stress (132, 133). Research has recently focused on modulating IRE1 as a pro-survival arm of the UPR with a view to reduce loss of these neurons. In fact Sado and colleagues have shown that overexpression of XBP1s demonstrated cytoprotective effects against three different in vitro Parkinson's models (134).

XBP1s is also linked to familial amyotrophic lateral sclerosis (fALS), a disease caused by mutations in superoxide dismutase-1 (SOD1). While increased XBP1s activity appears to be therapeutically beneficial to some neurodegenerative disorders this is not exclusively true as XBP1 deficient cells had increased autophagy resulting in reduced mutant SOD1 aggregation and were overall less likely to develop fALS (135). It is

clear that IRE1/XBP1 arm has potential as a viable therapeutic option in neurodegenerative conditions but whether the benefit lies in increasing or decreasing its activity appears to be condition specific.

1.2.5.3 Inflammatory diseases

XBP1s is linked to the immune system via its role in inflammation and as such it has a potential role in inflammatory diseases such as inflammatory bowel disease IBD (both forms: Crohn's disease and ulcerative colitis). In these inflammatory diseases, abnormalities in XBP1s levels was shown to have a causative effect (72). Dextran sodium sulphate induced mouse model of IBD showed that *IRE1β*$^{-/-}$ models developed colitis 3-5 days earlier than their wildtype counterparts suggesting a potential role of IRE1β in lessening ER deregulation prior to ulceration (136). As previously mentioned XBP1 is also linked to TLR signalling which is required for optimal pro-inflammatory cytokines in macrophages (86). Moreover XBP1 is reported to be essential for the survival and differentiation of dendritic cells (137).

1.2.5.4 Metabolic Diseases

Insulin metabolism has important roles in obesity and type 2 diabetes. Insulin resistance is thought to mainly develop during conditions of cellular stress and inflammation and common point of convergence of these pathways is XBP1s. A key player of insulin metabolism is phosphoinositide 3-kinase (PI3K), XBP1 can interact with the PI3K catalytic subunit P85α resulting in increased XBP1 nuclear translocation (138). Furthermore *Xbp1* deficient mice are reported to develop insulin resistance (139).

1.3 Targeting the UPR

As previously described the UPR has both pro-survival and pro-death potential. Therefore, targeting this response either by attenuating the adaptive arm or enhancing pro-death signalling may have clinical potential in the treatment of many conditions

including cancer. To date, drug development targeting the UPR has mainly focused on the inhibition of pro-survival IRE1α signals. This has led to the development of compounds such as STF-083010 and MKC-3646, which act by inhibiting the catalytic core of the RNase domain (140, 141). Directly targeting the endoribonuclease domain of IRE1α blocks its ability to splice XBP1 mRNA therefore reducing levels of the pro-survival transcription factor XBP1s. Attenuating signalling via this arm of the UPR, is thought to reduce pro-survival responses and push the cell towards death (inhibitors of IRE1/XBP1 will be discussed later in the text).

Increased levels of heat shock proteins is frequently reported in cancer and have been implemented in the modulation of the UPR response (142). Our lab showed that HSP72 can interact with IRE1α cytosolic domain and enhance IRE1α RNAse activity and splicing of XBP1(70), while others have reported how association of HSP90 effects IRE1α stability and activation (68). We can therefore hypothesise that specific targeting of HSPs for example, HSP72 or HSP90, by compounds such as MAL3-101, 17-AAG and radicicol may also help counteract IRE1 pro-survival signalling and enhance cell death pathway. In fact, 17-AAG showed increased cytotoxicity when used alongside IRE1 inhibitor MKC-3646 in MM cell lines (141).

PERK signalling contributes to pro-survival UPR responses by attenuating cap-dependent translation through the phosphorylation of eIF2α, thus preventing further protein accumulation in the ER. GSK2656157, an ATP competitive inhibitor of PERK catalytic activity, limits the ability of PERK to block translation thus can enhance cell death. Recent *in vivo* xenograft studies, using GSK2656157, have reported diminished growth in MM and pancreatic-derived tumours, thus highlighting the clinical potential of targeting PERK as an anti-cancer treatment (143). Additionally, during hypoxic conditions in pancreatic cell line KP4, PERK inhibition enhanced cytotoxicity. This confirms the importance of PERK arm for cellular adaption to hypoxia (144). PERK inhibition also shows clinical potential in hepatocellular carcinoma, a report from Vandewynckel *et al* explored the expression of UPR markers over time in a mouse

model of hepatocellular carcinoma and identified PERK as a promising drugable target. Interestingly, they showed that peak IRE1α activity occurred during tumour initiation but PERK activity was most predominant during tumour progression. Modulation of both these arms using chemical inhibition revealed that PERK inhibition had the greatest effect on reducing cell viability and overall tumour burden (145).

Studies have also looked at the therapeutic implications of modulating eIF2α in malignant conditions. Salubrinal is a selective inhibitor that blocks the dephosphorylation of eIF2α and was first characterised in neural cells (146). Further studies with this compound showed enhanced cell death when used in combination with proteasome inhibitors in leukemic and multiple myeloma cells (147-149). In addition, Salubrinal caused pancreatic beta cell dysfunction and apoptosis, and could enhance TRAIL induced hepatoma cell apoptosis (150, 151). On the other hand Peter Walter's group identified a small molecule inhibitor termed ISRIB that reverses the effects of eIF2α phosphorylation and restores cell's translation capacity (152). In prion diseased mice, a block in translation mediated by p-eIF2α is linked to synaptic failure and neuronal loss, however use of ISRIB was shown to induce neuro-protection without pancreatic toxicity which was previously observed with PERK inhibition (153). This suggests that in certain cases targeting downstream PERK targets may be a safer and more specific therapeutic approach.

Inhibitors of ATF6 are currently less developed than their IRE1/PERK counterparts and to our knowledge no ATF6 specific inhibitor is currently available. However, ATF6 has been implicated in modulation of chemoresistance and tumour cell dormancy suggesting that ATF6 inhibition is a currently untapped therapeutic avenue (52, 154). Activation of ATF6 relies upon cleavage by serine proteases in the Golgi and inhibitors of these proteases do exist. 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), a serine protease inhibitor, was shown to prevent ER stress-induced cleavage of ATF6 which resulted in transcriptional inhibition of downstream ATF6-target genes (155). However studies using this inhibitor to block ATF6 must be taken with caution as AEBSF inhibits a wide range of proteases and therefore the effects cannot be deemed

ATF6 specific. Chemical chaperones have also been linked to modulation of ATF6 activity. 4-Phenylbutyric acid (4-PBA) is a chemical chaperone capable of limiting the accumulation of unfolded protein in the ER. Next generation versions of 4-PBA were shown to selectively block the activation of ATF6 and IRE1 but not PERK during ER stress conditions. This is an interesting result as it identifies a chemical chaperone which seems to selectively target the predominantly pro-survival arms of the UPR (156).

Alternatively, rather than suppress the adaptive phase of the UPR another approach is to enhance the overall level of cellular ER stress and therefore push the cell towards cell death. Proteasome inhibitors, such as Bortezomib (also called Velcade), prevent the degradation of damaged/misfolded proteins, increasing the level of ER stress and committing the cell to death. Bortezomib has been successfully used as a single agent therapy in haematological conditions such as multiple myeloma and has reached clinical trials for other conditions such as metastatic breast cancer (157, 158). Alternate strategies for increasing ER stress levels include inhibitors of the ER associated degradation (ERAD) pathway. p97ATPase is involved in ERAD retrotranslocation and the ubiquitin fusion degradation pathway, inhibitors such as Eeyarestatin and DBeQ can block p97ATPase activity with the latter reported to induce rapid caspase activation and cell death in HeLa cells (159).

Overall, the UPR presents itself as a clinically relevant and druggable target as it is often the deciding factor between cell survival and cell death. Manipulation of these processes is only starting to be exploited with initial studies looking promising; however the full potential still remains to be exploited.

1.3.1 Targeting of IRE1/XBP1s

In recent times there has been increased focus on developing therapeutically viable inhibitors of IRE1/XBP1 axis due to the increasing amount of evidence that this arm plays an important role in malignant conditions. Inhibitor development has targeted two

main sites on IRE1; the catalytic core of the RNase domain and the ATP binding site of the kinase domain. Research has predominantly focused on developing inhibitors of this arm with limited investigation into compounds that can activate the RNase activity. A recent article by Peter Walter's group characterised a small molecule, IPA, which could bind IRE1's ATP binding pocket, stimulating oligomerisation and subsequent RNase activation. IPA was an interesting discovery as they report that unlike IRE1 activation which occurs at a range of concentrations, IPA could also activate PERK at low concentrations but paradoxically inhibit PERK at higher concentrations. This adds considerable implications to the use of kinase inhibitors therapeutically, suggesting more careful assessment of drug doses is need. The flavonal quercetin is also reported to activate IRE1 RNase domain and one can hypothesis that this may have clinical potential in neurodegenerative diseases or cases of IBD (160). As this body of research focuses on the application of IRE1 inhibition in breast cancer here we discuss the other commercially available inhibitors of IRE1 (summarised in table 1).

1.3.1.1 Salicylaldehydes

Salicaldehydes were first identified by researchers at Mannkind Corporation California in 2011. They began by expressing a cytosolic fragment of human IRE1 in insect cells and completing a fluorescence quenching based high throughput screen using a Cy-5 labelled XBP1 stem loop RNA substrate. After using this approach on a chemical library of 220,000 individual compounds salicylaldime and their hydrolysis products salicyaldehydes were identified as IRE1 inhibitors which did not inhibit the homologs of IRE1, RNase L or the unrelated RNase A and T_1 (161). The lead compound from this study was 3-ethoxy-5,6 dibromosalicylaldehyde which was the most potent compound based on IC_{50} analysis. This compound binds IRE1 reversibly and can inhibit the cleavage of XBP1 but was shown not to affect the phosphorylation of IRE1α following thapsigargin treatment in MM (161).

1.3.1.2 4μ8C

In 2012 Heather Harding in conjunction with David Ron identified a small molecule inhibitor of IRE1. Using recombinant human IRE1 cytosolic domain in a fluorescent based FRET-depression assay over 235,000 pure compounds and partially purified natural extracts were screened for IRE1 inhibition. This resulted in the identification of umbelliferones (7-hyroxy-4-methylcoumarin derivatives), as potent IRE1 inhibitors. CB5305630 was identified as their lead compound, however in aqueous conditions CB5305630 undergoes hydrolytic cleavage to form 8-formyl-7-hydroxy-4-methylcoumarin; this was identified as the active component and termed 4μ8C. 4μ8C can also be categorised as a salicaldehyde and therefore has have a similar mechanism of action as above. 4μ8C forms a Schiff base with lysine 907 in the active site of the IRE1 endonuclease domain, blocking substrate access to the active site and can inhibit XBP1 splicing and RIDD functions of IRE1 (162). While this inhibitor also shows affinity for lysine 599 present in the kinase domain of IRE1 and can block IRE1 kinase activity, it is worth noting that lysine 599 is a phosphate coordinating residue common to all kinases and so off-target effects seem likely.

1.3.1.3 MKC-3946

In 2012 Kenneth Anderson's group in collaboration with Mankind Corporation characterised MKC-3946 and explored its therapeutic potential in multiple myeloma. This inhibitor was a next generation version of the salicaldehydes identified in (161) and is a more potent and soluble version than its predecessor (163). MKC-3946 was shown to block endogenous XBP1s levels in patient derived MM cell lines and MM tumour xenograft models. This inhibitor could induce modest cytotoxicity in MM cells lines and enhance cytotoxicity to bortezomib and the HSP90 inhibitor 17-AAG. This enhanced cytotoxicity was observed to occur simultaneously to enhanced PERK signalling and IRE-JNK activity which the authors speculate are driving apoptosis in these conditions. The effect of MKC-3946 alone or in combination with bortezomib (which shows an additive effect) was recapitulated in an MM xenograft model (163).

1.3.1.4 STF-083010

Albert Koong's group in 2011 published their IRE1 inhibitor; STF-083010. This compound was identified using a high throughput chemical screen with a luciferase based XBP1 reporter construct stably expressed in HT1080 human fibrosarcoma cells. They showed that his compound could inhibit the endoribonuclease domain of IRE1 without affecting its kinase activity. STF-083010 could also induce a cytostatic and cytotoxic response in MM cell lines in a dose and time dependent manner and could reduce tumour growth in tumour xenograft models (140). Later Harding's 4μ8C paper would show that STF-083010 forms an aldehyde in water and also targets Lysine 907 of IRE1 RNase domain (162).

1.3.1.5 Toyocamycin

Lida's research group first began screening for compounds from cultured broths of microorganisms which could inhibit IRE1 in 2007 using a XBP1 luciferase reporter construct in HeLa cells. Initial studies revealed Trierixin (164) and Quinotrierixin (165) from *Streptomyces* culture broths as IRE1 inhibitors. Continuing this screening process Lida's group isolated Toyocamycin from a culture broth of *Actinomycete* strain (166). They showed that Toyocamycin could block IRE1α RNase activity without affecting the other arms of the UPR or IRE1α phosphorylation. Similarly to other IRE1 inhibitors, Toyocamycin could induce cytotoxicity in MM cell lines but also markedly reduced the proliferation of MM cell lines harbouring high XBP1 levels without eliciting the same effect in cells harbouring low XBP1s expression cells or non-MM cells. Toyocamycin also displayed anti-tumour activity when used alone and in combination with Bortezomib in human MM xenograft models (166). Moreover in 1968 a Phase I clinical trials tested the anti-tumour effect of Toyocamycin on solid tumours but the drug had little to no effect and so was not pursued further (167) . However it also revealed that as a single agent it had no systemic side effects, this is an intriguing result and lends strength to the pursuit of using Toyocamycin in the treatment of MM.

1.3.1.6 Compound 3

Compound 3 was identified by the Maly group at the University of California, San Francisco in 2013. The identification of this compound was based on previous findings which showed that specific ligands of the kinase domain can bypass the requirement of autotransphosphorylation and directly regulate the activity of the endoribonuclease domain (168). They reversed this hypothesis and explored the potential of kinase inhibitors to block the RNAse activity of IRE1α. Initially Wang *et al* explored two types of kinase inhibitors which can stabilise alternate kinase active site conformations –type I and type II. They screened a range of type II kinase inhibitors that competes for ATP binding and using recombinant human IRE1 construct with a FRET-quenched XBP1 RNA mini-substrate and identified Compound 3 as a kinase inhibitor with a dual ability to inhibit RNAse activity. This compound was validated in INS-1 insulinoma cell lines following treatment with ER stress inducer thapsigargin. However it must also be noted that as a kinase inhibitor it is not a completely selective inhibitor of IRE1α (169).

1.3.1.7 KIRA6

Kinase-Inhibiting RNase-Attenuator 6 (KIRA6) was characterised in 2014 Cell paper by Papa group. There are conflicting reports as to whether or not higher order IRE1 oligomers causes a decrease in substrate specificity of IRE1 RNase domain and RIDD activation (170, 171). Papa and colleagues have suggested that a higher order increase of the oligomeric state of IRE1, increases substrate range of the RNase domain and push the cell towards terminal UPR response. They report that KIRA6 is a type II IRE1 kinase inhibitor capable of breaking higher oligomers of IRE1 and thus limiting the substrate specificity of the RNase domain to XBP1. In line with this KIRA6 could significantly enhance photoreceptor survival in models of chronic ER stress induced retinal degradation (170).

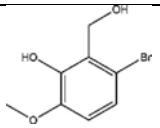
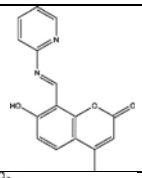
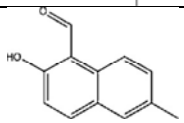
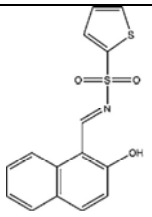
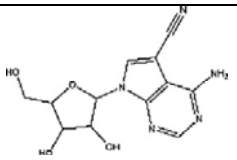
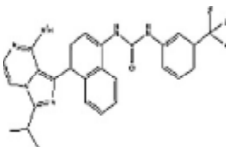
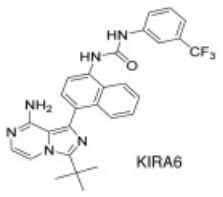
Name	Structure	RNAse Activity	Kinase Activity	Identified By
Salicylaldehydes		Inhibition	No reported effect	MannKind Corportation
4μ8C		Inhibition	Inhibition	Heather Harding / David Ron
MKC-3946		Inhibition	No reported effect	Mannkind Corportaion
STF-083010		Inhibition	No reported effect	Albert Koong
Toyocamycin		Inhibition	No reported effect	S. Lida
Compound 3		Inhibition	Inhibition	Dustin Maly
KIRA6		Inhibition	Inhibition	Papa

Table 1: IRE1 Inhibitors. Table listing name, structure, RNase activity, Kinase Activity of IRE1 inhibitors and name of those responsible for their identification.

1.4 Cell Death

When cells are exposed to severe or prolonged stress stimuli death can ensue. For many years this phenomenon was simply defined as unregulated (necrosis) or programmed cell death (apoptosis). While the original concept of cell death was first proposed as early as 1842 by Carl Voght, it wasn't until the 1960-1970s that the science community began to discover that cell death could occur in a regulated or programmed fashion (172). Since then the cell death research community has developed and expanded and now encompasses thirteen acknowledged forms of cell death including anoikis, apoptosis, autophagic death, cornification, entosis, mitotic catastrophe, necroptosis, parthanatosis, pyroptosis and ferroptosis (173). Apoptosis, the best characterised, and typically the most predominant form of programmed cell death, is described in further detail below.

1.4.1 Apoptosis

The term apoptosis was first coined by what is now referred to as a classic paper in the field published by Kerr, Wyllie, and Currie in 1972 (174). Later Horvitz would identify the reproducible death of 131 cells in the nematode *Caenorhabditis elegans* (1090 somatic cells total) during development. This finding was the first to show the remarkable accuracy and control of a programmed cell death system (175). Now we understand that apoptosis naturally occurs during development and aging and is necessary to maintain homeostasis of cell populations in tissue.

Apoptosis is characterised by condensation of nuclear chromatin, cytoplasmic shrinkage, membrane blebbing, nuclear fragmentation, and formation of apoptotic bodies. This evolutionarily conserved mechanism enables maintenance of homeostasis, development and morphogenesis, and removal of damaged, aged or unnecessary cells (176). Apoptotic deregulation is associated with a number of malignancies including cancer while excessive apoptosis is linked with neurodegenerative and neuromuscular

disorders thus highlighting the crucial importance of this pathway. Apoptosis can be subdivided into two pathways - extrinsic apoptosis and intrinsic apoptosis (177).

1.4.1.1 Extrinsic Apoptosis

Extrinsic signalling involves external stimuli that regulate transmembrane receptor (death receptor) mediated interactions. Death receptors are members of the tumour necrosis factor (TNF) receptor gene superfamily and include FAS, TNFR-1, DR-3, DR-4 and DR-5. These receptors contain a similar cysteine rich extracellular domain and an intracellular 80 amino acid sequence termed the death domain. Binding of the respective ligand to its receptor leads to oligomerisation of the receptor on the cell membrane and clustering of internal factors that transduce the death signal further into the cell (178). This is exemplified in the case of FAS/FAS-R where binding of FAS to its receptor results in recruitment of adaptor protein FADD (179). This binding leads to the recruitment of pro-CASPASE 8/10 via its death effector domain. A death inducing signalling complex (DISC) is now formed and leads to the catalytic activation of CASPASES 8/10 as shown in figure 5. This activation can be inhibited or limited via c-FLIP which has sequence homology to CASPASE 8 and 10. C-FLIP-L inhibits CASPASE 8 recruitment to the DISC subsequently blocking CASPASE 8 mediated apoptosis (180).

Until this point, the pathway can be referred to as the initiation phase of apoptosis which ends with activation of initiator CASPASES 8/10. The execution phase begins with direct downstream activation of CASPASES 3/7. However, CASPASES 8/10 can also amplify the internal death signal via cleavage of pro-apoptotic BCL-2 family member BID. The c-terminal truncated form of BID, tBID, migrates to the mitochondria where it inserts in the outer mitochondria membrane (OMM) (181) and causes BAX translocation and insertion into the OMM, resulting in BAX/BAK mediated OMM permeabilisation (182, 183). Extrinsic apoptosis and intrinsic apoptosis converge at this point, at the level of the mitochondria resulting in loss of mitochondrial membrane potential (ψm).

1.4.1.2 Intrinsic apoptosis

The intrinsic pathway is activated by internal cytotoxic signals such as viral infection, DNA damage, and growth factor deprivation, resulting in loss of mitochondrial membrane potential (ψm) and the opening of a mitochondrial permeability transition pore. This allows release of pro-apoptotic factors, including cytochrome *c*, SMAC/DIABLO and OMI (184-186). Cytochrome *c* binds APAF1 and together with pro-CASPASE 9 forms the apoptosome, a signalling platform that activates CASPASE 9 (187). SMAC/DIABLO and OMI enhance CASPASE activation though inhibition of inhibitors of apoptosis (IAPs) with studies overexpressing SMAC/DIABLO and OMI, showing enhancement of apoptotic signalling (185, 186). Activated CASPASE 9 can then continue to activate executioner CASPASES 3/7 and apoptosis ensues. Complete loss of mitochondrial membrane potential is considered the 'point of no return' in the apoptotic pathway, BCL-2 family members are the key regulators of this event and as such are critical regulators of cell fate.

1.4.1.3 BCL-2 family

BCL-2 family members are classified into anti-apoptotic members (BCL-2, BCL-X_L and MCL-1, BCL-w, BCL-2A1,) which have all four BH domains and pro-apoptotic multi-domain family members (BAX, BAK ,BOK) and BH3 domain only proteins BAD, BIK, BID, HRK, BIM, BMF, NOXA and PUMA which bind and regulate the anti-apoptotic members to promote apoptosis. BCL-2 family members are the key regulators of the intrinsic apoptotic pathway.

Pro-apoptotic BCL-2 family members are usually found in inactive state in viable cells but during cellular stress and/or death signals conformational changes can lead to their activation. For example in viable cells, BAX and BAK exist as monomers until upstream apoptotic signals trigger oligomerisation and insertion of active BAX and BAK into the mitochondrial membrane leading to OMM permeabilisation. The anti-apoptotic BCL-2 family members BCL-2/BCL-X_L can inhibit BAX and BAK but how

they elicit their anti-apoptotic effect exactly is debated. One theory suggests that BH3-only proteins bind directly to BAX and BAK (termed direct activation model), another theory postulates that it is the displacement of anti-apoptotic proteins from BAX and BAK thus neutralizing their inhibitory effect on BAK and BAX that leads to their activation (termed the indirect activation or displacement model) (188, 189).

NOXA and PUMA are two pro-apoptotic BCL-2 family members which have been shown to localise to the mitochondria where they are reported to interact and inhibit anti-apoptotic BCL-2, promoting BAX/BAK activation and resulting in the increased CASPASE 9 activation. These two proteins are thought to play an important role in genotoxic damage induced apoptosis as they can both be induced by p53 (190, 191). BIM is another similar anti-apoptotic protein that can exist in three different isoforms due to alternative splicing and can inhibit certain pro-survival BCL-2 family members thus promoting apoptosis (192).

1.4.1.4 CASPASES

In mammals apoptosis is mediated by cysteine dependent aspartic acid proteases (CASPASES) which are an evolutionary conserved family of enzymes which commit the cell to death irreversibly. First identified in *C. elegans* there are now 14 mammalian CASPASES identified, 11 of which in humans. CASPASES can be subdivided into two distinct groups; initiator CASPASES including CASPASE 2,8,9,10 and the effector CASPASES 3, 6 and 7. CASPASES are maintained as catalytically inactive zymogens in healthy cells and must undergo proteolytic activation during apoptosis to become active. The initiator CASPASES often require complex formation to facilitate auto-activation: for example DISC recruits adaptor proteins such as FADD to the death receptor leading to subsequent activation of CASPASE 8/10 through cleavage at internal residues separating the large and small subunits. These subunits associate to form as a CASPASE monomer, however the exact mechanism of activation of initiator CASPASES remains to be fully elucidated. On the other hand, effector CASPASE

activation is better understood through structural analysis of pro-CASPASE 7 (193, 194) which reveals that intra-chain cleavage by the initiator CASPASES causes increased catalytic activity by orders of magnitude. Overall these studies showed that L2' loop, a surface loop, becomes open ended after activation cleavage and adopts a supporting configuration for homodimerisation and exposes the catalytic active site of the enzyme.

Once activated the executioner CASPASES can activate cytoplasmic endonucleases and proteases that degrade nuclear material and cytoskeletal proteins. Executioner CASPASES also have direct cleavage substrates such as PARP, cytokeratins, alpha fodrin – a plasma membrane cytoskeletal protein, among many others (178). CASPASE 3 is considered the predominant executioner CASPASE and can induce cytoskeletal reorganisation and disintegration of the cell into apoptotic bodies. Moreover, it leads to the activation of the endonuclease CAD via cleavage of its inhibitor protein, ICAD. CAD can then degrade chromosomal DNA and causes chromatin condensation, a morphological hallmark of the apoptotic process (195).

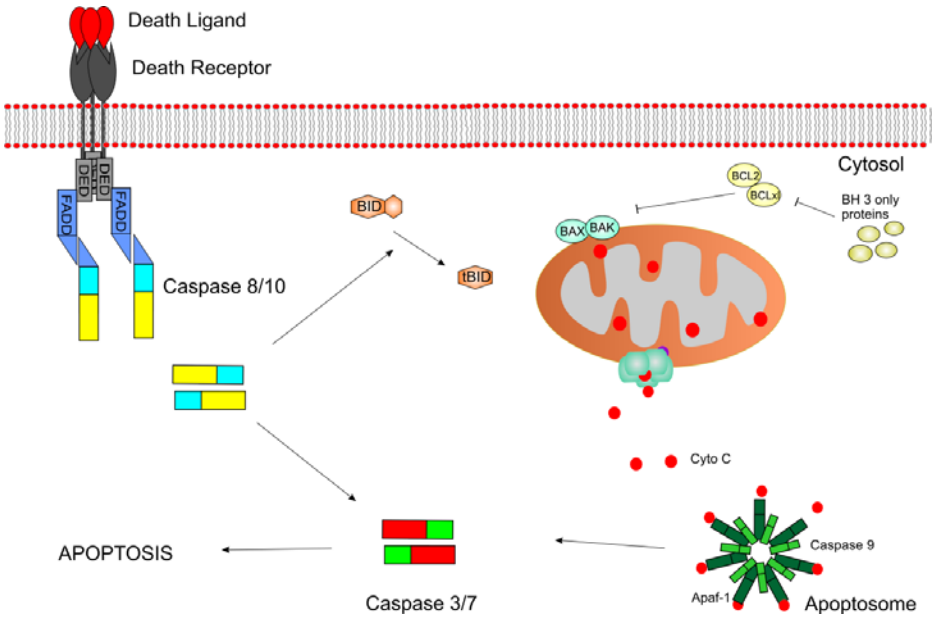


Figure 1.12: Apoptosis can occur via two distinct pathways. the extrinsic is mediated by ligand binding to death receptors and subsequent activation of CASPASES. The intrinsic pathway occurs via loss of mitochondrial potential and downstream apoptosome formation. Both pathways converge at the activation of executioner CASPASES

Cellular stress responses such as the UPR can often be insufficient to overcome the stress if it is prolonged or too severe, this can result in activation of the intrinsic apoptotic pathway. Next we will discuss in brief how ER stress is thought to signal to the intrinsic pathway and what key players are involved.

1.5 ER Stress induced apoptosis

While the UPR is predominantly thought to be a pro-survival response, it is capable of eliciting an apoptotic response if needed. One key link between the UPR and the mitochondrial pathway is through regulation of BCL-2 family members most notably pro-apoptotic BIM, PUMA and NOXA. As previously mentioned CHOP can up regulate pro-apoptotic BIM, with PUMA and NOXA linked to ER stress mediated apoptosis in a p53 dependent manner (196). *p53*$^{-/-}$ cells were partially protected from ER stress induced apoptosis which the authors attributed to PUMA and NOXA regulation (196). How ER stress is linked to p53 induced cell death is not fully understood but it is suggested to occur in a NF-κB dependent manner with all three UPR arms linked to NF-κB regulation (197-200). Through understanding the wide range of NF-κB downstream targets, it is likely that NF-κB mediates this response via upregulation of TNF as previously mentioned or through upregulation of pro-apoptotic BCL-2 family members. Moreover, further evidence of PUMA and NOXA importance to ER stress induced apoptosis is obvious as *Puma* and *Noxa* null MEFs display partial resistance to ER stress induced cell death (196).

The ER's role as a cellular store of free calcium is paramount to cell survival as calcium is an important intracellular signalling molecule. BCL-2 family members have been implicated in the regulation of ER calcium levels and coordinating ER mitochondria calcium signalling. Anti-apoptotic BCL-2 and BCL-xL can regulate and reduce ER free calcium levels, thus protecting the cell from ER stress induced apoptosis. This is thought to occur via direct interaction of BCL-2 with IP3R calcium channel at the ER

membrane (201, 202). However, phosphorylation of BCL-2 by kinases such as JNK can reverse this effect resulting in increased calcium release from the ER. This can lead to increased mitochondrial calcium uptake and pro-apoptotic signalling (203). Reports indicate that BI-1 can also inhibit ER calcium release with BI-1 overexpression causing reduced calcium release with corresponded with reduced BAX translocation to the mitochondria, suggesting a role of BI-1 in ER stress induced apoptosis (204). These studies, in addition to others reporting similar roles of PUMA (205) and BAX/BAK (206) in regulation of ER calcium levels, highlight not only the important crosstalk mediated by calcium from the ER to the mitochondria but also how BCL-2 family members are key regulators of this decision.

However at the level of the mitochondria, calcium transporters have low affinity for calcium and therefore require high levels of calcium to stimulate uptake. To overcome this requirement it is proposed that the ER and mitochondria have physical interacting sites in their membranes referred to as mitochondria associated ER membranes (MAMs). These contact sites are enriched for IP3 receptors and voltage dependent anion channel 1 (VDAC1) and ensure shuttling of sufficient calcium from the ER that will enable mitochondrial uptake (207). Also located in this region is sarcoplasmic reticulum calcium ATPase 1 (S1T) which is upregulated via the PERK arm during ER stress. SIT was shown to increase ER mitochondria contact sites, limit mitochondrial movement and cause increased calcium leak from the ER thus aiding ER stress induced apoptosis (208).

Calcium is a key intercellular signaling molecule and can lead to release of mitochondrial content via MOMP during cellular stress conditions. This occurs once calcium levels within the mitochondrial matrix surpasses a critical and sustained threshold resulting in activation of mitochondrial transition pore, and release of Cytochrome *c* with subsequent downstream apoptosome formation (207).

1.6 Additional Regulators of the ER stress response; microRNAs

microRNAs (miRNAs) are a small group of conserved non protein coding RNA (20-22 nucleotides in length) that play a key role in post transcriptional regulation of gene expression. miRNA down regulate gene expression via binding to 3'UTR (untranslated regions) of target mRNA with complimentary or semi complimentary sites altering regulation of mRNA stability and/or translational repression depending on the degree of complementarity (209). The role miRNAs play in ER stress induced cell death is only coming to light in recent years. miRNA-mediated regulation of the UPR may alter the balance between cell survival and cell death but only a limited amount have been determined.. 25141 mature miRNA products in 193 species have been identified to date with only a small fraction (approx 30%) with known function; this opens a new chapter of possibilities of mRNA regulation within the cell, which may include regulators of ER stress induced cell death (miRBase, release 19.0, http://www.mirbase.org).

miRNAs can directly modulate the ER stress response or themselves be regulated by ER stress. For instance, miR-122, found copiously in the liver and often repressed in hepatocellular carcinomas (HCC), can repress the oncoprotein proteasome 26S subunit non ATPase10 (PSMD10)/ p28-Gank (which potentiates the adaptive UPR and promotes cell survival) through its interacting partner CDK4 and promote apoptosis. ER chaperones GRP78, Calreticulin (Calr) and ER 29, in addition to UPR proteins, p-eIF2α and p-IRE1 were repressed following overexpression of miR-122, however these observations were partially attributed to the decrease in PSMD10 which has been previously reported to modulate the UPR response. Altogether the repression of ER chaperones, UPR proteins and PSMD10 lowers the protein folding capacity of the ER, attenuates the adaptive UPR and promotes apoptosis (210, 211). A study showed that HCC cells, which are partially resistant to ER stress, down regulate miR-221/222 in response to ER stress, which results in upregulation of $p27^{kip1}$ and G1 phase arrest. This study suggests that miR221/222 repression can help HCC cells avoid ER stress-induced apoptosis by augmenting G1 cell cycle arrest (212). miRNAs may also be directly regulated by the UPR as seen by the PERK dependent induction of miR-30c-2* upon ER stress. miR-30c-2* has been shown to increase repression of XBP1 mRNA thereby

reducing overall pro-survival signalling and aiding commitment to cell death (210). In addition, mir-106b-25 cluster and its host gene MCM-7, are repressed by PERK dependent transcription factors ATF4 and NRF2, which leads to increased expression of pro-apoptotic BIM and subsequent apoptosis (213).

MicroRNA array analysis revealed that approximately 13 miRNAs are regulated by ATF6 most probably via regulations of their host genes. In ischemic hearts, ATF6 is upregulated to protect against ER stress, causing miR-455 downregulation which causes an increase in *Calr,* an ER calcium-binding protein that acts as a molecular chaperone during ER stress. Neonatal rat ventricular myocyte cultures (NRVMCs) treated with ER stress inducer Tunicamycin, or overexpressing ATF6, also had increased CALR due to repression of miR-455. Reticulan 4 (RTN4) , an ER anchored protein, which has been shown to reduce the anti-apoptotic effect of BCL-2 and BCL- $X_L$and protect cells from ER stress-induced apoptosis, is also induced by ATF6 and is a predicted target of miR-455, however the correlation has not been functionally tested in response to ER stress (214, 215).

While miRNAs can play a significant role in ER stress induced cell death, having the ability to tip the balance between survival and cell death, more research into miRNAs is needed in this area to fully elucidate their role and determine if there may be a therapeutic use for them against disease in which ER stress occurs.

1.7 Autophagy

Autophagy (Greek ‘auto’ meaning oneself and ‘phagy’ to eat) is a regulated pathway in which components of the cell including proteins, protein complexes and organelles are degraded via lysosomal enzymes. Autophagic double membranes were first described in 1960s by de Duve and Wattiaux but it wasn’t until the late 1990s that the molecular components (most notably a range of autophagy-related genes (ATGs)) were first characterised in yeast by Yoshinori Ohsumi (216, 217). Since then approximately 34 ATGs have been identified with many conserved from yeast to humans. Nowadays at

least three forms of autophagy have been described; chaperone mediated autophagy, microautophagy and macroautophagy. These processes differ with regard to their physiological functions and mechanism of cargo delivery to lysosome. Here we will focus on macroautophagy (hereafter called autophagy), the degradation pathway involving delivery of cytoplasmic components sequestered in double membrane vesicles to the lysosome, a process widely used to degrade and recycle long lived proteins and organelles (218). This complex process can be divided into mechanistically distinct stages; autophagy induction, vesicle nucleation, autophagosome formation, autophagosome maturation, and cargo selection. Next, these stages will be briefly described to add clarity to the process.

1.7.1 Autophagy induction

Basal autophagy occurs at low levels under normal resting condition, so it is vital that during stressful conditions the cell is primed to up regulate autophagy. Induction requires activation of ULK1 complex which contains four known proteins; ULK1/2, ATG13, FIP200 and ATG101. Under basal conditions ULK1 complex activity is limited by mammalian target of rapamycin (MTOR) complex one (MTORC1) which phosphorylates ULK1/2 – ATG13 thereby inhibiting of ULK1/2 kinase activity (219). mTORC1 is a key sensor of cellular nutritional status and is a well-established inhibitor of autophagy (220, 221). Another sensor of cellular energy levels is AMPK, which can prompt the induction of autophagy via two possible mechanisms. Firstly, through inhibition of mTORC1 (222, 223) resulting in dephosphorylation of ULK1 and secondly through phosphorylation of ULK1 with numerous AMPK phosphorylation sites identified on ULK1 which can lead to ULK1 activation (224).

1.7.2 Vesicle Nucleation

Nucleation and assembly of the initial autophagophore requires phosphatidylinositol 3 Kinase complex (PI3K) formation. In mammals VPS34 is a class III PI3K and functions to produce phosphatidylinositol 3 phosphate which is crucial for early membrane

formation. VPS34 forms a stable complex with p150 and Beclin1. This serves as a binding platform and mediates the recruitment of several factors capable of promoting autophagy (e.g. ATG14L, UVRAG, AMBRA-1). ULK1 was shown to activate this formation through phosphorylation of Beclin1 and activation of VPS34 (225, 226). VPS34, in partnership with the factors mentioned, recruits two interrelated ubiquitin-like conjugation systems to the phagophore initiating autophagosome elongation.

1.7.3 Autophagosome elongation

The ubiquitin-like conjugation system is a stepwise conserved process of attributing a post translational modification to a protein. Autophagosome elongation requires two ubiquitin like conjugation systems; ATG12-ATG5 and LC3 conjugation system. During typical ubiquitination, an E1 activating enzyme, E2 conjugating enzyme, and E3 ligase function together to transfer a ubiquitin chain to a substrate. During autophagy ATG7 acts as the E1 activating enzyme for both ATG12 and LC3 systems.

Next, ATG12 is transferred to ATG10 (E2 conjugating enzyme) and is covalently attached to a lysine on ATG5. Unlike ubiquitination, this is an irreversible process and does not require an E3 ligase. ATG12-ATG5 then interacts with ATG16 which facilitates tetramer formation through self oligomerisation. This complex can then attach to the developing phagophore (227). During the LC3 conjugation system LC3 is activated by ATG7 and transferred to ATG3 (E2). LC3 is then attached via an amide bond to the target lipid phosphatidylethanolamine (PE). It is thought that ATG12-ATG5 can act as the E3 ligase to mediate LC3 attachment to PE and then acts as a platform to recruit lipidated LC3 to the autophagosomal membrane (226, 228).

1.7.4 Autophagosome maturation

Once the autophagosome is fully formed LC3 is cleaved from PE by ATG4 and recycled back into the cytosol (229). The precise mechanism of how the autophagosome

fuses with the lysosome is not fully understood but it is believed that the trafficking to the lysosome is dependent upon the microtubule network (230) with fusion occurring via lysosomal protein LAMP-2 and small GTPase RAB7 (231, 232). After fusion the cargo within the autophagosome is degraded by lysosomal hydrolases and proteinases. Following the degradation process the remaining small molecules (amino acids for example) are exported back into the cytosol for future use.

1.7.5 Cargo selection

While we have a good understanding of how the autophagosome is formed, how its cargo is selected and targeted is less clear. Initially it was thought that autophagy was a non-selective process but growing evidence of autophagy receptors including sequestosome 1, NBR1, BNIP3L, NDP52 suggests that this may be a more target-specific process than previously thought. These receptors recognise the target and via LC3 interacting region (LIR) mediate delivery to the autophagosome (230, 233). Moreover, increasing evidence of organelle specific autophagy for example mitophagy (mitochondria) ER-phagy (ER) and crinophagy (Golgi) also argue that a specific and selective process must be taking place (226, 234).

1.8 Breast Cancer

Breast cancer is a diverse and genetically heterogeneous disease and therefore standardised classification systems have been developed to organise this disease (235). These classification systems have continued to evolve over the years with further factors being examined and included to allow a greater stratification of the various types and allowing for better predictive outcomes. Classical clinical features include age, tumour size, auxillary node involvement, angio lymphatic invasion, alongside histological grading and hormone receptor/HER2 status (236). Histological classification of the breast cancer subtypes, which is a predominant analysis in clinic, broadly characterises breast cancer into in situ carcinoma (ductal or lobular) and invasive carcinoma (Tubular, Ductal Lobular, Invasive lobular, Infiltrating ductal,

Mucinous, Medullary, infiltrating ductal) (reviewed in (237)). While this classification system has been a valuable tool in clinic it focuses upon histology without taking into account additional molecular markers that can further define breast cancer.

Intrinsic molecular markers of breast cancer generally rely upon the expression of oestrogen receptor (ESR1), progesterone receptor (PR), and whether it is HER2/ErbB2 enriched. More extensive microarray gene analysis studies carried out by Perou *et al* in 2000 typically identified four reproducible molecular subtypes; Basal like, HER2/ErbB2^{+}, normal breast like, and luminal subtype (238). This system has facilitated greater outcome predictions, with triple negative/basal-like having the worst prognosis and shortest recurrence free survival (239). Triple negative breast cancers (TNBC) are defined by lack of ERS1-α, PR, or HER2 enrichment and is often used interchangeably with the term basal breast cancer, although his is not technically correct. While TNBC can fall within the basal subtype cluster it is not stereotypically true as it requires a more comprehensive gene expression analysis to define the basal subtype.

Luminal subtypes can now be further delineated into luminal A, B, and C. A has the highest expression of ERS1-α gene, in addition to high levels of XBP1, trefoil factor 3, hepatocyte nuclear factor 3 alpha and the oestrogen regulated LIV-1. Luminal B and C subtypes have moderate expression of the luminal specific genes including the oestrogen receptor cluster. The distinguishing feature of luminal subtype C is a high expression of a novel set of genes whose functions are unknown, but can also be linked to the basal-like and her subtypes (239, 240).

While the use of microarray analysis has greatly enhanced our ability to define breast cancer it is not a financially viable option in clinic at present. However, researchers have narrowed down a 50 gene signature termed PAM50 which is capable of identifying the intrinsic subtypes (241). This gene set was shown to significantly improve the predication of relapse compared to clinical characteristics alone, but it is it

noteworthy that using both in combination is suggested to give the most accurate prediction (241).

While we are gaining greater insight into breast cancer subtypes, another field of interest is emerging, with intense research focusing on breast cancer stem cells. The cancer stem cell hypothesis claims that a sub group of cells exist within the tumour that are responsible for initiation and tumour progression and are the main drivers of tumourigenesis (235). Whether the CSC is derived from normal cells within the stem cell hierarchy or from normal stromal cells remains to be elucidated. There are reported features that can identify this population in breast cancer. In 2003 Al-Hajj *et al* identified surface markers $CD44^{high}/CD24^{low}/Lin^{-}$ as capable of generating tumours when just 200 of these cells were inserted into SCID mice while 20,000 cancer cells that did not display these markers were incapable of forming tumours (242). Furthermore, aldehyde dehydrogenase 1 was later identified as an intercellular marker of normal and cancer stem cells (243).

In 2006 two groups publish papers in *Nature* identifying a subpopulation of mammary cells, where a single cell was capable of reconstituting a mammary gland *in vivo*. Visvander group identified the key cell surface markers as $Lin^{-}CD29^{high}CD24^{medium}$ (244) with Eaves group proposing cell surface markers $CD24^{medium}$, $CD49f^{high}$ (245). While these initial studies were completed in a mouse model they have also been utilised in the isolation of human mammary stem cells (246).

As we better understand the diversity of cancer, the idea of one size fits all therapeutic approach is becoming more apparently flawed. Improving our understanding of the molecular and histological characteristics of breast cancer will better equip clinicians to individualise treatment and help to identify new therapeutic approaches.

Chapter 2 Materials and Methods

2.1 Cell Culture

MCF10A are a nontumorigenic breast epithelial cell line and were purchased from ATCC and maintained in 5% horse serum, 20 ng/ml epidermal growth factor, 0.5 µg/ml Hydrocortisone, 100 ng/ml Cholera toxin, 10 µg/ml insulin and 50 U/ml penicillin, 50 µg/ml streptomycin. MCF7 (ECACC), MDA-MB231 (ECACC) and HEK293t (ATCC) and all were cultured in DMEM high glucose from sigma (D6429), supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, 50 µg/ml streptomycin, and 2mM L-glutamine(Sigma G7513). SKBR3 (ECACC) and HCT116 were cultured in McCoys5A (Sigma M9309) supplemented with 10% FBS, 50 U/ml penicillin, 50 µg/ml streptomycin. HCC1806 (ATCC) and T47D (ECACC) were cultured in RPMI medium (Sigma R0883) supplemented with 10% FBS and L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin.
All cells were cultured at 37^0C and 5% CO_2 in a humidified incubator.

2.2 Drug treatments

2.2.1 Drugs used in the treatment of breast Cancer

Placitaxel (taxol) (Sigma T7402) binds to the N-terminus of β-tubulin and and stabilizes microtubules arresting the cell cycle at the G2/M phase, this microtubule damsge leads to apoptosis. Doxirubicin (LC laboratories #D-4000) is an Anthracycline Topoisomerase. Inhibitor Bortezomib (Selleck Chemicals #PS-341), is a proteasome inhibitor in phase 2 clinical trials for breast cancer. Methotrexate is an inhibitor of tetrahydrofolate dehydrogenase and prevents the formation of tetrahydrofolate, necessary for synthesis of thymidylate, an essential component of DNA. Melphalan (Sigma M2011) forms DNA intrastrand crosslinks by bifunctional alkylation in 5′-GGC sequences. Tamoxifen (Sellek Chemicals S1972) is antagonist of the oestrogen receptor. 5-Fluorouracil (Sigma 6627) is a pyrimidine analog and therefore inhibits nucleic acid synthesis. These drugs were used for indicated times at indicated doses.

2.2.2 ER stress inducers

To induce ER stress Thapsigargin (Tg, Sigma T7765) is a non-competitive inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase. Brefeldin A (BFA, Sigma B7651) interferes with anterograde transport from the ER to the Golgi and tunicamycin (Tm, Sigma B7651) inhibits N-glycosylation in eukaryotes by blocking the transfer of GlcNAc-1-P from UDP-GlcNAc to dolichyl-P. Etoposide (Eto, Sigma E1383) inhibits topoisomerase II.

2.2.3 Pathway specific inhibitors

To inhibit the UPR, 20μM IRE1 inhibitor MKC-8866 (MannKind corp) or 300nM PERK inhibitor (Toronto Research chemicals -GSKG797800) were used. To inhibit the autophagic pathway cells were treated with 20μM Choloroquine (CQ Sigma C6628) or with 10μM Spautin-1 (Sigma #SML0440) every 24hours.

2.2.4 Neutralising Antibodies

All neutralising antibodies were purchased from R&D systems. IL-6 (MAB206-SP clone 6708), IL-8 (MAB208-SP clone 6217), Gm-csf (MAB215 clone 3209), TGFβ (MAB1835-SP clone 1D11), Gro-α (MAB 275-500)

2.3 Western Blotting

2.3.1 Protein Sample Preparation

At the end of treatment cells were scraped into the media and centrifuged at 7000rpm for 5 mins. Media was then removed and cells washed in 1ml of cold PBS and centrifuged once more for 5 mins. PBS was removed and cells were lysed in 2X lysis buffer (4% SDS, 120mM Tris HCL pH 6.8, 10% glycerol, 100nM DTT and dash of bromophenol blue). Volume of buffer was determined by cell number. Samples were boiled at 95^{o}C for 5mins and either stored at -20^{o}C or loaded directly onto an SDS-PAGE gel.

2.3.2 SDS-PAGE gel electrophoresis

Gels were cast the day prior to running the gel. The percentage of SDS-PAGE gel will determine the protein separation, lower percentage gels are better for resolving high molecular weight molecules, while higher percentages are needed to resolve smaller proteins. The SDS PAGE gels were then run in a tank with appropriate amounts of running buffer at 50V until the sample passes through the stacking gel and 80V until the dye front reached the end of the gel.

The gel was then sandwiched between a sponge, filter paper, gel, nitrocellulose paper, filter paper and sponge. The proteins were transferred onto the nitrocellulous paper for 90mins at constant volts of 110V in transfer buffer (10mM CAPS pH11 and 20% methanol). Membrane was blocked in 5% non fat milk in PBS containing 0.1% tween (PBS-T). The primary antibodies were incubated as described in table 2. After primary incubation the membranes were washed three times for five mins each in PBS-T. Membrane is then incubated in appropriate horseradich peroxidase conjugated secondary antibody in 5% non fat milk PBS-T for 2 h at room temperature with agitation. Antibodies were visualized using Western lightning ECL substrates (Perkin Elmer).

Protein	Company	Primary antibody conditions	Secondary Antibody conditions
XBP1	Abcam	1/2000 in 5% milk -T overnight 4°C	1/5000 anti rabbit in 5% milk-T 2 h RT
Actin	Sigma A2066	1/5000 in 5% milk -T 1 h RT	1/5000 anti rabbit in 5% milk-T 2 h RT
Perk	Cell signalling	1/1000 in 5% milk-T Overnight 4°C	1/5000 anti rabbit in 5% milk-T 2 h RT
ATF4	Cell signalling	1/1000 in 5% milk- T overnight 4°C	1/5000 anti rabbit in 5% milk-T 2 h RT
Chop	Cell signalling	1/1000 in 5% milk -T Overnight 4°C	1/5000 anti mouse in 5%milk 2 h RT
T-eif2α	Cell signalling	1/5000 in 5% milk -T Overnight 4°C	1/5000 anti rabbit in 5% milk-T 2 h RT
p-eif2α	Cell signalling	1/1000 in 5% BSA -T overnight at 4°C	1/5000 anti rabbit in 5% milk-T 2 h RT
ATF6	Cosmobio	1/1000 in 5% milk-T overnight 4°C	1/5000 anti mouse in 5% milk-T 2 h RT
LC3	Sigma	1/5000 in 5% milk-T Overnight 4°C	1/5000 anti rabbit in 5% milk-T 2 h RT
Sestrin2	Sigma	1/500 in 5% milk-T Overnight 4°C	1/5000 anti mouse in 5% milk-T 2 h RT
Caspase 3	Cell signalling	1/1000 in 5% milk-T overnight 4°C	1/5000 anti rabbit in 5% milk-T 2hr RT
PARP	Cell signalling	1/1000 in 5% milk-T overnight 4°C	1/5000 anti rabbit in 5% milk-T 2hr RT
IL-6	R&D systems	1/1000 in 5% milk-T overnight 4°C	1/5000 anti mouse in 5% milk-T 2hr RT
IL-8	R&D systems	1/1000 in 5% milk-T overnight 4°C	1/5000 anti mouse in 5% milk-T 2hr RT

Table 2: Antibodies. Summary of Antibodies and conditions of use.

2.4 Flow Cytometry

2.4.1 Sample Preparation

Supernatant was removed and kept. Cells were washed with Hanks Balanced salt solution and 1xTrypsin was added, volume was determined based on plate size. Plate was placed back into 37oC incubator until fully detached. The corresponding supernatant was then added to the well and the cells collected in eppendorf tubes. The cells were allowed to recover at 37oC for 20 mins. The cells were then processed according to specific flow cytometry assay needs.

2.4.2 TMRE

Mitochondrial membrane potential $\Delta\Psi_m$ occurs during apoptosis and this can be measured by flow cytometry. TMRE is a cell permeable, positively-charged, red-orange dye that readily accumulates in active mitochondria due to their relative negative charge. Depolarized or inactive mitochondria have decreased $\Delta\Psi_m$ and fail to sequester TMRE. Carbonyl Cyanide m-chlorophenyl hydrozone (CCCP) is a ionophore uncoupler of oxidative phosphorylation and therefore destroys $\Delta\Psi_m$. To generate a positive control cells were treated with 60 μM CCCP for 3 hrs prior to harvesting. After treatment detached cells were collected and combined with remaining cells following trypsinisatio and incubated with 100 nm TMRE at room temperature for 30 min in the dark and analysed by flow cytometry.

2.4.3 PI Staining

Promidium Iodide (PI) (Sigma, P4170) is a flouresent stain for nucleic acids. Live cells with an intact cell membrane exclude PI however during cell death process cells lose membrane intengrity allowing entry of PI into the cell thus allowing us to distinguish between live and dead cells. After harvesting the sample via trypsinisation, the cells

were spun down at 3000rpm for 5 mins and resuspended in 300µl PBS containing 4µl of PI (50µg/ml stock) and immediately analysed by flow cytometry.

2.4.4 Cell Cycle analysis

This assay is based on PI ability to bind the DNA, allowing quantification of cells in subG1, G1, S, G2M phases of cell cycle. Cells were fixed in 70% ice cold ethanol, after harvesting cells were washed in PBS and resuspended in 150µl ice cold PBS and then while on a vortex 350µl ice cold ethanol was added dropwise. Cells remained on ice for mimimum of 1 h and then either processed or stored at -20^{o}C. Cells were centrifuged for 5 mins at 2000rpm and washed in PBS. An RNase A containing PI mix was then prepared by adding 5µl of RNase A (10mg/ml stock) to 95µl PI (20µg/ml). Cell pellet was resuspended in 100µl of RNaseA/PI and incubated on ice in the dark for 30mins. 200µl PBS was added and measure by flowcytometry on linear scale Pe-A channel.

2.4.5 EdU incorportation Assay

This assay is a measure of cells in S phase within a given time, in this case we used 1 hour. 5-ethynyl-2'-deoxyuridine (EdU) (Berry & Associates PY7563) is a nucleoside analog of thymidine and is incorporated into DNA during active DNA synthesis. Measurement of EdU relies on a click reaction, which is a copper (I) catalyzed reaction between an azide and an alkyne. The EdU contains the alkyne which can be reacted with the azide-containing FITC, to form a stable, triazole ring. 10µM EdU was added to samples 1hour before harvesting. Cells are trypsinised, washed in 1xPBS and fixed in 70% ice cold ethanol and left on ice for approx 1hr before storing at -20^{o}C. After thawing 1ml PBS added to each sample and spun at 3000rpm for 10mins. Pellet washed with 1ml PBS. Spun at 2500rpm for 5mins. Pellet was washed in 2ml PBS (1%BSA) and spun at 2500rpm for 5mins. Pellet was resuspended in 1ml of click cocktail: 1ml PBS + 10µl 1M sodium ascorbate + 10µl of 10mM 5'FITC Azide + 20µl 0.1M Copper II sulphate (order of addition very important therefore added sequentially). The cells

were then incubated in the dark at room temperature for 30mins. 10ml of PBS, 0.5% tween, 1% BSA was added to the sample to quench signal for ten mins at room temperature. The samples were spun at 3000rpm for 5mins and washed twice with 2mls PBS spinning at 3500rpm in between washes. 300µl PBS was added to sample and measured on FITC channel on FACS.

2.5 RNA preparation

Total RNA was isolated by adding 500 µl of TRI Reagent (Invitrogen) onto the cell monolayer or if floating cells were observed, all cells were scraped, collected and centrifuged at 1500 rpm for 5 min. The pellet was then resuspended in 500 µl of TRI reagent. To separate RNA from DNA and protein, chloroform was added, mixed well and incubated for 2 min at room temperature. Samples were centrifuged at 12,000g for 15 min. The upper clear phase was carefully pipetted into freshly autoclaved 1.5 ml tubes, with special care taken not to disturb the interface. In order to precipitate the RNA, 250 µl of isopropanol was added to each sample and incubated at -20^{o}C overnight. Samples were then centrifuged at 12,000g for 10 min and the RNA pellet was washed in 75% Ethanol. All of the ethanol was carefully removed and the pellet was air dryed to allow all traces of ethanol to evaporate. The RNA was resuspended in 25 µl of DEPC water and stored at -80 ° C until ready to use.

2.6 Reverse transcription RT-PCR

To synthesise cDNA, 2 µg of RNA was subjected to DNase treatment with 1 µl DNase and 1 µl Buffer (Invitrogen) added per reaction and incubated for 15 min at room temperature. The DNase was inactivated using 1 µl of EDTA. DNase treatment is to ensure there is no contaminating genomic DNA present. The RNA was then reverse transcribed into cDNA using Superscript III first strand RT-PCR system and random hexamers (Invitrogen). To each reaction 1 µl of random hexamer was added, mixed well, and heated at 65 ^{o}C for 2 min, and then 25 ^{o}C for 2 min. First strand mastermix consisting of molecular grade water, first strand buffer, DTT, dNTPs and superscript III enzyme was added to each sample and mixed well. The samples were kept at 25 ^{o}C for

10 min followed by 50 °C for 50 min, and finally inactivated at 85 °C for 10 min. Conventional PCR for human GAPDH was carried out using 2 µl of cDNA to check cDNA quality with 2x Go Taq mastermix (Promega).

2.6.1 GAPDH

GAPDH FWD 5' ACCACAGTCCATGCCATC 3'
GAPDH REV 5' TCCACCCTGTTGCTG 3'.

PCR cycle conditions:

1. 94 °C for 3 min,
2. 94 °C for 30s
3. 55 °C for 30s
4. 72 °C for 30s
5. 72 °C for 7 min
6. 4 °C for ∞.

Repeat steps 2-4 for 25 cycles.

2.6.2 XBP1

FWD: TTACGAGAGAAAACTCATGGCC

REV: GGGTCCAAGTTGTCCAGAATGC

PCR cycle conditions:

1. 94 °C for 3 min,
2. 94 °C for 30s
3. 55 °C for 30s
4. 72 °C for 30s
5. 72 °C for 7 min
6. 4 °C for ∞.

Repeat steps 2-4 for 30 cycles.

2.7 Lentivirus production

Lentivirus integrate their genetic material into the host cell allowing for stable, long-term expression of the transgene or shRNA.

Lentivirus production was typically carried out in T175 flask and would be scaled up or down as necessary depending on flask size using the following as a point of reference. HEK 293T cells were seeded at $10x10^6$ cells per T175. The PSAx2.2 (14 µg), RSV (6 µg), DM2G (6 µg) and desired vector (14 µg) were incubated with 500µl NaCl buffer solution. Simultaneously 80µl JetPEI was incubated with 500 µl NaCl buffer solution. These were incubated at RT for 5 min before mixing. They were then incubated for a further 20mins at room temperature before added to antibiotic free media of 293T cells (15mls vol). The cells generated the virus over the next 48 hours and supernatant was collected at 24 and 48 h. This virus media is sterile filtered and either stored at -80^{o}C or added directly to cells. Before adding to cells the virus containing media was incubated with 5 µg/ml polybrene for 5 min at RT and then added to desired cells. Cells were seeded at 40% confluency in a 6 well plate. Plate is spun down at 1200rpm for 90 mins at 37^{o}C. 24h later virus containing media is removed and replaced.

2.8 Cell Viability assays – 96 well format

2.8.1 MTT

T74D, MDA-MB231 and MCF7 were seeded at 20,000 cells/ml while MCF10A were seeded at 100,000 cells/ml and 24 h later treated. 5 mg/ml concentration of MTT ((3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazonium bromide) was added to the wells and incubated at 37°C for 3 h. The reaction was stopped with a stop mix containing 20% SDS in 40% dimethyl formamide. Plate was incubated overnight in the dark at room temperature with agitation. Absorption read at 550nm, viability calculated relative to control.

2.8.2 Sytox Green

Sytox green is a nucleic stain and so works similarly to PI staining described above. In brief cells were set up and treated in a 96 well format. Sytox Green (5mM stock) was diluted one in one hundred in Hanks and then 2µl was added per well and read on plate reader. 1µl of 10% TritonX was added to each well and incubated for 1 h at 37oC to reach 100% lysis of cells, the plate was then read again on plate reader. Blanks without the addition of sytox green were also generated. The initial reading was calculated relative to 100% lysis (the second reading).

2.9 siRNA transfection

siRNA were purchase from Darmacon

2.9.1 HCC1806 / MCF7

Cells were seeded at 60-70% confluency in pen/strep free media. Transfection volumes differ based on flask size but for siRNA transfection of a T25 flask the following procedure was followed. 20µl of empty media was mixed with 8µl Lipofectamine 2000 (Fermentas) and at the same time siRNA (1µl Sestrin2 or 2.2 µl siXBP1 and control non coding in each case) was added to 20 µl empty media and incubated at RT for 5 min. The siRNA was then mixed with Lipofectamine and allowed to incubate at RT for 20mins. Media was replaced on cells and 2ml of pen/strep free media was added. The siRNA mixes were then added drop wise and mixed by gentle swirling of flask. Cells were incubated for 4 h at 37oC at which time the media was replaced. The next morning cells were reseeded for experiment and treated 24 h later. In total the cells were treated 36 h post transfection.

2.9.2 MDA-MB231

Cells were seeded at 60-70% confluency in pen/strep free media. Transfection volumes differ based on flask size but for siRNA transfection of a T25 flask the following procedure was followed. 20µl of empty media was mixed with 8µl Darmafect 4 and at the same time siRNA (4.5 µl siXBP1 and control non coding in each case) was added to 20 µl empty media and incubated at RT for 5 min. The siRNA was then mixed with Darmafect and allowed to incubate at RT for 30mins. Media was replaced on cells and 4ml of pen/strep free media was added. The siRNA mixes were then added drop wise and mixed by gentle swirling of flask. Cells were incubated for 4 h at 37^{o}C at which time the media was replaced. The next morning cells were reseeded for experiment and treated 24 h later. In total the cells were treated 36 h post transfection.

2.10 Reduced Serum conditions

The seeding densities and serum concentrations were optimised for each cell line prior to experiment. MCF7 and SKBR3 were replenished with fresh inhibitor on day 4 and day 8. (summarised in table 3)

Cell line	Seeding Density (per ml)	Serum Concentration (%)	Experiment length (days)
MDA-MB231	15, 000	2	5
MCF7	5,000	1	10
SKBR3	20,000	2	9
HCC1806	10,000	2	7
MDA-MB231 shXBP1	10,000	1	6

Table 3: Reduced serum conditions. Conditions of reduced serum experiment applied to each breast cancer cell lines

2.11 Supernatant swap experiments

To examine if our observations were due to extracellular factors we performed various supernatant swap experiments. To add clarity to this experiment each is described in detail here.

2.11.1 Proliferation

MCF7 and MDA-MB231 were seeded at 100,000 and 50,000 cells per well of 6 well plate respectively. Both were treated with 20μM MKC-8866 / DMSO control for 48 hours. Supernatant from MKC-8866/DMSO treated wells were pooled and put back onto the cells in various ratios indicated for a further 48 h at which point they were harvested and counted.

2.11.2 Reduced serum

MDA-MB231 were seeded and treated as previously described. On Day 2 supernatant from DMSO treated cells was put onto wells treated with MKC and PI anaylsed on day 5. To ascertain if the effect was dependent protein factors, supernatnant from DMSO treated cells was heated to 55^{o}C for 30mins and this supernatant was then put on MKC8866 treated cells or as a control put back onto DMSO treated cells.

2.11.3 Cytokine array

Human XL cytokine Array kit was purchased from R&D systems (ARY022) and was completed with supplied reagents as per manufacturers' recommendations. In brief; membranes were blocked with buffer 6 for 1 h at RT. Membrane was incubated with 2mls of supernatant overnight at 4^{o}C on shaker. Next day membranes were washed three times with wash buffer for 10 mins each. Detection antibody cocktail was then added to membrane for 1 h at RT and then washes were repeated. 2ml of Streptavidin-HRP was incubated with membrane for 30mins at RT. Again the washes were repeated before 1ml of chemi Reagent Mix and the array developed.

2.12 ELISA

All ELISAs were purchased from R&D systems. IL-6 (DY206), IL-8 (DY208), Gro-α (DY275).

ELISA were carried out as follows. Coat plate in 100 µl of diluted capture antibody. seal an incubate overnight at RT. Aspirate each well and wash three times in wash buffer, invert plate against clean paper towel. Block plate by adding reagent diluents to each well and incubate at RT for 1 h. Repeat washes as above. Add 100 µl sample or standard in reagent diluents and incubate at RT for 2h. Repeat wash steps as above. Add 100 µl detection antibody diluted in reagent diluents to each well, cover and incubate at RT for 2 h. Repeat wash steps as above. Add 100 µl substrate solution to each well. Incubate for 20 mins at RT in dark. Add 50 µl stop reagent to each well, gently top plat eo ensure mixing. Determine the optical density of each well using microtitre plate set to 540-570nM. Quantify sample from standard curve of standards

Chapter 3

Investigating IRE1/XBP1s inhibition by MKC-8866 in breast cancer

3.1 Introduction and research rationale

The cancer microenvironment is exposed to a range of adverse conditions such as hypoxia, nutrient starvation and oxidative stress. Despite this tumours have evolved strategies to thrive and survive these conditions, often through modulation of the UPR (247). The UPR has been implicated at multiple levels of cancer biology with increased protein levels of UPR markers often reported, for example increased levels of GRP78 has been observed in breast, colon, and adenocarcinoma cancer cell lines (14). In particular there is growing evidence linking enhanced UPR activation to breast cancer tumourigenesis. Reduced expression levels of GRP78 in a transgenic breast cancer model led to reduced cell proliferation, increased apoptosis and decreased angiogenesis resulting in prolonged tumour latency (248). Loss of PERK attenuated breast tumour development in a transgenic model, and PERK was implicated in limiting oxidative damage in tumour cells (249). In more recent times, research has focused on the potential role of IRE1/XBP1s in breast cancer.

XBP1 is reported to be over expressed across a range of breast cancer types in both cell lines and primary patient samples (250-252). Elevated XBP1s levels are observed across all breast cancer subtypes; however the highest levels are reported to occur within TNBC (125, 251). XBP1s is also directly linked to epithelial to mesenchymal transition (EMT), which is a vital process for the invasion of epithelial tumours. Li *et al* 2015 demonstrated that XBP1s could directly promote EMT through induction of the key EMT regulator, SNAIL (251). Furthermore, increasing evidence suggest that XBP1s may be involved in early tumourigenesis process with a report indicating that endoplasmic reticulum (ER) lipid raft-associated 2 (ERLIN2), a proposed oncogenic factor in breast cancer, is modulated by IRE1/XBP1s axis (253).

Laurie Glimchers group have spear headed the recent work implicating XBP1s in cancer progression. In 2014, her group reported reduced breast cancer tumour growth and relapse, in addition to reduced expression of breast cancer stem cell markers $CD44^{high}$ $CD24^{low}$ following XBP1 knockdown. While it was previously established

that XBP1 was required for cellular survival during hypoxia condition (126), this study also reported that XBP1s directly interacted with hypoxia inducible factor 1 (HIF1) and was required for complete downstream hypoxic response (125). This result was interesting as it places XBP1s at the epicentre of breast cancer tumour maintenance. This year Glimchers group further characterised the role of XBP1s in immunity; reporting that XBP1s upregulation by lipid peroxidation by-products in tumour associated dentritic cells (tDC) induces a triglyceride biosynthetic program, resulting in massive lipid accumulation in the cell (254). This accumulation inhibits the ability of tDC to recruit anti-tumour T-cells and so confers a survival advantage. Knockdown of XBP1 in tDC showed a decrease in tumour burden and 30% increase in survival across three preclinical models of ovarian cancer (254).

There are also strong links to associate XBP1s to overall breast cancer patient survival, with XBP1s induction linked to poor relapse-free survival in TNBC patients (125) and the ratio of XBP1u / XBP1s correlated to relapse free survival time in breast cancer patients (255). These reports plus others in the field demonstrate that XBP1s plays an important role in tumourigenesis and most likely confers a survival advantage to breast cancer cells. This establishes a prerequisite for the development of therapeutically relevant inhibitor of IRE1 RNase domain. Through collaboration with MannKind Corporation, our lab attained MKC-8866; which is a new generation IRE1 inhibitor of MKC-3946, previously shown by Anderson *et al* to have clinical potential in multiple myeloma cell lines (163). MKC-8866, unlike its predecessor, has to date shown no toxicity or off target effects in both *in vitro* and *in vivo* studies and so presents itself as a possible new and exciting clinical tool.

The aim of this study was to

- Characterise the effect of MKC-8866 on breast cancer cell lines
- To establish if any therapeutic effect could be observed using MKC-8866 as a single agent

3.2 Results

3.2.1 Basal levels of XBP1s across a range of breast cancer cell lines

At the outset of this work previous literature had shown that XBP1s is present at basal levels in a range of breast cancer cell lines at the mRNA level; however the literature failed to distinguish between basal levels of XBP1s from XBP1u at the protein level in breast cancer. This differentiation is vital, as splicing is required to generate the potent pro-survival transcription factor XBP1s. To this end I set out to assess the protein expression level of XBP1s in a range of breast cancer subtypes. As previously mentioned breast cancer subtypes are defined by the presence of one or more of the three receptors, receptor status of cell lines used is shown in table 4.

Cell line	ESR1	PR	HER2
MCF7	+	+	-
T47D	+	+	-
SKBR3	-	-	+
MDA-MB231	-	-	-
MDA-MB468	-	-	-

Table 4 Cell line receptor classification. ESR1, PR and HER2 status of each cell lined screened for basal XBP1s levels.

XBP1s protein is found basally across a range of breast cancer cell lines and is most highly upregulated in TNBC cell lines MDA-MB231 and MDA-MB468 as shown in figure 3.2.1.

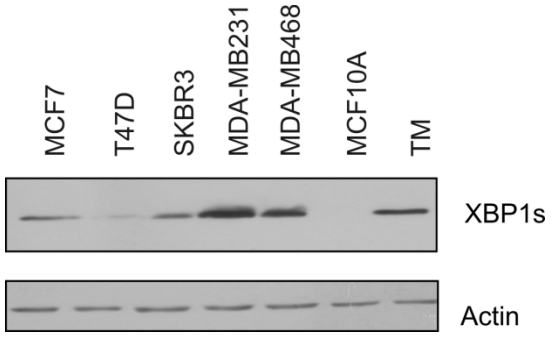


Figure 3.2.1 Basal levels of XBP1s across a range of breast cancer cell lines. Expression of XBP1s was assessed by Western blotting in MCF7, T47D, SKBR3, MDA-MB231, MBA-MB468 and MCF10A. MCF10A cells treated with 1 µg/ml of tunicamycin were used as a positive control. ACTIN was used as a loading control. Data is representative of three independent repeats.

3.2.2 *Characterization of MKC-8866*

MKC-8866 dose response was characterised in MCF10A cells. MCF10As were treated with a vehicle control or 1 µM of Thapsigargin (Tg) for 24 h along with 10 µM – 40 µM of MKC-8866. RNA samples were harvested and subjected to RT-PCR for XBP1 and GAPDH. It was and determined that 10 µM – 20 µM of MKC-8866 was sufficient to inhibit XBP1s (Fig 3.2.2B). MKC-8866 elicits its effect as quickly 4 h (Fig 3.2.2C) shown via qPCR time course analysis of XBP1s levels and downstream targets of XBP1s- ERDJ4 and HERP in MDA-MB231. Additional RT-PCR analysis in MDA-MB231 shows inhibition is maintained up to 4 days post treatment without the need for being replenished in the system (Fig 3.2.2D).

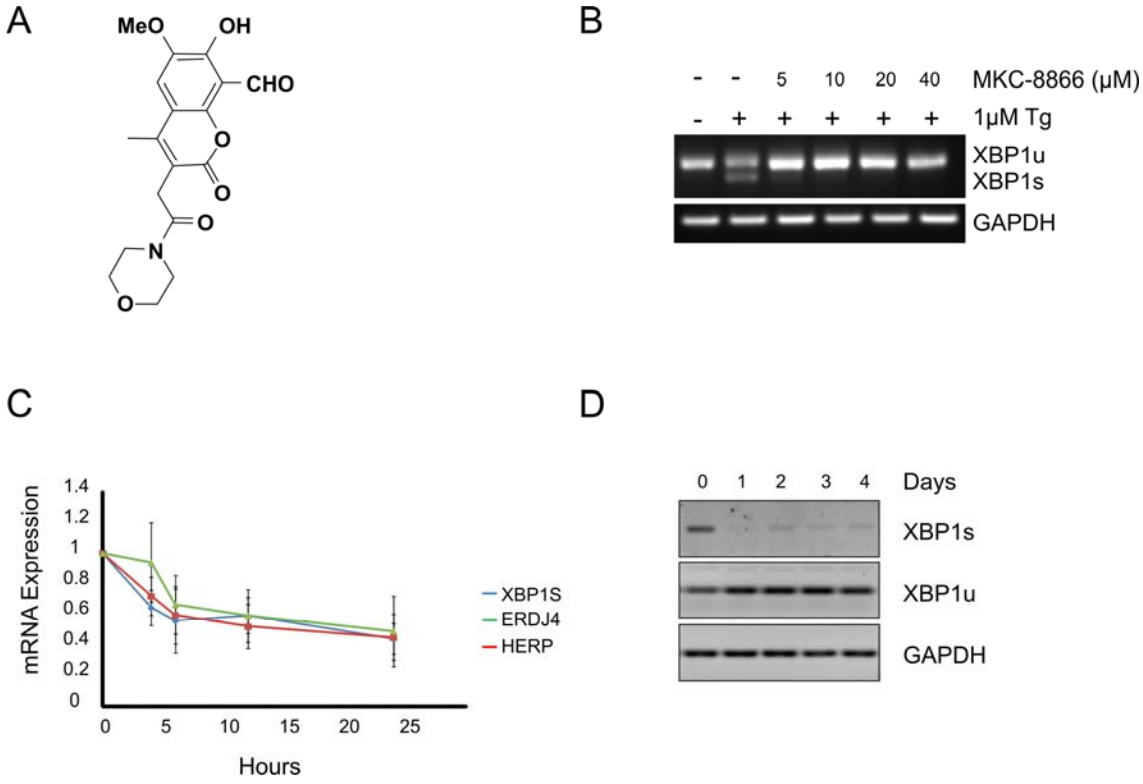


Figure 3.2.2 Characterization of MKC-8866. (A) Structure of MKC-8866. **(B)** Expression levels of XBP1 mRNA levels in MCF10A cells following 1µM of TG cotreated with 5-40 µM for 24 h was quantified by real-time RT-PCR, normalizing against GAPDH. **(C)** qPCR analysis of XBP1s and downstream targets ERDj4 and HERP over time following treatment with MKC-8866.(CREDIT to Eoghan McGrath for this qPCR result) **(D)** PCR analysis of XBP1s over time (1-4 days) following treatment with MKC-8866. Values shown ±SD are representative of three independent repeats.

3.2.3 MKC-8866 is specific to the IRE1 arm of the UPR

Confident that MKC-8866 could reduce XBP1s levels in this system, next the effect of MKC-8866 on the other arms of UPR was determined. In order to investigate this, T47D cells were treated with 1 μM of Tm alone or in combination with MKC-8866. MKC-8866 was specific to IRE1 arm as co-treatment with MKC-8866 and Tm did not augment UPR signalling as measured by expression of p-PERK, CHOP or cleaved ATF6 relative to Tm alone (Fig 3.2.3A). Therefore MKC-8866 is specific to the IRE1 arm of the UPR. Furthermore, MKC-8866 also inhibited basal levels of XBP1s (Fig 3.2.3B) across different cell lines, indicating that IRE1 activation by an external stimulus is not required for basal inhibition by MKC-8866.

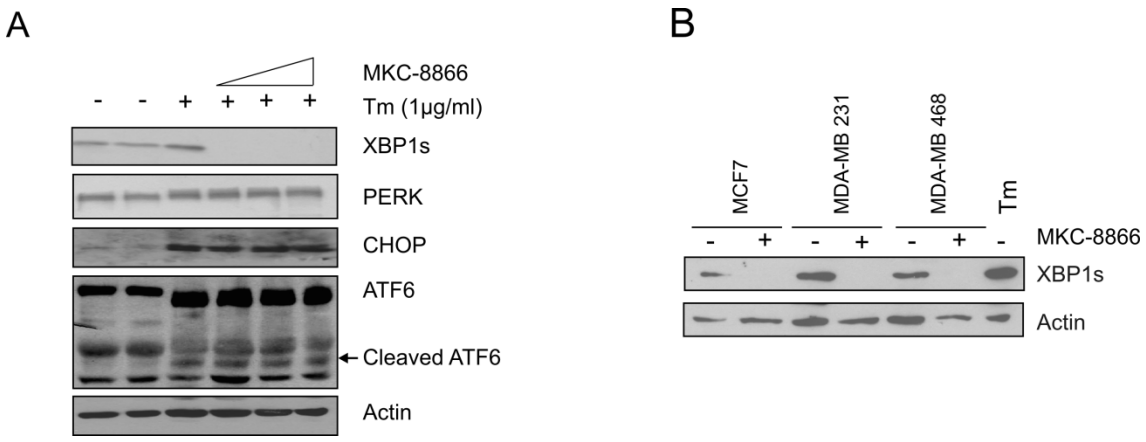


Figure 3.2.3 Effect of MKC-8866. (**A**). MKC-8866 can reduce XBP1s levels following 1 μg/ml Tm treatment for 24 h but did not affect the other UPR arms shown via p-PERK, CHOP and cleaved ATF6 induction. **(B).** MKC-8866 can reduce basal XBP1s levels in MCF7, MDA-MB231 and MDA-MB468 cell lines at 24 h. Blots are representative of 3 independent repeats

3.2.4 Effect of MKC-8866 in breast cancer

As basal levels of XBP1s were observed across various breast cancer cell lines I speculated that breast cancer cells may be addicted to this pro-survival transcription factor, therefore T47D and MDA-MB231 cell lines were initially treated with MKC-

8866 alone. To ascertain if cell survival was reliant upon XBP1s expression cell viability was first monitored by MTT. MKC-8866 over time reduced the viability of cells as measured by MTT assay (Fig 3.2.4 A-B). Contrary to this result however cells treated with MKC-8866 or vehicle DMSO control for 72 h did not result in significant cell death measured by annexin V/ PI staining as shown in Fig 3.2.4C. These cells are capable of exposing phosphatidylserine as 500 nM Taxol for 24 h shows both annexin V and PI positivity. Additionally MKC-8866 caused a decrease in migration rate of MDA-MB231 (Fig 3.2.4D).

While MTT may often be regarded as a cell viability assay this is not technically true as MTT assay is a colorimetric assay based on the metabolic activity of cellular enzymes that reduce the tetrazolium dye, MTT, to its insoluble formazan therefore the reduction in value observed in Fig 3.2.4 A-B could be due to cell number differences over time. Overall cell number was monitored following IRE1 inhibition as a possible explanation for a decrease in viability measured by MTT. Interestingly, both cell lines showed a significant reduction in cell number at 72 h following IRE1 inhibition (Fig 3.2.4 E-F)

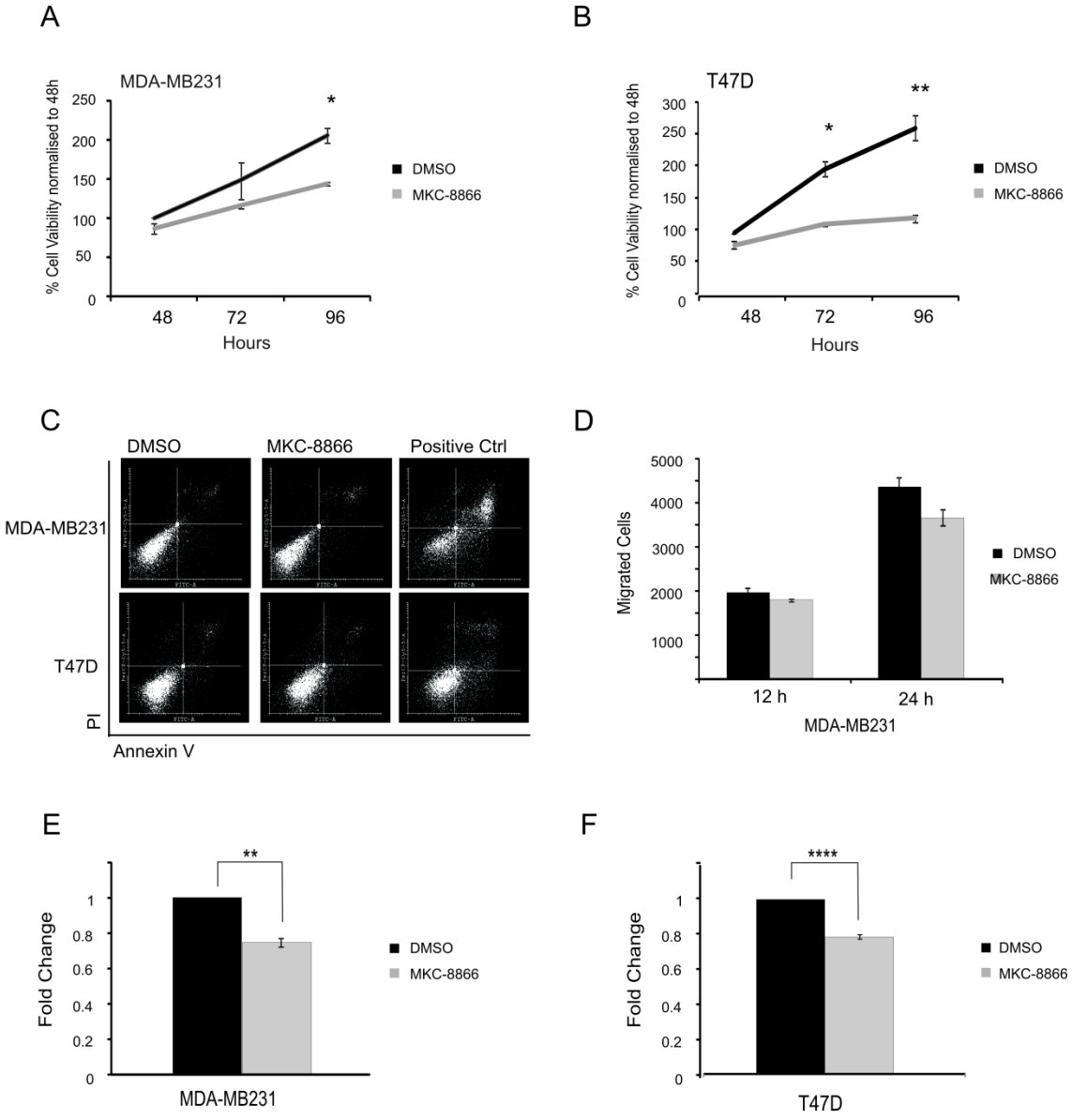


Figure 3.2.4 Effect of MKC-88 on breast cancer cells T47D and MDA-MB231. (**A-B)** MTT analysis over time following MKC-8866 treatment in MDA-MB231 **(A)** and T47D **(B). (C)** Annexin V /PI staining in MDA-MB231 and T47D following treatment with MKC-8866 for 72 h. 500nM Taxol for 24 h used as positive control **(D)** Transwell migration assay in MDA-MB231 shows MKC-8866 may reduce migration rate of these cells. Cell number at 72 h following MKC-8866 treatment, cell number represented as fold change relative to control cells MDA-MB231 **(E)** and T47D **(F).** Values shown are representative of three independent ±SEM P <0.05, ** P<0.01, *** P< 0.001 Students *t*-test

3.2.5 MKC-8866 decreases proliferation rate of breast cancer cell lines

Previous literature has suggested that IRE1 and its downstream target XBP1s may play a role in the proliferation rate of pancreatic and prostate cancer cell lines (256, 257). As a decrease in cell number was observed which did not correspond to cell death, the effect of MKC-8866 on the proliferation rate of the cells was determined. The cells were treated with MKC-8866 and counted every second day for six days. They were seeded in a 12 well plate and passaged when they became 70% confluent, therefore they did not reach a plateau phase but remain in logarithmic growth. At each timepoint trypan blue positivity was also assessed to ensure that a decrease in cell number could not be attributed to cell death. Cell proliferation in ESR1+ positive cell lines MCF7 and T47D was first investigated. There was a significant reduction in cell number over time that could not be attributed to cell death in these cell lines as shown in Fig 3.2.5A-B. As growth analysis is open to bias, a 5-ethynyl-2′-deoxyuridine (EdU) incorporation assay was performed. For this assay the cells are incubated with 10 μM EdU for 1 h pulse treatment prior to harvesting. During this hour EdU will be incorporated into cells undergoing DNA synthesis and is subsequently detected by a fluorescent azide through a Cu(I)-catalyzed [3 + 2] cyclo addition reaction ("click" chemistry). This azide is tagged with Fluorescein isothiocyanate (FITC) and therefore can be measured by flow cytometry. MCF7/T47D cells treated with 20 μM MKC-8866 demonstrated a significant reduction in S phase cells as shown in Fig 3.2.5 C-D. Etoposide a DNA damage inducer was used as a positive control to induce cell cycle arrest.

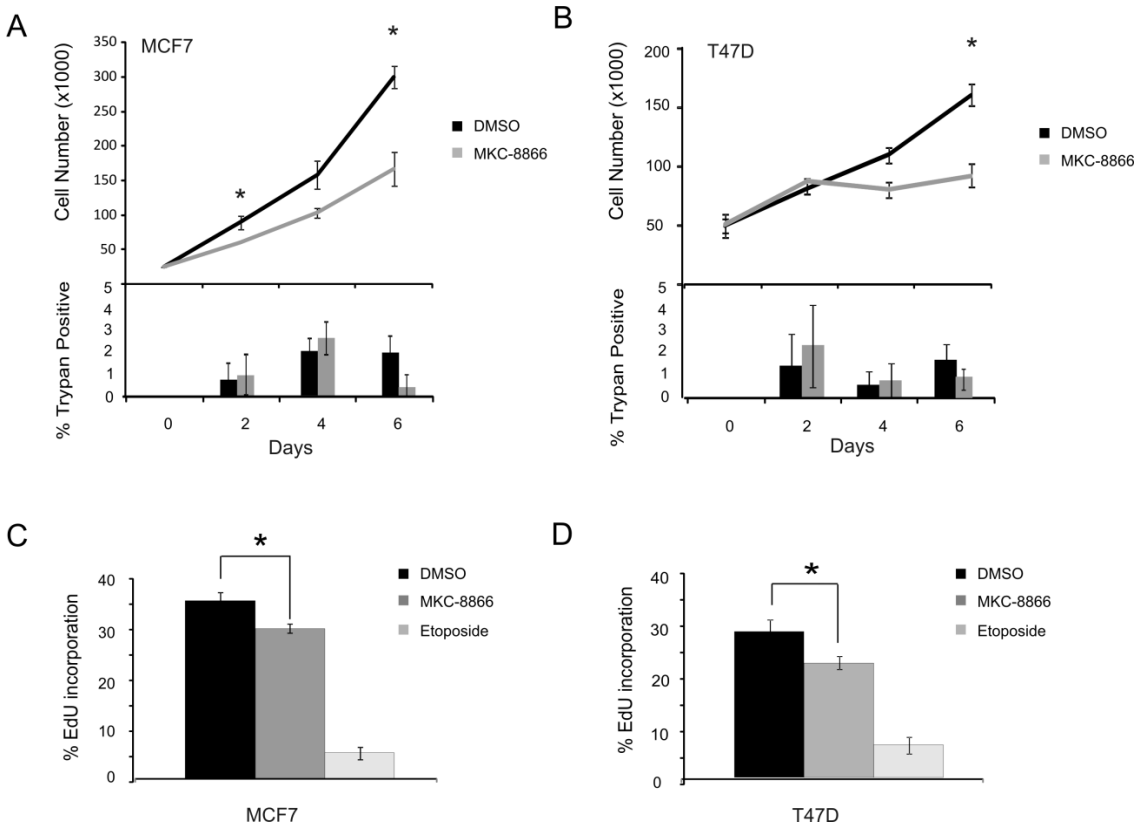


Figure 3.2.5 MKC-8866 reduces proliferation rate of ER positive cell lines. MKC-8866 reduces **(A)** MCF7 and **(B)** T47D cell number over time which is not due to cell death. EdU incorporation shows a reduction in S phase cells in **(C)** MCF7 and **(D)** T47D following MKC-8866 treatment. Etoposide acts a positive control of cell cycle arrest**.** Values shown are representative of three independent ±SEM P <0.05, ** P<0.01, *** P< 0.001 Students *t*-test

3.2.6 MKC-8866 reduces proliferation rate of TNBC cell lines

To avoid limiting this result to one particular breast cancer subtype, the effect of MKC-8866 on TNBC cell lines MDA-MB231 and MDA-MB468 was also determined. Taking the same approach described above cell number over time was monitored. MKC-8866 could reduce cell number in a manner that was not associated with cell death induction as trypan blue positivity remained comparable to vehicle treated cells (Fig 3.2.6A-B). Interestingly the cells continued to grow in the presence of MKC-8866 suggesting that an overall decrease in proliferation was occurring. Analysis of EdU incorporation confirms this that there is a reduction in S phase cells demonstrating a reduction in proliferation rate (Fig 3.2.6 C-D).

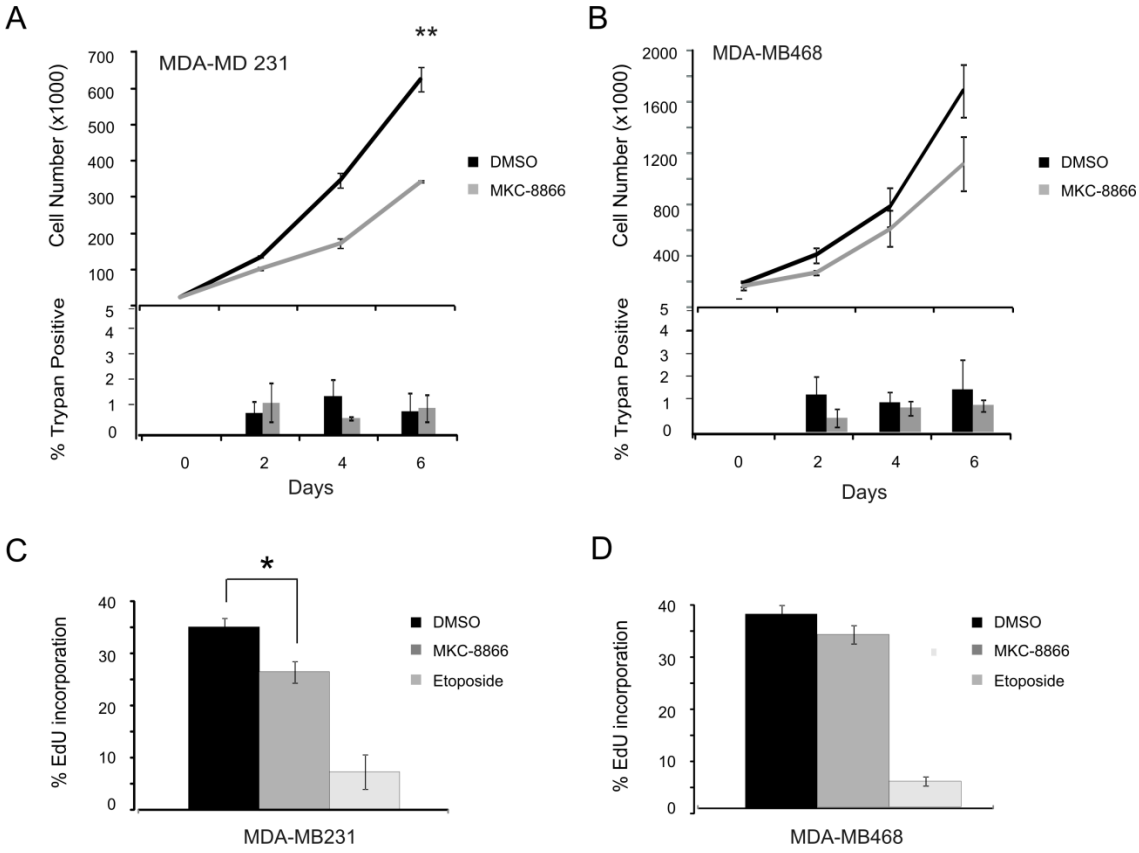


Figure 3.2.6 MKC-8866 reduces proliferation rate of TNBC cell lines. MKC-8866 reduces **(A)** MDA-MB231 and **(B)** MDA-MB468 cell number over time which is not due to cell death. EdU incorporation shows a reduction in S phase cells in **(C)** MDA-MB231 and **(D)** MDA-MB468 following MKC-8866 treatment. Etoposide is a positive control of cell cycle arrest. Values shown are representative of three independent ±SEM P <0.05, ** P<0.01, *** P< 0.001 Students *t*-test

3.2.7 MKC-8866 decreases proliferation in HER2 positive cells

As previous data demonstrated a decrease in proliferation rate of two subtypes ESR1+ positive and TNBC, a cell line representing the HER2 positive subtype, SKBR3 cells was also analysed. A comparable decrease in proliferation occurs that is not attributed to cell death induction monitored by trypan blue incorporation (Fig 3.2.7A). Reduction in proliferation rate was further confirmed by reduced EdU incorporation in SKBR3 cells following MKC-8866 treatment for 72 h (Fig 3.2.7B).

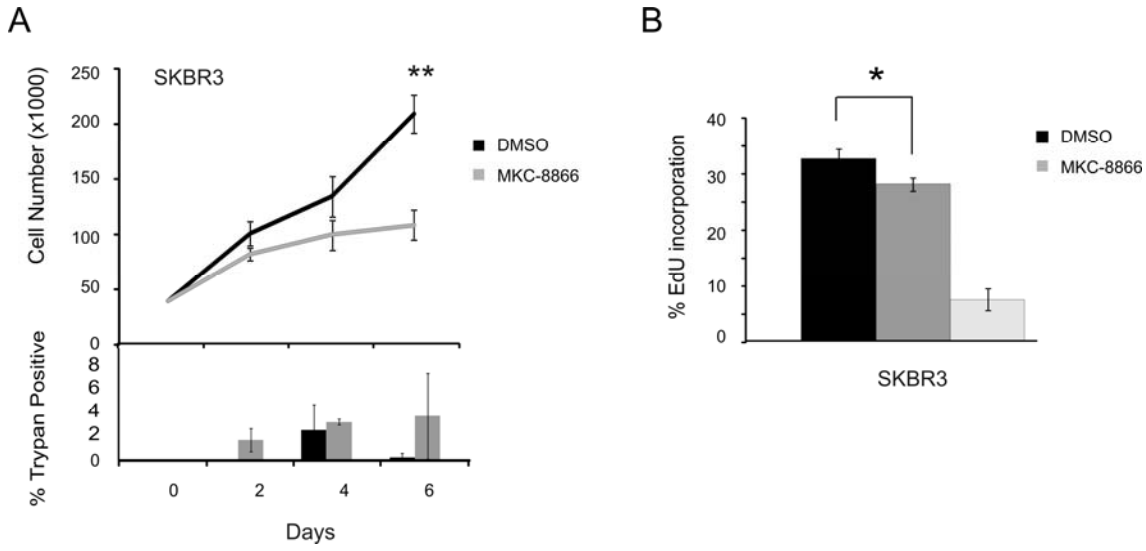


Figure 3.2.7 MKC-8866 reduces proliferation rate of HER2 positive cells. MKC-8866 reduces **(A)** SKBR3 cell number over time which is not due to cell death. **(B)** EdU incorporation shows a reduction in S phase cells in following MKC-8866 treatment. Etoposide is a positive control of cell cycle arrest. Values shown are representative of three independent ±SEM P <0.05, ** P<0.01, *** P< 0.001 Students *t*-test

3.2.8 MKC-8866 mediates its effect via IRE1/XBP1s.

To confirm whether the decrease in proliferation observed was mediated by XBP1s directly or was due to RIDD inhibition or alternatively an off target effect of the drug, a XBP1 knockdown cell line was generated. MDA-MB231 cell were virally transduced with either a short hairpin (sh) RNA targeting XBP1, or the corresponding empty vector control (PLKO). These cells were then selected via puromycin and basal knockdown

was confirmed via western blotting for XBP1s (Fig 3.2.8A). In line with previous data, MDA-MB231-shXBP1 cells, proliferate significantly slower than their PLKO expressing counterparts, suggesting that XBP1 has a positive effect on cell proliferation. Again trypan blue incorporation confirms that the reduced proliferation of XBP1 knockdown cells was not due to cell death. EdU incorporation results confirmed that XBP1 deficiency had an effect on level of S phase cells (Fig 3.2.8C). Additionally to ensure that the reduction in proliferation observed with MKC-8866 is truly mediated via IRE1/XBP1s and not a by product result of applying this inhibitor, the proliferation rate of a cell line known to lack basal XBP1s expression; MCF10A was analysed (Fig 3.2.8). MKC-8866 had no effect on cell number of MCF10A (Fig 3.2.8B) and did not alter number of cells in S phase (Fig 3.2.8D). This confirms that MKC-8866 reduces the proliferation rate of breast cancer cells via inhibition of IRE1/XBP1 axis.

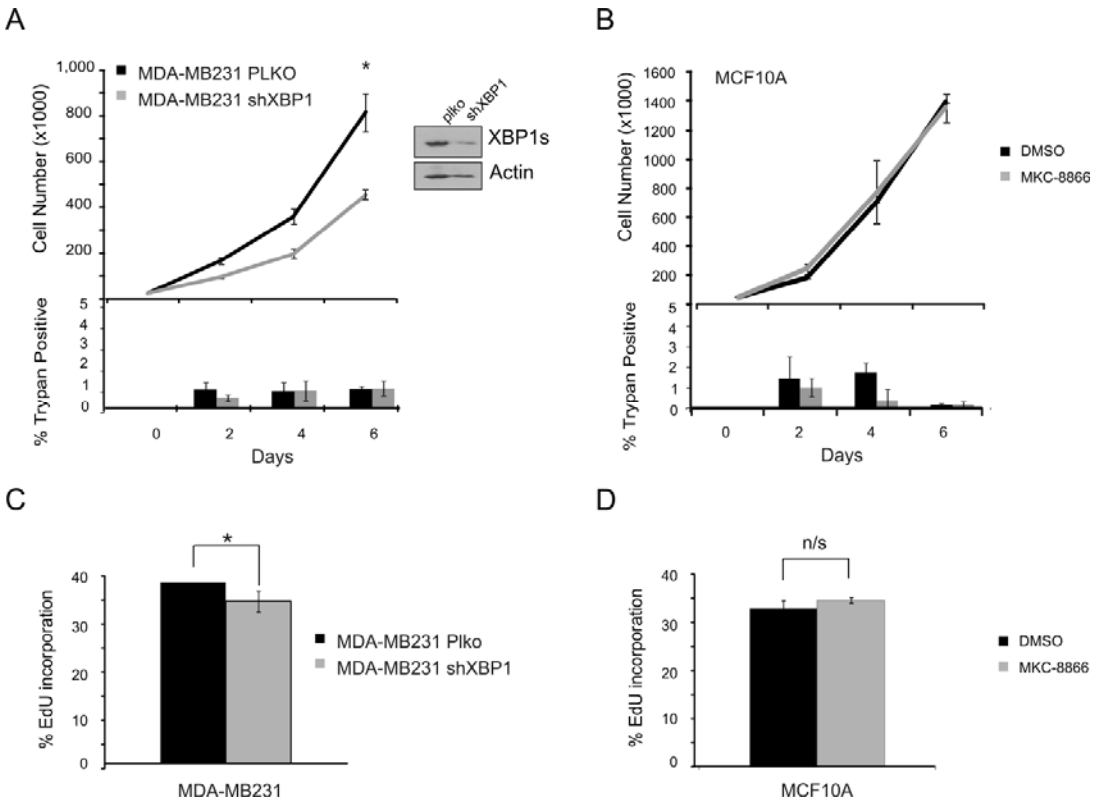


Figure 3.2.8 MKC-8866 mediates its effect via IRE1/XBP1s. (**A)** MDA-MB231 shXBP1 grow significantly slower than PLKO counterparts which could not be attributed to cell death shown via trypan blue incorporation. **(B)** MKC-8866 does not affect the growth of MCF10A cells which lack basal XBP1s levels. **(C)** EdU incorporation assay confirms that knockdown of XBP1 reduces proliferation of MDA-MB231. **(D)** MKC-8866 does not affect the proliferation of MCF10A cells as determined by EdU incorporation. Values shown are representative of three independent ±SEM P <0.05, ** P<0.01, *** P< 0.001 Students *t*-test

3.2.9 Overexpression of XBP1s promotes cell proliferation

Data thus far has demonstrated that inhibition of XBP1s could reduce the proliferation of breast cancer. Based on this observation the effect of overexpressing XBP1s was tested. HCC1806, a TNBC cell line, were virally transduced with pwpt-XBP1s or control empty vector pwpt (Fig 3.2.9C) and proliferation monitored as previously described. Similar to previous TNBC cells lines tested, MKC-8866 could reduce cell number over time of HCC1806 (Fig 3.2.9A). Significantly XBP1s overexpression increased the proliferation rate of HCC1806 (Fig 3.2.9B).

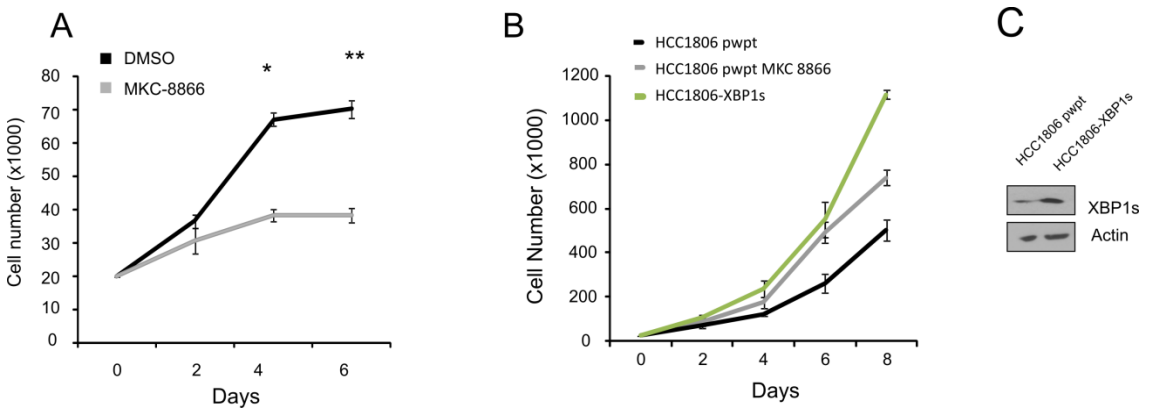


Figure 3.2.9 XBP1s drives proliferation. **(A)** MKC-8866 could reduce the proliferation rate of HCC1806. **(B)** Overexpression of XBP1s could increase proliferation while MKC-8866 could reduce proliferation of control cells. **(C)** Confirmation of XBP1s overexpression in HCC1806 via western blotting**.** . Values shown are representative of three independent ±SEM P <0.05, ** P<0.01, *** P< 0.001 Students *t*-test

3.2.10 Inhibition of the PERK pathway has no effect on cell proliferation

IRE1 is not the only arm of UPR linked to breast cancer biology, and a role for PERK is also reported (249). To determine if the observed effect on proliferation is solely linked to IRE1 axis or a general result of UPR inhibition, a PERK kinase inhibitor was applied to the cells and cell number monitored 72 h later. It is only through IRE1 inhibition and not via PERK inhibition that a decrease in proliferation is observed in MDA-MB231. (Fig 3.2.10)

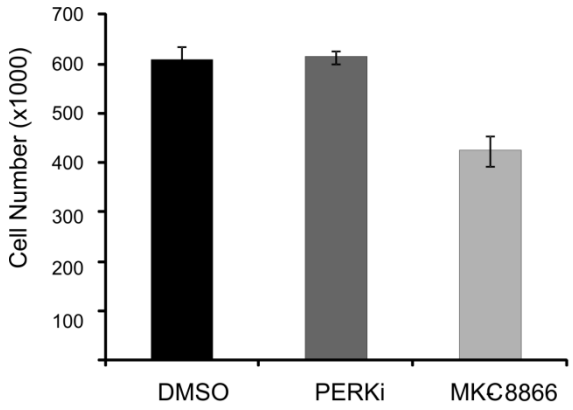


Figure 3.2.10 IRE1 but not PERK inhibition reduces proliferation. MKC-8866 can reduce cell number over time but similar results with PERK inhibition are not observed. . Values shown are representative of three independent ±SEM P <0.05, ** P<0.01, *** P< 0.001 Students *t*-test

3.2.11 XBP1 regulation of cell cycle

A decrease in EdU incorporation may suggest that MKC-8866 is altering cell cycle progression. IRE1/XBP1s role in cell cycle is not fully understood however one study suggests that XBP1 may regulate Cyclin A1 (258). To determine if MKC-8866 is affecting cell cycle PI staining was applied and overall DNA content measured via flow cytometry. In MDA-MB231 cells, a significant increase in cells in G1 phase is observed compared to control following MKC-8866 treatment for 72 h, however contradictory result are ascertained in T47D, in which we see a marked decrease in G1 phase cells (Fig 3.2.11 A, C). In line with the literature, a decrease in XBP1s expression can alter Cyclin A1 levels in MDA-MB231 (Fig 3.2.11B) however this is not recapitulated in T47D cells (Fig 3.2.11C). This suggests that additional factors downstream of XBP1s may also be capable of modulating proliferation.

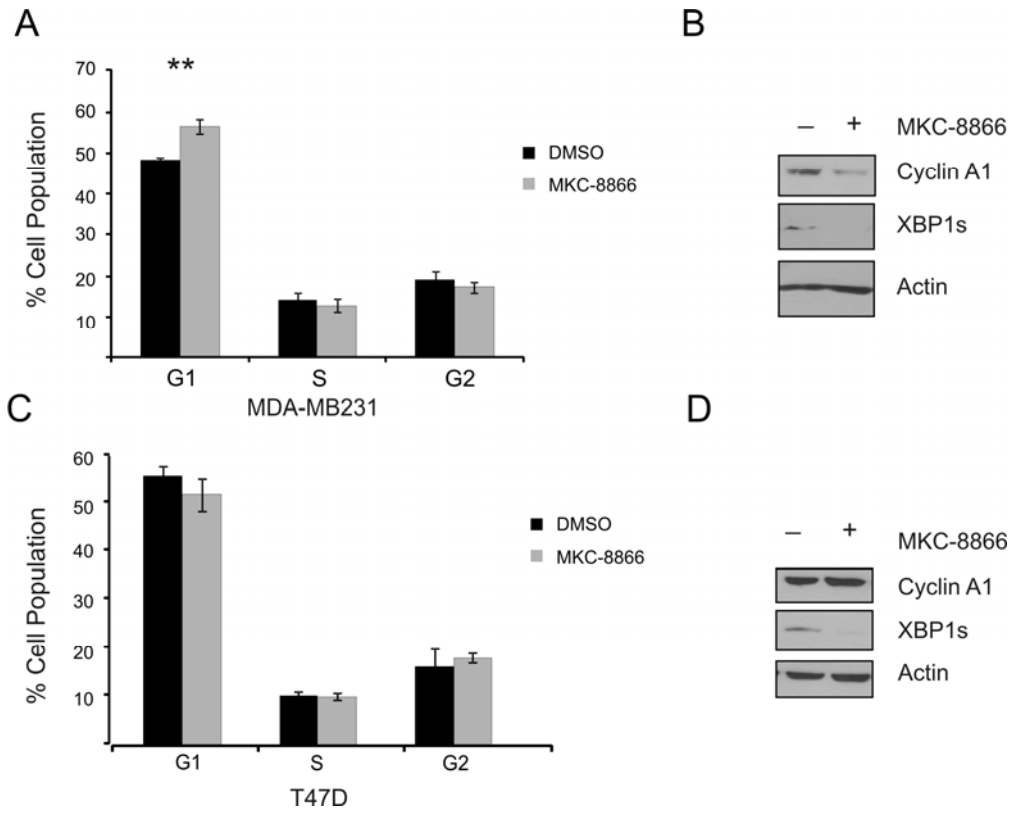


Figure 3.2.11 Cell Cycle analysis following MKC-8866 treatment. (A) Cell cycle analysis of MDA-MB231 following 48 h treatment with MKC-8866. **(B)** MKC-8866 can affect cyclin A1 levels in MDA-MB231. **(C)** Cell cycle analysis of T47D following 48 h treatment with MKC-8866. **(D)** MKC-8866 cannot affect cyclin A1 levels in T47D. Values shown are representative of three independent ±SEM P <0.05, ** P<0.01, *** P< 0.001 Students *t*-test

3.2.12 IRE1 inhibition can potentiate cell death during reduced nutrient conditions

XBP1 has been reported to be essential for tumour establishment and is an important pro-survival component of the UPR. I therefore wanted to consider the potential of down regulating XBP1s during nutrient deprivation, a classical condition of the tumour microenvironment. Two experimental models were established to analyse this hypothesis; reduced glucose levels and reduced serum conditions.

Tumours are often exposed to reduced glucose levels, and so to mimic this condition *in vitro*, cells were cultured in glucose free media but supplemented with 10% FBS. This allowed the cells to have a limited but diminished source of glucose as compared with

normal culture conditions. MKC-8866 or DMSO as a vehicle was added to the cells and cell death was monitored via PI incorporation. Surprisingly MKC-8866 did not enhance cell death but rather elicited a slight protective effect over time in MDA-MB231 (Fig 3.2.12A) and HCC1806 (Fig 3.2.12 B).

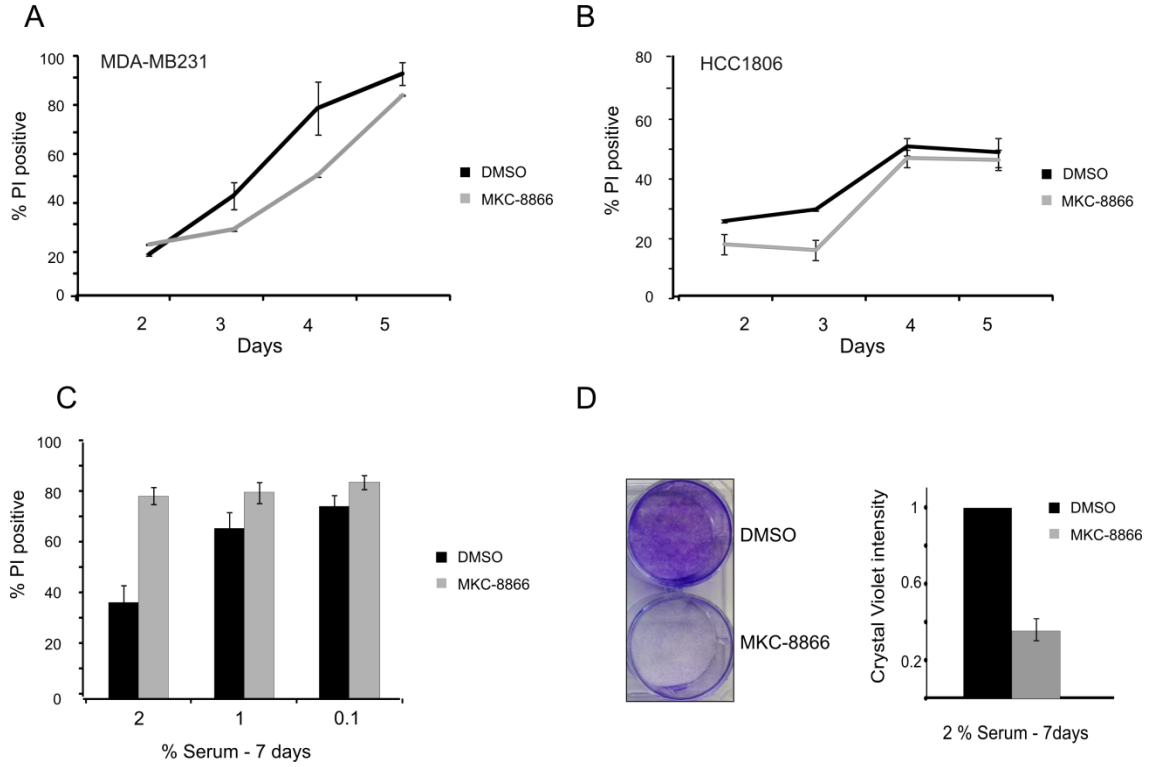


Figure 3.2.12; MKC-8866 protects cells during glucose reduction but enhances cell death during reduced serum levels. **(A)** MDA-MB231 **(B)** HCC1806 cells with/without MKC-8866 under reduced glucose conditions; PI incorporation is used as readout of cell death. **(C)** MKC-8866 enhanced cell death during reduced serum conditions in MDA-MB231**. (D)** crystal violet staining confirms cell death occurring at 2% serum in presence of MKC-8866. Values shown are representative of three independent ±SEM P <0.05, ** P<0.01, *** P< 0.001 Students *t*-test

3.2.13 MKC-8866 leads to cell death during reduced serum conditions.

As glucose is but one nutrient that may affect the tumour microenvironment, a reduction of global nutrient supply was next examined in the presence and absence of XBP1s activity. To achieve this reduction, a dose response to various levels of serum was completed with and without MKC-8866. As shown in Fig 3.2.12C IRE1 inhibition increased cell death across the three different serum concentrations in MDA-MB231, with the most predominant difference occurring at two percent serum. Fig 3.2.12D shows colony formation at day 7, with crystal violet intensity used as the readout of cell

viability. These data show a significant difference between MDA-MB231 cells treated with MCK-8866 versus vehicle control cells.

This observation was pursued by exploring the effect of MKC-8866 during reduced serum conditions across a range of cell lines. Analysis of PI incorporation over a five day time course in MDA-MB231 (Fig 3.2.13B), 10 day in MCF7 (Fig 3.2.13C), 7 day in HCC1806 (fig 3.2.13D) and 9 day in SKBR3 (Fig 3.2.13E) revealed that upon addition of MKC-8866, cells undergo cell death during reduced serum conditions. Most interestingly vehicle cells continue to proliferate during the course of the experiment, depicted in Fig 3.2.13A. This phenomenon occurred irrespective of breast cancer subtype and with such drastic differences, it warranted further investigation.

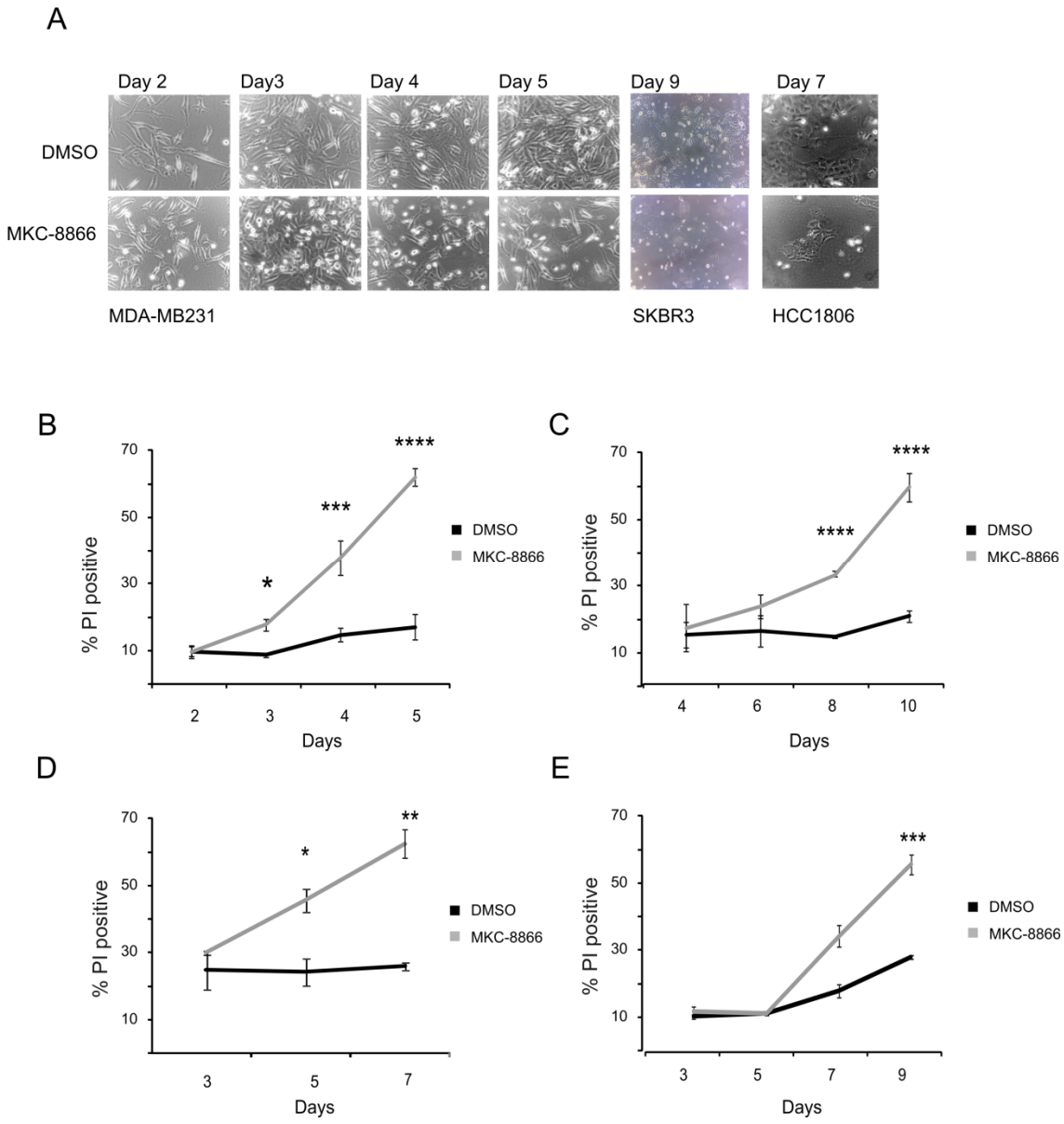


Figure 3.2.13; MKC-8866 leads to cell death during reduced serum conditions. (A) Cell images taken during reduced serum conditions in MDA-MB231, SKBR3 and HCC1806. MKC-8866 induced cell death measured by PI incorporation over time in **(B)** MDA-MB231 at 2% serum, **(C)** MCF7 at 1% serum, **(D)** HCC1806 at 2%serum and **(E)** SKBR3 at 2% serum. Values shown are representative of three independent ±SEM P <0.05, ** P<0.01, *** P< 0.001 Students *t*-test

3.2.14 MKC-8866 induces an apoptotic response during reduced serum conditions.

To better understand what was occurring in serum starved cells treated with MKC-8866 the mode of cell death occurring was determined. Apoptosis is the most common form of cell death and mitochondrial potential was measured via loss of TMRE. Addition of MKC-8866 caused approximately 50% increase in the proportion of cells with low mitrochondrial membrane potential (Fig 3.2.14A). These results are consistent with data obtained with PI analysis (Fig 3.2.14). A similar trend was observed in HCC1806 (Fig 3.2.14B). Collectively this data is suggestive of an apoptotic mode of cell death occurring in serum starved cells. This conclusion was further supported by the presence of CASPASE 3 cleavage, a classical executioner caspase, as measured by western blotting and noted a marked increase in cleaved CASPASE 3 in presence of MKC-8866 as compared to vehicle. PARP, a caspase substrate, also underwent enhanced cleavage in cells treated with MKC-8866 (Fig 3.2.14C-D). This confirms that MKC-8866 induces an apoptotic response during reduced serum conditions.

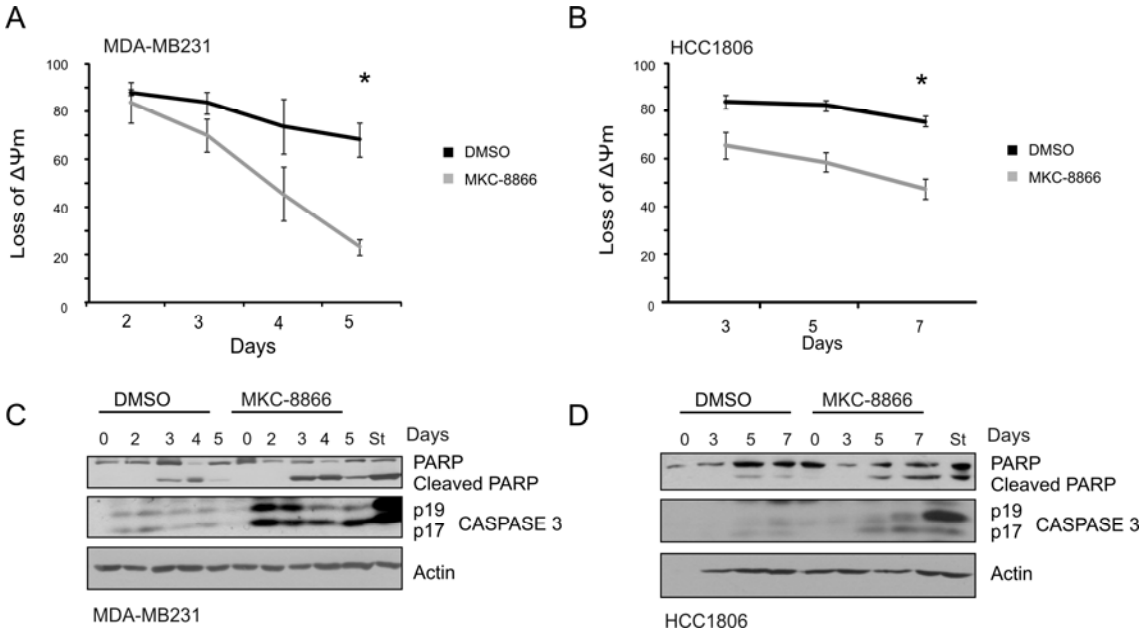


Figure 3.2.14 MKC-8866 induces an apoptotic response during reduced serum conditions. (A) MDA-MB231 and **(B)** HCC1806 cultured in reduced serum conditions were treated with 10 μM MKC-8866 or with DMSO control for 2-5 days. ΔΨm was assessed by TMRE staining and flow cytometry. PARP and caspase 3 cleavage were assessed by western blottingin **(C)** MDA-MB231 and **(D)** HCC1806 with MKC-8866 in reduced serum conditions. Actin was used as a loading control. Values shown are representative of three independent ±SEM P <0.05, ** P<0.01, *** P< 0.001 Students *t*-test

3.2.15 *MCK-8866 induced apoptosis in reduced serum conditions in a manner which is dependent on reduced XBP1 levels*

To ascertain whether induction of apoptosis during reduced serum conditions is mediated via XBP1s, induction of apoptosis in reduced serum condition was assayed in XBP1 knockdown cell line MDA-MB231 shXBP1 and control, MDA-MB231 PLKO. Cell death over time at one percent serum levels was measured by PI staining and flow cytometry. XBP1 knockdown cells showed enhanced cell death (Fig 3.15A-B). While the overall trend of enhanced cell death in XBP1s knockdowns was similar to that observed previously described it was not as profound as wild-type MDA-MB231 with addition of the inhibitor. This is owed to incomplete knockdown of XBP1s in this system (Fig 3.2.15C).

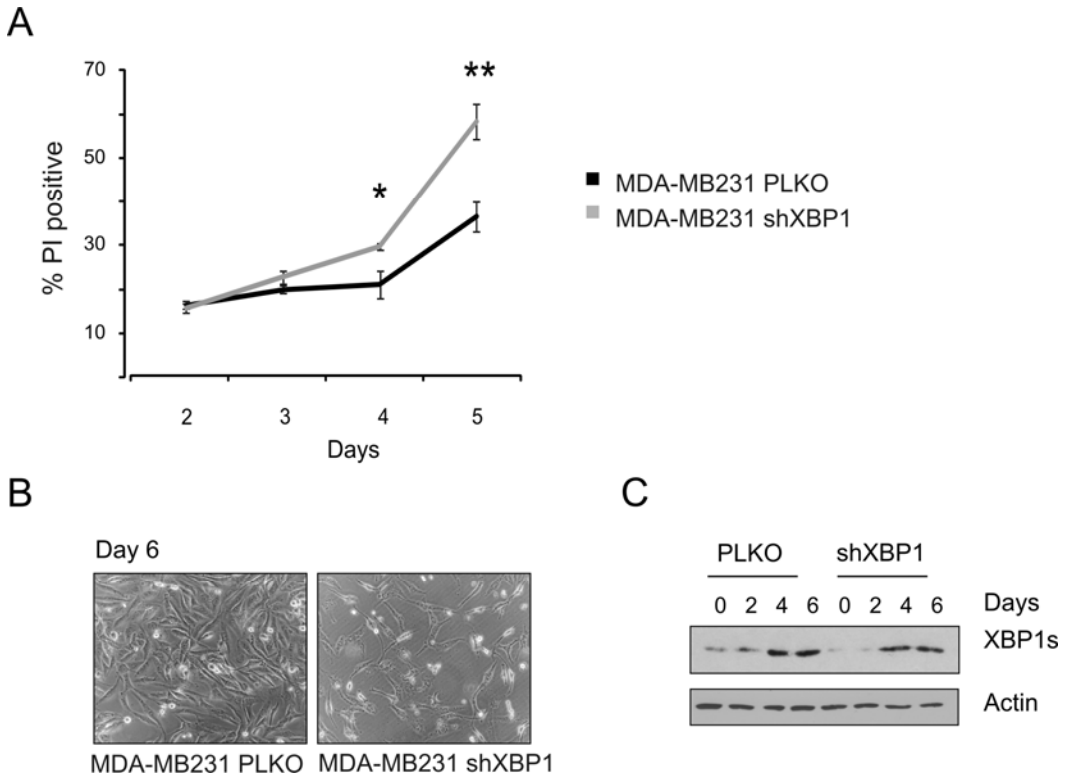


Figure 3.2.15 Apoptosis is due to reduced XBP1 levels. (A) PI incorporation analysis demonstrates enhanced cell death in MDA-MB231 XBP1 knockdown cells as compared to control. **(B)** Images taken on day 6 of experiment highlight the cellular difference between both cell lines. **(C)** Western blotting analysis confirms knockdown of XBP1s at initial stages of experiment is re-expressed over time. Values shown are representative of three independent ±SEM P <0.05, ** P<0.01, *** P< 0.001 Students *t*-test.

3.2.16 UPR activation during reduced serum levels is enhanced by MKC-8866

The induction of cell death was occurring due to the inhibition of IRE1 RNase domain, an important arm of the UPR therefore it was important to understand what was occurring at the level of the UPR during these conditions. To monitor UPR activity western blotting analysis of a range of UPR markers; XBP1s, IRE1, p-PERK (monitored by upshift), ATF4, CHOP and ATF6 cleavage was completed. HCC1806 and MDA-MB231 (Fig 3.2.16A-B) reveals similar UPR profiles occurring in both cell lines. XBP1s inhibition is maintained during the course of the experiment and MKC-8866 does not affect overall IRE1 levels. However earlier and enhanced activation of ATF4, CHOP and cleaved ATF6 occur with treatment of MKC-8866 in both cell lines. It is possible that the absence of pro-survival XBP1s drives the cell towards a quicker terminal UPR response.

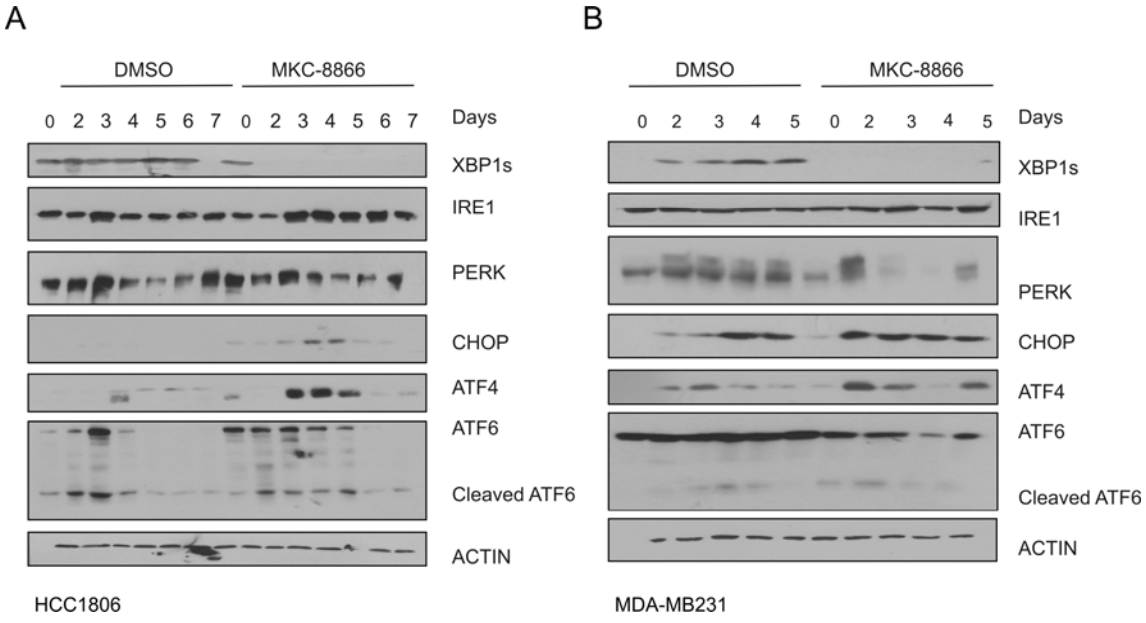


Figure 3.2.16 UPR activation during reduced serum levels is enhanced by MKC-8866. UPR profile of **(A)** HCC1806, and **(B)** MDA-MB231 during reduced serum conditions in the presence and absence of MKC-8866. Data shown are representative of three independent repeats

3.2.17 Extracellular factors mediate the effect of MKC-8866

In order to delineate how MKC-8866 is eliciting its effect on proliferation and survival; whether the mechanism was via intracellular or extracellular factors was assessed. Cells were treated with MKC-8866 or corresponding DMSO control and allowed to grow for 2 days, at which point the supernatant was removed from MKC-8866 treated cells and replaced with media from DMSO treated cells. Cells were re-incubated for a further 2 days and total cell number was counted on day 4 of the experiment. This would determine if MKC-8866 was altering the secretome and in doing so was affecting the proliferation rate of the cells. MKC-8866 significantly reduced cell number compared to DMSO control cells and this reduction was reversed in cells whose supernatant was replaced on day 2 with DMSO supernatant (Fig 3.2.17A-B). Moreover, when the DMSO supernatant was diluted with MKC-8866 supernatant in ratios of 80%DMSO: 20% MKC, 60%DMSO: 40%MKC and 40% DMSO: 60% MKC, the cell number reduced was rescued in a dose dependent manner in both cell lines (Fig 3.2.18 A-B). Critically the increase in cell number elicited by DMSO conditioned media was not observed when normal media was applied instead. This suggests that factors present within the DMSO conditioned media but absent in MKC-8866 conditioned media, may be able to modulate growth.

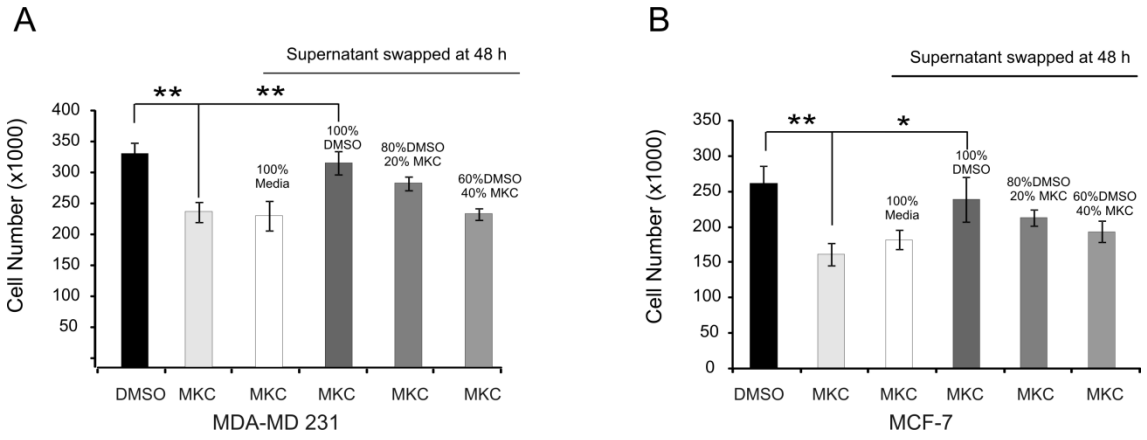


Figure 3.2.17: DMSO conditioned media rescue MKC-8866 mediated reduction in proliferation. Cells were treated with MKC-8866 or DMSO for 48 h. Supernatants from DMSO treated cells (or media) were swapped onto MKC-8866 treated cells in indicated ratios. Cell number for **(A)** MDA-MB231 and **(B)** MCF7 were counted on day 4. Values shown are representative of three independent ±SEM P <0.05, ** P<0.01, *** P< 0.001 Students *t*-test

3.2.18 MKC-8866 induction of cell death is elicited via extracellular factors.

It seemed likely that extracellular factors capable of modulating proliferation could also affect cell survival under reduced nutrient conditions. To test this hypothesis cells were treated with/without MKC-8866 during reduced serum condition but at day 2 in MDA-MB231 and day 4/5 in MCF7 (time points before the induction of cell death) the supernatant of MKC-8866 treated cells were discarded and replaced with supernatant from DMSO treated cells. Cell death was then measured by PI incorporation on day 5 or 10 respectively. it is evident that swapping supernatants on day 2 (from DMSO cells onto MKC-8866 cells) almost completely rescued the induction of cell death by MKC-8866 in MDA-MB231 cells (Fig 3.2.18 A-C). This was not due to removal of inhibitor from the cells as re-adding MKC-8866 to the cells still resulted in rescue. Moreover replacing the supernatant with media (i.e. not conditioned) continued to elicit an apoptotic response and showed no rescue.

A similar response was observed in MCF7 cells (Fig 3.2.18 D), supernatant swapped from DMSO treated cells onto MKC treated cells on day 4 or 5 lead to cell rescue. MKC-8866 was re-added to wells on day 4 and day 8 and so further replenishing was unnecessary. Again, replacing the supernatant with media did not afford any rescue to the cells, indicating a role of extracellular factors.

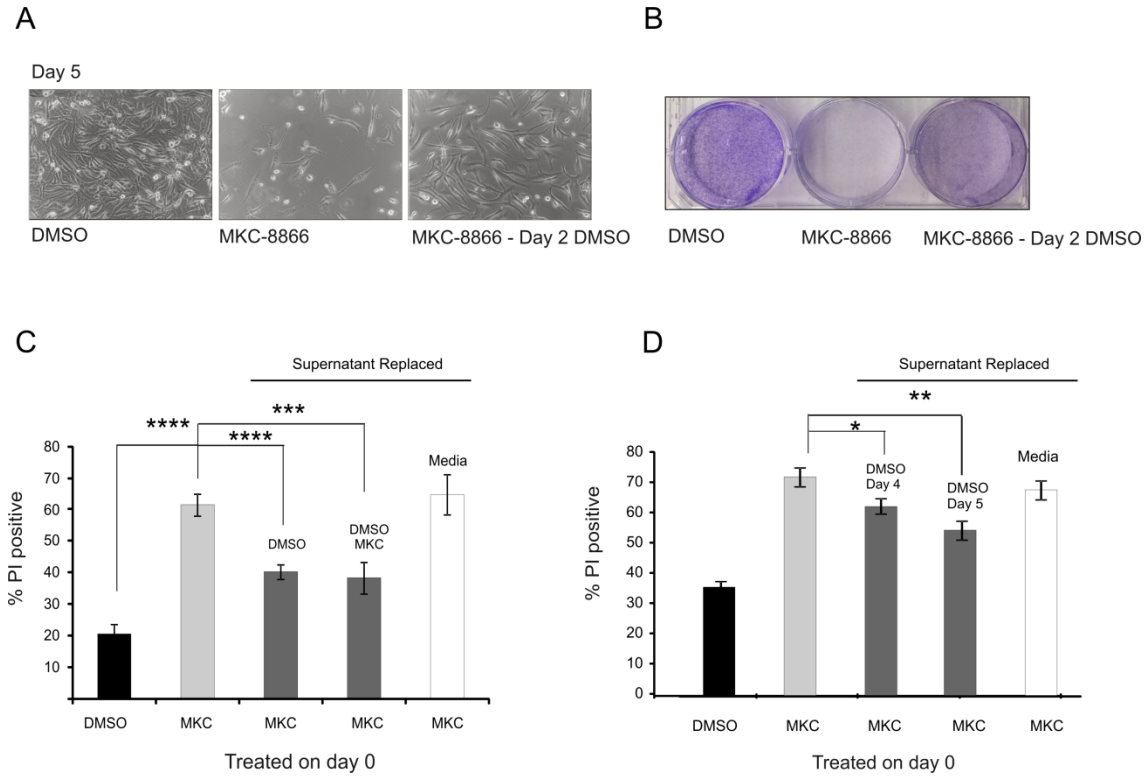


Figure 3.2.18 MKC-8866 induction of cell death is elicited via extracellular factors. (A) Images of MDA-MB231 at day 5 of experiment showing that death induced by MKC-8866 can be rescued by addition of conditioned supernatant on day 2. **(B)** Crystal violet staining of MDA-MB231 on day 5 post treatment showing that death induced by MKC-8866 can be rescued by addition of conditioned supernatant on day 2. **(C)** PI incorporation is used to quantify cell death in MDA-MB231 following treatment with MKC-8866 and subsequent replacement of supernatant on day 2 as indicated. **(D)** PI incorporation is used to quantify cell death in MCF7 following treatment with MKC-8866 and subsequent replacement of supernatant on day 4 or 5 as indicated. Values shown are representative of three independent ±SEM P <0.05, ** P<0.01, *** P< 0.001 Students *t*-test

3.2.19 Delineating the effect of MKC-8866 on extracellular factors

To ensure that this rescue was directly due to XBP1 mediated effects, I established a similar experimental set up with cells expressing XBP1 shRNA or control PLKO. A similar rescue of cell death demonstrated in Fig 3.2.19A was observed with the replacement of supernatant on day 2, confirming once more that results attained with MKC-8866 are mediated by XBP1s. In order to determine whether extracellular protein factors within the supernatants were responsible for rescuing cell death the supernatants were denatured by heating to 55^{o}C. Denatured supernatants (DS) were then added to

MDA-MB231/MCF7 cells and the capability of the DS to rescue cells from serum starvation induced cell death was assessed by PI staining and flow cytometry. MDA-MB231 and MCF7, in Fig 3.2.19B, are jointly portrayed and reveal that heating of DMSO conditioned supernatant to 55^{o}C for 30 minutes resulted in a loss of rescue afforded by this supernatant and cell death occurred once more in these cells. As an additional control to ensure that heating to 55^{o}C did not lead to cell death independently from MKC-8866, DMSO conditioned media heated to 55^{o}C was replaced onto DMSO treated cells, but had no effect on cell viability.

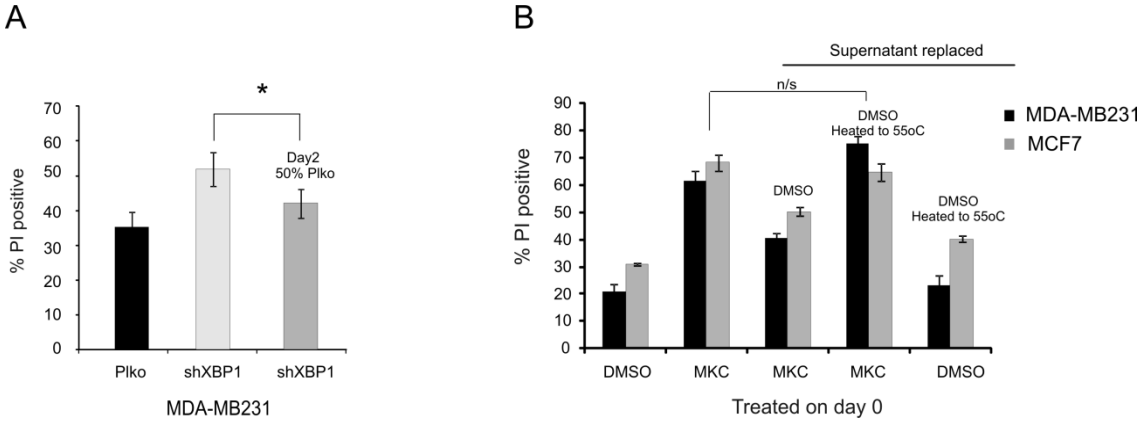


Figure 3.2.19: Delineating the effect of MKC-8866 on extracellular factors. **(A)** Results obtained with supernatant swaps using MKC-8866 can be recapitulated in XBP1 knockdown cells. **(B)** Heating of supernatants to 55^{o}C caused a loss of rescue afforded by the conditioned media. Heating to 55^{o}C did not independently induce cell death in MCF7 or MDA-MB231 cells. Values shown are representative of three independent ±SEM P <0.05, ** P<0.01, *** P< 0.001 Students *t*-test

3.2.20 Identification of extracellular targets from MKC-8866 treated cells

Having established that MKC-8866 may be modulating extracellular factors, I next aimed to identify which extracellular factors were being modulated in our system. Microarray analysis of MDA-MB231 cells treated with/without MKC-8866 for 24 h (described in Figure 3.2.20A) had revealed that interleukin pathway was down

regulated following treatment with MKC-8866 (Figure 3.2.20B) (Microarray credit to Eoghan McGrath).

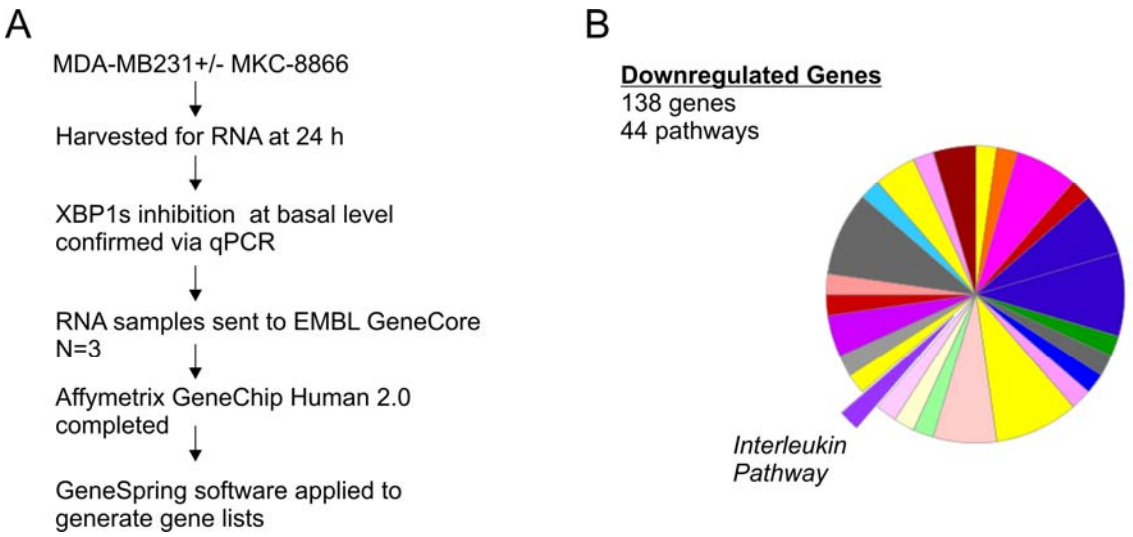


Figure 3.2.20: Microarray analysis of MDA-MB231 +/- MKC-8866. **(A)** Experimental outline of samples generated and outsourced to EMBL GeneCore for microarray analysis. Three independent repeats were generated and analysed **(B)** Pathway analysis of genes down regulated following MKC-8866 treatment revealed regulation of interleukin pathway (Credit to Eoghan McGrath)

This suggested that cytokines may be affected by MKC-8866 treatment and therefore could be playing a role in our system. To identify these factors a cytokine array was performed on supernatants (cells treated at 2% serum with/without MKC-8866) from cells harvested from day 2 of MDA-MB231 experiments. As cytokine arrays are a singular experiment and not open to repeats, I wanted to ensure that the supernatants selected were representative of previous observations therefore three aspects were examined before continuing with the cytokine array. Firstly, XBP1s levels were tested for inhibition indicating that the inhibitor was working correctly (Fig 3.2.21A). Secondly, the supernatants proposed to be use for the array were assessed to determine if they were eliciting a comparable induction of cell death and rescue (Fig 3.2.21B). Finally cell number was counted on day 2 in this system to rule out that differences in cytokine level could be attributed to cell number (Fig 3.2.21 C).

Confident that the supernatant generated were representative of previous experiments a cytokine array was preformed (Fig 3.2.21D). Four cytokines were immediately recognisable as being differentially regulated in MKC-8866 conditioned media namely IL-6, IL-8, GRO-α and GM-CSF.

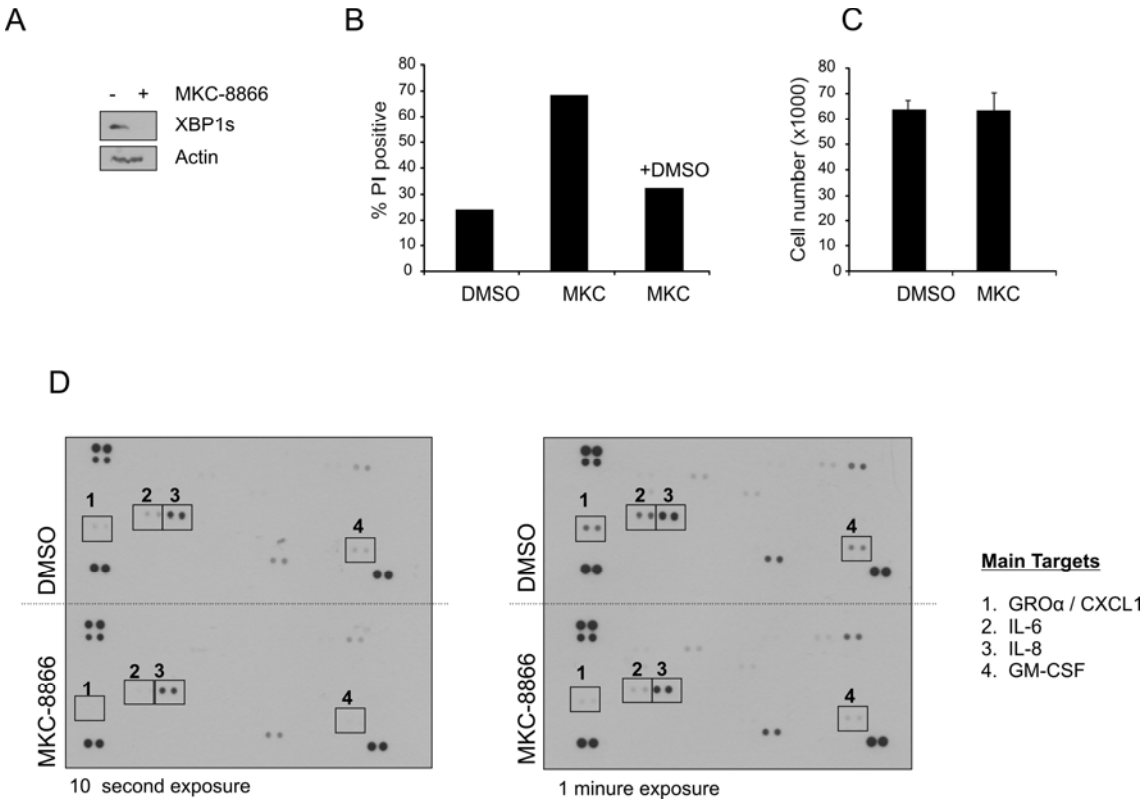


Figure 3.2.21 Cytokine array. (A) Confirmation that MKC-8866 inhibited XBP1s. **(B)** Confirmation that the supernatant collect elicited cell death and rescue comparable to previous experiments. **(C)** Cell number at 48 h does not differ between the samples. **(D)** Image of cytokine array including 10 second exposure and 1 min exposure

3.2.21 Quantitative analysis of IL-6, IL-8 and GRO-α following MKC-8866 treatment

Next secreted levels of IL-6, IL-8 and GRO-α were examined via ELISA in MDA-MB231 cells treated with/without MKC-8866 at 2% serum for 2 days. Addition of MKC-8866 demonstrated in Fig 3.2.22 A-C significantly reduces the secreted levels of these cytokines with greatest reduction observed with Gro-α levels. In line with

literature, IL-6 and IL-8 are transcriptionally regulated by XBP1s as western blotting analysis reveals lower protein levels in MKC-8866 treated cell lysates.

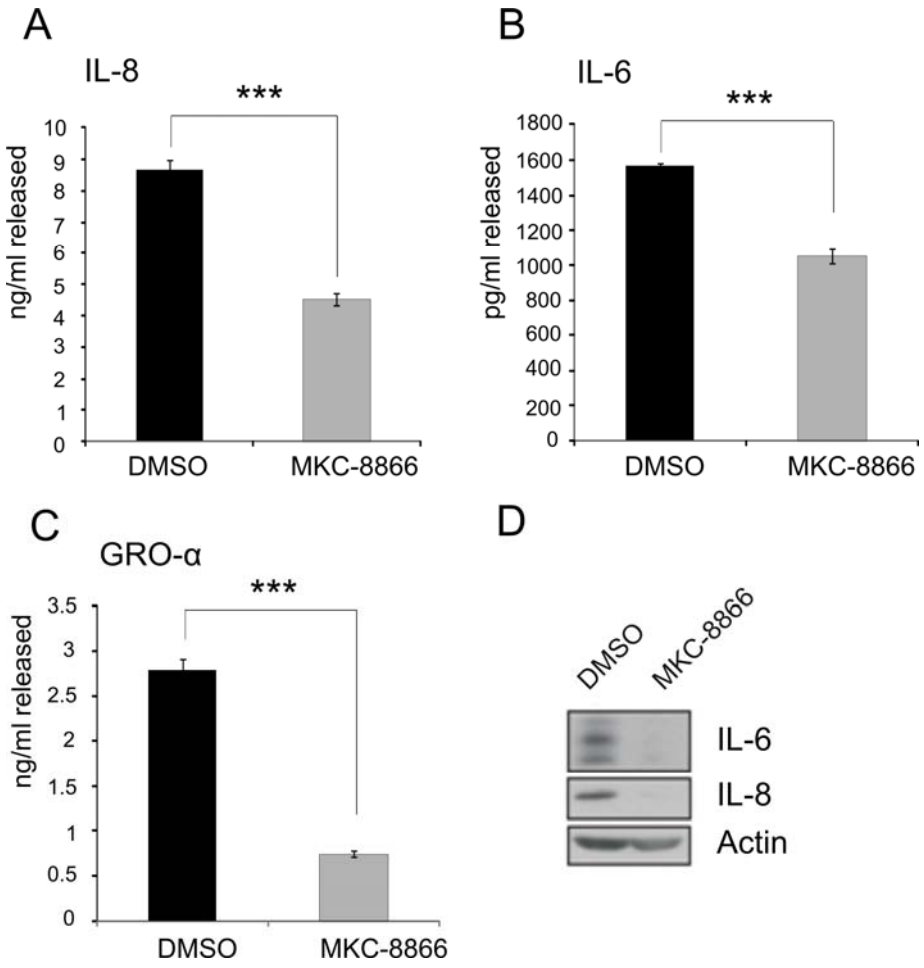


Figure 3.2.22 Quantitative analysis of IL-6, IL-8 and Gro-α following MKC-8866 treatment. ELISA analysis of secreted **(A)** IL-8 **(B)** IL-6 and **(C)** Gro-α show reduced levels following MKC-8866 treatment. **(D)** MKC-8866 also reduces protein levels of IL-6 and IL-8 within the cell. Values shown are representative of three independent ±SEM P <0.05, ** P<0.01, *** P< 0.001 Students *t*-test

3.2.22 Cytokines modulate cell growth and survival

To examine the functional effects of these cytokines, neutralising antibodies against each cytokine were added to MDA-MB231/MCF7 cells. Microarray analyses described above also implicated TGFβ as a possible downstream target of XBP1s, and due to an underlying connection to breast cancer metastasis it was also included in this study. These cytokines can affect the growth of MDA-MB231 cells with most pronounced effect observed with GRO-α and GM-CSF (Fig 3.2.23A). In MCF7 cells neutralising antibodies against IL-8, IL-6, GM-CSF and TGFβ could all significantly reduce proliferation (Fig 3.2.23B). While this observation is limited as it does not directly correlate these cytokines to MKC-8866 mediated reduction in proliferation, it is suggestive that these cytokines, which may be downstream of XBP1s contribute to proliferation rate of breast cancer cells.

These neutralising antibodies were next applied to the supernatant swap experiments (more specifically to DMSO conditioned media before swapping this supernatant onto MKC-8866 treated cells). This yielded interesting results depicted in Fig 3.2.23C. MKC-8866 induced cell death under these conditions; a result that was rescued by DMSO conditioned media. IL-6, IL-8, GRO-α and most noticeably TGFβ, all diminished the rescue afforded by this media implicating these specific cytokine in the effect of MKC-8866 on cell survival. Furthermore this observation strengthens the possibility that XBP1s drives production of these cytokines affording a survival advantage to breast cancer.

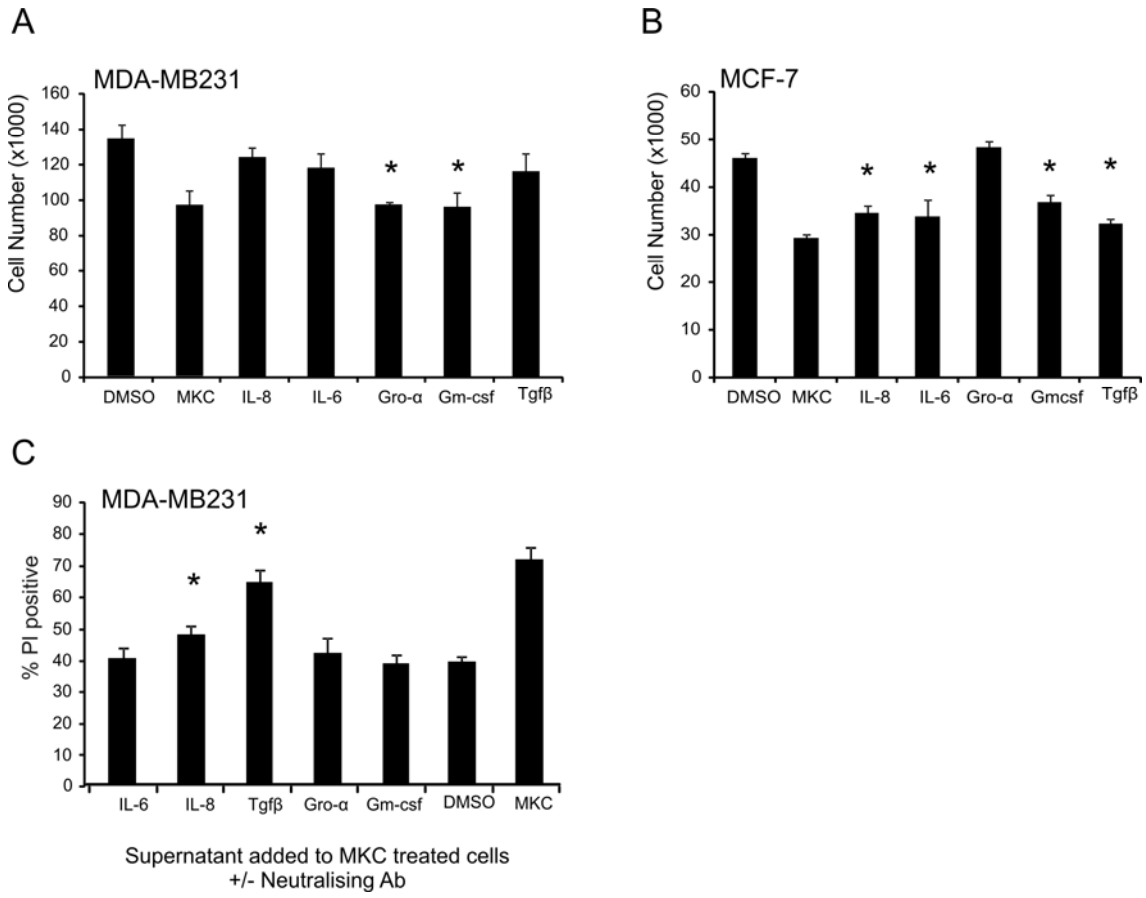


Figure 3.2.23 Cytokines modulate cell growth and survival. (A) MDA-MB231 cells counted 5 days post addition of cytokine neutralising antibodies reveal Gro-α and GM-CSF can significantly modulate proliferation. **(B)** MCF7 MB231 cells counted 5 days post addition of cytokine neutralising antibodies reveal IL-6, IL-8, TGFβ and GM-CSF can significantly modulate proliferation. **(C)** Cell death analysis of supernatant swaps including neutralising antibodies reveal IL-8, IL-6, Gro-α and TGFβ can modulate cell survival during reduced serum conditions in MDA-MB231. Values shown are representative of three independent ±SEM P <0.05, ** P<0.01, *** P< 0.001 Students *t*-test

3.2.23 MCK-8866 can reduce tumour volume in an in vivo xenograft model

Through collaboration with MannKind an MCF7 xenograft study was carried out on our behalf, based on my *in vitro* findings. This study revealed that MKC-8866 as a single agent could readily reduce the tumour volume (Figure 3.2.24). This *in vivo* work adds strength to the rationale that MKC-8866 may be a therapeutically relevant agent in the treatment of breast cancer.

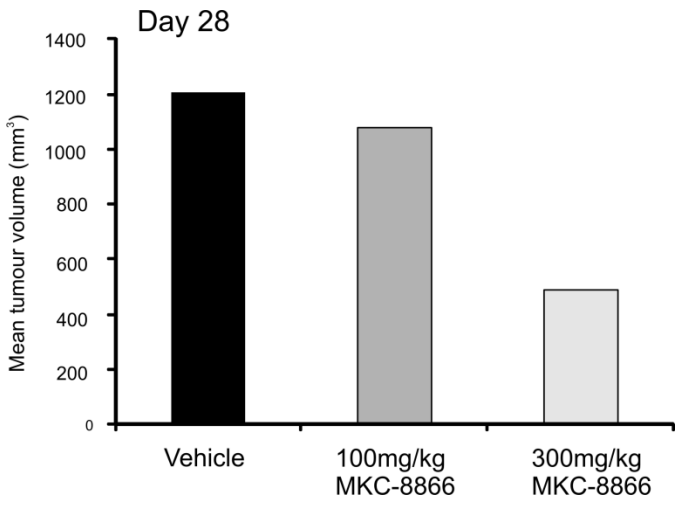


Figure 3.2.24 MCF7 xenograft study. MCF7 xenograft mice were treated with 100mg/kg or 300mg/kg or vehicle conttol and tumour volume recorded at day 28. Mean tumour volume is represented.

3.3 Discussion

Breast cancer is a growing problem worldwide with over 300,000 new cases diagnosed annually and causing approx 90,000 deaths in the EU each year, therefore research into its development and treatment options is imperative (259). Previous literature has begun to unwind the possible role of XBP1s in breast cancer with clinical research linking levels of XBP1s to breast cancer survival (125, 255). This study focuses on the therapeutic potential of MKC-8866 as a tool for IRE1/XBP1 inhibition and builds upon current research delineating XBP1s role in breast cancer. Here I demonstrate that XBP1s is over expressed at protein level across a range of breast cancer cell lines and is most associated with TNBC. XBP1s levels are currently used in the diagnosis of luminal A breast cancer (240); however the data presented here suggests that XBP1s may also have diagnostic potential for TNBC. This observation is in line with current literature indicating higher levels of XBP1s associated with TNBC (125).

While UPR has been implicated at multiple levels of breast cancer survival to date no studies have assessed the functional contribution of the IRE1 arm to breast cancer cell survival through application of an IRE1 inhibitor. MKC-8866 is a new and potent inhibitor of XBP1 splicing, with no off target effects shown to date. With a quick mechanism of action (<4 h) and maintained inhibition for up to 4 days without need of replenishment, MKC-8866 is a desirable IRE1 inhibitor. Reports suggest that inhibition or down regulation of individual UPR arms can affect the other UPR arms, as reduced XBP1 mRNA expression has been previously demonstrated in *PERK*$^{-/-}$ MEFs following tunicamycin treatment (260). As such it is important to determine if compensatory or crosstalk occurs following IRE1 inhibition; here I show that MKC-8866 does not induce a compensatory upregulation of other UPR arms and exclusively targets IRE1 signalling. Widespread UPR activation does not appear to occur basally in breast cancer cell lines but appears limited to IRE1/XBP1s activation. It is unknown how IRE1 is selectively activated in breast cancer however somatic mutations are considered prevalent in breast cancer, with a high probability of *ire1* containing a driving mutation.

It could be mutation that affords IRE1 its basal activity but further analysis of IRE1 is needed to conclusively explain this phenomenon.

In this study I show that the application of an IRE1 inhibitor but not a PERK inhibitor could significantly reduce the proliferation rate of breast cancer cells irrespective of subtype. This result confirms that MKC-8866 reduces proliferation via an IRE1/XBP1 axis specific effect rather than inhibition of general UPR mechanisms in breast cancer cell lines. Knockdown of XBP1 in MDA-MB231 cells using XBP1 shRNA, confirmed that the effect of MKC-8866 on proliferation was specific to XBP1s levels. Moreover, further enhancement of XBP1s levels using an overexpression system in HCC1806 demonstrates further the effect of XBP1s on growth. Furthermore to potentiate the rationale of using MKC-8866 on breast cancer, its effect on nontumorigenic breast epithelial cell line MCF10A was also examined. MKC-8866 could not modulate the growth of a nontumorigenic breast epithelial cell line (MCF10A) suggesting that the specificity of its effect is limited to cancerous subtypes.

XBP1s is previously reported to modulate proliferation of other cancer subtypes including pancreatic and prostate cancers (256, 257), this work strengthens further the rationale of XBP1s modulation of cancer growth. In breast cancer, XBP1s is reported to affect proliferation through regulation of Cyclin A1 levels (258). Cyclin A1 is normally only expressed in embryonic mammary gland and not in differentiated adult mammary tissue (261). Six1 is reported to drive Cyclin A1 re-expression in breast cancer and thus, afford a growth advantage (261). However, I observed conflicting cell cycle profiles and Cyclin A1 levels in MDA-MB231 and T47D following MKC-8866 treatment. This study confirms that Cyclin A1 may be regulated by XBP1s in MDA-MB231. However this is not a widespread breast cancer effect as MKC-8866 did not regulate Cyclin A1 in T47D cells; therefore it is not convincing that this is the only downstream target of XBP1s capable of modulating growth.

I investigated whether IRE1 inhibition was eliciting its effects via an intracellular or extracellular phenomenon by first examining the effects of conditioned media on cell

number. I identified that the reduction in proliferation rate observed by MKC-8866 could be rescued with substitution of vehicle conditioned supernatant, indicating a role of extracellular factors. This suggested that cellular dependence on pro-survival extracellular factors may be enhanced during periods of stress; and as the UPR is tightly implicated in adaption to tumour microenvironments, the effect of MKC-8866 as a single agent was explored in conditions mimicking tumour microenvironment, such as reduced nutrient supply. To our surprise, MKC-8866 protected the cells from reduced glucose conditions. Following closer examination of the literature, this result may be attributed to antigluconeogenic activity of XBP1s via FOXO1 degradation (262), therefore inhibition of XBP1s may infer a survival advantage under reduced glucose conditions. Contrary to this observation however, during an overall reduction in nutrient supply, mimicked by reduced serum levels, MKC-8866 was sufficient to elicit an apoptotic response in the cell. This phenomenon could also be attributed to modulation of extracellular factors, as similarly to my observation with proliferation; substitution of conditioned supernatant could rescue this result.

Through cytokine array analysis of supernatants, I identify IL6, IL8, GROα, GM-CSF alongside TGFβ as possible targets of MKC-8866 inhibition. While IL6 and IL8 have previously been reported as downstream targets of XBP1s, XBP1s ability to regulate these cytokines in breast cancer is unreported (86, 263). However in breast cancer, IL6 and IL8 are reported to be key regulators of TNBC growth (264). In this study I show that IRE1 inhibition could dramatically reduce secreted levels of IL6 and IL8, a result also observed at the protein level which suggests that IRE1/XBP1s plays an important role in the regulation of these cytokines in breast cancer. To my knowledge this work is the first to link IRE/XBP1s signalling to GROα (CXCL1) regulation. While it is possible that regulation of GROα could be a secondary consequence of IRE1 inhibition, further experiments would be needed to confirm a direct regulation, it is none the less an interesting observation. GROα is a chemokine associated with lung replapse in breast tumours (265, 266) and increasing the aggressiveness of circulating tumour cells (CTC) (267). Interestingly this study by Kim *et al* also identified IL6 and IL8 as CTC attractants (267). In 2012, Acharyya *et al* reported that GROα can promote metastasis

and chemoresistance in breast cancer via recruitment of myeloid cells which up regulate S100A8/9 inducing a paracrine signalling loop that confers resistance to chemotherapy and enhances metastatic potential (268). Here, I demonstrate the ability of MKC-8866 to reduce GROα levels almost four fold establishing a new untapped avenue for targeting GROα levels in breast cancer.

Furthermore, I observe a functional effect of TGFβ inhibition in this system. TGFβ is also linked to breast cancer metastasis with reports indicating that TGFβ plays an important role in EMT of breast cancer (269). Contradictory reports of the function of TGFβ in cancer exist; TGFβ is reported to play both tumour inhibiting and enhancing effects (270, 271). In this study we show that inhibition of TGFβ by neutralising antibody could reverse the rescue observed with conditioned medium, pushing the cells towards cell death, suggesting that TGFβ is important to cellular survival under these conditions.

I explored this observation further and employed an MCF7 xenograft model to observe any *in vivo* effects of MKC-8866. Indeed MCF7 xenograft showed promising results with overall reduction in tumour volume following MKC-8866 treatment. This *in vivo* study strengthens the clinical implication for the use of MKC-8866 in breast cancer treatment. Whether this reduction in tumour volume can be attributed to reduced cytokine level would be interesting to determine. Chen *et al* 2014 suggest that IRE1/XBP1s can modulate stem cell like cells in breast cancer which could also be attributing to reduced volume *in vivo* (125).

In conclusion this work demonstrates that MKC-8866 is a potent IRE1 inhibitor which can affect breast cancer proliferation and survival through modulation of extracellular factors such as IL6, IL8, GROα and TGFβ.

Chapter 4

Investigating the therapeutic potential of MKC-8866 as a combination therapy in the treatment of breast cancer

4.1 Introduction and research rationale

Breast cancer is a growing problem worldwide, improvement to current treatment options and/or development of new treatment strategies is imperative to overcome this disease. There is evidence to suggest that some current anticancer drugs can induce ER stress. Both Bortezomib, a proteasome inhibitor, and Resvertrol, a natural polyphenolic compound are potent inducers of ER stress and downstream UPR response in cancer cells (272, 273). Vorinostat and Romidepsin are two Histone deacetylase inhibitors (HDAC) inhibitors recently attaining FDA approval for treatment of cutaneous T cell lymphoma (274). HDAC are reported to lead to accumulation of misfolded proteins in cells (275). While these reports link anti-cancer drugs to activation of the UPR further studies also suggest that the UPR may play a role in development of chemoresistance (276, 277). Furthermore, increased expression of GRP78 was reported to contribute to chemoresistance of breast cancer to topoisomerase inhibitors (278).

XBP1 is linked to modulation of treatment response in breast cancer. XBP1s is mostly associated with oestrogen receptor responses and XBP1 splicing correlates with poor prognosis in breast cancer patients treated with anti-oestrogens therapy (279). In breast cancer cell lines, overexpression of XBP1s conferred resistance to anti-oestrogens by abrogating cell cycle arrest and reducing apoptosis (280). Moreover, physical interaction with XBP1s rendered oestrogens receptor α activity ligand-independent (281). Oestrogen treatment induced higher XBP1s levels and increased chemo-resistance in breast cancer cell lines (255).

Through the association of UPR to chemoresistance, and increasing evidence of XBP1s role in oestrogen response, I hypothesised that XBP1s may also be modulating the response of breast cancer to other commonly used anti-cancer drugs. Chapter 3 focused on the implications of MKC-8866 as a single agent in breast cancer and examined its effect on proliferation and survival; here I expand on this work and focus on how MKC-8866 could be applied in a clinical setting.

The aim of this study was to

- Identify if drugs used in the treatment of breast cancer induced XBP1s
- Determine if MKC-8866 could potentiate the effects of these drugs

4.2 Results

4.2.1 Anti Cancer drugs can induce XBP1s

To determine the clinical significance of IRE1 inhibition, MKC-8866 was applied in combination with established anti-cancer drugs and cell death monitored. In order to identify possible combinations a range of anti-cancer drugs were screened in breast cancer cell lines for their ability to induce XBP1s (drugs are listed in Table 5). This study was not limited to one breast cancer subtype but encompassed a range of breast cancer subtypes; oestrogen receptor positive (ESR1+) luminal (MCF7 and T47D), HER2 positive SKBR3 and TNBC MDA-MB231. Tamoxifen and Taxol can induce XBP1s in T47D. Melphalen and Taxol induce XBP1s in MDA-MB231. Tamoxifen induces XBP1s in MCF7 and Melphalan can induce XBP1s in SKBR but to a lesser extent (Fig 4.2.1D). As expected Bortezomib induced XBP1s across all cell lines tested.

Drug	**Effect**
Tamoxifen	ESR antagonist
Melphalan	an alkylating agent
5-Fluoruracil	a pyrimidine analog
Doxorubicin	a DNA intercalating agent
Taxol	a mitotic inhibitor
Bortezomib	a proteasome inhibitor

Table 5; Anticancer drugs Anti cancer drugs and their reported effects in breast cancer

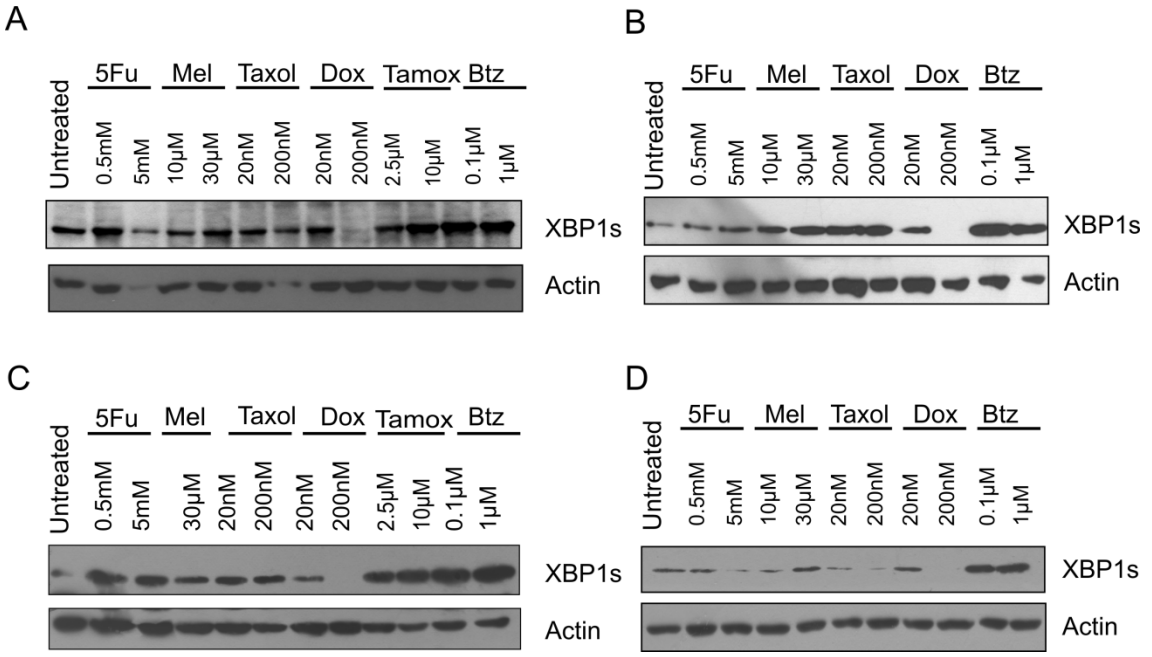


Figure 4.2.13 Anti cancer drugs induction of XBP1s. (A) T47D analysis of XBP1s induction following treatment with 5FU, Melphalan, Taxol, Doxorubicin, Tamoxifen, Bortezomib for 24 h. **(B)** MDA-MB231 analysis of XBP1s induction following treatment with 5FU, Melphalan, Taxol, Doxorubicin, Bortezomib for 24 h. **(C)** MCF7 analysis of XBP1s induction following treatment with FU, Melphalan, Taxol, Doxorubicin, Tamoxifen, Bortezomib for 24 h**. (D)** SKBR3 analysis of XBP1s induction following treatment with FU, Melphalan, Taxol, Doxorubicin, Bortezomib for 24 h. Data representative of 3 independent repeats.

4.2.2 Tamoxifen

As Tamoxifen induced XBP1s in both T47D and MCF7, the role of Tamoxifen was further examined in ERS1 positive cell lines. Western blotting analysis revealed that XBP1s is induced by Tamoxifen in a dose dependent manner in both T47D and MCF7 as shown in Fig 4.2.2 A-B. As XBP1s is induced in a dose dependent manner it could be stimulating a defensive mechanism aiding survival, and as such inhibiting XBP1s might potentiate Tamoxifen induced cell death. To test this hypothesis T47D cells were co-treated with Tamoxifen and MKC-8866 or DMSO with cell viability measured by MTT. Viability was assessed at 48 h, 72 h and 96 h post treatment and at all doses there

is a statistically significant decrease in viability with the addition of MKC-8866 as compared to control (Fig 4.2.2C).

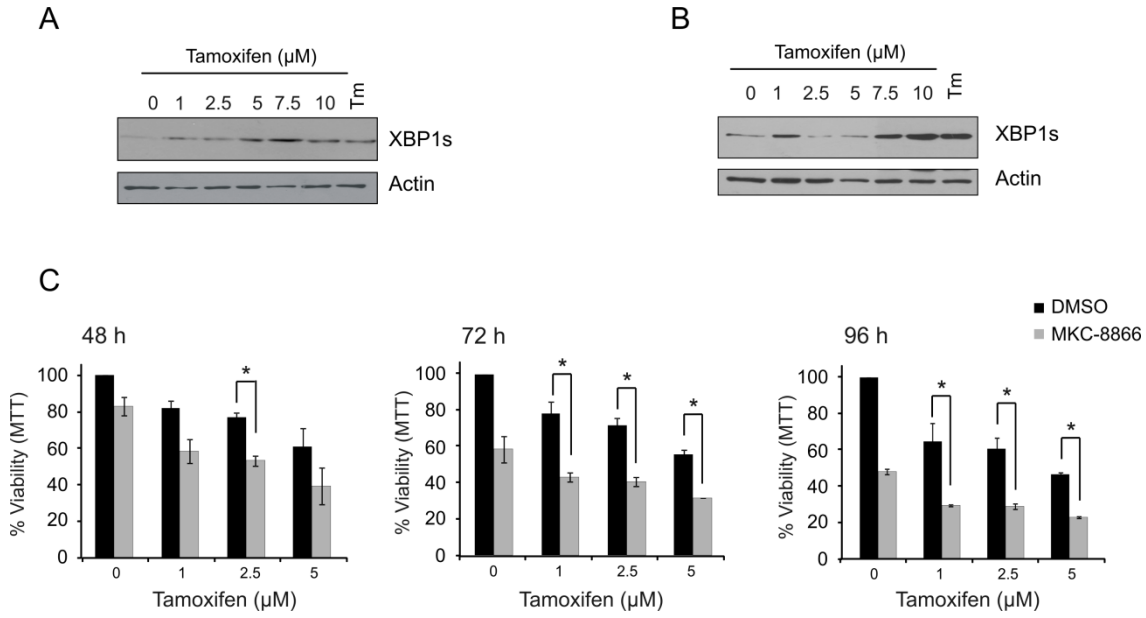


Figure 4.14.2 Role of XBP1s in ERS1 positive cell lines. Tamoxifen induces XBP1s in dose dependent manner in **(A)** T47D and **(B)** MCF7 at 24 h. **(C)** MTT analysis of tamoxifen treatment with/without MKC-8866 in T47D at 48h, 72h and 96 h. Values shown are representative of three independent ±SEM P <0.05, ** P<0.01, *** P< 0.001 Students *t*-test

This result is more apparent when we look at the time course analysis of 5 μM tamoxifen in both T47D and MCF7 cell lines. With MTT as a measure of cell viability it is clear that the addition of MKC-8866 is greatly increasing the effect of Tamoxifen in both T47D and MCF7 cell lines.

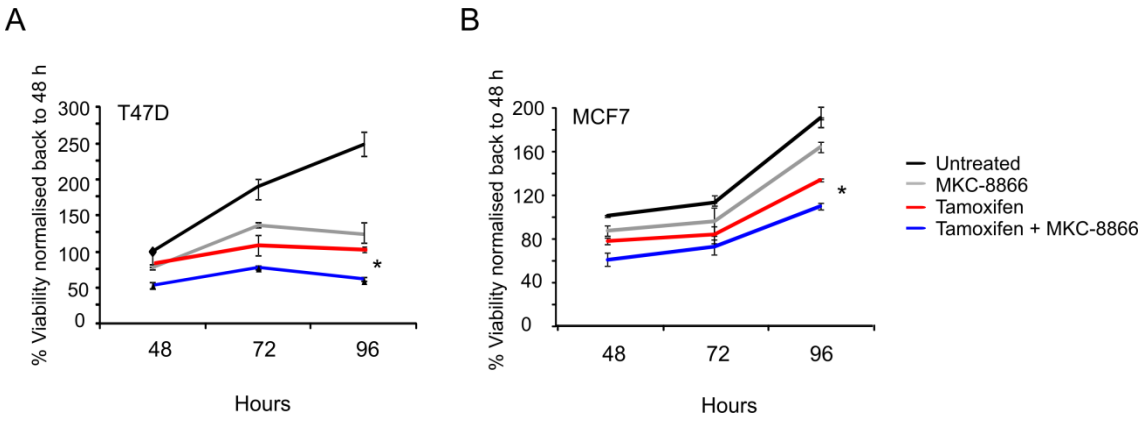


Figure 4.2.3 5μM Tamoxifen in combination with MKC-8866 in T47D and MCF7 cells over time. MTT analysis of **(A)** T47D and **(B)** MCF7 treated with MKC8866, Tamoxifen and in combination over time. Results presented is relative to 48 h treatment. Values shown are representative of three independent ±SEM P <0.05, ** P<0.01, *** P< 0.001 Students *t*-test

As MTT analysis indicated that MKC-8866 may potentiate the effect of Tamoxifen in T47D and MCF7 PI incorporation apoptotic assay was performed to confirm this result. T47D cells were co-treated with MKC-8866 and various concentrations of Tamoxifen for 72 h. Addition of the MKC-8866 subtly potentiated the effect of tamoxifen induced cell death in T47D (Fig 4.2.4A). While this result was not as profound an effect as observed with MTT assay, the biological trend remained. Protein samples were also taken from this experiment and probed via western blotting for apoptotic makers including CASPASE 3 and PARP cleavage as a third readout of viability under these conditions. Cleaved CASPASE 3 and PARP levels were clearly increased in samples co-treated with MKC-8866 as compared to control is evident (Fig 4.2.4B).

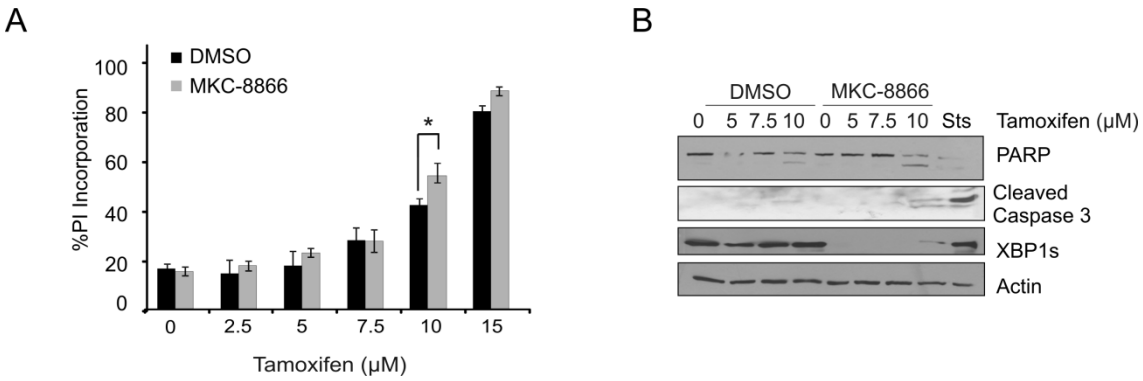


Figure 4.2.4 Cell death analysis of Tamoxifen and MKC-8866 in combination in T47D cells. (A) PI incorporation assay of MKC-8866 and tamoxifen used in combination at 72 h. **(B)** Western blotting analysis of cleaved caspase 3 and PARP cleavage in T47D following combination treatment with/without MKC-8866 during tamoxifen treatment at 72 h. Values shown are representative of three independent ±SEM P <0.05, ** P<0.01, *** P< 0.001 Students *t*-test

Next this combination was explored in an *in vivo* setting using an MCF7 xenograft model. Here under direction from our lab and in collaboration with MannKind MCF7 xenograft model was established and treated with tamoxifen alone, MKC-8866 alone, and in combination. While previously described in chapter 3 MKC-8866 alone could reduce the tumour volume, here tamoxifen could also reduce tumour size; however no further reduction in tumour volume was observed when tamoxifen was used in combination with MKC-8866 (Figure 4.2.5).

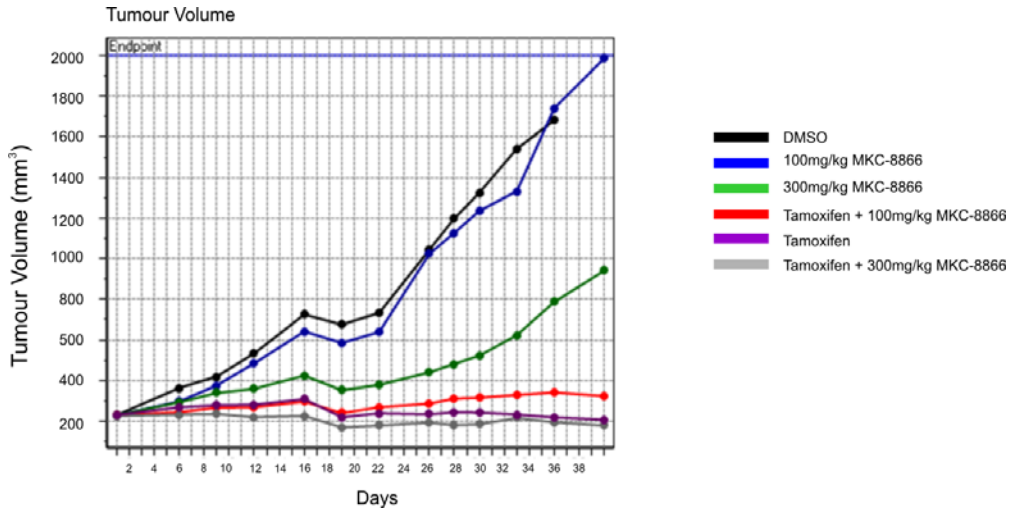


Figure 4.2.5 MCF7 Xenograft. MCF7 xenograft model monitoring tumour volume over time following treatment with MKC8866 with/without Tamoxifen

4.2.3 Melphalan

Melphalan inhibits DNA and RNA synthesis by alkylating the DNA, inducing genotoxic stress and subsequent cytotoxicity (282). It is currently used in clinic for the treatment of TNBC. Western blotting screening revealed that Melphalan could induce XBP1s in MDA-MB231 and SKBR3. This result was explored further to examine if IRE1 inhibition could potentiate Melphalan induced cell death *in vitro*. A Melphalan dose response was completed and MTT assay applied as a readout of cell viability at 24 h, 48 h and 72 h (Fig 4.2.6A). MKC-8866 had little to subtle effects on the cytotoxicity of Melphalan in MDA-MB231. To investigate this result a cytox green incorporation assay was applied as a measure of membrane permeability and therefore a measure of cell death. No effect on cell viability was recorded when used in combination with MKC-8866. Interestingly however, Melphalan could induce a robust UPR response in a dose dependent manner as shown via western blotting of the UPR markers (Fig 4.2.6C).

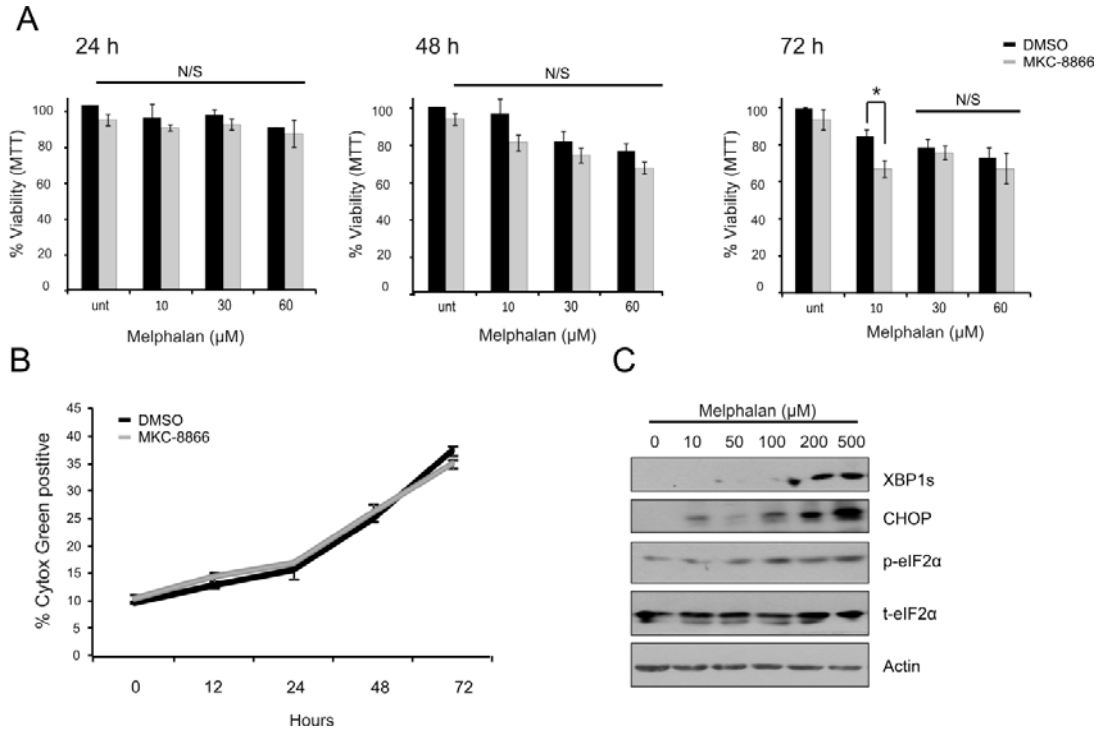


Figure 4.2. 6 Melphalan in combination with MKC-8866 in MDA-MB231.(A) MTT analysis of Melphalan in combination with MKC-8866 in MDA-MB231 measured at 24 h, 48 h and 72 h. **(B)** cytox green incorporation assay of 10μM Melphalan with/without MKC-8866 over time. **(C)** Western blotting analysis of UPR markers following treatment with Melphalan in MDA-MB231. Values shown are representative of three independent ±SEM P <0.05, ** P<0.01, *** P< 0.001 Students *t*-test

4.2.4 Bortezomib

Bortezomib is most commonly used in the treatment of blood cancer conditions but it has recently reached phase 2 clinical trials in breast cancer (157). As Bortezomib is an inducer of UPR response, the value of using Bortezomib in combination with MKC-8866 was briefly analysed in TNBC. MTT analysis of increasing doses of Bortezomib with/without MKC-8866 was performed in MDA-MB231 cells at 12 h, 24 h and 48 h post treatments. This revealed a reduction in viability when used in combination with MKC-8866 at 24 h and 48 h post treatment (Fig 4.2.7A). Further analysis via cytox green incorporation assay was completed but demonstrated that no additive effect is observed in combination in MDA-MB231 over time (Fig 4.2.7B). To validate that bortezomib could induce a UPR in breast cancer as similarly reported in other disease states; the induction of UPR markers following 24 h treatment with various concentrations of Bortezomib in MDA-MB231 was assessed via western blotting (Fig 4.2.7C).

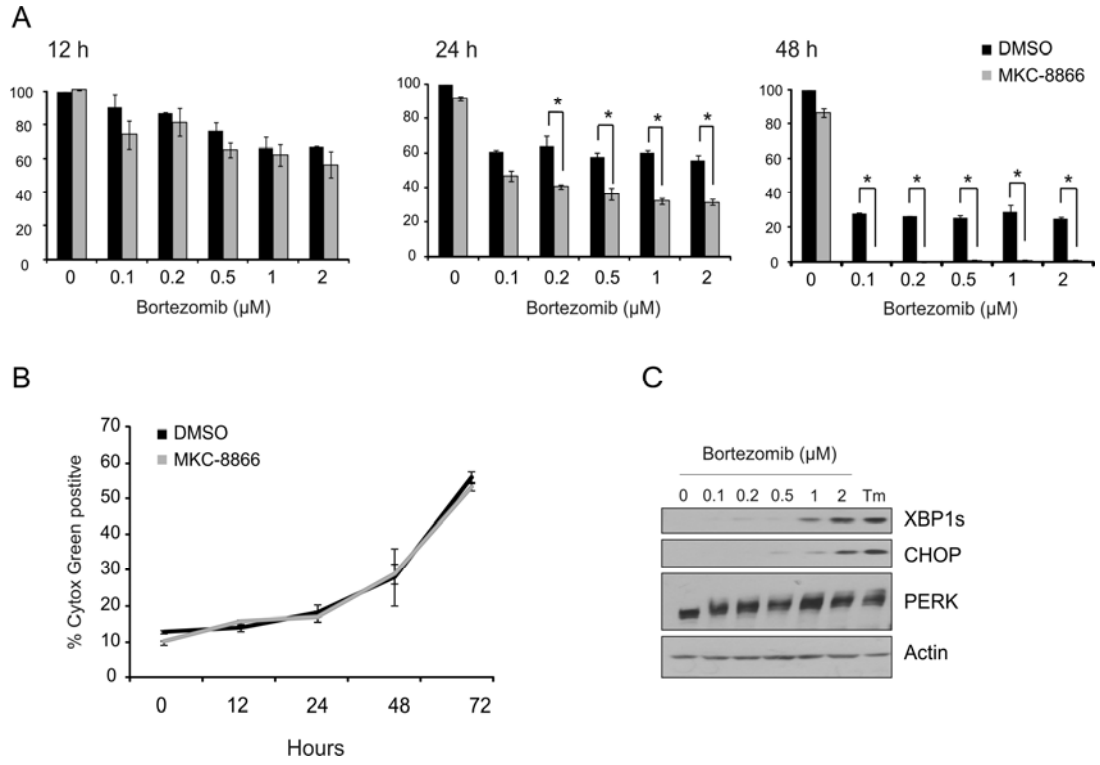


Figure 4.2.7 Bortezomib and MKC-8866 in MDA-MB231. (A) MTT analysis of Bortezomib doses with/without MKC-8866 measured at 12 h, 24 h, and 48 h **(B)** Cytox green analysis of 500nM Bortezomib in combination with MKC-8866 in MDA-MB231 over time. **(C)** Bortezomib induces UPR activation in dose dependent manner in MDA-MB231 at 24 h. Values shown are representative of three independent ±SEM P <0.05, ** P<0.01, *** P< 0.001 Students *t*-test

4.2.5 Taxol

Western blotting screening had revealed that Taxol could readily induce XBP1s in TNBC therefore this observation was explored further. Initially using MTT analysis cell viability over time was monitored following treatment with various doses of Taxol in combination with MKC-8866. Interestingly, this showed that MKC-8866 could potentiate the effect of Taxol in MDA-MB231 (Fig 4.2.8A). Taxol can induce a UPR response, with dose dependent increase of XBP1s, CHOP, p-PERK, and p-eIF2a in MDA-MB231(Fig 4.2.8B).This was an interesting observation that was not cell line specific as a similar effect was observed with Taxol in MDA-MB468 TNBC cell line (Fig 4.2.8C).

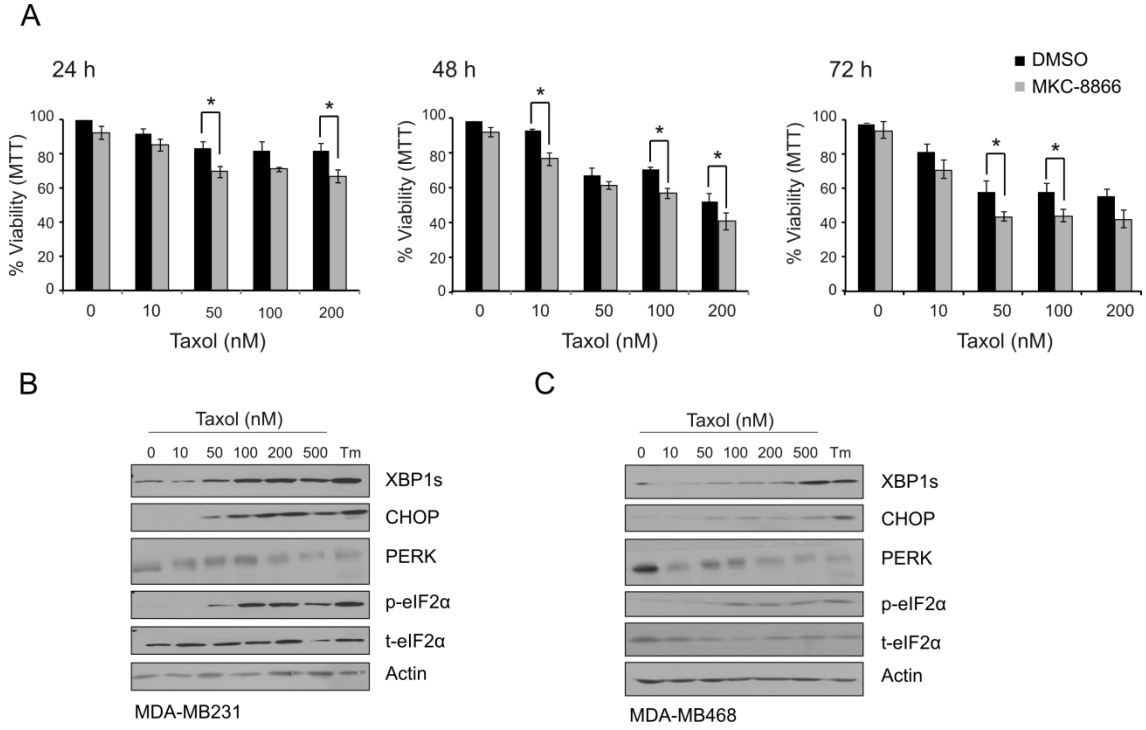


Figure 4.2.8 Taxol induces UPR and is enhanced by combination of MKC-8866. (A) MTT analysis of Taxol doses over time with/without MKC-8866 in MDA-MB231. Taxol induces UPR response in dose dependent manner at 24 h in **(B)** MDA-MB231 and **(C)** MDA-MB468. Data shown is representative of three independent ±SEM P <0.05, ** P<0.01, *** P< 0.001 Students *t*-test

To explore this observation, time course analysis of 50 nM Taxol and 200 nM Taxol was completed and revealed induction of UPR markers over time in MDA-MB231 (Fig 4.2.9 B and D). Cytox green incorpotation assay of 50 nM and 200 nM Taxol plus

MKC-8866 showed enhanced cytotoxicity over time in combination. This observation was most notable with 200 nM Taxol, lending further strength to the hypothesis that MKC-8866 could enhance Taxol induced cell death (Fig 4.2.9 A and C).

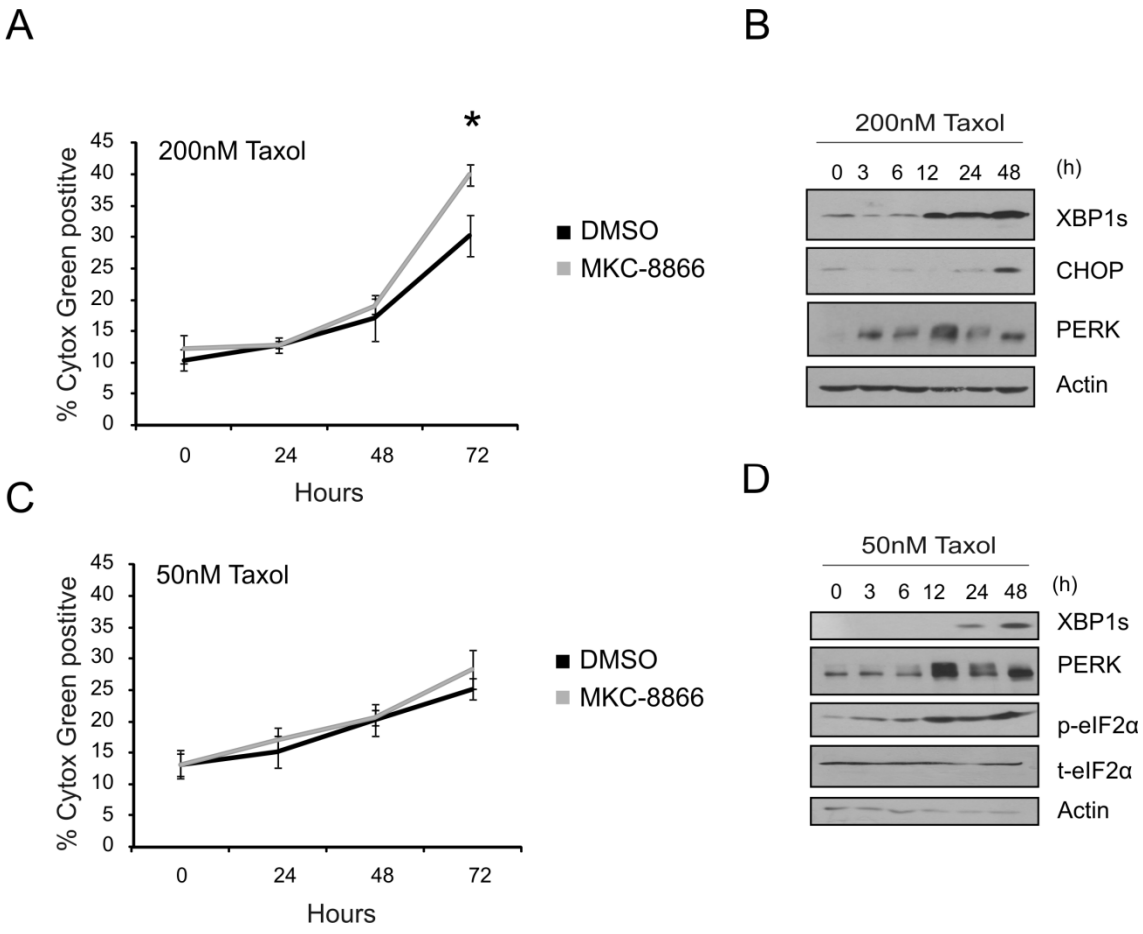


Figure 4.2.9 50nM and 200nM Taxol induce UPR and are enhanced by MKC-8866. (A) Cytox green incorporation analysis of 200nM with/without MKC-8866 over time in MDA-MB231. **(B)** 200nM induces UPR over time. **(C)** Cytox green incorporation analysis of 50nM with/without MKC-8866 over time in MDA-MB231. **(D)** 50nM induces UPR over time. Values shown are representative of three independent ±SEM P <0.05, ** P<0.01, *** P< 0.001 Students *t*-test

200 nM and 50 nM Taxol both looked promising combinations with MKC-8866 in MDA-MB231 cell line but whether co-treatment was the best approach to this combination remained to be determined. Initially a co treatment approach was applied and PI incorporation monitored over time (Fig 4.2.10 A-B). There was a biological

trend of enhanced cytotoxicity with MKC-8866 with 50 nM at 12 h, 24 h and 48 h but less so with 200 nM Taxol. Another combinational approach was post treatment, the rationale being that perhaps allowing enhanced activation of IRE1 followed by inhibition of this response may have a greater effect on cell viability. MDA-MB231 cells were treated for 12 h Taxol before addition of MKC-8866 with PI incorporation measured at 12, 24 and 48 h. 200nM Taxol showed enhanced cytotoxicity using this approach (Fig 4.2.10 (C-D))

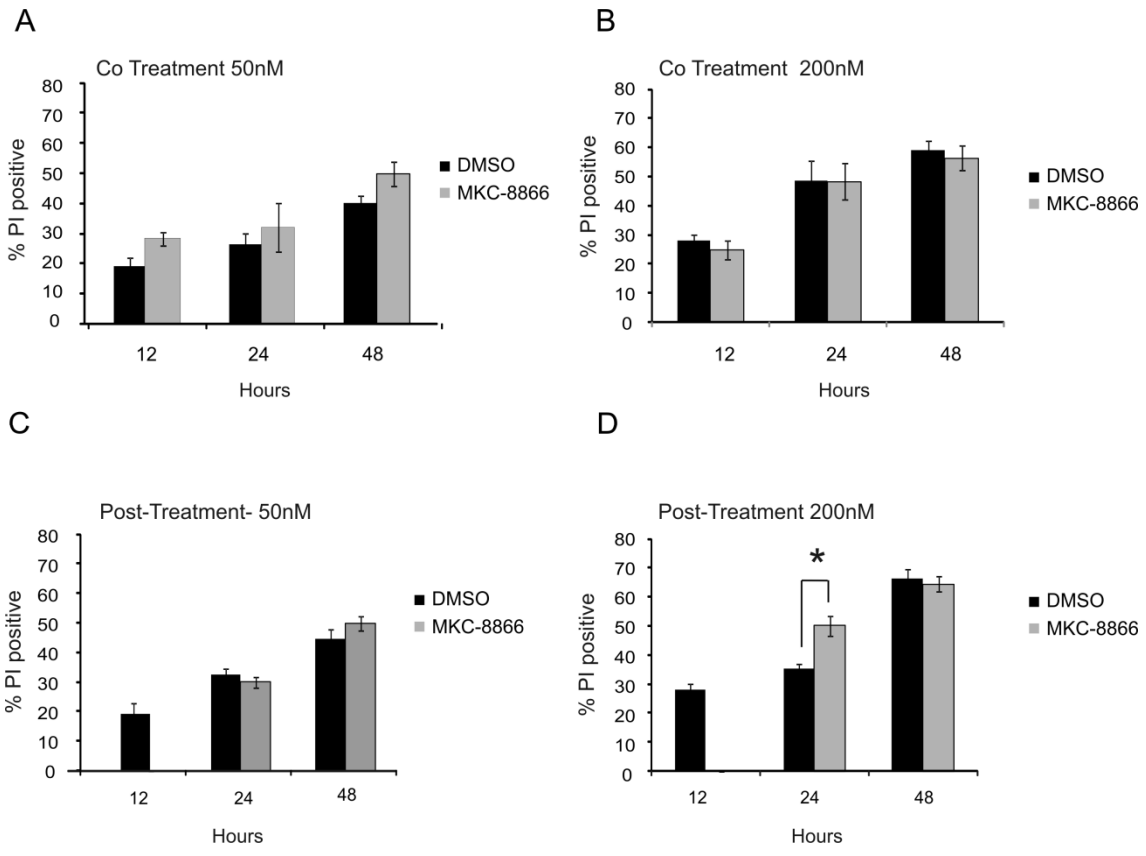


Figure 4.2.10 Co-treatment and Post treatment analysis of MKC-8866 and Taxol. (A) 50nM and **(B)** 200nM co treatment of Taxol plus MKC-8866. Post treatment anaylsis of combination of MKC-8866 and **(C)** 50nM and **(D)** 200nM. Values shown are representative of three independent ±SEM P <0.05, ** P<0.01, *** P< 0.001 Students *t*-test

Next a pre-treatment approach was applied. Cells were pre treated for 48 h with MKC-8866 before treating with 50 nM or 200 nM Taxol and monitoring PI incorporation at 12 h, 24 h and 48 h post Taxol treatment. Interestingly this warranted the greatest enhancement of cell death, with statistically significant enhancement of cytotoxicity observed with both 50 nM and 200 nM Taxol at 48 h post Taxol treatment (corresponded to 96 h post MKC-treatment) (Fig 4.2.11 A-B). Since previous work (chapter 4) had shown MKC-8866 induced cell death under reduced serum conditions, whether the effect of MKC-8866 on Taxol cytotoxicity would be further enhanced during these conditions was examined. Taking the same approach as pre-treatment above, MDA-MB231 were pre-treated with MKC-8866 for 48 h at 2% serum and then treated with 50 nM or 200 nM Taxol for 12 h, 24 h and 48 h. This resulted with a further enhancement of cell death at 48 h post Taxol treatment when used in combination with MKC-8866 (Fig 4.2.11 C-D).

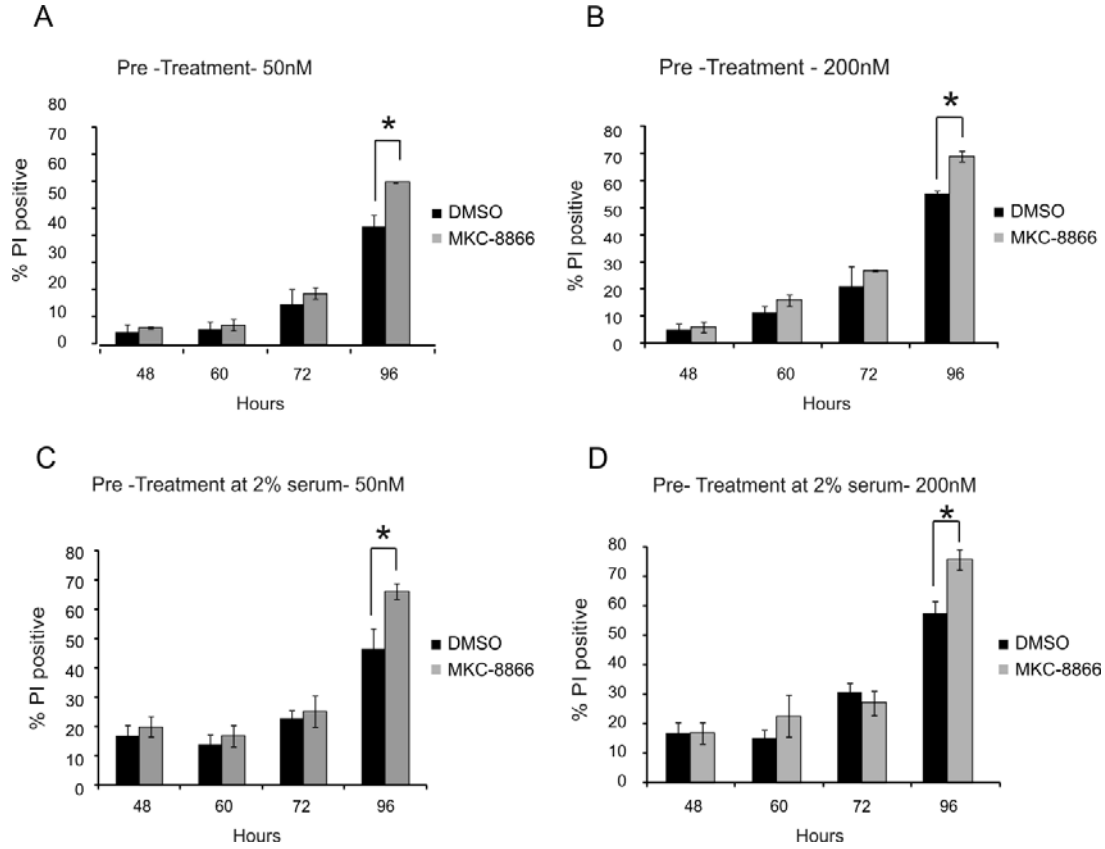


Figure 4.2.15 Pre treatment with MKC-8866 during Taxol treatment. Pre-treatment for 48h with MKC-8866 and subsequent treatment with **(A)** 50nM Taxol or **(B)** 200nM Taxol in MDA-MB231. MDA-MB231 cells were cultured in 2% serum with MKC-8866 for 48h before treatment with **(C)** 50nM Taxol and **(D)** 200nM Taxol. PI incorporation is used as a measure of cell death. Values shown are representative of three independent P <0.05, ** P<0.01, *** P< 0.001 Students *t*-test

While Taxol induced cell death could be enhanced through combination with IRE1 inhibitor MKC-8866, this was limited to *in vitro* environment. To validate this observation and to examine whether this would translate into an *in vivo* setting our lab, through collaboration with MannKind Corp, undertook a MDA-MB231 xenograft experiment. MDA-MB231 xenograft SCID mice were generated and once the tumour was established, mice were administered variants of three conditions; MKC-8866 alone, Taxol alone or Taxol and MKC-8866 in combination, and tumour volume was recorded until 8 days post treatment. To our surprise MKC-8866 alone had little to no effect on tumour size but did enhance Taxol mediated reduction of tumour volume (Figure 4.2.12). Additionally tumour regrowth measured at day 8 post administration of last dose, was reduced in mice treated in combination compared to those treated alone Figure 4.2.12).

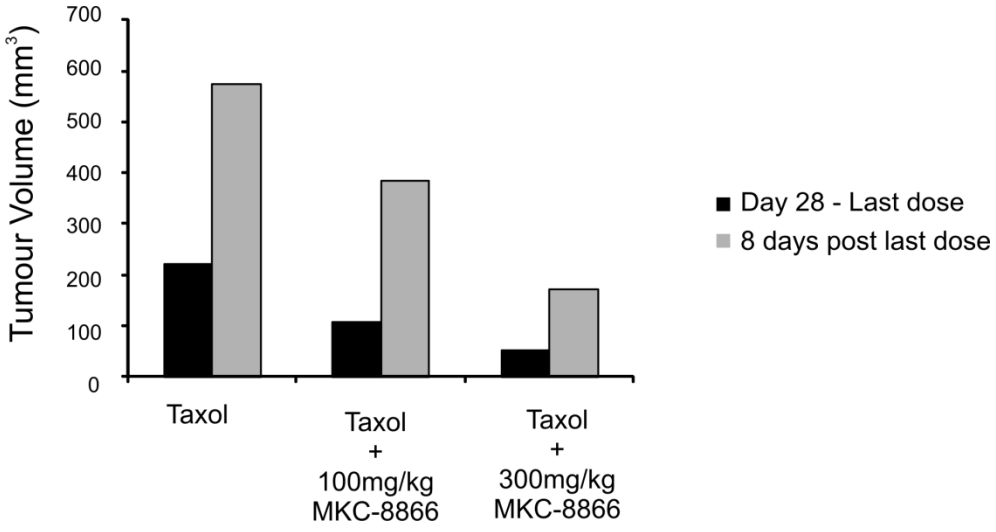


Figure 4.2.12 MDA-MB231 Xenograft model. Xenograft study demonstrating MKC-8866 enhances Taxol mediated reduction in tumour volume and limits the regrowth of tumour post treatment.

4.3 Discussion

Identifying a suitable anti cancer drug that could be therapeutically complimented by MKC-8866 would greatly strengthen the rationale for using MKC-8866 in breast cancer treatment. Screening of anticancer drugs for their ability to induce XBP1s identified Tamoxifen, Melphalan, Taxol and Bortezomib as possible inducers of XBP1s. Tamoxifen is an oestrogen receptor antagonists, and since its development has been a standard treatment option for ERS1 positive breast cancer patients (283, 284). Although being a common clinical drug, Tamoxifens' precise mechanism of action and how it elicits cell death at higher concentration is still debated with various pathways linked to its mechanism (reviewed in (285)). Here I show that tamoxifen can induce XBP1s in a dose dependent manner in MCF7 and T47D cell lines. Precisely how this induction is occurring is undefined. Tamoxifen could induce XBP1s through modulation of calcium signalling via calmodulin binding or induction of oxidative stress (286, 287). While overexpression of XBP1s is reported to protect from tamoxifen induced cell death (288) here I examine XBP1s inhibition mediated by MKC-8866. A subtle enhancement of tamoxifen effects was observed upon co treatment but *in vivo* work suggests that MKC-8866 may not be able to potentiate the effects of Tamoxifen.

Next I explored chemotherapeutic drugs Melphalan, Bortezomib and Taxol in TNBC. p-eIF2α was previously shown to be induced by Melphalan, but to my knowledge this is the first report to demonstrate a wider UPR activation following Melphalan treatment. While no additive effect was observed upon co-treatment with MKC-8866, how Melphalan, an alkylating agent, may be activating the UPR is intriguing. Bortezomib has reached phase II clinical trials for breast cancer treatment and is a known inducer of ER stress (157, 289). Combining IRE1 inhibition and bortezomib treatment has shown promise in multiple myeloma (163), here I observed little to no benefit to this combination in breast cancer.

Taxol is a microtubule stabiliser commonly used in the treatment of TNBC. Taxol has been linked to ER stress through modulation of calcium release from the ER and inhibition of Bcl-2 (290-292). I charachterise Taxol induction of UPR markers in two cell models of TNBC and further show a time dependent induction of UPR in MDA-MB231. Co treatment analysis of MKC-8866 and Taxol at clinically relevant doses of Taxol (293, 294) suggested a slight enhancement of cell death. Whether co treatment was the optimal combination of these drugs remained unknown and so I explored the use of MKC-8866 as a co-, post- or pre- treatment option. Pre-treatment with MKC-8866 significantly sensitised MDA-MB231 cells to Taxol induced cell death.

Through collaboration with MannKind we next pursued an MDA-MB231 xenograft to analyse *in vivo* the clinical potential of this two drugs in combination. MKC-8866 showed potential to enhance Taxol mediated effects as tumour growth at day 28 was reduced almost 50% further by addition of MKC-8866. Furthermore the ability of MKC-8866 to reduce tumour regrowth has extensive clinical implications. My previous work in chapter 1 could suggest that MKC-8866 is altering the secretome and reducing tumour growth in that capacity. Alternatively could this be as result of reduced capacity of cancer stem cells, as recently XBP1 knockdown in TNBC was reported to diminish breast cancer stem cell markers $CD44^{high}CD24^{low}$ (125).

In conclusion I demonstrate that anticancer drugs have the ability to elicit UPR in breast cancer cells and that MKC-8866 shows clinical potential as a combination therapy with Taxol.

Chapter 5

Endoplasmic Reticulum Stress-mediated induction of Sestrin 2 potentiates cell survival

5.1 Introduction and research rationale

Autophagy is an evolutionary conserved lysosomal pathway for degrading cytoplasmic proteins, macromolecules, and organelles (295). Two of the ER stress sensors, IRE1 and PERK, have been shown to play a role in the transcriptional regulation of components of autophagic machinery (reviewed in (296)). The PERK pathway mediated via ATF4 has been strongly linked to autophagy induction, with studies showing transcriptional upregulation of genes implicated in the formation, elongation and function of the autophagosome (297, 298). However, less is known about the contribution of IRE1 to this process. Conflicting reports have linked IRE1 to both the induction and inhibition of the autophagic process (299, 300). IRE1/JNK axis has been linked to Beclin1 induction (300, 301) while XBP1 deficient cells enhance autophagy through regulation of FOXO1(299).

Sestrin 2 is a member of a family of highly conserved stress-response proteins, transcriptionally activated by p53. Initially Sestrins were identified as antioxidants that control the activity of peroxiredoxins which scavenge reactive oxygen species (ROS), but further studies revealed that they were inhibitors of mammalian target of rapamycin complex 1 (mTORC1) signalling (302). In response to genotoxic stress, Sestrin 2 can activate AMPK by proximity-induced phosphorylation, causing phosphorylation of TSC2, and subsequent mTORC1 inhibition (303). Inhibition of mTORC1 is crucial for the activation of the autophagy induction complex, ULK1/2-Atg13-FIP200-Atg101 (224). Sestrin 2 was shown to be a positive regulator of autophagy pathway with overexpression of Sestrin 2 reported to increase LC3-I to LC3-II conversion rates (304). Sestrin 2 is thought to elicit a pro-survival effect through its ability to alleviate stress via induction of an autophagic response, but, in contrast, it may also inhibit cellular proliferation through its function as an inhibitor of mTORC1 activity. The overall cellular outcome may depend upon the stress stimulus, for example; 2-deoxyglucose activation of the PI3K-Akt pathway caused Sestrin 2-mediated induction of protective

autophagy (302), while the inactivation of the steroid-thyroid-retinoid nuclear receptor TR3 leads to Sestrin 2 mediated growth inhibition in lung cancer (305).

Initially it was believed that transcription of Sestrin 2 is controlled solely in p53 dependent manner (306) but more recent reports demonstrate that Sestrin 2 expression may also be mediated from the UPR. In 2013 Bruning *et al* reported that Sestrin 2 was induced during ER stress which they attributed to ATF4 activity, downstream of PERK. However, this study did not explore the potential of IRE1 in this system and heavily relied upon overexpression systems (307). In a model of obesity induced ER stress, Sestrin 2 induction was linked to PERK mediated CCAAT-enhancer-binding protein beta (c/EBPβ) expression. Interestingly this paper reported that Sestrin 2 attenuated protein translation via mTORC1, which they argued played a cytoprotective role during ER stress. Subsequent knockdown of Sestrin 2 lead to an increase in cytotoxicity via increased protein load causing an upregulation of UPR markers (308).

A previous study undertaken in our lab by Dr. Svetlana Saveljeva demonstrated that Sestrin 2 could be induced by ER stress inducers thapsigargain and Brefeldin A. While the literature linked Sestrin 2 expression to PERK arm during ER stress, Dr Saveljeva work also suggested that IRE1 may also be capable of inducing Sestrin 2 expression following ER stress. In light of my interest in IRE1/XBP1s role in breast cancer I was interested in exploring this observation further and to better charachterise the contribution of IRE1/XBP1s to Sestrin 2 expression. Moreover I wanted to explore the role of Sestrin 2 in cancer and examine the possibility of Sestrin 2 affecting breast cancer responses to treatment.

The aim of this study was to;

- Expand on data linking IRE1/XBP1s to Sestrin 2 regulation
- Investigate if any chemotherapeutic drugs could induce Sestrin 2 expression
- Explore whether Sestrin 2 can affect cellular response to chemotherapeutic drugs.

5.2 Results

5.2.1 Sestrin2 is induced by ER stress in a p53 independent manner

A previous study in our laboratory demonstrated that ER stress inducers thapsigargin and Brefeldin A could induce Sestrin 2 expression as early as 6 hours post treatment in MCF7 cells (Fig 5.2.1 A and B credit to SS). While Sestrin 2 was widely thought to be a p53 dependent gene, I show that Sestrin 2 can be induced by ER stress in a p53 independent manner. Using HCT116 p53 $^{-/-}$ and their corresponding wild type, Fig 5.2.1C demonstrates that ER stress inducer Tg could increase Sestrin 2 expression in both cell types. Moreover in MCF7 cells which express wild type p53, Tg and BfA do not induce active p53, indicated by p-p53 levels, at 12 h of treatment (Fig 5.2.1D) even though we see Sestrin 2 expression appearing at 6 h post treatment. This suggests that in the case of unresolved UPR another pathway must be capable of inducing Sestrin 2 expression.

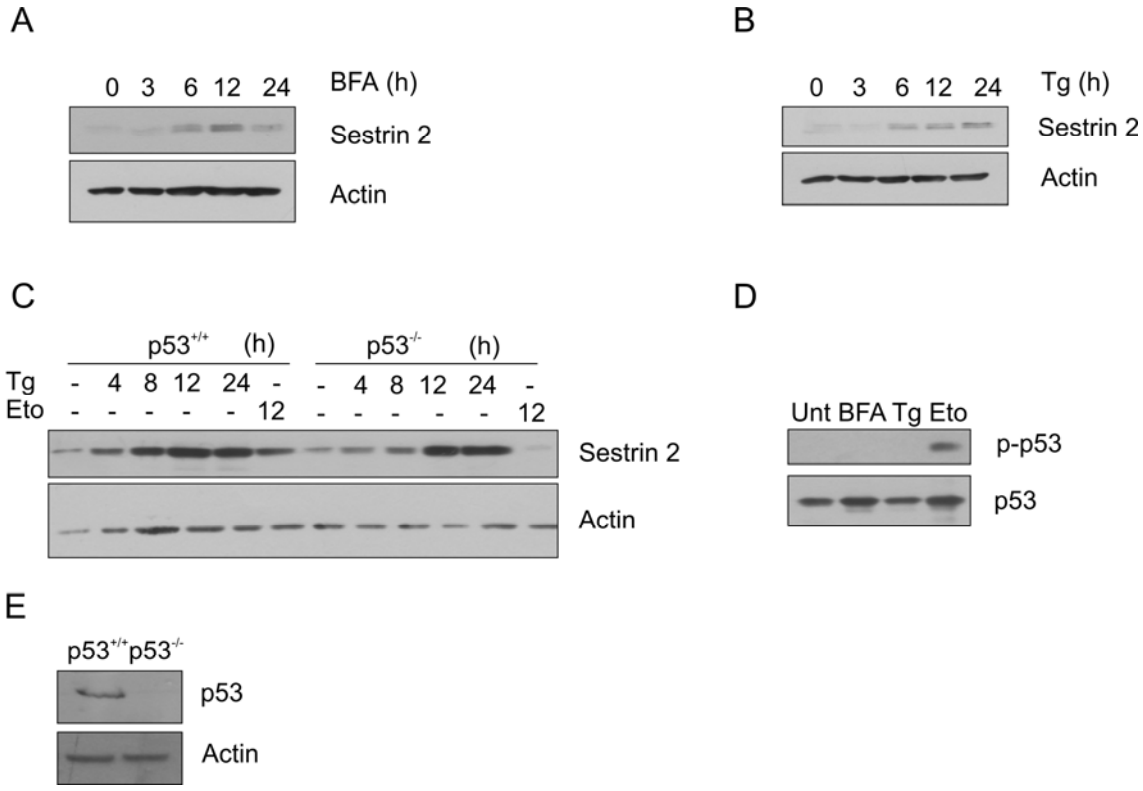


Figure 5.2.1 Sestrin 2 is induced by ER stress in p53 independent manner. MCF7 cells were treated with 0.5 μg/mL of BFA **(A)** or 1 μM of Tg **(B)** for indicated time and probed for Sestrin 2 expression (credit SS) **(C)** HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells were treated with 1 μM Tg for 4-24 h or 50 μM Etoposide for 12 h and lysates immunoblotted for Sestrin 2 **(D)** MCF7 cells were treated with 0.5 μg/mL BFA, 1 μM Tg or 50 μM Etoposide for 12 h after which lysates were immunoblotted for Ser15 p-p53 and total p53. **(E)** $p53^{+/+}$ and $p53^{-/-}$ cells were probed for total p53. Data representative of 3 independent repeats

5.2.2 Sestrin 2 expression is mediated from PERK and IRE1

While the literature focused on PERK mediated Sestrin 2 expression during ER stress I wanted to investigate if this was exclusively true or whether IRE1 also played a role. To ascertain this result IRE1 and PERK inhibitors were applied with ER stress induction in MCF7 cells, and Sestrin 2 expression monitored. Both arms of the UPR appear responsible for Sestrin 2 induction (Fig 5.2.2A). To confirm that our inhibitors were sufficient to block PERK and IRE1 under these conditions, downstream ATF4 and XBP1 expression levels were examined as controls. To verify that XBP1 could modulate Sestrin 2 expression, MCF7 cells were transfected with siRNA targeting XBP1 and monitored Sestrin 2 expression following Tg treatment. Knockdown of XBP1 Fig 5.2.2B reduced Sestrin 2 expression following Tg treatment, further validating the role of XBP1 in Sestrin 2 induction.

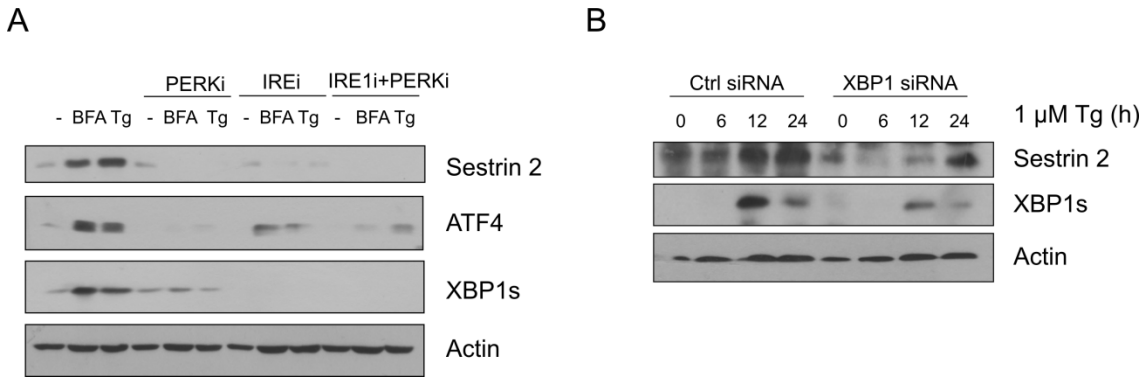


Figure 5.2.2 Sestrin 2 is mediated from bothe PERK and IRE1 arms of UPR. (A) Western blotting anayslis in MCF7 cells reveal that Perk and IRE1 inhibitors can reduces Sestrin 2 levels following 12 hour treatments with BFA or Tg. ATF4 and XBP1 confirm functionality of inhibitors. (B) siRNA knockdown of XBP1 reduce Sestrin 2 levels in MCF7 cells following Tg treatment at indicated time points.

5.2.3 *Investigating the role of Sestrin2 in Breast Cancer*

5.2.3.1 *Taxol*

Taxol had previously been identified as a potent inducer of ER stress (result chapter 2) therefore I was interested in investigating whether Taxol could also induce Sestrin 2 expression. Indeed this appeared true, as shown in Fig 5.3A; Taxol could induce Sestrin 2 expression in a dose dependent manner. HCC1806 a TNBC cell line, which harbours a p53 mutation, was selected as a cell model to allow us to examine other pathways which may contribute to Sestrin 2 regulation.

As our interest lay in exploring the effect of Sestrin 2 knockdown on chemotherapeutic response I determined if Taxol-mediated expression of Sestrin 2 could induce a potent and functional downstream effect. Downstream effect of Sestrin 2 induction was examined by looking at phosphorylation of mTOR (Fig 5.2.3B) however there was no reduction in p-mTOR levels suggesting additional complexity of Taxol mediated effects. While Taxol induction of Sestrin 2 expression was interesting, I wanted to explore if other chemotherapeutics could induce Sestrin 2 expression that would result in inactivation of mTORC pathway.

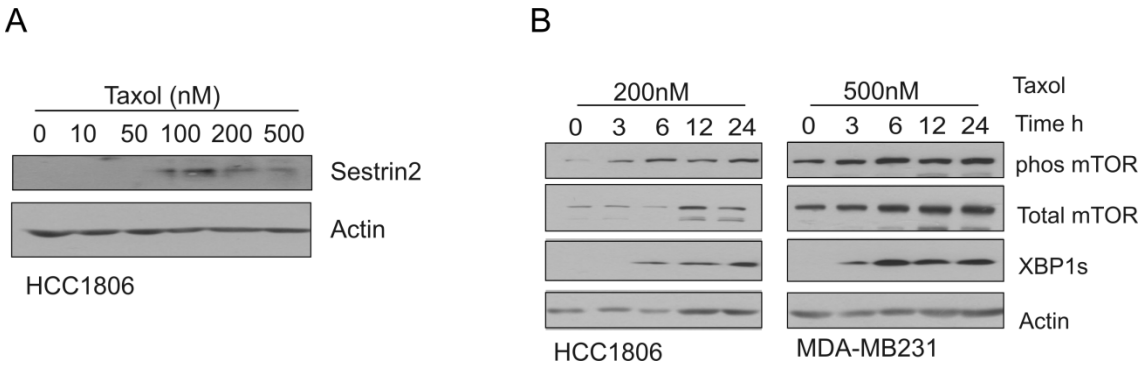


Figure 5.2.3 Taxol induces Sestrin 2. (A) Sestrin 2 is induced in a dose dependent manner by Taxol at 24 h in HCC1806 cells. **(B)** 200 nM Taxol in HCC1806 or 500 nM Taxol in MDA-MB231 could not reduce downstream p-mTor levels, had no effect on total mTOR. Taxol could still induce XBP1s. Result is representative of 3 independent repeats

5.2.3.2 Chemotherapeutics

To explore the ability of other chemotherapeutics to induce Sestrin 2 expression, a range of common breast cancer chemotherapeutic drugs were administered to both p53 wild type cells MCF7 and p53 mutant cells HCC1806 (Fig 5.2.4 A-B). Interestingly Melphalan, Bortezomib and Methotrexate could induce Sestrin 2 in p53 mutant cells HCC1806. To validate this observation, dose response analysis of all three drugs in HCC1806 cell line was performed and Sestrin 2 expression examined. All three drugs could induce a dose dependent increase of Sestrin 2 expression (Fig 5.2.4C-D). Melphalan appeared to be the weakest inducer of Sestrin 2 with stronger signals observed with Bortezomib and Methotrexate, therefore Bortezomib and Methotrexate were selected for further analysis.

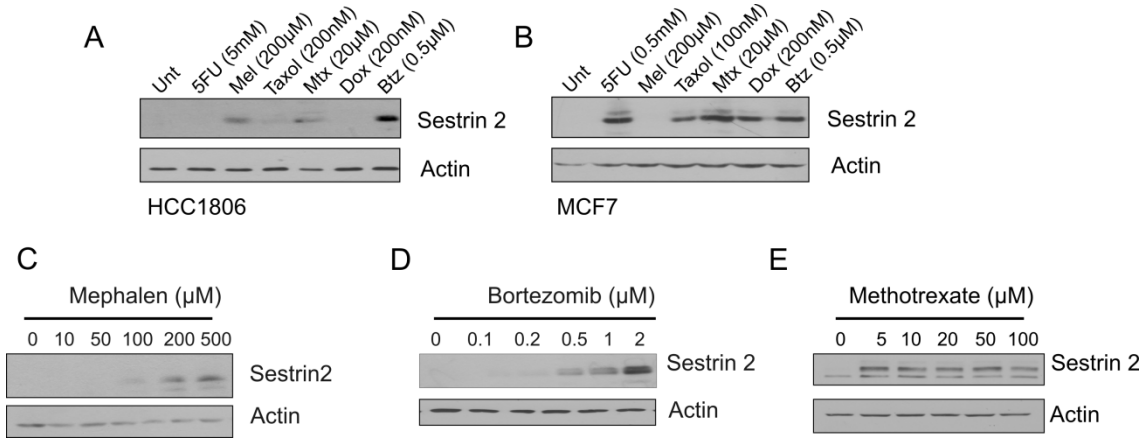


Figure 5.2.4 Chemotherapy drugs can induce Sestrin 2 expression. Examined a range of common chemotherapeutic for their ability to induce Sestrin 2 expression in (A) HCC1806 and (B) MCF7 cell lines. Sestrin 2 is induced in a dose-dependent manner in HCC1806 cells by (C) Melphalan, (D) Bortezomib, (E) Methotrexate. Results are representative of three independent repeats. Result is representative of 3 independent repeats.

5.2.3.3 Bortezomib (Btz)

Btz is a proteasome inhibitor most typically associated with treatment of multiple myeloma but has also reached phase 2 clinical trials in breast cancer (157). Btz has been widely demonstrated to induce UPR activation due to a build up of proteins in the cell following proteasome inhibition (309). Time course analysis of 0.5 μM Btz at 0, 3, 6, 12 and 24 h was conducted and Sestrin 2 expression and LC3I-II conversion monitored by western blotting. 0.5 μM Btz could induce Sestrin 2 expression by 12 h and as it also induced LC3I-II conversion, a downstream functional autophagic response was likely occuring. Next UPR activation was monitored and observed XBP1 and ATF4 activation occurred prior to Sestrin 2 expression, leading us to speculate that the UPR might be driving Btz induced Sestrin 2 expression. To ascertain the functionality of the Sestrin 2 expression, p-mTOR levels were examined and could see a reduction by 12 h post treatment (Fig 5.2.5B).

While reduction in p-mTOR levels is indicative of induction of autophagy, choloroquine was also used in combination with Btz to examine whether autophagy was inducing autophagic flux. An accumulation of LC3-I to LC3-II conversion with cholorquine (CQ) indicated that Btz was inducing a functional autophagic response (Fig 5.2.5C).

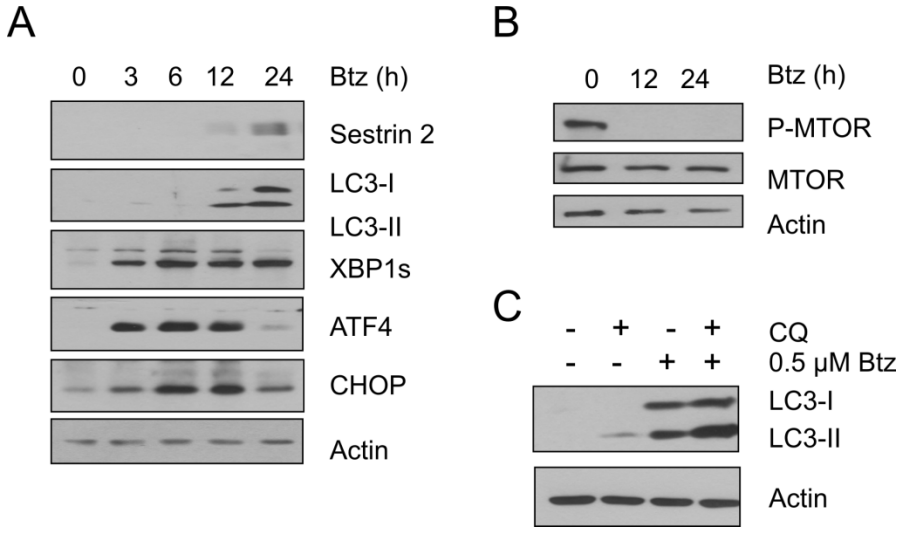


Figure 5.2.5 Btz induces Sestrin 2 and autophagy. **(A)** Timecourse analysis of 0.5μM Btz revealed that Sestrin 2 is induced by 12 h of treatment and is accompanied by LC3 conversion alongside UPR activation in HCC1806. **(B)** Btz could reduces p-MTOR levels by 12 h in HCC1806 (C) Btz induces functional autophagy downstream, shown here by accumulation of LC3 conversion upon addition of CQ. Results representative of 3 independent repeats.

5.2.3.4 Methotrexate (Mtx)

Mtx is an anti-folate drug used in the treatment of TNBC. Following the same rationale used with Btz, a timecourse experiment 0, 3, 6, 12, 24 and 48 h with 20μM Mtx was completed and Sestrin 2 expression and LC3I-II conversion monitored. Induction of Sestrin 2 at 24 h and 48 h was observed correlating with LC3I-II conversion. UPR analysis over this time course also showed activation of the IRE1 and PERK pathways simultaneously or prior to Sestrin 2 induction. The potency of Sestrin 2 expression was shown by reduction of phosphor-mTOR levels by 24 h post treatment (Fig 5.2.6B) Moreover this induction appeared to coincide with induction of a functional autophagic response as an increase in accumulation of LC3-II is apparent when treated in combination with CQ (Fig 5.2.6 C).

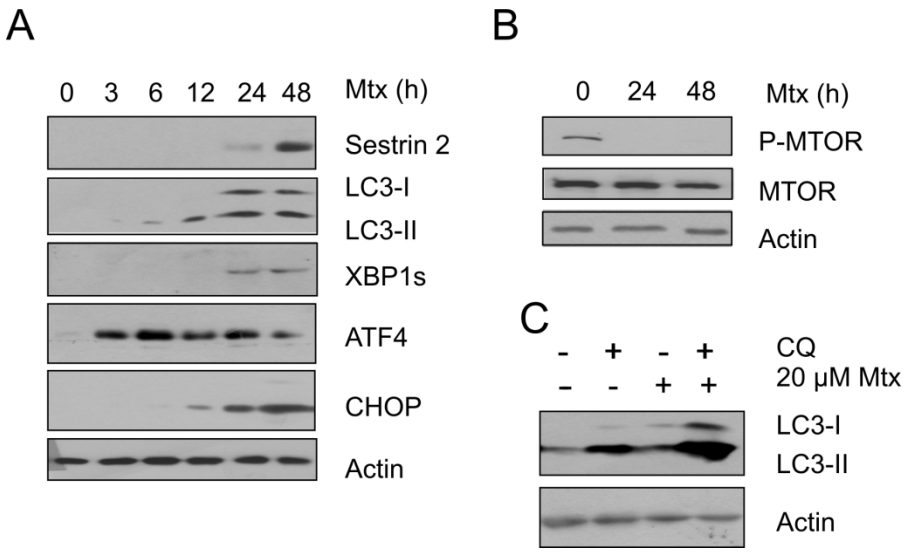


Figure 5.2.6 Methotrexate induces Sestrin2 and Autophagy (A) Timecourse analysis of 20μM Mtx revealed that Sestrin 2 is induced by 24 h of treatment and is accompanied by LC3 conversiona alongside UPR activation in HCC1806. **(B)** Mtx could reduces p-MTOR levels by 12 h in HCC1806 **(C)** Mtx induces functional autophagy downstream, shown here by accumulation of LC3 conversion upon addition of CQ. Result is representative of 3 independent repeats.

5.2.4 Sestrin 2 knockdown sensitises to Btz and Mtx

Confident that potent induction of Sestrin 2 was occurring with both Btz and Mtx treatment, the consequence of Sestrin 2 knockdown to the outcome of cell death was explored with Btz and Mtx. HCC1806 cells were transfected with siRNA against Sestrin 2 or non coding control siRNA and cell viability examined after treatment with these drugs. Sestrin 2 knockdown significantly reduced the cell viability of HCC1806 cells at 72 h with similar trend appearing at the earlier 24 h and 48 h timepoints (Fig 5.2.7A). A comparable result was attained using Btz in conjunction with Sestrin 2 knockdown, with significant differences recorded at 48 h and 72 h post treatment (Fig 5.2.7B). Confirmation of Sestrin 2 knockdown at 72 h is shown by western blot analysis of Sestrin 2 following Mtx treatment (Fig 5.2.7C).

Interestingly Sestrin 2 knockdown may also affect the long term survival of cells following Mtx treatment as shown in Fig 5.2.7 D. Cells with and without siRNA mediated knockdown of Sestrin 2 were treated for 48 h with Mtx at which point, the treatment was removed and the cells allowed to recover for a further 10 days. As crystal violet staining clearly shows absence of colonies with Sestrin 2 knockdown compared to non coding controls indicative of Sestrin 2 effects on long term survival of HCC1806 cells following Mtx treatment.

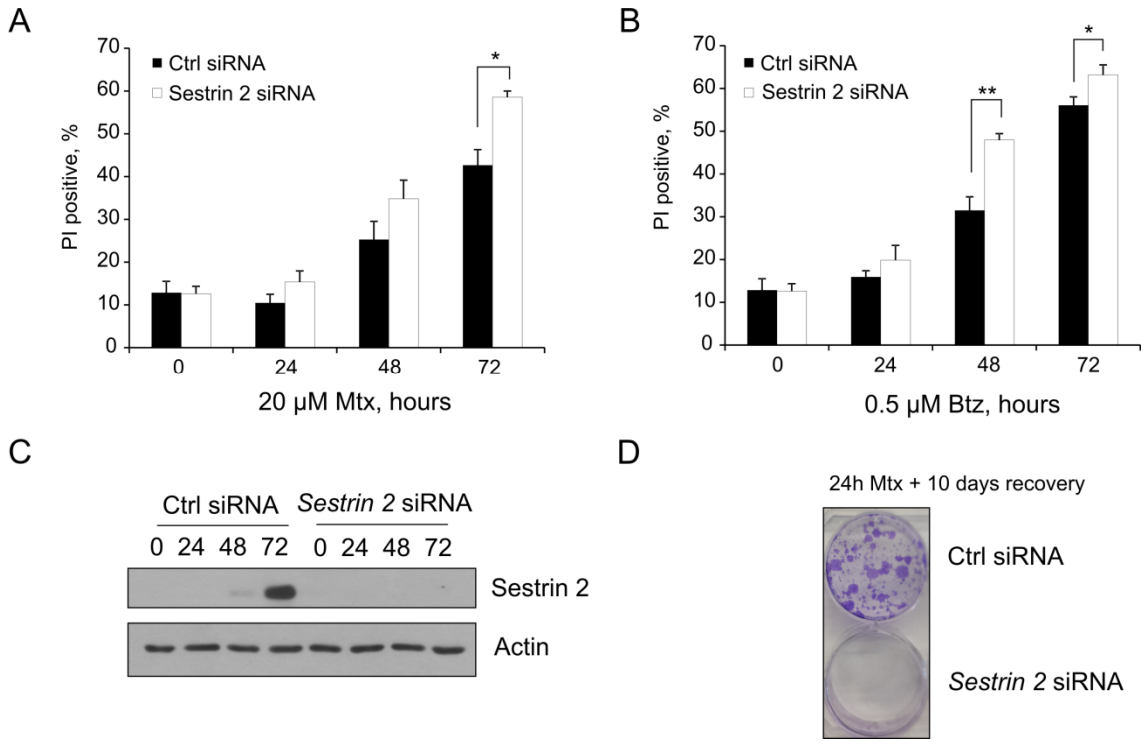


Figure 5.2. 7Sestrin 2 knockdown sensities to Mtx and Btz treatment in HCC1806 (A) Sestrin 2 knockdown sensities to Mtx induced cell death measured by PI incorporation. **(B)** Sestrin 2 knockdown sensitised to Btz induced cell death measured by PI incorporation. **(C)** Sestrin 2 knockdown is confirmed and maintained up to 72 h durin mtx treatment in HCC1806 **(D)** Crystal violet staining shows that Sestrin 2 knockdown can effect long term survival of HCC1806 following Mtx treatment. Values shown are representative of three independent ±SEM P <0.05, ** P<0.01, *** P< 0.001 Students *t*-test

5.2.5 Sestrin 2 induction during Mtx treatment is mediated from the UPR

As HCC1806 cells harbour a p53 mutation so it appears likely that induction of Sestrin 2 is mediated from an alternative pathway. To investigate if this expression was indeed linked to UPR activation, PERK and IRE1 inhibitors were employed and Sestrin 2 expression monitored during Mtx treatment. Both PERK and IRE1 inhibitors were capable of reducing Sestrin 2 levels following Mtx treatment as shown in Fig 5.2.8A. This corresponded to a similar reduction in LC3I-II conversion. In order to validate that these inhibitors were in fact functional, XBP1s and p-PERK levels (measured by upshift of PERK) were also examined and confirm functional inhibition of this pathways during treatment.

As previous literature had indicated that PERK knockdown could down regulate Sestrin 2 expression, HCC1806 cells were transfected with siRNA against XBP1 or corresponding control siRNA and repeated both Mtx and Btz treatments monitoring Sestrin 2 levels. siXBP1 reduced Sestrin 2 expression compared to control allowing us to confirm that Sestrin 2 expression is also mediated through IRE1/XBP1 axis (Fig 5.2.8B-C).

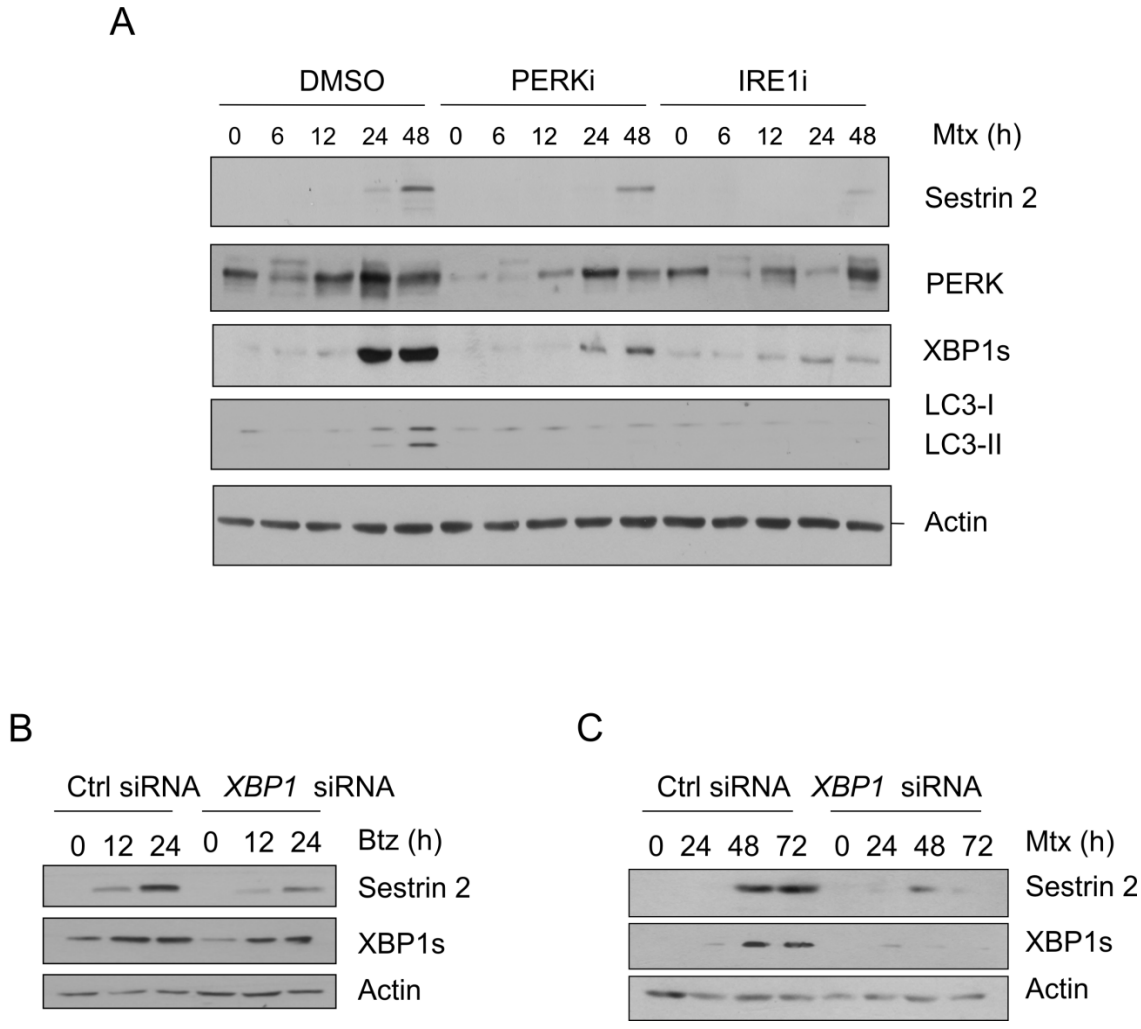


Figure 5.2.8 Mtx and Btz induction of sestrin 2 is mediated from the UPR. (A) PERK and IRE1 inhibition can reduce Sestrin 2 induction following Mtx treatment in HCC1806 cells. PERK and XBP1 confirm functional inhibition. **(B)** siRNA knockdown of XBP1 also reduces Btz induced Sestrin 2 expression **(C)** siRNA knockdown of XBP1 reduces Mtx induced Sestrin 2 expression in HCC1806 cells. Result is representative of three independent repeats

5.2.6 Mtx co-ordinates cell death via UPR

This study suggests that Sestrin 2 modulates cell survival during Mtx treatment; owing to UPR induction of Sestrin 2, I was interested in exploring the role of UPR arms during Mtx treatment. Inhibitors of IRE1 and PERK were applied during Mtx treatment in HCC1806 and cell death monitored by PI incorporation. Surprisingly inhibition of PERK and to a greater extent IRE1 inhibition protected the cells from Mtx induced cell death (Fig 5.2.9). This suggests that Mtx induces cell death via downstream UPR mechanisms. Considering the vast downstream targets of both arms it is uncertain how precisely it is elucidating its effect but most certainly warrants further investigation.

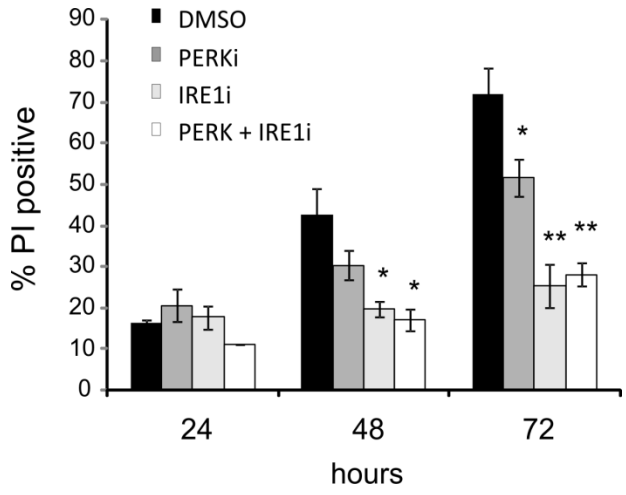


Figure 5.2.9 Inhibition of PERK and IRE1 protect from Mtx induced cell death. PERK and IRE1 inhibitors were used singly and in combination during Mtx treatment in HCC1806 cells. Cell death was assessed at 24, 48 and 72 h by PI incorporation. Values shown are representative of three independent ±SEM P <0.05, ** P<0.01, *** P< 0.001 Students *t*-test

5.2.7 Effect of Sestrin 2 is not mediated via Autophagy

Precisely how Sestrin 2 knockdown could be reducing the viability of Mtx treatment remained unknown and so initially I looked at the autophagic pathway. During Sestrin 2 knockdown, functional autophagy was reduced as shown with CQ combinations in Fig

5.2.10A-B. This led us to hypothesis that as Mtx was inducing functional autophagy and Sestrin 2 knockdown was reducing these levels, perhaps Sestrin 2 was elucidating a pro-survival autophagic response. To investigate if this was true a PI3K inhibitor Spautin 1 was applied to inhibit the early stages of the autophagic pathway to and monitored cell viability (Fig 5.2.10C). Surprisingly, inhibition of autophagy via Spautin 1 did not reduce the cell viability of HCC1806 unlike Sestrin 2 knockdown. To confirm the functionality of the Spautin 1 inhibitor autophagic flux was monitored via LC3I-II conversion in conjunction with CQ. As shown in this inhibitor was in fact functional and could inhibit autophagy in this system observed via diminished LC3I-II conversion Fig 5.2.10D.

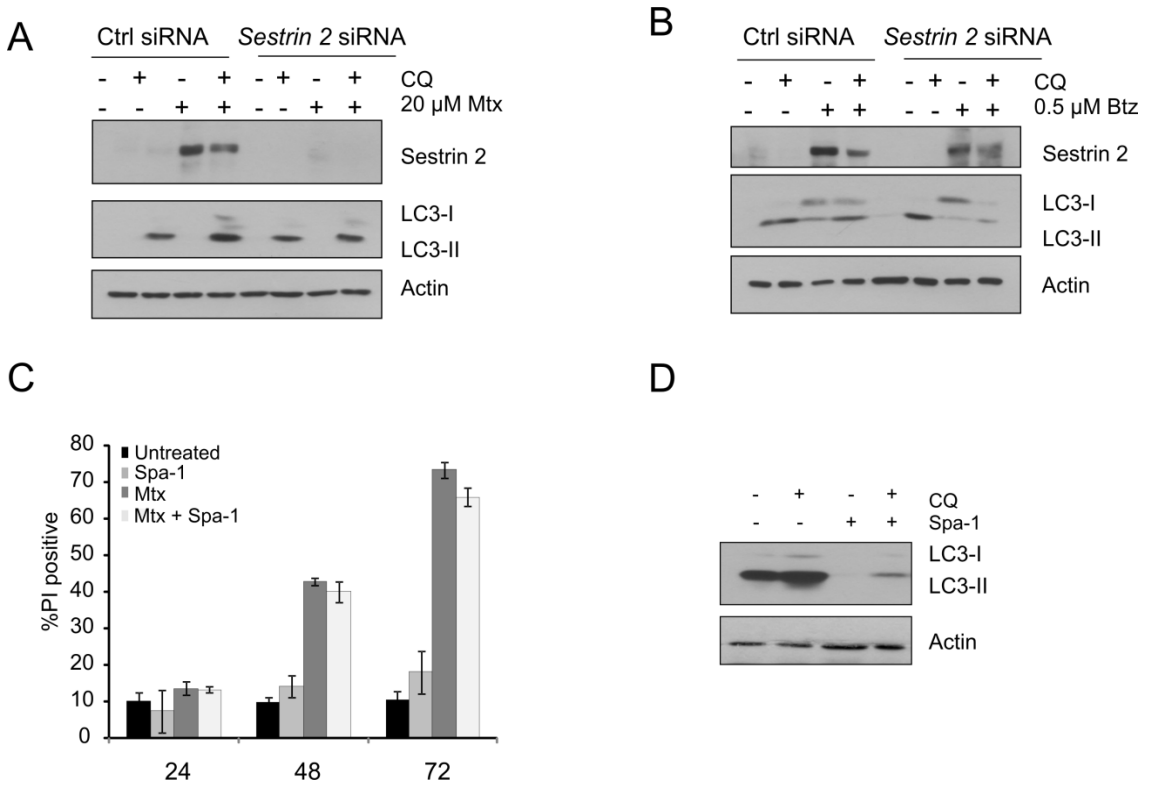


Figure 5.2.10 Sestrin 2 induces functional autophagy it does not mediate the effect of Sestrin 2 on Mtx viability. (A) Sestrin 2 knockdown reduces autophagy levels during Mtx treatment (B) Sestrin 2 knockdown reduces autophagy levels during Btz treatment. **(C)** inhibition of autophagy by Spa-1 does not affect Mtx induced cell death in HCC1806 cells. **(D)** Spa-1 is functional in this system, shown by reduced LC3 conversion levels. Values shown are representative of three independent ±SEM P <0.05, ** P<0.01, *** P< 0.001 Students *t*-test

5.2.8 Sestrin 2 sensitises to Mtx treatment via its effect on protein translation

As previously mentioned Sestrin 2 can elicit two downstream functions; induction of autophagy and inhibition of protein translation via mTOR. As autophagy did not appear to affect Mtx induced cell death I explored whether cell Sestrin 2 knockdown was modulating protein load in this system. Firstly, levels of p-MTOR and p-S6K following Sestrin 2 knockdown were examined, revealing that knockdown maintained elevated levels of these proteins (Fig 5.2.11).

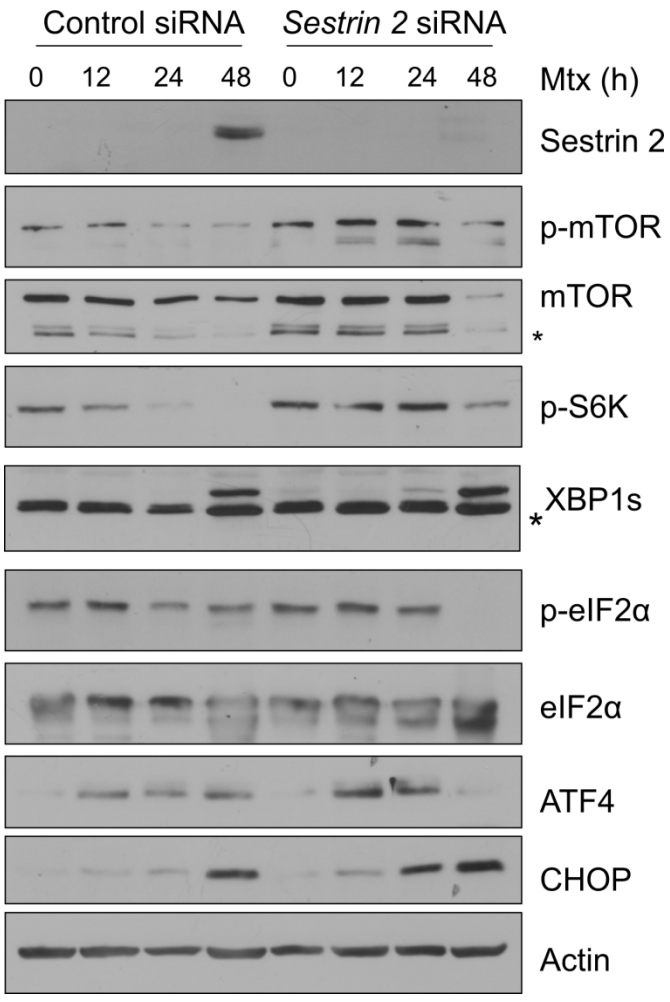


Figure 5.2.11 Sestrin 2 knockdown exacerbates ER stress during Mtx treatment. Knockdown of Sestrin 2 maintains p-mTOR and p-S6K levels during Mtx treatment and enhances induction of UPR markers, XBP1, CHOP, ATF4 and p-eIF2α. Result is representative of three independent repeats.

This suggests that in conditions of Sestrin 2 knockdown, mTOR is inactive and rates of protein translation remain unaffected. To determine whether reduced Sestrin 2 expression exacerbated Mtx-induced ER stress I examined XBP1, ATF4, CHOP and eIF2α phosphorylation in Mtx treated control versus Sestrin 2 siRNA transfected cells. Enhanced XBP1s, CHOP, ATF4 and eIF2α phosphorylation was observed in Mtx treated Sestrin 2 siRNA cells suggesting Sestrin 2 knockdown potentiates the ER stress response in Mtx treated HCC1806 cells

As previous data suggests that Mtx induces cell death via UPR, (PERK/IRE1inhibitors afford almost complete protection against Mtx induced cell death) and here ER stress is exacerbated by Sestrin 2 knockdown, one can postulate that it is Sestrin 2 effects on protein translation that enhances Mtx induced cell death.

5.3 Discussion

ER stress is known to activate transcription of a cohort of genes that aim to restore cellular homeostasis or, if conditions are too severe, to promote cell death. In a microarray analysis of the genes differentially expressed in HCT116 cells after treatment with ER stress inducers (NCBI, Gene Expression Omnibus, Accession GSE63252 and (297)) our lab identified Sestrin 2 as one of the genes that was induced in response to unresolved UPR. Sestrin 2 is a previously identified stress-responsive protein, that is involved in inactivation of mTORC1 and autophagy induction. (303) Therefore hypothesising that a novel link exists between UPR and Sestrin 2 -AMPK-mediated autophagy induction I investigated whether Sestrin 2 induction under ER stress conditions affects autophagy and cell survival. My results demonstrated that the UPR is capable of inducing Sestrin 2 levels and that this is independent of p53 status. At the conception of this work there was no published research linking the UPR to Sestrin 2 induction. I show that expression of Sestrin 2 can be mediated by the PERK and IRE1 arms of UPR and to my knowledge this is the first study to show regulation by both arms of UPR. During the course of this study, two other papers reported that PERK can mediate upregulation of Sestrin 2 in response to ER stress, however these described the involvement of C/EBP β and ATF4 in the transcriptional regulation of Sestrin 2, respectively (308, 310). My work agrees with the role of the PERK arm, and additionally, I clearly show that the IRE1 arm contributes to Sestrin 2 induction during ER stress using chemical inhibition of IRE1 and siRNA knockdown of XBP1. Furthermore, there is very little Sestrin 2 induction if either arm is blocked, suggesting that both arms are essential for a robust response. This might suggest that IRE1/XBP1 and the PERK arm act in synergy with a much tighter cross-talk occurring between the two arms of the UPR than previously thought.

A report by Lefort *et al* suggests that autophagy plays an important role in the progression and chemoresistance of TNBC (311). With this in mind I hypothesized that inhibiting Sestrin 2 upregulation and therefore autophagy induction, could be therapeutically relevant. I determined that both Btz and Mtx can activate the UPR and

induce Sestrin 2 in both MCF7 and HCC1806 cell lines. To maximise clinical relevance I explored Sestrin 2 inhibition in HCC1806, a TNBC cell line that harbours a p53 mutation. Importantly, knockdown of Sestrin 2 significantly sensitized the cells to treatment with both drugs; however this was not due to inhibition of autophagy but rather mediated by Sestrin 2 affects on protein translation. I demonstrate that Mtx can mediate cell death via the UPR as PERK and IRE1 inhibition protected cells from Mtx induced cell death. I show that Sestrin 2 knockdown maintains p-MTOR levels, and so block on protein translation fails to occur therefore increasing protein load in the cell. This increase in protein load exacerbates ER stress levels driving the cell further towards terminal UPR response and cell death.

The role of Sestrin 2 in cancer is not limited to breast cancer as a recent study shows the oncogenic role of Sestrin 2 in skin cancer, with data suggesting that the knockdown of Sestrin 2 can sensitize human squamous cell carcinoma and melanoma cells to the treatment with chemotherapeutics (312). These results, together with the results of our study, highlight the importance of Sestrin 2 in cell survival during stress and the clinical potential it harbours. Additionally, I believe that this work provides a novel mechanism for the sensitization of breast cancer to chemotherapeutics, suggesting a strong rationale for investigating combinations of ER stress inducing chemotherapeutic agents and Sestrin 2 modulating agents

Chapter 6

General Discussion

6.1.1 Understanding IRE1 activity in Breast cancer

XBP1 was first identified in 1990 as a bZIP transcription factor in B-cells. This initial study characterised its interaction with the conserved X2 boxes located in the promoters of MHC class II genes (313). Three years later two groups independently characterised IRE1 as a mediator of ER-nucleus crosstalk and a component of the UPR (314, 315). Through yeast studies in 1996, IRE1 RNase domain was first linked to Hac1 splicing and activation (316). However it wasn't until the early 2000s, over ten years since its first discovery, that XBP1 was identified as the Hac1 human homolog involved in ER stress responses (317, 318). Over the past fifteen years our understanding of IRE1/XBP1s axis has continued to evolve with emphasis more recently being placed on its pathological implications; this thesis builds upon this work.

6.1.1.1 IRE1 activation

In this study I develop our understanding of IRE1/XBP1s role in relation to breast cancer with a view that the underlying mechanisms may be applicable to a wider understanding of IRE1/XBP1s role in cancer. I confirm XBP1s overexpression across a range of breast cancer subtypes and explore its functional implications on the secretome and treatment responses. Through inhibition of IRE1 RNase domain by MKC-8866, this study confirms that it is through enhanced IRE1 activity that XBP1 becomes active in breast cancer. How IRE1 is first activated in this system remains elusive. Literature suggests that somatic mutations within IRE1 may drive its activity but with increasing studies identifying ER stress independent activation of IRE1 it is possible that currently unidentified mechanisms are at play (85, 88, 122). In chapter 3 of this thesis I identify a role of IRE1/XBP1s on the levels of secreted factors. While cytokine array analysis reveal regulation of particular cytokines (namely IL-6, IL-8 GRO-α, GM-CSf and TGFβ) this approach was limited in its capacity to identify new factors or regulators beyond the scope of the array. Previously, secreted VEGF was reported to drive IRE1

RNase activation. Zeng *et al* demonstrated that VEGF selectively activates IRE1 pathway; ligand binding leads to receptor internalisation where it then localises to the ER, directly binding to IRE1 and leading to its activation (88).Whether VEGF is the only secreted factor capable of eliciting such a result is unknown. Identification of IRE1/XBP1s regulated secreted factors inspires the question as to whether these secreted factors are capable of inducing a positive feedback loop leading to IRE1 activation? Alternatively could VEGF signalling itself be at play in this system? Further analysis of the crosstalk between IRE1 and the secretome will be needed to address this question.

6.1.1.2 RIDD

The second downstream mechanism of IRE1 RNase domain is RIDD, a less understood function (67). The discovery of RIDD less than ten years ago has opened a new and exciting avenue in the IRE1 field. MKC-8866 is a potent inhibitor of IRE1 RNase and therefore can also inhibit RIDD; this inhibitor will allow further characterisation of RIDD mechanism. Whether RIDD plays a functional role in cancer biology is currently unknown but with high basal IRE1 RNase activity (shown via enhanced XBP1 splicing) widely reported in cancer, it is therefore likely that RIDD is also functional and may have greater implications in cancer biology than currently understood. This two part downstream function of IRE1 RNase (XBP1 splicing/ RIDD) requires further research especially if we are to consider targeting IRE1 therapeutically in clinic. This thesis demonstrates this necessity, for instance MKC-8866 inhibition of XBP1s can elicit cell death under reduced serum conditions, but when used in combination with Methotrexate treatment can protect from Methotrexate-induced cell death. I cannot confirm that the latter result is mediated through RIDD inhibition, however as RIDD is linked to terminal UPR response and induction of cell death it is certainly a possibility (67).

RIDD targets are thought to be cell type specific therefore the potential impact of inhibiting RIDD may vary amongst cell types. However, as enhanced XBP1 splicing affords a survival advantage to cancer cells it is likely that RIDD should aid this process further rather than impede it. It is possible that RIDD targets may include mRNA of

tumour suppressor proteins or other negative regulators of tumourigenesis. Future studies would be needed to explore this hypothesis.

6.1.2 The role of XBP1s in malignant tumours

6.1.2.1 Tumour development

It is undisputed that XBP1s has implications in cancer biology, the most compelling evidence coming from the studies indicating that *XBP1* knockout cells cannot form tumours *in vivo* (126). The inability of XBP1 knockout to form tumours highlights its importance to tumourigenesis and begs the question; is XBP1 required for the early stage tumour development and transformation of cancer and therefore could it be an oncogenic factor? XBP1s is robustly linked to multiple myeloma and as previously mentioned contributes to tumourigenesis and therapy resistance (319, 320). XBP1s is also involved in the secretory differentiation of the precursor cells of this malignancy, plasma cells, and therefore plays a crucial role in its pathogenesis (321). Based on the similar secretory nature of breast epithelial cells and the expression of XBP1s across a range of breast cancer subtypes, XBP1s could be playing a causal role in breast cancer development. Moreover XBP1s is up-regulated in breast cancer cells but not in nontumorigenic MCF10A cells. I report that MKC-8866 has no effect on the proliferation of this cell lines suggesting that the function of XBP1s is limited to a breast cancer phenotype and not a 'normal' breast epithelial cell line. It would be interesting to determine if XBP1s can drive transformation of breast epithelial cells or is rather a consequence of transformation?

6.1.2.2 Tumour maintenance – surviving Hypoxia

Recently XBP1s has been shown to dimerise and regulate HIF1α transcriptional targets (125). As hypoxia is a classical feature of solid tumour microenvironment this would suggest the inhibition of XBP1s by MKC-8866 could potentially affect the ability of any solid tumour mass to survive within a hypoxic environment. This dimerisation was

reported in breast cancer but further studies are needed to determine if this interaction is a widespread cellular occurrence or cell type specific. However the importance of XBP1s to cellular survival during hypoxia was already established by Albert Koong's group in 2004. Here they showed that XBP1$^{-/-}$ cells were more sensitive to hypoxia environment than their wildtype counter parts (126). This paper strengthens further the potential XBP1/HIF1α crosstalk. Additionally HIF1α is linked to upregulation of autophagy genes including Sestrin 2, which was demonstrated in macrophages and murine fibroblast cells (322, 323). This would suggest that in conjunction with the results I present here, that Sestrin 2 is a common transcription factor of HIF1α and XBP1s thus potentially strengthening the possibility of HIF1α/XBP1 interplay in breast cancer.

Breast cancer patients usually present with an established tumour mass, therefore even if XBP1 drives tumour formation the concept of targeting XBP1s prior tumour establishment is futile. This thesis demonstrates that IRE1 inhibition by MKC-8866 can reduce the volume of an established tumour mass *in vivo* both alone and in combination with Taxol. Moreover, as XBP1s levels are currently used to help identify luminal A breast cancer subtypes in clinic and we observed reduced MCF7 tumour volume with MKC-8866, this establishes a foundation for the use of MKC-8866 on luminal A breast tumours.

6.1.3 Therapeutic potential of MKC-8866 as a combination treatment in breast cancer

Inhibitors are a commonly used approach in cancer treatments, and currently include but are not limited to tyrosine kinase inhibitors, proteasome inhibitors, mTOR inhibitors, PI3K inhibitors, histone deacetylase inhibitors and hedgehog pathway blockers (324-327). Therefore the concept of using UPR inhibitors and more specifically MKC-8866 is clinically unsurprising. This study provides compelling evidence for MKC-8866 clinical application as a single agent therapy in breast cancer. However, to attain maximum value of IRE1 inhibition it must be considered as a component of a larger treatment regime for patients. Here I provide evidence to suggest

that MKC-8866 could be valuable in TNBC treatment when used in combination with Taxol.

6.1.3.1 MKC-8866 in combination with Taxol- a promising result

First discovered in 1960s, Taxol was introduced into clinics as an anticancer drug in the 1990s and since then has shown clinical potential across a range of cancer types (328, 329). MKC-8866 appears a potential treatment option preceding Taxol administration, with significant increase in cell death observed in MDA-MB231 cell line during these conditions. MKC-8866 could enhance Taxol induced cell death even further during reduced nutrient conditions. This observation was developed further via xenograft model of TNBC where MKC-8866 enhanced the reduction in tumour volume when used in combination with Taxol compared to Taxol alone. The clinical potential of MKC-8866 in combination with Taxol was further exemplified by reduced tumour re-growth measured eight days post treatment. In clinic the recurrence and regrowth of cancerous tumours is one of the greatest difficulties faced. How MKC-8866 is precisely diminishing regrowth here is intriguing. One possibility is due to reduced cytokine production, as suggested in chapter 3 of this thesis. It is interesting to speculate that through reduced secreted growth factor levels, the tumour is unable to facilitate the same level of regrowth as vehicle counterparts. Alternatively diminished regrowth could be occurring via down regulation of cancer stem cells, as suggested by Chen *et al* (125).

Cancer stem cell hypothesis is based upon the idea that within a tumour volume lies a subset of cancer cells with stem cell like ability to self renew but also terminally differentiate which then constitutes the bulk of tumour volume. It is unlikely that these cells are derived from normal tissue stem cell but rather are cells that through transformation acquire stem cell like properties (330). Breast cancer stem cells can be identified through expression of cell surface markers $CD44^{high}CD24^{low}$ (242). It wasn't until very recently that XBP1 knockdown was reported to down regulate the expression of these cell surface markers in breast cancer and therefore decrease breast cancer stem cell population. If XBP1 knockdown can decrease cancer stem cell populations this has

vast clinical implications for MKC-8866, as the main challenge of cancer treatments is the ability of cancer to self renew and self propagate once treatment has ceased. Moreover studies have reported that it is the cancer stem cell population that propagates tumour establishment *in vivo,* with cells lacking these stem cell markers unable to establish tumours (242, 245). With research reporting the inability of *XBP1*$^{-/-}$ cells to form tumours *in vivo* and recent links between XBP1 and cancer stem cell maintenance, it is curious to speculate that these two observations may be linked. If this hypothesis is true the therapeutic potential of MKC-8866 far exceeds its ability to simply enhance Taxol induced cell death.

6.1.3.2 MKC-8866 in combination with Methotrexate- a cautionary result

While MKC-8866 appears a promising combinational approach with Taxol, it is clear from Methotrexate studies that combination treatments with MKC-8866 are not always a favourable approach. IRE1 inhibition could potentiate Taxol treatment but on the other hand could protect from Methotrexate induced death. This poses a concern that unless we fully understand the mechanism of drugs used in clinic, combination therapies could have devastating effects. I show Methotrexate induces a UPR response and is a potent inducer of XBP1s. Typically this would suggest that inhibiting the pro-survival mechanisms of UPR (i.e. XBP1s) should push the cell towards a quicker terminal response and therefore enhanced cell death. This hypothesis proved true for Taxol but contradictory for Methotrexate. Cell survival observed with Methotrexate and MKC-8866 highlights the importance of considering whether cell death or cell survival is mediated via IRE1 axis. Benefits of considering MKC-8866 as a combination therapy would need to be weighed against the possibility of affording a survival advantage as seen with Methotrexate.

6.1.3.3 Sestrin 2, a novel XBP1s target with clinical potential in breast cancer

Autophagy is linked to cancer biology at multiple levels and can provide the precursors for macromolecular synthesis aiding cancer survival during nutrient starvation and hypoxia. Sestrin 2 acts upstream in the autophagy pathway and while no Sestrin 2 inhibitors currently exist; this study provides a rationale for the inhibition of Sestrin 2 as therapeutic option in the treatment of breast cancer. Through an enhancement of oxidative stress autophagy can also promote genome instability and malignant transformation (331). Furthermore, autophagy can be induced in response to chemotherapeutic treatment and is reported to aid drug resistance (332). Previous studies have shown that autophagy inhibition can induce cell death in breast cancer (333). This strengthens the rationale of Sestrin 2 inhibition as an untapped therapeutic approach in the treatment of breast cancer. I demonstrate that Sestrin 2 knockdown can modulate autophagy levels in breast cancer but furthermore can also affect protein load during Methotrexate treatment, driving the cell towards cell death thus generating a dual downstream cytotoxic effect. Additionally I identify Sestrin 2 as a downstream target of XBP1s but show that by down regulating Sestrin 2 directly, one can bypass the survival advantage afforded by XBP1s inhibition in this system.

6.1.4 IRE/XBP1s can influence the tumour microenvironment through regulation of secreted factors

It is widely accepted that the interactions between tumour cells and tumour microenvironment can drive development and progression of a tumour toward a malignant phenotype. Maffini *et al* reported that neoplastic epithelial cells could be reprogrammed following recombination of mammary carcinoma cells with normal mammary gland stroma (334). Evidence also suggests that polymorphism in extracellular matrix proteins can increase the risk of breast cancer metastasis (335). Cytokines are proteins often found in the extracellular matrix (ECM) which mediate cell to cell communication and are shown to participate in cancer progression (336). Here I report that inhibition of IRE1 signalling by MKC-8866 can modulate secreted factors of

breast cancer cells including IL-6, IL-8, and GRO-α. IL-6 can led to induction of oxidative stress and is reported exerts pro-angiogenic effects in the tumour microenvironment of solid tumours (337). Additionally IL-8 is linked to colon cancer proliferation migration and angiogenesis while also accelerating EMT (338, 339). While XBP1s has been previously linked to IL-6 and IL-8 induction, this is the first report to demonstrate this phenomenon occurs in breast cancer (86, 263). Both GRO-α and XBP1 have independently been reported as important for lung metastasis in breast cancer, this body of research suggests that this contribution to metastasis may be one and the same (125, 268, 340). Metastasis is an extremely important clinical event, since a majority of cancer mortality is associated with the metastatic tumours, rather than the primary tumour. An inhibitor capable of down regulating factors that are so convincingly associated with cancer progression in addition to XBP1s has exciting clinical potential. It is clear that IRE1/XBP1 axis is linked to regulation of cytokines, however the precise role remains undetermined. XBP1 may be directly upregulating the transcription of these cytokines or a feedback loop may be occurring where XBP1s functions upstream, driving a set of cytokines which then lead to the upregulation of additional factors.

Multiple chemotherapeutic drugs are reported to have the potential of inducing expression and release of pro inflammatory cytokines from dying tumour cells (341). A report from Pusztai *et al* demonstrated elevated levels of IL-6 and IL-8 in plasma levels of breast cancer patients treated with paciltaxel every three weeks. This suggests that in addition to inhibition of pro-survival XBP1splicing with ER stress inducer Taxol, MKC-8866 could also be enhancing cytotoxicity of Taxol treatment through down regulation of IL-6 and IL-8 induction.

6.2 Conclusion

In conclusion this body of research strengthens the rationale of targeting IRE1/XBP1 axis as a therapeutic option in breast cancer both as a single agent approach and in combination with Taxol. However, it also suggests caution that MKC-8866 may not be suitable option in combination with all chemotherapeutics. Additionally this research strengthens our knowledge of ER-autophagy crosstalk in breast cancer, identifying Sestrin 2 as a novel downstream target of IRE1/XBP1s axis.

7 Future Directions

The first results chapter of this thesis discussed the effect of IRE1 signalling on the secretion of extracellular factors. This study could be pursued through various avenues. First of all whether the regulation of cytokines observed in MDA-MB231 is also true for other cell lines or is a cell line specific response remains to be determined. Additional breast cancer cell lines could be explored to resolve this issue. I would select MCF7 as a second breast cancer cell line as data from an *in vivo* xenograft model is already available and would complement the research. To complete this, a cytokine array could be undertaken on supernatant from MCF7 cells during reduced serum condition with and without addition of MKC-8866.

More information about MKC-8866 regulation of cytokine levels could also be drawn from the *in vivo* xenograft models; it would be interesting to monitor cytokine levels from blood samples throughout the *in vivo* experiments. Blood samples could be taken from the *in vivo* mouse models periodically during the study and cytokine levels assessed to determine if MKC-8866 can affect these levels *in vivo* and if so, establish the kinetics of their down regulation, affording a more comprehensive understanding of MKC-8866 regulation of these cytokines.

While this study has identified at least five cytokines which may be down regulated by MKC-8866 a larger proteomic analysis of the secretome could be undertaken to identify additional markers. While larger studies may be beneficial to identify new cytokine targets downstream of IRE1, ELISA analysis of all cytokine levels identified would be needed to quantify the extent of MKC-8866 regulation.

While identifying extracellular factors regulated by MKC-8866 is interesting, it would also be advantageous to examine how IRE1/XBP1 is regulating these factors. Initially western blotting timecourse analysis of each cytokine following addition of neutralising antibodies would help identify if any cytokine was driving regulation of another cytokine. If addition if neutralising antibodies were not affecting the regulation of the other cytokines this would suggest an independent regulator, possibly XBP1s, was

driving their regulation in this system. One experiment to determine if XBP1s was directly driving upregulation of these cytokines would be chIP. This would determine if XBP1s could bind to the promoter sequence of each cytokine. However chIP analysis has limitations as it would only establish the physical binding of XBP1s to the promoter region and not its activity. Knock in mutations to the identified regions of XBP1s binding within the promoter sequence and subsequent analysis of cytokine levels would allow the researcher to establish whether XBP1s was driving transcription at this site.

To enhance functional implications of the regulation of these extracellular factors, their impact on recruitment of immune cells could also be examined. To achieve this conditioned media with and without MKC-8866 could be used as attractant medium for migration and invasion chambers and the migration/invasion of Thp-1 cells assessed. Furthermore while xenograft studies are a common *in vivo* approach, spontaneous models of breast cancer would be more desirable as they would allow us to determine the effect on immune cells and better recapitulate metastasis.

There is also scope to pursue a more basic research approach and use MKC-8866 as a tool to identify the role of RIDD in breast cancer. To date no report has identified RIDD targets in breast cancer and in light of enhanced XBP1 splicing, RIDD is also likely to be functional. To identify targets a microarray analysis of RNA from cells treated with and without MKC-8866 could be performed. Pathway analysis would reveal if particular cellular pathways are exploited by IRE1 signalling. Potential RIDD targets would be initially identified as those significantly increased in the presence of MKC-8866 and then screened for RIDD cleavage consensus sequence. The most highly likely candidate could then be validated through qPCR analysis using MKC-8866 to block IRE1 RNase and actinomycin D to block transcription allowing the researcher to rule out the involvement of XBP1s targets. Once identified RIDD targets could be over expressed or knockdown to identify the functional importance of these targets in breast cancer.

The ability of MKC-8866 to reduce tumour regrowth *in vivo* is interesting and warrants further investigation. The cancer stem cell hypothesis is an interesting topic and there is mounting evidence to suggest that XBP1s may be an important factor in their biology. Previous literature has shown that XBP1 is required for tumour establishment *in vivo* and knockdown of XBP1 can reduce $CD44^{high}CD24^{low}$ expression (125, 126), which is a readout of breast cancer stem cells. Furthermore as XBP1 is also correlated to overall patient survival (125) and we observe that IRE1 inhibition affects tumour regrowth, could this phenomenon be linked to XBP1s regulation of breast cancer stem cells? To assess this hypothesis, expression of $CD44^{high}CD24^{low}$ could be determined in this system, however for a more accurate analysis of cancer stem cell population ALDH1 levels would also be included (243). Additionally mammosphere formation using cancer stem cell populations (as described in (342) and (343)) would allow us to determine the effect of MKC-8866 not alone on the expression of these markers but also extend to the effect of MKC-8866 on cancer stem cell proliferation and self renewal capacity.

The concept of XBP1s driven Sestrin 2 induction could be strengthen via chIP analysis of Sestrin 2 promoter for XBP1s binding. Similarly knock in mutation of the promoter sequence would also add clarity to this hypothesis.

References

1. Fagone P, Jackowski S. (2009) Membrane phospholipid synthesis and endoplasmic reticulum function. Journal of lipid research 50 Suppl:S311-316.
2. Jäger R, Bertrand MJ, Gorman AM, Vandenabeele P, Samali A. (2012) The unfolded protein response at the crossroads of cellular life and death during endoplasmic reticulum stress. Biology of the cell / under the auspices of the European Cell Biology Organization 104:259-270.
3. Matsuura S, Masuda R, Sakai O, Tashiro Y. (1983) Immunoelectron microscopy of the outer membrane of rat hepatocyte nuclear envelopes in relation to the rough endoplasmic reticulum. Cell structure and function 8:1-9.
4. Chen Y, Zhang Y, Yin Y, et al. (2005) SPD--a web-based secreted protein database. Nucleic acids research 33:D169-173.
5. High S. (1995) Protein translocation at the membrane of the endoplasmic reticulum. Progress in biophysics and molecular biology 63:233-250.
6. Braakman I, Bulleid NJ. (2011) Protein folding and modification in the mammalian endoplasmic reticulum. pp. 71-99.
7. Hurtley SM, Helenius A. (1989) Protein Oligomerization in the Endoplasmic Reticulum. Annual Review of Cell Biology 5:277-307.
8. Spurway TD, Dalley JA, High S, Bulleid NJ. (2001) Early events in glycosylphosphatidylinositol anchor addition. substrate proteins associate with the transamidase subunit gpi8p. The Journal of biological chemistry 276:15975-15982.
9. Ferguson MA. (1999) The structure, biosynthesis and functions of glycosylphosphatidylinositol anchors, and the contributions of trypanosome research. Journal of cell science 112:2799-2809.
10. Tatu U, Braakman I, Helenius A. (1993) Membrane glycoprotein folding, oligomerization and intracellular transport: effects of dithiothreitol in living cells. Embo j 12:2151-2157.
11. Pena F, Jansens A, van Zadelhoff G, Braakman I. (2010) Calcium as a crucial cofactor for low density lipoprotein receptor folding in the endoplasmic reticulum. The Journal of biological chemistry 285:8656-8664.
12. Braakman I, Helenius J, Helenius A. (1992) Role of ATP and disulphide bonds during protein folding in the endoplasmic reticulum. Nature 356:260-262.
13. Oslowski CM, Urano F. (2011) Measuring ER stress and the unfolded protein response using mammalian tissue culture system. Methods in enzymology 490:71-92.
14. Healy SJM, Gorman AM, Mousavi-Shafaei P, Gupta S, Samali A. (2009) Targeting the endoplasmic reticulum-stress response as an anticancer strategy. European Journal of Pharmacology 625:234-246.
15. Yoshida H. (2007) ER stress and diseases. FEBS Journal 274:630-658.
16. Roussel BD, Kruppa AJ, Miranda E, Crowther DC, Lomas DA, Marciniak SJ. (2013) Endoplasmic reticulum dysfunction in neurological disease. The Lancet Neurology 12:105-118.
17. Brodsky Jeffrey L. Cleaning Up: ER-Associated Degradation to the Rescue. Cell 151:1163-1167.

18. Nakatsukasa K, Brodsky JL. (2008) The Recognition and Retrotranslocation of Misfolded Proteins from the Endoplasmic Reticulum. Traffic (Copenhagen, Denmark) 9:861-870.
19. Nishikawa SI, Fewell SW, Kato Y, Brodsky JL, Endo T. (2001) Molecular chaperones in the yeast endoplasmic reticulum maintain the solubility of proteins for retrotranslocation and degradation. J Cell Biol 153:1061-1070.
20. Gillece P, Luz JM, Lennarz WJ, de La Cruz FJ, Romisch K. (1999) Export of a cysteine-free misfolded secretory protein from the endoplasmic reticulum for degradation requires interaction with protein disulfide isomerase. J Cell Biol 147:1443-1456.
21. Cormier JH, Tamura T, Sunryd JC, Hebert DN. (2009) EDEM1 recognition and delivery of misfolded proteins to the SEL1L-containing ERAD complex. Mol Cell 34:627-633.
22. Schäfer A, Wolf DH. (2009) Sec61p is part of the endoplasmic reticulum-associated degradation machinery. The EMBO Journal 28:2874-2884.
23. Greenblatt EJ, Olzmann JA, Kopito RR. (2011) Derlin-1 is a rhomboid pseudoprotease required for the dislocation of mutant alpha-1 antitrypsin from the endoplasmic reticulum. Nature structural & molecular biology 18:1147-1152.
24. Claessen JH, Kundrat L, Ploegh HL. (2012) Protein quality control in the ER: balancing the ubiquitin checkbook. Trends in cell biology 22:22-32.
25. Meusser B, Hirsch C, Jarosch E, Sommer T. (2005) ERAD: The long road to destruction. Nature Cell Biology 7:766-772.
26. Ruggiano A, Foresti O, Carvalho P. (2014) ER-associated degradation: Protein quality control and beyond. The Journal of Cell Biology 204:869-879.
27. Guerriero CJ, Brodsky JL. (2012) The delicate balance between secreted protein folding and endoplasmic reticulum-associated degradation in human physiology. Physiological reviews 92:537-576.
28. Gorman AM, Healy SJM, Jäger R, Samali A. (2012) Stress management at the ER: Regulators of ER stress-induced apoptosis. Pharmacology and Therapeutics 134:306-316.
29. Bertolotti A, Zhang Y, Hendershot LM, Harding HP, Ron D. (2000) Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. Nature Cell Biology 2:326-332.
30. Ye J, Rawson RB, Komuro R, et al. (2000) ER Stress Induces Cleavage of Membrane-Bound ATF6 by the Same Proteases that Process SREBPs. Molecular Cell 6:1355-1364.
31. Logue SE, Cleary P, Saveljeva S, Samali A. (2013) New directions in ER stress-induced cell death. Apoptosis : an international journal on programmed cell death 18:537-546.
32. Harding HP, Zhang Y, Ron D. (1999) Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. Nature 397:271-274.
33. Lu PD, Harding HP, Ron D. (2004) Translation reinitiation at alternative open reading frames regulates gene expression in an integrated stress response. J Cell Biol 167:27-33.

34. Harding HP, Novoa I, Zhang Y, et al. (2000) Regulated Translation Initiation Controls Stress-Induced Gene Expression in Mammalian Cells. Molecular Cell 6:1099-1108.
35. Szegezdi E, Logue SE, Gorman AM, Samali A. (2006) Mediators of endoplasmic reticulum stress-induced apoptosis. EMBO Reports 7:880-885.
36. Saito A, Ochiai K, Kondo S, et al. (2011) Endoplasmic reticulum stress response mediated by the PERK-eIF2(alpha)-ATF4 pathway is involved in osteoblast differentiation induced by BMP2. The Journal of biological chemistry 286:4809-4818.
37. Harding HP, Zhang Y, Zeng H, et al. (2003) An Integrated Stress Response Regulates Amino Acid Metabolism and Resistance to Oxidative Stress. Molecular Cell 11:619-633.
38. Cullinan SB, Diehl JA. (2006) Coordination of ER and oxidative stress signaling: the PERK/Nrf2 signaling pathway. Int J Biochem Cell Biol 38:317-332.
39. Zinszner H, Kuroda M, Wang X, et al. (1998) CHOP is implicated in programmed cell death in response to impaired function of the endoplasmicâ€‰reticulum. Genes & Development 12:982-995.
40. Shao D, Ni J, Shen Y, et al. (2015) CHOP mediates XBP1S-induced renal mesangial cell necrosis following high glucose treatment. European Journal of Pharmacology 758:89-96.
41. Yoshida H, Okada T, Haze K, et al. (2000) ATF6 activated by proteolysis binds in the presence of NF-Y (CBF) directly to the cis-acting element responsible for the mammalian unfolded protein response. Molecular and Cellular Biology 20:6755-6767.
42. McCullough KD, Martindale JL, Klotz L-O, Aw T-Y, Holbrook NJ. (2001) Gadd153 Sensitizes Cells to Endoplasmic Reticulum Stress by Down-Regulating Bcl2 and Perturbing the Cellular Redox State. Mol Cell Biol 21:1249-1259.
43. McCullough KD, Martindale JL, Klotz LO, Aw TY, Holbrook NJ. (2001) Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bc12 and perturbing the cellular redox state. Molecular and Cellular Biology 21:1249-1259.
44. Puthalakath H, O'Reilly LA, Gunn P, et al. (2007) ER Stress Triggers Apoptosis by Activating BH3-Only Protein Bim. Cell 129:1337-1349.
45. Ghosh AP, Klocke BJ, Ballestas ME, Roth KA. (2012) CHOP potentially co-operates with FOXO3a in neuronal cells to regulate PUMA and BIM expression in response to ER stress. PloS one 7:e39586.
46. Marciniak SJ, Yun CY, Oyadomari S, et al. (2004) CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. Genes and Development 18:3066-3077.
47. Li G, Mongillo M, Chin KT, et al. (2009) Role of ERO1-α-mediated stimulation of inositol 1,4,5-triphosphate receptor activity in endoplasmic reticulum stress-induced apoptosis. Journal of Cell Biology 186:783-792.
48. Novoa I, Zeng H, Harding HP, Ron D. (2001) Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2α. Journal of Cell Biology 153:1011-1021.
49. Gupta S, Giricz Z, Natoni A, et al. (2012) NOXA contributes to the sensitivity of PERK-deficient cells to ER stress. FEBS letters 586:4023-4030.

50. Haze K, Yoshida H, Yanagi H, Yura T, Mori K. (1999) Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. Mol Biol Cell 10:3787-3799.
51. Nadanaka S, Okada T, Yoshida H, Mori K. (2007) Role of Disulfide Bridges Formed in the Luminal Domain of ATF6 in Sensing Endoplasmic Reticulum Stress. Molecular and Cellular Biology 27:1027-1043.
52. Higa A, Taouji S, Lhomond S, et al. (2014) Endoplasmic reticulum stress-activated transcription factor ATF6alpha requires the disulfide isomerase PDIA5 to modulate chemoresistance. Mol Cell Biol 34:1839-1849.
53. Wu J, Rutkowski DT, Dubois M, et al. (2007) ATF6alpha optimizes long-term endoplasmic reticulum function to protect cells from chronic stress. Dev Cell 13:351-364.
54. Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K. (2001) XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Cell 107:881-891.
55. Morishima N, Nakanishi K, Nakano A. (2011) Activating transcription factor-6 (ATF6) mediates apoptosis with reduction of myeloid cell leukemia sequence 1 (Mcl-1) protein via induction of WW domain binding protein. Journal of Biological Chemistry 286:35227-35235.
56. Boot-Handford R, Briggs M. The unfolded protein response and its relevance to connective tissue diseases. Cell and Tissue Research 339:197-211.
57. Oikawa D, Kimata Y, Kohno K, Iwawaki T. (2009) Activation of mammalian IRE1α upon ER stress depends on dissociation of BiP rather than on direct interaction with unfolded proteins. Experimental Cell Research 315:2496-2504.
58. Lee KPK, Dey M, Neculai D, Cao C, Dever TE, Sicheri F. (2008) Structure of the Dual Enzyme Ire1 Reveals the Basis for Catalysis and Regulation in Nonconventional RNA Splicing. Cell 132:89-100.
59. Korennykh AV, Egea PF, Korostelev AA, et al. (2009) The unfolded protein response signals through high-order assembly of Ire1. Nature 457:687-693.
60. Knighton DR, Zheng J, Ten Eyck LF, et al. (1991) Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. Science 253:407-414.
61. Han D, Lerner AG, Vande Walle L, et al. (2009) IRE1α Kinase Activation Modes Control Alternate Endoribonuclease Outputs to Determine Divergent Cell Fates. Cell 138:562-575.
62. van Anken E, Pincus D, Coyle S, et al. (2014) Specificity in endoplasmic reticulum-stress signaling in yeast entails a step-wise engagement of HAC1 mRNA to clusters of the stress sensor Ire1,
63. Yanagitani K, Imagawa Y, Iwawaki T, et al. (2009) Cotranslational Targeting of XBP1 Protein to the Membrane Promotes Cytoplasmic Splicing of Its Own mRNA. Molecular Cell 34:191-200.
64. Lu Y, Liang F-X, Wang X. (2014) A Synthetic Biology Approach Identifies the Mammalian UPR RNA Ligase RtcB. Molecular Cell 55:758-770.
65. Sidrauski C, Walter P. (1997) The Transmembrane Kinase Ire1p Is a Site-Specific Endonuclease That Initiates mRNA Splicing in the Unfolded Protein Response. Cell 90:1031-1039.

66. Yanagitani K, Kimata Y, Kadokura H, Kohno K. (2011) Translational pausing ensures membrane targeting and cytoplasmic splicing of XBP1u mRNA. Science 331:586-589.
67. Maurel M, Chevet E, Tavernier J, Gerlo S. (2014) Getting RIDD of RNA: IRE1 in cell fate regulation. Trends in biochemical sciences 39:245-254.
68. Marcu MG, Doyle M, Bertolotti A, Ron D, Hendershot L, Neckers L. (2002) Heat shock protein 90 modulates the unfolded protein response by stabilizing IRE1alpha. Mol Cell Biol 22:8506-8513.
69. Ota A, Wang Y. (2012) Cdc37/Hsp90 protein-mediated regulation of IRE1α protein activity in endoplasmic reticulum stress response and insulin synthesis in INS-1 cells. Journal of Biological Chemistry 287:6266-6274.
70. Gupta S, Deepti A, Deegan S, Lisbona F, Hetz C, Samali A. (2010) HSP72 protects cells from ER stress-induced apoptosis via enhancement of IRE1α-xbp1 signaling through a physical interaction. PLoS Biology 8.
71. Tirasophon W, Lee K, Callaghan B, Welihinda A, Kaufman RJ. (2000) The endoribonuclease activity of mammalian IRE1 autoregulates its mRNA and is required for the unfolded protein response. Genes & Development 14:2725-2736.
72. Kaser A, Lee A-H, Franke A, et al. (2008) XBP1 Links ER Stress to Intestinal Inflammation and Confers Genetic Risk for Human Inflammatory Bowel Disease. Cell 134:743-756.
73. Lee A-H, Scapa EF, Cohen DE, Glimcher LH. (2008) Regulation of Hepatic Lipogenesis by the Transcription Factor XBP1. Science 320:1492-1496.
74. Gao B, Lee SM, Chen A, et al. (2008) Synoviolin promotes IRE1 ubiquitination and degradation in synovial fibroblasts from mice with collagen-induced arthritis,
75. Zhu X, Zhang J, Sun H, et al. (2014) Ubiquitination of inositol-requiring enzyme 1 (IRE1) by the E3 ligase CHIP mediates the IRE1/TRAF2/JNK pathway. The Journal of biological chemistry 289:30567-30577.
76. Lu G, Ota A, Ren S, et al. (2013) PPM1l encodes an inositol requiring-protein 1 (IRE1) specific phosphatase that regulates the functional outcome of the ER stress response. Molecular Metabolism 2:405-416.
77. Qiu Y, Mao T, Zhang Y, et al. (2010) A crucial role for RACK1 in the regulation of glucose-stimulated IRE1α activation in pancreatic β-cells. Science signaling 3:ra7-ra7.
78. Hetz C, Bernasconi P, Fisher J, et al. (2006) Proapoptotic BAX and BAK Modulate the Unfolded Protein Response by a Direct Interaction with IRE1α. Science 312:572-576.
79. Rodriguez DA, Zamorano S, Lisbona F, et al. (2012) BH3-only proteins are part of a regulatory network that control the sustained signalling of the unfolded protein response sensor IRE1[alpha]. EMBO J 31:2322-2335.
80. Bailly-Maitre B, Fondevila C, Kaldas F, et al. (2006) Cytoprotective gene bi-1 is required for intrinsic protection from endoplasmic reticulum stress and ischemia-reperfusion injury. Proceedings of the National Academy of Sciences of the United States of America 103:2809-2814.
81. Bailly-Maitre B, Belgardt BF, Jordan SD, et al. (2010) Hepatic Bax inhibitor-1 inhibits IRE1α and protects from obesity-associated insulin resistance and glucose intolerance. Journal of Biological Chemistry 285:6198-6207.

82. Lisbona F, Rojas-Rivera D, Thielen P, et al. (2009) BAX Inhibitor-1 Is a Negative Regulator of the ER Stress Sensor IRE1α. Molecular Cell 33:679-691.
83. Castillo K, Rojas-Rivera D, Lisbona F, et al. (2011) BAX inhibitor-1 regulates autophagy by controlling the IRE1α branch of the unfolded protein response. The EMBO Journal 30:4465-4478.
84. Rong J, Chen L, Toth JI, Tcherpakov M, Petroski MD, Reed JC. (2011) Bifunctional Apoptosis Regulator (BAR), an Endoplasmic Reticulum (ER)-associated E3 Ubiquitin Ligase, Modulates BI-1 Protein Stability and Function in ER Stress. Journal of Biological Chemistry 286:1453-1463.
85. Jwa M, Chang P. (2012) PARP16 is a tail-anchored endoplasmic reticulum protein required for the PERK- and IRE1α-mediated unfolded protein response. Nat Cell Biol 14:1223-1230.
86. Martinon F, Chen X, Lee A-H, Glimcher LH. (2010) TLR activation of the transcription factor XBP1 regulates innate immune responses in macrophages. Nat Immunol 11:411-418.
87. Liu Y, Shao M, Wu Y, et al. (2015) Role for the endoplasmic reticulum stress sensor IRE1α in liver regenerative responses. Journal of Hepatology 62:590-598.
88. Zeng L, Xiao Q, Chen M, et al. (2013) Vascular endothelial cell growth-activated XBP1 splicing in endothelial cells is crucial for angiogenesis. Circulation 127:1712-1722.
89. Lin JH, Li H, Yasumura D, et al. (2007) IRE1 Signaling Affects Cell Fate During the Unfolded Protein Response. Science 318:944-949.
90. Wang XZ, Harding HP, Zhang Y, Jolicoeur EM, Kuroda M, Ron D. (1998) Cloning of mammalian Ire1 reveals diversity in the ER stress responses. EMBO Journal 17:5708-5717.
91. Donovan N, Becker EBE, Konishi Y, Bonni A. (2002) JNK Phosphorylation and Activation of BAD Couples the Stress-activated Signaling Pathway to the Cell Death Machinery. Journal of Biological Chemistry 277:40944-40949.
92. Lei K, Davis RJ. (2003) JNK phosphorylation of Bim-related members of the Bcl2 family induces Bax-dependent apoptosis. Proceedings of the National Academy of Sciences of the United States of America 100:2432-2437.
93. Yang Q, Kim Y-S, Lin Y, Lewis J, Neckers L, Liu Z-G. (2006) Tumour necrosis factor receptor 1 mediates endoplasmic reticulum stress-induced activation of the MAP kinase JNK. EMBO Reports 7:622-627.
94. Hu P, Han Z, Couvillon AD, Kaufman RJ, Exton JH. (2006) Autocrine Tumor Necrosis Factor Alpha Links Endoplasmic Reticulum Stress to the Membrane Death Receptor Pathway through IRE1α-Mediated NF-κB Activation and Down-Regulation of TRAF2 Expression. Molecular and Cellular Biology 26:3071-3084.
95. Hollien J, Weissman JS. (2006) Decay of Endoplasmic Reticulum-Localized mRNAs During the Unfolded Protein Response. Science 313:104-107.
96. Clauss IM, Chu M, Zhao JL, Glimcher LH. (1996) The basic domain/leucine zipper protein hXBP-1 preferentially binds to and transactivates CRE-like sequences containing an ACGT core. Nucleic acids research 24:1855-1864.
97. Ono SJ, Liou HC, Davidon R, Strominger JL, Glimcher LH. (1991) Human X-box-binding protein 1 is required for the transcription of a subset of human class II

major histocompatibility genes and forms a heterodimer with c-fos. Proceedings of the National Academy of Sciences of the United States of America 88:4309-4312.
98. Yamamoto K, Sato T, Matsui T, et al. (2007) Transcriptional induction of mammalian ER quality control proteins is mediated by single or combined action of ATF6alpha and XBP1. Developmental cell 13:365-376.
99. Wang FM, Chen YJ, Ouyang HJ. (2011) Regulation of unfolded protein response modulator XBP1s by acetylation and deacetylation. Biochemical Journal 433:245-252.
100. Jiang Z, Fan Q, Zhang Z, et al. (2012) SENP1 deficiency promotes ER stress-induced apoptosis by increasing XBP1 SUMOylation. Cell Cycle 11:1118-1122.
101. Lee AH, Iwakoshi NN, Glimcher LH. (2003) XBP-1 Regulates a Subset of Endoplasmic Reticulum Resident Chaperone Genes in the Unfolded Protein Response. Molecular and Cellular Biology 23:7448-7459.
102. Masaki T, Yoshida M, Noguchi S. (1999) Targeted disruption of CRE-Binding factor TREB5 gene leads to cellular necrosis in cardiac myocytes at the embryonic stage. Biochemical and biophysical research communications 261:350-356.
103. Reimold AM, Etkin A, Clauss I, et al. (2000) An essential role in liver development for transcription factor XBP-1. Genes and Development 14:152-157.
104. Reimold AM, Iwakoshi NN, Manis J, et al. (2001) Plasma cell differentiation requires the transcription factor XBP-1. Nature 412:300-307.
105. Lee AH, Chu GC, Iwakoshi NN, Glimcher LH. (2005) XBP-1 is required for biogenesis of cellular secretory machinery of exocrine glands. EMBO Journal 24:4368-4380.
106. Bettigole SE, Lis R, Adoro S, et al. (2015) The transcription factor XBP1 is selectively required for eosinophil differentiation. Nat Immunol 16:829-837.
107. Simson L, Ellyard JI, Dent LA, et al. (2007) Regulation of carcinogenesis by IL-5 and CCL11: a potential role for eosinophils in tumor immune surveillance. Journal of immunology (Baltimore, Md : 1950) 178:4222-4229.
108. Chu VT, Frohlich A, Steinhauser G, et al. (2011) Eosinophils are required for the maintenance of plasma cells in the bone marrow. Nat Immunol 12:151-159.
109. Cyr DM, Langer T, Douglas MG. (1994) DnaJ-like proteins: molecular chaperones and specific regulators of Hsp70. Trends in biochemical sciences 19:176-181.
110. Qiu XB, Shao YM, Miao S, Wang L. (2006) The diversity of the DnaJ/Hsp40 family, the crucial partners for Hsp70 chaperones. Cellular and molecular life sciences : CMLS 63:2560-2570.
111. Lai CW, Otero JH, Hendershot LM, Snapp E. (2012) ERdj4 protein is a soluble endoplasmic reticulum (ER) DnaJ family protein that interacts with ER-associated degradation machinery. The Journal of biological chemistry 287:7969-7978.
112. Shen Y, Meunier L, Hendershot LM. (2002) Identification and characterization of a novel endoplasmic reticulum (ER) DnaJ homologue, which stimulates ATPase activity of BiP in vitro and is induced by ER stress. The Journal of biological chemistry 277:15947-15956.
113. Lee TG, Tomita J, Hovanessian AG, Katze MG. (1990) Purification and partial characterization of a cellular inhibitor of the interferon-induced protein kinase of Mr 68,000 from influenza virus-infected cells. Proc Natl Acad Sci U S A 87:6208-6212.

114. Melville MW, Tan SL, Wambach M, Song J, Morimoto RI, Katze MG. (1999) The cellular inhibitor of the PKR protein kinase, P58(IPK), is an influenza virus-activated co-chaperone that modulates heat shock protein 70 activity. Journal of Biological Chemistry 274:3797-3803.
115. Yu M, Haslam RHA, Haslam DB. (2000) HEDJ, an Hsp40 co-chaperone localized to the endoplasmic reticulum of human cells. Journal of Biological Chemistry 275:24984-24992.
116. Yamaguchi A, Hori O, Stern DM, Hartmann E, Ogawa S, Tohyama M. (1999) Stress-associated endoplasmic reticulum protein 1 (SERP1)/Ribosome-associated membrane protein 4 (RAMP4) stabilizes membrane proteins during stress and facilitates subsequent glycosylation. J Cell Biol 147:1195-1204.
117. Hosokawa N, Wada I, Hasegawa K, et al. (2001) A novel ER α-mannosidase-like protein accelerates ER-associated degradation. EMBO Reports 2:415-422.
118. Molinari M, Calanca V, Galli C, Lucca P, Paganetti P. (2003) Role of EDEM in the release of misfolded glycoproteins from the calnexin cycle. Science 299:1397-1400.
119. Fang Y, Yan J, Ding L, et al. (2004) XBP-1 increases ERalpha transcriptional activity through regulation of large-scale chromatin unfolding. Biochemical and biophysical research communications 323:269-274.
120. Vandewynckel YP, Laukens D, Geerts A, et al. (2013) The paradox of the unfolded protein response in cancer. Anticancer research 33:4683-4694.
121. Ma Y, Hendershot LM. (2004) The role of the unfolded protein response in tumour development: friend or foe? Nat Rev Cancer 4:966-977.
122. Greenman C, Stephens P, Smith R, et al. (2007) Patterns of somatic mutation in human cancer genomes. Nature 446:153-158.
123. Parsons DW, Jones S, Zhang X, et al. (2008) An Integrated Genomic Analysis of Human Glioblastoma Multiforme. Science (New York, NY) 321:1807.
124. Guichard C, Amaddeo G, Imbeaud S, et al. (2012) Integrated analysis of somatic mutations and focal copy-number changes identifies key genes and pathways in hepatocellular carcinoma. Nat Genet 44:694-698.
125. Chen X, Iliopoulos D, Zhang Q, et al. (2014) XBP1 promotes triple-negative breast cancer by controlling the HIF1[agr] pathway. Nature 508:103-107.
126. Romero-Ramirez L, Cao H, Nelson D, et al. (2004) XBP1 is essential for survival under hypoxic conditions and is required for tumor growth. Cancer research 64:5943-5947.
127. Zhang K, Wong HN, Song B, Miller CN, Scheuner D, Kaufman RJ. (2005) The unfolded protein response sensor IRE1α is required at 2 distinct steps in B cell lymphopoiesis. Journal of Clinical Investigation 115:268-281.
128. Carrasco DR, Sukhdeo K, Protopopova M, et al. (2007) The differentiation and stress response factor XBP-1 drives multiple myeloma pathogenesis. Cancer Cell 11:349-360.
129. Tay KH, Luan Q, Croft A, et al. (2014) Sustained IRE1 and ATF6 signaling is important for survival of melanoma cells undergoing ER stress. Cellular Signalling 26:287-294.
130. Lee JH, Won SM, Suh J, et al. (2010) Induction of the unfolded protein response and cell death pathway in Alzheimer's disease, but not in aged Tg2576 mice. Experimental & molecular medicine 42:386-394.

131. Casas-Tinto S, Zhang Y, Sanchez-Garcia J, Gomez-Velazquez M, Rincon-Limas DE, Fernandez-Funez P. (2011) The ER stress factor XBP1s prevents amyloid-β neurotoxicity. Human Molecular Genetics 20:2144-2160.
132. Ryu EJ, Harding HP, Angelastro JM, Vitolo OV, Ron D, Greene LA. (2002) Endoplasmic reticulum stress and the unfolded protein response in cellular models of Parkinson's disease. Journal of Neuroscience 22:10690-10698.
133. Shimura H, Hattori N, Kubo SI, et al. (2000) Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. Nature Genetics 25:302-305.
134. Sado M, Yamasaki Y, Iwanaga T, et al. (2009) Protective effect against Parkinson's disease-related insults through the activation of XBP1. Brain Research 1257:16-24.
135. Hetz C, Thielen P, Matus S, et al. (2009) XBP-1 deficiency in the nervous system protects against amyotrophic lateral sclerosis by increasing autophagy. Genes Dev 23:2294-2306.
136. Bertolotti A, Wang X, Novoa I, et al. (2001) Increased sensitivity to dextran sodium sulfate colitis in IRE1β-deficient mice. Journal of Clinical Investigation 107:585-593.
137. Iwakoshi NN, Pypaert M, Glimcher LH. (2007) The transcription factor XBP-1 is essential for the development and survival of dendritic cells. The Journal of experimental medicine 204:2267-2275.
138. Winnay JN, Boucher J, Mori MA, Ueki K, Kahn CR. (2010) A regulatory subunit of phosphoinositide 3-kinase increases the nuclear accumulation of X-box-binding protein-1 to modulate the unfolded protein response. Nature Medicine 16:438-445.
139. Özcan U, Cao Q, Yilmaz E, et al. (2004) Endoplasmic Reticulum Stress Links Obesity, Insulin Action, and Type 2 Diabetes. Science 306:457-461.
140. Papandreou I, Denko NC, Olson M, et al. (2011) Identification of an Ire1alpha endonuclease specific inhibitor with cytotoxic activity against human multiple myeloma. Blood 117:1311-1314.
141. Mimura N, Fulciniti M, Gorgun G, et al. (2012) Blockade of XBP1 splicing by inhibition of IRE1α is a promising therapeutic option in multiple myeloma. Blood 119:5772-5781.
142. Westerheide SD, Morimoto RI. (2005) Heat Shock Response Modulators as Therapeutic Tools for Diseases of Protein Conformation. Journal of Biological Chemistry 280:33097-33100.
143. Atkins C, Liu Q, Minthorn E, et al. (2013) Characterization of a Novel PERK Kinase Inhibitor with Antitumor and Antiangiogenic Activity. Cancer Research 73:1993-2002.
144. Cojocari D, Vellanki RN, Sit B, Uehling D, Koritzinsky M, Wouters BG. (2013) New small molecule inhibitors of UPR activation demonstrate that PERK, but not IRE1α signaling is essential for promoting adaptation and survival to hypoxia. Radiotherapy and Oncology 108:541-547.
145. Vandewynckel Y-P, Laukens D, Bogaerts E, et al. (2015) Modulation of the unfolded protein response impedes tumor cell adaptation to proteotoxic stress: a PERK for hepatocellular carcinoma therapy. Hepatol Int 9:93-104.

146. Boyce M, Bryant KF, Jousse C, et al. (2005) A selective inhibitor of eIF2alpha dephosphorylation protects cells from ER stress. Science 307:935-939.
147. Drexler HC. (2009) Synergistic apoptosis induction in leukemic cells by the phosphatase inhibitor salubrinal and proteasome inhibitors. PloS one 4:e4161.
148. Schewe DM, Aguirre-Ghiso JA. (2009) Inhibition of eIF2alpha dephosphorylation maximizes bortezomib efficiency and eliminates quiescent multiple myeloma cells surviving proteasome inhibitor therapy. Cancer research 69:1545-1552.
149. Jiang H-Y, Wek RC. (2005) Phosphorylation of the α-Subunit of the Eukaryotic Initiation Factor-2 (eIF2α) Reduces Protein Synthesis and Enhances Apoptosis in Response to Proteasome Inhibition. Journal of Biological Chemistry 280:14189-14202.
150. Cnop M, Ladriere L, Hekerman P, et al. (2007) Selective inhibition of eukaryotic translation initiation factor 2 alpha dephosphorylation potentiates fatty acid-induced endoplasmic reticulum stress and causes pancreatic beta-cell dysfunction and apoptosis. The Journal of biological chemistry 282:3989-3997.
151. Teng Y, Gao M, Wang J, et al. (2014) Inhibition of eIF2[alpha] dephosphorylation enhances TRAIL-induced apoptosis in hepatoma cells. Cell Death Dis 5:e1060.
152. Sidrauski C, Acosta-Alvear D, Khoutorsky A, et al. (2013) Pharmacological brake-release of mRNA translation enhances cognitive memory,
153. Halliday M, Radford H, Sekine Y, et al. (2015) Partial restoration of protein synthesis rates by the small molecule ISRIB prevents neurodegeneration without pancreatic toxicity. Cell Death Dis 6:e1672.
154. Schewe DM, Aguirre-Ghiso JA. (2008) ATF6α-Rheb-mTOR signaling promotes survival of dormant tumor cells in vivo. Proceedings of the National Academy of Sciences 105:10519-10524.
155. Okada T, Haze K, Nadanaka S, et al. (2003) A serine protease inhibitor prevents endoplasmic reticulum stress-induced cleavage but not transport of the membrane-bound transcription factor ATF6. The Journal of biological chemistry 278:31024-31032.
156. Zhang H, Nakajima S, Kato H, et al. (2013) Selective, potent blockade of the IRE1 and ATF6 pathways by 4-phenylbutyric acid analogues. British Journal of Pharmacology 170:822-834.
157. Yang CH, Gonzalez-Angulo AM, Reuben JM, et al. (2006) Bortezomib (VELCADE) in metastatic breast cancer: pharmacodynamics, biological effects, and prediction of clinical benefits. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO 17:813-817.
158. Roccaro AM, Vacca A, Ribatti D. (2006) Bortezomib in the treatment of cancer. Recent patents on anti-cancer drug discovery 1:397-403.
159. Chou T-F, Brown SJ, Minond D, et al. (2011) Reversible inhibitor of p97, DBeQ, impairs both ubiquitin-dependent and autophagic protein clearance pathways. Proceedings of the National Academy of Sciences 108:4834-4839.
160. Wiseman RL, Zhang Y, Lee KP, et al. (2010) Flavonol activation defines an unanticipated ligand-binding site in the kinase-RNase domain of IRE1. Mol Cell 38:291-304.

161. Volkmann K, Lucas JL, Vuga D, et al. (2011) Potent and Selective Inhibitors of the Inositol-requiring Enzyme 1 Endoribonuclease. Journal of Biological Chemistry 286:12743-12755.
162. Cross BCS, Bond PJ, Sadowski PG, et al. (2012) The molecular basis for selective inhibition of unconventional mRNA splicing by an IRE1-binding small molecule. Proceedings of the National Academy of Sciences 109:E869-E878.
163. Mimura N, Fulciniti M, Gorgun G, et al. (2012) Blockade of XBP1 splicing by inhibition of IRE1alpha is a promising therapeutic option in multiple myeloma. Blood 119:5772-5781.
164. Tashiro E, Hironiwa N, Kitagawa M, et al. (2007) Trierixin, a novel Inhibitor of ER stress-induced XBP1 activation from Streptomyces sp. 1. Taxonomy, fermentation, isolation and biological activities. The Journal of antibiotics 60:547-553.
165. Kawamura T, Tashiro E, Yamamoto K, Shindo K, Imoto M. (2008) SAR study of a novel triene-ansamycin group compound, quinotrierixin, and related compounds, as inhibitors of ER stress-induced XBP1 activation. The Journal of antibiotics 61:303-311.
166. Ri M, Tashiro E, Oikawa D, et al. (2012) Identification of Toyocamycin, an agent cytotoxic for multiple myeloma cells, as a potent inhibitor of ER stress-induced XBP1 mRNA splicing. Blood Cancer Journal 2:e79.
167. Wilson WL. (1968) Phase I study with toyocamycin (NSC-63701). Cancer chemotherapy reports Part 1 52:301-303.
168. Papa FR, Zhang C, Shokat K, Walter P. (2003) Bypassing a kinase activity with an ATP-competitive drug. Science 302:1533-1537.
169. Wang L, Perera BGK, Hari SB, et al. (2012) Divergent allosteric control of the IRE1α endoribonuclease using kinase inhibitors. Nat Chem Biol 8:982-989.
170. Ghosh R, Wang L, Wang Eric S, et al. (2014) Allosteric Inhibition of the IRE1α RNase Preserves Cell Viability and Function during Endoplasmic Reticulum Stress. Cell 158:534-548.
171. Tam Arvin B, Koong Albert C, Niwa M. (2014) Ire1 Has Distinct Catalytic Mechanisms for XBP1/HAC1 Splicing and RIDD. Cell Reports 9:850-858.
172. Curtin JF, Cotter TG. (2003) Apoptosis: Historical perspectives. Essays in biochemistry 39:1-10.
173. Kroemer G, Galluzzi L, Vandenabeele P, et al. (2009) Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell death and differentiation 16:3-11.
174. Kerr JF, Wyllie AH, Currie AR. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. British journal of cancer 26:239-257.
175. Ellis HM, Horvitz HR. (1986) Genetic control of programmed cell death in the nematode C. elegans. Cell 44:817-829.
176. Saraste A, Pulkki K. (2000) Morphologic and biochemical hallmarks of apoptosis. Cardiovascular Research 45:528-537.
177. Fulda S, Debatin KM. (0000) Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. Oncogene 25:4798-4811.
178. Elmore S. (2007) Apoptosis: a review of programmed cell death. Toxicologic pathology 35:495-516.

179. Ashkenazi A, Dixit VM. (1998) Death Receptors: Signaling and Modulation. Science 281:1305-1308.
180. Kataoka T, Schroter M, Hahne M, et al. (1998) FLIP prevents apoptosis induced by death receptors but not by perforin/granzyme B, chemotherapeutic drugs, and gamma irradiation. Journal of immunology (Baltimore, Md : 1950) 161:3936-3942.
181. Gross A, Yin XM, Wang K, et al. (1999) Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. The Journal of biological chemistry 274:1156-1163.
182. Eskes R, Desagher S, Antonsson B, Martinou JC. (2000) Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. Mol Cell Biol 20:929-935.
183. Wei MC, Lindsten T, Mootha VK, et al. (2000) tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. Genes Dev 14:2060-2071.
184. Garrido C, Galluzzi L, Brunet M, Puig PE, Didelot C, Kroemer G. (2006) Mechanisms of cytochrome c release from mitochondria. Cell Death Differ 13:1423-1433.
185. 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.
186. van Loo G, van Gurp M, Depuydt B, et al. (2002) The serine protease Omi/HtrA2 is released from mitochondria during apoptosis. Omi interacts with caspase-inhibitor XIAP and induces enhanced caspase activity. Cell Death Differ 9:20-26.
187. Chinnaiyan AM. (1999) The apoptosome: heart and soul of the cell death machine. Neoplasia (New York, NY) 1:5-15.
188. Willis SN, Fletcher JI, Kaufmann T, et al. (2007) Apoptosis Initiated When BH3 Ligands Engage Multiple Bcl-2 Homologs, Not Bax or Bak. Science 315:856-859.
189. Youle RJ. (2007) Cellular Demolition and the Rules of Engagement. Science 315:776-777.
190. Oda E, Ohki R, Murasawa H, et al. (2000) Noxa, a BH3-Only Member of the Bcl-2 Family and Candidate Mediator of p53-Induced Apoptosis. Science 288:1053-1058.
191. Liu FT, Newland AC, Jia L. (2003) Bax conformational change is a crucial step for PUMA-mediated apoptosis in human leukemia. Biochemical and biophysical research communications 310:956-962.
192. O'Connor L, Strasser A, O'Reilly LA, et al. (1998) Bim: a novel member of the Bcl-2 family that promotes apoptosis. Embo j 17:384-395.
193. Elliott JM, Rouge L, Wiesmann C, Scheer JM. (2009) Crystal Structure of Procaspase-1 Zymogen Domain Reveals Insight into Inflammatory Caspase Autoactivation. Journal of Biological Chemistry 284:6546-6553.
194. Riedl SJ, Fuentes-Prior P, Renatus M, et al. (2001) Structural basis for the activation of human procaspase-7. Proceedings of the National Academy of Sciences 98:14790-14795.
195. Sakahira H, Enari M, Nagata S. (1998) Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature 391:96-99.

196. Li J, Lee B, Lee AS. (2006) Endoplasmic reticulum stress-induced apoptosis: Multiple pathways and activation of p53-UP-regulated modulator of apoptosis (PUMA) and NOXA by p53. Journal of Biological Chemistry 281:7260-7270.
197. Lin W-C, Chuang Y-C, Chang Y-S, et al. (2012) Endoplasmic Reticulum Stress Stimulates p53 Expression through NF-κB Activation. PloS one 7:e39120.
198. Deng J, Lu PD, Zhang Y, et al. (2004) Translational repression mediates activation of nuclear factor kappa B by phosphorylated translation initiation factor 2. Molecular and Cellular Biology 24:10161-10168.
199. Kaneko M, Niinuma Y, Nomura Y. (2003) Activation signal of nuclear factor-κB in response to endoplasmic reticulum stress is transduced via IRE1 and tumor necrosis factor receptor-associated factor 2. Biological and Pharmaceutical Bulletin 26:931-935.
200. Yamazaki H, Hiramatsu N, Hayakawa K, et al. (2009) Activation of the Akt-NF-κB pathway by subtilase cytotoxin through the ATF6 branch of the unfolded protein response. Journal of Immunology 183:1480-1487.
201. White C, Li C, Yang J, et al. (2005) The endoplasmic reticulum gateway to apoptosis by Bcl-XL modulation of the InsP3R. Nature Cell Biology 7:1021-1028.
202. Pinton P, Ferrari D, Magalhaes P, et al. (2000) Reduced loading of intracellular Ca2+ stores and downregulation of capacitative Ca2+ influx in Bcl-2-overexpressing cells. Journal of Cell Biology 148:857-862.
203. Bassik MC, Scorrano L, Oakes SA, Pozzan T, Korsmeyer SJ. (2004) Phosphorylation of BCL-2 regulates ER Ca2+ homeostasis and apoptosis. EMBO Journal 23:1207-1216.
204. Chae HJ, Kim HR, Xu C, et al. (2004) BI-1 regulates an apoptosis pathway linked to endoplasmic reticulum stress. Mol Cell 15:355-366.
205. Luo X, He Q, Huang Y, Sheikh MS. (2005) Transcriptional upregulation of PUMA modulates endoplasmic reticulum calcium pool depletion-induced apoptosis via Bax activation. Cell Death and Differentiation 12:1310-1318.
206. Oakes SA, Scorrano L, Opferman JT, et al. (2005) Proapoptotic BAX and BAK regulate the type 1 inositol trisphosphate receptor and calcium leak from the endoplasmic reticulum. Proceedings of the National Academy of Sciences of the United States of America 102:105-110.
207. Grimm S. (2012) The ER-mitochondria interface: The social network of cell death. Biochimica et Biophysica Acta - Molecular Cell Research 1823:327-334.
208. Chami M, Oulès B, Szabadkai G, Tacine R, Rizzuto R, Paterlini-Bréchot P. (2008) Role of SERCA1 Truncated Isoform in the Proapoptotic Calcium Transfer from ER to Mitochondria during ER Stress. Molecular Cell 32:641-651.
209. Valencia-Sanchez MA, Liu J, Hannon GJ, Parker R. (2006) Control of translation and mRNA degradation by miRNAs and siRNAs. Genes and Development 20:515-524.
210. Byrd AE, Aragon IV, Brewer JW. (2012) MicroRNA-30c-2* limits expression of proadaptive factor XBP1 in the unfolded protein response. Journal of Cell Biology 196:689-698.
211. Yang F, Zhang L, Wang F, et al. (2011) Modulation of the unfolded protein response is the core of microRNA-122-involved sensitivity to chemotherapy in hepatocellular carcinoma 1,2. Neoplasia (New York, NY) 13:590-600.

212. Dai R, Li J, Liu Y, et al. (2010) MiR-221/222 suppression protects against endoplasmic reticulum stress-induced apoptosis via p27 Kip1- and MEK/ERK-mediated cell cycle regulation. Biological Chemistry 391:791-801.
213. Gupta S, Read DE, Deepti A, et al. (2012) Perk-dependent repression of miR-106b-25 cluster is required for ER stress-induced apoptosis. Cell Death and Disease 3.
214. Tagami S, Eguchi Y, Kinoshita M, Takeda M, Tsujimoto Y. (2000) A novel protein, RTN-x(s), interacts with both Bcl-x(L) and Bcl-2 on endoplasmic reticulum and reduces their anti-apoptotic activity. Oncogene 19:5736-5746.
215. Belmont PJ, Chen WJ, Thuerauf DJ, Glembotski CC. (2012) Regulation of microRNA expression in the heart by the ATF6 branch of the ER stress response. Journal of Molecular and Cellular Cardiology 52:1176-1182.
216. De Duve C, Wattiaux R. (1966) Functions of lysosomes. Annual review of physiology 28:435-492.
217. Ohsumi Y. (1999) Molecular mechanism of autophagy in yeast, Saccharomyces cerevisiae. Philosophical transactions of the Royal Society of London Series B, Biological sciences 354:1577-1580; discussion 1580-1571.
218. Levine B, Kroemer G. (2008) Autophagy in the Pathogenesis of Disease. Cell 132:27-42.
219. Hosokawa N, Hara T, Kaizuka T, et al. (2009) Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy. Molecular biology of the cell 20:1981-1991.
220. Wullschleger S, Loewith R, Hall MN. (2006) TOR signaling in growth and metabolism. Cell 124:471-484.
221. Sudarsanam S, Johnson DE. (2010) Functional consequences of mTOR inhibition. Current opinion in drug discovery & development 13:31-40.
222. Inoki K, Zhu T, Guan KL. (2003) TSC2 mediates cellular energy response to control cell growth and survival. Cell 115:577-590.
223. Gwinn DM, Shackelford DB, Egan DF, et al. (2008) AMPK phosphorylation of raptor mediates a metabolic checkpoint. Mol Cell 30:214-226.
224. Kim J, Kundu M, Viollet B, Guan K-L. (2011) AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. Nat Cell Biol 13:132-141.
225. Russell RC, Tian Y, Yuan H, et al. (2013) ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase. Nat Cell Biol 15:741-750.
226. He C, Klionsky DJ. (2009) Regulation mechanisms and signaling pathways of autophagy. Annual review of genetics 43:67-93.
227. Geng J, Klionsky DJ. (2008) The Atg8 and Atg12 ubiquitin-like conjugation systems in macroautophagy. 'Protein Modifications: Beyond the Usual Suspects' Review Series. EMBO Reports 9:859-864.
228. Hanada T, Noda NN, Satomi Y, et al. (2007) The Atg12-Atg5 conjugate has a novel E3-like activity for protein lipidation in autophagy. The Journal of biological chemistry 282:37298-37302.
229. Kirisako T, Ichimura Y, Okada H, et al. (2000) The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway. J Cell Biol 151:263-276.
230. Weidberg H, Shvets E, Elazar Z. (2011) Biogenesis and cargo selectivity of autophagosomes. Annual review of biochemistry 80:125-156.

231. Jahreiss L, Menzies FM, Rubinsztein DC. (2008) The itinerary of autophagosomes: from peripheral formation to kiss-and-run fusion with lysosomes. Traffic (Copenhagen, Denmark) 9:574-587.
232. Tanaka Y, Guhde G, Suter A, et al. (2000) Accumulation of autophagic vacuoles and cardiomyopathy in LAMP-2-deficient mice. Nature 406:902-906.
233. Shvets E, Fass E, Scherz-Shouval R, Elazar Z. (2008) The N-terminus and Phe52 residue of LC3 recruit p62/SQSTM1 into autophagosomes. Journal of cell science 121:2685-2695.
234. Marzella L, Ahlberg J, Glaumann H. (1981) Autophagy, heterophagy, microautophagy and crinophagy as the means for intracellular degradation. Virchows Archiv B, Cell pathology including molecular pathology 36:219-234.
235. Stingl J, Caldas C. (2007) Molecular heterogeneity of breast carcinomas and the cancer stem cell hypothesis. Nat Rev Cancer 7:791-799.
236. Bosch A, Eroles P, Zaragoza R, Vina JR, Lluch A. (2010) Triple-negative breast cancer: molecular features, pathogenesis, treatment and current lines of research. Cancer treatment reviews 36:206-215.
237. Malhotra GK, Zhao X, Band H, Band V. (2010) Histological, molecular and functional subtypes of breast cancers. Cancer Biology & Therapy 10:955-960.
238. Perou CM, Sørile T, Eisen MB, et al. (2000) Molecular portraits of human breast tumours. Nature 406:747-752.
239. Sorlie T, Perou CM, Tibshirani R, et al. (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 98:10869-10874.
240. Sorlie T, Tibshirani R, Parker J, et al. (2003) Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci U S A 100:8418-8423.
241. Parker JS, Mullins M, Cheang MCU, et al. (2009) Supervised Risk Predictor of Breast Cancer Based on Intrinsic Subtypes. Journal of Clinical Oncology 27:1160-1167.
242. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. (2003) Prospective identification of tumorigenic breast cancer cells. Proceedings of the National Academy of Sciences of the United States of America 100:3983-3988.
243. Ginestier C, Hur MH, Charafe-Jauffret E, et al. (2007) ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell stem cell 1:555-567.
244. Shackleton M, Vaillant F, Simpson KJ, et al. (2006) Generation of a functional mammary gland from a single stem cell. Nature 439:84-88.
245. Stingl J, Eirew P, Ricketson I, et al. (2006) Purification and unique properties of mammary epithelial stem cells. Nature 439:993-997.
246. Lim E, Vaillant F, Wu D, et al. (2009) Aberrant luminal progenitors as the candidate target population for basal tumor development in BRCA1 mutation carriers. Nat Med 15:907-913.
247. Hetz C, Chevet E, Harding HP. (2013) Targeting the unfolded protein response in disease. Nat Rev Drug Discov 12:703-719.
248. Dong D, Ni M, Li J, et al. (2008) Critical role of the stress chaperone GRP78/BiP in tumor proliferation, survival, and tumor angiogenesis in transgene-induced mammary tumor development. Cancer Res 68:498-505.

249. Bobrovnikova-Marjon E, Grigoriadou C, Pytel D, et al. (2010) PERK promotes cancer cell proliferation and tumor growth by limiting oxidative DNA damage. Oncogene 29:3881-3895.
250. Fujimoto T, Onda M, Nagai H, Nagahata T, Ogawa K, Emi M. (2003) Upregulation and overexpression of human X-box binding protein 1 (hXBP-1) gene in primary breast cancers. Breast cancer (Tokyo, Japan) 10:301-306.
251. Li H, Chen X, Gao Y, Wu J, Zeng F, Song F. (2015) XBP1 induces snail expression to promote epithelial- to-mesenchymal transition and invasion of breast cancer cells. Cell Signal 27:82-89.
252. Zhu Y, Singh B, Hewitt S, et al. (2006) Expression patterns among interferon regulatory factor-1, human X-box binding protein-1, nuclear factor kappa B, nucleophosmin, estrogen receptor-alpha and progesterone receptor proteins in breast cancer tissue microarrays. International journal of oncology 28:67-76.
253. Wang G, Liu G, Wang X, et al. (2012) ERLIN2 promotes breast cancer cell survival by modulating endoplasmic reticulum stress pathways. BMC Cancer 12.
254. Cubillos-Ruiz Juan R, Silberman Pedro C, Rutkowski Melanie R, et al. ER Stress Sensor XBP1 Controls Anti-tumor Immunity by Disrupting Dendritic Cell Homeostasis. Cell 161:1527-1538.
255. Davies MPA, Barraclough DL, Stewart C, et al. (2008) Expression and splicing of the unfolded protein response gene XBP-1 are significantly associated with clinical outcome of endocrine-treated breast cancer. International Journal of Cancer 123:85-88.
256. Xu T, Yang L, Yan C, et al. (2014) The IRE1[alpha]-XBP1 pathway regulates metabolic stress-induced compensatory proliferation of pancreatic [beta]-cells. Cell Res 24:1137-1140.
257. Sheng X, Arnoldussen YJ, Storm M, et al. (2015) Divergent androgen regulation of unfolded protein response pathways drives prostate cancer. EMBO molecular medicine 7:788-801.
258. Thorpe JA, Schwarze SR. (2010) IRE1α controls cyclin A1 expression and promotes cell proliferation through XBP-1. Cell Stress & Chaperones 15:497-508.
259. Malvezzi M, Bertuccio P, Levi F, La Vecchia C, Negri E. (2014) European cancer mortality predictions for the year 2014. Annals of Oncology 25:1650-1656.
260. Teske BF, Wek SA, Bunpo P, et al. (2011) The eIF2 kinase PERK and the integrated stress response facilitate activation of ATF6 during endoplasmic reticulum stress. Molecular biology of the cell 22:4390-4405.
261. Coletta RD, Christensen K, Reichenberger KJ, et al. (2004) The Six1 homeoprotein stimulates tumorigenesis by reactivation of cyclin A1. Proceedings of the National Academy of Sciences of the United States of America 101:6478-6483.
262. Zhou Y, Lee J, Reno CM, et al. (2011) Regulation of glucose homeostasis through a XBP-1-FoxO1 interaction. Nat Med 17:356-365.
263. Toosi S, Orlow SJ, Manga P. (2012) Vitiligo-Inducing Phenols Activate the Unfolded Protein Response in Melanocytes Resulting in Upregulation of IL6 and IL8. J Invest Dermatol 132:2601-2609.
264. Hartman ZC, Poage GM, den Hollander P, et al. (2013) Growth of triple-negative breast cancer cells relies upon coordinate autocrine expression of the proinflammatory cytokines IL-6 and IL-8. Cancer research 73:3470-3480.

265. Minn AJ, Gupta GP, Siegel PM, et al. (2005) Genes that mediate breast cancer metastasis to lung. Nature 436:518-524.
266. Minn AJ, Gupta GP, Padua D, et al. (2007) Lung metastasis genes couple breast tumor size and metastatic spread. Proceedings of the National Academy of Sciences of the United States of America 104:6740-6745.
267. Kim MY, Oskarsson T, Acharyya S, et al. (2009) Tumor self-seeding by circulating cancer cells. Cell 139:1315-1326.
268. Acharyya S, Oskarsson T, Vanharanta S, et al. (2012) A CXCL1 paracrine network links cancer chemoresistance and metastasis. Cell 150:165-178.
269. Xu J, Lamouille S, Derynck R. (2009) TGF-[beta]-induced epithelial to mesenchymal transition. Cell Res 19:156-172.
270. Zugmaier G, Ennis BW, Deschauer B, et al. (1989) Transforming growth factors type beta 1 and beta 2 are equipotent growth inhibitors of human breast cancer cell lines. Journal of cellular physiology 141:353-361.
271. McEarchern JA, Kobie JJ, Mack V, et al. (2001) Invasion and metastasis of a mammary tumor involves TGF-beta signaling. International journal of cancer Journal international du cancer 91:76-82.
272. Gu H, Chen X, Gao G, Dong H. (2008) Caspase-2 functions upstream of mitochondria in endoplasmic reticulum stress-induced apoptosis by bortezomib in human myeloma cells. Molecular cancer therapeutics 7:2298-2307.
273. Park JW, Woo KJ, Lee JT, et al. (2007) Resveratrol induces pro-apoptotic endoplasmic reticulum stress in human colon cancer cells. Oncology reports 18:1269-1273.
274. West AC, Johnstone RW. (2014) New and emerging HDAC inhibitors for cancer treatment. J Clin Invest 124:30-39.
275. Kawaguchi Y, Kovacs JJ, McLaurin A, Vance JM, Ito A, Yao TP. (2003) The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress. Cell 115:727-738.
276. Al-Rawashdeh FY, Scriven P, Cameron IC, Vergani PV, Wyld L. (2010) Unfolded protein response activation contributes to chemoresistance in hepatocellular carcinoma. European journal of gastroenterology & hepatology 22:1099-1105.
277. Yang F, Zhang L, Wang F, et al. (2011) Modulation of the unfolded protein response is the core of microRNA-122-involved sensitivity to chemotherapy in hepatocellular carcinoma. Neoplasia (New York, NY) 13:590-600.
278. Lee E, Nichols P, Spicer D, Groshen S, Yu MC, Lee AS. (2006) GRP78 as a novel predictor of responsiveness to chemotherapy in breast cancer. Cancer research 66:7849-7853.
279. Davies MP, Barraclough DL, Stewart C, et al. (2008) Expression and splicing of the unfolded protein response gene XBP-1 are significantly associated with clinical outcome of endocrine-treated breast cancer. Int J Cancer 123:85-88.
280. Gomez BP, Riggins RB, Shajahan AN, et al. (2007) Human X-box binding protein-1 confers both estrogen independence and antiestrogen resistance in breast cancer cell lines. FASEB J 21:4013-4027.
281. Ding L, Yan J, Zhu J, et al. (2003) Ligand-independent activation of estrogen receptor alpha by XBP-1. Nucleic Acids Res 31:5266-5274.

282. Samuels BL, Bitran JD. (1995) High-dose intravenous melphalan: A review. Journal of Clinical Oncology 13:1786-1799.
283. Howell A. (2008) The endocrine prevention of breast cancer. Best practice & research Clinical endocrinology & metabolism 22:615-623.
284. Goldhirsch A, Ingle JN, Gelber RD, et al. (2009) Thresholds for therapies: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2009. Annals of Oncology 20:1319-1329.
285. Mandlekar S, Kong AN. (2001) Mechanisms of tamoxifen-induced apoptosis. Apoptosis : an international journal on programmed cell death 6:469-477.
286. O'Brian CA, Ioannides CG, Ward NE, Liskamp RM. (1990) Inhibition of protein kinase C and calmodulin by the geometric isomers cis- and trans-tamoxifen. Biopolymers 29:97-104.
287. Gundimeda U, Chen ZH, Gopalakrishna R. (1996) Tamoxifen modulates protein kinase C via oxidative stress in estrogen receptor-negative breast cancer cells. The Journal of biological chemistry 271:13504-13514.
288. Gomez BP, Riggins RB, Shajahan AN, et al. (2007) Human X-Box binding protein-1 confers both estrogen independence and antiestrogen resistance in breast cancer cell lines. FASEB Journal 21:4013-4027.
289. Jones MD, Liu JC, Barthel TK, et al. (2010) A proteasome inhibitor, bortezomib, inhibits breast cancer growth and reduces osteolysis by downregulating metastatic genes. Clinical cancer research : an official journal of the American Association for Cancer Research 16:4978-4989.
290. Pan Z, Gollahon L. (2013) Paclitaxel attenuates Bcl-2 resistance to apoptosis in breast cancer cells through an endoplasmic reticulum-mediated calcium release in a dosage dependent manner. Biochemical and biophysical research communications 432:431-437.
291. Brichese L, Barboule N, Heliez C, Valette A. (2002) Bcl-2 Phosphorylation and Proteasome-Dependent Degradation Induced by Paclitaxel Treatment: Consequences on Sensitivity of Isolated Mitochondria to Bid. Experimental Cell Research 278:101-111.
292. Pan Z, Avila A, Gollahon L. (2014) Paclitaxel Induces Apoptosis in Breast Cancer Cells through Different Calcium—Regulating Mechanisms Depending on External Calcium Conditions. International Journal of Molecular Sciences 15:2672-2694.
293. Rowinsky EK. (1993) Clinical pharmacology of Taxol. Journal of the National Cancer Institute Monographs:25-37.
294. Pan Z, Gollahon L. (2011) Taxol directly induces endoplasmic reticulum-associated calcium changes that promote apoptosis in breast cancer cells. The breast journal 17:56-70.
295. Rubinsztein DC. (2012) Autophagy—alias self-eating—appetite and ageing,
296. Deegan S, Saveljeva S, Gorman A, Samali A. (2013) Stress-induced self-cannibalism: on the regulation of autophagy by endoplasmic reticulum stress. Cell Mol Life Sci 70:2425-2441.
297. Deegan S, Koryga I, Glynn SA, Gupta S, Gorman AM, Samali A. (2015) A close connection between the PERK and IRE arms of the UPR and the transcriptional regulation of autophagy. Biochemical and Biophysical Research Communications 456:305-311.

298. B'chir W, Maurin A-C, Carraro V, et al. (2013) The eIF2α/ATF4 pathway is essential for stress-induced autophagy gene expression. Nucleic Acids Research 41:7683-7699.
299. Vidal RL, Figueroa A, Court FA, et al. (2012) Targeting the UPR transcription factor XBP1 protects against Huntington's disease through the regulation of FoxO1 and autophagy. Human Molecular Genetics 21:2245-2262.
300. Corazzari M, Rapino F, Ciccosanti F, et al. (2014) Oncogenic BRAF induces chronic ER stress condition resulting in increased basal autophagy and apoptotic resistance of cutaneous melanoma. Cell Death Differ.
301. Cheng X, Liu H, Jiang CC, et al. (2014) Connecting endoplasmic reticulum stress to autophagy through IRE1/JNK/beclin-1 in breast cancer cells. International journal of molecular medicine 34:772-781.
302. Ben-Sahra I, Dirat B, Laurent K, et al. (2013) Sestrin2 integrates Akt and mTOR signaling to protect cells against energetic stress-induced death. Cell Death Differ 20:611-619.
303. Budanov AV, Karin M. (2008) p53 Target Genes Sestrin1 and Sestrin2 Connect Genotoxic Stress and mTOR Signaling. Cell 134:451-460.
304. Budanov AV, Lee JH, Karin M. (2010) Stressin' Sestrins take an aging fight. EMBO Molecular Medicine 2:388-400.
305. Lee SO, Andey T, Jin UH, Kim K, Sachdeva M, Safe S. (2012) The nuclear receptor TR3 regulates mTORC1 signaling in lung cancer cells expressing wild-type p53. Oncogene 31:3265-3276.
306. Maiuri MC, Malik SA, Morselli E, et al. (2009) Stimulation of autophagy by the p53 target gene Sestrin2. Cell Cycle 8:1571-1576.
307. Bruning A, Rahmeh M, Friese K. (2013) Nelfinavir and bortezomib inhibit mTOR activity via ATF4-mediated sestrin-2 regulation. Molecular oncology 7:1012-1018.
308. Park H-W, Park H, Ro S-H, et al. (2014) Hepatoprotective role of Sestrin2 against chronic ER stress. Nat Commun 5.
309. Fribley A, Wang CY. (2006) Proteasome inhibitor induces apoptosis through induction of endoplasmic reticulum stress. Cancer Biology and Therapy 5:745-748.
310. Brüning A, Rahmeh M, Friese K. (2013) Nelfinavir and bortezomib inhibit mTOR activity via ATF4-mediated sestrin-2 regulation. Molecular Oncology 7:1012-1018.
311. Lefort S, Joffre C, Kieffer Y, et al. (2014) Inhibition of autophagy as a new means of improving chemotherapy efficiency in high-LC3B triple-negative breast cancers. Autophagy 10:2122-2142.
312. Zhao B, Shah P, Budanov AV, et al. (2014) Sestrin2 Protein Positively Regulates AKT Enzyme Signaling and Survival in Human Squamous Cell Carcinoma and Melanoma Cells. Journal of Biological Chemistry 289:35806-35814.
313. Liou HC, Boothby MR, Finn PW, et al. (1990) A new member of the leucine zipper class of proteins that binds to the HLA DRα promoter. Science 247:1581-1584.
314. Cox JS, Shamu CE, Walter P. (1993) Transcriptional induction of genes encoding endoplasmic reticulum resident proteins requires a transmembrane protein kinase. Cell 73:1197-1206.

315. Morl K, Ma W, Gething M-J, Sambrook J. (1993) A transmembrane protein with a cdc2+CDC28-related kinase activity is required for signaling from the ER to the nucleus. Cell 74:743-756.
316. Cox JS, Walter P. (1996) A novel mechanism for regulating activity of a transcription factor that controls the unfolded protein response. Cell 87:391-404.
317. Calfon M, Zeng H, Urano F, et al. (2002) IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. Nature 415:92-96.
318. Lee K, Tirasophon W, Shen X, et al. (2002) IRE1-mediated unconventional mRNA splicing and S2P-mediated ATF6 cleavage merge to regulate XBP1 in signaling the unfolded protein response. Genes Dev 16:452-466.
319. Carrasco DR, Sukhdeo K, Protopopova M, et al. (2007) The Differentiation and Stress Response Factor XBP-1 Drives Multiple Myeloma Pathogenesis. Cancer Cell 11:349-360.
320. Bagratuni T, Wu P, Gonzalez de Castro D, et al. (2010) XBP1s levels are implicated in the biology and outcome of myeloma mediating different clinical outcomes to thalidomide-based treatments. Blood 116:250-253.
321. Shaffer AL, Shapiro-Shelef M, Iwakoshi NN, et al. (2004) XBP1, downstream of Blimp-1, expands the secretory apparatus and other organelles, and increases protein synthesis in plasma cell differentiation. Immunity 21:81-93.
322. Essler S, Dehne N, Brüne B. (2009) Role of sestrin2 in peroxide signaling in macrophages. FEBS Letters 583:3531-3535.
323. Essler S, Dehne N, Brune B. (2009) Role of sestrin2 in peroxide signaling in macrophages. FEBS letters 583:3531-3535.
324. Zagouri F, Sergentanis TN, Chrysikos D, Filipits M, Bartsch R. (2012) mTOR inhibitors in breast cancer: A systematic review. Gynecologic Oncology 127:662-672.
325. Zhang J, Yang PL, Gray NS. (2009) Targeting cancer with small molecule kinase inhibitors. Nat Rev Cancer 9:28-39.
326. Falkenberg KJ, Johnstone RW. (2014) Histone deacetylases and their inhibitors in cancer, neurological diseases and immune disorders. Nat Rev Drug Discov 13:673-691.
327. Sandhiya S, Melvin G, Kumar SS, Dkhar SA. (2013) The dawn of hedgehog inhibitors: Vismodegib. Journal of Pharmacology & Pharmacotherapeutics 4:4-7.
328. Long HJ. (1994) Paclitaxel (Taxol): a novel anticancer chemotherapeutic drug. Mayo Clinic proceedings 69:341-345.
329. Murray S, Briasoulis E, Linardou H, Bafaloukos D, Papadimitriou C. (2012) Taxane resistance in breast cancer: Mechanisms, predictive biomarkers and circumvention strategies. Cancer treatment reviews 38:890-903.
330. McDermott SP, Wicha MS. (2010) Targeting breast cancer stem cells. Molecular oncology 4:404-419.
331. Poillet-Perez L, Despouy G, Delage-Mourroux R, Boyer-Guittaut M. (2015) Interplay between ROS and autophagy in cancer cells, from tumor initiation to cancer therapy. Redox Biology 4:184-192.
332. Amaravadi RK, Lippincott-Schwartz J, Yin XM, et al. (2011) Principles and current strategies for targeting autophagy for cancer treatment. Clinical Cancer Research 17:654-666.

333. Sharma N, Thomas S, Golden EB, et al. (2012) Inhibition of autophagy and induction of breast cancer cell death by mefloquine, an antimalarial agent. Cancer Letters 326:143-154.
334. Maffini MV, Calabro JM, Soto AM, Sonnenschein C. (2005) Stromal Regulation of Neoplastic Development: Age-Dependent Normalization of Neoplastic Mammary Cells by Mammary Stroma. The American journal of pathology 167:1405-1410.
335. Hunter KW. (2004) Host genetics and tumour metastasis. British journal of cancer 90:752-755.
336. Zaromytidou A-I. (2011) Tumour microenvironment: Cytokines signal for invasion. Nat Cell Biol 13:1188-1188.
337. Middleton K, Jones J, Lwin Z, Coward JIG. (2014) Interleukin-6: An angiogenic target in solid tumours. Critical Reviews in Oncology/Hematology 89:129-139.
338. Ning Y, Manegold PC, Hong YK, et al. (2011) Interleukin-8 is associated with proliferation, migration, angiogenesis and chemosensitivity in vitro and in vivo in colon cancer cell line models. International Journal of Cancer 128:2038-2049.
339. Bates RC, Mercurio AM. (2003) Tumor necrosis factor-α stimulates the epithelial-tomesenchymal transition of human colonic organoids. Molecular biology of the cell 14:1790-1800.
340. Spiotto MT, Banh A, Papandreou I, et al. (2010) Visualization of the unfolded protein response in primary tumors reveals unique microenvironments that reflect metabolic variations and predict for tumor growth. Cancer research 70:78-88.
341. Galluzzi L, Senovilla L, Zitvogel L, Kroemer G. (2012) The secret ally: immunostimulation by anticancer drugs. Nat Rev Drug Discov 11:215-233.
342. Dontu G, Abdallah WM, Foley JM, et al. (2003) In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev 17:1253-1270.
343. To K, Fotovati A, Reipas KM, et al. (2010) YB-1 induces expression of CD44 and CD49f leading to enhanced self-renewal, mammosphere growth, and drug resistance. Cancer research 70:2840-2851.