The role of microRNAs during Endoplasmic Reticulum Stress-induced cell death.

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by

Karen Cawley

Apoptosis Research Centre, Discipline of Biochemistry, School of Natural Sciences,

National University of Ireland, Galway, Ireland.

Thesis Supervisor: Professor Afshin Samali

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LIST OF PUBLICATIONS

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ABSTRACT

Endoplasmic Reticulum (ER) stress occurs in response to negative insult or stimuli to the cell. It induces a pathway called the unfolded protein response (UPR) which can alleviate stress and restore the cell to its "healthy state" or if the stress cannot be overcome, the UPR induces pro-apoptotic signalling events to eliminate the stressed cell. Such pathways have been associated with many pathological conditions. In cancer for instance, pro-survival UPR signalling can be utilized to compromise apoptosis induction and enhance cell growth and survival. In other diseases like neurodegeneration and diabetes excessive apoptosis and ER stress is observed and enhances disease progression. The mechanisms controlling the switch between pro-survival and pro-apoptotic ER stress responses is not fully elucidated. We considered that the small non-coding RNAs called microRNAs (miRNAs) may have a role in ER stress induced cell death and may fine tune the signalling events that determine cell fate or that ER stress may regulate miRNA expression to modulate stress responses.

Two approaches for investigating the role of miRNA during ER stress induced cell death were employed. Firstly, a global approach was taken in which the biogenesis pathway of miRNAs was compromised to understand if miRNAs were important for ER stress induced apoptosis. This study revealed that cells with compromised miRNA biogenesis machinery were resistant to ER stress induced cell death but not other inducers of apoptosis. Although loss of global miRNA expression had no effect on the UPR, induction of the intrinsic apoptosis pathway was delayed, upstream of the mitochondria. This was due to altered levels of the BCL-2 family members which subsequently prevented BAX activation and permeabilisation of the outer mitochondrion membrane. Secondly, a candidate approach was used to determine if miRNA themselves are regulated during ER stress induced cell death. miRNA microarray analysis was used to screen for miRNA differentially regulated upon ER stress. This revealed that miRNA of the miR-17-92 cluster and their paralog cluster miR-106b-25 were downregulated during ER stress induced cell death in a PERK dependant manner. Further analysis revealed that the PERK regulated transcription factors NRF2 and ATF4 were responsible for this repression. The BH3 only protein, BIM a known target of these miRNA, increased in their absence and lead to apoptosis. This work illustrates an essential role for miRNA in ER stress - induced cell death.

ABBREVIATIONS

AGO	Argonaute
AIF	Apoptosis-inducing factor
APAF-1	Apoptotic peptidase activating factor 1
ARE	Antioxidant response element
ASK1	Apoptosis signal-regulation kinase 1
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 proteins
CAD	Caspase-activated DNase
CARD	Caspase recruitment domain
Caspases	Cysteine-dependent aspartate-directed proteases
CD95	Cluster of differentiation 95
c-FLIP	Cellular FLICE inhibitory protein
Cdkn2a	Cyclin-dependant kinase inhibitor 2a
CHD5	Chromodomain helicase DNA binding protein 5

CHOP	C/EBP-homologous protein
CLL	Chronic lymphocytic leukaemia
CO	Carbon monoxide
DD	Death domain
DED	Death effector domain
DGCR8	DiGeorge Syndrome Critical Region Gene 8
DISC	Death inducing signalling complex
DNA	Deoxyribonucleic acid
EDEM1	ER degradation enhancer, mannosidase alpha-like 1
eIF2α	Eukaryotic translation initiation factor 2 α
EMT	Epithelial mesenchymal transition
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
FADD	Fas-associated death domain
FAP 1	FAS associated phosphatase 1
FLICE	FADD-like IL-1β-converting enzyme
GLS	Golgi localization signals
GRP78/Bip	Glucose-regulated protein, 78
HCC	Hepatocellular carcinoma
HO-1	Heme oxygenase 1
HSP72	Heat shock protein 72

IAP	Inhibitor of apoptosis protein
ICAD	Inhibitor of caspase-activated DNase
IKK	Inhibitor of NFkB kinase
IRE1α	Inositol requiring enzyme-1
JNK	c-JUN terminal kinase
KEAP1	Kelch-like ECH-associated protein
M^7G	5' 7 methylguanosine
MCL-1	Myeloid cell leukemia sequence 1
MEF	Mouse embryonic fibroblast
miRNA	microRNA
MLC	Myosin regulatory light chain
MOMP	Mitochondrial outer membrane permeabilization
mRNP	Messanger ribonucleoprotein
NF-Y	Nuclear transcription factor Y
NQO1	NAD(P)H:quinone oxidoreductase
NRF2	Nuclear factor (erythroid-derived 2)-like 2
p58(IPK)	p58 inhibitor of protein kinase
PARP	Poly (ADP-ribose) polymerase
PB	Processing/P-body
PCR	Polymerase chain reaction
PDI	Protein disulphide isomerases
PERK	(PKR)-like ER kinase
PKR	Protein kinase R
Pre-miRNA	Precursor miRNA
Pri-miRNA	Primary miRNA
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
SGs	Stress granule
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
TRBP	TAR RNA binding protein
UPR	Unfolded protein response
UPRE	Unfolded Protein Response Element
UTR	Untranslated region
XBP1	X-box binding protein
XBP1s	X-box binding protein spliced
XIAP	X-linked inhibitor of apoptosis protein

CHAPTER 1

INTRODUCTION

1.0 Introduction

1.1 Programmed Cell death

Programmed cell death refers to the demise of cells by any means and was first proposed in 1964 to describe cell death of the intersegmental muscle of the silkmoth (Lockshin and Williams, 1964; Lockshin and Zakeri, 2001). There are several cell death modalities defined, based on their morphological, biochemical or immunological features (Galluzzi et al., 2007). On occasion different forms of cell death have overlapping features and characteristics and can be difficult to distinguish from one another. They can occur separately but also simultaneously as seen with apoptosis and autophagic cell death where autophagy is thought to occur alongside apoptosis (Zeiss, 2003). Also one form of cell death may follow on from another as seen with late apoptotic, early necrotic cells (Levine and Yuan, 2005). It is thought that the way in which a cell dies can depend on the severity or strength of the incoming death promoting signal. Different modes of cell death include apoptosis, necrosis, autophagy regulated cell death, mitotic catastrophy, Granzyme mediated, paraptosis and more recently, necroptosis (Galluzzi et al., 2007; Galluzzi et al., 2012; Kroemer et al., 2009). Cell death is a vital process to any organism and for any one cell that divides to produce a new cell, one cell must die (Lockshin and Zakeri, 2007). Considering this, a huge amount of rapid cell death occurs in the body every day. Thus, deregulation of cell death is massively involved in pathologies where too much or too little cell death leads to disease progression e.g. cancer, neurodegeneration, etc. (Carson and Ribeiro, 1993; Favaloro et al., 2012).

1.2 Apoptotic cell death

Apoptosis is a mode of programmed cell death that brings about the destruction of the cells in a sequential step-wise fashion i.e. apoptosis is a type of cell suicide. Due to the “programmed” nature of this process cells die without causing damage to other cells or tissues (Kerr et al., 1972). Initially this process was considered a genetically determined process in which cells die at certain stages of development e.g. the formation of singular digits such as fingers

(Zaleske, 1985). Although apoptosis is vital during development, it also occurs for other reasons. For instance, apoptosis is a means of destroying defective or damaged cells. Often cells that have developed a genetic abnormality will be forced to commit cell suicide (Norbury and Hickson, 2001). Apoptotic cells have a set of morphological characteristics that set them apart from healthy cells and can readily be detected using electron microscopy (Kerr et al., 1972). They also have distinct biochemical features and complex pathways that direct the controlled demolition of the cell (Galluzzi et al., 2012; Hengartner, 2000).

1.2.1 Apoptotic morphology

The morphological characteristics of an apoptotic cell include cell shrinkage where cells appear smaller with tightly packed organelles, chromatin condensation in which chromatin appears fragmented or broken up. Membrane blebbing occurs due to Rho mediated phosphorylation of myosin regulatory light chain (MLC) and its interaction with Actin which allows cell contraction and membrane blebbing. Other actin interacting proteins have been implicated in this and one such cytoskeletal protein, fodrin is cleaved by the aspartic proreases known as caspases, during apoptosis (Mills et al., 1998; Vanags et al., 1996). Kayorrhexis or disruption of the nuclear membrane follows this and allows fragmented chromatin to diffuse through the cytoplasm. The final morphological feature is the formation of apoptotic bodies. These are engulfed by macrophages and destroyed limiting any inflammatory response. An important feature of apoptotic cells is the presence of phosphatidylserine (PS) on the cell surface; this is thought to act as a signal to macrophages that these cells should be phagocytosised (Kurosaka et al., 2003). Healthy cells do not display PS but instead it is retained inside the cell by aminophospholipid translocase. Upon apoptotic stimulus, this enzyme is inactivated and PS flips to the outer leaflet of the plasma membrane (Bratton et al., 1997). This exposure has been exploited to develop Annexin V-FITC staining as a way to measure percentage apoptosis by (Logue et al., 2009), as described in materials and methods section.

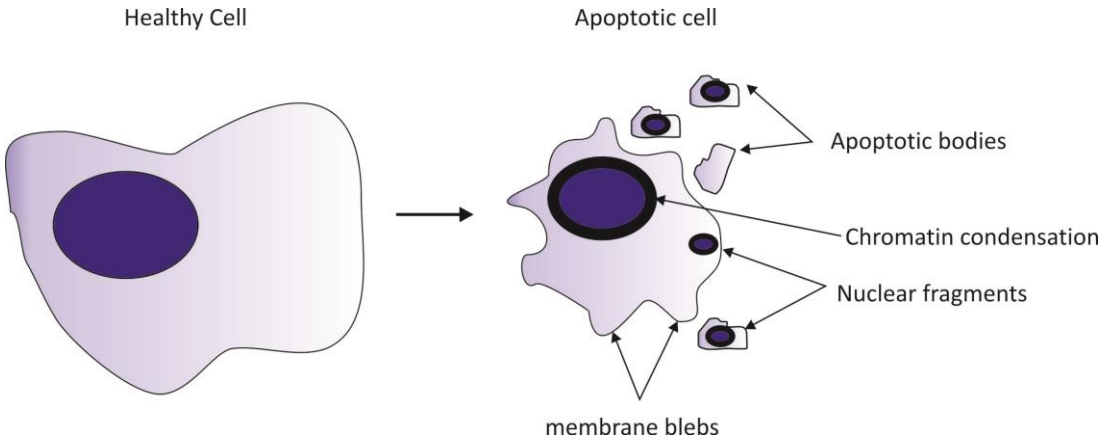

Figure 1.1 Apoptotic morphology
Apoptotic cells are known to shrink and chromatin in the nucleus often condenses at the nuclear membrane. Blebbing of the cell membrane occurs due to a breakdown of cytoskeletal proteins followed by karyorrhexis and budding of apoptotic bodies containing nuclear fragments or even tightly packed organelles.

1.2.2 Molecular basis of apoptosis

Depending on the type of insult to the cell, there are two main apoptotic pathways termed intrinsic (mitochondrial) and extrinsic (death receptor) apoptosis. The components of either pathway can overlap into the other pathway to ensure death of the cell (Elmore, 2007). There are two very important groups of proteins involved in these pathways, the B cell lymphoma 2 (BCL-2) family and **c**ysteine-dependent **asp**artate-directed prote**ases** (Caspases) (Cory and Adams, 2002; MacKenzie and Clark, 2012).

1.2.3 The BCL-2 family

These are a family of proteins made up from a combination of 4 domains called BCL-2 homology (BH) domains along with a transmembrane (TM) domain. They are sub divided into groups based on function, and/or domain and have been shown to interact with one another and form heterodimers (Lama and Sankararamakrishnan, 2008; Moroy et al., 2009; Oltvai et al., 1993). There are the pro-survival BCL-2 proteins that generally contain all 5 domains and

include BCL-2, myeloid cell leukemia sequence 1 (MCL-1), BCL-xL BCL-w BCL-B and NRH/Nr-13. Conversely, pro-apoptotic family members generally lack one or more of the BH domains and so are subgrouped into pro-apoptotic multidomain (generally share BH1, 2, 3 and TM domains) and proapoptotic BH3 only proteins (BOPs) which as the name suggests carry only the BH3 domain and/or the TM domain. Multidomain proteins include BAX, BAK and BOK while BH3 only proteins include BAD, BIK, BIM, BMF, BNIP3, HRK, NOXA, PUMA, SPIKE and tBID (Leber et al., 2007). There is much debate on the actual mode in which these proteins regulate the apoptotic threshold, and several models have been put forward e.g. the rheostat model, the direct activation model and the neutralization model. However one thing is clear, predominance of pro-apoptotic family members leads to translocation of BAX to the outer mitochondrial membrane (OMM) where it heterodimerises with BAK leading to permeabilisation of the OMM and apoptosis. Some studies have suggested that the conformation of BAX is a transient state easily shifting between different conformational shapes. Apparently interaction with but not insertion into the mitochondrial membrane causes a conformational change in BAX to its more thermo-stable and active form where it exposes an amino-terminal epitope called 6A7 which is readily detected by the 6A7 monoclonal antibody as described in chapter 2: materials and methods (Leber et al., 2007). From the literature, it appears that the BCL-2 regulatory models can overlap and perhaps there are multiple layers to the regulation of these proteins. For example the rheostat model suggests that it is the balance between pro- and anti-apoptotic proteins that decides if the cell lives or dies. If there are higher levels of pro-apoptotic proteins, then this would tip the cell towards an apoptotic state or vice versa (Ploner et al., 2008). The neutralization model suggests that pro-apoptotic proteins bind anti-apoptotic proteins and neutralize their effects (Nickells, 2010). Again this sounds very similar to the rheostat model. Perhaps the ratio between pro- and anti-apoptotic proteins affects the ability to neutralize. Finally some have suggested the direct activation model in which BOPs have further been subdivided into activators and sensitizers. This model suggests that sensitizer BOPs bind to pro-survival proteins which then can no longer bind to activator BOPs. Activators are then free to directly interact with BAX and BAK to promote their translocation to the mitochondria (Chipuk et al., 2010; Danial, 2007).

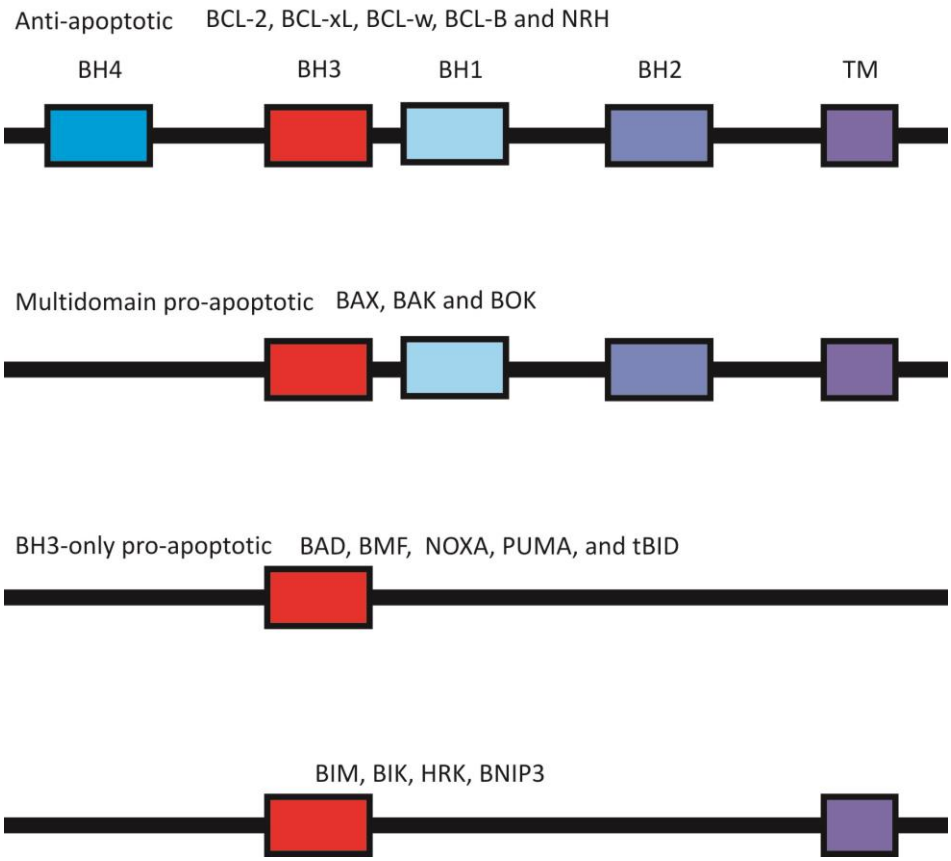

Figure 1.2 BCL-2 family classification
BCL-2 family members are classified based on structure and function. They can consist of one or more of 4 homology domains (BH) and/or a transmembrane (TM) domain. Pro-survival family members generally have all 5 domains and include BCL-2, MCL-1, BCL-w, BCL-B and NRH. Pro-apoptotic members are sub-grouped into multi-domain members such as BAX, BAK AND BOK and generally have domains BH 1-3 and the TM; and BH3 only members such as BAD, BMF, NOXA, PUMA, TBID, BIM, BIK, HRK, AND BNIP3 which all carry the BH3 domain but only a few carry both the BH3 and the TM domains.

1.2.4 Caspases

Caspases are a group of cysteine proteases characterised by a conserved QACXG pentapeptide sequence and three catalytic residues with a Cys285 active site. They are also known to specifically cleave substrates after an Asp residue (Fischer et al., 2003). Caspases are all generated as inactive zymogens consisting of a pro-domain, a p20 large fragment and

a p17 small fragment. Activation of caspases occurs when the zymogen precursor is cleaved to separate the large and small fragments and subsequent removal of the pro-domain (Li and Yuan, 2008). Caspases have been classified into several subgroups based on criteria such as, function and location within a signalling pathway, their pro-domain length, substrate preference etc… For instance some caspases are considered inflammatory (caspase-1, -4, -5, -11, -13) with major roles in cytokine maturation and T-cell proliferation. Others are considered apoptotic with their activation resulting in the cells demise. These are grouped into initiator caspases (caspases-2, -8, -9, -10, -12) which effect upstream signalling events and often activate downstream caspases referred to as effector caspases (caspases-3, -6,-7) (Earnshaw et al., 1999). Initiators can often self-cleave or auto-activate themselves and are recognised by long pro-domains that contain a caspase recruitment domain (CARD) or a death effector domain (DED). Effector caspases are then recognized by short pro-domains and are activated by direct cleavage from initiator caspases. Executioners are suitably named as they are responsible for many of the biochemical and morphological changes that bring about the death of the cell (Shi, 2004). This is seen when inhibitor of caspase-activated DNase (ICAD) is cleaved by caspase-3 resulting in activation of caspase-activated DNase (CAD) which degrades DNA (Sakahira et al., 1998; Tang and Kidd, 1998). poly(ADP-ribose) polymerase-1 (PARP-1) is cleaved by caspase-3 and -7 during apoptosis resulting in its inactivation, as such it no longer detects DNA damage (Kaufmann et al., 1993). Different caspases are known to preferentially cleave after certain tetrapeptide sequences, for example, caspase-2, -3 and -7 preferably cleave after DEXD while caspase-6, -8, and -9 cleave (L/V)EXD where X has been seen as V, T or H (Crawford and Wells, 2011; Fischer et al., 2003).

1.2.5 Intrinsic apoptosis

The intrinsic or mitochondrial pathway is initiated by intracellular signals that regulate transcriptional, translational and post-translational events, to promote apoptosis by altering the permeability of the mitochondrion. For instance, the removal of factors needed for cell survival such as glucose, hormones, growth factors or exposure to toxins, certain viruses,

radiation, thermal stress, and an accumulation of reactive oxygen species can force the cell into apoptosis (Elmore, 2007).

Any of these stresses can affect the levels of BCL-2 family proteins, usually increasing the pro-apoptotic members of this family while decreasing the pro-survival members. This results in the translocation of pro-apoptotic BCL-2 proteins BAX to the mitochondria, where it dimerises with BAK to form pores in the OMM. Within the intermembrane space, the apoptotic proteins, cytochrome *c*, Smac/DIABLO, Apoptosis-inducing factor (AIF), endonuclease G, and Omi/HtrA2 are stored. Pore formation permeabilises the OMM and thus releases these apoptotic proteins into the cytoplasm (Green and Kroemer, 2004). Some of these proteins subsequently activate caspases while others are caspase-independent death effectors. Cytochrome *c* is a component of the apoptosome, a death promoting platform also consisting of apoptotic peptidase activating factor 1 (APAF-1) and caspase-9. In resting conditions APAF-1 is in an inactive conformational state and bound to dATP. Binding of cytochrome *c* causes a conformational change, where APAF-1 undergoes nucleotide exchange, and its AAA+ ATPase domain is exposed allowing oligomerization of several APAF-1 proteins to form a circular apoptosome complex to which caspase-9 is recruited. Crystallisation of apoptosome complexes has revealed a complex consisting of 7 APAF-1 molecules, 7 cytochrome *c* molecules forming a wheel shaped platform to which caspase-9 binds apically (Bratton and Salvesen, 2010). Upon binding to APAF-1, caspase-9 becomes activated via CARD-CARD interaction; caspase-3 is then recruited to the apoptosome and directly cleaved and activated by caspase-9 (Cain, 2003). Active caspase-3 has many cellular substrates, cleavage of which brings about destruction of the cell (Saikumar et al., 2007). Other factors released from the intermembrane space such as Smac/DIABLO and Omi/HtrA2 promote apoptosis by sequestering inhibitor of apoptosis proteins (IAPs) (Shiozaki and Shi, 2004). Omi/HtrA2 is also thought to have protease activity which in vitro, degradation of XIAP, cIAP1, cIAP2 and anti-apoptotic PED/ PEA-15 was observed (Vande Walle et al., 2008). AIF and endonuclease G are released into the cytoplasm and then translocate to the nucleus and participate in nuclear chromatin condensation and DNA fragmentation (Elmore, 2007; Lorenzo and Susin, 2007).

1.2.6 Extrinsic apoptosis

The extrinsic or death receptor (DR) pathway is initiated by binding of an extracellular ligand to a corresponding death receptor. Death receptors are members, along with approximately 20 other proteins, of the tumour necrosis factor (TNF) family (Locksley et al., 2001). TNF receptor family proteins share common structural characteristics including their cysteine rich extracellular domain, and a cytoplasmic domain which is referred to as the 'death domain' (DD) (Ashkenazi and Dixit, 1998). TNF-R1: TNF-L, cluster of differentiation 95/ (CD95 /Fas**):** FasL, TNF-related apoptosis-inducing ligand (TRAIL) -R1 (DR4): TRAIL-L, TRAIL-R2(DR5): TRAIL-L and DR3: TNF-related weak inducer of apoptosis (Tweak)-L, where L represents the ligand, are the most well characterised receptor: ligand couples in relation to apoptosis and all transmit their death-inducing signal through the DD (Fulda and Debatin, 2004). Upon ligand binding, these receptors trimerise, and recruit adaptor proteins such as fas-associated death domain (FADD) followed by recruitment of caspase-8, which all together form a death inducing signalling complex (DISC). DISC formation results in caspase-8 oligomerisation and auto-activation via polyubiquitination of its p10 catalytic domain by Cul3-E3 ligase (Jin et al., 2009). Active caspase-8 proceeds to cleave and activate caspase-3 resulting in cell death (Kischkel et al., 1995). Signalling through caspase-8 can be "shut-down" via K48-linked polyubiquitination of its large catalytic domain by TNF-receptor associated factor 2 (TRAF2) which essentially targets caspase-8 for 26S proteosomal degradation (Gonzalvez et al.). Another inhibitor of caspase-8 is cellular FLICE inhibitor protein (c-FLIP) which is strongly homologous to caspase-8 but lacks protease function. Like caspase-8, c-FLIP is recruited to the DISC and cleaved between its p18 and p10 subunits leaving a 43 kDa fragment at the DISC. This blocks caspase-8 activation and extrinsic apoptosis (Scaffidi et al., 1999). It must be noted, another study reported c-FLIP can enhance apoptosis at more physiologically relevant levels and only when exceeding these levels can it block caspase-8 activation (Chang et al., 2002). The extrinsic pathway can also be blocked at the receptor level by FAS associated phosphatise 1 (FAP 1) which binds CD95/FAS, and dephosphorylates its phospho-tyrosine 275 residue to inhibit FAS-L induced apoptosis (Foehr et al., 2005). FAP 1 can also bind intracellular CD95 and prevent its export to the cell surface (Ivanov et al., 2003). In some cases, the accumulation of caspase-8 at the DISC is not

adequate to activate caspase-3 and so the extrinsic pathway, amplifies the intrinsic pathway. Essentially, caspase-8 cleaves the BCL-2 family member, BID to form a truncated version tBID (Scaffidi et al., 1998). tBID, translocates to the mitochondria, where it binds to cardiolipin present on the mitochondrion surface and is thought to be involved in BAX/BAK oligomerization and pore formation (Kim et al., 2009; Korytowski et al., 2011).

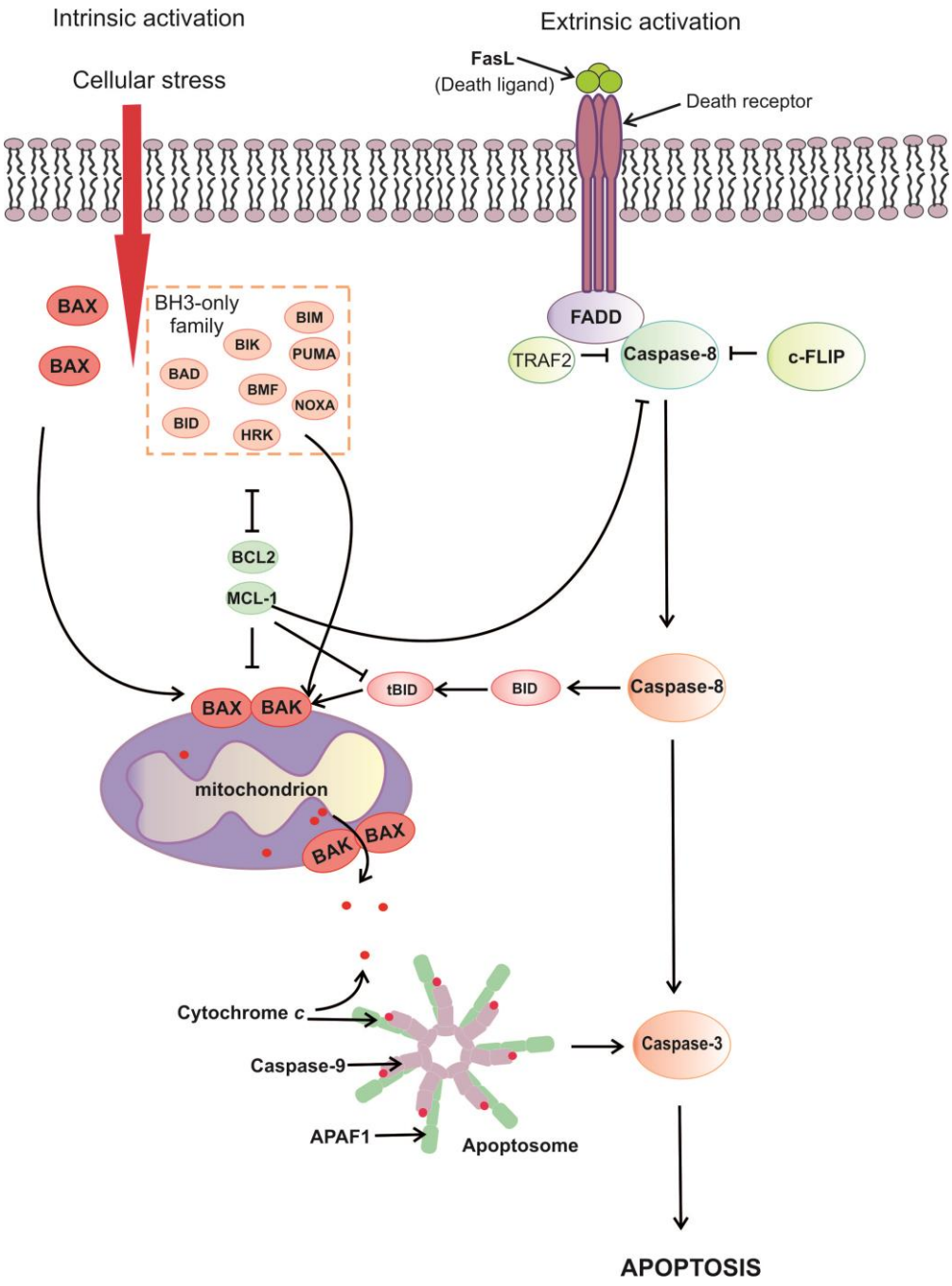

Figure 1.3 Intrinsic and extrinsic apoptotic pathways

The intrinsic apoptosis pathway is initiated by toxic stimuli that trigger the cells stress responses and cause changes to the OMM. BCL-2 family members are activated in response to stress signals and determine if the mitochondrial membrane is permeabilised based on the balance between pro-apoptotic and anti-apoptotic family members. BAX translocates to the mitochondria, and heterodimerises with BAK to form pores in the OMM to mediate the release of cytochrome *c* from the mitochondrial intermembrane space into the cytosol where it stimulates formation of the apoptosome. This leads to activation of caspase-9 and downstream caspase-3. The extrinsic pathway is activated by ligand binding to death receptors, which recruits adaptor proteins such as FADD and leads to activation of caspase-8 and downstream caspase-3. Ultimately both pathways induce apoptosis.

1.3 Endoplasmic reticulum stress

The Endoplasmic Reticulum (ER) is an organelle in the cell in which secreted, membrane bound are synthesized and correctly folded (English et al., 2009; Voeltz et al., 2002). Newly Synthesized proteins must undergo several types of post-translational modifications some of which are necessary for folding them into their correct conformation. The unique oxidative and calcium rich environment of the ER is a requirement of the multitude of chaperone and foldase proteins that bring about the necessary post-translational modifications and fold proteins into their native most stable conformation (Braakman and Bulleid, 2011; Stevens and Argon, 1999). The most noteworthy of the ER chaperones are, Glucose-regulated protein, 78 (GRP78/Bip) which is a sensor for misfolded proteins, or the chaperone-like lectins Calnexin and Calreticulin recognize N-linked oligosaccharides with one terminal glucose, when the last glucose molecule is removed and the protein is in its folded confirmation, only then is the protein allowed to exit the ER. Some well-known foldases include Protein disulphide isomerases (PDI) family members, ER oxidoreductin 1 (Ero-1) and peptidyl–prolyl isomerases, which enhance formation of disulphide and peptidyl–prolyl bonds respectively (Araki and Nagata, 2011). Abnormal changes in the cells Adenosine triphosphate (ATP) supply, imbalance in the intracellular redox state, disruption of calcium homeostasis, accumulation of unfolded proteins, and inhibition of protein glycosylation, can cause the ER to become stressed. This causes the induction of a series of signalling events, collectively termed the unfolded protein response (UPR) (Kaufman, 2002).

1.4 The unfolded protein response

The UPR (Figure 1.5) can be classed into two separate phases termed the adaptive UPR and the apoptotic UPR. Adaptive responses aim to return order to the stressed ER by lowering the amount of proteins being synthesised, increasing the levels of chaperones to help fold unfolded proteins or directing misfolded proteins to the proteosome for degradation and thus alleviating the burden of accumulated protein in the ER. However, if stress is too severe then the UPR switches to promote cell death (Hetz, 2012; Rasheva and Domingos, 2009). There are three pathways of the UPR. The protein kinase R (PKR)-like ER kinase (PERK), activating transcription factor-6 (ATF6) and Inositol requiring enzyme-1 α (IRE1α) pathways. Under

normal resting conditions, these transmembrane proteins remain bound and inactive to GRP78 when the ER is stressed however, GRP78's chaperoning activity is required and so it dissociates from PERK, ATF6 and IRE1 to attend to unfolded proteins. PERK, ATF6 and IRE1 become activated and all three UPR effector proteins illicit their downstream effects (Szegezdi et al., 2006), (Schroder and Kaufman, 2005). However, research has shown increasing complexity to the activation of these receptors and requires further events after GRP78 dissociation. It has been shown in yeast that a mutation of GRP78 binding site in the luminal domain of IRE1 does not result in constitutive activation of the receptor (Oikawa et al., 2007), suggesting that other factors are required for its activation. Further to this dimerization of human IRE1 core stress sensing region (CSSR) in the ER lumen creates a major histocompatibility complex (MHC)-like groove. It is believed that unfolded proteins bind to this MHC-like grove, promoting the formation of higher order oligomers required for UPR activation (Zhou et al., 2006). In fact, it has been shown in yeast that unfolded proteins act as ligands binding to the core ER-luminal domain of IRE1 resulting in it oligomerisation and activation in vitro (Gardner and Walter, 2011). However it must be noted that the mechanism of activation for IRE1 between yeast and human cells differs some what. While yeast Ire1 activation, is regulated by both GRP78 dissociation and direct association with unfolded proteins, the activation of mammalian IRE1α is mainly regulated by GRP78 disscociation. Conversely, human IRE1β activation requires association with misfolded proteins rather than GRP78 dissociation (Maurel et al., 2013). The luminal domain of PERK and IRE1 shows similar features and could possibly be activated in a similar manner (Zhou et al., 2006). All three arms have adaptive and apoptotic signalling features which will now be described.

1.4.1 PERK Signalling:

PERK is a serine/threonine kinase of the eukaryotic initiation factor 2 (eIF2) kinase family (Wek et al., 2006). It is activated upon release from GRP78 through homodimization via its luminal domain and subsequently trans-autophosphorylates its respective cytoplasmic domain at residue threonine 980 (Schroder and Kaufman, 2005). PERK then phosphorylates eIF2α (eukaryotic translation initiation factor alpha) and in doing so blocks cap dependent protein translation. This inadvertedly results in cap independant translation of certain mRNAs with

short open reading frames in their 5' UTR (5' untranslated region) (Wek et al., 2006). Blocking translation of so many proteins, serves to relieve the pressure placed on the stressed ER and give it time to restore its protein folding capacity to normal levels. The transcription factor Activated transcription factor 4 (ATF4) is one protein translated in a cap-independent manner. This is due to the fact that, ATF4 has two upstream open reading frames (uORFs) that are inhibitory and normally block downstream ATF4 expression. During enhanced eIF2α phosphorylation, the inhibitory function of the uORFs is bypassed, and instead there is increased ATF4 translation (Wek et al., 2006). ATF4 signalling is initially adaptive and it induces transcription of pro-survival genes such as those needed for amino acid import and metabolism e.g. Cystathionine γ-lyase (Cth), and methylenetetrahydrofolate (Mthfd) which are need for metabolism of sulphur containing amino acids and Glycine transporter (Glyt1) which provides glycine for gluthione biosynthesis which reduces endogenous peroxidase levels ATF4 signalling helps to maintain the ERs oxidative environment (Harding et al., 2003). Nuclear factor (erythroid-derived 2)-like 2 (NRF2), a b-ZIP cap "n" collar transcription factor can also be induced by PERK. NRF2 is normally bound to its negative regulator Kelch-like ECH-associated protein (KEAP1) in the cytoplasm (Kobayashi and Yamamoto, 2005; Lee et al., 2007). It is activated by PERK-mediated phosphorylation, dissociates from KEAP1 (Cullinan et al., 2003) and translocates to the nucleus and binds to genes with an antioxidant response element (ARE) in their promotors i.e. anti-oxidant associated genes the code for phase-II detoxifying enzymes such as heme oxygenase-1 (HO-1) (Alam and Cook, 2003; Alam et al., 1999) and NAD(P)H:quinone oxidoreductase (NQO1) (Ishii et al., 2000; Kwak et al., 2003). HO-1 catabolizes heme to biliverdin, iron and carbon monoxide (CO). Biliverdin reductase further converts biliverdin into bilirubin which, mops up peroxy radicals and block the oxidation of lipids (Ryter and Choi, 2009). It has been shown that during ER stress in vascular smooth muscle cells, NRF2 activates HO-1; perhaps this is an adaptive action of the cell to maintain a healthy oxidative environment within the ER. In the same study, exogenous addition CO can prevent ER stress-induced apoptosis which may be due to decreased C/EBP-homologous protein (CHOP) expression (Liu et al., 2005b), so perhaps endogenous HO-1 mediated CO production can stall CHOP activation to restore ER homeostasis. Similarly there may exist a feedback mechanism in which CO can directly regulated PERK-NRF2 signalling to activate HO-1 but has no effect on other arms of the UPR, in this instance pretreatment of CO could prevent ER stress induced cell death (Kim et al., 2007). If the protein burden on the cell is too severe, PERK signalling can switch from its adaptive mode to

an apoptotic mode. Firstly, ATF6 can upregulate p58 inhibitor of protein kinase (p58(IPK)) which encodes the p58(IPK) protein that binds to the kinase domain of PERK, preventing further eIF2α phosphorylation and thus restores cap-dependant protein translation (van Huizen et al., 2003). The three arms of the UPR can induce CHOP however PERK induction of CHOP through ATF4 appears crucial for upregulation of CHOP at the protein level (Szegezdi et al., 2006). The exact mechanism in which CHOP induces apoptosis is still controversial but is would seem CHOP regulates multiple targets. Reports have shown that CHOP can upregulate the pro-apoptotic BCL-2 proteins BIM, and PUMA as well as extrinsic mediator DR5 (Cazanave et al., 2010; Ghosh et al., 2012; Yamaguchi and Wang, 2004), and to downregulate the anti-apoptotic protein BCL-2 to promote apoptosis (McCullough et al., 2001). It can also increase the cellular level of ROS, by causing a decrease in gluthione (Marciniak et al., 2004). Furthermore, CHOP transcriptionally induces ERO1α which reoxidizes PDI and forms H202. CHOP induced ERO1α also causes the ER calcium release channel IP3R1 to become active and release calcium into the cytoplasm which subsequently leads to apoptosis (Li et al., 2009). Another target of ATF4 is DNA damage-inducible 34 (GADD34), which negatively controls eIF2α phosphorylation by interacting with protein phosphatase 1C (PP1C), restoring protein synthesis (Hetz et al., 2012).

1.4.2 ATF6 Signalling:

There are two known subtypes of ATF6, designated ATF6α and ATF6β (Haze et al., 1999). Knockout of both subtypes separately did not impede development in mice; however knockout of both simultaneously was embryonically lethal (Wu et al., 2007; Yamamoto et al., 2007). The C-terminus of ATF6 is embedded within the ER while the N-terminus protrudes into the cytoplasm. In unstressed conditions, ATF6 is found as a monomer, dimer or oligomer due to the presence of intra- and inter- molecular disulphide bonds. Upon exposure to chemical and physiological inducers of ER stress, the disulphide bonded ATF6 is reduced to a monomer form (Nadanaka et al., 2006) and dissociates from GRP78. Once ATF6 is free from GRP78, a region containing two golgi localization signals called GLS1 and GLS1 is exposed and the reduced monomer form of ATF6 is transported to the Golgi apparatus (Shen et al., 2002). It is in the Golgi that ATF6 is reduced and its N-terminal fragment known as ATF6-N is cleaved from the total or parent protein known as ATF6-P by proteases specific to the Golgi known as

Site-1 protease (S1) and Site-2 protease (S2) (Ye et al., 2000). ATF6-N is translocated to the nucleus and exhibits its defining role in transcriptional activation of genes that encode for chaperone proteins, folding enzymes and proteins associated with ER-associated degradation (ERAD) to help restore the ER to its normal functioning state, notably it induces transcription of X-box binding protein (XBP1) which requires further processing by IRE1 to become active (Kim et al., 2008; Szegezdi et al., 2006). ATF6 regulates the transcription of these genes by binding to the cis-acting ER Stress response Element I and II (ERSE, ERSE II) and Unfolded Protein Response Element UPRE, which were identified in the promoters of several UPR associated genes, such as GRP78 which acts as a sensor for ER stress and maintains the ER calcium store; and ERP72 (Endoplasmic reticulum resident protein 72) which assists protein folding; GRP94; and Calreticulin (Yamamoto et al., 2004b).

1.4.3 IRE1 signalling:

In mammals, there are two active isoforms of IRE1, termed IRE1α and IRE1β which, similar to the activation of PERK, and upon release form GRP78 and via binding of unfolded proteins, IRE1 dimerizes, autophosphorylates and activates itself (Gardner and Walter, 2011; Szegezdi et al., 2006). IRE1 consists of two domains, an endoribonuclease domain and a serine/threonine kinase domain. The endoribonuclease domain directly cleaves XBP1 mRNA to from a smaller mRNA product called spliced XBP1 (XBP1s) (Figure 1.4). Splicing of XBP1 differs from the majority of other splicing events and is termed “unconventional”. When compared to the classical splicing via the splicosome which recognizes a sequence at an exon-intron boundary that generally follows chambons rule i.e splices a site beginning with AU that ends in AG, GU-AG or AU-AC (Tarn and Steitz, 1997; Uemura et al., 2009); unconventional splicing is completely independent from splicosome processing and depends solely on IRE1 and an RNA ligase that has not yet been identified in mammals. The stem loop structure in XBP1 replaces the typical chambons sequence and is spliced out by IRE1 and the XBP1 mRNA is then ligated by the RNA ligase and translated to produce XBP1s with a shift in its codon reading frame. Once XBP1 has been spliced it encodes for its transcription factor that has been identified to target many UPR and ERAD associated chaperones such as p58(IPK) which, as already mentioned blocks PERK-mediated eIF2α phosphorylation and

ends the translational block, ERdj4/ HSP40 and EDEM1 (Kawahara et al., 1998; Lee et al., 2003). It has been shown that ATF6 and XBP1 are translocated to the nucleus upon ER stress and only when the transcription factor NF-Y (nuclear transcription factor Y) binds to the sequence CCAAT can ATF6 and XBP1 bind to the CCACG region of the ERSE and initiate transcription of the ERSE containing chaperones and ERAD associated genes (Roy and Lee, 1999; Yamamoto et al., 2004a). XBP1s is also involved in the observed expansion of the ER during stress by increasing phospholipid synthesis (Sriburi et al., 2004). Splicing of XBP1 is part of IRE1's adaptive response and as such has been shown to promote cell survival. For instance overexpression of Heat shock protein 72 (HSP72) was reported to prolong splicing of XBP1 and confer resistance to ER stress induced cell death (Gupta et al., 2010). The Rnase activity of IRE1 also splices a subset of mRNA through a process known as Regulated IRE1 Dependent Decay (RIDD). The specific RNAs degraded by RIDD activity depends on the cell type affected and mostly targets mRNAs encoding for proteins of the secretory pathway. However other targets have been implicated in apoptosis regulation (Hollien et al., 2009). It is thought that the presence of a conserved nucleotide sequence accompanied by a defined secondary structure may effect the selectivity of IRE1 to degrade particular RIDD substrates (Maurel and Chevet., 2013).

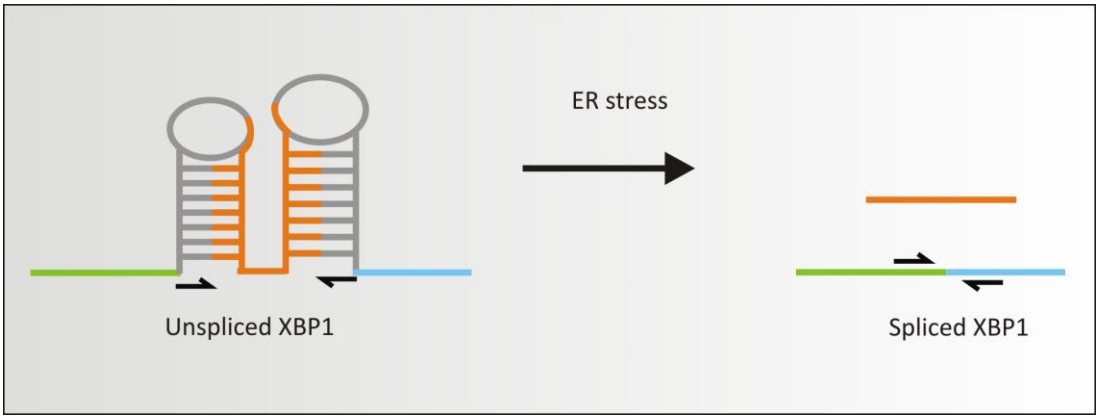

Figure 1.4 splicing of XBP1

Upon ER stress ATF6 transcriptionally activates XBP1 mRNA and is spliced by the RNase activity of IRE1, removing the mRNAs hairpin structure to form a smaller transcript with a shift in the open reading frame that encodes the active transcription factor XBP1s.

IRE1 signalling can also switch from adaptive signalling to apoptotic signalling if the stress on the ER cannot be averted. IRE1 can recruit TRAF2 which, activates apoptosis signal-regulation kinase 1 (ASK1) which induces a series of phosphorylation events, ultimately leading to phosphorylation and activation of c-JUN terminal kinase (JNK 1/2) (Urano et al., 2000b). The exact outcome of JNK activation during ER stress is not well characterised, however it has been shown that IRE1-JNK signalling is activated through mammalian target of rapamycin complex 1 (mTORC1)-mediated reduction in AKT phosphorylation. In the same study use of rapamycin an inhibitor of mTORC1 blocked IRE1-JNK signalling and apoptosis in vitro and could reduce tunicamycin induced tubular injury and apoptosis in mice (Kato et al., 2012). JNK has also been reported to be pro-apoptotic by translocating to the nucleus and transactivating transcription factors that subsequently regulate pro- or anti- apoptotic genes. One of the better characterized targets of JNK is c-Jun, which is phosphorylated by JNK and brings about AP-1 (activating protein 1) dimerization (Dhanasekaran and Reddy, 2008). AP-1 dimers can consist of Jun, Fos or ATF subunits that bind to a common gene and regulate its transcription (Karin et al., 1997) e.g. TNF-alpha (α), Fas-L, and BAK (Fan and Chambers, 2001). JNK has also been reported to phosphorylate p53 at Ser 6. This stabilizes p53 and blocks its degradation by the proteasome (Fuchs et al., 1998). p53 is a tumour suppressor that can induce a cell cycle checkpoint via upregulation of p21 which binds to and inhibits cyclin-dependant kinase 2 (CDK2) a protein required for G1-S phase transition (Di Leonardo et al., 1994). p53 is also a transcription factor and has been reported to upregulate pro-

apoptotic BCL-2 family proteins such as BAX, PUMA, NOXA and BID (Gurzov et al., 2010; Oda et al., 2000; Sax et al., 2002; Zhan et al., 1994). Downstream of the mitochondria p53 can transcriptionally upregulate APAF-1 (Moroni et al., 2001) which may be more an enhancing effect in apoptosis rather than an initiating event. p53 can upregulate DR5 (Wu et al., 1997) and CD95 (Muller et al., 1998) death receptors to potentiate extrinsic apoptosis. p53 signalling was also reported to disrupt pro-survival pathway P13K via upregulation of another tumor supressor PTEN (Stambolic et al., 2001). Aside from nuclear functions of JNK, it may also play a part in OMM permeabilisation where MEFs lacking JNK1/ 2 failed to release cytochrome *c* (Tournier et al., 2000). The exact role for JNK in cytochrome *c* release is not yet known but it may be via its induction of the extrinsic death receptors or caspase-8 mediated BID cleavage (Dhanasekaran and Reddy, 2008) . JNK has also been shown to enhance the activity of pro-apoptotic BCL-2 family proteins BIM and BMF via direct phosphorylation (Lei and Davis, 2003). This releases them from sequestering proteins dynein and myosin V motor complexes and can lead to intrinsic apoptosis. JNK phosphorylation of BAD at Ser 128 and Ser 148 blocks it interaction with neutralizing 14-3-3 proteins and thus BAD can block pro-survival BCL-2 family members and enhance apoptosis (Donovan et al., 2002; Wang et al., 2007). TNFα induced apoptosis has also been reported to involve JNK-mediated cleavage of BID where JNK cleaves BID, forming jBID which translocates to the mitochondria and mediates specific Smac/DIABLO release with no release of cytochrome *c*. The release of Smac/DIABLO ultimately potentiates cell death by disrupting TRAF2-cIAP complexes that otherwise block caspase-8 activation (Deng et al., 2003).

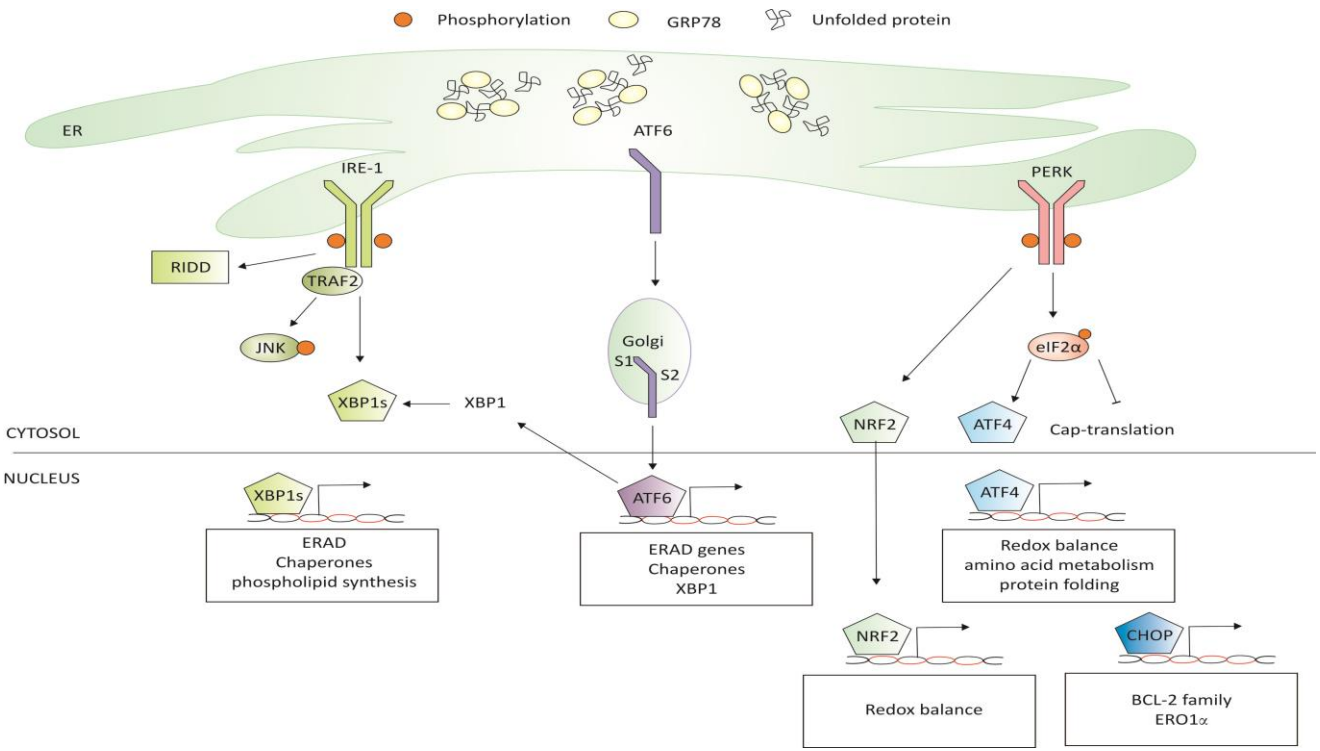

Figure 1.5 Unfolded protein response

Upon stress, IRE1, ATF6 and PERK dissociate from GRP78 and become active. PERK phosphorylates eIF2α, blocking cap-dependant translation and enhancing cap-independent translation of ATF4 which, upregulates genes for amino acid synthesis, redox balance and CHOP. PERK also phosphorylates NRF2 which induces antioxidant response genes. CHOP promotes apoptosis by upregulation of BIM and ERO1α and downregulation of BCL-2. ATF6 translocates to the Golgi, is cleaved to form its active N-terminal fragment which upregulates XBP1, ERAD and chaperone genes. IRE1 can cleave mRNAs via RIDD, or recruit TRAF2 which leads to JNK phosphorylation. IRE1's RNase domain can splice XBP1 which encodes the protein XBP1s, which upregulates ERAD, chaperones and genes needed for phospholipid synthesis.

1.5 microRNA

microRNAs (miRNAs) are evolutionary conserved, small non-coding RNAs that are roughly 22nt in length. Their main and defining role in the cell is as the endogenous effectors of RNA interference (RNAi), a pathway in which miRNAs "interfere" with mRNA to protein translation via direct degradation or repression of mRNA, and as such control gene expression (Montgomery et al., 1998; Siomi and Siomi, 2009). In fact, miRNA regulate at least one third of all genes (Lewis et al., 2005) and are important regulators of all major processes such as development (Shi and Jin, 2009), metabolism (Rottiers et al., 2011) and apoptosis (Lynam-Lennon et al., 2009). According to mirBase (release 19) there are now 2042 known mature miRNA found in humans and it is well known that, a single miRNA can have multiple targets (Bartel, 2009), thus, altered expression of one miRNA may have massive consequences in the cell, tissues and throughout the body. As such, deregulated miRNA expression is seen in many disease conditions such as cardiac, neurodegenerative, autoimmune diseases, diabetes and in tumourogenesis (Bushati and Cohen, 2008; Ha, 2011; Tang et al., 2008b; Thum and Bauersachs, 2008). As a result miRNA may serve as potential therapeutic targets.

1.5.1 Biogenesis of miRNA

1.5.1.1 Transcription

miRNA are found in introns of mRNA, coding regions and even intergenic regions. Transcription of miRNA located in introns and coding regions often occurs simultaneously to the host gene as observed with the miRNA miR-211 and its host gene melastatin (Levy et al., 2010) while transcription of those miRNA situated in the intergenic regions of the genome is thought to occur through their own promoters (Faller and Guo, 2008; Kim, 2005). miRNA were also found as clusters on polycistronic transcripts and so more than one miRNA can be transcribed from one transcriptional initiation event (Bartel, 2004; Kim, 2005). Those miRNA found as clusters often have an analogous sequence but not always, however it has been

seen that miRNA of the same cluster can coordinate their efforts for regulation of a particular task or process (Yu et al., 2006). This can be seen by the coordinated functioning of the mir-17-92 cluster which is up regulated in some human cancers and has oncogenic function (Hayashita et al., 2005).

The formation of mature miRNA begins with a long RNA transcript known as the primary miRNA (Pri-miRNA). These transcripts are usually transcribed by RNA polymerase II, (Pol II) however there have been reports that show some miRNAs are transcribed by RNA polymerase III (Pol III). The majority of miRNA are transcribed by Pol II due to the presence of four Uracil (U) bases which correspond to four Thymine (T) bases in the miRNA gene sequence which is essentially a termination sequence for Pol III. Furthermore many pri-miRNA carry a 5' M^7G cap and a poly (A) tail, which are features associated with Pol II mediated transcription (Lee et al., 2004). Some miRNAs e.g. the resident miRNA of chromosome 19, are situated between the retrotransposons known as Alu elements which are transcribed by Polymerase III, and so too where miRNA located between such elements e.g. miR-515-l, miR-515a, miR-519a-1 and miR-517c (Borchert et al., 2006). Alu elements move throughout the genome, they leave a particular site by their transcription into RNA and are inserted into a different part of the genome through reverse transcription. It is through such insertion that these Alu elements can cause mutations in certain genes and the reason why they are implicated in many genetically associated diseases (Hancks and Kazazian, 2012). Other miRNA particularly those encoded by viruses such as the herpes family viruses lie between tRNA promoter regions and may also be transcribed by Pol III. (Liu et al., 1995) By using bioinformatics techniques further analysis showed that more miRNA are located between Alu elements, tRNA promoter regions and even mammalian wide interspersed repeat sequences (MWIR) then originally believed. Results indicate that approximately 18 such miRNA could be transcribed by Pol III (Borchert et al., 2006).

The transcription of miRNA is regulated by many factors. For instance various transcription factors have been shown to recognise particular miRNA and either increase or decrease their promoter activity (Breving and Esquela-Kerscher, 2009). The oncogenic transcription factor, c-MYC has been shown to regulate the transcription of about 13 miRNA clusters (Frenzel et al., 2010) including mir-17-92 cluster where it upregulates the clusters transcription via

recognition of its upstream CATGTG sequence (O'Donnell et al., 2005). However Myc over expressing B-cells exhibited repression of a vast majority of miRNA and display enhanced tumourgenecity (Chang et al., 2008). This suggests that Myc or downstream targets of Myc can repress miRNA expression. Other studies have shown that epigenetic regulation is also a contributing factor to miRNA expression. A small number of miRNA are actually found in CpG islands and can only be transcribed if they have been methylated by DNMT1/ DNMT3b (Breving and Esquela-Kerscher, 2009).

Transcription if the miRNA gene generates an RNA transcript referred to as the primary or pri-miRNA and as mentioned can be a single or clustered transcript (Bartel, 2004).

1.5.1.2 Maturation:

Once it is transcribed, the pri-miRNA is processed by a protein complex called the microprocessor. This consists of the ribonuclease III enzyme called DROSHA and the protein DGCR8 (DiGeorge Syndrome Critical Region Gene 8) and other other cofactors, e.g., the DEADbox RNA helicases p68/DDX5 and p72/DDX17 (Gregory et al., 2004). This complex cleaves the pri-miRNA into a smaller transcript of approximately 70nt called the precursor miRNA (pre-miRNA). The cleavage results in the pre-miRNA having a 5' phosphate and a 3' overhang at the base of its stem (Bartel, 2004). DROSHA and DGCR8 co-ordinate their efforts and post-trancriptionally regulate each other with the interaction between the two proteins contributing to the optimal functioning of DROSHA (Tang et al., 2010), while DROSHA cleaves and destabilizes the DGCR8 mRNA hairpin therefore negatively regulating its own function (Han et al., 2009) Recognition of the pri-miRNA seems to depend on cooperative binding of DGCR8 and the pri-miRNA with one model suggesting that DGCR8 forms higher order structures e,g, DGCR8 dimers forming trimers and increasing the cooperative binding to pri-miRNAs (Faller et al., 2010). Binding of heme to DGCR8 has shown to catalyze trimerization upon interaction with pri-miRNA and the activity of the microprocessor complex greatly increases resulting in more efficient processing of the transcript (Faller et al., 2007). The smaller pre-miRNA is transported out of the nucleus through the karyopherin nucleocytoplasmic transport factor known as Exportin-5 (XPO5) which is dependent on Ran- GTPase activity. The RanGTpase allows for cytoplasmic binding

of the molecule being transferred while hydrolysis to RanGDP allows release of the molecule (Yi et al., 2003). Once in the cytoplasm the pre-miRNA is further cleaved by another ribonuclease III called DICER which is thought to recognise the 5' phosphate and 3' overhang portion of the transcript. As with DROSHA, DICER also cleaves both strands of the double stranded transcript, however this cleavage event results in the loss of the stem loop structure and the formation of a miRNA:miRNA* duplex consisting of two miRNA strands one of which is degraded while the other becomes incorporated into the "mature" RNA induced silencing complex (RISC). When the mature RISC is described, the consensus opinion is that only one strand is selected into the RISC in an ATP-dependant manner. However it has been shown that prior to RISC maturation the miRNA;miRNA* duplex is loaded onto the RISC in an ATP dependant manner and with the cooperation of the chaperone proteins Hsc70 and Hsp90 which possible direct a conformational change in AGO proteins. However the unwinding of both strands has been reported to occur independently of ATP hydrolysis and does not require Hsc70 or Hsp90. It has not been fully elucidated how unwinding occurs. Some reports suggest the miRNA helix is cleaved thus releasing one strand while others have suggested that unmatched base pairs in the two strands allow for unwinding. Another group have proposed the "rubber band" model by which AGO proteins are forced into a conformational change by ATP hydrolysis to allow space for miRNA uptake, generating structural tension. They propose that the AGO protein snaps down into a more relaxed structure around the miRNA duplex and that release of tension would provide the energy to unwind the helix (Iwasaki et al., 2010). The single miRNA strand seleted by RISC, acts a guide for the silencing complex by directing it towards the target mRNA. The RISC-miRNA complex generallytargets the 3' UTR (untranslated region) of the mRNA and induces silencing by one of two mechanisms, a translational block or mRNA degradation. However it must be noted that there have been reports showing unconventional miRNA-mediated silencing via the 5' UTR of the mRNA. Some miRNAs recruit epigenetic silencing markers to the 5' UTR to essentially "turn off" gene expression e.g. miR-211 (Chitnis et al., 2012). Complementarity between the miRNA and the target mRNA is thought to be involved in the decision of which mechanism is used for RNAi-mediated silencing (Bartel, 2004). For some reason the processing of a select few of miRNA differs from that of other miRNA and requires additional adaptor proteins for efficient processing. This is seen with the regulation of miR-21 via SMAD proteins. The expression of miR-21 is increased via Transforming growth factor β (TGFβ) and bone morphogenetic protein (BMP) signalling which results in the contractile phenotype of vascular smooth muscle cells.

TGF β and BMP recruit SMAD proteins to the microprocessor complex where it interacts with the microprocessor component p68 helicase and enhances DROSHA's processing capabilities resulting in increased levels of miR-21 (Davis et al., 2008). miR-155, a miRNA that promotes epithelial mesenchymal transition (EMT) and metastasis, is also reported to be regulated by TGF β signalling and the recruitment of SMAD proteins (Kong et al., 2008). Biogenesis of miRNA let-7 can be abolished by Lin-28 binding to the loop region of pre-let-7a and blocking DROSHA recognition, an important process for embryonic development (Newman et al., 2008). There are also factors that recognise a particular sequence in the terminal loop of the pri-miRNA as well as mature miRNA. This has been seen for pri-mir-18a to which heterogenous nucleoprotein A1 (hnRNPA1) binds to its terminal loop and stem and alters its structure to accommodate DROSHA processing. Interestingly, other members of the 17-18a-19a cluster do not interact with this protein because unlike 18a they lack the appropriate sequence UAGGGA/U which hnRNPA1 can recognise (Guil and Caceres, 2007). Other miRNA also interact with KH-type splicing regulatory protein (KHSRP) via their terminal loops which enhances processing by DROSHA in the nucleus or DICER (van Kouwenhove et al., 2011) in the cytoplasm particularly miRNA that contain a triple GGG sequence in their stem loop with is specifically recognised by KHSRP, such as Let-7a (Trabucchi et al., 2010).

1.5.1.3 Non-Canonical biogenesis

On occasion alternative miRNA biogenesis pathways occur in which processing by either DROSHA or DICER is by-passed. The mirtron pathway consists of intronically derived miRNAs, referred to as mirtrons that lack the typical helical formation at their base which is necessary for recognition and processing by DROSHA. Instead these mirtrons have canonical splice sites that are recognized by the spliceosome. The pre-miRNA formed as a result of splicesome processing must first be linearized and folded into hairpins before then can be transported to the cytoplasm via Exportin V (XPO5). Once in the cytoplasm the mirtron hairpins are processed by DICER the same as canonically generated miRNAs (Ruby et al., 2007). Another group of miRNA were reported to by-pass DROSHA but differed from mirtons in that miRNA in this subgroup were derived from long introns of protein coding regions, and pre-miRNAs formed were typically longer than conventional miRNAs. It is thought that an as of yet, unidentified RNase enzyme must be responsible for the processing of these miRNAs

that by pass DROSHA or that there may be possible unidentified splice sites embedded (Chong et al., 2010). More recently, 2 splicing-independent mirtron-like miRNAs (simtrons), miR-1225 and miR-1228 where identified. These are intronically located but are not produced by splicosome processing, but do require DROSHA processing. The same study showed DGBR8, DICER and XPO5 are not required for biogenesis of these simtrons and suggest other proteins with RNase activity may be involved in their maturation (Havens et al., 2012). The miR-451/AGO2 pathway is thought to follow the canonical pathway initially with processing of miR-451 by DROSHA. However it seems that the pre-miRNA produced is too short at only ~17 nt and the two strands of the miRNA: miRNA* are highly complementary which is thought to hinder unwinding for DICER recognition. Instead, miR-451 is processed by the slicer activity of Argonaute (AGO2) (Cheloufi et al., 2010).

1.5.2 miRNA-mediated RNAi

1.5.2.1 The RISC complex

The RISC is a type of ribonucleoprotein complex that has been identified in several species with varying sizes, and varying functional components (Martinez and Tuschl, 2004). Argonaute (AGO) proteins however are considered as the main components of the RISC (Tan et al., 2011). There are 8 members of the human AGO protein family which are divided into two subfamilies, the AGO subfamily consisting of AGO proteins 1 to 4 and the PIWI subfamily which consists of HIWI 1 to 3 and HILI (Peters and Meister, 2007). AGO proteins generally consist of 2 structural domains known as PAZ (Piwi- Argonaute- Zwille) and PIWI domains. PAZ is thought to be responsible for anchoring miRNA due the presence of a specialised binding domain that recognises the 3' overhang of the DICER processed miRNA (Hock and Meister, 2008). Only one AGO protein has been identified to have endonuclease activity in humans which is AGO-2 and has been designated the nickname "Slicer". It is thought that AGO-2 is involved in miRNA-mediated silencing by cleaving mRNA (Hock and Meister, 2008; Tan et al., 2011). It was shown that AGO-2 must undergo prolyl 4-hydroxylation otherwise it becomes destabilised and the silencing effect is not as efficient (Qi et al., 2008). One study revealed that AGO-2, DICER and the DICER interacting protein TRBP (TAR RNA binding protein) which is necessary for recruitment of miRNA to AGO2, interact together to form a RISC complex of approx 500 kDa with ATP independent assembly and functionality (Gregory et al., 2005). The double stranded RNA binding protein (dsRBP) PACT is also thought to be a

RISC component. Although there are no reports connecting PACT to AGO-2, it is reported that PACT can in fact associate with both TRBP and DICER directly. It was also shown that DICER function is compromised in the absence of one or both of these proteins which shows that both PACT and TRBP are necessary for DICER's ability to produce miRNA and siRNA (Kok et al., 2007).

1.5.2.2 Processing-bodies: the site of silencing

Processing (P)bodies (PBs) are essentially mRNP (messanger ribonucleoprotein) - protein complexs consisting of the mRNA transcript to be silenced along with various proteins required for the degradation process (Hieronymus and Silver, 2004). It is thought that the miRNA-RISC complex directs mRNA to the components necessary to form the mRNP complexes which then aggregate into PBs and thus the mRNA can be degraded (Liu et al., 2005a). These foci are located in the cytoplasm, the full protein composition is not yet known, but it is known that components associated with degradation or translational inhibition accumulate in these foci e.g. the decapping enzyme Dcp1p/Dcp2p, and the CCR4/POP2/NOT complex which will be describe with more detail later (Cougot et al., 2004; Parker and Sheth, 2007) It has been shown that AGO proteins become highly concentrated in PBs during times of cellular stress and one study showed that an interaction between AGO proteins and RNA binding proteins during serum and amino acid starvation conditions causes activation of normally inactive miRNA (Parker et al, 2007). The family of proteins referred to as GW182 which associates with the miRNA-RISC are also found to accumulate in PBs and their purpose may be to direct the mRNA-miRNA-RISC to PBs for processing. Knockout of GW182 revealed its importance for effective mRNA degradation or translational inhibition, because it caused attenuation of PB assembly, as well as the failure of mRNA-miRNA–RISC association with PBs and thus ineffective silencing (Ding and Han, 2007). One study showed that targeting of mRNA to PBs require the presence of miRNA (Liu et al., 2005a).

1.5.2.3 RNAi: mRNA Degradation or translational inhibition

As discussed mRNA is targeted to PBs and are either degraded or translationally repressed (Bartel, 2004). Cap-dependant translation is controlled by the eIF4F complex which consists of the cap binding protein eIF4E, the RNA helicase eIF4A and the scaffold protein eIF4G which acts as a platform for 40S ribosome interaction to induce translation (Parker and Sheth, 2007). One hypothesis suggests that the AGO proteins lacking slicer activity may have a motif that can interact with eIF4E and in doing so block the interaction between eIF4E and the mRNA and thus block translation. However, a more recent publication has reported that AGO2 can directly bind to the cap binding protein complex via the translation factor eIF4GI to mediate translational repression (Ryu et al., 2013). Translational repression of mRNA has also been reported to a reversible process since when PBs are formed during conditions of stress, that mRNA can be released from these PBs if the stress has been alleviated and can then be taken up by a polysome (cluster of ribosomes) and translated into a functional protein (Bhattacharyya et al., 2006). The choice to degrade mRNA or inhibit its translation may be determined by the degree of complementarity between the miRNA and the 3' UTR of the mRNA. Full complementarity is thought to result in cleavage by AGO-2 but partial complementarity is thought to initiate a translational block (Zhang et al., 2007). If degraded, deadenylation of the polyA tail is mainly carried out by the deadenylase complex consisting of Ccr4p/ pop2p/ not (Parker and Sheth, 2007). At this stage the pathway can take on two different directions: The first pathway involves the removal of the (5' 7 methylguanosine) M^7G cap by the enzyme Dcp1p/Dcp2p resulting in the 5'- 3' cleavage of the remaining transcript by an exonuclease called xrn1p (Ingelfinger et al., 2002) The alternative secondary pathway to this involves deadenylation followed by 3'-5' cleavage of the mRNA by the exosome complex (Parker and Sheth, 2007).

1.5.3 miRNA turnover:

Little is known about degradation of miRNA but it has been reported that the small RNA degrading nuclease SDN-1 has 3' to 5, exonuclease activity and can actually degrade miRNA in Arabidopsis (Ramachandran and Chen, 2008). It was seen that 3' methylation via HEN1 methyltransferase may protect against such degradation by stabilizing the miRNA. In fact levels of mature miRNA are lower in HEN1 mutants. Similarly, 3' adenylation of mir-122 by GLD-2 which is a poly-(A) polymerase found in the cytoplasm stabilises the mature miRNA and prevents its depletion. In mammals XRN-2 behaves a lot like SDN but with 5'-3' exonuclease activity that only degrades single stranded miRNA. This suggests that the passenger strand which is not protected by Argonaute will be a target for XRN-2 (Kai and Pasquinelli, 2010). There is some debate over miRNA half-life however it is thought to vary from miRNA to miRNA and from response to response, in some instances the half-life being as little as half an hour to others remaining active and present in the cell for days (Gantier et al., 2011). The stability of miRNAs is also determined by the presence of GW128, for only in its absence can miRNA be degraded (Yao et al., 2012).

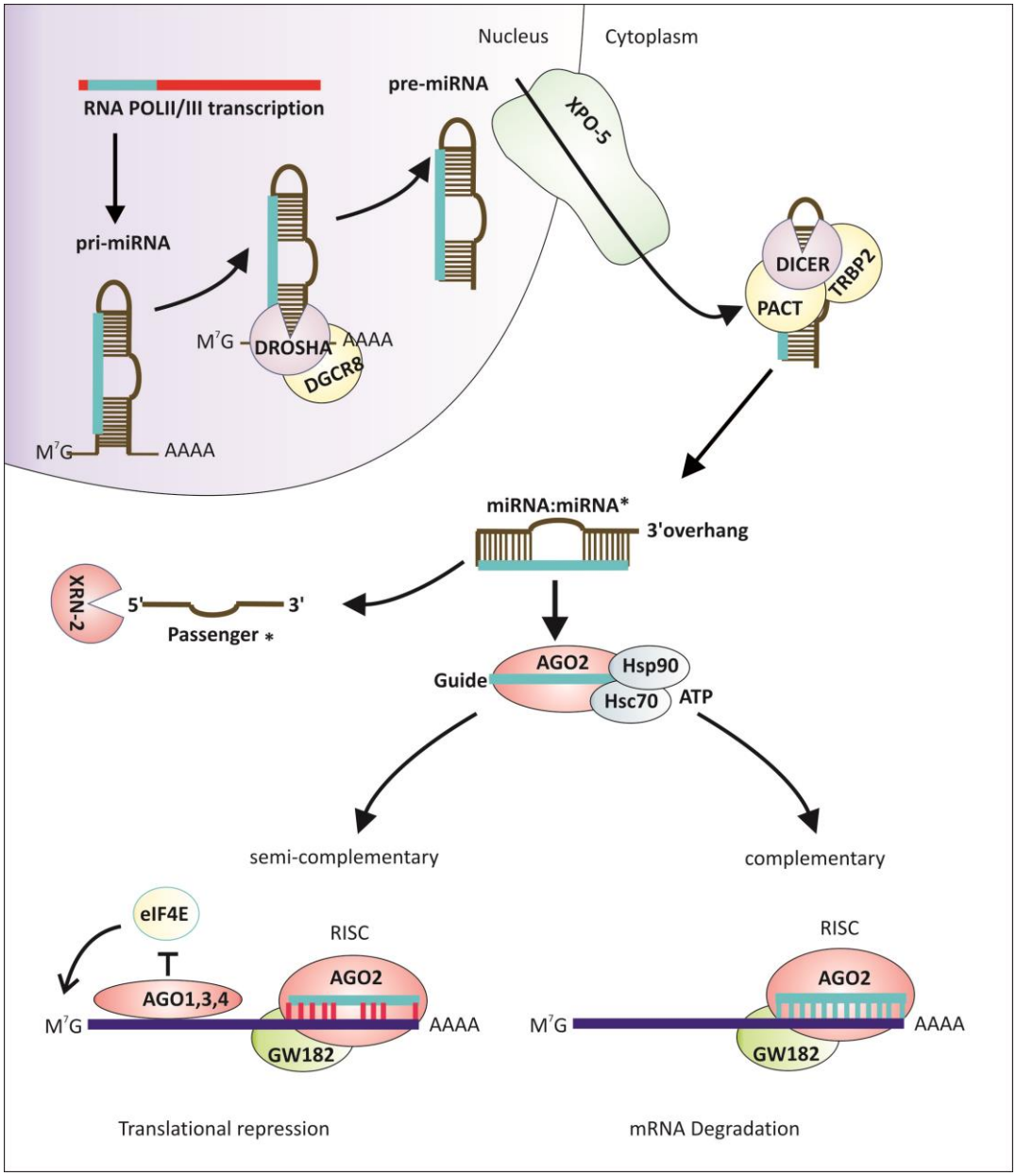

Figure 1.6 miRNA biogenesis

miRNA Biogenesis begins in the nucleus where a miRNA gene is transcribed by RNA Pol II/ III forming a single pri-miRNA or clustered pri-miRNA transcript. This is processed by DROSHA at the base of its hairpin stem to form a pre-miRNA transcript of ~ 70 nt which is transported out of the nucleus by XPO-5. In the cytoplasm it is further processed by DICER which removes it's hairpin loop to form a miRNA:miRNA* duplex of ~ 21 nt. The duplex is loaded onto the RISC via ATP hydrolysis and chaperones Hsc70/Hsp90. The duplex is separated and one strand is retained within the RIS and mediates the translational repression or degradation of its target mRNA based on the degree of complementarity between miRNA and mRNA. The second strand of the miRNA: miRNA* duplex is usually degraded via XRN-2

1.6 Apoptosis and cancer

Cancer is a disease in which cells are reprogrammed for enhanced proliferation, continuous replication, evasion of apoptosis and many more characteristics that allow cells to proliferate and grow so much that they can form a solid mass or tumour and/ or metastasize to distant tissues and organs (Hanahan and Weinberg, 2011). In healthy tissues and organs cells continually die and proliferate. However sometimes apoptosis is required for other reasons for instance cells that acquire unwanted mutations in their genome are normally detected and destroyed before any dangerous material can be passed on to further generations of cells (Elmore, 2007). However it is common that cancers cells acquire mutations that allow for enhanced survival e.g. mutations in tumour suppressor genes such as p53 can be acquired or upregulation of cell survival signalling pathways such as phosphoinositide-3-kinase (PI3K) and Extracellular signal-regulated kinase (ERK) to promote tumour development (Hanahan and Weinberg, 2011). Other times or even along with amplification of survival signals, cells can modulate apoptotic responses to bypass cell death and enhance survival. Pro-survival BCL-2 and is often upregulated in cancer as seen in Lung carcinoma, CLL, multiple myeloma, breast, colorectal, prostate cancers etc…(Higashiyama et al., 1996; IERVOLINO et al., 2002; McDonnell et al., 1992; Real et al., 2002; Robertson et al., 1996; Sinicrope et al., 1995). However high BCL-2 expression has conflicting prognostic implications, e.g., it has been associated with poor and favourable prognosis in acute myeloid leukaemia and breast cancer respectively (Dawson et al., 2010; Karakas et al., 1998). In fact, BCL-2 was initially discovered from its aberrant chromosomal translocation t(14;18) in follicular lymphoma. This translocation results in fusion of BCL-2 to immunoglobulin heavy chain enhancer thus upregulating BCL-2 transcription (Tsujimoto et al., 1985). Higher MCL-1 levels have been detected in haemopoetic, lymhoma and multiple myeloma cancers (Campbell et al., 2010; Wenzel et al., 2012; Wuilleme-Toumi et al., 2005). In cervical cancer it was shown that MCL-1 is upregulated via PI3K/AKT signalling (Wei et al., 2001) which is already associated with carcinogenesis, acting downstream of many receptor tyrosine kinases and provides powerful pro-survival signals via AKT-dependent phosphorylation of transcription factors such as

nuclear factor-kappa B (NF-κB) and forkhead, as well as direct phosphorylation of anti-apoptototic proteins including BCL-2 family members (Hafsi et al., 2012). High levels of MCL-1 as well as upregulation of pro-survival XIAP and c-FLIP are reported to provide resistance to TRAIL and chemotherapeutic therapies (Wang et al., 2008b). However, questions have been raised regarding the anti-apoptotic effects of XIAP, and one study revealed that expression of XIAP to similar level of what is physiologically relevant does not in fact provide any resistance and only when expressed alongside a stable knockdown of Smac/diablo was resistance seen (Seeger et al., 2010). Conversely, pro-apoptotic BCL-2 family members such as BIM have been reported to be downregulated in cancers including colorectoral cancer where activated ERK 1/2 which phosphorylates BIM and directs it's degradation by the proteosome, thus enhancing cell survival (Greenhough et al., 2010). BIM was also reported to be important for leukaemia cell death when PI3K/AKT and ERK 1/2 pathways have been compromised, where levels of BIM increased and MCL-1 decreased suggesting BIM is lower in leukaemias which active survival pathways (Rahmani et al., 2009). In fact, deletions of BIM in mice lead to development of B-cell lymphoma in Eμ-*Myc*-transgenic mice (Egle et al., 2004). Deletion of BID and BAD in mice also resulted in development of malignancy resembling chronic myelomonocytic leukemia and B-cell lymphoma respectively (Ranger et al., 2003; Zinkel et al., 2003). Deletion or inactivation of other BH3 only proteins has also been reported in cancers. The locus carrying PUMA undergoes frequent mutations as seen in B cell lymphomas, gliomas and neuroblastomas (Garrison et al., 2008). Deletion of the locus carrying BMF was observed in breast, lung and colon cancers. Loss of BIK or methylation-mediated silencing has been reported in renal carcinomas (Sturm et al., 2006). Methylation and silencing of HRK was shown to occur in several cancers including gastric, colorectal and glioblastoma cancers (Nakamura et al., 2005; Obata et al., 2003). It has to be noted however that more recent publications have shown that the usually pro-apoptotic BIM and BAD may act as pro-survival proteins in cancer. In the case of BIM, it was shown to be constitutively overexpressed in prostate and breast cancer cell lines and primary tumour cells where its upregulation was shown to be driven by the transcription factor E2F1 (Gogada et al., 2012). The ability of cells to cope to high levels of BIM without undergoing apoptosis was elaborated when the phosphorylation status of BIM at Ser69 or Ser87 was examined and higher phosphorylated BIM was observed in these prostate and breast cancer models. Previously phosphorylation of BIM at Ser69 or Ser87 was reported to enhance cancer survival by promoting BIM degradation or inactivation (Paterson et al., 2012; Qi et al., 2006; Wang et al.,

2011). Further to this, MCL-1 and BCL-xL where upregulated and shown to interact with BIM, and were thought to sequester BIM at membrane structures like the mitochondria, and perhaps blocked from interacting with BAX (Gogada et al., 2012). It would appear that it is merely neutralized by phosphorylation and/or sequestration by pro-survival proteins. Similarily, high levels of phosphorylated BAD were detected in prostate cancer and was surprisingly found to promote proliferation of prostate cells in vitro and of prostate tumors in vivo (Smith et al., 2009). Another study revealed that BAD can heterodimerize with BCL-xL and allow cells to avoid cell cycle checkpoint to promote proliferation (Chattopadhyay et al., 2001), perhaps this is a possible mechanism of how higher BAD may be advantageous to cancer cells.

1.7 ER stress and cancer

Deregulation of ER stress has been reported to occur in many cancers including, hepatocellular carcinoma (HCC), breast, skin etc. (Moenner et al., 2007). Within the tumour microenvironment for example, cancer cells are often subjected to inflammatory stress, tightly packed and can be nutrient deprived, subjected to low pH and hypoxic (Hanahan and Weinberg, 2011; Lorusso and Ruegg, 2008), as such ER stress signalling pathways are subsequently activated. Often these cells can utilize ER stress to promote apoptosis and provide room for new proliferating cells but also cancer cells can commandeer the adaptive UPR responses to promote cell survival (Feldman et al., 2005; Koumenis and Wouters, 2006; Spiotto et al., 2010).

As seen with the upregulation of pro-survival apoptotic proteins in cancer, there is also reported to be upregulation of ER stress associated genes in cancer such as colon, breast, lung, as well as in HCC (Wang et al., 2010). ATF6 and GRP78 mRNA showed increased expression in HCC (Shuda et al., 2003). While IRE1 has been shown to be regulated by tyrosine phosphatase 1B (PTP-1B) (Gu et al., 2004) where PTP-1B expression was shown to be necessary for complete IRE1 signaling. PTP-1B is a phosphatase downregulated in HCC and associated with a more aggressive form of the disease (Zheng et al., 2012). Loss of PTP-1B attenuated IRE1 signalling and ER stress induced apoptosis which could explain why loss of PTP-1B in HCC enhances tumourigenesis (Gu et al., 2004). PTP-1B has also been

reported to delay tumourigenesis in ErbB2 breast cancers however (Julien et al., 2007) and perhaps in some scenarios, if PTP-1B induced IRE1 this could enhance XBP1s levels to promote survival. In fact, XBP1s is associated with poor survival in ERα positive breast cancer (Davies et al., 2008) and in multiple myeloma (Bagratuni et al., 2010). Interestingly, use of an inhibitor of the IRE1 endoribonuclease domain (Blocks splicing of XBP1) was published to have sensitized multiple myeloma to treatment and may be a potential therapeutic in this and other cancer types in the future (Mimura et al., 2012). There have also been major implications for the upregulation of GRP78 in cancer, where approximately ten different cancer types including lung, breast, colon and HCC had increased expression of GRP78 (Moenner et al., 2007). Interestingly, it is not just the typical ER localised GRP78 that may be important for cancer survival but also cell surface bound GRP78 which is thought to function as a surface receptor and has been detected on lymphoma, Leukaemia's, neuroblastoma's, colon adenocarcinomas and many other cancer cells (Ni et al., 2011). Several pro-tumourigenic molecules can bind to the GRP78 receptor and promote survival for example, α2-macroglobulin which activates ERK1/2, AKT and NFκB to enhance proliferation and has been reported to also amplify metastasis associated motility (Misra et al., 2006). Signalling through α2-macroglobulin: GRP78 has also been reported to promote metastasis associated motility via p21-activated kinase-2 (PAK-2) and LIM domain kinase-1(LIMK1)-mediated cofilin phosphorylation (Misra et al., 2005). PAK-2 is a regulator of cytoskeleton remodelling, is a known caspase substrate that when cleaved to its p34 fragment induces apoptosis through JNK (Bokoch, 1998), on the other hand, full length PAK-2 has been implicated in cancer survival as it can lead to BAD phosphorylation and inactivation (Kumar et al., 2006). LIMK1 is also involved in cytoskeleton remodelling and associated with metastsasis through modulation of cofilin phosphorylation (Scott and Olson, 2007). The oncogenic cell surface protein CRIPTO is another GRP78 binding partner and was shown to inhibit TGF-β to promote tumour development (Shani et al., 2008). Interestingly a unique isoform of GRP78 known as GRP78va which is localized only in the cytosol may also promote survival. GRP78va is a truncated version of the parent protein, that upon stress, intron1 of GRP78 is retained and thus the protein loses its ER-localisation signal peptide (Ni et al., 2011). Little is known about the interacting partners of this protein but it has been shown to bind to and inhibit p58(IPK) and thus block it from inhibiting PERK, thus the PERK adaptive responses are prolonged to promote survival (Ni et al., 2009). The role of PERK in cancer survival is somewhat controversial with some reports suggesting a pro-survival role e.g. PERK was shown to

reduce ROS (reactive oxygen species)-mediated DNA damage and initiate and enhance tumour growth where loss of PERK in mammary carcinoma cells caused accumulation of ROS and induction of a DNA damage checkpoint (Bobrovnikova-Marjon et al., 2010). Further to this, RAS-transformed tumours could not form subcutaneous transplants in the absence of PERK, again suggesting PERK promotes tumour formation (Blais et al., 2006). Other studies suggest anti-survival PERK signalling, where a dominant negative PERK overexpression in normal mammary epithelial cells caused development of neoplastic characteristics indicative of increased cell proliferation (Sequeira et al., 2007). Similarly, drug-inducible dimerization of PERK in squamous carcinoma T-Hep3 and colon carcinoma SW620 caused G0-G1 arrest to block proliferation (Ranganathan et al., 2008). Downstream of PERK, transcription factors ATF4 and NRF2 may also contribute to cancer progression.

ATF4 mediates oxidative homeostasis as a pro-survival mechanism downstream of PERK. Within the tumour microenvironment, cells are in a severe state of cellular stress often lacking important nutrients such as glucose, experiencing fluctuations in oxygen and pH levels and often hypoxic (Hanahan and Weinberg, 2011). ATF4 is often activated in these stressed cells within the tumour to enhance their ability to cope within such harsh conditions. In fact mouse embryonic fibroblast (MEF) cells lacking ATF4 are over-sensitive to hypoxia and mice lacking ATF4 were shown to be hypoglycaemic (Blais et al., 2004) (Seo et al., 2009). In primary embryonic fibroblasts transformed by co-expression of H-Ras and SV40, ATF4 was shown to have a vital role by repressing cyclin-dependant kinase inhibitor 2A (p16/cdkn2a) a known tumour suppressor to enhance proliferation and survival. The same cells lacking ATF4 resist cellular transformation and become senescent due to an increase in cdkn2a targets p16INK4A and p19ARF (Horiguchi et al., 2012). ATF4 expression is also associated with drug resistance in cancer cells such as cisplatin, doxorubicin, etoposide, SN-38 and Vincristine (Tanabe et al., 2003; Zhu et al., 2012b).

NRF2 signalling is thought as a double edged sword, with authors referring to the good and bad side to NRF2 signalling (Sporn and Liby, 2012). Generally NRF2 signalling is considered as an adaptive UPR response, activating pro-survival genes and aiding the cells recovery from stress, however, as with most pro-survival signalling pathways, hyperactivation can lead to enhance cell survival (Alam et al., 1999), in this instance by limiting ROS build up (Motohashi et al., 2010). In carcinomas of the lung, breast, gallbladder and liver, mutations in negative regulator of NRF2, KEAP1 were observed (Yoo et al., 2012) and not only

hyperactivate NRF2 (Konstantinopoulos et al., 2011) but mutations can cause aberrant binding between KEAP1 and other KEAP1 interacting proteins. Under normal circumstances KEAP1 acts as a platform for the Cul3-E3 ligase complex in which KEAP1 targets proteins including IKK (inhibitor of NF-κB kinase) and BCL2 for degradation (Tian et al., 2012). KEAP1 mutations can prevent complex formation leading to stabilization of IKK which activates pro-survival NF-κB (Lee et al., 2009) or stabilization of BCL2 which is free to block BAX activation and apoptosis (Niture and Jaiswal, 2010). Mutations in NRF2 itself has shown to enhance its activity to promote cancer e.g. skin, oesophagus, and lung cancers (Kim et al., 2010b; Shibata et al., 2008). Further to enhancing cancer progression, high NRF2 has also been associated with resistance to many chemotherapeutics including etoposide, doxorubicin, cisplatin and 5' fluorouracil (Wang et al., 2008a). In saying that, some reports have reported that NRF2 activation in cancers and other pathologies has preventative characteristics. Some chemotherapeutics are said to activate NRF2 to a beneficial end and many drugs that induce NRF2 such as sulphoraphane and curcumin (for the prevention of cancer), dimethyl fumarate (for the treatment of multiple sclerosis), bardoxolone methyl (for the treatment of diabetic nephropathy) and resveratrol (for multiple pathologies) are currently in clinical trial (Sporn and Liby, 2012).

Due to the ramifications of deregulated UPR signalling in cancer (and other diseases) much emphasis has been placed on the development of UPR inhibitors. More specifically, the development of inhibitors that specifically "shut off" one arm of the UPR without effecting the other two arms. For instance Mannkind Corporation has been developing several IRE1 inhibitors that appear to specifically bind to IRE1, and inhibit its RNase activity, for example they have developed a range of salicylaldehyde analogs. These compounds inhibited XBP-1 splicing and blocked transcriptional up-regulation of known XBP-1 targets as well as mRNAs targeted for degradation by IRE1 (Volkmann et al., 2011). A similar type of IRE1 inhibitor developed by Mannkind has shown promising effects in that treatment of multiple myeloma cells (Mimura et al., 2012). The mannkind IRE1 inhibitor used in this study MKC4485 is a new variant of the compound used in (Mimura et al., 2012) and has been shown to have more specificity for inhibition of the RNase activity of IRE1 and has no toxicity to cells used in study when applied alone. Another group screened for IRE1 inhibitors based on the fact that efficient RNase function of IRE1 is dependent upon autophosphorylation of the kinase domain and thus screened for known kinase inhibitors. This lead to the discovery of ATP-mimetic

compounds, which could inhibit expression of XBP-1s. In addition transcriptional targets of XBP-1s and phosphorylation of IRE1 were also negatively affected by these compounds. One particular compound (OICR000287A), was significantly more toxic to cells treated with ER stress (Griffen et al.,2012). However it must be noted that inhibition of both the kinase and the RNase activity of IRE1 may have unwanted consequences. Inhibitors of the PERK arm of the UPR are also being persued for therapeutic assessment. For example the most recently developed inhibitor produced by GlaxoSmithKline (GSK), GSK2656157 is an ATP-competitive inhibitor of PERK enzyme activity inhibits PERK activity by inhibiting ER stress-induced PERK autophosphorylation, eIF2α substrate phosphorylation, together with corresponding decreases in ATF4 and CHOP in multiple cell lines. Administration of GSK2656157 to mice caused the growth inhibition of multiple human tumor xenografts in mice. It also caused altered amino acid metabolism, decreased blood vessel density, and vascular perfusion which may be responsible for the observed antitumor effect (Atkins et al., 2013). However due to the contradictory evidence reported for deregulated PERK in cancer much caution would be required when using a PERK inhibitor in as a cancer therapy.

1.8 miRNAs in apoptosis and cancer

As mentioned, one way in which cancer develops is by evasion of apoptosis. miRNAs are major regulators in the biological processes of cell differentiation, growth, metabolism and apoptosis (Crippa et al., 2012; Lynam-Lennon et al., 2009; Pfaff et al., 2012; Zaravinos et al., 2012). Like other regulators of these processes, miRNA expression and functions can be altered to promote excess proliferation of cells and to disrupt apoptosis, ultimately resulting in cancer development and even tumourigenesis (Lynam-Lennon et al., 2009). More than 50% of known miRNA genes are located in fragile sites and regions of the genome that are linked to cancer development. Alterations to normal miRNA expression can cause the downregulation of target tumour suppressor genes, proapoptotic genes and/or the up-regulation of target oncogenes and pro-survival proteins, and thus are important in the development and progression of cancer (Zhang et al., 2007). The balance in the levels of BCL-2 family members is crucial in determining apoptotic signalling, and recent reports demonstrating their regulation by miRNAs represents a layer of regulation that is important in

cancer cell survival. BCL-2 itself is a target of miR-15/-16-1 and their downregulation can lead to increased BCL-2 levels. Interestingly, chronic lymphocytic leukaemia, which is characterised by the presence of non-dividing malignant B cells that overexpress BCL-2, also exhibits down-regulation of miR-15a/-16-1 cluster in 65% of cases (Calin et al., 2008). The BH3-only proteins are largely regulated by changes in expression and the role of miRNA implementing these changes is now coming to light. The miR-17-92 cluster has been shown to regulate apoptosis through its ability to repress BIM translation. BIM regulation by this cluster is critical in early B cell development where the miR-17-92 cluster promotes the survival of early B cell progenitors (Ventura et al., 2008). Conversely, upregulation of miR-17-92 has been linked to lymphoproliferative disease. Transgenic mice with a synthetic miR-17-92 transgene' develop greatly enlarged lymph nodes and spleens (Calin et al., 2008; Xiao et al., 2008). The miR-221/222 cluster, a group of miRNA often upregulated in cancers and linked to estrogen receptor status in breast cancer, where ERα negative cells which are highly resistant to therapy, have higher levels of miR-221/222 which enhances growth and survival (Di Leva et al., 2010; Zhao et al., 2008). These miRNA have been shown to downregulate BOPs PUMA and BIM, and also the tumour suppressor PTEN in glioblastoma, breast and gastric cancers respectively, to promote survival (Chun-zhi et al., 2010; Terasawa et al., 2009; Zhang et al., 2010). They have also been associated with enhanced resistance to extrinsic apoptosis, where overexpression in TRAIL-sensitive cells H460s, caused 40% increase in resistance to TRAIL. This resistance could be afforded to the miR-221/222-mediated repression of tumour suppressor p27kip (Garofalo et al., 2008). Repression of p27kip1 by these miRNA has also provided resistance to the chemotherapeutic tamoxifen in breast cancer cells (Miller et al., 2008). MCL-1 a pro-survival BCL-2 family member is often overexpressed in cancers and has been linked to TRAIL resistance. Interestingly miR-29 expression inversely correlates with MCL-1 i.e. while MCL-1 is highly expressed, miR-29 is downregulated. Forced expression of miR-29 reduced MCL-1 and sensitized cells to TRAIL (Mott et al., 2007). In neuronal maturation, it was shown that miR-29b can target multiple BH3-only proteins including BIM, PUMA, BMF, HRK and neuronal-BAK to prevent apoptosis. This may have implications in cancer were miR-29b is highly expressed (Kole et al., 2011). However the literature has shown that miR-29b is often downregulated in cancers such as CLL and multiple myeloma to enhance cell growth and survival and overexpression induces apoptosis (Amodio et al., 2012; Pekarsky et al., 2006; Zhang et al., 2011). miR-29b has also been shown to repress a variety of pro-survival molecules including MCL-1 and PI3K (Li et al.,

2012; Zhang et al., 2011), as such, its downregulation in cancer would result in increased expression of these pro-survival proteins to enhance cancer progression. Perhaps miR-29b repression of pro-apoptotic proteins is specific to neuronal maturation. Indirect regulation of BCL-2 family members by miRNAs also occurs, for instance, the miR-34a is induced by p53 (Tarasov et al., 2007), a tumour suppressor that is commonly mutated in human cancers (Frezza and Martins, 2012). In normal cells, miR-34a negatively regulates the expression of Silent information regulator 1 (SIRT1), a class III histone deacetylase that can catalyse the deacetylation of p53, thereby inactivating it. Thus, p53 increases miR-34a which leads to increased p53 (Yamakuchi and Lowenstein, 2009). This positive feedback loop could potentially cause increased expression of p53 target genes, which include BH3-only proteins, BAX, PUMA and NOXA (Gurzov et al., 2010; Oda et al., 2000; Zhan et al., 1994). Both intrinsic and extrinsic apoptosis pathways converge on the activation of caspases (Galluzzi et al., 2012). Although caspases are mainly regulated by cleavage, their intracellular concentrations also influence their ease of activation (Chang and Yang, 2000) thus miRNA-mediated silencing of these proteases can effect downstream death pathways. Caspase-9 is a direct target of miR-133, miR-24a and miR-21 in cardiomyocytes, the retina of the Xenopus eye and in glioma respectivly (Walker and Harland, 2009; Xu et al., 2007; Zhou et al., 2010). Caspase-3 is a target of miR-Let-7a, and by repressing caspase-3, miR-Let-7a renders cells resistant to several apoptosis inducing therapeutic drugs including doxorubicin, paclitaxel and interferon-γ (Tsang and Kwok, 2008). Caspase-3 can also be silenced by miR-21, where loss of miR-21 resulted in apoptosis of glioma cells via increased caspase-3 and -9 (Zhou et al., 2010). miRNAs may directly or indirectly regulate components of the extrinsic pathway to enhance tumour survival and resistance to TRAIL. For example, FasL downregulation by miR-21 downstream of AKT signalling represses apoptotic signalling (Sayed et al., 2010). Increased expression of certain miRNAs, such as miR-24, and miR-221/222, that can repress expression of target mRNAs, Fas associated factor-1 (FAF-1), and PTEN respectively (Chun-zhi et al., 2010; Qin et al., 2010), can lead to a reduction in apoptotic signals since FAF-1 would otherwise promote apoptosis (Chu et al., 1995) or PTEN would inhibit pro-survival pathways such PI3K/AKT (Weng et al., 2001). Conversely, a downregulation of miRNAs that target genes that block death, such as Fas-associated phosphatase 1 (FAP1)-targeting by miR-200c, MCL-1 repression by miR-29 and c-FLIP downregulation by miR-512-3p, leads to an increased resistance to cell death (Chen et al., 2010; Mott et al., 2007; Schickel et al., 2010).

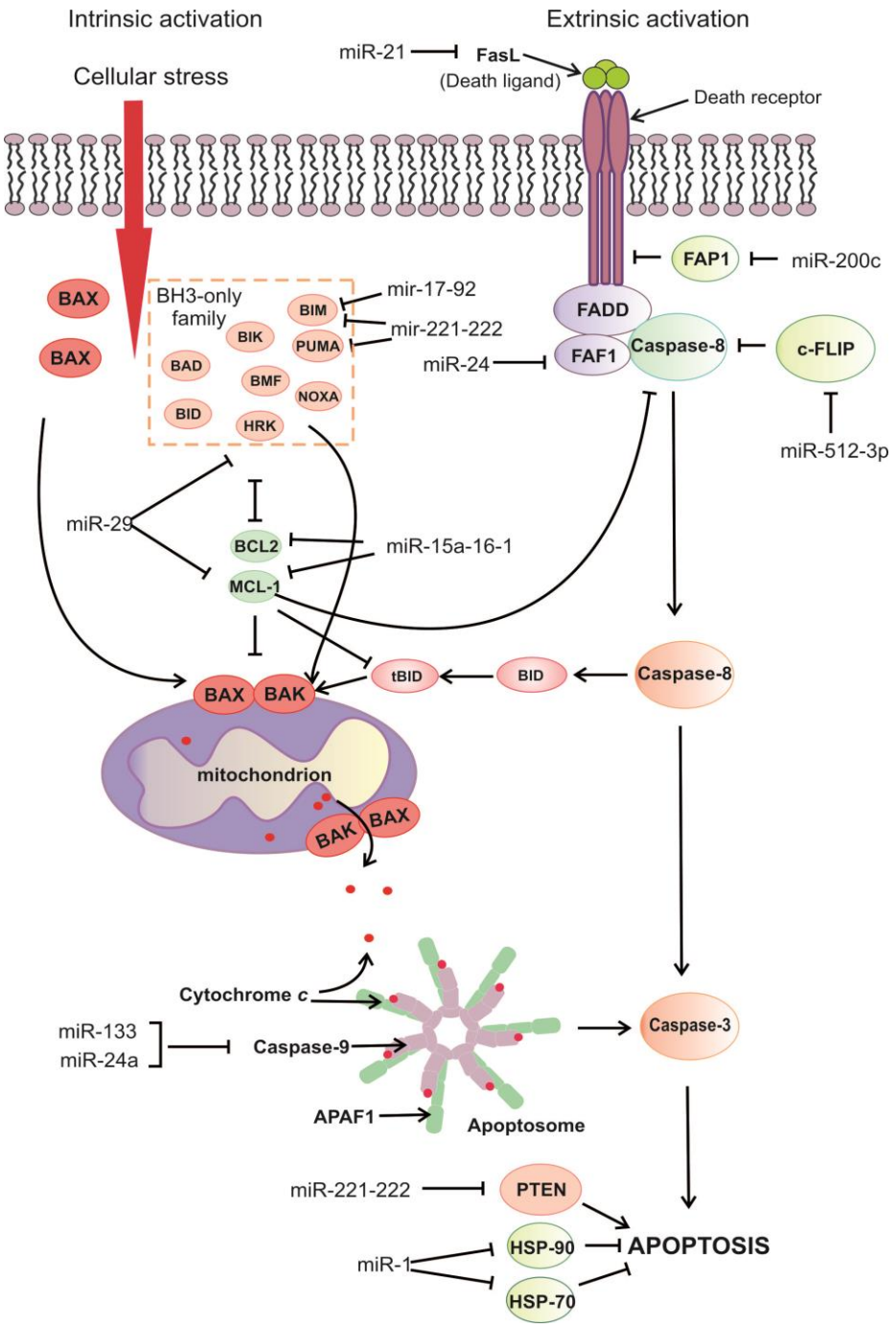

Figure 1.7 miRNAs in apoptosis and cancer

The above depicts the various miRNAs that modulate the expression of pro-apoptotic or pro-survival proteins to by-bass apoptosis and promote cancer. During intrinsic apoptosis, miR-29 can target multiple BOPs while BIM is silenced by both miR-17-92 and miR-221/222. Puma and tumor suppressor PTEN are also targets of miR-221/222. Pro-survival BCL-2 and MCL-1 are targets of miR-15a-16-1. Caspase-9 can be silenced by miR-133 or miR-24a. Hsp-90 and -70 can be silenced by miR-1. During Extrinsic apoptosis, FasL and FAF1 are targets of miR-21 and miR-24 respectively. FAP1 and c-FLIP are targets of miR-200c and miR-512-3p respectively.

1.9 miRNA Biogenesis and cancer

It must be noted that there is a global loss of miRNA observed in most cancers and some have suggested that most miRNA act as tumour suppressors. More recently profiling of proteins associated with miRNA biogenesis such as DICER, DROSHA, Exportin 5, TARBP2 and AGO-2 has been carried out in a variety of cancers and are aberrantly expressed compared to normal tissue, often promoting cancer development. For instance lower DICER expression has been observed in cancers of different origin, e.g. colon, lung, breast, ovarian and endometrial (Dedes et al., 2011; Faggad et al., 2012; Karube et al., 2005; Merritt et al., 2008; Torres et al., 2011) and 27% of tumours tested showed deletions in the gene encoding DICER and is associated with increased tumourigenesis and poor patient prognosis (Farazi et al., 2011). One study actually independently knocked down DICER, DROSHA and DGBR8 in mouse adenocarcinoma cell line LKR13 and showed these cells were more tumourigenic and had increased expression of oncogenic genes K-, N- , H-Ras, c-Myc, and E2F1. DICER knockdown cells also formed larger tumours in vivo as seen with K-Ras driven lung cancer (Kumar et al., 2007) and Rb-driven retinoblastoma (Lambertz et al., 2010). DICER has also been reported to act as a haploinsufficent tumour suppressor, where deletion of a single allele of DICER promotes tumour development (Kumar et al., 2009; Lambertz et al., 2010). Loss of DICER has also been linked to cancer development where, the anti-diabetic agent Metformin was shown to have anti-cancer effects, by increasing DICER mRNA and protein via E2F transcription factors, along with the repression of oncogenic c-MYC via miR-33a (Blandino et al., 2012). Several components of the miRNA biogenesis pathway were reported to undergo inactivating mutations that reduce miRNA processing and enhance tumourigenesis, e.g., XPO5 the gene encoding Exportin 5 which transports miRNA from the nucleus to the cytoplasm was reported to undergo inactivating mutations that trap miRNAs in the nucleus and formed tumours rapidly in nude mice compared to WT cells (Melo et al., 2010). TARBP2 a DICER interacting protein, that stabilizes DICER and enhances miRNA processing (Daniels et al., 2009) also undergoes truncating mutations which reduce TARBP2 protein expression cause DICER protein destabilization, compromised pre-miRNA levels and increased viability and tumour development (Melo et al., 2009). TARBP2 also seems to be a focal point in survival or death promoting effects of the miRNA biogenesis pathway. ERK mediated phosphorylation of TARBP2 increases processing of a subset of oncogenic pre-miRNA such

as pre-miR-17, -20a and -92a and decreased processing of tumour suppressor like pre-miR-let7 (Paroo et al., 2009). Somatic mutations in AGO2 causing loss of expression have been detected in gastric and colorectal cancers where high microsatellite instabilities occur and have been linked to cancer progression (Kim et al., 2010a). In fact, low AGO2 expression was observed in Lung adenocarcinoma compared to normal tissue and overexpression of AGO2 in lung adenocarcinoma cell line H1299 decreased proliferation and tumour development in nude mice (Zhang et al., 2012). However, other studies have shown that loss of AGO2 induces apoptosis of myeloid leukaemia cells (Naoghare et al., 2011). It has also been reported that AGO2 is highly expressed in aggressive cancers for instance, in ERα negative breast cells cell lines and tumours, which as mentioned earlier are resistant to most therapies, AGO2 is highly expressed via epidermal growth factor receptor (EGFR) and Mitogen-activated protein kinase (MAPK) signalling (Adams et al., 2009).

1.10 miRNAs in ER stress and cancer

The way in which cells cope with stress is generally referred to as cellular stress responses. There are a variety of factors that cause the cell to become stressed. Maintaining the cells ideal environmental conditions is vital for normal cell functioning and survival however perturbations in such conditions can lead to cell stress and if stress persists, apoptosis (Fulda et al., 2010; Leung and Sharp, 2007). ER stress is one such response. As previously described ER stress induces the UPR response which initially tries to de-stress the cell and allow for recovery and if unsuccessful and stress is too severe signalling switches to promote apoptosis (Szegezdi et al., 2006). In any stress response the cell responds by altering the levels of various proteins via the regulation of gene expression. The fact that miRNA can regulate gene expression at the mRNA level makes them, vital components of cell stress responses. If miRNA transcription is up regulated a corresponding mRNA target will be degraded or translationally blocked, this may be necessary when a cell stress response pathway needs to inhibit a particular protein in order to cope with stress while if miRNA transcription is decreased, mRNA levels will increase and thus protein levels will increase or vice versa (Babar et al., 2008). The exact role miRNA may play during ER stress or indeed if miRNA are regulated by the transcriptional programs of the UPR is not fully elucidated, however miRNA have been shown to be major players in other cellular stress responses

where they can augment the switch between survival and death of the cell (Babar et al., 2008). From what has been reported regarding the role of miRNA during ER stress, it seems that miRNA can fine tune the balance between cell death and survival.

For example, miR-221/222 as discussed is associated with enhanced tumourigenesis and resistance to cancer therapies via downregulation of tumour suppressor $p27^{kip1}$ (Galardi et al., 2007). However, in HCC cells subjected to ER stress, miR-221/222 were downregulated and $p27^{kip1}$ increase was shown to enhance resistance to ER stress induced apoptosis by inducing G1 phase arrest in the cell cycle (Dai et al., 2010). Perhaps blocking apoptosis allows for prolonged adaptive UPR responses. The miRNA, miR-205 is downregulated in both oxidative and ER stress responses and increases sensitivity to both stresses with increased ROS and decreased expression of anti-oxidant response genes. The protein prolyl hydroxylase 1 (PHD1), a controller of ROS levels and involved in oxidative-ER stress crosstalk is downregulated by miR-205 acting to protect cells from oxidative and/ or ER stress induced cell death (Muratsu-Ikeda et al., 2012). This suggests that miR-205 could potentially be deregulated in cancers to reduce oxidative or ER stress to promote cell survival. It has already been reported that miR-205 is highly expressed in cervical cancer tissues and promotes growth and migration through downregulation of CCN (Cyr61 (cysteine-rich protein 61), CTGF (connective tissue growth factor), Nov (nephroblastoma overexpressed)) proteins, CYR61 and CTGF (Xie et al., 2012) which are known for their role in cell proliferation, angiogenesis and tumourigeneis (Brigstock, 2003).

miRNAs are not just regulated by ER stress but can themselves regulate various components of the ER stress response. For example, ATF4 is downregulated by miR-214 leading to inhibition of bone formation (Wang et al., 2013). Additionally, the transmembrane transcription factor OASIS is repressed by miR-140 resulting in increased expression of extracellular matrix encoding genes in pancreatic beta cells subjected to stress (Vellenki et al., 2012). More recently, IRE1 has been shown to be targeted by miR-1291 resulting in increased expression of oncogenic Glypican-3 (Maurel et al., 2013).

1.10.1 PERK-regulated miRNAs:

During conditions of ER stress, miR-211 is upregulated in a PERK-dependant manner to downregulate CHOP. Atypically, miR-211 binds to the 5' UTR of CHOP, promotes silence-inducing histone methylation and subsequent repression of CHOP (Chitnis et al., 2012). This reveals a new level to the adaptive responses of PERK, where not only is cap-dependant translation blocked but pro-apoptotic protein CHOP induction is blocked to allow cells some time to manage unfolded proteins and recover from stress. As mentioned above, PERK signalling has been reported to promote tumourigenesis, it could be possible that upregulation of miR-211 contribute to this process. In fact, miR-211 was shown to promote tumourigenesis in colon cancer cells by orchestrating the CpG island hypermethylation of the CHD5 (chromodomain helicase DNA binding protein 5) promoter (Cai et al., 2012). CHD5 is a known tumour suppressor often deleted in cancers due to its location on 1p36.3 which has been reported to be frequently deleted in cancers e.g. breast, and neuroblastoma (Bagchi et al., 2007; Fujita et al., 2008; Wu et al., 2012b). Promotor hypermethylation of CHD5 was also reported in Lung and gastric carcinomas but was not linked to miR-211, but it is possible miR-211 is responsible in these cases (Wang et al., 2009; Zhao et al., 2012). It must be noted that miR-211 may not always promote tumourigenesis. miR-211 is encoded within an intron of host gene melastatin which is considered a tumour suppressor and is often repressed in melanomas as is miR-211. As expected, forced expression of miR-211 reduced metastatic ability and tumour growth of melanoma (Levy et al., 2010). PERK also causes, miR-30c-2* upregulation during ER stress and downregulates XBP1 (Byrd et al., 2012). This mechanism may act as a switch between the adaptive and apoptotic responses of PERK signalling by which PERK lowers XBP1 mRNA, therefore less pro-survival XBP1 splicing would occur. Downstream of PERK, pro-apoptotic CHOP has been shown to regulate miR-708 in the brain and eyes of mice, where it inhibits neuronatin (NNAT) (Ryu et al., 2013) and rhodopsin (RHO) (Behrman et al., 2011) respectively. By inhibiting NNAT, intracellular calcium levels are disrupted causing metastasis in brain cancer. While inhibition of RHO, is likely to prevent excessive production of the protein to enter the ER.

1.10.2 ATF6-regulated miRNAs

It was reported from miRNA array analysis of RNA from the hearts of ATF6 transgenic (TG) mice that there are 13 ATF6-regulated miRNAs. It is well known that ATF6 is upregulated during ischemia as protective mechanism to limit damage to the heart. Interestingly, in the same study, ATF6 was shown to downregulate miR-455 which would otherwise repress Calreticulin. In absence of this miRNA, there is an increase in Calreticulin in the pathologic heart and subsequent decrease of hypertrophic growth (Belmont et a., 2012).

1.10.3 IRE1-regulated miRNAs

As previously discussed XBP1s is highly expressed and promotes cancer survival (Bagratuni et al., 2010). More recently it has been shown in HCC tissues; the miR-199a/214 cluster is downregulated resulting in increased expression of XBP1s and enhanced cell proliferation and tumour formation. The same study showed this cluster was downregulated in response to ER stress treatment via pro-survival NF-*κ*B signalling and may be a component of the adaptive UPR responses (Duan et al., 2012). Certain miRNAs (premiRs -17, -34a, -96, and -125b) have recently been shown to be cleaved by IRE1 causing derepression of caspase-2 and apoptosis (Upton et al., 2012). In addition to this, IRE1–mediated destabilization of miR-17 was shown to increase *TXNIP* mRNA stability. In turn, elevated TXNIP protein leads to the activation of the NLRP3 inflammasome, causing procaspase-1 cleavage and interleukin 1β (IL-1β) secretion, thereby increasing systemic or local inflammatory response (Lerner et al., 2012). Little is known about IRE1s RIDD function (Hetz, 2012; Hollien et al., 2009) or what determines if IRE1s RNase domain cleaves XBP1 as a pro-survival mechanism or if it degrades mRNA/ miRNA as a pro-apoptotic mechanism but perhaps this switch is deregulated in cancer to deter RIDD and enhance XBP1 splicing. Perhaps XBP1 is a more favourable substrate for IRE1 and only when it is no longer abundant, and RIDD substrates are available this switch occurs.

1.11 Summary

This thesis details the investigation of miRNAs and their role in ER stress induced cell death and also shows that loss of global miRNAs confers resistance to ER stress induced cell death in colon cancer cells. As such this introduction has discussed the molecular and biological features of apoptosis, ER stress and the UPR response that is activated to deal with this stress and the mechanisms of miRNA biogenesis and function. Particular focus has been given to how these pathways can be deregulated in cancer individually and simultaneously to help cells evade apoptosis and enhance cell growth, and tumourigenesis.

1.12 Rationale and objective

The work described hereafter began as a consequence of the lack of evidence supporting a role for miRNAs during ER stress-induced cell death. The first indications that miRNAs may be important in the ER stress response came from the reports showing that during conditions of cellular stress, including, ER stress, there is an increased accumulation of miRNA-AGO2-p-body foci formation (Read et al.,2012; Parker et al., 2007)). Additionally it was shown that the miRNA biogenesis machinery is located on the ER. For instance, DICER was shown to co-localize with calreticulin at the ER surface (Provost et al., 2002) and more recently, it was shown that cytoskeleton-linking endoplasmic reticulum (ER) membrane protein of 63 kDa (CLIMP-63) is a novel Dicer-interacting protein. CLIMP-63 interacts with Dicer to form a high molecular weight complex. Rapidly following its synthesis, DICER binds with a portion of the luminal domain of CLIMP-63, transits through the ER, is glycosylated and can be secreted by cultured human cells with CLIMP-63. This interaction is thought to regulate DICER function (Pepin et al., 2012). Thus the rationale for investigating miRNAs in ER stress arose, where the potential for miRNAs as important regulators of ER stress and the UPR was hypothesised. It was considered that, as regulators of gene expression, miRNAs could potentially decide cell fate in response to ER stress by "tipping" the balance between adaptive and apoptotic signalling events.

Objective:

To investigate the importance of miRNAs in ER stress induced cell death and to determine if indeed they may modulate cell fate decisions during this response.

CHAPTER 2

MATERIALS AND METHODS

2.0 Materials and Methods

2.1 Cell culture

All cells described in this section are adherent and so grow while attached to the flask or dish surface.

HCT116, and RKO, WT and DICER Exn5/Exn5 cells were a kind gift from Bert Vogelstein and Victor E. Velculescu (Cummins et al., 2006). These cells are colorectoral carcinoma with epithelial morphology. They were maintained in T75 flasks in McCoys 5A modified medium with 10% heat inactivated fetal bovine serum (FBS) and 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma) with 5% CO2 at 37 ^{o}C. These were used as they had pre-designed loss of DICER allowing for quick and accurate analysis of the effects of ER stress on cells with global loss of miRNAs.

MCF7 cells were obtained from ATCC and are estrogen receptor positive Breast adenocarcinoma with epithelial morphology. They were maintained in Dulbecco's modified eagles medium (DMEM) with 10% heat inactivated fetal bovine serum (FBS) and 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma) with 5% CO2 at 37 ^{o}C.

When the cells had grown to approximately 80% confluency, they were trypsinised using 2mls 1X Trypsin/ EDTA in HANKS balanced salt solution at 37^{o}C for 3 minutes to remove them from their culture flask. Cells were collected in 10mls of medium and mixed until a single cell suspension was formed. Of this suspension, 10 µl was added to one chamber on a Haemocytometer to count and estimate how many cells were present per ml.

HEK 293t cells were obtained from ATCC and are derived from human embryonic kidney. They were maintained in Dulbecco's modified eagles medium (DMEM) with 10% heat inactivated fetal bovine serum (FBS) and 50 U/ml penicillin and 50 mg/ml Streptomycin (Sigma) with 5% CO2 at 37 ^{o}C.

When the cells had grown to approximately 80% confluency, they were trypsinised using 2mls 1X Trypsin/ EDTA in HANKS balanced salt solution at 37^{o}C for 3 minutes to remove them from their culture flask. Cells were collected in 10 ml of medium and mixed until a single cell suspension was formed. Of this suspension, 10 µl was added to one chamber on a Haemocytometer to count and estimate how many cells were present per ml.

2.2 Drug treatments

The pharmacological inducers of ER stress, Thapsigargin (Tg), Tunicamycin (Tm) and Brefeldin A (Bfa) were used in this study to investigate the role of miRNA during ER stress induced cell death.

Thapsigargin is derived from the plant *Thapsia garganica. Linnaeus*, a weed found on the Mediterranean. It is a well-established inhibitor of ubiquitously expressed sarco-endoplasmic reticulum Ca2+-ATPases (SERCAs). By blocking SERCAs, Tg causes release of Ca2+ from ER calcium stores and also prevents the re-uptake of Ca2+ into these stores resulting in an increased cytosolic Ca2+ load. Loss of Ca2+ from the ER can affect the proper functioning of some ER resident proteins but more importantly Ca2+ overload of the cytosol results in apoptosis (Treiman et al., 1998).

Tunicamycin (Tm) is an antiviral antibiotic derived from *Streptomyces lysisuperificus*. It inducers ER stress by blocking DPAGT1, a protein involved in the first step of N-linked glygosylation of proteins. Proteins that are not glycosylated are not carried out of the ER and so many misfolded proteins accumulate within the ER lumen incurring stress and subsequent activation of the UPR (Heifetz et al., 1979; Takatsuki et al., 1971).

Brefeldin A (Bfa) also referred to a Cyanein and Decumbin although less regularly, is produced by some fungi such as Penicillium, Penicillium Brefeldianium, and Penicillium cyanium. One of the most recognised effects of Bfa is the dismantling of the trans-golgi network. Bfa causes dissociation of coat protein from the golgi and tran-golgi membrane and subsequent break down of the trans-golgi subcompartments which then fuse to the ER. Thereafter, transport of proteins from the ER to golgi is blocked thus proteins accumulate in the lumen of the ER.

Etoposide (Etop) is a podophyllotoxin, a type of plant alkaloid. It inhibits topoisomerase II an enzyme required for proper winding of DNA strands and as such can effect DNA replication and induce cell cycle arrest and apoptosis.

Staurosporin (Sts) is a microbial alkaloid that functions as a broad range protein kinase inhibitor e.g. Sts can inhibit Protein kinase C (PKC), myosin light chain (MLC) kinase and tyrosine kinases. Due to the non-specific effects of Sts on kinases, many proteins involved in cell cycle, proliferation and pro-survival signalling may not be activated via phosphorylation resulting in apoptosis (Omura et al., 1995). It can also inhibit mTOR-associated translation factors such as p70 S6 kinase and 4E-BP1 and causes increase eIF2α

phosphorylation due to PKC inhibition to bring about apoptosis (Tee and Proud, 2001).

Cells were either seeded to 6 well plates or T25 flasks with $0.4x10^6$ cells or $2x10^6$ cells respectively, depending on the experiment and quantity of cells required. The following day cells were treated with 500 ng/ml Bfa, 500 ng/ml Tm, 200 nM Sts, or 100 μM Etop for the indicated time periods. All reagents were from Sigma-Aldrich unless otherwise stated.

2.3 Transformation of Plasmids

Transformation is a technique used to introduce foreign DNA in the form of a plasmid into competent bacterial cells. For the generation of all plasmids, DH5α E. coli cells were used. Plasmid DNA was combined with the cells and incubated on ice for 30 min. The cold temperature aids the permeabilisation of the cells to ready them for taking up DNA. The mixture was then heat shocked at 42 ^oC for 50 s. This results in the movement of a thermal gradiant across the cell membrane which draws the DNA across with it. Cells are quickly placed on ice for 2 min and combined with 500 μl of LB Broth. Cells are allowed to recover and start to grow at 37 ^oC for 1 hour. The cells were spread on LB agar plate containing the correct antibiotic and grown overnight at 37 ^oC. The next day, bacteria from a single colony, was further inoculated into LB broth plus antibiotic. Again this was incubated overnight at 37 ^oC while shaking. The resulting culture was centrifuged at 5000 g for 20 min to pellet the bacterial cells containing the plasmid of interest. Plasmid extraction was carried out using Qiagen maxi-prep kit (Cat. No. 12163).

2.4 Transfections

This is a method used to transport DNA or a gene of interest in the form of a plasmid, into the cell. There are many reagents and methods used and all are designed to allow DNA to pass through the negatively charged cell membrane. Often cell lines respond differently to different transfection reagents or methods with one giving higher transfection efficiency than another.

For the transfection of MCF7 cells, Turbofect (Fermentas) was used. It is a cationic polymer based transfection reagent that forms positively charged complexes with DNA to allow efficient delivery of DNA into cells. Cells were seeded at the appropriate density and 24 h later media was changed to DMEM with FBS minus pen/strep and then transfected with the plasmid of interest in a, 1:2 ratio of DNA to Turbofect. In other words, 1ug of DNA was mixed with 50 µl of DMEM and 2 µl of Turbofect was added to 50 µl DMEM and incubated for 5 mins at room temperature. The Turbofect mixture was added to the DNA mixture and pipetted gently to mix. This was incubated at for 20 mins at room temperature to allow formation of DNA:Turbofect complexes. The complexes were added drop by drop to the well of cells and mixed gently by swirling the plate, and incubated for 4 h at 37 ^{o}C. Media was changed and 24 h later cells were harvested for experiments.

For the transfection of 293t cells, JetPEI (PolyPlus) was used. It is another polymer based transfection reagent that gives high transfection efficiency for these cells. Cells were seeded at the appropriate density and 24 h later media was changed to DMEM with FBS minus pen/strep and then transfected with the plasmid of interest in a, 1:2 ratio of DNA to JetPEI. In other words, 1ug of DNA was mixed with 50 µl of 150 mM NaCL and 2 µl of JetPEI was added to 50 µl 150 mM NaCL and incubated for 5 mins at room temperature. The JetPEI mixture was added to the DNA mixture and pipetted gently to mix. This was incubated at for 20 minutes at room temperature to allow formation of DNA:JetPEI complexes. The complexes were added drop by drop to the well of cells and mixed gently by swirling the plate, and incubated for 4 h at 37 ^{o}C. Media was changed and 24 h later cells were harvested for experiments.

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2.5 Lentivirus generation

Lentiviruses are a more complex type of retrovirus, with the ability to infect dividing and non-dividing cells. Lentiviral gene transfer vectors used in this study are HIV based. For generation of all lentivirus used here, the 2nd generation system from Addgene was used. Viral auxilliary genes such as vpr, vif, vpu and nef are not included in this system making it safer to handle. pTIG-U6tetO-shDR-Drosha shRNA a gift received from John Rossi (Aagaard et al., 2007), pSicoR-Empty Vector, pSicoR-Dicer shRNA, pTRIBZ-Keap 1 shRNAs were co-transfected into HEK 292t cells along with the packaging plasmids from Addgene using JetPEI tranfection method as described in the section 2.4 Transfections. pMD2.G Cat#12259 which is the envelope plasmid required for the production of viral particles, psPAX2 Cat#12260 provides the CAG promoter which enhances expression of packaging proteins, pRSV-Rev Cat#12253 provides Rev which is needed for export of viral RNA. 24 h post transfection the supernatant (containing lentivirus) of the transfected cells was harvested and replaced with 15ml of fresh growth medium. The second round of lentivirus harvest was carried out 24 h later. The supernatant containing the lentivirus was filtered through a 0.45 nm Nalgene filter to remove any cells. The virus can be used immediately for transduction of cells, stored at 4 °C for 1 week or aliquoted into cryotubes and frozen in -80 °C.

2.6 Lentivirus transduction

The advantage of lentivirius production to create stable overexpressing or knockdown cell lines was utilized for this study. This is powerful tool for monitoring protein function. Cells were seeded in 6 well plates at ~ 40% confluency. The next day cells were tranduced with 1.5 mls of lentivirus per well. The plates were then centrifuged for 90 min at 1500rpm (400g) at room temperature. Following centrifugation cells were placed directly into the incubator and left overnight. The next day, virus was removed and replaced with fresh complete medium. HCT116 WT cells were transduced with pTIG-U6tetO-shDR-Drosha shRNA to generate stable DROSHA knockdown cell lines. Once transduced, cells underwent cell sorting to isolate DROSHA knockdown cells from cells in the population that may not have been transduced. This was done based on GFP positivity by gating out cells that were GFP negative. HCT116 WT cells were transduced with pSicoR-Empty Vector and pSicoR-Dicer shRNA (Addgene 14763_Tyler Jacks lab). These cells were then selected with, 3 µg/ml of puromycin to enrich for DICER knockdown cells. Cells were further maintained in 500 ng/ml of puromycin.

2.7 Annexin V staining

In the beginning stages of apoptosis, the phospholipid phosphotidylserine (PS) is translocated from the inner side of the plasma membrane to the outside without compromising the membranes integrity. Annexin V is vascular protein known to have strong binding affinity for phospholipids like PS but not for other phospholipids abundant on the plasma membrane such as phosphatidyl choline or sphingomyeline. This makes the expression, purification and use of fluorescein isothiocyanate (FITC) conjugated recombinant Annexin V an excellent tool for analysis of apoptotic cells. It must be noted that late apoptotic cells or early necrotic cells will have a permeabilized outer membrane and will also become Annexin V positive. To distinguish between early and late apoptotic cells a dye that only enters permeabilized cells is used along with Annexin V. In my experiments I have used Propidium Iodide (PI), a DNA intercalating dye that fluoresces in the red channel (PE). After treatment, floating cells were

collected and remaining cells were trypsinised, combined with floating cells and allowed to recover for 10 minutes at 37°C. The cells were collected by centrifugation at 5000g for 5 min, washed once in ice-cold calcium buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$), and incubated with Annexin V-FITC for 15 min on ice. Prior to analysis 300 µl of binding buffer containing 4 µl of PI (50 µg/ml) was added and analyzed on a FACSCanto flow cytometer.

2.8 Measurement of ΔΨm by TMRE staining

Mitochondrial membrane potential $\Delta\Psi_m$ is essentially related to the pumping of hydrogen ions across the inner membrane during ATP generation, a process involving electron transport and oxidative phosphorylation. Mitochondrial membrane potential was determined by using the fluorescent probe tetramethylrhodamine ethyl ester (TMRE, Molecular Probes). TMRE is a Red/orange dye easily detected by flow cytometry in the PE channel. It permeates the plasma membrane and due to its positive charge accumulates in the highly negative mitochondrial membrane. Depolarized mitochondria thus exclude the dye, and in doing so provide an efficient, and straight forward assay for measuring the loss of $\Delta\Psi_m$ during Apoptosis. As a ppositive control for this assay I used the protonophore Carbonyl Cyanide m-chlorophenyl hydrozone (CCCP) which uncouples the electron transport chain from ATP generation. It enters the mitochondria in its protonated form, alters the pH gradient, and quickly leaves again in its anionic form, destroying the membrane potential. This can occur repeatedly. CCCP treated cells thus show loss of TMRE positivity. After treatment, floating cells were collected and remaining cells were trypsinised, combined with floating cells in medium and incubated with TMRE at room temperature for 30 minutes in the dark and analyzed by flow cytometry in the PE channel using a FACScanto flow cytometer.

2.9 Analysis of DEVDase Activity

Caspase-3 and Caspase-7 show specificity for cleavage at the C-terminal side of the aspartate residue of the sequence DEVD (Asp-Glu-Val-Asp). As these caspases are active during apoptosis and lead to the demise of the cells, an assay such as this is a good readout for cells undergoing apoptosis. Cells were harvested and pelleted by centrifugation at 350 g. After washing in PBS, cell pellets were re-suspended in 50 µl of PBS and 25 µl was transferred to duplicate wells of a microtiter plate and snap-frozen in liquid nitrogen. To initiate the reaction, 50 µM of the caspase substrate carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-m ethyl-coumarin (DEVD-AMC, Peptide Institute Inc.) in assay buffer (100 mM HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS, 5 mM DTT and 0.0001% Igepal-630, pH 7.25) was added to cell lysates. Liberated free AMC was measured by a Wallac Victor 1420 Multilabel counter (Perkin Elmer Life Sciences) using 355 nm excitation and 460 nm emission wavelengths at 37°C at 60 s intervals for 25 cycles. The data were analyzed by linear regression and enzyme activity was expressed as nM of AMC released × min^{-1}×mg^{-1} total cellular protein.

2.10 RNA preparation

RNA can be utilised to determine changes in gene expression and can be an indicator of a proteins importance in a particular signalling pathway. The main objective of isolating RNA in my studies was to determine changes in gene expression in response to loss of mature miRNA during ER stress. RNA is converted to cDNA which can then be analysed by QRT-PCR.

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 minutes. 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 minutes at room temperature. Samples were centrifuged at 12,000g for 15 minutes. The upper clear phase was carefully pipette into freshly autoclaved 1.5 ml tubes, with special care taken not to disturb the interface. To precipitate the RNA, 250 µl of isopropanol was added to each sample

and incubated at -20 °C overnight. Samples were then centrifuged at 12,000g for 10 minutes 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.

2.11 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 minutes 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°C for 2 minutes, and then 25°C for 2 minutes. 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°C for 10 minutes followed by 50°C for 50 minutes, and finally inactivated at 85°C for 10 minutes. Conventional PCR for human GAPDH was carried out using 2 µl of cDNA to check cDNA quality with 2x Go Taq mastermix (Promega). Primers to amplify GAPDH:

GAPDH Fwd 5' ACCACAGTCCATGCCATC 3' and
GAPDH Rev 5' TCCACCCTGTTGCTG 3'.

PCR cycle conditions: 94 °C for 3 minutes, 94 °C for 30s, 55 °C for 30 s, 72 °C for 30 s, 72 °C for 7 minutes, 4 °C for ∞. Repeat steps 2-4 for 25 cycles. Conventional PCR for human DICER was carried out on cDNA using 2x Go Taq mastermix and with the following primers:

DICER Fwd 5'TCAAACCTAGAAGTAAATGCAT 3' and
DICER Rev 5' TCTTCCAATTCCTCTGGATCAC 3'.

PCR cycle conditions: 94 °C for 3 min, 94 °C for 30s, 55 °C for 30 s, 72 °C for 30 s, 72 °C for 7 mins, 4 °C for ∞. Repeat steps 2-4 for 40 cycles.

2.12 miRNA specific RT-PCR

Due to their small size, miRNA are not amplified by conventional reverse transcription using oligo dT or random primers. Instead Applied Biosystems have designed specialized primers that individually recognise each miRNA separately. The beauty of these primers is their stem loop structure which binds to the single stranded mature miRNA with higher affinity then a conventional primer. This is because, miRNA precursor structure originally possess a hairpin. As such a single reaction for each miRNA and each RNA samples must be set up. To start, RNA was diluted to 100 ng/ul and reverse transcribed into cDNA using miRNA specific primers and a miRNA reverse transcription kit (Applied Biosystems). PCR conditions: 16 ^{o}C for 30 mins, 42 ^{o}C for 30 mins and 85 ^{o}C for 5 mins.

2.13 Microarray

Total RNA was isolated from HCT116 WT and Exn5/Exn5 cells treated with 500 ng/ml of Bfa or 500 ng/ml of Tm for 24 hours. RNA was prepared for analysis by DNA vision as previously described. Briefly, RNA was reverse transcribed into cDNA which is in vitro transcribed into cRNAs in the presence of biotinylated ribonucleotides which are then fragmented. Each labelled cRNA target is hybridized onto a separate chip. Arrays consist of a set of multiple 25-mer oligonucleotide probe pairs for each gene which are 3' biased. After hybridization, arrays are stained with phycoeerythrin-conjugated streptavivdin, washed and scanned. Gene expression profiling was performed by DNA Vision (Charleroi, Belgium, EU, using affymetrix array technology. http://www.dnavision.com. For clustering of normalized results, the raw data have been normalized together using the RMA algorithm, with principal component analysis using all the 54675 probe sets of the GeneChips after RMA normalization. The groups of interest have been compared using moderated t-test where p-values of less than or equal to 0.05 have been considered significant. The data shown has been represented as a heatmap (Figure 3.2) and as line graphs (Figure 3.10).

2.14 miRNA microarray

Total RNA was isolated from H9c2 cells treated with thapsigargin or tunicamycin for 24 hours. MicroRNA expression profiling was performed by LC Sciences (Houston, TX, USA, http://www.lcsciences.com) using a custom µ-Paraflo array containing probes for 350 rat miRNAs from Sanger miRBase. Data was represented by histogram graphs (Figure 4.1) where fold change in miRNA expression in H9c2 cells upon treatment with Tg and Tm was compared to that of H9c2 cells under untreated conditions. Fold change was calculated from mean fluorescent signal intensities of three independent repeats. P-values representing the significance when comparing treated and untreated samples were also calculated and only samples with p-values less than or equal to 0.05 were considered significant.

2.15 Real time Quantitative RT-PCR

Real time QRT PCR is simple and yet quantitative way of determining differences in gene expression between samples. There are two types of chemistry used for QRT-PCR Syber green and Taqman. For the majority of my analysis I used Taqman chemistry to avoid the limitations of syber green chemistry. Mainly, Taqman allows for the avoidance of non-specific amplification during PCR and does not require primer optimization steps. PCR using Taqman chemistry also referred to as the 5' nuclease assay enlists the participation of a forward and reverse primer designed to amplify the target of interest and a MGB (Minor groove binder) probe which hybridizes to the target between the two primers. It is this probe that allows for Real time detection of product amplification. The probe has a fluorescent reporter dye attached to its 5' end and the MGB and Non Fluorescent Quencher (NFQ) dye tagged at its 3' end. As DNA polymerase ampliys the target it begins to cleave the probe separating the fluorescent dye from the NFQ allowing realease of a fluorescent signal detected by the QRT-PCR machine. For QRT-PCR, cDNA is synthesised and diluted to a concentration of 40 ng per 10 µl reaction; it is then combined with Brilliant III Ultra-Fast QPCR Master Mix (Agilent) and 20X Taqman gene expression assays (Applied Biosystems and IDT) and accurately dispensed to a fast optical MicroAmp 96-well plate (Applied Biosystems) in triplicate The PCR is run for 40 cycles on an Applied Biosystems fast 7500 machine using the following cycling

conditions: Hold at 95 ^{o}C for 3 minutes, then 40 cycles at 95 ^{o}C for 12 s, and 60 ^{o}C for 30 s. The relative expression levels (relative to GAPDH) are calculated using the $\triangle\triangle$Ct method when comparing a treated sample to an untreated sample.

2.16 Western Blotting

Western blotting is a technique to separate proteins from a pool of many based on their size by gel electrophoresis. The separated proteins are then transferred to a nitrocellulose membrane which is then incubated with a primary antibody that specifically recognises a protein of interest. Another antibody the secondary, tagged with HRP (Horseradish peroxidase) binds to the primary antibody and with addition of

an HRP substrate, a chemilluminiscent signal is read as a sharp band on x-ray film. The process begins with sample preparation where cells were washed once in ice-cold PBS and lysed in whole cell lysis buffer (20 mM HEPES pH 7.5, 350 mM NaCl, 0.5 mM EDTA, 1 mM $MgCl_2$, 0.1 mM EGTA, and 1% NP-40) after stipulated time of treatments and boiled at 95°C with Laemmli's SDS-PAGE sample buffer for 5 min. Protein concentration was determined by Bradford method. Equal amounts (20 µg/lane) of protein samples were run on an SDS polyacrylamide gel. The proteins were transferred onto nitrocellulose membrane and blocked with 5% milk in PBS-0.05% Tween. The membrane was incubated with the primary antibody, Caspase-3 (Cell Signaling Technology, Cat# 9662), sXBP1 (Biolegend), CHOP (Santa Cruz Biotechnology, Inc, Cat# sc-793), phosphorylated eIF2α (Cell Signaling Technology, Cat# 3597), total eIF2α (Cell Signaling Technology, Cat# 2103), IRE1α (Cell Signaling Technology, Cat# 3294S), Bim (Stressgen Cat # AAP-330), BAD (Santa Cruz Cat # sc-7869), BID (Santa Cruz cat # sc-11423), Bak (Santa Cruz Cat # sc-832), BCL-2 (Santa Cruz Cat # sc-509), MCL-1 (Cell signalling technologies Cat # 4572), BCL-x_L (Santa Cruz Cat # sc-8392), cytochrome C (BD Biosciences Cat # 556433), Smac/ Diablo (Cell signalling technologies Cat # 2934), Cox IV (Cell signalling technologies Cat # 4844) or β-Actin (Sigma, Cat# A-5060) for 2 h at room temperature or overnight at 4°C. The membrane was washed 3 times with PBS-0.1% Tween and further incubated in appropriate horseradish peroxidase-conjugated secondary antibody (Pierce) for 90 min. Signals were detected using West pico chemiluminescent substrate (Pierce).

2.17 Detection of active Bax by flow cytometry

Bax is a pro-apoptotic member of the BCL-2 family of proteins. In healthy cell it is mainly in the cytoplasm however upon induction of apoptosis Bax translocates to the mitochondria and dimerizes to from pores in the mitochondrial membrane. As such it serves as as an excellent protein to determines at what stage of the apoptotic pathway cells are at. To harvest cells, they were trypsinised and then fixed in 2% formaldehyde for 10 mins at room temperature. Washed cells were resuspended in PBS and stored at 4 ^{o}C overnight. Anti-BAX antibody (BS Biosciences clone 6A7, 2 µl of 0.5 µg/ml stock) was added to 100 µl of permeabilisation buffer (0.1% saponin, 0.5% BSA, 1x PBS) in which, cells were incubated for 1 h at 4 ^{o}C. Mouse IgG isotype control (Biolegend).

served as an autofluorescent control. Samples were washed and incubated with 1:200 dilution of FITC-conjugated anti-mouse antibodies in PBS for 1 h at 4 ^{o}C. Samples were washed and resuspended in 300 µl PBS and analysed by Flow cytometry. Histograms were overlayed using Cyflogic.

2.18 Luciferase reporter Assays

In promoter assays, MCF-7 cells were transfected with 0.8 µg of firefly luciferase vectors (empty pGL4 or pGL4prom17M vector), in combination with a *Renilla* luciferase vector (0.2 µg) as internal control. Twenty four hours post-transfection cells were treated with Tg or Tm for 24 h. Firefly luciferase and Renilla luciferase activities were measured 48 h after transfection at 560 nm using 10s luminescence protocol on Wallac plate reader and then normalized for *Renilla* luciferase activity. Alternatively, in Bim 3' UTR reporter assays, MCF-7 or PC12 cells were transfected with 1.0 µg of psiCHECK2 (Promega) reporter plasmid containing the Bim 3' UTR. 24 h post-transfection cells were treated with thapsigargin or tunicamycin for 24 h. Firefly luciferase and Renilla luciferase activities were measured 48 h after transfection at 560 nm using 10s luminescence protocol on Wallac plate reader and then normalized for *Firefly* luciferase activity.

2.19 Site directed mutagenesis PCR

NRF2 binding sites were detected using online software Transfac. (http://www.biobase-international.com/product/transcription-factor-binding-sites). Site-directed mutagenesis was performed with the QuikChange® system (Stratagene®, La Jolla, U. S. A.) as per the manufacturer's instructions with minor changes. All primers were synthesized by Sigma Aldrich and 1% (v/v) Dimethylsulfoxide (DMSO) was added to mutagenic reactions in which strong secondary interactions were likely. Site-directed mutagenesis primers used were:

Site 1:
mir 17-92 site 1 FP

5'CGGGCGGGCGGGGAGGT**CTTAAGT**ACTTTGTTTTTTATGC 3'

mir 17-92 site 1 RP
5' GCATAAAAAACAAAGT**ACTTAAG**ACCTCCCCGCCCGCCCG 3'

Site 2:
mir 17-92 site 2 FP

5'CCATTGGAAGAGCCA**CCACT**TAAAGTGCTAGTTGGATGGTTGG 3'
mir 17-92 site 2 RP

5' CCAACCATCCAACTAGCACTTTAAGT**GGTGG**CTCTCCCAATGG 3'

Predicted NRF2 binding sites were mutated from CGGAAG –CTTAAG. Mutation of site 1 resulted in addition of AFLII restriction site while mutation of site 2 resulted in deletion of BSR1 restriction site. Introduction or deletion of either restriction site allows for easy screening of colonies carrying possible mutated DNA.

2.20 Chromatin Immunoprecipitation (ChIP) assay

ChIP is a technique that essentially detects protein: DNA interactions. For instance, ChIP is routinely used to prove interactions between transcription factors and DNA, as done in this study. The protein of interest is immunoprecipitated as with traditional methods however samples are fixed prior to lysis to maintain the protein: DNA interaction. As such, any DNA the protein has bound to will be pulled down with it. Protein is degraded and PCR is carried out on Purified, sheared DNA. For this study the interaction between the transcription factors ATF4 and NRF2 to the promoter of the miR-17-92 cluster was investigated. This assay involves many steps that must be fully optimized to obtain sufficient chromatin to detect the site of interest.

Optimization of IP conditions was carried out before isolating chromatin. Antibodies previously reported to work in ChIP assays for NRF2 (Epitomics_Cat #2178-1) and ATF4 (Proteintech_Cat # 60035-1) were selected for the investigation. The ChIP-IT enzymatic kit (Active Motif_Cat # 53006) was used which provides almost all reagents required for successful ChIP, including IgG and RNA pol II antibodies for negative and positive control immunoprecipitation reactions. Conventional IP was carried out to check pull down efficiency of the chosen antibodies where the IP and input were western blotted using the same antibodies as used for the pull down and also with a second set of antibodies for NRF2 (Santa Cruz_Cat # Sc- 13032) and ATF4 (Santa Cruz_Cat # Sc- 22800).

Enzymatic shearing was optimized by incubating chromatin (For description of preparation see below) with and without shearing enzyme for 5, 10, 15 and 30 min at 37^{o}C. Cross linking was reversed by adding 150 µl dH_20, 8 µl 5 M NaCl and 1 µl RNase for 4 h at 65^{o}C. Once at room temperature, samples were briefly centrifuged and 2 µl of proteinase K was added to digest proteins for 1.5 h at 42 ^{o}C. DNA was then electrophoresed on a 1% Agarose gel. PCRs for Site 2 were optimized on the full length miR-17-92 promotor plasmid. Site 2 PCR with primers below worked with the calculated Tm of 53 ^{o}C.

Site 2 Fwd: 5' GTGTCAATCCATTTGGGAGAG 3'
Site 2 Rev: 5' TGGTCACAATCTTCAGTTTTAC 3'

PCR cycle conditions: 94 ^{o}C for 3 min, 94 ^{o}C for 30s, 53 ^{o}C for 30 s, 72 ^{o}C for 30 s, 72 ^{o}C for 7 mins, Repeat steps 2-4 for 40 cycles.

Once all parameters were optimized 3X T75 flasks of MCF7 cells were seeded at 3X10^{6} cells per flask. The next day, cells were transfected with 3 µg of NRF2 plasmid, 3 µg of ATF4 plasmid and 1 µg of GFP plasmid per flask. 24 h post transfection, cells were checked for GFP positivity and 10 ml of **Fixation** solution (1.62 ml of 37% formaldehyde in 50 ml DMEM) was added per flask and incubated for 10 minutes at room temperature while shaking; to crosslink protein: DNA interactions. Cells were washed with 10mls ice cold 1X PBS. Fixation was terminated using 10 ml Glycine stop fix solution (3 ml 10X Glycine, 3 ml 10X PBS and 24 ml autoclaved dH20). Cells were washed in 10 mls ice cold 1X PBS. 2 mls of scraping solution (600 µl 10X PBS in 5.4 mls Autoclaved dH_20 plus 30 µl PMSF) was added into which cells were scraped. Cells from all 3 flasks were pooled into a 15 ml tube and centrifuged at 2500 rpm for 10 min at 4 ^{o}C. Pellet was resuspended in 1ml ice cold lysis buffer to which 5ul PMSF and 5ul PIC have been added. Incubate on ice for 30 min. With a 23 gauge syringe the lysate was gently syringe up and down along the side of tube ~30 times to release nuclei and then centrifuge at 5000 rpm for 10 min at 4 ^{o}C to pellet nuclei. The pellet was resuspended in 1 ml digestion buffer with 5ul PMSF and 5ul PIC and pre-warmed for 5mins at 37 ^{o}C.

For **enzymatic shearing**, a 1:100 dilution of the shearing enzyme was made (0.5 µl to 49.5 of 50% glycerol in autoclaved dH_20). 50 ul of diluted enzyme was added to pre-warmed nuclei, vortexed to mix and incubate at 37^{o}C for optimized time 30 minutes as previously optimized). The reaction was stopped by adding 20 µl ice cold EDTA for 10mins on ice. The sample was centrifuged at 10,000 rpm for 10mins at 4^{o}C and supernatant collected. 25 µl was kept for DNA purification and confirmation of shearing on a 1% agarose gel. The remainder was aliquoted into 250 µl volumes and stored at -80^{o}C (each aliquot can be used for 4 chip reactions).

Before beginning the IP, chromatin was **pre-cleared**. Per IP, 100 µl of Protein G beads were briefly centrifuged, excess liquid was removed and the beads were combined with 50ul chromatin, 59 µl ChIP buffer and Protease inhibitor cocktail (PIC). Samples were rotated at 4 ^{o}C overnight. They were centrifuged at 4000 rpm for 2 minutes and incubated on ice for 2 minutes to allow beads to fully settle. The supernatant was transferred to a fresh tube taking care not to disturb beads and centrifuged again to ensure removal of all beads.

A small amount of chromatin (10 µl) was kept as the input sample at -20 ^{o}C until the IP samples were complete. To IP ATF, NRF2, IgG and RNA Pol II, 170 µl / IP of pre-cleared chromatin was added to 0.65 ml siliconized tubes and incubated with 2 µg of the NRF2, ATF4, and the kit supplied IgG and RNA Pol II antibodies, at 4^{o}C overnight while rotating. 100 µl of Protein G beads were added to each IP, mixed well and rotated for 2 h at 4^{o}C.

Samples were centrifuged at 4000 rpm for 2 minutes and Let stand for 30 s to allow beads to fully settle. The supernatant was removed without disturbing beads and discarded. All remaining pelleting steps were done in this manner. The following buffers were made for subsequent wash steps.

Buffer	**ChIP reaction X 1**
Chip IP Buffer	400 µl Chip IP Buffer + 2 µl PIC
Wash Buffer 1	1.6mls Wash buffer 1 + 1.6 µl PIC
Wash Buffer 2	400 µl Wash buffer 2 + 0.4 µl PIC
Wash Buffer 3	Premade (place on ice)

Each chip reaction was washed as follows:

- Resuspend beads in 400 µl ChIP IP buffer+ PIC: **x1**, vortexed and rotated for 2 minutes and pellet beads.
- Resuspend beads in 400 µl Wash Buffer 1+PIC: **x4**, vortexed and rotated for 2 minutes and pellet beads.
- Resuspend beads in 400 µl Wash Buffer 2+PIC: **x1,** vortexed and rotated for 2 minutes and pellet beads.
- Resuspend beads in 400 µl Wash Buffer 3: **x2,** vortexed and rotated for 2 minutes and pellet beads.
- On final wash with Buffer 3 remove as much buffer as possible without disturbing the beads.

To elute DNA from the beads, 50 µl of ChIP elution Buffer (5 µl of 1M NaHCO3 and 100 µl 1% SDS per IP) was added to each pelleted chip reaction, vortexed briefly and incubated for 15 min at room temperature, while rotating. Samples were centrifuged at 4000 rpm for 2 min, and let stand for 30 s to allow beads to fully settle. The supernatant was transferred to a 1.5ml tube and the beads were centrifuged again and the remaining supernatant recovered.

The input sample was thawed and 90 µl of autoclaved dH_20 was added and along with IP'd samples cross-links were reversed and RNA removed by adding 4 µl 5 M NaCl and 1 µl RNase A to each sample. Samples were incubated at 65^oC overnight.

Proteins were removed by treating samples with 2 µl Proteinase K, 2 µl 1 M Tris-Cl pH 6.5 and 2 µl 0.5 M EDTA at 42 ^oC for 2 h.

DNA was then purified using columns and buffers supplied by the kit. Eluted DNA was stored at -20^oC until PCRs were carried out.

Prior to PCR, input DNA was diluted 1:10 and then 5 µl of DNA was added per reaction. Primers and PCR cycle conditions used to detect Site 2 were given above. GAPDH and negative control primers were supplied by the kit.

CHAPTER 3:

RESULTS CHAPTER 1

LOSS OF DICER CONFERS RESISTANCE TO ER STRESS INDUCED CELL DEATH

3.0 Results chapter 1

Loss of DICER confers resistance to ER stress induced cell death

In this chapter, the effects of, global loss of miRNAs during ER stress-induced cell death was investigated in colon cancer cell lines RKO and HCT116. This subject is of particular interest due to the fact that miRNAs regulate a range of stress responses and can themselves be regulated under stress conditions, such as nutrient deprivation, serum starvation, UV or radiation induced DNA damage, hypoxia, and stress-induced immune responses (Chaudhry et al., 2012; Dresios et al., 2005; Leung and Sharp, 2007; Marsit et al., 2006; Patella and Rainaldi, 2012; Tang et al., 2008a). However, little is known about the role of miRNAs during ER stress. In addition to this, miRNA are differentially expressed in a variety of cancers, which in turn can enhance tumourigenesis and modulate cells responsiveness to therapies (Farazi et al., 2011). In fact, a global loss of miRNA is observed in many cancers (Calin and Croce, 2006; Lu et al., 2005). Complementary to this, lower expression of DICER, an RNase III enzyme required for the processing and maturation of miRNA, has been observed in lung, breast, ovarian and endometrium cancers (Dedes et al., 2011; Karube et al., 2005; Merritt et al., 2008; Torres et al., 2011). Lower DICER expression has enhanced tumourigenesis (Kumar et al., 2007) and is associated with poor patient prognosis (Sand et al., 2010; Wu et al., 2012a; Zhu et al., 2012a). Similarly DICER is considered a haploinsufficient tumour suppressor, where deletion of a single allele of DICER promotes tumour development (Kumar et al., 2009; Lambertz et al., 2010). DICER expression is also modulated by an assortment of stresses. Reactive oxygen species, phorbol esters, Ras oncogene signalling, type 1 interferons and serum withdrawal block DICER expression while IFN-γ induces DICER expression (Asada et al., 2008; Wiesen and Tomasi, 2009). More recently, the antidiabetic agent Metformin was shown to have anti-cancer effects, by inducing AMPK/E2F-mediated increase in DICER mRNA and protein as well as the repression of oncogenic c-MYC via miR-33a (Blandino et al., 2012). Interestingly DICER co-localization with calreticulon on the ER has been observed. The functional significance of this is unknown but the authors of this study suggested that RNAi may occur where the ER meets the cytoplasm (Martello et al., 2010). As discussed earlier, the ER stress and UPR pathways have been well characterised and detected in a variety of disease conditions including neurodegenerative disease, diabetes and cancer (Eizirik et al., 2008; Moenner et al., 2007; Stefani et al., 2012; Zhong et al., 2012)

In cancer, ER stress occurs as a result of the hypoxic tumour microenvironment and the survival responses of the UPR can be manipulated to enhance cancer cell survival (Bi et al., 2005; Moenner et al., 2007; Schonthal, 2012). However, the full extent of ER stress signalling in cancer cell apoptosis is not fully elucidated. A greater appreciation of how ER stress is regulated in cancer, is critical for advancement of therapeutic stratagies (Healy et al., 2009). We hypothesized a role for DICER and miRNA in ER stress-induced cancer cell death. Here we report an increase in DICER mRNA during ER stress-induced cell death and loss of DICER and thus mature miRNA rendered cancer cells resistant to ER stress. This resistance was through the modulation of BCL-2 family proteins. miRNAs may tip the balance between survival and apoptosis and ultimately determine cell fate.

3.1 Bfa and Tm cause a concentration dependant increase in cell death in HCT116 cells

To establish the correct dose of ER stress inducing agents Tm and Bfa, to use for all subsequent experiments, HCT116 cells were treated with increasing doses 0.5, 1 and 5 µg/ml of each compound. Increasing levels of cell death were observed with increasing concentration of Bfa at 24 h and with increasing concentration of Tm at 48 h. A dose of 500 ng/ml, was chosen of both Tm and Bfa for further experiments, as it induces approximately 50% cell death at 24 and 48 h respectively. The percentage cell death was measured using Annexin V-FITC/(PI) staining. Annexin V binds to phophatidylserine (PS) which is only exposed on the surface of dying cells while PI only stains the nuclei of late apoptotic early necrotic cells as such combination of Annexin V/ PI staining is indicative of % cell death. Note the kinetics of both compounds are quite different in HCT116 cells. Bfa had a more potent effect, inducing cell death much earlier than Tm which does not induce cell death until 48 h.

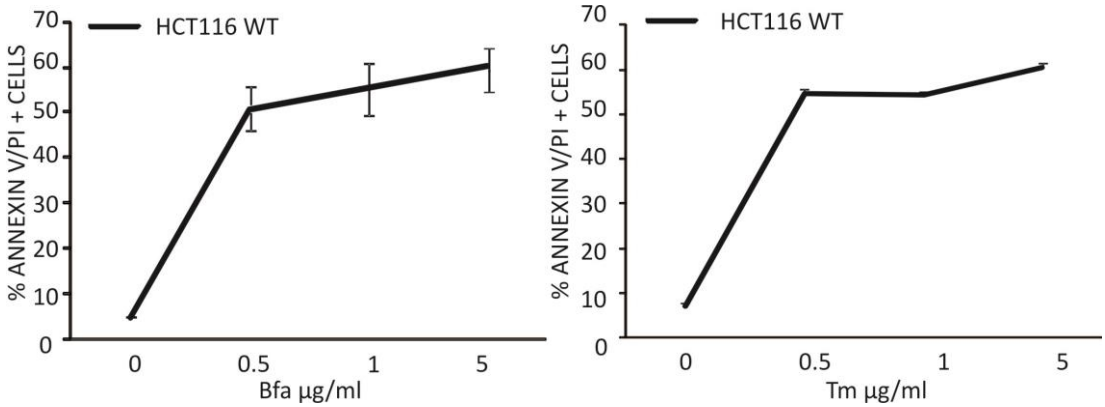


Figure 3.1 Bfa and Tm cause a concentration dependant increase in cell death in HCT116 cells. HCT116 cells were treated with increasing concentrations of Bfa and Tm (0.5-5 µg/ml) for 24 h and 48 h respectively. Cell death was assessed by Annexin V/PI staining where Annexin V/ PI positive cells were taken as the % of dead cells. Data represents the mean of three independent experiments with error bars based on SEM of the repeats.

3.2 Genes associated with miRNA biogenesis are increased during ER stress

During ER stress, many downstream effects are due to the activation of transcriptional events that either promotes cell survival or cell death. In an attempt to identify new genes regulated during ER stress-induced cell death, HCT116 cells were treated with 500 ng/ml of Tm and 500 ng/ml of Bfa for 24 h. RNA was isolated and Microarray analysis carried out (DNA Vision). Interestingly, an increase in the expression of genes associated with miRNA biogenesis was observed including DROSHA, DICER, AGO2, AGO3, AGO4, TARBP1, DCP1A, and DCP2.

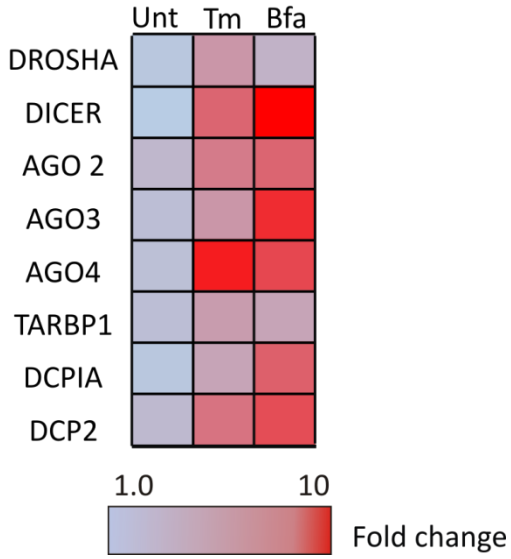


Figure 3.2 Genes associated with miRNA biogenesis are increased during ER stress. HCT116 cells were treated with 500 ng/ml of Tm, and 500 ng/ml of Bfa for 24 h. Affymatrix Microarray was used to measure gene expression. Fold change in gene expression between 1 and 10 is represented by a heatmap. Data represents the mean values of three independent repeats with p values less than or equal to 0.05.

3.3 Disruption of DICER impairs biogenesis of miRNA

Increased expression of genes associated with miRNA biogenesis during ER stress, suggested an important role for miRNA during ER stress, to investigate this further, we used cells in which miRNA biogenesis was compromised, RKO and HCT116 WT and DICER hypomorphic cells (Exn5/Exn5). Exn5/Exn5 cells have an in-frame 43-amino acid insertion into Exon 5 of DICER adjacent to DExH motif (Cummins et al., 2006; Soifer et al., 2008), an important motif in RNA helicases (Schwer, 2001). The insertion mutation results in a mutant DICER protein that has defects in processing of most endogenous pre-miRNAs into mature miRNAs (Soifer et al., 2008). Visually, HCT116 cells behave similarly to WT with no noticeable difference in proliferation rate or morphology while RKO Exn5/Exn5 cells appear to proliferate faster than WT cells. Due to the insertion made to DICER, conventional PCR on cDNA from RKO and HCT116 Exn5/Exn5 cells should give a higher band when compared to WT DICER. However, the PCR conditions optimized here could only detect WT DICER (Figure 3.3 A). Primers used to amplify DICER were designed to flank exon 5 and therefore should detect both WT and hypomorphic DICER. However, it was verified that DICER function was indeed compromised in these cells by measuring the levels of mature miRNA in WT and Exn/Exn5 cells and clearly there is a significant loss of mature miRNA in Exn/Exn5 cells (Figure 3.3 B).

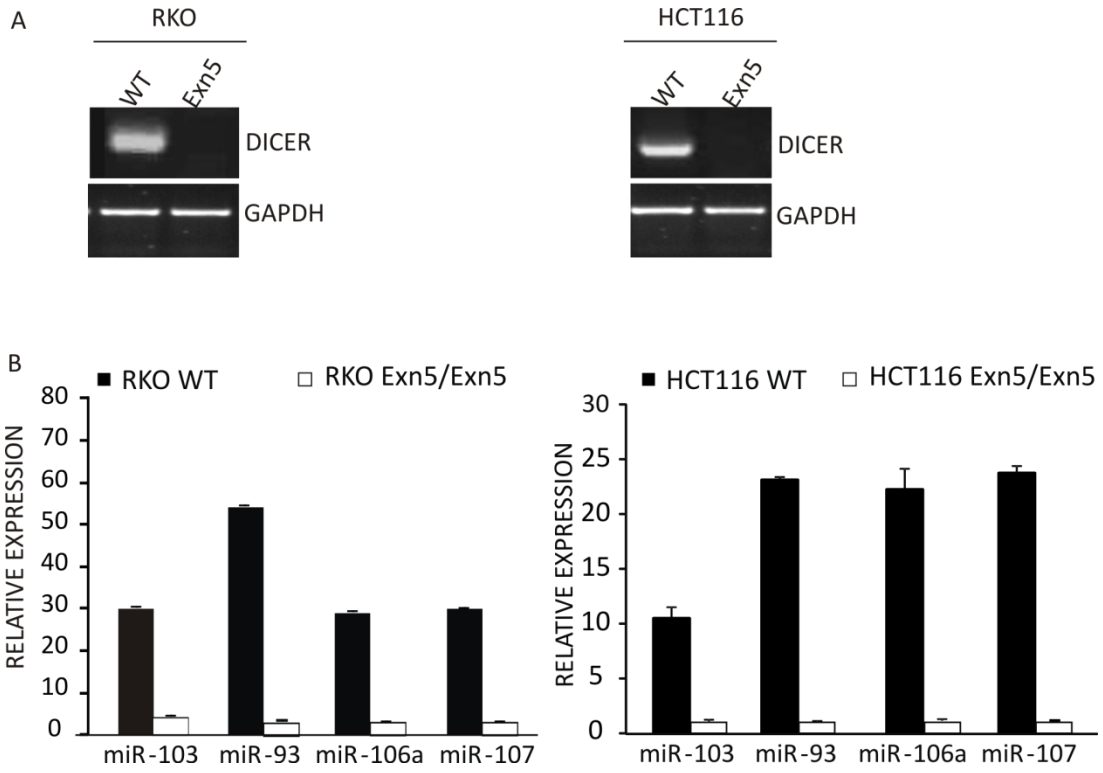


Figure 3.3 Disruption of DICER impairs biogenesis of miRNA. A) Conventional PCR for DICER on cDNA from RKO WT and Exn5/Exn5 cells (LHS) and from HCT116 WT and Exn5/Exn5 cells (RHS). GAPDH was used as a loading control. B) QRT-PCR for miRNA in RKO and HCT116 WT and Exn5/Exn5 cells. Each sample was loaded in triplicate and error bars are representable of the SD between triplicates wells.

3.4 Exn5/Exn5 cells are resistant to ER stress-induced cell death

The consequences of compromising DICER and in turn, miRNA biogenesis were investigated during ER stress. When HCT116 Exn5/Exn5 cells were treated with increasing doses of Tm or Bfa (0.5- 5 μg/ml), the percentage of cell death was considerably decreased when compared to WT cells treated under the same conditions. There was a notable resistance to ER stress-induced cell by 500 ng/ml Tm at 48 h (p= 0.0001 n=3), as well as with 500 ng/ml Bfa at 24 h (p=0.001 n=3). Even at higher doses of either compound, cells remain resistant. 1 μg/ml Tm at 48 h (p= 0.001 n=3), as well as with 1 μg/ml Bfa at 24 h (p=0.0003 n=3). 5 μg/ml Tm at 48 h (p= 0.002 n=3), as well as with 5 μg/ml Bfa at 24 h (p=00001 n=3). Similar results were obtained in RKO WT and Exn5/Exn5 cells treated with increasing doses of Bfa (0.5-5 μg/ml). 500 ng/ml Bfa (p=0.026 n=3), 1 μg/ml Bfa (p=0.001 n=3), 5 μg/ml Bfa at 24 h (p=0.05 n=3).

3.5 Exn5/Exn5 cells are not protected against other cellular stress inducers

Since dose response curves (Figure 3.4) showed there was a notable resistance to ER stress-induced cell death by Bfa and Tm at 24 h and 48 h respectively, resistance to other cell death inducers Etoposide (Eto) and Staurosporin (Sts) was investigated. Surprisingly, Exn5/Exn5 cells exhibited only marginal protection to Etop at 24 h and 48 h or Sts for 24 h and 48 h when compared to WT cells (Figure 3.5 A). While yet again Exn5/Exn5 cells were significantly more resistant to Tm at 24 h (p=0.01) and 48 h (p= 0.0001), and Bfa at 24 h (p=0.0005) and 48 h (p=0.02). Similar results were obtained in RKO WT and Exn5/Exn5 cells treated with 500 ng/ml of Tm (p=0.05) and 500 ng/ml of Bfa (p=0.001) for 24 h (Figure 3.5 B). Reduced processing of pro-caspase-9, pro-caspase-3 and the caspase substrate PARP was evident in Exn5/Exn5 HCT116 cells following 24 h Bfa treatment confirming protection of these cells against ER stress-induced apoptosis (Figure 3.5 C).

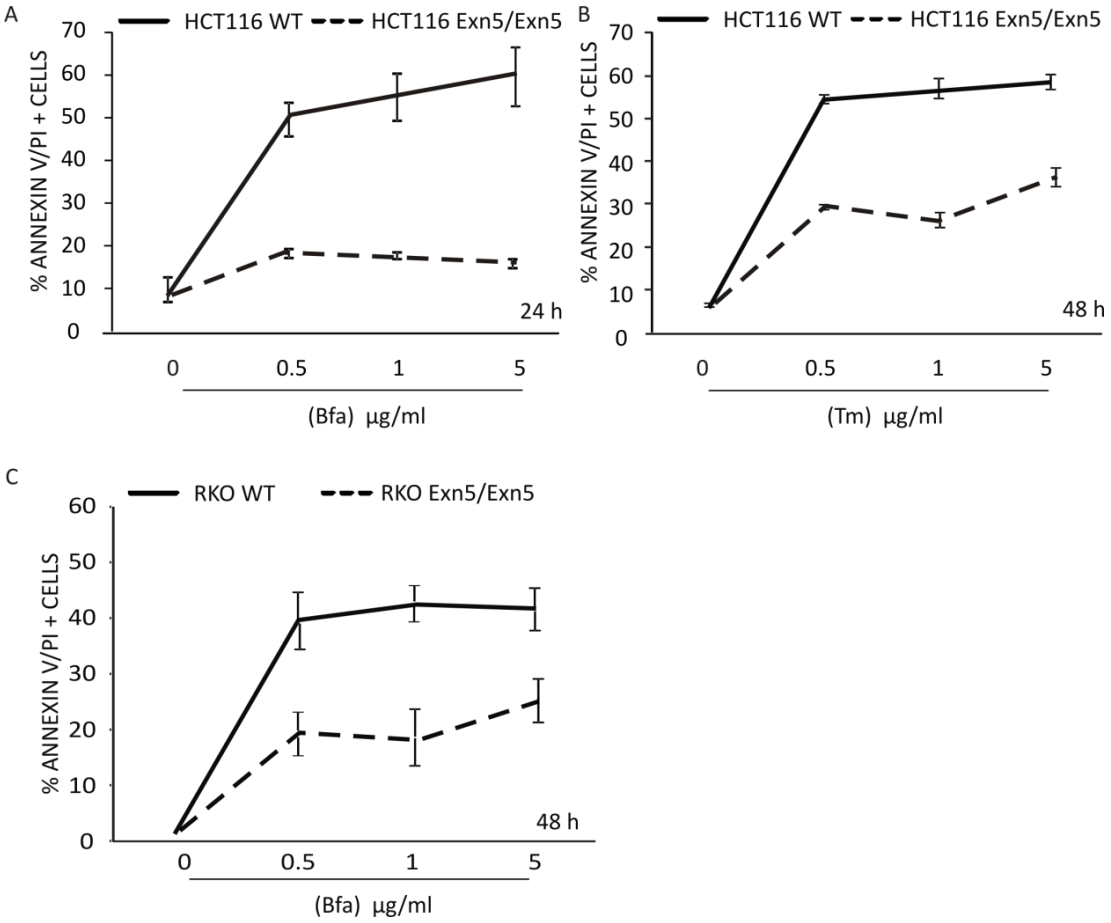


Figure 3.4 Exn5/Exn5 cells are resistant to ER stress-induced cell death. A) Dose response in HCT116 WT and Exn5/Exn5 cells for Bfa at 24 h. Doses used were 0.5, 1 and 5 µg/ml. B) Dose response in HCT116 WT and Exn5/Exn5 cells for Tm at 48 h. Doses used were 0.5, 1 and 5 µg/ml. C) Dose response in RKO WT and Exn5/Exn5 cells for Bfa at 48 h. Doses used were 0.5, 1 and 5 µg/ml. Flow cytometry based measurement, of Annexin V/ PI positive cells was used to estimate % cell death for all of the above.

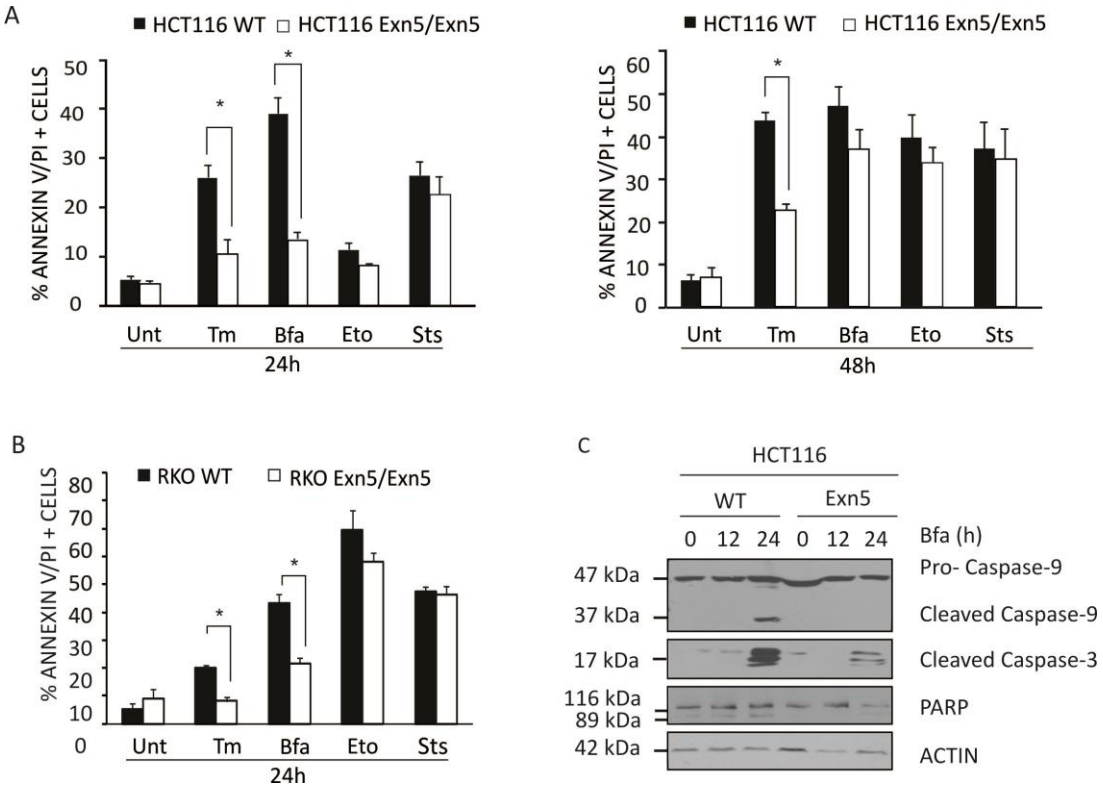


Figure 3.5 Exn5/Exn5 cells show only marginal protection against other cellular stress inducers. A) HCT116 WT and Exn5/Exn5 cells treated with 500 ng/ml Tm, 500 ng/ml Bfa, 100 µM Etop and 250 nM Sts for 24 and 48 h time points. Flow cytometry based measurement, of Annexin V/ PI positive cells was used to estimate % cell death. Exn5/Exn5 cells were significantly more resistant to Tm at 24 h (p=0.01) and 48 h (p= 0.0001), and Bfa at 24 h (p=0.0005) and 48 h (p=0.02). B) RKO WT and Exn5/Exn5 cells treated with 500 ng/ml Tm, 500 ng/ml Bfa, 100 µM Etop and 250 nM Sts for 24 and 48 h time points. Flow cytometry based measurement, of Annexin V/ PI positive cells was used to estimate % cell death. Exn5/Exn5 cells were significantly more resistant to Tm (p=0.05) and Bfa (p=0.001) at 24 h C) Western blots for caspase-9, cleaved caspase-3, PARP in HCT116 WT and Exn5/Exn5 cells treated with 500 ng/ml of BFA for 12 h and 24 h. ACTIN was used as a loading control.

3.6 Knockdown of DICER impairs biogenesis of miRNA

DICER was also independently knocked down using a pSicoR-DICER shRNA to confirm the effects we see in Exn5/Exn5 cells were not due to the way in which DICER was disrupted but are in fact a result of compromised miRNA processing. Knock down efficiency was checked using QRT-PCR to measure the relative expression of DICER mRNA in DICER shRNA cells compared to EV control cells. DICER shRNA was effective in knocking down DICER, where levels were substantially decreased compared to EV (Empty vector) control cells (Figure 3.6 A). Consistent with this, DICER shRNA cells have ~ 50% lower expression of mature miRNAs, thus confirming that miRNA biogenesis has been compromised. (Figure 3.6 B).

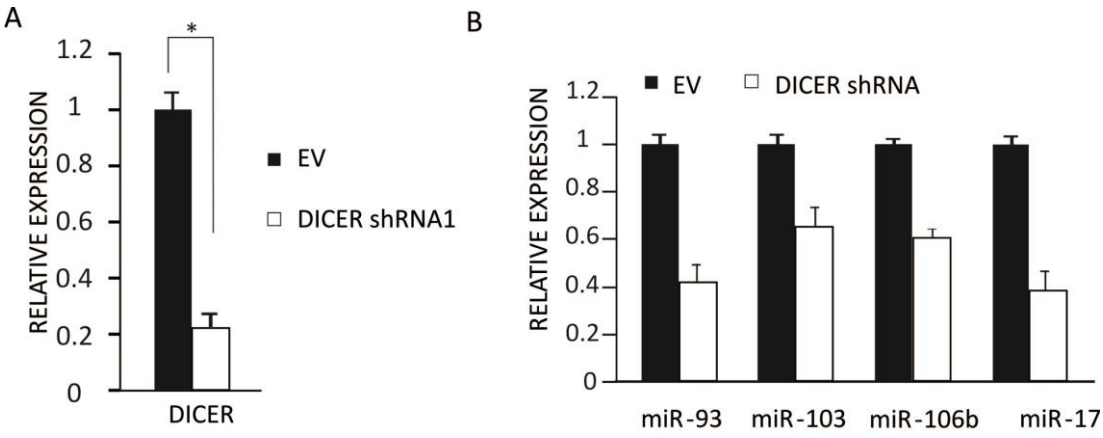


Figure 3.6 Knockdown of DICER impairs miRNA biogenesis. A) HCT116 EV and DICER shRNA cells were harvested from resting conditions and QRT-PCR for DICER was carried out. Relative expression corresponds to the expression of DICER, relative to GAPDH when expression in EV cells is equal to one. B) miRNA QRT-PCR in HCT116 EV and DICER shRNA cells taken from resting conditions. Relative expression corresponds to the expression of DICER, relative to Sn U6 when expression in EV cells is equal to one.

3.7 DICER shRNA cells are resistant to ER stress induced cell death

To examine the effects of compromising miRNA biogenesis via DICER knockdown during ER stress, cells were treated with, 500 ng/ml of Tm and 500 ng/ml of Bfa for 24 h and Annexin V/ PI staining was carried out to determine the % cell death. Agreeing with previous data, the level of cell death was lower in DICER shRNA cells treated with Tm for 24 h (p=0.004) and Bfa for 24 h (p=0.003) (Figure 3.7). These results confirm that it is not just the way in which DICER was disrupted in Exn5/Exn5 cells that provides resistance to ER stress induced cell death but through disruption of miRNA biogenesis in general.

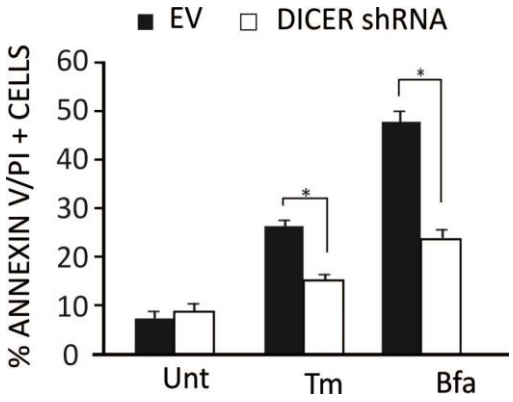


Figure 3.7 DICER shRNA cells are resistant to ER stress induced cell death. HCT116 EV and DICER shRNA cells were treated with 500 ng/ml of Tm and 500 ng/ml of Bfa for 24 h and the % cell death was estimated by flow cytometry based measurement, of Annexin V/ PI staining. DICER shRNA cells were significantly more resistant to treatment with Tm (p=0.004) or Bfa (p=0.003) for 24 h and Bfa for 24 h (p=0.003).

3.8 DROSHA knockdown impairs miRNA biogenesis

To fully ensure the resistant phenotype of Exn5/Exn5 cells is due to compromised miRNA biogenesis, and not a result of interruption of DICER function, the biogenesis pathway was compromised using a TET-inducible shRNA targeting DROSHA. To activate transcription of the shRNA, 500 ng/ml of doxycycline was added to HCT116 DROSHA shRNA cells for 1, and 3 days after which DROSHA expression was detected via QRT-PCR. It must be noted, this shRNA is leaky and shows marginal knockdown even in the absence of doxycycline (Aagaard et al., 2007). However it is clear that there is a much greater knockdown of DROSHA following three days doxycycline treatment (Figure 3.8 A). Expression of mature miRNA was determined in DROSHA shRNA cells and there was a maximum decrease in miRNA expression following 3 days of doxycycline (Figure 3.8 B). These results confirm that knockdown of DROSHA disrupts processing of miRNA.

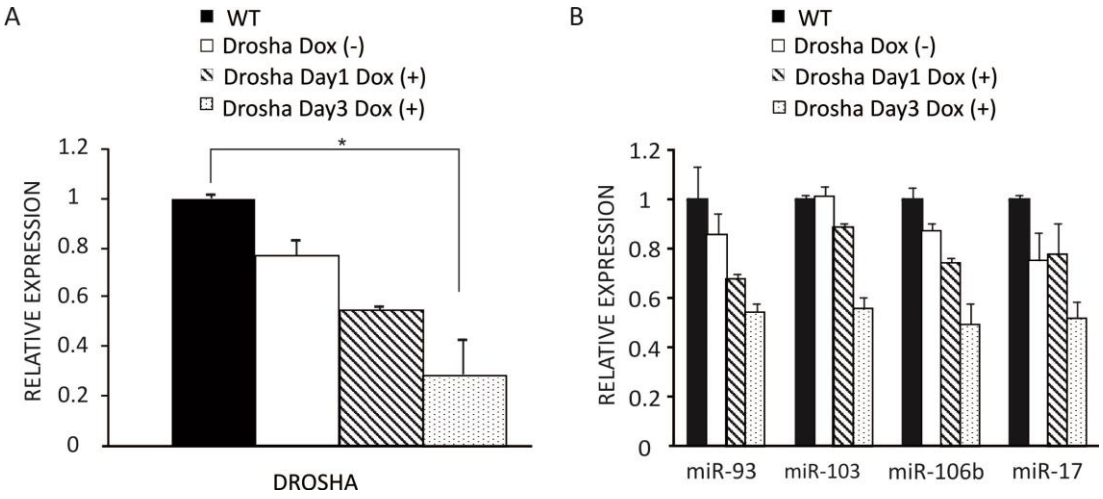


Figure 3.8 DROSHA knockdown impairs miRNA biogenesis. A) DROSHA cells were grown with or without 500 ng/ml doxycycline for up to 3 days, QRT-PCR for DROSHA was carried out and relative expression determined when expression relative to GAPDH in WT cells was equal to one. B) DROSHA cells were grown with or without 500 ng/ml doxycycline for up to 3 days, miRNA QRT-PCR was carried out and relative expression determined when expression relative to Sn U6 in WT cells was equal to one.

3.9 DROSHA shRNA cells are resistant to ER stress induced cell death

To examine the effects of compromising miRNA biogenesis via DROSHA knockdown during ER stress, sensitivity of DROSHA shRNA cells to Tm and Bfa was determined using TMRE staining, where the percentage of cells that lose TMRE staining is an indicator of cell death. TMRE only binds when the OMM potential is stable, dying cells generally have significant loss of mitochondrial membrane potential and as such lose TMRE staining. Again agreeing with data shown in Exn5/Exn5 cells as well as DICER shRNA cells, DROSHA shRNA cells are also resistant to ER stress-induced cell death induced by Bfa at 24 (p=0.004) and 48 h (p=0.005) and Tm at 48 h (p=0.02) where DROSHA shRNA cells retain TMRE staining when compared to WT cells (Figure 3.9 A). Western blotting also revealed there was lower expression of cleaved caspase-3 in DROSHA shRNA cells compared to WT cells, following 24 h and 48 h Bfa treatment again confirming cells are resistant to ER stress-induced cell death. (Figure 3.9 B).

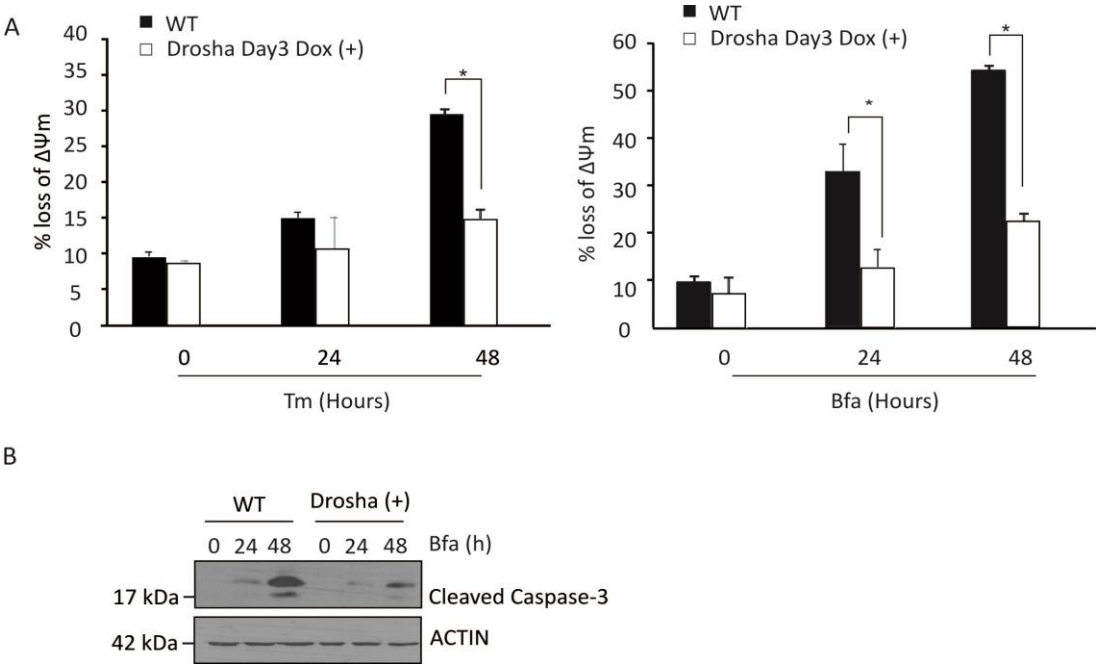


Figure 3.9 DROSHA shRNA cells are resistant to ER stress induced cell death. A) HCT116 WT and DROSHA shRNA cells + Doxycycline for 3 days were treated with 500 ng/ml of Tm and 500 ng/ml of Bfa for 24 and 48h. Flow cytometry based measurement; of TMRE staining was used to estimate % cell death. DROSHA shRNA cells were significantly more resistant to treatment with Tm at 48 h (p=0.02) or Bfa at 24 (p=0.004) and 48 h (p=0.005). B) Western blot for cleaved caspase-3 in HCT116 WT and DROSHA shRNA cells + Doxycycline for 3 days and treated with 500 ng/ml of Tm and 500 ng/ml of Bfa for 24 and 48h. ACTIN used as a loading control.

3.10 Microarray analysis of genes associated with UPR and ER stress

To investigate why cells with compromised miRNA biogenesis are resistant to ER stress-induced cell death, microarray analysis was carried out on cells treated with 500 ng/ml of Tm and 500 ng/ml of Bfa for 24 h. Three independent repeats were set-up; RNA was isolated and further sample processing and analysis was performed by DNA Vision. To identify potential candidates that may explain the resistant phenotype, screening of data was based on, genes that are differentially expressed at basal levels as well as under both treatments simultaneously that are associated with the UPR and apoptosis were screened, values with a Log FC value of 2 or more were short listed. For instance, pro-apoptotic proteins that were downregulated in Exn5/Exn5 cells, or pro-survival proteins, that were upregulated in Exn5/Exn5 cells. Genes that showed the most promising differences in expression included EDEM1, GRP78, ERP72, CHOP, ATF4, p58IPK, ERO1β, ERDJ4, PERK and NOXA (Figure 3.8).

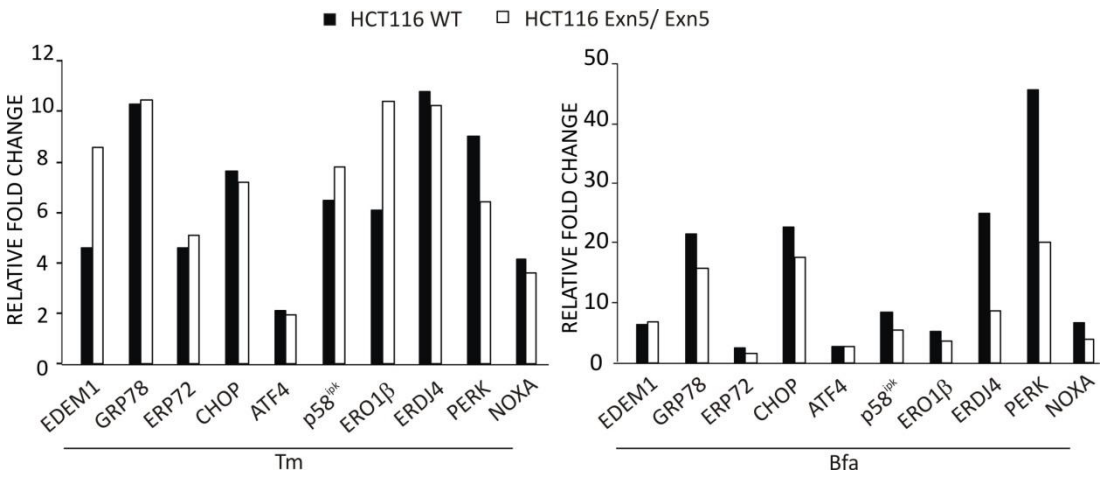


Figure 3.10 Microarray analysis of genes associated with the UPR and ER stress.
Microarray analysis of gene expression in HCT116 WT and Exn5/Exn5 cells were treated with 500 ng/ml of Tm and 500 ng/ml of Bfa for 24 h. Affymetrix microarray analysis was carried out on triplicate experiments by DNA Vision. Relative Fold change as represented above was calculated from Log FC values and made relative to WT untreated. Data shown gave p-values less than or equal to 0.05.

3.11 Expression of pro-apoptotic PERK, CHOP and NOXA mRNA is lower in Exn5/Exn5 cells

To validate the results obtained from the microarray, HCT116 WT and Exn5/Exn5 cells were treated with 500 ng/ml of Tm and 500 ng/ml of Bfa for 12 h and 24 h. RNA was reverse transcribed into cDNA and gene expression was analysed via QRT-PCR using Taqman chemistry based assay sets described in chapter 2; The pro-apoptotic associated PERK, CHOP and NOXA all displayed lower mRNA expression in Exn5/Exn5 cells compared to WT cells.

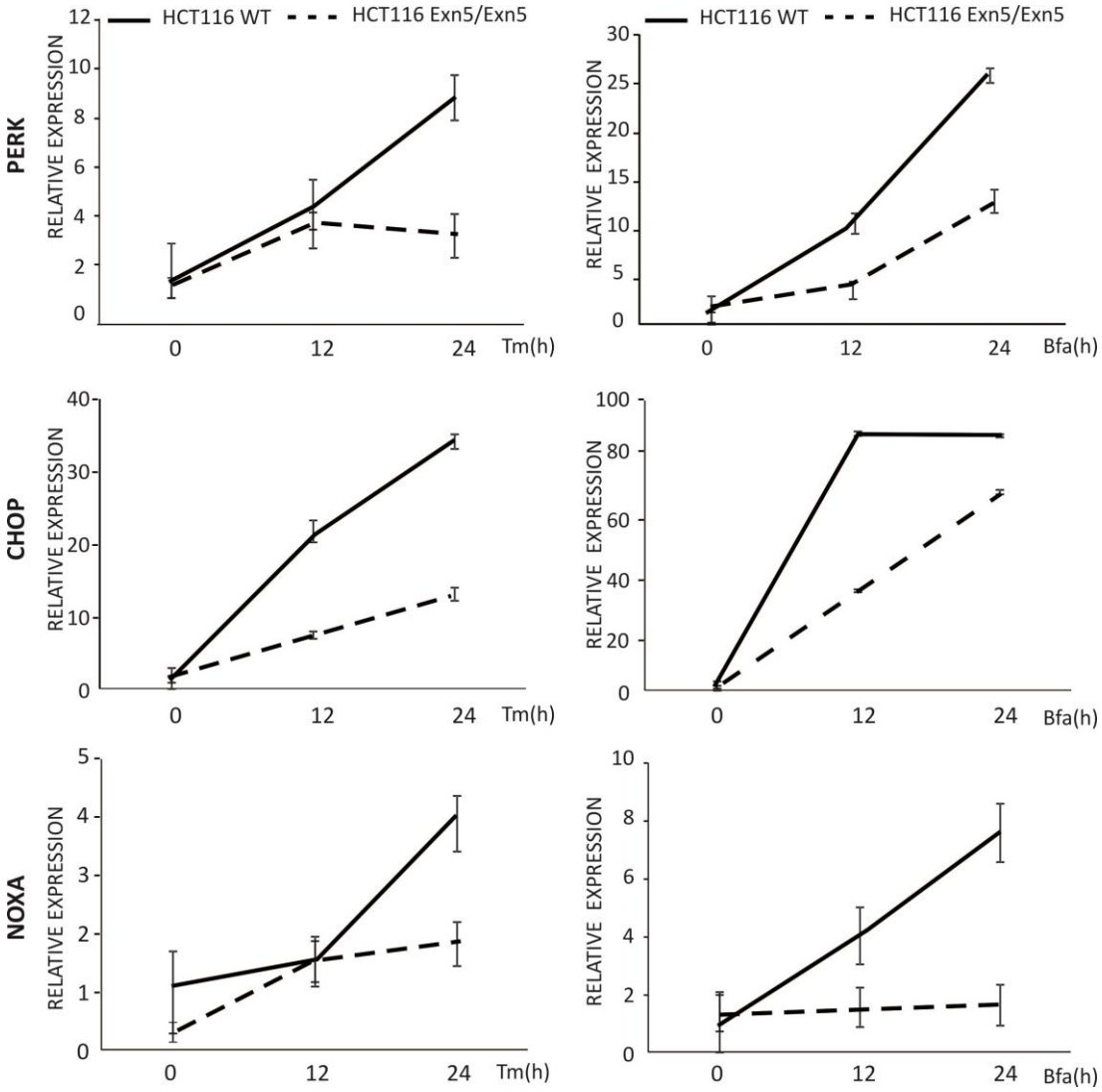


Figure 3.11 Expression of pro-apoptotic PERK, CHOP and NOXA mRNA is lower in Exn5/Exn5 cells. QRT-PCR for PERK, CHOP and NOXA in HCT116 WT and Exn5/Exn5 cells treated with 500 ng/ml of Tm for 12 h and 24 h (LHS) or 500 ng/ml of Bfa for 12 h and 24 h (RHS).

3.12 The Unfolded protein response proceeds in the absence of miRNA

Differences in PERK, CHOP and NOXA at the mRNA level in Exn5/Exn5 cells led to an investigation of the levels of these at protein level and of other well-known proteins of the UPR. Levels of CHOP, NOXA, phospho- eIF2α, Total- eIF2α, IRE1, ATF6, and sXBP1 were examined by western blotting. Differences seen at mRNA level with PERK, CHOP and NOXA were not evident at protein level where total and phospho- eIF2α (representative of PERK activity) displayed a similar level of expression in WT and Exn5/Exn5 cells, and both CHOP and NOXA protein levels were also comparable between the two cell lines. Interestingly, sXBP1 levels were consistently decreased in Exn5/Exn5 cells (Figure 3.9).

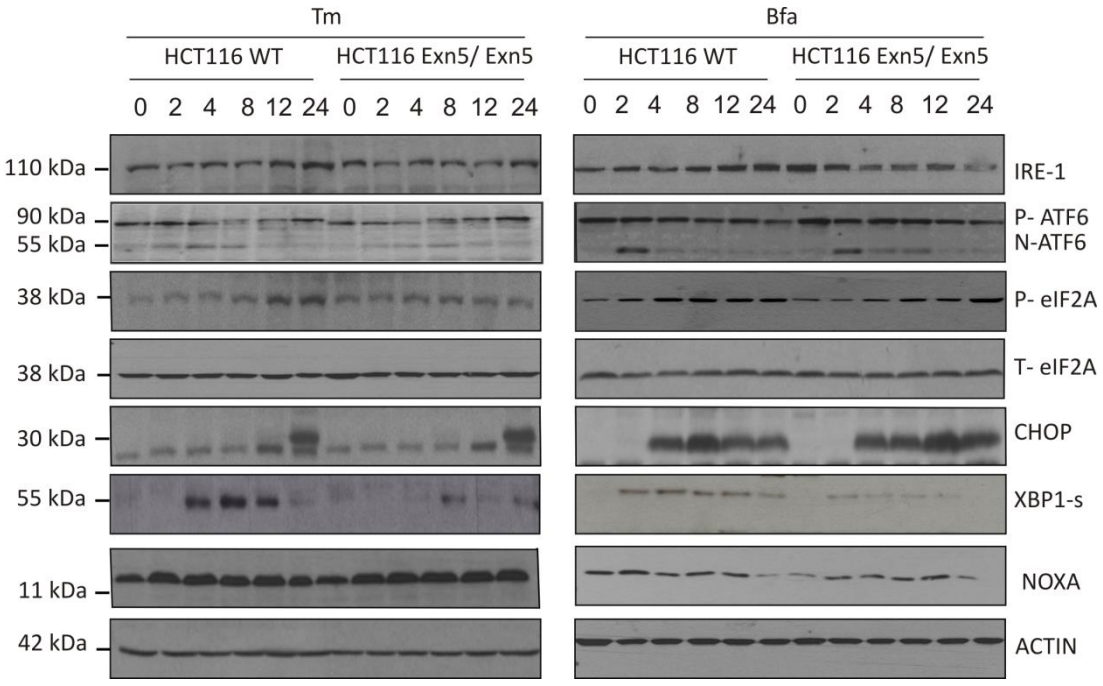


Figure 3.12 The Unfolded protein response proceeds in the absence of miRNA. A) Western blots to detect UPR/apoptosis-associated proteins in HCT116 WT and Exn5/Exn5 cells treated with 500 ng/ml Bfa and 500 ng/ml Tm for 2, 4, 8, 12 and 24 h. Proteins analysed include IRE1, ATF6, P-eIF2α and T-eIF2α, CHOP, XBP1s and NOXA. ACTIN was used as a loading control.

3.13 IRE1 ribonuclease-mediated signalling is not important for resistance of Exn5/Exn5 cells

Lower levels of XBP1s in a cell line resistant to ER stress induced cell death was surprising since, prolonged sXBP1 levels are associated with cell survival. The role of XBP1s was further investigated to determine if it contributed to the resistant phenotype of Exn5/Exn5 cells. We used an inhibitor of IRE1, MKC4485, designed by Mannkind Corporation to specifically block the RNase domain of IRE1 and thus preventing XBP1 from being spliced. An earlier version of this compound was previously described to block splicing of XBP1 (Mimura et al., 2012). A dose response showed 5 μM of inhibitor was enough to prevent splicing of XBP1 and further experiments were carried out with this dose (Figure 3.13 A). Sensitivity of cells to ER stress was analyzed following the addition of the IRE1 inhibitor. Blocked splicing of XBP1 with this inhibitor did not confer resistance to ER stress-induced cell death in WT cells. Although splicing of XBP1 is generally a pro-survival event, the lower levels in Exn5/Exn5 cells suggested the opposite since these cells are resistant to ER stress. Use of the inhibitor proved lower XBP1s has no role in the resistant phenotype of Exn5/Exn5 cells. (Figure 3.13 B).

3.14 IRE1 signalling is not important for the resistant phenotype of Exn5/Exn5 cells

This result shows that the lower levels of XBP1s in Exn5/Exn5 cells, is not a contributing factor to their resistance to ER stress. Although low XBP1s did not confer resistance to ER stress, there was still, a difference in its expression between WT and Exn5/Exn5 cells. This suggested IRE1 signalling in general may be altered in Exn5/Exn5 cells. The use of the IRE1 inhibitor and no subsequent change in the phenotype of Exn5/Exn5 cells suggests that the RNase activity of IRE1 is not required; we investigated signalling of IRE1 which does not require the RNase domain. Hence, we examined JNK phosphorylation which is activated through IRE1 kinase signalling and TRAF2. However, there were no differences in Total or phospho-JNK levels bwtween WT and Exn5/Exn5 cells. These results show that IRE1 signalling does not contribute to the resistance of Exn5/Exn5 cells (Figure 3.14).

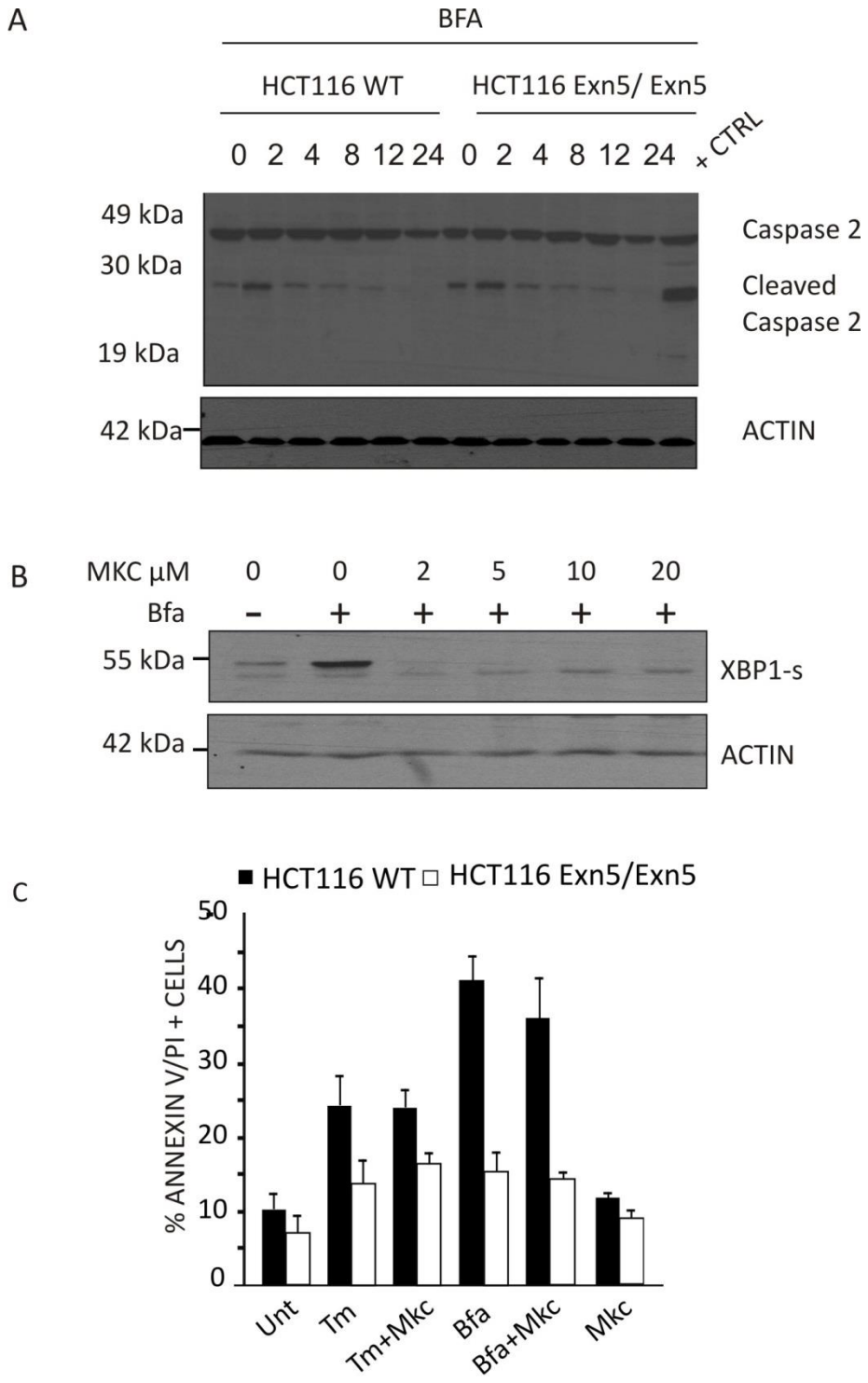


Figure 3.13 IRE1 ribonuclease-mediated signalling is not important for resistance of Exn5/Exn5 cells. A) Western blot for XBP1s in HCT116 WT cells treated with 0-20 µM MKC4485 and 500 ng/ml of Bfa for 24 h. ACTIN was used as a loading control. B) Annexin V/PI staining was used to assess the % cell death of HCT116 WT and Exn5/Exn5 cells treated with 5 µM MKC4485 and 500 ng/ml of Bfa for 24 h.

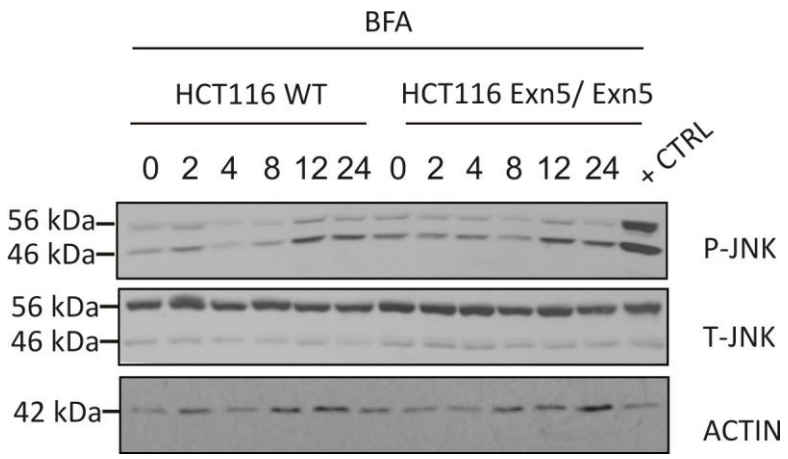


Figure 3.14 IRE1 signalling is not important for the resistant phenotype of Exn5/Exn5 cells. Western blots to detect total (T) and phospho (P) JNK1/2 in HCT116 WT and Exn5/Exn5 cells treated with 500 ng/ml Bfa and 500 ng/ml Tm for 2, 4, 8, 12 and 24 h. ACTIN was used as a loading control.

3.15 Heat shock proteins are not important for the resistant phenotype of Exn5/Exn5 cells

Recently reports have been published showing miRNA regulation of heat shock proteins (Yin et al., 2009). Our lab has published that high expression of Hsp72 protects against ER stress (Gupta et al., 2010), as does high expression of other heat shock components (Unpublished data). And one study in drosophila reported that loss of components of the miRNA biogenesis pathway including DCR2 and AGO2 resulted in transcriptional defects especially during heatshock where lack of these proteins resulted in increased expression of Hsp70A and Hsp70B (Cernilogar et al., 2011). From this we hypothesised that disruption of miRNA in our model may confer resistance to ER stress induced cell death via upregulation of heat shock proteins. To investigate this further we blotted for Hsp72 and Hsp27 proteins in HCT116 WT and Exn5/Exn5 cells. If our hypothesis was true we would have expected to see higher levels of these heat shock proteins in Exn5/Exn5 cells. However this was not the case as levels of Hsp72 and Hsp27 were comparable in both cell lines.

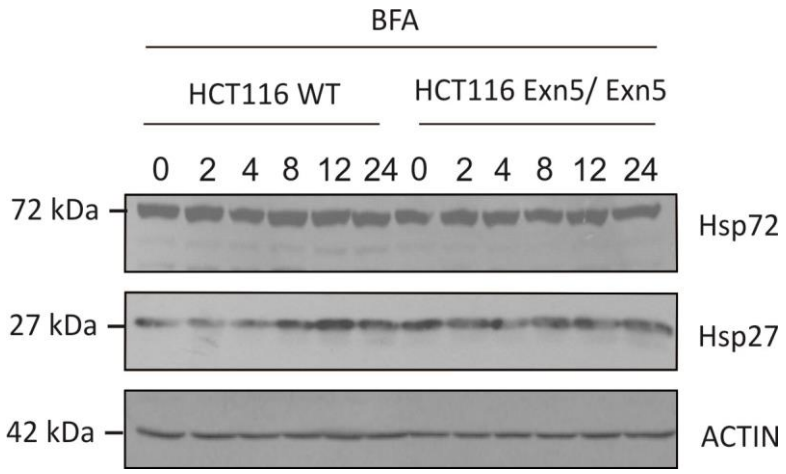


Figure 3.15 Heat shock proteins 72 and 27 are not important for the resistant phenotype of Exn5/Exn5 cells. Western blots for Hsp72 and Hsp27 in HCT116 WT and Exn5/Exn5 cells treated with 500 ng/ml of Bfa for 2-24 h. ACTIN was used as a loading control.

3.16 Exn5/Exn5 cells retain their outer mitochondrial membrane potential

As shown, the UPR is not affected by compromised DICER function and so can proceed in the absence of miRNA regulation. However, the results we see appear to be specific to ER stress-induced cell death. This indicates that, the intrinsic apoptosis pathway itself must be compromised for these cells to survive. This indicates that miRNAs are important for the cells response to ER stress-induced cell death but not as vital for other cell death induced signalling, such as that induced by Etop or Sts. As the intrinsic pathways is centred around the functionality of the mitochondria, we investigated the status of the OMM potential using TMRE staining, to indicate the point in the intrinsic apoptotic pathway resistance may occur. Exn5/Exn5 cells retain TMRE positivity when compared to WT cells following 36 h Tm treatment ($p=0.042$ $n=3$) and 24 h treatment with Bfa ($p= 0.012$ $n=3$), suggesting resistance may occur upstream of the mitochondria (Figure 3.16 A). Graphs are representative of the data shown in A (Figure 3.16 B).

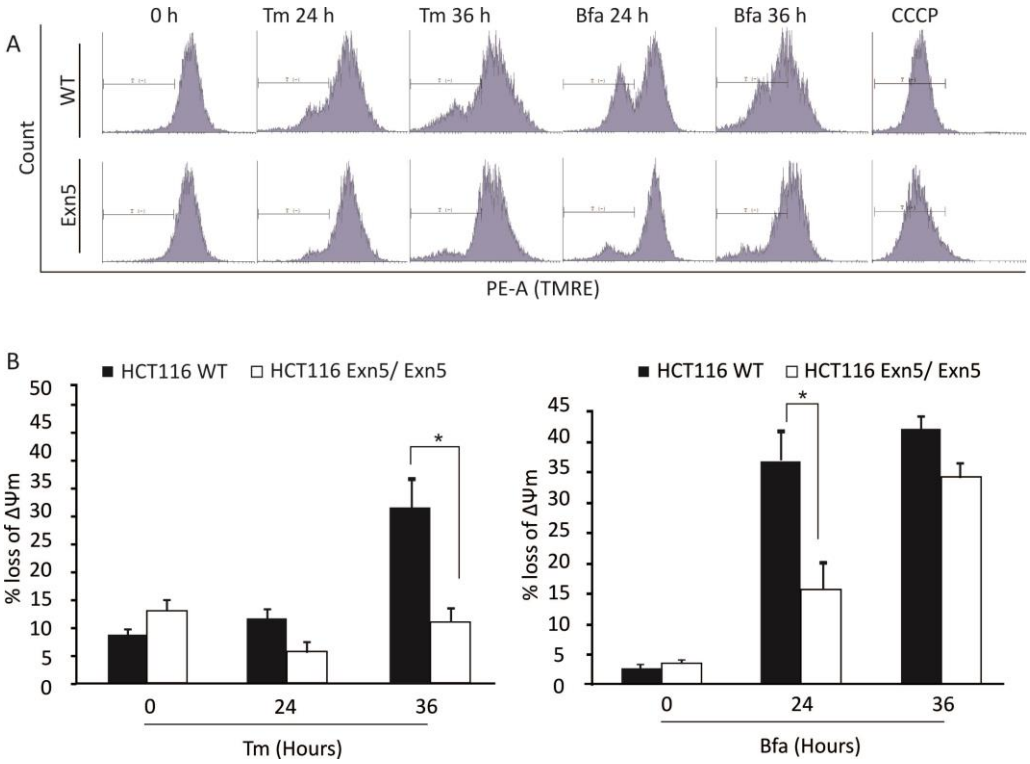


Figure 3.16 Exn5/Exn5 cells retain their outer mitochondrial membrane potential. A) Flow cytometry profiles of TMRE staining in HCT116 WT and Exn5/Exn5 cells treated with 500 ng/ml Bfa and 500 ng/ml Tm for 24 and 36 h. Histogram plots (purple) show loss of TMRE staining in the PE channel. B) The graphed representation of % of cells with loss of TMRE is also shown (lower graphs). Exn5/Exn5 cells retain TMRE positivity when compared to WT cells following 36 h Tm treatment (p=0.042) and 24 h treatment with Bfa (p= 0.012).

3.17 Resistance of Exn5/Exn5 cells occurs upstream of the mitochondrion

To prove that the resistant phenotype of Exn5/Exn5 cells occurs upstream of the mitochondria, translocation of BAX from the cytoplasm to the mitochondria was inspected using an antibody that specifically detects the active form of BAX. Agreeing with the hypothesis, less active BAX was detected by flow cytometry in Exn5/Exn5 cells treated with 500 ng/ml of Bfa for 12 h (p=0.05 n=3) and 24 h (p= 0.026 n=3) compared to WT cells (Figure 3.17 A). The Graph represents the flow cytometry data shown in A (Figure 3.17 B).

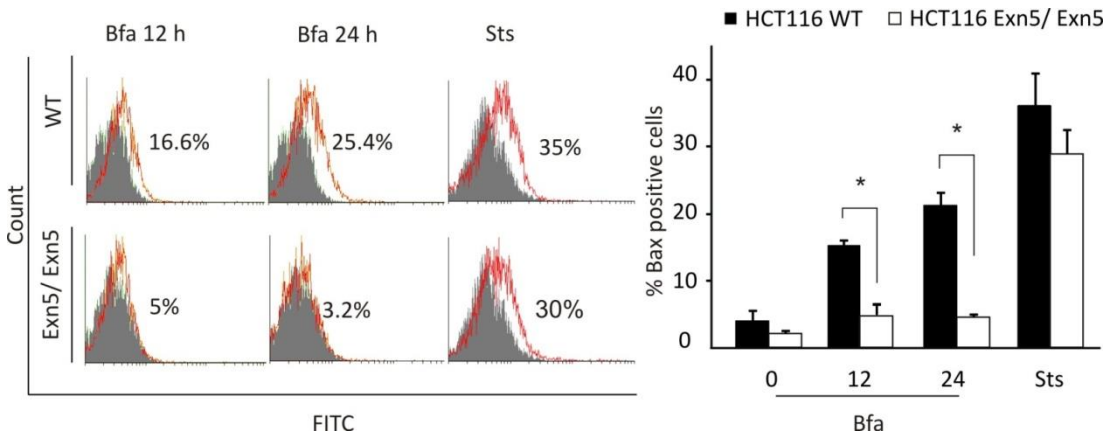


Figure 3.17 Resistance of Exn5/Exn5 cells occurs upstream of the mitochondrion. A) Flow cytometry based measurement of the active conformation of BAX in HCT116 WT and Exn5/Exn5 cells treated with 500 ng/ml of Bfa for 12 h and 24 h or 100 nM Sts for 24 h. Histogram overlays were used to represent the data shown, where treated samples (red peak) were compared to untreated samples (Grey peak). B) The graphed representation of % cells with active BAX is also shown (RHS). Significantly less active BAX was detected by flow cytometry in Exn5/Exn5 cells treated with Bfa for 12 h ($p=0.05$) and 24 h ($p= 0.026$).

3.18. Altered expression of BCL-2 family proteins may attenuate cell death in Exn5/Exn5 cells.

The balance between pro-survival and pro-apoptotic BCL-2 family proteins, is known to modulate mitochondrial apoptosis (Breckenridge and Xue, 2004; Cory and Adams, 2002). In other words, if pro-apoptotic BCL-2 proteins are more highly expressed than pro-survival BCL-2 proteins they thus neutralize pro-survival proteins, BAX and BAK are free to dimerize, and form pores within the OMM. Considering that there is lower BAX activation in Exn5/Exn5 cells, we examined the levels of BCL-2 family members in WT and Exn5/Exn5 cells. There is in fact a different expression pattern of BCL-2 family proteins in Exn5/Exn5 cells, where levels of pro-survival MCL-1 and BCL-2 are prolonged to 4 h and more highly expressed respectively; and pro-apoptotic BAD is much lower while interesting BIM seems higher. There was no notable difference in the expression of BAK, BCL-xL, or BID (Figure 3.18).

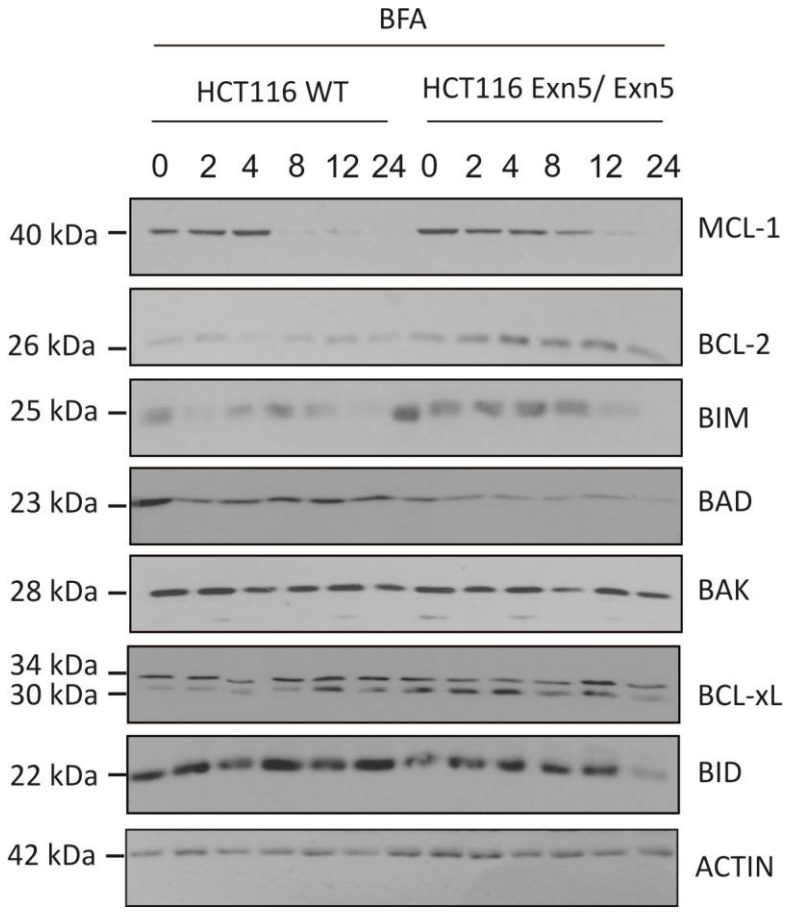


Figure 3.18 Altered expression of BCL-2 family proteins may attenuate cell death in Exn5/Exn5 cells. Western blots for BCL-2 family proteins in HCT116 WT and Exn5/Exn5 cells treated with 500 ng/ml of Bfa for 2, 4, 8, 12 and 24 h. Proteins analyzed were MCL-1, BCL-2, BIM, BAD, BAK, BCL-xl, and BID. ACTIN was used as a loading control.

3.19 The expression profile of BCL-2 family member's differs between Etop and Bfa treatments

As shown, in figure 3.5 Exn5/Exn5 cells are resistant to ER stress inducers Tm and Bfa but not other cell death inducers Etop and Sts. We hypothesized that altered expression of BCL-2 family proteins in Exn5/Exn5 cells provides resistance to ER stress induced cell death. We found that BCL-2 proteins were differentially modulated in ER stress including BCL-2, MCL-1, BIM and BAD (Figure 3.18). As cells induce different stress response pathways to deal with different stresses, we hypothesized that BCL-2 family proteins would as such be differentially regulated depending on the stress response. To investigate this further, we treated HCT116 WT and Exn5/Exn5 cells with Etop for 3-36 h and analysed the levels of BCL-2 proteins. Interestingly, and agreeing with our hypothesis, Etop treatment results in a different BCL-2 expression profile in Exn5/Exn5 cells than when the same cells are treated with ER stress inducers. For instance, MCL-1 is still higher in Exn5/Exn5 cells upon Etop treatment, but BCL-2 levels are comparable between WT and Exn5/Exn5 cells, while however there are also higher levels of pro-apoptotic BOPs BIM and BAD.

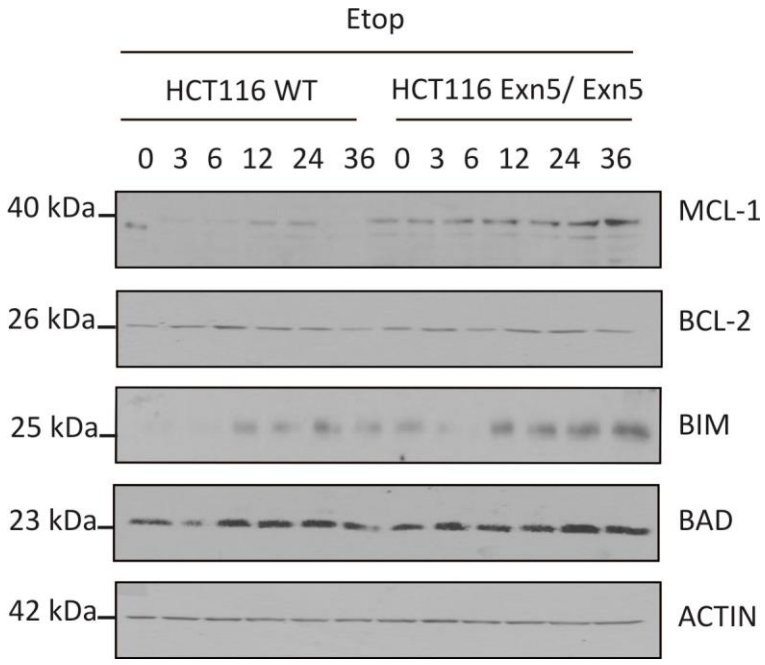


Figure 3.19 The expression profile of BCL-2 family member's differs between Etop and Bfa treatments. Western blot for MCL-1, BCL-1, BIM and BAD in HCT116 WT and Exn5/Exn5 cells treated with 100 µM of Etop for 3-36 h. ACTIN was used as a loading control.

CHAPTER 4:

RESULTS CHAPTER 2

IDENTIFICATION OF ER STRESS REGULATED MIRNA

4.0 Results chapter 2

Identification of ER stress regulated miRNA

This chapter describes the investigation of miRNAs that are regulated during ER stress-induced cell death. ER stress was shown to downregulate miRNAs of the miR-17-92 cluster and its paralog cluster miR-106b-25 in three different cells lines, human breast cancer-derived cell line MCF7s, rat adrenal pheochromocytoma-derived PC12 cells and mouse cardiomyocyte-derived cell line H9C2s. miRNAs were first implicated in stress responses when AGO2 was shown to relocate from the cytoplasm into PBs upon cellular stress in a miRNA dependant fashion (Leung et al., 2006; Liu et al., 2005a). miRNAs can be regulated in response to a variety of stimuli including stress-inducing stimuli e.g. hypoxic, oxidative, temperature and inflammatory stress responses, which often leads to enhanced disease pathogenesis (Kulshreshtha et al., 2007; Leung and Sharp, 2010; Xu et al., 2012; Yin et al., 2009). Like genes, miRNA expression can be controlled through transcription factor binding, which can either enhance or deter transcription (Levine and Tjian, 2003). As a single miRNA can bind an array of mRNAs and prevent their translation, the increase or decrease in miRNA due transcription factor binding can have major consequences on the cell. Essentially, transcription factor-mediated modulation of miRNA expression can decide if the cell lives or dies (Ding et al., 2009). For example, hypoxia inducible transcription factor 1α (HIF1α), the key transcription factor activated during hypoxia, induces several miRNAs including miR-210a, miR-210b and miR-26a (Kulshreshtha et al., 2007). Multiple stress responses activate p53 which in turn activates members of the miR-34 family resulting in cell cycle arrest and eventually apoptosis (Chang et al., 2007). More recently, it has been reported that miRNA can be directly regulated by ER stress. The miR-221-222 cluster is downregulated during ER stress and brings about apoptosis through increased p27 kip1, a cyclin dependant kinase inhibitor (Dai et al., 2010). XBP1s can induce miR-346 during the adaptive phase of ER stress to repress TAP1 (antigen peptide transporter 1), and prevents presentation of MHC class I proteins (Bartoszewski et al., 2011). NFκB activated by ER stress in HCC cells, blocked transcription of the and resulted in increased XBP1s, a direct target of miR-214 (Duan et al., 2012).

The miR-17-92 cluster which is highly oncogenic and has been designated the nickname "Oncomir-1" consists of six miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1) on chromosome 13 that are transcribed as a single polycistronic unit (Mendell, 2008). However, it must be noted that although all miRNA of the cluster are transcribed together, the kinetics and processing efficiency of individual miRNAs from that cluster can differ. The differences in processing efficiency has recently been attributed the tertiary formation the cluster transcript adopts (Chakraborty et al., 2012). Two miR-17-92 cluster paralogs exist in mammals: the miR-106b-25 cluster which is located on human chromosome 7 and the miR-106a-363 cluster which located on the X chromosome (Tanzer and Stadler, 2004). The miR-106b-25 cluster is located intron 13 of the protein-coding gene miniature chromosome maintenance 7 (MCM7) and consists of three miRNAs, (miR-106b, miR-93, and miR-25) (Mendell, 2008). MCM7 overexpression has has been linked cancer where its function in DNA replication and cell cycle progression may function cooperatively with miR-106b-25 to enhance cancer progression (Luo, 2011). The second paralog cluster, miR-106a-363 is unlike the miR-17-92 and miR-106b-25 clusters, which are both abundantly expressed across many tissues and cell types, instead it is generally undetectable or expressed at trace levels (Ventura et al., 2008). Although the miR-17-92 and miR-106b-25 clusters have many similar targets and are considered highly tumourigenic, they have also been shown to have very separate roles especially in regards to development. For instance, several members of the miR-17 family, including miR-17-5p, miR-20a, miR-93, and miR-106a have been shown to be specifically expressed in undifferentiated or differentiating embryonic stem cells (Foshay and Gallicano, 2009). Deletion of the miR-17-92 cluster results in smaller embryos and immediate postnatal death of all animals associated severe lung hypoplasia and defective ventricular septum while deletion of the miR-106a-363 and miR-106b-25 clusters, either alone or in combination, does not result in any obvious phenotype (Ventura et al., 2008). Furthermore, loss of both miR-106b-25/miR-17-92 clusters results in embryonic death associated with severe cardiac developmental abnormalities, consisting of defective ventricular and atrial septation and thinner ventricles (Ventura et al., 2008).

In the context of carcinogenesis, amplification and overexpression of miR-106b-25/miR-17-92 cluster has been documented in B-cell lymphomas, lung cancer and gastric cancer (Mendell, 2008). The oncogenic transcription factors c-Myc and E2F3 induce the expression of the miR-17-92 cluster and E2F1 induces the expression of miR-106b-25 (Aguda et al., 2008). Inversely, p53 can repress miR-17-92 during conditions of hypoxia, which could have

implications for cancers in which p53 is deleted or mutated (Yan et al., 2009). As with all miRNAs, the individual miRNAs of the miR-17-92 cluster can potentially target hundreds of mRNAs within the cell, however in relation to cancer progression the most well characterised targets are the tumour suppressor PTEN and the pro-apoptotic BOP, BIM. In fact, high expression of miR-17-92 enhanced lymphoproliferative disease and autoimmunity in mice due to their silencing of the PTEN and BIM (Xiao et al., 2008). Furthermore, miR-17-92-mediated repression of BIM has also been shown to cause resistance to Paclitaxel in human ovarian carcinoma SKOV3-TR30 cells (Shuang et al., 2013). Although much is known regarding the biological effects of the miR-17-92 cluster, little is known about its regulation.

This study has revealed that the miRNAs of the miR-17-92 and miR-106b-25 cluster are down regulated during ER stress in a PERK-dependant manner, where NRF2 and ATF4 block their transcription. This results in upregulated BIM mRNA and protein and subsequent apoptosis. Although it has been already reported that BIM is a direct target of these miRNA, the significance of this during ER stress has never been shown. BIM above other BOPs, is a hugely important effector of ER stress-induced apoptosis (Puthalakath et al., 2007). Our results show that miRNA as important regulators of cell fate in response to ER stress and shows a novel aspect of NRF2 signalling, separating it from its role in oxidative pro-survival signalling. Deviating from its normal function in cell survival, the observations made here would suggest that NRF2 can under certain conditions, promote apoptosis.

4.1 miRNA of the miR-17-92 and miR-106b-25 cluster are downregulated during ER stress

With the aim of identifying novel miRNAs regulated during ER stress induced cell death, H9C2s where treated with Tg and Tm and a miRNA expression microarray was carried out. Several hundred miRNA where analysed and multiple members of the miR-17-92 and miR-106b-25 clusters where shown to be downregulated (Figure 4.1). These are considered highly oncogenic miRNA and so their repression was of significant interest in a model of apoptosis.

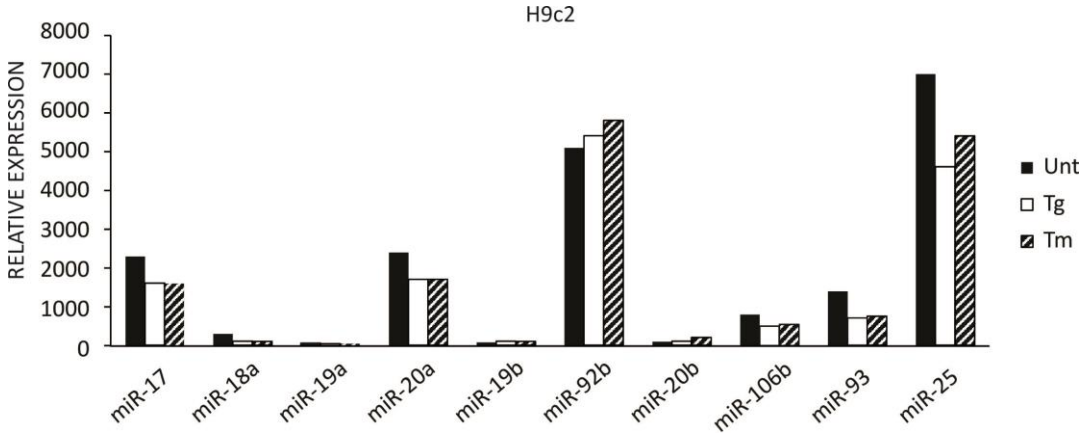


Figure 4.1 miRNA of the miR-17-92 and miR-106b-25 cluster are downregulated during ER stress. H9c2 cells treated with 1 μM of Tg, and 1 μg/ml of Tg for 24 h. Affymatrix Microarray was used to measure gene expression. Graph shows the expression of miRNAs of the miR-17-92 and miR-106b-25 clusters. Data represents the mean values of three independent repeats with a p- value of <0.01.

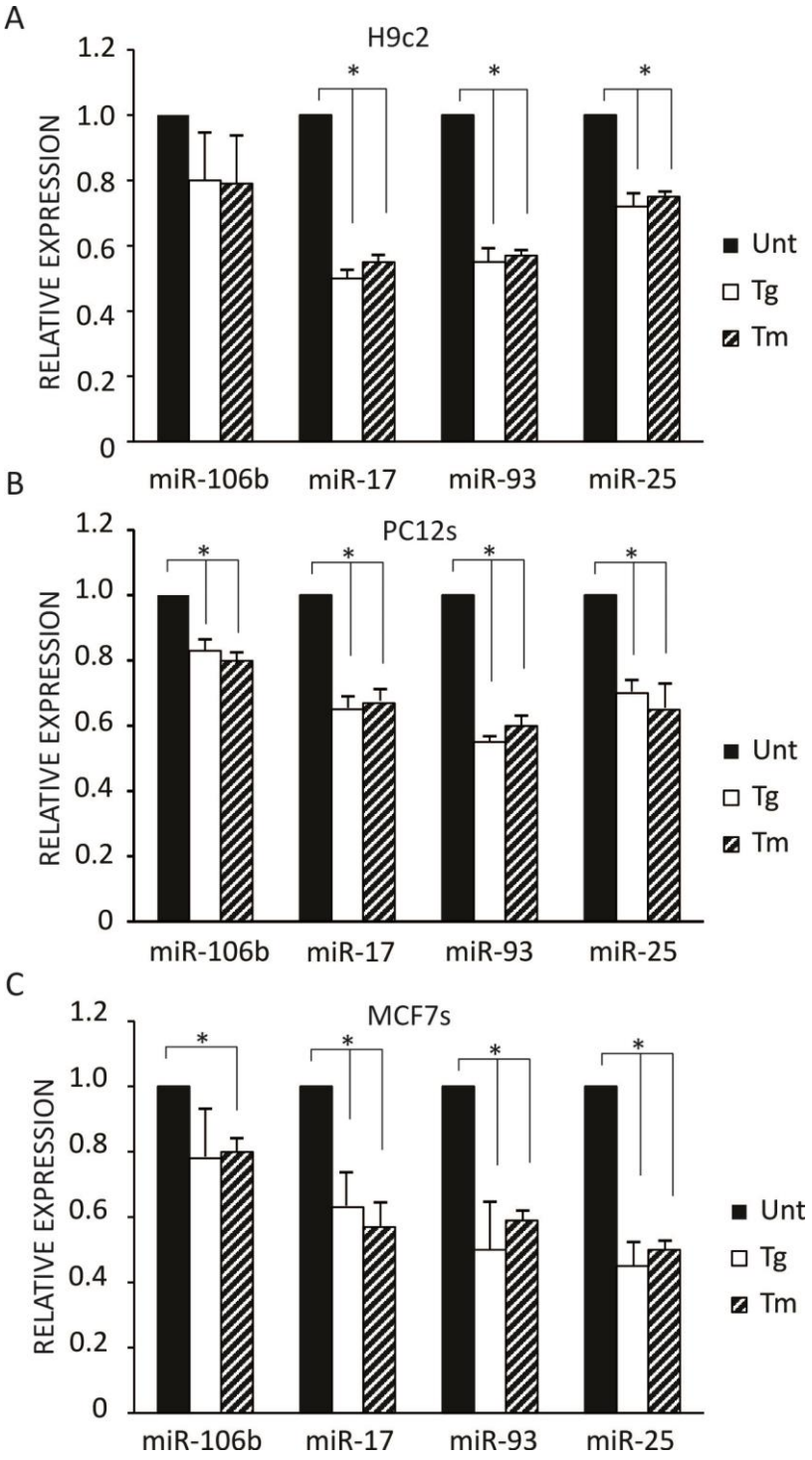
4.2 miR-106b, miR-17, miR-93 and miR-25 are downregulated during ER stress

To further investigate this observation and validate results seen in (Figure 4.1), H9c2 cells were treated with Tg and Tm and miRNA QRT-PCR was carried out. Of the miRNAs downregulated in the microarray, miR-106b, miR-17, miR-93 and miR-25 were validated to be downregulated in H9c2 cells upon ER stress (Figure 4.2 A). To determine if this observation was cell type specific or not, PC12 cells were treated with Tg and Tm and again the expression of miR-106b, miR-17, miR-93 and miR-25 was checked via QRT-PCR. All four miRNA were again validated to be downregulated during ER stress (Figure 4.2 B). It has been shown that DICER is specifically cleaved by caspase-3 at two sites STTD1476 and

CGVD1538 within the RNase IIIa domain during apoptosis. Caspase-3-mediated cleavage of DICER during apoptosis reduces its catalytic activity, and reduces the production of mature miRNAs. However, no cleavage of DICER was observed in caspase-3-deficient, MCF-7 cells (Nakagawa et al., 2010). Therefore to exclude the possibility that reduced levels of miR-106b, miR-17, miR-93 and miR-25 observed during conditions of ER stress is due to the proteolytic inactivation of DICER levels of miR-106b, miR-17, miR-93 and miR-25 in MCF-7 cells upon exposure to ER stress were analysed. Agreeing with previous results miR-106b, miR-17, miR-93 and miR-25 were downregulated during ER stress (Figure 4.2 C). All of the above findings suggest that repression of these miRNA is not isolated to a particular disease model but a more general ER stress-related phenomenon. Furthermore as MCF7 cells are a human-derrived cell line, this indicates that our observations are evolutionary conserved.

4.3 The miR-17-92 promotor is responsive to ER stress

To understand how these miRNA are regulated by ER stress, a PGL4-reporter construct carrying the full length promotor of the miR-17-92 cluster, cloned at the 5' site of the luciferase gene (PGL4-miR-17-92-FL) was used. See schematic of PGL4-miR-17-92-FL (Figure 4.3 A). The reporter was confirmed to be responsive to Tg and Tm in MCF7 cells where low luciferase activity was detected while treatment with H202 did not lead to a decrease in luciferase activity (Figure 4.3 B). This suggested that decreased expression of miRNAs of this cluster occurs at the transcriptional level.



4.2 miR-106b, miR-17, miR-93 and miR-25 are downregulated during ER stress.

A) QRT-PCR for miRNAs miR-106b, miR-17, miR-93, and miR-25 in H9c2 cells treated with 1 µM of Tg, and 1 µg/ ml of Tm for 24 h. B) QRT-PCR for miRNAs miR-106b, miR-17, miR-93, and miR-25 in PC12 cells treated with 0.25 µM of Tg and 2 µg/ml of Tm for 24 h. C) QRT-PCR for miRNAs miR-106b, miR-17, miR-93, and miR-25 in MCF7 cells treated with 2 µM of Tg and 2 µg/ml of Tm for 24 h. Each sample was loaded in triplicate and normalised to Sn U6 or Sno RNA. Error bars represent the SEM between three independent experiments.

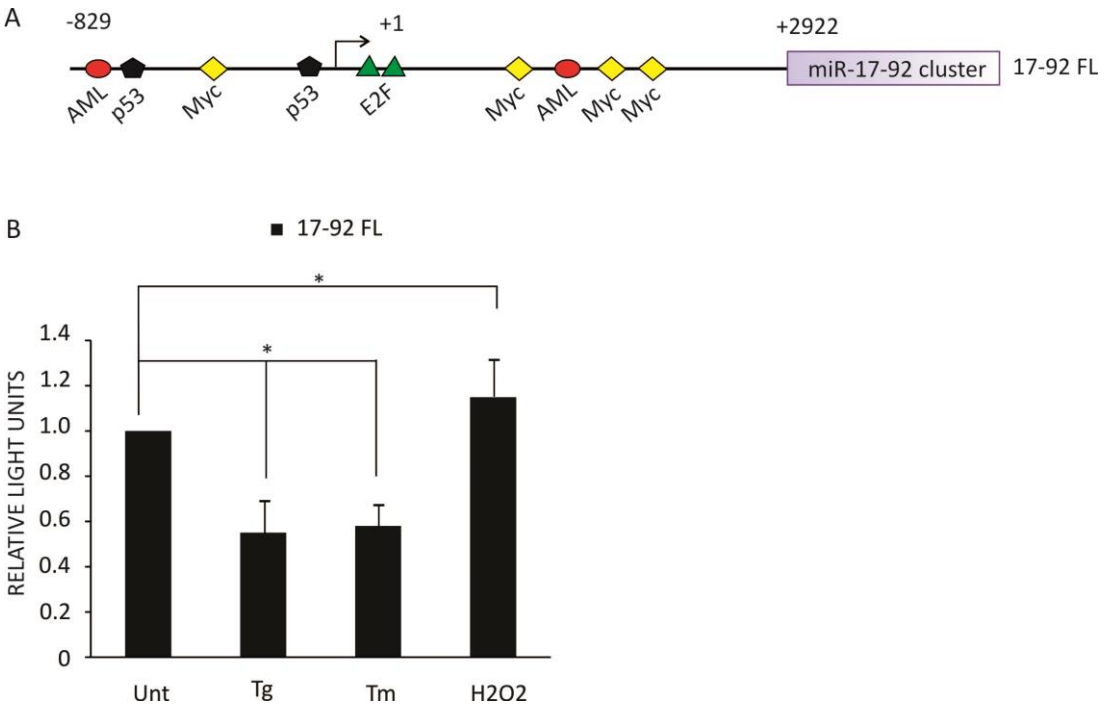
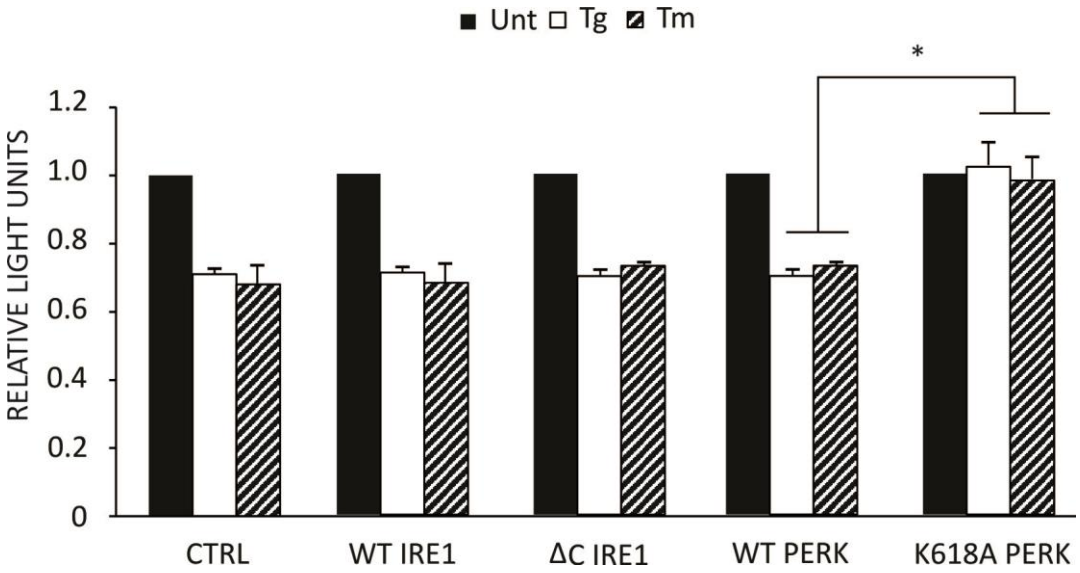


Figure 4.3 The miR-17-92 promotor is responsive to ER stress. A) Schematic representation of the miR-17-92 full length (FL) promoter construct, depicting previously reported transcription factor binding sites, such as AML, p53, E2F, and Myc. B) MCF7 cells transfected with 17-92 FL and Renilla luciferase, 24 h post-transfection cells were treated with 2.0 µM Tg, 2.0 µg/ml Tm or 600 µM H2O2 for 24 h. Promotor activity was assessed using a luciferase assay where firefly luciferase activity was

4.4 Repression of the miR-17-92 cluster is dependent on PERK

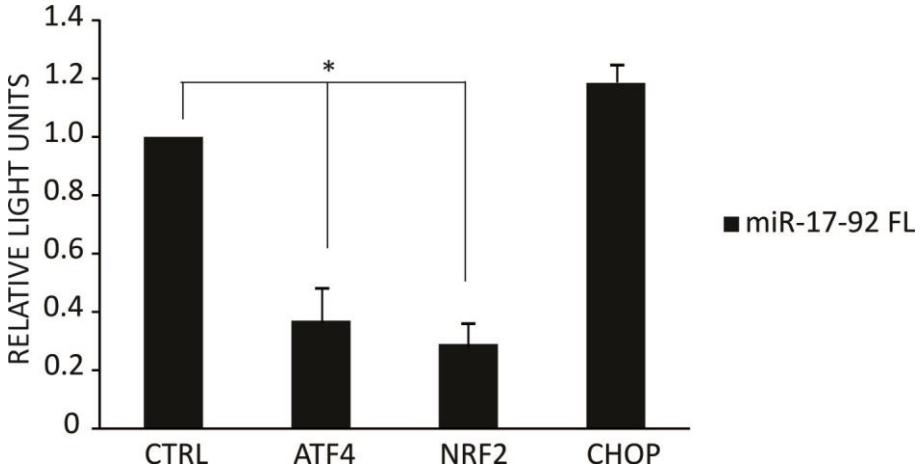
To determine what arm of the UPR is responsible for repression of the miR-17-92 cluster, MCF7 cells were co-transfected with the miR-17-92 promotor construct, PERK cDNA, IRE1α cDNA, PERK (K618A), a dominant-negative PERK mutant that blocks transphosphorylation and activation of the PERK kinase or IRE1α ΔC, a dominant-negative IRE1 mutant containing the lumenal and transmembrane domains but lacking the cytosolic effector domains. Of these only the dominant negative of PERK (K618A) blocked ER stress-induced repression of the miR-17-92 FL reporter (Figure 4.2 B). This demonstrates that repression of these miRNA is dependent on the PERK arm of the UPR.



4.4 Repression of the miR-17-92 cluster is dependent on PERK. MCF7 cells co-tranfected with 17-92 FL, Renilla and either pcDNA 3.1, WT PERK or K618A dominant negative PERK plasmids. 24 h post-transfection cells were treated with 2.0 μM Tg, or 2.0 μg/ml Tm for 24 h. Promotor activity was assessed using a luciferase assay where firefly luciferase activity was normalized to Renilla luciferase. Error bars represent the SEM of three independent repeats performed in duplicate.

4.5 The miR-17-92 promotor is responsive to overexpression of ATF4 or NRF2

As described, the UPR induces a massive transcriptional response to deal with the unfolded protein burden in the ER, and genes required for chaperoning, folding, and ERAD are activated as a result. As miRNA can be transcriptionally regulated by transcription factors, and we have shown that repression is mediated through PERK, PERK regulated transcription factors NRF2, ATF4 and CHOP were investigated as mediators of miR-17-92 repression. Again the miR-17-92 FL reporter was co-transfected with NRF2, ATF4 and CHOP expression vectors into MCF7 cells. Both NRF2 and ATF4 could reduce the activity of the promoter but CHOP could not (Figure 4.5).



4.5 The miR-17-92 promotor is responsive to overexpression of ATF4 or NRF2.

MCF7 cells co-transfected with miR-17-92 FL luciferase reporter, Renilla luciferase and either pcDNA 3.1 CTRL, ATF4, NRF2 or CHOP. 48 h post-transfection a luciferase assay was carried out where firefly luciferase activity was normalized to Renilla luciferase. Error bars represent the SEM of three independent repeats performed in duplicate.

4.6 Overexpression of NRF2 and ATF4 can downregulate miRNA of the miR-17-92 cluster

Furthermore, overexpression of NRF2 and ATF4 for 24 h and 48 h decreased the expression of individual miRNA of the miR-17-92 cluster, including miR-106b, miR-17, miR-25, miR-93, miR-18a, miR-20a, as detected by miRNA QRT-PCR (Figure 4.3 B). This shows that the miR-17-92 miRNAs are downregulated in response to ER stress via PERK-NRF2-ATF4 signalling.

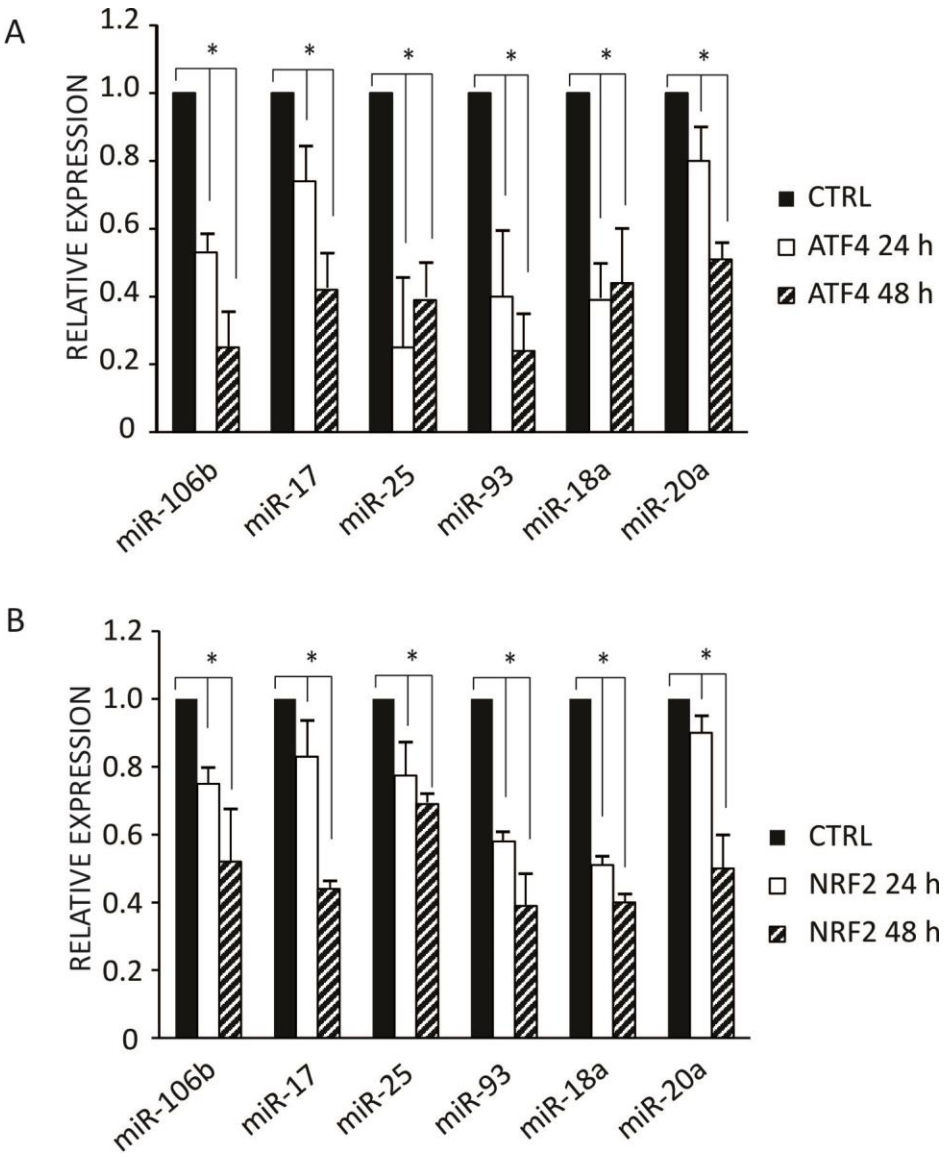


Figure 4.6 Overexpression of NRF2 and ATF4 can downregulate miRNA of the miR-17-92 cluster. A) miRNA QRT-PCR for miR-106b, miR-17, miR-25, miR-93, miR-18a, miR-20a in MCF7 cells were transfected with ATF4 for 24 h and 48 h and normalised against Sn U6 RNA. B) miRNA QRT-PCR for miR-106b, miR-17, miR-25, miR-93, miR-18a, miR-20a in MCF7 cells were transfected with NRF2 for 24 h and 48 h and normalised against Sn U6 RNA. Error bars represent SEM from three independent experiments performed in triplicate

4.7 Identification of NRF2 and ATF4 responsive regions on the miR-17-92 promotor

To determine the region on the miR-17-92 promotor that is responsive to NRF2 and ATF4, short promotor constructs corresponding to different regions of the full length promotor where used. A schematic illustrates the full length promotor (17-92 FL) as well as the three short promotors (17-92/1, 17-92, and 230) and where they are found in relation to the full length promotor (Figure 4.7 A). In MCF7 cells all four promotor constructs showed decreased activity in response to ER stress-induced by Tg and Tm for 24 h and agreeing with earlier data all four constructs were downregulated via overexpression of NRF2 and ATF4 (Figure 4.7 B). This would suggest that the miR-17-92 cluster can be regulated at two different sites in on its promotor, one located on the "17-92" region and another on the "230" region. This also suggests that interaction of NRF2 and ATF4 on only one of these sites is required for the repression of the cluster.

4.8 There is direct recruitment of NRF2 on the miR-17-92 promotor

In an attempt to show that NRF2 and ATF4 directly regulate the miR-17-92 cluster at sites within the 17-92 or 230 region of the promotor, the online software genomatix was used to identify possible binding sites for NRF2 or ATF4 within these regions. Interestingly two consensus sites where identified for NRF2, one (GTCGGAAGTA) located from -82 to -73 bp and the other (CACTTCCAGT) located from +2699 to + 2708 bp on the promotor. These sites were then termed, Site 1 (within 17-92 region) and Site 2 (within 230 region). To show that these were in fact the sites at which NRF2 could bind, site directed mutagenesis was used to separately mutate these sites on the 17-92 or 230 promotor constructs. Sequence analysis was used to confirm that the sites were indeed mutated. However, when luciferase assays were done with wt and mutant (mt) promotor constructs, the activity was still repressed upon ER stress and overexpression of NRF2 and ATF4 (Figure 4.8 A). When less stringent analysis of possible NRF2 binding sites was carried, several other possible sites were identified, which may explain why mutagenesis of site 1 and site 2 had no effect. To prove the interaction

between NRF2, ATF4 and the miR-17-92 promotor, a ChIP assay was done, in which NRF2 and ATF4 were overexpressed, and were immunoprecipitated, PCR for site 2 carried out on DNA pulled down with either protein. Direct binding could be seen with NRF2 but not ATF4. Although interaction between ATF4 and known target CHOP could be seen. This suggests that NRF2 is sufficient for miR-17-92 repression. However we have already shown that overexpression of ATF4 alone can repress the cluster. NRF2 and ATF4 have previously been shown to heterodimerize and bind to an StRE in the HO-1 gene to regulate its expression (He et al., 2001), and more recently it was reported that ATF4 requires NRF2 for its transcriptional activation (Miyamoto et al., 2011). Therefore it is possible that overexpression of ATF4 somehow activates NRF2 and both heterodimerize to modulate gene expression. This has previously been seen for the activation of HO-1. Binding with RNA pol III was used as a positive control and IgG was used as a negative control. Furthermore the known target of NRF2, HO-1 was detected by PCR in the appropriate pull down and serves as an extra positive control (Figure 4.8 B).

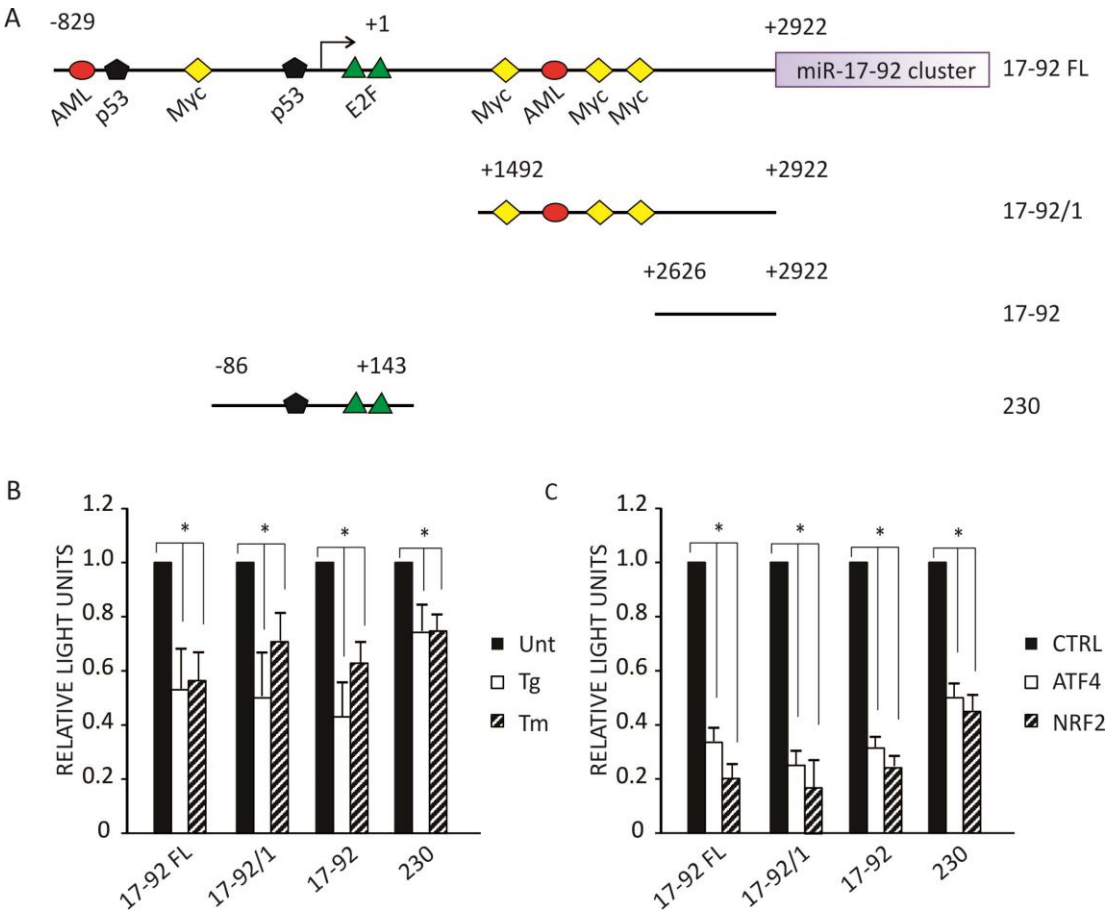


Figure 4.7 Identification of NRF2 and ATF4 responsive regions on the miR-17-92 promotor. A) Schematic representation of the miR-17-92 full length (FL) promoter construct, and the corresponding shortened promotor constructs (17-92/1, 17-92 and 230). B) MCF7 cells were transfected with one of the four reporter constructs and Renilla luciferase. 24 h post transfection, cells were treated with 2.0 µM Tg, or 2.0 µg/ml Tm for 24 h. Promotor activity was assessed using a luciferase assay where firefly luciferase activity was normalized to Renilla luciferase. C) MCF7 cells were co- transfected with one of the four reporter constructs, Renilla luciferase and either pcDNA 3.1, ATF4 and NRF2 plasmids. 24 h post transfection, promotor activity was assessed using a luciferase assay where firefly luciferase activity was normalized to Renilla luciferase. Error bars represent the SEM of three independent repeats performed in duplicate.

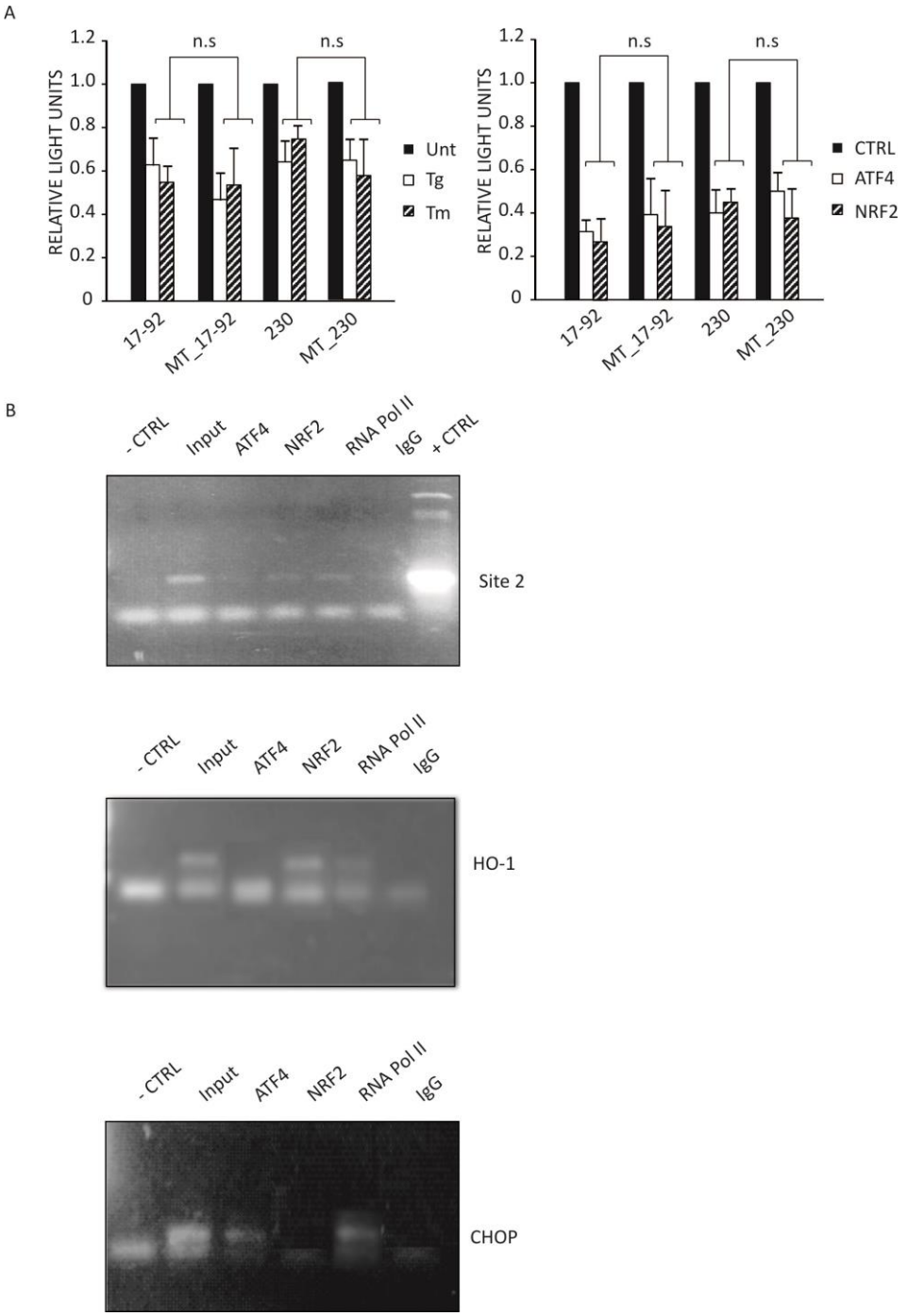


Figure 4.8 There is direct recruitment of NRF2 on the miR-17-92 promotor. A) MCF7 cells were co- transfected with one of the four reporter constructs, Renilla luciferase and either pcDNA 3.1, ATF4 and NRF2 plasmids. 24 h post transfection, promotor activity was assessed using a luciferase assay where firefly luciferase activity was normalized to Renilla luciferase. Error bars represent the SEM of three independent repeats performed in duplicate. B) ChIP assay for Site 2 on DNA associated to ATF4 or NRF2 in MCF7 cells co- transfected with ATF4 and NRF2 plasmids. RNA pol II and IgG were used as positive and negative controls respectively. CHOP and HO-1 also served as positive controls for ATF4 and NRF2 ChIPs respectively.

4.9 ER stress leads to increased expression of BIM

To examine the functional significance of the repression of the miR-17-92 or 106b-25 clusters, during ER stress, the levels of BH3 only protein BIM, a known target of miRNA from these clusters and previously been reported to be important for ER stress-induced cell death; was investigated. Firstly Mrna level of Bim and the host gene of the miR-106b-25 cluster were checked via QRT-PCR of MCF7 cells treated with Tg and Tm for 24 h. Interesting the host gene decreased with ER stress, while BIM levels increased, suggesting a correlation in the two observations (Figure 4.9 A). Next, to show BIM is directly regulated by miRNA of the cluster, a psiCHECK luciferase construct, carrying the 3'UTR of BIM which contains the putative binding sites for the miR-17-92 cluster miRNAs (WT_BIM UTR) was used. Similarly, the same construct in which the miR-17-92 binding sites were abolished was used as a control (MT_BIM UTR). Upon ER stress there is increased activity of the WT_BIM UTR but not the MT_BIM UTR, showing mutation of these sites abolishes the regulation of BIM through these miRNA. Treatment with Etop and H2O2 had no significant effect on the WT_BIM UTR suggesting this effect may be specific to ER stress responses (Figure 4.9 B).

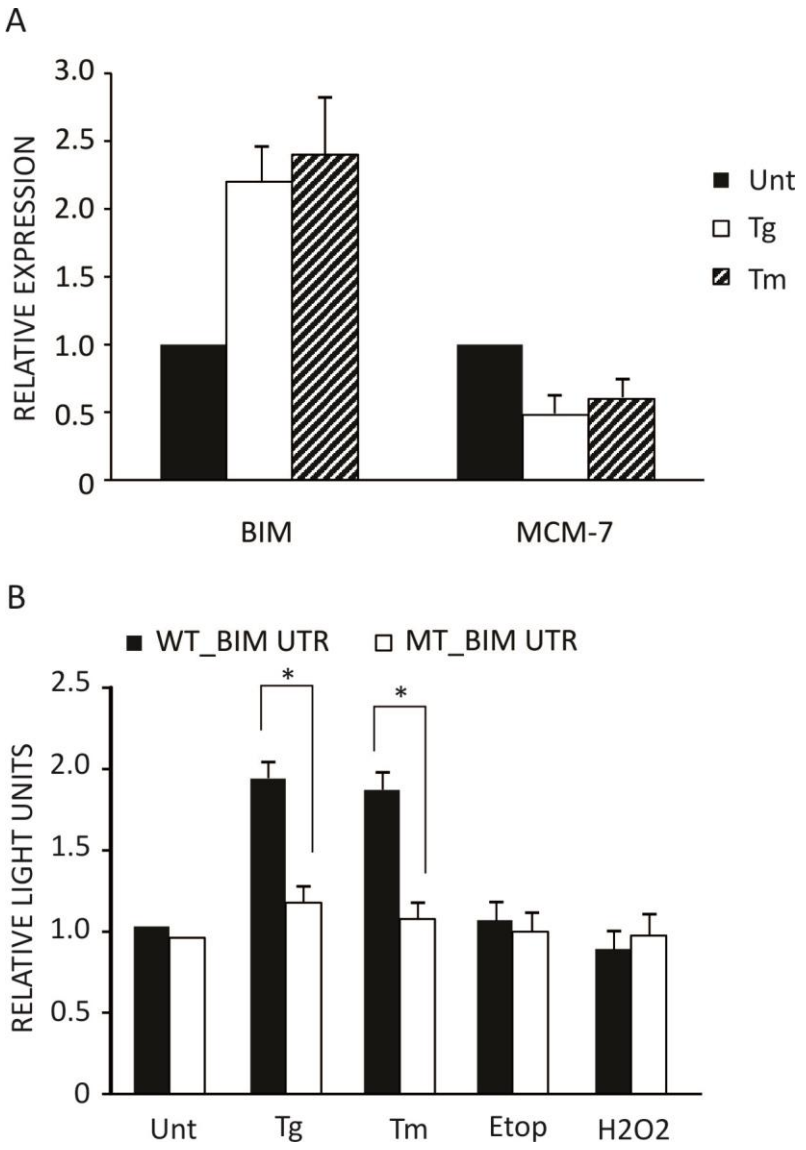


Figure 4.9 ER stress leads to increased expression of BIM. A) MCF7 cells treated with 2.0 µM Tg and 2.0 µg/ml Tm for 24 h. QRT-PCR for BIM and MCM-7 was carried out and normalized to GAPDH. B) MCF7 cells transfected with WT/MT_BIM 3' UTR. 24 h post transfection, cells were treated with 2.0 µM Tg and 2.0 µg/ml Tm for 24 h. promotor activity was assessed using a Renilla luciferase assay where firefly luciferase activity was normalized to Firefly luciferase.

4.10 Forced expression of miR-17-92 reduces ER stress-mediated increase in BIM

Up to this point all data suggested that ER stress leads to repression of the miR-17-92 or 106b-25 cluster miRNAs, which in turn leads to an increase in BIM levels. To investigate this further MCF7 cells were transfected with empty vector PCXN2 (EV) or PCXN2 miR-17-92 (miR-17-92) and treated with 2.0 μM of Tg for 24 h. BIM protein levels were detected by western blotting, where it increased with ER stress in EV cells while the increase was greatly reduced in miR-17-92 cells (Figure 4.10). Previously it has been reported that CHOP can upregulate BIM. Therefore, CHOP levels were compared between EV and miR-17-92 cells, where no notable difference was observed. These results indicate that changes in BIM are not due to changes in CHOP, but as a result of miR-17-92 overexpression.

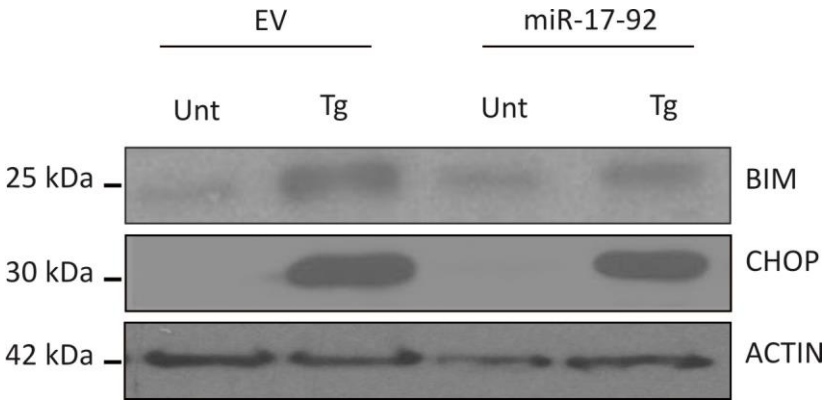


Figure 4.10 Forced expression of miR-17-92 reduces ER stress-mediated increase in BIM. Western blot for BIM and CHOP in PC12 cells transfected with EV or miR-17-92 plasmids, 24 h post transfection cells were treated with 2.0 μM Tg and 2.0 μg/ml Tm for 24 h. ACTIN was used as a loading control. Please note the results shown in this figure were generated by Dr. Ayswaria Deepti.

4.11 Forced expression of miR-17-92 reduces ER stress induced cell death

BIM has been reported to be an important mediator of ER stress induced cell death. As such we hypothesised that deterring the induction of BIM during ER stress via forced expression of miR-17-92 may also deter ER stress-induced cell death. To examine our hypothesis, MCF7 cells were transfected with EV or miR-17-92 overexpression plasmids, 24 h post transfection, cells were treated with Tg and Tm. Cell viability was accessed using a β-Galactosidase activity assay and as expected ectopic expression of miR-17-92 enhanced resistance to ER stress-induced cell death.

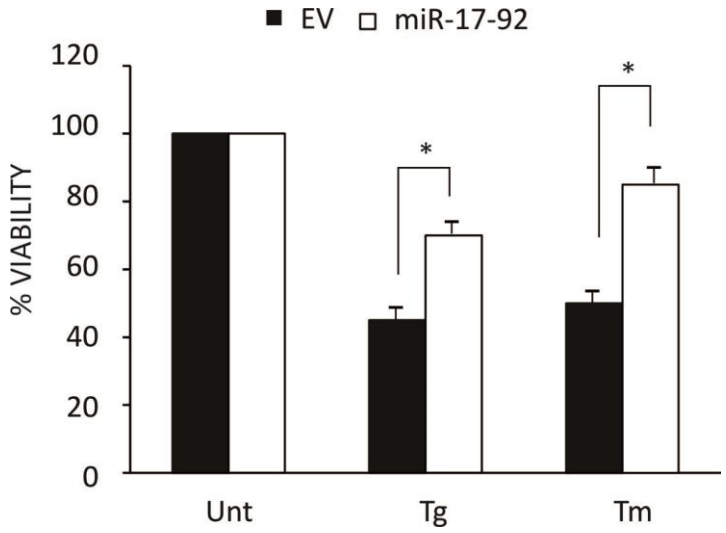


Figure 4.11 Forced expression of miR-17-92 reduces ER stress induced cell death. B-Galactosidase activity assay was used to measure % cell viability in MCF7 cells were transfected with EV or miR-17-92 plasmids, and treated with 2.0 μM Tg and 2.0 μg/ml Tm for 24 h.

4.12 ER stress-mediated increase in BIM is PERK-NRF2-ATF4 dependant

As shown in Figure 4.4, repression of the miR-17-92 cluster is PERK dependant. Therefore it was necessary to show that increase in BIM was also dependant on PERK signalling. To examine this, MCF7 cells were co-transfected with the WT_BIM 3' UTR and either pcDNA 3.1, WT PERK, PERK K618A, WT IRE1 or ΔC IRE1. Cells were treated with Tg and Tm for 24 h and a luciferase assay was used to assess changes in the UTR activity. Dominant negative IRE1 (ΔC IRE1) showed no difference to BIM 3' UTR activity, however, the dominant negative PERK (K618A PERK) showed significant decrease in UTR activity, showing that PERK is required for ER stress mediated BIM upregulation (Figure 4.12 A). Next the effects of ectopic expression of ATF4 and NRF2 on BIM were investigated. MCF7 cells were then transfected with ATF4 and NRF2 and QRT-PCR for CHOP, BIM and MCM7 carried out. Again increased BIM mRNA was seen as MCM7 decreased while no change in CHOP levels were observed. GAPDH was used to normalize (Figure 4.12 B). The Protein level of BIM was also analysed via western blotting following overexpression of NRF2 and ATF4 and again an increase in BIM was observed (Figure 4.12 C)

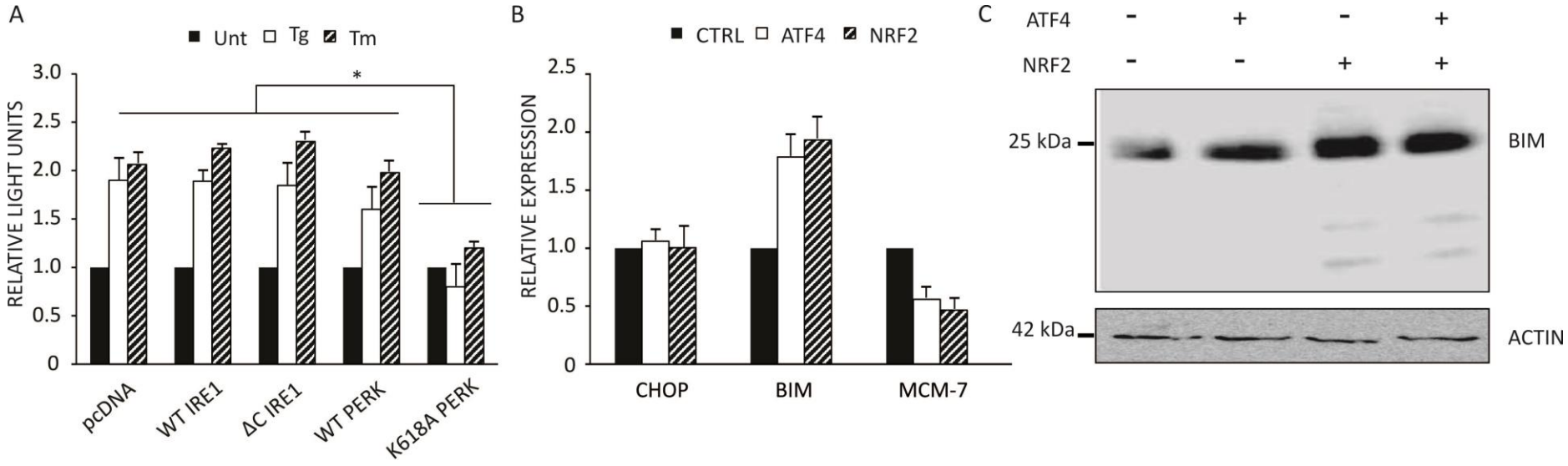


Figure 4.12 ER stress-mediated increase in BIM is PERK-NRF2-ATF4 dependant. A) MCF7 cells were co-transfected with WT_BIM UTR and either pcDNA 3.1, WT PERK, K618A PERK, WT IRE1 or ΔC IRE1 plasmids. 24 h post transfection, cells were treated with 2.0 µM Tg and 2.0 µg/ml Tm for 24 h. To assess UTR activity, Renilla Luciferase assay was carried out and normalized to firefly luciferase. B) MCF7 cells were transfected with either ATF4 or NRF2 or pcDNA 3.1 as CTRL. 24 h post transfection RNA was isolated. QRT-PCR was used to determine mRNA levels of CHOP, BIM and MCM-7. C) (To be inserted) MCF7 cells were transfected with either ATF4 or NRF2 or pcDNA 3.1 as CTRL. 24 h post transfection cells were lysed and western blotting was used to determine protein level of BIM. ACTIN was used as a loading control.

CHAPTER 5:

DISCUSSION

5.0 DISCUSSION

The work in this thesis illustrates an important role for miRNAs during ER stress induced cell death. Two approaches were taken in this investigation. The first has been described in chapter 3 were global miRNA levels were compromised and the effects of such during ER stress induced cell death examined. The second study as detailed in chapter 4 involved a candidate approach were changes in miRNA expression patterns during ER stress induced cell death were investigated. Below the significance of the finding of both chapters are discussed individually.

5.1 Loss of DICER confers resistance to ER stress induced cell death

This study shows that, a functional miRNA biogenesis pathway is required for efficient induction of ER stress-mediated apoptosis. Three model systems in which miRNA biogenesis was compromised were used, DICER hypomorphic (Exn5/Exn5) cells, DICER knockdown cells and DROSHA knockdown cells, all of which were resistant to ER stress induced cell death. Further investigation of the UPR response showed there was no major difference in the expression of typical markers of the UPR. However, the intrinsic apoptotic pathway was delayed in these cells upstream of the mitochondria due to altered expression of the BCL-2 family proteins.

In preparation for microarray analysis of gene expression during ER stress induced cell death, dose response treatments of 0-5 μg/ml Tm and Bfa were carried out on HCT116 cells (Figure 3.1) and a subsequent dose of 0.5 μg/ml was chosen because we were looking for the lowest dose that induced ~ 50% cell death. With the aim of identifying novel proteins involved in ER stress induced cell death via microarray analysis, an increased expression in several genes associated with microarray biogenesis was observed (Figure 3.2). DICER expression has previously been reported to be differentially expressed by different cell stress inducing agents (Asada et al., 2008; Wiesen and Tomasi, 2009). The modulation of multiple components of the biogenesis pathway during cellular stress however has not. This immediately suggested that miRNA could be important during ER stress induced cell death. The functional significance of this was investigated using colorectoral cancer cell lines, RKO and HCT116

made hypomorphic for DICER (Exn5/Exn5) (Calin and Croce, 2006)where levels of mature miRNA are severely compromised (Figure 3.3 a&b). When exposed to increasing doses of Tm and Bfa treatments, HCT116 and RKO Exn5/Exn5 cells were resistant to ER stress induced apoptosis even at the highest dose of 5 μg/ml of each compound (Figure 3.4). Although loss of miRNA biogenesis provided resistance to ER stress induced cell death, it only provided a maximum protection of ~10% to the same cells (HCT116 and RKO Exn5/Exn5) treated with Etop and Sts (Figure 3.5). This may be explained by the fact that cells can respond differently to different stress stimuli and DICER expression or indeed the expression of individual miRNA does not always correlate with enhanced survival even in the same cell type. The fact that DICER or miRNA expression can promote or deter survival depending on cell type or stimulus, suggests that each stimulus induces its own stress response, with each response regulating a different arsenal of miRNA or indeed reducing certain miRNA to fine-tune gene expression and thus determine the cells fate.

In HCT116 cells DICER was independently knocked down and levels of mature miRNA compromised (Figure 3.6). Again by compromising miRNA expression, ER stress induced cell death was attenuated (Figure 3.7). The upstream component of the miRNA biogenesis pathway, DROSHA was also knocked down and the expression of miRNA compromised (Figure 3.8). As with DICER compromised cells this model was also resistant to ER stress induced cell death (Figure 3.9). Thus resistance to ER stress is not restricted to disruption of DICER results from loss of mature miRNA. These findings have relevance to cancers where miRNAs are increasingly being implicated in disease pathogenesis, also where DICER and other components of the miRNA biogenesis machinery are reported to have altered expression. Loss of DICER is associated with increased tumourigenicity and with poor patient prognosis in several cancers (Kumar et al., 2007; Sand et al., 2010; Wu et al., 2012a; Zhu et al., 2012a). More importantly for the present study, down-regulation of DICER has been observed in tissue samples from patients with colorectal cancer. Low DICER expression is more common in advanced, high grade tumours and is associated with low patient survival (Faggad et al., 2012). How loss of miRNAs promotes tumour development is not clearly understood. Following initiation of malignancy, poor vascularisation of the tumour mass leads to stressful conditions in the tumour microenvironment, including low oxygen supply, nutrient deprivation and pH changes. These conditions activate a range of cellular stress-response pathways, including the UPR (Lorusso and Ruegg, 2008). At present it is unclear how cancer

cells adapt to long-term ER stress whether the protective elements of the response are enhanced, or the destructive components suppressed. On the basis of our results showing that loss of miRNA biogenesis abrogates ER stress-induced apoptosis we speculate that one mechanism by which the down-regulation of miRNA biogenesis contributes to increased tumourigenesis is by inhibiting ER stress-induced apoptosis under stressful conditions in the tumour microenvironment.

However, it is important to note that loss of DICER is not always associated with enhanced cancer cell survival or resistance to cell death. Some cancers have high DICER expression and this correlates with increased cancer proliferation and metastasis (Ma et al., 2011; Noh et al., 2011; Stratmann et al., 2011). Inactivation of DICER has been associated with increased apoptosis where TNFα and PKC inhibitors result in caspase-3-mediated cleavage of DICER, which disrupts its miRNA processing function and enhanced cell death of Hela cells (Ghodgaonkar et al., 2009; Matskevich and Moelling, 2008). The exact mechanism of how disruption of DICER leads to apoptosis, is yet unknown in mammals. One report in *C. elegans* showed DCR-1 was cleaved by CED-3 disrupting its miRNA processing function, and converting DCR-1 to a DNase that produced 3'hydroxyl DNA breaks i.e. DNA fragmentation and subsequent apoptosis (Nakagawa et al., 2010) All of this suggests that levels of global miRNA expression can be specific to the type of cell, disease or stress stimuli and indicates the importance of uncovering the implications or the mechanisms of such, under different conditions.

The fact that loss of miRNA conferred resistance to ER stress but not other compounds, suggested that the mechanism of resistance must be specific to the signalling induced by ER stress. As the UPR is activated during ER stress but not by other cell death inducers, we hypothesized that miRNAs could be important for UPR signalling and that lack of miRNAs may compromise these pathways to enhance survival. Gene expression analysis via microarray and QRT-PCR revealed lower expression of pro-apoptotic proteins PERK, CHOP and NOXA in Exn5/Exn5 cells (Figure 3.10). We had speculated that if the altered expression of these genes followed through to the protein level then, they would be good candidates to explain the resistant phenotype of Exn5/Exn5 cells. However, analysis of these molecules at protein level as well as other known markers of the UPR showed no difference in expression

compared to WT cells (Figure 3.11), indicating that the UPR proceeds as normal in the absence of miRNA.

Of the markers of the UPR examined at protein level, one protein did appear to have altered expression, and that was XBP1s. Surprisingly levels of XBP1s were consistently lower in Exn5/Exn5 (Figure 3.12). Prolonged splicing of XBP1 is associated with enhanced survival of cancer cells (Bagratuni et al., 2010) and has previously been reported to protect against ER stress (Gupta et al., 2010), so the lower levels we have observed in cells that are resistant to ER stress induced cell death was surprising. However there was a report by the Hetz group, were XBP1 deficiency in the nervous system could protect against the ER stress associated disease amyotrophic lateral sclerosis (ALS). Loss of XBP1 essentially compromises the induction of ERAD, a pathway that directs proteins from the ER to the proteosome for degradation. Subsequent accumulation of misfolded proteins at the ER activates autophagy to deal with the burden of misfolded proteins to enhance cell survival (Hetz et al., 2009). To investigate if lower XBP1s levels in our model could be protective, we used an inhibitor of IRE1, MKC4485, which specifically blocks the RNase function of IRE1 to block splicing of XBP1 in HCT116 WT and Exn5/Exn5 cells. We postulated that if loss of XBP1 had a protective role in Exn5/Exn5 cells, then blocking splicing in WT cells would confer resistance to WT cells. Although the inhibitor could block XBP1 splicing (Figure 3.13 A), it had no effect on cell viability in HCT116 WT cells (Figure 3. 13 B). Although lower XBP1 splicing is not important for resistance in this model, the lower levels still suggested that IRE1 signalling may still be compromised. IRE1/TRAF2/ASK1 signalling is known to phosphorylate and activate JNK1/2 to promote apoptosis (Urano et al., 2000a). This lead us to hypothesis that perhaps JNK1/2 are important for ER stress induced cell death in our model and that if IRE1 signalling was somehow compromised in Exn5/Exn5 cells so too would be JNK induced cell death. However, there was no difference in the level of total and phospho- JNK1/2 between WT and Exn5/Exn5, ultimately ruling out a role for IRE1 signalling in the resistance of Exn5/Exn5 cells to ER stress induced cell death Figure 3.15).

Since components of the UPR did not appear to be compromised in Exn5/Exn5 cells, we next sought to examine the main intrinsic apoptotic pathway. The intrinsic pathway revolves around the state the OMM (Elmore, 2007). If this is compromised, i.e. by loss of OMM potential or if

the OMM becomes permeabilized is indicative of activation of apoptosis. (Figure 3.16) shows that in Exn5/Exn5 cell that are resistant to ER stress induced cell death, retain TMRE positive staining indicating that they retain their OMM potential in response to treatment while WT cells show a much greater loss of TMRE staining (Figure 3.17). Although more of a readout of % of cell undergoing apoptosis, the difference in OMM potential between WT and Exn5/Exn5 cells also suggested that the intrinsic pathway may be compromised upstream of the mitochondria. Translocation of BAX is associated with changes in mitochondrial integrity because it form pores in the OMM along with BAK, permeabilizing the OMM and releasing pro-apoptotic factors into the cytoplasm (Leber et al., 2007). To determine if resistance was in fact provided upstream of the mitochondria, an antibody that only detects the active conformation of BAX (6A7) was used to determine the levels of active BAX in WT and Exn5/Exn5 cells via flow cytometry. As hypothesised there was less active BAX in Exn5/Exn5 cells (Figure 3.18), supporting the idea that resistance to ER stress in this model occurs upstream of the mitochondria.

Translocation of BAX is under the control of other BCL-2 family members, where the balance between pro-survival and pro-apoptotic proteins is a deciding factor where higher levels of pro-apoptotic protein can neutralize pro-survival proteins, allowing freedom of BAX and its translocation to the OMM (Cory and Adams, 2002). Due to lower levels of active BAX in Exn5/Exn5 cells we next examined if levels of BCL-2 family members could explain differences in BAX levels and discovered that there was differential expression of these proteins between WT and Exn5/Exn5 cells. Of particular interest, pro-survival BCL-2 and MCL-1 are higher and prolonged in these cells respectively, while pro-apoptotic BAD decreased during ER stress in Exn5/Exn5. Surprisingly, there were quite high levels of pro-apoptotic BIM in Exn5/Exn5 cells even though these cells are resistant to cell death (Figure 3.19). Many reports describing the regulation of miRNA during apoptosis, indirectly show that such regulation occurs upstream of the mitochondria at the level of the BCL-2 family proteins. The BH3 only protein BIM can be targeted by miR-17-92, while miR-221-222 can target both BIM and PUMA. miR-29 can target multiple BH3 only proteins as well as nuclear specific N-BAK. miR-15a-16-1 can downregulate BCL-2 while MCL-1 can be repressed by miR-29 and miR-15a-16-1 (Cawley et al., 2012; Lynam-Lennon et al., 2009). This study shows that loss of DICER and thus mature miRNA results in dramatic differences in the expression patterns of certain BCL-2 family members. Global loss of mature miRNA in this model means that the

miRNA mentioned above cannot be induced to repress BCL-2, MCL-1 or BIM which may explain the elevated levels in response to stress. BCL-2 family proteins are thought to act as a rheostat with pro- and anti- apoptotic members competing with each other to either promote or block apoptosis. Our results suggest that loss of DICER causes higher levels of pro-survival BCL-2 and MCL-1 that may neutralise pro-apoptotic BH3 only proteins in response to ER stress, delay mitochondrial apoptosis and thus, enhance survival. BIM is conventionally a pro-apoptotic protein and is an important inducer of ER stress induced cell death (Puthalakath et al., 2007). Therefore, a high level of BIM in a model resistant to ER stress does raise questions as to why these cells are not dying. The rheostat model proposed above can further be supported by the study carried out by the Chandra group which reported higher BIM expression in prostate and breast cancer but BIM was phosphorylated at serine 69 and serine 87, which are thought to neutralize the apoptotic function of BIM, furthermore, BIM was sequestered by pro-survival BCL-xL and MCL-1 (Gogada et al., 2012).

Since it is the main apoptotic pathway that is altered in Exn5/Exn5 cells, then the same cells should technically be resistant to other inducers of apoptosis but this was not the case. We hypothesised that, BCL-2 family proteins are modulated differently in response to different stimuli. To investigate this, the levels of the BCL-2 family proteins in HCT116 WT and Exn5/Exn5 treated with Etop were examined and indeed levels of BCL-2 family members differed upon Etop treatment. Although levels of MCL-1 seem to increase there appears to be no change in BCL-2 or BAD. Yet again however, levels of BIM were higher. Perhaps in Etop treatment high MCL-1 alone is not enough to block the BIM/BAD-mediated cell death. Increased expression of these BCL-2 and MCL-1, is often seen in cancers and is known to provide resistance to therapy (Campbell et al., 2010; Higashiyama et al., 1996; Karakas et al., 1998; Real et al., 2002; Wuilleme-Toumi et al., 2005), this may explain why Exn5/Exn5 cells are more specifically resistant to ER stress-induced cell death and not death induced by Etop or Sts. It would be interesting to look at human cancer samples for correlation between DICER expression, tumour progression and levels of BCL-2 family proteins. It may be possible that cancers with low DICER, and enhanced tumourigenesis could have altered BCL-2 family expression to enhance survival.

5.2 Identification of ER stress regulated miRNA

This study shows that miRNAs belonging to the miR-17-92 cluster have a pivotal role in the UPR. The expression of these miRNAs is markedly downregulated during ER stress conditions. The repression of miR-17-92 cluster during ER stress is dependent on PERK signaling. Furthermore, our results show that miR-17-92 cluster is a novel target for ATF4 and NRF2-mediated transcriptional repression under conditions of ER stress. Ectopic expression of ATF4 or NRF2 can lead to a decrease in levels of mature miRNAs belonging to miR-17-92 cluster as well as reduction in miR-17-92 promoter reporter activity. Their functional role during ER stress was demonstrated by overexpression experiments – an increased level of members of miR-17-92 cluster inhibits ER stress- induced apoptosis by repressing BIM.

In a bid to determine novel miRNAs regulated during ER stress induced cell death, a candidate approach was taken where microRNA microarray analysis was carried out on cells treated with Tm and Tg. This revealed that miRNAs of the miR-17-92 and miR-106b-25 cluster were downregulated during ER stress (Figure 4.1) which was validated by QRT-PCR again showing that miR-106b, -17, -93 and -25 are downregulated, upon ER stress in H9c2, PC12 and MCF7 cell lines (Figure 4.2). The miR-17-92 and miR-106b-25 clusters are considered as oncogenic clusters with miR-17-92 even being “nicknamed” oncomiR-1 and they are often highly expressed to promote cancer progression (Hayashita et al., 2005; van Haaften and Agami, 2010). The miR-17-92 cluster consists of 6 individual miRNAs miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1 while the miR-106b-25 cluster consists of three individual miRNAs miR-106b, miR-93 and miR-25. Therefore one transcriptional event can produce several miRNAs at once, and any one of these miRNAs can have hundreds of potential targets, suppression of which generally promotes cancer survival. Some of well characterised targets of these clusters include the tumour suppressor PTEN, the cell cycle checkpoint regulator p21 and the pro-apototic proteins BIM and E2F1 whose repression allows cancer cells to procedd through the cell cycle, increase proliferation, and by-pass apoptosis to enhance survival (Ivanovska et al., 2008; O'Donnell et al., 2005; Ventura et al., 2008) These miRNAs are thought to work separately and also in a coordinated effort to enhance their oncogenic potential (Olive et al., 2010). For instance miR-18a is highly expressed in nasopharyngeal carcinoma (NPC) and can enhance tumourigenesis by

repressing DICER, which subsequently effects global miRNA expression and increases oncogenic and EMT associated genes (Luo et al., 2012). miR-19 overexpression alone is enough to block apoptosis to enhance c-myc lymphomagenesis (Olive et al., 2009) while overexpression of miR-17 alone has an opposite role to that of the full cluster, where it has been reported to limit cell adhesion, migration and proliferation in mice (Shan et al., 2009), suggesting that the individual members may be in delicate balance with each other to control cell survival. Therefore their repression during ER stress suggested they may be important in the regulation of cell fate decisions.

To understand how these miRNA are regulated during ER stress, a luciferase construct carrying the miR-17-92 promotor (Figure 4.3 A) was used. Increased luciferase activity was detected during ER stress but not H_2O_2 (Figure 4.3 B) treatment suggesting that these miRNA are directly regulated by ER stress signalling events. Repression of this cluster was shown to be dependent on PERK signalling where dominant negative PERK overexpression restored luciferase activity (Figure 4.3 C). PERK is known to activate several transcription factors to control UPR responses (Szegezdi et al., 2006) and miRNA can be transcriptional regulated by transcription factors (Breving and Esquela-Kerscher, 2009), thus we investigated PERK regulated transcription factors in the control of this cluster. While overexpression of CHOP had no effect on the cluster expression, overexpression of both ATF4 and NRF2 could repress the cluster (Figure 4.4). Further to this overexpression of NRF2 or ATF4 reduces the levels of individual miRNAs of the cluster (Figure 4.5). We also determined two regions within the miR-17-92 cluster that were responsive to ER stress and overexpression of ATF4 or NRF2 (Figure 4.6) however mutation of predicted binding sites for NRF2 within these regions did not restore promoter expression (Figure 4.7) but we did confirm direct recruitment between ATF4 or NRF2 and the miR-17-92 promotor within the 17-92 region (Figure 4.8). On the basis of bioinformatic analysis, we hypothesized that the functional effects of miR-17-92 cluster may be mediated by BIM. True to the prediction, BIM mRNA increased upon ER stress treatment, while expression the host gene of the miR-106b-25 cluster decreased. During ER stress activity of the 3' UTR for BIM carrying sites for the miR-17-92 cluster increased but other treatments such as H_2O_2 and Etop had no effect on its activity. However when binding sites for the miR-17-92 cluster were abolished UTR activity remained unchanged (Figure 4.9), again suggesting repression of the miR-17-92 cluster and subsequent increase in BIM is specific to ER stress and that BIM translation is increased during ER stress and is dependent

on presence of functional binding sites for members of miR-17-92 cluster in its 3' UTR. As with repression of the cluster, increased activity of the BIM 3' UTR is dependant in PERK and overexpression of NRF2 and/or ATF4 could upregulate BIM mRNA with this increase following through to the protein level (Figure 4.10). Forced expression of the miR-17-92 could abolish ER stress-mediated induction of BIM and increase cell viability by ~ 20% further confirming that downregulation of the miR-17-92 cluster during ER stress is important for ER stress-BIM-mediated apoptosis (Figure 4.11). BIM is essential for ER stress-induced apoptosis in a broad range of cell types, including thymocytes, macrophages and epithelial cells. We also observed a good correlation between induction of BIM and ER stress-induced apoptosis, further confirming the role for BIM in ER stress induced apoptosis. Although the role of BIM in ER stress induced apoptosis has been elucidated, our study provides a novel mechanism to how BIM can be regulated during ER stress since previously little was known about the control of its expression by miRNAs in cell death.

As mentioned, transcription factors, ATF4 and NRF2 are activated during ER stress in a PERK-dependent manner (Cullinan et al., 2003; Wek et al., 2006). In general NRF2 is associated with activation phase II antioxidant induction, preventing accumulation of ROS helping cells recover from stress or in cancer often enhancing unfavourable survival of cancer cells (Sporn and Liby, 2012). ATF4 has a double sided role in cell fate decisions, initially promoting survival through enhancing expression of pro-survival genes such as those needed for amino acid metabolism, and then inducing pro-apoptotic associated protein CHOP (Szegezdi et al., 2006). Similar to NRF2, ATF4 expression has been implicated in cancer in particularly in the hypoxic tumour environment (Blais et al., 2004; Horiguchi et al., 2012). However this study illuminates a novel role for NRF2 in promoting cell death. This leads to the question, what is the biological significance of transcriptional repression of miR-17-92 cluster by ATF4 and NRF2? Reports have demonstrated that PERK signalling can confer both protective and pro-apoptotic in different cancer modals but also during ER stress. For instance, PERK knockout MEFs are more sensitive to ER stress induced apoptosis via increased NOXA expression (Gupta et al., 2012a). However, PERK was shown to be a requirement for ROS-mediated ER stress-induced apoptosis via increased CHOP, where PERK knockout cells displayed resistance to ROS-induced ER stress (Verfaillie et al., 2012). Alternatively, PERK signalling has been shown to impair cell proliferation and promote apoptosis (Lin et al., 2009; Sequeira et al., 2007). ATF4 has been shown to be activated in

response to oxidative stress, promote cell death in the nervous system in vitro and to promote stroke in vivo (Lange et al., 2008). Fenretinide or bortezomib are used in the treatment of neuroectodermal tumour cells and it has been reported that these compounds induce ATF4-mediated ER stress-induced apoptosis, which unusually is not dependant on PERK (Armstrong et al., 2010). KEAP1, is a negative regulator of NRF2 and in conjunction with Cullin3, directs NRF2 poly-ubiquitination in unstressed cells (Furukawa and Xiong, 2005; Itoh et al., 1999). As expected, KEAP1 deficient mice show increased activity of NRF2 but unexpectedly died perinatally (Wakabayashi et al., 2003). Further sustained activation of NRF2 in ATG5-deficient mouse livers due to p62 mediated stabilization of NRF2 has been reported to be a major cause of toxicity in autophagy-impaired livers (Komatsu et al., 2010). The molecular mechanisms by which ATF4 and NRF2 exert their pro-apoptotic effects are still not fully elucidated as such; the data presented here provides the molecular mechanism underlying the PERK- mediated cell death induction.

CHAPTER 6:

FUTURE DIRECTIONS

6.0 Future directions

Resistance of HCT116 Exn5/Exn5 cells to ER stress induced cell death, has been attributed to altered BCL-2 family protein expression, upstream of the mitochondria.

To investigate the significance of these findings further it would be interesting to examine colon cancer-derrived patient samples in order to gain the closest human model to the Exn5/Exn5 model used here. Other studies have reported low DICER expression in various cancers including colon cancer and have correlated this to enhanced and more aggressive tumourigenesis and poor patient prognosis. However, no reports have linked BCL-2 family proteins with low DICER expression and increased cancer cell survival. I would be interested in examining colon cancer patient samples in which low DICER expression was associated with enhanced tumourigenesis, for BCL-2 family protein expression. If these parameters did then correlate with one another, it may suggest that altered BCL-2 expression due to low DICER expression affects the longevity of colon cancer cells and may help cancer cells cope with cellular stress associated with tumour growth and expansion.

Further to this it may be important to understand more clearly how the BCL-2 family are modulated in the HCT116 Exn5/Exn5 model. One possibility would be to test if, changes in the expression of the BCL-2 family proteins occurs directly as a result of global loss of miRNA or indirectly from de-repression of miRNA targets which can then enhance or deter expression of the BCL-2 family proteins.

To investigate direct involvement of miRNA in the regulation of BCL-2 family proteins, luciferase reporter constructs carrying the 3'UTR of individual BCL-2 family proteins could be designed specifically for the proteins shown to be differential in our system including BCL-2, MCL-1, BIM and BAD. If there was increased or decreased expression of the 3'UTRs during ER stress, this would indicate that particular miRNA are decreased or increased respectively during ER stress and allow or block translation of the BCL-2 protein being assessed. If the 3'UTRs were indeed responsive to ER stress, they could be mapped to detect potential binding sites for miRNAs. Thus specific miRNAs that may be responsible for regulation of these proteins during ER stress could be identified.

If regulation of BCL-2 family members occurs through a more indirect route, pre- and post-translational modes of regulation could be investigated. For instance mRNA levels of these BCL-2 proteins could be checked via QRT-PCR. If differences were evident between WT and Exn5/Exn5 cells, this could indicate that regulation of these proteins occurs at the transcriptional level. The various transcription factors known to regulate these proteins could be examined. However if differences in expression between both cell lines was only observed at protein level, this would indicate that post-translational modifications were involved. For instance phosphorylation of several members of the family is known to occur and can either enhance or deter their function depending on the amino acid residue that is phosphorylated or the kinase responsible. Therefore phosphorylation status of the proteins showing altered expression e.g. BIM, BAD, BCL-2 and MCL-1, could be examined, for example antibodies that can detect phosphorylation of a particular residue could be used. Furthermore, expression of the kinase associated with phosphorylation of a particular residue could be compared between HCT116 WT and Exn5/Exn5 cells. A chemical inhibitor of the kinase of interest could be used to show it effects protein expression and cell viability.

The second results chapter discussed the importance of the miR-17-92 cluster during ER stress where it was downregulated to modulate BIM expression and enhance cell death. A process that is dependent on PERK-NRF2 signalling.

To continue this work it would be interesting to focus these finding to a more clinically relevant model. We have already shown that the miR-106b-25 cluster is downregulated in SOD1G86R mice. These mice represent a model for the motor neurodegenerative disease, amyotrophic lateral sclerosis (ALS) in which ER stress and increased BIM expression have been linked with disease progression (Gupta et al., 2012b). Perhaps addition of miR-106b-25 mimics in this model could deter disease progression by lowering BIM levels.

To further the molecular understanding of NRF2 mediated repression of the miR-17-92 cluster it would be intriguing to determine if the presence of ATF4 is required to mediate repression of the cluster. The results obtained from the ChIP assay experiment (Figure 4.8) show that although we can IP ATF4 and PCR amplify its positive target CHOP, direct interaction with miR-17-92 could not be detected. This suggests that NRF2 is the direct interacting transcription factor regulating miR-17-92. However previous data showed overexpression of ATF4 alone could downregulate miR-17-92, suggesting that NRF2 and ATF4 heterodimerize to collectively regulate the miRNA cluster. To test if the presence of ATF4 is required to regulate the cluster, transient transfection of the miR-17-92 promotor and overexpression of NRF2 in a cell line lacking ATF4, or in which a dominant negative could be used to block ATF4 expression could be used. The sites on NRF2 required for ATF4 binding could also mutated thus preventing formation of NRF2:ATF4 heterodimers thus elucidating if NRF2 alone can repress the miR-17-92 cluster.

Another approach would be to investigate the significance of miR-17-92 cluster repression and subsequent increased BIM expression in disease models in which the role of NRF2 can have negative consequences on disease progression and outcome e.g. the Bad side of NRF2. I would question, what decides if NRF2 signalling promotes or deters cancer progression and could our findings have a role to play in these decisions. Perhaps NRF2 signalling is similar to that of the UPR in which at a certain stage of stress signalling can switch from adaptive to pro-apoptotic but in cancers, perhaps that adaptive or pro-survival signals are hyperactive and the “switch” to promote apoptosis does not occur. Perhaps this switch only happens in the presence of ER stress or more particularly via PERK-ATF4

signalling. It may be interesting to use a clinical ER stress treatment such as bortezomib on samples with high NRF2 activity and enhanced survival to determine if this could promote NRF2-ATF4 pro-apoptotic signalling. Perhaps patient samples in which NRF2 promotes cancer could be compared to those in which NRF2 signalling can deter cancer and check the expression levels of the miR-17-92 cluster and BIM and determine if these factors correspond to the different phenotypes. Perhaps in settings where NRF2 survival signalling is hyperactive and cannot heterodimerise with ATF4, miR-17-92, an oncogenic cluster, could remain active and silence its many tumour suppressor or pro-apoptotic targets thus adding an additional survival advantage to cancer cells. In such a scenario, delivery of miR-17-92 antagomirs or sponges could be a potential therapeutic target to overcome the bad effects of NRF2 signalling in cancer.

CHAPTER 7

APPENDIX

Reagents

7.0 APPENDIX

7.1 Taqman assays

Gene	Catalogue number	Supplier
CHOP	Hs00358796_g1	Applied Biosystems
NOXA	Hs00560402_m1	
GAPDH	Hs99999905_m1	

PERK	Integrated DNA technologies
Probe sequence	5'-/56-FAM/AGCAGTGGG/ZEN/ATTTGGATGTGGGAT/3IABkFQ/-3'
Primer 1	5'-GAACCAGACGATGAGACAGAG-3'
Primer 2	5'-GGATGACACCAAGGAACCG-3'

miRNA	Catalogue number	Supplier
miR-17	2308	Applied Biosystems
miR-18a	002422	
miR-20a	580	
miR-25	000403	
miR-93	001090	
miR-103	439	
miR-106a	002169	
miR-106b	000442	
miR-107	443	
U6 SnRNA	001973	
SnoRNA	1718	

7.2 Plasmids

Name	Vector	Source
DICER shRNA	pSicoR-Puro	Addgene
Empty Vector	pSicoR-Puro	Addgene
DROSHA shRNA	pTIG-U6tetO-shDR	Tyler Jacks
ATF4	pEF/Myc/Mito	Jawed Alam
NRF2	pCI	Alan Diehl
CHOP		Andreas Strasser
PERK	pCDNA 3.1	David Ron
PERK DN (K618A)	pcDNA 3.1	David Ron
IRE1	pcDNA 3.1	Kazunori Imaizumi
IRE1 DN (ΔC)	pcDNA 3.1	Kazunori Imaizumi
17-92 FL promotor	PGL4	Laura Fontana
17-92	PGL4	Laura Fontana
17-1	PGL4	Laura Fontana
Pro 230	PGL4	Scott Hammond
17-92 expression vector	pCXN	Klaus Rajewsky
Empty vector	pCXN	Klaus Rajewsky
Bim 3' UTR WT & MT	PsiCHECK	Klaus Rajewsky

7.3 Antibodies for Western blotting

Antibody	Catalogue number	Supplier
Caspase-3 (Cleaved)	9644	Cell signalling technologies
Caspase-9	9506	Cell signalling technologies
PARP	G542	Cell signalling technologies
IRE1	3294	Cell signalling technologies
ATF6	73-500	CosmoBio
P-eIF2α	97215	Cell signalling technologies
T-eIF2α	9722	Cell signalling technologies
CHOP/ GADD154	Sc-793	Santa Cruz
XBP1s	619501	Biolegend
NOXA	114C307	Calbiochem
MCL-1	4572	Cell signalling technologies
BCL-2	Sc-509	Santa Cruz
BIM	AAP-330	Stressgen
BAD	Sc-7869	Santa Cruz
BAK	Sc-832	Santa Cruz
BCL-xL	Sc-8392	Santa Cruz
BID	Sc-11423	Santa Cruz
PUMA	4972	Cell signalling technologies
NRF2	sc-13032	Santa Cruz
ATF4	sc-200	Santa Cruz
LCIII	L7543	Sigma Aldrich
β-ACTIN	A2066	Sigma Aldrich

7.4 Antibodies to detect Active BAX by flow cytometry

Antibody	Catalogue number	Supplier
BAX (6A7)	556467	BD Biosciences
Mouse Isotype Control	A3574	Biolegend
Mouse-FITC secondary	F2012	Sigma Aldrich

7.5 Antibodies for ChIP-IT assay

Antibody	Catalogue number	Supplier
NRF2	2178-1	Epitomics
ATF4	60035-1	Proteintech
RNA POL II (+ CTRL)	61081	Active Motif
IgG (- CTRL)	53011	Active Motif

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